RESEARCH PAPER

The utilization and desaturation of oleate and linoleate during glycerolipid biosynthesis in olive (Olea europaea L.) callus cultures

M. Luisa Hernández1,2, Irina A. Guschina2, José M. Martínez-Rivas1, Manuel Mancha1 and John L. Harwood2,*

1 Instituto de la Grasa (CSIC), Av. Padre Garcia Tejero 4, E-41012 Seville, Spain
2 School of Biosciences, Cardiff University, Cardiff CF10 3AX, UK

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Abstract

Callus cultures from olive (Olea europaea L.) were used to study characteristics of desaturation in this oil-rich tissue. The incorporation of [1-14C]oleate and [1-14C]linoleate into complex lipids and their further desaturation was followed in incubations of up to 48 h. Both radiolabelled fatty acids were rapidly incorporated into lipids, especially phosphatidylcholine and triacylglycerol. Radiolabelling of these two lipids peaked after 1–4 h, after which it fell. In contrast, other phosphoglycerides and the galactosylglycerides were labelled in a more sustained manner. [1-14C]Linoleate was almost exclusively found in the galactolipids. With [1-14C]oleate as a precursor, the only significant desaturation to linolenate was in the galactolipids. Monogalactosyldiacylglycerol was the first lipid in which [1-14C]linoleate and [1-14C]linolenate appeared after incubation of the calli with [1-14C]oleate and [1-14C]linoleate, respectively. The presence of radioactivity in the plastidial lipids shows that both [1-14C]oleate and [1-14C]linoleate can freely enter the chloroplast. Two important environmental effects were also examined. Raised incubation temperatures (30–35 °C) reduced oleate desaturation and this was also reflected in the endogenous fatty acid composition. Low light also caused less oleate desaturation. The data indicate that lysophosphatidylcholine acyltransferase is important for the entry of oleate and linoleate into olive callus lipid metabolism and phospholipid: diacylglycerol acyltransferase may be involved in triacylglycerol biosynthesis. In addition, it is shown that plastid desaturases are mainly responsible for the production of polyunsaturated fatty acids. Individual fatty acid desaturases were differently susceptible to environmental stresses with FAD2 being reduced by both high temperature and low light, whereas FAD7 was only affected by high temperature.

Key words: Callus culture, chloroplast lipids, desaturation, light, linoleate, microsomal lipids, oleate, olive, temperature.

Introduction

Olive oil is one of the world’s major edible oils and is pre-eminent in the Mediterranean region (Gunstone et al., 2007). The olive tree (Olea europaea L.) produces an oil in its fruits which is highly enriched in oleic acid (55–83%), although it also contains variable amounts of linoleic acid (3.5–21%) but less than 1% of α-linolenic acid (European Commission Regulation, 2003). The oil is highly prized for its sensory and nutritional properties (Harwood and Aparicio, 2000) and, as such, the best quality ‘virgin’ oils attract premium prices. The polyunsaturated fatty acids have an important effect on the oil quality. Elevated linoleic acid content negatively affects the technological properties of the oil, such as the oxidative stability, and also has important nutritional characteristics (Cunnane, 2003). On the other hand, the low levels of α-linolenic acid are essential for aroma biogenesis during the milling and malaxation processes to obtain virgin olive oil (Olias et al., 1993).

Fatty acid synthesis in plants begins with the de novo formation of palmitate and stearate in the plastid. The stearate is then desaturated by stearoyl-ACP Δ9-desaturase. The oleate product can either be utilized within the plastid for lipid assembly and further desaturation, or

* To whom correspondence should be addressed. E-mail: harwood@cardiff.ac.uk

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exported to allow desaturation on the endoplasmic reticulum as well as lipid formation via the Kennedy pathway (Gunstone et al., 2007) as well as new, alternative, acyl-CoA-independent mechanisms such as those involving phospholipid:diacylglycerol acyltransferase (PDAT) (Dahlqvist et al., 2000) or diacylglycerol:diacylglycerol transacylase (DGTA) activities (Stobart et al., 1997). The individual desaturases responsible for the formation of linoleate and α-linolenate from oleate have been identified, and utilize complex lipid substrates (Wallis and Browse, 2002). The principal substrates are phosphatidylcholine (PC) in the endoplasmic reticulum and monogalactosyldiacylglycerol (MGDG) in the plastid.

Unlike oilseeds, little is known of the fatty acid and glycerolipid biosynthesis in oil fruits. Olive fruit represents an interesting system to investigate fatty acid and lipid metabolism, since it possesses the remarkable characteristic of having high triacylglycerol (TAG) content together with active chloroplasts (Sánchez, 1994). Moreover, because the olive mesocarp is photosynthetic and, indeed, provides directly about half of the total carbon for oil synthesis (Harwood and Sánchez, 2000), then it contains membranes which are highly unsaturated. However, the olive fruit is a difficult plant material to work with since great physiological changes take place in the mesocarp during the long developing and ripening period. The initial green tissue varies to green-purple and then to dark purple while oil accumulation occurs.

Previously, callus cultures derived from olive seeds have been used as a convenient way of studying different aspects of fatty acid and lipid metabolism. The advantages of using such cultures and the validity of results obtained with them have been discussed (Williams and Harwood, 2000). They have been used to examine fatty acid biosynthesis, phospholipid metabolism (Salas et al., 2000) and the generation of volatiles via the lipoxygenase pathway (Sánchez and Salas, 2000). Photomixotrophic olive callus cultures are characterized by the presence of the typical chloroplastic and non-chloroplastic membrane lipids, together with the accumulation of TAG, which is unusual in plant tissue cultures (Williams et al., 1993). Thus, callus cultures can be a good model system to study lipid metabolism using radioactive precursors as an alternative to young olive mesocarp, since the use of tissue slices has the disadvantage of introducing disturbing factors into the results as a consequence of the wounding metabolism elicited upon excising the mesocarp tissue (Sánchez, 1994). Accordingly, in order to determine aspects of the biosynthesis of the polyunsaturated fatty acids, linoleate and α-linolenate, callus cultures have been used to study the desaturation pathways, with oleate and linoleate as radioactive precursors. These results clearly demonstrate the important contribution of plastidial fatty acid desaturation in olive and also the influence of light and temperature on this process.

### Materials and methods

#### Plant material

Olive (Olea europaea L. cv. Picual) fruits were collected 22 weeks after flowering and used to establish photomixotrophic olive calli, as reported by Williams et al. (1993). Olive callus cultures were grown on modified MS medium (Murashige and Skoog, 1962), supplemented with 6 μM 2,4-dichlorophenoxyacetic acid and 0.28 μM benzylaminopurine. The cultures were grown at 25 °C with a 12/12 h light/dark cycle and the light intensity was 11.5 μmol m⁻² s⁻¹ (standard conditions). Calli were subcultured every 28 d and used for experiments 20–25 d after subculturing.

For temperature experiments, the calli were cultured in the range 20–35 °C at the standard light intensity. To assess the effect of light, illumination was at 130 μmol m⁻² s⁻¹ or at 1.42 μmol m⁻² s⁻¹, and the calli maintained at the standard temperature. Both incubations were for 48 h.

#### Radiolabelling protocol

Callus cultures were selected for uniformity of mass and morphological appearance. Replicated samples of about 300 mg fresh weight (FW) were used for each treatment. The [1-¹⁴C] fatty acids were dissolved in ethanol, and 3 μl of this solution containing the appropriate amount of the radioactive precursor were diluted in 120 μl of 100 mM sorbitol and placed on to the surface of the callus. Incubations were conducted under the standard growing conditions or under different temperatures and light intensities. At the end of the incubation period, the calli were rinsed briefly with distilled water to remove any unimbibed radiolabel and transferred into screw-capped glass tubes. Two ml isopropanol were added to the calli before heating at 70 °C for 30 min, to inactivate endogenous lipase activity.

#### Lipid extraction and analysis

Lipids were extracted by a high-salt extraction method (Garbus et al., 1963) as modified for plant tissues (Smith et al., 1982). This extraction method has been shown to be very efficient for plant acyl lipids, including highly polar components. Lipid separation was carried out by thin-layer chromatography (TLC) using pre-coated (E Merck, Darmstadt, Germany) silica gel plates, which were activated at 110 °C for 1 h prior to use. Non-polar lipids were purified by one-dimensional TLC, using toluene/hexane/formic acid (70:30:0.5, by vol.) for the first development to whole plate height, followed by a second development with hexane/diethyl ether/formic acid (60:40:1, by vol.) to half height. Polar lipids were separated by two-dimensional TLC using chloroform/methanol/water (65:25:4, by vol.) in the first dimension, and then chloroform/acetone/methanol/acetic acid/water (25:10:5:5:2.5, by vol.) for the second direction.

Individual lipids were visualized under iodine vapour and the identification was made by reference to authentic standards. After scraping off the corresponding bands, the radioactivity was determined by liquid-scintillation counting, and the fatty acid composition was determined as described below.

Fatty acid methyl esters (FAMEs) of the total lipid fraction and the individual lipid classes were produced by acid-catalysed transesterification (Williams et al., 1993). In the time-course experiments, the FAMEs were separated on AgNO₃-TLC. The plates were prepared by pouring 10 ml of 10% AgNO₃ in acetonitrile onto silica gel 60 plates, activating at 110 °C for 1 h, and developing with hexane/diethyl ether (75:25, v/v). The percentage of [1-¹⁴C] fatty acids was determined on the TLC plates by autoradiography, using an Instant Imager (Packard Instrument Co., Meriden, CT, USA). In the different temperature and light intensity experiments,
the FAMEs were analysed by radio-GLC, using a Pye Unicam GCD gas chromatograph connected to a Raytest Raga gas flow proportional counter (LabLogic Systems Limited, Sheffield, UK). A glass column (1.5 m × 3.0 mm) packed with 10% SP-2330 on 100/120 mesh Supelcoport (Supelco, Faffron Walden, Essex, UK) was used to separate individual FAMEs. The system was normally operated with an argon/CO₂ [95:5, v/v, 45 ml min⁻¹] gas carrier using an oven temperature of 180 °C.

**Determination of radioactivity**

Radioactivity was determined by liquid-scintillation counting. The samples were assayed in 10 ml of Optiphos scintillant (Canberra Packard, Pangbourne, Berks., UK). Quench correction was made automatically by the external-standard channels-ratio method.

**Results**

**Uptake and incorporation of [1-14C]oleate into callus lipids**

When [1-14C]oleate was added to the calli, it was rapidly taken up so that, by 4 h, the total lipids were labelled to the maximum extent (Fig. 1), and the labelled free fatty acid content was negligible after 1 h (data not shown). Radioactivity also accumulated in the aqueous phase (probably due in part to labelling of acyl-thiolesters) and was maintained at low but constant levels throughout the incubations (data not shown).

The general incorporation of radioactivity is reflected in the labelling of individual lipid classes (Fig. 1). After 10 min of incubation [1-14C]oleate was incorporated into all major lipids. Within the polar lipids, phosphatidylcholine (PC) was the most highly labelled and this radiolabelling peaked at 4 h. Thereafter, there was a significant loss of label (about 50%) within the remaining incubation period (Fig. 1A). The other major polar lipid classes labelled were phosphatidylethanolamine (PE), phosphatidate (PA), phosphatidylinositol (PI), monogalactosyldiacylglycerol (MGDG), and diacylgalactosyldiacylglycerol (DGDG). All of these classes were also rapidly labelled within the first 4 h of incubation but none showed the significant loss of label seen by PC in the remaining 44 h of incubation.

For the non-polar neutral lipids, triacylglycerol (TAG) was best labelled. It showed its highest radioactivity at 4 h, followed by a 50% reduction by 24 h (Fig. 1B). Both monoacylglycerol (MAG) and diacylglycerol (DAG) were significantly labelled. DAG showed a peak in labelling by 1 h, followed by a decline to 4 h, after which radioactivity remained fairly constant.

Once [1-14C]oleate is incorporated into complex lipids, it has the potential for desaturation by the different desaturases that use acyl lipids as substrates. In Fig. 2A the desaturation to linoleate (Δ12-desaturation) and to α-linolenate (Δ15-desaturation) is shown. For the phosphoglycerides, only PC and PE showed any significant desaturation of oleate to linoleate within the first 8 h of incubation. By 24 h PA and PI also contained significant [1-14C]linoleate and this increased for all phosphoglycerides by 48 h. Only small amounts of radiolabelled α-linolenate were found in any phosphoglyceride by 48 h.

For the galactosyglycerides, there was much more rapid desaturation. In MGDG, [1-14C]oleate rapidly declined so that, by 1 h a peak in the formation of [1-14C]linoleate was seen which then rapidly declined (Fig. 2A). This decrease in the formation of [1-14C]linoleate was followed by a rapid accumulation of [1-14C]α-linolenate. Thus, MGDG was the first lipid in which [1-14C]linoleate and [1-14C]α-linolenate were detected. The accumulation of polyunsaturated fatty acids, especially [1-14C]linoleate and [1-14C]α-linolenate, in DGDG was also noticeable but was less than (and lagged behind) that of MGDG (Fig. 2A).

For the non-polar lipids, [1-14C]linoleate peaked in both DAG and MAG by 8 h with the former being better labelled (Fig. 2B). Very little [1-14C]linoleate was found in TAG, even by 48 h. In addition, no accumulation of [1-14C]-linolenate was detected in non-polar lipids.
Uptake and incorporation of [1-14C]linoleate into callus lipids

In the case of [1-14C]linoleate precursor, the radioactivity was also low in the aqueous phase and the content of labelled free fatty acid negligible after 1 h (data not shown). [1-14C]Linoleate was incorporated after 10 min of incubation into all the major lipids, showing a pattern of incorporation into lipid classes (Fig. 3) broadly similar to that of [1-14C]oleate (Fig. 1).

Thus, of the phosphoglycerides, PC was rapidly and well labelled (Fig. 3A). Maximum labelling was by 2 h, after which about half of the total label was lost by 48 h. As with [1-14C]oleate labelling (Fig. 1A), PE was the next best labelled polar lipid. It showed similar labelling to PC by 24 h (Fig. 3A). Unlike PC, none of the other polar lipids lost large amounts of radioactivity after 2 h of incubation. In fact, the two galactosylglycerides (MGDG and DGDG) accumulated more radiolabel within this period.

Of the non-polar lipids, TAG was rapidly labelled from [1-14C]linoleate (Fig. 3B) and, as with [1-14C]oleate labelling (Fig. 1B), had lost about half its radioactivity by 24 h. Although DAG and MAG were appreciably labelled, their total radioactivity was considerably less than that of TAG.

Regarding [1-14C]linolenate desaturation, after its incorporation into complex lipids, only small amounts of [1-14C]linolenate were found in the various phospholipid fractions (Fig. 4). By contrast, [1-14C]linolenate rapidly accumulated in MGDG, reaching over 50% of the total radiolabelled fatty acids by 4 h and over 80% at 48 h. The same pattern of [1-14C]linolenate accumulation was seen in DGDG which had a final composition with equal amounts of radioactive linoleate and linolenate (Fig. 4). No detectable accumulation of [1-14C]linolenate was found in any non-polar lipid fraction (data not shown), in agreement with the data for [1-14C]oleate incorporation (Fig. 2B).

Effect of growth temperature on fatty acid desaturation

Olive calli were incubated at temperatures in the range 20–35 °C with [1-14C]oleate for 48 h. The results for different temperatures were compared statistically with those at 25 °C (standard incubation conditions). Increases in temperature to 30 °C or 35 °C caused a significant decrease in the levels of [1-14C]linoleate, which was
paralleled by a rise in [1-14C]oleate (Fig. 5A). This alteration was also reflected in the pattern of total endogenous fatty acids, where, in addition, α-linolenate was also decreased at 30 °C and 35 °C (Fig. 5B).

The major lipid classes were separated and their radioactive acyl components analysed following incubation of calli with [1-14C]oleate at different temperatures for 48 h (Table 1). Compared with cultures at 25 °C, incubation temperature had little effect on the total radioactive labelling of individual lipid classes (data not shown). Although the data for fatty acid radiolabelling in individual lipid classes varied, a general trend for less desaturation of [1-14C]oleate at 30 °C or 35 °C compared with 25 °C was found. A different temperature effect on extra-plastidial (microsomal) compared to plastidial lipids was observed (Table 1). The percentage of [1-14C]linoleate decreased in those lipids mainly occurring in the extra-plastidial compartment (PC, PE, PA, DAG, and TAG) as the temperature rose, but remained essentially unchanged in the plastidial galactolipids (MGDG and DGDG). In addition, the percentage of [1-14C]linoleate increased in PE, and to a lesser degree in PC and PA, as the temperature rose, but decreased in MGDG and DGDG.

The endogenous fatty acid patterns for different lipid classes in calli are often quite distinct and, not surprisingly, the effects of growth temperature varied from lipid to lipid (Table 2). A general observation was that the proportion of oleate tended to increase at 30 °C and 35 °C, commensurate with a decrease in PUFAs. In the phosphoglycerides, the increase in oleate was accompanied by a decrease in linoleate, but there was little alteration in the percentage of α-linolenate (Table 2). These changes were similar to those found for radioactive labelling (Table 1).

Effect of light intensity on fatty acid desaturation

Light is known to affect fatty acid synthesis in plants and there have been a number of studies on its overall influence on the rate of biosynthesis (Harwood, 1998). However, less is known about any detailed effects on fatty acid desaturation. When the olive calli were incubated under two distinct light regimes, there was a noticeable increase in conversion of [1-14C]oleate to [1-14C]linoleate
The endogenous fatty acids were also analysed. The most consistent effect of light was an increase in the proportion of linoleate under high light intensity (Table 4).

Discussion

Oleate and linoleate are utilized for glycerolipid biosynthesis in olive callus cultures with a significant contribution of LPCAT and PDAT activities

Olive calli were rapidly labelled from both [1-14C]oleate and [1-14C]linoleate under the experimental conditions, with the highest incorporation into both the major labelled polar lipid, PC, and the main non-polar lipid, TAG, occurring by 2–4 h (Figs 1, 3). These two lipid classes lost significant amounts of radioactivity in the remaining incubation period, in agreement with the central role of PC as a key intermediate in lipid metabolism and the possible reutilisation of TAG through the reaction
Table 2. Effect of temperature on the fatty acid composition of endogenous lipid classes in olive calli

| Lipid class | Temperature (°C) | 16:0 | 18:0 | 18:1 | 18:2 | α-18:3 |
|-------------|-----------------|------|------|------|------|--------|
|             |                 | 16:0 |      |      |      |        |
|            |                 | 18:0 |      |      |      |        |
| DAG         | 20              | 45.6±1.9 | 19.8±1.9<sup>a</sup> | 15.0±1.5<sup>a</sup> | 5.0±1.0 | 14.7±0.6<sup>a</sup> |
|             | 25              | 44.6±0.3 | 12.6±2.9 | 19.0±0.5 | 5.4±1.0 | 18.5±1.1 |
|             | 30              | 51.2±2.5<sup>a</sup> | 16.2±0.1<sup>a</sup> | 16.4±0.8<sup>a</sup> | 2.5±0.2<sup>a</sup> | 13.8±1.6<sup>a</sup> |
|             | 35              | 45.0±5.1 | 5.5±1.8<sup>a</sup> | 21.9±1.1 | 2.8±0.8<sup>a</sup> | 24.8±4.9<sup>a</sup> |
| TAG         | 20              | 45.6±4.2 | 17.6±0.8 | 16.9±3.8 | 2.6±0.2 | 17.4±2.6 |
|             | 25              | 40.0±1.1 | 16.9±2.9 | 19.1±0.6 | 2.5±0.8 | 21.5±2.5 |
|             | 30              | 37.7±3.1 | 18.2±1.5 | 19.3±1.9 | 2.0±0.2 | 22.8±3.7 |
|             | 35              | 28.6±3.1<sup>a</sup> | 10.2±2.4<sup>a</sup> | 28.4±4.5<sup>a</sup> | 2.2±0.3 | 30.6±0.7<sup>a</sup> |
| PA          | 20              | 15.6±6.8 | 7.3±1.4 | 38.2±3.9 | 15.8±1.8<sup>a</sup> | 23.1±1.7 |
|             | 25              | 23.3±1.6 | 7.0±0.7 | 34.6±0.8 | 12.1±1.0 | 23.9±1.2 |
|             | 30              | 20.3±4.0 | 11.0±2.3<sup>a</sup> | 42.0±3.6<sup>a</sup> | 7.1±1.4<sup>a</sup> | 19.7±2.4 |
|             | 35              | 24.1±1.0 | 5.0±0.3<sup>a</sup> | 43.5±1.0<sup>a</sup> | 4.4±0.2<sup>a</sup> | 23.8±0.5 |
| PC          | 20              | 22.8±4.8 | 7.6±1.2 | 36.1±4.8 | 12.6±1.7 | 20.9±2.9 |
|             | 25              | 20.3±0.9 | 7.0±1.5 | 35.2±2.2 | 12.5±1.7 | 25.0±3.1 |
|             | 30              | 14.5±4.2 | 7.8±2.0 | 46.7±1.4<sup>a</sup> | 7.6±1.1<sup>a</sup> | 23.5±1.4 |
|             | 35              | 28.6±0.2<sup>a</sup> | 4.9±0.6 | 43.4±1.8<sup>a</sup> | 3.7±0.9<sup>a</sup> | 19.5±2.3 |
| PE          | 20              | 30.1±1.0<sup>a</sup> | 17.2±0.1<sup>a</sup> | 14.8±0.4<sup>a</sup> | 17.7±1.2<sup>a</sup> | 20.2±0.1<sup>a</sup> |
|             | 25              | 24.0±1.1 | 11.8±1.4 | 19.3±1.9 | 15.9±0.9 | 29.0±0.7 |
|             | 30              | 30.3±5.1<sup>a</sup> | 8.1±1.0<sup>a</sup> | 28.1±2.4<sup>a</sup> | 10.2±1.6<sup>a</sup> | 23.4±0.1<sup>a</sup> |
|             | 35              | 29.0±1.8<sup>a</sup> | 4.0±0.2<sup>a</sup> | 24.8±0.7<sup>a</sup> | 8.5±0.1<sup>a</sup> | 33.7±2.2<sup>a</sup> |
| MGDG        | 20              | 5.3±0.7 | 3.2±0.5 | 3.7±1.7 | 1.5±10.8 | 86.3±2.8 |
|             | 25              | 6.4±1.9 | 3.2±1.1 | 3.4±0.4 | 1.3±0.4 | 85.7±3.6 |
|             | 30              | 6.5±1.0 | 3.8±0.4 | 4.3±0.2<sup>a</sup> | 1.1±0.3 | 84.3±2.0<sup>a</sup> |
|             | 35              | 6.7±0.3 | 1.8±0.5<sup>a</sup> | 7.6±0.7<sup>a</sup> | 7.7±0.0<sup>a</sup> | 76.2±1.6<sup>a</sup> |
| DGDG        | 20              | 19.9±2.7 | 8.0±0.1 | 9.5±2.0 | 2.1±0.7 | 60.5±0.1 |
|             | 25              | 21.5±1.2 | 7.1±1.6 | 8.6±0.0 | 2.2±0.0 | 60.7±0.4 |
|             | 30              | 25.3±1.7<sup>a</sup> | 14.5±0.1<sup>a</sup> | 7.6±0.3 | 1.6±0.1 | 51.0±1.4<sup>a</sup> |
|             | 35              | 25.9±1.5<sup>a</sup> | 9.7±1.5 | 12.1±0.3<sup>a</sup> | 4.2±0.4<sup>a</sup> | 48.1±2.2<sup>a</sup> |

<sup>a</sup> Indicates significantly different from 25 °C incubations (P < 0.05) by Student’s t test.

catalysed by diacylglycerol transacylase (DGTA) (Stobart et al., 1997). However, not all the decrease observed in radiolabelled PC and TAG could be accounted for by transfer to other lipid classes (Figs 1, 3) or by accumulation of radioactivity in the aqueous fraction (data not shown). This suggests that catabolism of these radioactive lipids is occurring, possibly by β-oxidation.

The rapid labelling of PC, but not of intermediates of the Kennedy pathway, suggests that lysophatidylcholine acyltransferase activity (LPCAT) is mainly responsible for the entry of the radioactive precursors into lipid metabolism by the olive calli. This is in agreement with previous data obtained using microsomal preparations from olive mesocarp (Sanchez et al., 1992) and with the high activity of this enzyme in other plants (Kjellberg et al., 2000; Montgrand et al., 2000). The low rate of labelling of DAG at the same time that TAG was being rapidly labelled could indicate that the contribution of the phospholipid: diacylglycerol acyltransferase (PDAT) reaction to TAG biosynthesis is significant (Weselake, 2005) although it has to be noted that measurement of this enzyme in vitro in subcellular fractions from olive calli indicated only low activity (Ramli et al., 2005).

Certainly the present data are in agreement with the conclusion that diacylglycerol acyltransferase (DAGAT) exerts significant flux control over TAG formation in olives (Ramli et al., 2005).

**Plastids are the preferential site for oleate and linoleate desaturation in olive callus culture**

MGDG was the first lipid in which [1-14C]linoleate appeared after incubation of the calli with [1-14C]oleate (Fig. 2) indicating a preferential site for oleate desaturation in the plastids. This would use the plastidial oleate desaturase (FAD6) rather than the endoplasmic reticulum oleate desaturase (FAD2) (Wallis and Browse, 2002). Afterwards, the proportion of [1-14C]linoleate in MGDG decreased, commensurate with the rise in [1-14C]linolenate, indicating a high plastidial linoleate desaturase (FAD7) activity. Labelling of DGDG for both [1-14C]linoleate and [1-14C]linolenate lagged behind that of MGDG, in keeping with its formation from the latter (Dörmann, 2005). The presence of radioactivity in the plastidial lipids indicates that both [1-14C]oleate and [1-14C]linoleate can enter the chloroplast, although details of this process remain to be elucidated. This is in agreement with the fact that olive is a so-called 18:3 plant, in which chloroplast lipids are synthesized using fatty acids produced in the organelle, but where import of fatty acids, especially linoleate, from the endoplasmic
through oleate is the main product of the de novo and Browse, 1996; Miquel and Browse, 1992) Even et al. reticulum is also important (Slack FW) under standard temperature for 48 h at high (130 μmol m⁻² s⁻¹) or under high light (130 μmol m⁻² s⁻¹). Means ±SDs for five independent experiments are shown.

Table 3. Effect of light intensity on the incorporation of [1-¹⁴C]oleate into lipids and further desaturation in olive callus cultures

Calli were incubated with [1-¹⁴C]oleate as indicated for Fig. 6. Incubations were for 48 h at low light intensity (1.42 μmol m⁻² s⁻¹) or under high light (130 μmol m⁻² s⁻¹). Means ±SDs for five independent experiments are shown.

| Lipid class | Light intensity | [¹⁴C] fatty acid labelling (%) |
|-------------|----------------|-----------------------------|
|             | 18:1           | 18:2                        | α-18:3 |
| DAG         | High           | 78.5±5.4                    | 21.5±3.5 | ND² |
|             | Low            | 86.4±5.7⁴                   | 13.6±2.2 | ND² |
| TAG         | High           | 82.1±1.3                    | 17.9±1.3 | ND² |
|             | Low            | 87.5±0.9⁵                   | 12.5±1.0 | ND² |
| PA          | High           | 64.0±1.8                    | 29.4±0.4 | 6.6±1.9 |
|             | Low            | 65.6±2.8                    | 24.0±0.8 | 10.4±2.2⁴ |
| PC          | High           | 71.1±5.2                    | 27.0±5.0 | 1.9±0.4 |
|             | Low            | 69.7±6.5                    | 24.2±6.4 | 6.1±1.0⁴ |
| PE          | High           | 34.8±3.1                    | 47.7±5.4 | 17.6±3.6 |
|             | Low            | 47.3±2.5⁴                   | 37.3±1.5 | 15.4±3.1 |
| MGDG        | High           | 21.6±4.5                    | 9.2±2.2  | 69.2±6.8 |
|             | Low            | 15.5±1.3⁴                   | 6.35±1.6 | 78.2±6.1 |
| DGDG        | High           | 32.5±4.1                    | 11.9±0.2 | 55.6±4.3 |
|             | Low            | 37.6±1.5⁵                   | 10.3±0.4 | 82.1±1.3 |

⁴ Indicates significantly different for the two light regimes (P < 0.05) by Student’s t test.
² ND, not detected.

Fig. 6. Effect of light intensity on the fatty acid composition of the radiolabelled (A) and non-radioactive total lipids (B) of olive callus cultures. Calli were incubated with [1-¹⁴C]oleate (1 μCi; 60 pmol mg⁻¹ FW) under standard temperature for 48 h at high (130 μmol m⁻² s⁻¹) (white bars), and low (1.42 μmol m⁻² s⁻¹) light intensity (black bars). Results are means ±SD of five independent experiments. Statistically significant differences (ANOVA test) (P < 0.05) comparing light intensities are indicated by an asterisk.

indicate that PE could also be a substrate as suggested for oleate desaturation in safflower (Sanchez and Stumpf, 1984). Alternatively, linoleate could be transferred preferentially from PC to PE to allow its accumulation in the latter.

Within the non-polar lipids, [1-¹⁴C]linoleate was found in both MAG and DAG but hardly at all in TAG. No significant [1-¹⁴C]linolenate was found in any of the non-polar lipids showing that there was negligible transfer of this fatty acid from its site(s) of formation. There are two explanations for the presence of [1-¹⁴C]linoleate in DAG but not in TAG. First, both DAGAT and PDAT may have a low selectivity for substrates containing linoleate. However, TAG was rapidly labelled when [1-¹⁴C]linoleate was used (see below) making this explanation unlikely. Second, the [1-¹⁴C]linoleate-labelled DAG may originate in the plastid and, therefore, not be available for TAG formation on the endoplasmic reticulum. This would also be consistent with the rapid formation of [1-¹⁴C]linoleate via the plastidial oleate desaturase, as discussed above.

Exogenously-supplied [1-¹⁴C]linoleate was rapidly incorporated into both PC and TAG (Fig. 3). Radioactivity was lost from both of these lipids at later incubation times but without transfer to other lipid classes, again indicating significant catabolism, as noted before. Compared to the [1-¹⁴C]oleate time-course, PE was rather better labelled by [1-¹⁴C]linoleate. This agreed with the preferential accumulation of [1-¹⁴C]linoleate in PE rather than PC during the [1-¹⁴C]oleate time-course (Fig. 2).
The rapid labelling of TAG during the $[1-^{14}C]$linoleate time-course (Fig. 3) shows that any $[1-^{14}C]$linoleoyl-CoA generated from exogenous sources within the cytosol is easily available. Since DAG is not well labelled, there is either a high turnover of this intermediate or, more likely, TAG can be formed from radiolabelled PC using PDAT. The negligible labelling of $[1-^{14}C]$linoleate in TAG during the $[1-^{14}C]$oleate time-course but excellent labelling from exogenous $[1-^{14}C]$linoleate is fully consistent with a preferential site for oleate desaturation in the plastids of olive calli, and also indicates that $[1-^{14}C]$linoleate-labelled DAG should be generated in the plastid, as discussed above.

Generation of $[1-^{14}C]$linoleate-labelled MGDG allowed a rapid formation of $[1-^{14}C]$linolenate, in keeping with this galactolipid being a substrate for the plastid linoleate desaturase FAD7 (Jones and Harwood, 1980). Again, as with the $[1-^{14}C]$oleate time-course (Fig. 2), the labelling of DGDG was consistent with it being formed from MGDG (Dörmann, 2005) rather than the former lipid being a desaturase substrate itself (Fig. 4). No other lipid class contained any significant $[1-^{14}C]$linoleate during the $[1-^{14}C]$linoleate time-course, commensurate with the formation of $[1-^{14}C]$linolenate on MGDG (Jones and Harwood, 1980) and the preferential accumulation of linolenate in the galactosylglycerides (Hitchcock and Nichols, 1971). In addition, the high level of $[1-^{14}C]$linolenate detected in galactolipids, compared with the low level found in microsomal lipids indicates that the activity of the FAD7 enzyme is much higher in olive than that of the microsomal linoleate desaturase (FAD3).

**Temperature and light intensity differentially affect FAD2 and FAD7**

The effect of temperature on fatty acid desaturation in olive calli was compared to incubations at 25 °C. The desaturation of $[1-^{14}C]$oleate to $[1-^{14}C]$linoleate was significantly reduced at 30 °C and 35 °C but, interestingly, without effect on the proportion of $[1-^{14}C]$linolenate (Fig. 5A). Both linoleate and $\alpha$-linolenate were reduced in the endogenous lipids (Fig. 5B). A reduction in total unsaturation at higher temperatures is frequently seen in poikilotherms (Guschina and Harwood, 2006a). It may be due to a number of factors including alterations in gene expression and oxygen availability (Rutter et al., 2002; Rolletschek et al., 2007).

A more detailed breakdown of the effect of incubation temperature for different lipid class desaturation is shown in Table 2. In general, a reduction in the proportion of $[1-^{14}C]$linoleate was found for the phospholipids and DAG and TAG (though to a different extent) with rising temperature, but not in galactolipids. This indicates that high temperatures negatively affect the FAD2, possibly by thermal inactivation of the enzyme, but not the FAD6. The low thermal stability of the FAD2 has been described (Martínez-Rivas et al., 2002; Rolletschek et al., 2004) as temperature rose. This general decrease in unsaturation at higher ambient temperatures is also mimicked in a number of crop plants (Weselake, 2005) including for olives grown in different locations (Aguilera et al., 2005).

Rather little is known about the influence of light on fatty acid patterns although some discussion of general
aspects has been made for plants (Harwood, 1998) and, 
much more recently, for algae (Guschina and Harwood, 2006b). 
Sánchez (1994) has discussed a number of effects of light in 
lipid synthesis and accumulation in olive. While 
α-linolenate labelling and endogenous levels were pre-
erved well at the two very different light levels, there was 
clearly a reduction in the desaturation of oleate to linoleate 
at low light (Fig. 6; Tables 3, 4). Little is known about the 
enzymic characteristics of the fatty acid Δ12-desaturases, 
although a reduced cofactor is required. Thus, the effect of 
light may be indirect in that, at low light, any production 
of reduced equivalents through photosynthesis would be 
impaired and thus the plastid FAD6 desaturase might 
be especially vulnerable. However, this would not be 
a general phenomenon because conversion of linoleate to 
linolenate by Δ15-desaturation is unaffected. Our results 
indicate that the enhanced oleate desaturation at high light 
was caused by increasing FAD2 activity and, possibly, 
FAD6. Although no light effect on linolenate desaturases 
was observed, a light-sensitive mechanism that differently 
regulates transcription and transcript stability of micro-
smal (FAD3) and plastidal (FAD7) linoleate desaturases 
has been described in photosynthetic soybean cell sus-
pensions submitted to dark (Collados et al., 2006). Further 
experiments are needed to clarify the effects of light on 
fatty acid desaturation in olive at the molecular level.

Conclusions

The present study has shown the key role of LPCAT, 
which seems to be mainly responsible for the entry of 
oleate and linoleate into olive callus lipid metabolism, and 
also the involvement of PDAT in TAG biosynthesis. The 
contribution of these two enzymes has been shown to 
deend on the plant species. In addition, the results also 
emphasize the importance of plastidial fatty acid desatura-
tion in generating polyunsaturated fatty acids for complex 
lipid biosynthesis in olive callus cultures as a model for 
the young olive mesocarp. This is also interesting in view 
of the major role that plastids play in the general supply of 
carbon for lipid accumulation in this important oil fruit 
(Salas et al., 2000). Our results have also shown that 
olive, like other crop plants, is sensitive to environmental 
temperature and, in addition, its fatty acid unsaturation is 
affected by ambient light. In particular, FAD2 is regulated 
by temperature and light intensity, while FAD7 is affected 
by high temperature. Explaining the molecular mecha-
nisms by which external stresses can regulate fatty acid 
desaturation would be an obvious extension to our study.

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