RESEARCH PAPER

Cold affects the transcription of fatty acid desaturases and oil quality in the fruit of *Olea europaea* L. genotypes with different cold hardiness

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Abstract

The olive tree lacks dormancy and is low temperature sensitive, with differences in cold tolerance and oil quality among genotypes. The oil is produced in the drupe, and the unsaturated fatty acids contribute to its quality. The aim of the present research was to investigate the relationship among development, cold response, expression of fatty acid desaturase (*FAD*) genes, and unsaturated fatty acid composition in drupes belonging to genotypes differing in leaf cold tolerance, but producing good oil (i.e. the non-hardy Moraiolo, the semi-hardy Frantoio, and the hardy Canino). In all genotypes, cold sensitivity, evaluated by cold-induced transient increases in cytosolic calcium, was high in the epi-mesocarp cells before oil body formation, and decreased during oil biogenesis. However, genotype-dependent differences in cold sensitivity appeared at the end of oil production. Genotype-dependent differences in *FAD2.1*, *FAD2.2*, *FAD6*, and *FAD7* expression levels occurred in the epi-mesocarp cells during the oleogenic period. However, *FAD2.1* and *FAD7* were always the highest in the first part of this period. *FAD2.2* and *FAD7* increased after cold applications during oleogenesis, independently of the genotype. Unsaturated fatty acids increased in the drupes of the non-hardy genotype, but not in those of the hardy one, after cold exposure at the time of the highest *FAD* transcription. The results show a direct relationship between *FAD* expression and lipid desaturation in the drupes of the cold-sensitive genotype, and an inverse relationship in those of the cold-resistant genotype, suggesting that drupe cold acclimation requires a fine *FAD* post-transcriptional regulation. Hypotheses relating *FAD* desaturation to storage and membrane lipids, and genotype cold hardiness are discussed.

Key words: Cold response, fatty acid desaturase genes, fruit development, oil composition, olive tree, unsaturated fatty acids.

Introduction

The olive tree (*Olea europaea* L.) is an evergreen species that ranks sixth in the world’s production of vegetable oils (Alagna et al., 2009). Since ancient times it has been cultivated in the Mediterranean Basin, where ~2600 cultivars have been identified based on morphological traits (Hatzopoulos et al., 2002). In more recent years, interest in olive oil production has been extended to countries outside the Mediterranean region such as Argentina, the USA, Mexico, South Africa, western Africa, Australia, Azerbaijan, China and Japan (Rugini et al., 2000; Mannina et al., 2001).

Olive trees lack dormancy, and are sensitive to low temperatures (D’Angeli et al., 2003, and references therein). For cold acclimation to occur, the plant needs to be subjected to gradual decreases in temperature, with the first acclimation changes starting in summer (Rugini and Fedeli, 1990). Fruit growth takes place in ~4–5 months, and fruits are harvested at ripening (Conde et al., 2008); that is, late autumn/early winter. During this long growth period, the fruits may be exposed to sudden and sharp decreases in temperature. This cold stress may not be dangerous,
because it has been demonstrated that winter chilling results in the slowing down of the post-maturation (ripening) process of the fruit, which improves the quality of the oil (Palliotti and Bongi, 1996). Moreover, it is traditionally known that the oil produced in Mediterranean regions characterized by colder winters is of better quality (Bernardi et al., 2008). Cold stress in olive tree plants is characterized by changes intervening at different cell and tissue levels (D’Angeli and Altamura, 2007). It is known that the cell membranes are a primary site of cold-induced injury, and that cold acclimation includes the activation of mechanisms that protect membrane fluidity by ensuring an optimal activity of associated enzymes (Thomashow, 2001). Among these mechanisms, there are changes in lipid composition, accumulation of cryoprotectants, and transient increases in second messengers (e.g. cytosolic calcium ions) involved in signal transduction leading to the activation of cold-responsive and acclimation-related genes (Thomashow, 2001; D’Angeli and Altamura, 2007). The capability for cold acclimation of olive tree cultivars has been characterized experimentally by monitoring cold-induced cytosolic calcium changes in leaf protoplasts (D’Angeli et al., 2003; D’Angeli and Altamura, 2007).

More than 90% of the olive oil is produced in the mesocarp of the drupe (Connor and Fereres, 2005). Phenols and lipids contribute to determine oil quality, but the unsaturated fatty acid (FA) fraction is also very important. The latter includes oleic acid (55–83%), linoleic acid (3.5–21%), and ω-linolenic acid (<1%) (European Commission, 2003). Moreover, olive oil has a low content of saturated FAs, which makes it appropriate for human consumption as well as an excellent fat in diets designed to reduce cardiovascular disease (Salas et al., 2000). The quality of olive oil has been reported to be influenced by fruit ripening, which, in turn, is affected by environmental conditions and agricultural practices, even if it seems to be mostly under genetic control (Conde et al., 2008).

The ability to adjust membrane lipid fluidity by changing the levels of unsaturated FAs is a feature of cold-responsive species, and is provided, as for other abiotic and biotic stresses, mainly by the regulated activity of fatty acid desaturases (FADs) (Upchurch, 2008). For example, the Arabidopsis fad6 mutant, which lacks an active ω6 FAD and accumulates palmitoleic and oleic acids, has reduced levels of polyunsaturated FAs in the chloroplast lipids and exhibits a decreased number of thylakoid membranes under chilling stress. Moreover, the Arabidopsis fad2 mutant, deficient in an endoplasmic reticulum (ER)-localized ω6 FAD, has decreased polyunsaturates in the extrachloroplast membrane lipids, and long exposure to low temperatures results in plant withering (Miquel et al., 1993).

The FADs responsible for the formation of linoleic and ω-linolenic acids from oleic acid have been identified in olive trees. They utilize complex lipid substrates (Wallis and Browse, 2002), the principal ones being phosphatidylcholine in the ER and monogalactosyl-diaclylglycerol in the plastid (Hernandez et al., 2008, and references therein). The activities of two ω6 FADs—FAD6 in the chloroplast and FAD2 in the ER—are necessary for the production of linoleic acid from oleic acid (Banilas et al., 2005; Hernandez et al., 2005, 2008). In the cv. Picual, two isoforms of FAD2—OepFAD2.1 and OepFAD2.2—have been identified, and OepFAD2.2 has been suggested to be the enzyme involved in lipid desaturation in the fruit pulp (Hernandez et al., 2005). Moreover, in the cv. Koroneiki both FAD2 and FAD6 are expressed in various organs including flowers, fruits, and seeds (Hatzopoulos et al., 2002; Banilas et al., 2005).

In some species, there is also a positive relationship between the composition of trienoic FAs (hexadecatrienoic and linolenic acids) in chloroplast membranes, low temperature tolerance, and growth stage. In fact, by overexpressing the Arabidopsis chloroplast ω3 FAD7 gene in tobacco, trienoic FAs increase in leaf tissues, and the transgenic seedlings are chilling tolerant (Kodama et al., 1995). In contrast, AtFAD7 in Arabidopsis is not affected by temperature variations, whereas its isoform AtFAD8 is induced by low temperatures (<10 °C) (Matsuda et al., 2005). FAD8 is also induced by low temperatures in tobacco, maize, and rice (Gibson et al., 1994; Kodama et al., 1994, 1997; Berberich et al., 1998). In Olea europaea cv. Koroneiki drupes, cDNAs coding for a plastidial FAD (OeFAD7), responsible for the production of trienoic acids, and for a microsomal linoleate FAD3, have been identified (Banilas et al., 2007). Recently, FAD7 expression in cv. Picual callus has been demonstrated to be affected by high temperatures (Hernandez et al., 2008).

The drupe of the olive tree represents an interesting system to investigate FA metabolism and possible changes related to cold. In fact, its remarkable characteristic of lacking oleosins does not hamper the coalescence of oil droplets, making oil extraction from the pulp easy. In addition, the absence of oleosins avoids the use of triacylglycerols (TAGs; i.e. FAs attached to the glycerol backbone) as nutrition for the embryo (Salas et al., 2000). Moreover, the olive possesses photosynthetic activity which provides about half of the total carbon for oil synthesis (Harwood and Sanchez, 2000). However, there is scarce information about the morpho-physiological changes in response to cold stress that take place during drupe development, and about the FADs involved, their regulation by cold, and the final effects on oil quality.

Moreover, improving orchard management is an important goal of agriculture and agrobiotechnology of olive trees (Fernández et al., 2008; Tognetti et al., 2008), especially in the countries which have recently begun cultivation. Understanding the relationship between FAD gene expression, oil quality, and genotype resistance to cold might be a determinant for the selection of the cultivars to be planted to optimize oil production in new orchards exposed to sharp drops in temperature.

The aim of the present research is to shed light on the relationship among development, cold response, FAD expression, and oil composition in the olive tree drupe, and to find possible genotype-dependent differences.
Towards this aim, a cytobehistorical analysis was performed on the epi-mesocarp cells of the drupe at successive stages from fertilization to ripening in cultivars known to have different cold responsiveness in the leaves (D’Angeli et al., 2003; D’Angeli and Altamura, 2007), and good oil quality (Salter et al., 1997; Gucci and Servili, 2005). The selected genotypes were the cold-tolerant (i.e. hardy) cv. Canino, endemic to the volcanic areas of Central Italy, the weakly tolerant (i.e. semi-hardy) cv. Frantoio, common in Tuscany, and the cold-sensitive (i.e. non-hardy) cv. Moraiolo, from Central and Southern Italy. The transcript levels of FAD genes involved in FA desaturation were investigated in parallel during drupe growth, under natural seasonal conditions, and in the presence of cold shocks. The unsaturated FA composition in the epi-mesocarp cells was evaluated and compared among the cultivars.

The results show that the epi-mesocarp cells are cold sensitive in all the genotypes before the first oil bodies are formed in the drupe. Cold sensitivity decreases during the oil production phase, with genotype-dependent differences at the end of oil biogenesis. Differences in FAD transcript levels occur in response to development and genotype. Cold affects FAD transcription during oil biogenesis. Unsaturated FAs change in a genotype-dependent manner in drupes exposed to cold at the time of the highest FAD transcription. The relationship between FAD expression, FA desaturation, and genotype-dependent cold sensitivity is discussed.

Materials and methods
Genotype selection
Italian genotypes of O. europaea L., known for good oil quality and for differences in cold acclimation determined by the epifluorescence analysis of cytosolic calcium transients on leaf protoplasts (D’Angeli et al., 2003; D’Angeli and Altamura 2007), were chosen for the study. They were the hardy cv. Canino, the semi-hardy cv. Frantoio, and the non-hardy cv. Moraiolo.

Protoplast isolation and fluorescence measurements
Protoplasts were obtained from the epi-mesocarp tissue of 10 drupes collected, at specific times after flowering, from five randomly selected 5-year-old trees per cultivar. The trees were grown in pots under the same conditions (i.e. standard sandy loam soil, daily irrigation) in the Botanical garden of the Environmental Biology Department of Sapienza University (Rome: 41°53′3.5″ latitude North, 12°29′31″ longitude West, 20 m a.s.l.) for three crop years (2006–2008). For protoplast isolation, the drupes were cut into thin sections, and incubated in an enzymatic solution (10% w/v) according to D’Angeli et al. (2003) overnight in darkness (23 °C) with orbital shaking at 100 rpm. The cellular suspension was filtered through a nylon grid (200 μm) before use.

The protoplast solution (1 ml) was incubated with Calcium-Crimson AM fluorochrome (5 μM, Invitrogen, Italy) to detect cytosolic calcium ([Ca^{2+}]_c) after cold shocks of a cooling rate (ΔT/ Δt) of 10 °C within 60 s starting from room temperature. The shocks were single or repeated twice (the second shock applied after the protoplast solution had returned to room temperature). Aliquots of 100 μl of the protoplast solution were used for measuring [Ca^{2+}]_c (three protoplasts per aliquot were analysed, out of an average of 30 protoplasts). Slide preparation was performed according to D’Angeli et al. (2003). For the detection of Calcium-Crimson-AM fluorescence, a Zeiss Axioslab microscope (Zeiss, Germany) equipped with an excitation filter (BP) of 510–560 nm and an emission filter (LP) of 590 nm was used.

Protoplast solution (1 ml) was also marked with the apoptosis detection kit Vybrant Apoptosis assay (Invitrogen, Italy) to allow simultaneous imaging of viable, dead, and apoptotic protoplasts present in the same sample according to D’Angeli and Altamura (2007). Aliquots of 50 μl were counted under a DMIRB Leica microscope (Leica, Germany) equipped with a UV filter set (excitation 340–380 nm, emission 425 nm), a Texas Red filter set (excitation 560–590 nm, emission 615–655 nm), and a FITC filter set (excitation 450–490 nm, emission 515 nm) for propidium iodide, YO-PRO, and Hoechst 33342 dyes, respectively.

The fluorescence images were acquired by means of a Zeiss Axioslab microscope, equipped with a Leica DFC 320 video camera, for [Ca^{2+}]_c detection, and by means of a DMRB Leica microscope, equipped with a Leica DC 500 video camera, for Vybrant Apoptotic assay, and analysed with ImageJ as image analysis software (National Institute of Health, USA).

Histological analysis
Ten drupes were collected three times a week from full flowering to ripening from the same trees and genotypes used for the protoplast isolation. The samples were fixed in 70% ethanol, dehydrated, embedded in a resin, sectioned at 5 μm thickness with a Microm Zeiss HM 350 SV micrometre (Zeiss, Germany), and stained with toluidine blue. Fresh drupes were also included in agar (5%, Sigma, Italy), cut at 30 μm thickness with a vibratome (Vibratome Series 1000, Technical Products International, UK), and stained with Sudan IV for lipid detection (Wigglesworth, 1975).

Hand-cut epi-mesocarp sections of the drupe were incubated in an acid (pH 4.0) buffer and directly observed under light microscopy for anthocyanin detection.

The samples for scanning electron transmission microscopy were fixed in 70% ethanol, dehydrated, embedded in resin, sectioned at 5 μm and 30 μm with a Microm Zeiss HM 350 SV micrometre, and attached on a scanning electron microscopy stub with an adhesive (Agar Scientific, Stanstead, UK), stored in a desiccator for at least 24 h prior to examination, and sputter coated with palladium–gold for 90 s in an Polaron SEM Coating System.

The histological images were examined under a Zeiss Axioslab microscope, equipped with a Leica DFC 320 video camera. The scanning electron transmission images were examined under an LEO SE EVO 40 electron microscope (Zeiss, Germany), at an accelerating voltage of 15 kV, and acquired with Zeiss Smart SEM (Leo-32) image software.

RNA extraction, cDNA sequencing, and real-time quantitative PCR (QRT-PCR)
Total RNA was isolated using the protocol described by Lopez-Gomez (1992), pooling the plant material from 10 drupes at the third week after flowering (WAF), and from the epi-mesocarp of 10 drupes collected once a week from the 10th to the 20th WAF (the same trees and genotypes as used for the protoplast isolation). For cold experiments, three trees in pots per replicate and genotype (cv. Moraiolo and cv. Canino) were put in a Lab Companion Plant Growth Chamber (Vernon Hills, Illinois, USA) at 6 °C for 24 h (at WAF 10) and 72 h (at WAF 19) under a 16 h/8 h (light/dark) photoperiod (300 μE m⁻² s⁻¹). Control plants of the same age were grown in a similar way at 30 °C (WAF 10) and 25 °C (WAF 19), under the same photoperiod. The epi-mesocarp of 10, randomly collected, drupes per sampling time was used.

Tri-Reagent Solution (RNA-DNA/Protein isolation Reagent Applied Biosystem) was used to isolate total RNA after the cold shock experiments, following the manufacturer’s recommendations.
First-strand cDNA was synthesized from 1 μg of total RNA in 30 μl with oligo(dT) and Supercript II (Invitrogen), according to the manufacturer’s instructions and quantified using a NanoDrop spectrophotometer (ND-1000 UV-Vis, NanoDrop Technologies) and gel electrophoresis.

Primers F1–F32 (Supplementary Table S1 available at JXB online), designed by OligoExplorer 1.2 software (GENELINK, New York, USA), were used in an RT-PCR from the total RNA of epi-mesocarp cells. The nucleotide sequences of cDNA of olive FAD transcripts were determined by dideoxynucleotide chain termination sequencing (Sanger et al., 1977) using an ABI Prism 3730 DNA Sequencer in UTAAGRI-GEN Casaccia Enea (Rome, Italy). The cDNA sequences were analysed by CodonCod Aligner 3.6.1 software (CodonCod Corporation, Maryland, USA).

Aliquots of 3 μl of cDNA (3.33 ng μl⁻¹) were used for QRT-PCR experiments, carried out with gene-specific primers, using an ABI PRISM 7900 instrument (Applied Biosystem) and a Platinum SYBR-Green master Kit (Invitrogen). PCR conditions were: 5 min at 95 °C followed by 45 cycles of incubations at 95 °C for 1 s and 58 °C for 30 s. Quantification was performed using standard dilution curves for each studied gene fragment and the data were normalized for the quantity of actin transcript (Livak and Schmittgen, 2001). The housekeeping gene is selected after QRT-PCR experiments with different candidate genes: the 18S rRNA subunit (O. europaea L.), elongation factor (Solanium esculentum Mill.), the Olex34 gene (O. europaea L.), and β-actin (O. europaea L.).

At least three PCR runs were carried out for each cDNA to serve as technical replicates, and two independent experiments were carried out by using two independent RNAs for each sample. Means from two independent experiments were subjected to SEM calculation.

The genes of the olive tree, and of other plants, used for the design of primers are available in GenBank (www.ncbi.nlm.nih.gov/GenBank/EMBL/DDJB), and the primer sequences for QRT-PCR amplification are: FAD2.1 (N. AY733076), GCAATCAAGCCAC-TATTAGTG and GCCATACAAGCCTCTTGCC; FAD2.2 (N. AY733077), GCAATTAAGCGCATATTAGG and CATTCC TCTCGCTTCTCC; FAD3 (N. DQ788673), GTCATGGGGA TGTATTACAGAG and CAAATGGGCTCTTATTTAAGGC; FA D6 (N. AY772187), GAACCAGGTCTACAGAACGAGG and GTTG TGAATCTTGGTTGTC; FAD7 (N. DQ788673), CCCATGTC ATACATCACCTC and GCAATACAAGGGAAGGAGG; FA D8a (N. AT5G05580–GDB167603), CTCTTTGCTCTCTAACC TA and TCCCATATCATGCCAGT; FAD8b (N. AT5G055 80–GDB167603), GAACCTGGGCATTGAATGTGG and CCTG GGTCAATCTCTCC; ACT1 (N. AY788899.1), AGAAAGGAT CTCTATTGTGTAATAC and CAACCTCAAATAGAAGTG ATG, and ACT2 (N. AY788899.1), TGAAGTGTGATGTGGATA TTAGG and CTTCTGATTCTCTGCAAG.

Nuclear magnetic resonance (NMR) sample preparation

Proton NMR spectra (D’Imperio et al., 2007) of epi-mesocarp tissues of drupes at WAF 6 and WAF 10 were acquired by a high resolution magic angle spinning (HRMAS) technique at a spinning rate of 6 KHz. A 45 ° pulse of 4.0 μs, spectral width of 12.7 ppm, relaxation delay of 1.5 s, and a 32K complex point were used during acquisition. The free induction decay (FID) was zero filled to the 64K complex point and Fourier transformed after apodization with an exponential function of 0.5 Hz.

To evaluate possible variations in FA composition of the lipids after cold stress, three potted trees per replicate and genotype were put in a Lab Companion Plant Growth Chamber (Vernon Hills, Illinois, USA) either at 6 °C for 72 h or at 0 °C for 2 h followed by 10 °C for 14 d overall, under a 16 h/8 h (light/dark) photoperiod (300 μE m⁻² s⁻¹). The control plants were grown in a similar way at 30 °C under the same photoperiod.

The epi-mesocarp of 10 drupes per replicate and genotype, collected from the end of WAF 10 to the end of WAF 12, was extracted from liquid nitrogen and 10 ml of protonated chloroform, rotary evaporated, and re-suspended in 400 μl of deuterated chloroform. Anther two drupes per replicate and genotype were subjected to histological analysis (see above) to test possible cold-induced damage.

High-resolution 13C-NMR spectra of chloroform epi-mesocarp extracts of the drupes collected from stressed and unstressed plants were obtained at 150 MHz on a Bruker 600 Avance spectrometer (Bruker, Italy) with a 5 mm dual 13C/H probe. Each FID was acquired over a spectral width of 220 ppm at 27 °C using a 90 ° pulse of 9.60 μs, and inverse-gated decoupling to avoid a nuclear Overhauser effect to the signals. To avoid signal saturation, a delay of 20 s between acquisitions was used. The spin-lattice relaxation rate of the slowest relaxing nuclei (the carbonyls) was estimated to be 5 s.

Results

The cold sensitivity of the drupe is high, and genotype independent, before oil body formation

Drupe development and maturation was monitored histologically in specimens of cv. Moraiolo, cv. Frantoio, and cv. Canino grown under the same conditions for three crop years (see Materials and methods).

Genotype-dependent differences in the flowering time were observed. Frantoio and Canino specimens showed early flowering and late flowering, respectively, in each year, with the full blooming of Frantoio occurring in the third week of May, and that of Canino 2 weeks later. In contrast, Moraiolo specimens showed differences in flowering time depending on the seasonal thermo-pluvial regime of the year (www.arsial.it/portalearsial/agrometro), being early flowering (full blooming in the second week of May) in low rainfall years (2007 and 2008) and late flowering (full blooming in the fourth week of May) in the high rainfall year (2006). The timing of drupe development and maturation was constant in the crop years for each genotype, lasting 20 WAFs in Canino and Moraiolo, and 18 WAFs in Frantoio. During this period, the drupe passed through four developmental stages. The first stage started soon after fertilization, and was characterized by ovary growth through cell division (Supplementary Fig. S1A, B at JXB online). The seed coat, the inner epidermis, and the first groups of sclereids in the mesocarp and in the external endocarp also differentiated at this stage (WAF 2) (Fig. 1A, and Supplementary Fig. S1C). The second stage lasted up to WAF 6, and was characterized by drupe growth by cell expansion. Lignification of the endocarp and vascular bundle differentiation in the mesocarp also occurred (Fig. 1B). The third stage extended from WAF 7 to WAF 10. During this stage, the fruit continued to grow by cell expansion, and showed cell to cell separation in the mesocarp, and increasing endocarp lignification. Lipids were stored in oil bodies in the mesocarp cells. The first oil bodies (2–4 per cell, on average) were evidenced by lipid-specific histological staining at WAF 7 (Fig. 1C), increased in number and began to coalesce at WAF 8, and continued to coalesce at WAF 9, giving rise to a main oil droplet per cell, occupying ~40% of the cell volume, and to minor oil
Fig. 1. Developmental events related to olive tree drupe growth and maturation in cv. Frantoio, cv. Canino, and cv. Moraiolo. (A, B, D, F, G, H, I, K, and L) Histological, longitudinal sections observed under light microscopy after toulidine blue staining. (C and inset) Vibratome sections observed under light microscopy after Sudan IV staining. (E, J, and inset in H) Sections observed under scanning electron microscopy. (M) Hand-cut tangential section after immersion in an acid (pH 4.0) buffer. (A) Young drupe showing the differentiation of the internal endocarp (ie), external endocarp with lignified cells (ee), mesocarp (me), and epicarp (ep) (end of WAF 2, cv. Canino). (B) Conspicuous number of sclereids in the endocarp and vascular bundle differentiation in the mesocarp of a cv. Frantoio drupe at WAF 6. (C) Detail of the mesocarp cells of cv. Canino treated with the lipid-specific stain. The oil bodies are stained in red. One oil droplet per cell, and minor oil bodies, are shown in the inset (WAF 9). (D) Detail of expanded mesocarp cells containing an oil droplet, and chloroplasts with plastoglobuli-like structures inside (arrows) confining with small oil bodies (arrowheads) (WAF 9, cv. Frantoio). (E) Formation of the oil droplet by fusion of large oil bodies (arrow) in mesocarp cells (WAF 10, cv. Canino). (F and G) Details of mesocarp cells showing chromoplasts (F, and inset), and chloroplasts (G) located around the oil droplet (o). Plastoglobuli are shown by the arrows in the inset of F, and in G (WAF 10, cv. Moraiolo). (H) Mesocarp cells of cv. Moraiolo with chloroplasts bordering the oil droplet (o) (WAF 11), and granules (arrow) between them and the oil droplet. In the inset, the granules are magnified under scanning electron microscopy, and appear as clumps of plastoglobuli-like structures blebbing from the plastid. (I) Detail of the external mesocarp showing a very large oil

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bodies (Fig. 1C, inset). These small oil bodies were observed near the chloroplasts (Fig. 1D, arrowheads), which started to exhibit plastoglobuli-like structures (Fig. 1D, arrows). By the completion of endocarp lignification (i.e. pit hardening) (WAF 10), oil bodies had fused into a large oil droplet comprising ~60% of the cell volume (Fig. 1E). At WAF 10 some chloroplasts had already turned into chromoplasts in the epi-mesocarp cells (Fig. 1F). Both chromoplasts and chloroplasts were located around the central oil droplet (Fig. 1F, G), and showed plastoglobuli (Fig. 1F, inset, and G, arrows).

The lipid content of the epi-mesocarp tissues of the drupe at WAF 10 was also verified by NMR analysis. As shown for Moraiolo in Fig. 2, the comparison of the carbohydrates and lipids present at the end of stage 2 (WAF 6) and at the end of stage 3 (WAF 10) shows that the lipids were many fold higher at WAF 10 than at WAF 6, whereas the carbohydrate content remained constant.

The cold sensitivity of protoplasts from epi-mesocarp cells was tested by monitoring cytosolic calcium variations caused by the application of a temperature decrease of 10 °C in 1 min (i.e. a cooling rate of ΔT/Δt=10 °C in 60 s). This cooling rate was selected because it had been useful for showing differences in cold responsiveness of leaf protoplasts of the same cultivars (D’Angeli et al., 2003; D’Angeli and Altamura, 2007). The cold shock was applied at WAF 6 and at WAF 10 according to the procedure described in the Materials and methods. It was observed that cold sensitivity was similarly high in all genotypes (i.e. 50% calcium transient increases, on average) before the onset of oil production (WAF 6), whereas it decreased significantly (P <0.01) at WAF 10 (i.e. 30% calcium increases, on average), as shown in Fig. 3 for cv. Frantoio.

The cold sensitivity of the drupe decreases during stage 4, but in a genotype-dependent manner only at the end of the stage.

During the fourth stage, lasting 8 WAFs in Frantoio, and 10 WAFs in Moraiolo and Canino, oil accumulation continued, ceasing at the onset of the change in colour of the epicarp (i.e. at WAF 16 in Frantoio, WAF 17 in Canino and Moraiolo). During the first 2 weeks of this stage (WAFs 11–12), chromoplasts and chloroplasts (Fig. 1H) continued to border the central oil droplet, and the cytoplasm between them and the droplet exhibited a granulate appearance (Fig. 1H, arrow). These granules were evidenced by electron microscopy, and appeared as clumps of plastoglobuli-like structures exuded from the plastids (Fig. 1H, inset). At the beginning of the change in colour of the epicarp the central droplet (o) occupying almost all the cell volume (WAF 16, cv. Frantoio). (J) Very large oil droplet (o) in a mesocarp cell at the end of oil storage (WAF 17, cv. Moraiolo). (K) Detail of the mesocarp showing expanded cells with a central oil droplet (o) occupying ~80% of the cell volume, and degenerating nuclei (WAF 17, cv. Moraiolo). (L) Detail of the mesocarp of a cv. Canino drupe at WAF 17 showing expanded cells with chromoplasts (arrows) and a few chloroplasts (arrowheads). Senescing chloroplasts are magnified in the inset. (M) Detail of the epicarp and external mesocarp cells of cv. Canino showing anthocyanin deposition (in red) in the vacuole at WAF 18. (The tonoplast is shown by the arrows. The oil-droplet is evident at a different focal plane.) Bars=200 μm (A), 50 μm (B), 10 μm (C–M, and inset in C), and 1 μm (inset in H).

Fig. 2. HRMAS proton NMR spectra of the epi-mesocarp tissue of cv. Moraiolo drupes at the end of stage 2 of fruit development (WAF 6) (upper panel), and at the end of stage 3 (WAF 10) (lower panel). In the second spectrum the water signal is not visible due to solvent suppression by pre-saturation. A.U., arbitrary units.

Fig. 3. Variations in the cytosolic Ca²⁺ concentration ([Ca²⁺]c), expressed as pixel intensities (A.U., arbitrary units), in cv. Frantoio protoplasts from epi-mesocarp olive cells incubated with Calcium-Crimson-AM after a cold shock of ΔT/Δt=10 °C in 60 s at WAF 6 (end of stage 2, filled circles) and WAF 10 (end of stage 3, filled squares), n=30 at WAF 6 and at WAF 10. Protoplasts representative of the mean response are shown (i.e. protoplasts with a 50% rise in the epifluorescence of the [Ca²⁺]c signal at WAF 6 and a 30% rise at WAF 10, P <0.01 difference).
oil droplet occupied ~80% of the cell volume (corresponding to a mean diameter of ~36 μm in all genotypes) (Fig. 1I, J), some nuclei were degenerating (Fig. 1K), but chromoplasts were still evident (Fig. 1L, arrows), and chloroplasts, in particular, were highly senescent (Fig. 1J, arrowheads, and inset). During the last WAFs of this stage, the drupe progressively became purple. The purple colour was due to intense anthocyanin deposition in the vacuole (Fig. 1M) of the epicarp and external mesocarp cells. The drupe reached maturation at full purple coloration, when the mesocarp cells were very expanded, with large intercellular spaces, but contained an oil droplet which had not expanded further (Supplementary Fig. S1D at JXB online).

To determine how many cells still exhibited a functional nucleus during the last 3 weeks of stage 4, cell viability was tested at the onset of the change in colour of the epicarp and at its end. The results, monitored by the epifluorescence signal of the Vybrant Apoptotic test (D’Angeli and Altamura, 2007) in epi-mesocarp protoplasts, are shown for cv. Canino and cv. Moraiolo in Fig. 4, but similar results were also obtained for cv. Frantoio protoplasts (data not shown). Endocarp cells were not evaluated because at this stage they had already differentiated into sclereids, which are usually described as non-living cells at maturity (Fahn, 1990). The test showed that cell viability was ~60–70% at the onset of the change in colour of the epicarp (i.e. at WAF 17 for the cultivars in Fig. 4A, B), whereas it was reduced to ~20% at its end (i.e. at WAF 20 in Fig. 4A, B) without significant differences among genotypes. There were also no significant difference among genotypes in the percentages of apoptotic and dead cells (Fig. 4A, B).

Post-maturation (i.e. ripening) followed stage 4, and was characterized by mesocarp cell softening, whereas the size of the oil bodies did not increase further (all genotypes). The duration of the ripening before harvest changed with the genotype and was affected by the thermo-pluvial regime of the year (data not shown).

Single and repeated shocks of ΔT/Δt=10 °C in 60 s were applied to epi-mesocarp protoplasts at the onset of the change in colour of the drupe. The protoplasts responded to single shocks with cytosolic calcium peaks lower than those observed at stage 3 (see Fig. 3, and 5A, in comparison, for cv. Frantoio). When the shocks were repeated, weak transient rises continued to be observed at the second shock in the protoplasts of Moraiolo, and to a lesser extent in those of Frantoio, whereas they were not observed in the protoplasts of Canino (Fig. 5B). This shows that the experimental procedure was sufficient to cause cold acclimation in the still living pulp cells of the hardy genotype.

![Fig. 4. Viability of the epi-mesocarp cells at the end of stage 4, expressed as mean percentages of live, apoptotic, and dead protoplasts at the onset of the change in colour of the epicarp (WAF 17, open bars) and at the end (WAF 20, filled bars) in cv. Canino (A) and cv. Moraiolo (B). n=1000. (Results monitored by the epifluorescence signal after the Vybrant Apoptotic test.) Small bars show the SEM.](image-url)

![Fig. 5. Variations in cytosolic Ca^{2+} concentration ([Ca^{2+}]_c), expressed as pixel intensities (A.U., arbitrary units), in cv. Canino, Frantoio, and Moraiolo protoplasts from epi-mesocarp cells incubated with Calcium-Crimson-AM after a single (A), and a repeated (B) cold shock of ΔT/Δt=10 °C in 60 s applied at the onset of the change in colour of the drupe (i.e. at WAF 16 in Frantoio, and WAF 17 in Canino and Moraiolo), n=30. Protoplasts representative of the mean responses are shown [i.e. with a 16, 15.9, and 2.2% increase in (A), and with a 12.5, 15.9, and 6.6% increase, after the first shock, and 6.9, 7.4, and 0% increase, after the second shock, in (B), in cv. Moraiolo, Frantoio, and Canino, respectively].](image-url)
(Canino), but not in those of the non-hardy (Moraiolo) and semi-hardy (Frantoio) genotypes.

**FAD transcript levels increase between the end of stage 3 and the first weeks of stage 4 and cease at the completion of the change in colour of the drupe (end of stage 4)**

The production of linoleic and linolenic unsaturated FAs occurs through Ω3 and Ω6 FAD enzyme activities (see Introduction). The expression of FAD genes during oil production is important to understand the metabolism of unsaturated FAs, and for this reason QRT-PCR analyses were carried out. In the forming drupes at WAF 3, FAD2.1, FAD2.2, FAD3, FAD6, and FAD7 transcripts were present at very low levels, and that of FAD8 only in traces, as shown for cv. Frantoio in Supplementary Fig. S2A at JXB online. The transcript levels of FAD2.1 and FAD7 increased at the end of stage 3 (WAF 10), for example in cv. Frantoio (Fig. 6B and Supplementary S2A, for comparison), whereas those of FAD6 and FAD2.2 remained quite constant. In contrast, FAD3 levels greatly decreased, becoming hardly detectable like those of FAD8, as shown for cv. Frantoio in Supplementary Fig. S2B. During stage 4, the highest FAD levels were observed at WAF 11–12, and, secondly, around WAF 17 in Canino and Frantoio (Fig. 6A, B). FAD levels in Moraiolo were always much lower than in the other two genotypes, but the general trend of FAD transcription was maintained (Fig. 6A–C). The transcript abundance of FAD2.1, FAD2.2, and FAD6 was higher in cv. Canino than in cv. Frantoio and cv. Moraiolo (Fig. 6A–C). Moreover, in Canino and Frantoio, FAD2.1 transcripts were many fold more abundant than those of FAD2.2. The expression profile of FAD2.1 was the most abundant in comparison with those of FAD2.2, FAD6, and FAD7 in the epi-mesocarp cells of cv. Canino (Fig. 6A), whereas it was as abundant as that of FAD7 in cv. Frantoio (Fig. 6B). No prevalent FAD was observed in cv. Moraiolo after WAF 10 (Fig. 6C). However, FAD2.1 transcript levels were higher than those of FAD2.2 by ~10-fold at WAF 10, 11, and 15, respectively (Fig. 6C). FAD3 was not expressed at stage 4 in any genotype, and FAD8 was present only in traces (data not shown). All FAD transcripts were no longer observed at the completion of the change in colour of the drupe (Fig. 6A–C).

FAD coding sequences share an identity score spanning from 96% (FAD 2.1) to 99% (FAD 7), as shown in Supplementary Fig. S3A–D at JXB online. The percentage identity was also near 100% when the FAD transcripts of the cultivars were compared with the corresponding transcripts of cv. Picual and Koroneiki (AY083163, AY733076, AY733077, AY733075, and DQ788674).

**FAD gene expression changes after cold stress**

To detect whether FAD gene expression changed after exposure to low temperatures, cold shocks were applied to the hardy and non-hardy cultivars. It has been reported that long-lasting cold treatments must be applied to leaves of *O. europaea* cv. Leccino (hardy) in order to monitor changes in gene expression (Bernardi et al., 2008). Thus, it was decided to apply shocks more severe and longer lasting than those applied to the single cells to intact drupes in order to monitor calcium transients (Figs 3, 5).

After exposure at 6 °C for 24 h at WAF 10, FAD2.2 and FAD7 expression profiles increased similarly, FAD2.1 and FAD6 decreased similarly (Fig. 7), whereas the FAD3 and FAD8 profiles remained at a similar very low level in both genotypes, as shown for FAD3 in Supplementary Fig. S4A at JXB online. In contrast, after exposure at 6 °C for 72 h at WAF 19, differences between the genotypes were found. In fact, FAD2.2 expression progressively increased in Canino drupes, but increased only during the first 6 h in
those of Moraiolo (Fig. 8A, B). FAD2.1 expression increased slightly, and only during the first 6 h of treatment, in Canino, whereas it remained constantly at a very low level in Moraiolo (Fig. 8A, B). FAD7 transcripts were the most abundant in Canino, but not in Moraiolo, and in the former, but not in the latter, showed the highest rise after 6 h, whereas FAD6 showed a similar expression in response to cold in both genotypes (Fig. 8A, B). Moreover, cold stress caused the expression of FAD3, which was absent without the stress at this stage. In fact, FAD3 was observed already after 6 h of treatment in Moraiolo drupes, and at the end of the treatment (72 h) in Canino drupes (Supplementary Fig. S4B), albeit at very low levels. Only a slight increase in FAD8 transcripts was observed after 6 h of treatment in both genotypes, and was not followed by any further significant change (data not shown). The comparison of the transcript levels between the two shocks (Figs 7, 8) shows that even if the shock at WAF 10 lasted one-third of the time of that at WAF 19, the enhancement in the transcripts of specific FAD genes (i.e. FAD 2.2 and FAD7) occurred similarly at WAF 10 and WAF 19 in both genotypes.

The unsaturated FA composition changes in the drupe after a prolonged cold stress, and in a genotype-dependent manner

After exposure at 6 °C for 72 h at WAF 10, the FA pattern in the drupes of Canino and Moraiolo was evaluated by 1H-NMR spectroscopy; however, the contents of linoleic and
linolenic acids remained similar to those of untreated controls (data not shown), suggesting that a stronger and longer stress had to be applied.

Thus, a cold treatment at 0 °C for 2 h, followed by a period at 10 °C for overall 14 d, was applied to olive trees from WAF 10 to WAF 12; that is, during the oil production interval characterized by the highest FAD values (Fig. 6A, C). The freezing pre-treatment was applied to facilitate induction of acclimation in Canino drupes, and the acquired acclimation was verified by the absence of calcium transients in their protoplasts at 24 h (data not shown). At this time, macroscopic differences were shown by Canino and Moraiolo drupes; that is, oil droplets were present on the epicarp of Moraiolo drupes, but no exudate was present on Canino fruits. The histological control on the epicarp of the drupes of both genotypes did not show cellular damage (data not shown).

To evaluate the impact of cold stress on lipid desaturation, 13C-NMR spectroscopy was used on drupe extracts. The FA pattern during the whole stress period was monitored. The mean percentages of oleic, linoleic, and linolenic acids were evaluated on the extracts from the unstressed drupes (t₀, i.e. the end of WAF 10), and differences between the cultivars were observed in the polyunsaturated FAs, in particular (i.e. 75.8 and 70.0 for oleic acid, 5.9 and 11.5 for linoleic acid, and 6.1 and 7.5 for linolenic acid, in Canino and Moraiolo, respectively). No significant change in FAs occurred in any genotype under 0 °C for 2 h, followed by 10 °C, at WAF 11 (i.e. from t₀ to day 7 of treatment). After another 7 d at 10 °C [i.e. at the end of WAF 12, i.e. day 14 of treatment (Figs 9, 10)], the relative ratio among the different FAs was that typical of extra virgin olive oil (i.e. 70–80% oleic acid, 10–20% linoleic, and ~5% linolenic acid). However, from Fig. 9 it appears that no significant change continued to occur in the amount and composition of FAs in cv. Canino. In contrast, in the case of cv. Moraiolo (Fig. 10), a significant difference in the amount of polyunsaturated FAs, and mainly in linolenic acid, was observed between stressed and control drupes at both day 10 and day 14, indicating a positive correlation between cold stress, degree of saturation in the lipids, and genotype.

**Discussion**

The results show that the cold sensitivity of the epi-mesocarp cells of the olive tree drupe is developmentally and genetically controlled. Cold induces changes in FAD transcription during oil biogenesis. The unsaturated FAs increase in the drupes exposed to cold at the time of the highest FAD transcription, but this occurs in the cold-sensitive genotype and not in the hardy genotype.

![Fig. 9. Fatty acid (FA) distribution, determined by 13C-NMR, in epi-mesocarp extracts from cv. Canino drupes, either stressed by exposure to 10 °C from day 7 (end of WAF 11) to day 14 (end of WAF 12), after a pre-treatment at 0 °C for 2 h followed by 10 °C from t₀ (end of WAF 10) to day 7, or grown under normal conditions (30 °C) for the same period (control drupes). Bars represent fitting parameter standard deviations. The symbol size is bigger than the corresponding bar.](image9)

![Fig. 10. Fatty acid (FA) distribution, determined by 13C-NMR, in epi-mesocarp extracts from cv. Moraiolo drupes, either stressed by exposure to 10 °C from day 7 (end of WAF 11) to day 14 (end of WAF 12), after a pre-treatment at 0 °C for 2 h followed by 10 °C from day 0 (end of WAF 10) to day 7, or grown under normal conditions (30 °C) for the same period (control drupes). Bars represent fitting parameter standard deviations. The symbol size is, in some cases, bigger than the corresponding bar.](image10)
Oil accumulation is completed before the end of maturation, when most of the epi-mesocarp cells are still alive

It is known that the morphological characteristics of the mature drupe of *O. europaea* vary greatly among cultivars, are dependent on environmental and cultural conditions, and on the alternate fruiting, which is governed by both endogenous (e.g. ageing) and external factors (Lavee and Wodner, 1991; Conde et al., 2008). Moreover, different oil accumulation patterns have been reported for different cultivars (Lavee and Wodner, 1991, and references therein). Here it is shown that the period and the phases of the development and maturation of the drupe are genotype independent in the specimens of the three cultivars selected for the different cold sensitivity of their leaves.

Five main phases of drupe growth, from fertilization to ripening, have been described in the literature, with mesocarp development by cell expansion representing the second period of growth. It has been generalized that it starts after the completion of endocarp lignification (i.e. pit hardening), and is characterized by the accumulation of the oil (Hernandez et al., 2005; Conde et al., 2008, and references therein). However, the present results show that mesocarp expansion starts concomitantly with endocarp lignification in Canino, Frantoio, and Moraiolo, and continues during the third stage, in parallel with oil body formation and coalescence. It is possible that exogenous (cultural and environmental) and endogenous (e.g. ageing) factors may affect the timing of sclereid differentiation in the endocarp (i.e. lignification) and of cell expansion in the mesocarp, altering the coordination between these developmental processes, with a consequent shift in the start of oil production. This means that, even if widely accepted to date, a completely lignified endocarp is not the universal marker of the start of oil biogenesis. The present histological results show that the pit hardening occurs only at the end of the third stage (WAF 10) in the cultivars under study. In contrast, oil bodies appear in the mesocarp cells before (i.e. at WAF 7), and a large oil droplet, deriving from their fusion, is already present in each cell at WAF 10. Accordingly, the epi-mesocarp lipid content observed by the HRMAS technique at WAF 10 (Fig. 2), being many fold higher than at WAF 6, might be evidence of the storage lipids of the oil droplets (i.e. TAGs; Salas et al., 2000, and references therein), in addition to the membrane lipids.

There is no general agreement in the literature about the developmental event marking the end of oil accumulation in the drupe. Some studies show that oil continues to accumulate during ripening (Beltrán et al., 2004). Other studies show that the end of oil accumulation occurs before (i.e. during maturation), either at the end of the change in colour of the epicarp (Lavee and Wodner, 1991) or at its beginning (Hartmann, 1949). The present histological and scanning electron microscopy analyses show that a single, very expanded (i.e. ~80% of the cell volume, and 36 µm in mean diameter) oil droplet per mesocarp cell is present at the beginning of the change in colour of the epicarp, and that its volume does not increase later. This is confirmation that oil accumulation is complete at the beginning of the fruit’s change in colour in the investigated cultivars. In fact, even if the droplet volume/diameter is a non-quantitative measure of oil accumulation, it is used to determine the end point of oil biogenesis. It is known that oil biogenesis in the drupe reaches a plateau at the change in colour (Sanchez and Harwood, 2002; Alagna et al., 2009), and this corresponds to an oil droplet diameter per mesocarp cell of ~30 µm (Rangel et al., 1997; Salas et al., 2000).

In warm-temperature trees without dormancy, leaf cell viability is essential to low temperature sensing, and this is also the case for olive trees (D’Angeli and Altamura, 2007). In this species, in contrast to the leaves, the drupes at the same time experience temperature lowering and complete development, which includes events of programmed cell death, as in other fleshy fruits (Xu and Chye, 1999). In the present study, it was demonstrated that the majority of pulp cells are dead at the completion of the change in colour of the epicarp; that is, at the end point of maturation, whereas a consistent percentage is still alive at the onset of the change in colour; that is, at the end point of oil accumulation. This means that the drupe contains potentially cold-sensitive cells at least up to the end of oil biogenesis.

The cold sensitivity of epi-mesocarp cells is developmentally and genetically controlled

Like other woody species without dormancy, the olive tree takes weeks to express cold symptoms at the anatomical level. In the stem, the symptoms include death of cambial cells, and suberification and lignification of the cell walls (D’Angeli et al., 2003, and references therein). Leaf protoplasts have been considered as a good system to measure cold responses and acclimation in various plants (Mazars et al., 1997; Orvar et al., 2000). In olive tree, it has been demonstrated that mesophyll protoplasts respond to rapid temperature decreases with transient increases in cytosolic calcium ([Ca$^{2+}$]c), and that in acclimated protoplasts [Ca$^{2+}$]c transients are reduced or inhibited, depending on the cold susceptibility of the cultivar (D’Angeli et al., 2003). However, this is the first study to document the involvement of [Ca$^{2+}$]c signalling in the cold response of the cells of an oleogenic fruit. Here it was shown that the pulp protoplasts of olive tree drupes of the investigated genotypes, exposed to the same cooling rate as the leaf protoplasts (D’Angeli et al., 2003), were cold sensitive before the onset of oil production and during the production phase. This indicates that in the fruit no cold acclimation had been experienced by the cells during this developmental period, because otherwise differences between genotypes would have been observed dependent on their different leaf cold acclimation. The results regarding [Ca$^{2+}$]c signalling after single and repeated shocks applied to pulp protoplasts at the completion of oil accumulation (i.e. at the onset of the epicarp changing colour) show, instead, differences between the genotypes which agree well with those observed in the leaf protoplasts of the same cultivars exposed to similar shocks.
depends on the genotype. Acclimation is reached at the end of the oil accumulation phase under field temperature lowering, but the acclimation depends on the genotype.

The transcription of FAD2.1 and FAD7 characterizes oil biogenesis

Although gene expression at the mRNA level does not necessarily parallel enzyme activity, there is a wide body of research showing that FAD gene expression and mRNA accumulation during the growth of olive tree fruit is important for the FA composition of the oil (Hernandez et al. 2009, and references therein). Moreover, the desaturation of oleate and linoleate during TAG biosynthesis in olive callus cultures has been demonstrated (Hernandez et al., 2008).

The present results show that the gene transcripts of FAD2.1 and FAD2.2, ω6 gene isoforms of the ER, and of FAD6 and FAD7, ω6 and ω3 FAD genes, respectively, of the chloroplast, appeared in all genotypes at the onset of storage lipid biosynthesis in the fruit, and were present up to the completion of the change in colour of the epicarp, when oil accumulation was completed. OeFAD2 encodes oleate FAD. Based on RT-PCR analysis, a housekeeping expression pattern of this gene has been found in very young drupes of O. europaea cv. Koroneiki, and relatively high levels in the maturing and ripening mesocarps, suggesting a spatial and temporal expression of this FAD gene in the drupe, with an early role for the high demand of membrane biosynthesis and a late role for the desaturation of storage lipids (Banilas et al., 2005). Moreover, two isoforms, OepFAD2.1 and OepFAD2.2, have been isolated by northern blot analysis in another cultivar, cv. Picual (Hernandez et al., 2005). It has been demonstrated that the percentage of linoleic acid is very low in very young mesocarps, increasing a little during fruit development, and slightly more during ripening (Hernandez et al., 2005), indirectly confirming the temporal differences in FAD2 expression observed in cv. Koroneiki (Banilas et al. 2005). Moreover, very low FAD2.1 expression has been found in the mesocarp of cv. Picual independently of the developmental stage, whereas FAD2.2 has been found to be moderately expressed, but in ripening mesocarp only (Hernandez et al., 2005). The present results show the existence of the two isoforms (OeFAD2.1 and OeFAD2.2) also in cvs Frantoio, Canino, and Moraioi, and even in the young drupes of WAF 3. The transcripts of both the isoforms continued to be present in the epi-mesocarp, even if with fluctuations, from the stage at which an oil droplet per cell was first observed (WAF 10) to the end of maturation, suggesting a role for both enzymes, and perhaps for FAD2.1 in particular (see below), in the desaturation of storage lipids. The differences in the expression patterns of cv. Picual might be explained by the low linoleate content present in the mesocarp of this cultivar, and by its extremely long duration of drupe development and ripening (Hernandez et al., 2005). However, some genotype-dependent differences in the expression profiles of the FAD2 isoforms were also present among Canino, Frantoio, and Moraioi. In fact, FAD2.1 transcripts were always more abundant than those of FAD2.2 in Canino and Frantoio, but only up to WAF 15 in Moraioi. Moreover, FAD2.1 was the most expressed FAD only in Canino. It is reported here that FAD2.1 and FAD2.2 coding sequences of Canino and Moraioi are identical to their counterparts in Picual and Koroneiki. FAD2.2 has been recently suggested to be the main gene responsible for the linoleic acid content in the olive fruit mesocarp of cv. Picual, Arbequina, and Picudo, FAD2.1 expression being very low (Hernandez et al., 2009). FAD gene promoters were not investigated in the present study nor is this information on olive trees available in the literature. Thus, differences among olive tree genotypes in the promoter sequences, and/or in the interaction with specific transcription factors, might be taken into account to explain the observed differences in linoleic acid-related gene expression during drupe development/oil biogenesis.

The expression of the plastidial OeFAD6, another ω6 FAD gene, has been demonstrated in cv. Koroneiki (Banilas et al., 2005), and here confirmed for Canino, Moraioi, and Frantoio. In cv. Koroneiki it has been suggested that unsaturated FAs could result mainly from FAD6 activity at early stages of mesocarp development (WAF 5–11), and that the high levels of OeFAD6 mRNA in young drupes could also be due to the epicarp cells, which are rich in chloroplasts and lipids (Banilas et al., 2005, and references therein). In the present experiments, epicarp cells were in a minority in comparison with the mesocarp cells. The mesocarp cells were rich in plastids for a long period of drupe development; however, the expression levels of FAD6 were quite low at any stage from early drupe development to maturation, at least in Canino and Frantoio. In accordance with this, it has recently been demonstrated that the FAD6 gene also exhibits low expression levels in young, developing, and ripening drupes in five other cultivars of olive tree (Hernandez et al., 2009). Thus, it is likely that OeFAD6 is marginally involved in linoleic acid production in Canino and Frantoio. As a consequence, FAD2.1 seems to be the most important enzyme for linoleic acid production in the two genotypes. The absence of significant differences in FAD6 compared with FAD2.1 and FAD2.2 transcript abundances during the oleanogenic period in the drupes of Moraioi does not exclude a contribution of FAD6 to linoleic acid production in this genotype. However, the observation that at the beginning of oil production FAD2.1 transcription was more abundant than that of FAD6 and FAD2.2 suggests a major role for FAD2.1 for linoleic production in this genotype too.

As in A. thaliana (Iba et al., 1993), ω3 FAD genes (i.e. the microsomal FAD3 and the plastidial FAD7) encoding FADs responsible for catalysing the conversion of linolenic into linoleic acid are present in olive tree; that is, in the drupe of cv. Koroneiki (Banilas et al., 2007), and of Canino, Frantoio, and Moraioi (this study). Another ω3 FAD gene
of the plastidial type, \textit{FAD8}, has been reported to be a cold-induced isozyme of \textit{FAD7} in many species (Matsuda \textit{et al.}, 2005, and references therein). Indeed, \textit{FAD8} transcripts were found here for the first time also in olive trees; however, their levels were too low to suggest a role for this gene in oil biogenesis.

In \textit{O. europaea} cv. Koroneiki, the expression of \textit{OeFAD3} has been reported to be high at early stages of drupe development (WAF 5), becoming almost undetectable from WAF 11 (Banilas \textit{et al.}, 2007). The present results confirm this temporal regulation. In fact, in the mesocarp of Canino, Frantoio, and Moraiole \textit{FAD3} was present, but at a low level, early in development (WAF 3), and it disappeared during stage 4 (i.e. already at WAF 11).

The absence of \textit{OeFAD3} transcripts in the epi-mesocarp tissue during the crucial period for oil biogenesis suggests a negligible contribution of \textit{FAD3} to the FA desaturation of storage lipids without significant differences related to genotype.

In contrast, \textit{OeFAD7} seems to be the \(\omega 3\) FAD mainly involved in linolenic acid production. In fact, \textit{OeFAD7} transcripts increased in the drupe during early oil biogenesis (WAFs 10–12), also showing a second, minor, increase near the end of the production phase (WAF 17). The histological observations about cell viability and the presence of chloroplasts provide support that an enhanced transcription of this plastidial \textit{FAD} occurs over time in the drupe, highlighting the importance \textit{FAD7} for olive oil polyunsaturation.

An important question is how this FAD, localized in the plastid, can provide storage lipids to the oil droplet in the cytoplasm, in contrast to their well known production in the ER (Ohlrogge and Browse, 1995). It is known that the galactolipids are the main components of plastid membranes, and are substrates for \textit{FAD7} activity (Hernandez \textit{et al.}, 2008, and references therein). The olive tree is an 18:3 plant; that is, it mainly contains linolate in the galactolipids of chloroplast membranes (Ohlrogge and Browse, 1995). It is known that the chloroplast is the first organelle to show senescence symptoms in the plant (Matile, 1992), and that the formation of oversized plastoglobuli is an early symptom of senescence (Murphy, 2001, and references therein). The present and previous (Rangel \textit{et al.}, 1997) cytohistological/ultrastructural results confirm the presence of plastoglobuli in the chloroplasts of the drupe. Moreover, it is shown in the present study that the plastoglobuli of the epi-mesocarp show plastoglobuli, large enough to be detectable by light microscopy, already at the first weeks of oleogenesis; that is at about the time of the highest \textit{FAD} transcription. Plastoglobuli are the oil bodies of the plastid, and contain TAGs, which are rich in linolenic acid (e.g. in \textit{Arabidopsis}; Kaup \textit{et al.}, 2002). Not only do their number and size increase with plastid senescence (Murphy 2001, and references therein), but, concomitantly, the activities of most of the lipases increase (e.g. in soybean; Guiamet \textit{et al.}, 1999). Thus, it is possible that in the plastoglobuli of the pulp cells of the olive the \textit{FAD7} desaturation of galactolipids is followed by the release of free linolenic acid by the activity of a galactolipid-hydrolysing enzyme (galactolipase). Galactolipase is known to liberate free linolenic acid in the chloroplasts of spinach induced to senescence by ozone treatment (Sakaki \textit{et al.}, 1990), and in normally senescing \textit{Arabidopsis} chloroplasts (Kaup \textit{et al.}, 2002). Because this free polyunsaturated FA acts as a potent inhibitor of the Hill reaction, as an uncoupler of photophosphorylation, and a mediator of thylakoid disintegration (Sakaki \textit{et al.}, 1990, and references therein), it might be sequestered into the TAGs of the plastoglobuli of the drupe’s chloroplasts to diminish its toxic functions. This hypothesis is sustained by the observation that a diacylglycerol acyltransferase (DGAT), the enzyme catalysing the terminal step of TAG biosynthesis, normally present in the ER, is active in the \textit{Arabidopsis} senescing chloroplasts, where it plays a role by sequestering FAs de-esterified from galactolipids into TAGs (Kaup \textit{et al.}, 2002). In accordance with this, two DGATs have been recently found in olive trees, with DGAT-2 particularly active in the late stages of mesocarp development (Banilas \textit{et al.}, 2010). In the ultrastructural study of Rangel and co-workers (1997), a granulate cytoplasm has been described near the chloroplasts. The present analyses showed that the granulate appearance of the cytoplasm was due to clumps of plastoglobuli exuded from the plastids. In agreement with this, exudation of plastoglobuli from senescing chloroplast membranes has been reported in spinach and soybean (Sakaki \textit{et al.}, 1990; Guiamet \textit{et al.}, 1999). Moreover, the present histological analysis shows that some chloroplasts turn into chromoplasts, confirming previous results in \textit{Arbequina} drupes (Gandul-Rojas \textit{et al.}, 1999), and that they too contain plastoglobuli. Plastoglobuli in the chromoplasts have also been observed in other plants [e.g. \textit{Fagus} and \textit{Spinacia}, Steinmüller and Tevini (1985)], and it has been reported that they are enriched in TAGs (Steinmüller and Tevini, 1985; Murphy, 2001, and references therein). Thus, plastoglobuli exuded from chloroplasts and chromoplasts might provide TAGs to the oil droplets in the cytoplasm in the olive pulp. On the other hand, \textit{Arabidopsis} mutants deficient in ER FADs contain polyunsaturated FAs derived from the chloroplasts (Ohlrogge and Browse, 1995), and linolenic acid is present in olives (Figs 9, 10) even if \textit{FAD3} is not expressed during oleogenesis (Banilas \textit{et al.}, 2007; this study).

\textbf{Cold regulates FAD expression during oil biogenesis, and a direct relationship between lipid polyunsaturation and genotype cold sensitivity seems to exist}

In \textit{Arabidopsis}, as in other plants, a large body of research suggests that temperature regulation of \textit{FAD} expression occurs at the transcriptional, post-transcriptional, and post-translational levels (Matsuda \textit{et al.}, 2005; Kargiotidou \textit{et al.}, 2008; Upchurch, 2008, and references therein). A transcriptional regulation of \textit{FAD} gene expression by lowering of the temperature is known for numerous species. An increase in the mRNA levels of \textit{FAD2} is induced under cold stress in cotton (Kargiotidou \textit{et al.}, 2008). The \textit{Arabidopsis fad2} mutant has decreased polyunsaturates in
membrane lipids, and long-term cold application results in its death (Miquel et al., 1993). The overexpression of Aifad7 in tobacco increases linolenic acid in leaf tissues and induces chilling tolerance (Kodama et al., 1995).

In addition, evidence is provided with the present results that cold stress affects the transcription of FAD genes during olive drupe development. During the first weeks of oil biogenesis, when FAD2.1, FAD2.2, FAD6, and FAD7 show high mRNA levels, an increase in FAD2.2 and FAD7 transcripts and a decrease in FAD2.1 and FAD6 transcripts occurred after a stress at 6 °C for 24 h. The results were similar in the compared genotypes (Canino and Moraiolo), and this similarity was in accordance with the fact that the mesocarp cells were not yet cold acclimated in both genotypes, as demonstrated by the presence of active calcium signalling in both.

Regarding the inverse effect of cold shock on FAD2.2 and FAD2.1, it has been reported that cold stress may also affect the transcription of the isoforms of the same gene in opposite ways, as in the case of Aifad7 and Aifad8 in Arabidopsis (Matsuda et al., 2005). Also when the cold stress was applied during the lowering of FAD transcription (WAF 19), the still living epi-mesocarp cells of Canino and Moraiolo showed rapid increases in FAD2.2 and FAD7, independently of their differences in cold acclimation. In fact, the cells of Canino were already cold acclimated, whereas this was not the case for those of Moraiolo, as shown by the presence of calcium signalling in the latter and not in the former.

To better understand the drupe response in terms of FAD transcription, genotype cold sensitivity, and the presence of unsaturated FAs, cold acclimation was experimentally induced in Canino cells, and the severe cold treatment was extended to the whole period crucial for FAD transcription (WAF 10–12) for both genotypes. Significant differences in linoleic and linolenic acids between the acclimated drupes of Canino and the non-acclimated drupes of Moraiolo were observed, with both linoleic and, mainly, linolenic acids increasing in the non-cold-tolerant genotype only. Considering that FAD2.2 and FAD7 are the FAD transcripts increasing with cold in both genotypes, and that they are conserved between the genotypes, the genotype-specific FA polyunsaturation pattern occurring in the drupes must be the effect of some sort of post-transcriptional control(s) between the genotypes. For example, a low temperature-dependent translational regulation of an FAD gene has been reported in wheat root tips (Horiguchi et al., 2000). Moreover, a temperature-sensitive mechanism that regulates the post-translational stability of FAD proteins is active in Arabidopsis leaf tissues (Matsuda et al., 2005). Also the half-life of FAD proteins of Brassica napus and tung tree increases at cooler temperatures, and this is due to a post-translational regulation involving degradation pathways (O’Quin et al., 2010). Coupled with the present results, it is possible that a higher production and/or stability of FAD2.2 and FAD7 are present in the drupes of Moraiolo in comparison with Canino, causing higher levels of polyunsaturates in the former and not in the latter. It is known that in the cells of numerous plants an increase in the production of polyunsaturated lipids after cooler temperatures might help them to survive because of their lower melting temperatures (Guschina and Harwood, 2006). Thus, the higher production of both these unsaturated FAs in the cold-shocked drupes of Moraiolo, resulting from increased activity of specific FADs, might be interpreted as a mechanism of homeoviscous adaptation directly related to the cold susceptibility of the genotype, and mainly involving storage lipids.

The absence of variation in unsaturated FAs in the extracts of Canino drupe is in accordance with the results obtained on virgin oil composition for another hardy genotype, cv. Arbequina (Ortega-García and Peragón, 2009), grown in the field with exposure to frost (Morello et al., 2003). In both cultivars (Arbequina and Canino), the cold acclimation machinery, still largely unknown in fleshy fruits, might act post-transcriptionally, causing a homeostasis of FAD proteins. This suggests that, when acquired, cold hardiness does not need further homeoviscous adaptation, resulting in no change in oil, and, possibly, membrane lipid unsaturation.

The NMR analysis adopted in the present study does not allow discrimination between storage and membrane lipids. However, it seems to reflect the FA composition of the storage lipids, because a large oil droplet occupying 60% of the cell volume was already present at the time of sampling (WAF 10) in the mesocarp cells. Nevertheless, in the cases in which cold had caused substantial changes in the unsaturated FAs of the membrane lipids, these changes should have been observed. Thus, it is possible that the membrane lipid composition of the pulp cells of the hardy Canino did not change in response to cold acclimation. Accordingly, no changes in FA unsaturation are detected in cold-acclimated needles of pine seedlings (Hellgren et al., 1984), and in the FA composition of the plasma membrane of the crown tissues of orchard grass (Yoshida and Uemura, 1984). Moreover, linolenic acid has been demonstrated not to be directly involved in the acquisition of freezing tolerance in the cytomembranes of the needles of the extremely hardy red pine (Martz et al., 2006). In contrast, FA unsaturation paralleled the increase in freezing tolerance during cold acclimation in willow stems (Hietala et al., 1998), revealing that there is controversy about the possible roles of changes in FAs, and unsaturation, in the membranes during cold acclimation. Moreover, it has been proposed that changes in FAs associated with cold acclimation might include increases in linoleic acid, and decreases in linolenic acid, as occurs in the plasma membrane phospholipids of Solanum species (Palta et al., 1993). The modulation of specific unsaturated FAs in lipid membranes by cold acclimation might also affect the functioning of integral membrane proteins, such as ATPases of the plasma membrane and internal membranes (Martz et al., 2006). However, both polyunsaturated FAs were stable under cold treatment in the extracts from the cold-acclimated drupes of Canino (Fig. 9). It might be possible that the resistance to cold in the membranes of the Canino drupe involves the
activity of membrane-associated proteins with a cryoprotectant function. For example, in Arabidopsis, the COR15a gene encodes a protein which interacts with lipids of the chloroplast envelope to maintain membrane function during freezing (Steponkus et al., 1998).

Moreover, a relationship might link the cold acclimation effects in membrane lipids, in particular of the chloroplast, to storage lipids, and alternatives to the hypothesis of post-transcriptional control(s) of FAD2.2 and FAD7 in the cold-acclimated Canino drupes exposed to cold are possible. Since the activity of galactolipase is enhanced under various stresses (Sakaki et al., 1990, and references therein), a higher amount of free linolenic acid might be produced in the chloroplast by a cold-enhanced galactolipase activity. This could cause, in turn, the activity of the microsomal FAD2.2 and of the plastidial FAD7 to restore the desaturated FA composition of the chloroplast membranes. The galactolipase-produced linolenic acid might be toxic, and, instead of being sequestered into chloroplast TAGs, and then into the cytosolic oil droplets (see above), as seems to occur in Moraiolo (Fig. 10), might either be converted to phloem-mobile sucrose (Lung and Weselake, 2006, and references therein) or become the substrate of lipoxygenase (LOX) activity (Stelmach et al., 2001). In both cases, the levels of the desaturated FAs in the drupe extracts of Canino should remain constant, as in fact observed (Fig. 9). In accordance with this, low levels of linolenic acid are required in chloroplasts for protecting photosynthesis from low-temperature damage (Routaboul et al., 2000). It is also known that cold acclimation requires high levels of sucrose to sustain growth, but also less abundant levels of its galactoside, raffinose, as a cryoprotectant (Welling and Palva, 2006). Moreover, LOX activity is known to produce oxylipins, such as jasmonates, as mediators in the stress response (Stelmach et al., 2001). Two LOXs have been recently characterized in the drupes of Arbequina (hardy) and Picual (cold-tolerant; Ortega-Garcia and Peragon, 2009). Both OepLOXs contain a chloroplastic transit peptide, and both utilize linolenic and linoleic acids as substrates (Padilla et al., 2009). The transcript levels of one of the two (Oep2LOX2), after an increase in both cultivars, dramatically decrease only in the cold-tolerant cultivar, showing differences between the genotypes related to cold hardiness.

In conclusion, in the oleogenic fruit of this species, the response to cold also induces changes in FAD transcription in the presence of cold acclimation. A direct relationship between FAD expression and lipid desaturation seems to exist in the drupes of the cold-sensitive genotype, and an inverse relationship in those of cold-resistant genotype, suggesting that cold acclimation requires a fine post-transcriptional regulation of FAD.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Early (A–C) and late (D) events related to drupe growth and maturation in cv. Frantoio, cv. Canino, and cv. Moraiolo (histological, longitudinal sections observed under light microscopy after toulidine blue staining). A. Ovary growth by cell division soon after fertilization (stage 1, WAF 1, cv. Frantoio). B. Detail of mesocarp dividing cells at WAF 2 (cv. Frantoio). C. Detail of the internal endocarp with the inner epidermis and the seed coat differentiation at the end of WAF 2 (cv. Canino). D. Detail of the mesocarp at the drupe maturation stage. The expanded cells exhibit the central oil droplet (o) and are separated by wide intercellular spaces. (WAF 20, cv. Moraiolo). Bars=100 μM (A), 50 μM (B, C), 10 μM (D).

Figure S2. (A) Expression profiles by QRT-PCR of the transcripts of FAD2.1, FAD2.2, FAD3, FAD6, FAD7, and FAD8 (in the inset) in drupes of Olea europaea cv. Frantoio at the onset of stage 2 (WAF 3). (B) Expression profiles by QRT-PCR of the transcripts of FAD3 and FAD8 in the epi-mesocarp tissues from O. europaea cv. Frantoio drupes at the end of stage 3 (WAF 10). Data are reported as mean values of RNA abundance, expressed on a log scale, after normalization with β-actin, from two independent experiments and three technical replicates for each experiment. Error bars represent the SEM; when not shown, they are too small to be seen.

Figure S3. Sequence alignment of FAD2.1 (A), FAD2.2 (B), FAD6 (C), and FAD7 (D) in the epi-mesocarp of cv. Canino (Can) and cv. Moraiolo (Mor) drupes (Accession Number GenBank FAD2.1 cv. Moraiolo HQ908422; FAD2.2 cv. Canino HQ908423; FAD6 cv. Canino HQ889831; FAD7 cv. Canino HQ889832). Nucleotides differing between the corresponding sequences in the two genotypes are marked in grey. Sequences were analysed by CodonCode Aligner 3.6.1 and BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Figure S4. Expression profiles by QRT-PCR of FAD3 transcripts in the epi-mesocarp tissues of drupes of cv. Canino and cv. Moraiolo after exposure at 6 °C for 24 h at WAF 10 (A), and after exposure at 6 °C for 72 h at WAF 19 (B) (white columns). Black columns show the expression profiles in control drupes grown at 30 °C, at the onset of the experiment (0 h) and at 24 h in A. Control drupes grown at 25 °C for 0, 6, and 72 h at WAF 19 (B) did not show any FAD3 transcript. Data are reported as mean values of RNA abundance, expressed on a log scale, after normalization with β-actin, from two independent experiments and three technical replicates for each experiment. Error bars represent the SEM; when not shown they are too small to be seen.

Table S1. Primers (F1–F27) used to amplify FAD transcripts by RT-PCR from the total RNA of epi-mesocarp cells of olive tree drupes.

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