Title: Specialized Functions of Olive *FAD2* Gene Family Members Related to Fruit Development and the Abiotic Stress Response

Running head: Specialized roles of *FAD2* genes in olive fruit

Corresponding author: M. L. Hernández. Departamento de Bioquímica Vegetal y Biología Molecular, Universidad de Sevilla. Avda. Reina Mercedes s/n. 41012, Sevilla.
Tel: +34-95-5557076;
Email: mhjimenez@us.es

Subject areas: (3) Regulation of gene expression; (4) proteins, enzymes and metabolism.

Number of figures: 7

Number of tables: 2

Supplemental information: 4 figures and 5 tables

Specialized Functions of Olive *FAD2* Gene Family Members Related to Fruit Development and the Abiotic Stress Response

Running head:

Specialized roles of *FAD2* genes in olive fruit

M. Luisa Hernández*†, M. Dolores Sicardo, Patricia M. Arjona, José M. Martínez-Rivas

mhjimenez@us.es, lola.sicardo@ig.csic.es, patricia.arjona.prieto@gmail.com, mrivas@cica.es

Department of Biochemistry and Molecular Biology of Plant Products, Instituto de la Grasa (IG-CSIC), Campus Universidad Pablo de Olavide, Building 46, Ctra. Utrera Km.1, 41013 Sevilla, Spain.

*To whom correspondence should be addressed: M. L. Hernández. Departamento de Bioquímica Vegetal y Biología Molecular, Universidad de Sevilla. Avda. Reina Mercedes s/n. 41012, Sevilla.
Tel: +34-95-5557076;
Email: mhjimenez@us.es

† Present address: Departamento de Bioquímica Vegetal y Biología Molecular, Universidad de Sevilla. Avda. Reina Mercedes s/n. 41012, Sevilla.

The nucleotide sequences reported in this paper has been submitted to GenBank/EMBL/DDBJ database with accessions numbers MN103339 (OepFAD2-3), MN103340 (OepFAD2-4) y MN103341 (OepFAD2-5). Accessions numbers of OepFAD2-1 and OepFAD2-2 are AY733076 and AY733077, respectively.

Abstract

Three different cDNA sequences, designated *OepFAD2-3*, *OepFAD2-4* and *OepFAD2-5*, encoding three microsomal oleate desaturases (FAD2) have been isolated from olive (*Olea europaea* cv. Picual). Sequence analysis and functional expression in yeast of the corresponding cDNAs confirm that they encode microsomal oleate desaturases. Gene expression and lipid analysis indicate that these three genes are not involved in the linoleic acid present in seed lipids, while *OeFAD2-5,* together with *OeFAD2-2,* contribute mostly to the linoleic acid present in the mesocarp and, therefore, in the olive oil. Our results have also shown that olive *FAD2-3*, *FAD2-4* and *FAD2-5* genes expression is not only spatially and temporally regulated in olive fruit, but also is cultivar-dependent, as well as regulated by water regime, temperature, light, and wounding. All these data suggest specialized physiological roles for the olive *FAD2* gene family members with respect to both aspects of the biosynthesis of the linoleic acid, either present in storage lipids, that constitute the olive oil, or being part of membrane lipids, which are involved in the response to abiotic stresses, and highlight the differences on *FAD2* genes regulation between oilseeds and oil fruits.

Key words: abiotic stresses, FAD2, gene expression, linoleic acid, *Olea europaea*, olive.

Introduction

Polyunsaturated fatty acids (PUFA), mainly linoleic and linolenic acids, exhibit key functions in plant metabolism as reserve metabolites mostly in the form of triacylglycerols (TAG) and as structural components of membrane lipids (Ohlrogge and Browse, 1995). In addition, the synthesis of polyunsaturated fatty acids is also crucial for signal transduction pathways (Kachroo and Kachroo, 2009), including the jasmonate pathway which is involved in plant defence and male fertility (Wasternack and Feussner, 2018).

Fatty acid biosynthesis in higher plants begins in the plastid, mainly leading to the synthesis of palmitoyl-acyl carrier protein (ACP) and stearoyl-ACP by successive additions of two carbon atoms from acetyl-CoA (Harwood, 2005). Still in the plastid, these fatty acids can be desaturated by the soluble stearoyl-ACP desaturase (SAD) to produce palmitoleoyl-ACP and oleoyl-ACP, the latter being the main product of the plastidial fatty acid biosynthesis. These acyl-ACPs can either be utilized within the plastid for lipid assembly and further desaturation, or cleaved by specific thioesterases to free fatty acids, activated to acyl-CoAs, and exported to the cytosol. In this way, they are available in the endoplasmic reticulum (ER) for incorporation into glycerolipids to allow lipid formation via the Kennedy pathway, where diacylglycerol acyltransferase (DGAT) is the enzyme that catalyse the final acylation of diacylglycerol (DAG) to give TAG (Weselake et al., 2005). In addition, two enzyme activities catalysing acyl-CoA-independent synthesis of TAG have also been described: phospholipid:diacylglycerol acyltransferase (PDAT; Dahlqvist et al., 2000) and DAG:DAG transacylase (Mancha and Stymne, 1997). Furthermore, oleic acid present in plastidial or microsomal lipids can be further desaturated to linoleic and linolenic acids by the activity of membrane-bound fatty acid desaturases (FAD). These enzymes differ in their cellular localizations, lipid substrates, and electron donor systems (Shanklin and Cahoon, 1998). The microsomal Δ12 and Δ15 desaturases (FAD2 and FAD3, respectively) are located in the endoplasmic reticulum and use phospholipids as acyl substrates, as well as NADH, NADH-cytochrome b5 reductase, and cytochrome b5 as electron donors. On the other hand, the plastidial Δ12 and Δ15 desaturases (FAD6 and FAD7/8, respectively) are located in the chloroplast and use primarily glycolipids as acyl carriers, as well as NAD(P)H, ferredoxin-NAD(P) reductase, and ferredoxin as electron donors.

Because the FAD2 enzyme is responsible for the desaturation of oleic to linoleic acid which will be accumulated in storage lipids (TAG) and, therefore, controls the relative proportions of both fatty acids in edible oils, the *FAD2* gene has been extensively studied in oilseed crops. Since the cloning of the first plant *FAD2* gene in Arabidopsis (Okuley et al., 1994), it has been isolated and characterized from many oilseeds such as soybean (Heppard et al., 1996), sunflower (Martínez-Rivas et al., 2001), and safflower (Cao et al., 2013). With the exception of Arabidopsis where a single *FAD2* gene is present, FAD2 is encoded by gene families in most other plant species, with each member exhibiting distinct spatial and temporal expression patterns in different tissues (Dar et al., 2017). Many efforts have been made in oilseeds in order to obtain high oleic lines by reducing FAD2 expression and/or activity, since plant oils with a higher oleic/linoleic ratio exhibit better nutritional properties (Vos et al., 2003), and enhanced oxidative stability (Márquez-Ruiz et al., 1999). However, information about the regulation of oleic/linoleic ratio in oil fruits is still scarce.

Temperature is the main environmental stress that affects the oleic/linoleic ratio of oilseed storage lipids. Specifically, high temperatures decrease the linoleic acid content of oilseed oils (Canvin et al., 1965).

Different mechanisms have been proposed to explain how temperature regulates FAD2 in oilseeds, including transcriptional regulation in soybean (Byfield and Upchurch, 2007), and its effect on FAD2 activity levels in sunflower due to the low thermal stability of the enzyme (Sánchez-García et al., 2004) and through the effect of temperature on oxygen availability (Rolletschek et al., 2007).

In green tissues such as leaves or green cotyledons, given that the FAD2 enzyme is the key step in the synthesis of PUFA, its role regulating the unsaturated fatty acid composition of membrane lipids in response to different stresses has also been studied. *FAD2* gene expression is induced by different stimuli such as low temperature or darkness in cotton (Kargiotidou et al., 2008), drought or high salt in lima bean (Zhang et al., 2011), wounding in purslane (Teixeira et al., 2009) and pathogen attack in parsley (Kirsch et al., 1997).

Olive fruit represents an interesting system to investigate FAD2 regulation, since it contains two oil accumulating tissues: the mesocarp, with a major contribution to the final composition of the olive oil, and the seed, which is enclosed by a woody endocarp. Moreover, the olive mesocarp can be considered an alternative model to oilseeds to study fatty acid and lipid metabolism because it possesses the remarkable characteristic of having high TAG content together with photosynthetically active chloroplasts that contain membranes which are highly unsaturated and are capable of fixing atmospheric CO_2 (Sánchez, 1994).

Olive oil is one of the world's major edible oils and is highly enriched in oleic acid (55-83%), while linoleic acid account for 3.5-21% and linolenic acid for less than 1%. As previously mentioned for oilseed oils, the relative proportions of these unsaturated fatty acids have a significant effect on olive oil quality. Elevated linoleic acid content has important consequences in the nutritional characteristics of the olive oil (Cunnane, 2003), and negatively affects the technological properties, such as the oxidative stability (Aparicio et al., 1999). In fact, the search for new olive cultivars with high oleic acid content as a quality trait is considered a priority in olive breeding programs. Recently, the first Quantitative Trait Loci (QTLs) controlling fatty acid composition have been reported in olive, including the co-localization of QTLs for oleic and linoleic acids in the same linkage group (Hernández et al., 2017). On the other hand, it is well known that the linoleic acid content depends mainly on the variety. Picual and Arbequina, the two cultivars chosen in the present study, are characterised by a distinct linoleic acid percentage, low (4-5%) and moderately high (11-14%), respectively. Environmental and culture conditions also affect the linoleic acid content of the olive oil (Beltrán et al., 2004).

In olive, two genes encoding microsomal oleate desaturases (*OeFAD2-1* and *OeFAD2-2*) have been reported by Hernández et al. (2005), whereas only one plastidial gene (*OeFAD6*) has been described so far (Banilas et al. 2005, Hernández et al. 2011). Hernández et al. (2009) suggested that *OeFAD2-2* is the main gene that determines the linoleic acid content in the olive mesocarp and, therefore, in the virgin olive oil. In addition, the transcriptional regulation of *OeFAD2-1* and *OeFAD2-2* genes in olive mesocarp in response to different environmental stresses and culture water conditions has been described (Hernández et al., 2009; 2011; 2018; Matteuci et al., 2011; Moretti et al., 2019).

In the present work, we have isolated and characterised three novel members of the olive *FAD2* gene family, and its functional identity have been confirmed by heterologous expression in *S. cerevisiae* cells.

Transcriptional and lipid analysis in olive fruit from two different olive cultivars was performed in order to investigate the physiological role of each gene, their specific contribution to the linoleic acid content in the olive oil, and also to determine how different environmental factors regulate linoleic acid biosynthesis in olive mesocarp.

Results and Discussion

cDNA isolation and sequence analysis of three novel olive microsomal oleate desaturase genes

Three contigs were selected from the olive EST database (Muñoz-Mérida et al., 2013), which exhibit a high degree of similarity to the olive *FAD2-1* and *FAD2-2* genes previously isolated and characterized in our group (Hernández et al., 2005; 2009). Based on these contigs sequences, pairs of specific primers were designed and used for PCR amplification, together with an aliquot of an olive fruit (13 weeks after flowering, –WAF-) cDNA library, cv. Picual. We obtained three full length cDNA clones that were designated as *OepFAD2-3*, *OepFAD2-4* and *OepFAD2-5*, with sizes of 1425, 1398 and 1252 bp, respectively. They contained ORFs encoding predicted proteins of 381, 380 and 383 amino acid residues, which correspond to calculated molecular masses of 44.2, 43.7 and 44.1 kDa, respectively, and p*I* values of 8.4 for OepFAD2-3 and OepFAD2-4, and 8.9 for OepFAD2-5. Alignment of the olive FAD2 deduced amino acid sequences (Fig. S2) showed that OepFAD2-3 displayed 92% identity to the previously described OepFAD2-1, while OepFAD2-4 and OepFAD2-5 exhibited 79% and 82% identity to OepFAD2-2, respectively. Among them, OepFAD2-3 shared 73% and 74% identity with OepFAD2-4 and OepFAD2-5, respectively, while these last two shared 88% identity. Among the conserved amino acids in the five microsomal oleate desaturase sequences were eight histidines that have been shown essential for desaturase activity (Shanklin et al., 1994). These invariant residues are arranged in three histidine boxes (HXXXH, HXXHH, and HXXHH) with conserved spaces between them (Fig. S2), which are characteristic of all membrane-bound desaturases. These histidine boxes are thought to comprise the catalytic centre of the enzyme, since they form ligands to a diiron-oxygen cluster in the catalytic site (Shanklin and Cahoon, 1998). On the other hand, similar to the previously reported OepFAD2-1 and OepFAD2-2 proteins, the novel olive FAD2 sequences presented an aromatic amino acid enriched signal at the C-terminus (Fig. S2), which have reported to be both necessary and sufficient for maintaining localization of the enzymes in the ER (McCartney *et al*., 2004).

The hydropathy plots of the novel olive FAD2 amino acid sequences were generated by the method of Kyte and Doolittle (1982). Four different hydrophobic regions were found in OepFAD2-3 sequence (Fig. S2), similarly to previously reported for OepFAD2-1 and OepFAD2-2 (Hernández et al., 2005). However, OepFAD2-4 and OepFAD2-5 hydropathy plots showed five hydrophobic regions, due to that the first one detected in OepFAD2-1, OepFAD2-2 and OepFAD2-3 was divided into two hydrophobic domains by a hydrophilic region of ten residues (amino acids 71-80; Fig. S2). Although the most common observation for plants FAD2 enzymes has been four hydrophobic regions, five potential hydrophobic domains were also detected as in the case of BjFAD2 (Suresha et al., 2012). However, in all cases, the flanking hydrophobic domains are long enough to span the membrane twice, and the other two are too short, thus

may be single-pass monolayer segments, which fits with the predicted model for desaturase integral membrane proteins (Shanklin et al., 1994). According to this topological model, the three conserved histidine boxes were located in hydrophilic regions exposed to the cytoplasmic side (Los and Murata, 1998).

A phylogenetic tree based on deduced amino acid sequences of all known and characterised plant microsomal oleate desaturases (Table S2) was constructed to elucidate the phylogenetic relationship of olive microsomal oleate desaturase genes (Fig. 1). Interestingly, OepFAD2-3 was positioned close to OepFAD2-1, in the group of FAD2 enzymes specifically or highly expressed in developing seeds. On the other hand, OepFAD2-4 and OepFAD2-5 were positioned in a branch together, close to OepFAD2-2, with the FAD2 enzymes that have a constitutive expression. Therefore, the "seed-type" FAD2 enzymes had formed a distinct clade from the "housekeeping-type", indicating that they have evolved independently after separation by duplication from those *FAD2* genes that show a constitutive expression (Martínez-Rivas et al., 2001).

Finally, in order to investigate the genomic structure of the *FAD2* genes, we compared our five cDNA clones nucleotide sequences with the oleaster genome data assembly deposited in GenBank (Unver et al., 2017). The corresponding five genomic sequences of *FAD2* homologs in the wild olive tree (*Olea europaea* cv. sylvestris) were found and located in different chromosomes (Fig. 2). The fact that we have isolated the five olive *FAD2* clones from cDNA synthesized from mRNA confirms that the five olive *FAD2* genes are transcribed. Interestingly, no additional *FAD2* genes were identified in the oleaster genome. As reported before for many plant *FAD2* genes, we found the single large intron in the 5'-untranslated region (Fig. 2), (Dar et al., 2017). However, the most striking observation was the absence of the 5'-UTR intron in the *OeFAD2-4* sequence, at least in the 225 bp 5'-UTR cDNA sequence cloned. Cao et al. (2013) isolated eleven *FAD2* clones from safflower, and the intron was not amplified from three of them (*CtFAD2-6*, *-8* and *-9*). They suggested that the 5'-UTR in which the introns reside was incomplete in their clones. The relative positions of the 5'-UTR introns varied from 4 bp upstream from the ATG translation initiation codon, reported in Arabidopsis *FAD2* gene (Okuley et al., 1994) to 38 bp in the case of *CtFAD2-10* (Cao et al., 2013). In olive, the 5'-UTR introns were located at -30 and -31 bp in the case of *OepFAD2-1* and *OpeFAD2-3*, respectively, and -14 bp in the case of *OepFAD2-2* and *OepFAD2-5*. Therefore, it seems improbable that the intron in *OepFAD2-4* gene was present upstream the 225 bp 5'-UTR sequence cloned.

The occurrence of introns within the 5'-UTR of plant *FAD2* genes appears to have been evolutionary conserved. Interestingly, 5'-UTR intron-mediated enhancement of gene expression has been observed for many plant genes, including Arabidopsis and sesame *FAD2* genes (Kim et al., 2006). Furthermore, in the same study it has been demonstrated that the sesame *FAD2* intron exhibits promoter-like activity. In agreement with these results, the 5'-UTR intron of *Brassica napus FAD2* gene has also been demonstrated to have promoter activity and be involved in the enhancement of gene expression (Xiao et al. 2014). The variations in the relative positions and the substantial differences in the sizes of *FAD2* genes 5'-UTR introns, and the absence of this intron in the case of *OeFAD2-4* gene, are distinguishing structural differences among olive *FAD2* olive genes (Fig. 2), which could be important in differential expression of the genes, as suggested for other plant *FAD2* gene families like in safflower (Cao et al., 2013).

Functional expression of novel olive microsomal oleate desaturase genes in S. cerevisiae

Yeast has been proven as a suitable heterologous expression system for studying the functionality of ER desaturases. In this work, we have functionally overexpressed the novel olive *FAD2* genes in *S. cerevisiae* cells under the galactose-inducible yeast promoter of the pYES2 vector, to confirm that they encode for active microsomal oleate desaturases. The fatty acid analysis of the pYES2-OepFAD2-3, pYES2-OepFAD2-4 and pYES2-OepFAD2-5 transformed yeast cells revealed the presence of linoleic acid, which was not present in the control cells transformed with the empty vector (Table 1). The percentage of linoleic acid obtained with the inducible pYES2 vector at standard yeast growth temperature (30ºC) was considerably higher in the case of OepFAD2-3 (10.74%) and OepFAD2-5 (11.5%), compared to OepFAD2-4 (1.09%) (Table 1). These results indicate that the olive *FAD2* genes are functional, and they code for microsomal oleate desaturase isoforms capable of desaturating endogenous oleate to linoleate.

As has been reported before for many plant FAD2 expressed in *S. cerevisiae* (Covello and Reed, 1996; Kirsch et al., 1997; Martínez-Rivas et al., 2001) the transformant yeast cells were able to desaturate palmitoleic acid to produce palmitolinoleic acid, suggesting that the FAD2 enzyme expressed in yeast is able to recognize both oleic and palmitoleic acid as substrates. Interestingly, although yeast cells contain approximately equal quantities of both monounsaturated fatty acids, linoleic acid is always the major product, indicating that oleic acid is the preferred substrate of FAD2. Palmitolinoleic acid does not normally accumulate in abundance in plant tissues, although its appearance in olive mesocarp in response to wounding has been reported (Hernández et al., 2011). The production of this dienoic fatty acid by plant FAD2 enzymes should not be just as side effect of the synthesis of linoleic acid, considering that Cao et al. (2013) described the first FAD2 enzyme that specifically produced palmitolinoleic acid. However, it remains to be revealed the physiological role of this fatty acid in plants.

When yeast cells transformed with the three novel olive *FAD2* genes were grown at 15 ºC, the percentage of dienoic acids increased considerably in comparison to the standard grown temperature (30 ºC) (Table 1). This effect of temperature on plant FAD2 enzymes overexpressed in *S. cerevisiae* cells was reported for the first time in the case of the Arabidopsis FAD2 (Covello and Reed, 1996), and it has been attributed to changes in the desaturase activity due to the thermal stability of the enzyme, as reported for the FAD2 isoforms from sunflower (Sánchez-García et al., 2004). Interestingly, OepFAD2-4 showed similar dienoic acids levels to OepFAD2-3 and OepFAD2-5 when yeast cells were grown at 15 ºC, while these levels were much lower at 30 ºC, indicating a lower thermal stability for OepFAD2-4 isoform in comparison to the others.

Tissue specificity and developmental expression of olive microsomal oleate desaturase genes in relation to the linoleic acid content

In order to elucidate the different physiological roles of the olive *FAD2* genes, we have determined their expression levels by qRT-PCR using the $2^{-\Delta Ct}$ method, to make comparisons between the gene expression levels across developmental stages, cultivars and genes, and in relation to the linoleic acid content. With this purpose, we have used olive tissues from Picual and Arbequina cultivars, characterised by a highly

active lipid biosynthesis. In particular, we have studied young drupes before the lignification of the stone, when the mesocarp and seed tissues have not been differentiated yet; developing seeds, with a high rate of accumulation of storage lipids; mesocarp tissue, which possesses both, active chloroplasts and a high accumulation of TAG; and finally young leaves, where the biosynthetic machinery is directed towards the synthesis of membrane lipids.

As shown in Fig. 3, *OeFAD2-3* presented a similar expression pattern to *OeFAD2-1*, showing the highest transcript levels in young drupes and green seeds, although *OeFAD2-3* expression levels were considerably lower than those of *OeFAD2-1*. Regarding *OeFAD2-2*, this gene showed the highest expression levels observed in matured seed, although also showed moderate expression levels in the mesocarp at 31 WAF. On the contrary, *OeFAD2-4* transcripts were mainly detected in young drupes, being almost undetectable in the rest of the tissues studied. In the same way, *OeFAD2-5* gene expression were very low in all tissues studied, apart from mesocarp tissue, where the highest levels were observed at matured stage (31 WAF). These results clearly indicate that olive *FAD2* genes are differentially expressed in all tissues studied, pointing out a complex spatial regulation.

In young drupes, *OeFAD2-4* and mainly *OeFAD2-1* could be responsible for the linoleic acid present in this tissue (Hernández et al., 2009), while *OeFAD2-1* and *OeFAD2-2* seems to be involved in the synthesis of the linoleic acid in leaves (Hernández et al., 2009). In the case of the mesocarp, *OepFAD2-5* transcripts were majorly detected, suggesting its primary role in controlling the linoleic acid levels in this tissue.

Interestingly, unlike it has been reported for many oilseeds such as soybean (Heppard et al., 1996), sunflower (Martínez-Rivas et al., 2001), or safflower (Cao et al., 2013), where one or more seed-specific genes are responsible for the linoleic acid present in storage lipids, none of the olive *FAD2* genes were specifically or majorly expressed in developing seeds. Otherwise, those mainly expressed in seeds (*OeFAD2-1*, *OeFAD2-2* and, to a lesser extent *OeFAD2-3*) are also expressed in other olive tissues

Subsequently, we have analysed in more detail the expression levels of olive *FAD2* genes in mesocarp and seed tissues from Picual and Arbequina cultivars during olive fruit developing and ripening, to identify which of them are responsible for the linoleic acid content of these two oil accumulating tissues in olive fruit (Fig. 4).

With respect to developing seeds (Fig. 4A), the transcript levels of *OeFAD2-3*, *OeFAD2-4* and *OeFAD2-5* genes were very low, compared to *OeFAD2-1* and *OeFAD2-2*. As reported previously (Hernández et al., 2009), the high expression level detected for the *OeFAD2-1* gene at the beginning of seed development correlated well with the observed increase in linoleic acid, and the increase in the *OeFAD2-2* gene expression level detected in the seed during fruit ripening could explain the maintenance of high linoleic acid levels in this tissue. In addition, these expression levels were higher in Picual than in Arbequina seed, which correlated well with the slightly higher levels of linoleic acid reported in Picual compared to Arbequina (Hernández et al., 2009). Therefore, these data indicate that the contribution of the three novel *FAD2* genes to the linoleic acid present in the seed is minimal, and confirm *OeFAD2-1* and *OeFAD2-2* as the main genes responsible for the biosynthesis of this fatty acid in the seed, as it has been previously suggested (Hernández et al., 2005; 2009).

Regarding olive mesocarp, the highest expression was observed for the *OeFAD2-5* gene, particularly in Arbequina cultivar, where their transcripts levels showed a peak at 23 WAF, reaching the highest expression levels detected for any of the *FAD2* genes (Fig. 4B). These results suggest that, the gene responsible for the linoleic acid synthesis in olive mesocarp may be, not only *OeFAD2-2* as it has been previously proposed (Hernández et al., 2009), but also *OeFAD2-5*. In fact, while in Picual mesocarp *OeFAD2-2* and *OeFAD2-5* expression levels increased at the onset of the maturation period, in Arbequina mesocarp *OeFAD2-2* transcript levels were higher at the beginning and the end of fruit development. In addition, *OeFAD2-5* transcripts increased in the middle of the developmental process (Fig. 4B), compensating the decrease in *OeFAD2-2* expression levels. Taken as a whole, both expression patterns correlate well with the reported gradual increase of linoleic acid in the mesocarp tissue during olive fruit developing and ripening, and also explain the significant increase in linoleic acid detected before ripening in Arbequina cultivar (Hernández et al., 2009). In addition, the higher transcript levels detected for olive *FAD2-2* and *FAD2-5* genes in Arbequina mesocarp compared with Picual (Fig. 4B), are in agreement with the higher linoleic acid content reported for the mesocarp tissue from Arbequina with respect to that of Picual (Hernández et al., 2009).

All of these data reveal a temporal regulation of olive *FAD2* genes expression. Moreover, both olive mesocarp and seed tissues seems to have two genes that mostly control the PUFA levels, *OeFAD2-2* and *OeFAD2-5*, and *OeFAD2-1* and *OeFAD2-2*, respectively. The fact that both olive tissues that accumulate oil have two genes responsible for the oleic desaturation could be to ensure the linoleic acid synthesis throughout the long development period of the olive fruit, which takes about 35-40 weeks (Sánchez, 1994). Furthermore, because the fatty acid composition of the mesocarp, with minor contribution of the seed, determines the fatty acid composition of the oil, *OeFAD2-2* and *OeFAD2-5* seems to be the genes mainly responsible for the linoleic acid content of virgin olive oil.

To further investigate the metabolic pathways for linoleic acid production and accumulation in olive mesocarp, we determined the linoleic acid content in the different lipid classes from Picual and Arbequina cultivars at different stages of fruit development and ripening. As expected, the main differences between both cultivars were observed in neutral lipids, where the linoleic acid content was considerably higher in Arbequina than in Picual (Table S3). In addition, while in Picual mesocarp the linoleic acid content of DAG and TAG increased gradually during olive fruit development, in Arbequina it showed a maximum at 31 WAF, and then slightly decreased when the olive fruit is completely matured (Table S3). These results correlate well with the *OeFAD2-5* gene expression pattern, further confirming the contribution of this novel *FAD2* gene to the PUFA levels of olive mesocarp oil.

However, the most striking observations were the differences observed between both cultivars when we analysed the percentage of linoleic acid in lipid classes (Fig. 5). Again, we observed that linoleic acid proportion increased slight and gradually in DAG and TAG in Picual mesocarp, whereas the increase in Arbequina was rapid and significant at the onset of ripening (Fig. 5A). Curiously, the phospholipids analysis revealed that linoleic acid percentage increased considerably in all of them in Picual mesocarp during development and ripening, while in Arbequina, linoleic acid decreased in PC, PE and PA, and did not experiment significant changes in PI and PS (Fig. 5B). The similar trend of linoleic acid content in PC and

PA observed for each cultivar, increase in Picual and decrease in Arbequina, suggests that the linoleic acid synthesized in PC is mainly entering into DAG via lisophosphatidylcholine acyltransferase to form linoleoyl-CoA which is incorporated into the Kennedy pathway, and not via CDP choline:DAG cholinephosphotransferase and/or PC:DAG cholinephosphotransferase (Weselake, 2005). In addition, these results suggest that the incorporation of linoleic acid into TAG may occur preferentially via the Kennedy pathway in olive mesocarp, with minor contribution of PDAT activity. The observation that in Picual the linoleic acid percentage increased strongly in phospholipids but slightly in TAG, whereas in Arbequina is highly decreased in phospholipids and strongly increased in TAG, could be explained by a higher preference for linoleic acid of DGAT enzyme in Arbequina compared to Picual. It has been previously reported that plant DGAT displays a broad acyl-CoA preference. In olive mesocarp, once linoleic acid is synthesized in PC in Arbequina, is directed into TAG by the action of the enzymes of the Kennedy pathway. In contrast, in Picual mesocarp a lower preference of DGAT enzyme for linoleoyl-CoA could explain the accumulation of this fatty acid in other lipids, intermediates for TAG biosynthesis. Similarly, it has been reported that the selectivity of cocoa DGAT for stearoyl-CoA (Griffiths and Harwood, 1991) and the strong preference of DGAT from certain rape varieties for erucoyl-CoA (Bernerth and Frentzen, 1990) and DAG species with very long-chain acyl moieties (Taylor et al., 1992), play a decisive role in the specific incorporation of certain fatty acid into TAG molecules.

Transcriptional regulation of olive FAD2-5 gene in response to water stress

The general concept that different irrigation regimes affect olive oil fatty acid composition is supported by several studies (Gómez del Campo and García, 2013; Caruso et al., 2014). However, data related to the effect of water stress on olive fatty acid desaturases at transcriptional levels are scarce. Previous work from our group reported a small decrease of linoleic acid in Arbequina cultivated in rain-fed conditions compared to irrigation, which correlated with the decrease detected for the *OeFAD2-2*, but not *OeFAD2-1* and *OeFAD6*, gene expression levels (Hernández et al., 2009). More recently, we studied the effect of three different irrigation treatments on the Arbequina mesocarp fatty acid composition and fatty acid desaturase genes expression levels. We reported that the 30RDI irrigation treatment, which produced the higher level of water stress, caused a decrease in the linoleic acid content of olive mesocarp during fruit maturation, although the lack of correlation observed with *OeFAD2-1*, *OeFAD2-2* and *OeFAD6* genes expression levels pointed out to an un-identified *OeFAD2* gene that could be repressed by water stress, although the existence of post-transcriptional regulatory mechanism could not be discarded (Hernández et al., 2018). Interestingly, in the present work, when we have extended this last study analysing the expression levels of the three novel *OeFAD2* genes (Fig. 6), we found that the transcript levels of *OeFAD2-5* showed a significant reduction during the olive mesocarp development under water stress conditions (30RDI), which could explain the reported decrease in linoleic acid content previously observed with this treatment (Hernández et al., 2018). Therefore, although *OeFAD2-2* and *OeFAD2-5*, the main genes involved in linoleic acid synthesis in olive mesocarp (Fig. 4B), appears to be transcriptionally down regulated by water stress leading to a reduction in the linoleic acid content in Arbequina mesocarp (Hernández et al. 2009; 2018), *OeFAD2-5* seems to be more sensitive to this stress condition than *OeFAD2-2*.

Unlike the down regulation of *FAD2* genes expression observed in olive mesocarp, an increase in the transcript levels of *FAD2* gene in response to water stress has been reported in mandarin (Gimeno et al., 2009) and purslane (D'Andrea et al., 2015) leaves. These contrasting results could suggest the existence of a tissue-dependent transcriptional mechanism of *FAD2* genes in the water stress response.

Temperature regulation of novel microsomal oleate desaturase genes and linoleic acid content in olive mesocarp

To investigate the effect of temperature on the expression of novel olive *FAD2* genes in olive mesocarp from Picual and Arbequina cultivars, olive branches with 28 WAF olive fruit were incubated at low (15 ºC) and high (35 ºC) temperature with a 12 h light/12 h dark cycle, for 24 h. Although *OeFAD2-3* gene transcripts remained almost undetected during the treatment, *OeFAD2-4* and *OeFAD2-5* genes expression levels experimented an increase at 15 ºC (Fig. 7A), and a decrease at 35 ºC (Fig. 7B). This is the general response to temperature changes for fatty acid desaturases, since it is generally accepted that, among other changes, cold adaptation involves an increase in the unsaturation degree of fatty acids, to influence membrane fluidity (Los and Murata, 1998). In fact, the transcriptional regulation of *FAD2* gene expression by shifting of the temperature has been described for numerous plant species. Particularly, an increase in *FAD2* transcript levels under cold treatment and a reduction under the higher temperature have been reported in soybean and *Brassica juncea* seeds (Byfield and Upchurch, 2007; Suresha et al., 2012) and *Ginkgo biloba* leaves (Wang et al., 2013).

Our results also showed that the increase of *OeFAD2-4* and *OeFAD2-5* genes expression with decreasing temperature is considerably higher in Arbequina than in Picual (Fig. 7A), indicating a cultivar-dependent differential transcriptional response to cold of olive *FAD2* genes. This is in agreement with the results previously reported for *OeFAD2-1* and *OeFAD2-2* genes in olive mesocarp from different cultivars under low temperatures. (Hernández et al., 2011; Matteuci et al., 2011). These authors reported that the increase in *OeFAD2-1* gene transcripts at low temperature was lower in Picual and Moraiolo cultivars than in Arbequina and Canino ones, while the expression levels of *OeFAD2-2* showed a high transient induction with a peak between 3-6 h of incubation at low temperature in Picual and Moraiolo cultivars, but progressively increased in Arbequina and Canino mesocarp. Overall, these data indicate that *OeFAD2-2* seems to be the main microsomal oleate desaturase gene induced in response to cold in Picual mesocarp, while *OeFAD2-1*, *OeFAD2-2*, *OeFAD2-4*, and *OeFAD2-5* are induced in the case of Arbequina.

To better understand the olive mesocarp response to temperature, we analysed the linoleic acid content from different lipid classes in the mesocarp tissue from both cultivars incubated at 15 and 35 ºC, to look for correlations with the mentioned changes in *FAD2* transcript levels. Interestingly, we observed differences between the linoleic acid pattern in microsomal lipids from mesocarp tissue incubated at 15 ºC in both cultivars (Table 2). In Arbequina mesocarp we observed a considerable increase in the linoleic acid content in PC, PA and TAG throughout the incubation at 15 ºC, suggesting that the enhanced expression of *OeFAD2-1*, *OeFAD2-2*, *OeFAD2-4*, *OeFAD2-5* under low temperature observed in this cultivar (Hernández *et al*., 2011; Fig. 7A), led to a higher rate of desaturation of oleic acid in PC, the main substrate

for FAD2 and a major precursor for TAG biosynthesis (Bates *et al*., 2009). In contrast, in Picual mesocarp we detected that the linoleic acid levels decreased in TAG and increased in most of the membrane lipids as PI, PE, PC, PA (Table 2), suggesting that the *OeFAD2-2* up-regulation at low temperature in this cultivar (Hernández *et al*., 2011; Fig. 7A), led to an increase of linoleic acid content in membrane lipids, which it is accompanied by a mobilization of linoleic acid from storage lipids (TAG) to membrane lipids, probably to maintain membranes fluidity and integrity. Taken together these data allow us to propose a cultivar-dependent response to low temperature in olive mesocarp.

In addition, the mobilization of linoleic acid to membrane lipids observed only in Picual mesocarp, might indicate a higher tolerance of this cultivar to low temperature. The most common mechanism in response to low temperature stress described in poikilotherms is an increase in fatty acid desaturation (Guschina and Harwood, 2006). In the case of the Arabidopsis *fad2* mutant, which exhibit a decreased polyunsaturated fatty acid content in membrane lipids, long-term cold application results in its death, indicating that high levels of linoleic acid in membrane lipids are essential for plants resistance to cold (Miquel et al., 1993). Particularly in olive leaf, Gulen et al. (2009) concluded that polar lipids composition is clearly important in the ability of olive cultivars to stand against cold stress. In addition, the hypothesis of the higher cold-tolerance of Picual mesocarp than Arbequina one, is further supported by previous observations reported by Hernández et al. (2019). These authors demonstrated that low temperature transcriptionally regulates *SAD* genes in Picual mesocarp, leading to a modification of the unsaturated fatty acids content in Picual microsomal membrane lipids, while those changes were not observed in the case of Arbequina. The role of *FAD2* genes in plant cold tolerance has been demonstrated in hybrid poplar plants expressing a poplar *FAD2* gene, which exhibited enhanced freezing tolerance (Zhou et al., 2010). In the same way, a rice *FAD2* gene increased tolerance to low temperature when overexpressed in rice (Shi et al., 2012). In addition, in olive, a role for *FAD2* genes in the cold response of the seed coat and embryo has been proposed (D'Angeli et al., 2013).

With respect to high temperature, we could not find any correlation between the down-regulation of *FAD2* genes expression levels (Hernández et al., 2011; Fig. 7B) and the linoleic acid content in the different lipids classes (Table S4). These results could suggest a post-transcriptional regulation of *FAD2* genes. In soybean seeds, Heppard et al. (1996) observed an increase in oleic acid content with increasing temperature but found no corresponding decrease in *FAD2* transcripts accumulation, suggesting a post-transcriptional regulation of microsomal oleate desaturase enzyme activity. In sunflower seeds, it has been described that changes in temperature bring about shifts in the very low endogenous oxygen concentration, which affect FAD2 activity reversibly, without having an effect on gene transcription (Rolletschek et al., 2007). However, in olive mesocarp where a diffusion barrier such as the seed coat is absent and with the presence of active chloroplasts, a low oxygen level is not expected, preventing the occurrence of such a mechanism regulating FAD2. Interestingly, in olive callus cultures incubated with [1-^{14}C]oleate at high temperatures, a reduction in the proportion of [1-^{14}C]linoleate was found in the phospholipids but not in galactolipids, which indicates that high temperature negatively affect FAD2 (Hernández et al., 2008). Therefore, high temperature regulation in olive might be mediated by transcriptional as well as post-transcriptional mechanisms.

Transcriptional regulation of novel olive microsomal oleate desaturase genes by darkness

To study the effect of darkness on the transcript levels of olive *FAD2-3*, *FAD2-4* and *FAD2-5* genes in Picual and Arbequina mesocarp tissues, olive branches with 28 WAF olive fruits were incubated at 25 ºC in the darkness for 24 h. Expression analysis showed that, while *OeFAD2-3* transcript levels remained almost undetectable during all the darkness treatment, in the case of *OeFAD2-4* and *OeFAD2-5* they were reduced in both cultivars, although *OeFAD2-5* expression levels recovered to initial values after 24 h (Fig. 7C). In the same way, we reported a down-regulation of olive *FAD2-1* and *FAD2-2* genes under the same darkness conditions (Hernández et al., 2011). Similarly, Kargiotidou *et al*. (2008) observed that *FAD2* mRNA levels were reduced in cotton cotyledons after 6 h of incubation in dark conditions. In agreement with these results, Arabidopsis *FAD2* promoter-GUS activity of Arabidopsis seedlings grown in darkness conditions for 24 h has been reported to be decreased in cotyledons, but not in roots (Yuan et al., 2012).

However, the light-dependent transcriptional regulation of *FAD2* genes seems not to be well correlated with the linoleic acid content. Although it is generally accepted that light induces desaturation and return to dark causes a decline in desaturation activity (Kislyuk et al., 2013), it seems that this increase occurs mainly in the linolenic acid of green tissues, presumably due to the transcriptional regulation of linoleate desaturases (Collados et al., 2006), while linoleic acid remained practically unchanged (Hernández et al., 2011; Kislyuk et al., 2013). For that reason, we decided to look for correlations between the observed down-regulation of olive *FAD2* genes and the linoleic acid content in the different lipid classes in Picual and Arbequina mesocarp incubated under darkness conditions for 24 h. No relevant modifications in the amount of linoleic acid in the different lipids were observed throughout the darkness incubation, apart from a decrease in galactolipids detected in Picual mesocarp (Table S5). In addition, we previously observed in olive mesocarp incubated under darkness conditions that *SAD* genes down-regulation was the responsible for the reduction in the unsaturated fatty acid content, mainly oleic acid, in the chloroplast lipids MGDG and DGDG, being these effect restored in Arbequina mesocarp after 24 h of incubation, but not in Picual (Hernández et al., 2019). Therefore, the decreased in linoleic acid detected in galactolipids in Picual mesocarp (Table S5) might be just a consequence of the oleic acid reduction observed in these lipids (Hernández et al., 2019) and not a direct effect of *FAD2* genes down-regulation. The fact that the changes in linoleic acid were observed only in galactolipids confirmed that the effect of light/darkness is associated with changes in thylakoid formation, inducing changes in the accumulation and degree of unsaturation of chloroplast-localized lipids (Kislyuk et al., 2013).

Cultivar-dependent regulation of novel olive FAD2 genes expression levels and microsomal lipids dienoic acids content in response to wounding

The effect of wounding on the *OeFAD2-3*, *OeFAD2-4* and *OeFAD2-5* genes transcript levels in olive mesocarp was studied by incubating olive branches at standard conditions, except that the olive fruits (28 WAF) were subjected to mechanical damage. We verified that the wounding treatment of the mesocarp tissue was correctly performed since olive 13-lipoxygenase and 13-hydroperoxide lyase genes, which have

shown previously to be wound-inducible in plant tissues (Padilla et al., 2014), were transiently induced (Fig. S3). qRT-PCR analysis revealed an increase in *OeFAD2-3* and *OeFAD2-4* expression levels after wounding (Fig. 7D). In particular, while *OeFAD2-3* gene induction seems to be a long-term effect, *OeFAD2-4* gene transcripts were transiently induced, reaching a maximum at 3 and 6 h in Picual and Arbequina cultivars, respectively, as it has been described for olive *FAD2-1* and *FAD2-2* genes (Hernández et al., 2011). Interestingly, whereas *OeFAD2-1* and *OeFAD2-2* transient induction was higher in Arbequina cultivar, *OeFAD2-4* transcripts reached much higher levels at 6 h after wounding in Picual than in Arbequina mesocarp (Fig. 7D). The increase of plants *FAD2* genes expression in response to wounding has been reported before in cotton and purslane leaves (Kargiotidou et al., 2008; Teixera et al., 2009). In addition, an increase in PUFA after wounding was described in tomato and grapevine leaves (Conconi et al., 1996; Chitarrini et al., 2017). Therefore, the induction of oleate desaturase and fatty acid desaturation has been suggested to be an early component of the complex of responses associated with defence against wounding. PUFA can be transformed to lipid peroxides that could act as antimicrobial compounds (Wang et al., 1998), or they may act as precursor of signal molecules involved in plant defense, as in the case of jasmonates (Wasternack and Feussner, 2018).

In previous work from our group, we observed that the transient induction of *OeFAD2-1* and *OeFAD2-2* genes caused by wounding was accompanied by a slight increase in palmitolinoleic acid in microsomal lipids of both cultivars, whereas linoleic acid was increased only in Arbequina (Hernández et al., 2011). In order to identify possible cultivar-dependent differences in the olive mesocarp wounding response mechanism, we analysed the fatty acid composition of the different lipid classes in Picual mesocarp after wounding, and compared with that of Arbequina. We observed that palmitolinoleic and linoleic acids content increased in microsomal lipids of both cultivars, mainly in phospholipids and DAG. However, while linoleic acid was enhanced mostly in Arbequina lipids, the palmitolinoleic acid content was increased mainly in Picual lipids (Fig. S4). In addition, we recently reported that an olive stearoyl-ACP desaturase gene (*OeSAD3*), which could be responsible for the synthesis of palmitoleic from palmitic acid, was induced after wounding in mesocarp only in Picual cultivar (Hernández et al., 2019). These results together might suggest a cultivar-dependent wounding response in olive mesocarp, involving the synthesis of different sets of signal molecules via lipoxygenase pathway. Although the involvement of C16 PUFA in the oxylipins biosynthesis is less documented compared to its C18 homologs (Wasternack and Feussner, 2018), the enhanced resistance to powdery mildew observed in tomato plants transformed with the yeast Δ9 desaturase gene was apparently associated with the increase in palmitoleic and palmitolinoleic acids (Wang *et al*., 1998). Therefore, further investigations need to be done in order to elucidate the possible role of C16 derived oxylipins in plant defence response.

Conclusions

We have isolated and characterized three new members of the olive *FAD2* gene family. Sequence analysis of these genes (*OepFAD2-3*, *OepFAD2-4* and *OepFAD2-5*) indicates that they code for microsomal oleate desaturase enzymes. Their identity was confirmed by functional expression in yeast, with the three isoforms showing a temperature-dependent conversion of oleic and palmitoleic acids to

linoleic and palmitolinoleic acids, respectively. Gene expression profiling and lipid analysis during olive fruit development and ripening confirm *OeFAD2-1* and *OeFAD2-2* as the main genes responsible for the biosynthesis of linoleic acid in TAG of the seed, whereas *OeFAD2-2* and *OeFAD2-5* contributes mainly to the linoleic acid present in TAG of the mesocarp and, therefore, in the olive oil. Our results have also shown that olive *FAD2* genes expression is not only spatially and temporally regulated in olive fruit, but also is regulated by water regime, temperature, light, and wounding in a cultivar-dependent manner. All these data indicate specialized physiological roles for *FAD2* gene family members in olive and point out to the genes mainly responsible for linoleic acid content, either present in storage lipids, that constitute the olive oil, or being part of membrane lipids, which are involved in the response to abiotic stresses. Moreover, our results highlight the existing differences on *FAD2* genes regulation between oilseeds and oil fruits, especially in the case of olive fruit, which possesses both characteristics, reserve oil accumulation together with an active membrane lipids biosynthesis for the photosynthetic machinery. The identification and functional characterization of the complete olive *FAD2* gene family provides a significant insight into the principal determinants of synthesis of linoleic acid in olive fruit. In the future, RNAi and phenotype of transgenic olive plants will help to clarify the roles of specific FAD2 isoforms in olive mesocarp. In addition, this information will allow the development of molecular markers for the high oleic character to be used in the marker-assisted selection of new olive cultivars.

Materials and methods

Plant material and stress treatments

For tissues and developmental studies, olive (*Olea europaea* L. cv. Picual and Arbequina) trees were grown in the experimental orchard of Instituto de la Grasa, Seville (Spain), with drip irrigation and fertirrigation (irrigation with suitable fertilizers in the solution) from the time of full bloom to fruit maturation. Young drupes, developing seeds, and mesocarp tissue were harvested from at least three different olive trees at different weeks after flowering (WAF) corresponding to different developmental stages of the olive fruit: green (9, 12, 16, and 19 WAF); yellowish (23 WAF); turning or veraison (28 and 31 WAF); and mature or fully ripe (35 WAF). Immediately after harvesting, olive tissues were frozen in liquid nitrogen, and stored at -80 ºC. Young leaves were collected similarly.

The study of water deficit was conducted at the Sanabria orchard, a commercial super high density olive (cv. Arbequina) orchard near Seville (Spain). The full irrigation (FI) and two regulated deficit irrigation (RDI) treatments (60RDI and 30RDI) were applied as defined by Fernández *et al*. (2013). Olive mesocarp tissue was sampled at different WAF as described by Hernández *et al*. (2018).

Stress treatments were carried out according to Hernández et al. (2019). Olive branches from Picual and Arbequina cultivars with about 100 olive fruit at turning stage (28 WAF) were collected from different olive trees and incubated in a growth chamber at 25 ºC with a 12 h light/12 h dark cycle, and a light intensity of 300 µmol m^{-2} s^{-1}. These incubation parameters attempted to mimic physiological conditions of the tree, and were considered the standard conditions. No significant alterations in the fatty acid composition or

FAD2 genes expression levels were detected in the mesocarp tissue when olive fruits were incubated under the above mentioned standard conditions (Fig. S1). For stress treatments, standard conditions were modified depending on the effect studied. For low and high temperature experiments, the branches containing the olive fruit were incubated at 15 or 35 ºC, respectively, at the standard light intensity. To assess the effect of the darkness, light was turned off and the standard temperature was maintained. To study the effect of wounding, the whole surface of the olive fruit was mechanically damaged affecting mesocarp tissue, with pressure at zero time using forceps with serrated tips. The zero time of each experiment was selected 2 h after the beginning of the light period to maintain the natural photoperiod day/night of the olive fruit. When indicated, olive mesocarp tissues were sampled, frozen in liquid nitrogen, and stored at -80 ºC.

Isolation of novel microsomal oleate desaturase full-length cDNA clones

Candidate novel olive *FAD2* sequences were found in the olive EST database (Muñoz-Mérida et al., 2013) using the tblastn algorithm together with the amino acid sequences of olive FAD2-1 and FAD2-2, previously reported by our group (Hernández et al., 2005). Based on these new sequences, specific pairs of primers for each gene were designed and utilized for PCR amplification with ACCUZYME™ DNA polymerase (Bioline, Spain), which has proofreading activity. An aliquot of an olive Uni-ZAP XR cDNA library constructed with mRNA isolated from 13 WAF olive fruit of cultivar Picual (Haralampidis et al., 1998), was used as DNA template. One fragment with the expected size was generated in each reaction, subcloned into the vector pSpark® I (Canvax, Spain) and sequenced in both directions.

DNA sequence was performed by GATC (Biotech, Germany). The DNA sequence data were compiled and analysed with the LASERGENE software package (DNAStar, Madison, WI). Hydropathy plots were generated by the method of Kyte and Doolittle (1982). The multiple sequence alignments of olive FAD2 amino acid sequences were calculated using the ClustalX program and displayed with GeneDoc. Phylogenetic tree analysis was performed using the neighbor-joining method implemented in the Phylip package using Kimura's correction for multiple substitutions and a 1000 bootstrap data set. TreeView was used to display the tree.

Total RNA isolation and cDNA synthesis

Total RNA isolation was performed as described by Hernández et al. (2005) using 1.5 g of frozen olive tissue. RNA quality verification, removal of contaminating DNA and cDNA synthesis were carried out according to Hernández et al. (2009).

Expression analysis of microsomal oleate desaturase genes

The expression levels of the olive *FAD2* genes were determined by quantitative real-time PCR (qRT-PCR) using a CFX Connect real-time PCR System and iTaq Universal SYBR Green Supermix (BioRad,

California, USA) as previously described (Hernández et al., 2019). Primers for gene-specific amplification for *OeFAD2-1*, *OeFAD2-2* were previously designed by Hernández et al. (2009). For *OeFAD2-3*, *OeFAD2-4* and *OeFAD2-5* genes expression analysis, specific primers were designed using the Primer3 program (http://bioinfo.ut.ee/primer3/) and the Gene Runner program (Supplementary Table S1). The housekeeping olive ubiquitin2 gene (*OeUBQ2*, AF429430) was used as an endogenous reference to normalize (Hernández et al., 2009). For tissues and developmental studies, the relative expression level of each gene was calculated using the equation $2^{-\Delta Ct}$ where $\Delta Ct = (Ct_{GOI} - Ct_{UBQ2})$ (Livak and Schmittgen, 2001; Pfaffl, 2004). This method gave us an advantage to make comparisons in the level of gene expression across developmental stages, cultivars and genes. Regarding irrigation studies and stress treatments, the qRT-PCR data were calibrated relative to the corresponding gene expression level at 13 WAF from full irrigation treatment and zero time for each stress treatment and cultivar, respectively, as calibrator. In these cases, the $2^{-\Delta\Delta Ct}$ method for relative quantification was followed (Livak and Schmittgen, 2001). The data are presented as means ± SD of three biological replicates, each having two technical replicates per 96 well plate.

Functional expression of novel microsomal oleate desaturase genes in Saccharomyces cerevisiae

The corresponding ORF of the olive *FAD2* genes were amplified by PCR using ACCUZYME™ DNA polymerase (Bioline, Spain) and the following pairs of primers: LH165 (5'-ATGAATTCACACAATGGGAGCAGGAGGCCGT-3') and LH166 (5'-ATCGCGGCCGCTCAAAACTTGTTTTTATACCAGA-3') for *OeFAD2-3*; LH167 (5'-ATGAATTCACACAATGGGTGCTGGAGGCCGA-3') and LH168 (5'-ATCGCGGCCGCCTAAAGCTTGTTTTTGTACCAG-3') for *OeFAD2-4*; and LH167 and LH169 (5'-ATCGCGGCCGCTTAAAGCTTGTTACGATACCAG-3') for *OeFAD2-5*. For directional ligation behind the inducible *GAL1* gene promoter of the yeast expression vector pYES2 (Invitrogen), the primers were extended by a *Eco*RI (in the forward primer) and a *Not*I (in the reverse primer) restriction site (underlined). The resulting PCR product for each specific olive *FAD2* gene was double digested with *Eco*RI and *Not*I, and ligated into the digested destination vector. All constructs were checked by sequencing. Transformation of *S. cerevisiae* strain UTL-7A with the corresponding plasmids, yeast cells growth, and induction of gene expression was performed as previously described by Román et al. (2012), omitting the cultures supplementation with linoleic acid.

Lipid analysis

Olive fruit mesocarp tissue was heated at 70 ºC for 30 min with isopropanol to inactivate endogenous lipase activity. Lipids were extracted as described by Hara and Radin (1978) and lipid separation was carried out by TLC (Hernández et al., 2008). Fatty acid methyl esters of the different olive tissues and lipid preparations were produced by acid-catalysed transmethylation (Garcés and Mancha, 1993) and analysed by GC (Román et al., 2015). Heptadecanoic acid was used as internal standard to calculate the lipid and fatty acid content

in the samples. Results are expressed either in mol percent of the different fatty acids or in µg of linoleic acid per mg of FW, and are presented as means ± SD of three biological replicates.

Total lipid content and fatty acid composition of whole yeast cells were determined using the one-step method of Garcés and Mancha (1993). Fatty acid methyl esters were analysed by GC as described above.

Funding

This work was supported by the Spanish Ministry of Science, Innovation and Universities through Research Grants AGL2014-55300-R and AGL2017-87871-R (AEI/FEDER, UE).

Disclosures

Conflict of interest: No conflict of interest declared

Acknowledgments

Thanks to Drs. José Enrique Fernández and Antonio Díaz-Espejo for the availability of the Sanabria olive orchard to harvest olive fruit samples with different irrigation treatments.

Supporting information

Table S1. Gene accession numbers and sequences of primers pairs used for gene expression analysis by qRT-PCR in the present study.
Table S2. Accession number of the different microsomal oleate desaturases included in the phylogenetic tree analysis. xpression analysis by qRT-PCR in the present study.
Table S3. Linoleic acid content of different lipid classes from Picual and Arbequina mesocarp at different stages of fruit development.
Table S4. Linoleic acid content of different lipid classes from mesocarp tissue of Picual and Arbequina cultivars at different times of incubation using standard conditions except that the temperature was 35ºC.
Table S5. Linoleic acid content of different lipid classes from Picual and Arbequina mesocarp at different times of incubation at 25 ºC under darkness conditions.
Fig. S1. Effect of incubation under the standard conditions on the relative expression levels of olive FAD2-3, FAD2-4 and FAD2-5 genes in the mesocarp tissue from Picual and Arbequina cultivars.
Fig. S2. Comparison of the deduced amino acid sequences of olive microsomal oleate desaturase genes.
Fig. S3. Effect of wounding on the relative expression levels of olive *1LOX2* and *HPL* genes in the mesocarp tissue from Picual and Arbequina cultivars.
Fig. S4. Effect of wounding on the palmitolinoleic (A) and linoleic (B) acid percentage in microsomal lipid classes in the mesocarp tissue from Picual and Arbequina cultivars.

References

Aparicio, R., Roda, L., Albi, M.A., Gutiérrez, F. (1999) Effect of various compounds on virgin olive oil stability measured by rancimat. J. Agric. Food Chem. 47: 4150-4155.

Banilas, G., Moressis, A., Nikoloudakes, N., Hatzopoulos, P. (2005) Spatial and temporal expressions of two distinct oleate desaturases from olive (*Olea europaea* L.). Plant Sci. 168: 547-555.

Bates, P.D., Durrett, T.P., Ohlrogge, J.B., Pollard, M. (2009) Analysis of acyl fluxes through multiple pathways of triacylglycerol synthesis in developing soybean embryos. Plant Physiol. 150: 55-72.

Beltrán, G., Del Rio, C., Sánchez, S., Martínez, L. (2004) Influence of harvest date and crop yield on the fatty acid composition of virgin olive oils from cv. Picual. J. Agric. Food Chem. 52: 3434-3440.

Bernerth, R., Frentzen, M. (1990) Utilization of erucoyl-CoA by acyltransferase from developing seeds of *Brassica napus* (L.) involved in triacylglycerol biosynthesis. Plant Sci. 67: 21-28.

Byfield, G.E., Upchurch, R.G. (2007) Effect of temperature on delta-9 stearoyl-ACP and microsomal omega-6 desaturase gene expression and fatty acid content in developing soybean seeds. Crop Sci. 47: 1698-1704.

Cao, S., Zhou, X., Wood, C.C., Green, A.G., Singh, S.P., Liu, L., Liu, Q. (2013) A large and functionally diverse family of *FAD2* genes in safflower (*Carthamus tinctorius* L.) BMC Plant Biol. 13: 5.

Caruso, G., Gucci, R., Urbani, S., Esposto, S., Taticchi, A., Di-Maio, I., et al. (2014) Effect of different irrigation volumes during fruit development on quality of virgin olive oil of cv. Frantoio. Agric. Water Manag. 134: 94-103.

Canvin, D.T. (1965) The effect of temperature on the oil content and fatty acid composition of the oils from several oil seed crops. Canadian J. Bot. 43: 63-69.

Chitarrini, G., Zulini, L., Masuero, D., Vrhovsek, U. (2017) Lipid, phenol and carotenoid changes in 'Bianca' grapevine leaves after mechanical wounding: a case of study. Protoplasma 254: 2095-2106.

Collados, R., Andreu, V., Picorel, R., Alfonso, M. (2006) A light-sensitive mechanism differently regulates transcription and transcript stability of ω3 fatty-acid desaturases (FAD3, FAD7 and FAD8) in soybean photosynthetic cell suspensions. FEBS Lett. 580: 4934-4940.

Conconi, A., Miquel, M., Browse, J.A., Ryan, C.A. (1996) Intracellular levels of free linolenic and linoleic acids increase in tomato leaves in response to wounding. Plant Physiol. 111: 797-803.

Covello, P.S., Reed, D.W. (1996) Functional expression of the extraplastidial Arabidopsis thaliana oleate desaturase gene (FAD2) in Saccharomyces cerevisiae. Plant Physiol. 111: 223-226.

Cunnane, S. (2003) Problems with essential fatty acids: time for a new paradigm? Progr.Lipid Res. 42: 544–568.

D'Andrea, R.M., Triassi, A., Casas, M.I., Andreo, C.S., Lara, M.V. (2015) Identification of genes involved in the drought adaptation and recovery in *Portulaca oleracea* by differential display. Plant Physiol. Biochem. 90: 38-49.

D'Angeli, S., Falasca, G., Matteucci, M., Altamura, M.M. (2013) Cold perception and gene expression differ in *Olea europaea* seed coat and embryo during drupe cold acclimation. New Phytol. 197: 123-138.

Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., et al. (2000) Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc. Natl. Acad. Sci. USA 97: 6487–6492.

Dar, A.A., Choudhury, A.R., Kancharla, P.K., Arumugam, N. (2017). The *FAD2* gene in plants: Occurrence, regulation, and role. Front. Plant Sci. 8: 1789.

Fernández, J.E., Pérez-Martín, A., Torres-Ruiz, J.M., Cuevas, M.V., Rodríguez-Domínguez, C.M., Elsayed-Farag, S., et al. (2013) A regulated deficit irrigation strategy for hedgerow olive orchards with high plant density. Plant and Soil 372: 279-295.

Garcés, R., Mancha, M. (1993) One-step lipid extraction and fatty acid methyl esters preparation from fresh plant tissues. Anal. Biochem. 211: 139-143.

Gimeno, J., Gadea, J., Forment, J., et al. (2009) Shared and novel molecular responses of mandarin to drought. Plant Mol. Biol. 70: 403-420.

Gómez del Campo, M., García, J.M. (2013) Summer deficit-irrigation strategies in a hedgerow olive cv. Arbequina orchard: effect on oil quality. J. Agric. Food Chem. 61: 8899-8905.

Griffiths, G., Harwood, J.L. (1991) The regulation of triacylglycerol biosynthesis in cocoa (*Theobroma cacao* L.). Planta 184: 279-284.

Gulen, H., Cansev, A., Eris, A. (2009) Cold hardiness of olive (*Olea europaea* L.) cultivars in cold-acclimated and non-acclimated stages: seasonal alteration of soluble sugars and phospholipids. J. Agric. Sci. 147: 459-467.

Guschina, I.A., Harwood, J.L. (2006) Mechanisms of temperature adaptation in poikilotherms. FEBS Lett. 580: 5477-5483.

Hara, A., Radin, N.S. (1978) Lipid extraction of tissues with a low-toxicity-solvent. Anal. Biochem. 90: 420-426.

Haralampidis, K., Milioni, D., Sánchez, J., Baltrusch, M., Heinz, E., Hatzopoulos, P. (1998) Temporal and transient expression of stearoyl-ACP carrier protein desaturase gene during olive fruit development. J. Exp. Bot. 49: 1661-1669.

Harwood, J.L. (2005) Fatty acid biosynthesis. In: Plant Lipids. Murphy DJ (ed.), Oxford: Blackwell Publishing: 27-101.

Heppard, E.P., Kinney, A.J., Stecca, K.L., Miao, G.H. (1996) Developmental and growth temperature regulation of two different microsomal ω-6 desaturase genes in soybeans. Plant Physiol. 110: 311-319.

Hernández, M.L., Belaj, A., Sicardo, M.D., León, L., de la Rosa, R., Martín, A., et al. (2017) Mapping quantitative trait loci controlling fatty acid composition in olive. Euphytica 213: 7.

Hernández, M.L., Guschina, I.A., Martínez-Rivas, J.M., Mancha, M., Harwood, J.L. (2008) The utilization and desaturation of oleate and linoleate during glycerolipid biosynthesis in olive (*Olea europaea* L.) callus cultures. J. Exp. Bot. 59: 2425-2435.

Hernández, M.L., Mancha, M., Martínez-Rivas, J.M. (2005) Molecular cloning and characterization of genes encoding two microsomal oleate desaturases (FAD2) from olive. Phytochemistry 66: 1417-1426.

Hernández, M.L., Padilla, M.N., Mancha, M., Martínez-Rivas, J.M. (2009) Expression analysis identifies *FAD2-2* as the olive oleate desaturase gene mainly responsible for the linoleic acid content in virgin olive oil. J. Agric. Food Chem. 57: 6199-6206.

Hernández, M.L., Padilla, M.N., Sicardo, M.D., Mancha, M., Martínez-Rivas, J.M. (2011) Effect of different environmental stresses on the expression of oleate desaturase genes and fatty acid composition in olive fruit. Phytochemistry 72: 178-187.

Hernández, M.L., Sicardo, M.D., Alfonso, M., Martínez-Rivas, M.D. (2019) Transcriptional regulation of stearoyl-acyl carrier protein desaturase genes in response to abiotic stresses leads to changes in the unsaturated fatty acids composition of olive mesocarp. Front. Plant Sci. 10: 251.

Hernández, M.L., Velázquez-Palmero, D., Sicardo, M.D., Fernández, J.E., Diaz-Espejo, A., Martínez-Rivas, J.M. (2018) Effect of a regulated deficit irrigation strategy in a hedgerow 'Arbequina' olive orchard on the mesocarp fatty acid composition and desaturase gene expression with respect to olive oil quality. Agric. Water Manag. 204: 100-106.

Kachroo, A., Kachroo, P. (2009) Fatty Acid–Derived Signals in Plant Defense. Annu. Rev. Phytopathol. 47: 153-179.

Kargiotidou, A., Deli, D., Galanopoulou, D., Tsaftaris, A., Farmaki, T. (2008) Low temperature and light regulate delta 12 fatty acid desaturases (FAD2) at a transcriptional level in cotton (*Gossypium hirsutum*). J. Exp. Bot. 59: 2043-2056.

Kim, M.J., Kim, H., Shin, J.S., Chung, C., Ohlrogge, J.B., Suh, M.C. (2006) Seed-specific expression of sesame microsomal oleic acid desaturase is controlled by combinatorial properties between negative cis-regulatory elements in the SeFAD2 promoter and enhancers in the 5'-UTR intron. Mol. Genet. Genom. 276: 351-368.

Kirsch, C., Hahlbrock, K., Somssich, I.E. (1997) Rapid and transient induction of a parsley microsomal Δ12 fatty acid desaturase mRNA by fungal elicitor. Plant Physiol. 115: 283-289.

Kislyuk, I.M., Bubolo, L.S., Bykov, O.D., Kamentseva, I.E., Kotlova, E.R., Vinogradskaya, M.A. (2013) Strong light elevates thermotolerance of photosynthetic apparatus and the content of membranes and polar lipids in wheat leaves. Russ. J. Plant Physiol. 60: 611-622.

Kyte, J., Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105-132.

Livak, K.J., Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25: 402-408.

Los, D.A., Murata, N. (1998) Structure and expression of fatty acid desaturases. Biochim, Biophys. Acta-Lipids and Lipid Metabolism 1394: 3-15.

Mancha, M., Stymne, S. (1997) Remodelling of triacylglycerols in microsomal preparations from developing castor bean (*Ricinus communis* L.) endosperm. Planta 203: 51-57.

Márquez-Ruiz, G., Polvillo, M.M., Jorge, N., Ruiz-Méndez, M.V., Dobarganes, M.C. (1999) Influence of used frying oil quality and natural tocopherol content on oxidative stability of fried potatoes. J. Amer. Oil Chem. Soc. 76: 421-425.

Martínez-Rivas, J.M., Sperling, P., Lühs, W., Heinz, E. (2001) Spatial and temporal regulation of three different microsomal oleate desaturase genes (*FAD2*) from normal-type and high-oleci varieties of sunflower (*Helianthus annuus* L.). Mol. Breeding 8: 159-168.

Matteucci, M., D'Angeli, S., Errico, S., Lamanna, R., Perrotta, G., Altamura, M. (2011) Cold affects the transcription of fatty acid desaturases and oil quality in the fruit of *Olea europaea* L. genotypes with different cold hardiness. J. Exp. Bot. 62: 3403-3420.

McCartney, A.W., Dyer, A.W., Dhanoa, P.K., Kim, P.K., Andrews, D.W., McNew, J.A., Mullen, R.T. (2004) Membrane-bound fatty acid desaturases are inserted co-translationally into the ER and contain different ER retrieval motifs at their carboxy termini. Plant J. 37: 156-173.

Miquel, M., James, D., Dooner, H., Browse, J. (1993) Arabidopsis requires polyunsaturated lipids for low-temperature survival. Proc. Natl. Acad. Sci. USA 90: 6208-6212.

Moretti, S., Francini, A., Hernández, M.L., Martínez-Rivas, J.M., Sebastiani, L. (2019) Effect of saline irrigation on physiological traits, fatty acid composition and desaturase genes expression in olive fruit mesocarp. Plant Physiol. Biochem. 141: 423-430.

Muñoz-Mérida, A., González-Plaza, J.J., Cañada, A., et al. (2013) De novo assembly and functional annotation of the olive (*Olea europaea*) transcriptome. DNA Res. 20: 93-108.

Ohlrogge, J., Browse, J. (1995) Lipid biosynthesis. Plant Cell 7: 957-970

Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E., Browse, J. (1994) Arabidopsis *FAD2* gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell 6: 147-158.

Padilla, M.N., Hernández, M.L., Sanz, C., Martínez-Rivas, J.M. (2014) Stress-dependent regulation of 13-lypoxygenases and 13-hydroperoxide lyase in olive fruit mesocarp. Phytochemistry 102: 80-88.

Pfaffl, M.W. (2004) Quantification strategies in real-time PCR. In A–Z of Quantitative PCR. Edited by Bustin, S.A., 87–112. International University Line, La Jolla.

Rolletschek, H., Borisjuk, L., Sánchez-García, A., Gotor, C., Romero, L.C., Martínez-Rivas, J.M., Mancha, M. (2007) Temperature-dependent endogenous oxygen concentration regulates microsomal oleate desaturase in developing sunflower seeds. J. Exp. Bot. 58: 3171-3181.

Román, A., Andreu, V., Hernández, M.L., Lagunas, B., Picorel, R., Martínez-Rivas, J.M., Alfonso, M. (2012) Contribution of the different omega-3 fatty acid desaturase genes to the cold response in soybean. J. Exp. Bot. 63: 4973-4982.

Román, A., Hernández, M.L., Soria-García, A., López-Gomollón, S., Lagunas, B., Picorel, R., et al. (2015) Non-redundant contribution of the plastidial FAD8 ω-3 desaturase to glycerolipid unsaturation at different temperatures in Arabidopsis. Mol. Plant 8: 1599-1611.

Sánchez, J. (1994) Lipid photosynthesis in olive fruit. Prog. Lipid Res. 33: 97-104.

Sánchez-García, A., Mancha, M., Heinz, E., Martínez-Rivas, J.M. (2004) Differential temperature regulation of three sunflower microsomal oleate desaturase (FAD2) isoforms overexpressed in *Saccharomyces cerevisiae*. Eur. J. Lipid Sci. Technol. 106: 583-590.

Shanklin, J., Cahoon, E.B. (1998) Desaturation and related modifications of fatty acids. Ann. Rev. Plant Physiol. 49: 611-641.

Shanklin, J., Whittle, E., Fox, B.G. (1994) Eight histidine residues are catalytically essential in a membranse-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. Biochemistry 33: 12787-12794.

Shi, J., Cao, Y., Fan, X., Li, M., Wang, Y., Ming, F. (2012) A rice microsomal delta-12 fatty acid desaturase can enhance resistance to cold stress in yeast and Oryza sativa. Mol. Breeding 29: 743-757.

Suresha, G.S., Rai, R.D., Santha, I.M. (2012) Molecular cloning, expression analysis and growth temperature dependent regulation of a novel oleate desaturase gene (*fad2*) homologue from *Brassica juncea*. Aust. J. Crop Sci. 6: 296-308.

Taylor, D.C., Weber, N., Hogger, L.R., Underhill, E.W., Pomeroy, M.K. (1992) Formation of trierucoylglycerol (trierucin) from 1,2-dierucoylglycerol by a homogenate of microspore-derived embryos of *Brassica napus* L. Plant Physiol. 69: 355-358.

Teixera, M.C., Coelho, N., Olsson, M.E., Brodelius, P.E., Carvalho, I.S., Brodelius, M. (2009) Molecular cloning and expression analysis of three omega-6 desaturase genes from purslane (*Portulaca oleracea* L.). Biotechnol. Lett. 31: 1089-1101.

Unver, T., Wu, Z., Sterck, L., Turktas, M., Lohaus, R., Li, Z., et al. (2017) Genome of wild olive and the evolution of oil biosynthesis. Proc. Natl. Acad. Sci. USA 44: E9413-E9422.

Vos, E. (2003) Linoleic acid, 'vitamin F6'- is the western world getting too much? Probably. Lipids Technol. 15: 81-84.

Wang, H., Cao, F., Zhang, W., Wang, G., Yu, W. (2013) Cloning and expression of stearoyl-ACP desaturase and two oleate desaturases genes from *Ginkgo biloba* L. Plant Mol. Biol. Rep. 31: 633-648.

Wang, C., Chin, C.K., Chen, A.. (1998) Expression of the yeast Δ-9 desaturase gene in tomato enhances its resistance to powdery mildew. Physiol. Mol. Plant Pathol. 52: 371-383.

Wasternack, C., Feussner, I. (2018) The oxylipin pathways: biochemistry and function. Ann. Rev. Plant Biol. 69: 363-386.

Weselake, R.J. (2005) Storage lipids. In: Murphy DJ, ed. Plant lipids: biology, utilisation and manipulation. Oxford: Blackwells: 162-225.

Xiao, G., Zhang, Z.Q., Yin, C.H., Liu, R.Y., Wu, X.M., Tan, T.L., et al. (2014) Characterization of the promoter and 5'-UTR intron of oleic acid desaturase (*FAD2*) gene in *Brassica napus*. Gene 545: 45-55.

Yuan, S., Wu, X., Liu, Z., Luo, H., Huan, R. (2012) Abiotic stresses and phytohormones regulate expression of *FAD2* gene in *Arabidopsis thaliana.* J. Integrative Agric. 11: 62-72.

Zhang, Y.M., Wang, C.C., Hu, H.H., Yang, L. (2011) Cloning and expression of three fatty acid desaturase genes from cold-sensitive lima bean (*Phaseolus lunatus* L.). Biotechnol Lett. 33: 395-401.

Zhou, Z., Wang, M-J., Hu, J-J., Lu, M-Z., Wang, J.H. (2010) Improve freezing tolerance in transgenic poplar by overexpressing a ω-3 fatty acid desaturase gene. Mol. Breeding 25: 571-579.

Tables

Table 1. Fatty acid composition of *S. cerevisiae* cells overexpressing olive *FAD2* genes and grown at two different temperatures.

Plasmid	Temperature (ºC)	Fatty acid composition (mol %)					
		16:0	16:1	16:2	18:0	18:1	18:2
pYES2	30	15.86 ± 1.31	39.58 ± 2.69	ND	9.49 ± 2.99	35.07 ± 1.61	ND
	15	10.93 ± 2.59	20.86 ± 7.06	ND	25.30 ± 8.57	42.92 ± 4.11	ND
pYES2-OepFAD2-3	30	19.35 ± 0.53	37.26 ± 0.84	3.87 ± 0.36	6.84 ± 0.24	21.95 ± 0.61	10.74 ± 0.24
	15	13.87 ± 0.91	22.39 ± 2.12	9.91 ± 1.41	12.31 ± 1.93	20.92 ± 1.89	20.59 ± 0.62
pYES2-OepFAD2-4	30	15.81 ± 0.05	44.10 ± 0.68	0.60 ± 0.05	6.68 ± 0.26	31.71 ± 0.34	1.09 ± 0.05
	15	12.21 ± 1.00	20.00 ± 4.17	8.25 ± 1.18	14.52 ± 3.27	24.82 ± 1.66	20.21 ± 1.23
pYES2-OepFAD2-5	30	18.90 ± 0.29	36.26 ± 0.54	4.01 ± 0.11	7.36 ± 0.43	21.97 ± 0.28	11.50 ± 0.09
	15	11.72 ± 0.81	18.59 ± 1.87	11.04 ± 0.29	12.67 ± 0.58	23.16 ± 1.49	22.83 ± 0.42

Data are mean ± SD from three independent experiments, with duplicate determinations of fatty acid composition. 16:0, palmitic acid; 16:1, palmitoleic acid; 16:2, palmitolinoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid. ND, not detected.

Table 2. Linoleic acid content of different lipid classes from mesocarp tissue of Picual and Arbequina cultivars at different times of incubation using standard conditions except that the temperature was 15 ºC.

Cultivar	Time (h)	Linoleic acid content (µg/mg FW)						
		DAG	TAG	PI	PS	PE	PC	PA
Picual	0	0.0152 ± 0.0027	2.0551 ± 0.0408	0.0006 ± 0.0002	0.0004 ± 0.0001	0.0003 ± 0.0000	0.0022 ± 0.0003	0.0017 ± 0.0001
	1	0.0151 ± 0.0004	1.7218 ± 0.0541*	0.0007 ± 0.0001	0.0004 ± 0.0000	0.0008 ± 0.0000*	0.0016 ± 0.0001	0.0022 ± 0.0001
	6	0.0183 ± 0.0018	1.7220 ± 0.0788*	0.0007 ± 0.0001	0.0005 ± 0.0000	0.0010 ± 0.0000*	0.0023 ± 0.0001	0.0024 ± 0.0000
	24	0.0202 ± 0.0009	1.5073 ± 0.1927*	0.0011 ± 0.0001*	0.0005 ± 0.0001	0.0011 ± 0.0002*	0.0029 ± 0.0000*	0.0031 ± 0.0000*
Arbequina	0	0.1574 ± 0.0108	8.2567 ± 0.2923	0.0007 ± 0.0001	0.0009 ± 0.0004	0.0010 ± 0.0004	0.0032 ± 0.0001	0.0105 ± 0.0014
	1	0.1465 ± 0.0052	8.1823 ± 0.5283*	0.0011 ± 0.0004	0.0007 ± 0.0001	0.0010 ± 0.0001	0.0094 ± 0.0025*	0.0143 ± 0.0019*
	6	0.1511 ± 0.0001	9.8868 ± 0.2173*	0.0008 ± 0.0002	0.0008 ± 0.0000	0.0015 ± 0.0006	0.0093 ± 0.0001*	0.0201 ± 0.0013*
	24	0.1750 ± 0.0114	10.1309 ± 0.5701*	0.0008 ± 0.0003	0.0007 ± 0.0004	0.0006 ± 0.0000	0.0090 ± 0.0001*	0.0164 ± 0.0022*

Data are presented as means ± SD of three biological replicates. DAG, diacylglycerol; TAG, triacylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidate.
* indicates significantly different ($p < 0.05$) to time 0 h by two-way ANOVA with a Bonferroni post-test.

Figure legends

Fig. 1. Phylogenetic tree analysis of plant microsomal oleate desaturases. Alignments were calculated with ClustalX and the analysis was performed using the neighbor-joining method implemented in the Phylip package using Kimura's correction for multiple substitutions, and a 1000 bootstrap dataset. The *Chlamydomonas reinhardtii* FAD2 sequence was defined as outgroup. TreeView was used to display the tree. Positions of the olive FAD2 are in bold and olive *FAD2* genes isolated in this work are also underlined.

Fig. 2. Structure of *OeFAD2* genes in olive genome. Grey boxes indicate genomic locus. White boxes denote 5'- and 3'-UTRs. Black boxes indicate open reading frames. Lines denote introns.

Fig. 3. Relative transcript abundance of olive *FAD2* genes in different tissues of Picual and Arbequina cultivars. The relative transcript abundance was determined by qRT-PCR at the indicated tissues as described under Materials and methods. Data are presented as means ± SD of three biological replicates. *Indicates significantly different ($p < 0.05$) to Picual by two-way ANOVA with a Bonferroni post-test in Arbequina tissues.

Fig. 4. Relative transcript abundance of olive *FAD2* genes in the seed (A) and mesocarp tissue (B) from Picual and Arbequina cultivars. The beginning of fruit ripening corresponding to the appearance of pink-purple color is marked by an arrow. The relative transcript abundance was determined by qRT-PCR at the indicated stages of fruit development as described under Materials and methods. Data are presented as means ± SD of three biological replicates. *Indicates significantly different ($p < 0.05$) to Picual by two-way ANOVA with a Bonferroni post-test in Arbequina tissues.

Fig. 5. Linoleic acid percentage in neutral lipids (A) and phospholipids (B) from Picual and Arbequina mesocarp tissue at different stages of development. The linoleic acid percentage was determined as described under Materials and methods. Data are presented as means ± SD of three biological replicates. DAG, diacylglycerol; TAG, triacylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidate. *Indicates significantly different ($p < 0.05$) to time 16 weeks after flowering (WAF) by two-way ANOVA with a Bonferroni post-test.

Fig. 6. Effect of regulated deficit irrigation treatments on the relative expression levels of olive *FAD2-3*, *FAD2-4* and *FAD2-5* genes in the mesocarp tissue from cultivar Arbequina during olive fruit development and ripening. The relative expression levels were determined by qRT-PCR at the indicated stages of fruit development as described in Materials and methods, using the expression level of the corresponding gene at 13 weeks after flowering (WAF) from FI treatment as calibrator. Data are presented as means ±SD of

three biological replicates. *Indicates that 60 RDI is significantly different ($p < 0.05$) to FI by two-way ANOVA with a Bonferroni post-test. **Indicates that 30 RDI is significantly different ($p < 0.05$) to FI by two-way ANOVA with a Bonferroni post-test.

Fig. 7. Effect of different abiotic stresses on the relative expression levels of olive *FAD2-3*, *FAD2-4* and *FAD2-5* genes in the mesocarp tissue from Picual and Arbequina cultivars. Branches with about 100 olive fruits (28 weeks after flowering, –WAF-) were incubated using standard conditions except that the temperature was 15 ºC (A) or 35 ºC (B), the olive fruits were incubated under darkness (C) or were mechanically damaged (D). At the indicated times, relative expression levels were determined by qRT-PCR as described in Materials and methods, using the expression level of the corresponding gene at zero time as calibrator. Data are presented as means ±SD of three biological replicates. *Indicates significantly different ($p < 0.05$) to time 0 h by two-way ANOVA with a Bonferroni post-test in Picual mesocarp. **Indicates significantly different ($p < 0.05$) to time 0 h by two-way ANOVA with a Bonferroni post-test in Arbequina mesocarp.

Fig. 1

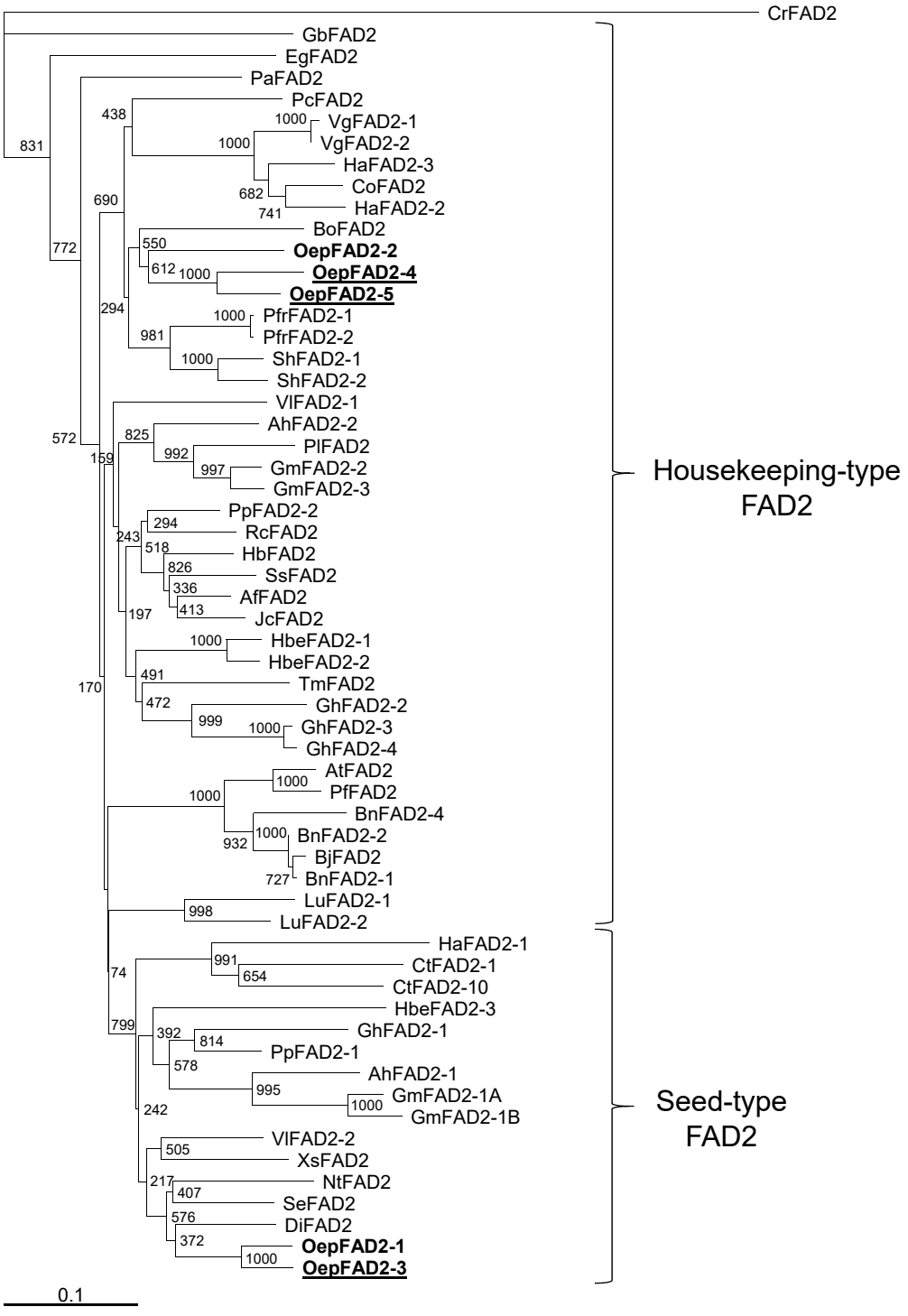

CrFAD2
GbFAD2
EgFAD2
PaFAD2
PcFAD2
VgFAD2-1
VgFAD2-2
HaFAD2-3
CoFAD2
HaFAD2-2
BoFAD2
OepFAD2-2
OepFAD2-4
OepFAD2-5
PfrFAD2-1
PfrFAD2-2
ShFAD2-1
ShFAD2-2
VlFAD2-1
AhFAD2-2
PlFAD2
GmFAD2-2
GmFAD2-3
PpFAD2-2
RcFAD2
HbFAD2
SsFAD2
AfFAD2
JcFAD2
HbeFAD2-1
HbeFAD2-2
TmFAD2
GhFAD2-2
GhFAD2-3
GhFAD2-4
AtFAD2
PfFAD2
BnFAD2-4
BnFAD2-2
BjFAD2
BnFAD2-1
LuFAD2-1
LuFAD2-2
HaFAD2-1
CtFAD2-1
CtFAD2-10
HbeFAD2-3
GhFAD2-1
PpFAD2-1
AhFAD2-1
GmFAD2-1A
GmFAD2-1B
VlFAD2-2
XsFAD2
NtFAD2
SeFAD2
DiFAD2
OepFAD2-1
OepFAD2-3
0.1
Housekeeping-type FAD2
Seed-type FAD2

Fig. 2

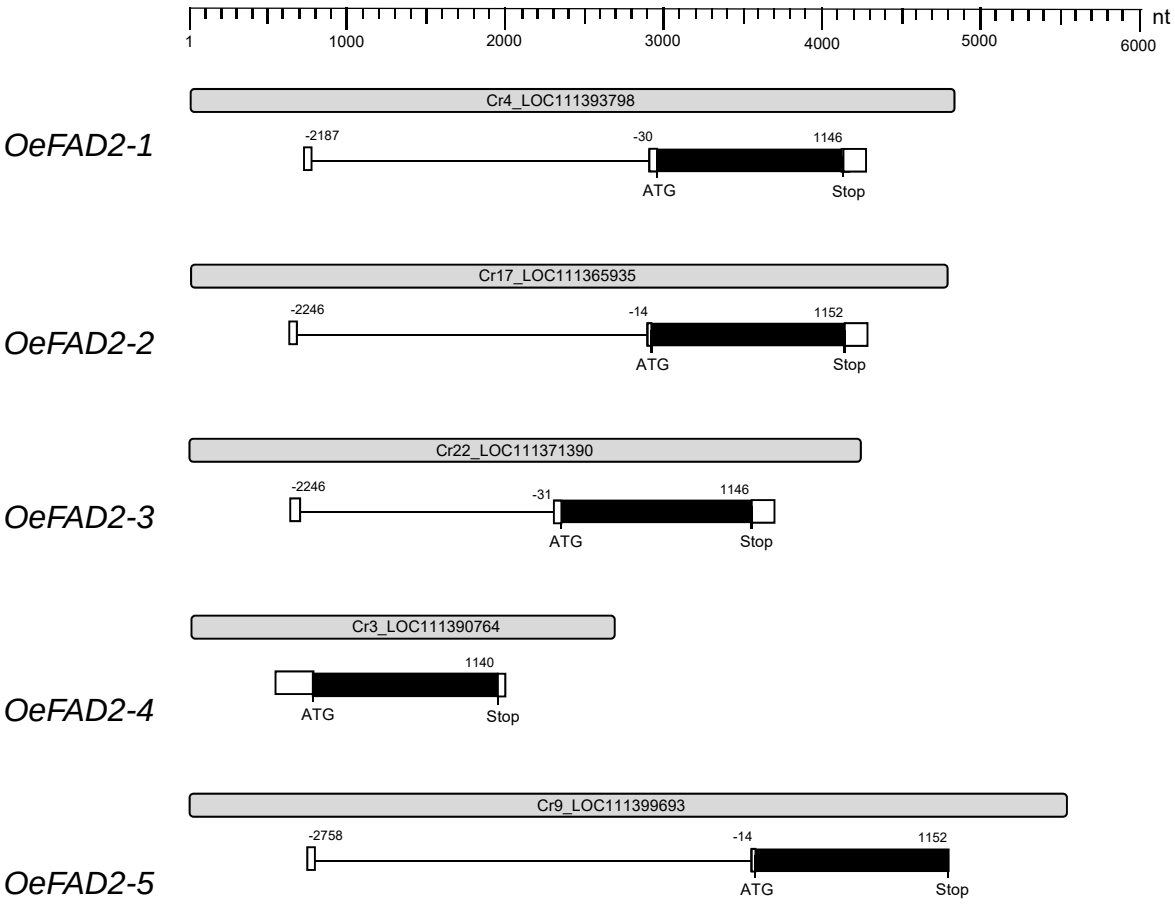

1
1000
2000
3000
4000
5000
6000
nt
Cr4_LOC111393798
OeFAD2-1
-2187
-30
1146
ATG
Stop
Cr17_LOC111365935
OeFAD2-2
-2246
-14
1152
ATG
Stop
Cr22_LOC111371390
OeFAD2-3
-2246
-31
1146
ATG
Stop
Cr3_LOC111390764
OeFAD2-4
1140
ATG
Stop
Cr9_LOC111399693
OeFAD2-5
-2758
-14
1152
ATG
Stop

Fig. 3

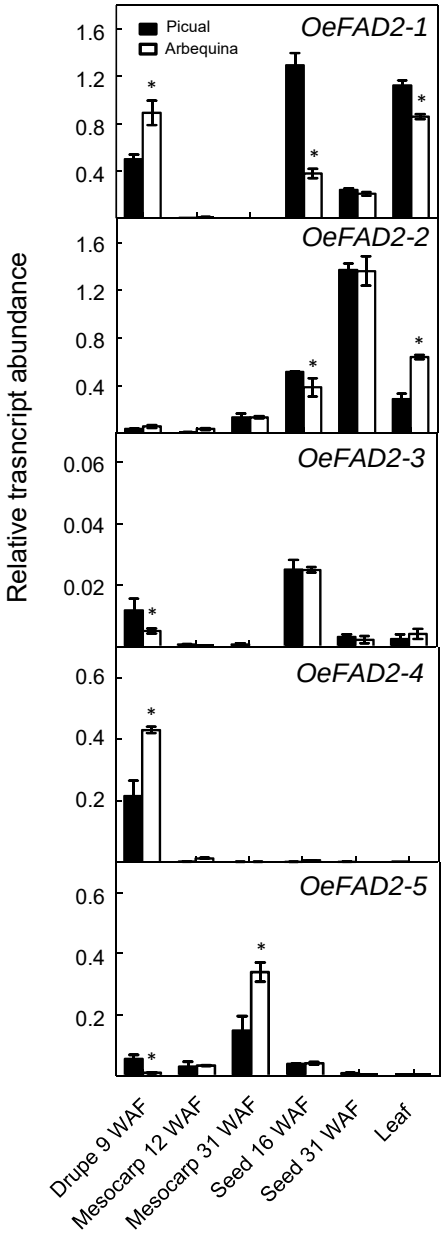

Picual
Arbequina
OeFAD2-1
OeFAD2-2
OeFAD2-3
OeFAD2-4
OeFAD2-5
Relative trasncript abundance
1.6
1.2
0.8
0.4
0.06
0.04
0.02
0.6
0.2
Drupe 9 WAF
Mesocarp 12 WAF
Mesocarp 31 WAF
Seed 16 WAF
Seed 31 WAF
Leaf

Fig. 4

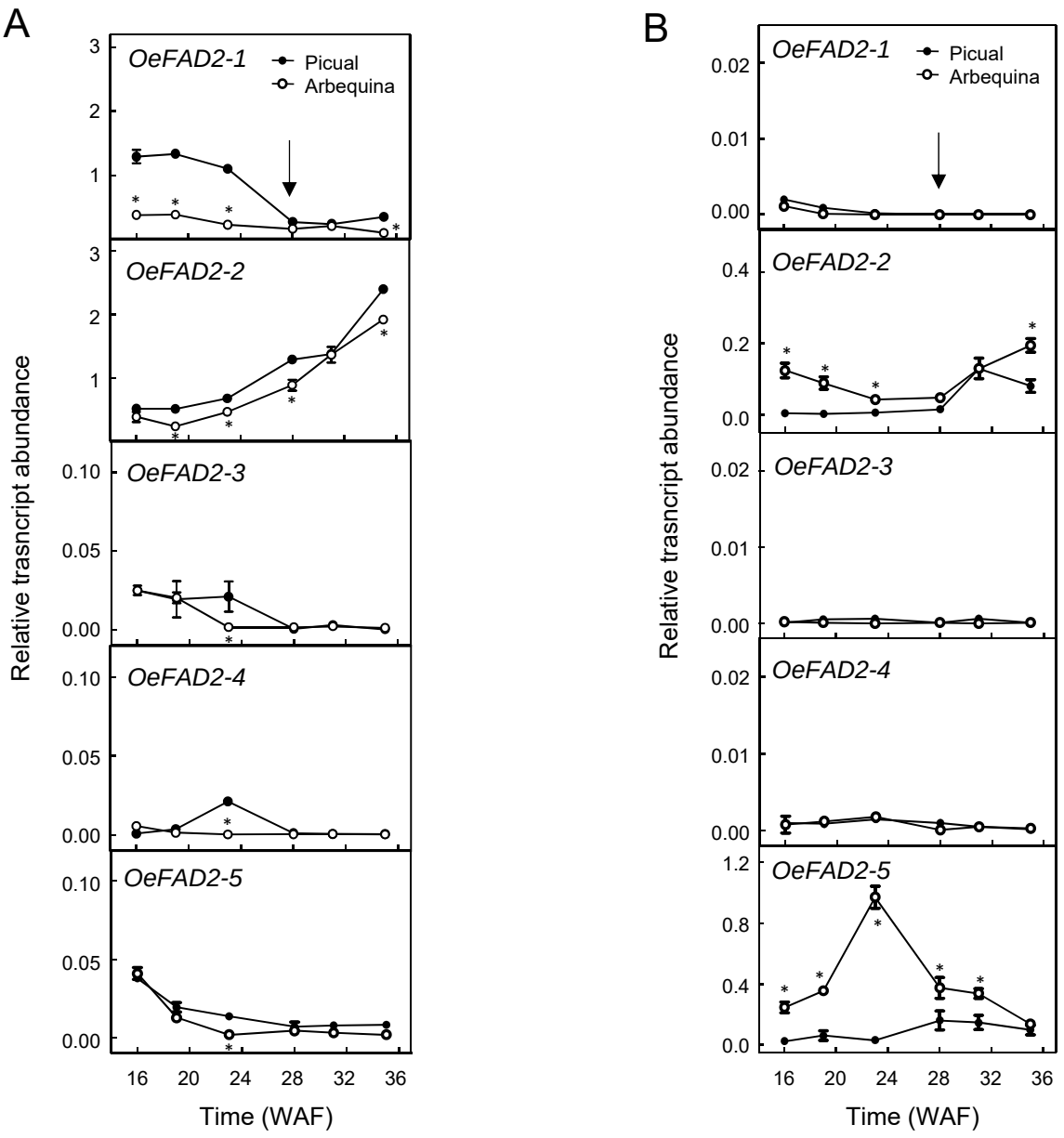

A
B
OeFAD2-1
Picual
Arbequina
OeFAD2-2
OeFAD2-3
OeFAD2-4
OeFAD2-5
Relative trasncript abundance
Time (WAF)
16
20
24
28
32
36

Fig. 5

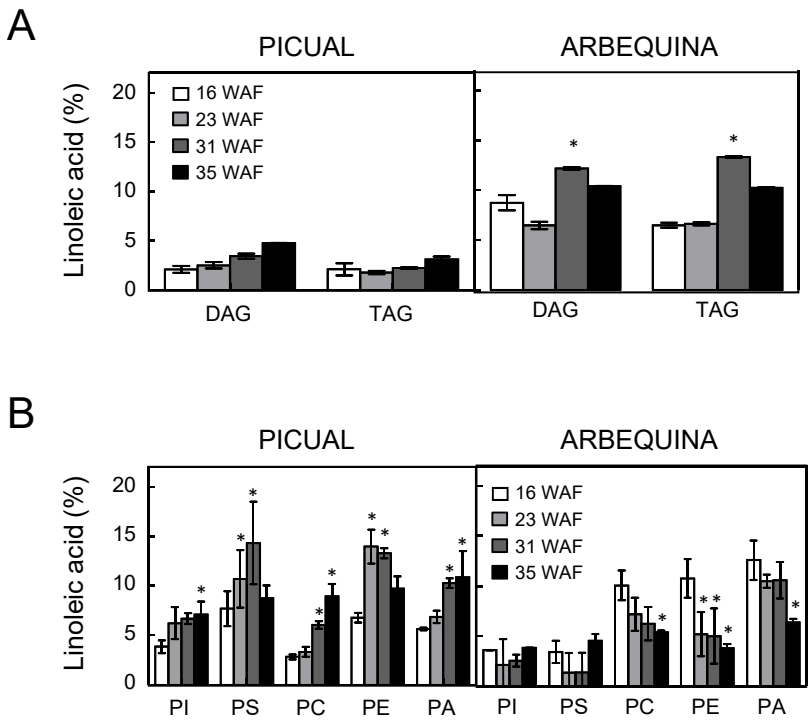

A
PICUAL
ARBEQUINA
Linoleic acid (%)
16 WAF
23 WAF
31 WAF
35 WAF
DAG
TAG
DAG
TAG
B
PICUAL
ARBEQUINA
Linoleic acid (%)
16 WAF
23 WAF
31 WAF
35 WAF
PI
PS
PC
PE
PA
PI
PS
PC
PE
PA

Fig. 6

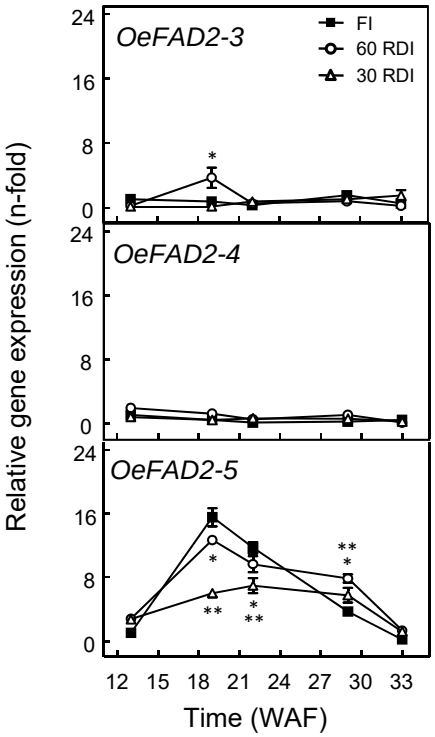
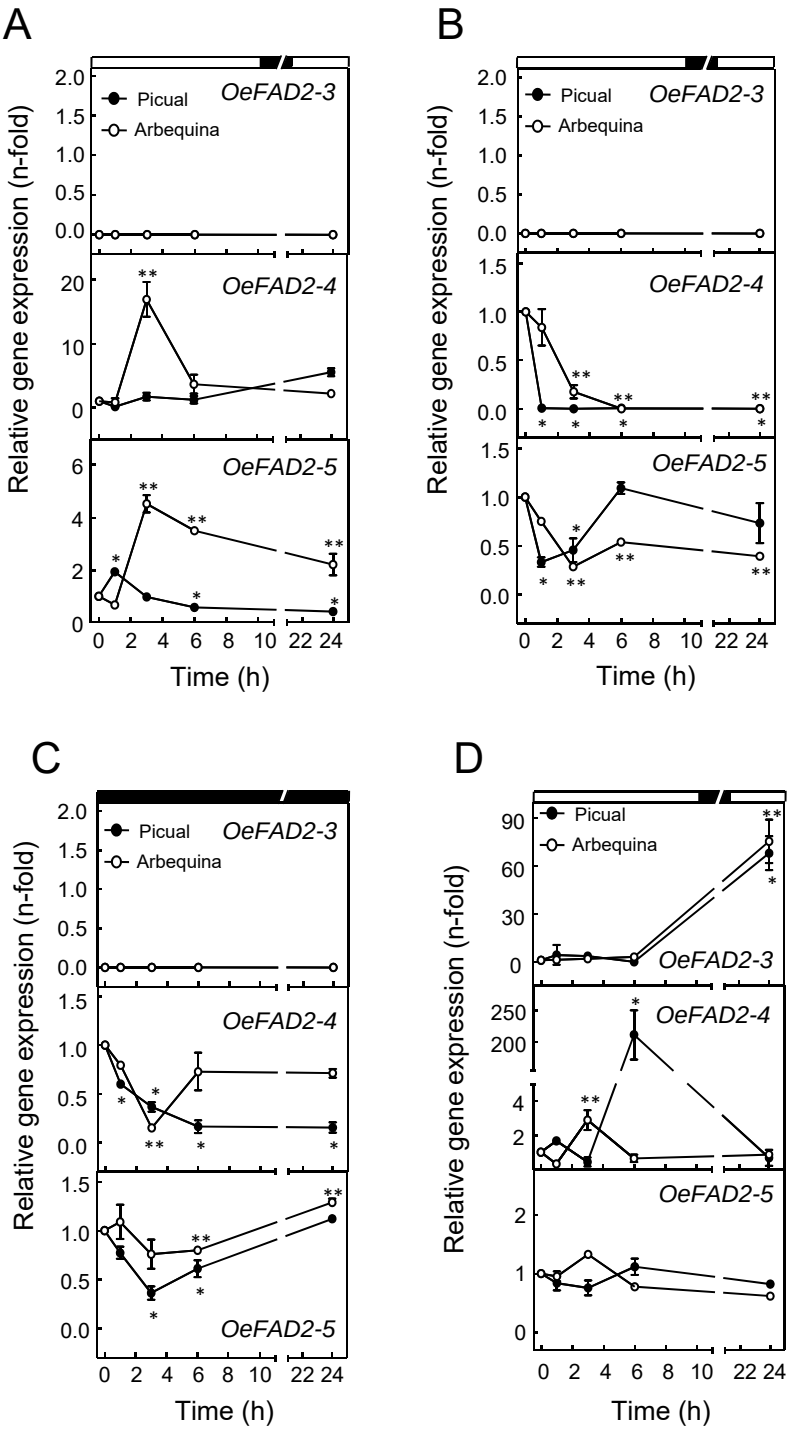

OeFAD2-3
OeFAD2-4
OeFAD2-5
FI
60 RDI
30 RDI
Relative gene expression (n-fold)
Time (WAF)
0
8
16
24
12 15 18 21 24 27 30 33

Fig. 7


A
B
C
D
Picual
Arbequina
OeFAD2-3
OeFAD2-4
OeFAD2-5
Relative gene expression (n-fold)
Time (h)
0 2 4 6 8 10 22 24