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Expression Analysis Identifies *FAD2-2* as the Olive Oleate Desaturase Gene Mainly Responsible for the Linoleic Acid Content in Virgin Olive Oil

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The effect of ripening stage and water regimen on oleate desaturase gene expression levels in the fruit of different olive (*Olea europaea* L.) varieties was investigated to elucidate the contribution of each to the linoleic acid content in virgin olive oil. To this end, fatty acid analysis and quantitative real time PCR were performed using distinct olive tissues and different developmental stages from the Picual and Arbequina cultivars. The results showed that the olive *FAD2-1*, *FAD2-2*, and *FAD6* genes were spatial and temporally regulated. In addition, the data indicated that *FAD2-2* seems to be the main gene responsible for the linoleic acid content in the olive fruit mesocarp tissue. This conclusion was also confirmed when the study was extended to Hojiblanca, Picudo, and Manzanilla varieties. With regard to the water regimen, unlike the Picual cultivar, a small increase of linoleic acid was observed in the Arbequina variety cultivated with irrigation, which correlated well with the increase detected for the *FAD2-2* gene expression level. All of these data strongly suggest that *FAD2-2* is the main gene that determines the linoleic acid content in the virgin olive oil.

KEYWORDS: *Olea europaea*; olive fruit; oleate desaturase; *FAD2*; *FAD6*; gene expression; quantitative real-time PCR; oleic acid; linoleic acid; water regimen; olive oil

INTRODUCTION

Virgin olive oil is a natural fruit juice with exceptional organoleptic and nutritional properties. These properties are determined by the metabolites initially present in the olive fruit and by the transformations of some of them occurring during the fruit processing to obtain the oil. Among those metabolites, fatty acids and tocopherols are involved in nutritional and technological properties of the virgin olive oil, such as its oxidative stability, and do not suffer any relevant transformation during processing (*1*). Linoleic and linolenic acids are the substrates of the lipoxygenase pathway, which is active during the milling and malaxation steps, generating the volatile compounds responsible for the aroma (*2*). Phenolic compounds, which come directly from the olive fruit or after their transformation, are also important for virgin olive oil quality, not only from a nutritional point of view but also in terms of technological and organoleptic properties, such as its oxidative stability and bitterness, respectively (*3*). Finally, pigments such as carotenoids and chlorophylls, which usually suffer modifications during the process to obtain the oil, are responsible for nutritional and organoleptic properties, such as the oils' color (*4*).

Despite the enzyme activities involved in the biosynthesis and further transformation of these metabolites seem to determine the final quality of virgin olive oil, biochemical studies are scarce due to the low enzyme activity levels detected in crude extracts obtained from olive fruit tissues. In addition, the presence of isoenzymes with different subcellular localization complicates the determination of the contribution and physiological role for each (*5*). For this reason, we have initiated a program using a molecular approach to identify genes codifying those enzymes and to analyze the evolution of their expression levels during the development and ripening of the olive fruit, looking for correlations with the corresponding metabolites. This information will help to determine the optimal moment for harvesting each cultivar. Furthermore, the identification of genes responsible for virgin olive oil quality will allow the development of molecular markers for marker-assisted selection of new varieties with improved properties in their oils.

Oleic acid is the major fatty acid in the olive oil (55−83%), whereas linoleic acid accounts for 3.5−21% and α-linolenic acid for < 1%. The relative contents of oleic, linoleic, and α-linolenic acids depend mainly on the variety but also on pedoclimatic and culture conditions (*6*), affecting the nutritional properties of the olive oil. In this respect, recent studies indicate that the linoleic acid consumption might be excessive, due to the high proportion of seed oils in the diet (*7*), being associated with a higher risk of hypertension and cardiovascular and carcinogenic diseases (*8*, *9*). Thus, it is currently recommended that the intake of ω-3 fatty acids, such as α-linolenic acid, be increased and that of ω-6 fatty acids, such as linoleic acid, be decreased to improve the equilibrium among eicosanoids derived from both classes of polyunsaturated fatty acids. The oleic, linoleic, and α-linolenic acids ratio has also important consequences in the technological properties

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of the olive oil. In fact, the low percentage of linoleic acid in the Picual variety and, especially, its high content of phenolic compounds, are responsible for the high oxidative stability exhibited by virgin olive oils of this variety, in comparison to those of other cultivars such us Arbequina or Hojiblanca (*10*, *11*). Most of the present study has been done using the cultivars Picual and Arbequina, which are characterized by a distinct linoleic acid content, low (4−5%) and moderate (11−14%), respectively, although some other cultivars were also included for comparison.

In higher plants, fatty acid biosynthesis occurs in the plastid, yielding primarily palmitic and stearic acids by successive additions of two carbon atoms from acetyl-CoA (*12*). Still in the plastid, most of the stearic acid is desaturated by the soluble stearate desaturase to oleic acid, which is the main product of the plastidial fatty acid synthesis. The oleic acid is then incorporated into glycerolipids and can be further desaturated to linoleic acid by two different Δ12-desaturases, which differ in their cellular localization, lipid substrates, and electron donor systems (*13*). The microsomal oleate desaturase (FAD2) is located in the endoplasmic reticulum and uses phospholipids as acyl substrates and NADH, NADH-cytochrome b_5 reductase, and cytochrome b_5 as electron donors. In contrast, the plastidial oleate desaturase (FAD6) is located in the chloroplast and uses primarily glycolipids as acyl carriers and NAD(P)H, ferredoxin-NAD(P) reductase, and ferredoxin as electron donors. In olive, two genes encoding two microsomal oleate desaturases (*OepFAD2-1* and *OepFAD2-2*) have been isolated and characterized (*14*), whereas only one gene corresponding to the plastidial oleate desaturase (*OeFAD6*) has been reported (*15*). The regulation and contribution of both types of oleate desaturases to the final linoleic acid content and distribution in plants is still poorly understood.

In this work, we have investigated the correlation between the expression level of olive microsomal (*FAD2-1* and *FAD2-2*) and plastidial (*FAD6*) oleate desaturase genes and the linoleic acid content in different olive tissues, varieties, and water regimens to determine the specific contribution of each gene and to identify the main olive oleate desaturase gene or genes responsible for the linoleic acid content in the olive fruit. To our knowledge, this is the first time that the expression level of a specific olive gene has been associated with the content of a chemical compound involved in olive oil quality, such as linoleic acid.

MATERIALS AND METHODS

Plant Material. Olive (*Olea europaea* L. cv. Picual and Arbequina) trees were grown in an orchard near Sevilla (Spain), which had been given drop irrigation and fertirrigation from the time of full bloom to fruit ripening (April−December). In the case of no irrigation, the olive trees received only natural rainfall. Young drupes, developing seeds, and mesocarp tissue were harvested at different weeks after flowering (WAF) corresponding to different developmental stages and maturation indices (MI) (*16*) of the olive fruit (12, 16, and 19 WAF, MI 0; 23 WAF, MI 1; 28 WAF, MI 2; 31 WAF, MI 3; 35 WAF, MI 4−5), chilled in liquid nitrogen, and stored at −80 °C. Young leaves were also collected in the same way. Dry weight (DW) was determined by incubating 25 g of olive fruits in the case of mesocarp tissue or 0.5 g of seeds at 110 °C until constant weight.

Fatty Acid Analysis. Fatty acid composition of olive tissues was determined using the one-step method of Garcés and Mancha (*17*). Following the addition of 13.2 mL of methanol/toluene/dimethoxypropane/H_2SO_4 (39:20:5:2, v/v/v/v) and 6.8 mL of heptane to 300 mg of olive tissue, the mixture was incubated for 1 h at 80 °C, forming a single phase. After cooling, the upper phase containing the fatty acid methyl esters was analyzed by gas−liquid chromatography using a HP-5890 (Hewlett-Packard, Palo Alto, CA) fitted with a capillary column (30 m length; 0.25 mm id; 0.20 μm film thickness) of fused silica (Supelco, Bellefonte, PA) and a flame ionization detector. Hydrogen was used as carrier gas with a lineal flux of 1.34 mL min^{-1} and a split ratio of 1:50. The injector and detector temperature was 220 °C, and the oven temperature was 170 °C. Heptadecanoic acid was used as internal standard to calculate the content of linoleic acid in the samples. Results are expressed either in mol percent of the different fatty acids or in milligrams of linoleic acid per gram of DW and are presented as means ± SD of three independent determinations.

Total RNA Extraction and cDNA Synthesis. Total RNA isolation was performed from 1−2 g of frozen olive tissues as described by Hernández et al. (*14*). The quality of RNA was verified by demonstration of intact ribosomal bands following agarose gel electrophoresis in addition to the absorbance ratios ($A_{280/260}$) of 1.8−2.0. Contaminating DNA was removed from RNA samples (10 μg) using the TURBO DNA-free kit (Ambion, USA). First-strand cDNA was synthesized from 5 μg of DNA-free total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with oligo $(dT)_{20}$ primer, following the manufacturer's instructions.

Quantitative Real Time PCR (qRT-PCR). Gene expression analysis was performed by qRT-PCR using an Mx3000P real-time PCR System and Brilliant SYBR Green Q-PCR Master Mix (Stratagene, La Jolla, CA). Primers for gene-specific amplification (**Table 1**) were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to generate a product of 100−200 bp and to have a T_m (melting temperature) of 60 ± 1 °C and a length of 19−23 bp. PCR reactions were carried out in duplicate in 96-well plates. Reaction mix (25 μL per well) contained 1× Brilliant SYBR Green Q-PCR Master Mix, 100 nM forward and reverse primers, and 1 μL of cDNA of the appropriate dilution, which was selected according to the primers amplification efficiency. The thermal cycling conditions consisted of an initial denaturation step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. The specificity of the PCR amplification was monitored by melting curve analysis following the final step of the PCR, from 55 through 95 °C at 0.1 °C s^{-1}. Additionally, PCR products were also checked for purity by agarose gel electrophoresis and by sequencing. PCR efficiencies (E) of all primers were calculated using dilution curves with eight dilution points, two-fold dilution, and the equation $E = [10^{(-1/\text{slope})}] - 1$. The housekeeping olive ubiquitine2 gene (*OeUBQ2*) was used to normalize as endogenous reference. The real-time PCR data were calibrated relative to the corresponding gene expression level in 12 WAF mesocarp tissue from Picual, following the $2^{-\Delta\Delta Ct}$ method for relative quantification (*18*). The data are presented as means ± SD of three reactions performed in different 96-well plates, each having two replicates in each plate.

Table 1. Gene Accession Numbers and Sequences of Primer Pairs Used for Gene Expression Analysis by qRT-PCR in the Present Study

gene	accession no.	sequence	amplicon size (bp)
OepFAD2-1	AY733076	forward: 5′-AGTCACAGACGACACCATTCC-3′ reverse: 5′-CGTTCAAGGCCAAGTACAGAG-3′	175
OepFAD2-2	AY733077	forward: 5′-CTTGTGGGCTTTACCGTCTC-3′ reverse: 5′-AGGGAGGGATGTGTATGCTG-3′	130
OepFAD6	AY733075	forward: 5′-TCACATTTCATCCCGAATACC-3′ reverse: 5′-CTTCATCAATCGCCAGTTCC-3′	112
OeUBQ2	AF429430	forward: 5′-AATGAAGTCTGTCTCTCCTTTGG-3′ reverse: 5′-AAGGGAAATCCCATCAACG-3′	132

RESULTS

To investigate the contribution of the three olive oleate desaturase genes (*FAD2-1*, *FAD2-2*, *FAD6*) to the linoleic acid content in different olive tissues, we have used leaves, where the biosynthetic machinery is directed toward the synthesis of membrane lipids, largely represented by galactolipids; developing seeds, with a high rate of accumulation of storage lipids; mesocarp tissue, which possesses both a high accumulation of triacylglycerols, which are the major components of the olive oil, and active chloroplasts, where the lipid biosynthesis of the thylakoid membranes takes place; and finally young drupes, which can be considered a tissue with intermediate characteristics between leaves and mesocarp. Particularly, we have studied the fatty acid composition and the relative expression levels of the *FAD2* and *FAD6* genes in the previously mentioned tissues from Picual and Arbequina varieties, looking for correlations between the linoleic acid content and the transcript level of the olive oleate desaturase genes. Unfortunately, attempts to assay the enzyme activities have been unsuccessful, possibly due to the occurrence of low levels of specific activity in the olive fruits compared to either oil seeds in the case of FAD2 (*19*) or to spinach leaves for FAD6 (*20*).

After lignification of the endocarp in the olive fruit (10−12 WAF), proper and differentiated seed and mesocarp tissues appear, and TAG biosynthesis and accumulation take place in both tissues, with no oil deposition detected during the first 10 WAF in the young drupe (*21*). As shown in **Figure 1A**, the young drupe exhibits a fatty acid composition with oleic, linoleic, and α-linolenic acid showing percentages around 20% in both varieties. The major fatty acid in mesocarp tissue and developing seeds was oleic acid, although with a higher percentage of linoleic acid in the seed, in either Picual or Arbequina varieties. On the contrary, leaves showed a moderate content of oleic and linoleic acid and a high percentage of α-linolenic acid in both varieties. When the expression levels of the oleate desaturase genes were analyzed in the above-mentioned tissues from Picual and Arbequina varieties (**Figure 1B**), it was observed that the *FAD2-1* gene was highly expressed in young drupes, developing seeds, and leaves, whereas its expression level was very low in mesocarp tissue. In both varieties, the *FAD2-2* gene showed higher expression levels in developing seeds and leaves than in young drupes and mesocarp. Finally, the *FAD6* gene exhibited low expression levels in young drupes, mesocarp tissue, and developing seeds from both cultivars, whereas in leaves they were slightly higher.

To identify the olive oleate desaturase gene or genes responsible for the linoleic acid content in the olive fruit, we have measured their expression levels and the linoleic acid content in the mesocarp tissue and developing seeds from Picual and Arbequina varieties during olive fruit development and ripening. The content and evolution of linoleic acid in the mesocarp of both cultivars were very different (**Figure 2A**). The Arbequina mesocarp showed always a higher linoleic acid content than that of Picual. In addition, the increase observed during development occurred at different moments, because in Arbequina it started immediately after the lignification of the stone, maintaining high levels during ripening, whereas in Picual the increase of linoleic acid occurred gradually, although slightly higher during olive fruit ripening. Regarding expression levels in the mesocarp tissue (**Figure 2B**), in the case of the *FAD2-1* gene it was decreased during fruit development in both varieties, being undetectable during ripening. The *FAD2-2* gene of the Picual variety showed an increase in its expression level that coincides with the beginning of ripening. However, in the Arbequina variety, the transcript level of the *FAD2-2* gene was always higher than in Picual. Furthermore, this level was high immediately after lignification

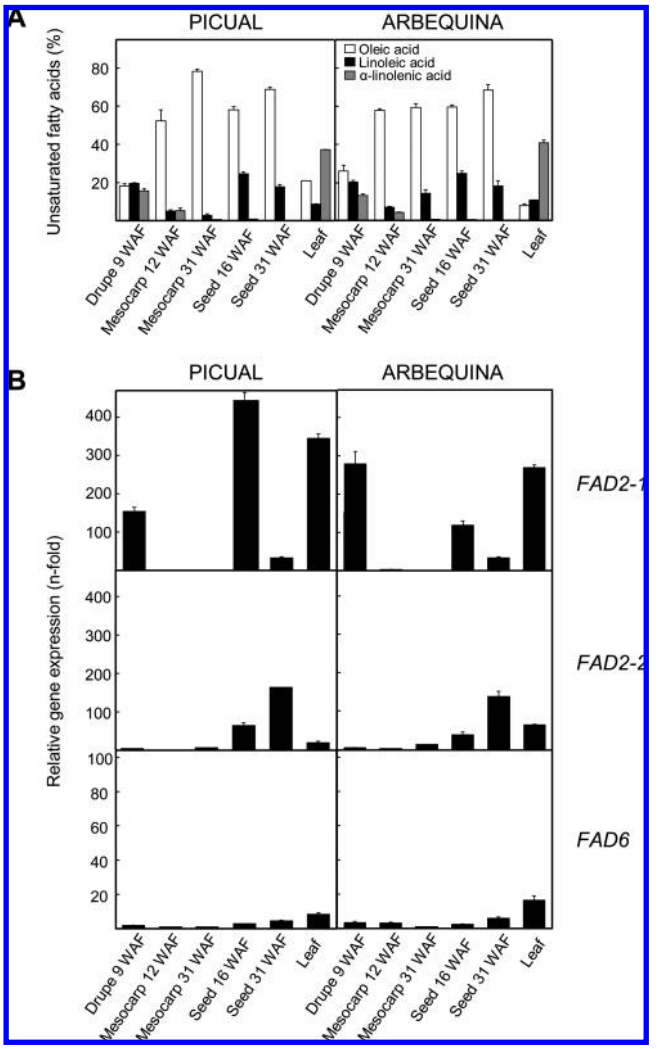

Figure 1. Unsaturated fatty acid composition (**A**) and relative expression levels of olive *FAD2-1*, *FAD2-2*, and *FAD6* genes (**B**) in different tissues of Picual and Arbequina varieties. Fatty acid composition and relative expression levels were determined in the indicated tissues as described under Materials and Methods.

and was increased at the onset of ripening. With respect to *FAD6*, low expression levels were detected, showing a small decrease during the development of the Arbequina variety.

A similar study was conducted in developing seeds. The linoleic acid content showed a fast and important increase in both varieties at the beginning of fruit development, immediately after lignification of the stone (**Figure 3A**). Then, it decreased very slightly, being a little higher in Picual than in Arbequina cultivar at 19 WAF. The expression analysis of the olive oleate desaturase genes (**Figure 3B**) revealed that the *FAD2-1* gene was highly expressed in developing seeds of 16−19 WAF of the Picual variety and, then, the expression level decreased rapidly to remain at constant low level during fruit ripening. A similar evolution for this gene was detected in seeds of the Arbequina cultivar, although its expression level was always lower than those of the Picual variety. The *FAD2-2* gene increased its expression during seed development in both varieties. With regard to *FAD6*, it exhibited always low and constant level in seeds, in either Picual or Arbequina cultivars.

The present study was extended to other varieties of economic importance (Picudo, Hojiblanca, and Manzanilla) using mesocarp tissue from those cultivars corresponding to three different

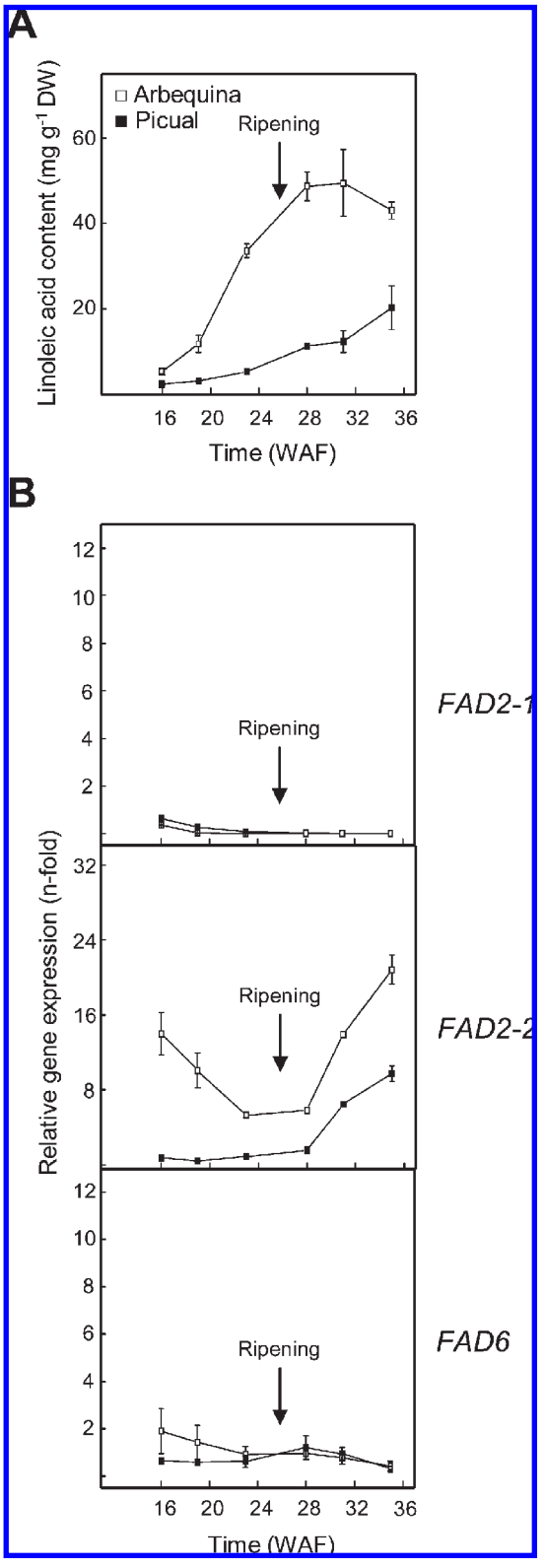

Figure 2. Evolution during olive fruit development and ripening of the linoleic acid content (**A**) and relative expression levels of olive *FAD2-1*, *FAD2-2*, and *FAD6* genes (**B**) in the mesocarp tissue of Picual and Arbequina varieties. The beginning of fruit ripening, corresponding to the appearance of pink-purple color, is marked by an arrow. Fatty acid composition and relative expression levels were determined in the indicated tissues as described under Materials and Methods.

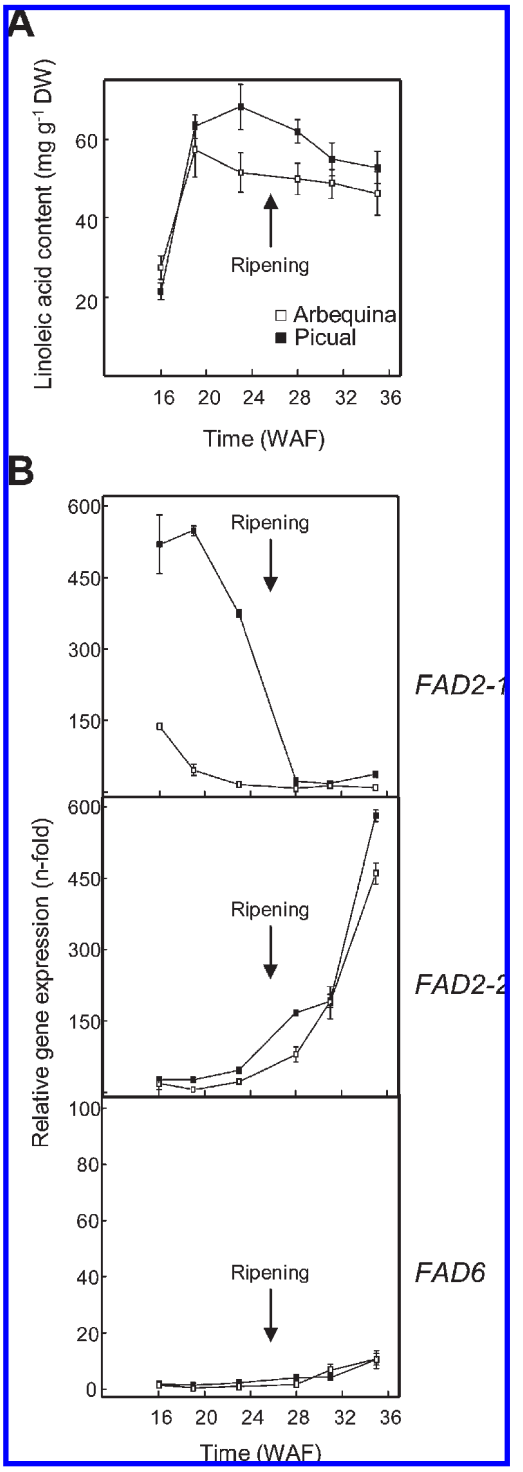

Figure 3. Evolution during olive fruit development and ripening of the linoleic acid content (**A**) and relative expression levels of olive *FAD2-1*, *FAD2-2*, and *FAD6* genes (**B**) in seeds of Picual and Arbequina varieties. The beginning of fruit ripening, corresponding to the appearance of pink-purple color, is marked by an arrow. Fatty acid composition and relative expression levels were determined in the indicated tissues as described under Materials and Methods.

stages of fruit ripening. The percentage of linoleic acid in the mesocarp of the cultivars Hojiblanca and Manzanilla during the ripening period remained practically constant, with values of approximately 5% (**Figure 4A**). On the contrary, in the Picual, Arbequina, and Picudo varieties, the percentage of linoleic acid increased during the ripening process. The *FAD2-1* and *FAD6* gene expression levels in the five varieties were very low during mesocarp ripening, showing no significant changes (**Figure 4B**).

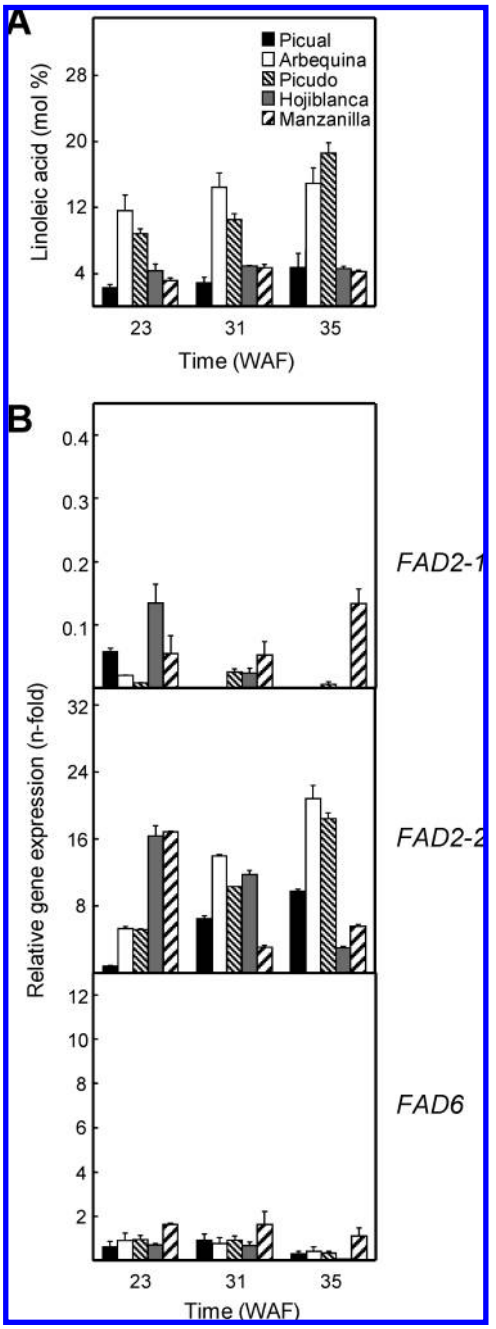

Figure 4. Linoleic acid percentage (**A**) and relative expression levels of olive *FAD2-1*, *FAD2-2*, and *FAD6* genes (**B**) in the mesocarp tissue from different olive fruit varieties at three ripening stages. Fatty acid composition and relative expression levels were determined in the indicated tissues as described under Materials and Methods.

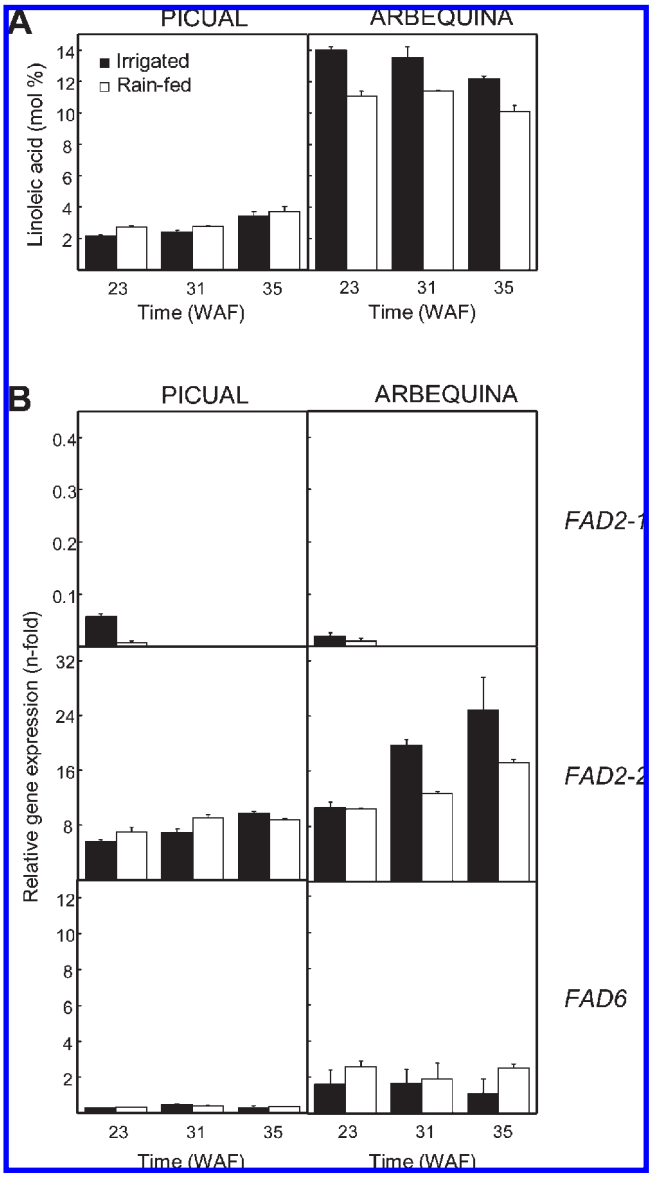

Figure 5. Effect of water regimen on the linoleic acid percentage (**A**) and relative expression levels of olive *FAD2-1*, *FAD2-2*, and *FAD6* genes (**B**) in the mesocarp tissue of Picual and Arbequina varieties. Fatty acid composition and relative expression levels were determined in the indicated tissues as described under Materials and Methods.

In contrast, the *FAD2-2* gene expression level showed an important increase during ripening in the Picual, Arbequina, and Picudo varieties, whereas a decrease in its expression level was detected in the cultivars Hojiblanca and Manzanilla.

To analyze the effect of the water regimen on the linoleic acid percentage and the olive oleate desaturase gene expression, the fatty acid composition and the expression level of the *FAD2-1*, *FAD2-2*, and *FAD6* genes were determined in mesocarp tissue from olive fruits of the Picual and Arbequina varieties cultivated with irrigation or natural rainfall (**Figure 5**). In the mesocarp of the cultivar Picual, no significant differences were detected due to the different water regimens in either linoleic acid percentage or gene expression levels. However, in the Arbequina variety, the percentage of linoleic acid and the *FAD2-2* gene expression level were higher in the mesocarp tissue of olive fruits harvested from olive trees cultivated with irrigation, whereas the transcript levels of *FAD2-1* and *FAD6* genes showed similar low levels in both watering conditions.

DISCUSSION

The physiological role of the three olive oleate desaturase genes has been studied by comparing their expression levels with the linoleic acid content in different olive tissues characterized by a highly active lipid biosynthesis, such as young drupes, mesocarp tissue, developing seeds, and leaves (**Figure 1**). The olive *FAD2-1*, *FAD2-2*, and *FAD6* genes were expressed in all studied tissues, although at different expression levels, pointing out their spatial regulation. In agreement with previous results (*14*), the expression pattern of the two *FAD2* genes found in this oil fruit, where none of them is specific or majorly expressed in developing seeds,

differs from that reported in oil seeds, with one *FAD2* gene showing predominant expression in seeds and the other, low expression levels in all plant tissues (*22*, *23*). On the other hand, the high α-linolenic acid content observed in young drupes and leaves is characteristic of photosynthetic tissues, where the plastidial lipids show high percentages of this fatty acid. Interestingly, the high expression level detected for the *FAD2-1* gene in these two tissues indicates that not only *FAD6*, as proposed in other plants (*24*), but also *FAD2-1* could be involved in the synthesis of the linoleic acid present in the plastidial lipids, where is further desaturated to α-linolenic acid. The linoleic acid synthesized in the endoplasmic reticulum by the FAD2-1 could enter the chloroplast and be incorporated into plastidial lipids, as has been demonstrated in olive callus cultures (*25*).

Fatty acid analysis, together with the determination of the olive oleate desaturase gene expression levels during fruit development and ripening, allows us to propose which of them could be responsible for the linoleic acid content in the mesocarp and seed of olive fruit and, therefore, in the virgin olive oil. Unlike *FAD2-1* and *FAD6* genes, which are characterized by a decrease in their expression levels in the mesocarp tissue during the development and ripening of the olive fruit, the expression pattern of the *FAD2-2* gene correlates adequately with the evolution of the linoleic acid in this tissue, in both the Picual and Arbequina varieties (**Figure 2**). Furthermore, the fact that the expression levels of this gene were always higher in the Arbequina than in the Picual variety could explain the lower linoleic acid content observed in the latter. In sunflower seeds it has been reported that very low internal oxygen concentration reversibly inactivates FAD2 activity (*26*). However, it is not likely that this mechanism regulates FAD2-2 in olive mesocarp, because a very low oxygen level or a sharply decreasing gradient is not expected in this tissue due to the presence of active chloroplasts and the lack of a diffusional barrier such as the seed coat. It is also interesting to mention that the beginning of the increase of the stearoyl-ACP desaturase gene expression level in the mesocarp tissue (*27*) occurs at early stages of development (19 WAF), and before that of the *FAD2-2*, explaining the fact that the mesocarp has high oleate content before it begins the ripening process, hence yielding olive oils richer in oleic acid when the olive fruit is harvested before maturation starts and, on the contrary, with enhanced linoleic acid content when it is extracted from late-ripening fruits (*11*).

With regard to the developing seeds (**Figure 3**), the increase in linoleic acid observed at the beginning of seed development in both varieties correlates well with the higher expression level detected for the *FAD2-1* gene. On the other hand, the increase in the *FAD2-2* gene expression level observed in the seed during fruit ripening could explain the maintenance of high linoleic acid levels in this tissue. These data suggest that both *FAD2-1* and *FAD2-2* genes contribute to the linoleic acid content in the seed, in such a way that the first one could be responsible for the high percentage of this fatty acid at the beginning of fruit development, whereas the second is involved in maintaining the high linoleic acid content during the final period of seed development when the *FAD2-1* gene expression decreases sharply. Unlike mesocarp tissue, oxygen availability could regulate FAD2 activity in olive seed, as reported in sunflower seeds (*26*).

All of these data on the evolution of the olive oleate desaturase gene expression levels during the development and ripening of the olive fruit reveal their temporal regulation, in either mesocarp tissue or developing seed, and suggest distinct physiological roles for each. The *FAD2-1* gene seems to be involved in the biosynthesis of the linoleic acid that is later incorporated both into storage lipids of young seeds and into plastidial lipids of young drupes and leaves, whereas the *FAD2-2* gene could be possibly responsible for the linoleic acid content of storage lipids in the mature seed and the mesocarp tissue and the *FAD6* gene could be implicated in the oleic acid desaturation in the plastidial lipids of the leaves. Furthermore, because the fatty acid composition of the mesocarp, with minor contribution of the seed, determines the fatty acid composition of the oil, the *FAD2-2* gene seems to be mainly responsible for the linoleic acid content of virgin olive oil.

To confirm this hypothesis, the percentage of linoleic acid and the expression level of the olive oleate desaturase genes were determined in the mesocarp tissue of three more varieties (Picudo, Hojiblanca, and Manzanilla), at three different stages covering the harvest period of the olive fruit for olive oil production (**Figure 4**). The expression level of the *FAD2-2* gene decreased during the ripening process in the Hojiblanca and Manzanilla varieties, which correlates with the unchanged linoleic acid content observed in these cultivars. On the contrary, in the Picual, Arbequina, and Picudo varieties, in which an increase in the linoleic acid percentage was detected during ripening, the corresponding increase in the *FAD2-2* gene expression level was also observed. No significant differences were detected in the expression level of the other two oleate desaturase genes. All of these data confirm that the *FAD2-2* gene is mainly responsible for the linoleic acid content in the mesocarp of olive fruit and, thus, in the virgin olive oil. They also show that during the development and ripening of the olive fruit, both the evolution of the linoleic acid content as described in other cultivars (*6*, *11*, *28*) and the oleate desaturase gene expression levels are dependent on the variety.

Several studies indicate that different water regimens could affect the composition and quality of virgin olive oil, as has been reported for the volatile (*29*, *30*), phenolic (*29*−*32*), and pigment composition (*28*). For this reason, the effect of two different water regimens (natural rainfall and additional irrigation) on the expression levels of the olive oleate desaturase genes and the linoleic acid content has also been studied. No significant changes were detected in the linoleic acid percentage in the mesocarp tissue of the Picual variety, in agreement with that described for the cultivars Carolea (*33*), Koroneiki (*34*), and Kalamata (*35*). In contrast, in olive fruits of the cultivar Arbequina harvested from olive trees cultivated with irrigation, a slight increase in the percentage of linoleic acid was observed (**Figure 5A**). This result is in agreement with previous results for the same variety (*36*) or in the cultivars Cornicabra (*37*) and Chetoui (*28*), although in Arbequina it has also been shown that different irrigation treatments did not affect significantly the fatty acid composition (*31*, *38*). Interestingly, an opposite behavior has been reported for the cultivar Leccino (*30*). With regard to the expression levels of the oleate desaturase genes, they were similar in both water regimens for the Picual variety (**Figure 5B**), as was observed for the linoleic acid percentage. On the contrary, a higher expression level was detected for the *FAD2-2* gene in the Arbequina variety cultivated with irrigation, which correlates well with the observed higher linoleic acid content, indicating that in this cultivar irrigation produces an increase in the *FAD2-2* transcript level and, thus, in the linoleic acid content. These results point out that the water regimen affects the fatty acid composition and gene expression levels depending on the variety and confirm again that the *FAD2-2* gene is mainly responsible for the linoleic acid content in the mesocarp of olive fruit.

Although all of this study has been focused on the regulation of the olive oleate desaturase genes at transcriptional level, we cannot discount the existence of translational or post-translational regulatory mechanisms, as reported for the soybean seed-specific oleate desaturase isoform (FAD2-1), wherein the

serine-185 residue is phosphorylated (*39*). This serine residue is located within a motif that could serve as a potential target for phosphorylation by calcium-dependent kinases (*40*). This motif is conserved in the deduced amino acid sequence of numerous plant FAD2, including the olive FAD2-1, but it is not present in the FAD2-2 protein sequence.

Finally, the identification of *FAD2-2* as the gene mainly responsible for the linoleic acid content in the olive mesocarp, and therefore in the virgin olive oil, will allow the development of molecular markers to be used in the marker-assisted selection of new olive varieties with improved linoleic acid content in their oils. Furthermore, knowledge of the factors involved in the regulation of its biosynthesis will help to establish optimum conditions for olive tree cultivation and olive fruit harvesting.

ABBREVIATIONS USED

DW, dry weight; FAD2, microsomal oleate desturase; FAD6, plastidial oleate desaturase; MI, maturation index; WAF, weeks after flowering.

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LITERATURE CITED

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