Control mechanisms operating for lipid biosynthesis differ in oil-palm (*Elaeis guineensis* Jacq.) and olive (*Olea europaea* L.) callus cultures

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As a prelude to detailed flux control analysis of lipid synthesis in plants, we have examined the latter in tissue cultures from two important oil crops, olive (*Olea europaea* L.) and oil palm (*Elaeis guineensis* Jacq.). Temperature was used to manipulate the overall rate of lipid formation in order to characterize and validate the system to be used for analysis. With [1-¹⁴C]acetate as a precursor, an increase in temperature from 20 to 30 °C produced nearly a doubling of total lipid labelling. This increase in total lipids did not change the radioactivity in the intermediate acyl-(acyl carrier protein) or acyl-CoA pools, indicating that metabolism of these pools did not exert any significant constraint for overall synthesis. In contrast, there were some differences in the proportional labelling of fatty acids and of lipid classes at the two temperatures.

The higher temperature caused a decrease in polyunsaturated fatty acid labelling and an increase in the proportion of triacylglycerol labelling in both calli. The intermediate diacylglycerol was increased in olive, but not in oil palm. Overall the data indicate the suitability of olive and oil-palm cultures for the study of lipid synthesis and indicate that *de novo* fatty acid synthesis may exert more flux control than complex lipid assembly. In olive, diacylglycerol acyltransferase may exert significant flux control when lipid synthesis is rapid.

Key words: fatty acid biosynthesis, lipid accumulation, metabolic control, oil fruit.

INTRODUCTION

Plants accumulate triacylglycerols (TAGs) as their major energy stores, which can account for over half the dry mass of some seeds [1]. This storage lipid is one of the world's most important plant commodities, with enormous potential for nutritional and industrial exploitation. The quality of the oil synthesized depends, among other factors, on the composition of its constituent TAGs and is influenced by the activity of a number of enzymes involved in the biosynthesis of storage TAGs during maturation [2]. Such control is exerted at the level of fatty acid synthesis *de novo* in the plastids, in subsequent modification reactions on these fatty acids and at the level of microsomal acyltransferases during storage lipid assembly. We know much about the individual enzymes and of genes coding their activities [3–6], and useful changes in TAG quality have been made by genetic manipulation [7]. However, as pointed out by Ohlrogge and Jaworski [8], much less is known about the overall regulation of carbon flux to TAG. This quantitative aspect of lipid accumulation is important for attempts to raise overall oil yields in crops and for adjusting the balance between TAGs and other storage materials. Moreover, recently, there have been notable decreases in oil yields for some commercial crops, such as oil palm (*Elaeis guineensis* Jacq.) [9], which lends such studies on carbon flux an immediate application to agriculture.

Most plant oils of commercial value are accumulated in seeds [5]. However, two fruits, oil palm and olive (*Olea europaea* L.) [10], are important. Palm oil is the second largest oil or fat traded in the world market and is, in fact, the most important crop

grown mainly for its oil [11]. Olive is one of the most significant oil crops in Europe and ranks sixth in the world production of vegetable oils [12]. Moreover, olive oil has a high commercial value compared with most other plant oils or fats. Palm oil derives from the mesocarp and contains about 45 % palmitic acid and 40 % oleic acid as its major constituents. A second oil, deriving from the seed kernel (palm kernel oil) is rich in medium-chain fatty acids [13,14]. By contrast, olive oil contains only small amounts of palmitate (about 10 %), but is particularly enriched (70–80 %) in the monoenoic acid oleate [12]. Because of the importance of plant lipids, an understanding of the regulation of their metabolism is clearly needed, but is currently lacking [1,6]. In order to try to redress this deficiency, we have conducted experiments using flux control analysis in order to quantify parts of the biosynthetic pathway to TAG. The methods of control analysis are particularly appropriate for such studies [15]. We chose to use two model systems – tissue cultures of oil palm and olive – because of the great importance of these two crops. Furthermore, previous studies in our laboratory with olive callus cultures had demonstrated their experimental advantages and applicability to *in vivo* situations [16].

In the first part of our studies, reported in the present paper, we describe background experiments with the two cultures which characterize basic parameters and also show how lipid biosynthesis can be changed by temperature. The data reveal some differences in the control mechanisms operating between olive and oil palm. Such experiments also provided information about possible flux constraints within the TAG accumulation pathway in the two crops and thus gave some indication of the overall

Abbreviations used: DAG, diacylglycerol; TAG, triacylglycerol; NEFA, non-esterified fatty acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid; ACP, acyl carrier protein; FAME, fatty acid methyl ester; KAS, β -ketoacyl-ACP synthase (β -oxoacyl-ACP synthase); TDCA, Top-Down Control Analysis.

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control structure of TAG biosynthesis, which has been tested more fully in further experiments.

EXPERIMENTAL

Materials

The oil-palm callus cultures used in this study were obtained from Dr John Eeuwens (Unifield T.C., Cambridge, U.K.). The cultures were routinely subcultured on Murashige and Skoog medium [17] in the presence of 10 mg/litre naphthaleneacetic acid and incubated at 30 °C in the dark. Subculturing was performed at 4-weekly intervals. Olive calli that were used in the present study were established by the following procedure. Olive fruits were broken and the pericarp was removed. The kernel was surface-sterilized with 4% (w/v) sodium hypochlorite for 20 min and allowed to imbibe in cold running water for 6 h. The kernels were then rinsed in sterile water and germinated aseptically at 25 °C. Etiolated embryos were dissected so that the cotyledons were cut transversely into two basal and two apical pieces. The individual segments were incubated on Murashige and Skoog medium [17] supplemented with an auxin/cytokinin combination [2,4-dichlorophenoxyacetic acid (2 mg/litre) and benzylamino-purine (0.1 mg/litre)] and subcultured at 4-weekly intervals.

[1-¹⁴C]Acetate (2.15 Gbq/mmol) and [U-¹⁴C]glycerol (5.51 Gbq/mmol) were purchased from Amersham International p.l.c., (Amersham, Bucks, U.K.). Lipid standards were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) or NuChek Prep (Elysian, Mn 56028, U.S.A.). All of the chemicals and solvents used were of the best available analytical grades.

Radiolabelling protocol

Callus cultures were selected for uniformity of mass and physiological appearance. Triplicate samples (0.5–1.0 g fresh mass) were used for each treatment unless otherwise stated. Carrier-free [1-¹⁴C]acetate (74 kbq) or [U-¹⁴C]glycerol (37 kbq) diluted in 0.2 ml of sorbitol (100 mM) was placed on to the surface of the callus and incubations were conducted as stated in the Tables. Incubations with [1-¹⁴C]acetate and [U-¹⁴C]glycerol were carried out for 24 and 8 h respectively. These times were within the linear period of radiolabel incorporation. Independent experiments showed that tissue penetration of both radiolabelled precursors was extremely rapid and there was no significant difference between the upper surface or lower parts of the tissue. Thus we are very confident that the callus cultures were uniformly labelled. Furthermore, tissue penetration was equally rapid at the two temperatures, so that any temperature effects could not be attributed to different availability of the precursors. At the end of the incubation period, the calli were rinsed briefly with distilled water to remove any unimbibed radiolabel and transferred into Pyrex methylating tubes. Propan-2-ol (2 ml) (to inactivate lipid-degradative enzymes) was added to the calli before heating at 70 °C in a heating block for 30 min to inactivate endogenous lipases.

Lipid extraction and analysis

Lipids were extracted by a high-salt extraction method [18] as modified by Smith et al. [19]. This extraction method has been shown previously to be very efficient for plant acyl lipids, especially polar components [20]. Lipid samples were purified by TLC on pre-coated (E. Merck, Darmstadt, Germany) silica gel plates. Non-polar lipids were separated using a solvent system consisting of light petroleum (60–80 °C fraction)/diethyl ether/acetic acid (80:20:2, by vol.). Polar lipids (glycosylacylglycerols,

phosphoacylglycerols) were separated with a solvent of chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). Individual lipids were identified by co-chromatography with authentic standards and revealed under UV light after spraying with 0.2% (w/v) 8-anilino-1-naphthalenesulphonic acid in anhydrous methanol.

Fatty acid components were analysed by GLC in the form of fatty acid methyl esters (FAMES). FAMES were prepared by acid-catalysed transmethylation and analysed by radio-GLC using a Pye Unicam GCD gas chromatograph connected to a Raytest Raga gas flow proportional counter (LabLogic Systems Limited, Sheffield, U.K.). A glass column (1.5 m × 3.0 mm) packed with 10% SP-2330 on 100/120 mesh Supelcoport (Supelco, Saffron Walden, Essex, U.K.) was used to separate individual FAMES. The system was normally operated with an argon/CO₂ [95:5 (v/v); 45 ml/min] gas carrier using an oven temperature of 180 °C. Quantification of mass and of the radioactive peaks was made using a Rachel software package (LabLogic) and pentadecanoic acid as an internal standard.

Separation and analysis of acyl-CoAs and acyl-(acyl carrier proteins) (acyl-ACPs)

Acyl-CoAs and acyl-ACPs were extracted from the aqueous phase obtained from the total lipid extraction by rapid reverse-phase partition chromatography through a Waters Sep-Pak C₁₈ cartridge as described by Stymne and Glad [21]. The column was pre-equilibrated successively with 2 ml of methanol, 2 ml of water and 2 ml of Garbus et al. [18] solution (2 M KCl in 0.5 M phosphate buffer, pH 7.4). Following sample loading, the column was washed with 15 ml of water to remove unbound materials. Fractions (1 ml each) were collected from the time of sample application and their radioactivity was determined. The unbound materials in eluate I contained acyl-ACPs and other water-soluble substances/proteins. Once the eluted radioactivity had returned to baseline, bound acyl-CoAs were eluted from the column with 15 ml of 0.4 M NH₄OH in methanol/water (4:1, v/v; eluate II). Fractions containing significant radioactivity in eluate II were pooled together and immediately evaporated to dryness in a rotary evaporator at 30 °C. The residue was then redissolved in 0.1 ml of water followed by 0.4 ml of methanol. Eluates I and II were then analysed for acyl-ACPs and acyl-CoAs respectively. Intact acyl-CoAs (from eluate II) were analysed by TLC using butan-1-ol/acetic acid/water (5:3:2, by vol.) as solvent. The purity of the acyl-CoAs was confirmed by comparing the *R_F* values with those of authentic acyl-CoA samples. No other compounds were detected. After spraying with water, acyl-CoAs from metabolic experiments were revealed as white bands and removed from the plate with a scalpel on to silver foil and transferred to liquid-scintillation vials for radioactivity counting.

The efficiency of the method with regard to adequate separation of the acyl-thioester fractions and quantitative recovery was examined using radiolabelled acyl-CoAs or acyl-ACPs. Complete separation of acyl-CoAs from acyl-ACPs was achieved using the method of Stymne and Glad [21], with no detectable cross-contamination. Recovery of acyl-CoAs and acyl-ACPs from the initial lipid extraction through the Sep-Pak separation was 93% and > 80% respectively.

Acyl-CoAs purified from Sep-Pak C₁₈ cartridges were hydrolysed with 40% (w/v) KOH in methanol at 70 °C for 30 min in a Grant heating block when fatty acid analysis was required. Subsequently, the sample was acidified with 30% (v/v) H₂SO₄ and the non-esterified fatty acids were extracted three times each with 3 ml of light petroleum (60–80 °C b.p.). The combined

upper organic phases were evaporated to dryness under a stream of nitrogen and methylated for the analysis of acyl chains.

Quantification of glycerol 3-phosphate

Glycerol 3-phosphate was quantified by an enzymic method after tissue extraction with HClO_4 [22].

Determination of radioactivity

Radioactivity was determined using a LKB Wallac 1209 Rack-beta (Wallac Oy, Turku, Finland) liquid-scintillation counter. All samples were evaporated to dryness under a stream of nitrogen in polyethylene disposable vials. The samples were counted in 10 ml of Optifluor scintillant (Canberra Packard, Pangbourne, Berks., U.K.). Quench correction was made automatically by the external-standard channels-ratio method.

RESULTS AND DISCUSSION

Preliminary experiments

Initial experiments were carried out in order to check that adequate labelling of acyl-thioester pools and individual lipid classes occurred under the standard incubation conditions. This was confirmed with up to 28 % of the total radiolabel added as $[1-^{14}\text{C}]$ acetate to the cultures being incorporated into lipids.

We also checked the specific radioactivity of the acyl-ACP and acyl-CoA pools to ensure that increased lipid labelling rates were not due merely to changes in the specific radioactivity of these pools. This showed that, for a given set of experiments, the specific radioactivity of thioester pools was similar showing that increased radioactivity if seen, was matched by an increase in endogenous mass.

Finally, for flux-control experiments, interpretation is made much simpler if a single metabolic pathway without many branch points is being used. Because of the complexity of plant lipid metabolism (particularly the co-operation of different subcellular compartments [1]) we wanted to measure the alternative metabolic pathways of (a) incorporation of newly synthesized fatty acids directly into chloroplast lipids and (b) the release of the non-esterified fatty acids (NEFAs) from thioester pools or complex lipids by thioesterase or acyl hydrolase activity. For the so-called '18:3' plants (like oil palm and olive), the only chloroplast lipids directly labelled by newly synthesized fatty acids are sulpholipid and phosphatidylglycerol [1,3]. Labelling of these complex lipids from $[1-^{14}\text{C}]$ acetate never exceeded 2 % in either oil-palm or olive cultures. The NEFA fraction accounted for 2–7 % of the total labelling in both tissues, showing that hydrolytic release of fatty acids was relatively minor.

Thus the preliminary experiments showed that over 90 % of the radioactivity from $[1-^{14}\text{C}]$ acetate was incorporated into complex lipids via *de novo* synthesis of fatty acids in the chloroplast followed by transfer to acyl-CoAs on the cytosolic envelope and acyltransfer in the cytosolic compartment – as expected for an 18:3-plant [3].

Effect of temperature on incorporation of radioactivity from $[1-^{14}\text{C}]$ acetate into lipids

The use of radiolabelled $[1-^{14}\text{C}]$ acetate as a precursor for studying fatty acid and lipid biosynthesis is well established [23]. Another advantage of the use of $[1-^{14}\text{C}]$ acetate as a precursor is that it labels almost entirely the acyl moieties of lipids and this simplifies interpretation of flux compared, say, to using glucose as a precursor, when polar moieties of lipids may be labelled significantly. During the incubation acetate is rapidly activated to

Table 1 Effect of temperature on the incorporation of radioactivity from $[1-^{14}\text{C}]$ acetate into lipid or non-lipid phases in oil-palm and olive callus cultures

Oil-palm or olive callus (0.5–1.0 g fresh mass) were labelled with $[1-^{14}\text{C}]$ acetate by incubation for 24 h at 20 and 30 °C respectively. Total lipids were extracted and incorporation of radiolabel into lipid and non-lipid phases was determined. *T*, temperature. Results are means $\pm$ S.D. for triplicate incubations. *Significantly different compared with 20 °C value by Student's *t* test ($P < 0.05$).

Callus	<i>T</i> (°C) ...	$10^{-5} \times$ Radioactivity in total lipids (d.p.m./g fresh mass)		$10^{-4} \times$ Radioactivity in non-lipids (d.p.m./g fresh mass)	
		20	30	20	30
Oil palm		2.8 ± 0.4	$5.4 \pm 0.6^*$	1.3 ± 0.5	1.5 ± 0.6
Olive		6.9 ± 0.9	$11.0 \pm 2.3^*$	3.6 ± 0.5	4.0 ± 0.6

acetyl-CoA and then carboxylated to yield malonyl-CoA, which serves as precursor for *de novo* fatty acid synthesis in the plastid. The fatty acid products (as acyl-ACPs) are converted into acyl-CoAs, which are then utilized by cytosolic acyltransferases for the synthesis of TAG and complex lipids. Of the total $[1-^{14}\text{C}]$ acetate taken up by tissue cultures, typically about 95 % is incorporated into lipids (see Table 1).

Olive and oil-palm calli were incubated at different incubation temperatures, and incorporation of radiolabel from $[1-^{14}\text{C}]$ acetate into lipids was monitored. The temperatures chosen represented a range to which oil palm and olive trees might normally be subjected. It is clearly evident that temperature altered the incorporation of radiolabel from $[1-^{14}\text{C}]$ acetate into total lipids (Table 1). Raising the temperature from 20 to 30 °C approximately doubled total lipid labelling. Stimulation of lipid synthesis at the higher temperature would be expected for metabolic systems in the absence of any inactivation of enzyme activity at 30 °C. Indeed, a Q_{10} (temperature coefficient of a reaction) of about 2 is usual for enzyme-catalysed reactions (see [24]). However, analysis of the non-lipid fraction indicated that a higher percentage incorporation of radioactivity from the $[1-^{14}\text{C}]$ acetate taken up by both tissues occurred in total lipids. Thus for oil palm the percentage incorporation into lipids increased from 95.5 to 97.3 % at the two temperatures, and for olive from 95.0 to 96.5 % of the total $[1-^{14}\text{C}]$ acetate taken up. Actually, the changes are even more significant, since, as will be seen below, acyl-thioesters are significant components of the aqueous fraction. Clearly, activation of acetate to acetyl-CoA did not limit incorporation of radiolabel into lipids to any great extent at either temperature.

The major lipid classes labelled from $[1-^{14}\text{C}]$ acetate by each tissue culture are shown in Table 2. As expected for non-photosynthetic plant tissues, phosphatidylcholine (PtdCho) was the highest labelled class. TAG was appreciably labelled as were phosphatidylethanolamine (PtdEtn) and phosphatidate (PtdOH). The lack of significant labelling of the chloroplast glycosylacylglycerols or phosphatidylglycerol during the incubation period showed that the main route for complex lipid assembly was the Kennedy pathway with the diacylglycerol (DAG) intermediate being used both for zwitterionic phosphoglyceride and for TAG assembly.

Incubation at 30 °C caused significant changes in the pattern of lipid labelling compared with 20 °C (Table 2), in addition to the doubling of total labelling (Table 1). For both oil palm and olive, incubation at 30 °C increased the proportion of TAG labelling and decreased that in NEFAs, PtdEtn and PtdOH. For olive, the percentage labelling of DAG was also increased,

Table 2 Effect of temperature on the incorporation of radioactivity from [1-¹⁴C]acetate into lipid classes in oil-palm and olive callus cultures

Oil-palm and olive calli were incubated with [1-¹⁴C]acetate for 24 h at 20 or 30 °C respectively. Total lipids were extracted and analysed by TLC. Lipid bands were scraped into scintillation vials and the radioactivity was determined. *T*, temperature. Results are means ± S.D. for triplicate incubations; tr, trace (< 0.05%). *Significantly different from 20 °C samples by Student's *t* test with *P* < 0.05.

	<i>T</i> (°C)	Lipid class ...	Radiolabel incorporated (% of total)					
			DAG	NEFA	TAG	PtdCho	PtdEtn	PtdOH
Oil palm	20		7.5 ± 1.0	6.9 ± 0.8	15.0 ± 2.0	39.9 ± 3.2	17.5 ± 1.5	11.2 ± 0.9
	30		5.0 ± 1.0	3.0 ± 0.9*	21.0 ± 2.2*	60.0 ± 4.0*	6.3 ± 0.8*	2.8 ± tr.*
Olive	20		9.8 ± 2.0	6.3 ± 1.3	11.0 ± 1.4	31.4 ± 4.3	14.9 ± 2.4	22.0 ± 2.6
	30		15.0 ± 2.2*	2.0 ± 0.8*	17.5 ± 2.3*	34.4 ± 2.9	8.9 ± 0.7*	15.8 ± 1.5*

Table 3 Effect of temperature on the incorporation of radioactivity from [1-¹⁴C]acetate into fatty acids in oil-palm and olive callus cultures

Oil-palm and olive calli were incubated with [1-¹⁴C]acetate for 24 h at 20 or 30 °C respectively. FAMES produced from lipid extracts were analysed by radio-GLC. *T*, temperature. Results are means ± S.D. for triplicate incubations; tr, trace (< 0.05%); ND, not detected. The significance of changes (between temperatures) was analysed by Student's *t* test (**P* < 0.05). Fatty acid abbreviations: C_{16:0}, palmitate; C_{18:0}, stearate; C_{18:1}, oleate; C_{18:2}, linoleate; C_{18:3}, α-linolenate.

Callus	<i>T</i> (°C)	Fatty acid ...	Radiolabelling (% of total)				
			C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}
Oil palm	20		32.0 ± 2.0	5.0 ± tr.	36.0 ± 2.0	22.0 ± 2.0	5.0 ± tr.
	30		40.0 ± 2.0*	2.0 ± tr.*	43.0 ± 2.0*	12.0 ± 1.0*	3.0 ± tr.*
Olive	20		39.0 ± 2.1	2.3 ± tr.	52.5 ± tr.	5.2 ± tr.	1.0 ± tr.
	30		36.4 ± 1.8	1.5 ± 1.2	60.0 ± 3.0*	2.1 ± tr.*	ND

whereas in oil palm that of PtdCho was higher. The increased percentage labelling of PtdCho and decreased labelling of PtdEtn, especially in oil palm, suggests that the cholinephosphotransferase activity is increased relative to ethanolaminephosphotransferase at 30 °C. There might also be a change in the relative activity of lysophosphatidylcholine acyltransferase [25,26] or in the methylation of PtdEtn to PtdCho. However, the latter is unlikely to be significant, especially in view of the data already obtained for olive cultures [27].

A significant increase in TAG labelling was seen at 30 °C for both cultures. In olive, but not in oil palm, there was a build up of label in DAG. In separate experiments we found also that the mass of TAG in oil palm and olive was increased by incubation at 30 °C from 0.35 to 0.50 and from 0.28 to 0.55 mg/g fresh weight respectively. These percentage increases are of the same order as those seen in Table 2, although the actual values are not directly comparable, since they were made with different tissue samples. In oil palm the level of DAG was low (50 µg/g fresh weight) and was not increased at 30 °C. However, most interestingly, the DAG level was increased from 0.16 to 0.30 mg/g fresh weight by incubation at 30 °C in olive calli. This might indicate that, under increased carbon flux to TAG, the DAG acyltransferase could exert significant flux control. However, it must be stressed that these were preliminary experiments to get a feel for the characteristics of the system, and specific flux-control data might show otherwise (see [15]). Nevertheless, it has been suggested that DAG acyltransferase could exert significant flux control over TAG accumulation in oilseed rape during periods of high oil accumulation [28]. This suggestion was confirmed in mutants of *Arabidopsis thaliana* (thale cress) with changed DAG acyltransferase activity [29]. It is also noteworthy that the increased lipid labelling found for both tissues at 30 °C was accompanied by a decrease in PtdOH labelling, which may indicate that phosphatidate phosphohydrolase, which has been

described as a key enzyme for mammalian TAG synthesis [30], does not exert major flux control in olive or oil-palm calli.

Effect of temperature on fatty acid labelling from [1-¹⁴C]acetate

Typically, plant tissues synthesize mainly C₁₆ and C₁₈ fatty acids with polyunsaturated C₁₈ products accumulating in photosynthetic tissues [23]. Likewise, both calli incorporated radioactivity from [1-¹⁴C]acetate mostly into C₁₆ and C₁₈ fatty acids (Table 3). Of these, palmitate (C_{16:0}) and oleate (C_{18:1}) were the most heavily labelled (Table 3), but with significant though small amounts of polyunsaturated products. At shorter time intervals, less polyunsaturated acids were labelled, but equilibrium had been reached by about 24 h (results not shown). In olive there was a relative increase in oleate percentage labelling under conditions which stimulate lipid synthesis (i.e. higher temperatures), which may reflect a constraint of carbon flux at the level of the Δ¹²-desaturase enzyme. Alternatively, membrane-bound fatty acid desaturases often show decreased activity at higher temperatures [24]. No statistically significant effect of temperature on the proportion of saturated-fatty-acid labelling or on ratio of C₁₆/C₁₈ products was found.

In oil-palm calli incubation at 30 °C again significantly increased the proportion of the radioactivity found in oleate. A significant increase in the relative labelling of palmitate was also seen. This altered the C₁₆/C₁₈ product ratio. The balance between palmitate and stearate products of fatty acid synthase is determined by the relative activity of condensing enzymes β-ketoacyl-ACP synthase (β-oxoacyl-ACP synthase, KAS) I and II of the fatty acid synthase. KAS II is responsible for the condensation of palmitate with a C₂ unit to form stearate. Thus the significant increase in C₁₆/C₁₈ products may indicate a constraint in the fatty acid synthase reactions at the level of KAS II.

Table 4 Effect of temperature on the incorporation of radioactivity from [1-¹⁴C]acetate into acyl-thioester pools and acyl-chains derived from the acyl-CoA pool in oil-palm and olive callus cultures

Oil-palm and olive calli were incubated with [1-¹⁴C]acetate for 24 h at 20 or 30 °C respectively. Lipids were extracted and acyl-thioester pools were purified from the aqueous phase obtained after total lipid extraction using Sep-Pak C₁₈ cartridges (see the Experimental section). Results are means $\pm$ S.D.s for triplicate incubations, except for *, where $n = 1$. *T*, temperature.

Callus	<i>T</i> (°C)	10 ⁻² $\times$ Radioactivity in acyl-CoA (d.p.m.)	10 ⁻⁴ $\times$ Radioactivity in acyl-ACP (d.p.m.)	Acyl composition (%) of acyl-CoAs			
				Saturated	Monoenes	Dienes	Trienes
Oil palm	20	5.8 $\pm$ 1.8	6.6 $\pm$ 2.0	12 $\pm$ 3	21 $\pm$ 8	35 $\pm$ 5	32 $\pm$ 7
	30	6.4 $\pm$ 2.3	7.0 $\pm$ 2.3	15 $\pm$ 4	29 $\pm$ 7	22 $\pm$ 6	34 $\pm$ 8
Olive	20	8.6 $\pm$ 2.0	15.5 $\pm$ 2.5	20*	12*	25*	43*
	30	8.9 $\pm$ 1.9	17.4 $\pm$ 2.0	21 $\pm$ 2	18 $\pm$ 1	29 $\pm$ 3	32 $\pm$ 3

Little accumulation of radiolabel in stearate is common for most plant systems and is usually thought to be due to high activity of stearyl-ACP Δ^9 -desaturase. In oil palm, as with olive, temperature modified the relative proportions of the C₁₈ unsaturated fatty acids. Particularly noticeable was the enrichment of polyunsaturated-fatty-acid labelling at low temperature (Table 3). This may indicate a major effect of temperature on Δ^{12} -desaturase (and probably also on Δ^{15} -desaturase) activity. It is generally accepted that, by several possible means, cold temperatures favour desaturation [24]. For example, desaturase activity can be decreased at higher temperatures through an effect of oxygen solubility, inactivation of the membrane-located desaturase or decrease in its gene expression [31]. Alternatively, as discussed above, the increased overall carbon flux towards fatty acid synthesis at 30 °C may be limited by the activity of the Δ^{12} - and Δ^{15} -desaturases under the experimental conditions.

Effect of temperature on the acyl-CoA and acyl-ACP pools

The effect of incubation temperature on acyl-CoA and acyl-ACP pools was determined in order to reveal the size of these pools compared with lipid classes and also whether they changed in amount or composition following temperature-induced alterations of lipid synthesis. Table 4 shows that total labelling of neither the acyl-CoA nor the acyl-ACP pools was affected by temperature. This was expected, as it has already been observed that temperature did not change the accumulation of radiolabel from [1-¹⁴C]acetate in the aqueous phase, which contained these acyl-thioesters as major components (Table 1). Furthermore, measurement of the total quantity of oil-palm-calli acyl-CoAs following separation on Sep-pak cartridges [21] revealed that the total pool did not change in mass during temperature manipulation. Total acyl-CoAs were 50–60 nmol/g fresh weight, a value significantly higher than that reported for *A. thaliana* leaves or germinating seedlings [32]. Whether all of the acyl-CoAs were bound to an acyl-CoA binding protein (such as has been reported in *Arabidopsis* [33]) is not known, but we assume that all of the acyl-CoAs would be effectively extracted by the method used [18].

We have suggested that the total cytosolic acyl-CoA pool is suitable as a system intermediate for the type of Top-Down (Metabolic) Control Analysis (TDCA) we are proposing and have explored in the accompanying paper [34]. Moreover, the binding of acyl-CoA to acyl-CoA binding proteins would not affect the proposed control analysis, described in detail in the accompanying paper [34] for the reasons outlined below.

In brief, to apply the top-down approach of Metabolic Control Analysis, the system under investigation is conceptually divided into those reactions that produce and those that consume a

suitable pathway intermediate which is uniquely related to the producing and consuming blocks of reactions. Fractional changes in steady-state fluxes through the blocks of reactions, in response to small fractional changes in steady-state intermediate levels, are calculated. These data are then used to calculate absolute group elasticities and, hence, absolute group flux control coefficients, allowing the distribution of control (among the blocks of reactions either side of the intermediate) within this simple system to be solved.

However, when calculating the group flux control coefficients for simple linear systems, it is not always necessary to measure the system intermediate, as the coefficients can be expressed as a function of fluxes alone. Therefore, if methods are available to measure independently the small fractional changes in steady-state fluxes through the two blocks of reactions in response to the changes in the levels of the chosen intermediate, it is possible to calculate the *relative* group elasticities and, hence, the *absolute* flux control coefficients by measuring the fluxes alone, under different conditions [35]. A detailed worked example of such a calculation is given in the Appendix of [36]. There, the group flux control coefficients are calculated from fluxes alone specifically to avoid or eliminate potential systematic and random errors which might arise from calculations and measurements of the intermediate. Moreover, in the work described in [36] the system intermediate is heterogeneous.

By applying the same method, justified and described in the above two papers [35,36] to the system described in the accompanying paper [34], the group flux control coefficients can be calculated directly as a function of fluxes alone, where changes in fluxes would be expressed relative to the steady-state fluxes in the unperturbed system. This ensures that the resulting group flux control coefficients describe the unperturbed system of immediate interest to us and demonstrate that any concerns associated with possible complications in the interpretation of intermediate values (because of the presence of acyl-CoA-binding proteins) are irrelevant to the outcome of the proposed TDCA.

It is noteworthy that the acyl-ACP pool was much better labelled than acyl-CoAs. This indicated that acyl-CoAs are used very rapidly for lipid assembly. Analysis of the acyl composition of the acyl-CoAs did not show any major difference for the two temperatures (Table 4). However, when compared with the total lipid composition (Table 3), polyunsaturated fatty acids were well labelled at both temperatures in the acyl-CoAs. Saturated fatty acids were also decreased in the acyl-CoAs, and these results may have been due, for example, to efficient transfer of palmitate and oleate via acyltransferases into complex lipids. In fact, previous work in our laboratory (see, e.g. [37,38]) has shown that, while all acyl-CoAs tested were capable of being used by the acyltransferases of the Kennedy pathway in olive or oil palm,

Table 5 Effect of temperature on the incorporation of radioactivity from [U-¹⁴C]glycerol into lipid classes in oil-palm and olive callus cultures

Oil-palm and olive callus were labelled with [U-¹⁴C]glycerol by incubation for 8 h at 20 or 30 °C respectively. Total lipids were extracted and radioactivity determined. Lipid separation was carried out by TLC. Results are means ± S.D. from triplicate incubations. Abbreviation: lysoPtdOH, lysophosphatidate (1-acylglycerol 3-phosphate). Detectable radioactivity was only present in the four lipids indicated. *Significantly different from 20 °C values by Student's *t* test (*P* < 0.05). *T*, temperature.

Calli	<i>T</i> (°C)	10 ⁻⁴ × Radioactivity in total lipids (d.p.m.)/g fresh weight	Lipid class labelling (% of total)			
			LysoPtdOH	PtdOH	DAG	TAG
Oil palm	20	3.0 ± 0.5	18 ± 2	48 ± 4	25 ± 3	9 ± 1
	30	5.1 ± 0.5*	12 ± 1*	49 ± 1	25 ± 4	14 ± 2*
Olive	20	3.9 ± 0.2	39 ± 5	33 ± 6	18 ± 1	10 ± 2
	30	7.1 ± 0.1*	33 ± 3	17 ± 3*	28 ± 4*	22 ± 3*

the use of palmitate and oleate respectively is selectively favoured for the first two acylations. Thus, for the main labelled lipids (Table 2), one would expect these fatty acids to be used preferentially. As mentioned under 'Preliminary experiments' above, the amount of radiolabelling of the acyl-CoA pool reflected well its mass. Therefore the small size of the acyl-CoA pool compared with lipids suggested strongly that such thioesters are used very efficiently for lipid formation.

Effect of temperature on glycerolipid synthesis

In order to carry out flux control analysis it is often desirable to label portions of a metabolic pathway selectively. So far as complex lipid formation is concerned, whereas [1-¹⁴C]acetate labels fatty acids and can, therefore, be used to study the whole pathway, radiolabel from [U-¹⁴C]glycerol would only be expected to label complex lipids significantly. In order to check that this was the case, incubations were carried out with both olive and oil-palm calli with [U-¹⁴C]glycerol for up to 48 h. After extraction of total lipids, the labelled products were deacylated and the amount of radiolabel in the liberated fatty acids compared with the water-soluble deacylated lipid moieties. Less than 2% of the total radioactivity was found in the fatty acid portions (results not shown), demonstrating clearly that [U-¹⁴C]glycerol could be used specifically to measure complex lipid assembly. We also measured the glycerol 3-phosphate pool [22], which was found to be about 0.3 µM and was not changed by temperature, thus confirming that changes in availability of glycerol 3-phosphate would be unlikely to give rise to any alterations in labelling.

We then used [U-¹⁴C]glycerol as a direct precursor for TAG synthesis [39]. Radioactivity only accumulated in four lipids – the intermediates and product (TAG) of the Kennedy pathway. Table 5 shows that raising the incubation temperature from 20 to 30 °C, not only doubled the total incorporation, but also changed the proportion of label in the individual Kennedy-pathway lipids. In both calli there was a significant increase in total lipid labelling at 30 °C. The increase was of a similar order to that seen with [1-¹⁴C]acetate (Table 1), i.e. *Q*₁₀ values of 1.7–1.8 compared with 1.6–1.9. In addition, the proportion of label in TAG was significantly increased by incubation at the higher temperature (Table 5), as it had been for [1-¹⁴C]acetate (Table 2) with, again, a bigger increase in the percentage labelling of DAG from [U-¹⁴C]glycerol seen for olive but not for oil palm (Table 5). This might be due to significant flux control at the level of DAG acyltransferase, as discussed above. Broadly, changes in lipid intermediates were rather small in oil palm and, therefore, it was concluded that carbon flux through the Kennedy pathway was not limited, even under conditions which stimulated TAG synthesis significantly. Instead, the overall rate of oil formation in oil palm would be likely to be controlled by the entry of

carbon into the Kennedy pathway and, perhaps, indicated an important role for glycerol 3-phosphate acyltransferase in this tissue. In general, these data fully agreed with the results of temperature-manipulation studies using [1-¹⁴C]acetate in both calli.

Conclusions

We have shown that callus cultures can be used for the study of lipid metabolism, in keeping with previous work (see, e.g., [16,38,40]). Both the oil-palm and olive calli used in the present study contained palmitate and oleate as the major constituents [41], with olive containing higher percentages of the latter – thus, mimicking the fatty acyl compositions of these plants *in vivo*. Moreover, some of the cultures, depending on conditions, were capable of accumulating TAG at up to 20% dry mass, in keeping with their utility for lipid-synthesis experiments (see [10,16]). Nevertheless, it is important to mention that each individual experiment has its own variations due to subtle differences between the callus tissues which were used. However, in general, these variations were relatively small and did not seem to have a significant effect on the reproducibility of the results. Therefore, together with their demonstrated high metabolic activities, cultures proved useful in our studies of storage-oil synthesis and should be of further utility in the future.

Our results indicated that olive and oil-palm callus cultures are capable of activating radiolabelled [1-¹⁴C]acetate, synthesizing fatty acids, and subsequently incorporating them into complex (including storage) lipids. Both calli resemble their respective fruits with regard to their ability to form distinctive patterns of fatty acids.

We manipulated the overall flux of carbon into lipid by raising the incubation temperature. Increasing the temperature from 20 to 30 °C (which is within the physiological range for olive trees and oil palms) caused about a doubling of total lipid labelling. Interestingly, this increase in lipid labelling did not change the total radioactivity (or mass) in the acyl-CoA pool significantly, indicating that metabolism of these thioesters did not constrain the overall rate of TAG and complex lipid assembly. Moreover, the small size of the acyl-CoA pool suggested strongly that such thioesters are used very efficiently for lipid formation. Indeed, it is well known that the long-chain-acyl-CoA pool is small in plant tissues in general [8,32].

Considering the evidence that DAG accumulates during stimulation of TAG formation at the expense of earlier intermediates of Kennedy pathway in olive, this may indicate that diacylglycerol acyltransferase activity exerts strong flux control in this tissue. In contrast, although there is a large increase in TAG labelling at 30 °C in oil palm, only a small increase in the amount of DAG was seen, and this suggested that diacylglycerol acyltrans-

ferase was not so important for limiting flux at high rates of lipid synthesis in oil palm. This potential difference in the role of the last Kennedy-pathway enzyme in the two tissues makes it an obvious target to probe for significance in flux control experiments.

The lack of changes in the acyl-CoA pool when lipid synthesis was doubled at higher temperatures suggested that the fatty acid synthesis block of reactions exerted the main control over lipid synthesis rates rather than complex lipid assembly. This finding is in keeping with other results for plant tissues where fatty acid addition has been able to increase total lipid formation [39,42]. Subtle changes in the relative activity of KAS I and KAS II in oil-palm calli were also possible when a significant increase in the proportional labelling of palmitate was observed at the higher temperature. In both calli, results from labelling studies with [U-¹⁴C]glycerol were consistent with those from [1-¹⁴C]-acetate labelling in showing approaching a doubling of lipid synthesis for a 10 °C rise in temperature. Also, in both instances, the increase in total lipid labelling on raising the incubation temperature reflected changes in the relative activities of individual enzymes during TAG synthesis via the Kennedy pathway.

In conclusion, oil palm and olive displayed similar responses to temperature in terms of overall total lipid synthesis and in the increased amounts of TAG made at 30 °C. However, there appeared to be detailed differences with regard to control mechanisms for the regulation of lipid biosynthesis in these two tissues. In view of these differences, further experiments were then conducted using Metabolic Control Analysis in order to provide quantitative data on the control of these carbon fluxes.

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REFERENCES

- Harwood, J. L. (1998) What's so special about plant lipids? In *Plant Lipid Biosynthesis. Fundamentals and Agricultural Applications* (Harwood, J. L., ed.), pp. 1–26, Cambridge University Press, Cambridge
- Sanchez, J., Cuvello, T. D. and Harwood, J. L. (1992) Glycerolipid biosynthesis by microsomal fractions from olive fruits. *Phytochemistry* **31**, 129–134
- Browse, J. and Somerville, C. (1991) Glycerolipid synthesis: biochemistry and regulation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 467–506
- Harwood, J. L. (1996) Recent advances in the biosynthesis of plant fatty acids. *Biochim. Biophys. Acta* **1301**, 7–56
- Murphy, D. J. (ed.) (1994) *Designer Oil Crops*, VCH, Weinheim
- Ohlrogge, J. B. and Browse, J. (1995) Lipid biosynthesis. *Plant Cell* **7**, 957–970
- Kinney, A. J. (1998) Production of specialised oils for industry. In *Plant Lipid Biosynthesis, Fundamentals and Agricultural Applications* (Harwood, J. L., ed.), pp. 273–285, Cambridge University Press, Cambridge
- Ohlrogge, J. B. and Jaworski, J. G. (1997) Regulation of fatty acid biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 109–136
- Amiruddin, M. N. and Embong, H. (1999) Supply and disappearance of Malaysian palm oil: a review. *Palm Oil Dev.* **30**, 49–56
- Salas, J., Sanchez, J., Ramli, U. S., Manaf, A. M., Williams, M. and Harwood, J. L. (2000) Biochemistry of lipid metabolism in olive and other oil fruits. *Prog. Lipid Res.* **39**, 151–180
- Salleh, M. and Chandramohan, D. (1999) Status and prospectus of the palm oil industry in selected countries. In *Proceedings of Sourcing of Oil Palm Planting Materials for Local and Overseas Joint Ventures*, pp. 8–27, Palm Oil Research Institute of Malaysia, Kuala Lumpur
- Kiritsakis, A. K. (1991) *Olive Oil*, American Oil Chemists Society, Champaign, IL
- Tan, B. K. and Oh, C. H. (1981) Malaysian palm oil: chemical and physical characteristics. *Palm Oil Res. Inst. Malaysia Technol.* **31**, 1–5
- Gunstone, F. D., Harwood, J. L. and Padley, F. B. (eds.) (1994) *The Lipid Handbook*, 2nd ed., Chapman and Hall, London
- Fell, D. A. (1997) *Understanding the Control of Metabolism*, Portland Press, London
- Williams, M. and Harwood, J. L. (2000) Use of tissue cultures as model systems for the study of lipid biochemistry in olives. In *Handbook of Olive Oil* (Harwood, J. L. and Aparicio, R., eds.), pp. 101–128, Aspen Publishers Inc, Gaithersburg, MD
- Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497
- Garbus, J., De Luca, H. F., Loomans, M. E. and Strong, F. M. (1963) The rapid incorporation of phosphatidate into mitochondrial lipids. *J. Biol. Chem.* **238**, 59–64
- Smith, K. L., Douce, R. and Harwood, J. L. (1982) Phospholipid metabolism in the brown alga, *Fucus serratus*. *Phytochemistry* **21**, 569–573
- Harwood, J. L. (1980) Plant acyl lipids; structure, distribution and analysis. In *The Biochemistry of Plants* (Stumpf, P. K. and Conn, E. E., eds.), vol. 4, pp. 1–55, Academic Press, New York
- Stymne, S. and Glad, G. (1981) Acyl exchange between oleoyl-CoA and phosphatidylcholine in microsomes of developing soya bean and its role in fatty acid desaturation. *Lipids* **16**, 298–305
- Lang, G. (1984) Glycerol 3-phosphate. In *Methods of Enzymatic Analysis* (Bergmeyer, H. U. and Grassl, M., eds.), vol. 6, pp. 525–531, Verlag Chemie, Weinheim
- Roughan, P. G. and Slack, C. R. (1992) Cellular organisation of glycerolipid metabolism. *Annu. Rev. Plant Physiol.* **32**, 97–132
- Harwood, J. L., Jones, A. L., Perry, H. J., Rutter, A. J., Smith, K. L. and Williams, M. (1994) Changes in plant lipids during temperature adaptation. In *Temperature Adaptation of Biological Membranes* (Cossins, A. R., ed.), pp. 107–139, Portland Press, London
- Stymne, S. and Stobart, A. R. (1987) Triacylglycerol biosynthesis. In *The Biochemistry of Plants* (Stumpf, P. K. and Conn, E. E., eds.), vol. 9, pp. 175–214, Academic Press, New York
- Stobart, A. R., Stymne, S., Shewry, P. R. and Napier, J. (1998) Triacylglycerol biosynthesis. In *Plant Lipid Biosynthesis: Fundamentals and Agricultural Applications* (Harwood, J. L., ed.), pp. 223–245, University Press, Cambridge
- Williams, M. and Harwood, J. L. (1994) Alternative pathways of phosphatidylcholine synthesis in olive callus cultures. *Biochem. J.* **304**, 463–468
- Perry, H. J. and Harwood, J. L. (1993) Use of [2-³H]glycerol precursor in radiolabelling studies of acyl lipids in developing seeds of *Brassica napus*. *Phytochemistry* **34**, 69–73
- Katavic, V., Reed, D. W., Taylor, D. C., Giblin, E. M., Barton, D. R., Zon, J., Mackenzie, S. L., Covello, P. S. and Kunst, L. (1995) Alteration of seed lipid composition by an ethyl methanesulphonate-induced mutation in *Arabidopsis thaliana* affecting diacylglycerol acyltransferase activity. *Plant Physiol.* **108**, 399–409
- Brindley, D. N. (ed.) (1988) *Phosphatidate Phosphohydrolase*, vol. 1, CRC Press, Boca Raton, FL
- Harwood, J. L. (1994) Environmental factors which can alter lipid metabolism. *Prog. Lipid Res.* **33**, 198–202
- Larson, T. R. and Graham, I. A. (2000) Application of a new method for the sensitive detection and quantification of acyl-CoA esters in *Arabidopsis thaliana* seedlings and mature leaves. *Biochem. Soc. Trans.* **28**, 575–577
- Johnson, P. E., Fox, S. R., Rawsthorne, S. and Hills, M. J. (2000) Inhibition by long-chain acyl-CoAs of glucose-6-phosphate metabolism in plastids isolated from developing embryos of oilseed rape. *Biochem. J.* **348**, 145–150
- Ramli, U. S., Baker, D. S., Quant, P. A. and Harwood, J. L. (2002) Control analysis of lipid biosynthesis in tissue cultures from oil crops shows that flux control is shared between fatty acid synthesis and lipid assembly. *Biochem. J.* **364**, 393–401
- Quant, P. A. (1993) Experimental applications of top-down control analysis to metabolic systems. *Trends Biochem. Sci.* **18**, 26–30
- Quant, P. A., Robin, D., Robin, P., Girard, J. and Brand, M. D. (1993) A top-down control analysis in isolated rat liver mitochondria: can the 3-hydroxy-3-methylglutaryl-CoA pathways be rate-controlling for ketogenesis? *Biochim. Biophys. Acta* **1156**, 135–143
- Manaf, A. M. and Harwood, J. L. (2000) Purification and characterisation of acyl-CoA:glycerol-3-phosphate acyltransferase from oil palm tissues. *Planta* **210**, 318–328
- Rutter, A. J., Sanchez, J. and Harwood, J. L. (1997) Glycerolipid synthesis by microsomal fractions from fruits and tissue cultures of olives. *Phytochemistry* **46**, 265–272
- Bafor, M. E., Johnson, L., Stobart, A. K. and Stymne, S. (1990) Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations for developing castor-bean endosperm. *Biochem. J.* **280**, 507–514
- Shintani, D. and Ohlrogge, J. B. (1995) Feedback inhibition of fatty acid synthesis in tobacco suspension cells. *Plant J.* **7**, 577–587
- Ramli, U. S. (1999) Ph.D. Thesis, University of Wales, Cardiff
- Bao, X. and Ohlrogge, J. B. (1999) Supply of fatty acid is one limiting factor in the accumulation of triacylglycerol in developing embryos. *Plant Physiol.* **120**, 1057–1062