Control analysis of lipid biosynthesis in tissue cultures from oil crops shows that flux control is shared between fatty acid synthesis and lipid assembly

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Top-Down (Metabolic) Control Analysis (TDCA) was used to examine, quantitatively, lipid biosynthesis in tissue cultures from two commercially important oil crops, olive (*Olea europaea L.*) and oil palm (*Elaeis guineensis* Jacq.). A conceptually simplified system was defined comprising two blocks of reactions: fatty acid synthesis (Block A) and lipid assembly (Block B), which produced and consumed, respectively, a common and unique system intermediate, cytosolic acyl-CoA. We manipulated the steady-state levels of the system intermediate by adding exogenous oleic acid and, using two independent assays, measured the effect of the addition on the system fluxes (J_A and J_B). These were the rate of incorporation of radioactivity: (i) through Block A from [1-^{14}C]acetate into fatty acids and (ii) via Block B from [U-^{14}C]glycerol into complex lipids respectively. The data showed that fatty acid formation (Block A) exerted higher control than lipid assembly (Block B) in both tissues with the following group flux control coefficients (C):

(i) Oil palm: $^{*}C^{J_{TL}}_{BlkA} = 0.64 \pm 0.05$ and $^{*}C^{J_{TL}}_{BlkB} = 0.36 \pm 0.05$
(ii) Olive: $^{*}C^{J_{TL}}_{BlkA} = 0.57 \pm 0.10$ and $^{*}C^{J_{TL}}_{BlkB} = 0.43 \pm 0.10$

where $^{*}C$ indicates the group flux control coefficient over the lipid biosynthesis flux (J_{TL}) and the subscripts BlkA and BlkB refer to defined blocks of the system, Block A and Block B. Nevertheless, because both parts of the lipid biosynthetic pathway exert significant flux control, we suggest strongly that manipulation of single enzyme steps will not affect product yield appreciably. The present study represents the first use of TDCA to examine the overall lipid biosynthetic pathway in any tissue, and its findings are of immediate academic and economic relevance to the yield and nutritional quality of oil crops.

Key words: oil palm (*Elaeis guineensis* Jacq.), olive (*Olea europaea L.*), Top-Down Control Analysis, triacylglycerol production.

INTRODUCTION

Considerable work has been carried out on the biochemistry of lipid metabolism in plants (see [1–6]). Hence it is well documented that the process in the so-called '$C_{18:3}$' plants, such as olive (*Olea europaea L.*) and oil palm (*Elaeis guineensis* Jacq.) involves the co-operation of two different subcellular compartmentations – the plastid and the endoplasmic reticulum. Fatty acids are produced by *de novo* synthesis in the plastid and then exported mainly to the cytosol as acyl-CoAs. This gives rise to 'eukaryotic lipids', which have C_{18} fatty acids at the *sn*-2 position, while the small amount of complex-lipid formation in the plastid gives rise to 'prokaryotic lipids' with C_{16} fatty acids at the *sn*-2 position [3]. Synthesis of storage lipid triacylglycerol (TAG) (as well as 'eukaryotic' membrane lipids) takes place predominantly via the glycerol 3-phosphate (or Kennedy) pathway [7] in the endoplasmic reticulum. Moreover, this spatially separate synthesis of prokaryotic and eukaryotic lipids (see [6]) has been confirmed recently by stable-isotope methodology [8].

Despite a detailed understanding of many aspects of the biochemical pathways of plant lipid metabolism, and even of the genes coding for many of the enzymes involved [5,6,9–13], our knowledge of their regulation and control is far from clear and it remains a relatively unexplored field [14]. This paucity of knowledge about control sites has been revealed when manipulation of isolated genes in many crops has led to little or no change in the lipids of the transgenic plants [14]. Thus overexpression of a so-called 'regulatory' enzyme may not lead to an increase of flux through a pathway. In fact, treatment with antisense RNA has often given very modest, or even no, changes [15].

Part of the problem with trying to identify simple steps in metabolism which can 'regulate' pathways is that the concept is misguided. Indeed, as has been emphasized in discussions about Metabolic Control Analysis (MCA), all reactions and hence all enzymes, can be important in a pathway. Moreover, depending on (physiological) conditions, different reaction steps can exert various amounts of control. For a detailed discussion of these concepts, see [16,17]. The theory of MCA was put forward many years ago [18,19], but has been unfairly and irrationally ignored by many biochemists. The theory shows that control of flux is distributed throughout a pathway and, therefore, that the concept of a single 'rate-limiting' reaction is an over-simplification. Therefore, it is essential to carry out MCA of important primary pathways of metabolism, such as lipid biosynthesis, in order to identify regions (and, perhaps, enzyme steps) where strong control is exerted and which may be worth manipulating genetically. This is especially important in view of the economic potential of genetically manipulated oil crops for improved nutritional quality or as renewable sources of petrochemical substitutes [6,20]. To emphasize the importance of carrying out proper MCA, a detailed study of the problem of achieving increases in metabolite production showed that alteration of a single reaction step seldom results in a significant change in primary product yield [21].

Tissue cultures represent a convenient experimental system for studying lipid biosynthesis and its regulation. Indeed, the justifi-

Abbreviations used: MCA, Metabolic Control Analysis; TDCA, Top-Down Control Analysis; TAG, triacylglycerol; DAG, diacylglycerol; PtdOH, phosphatidic acid; lysoPtdOH, lysophosphatidate; PtdCho, phosphatidylcholine; ACP, acyl carrier protein; FAME, fatty acid methyl ester.

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cation for the use of olive cultures and their applicability to *in vivo* situations has been discussed thoroughly [22,23]. In the previous paper, we carried out preliminary experiments using callus cultures from both olive and oil-palm tissues in order to delineate the use of different radiolabelled precursors and their labelling characteristics as well as to check that all the analytical methods were appropriate [24].

Temperature-manipulation studies suggested that the main control of carbon flux for lipid biosynthesis is exerted by enzymes of fatty acid biosynthesis rather than those involved in complex lipid assembly via the Kennedy pathway [24]. Therefore the present experiments were carried out in order to gain more information about the control structure of storage lipid accumulation of these tissues using MCA. Several techniques of MCA can be used to measure the degree of flux control in a metabolic pathway [16,17]. The first application of MCA to plant lipid metabolism was the demonstration (by Bottom-Up Control Analysis using a specific inhibitor) that acetyl-CoA carboxylase exerted strong flux control during light-stimulated fatty acid biosynthesis in leaves [25]. Another method that was of interest to us is Top-Down Control Analysis (TDCA) [26–28], because it can provide an immediate overview of the control structure of the whole pathway, thus giving information about the control exercised by large sections of a complex pathway. Thus, because we know so little about the regulation of plant lipid metabolism [14], we decided to apply TDCA as an appropriate approach to gain an initial picture for the control structure of the whole lipid-biosynthetic pathway.

In these experiments we made use of exogenous fatty acids in order to manipulate the fatty-acid-biosynthetic and the lipid-assembly pathways via changes in the steady-state levels of the cytosolic acyl-CoA pool. The new steady-state levels of the two system fluxes were then measured independently. Although exogenous fatty acids have not been used before for MCA experiments, we thought that they might be suitable because of some previous observations. First, fatty acids were shown to decrease fatty acid synthesis in tobacco (*Nicotiana tabacum*) suspension cells [29]. Secondly, they were also shown to stimulate the Kennedy pathway in *Cuphea lanceolata* (cigar flower) [30] and, recently, this result was confirmed for other embryos [31]. Having shown that exogenous oleate gave both these effects in callus cultures of oil palm and olive, we could then apply MCA to detailed experiments on lipid formation in these two important oil crops. To our knowledge, this is the first application of TDCA to lipid biosynthesis in any organism.

EXPERIMENTAL

Materials

Oil-palm and olive callus cultures were established and maintained on modified Murashige and Skoog [32] medium as described previously [24].

Sodium [1-^{14}C]acetate (sp. radioactivity 2.11 GBq/mmol), [U-^{14}C]glycerol (sp. radioactivity 5.51 GBq/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). TLC was performed on silica-gel G plates (Merck Ltd, Lutterworth, Leicestershire, U.K.). Phospholipids and fatty acid methyl ester (FAME) standards were obtained from Sigma (Poole, Dorset, U.K.) and NuChek (Elysian, MN, U.S.A.), respectively. Sep-pak C_{18} cartridges were from Waters (Milford, MA, U.S.A). All other chemicals and solvents used in lipid extraction and analysis were from Sigma or BDH (Poole, Dorset, U.K.). The best available analytical grades were used.

Radiolabelling studies

Callus cultures (20–25 days after previous subculturing) were selected for uniformity of mass and morphological appearance. Fresh masses before incubation were recorded and all results are presented on a per-gram-of-fresh-mass basis. Oil-palm or olive callus cultures (1–2 g fresh mass) were first pre-incubated with 400 μM oleic acid prepared as an emulsion in 100 mM sorbitol and 1% Tween-20 (Sigma) for 1 h followed by a further incubation for 8 h with 1 μCi of [1-^{14}C]acetate or [U-^{14}C]glycerol at 30 °C respectively. The pre-incubation period was found to be sufficient for oleate to enter the tissue significantly, as judged by a modification of the endogenous total fatty acid profile (results not shown). During the experiments there was no detectable release of $^{14}CO_2$, so we do not believe that fatty acid oxidation was a complication in our analysis. Such oxidation can sometimes be induced if fatty acids (though usually atypical ones) build up in plant tissues [33]. Control experiments were also carried out to ensure that Tween/oleate treatment did not alter penetration of the radiolabelled precursors throughout the callus tissues (see [24]). After incubation, the calli were rinsed briefly in 100 mM sorbitol and then inactivated by heating the calli in 1.25 ml propan-2-ol for 30 min at 70 °C, and quantitative extraction of lipids was carried out as described by Garbus et al. [34], and modified for plant tissues [35].

Radioactive samples were measured in a LKB Wallac 1209 Rackbeta counter (Wallac Oy, Turku, Finland) using Opti-Fluor scintillant fluid (Packard Bioscience, Groningen, The Netherlands). Quench corrections were made by the external-standard channels-ratio method.

Analytical procedures

Lipid, acyl-CoA, fatty acid and glycerol 3-phosphate analyses were carried out using the methods as described previously [24]. No change in the glycerol 3-phosphate pool size could be detected following Tween/oleate treatment.

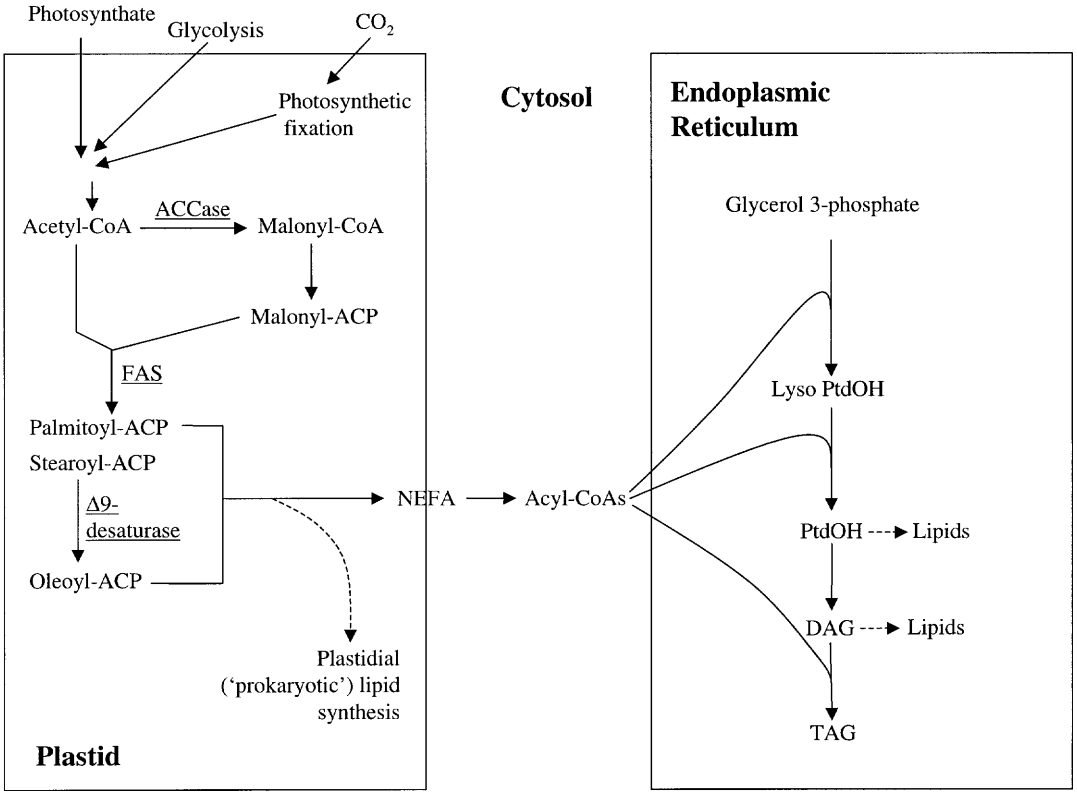
THEORY AS USED FOR TDCA OF LIPID BIOSYNTHESIS

Defining the system

To perform TDCA we needed to simplify conceptually the whole lipid biosynthetic pathway (Scheme 1) into two blocks of reactions connected uniquely by a single intermediate pool. Therefore, the lipid-biosynthesis pathway was divided into two blocks – i.e. the fatty-acid-synthesis block in the plastid (designated Block A) and the complex-lipid-assembly (TAG synthesis) block in the endoplasmic reticulum (designated Block B) with the cytosolic acyl-CoA pool designated as the system's intermediate (χ) (Scheme 2).

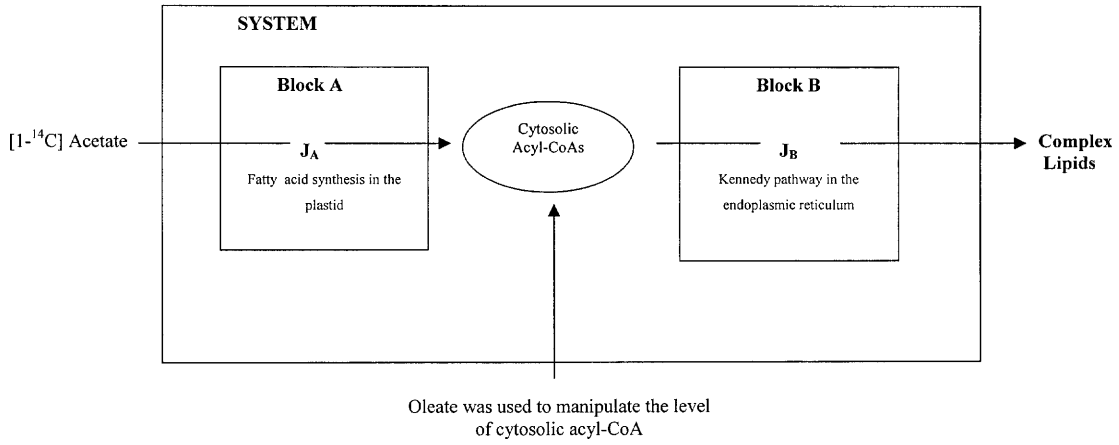
We treated all reactions leading to cytosolic acyl-CoA production in the plastid as one group, defined as Block A (with J_A as the flux through it). Thus Block A consisted of a number of enzymes which would be involved in [1-^{14}C]acetate metabolism – acetyl-CoA synthase, acetyl-CoA carboxylase, the Type II fatty acid synthase reactions, stearoyl-ACP desaturase, acyl-ACP thioesterases and acyl-CoA synthase (Scheme 1). The activity of the plastidic acyltransferases, if significant, would constitute a branch in the system. However, under the conditions of the analysis we had previously found [24] that incorporation of radioactivity by plastidic acyltransferases was very low and the system could be considered essentially as having a linear two-block organization.

Block B (with J_B as the flux through it) represents the group of enzymes in the endoplasmic reticulum which synthesize complex lipids from the cytosolic acyl-CoA pool. For storage-lipid



Scheme 1 Simplified plant lipid biosynthesis pathway showing fatty acid formation in the plastid

The acyl-ACPs produced by this process are either used directly for plastidic lipid production or hydrolysed and exported to the cytosolic compartment as acyl-CoAs. The latter are used by the acyltransferases of the Kennedy pathway on the endoplasmic reticulum. Abbreviations: ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; FAS, fatty acid synthase.



Scheme 2 Pathway reactions in lipid biosynthesis grouped together to allow the conceptually simplified system described in the text

Carbon flux from substrate pool, acetate, enters Block A to produce fatty acids. These fatty acids are then exported to the cytosol as acyl-CoA esters, which then serve as substrates for the Kennedy-pathway enzymes during TAG synthesis on the endoplasmic reticulum. Therefore, the two blocks of reactions are connected via a single pool of cytosolic acyl-CoA (as the system's intermediate). For the purpose of measuring kinetics of each block (i.e. J_A or J_B) independently, [U-^{14}C]acetate was used as carbon precursor for fatty acid synthesis in the plastid, while complex lipid assembly was followed conveniently with [U-^{14}C]glycerol, which proved to be selective for endoplasmic reticulum activities.

assembly, the enzyme reactions involved via the Kennedy pathway are: glycerol 3-phosphate acyltransferase, lysophosphatidate acyltransferase, phosphatidate phosphohydrolase and diacylglycerol (DAG) acyltransferase. Other reactions which were also active, as revealed by [1-^{14}C]acetate labelling, included those of phosphoacylglycerol or glycosylacylglycerol synthesis {via the eukaryotic pathway (see [3,6]) in olive and oil palm}. Additionally (although the situation is not known for olive or oil palm), a phosphatidylcholine:DAG acyltransferase [36] may be present. All such reactions are included in Block B.

For the purpose of TDCA, the fatty-acid-synthesis (Block A) and the TAG-assembly (Block B) pathways were manipulated indirectly via changes in the level of the system intermediate, acyl-CoA. These changes in the steady-state levels of the intermediate were achieved directly by manipulation with exogenous oleate and they were monitored by incorporation of radiolabel from labelled substrate, [1-^{14}C]acetate, which was used as precursor for fatty acid synthesis. In our experiments, the fluxes through the two blocks of reactions were measured independently: J_A was measured by [1-^{14}C]acetate labelling and TAG assembly (J_B) was monitored directly by using [U-^{14}C]glycerol. Division of the lipid-biosynthesis pathway at the cytosolic acyl-CoA pool had an additional advantage which related to lipid production in $C_{18:3}$ plants [6]. That is, as mentioned in the Introduction, cytosolic acyl-CoAs are the form in which lipids are exported from the plastid [3,9] and, hence, provided the unique connection between the two co-operating subcellular compartments (Scheme 1).

Equations for calculating group elasticity and group flux control coefficients

Application of the TDCA and detailed explanations for the equations used for calculating group elasticity and group flux control coefficients have been described [27]. The flux control summation theorem (of MCA) [18,19] states that all the flux control coefficients in a pathway sum to one.

Thus, for the two blocks of reactions of the lipid biosynthesis pathway (Scheme 2), the sum of group flux control coefficients for Block A and Block B is one, i.e.:

$$*C^{J_{TL}}_{BlkA} + *C^{J_{TL}}_{BlkB} = 1 \quad (1)$$

where $*C$ indicates the group flux control coefficient over the lipid biosynthesis flux (J_{TL}) and the subscripts BlkA and BlkB refer to defined blocks of the system, Block A and Block B.

The connectivity theorem [18,19], which holds for TDCA [26], expresses the relationship between flux control coefficients and elasticity coefficients. Thus, in our system:

$$(*C^{J_{TL}}_{BlkA})(*\epsilon^{BlkA}_{\chi}) + (*C^{J_{TL}}_{BlkB})(*\epsilon^{BlkB}_{\chi}) = 0 \quad (2)$$

Where $(*\epsilon^{BlkA}_{\chi})$ and $(*\epsilon^{BlkB}_{\chi})$ are group elasticity coefficients for Block A and Block B with respect to the system intermediate cytosolic acyl-CoA (χ).

Therefore, substitution of eqn (2) into eqn (1) gives us the equations for calculating group flux control coefficients for Block A as:

$$*C^{J_{TL}}_{BlkA} = \frac{(*\epsilon^{BlkB}_{\chi})}{(*\epsilon^{BlkB}_{\chi}) - (*\epsilon^{BlkA}_{\chi})} \quad (3)$$

and for Block B as:

$$*C^{J_{TL}}_{BlkB} = \frac{(*\epsilon^{BlkA}_{\chi})}{(*\epsilon^{BlkA}_{\chi}) - (*\epsilon^{BlkB}_{\chi})} \quad (4)$$

Elasticity coefficients are defined [18,19] as:

$$*\epsilon^{Step}_{\chi} = \frac{\delta v_{step}}{v_{step}} \cdot \frac{\chi}{\delta_{\chi}} \quad (5)$$

where v is the flux through the step, or, in this case, Block A and Block B (J_A or J_B). Therefore each of the two group elasticity coefficients can be expressed in terms of steady-state fluxes and intermediate levels, as:

$$*\epsilon^{BlkA}_{\chi} = \frac{\delta J_A}{J_A} \cdot \frac{\chi}{\delta_{\chi}} \quad (6)$$

where $\delta J_A/J_A$ is the fractional change in the steady-state flux through Block A of the pathway in response to a small fractional change in the level of $\chi(\delta\chi/\chi)$.

Similarly, the group elasticity coefficient of Block B to χ may be expressed as:

$$*\epsilon^{BlkA}_{\chi} = \frac{\delta J_B}{J_B} \cdot \frac{\chi}{\delta_{\chi}} \quad (7)$$

where $\delta J_B/J_B$ is the fractional change in the steady-state flux through Block B of the pathway in response to a small fractional change in the level of χ.

Therefore, substitution of eqns (6) and (7) into both eqns (3) and (4) permits us to calculate $(*C^{J_{TL}}_{BlkA})$ and $(*C^{J_{TL}}_{BlkB})$ from the steady-state flux and intermediate data resulting from single manipulation experiments.

RESULTS AND DISCUSSION

Inhibition of [1-^{14}C]acetate incorporation by exogenous oleate

Mindful of previous data from tobacco (*Nicotiana tabacum*) suspension cultures [29] or isolated embryos [30], which showed that lipid synthesis could be changed by exogenously supplied fatty acids, we decided to try similar techniques to manipulate carbon flux indirectly (via manipulation of the system intermediate with exogenous oleic acid) in our cultures. We chose to

Table 1 Effect of exogenous oleate on the incorporation of radioactivity from [1-^{14}C]acetate into total lipids and the cytosolic acyl-CoA pool of oil-palm calli (a) and olive calli (b)

Data in parentheses represent results as percentages of the untreated control values. Calli were pre-incubated with 400 μM oleate for 1 h prior to a further 8 h incubation with 1 μCi of [1-^{14}C]acetate at 30 °C. For further details and lipid analytical methods, see the Experimental section. Results are means ± S.D.s (n = 3, except *, where $n = 4$).

(a)

Treatment	10^{-5} × Radioactivity in total lipids (d.p.m.) (J_{A0})	10^{-3} × Radioactivity in acyl-CoAs (d.p.m.) (φ_0)
No addition		
Expt. 1	6.6 ± 1.9	3.6 ± 0.5
Expt. 2	6.7 ± 0.8	7.7 ± 1.2
	10^{-5} × Radioactivity in total lipids (d.p.m.) (J_{A1})	10^{-3} × Radioactivity in acyl-CoAs (d.p.m.) (φ_1)
+ 400 μM oleate		
Expt. 1	4.9 ± 0.7 (74%)	3.1 ± 0.8 (86%)
Expt. 2	4.1 ± 0.4 (61%)	5.9 ± 0.3 (77%)

(b)

Treatment	10^{-5} × Radioactivity in total lipids (d.p.m.) (J_{A0})	10^{-3} × Radioactivity in acyl-CoAs (d.p.m.) (φ_0)
No addition		
Expt. 1	9.2 ± 1.5	4.8 ± 1.2
Expt. 2	2.4 ± 0.3	4.4 ± 0.9
Expt. 3	1.5 ± 0.3	6.6 ± 0.6
	10^{-5} × Radioactivity in total lipids (d.p.m.) (J_{A1})	10^{-3} × Radioactivity in acyl-CoAs (d.p.m.) (φ_1)
+ 400 μM oleate		
Expt. 1	4.1 ± 0.7 (45%)	3.4 ± 0.4 (71%)
Expt. 2	2.1 ± 0.1 (88%)	4.1 ± 0.6 (93%)
Expt. 3*	1.2 ± 0.3 (80%)	5.8 ± 0.9 (88%)

Table 2 Effect of exogenous oleate on the *de novo* labelling of fatty acids from [1-^{14}C]acetate in oil-palm calli (a) and olive calli (b)

Incubations were carried out as detailed in the Experimental section. Fatty acid methyl esters were produced from total lipid extracts and analysed by radio-GLC. Incorporation of radiolabel into fatty acids was determined as percentage of total labelling using a Rachel software package [24]. Fatty acids are indicated by abbreviations showing the carbon chain separated by a colon from the number of double bonds. $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ were further identified as oleate, linoleate and α-linolenate respectively. Data are means ± S.D. ($n = 3$, except *, where $n = 4$). tr. = < 0.5%.

(a)

		Radioactivity (% of total)				
Treatment	Fatty acid ...	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Expt. 1						
No addition		36 ± 2	10 ± 2	43 ± 3	8 ± 1	3 ± tr.
+ 400 μM oleate		40 ± 4	17 ± 2	37 ± 4	5 ± 2	1 ± tr.
Expt. 2						
No addition		34 ± 2	8 ± 1	46 ± 3	9 ± 1	3 ± tr.
+ 400 μM oleate		37 ± 4	16 ± 2	39 ± 2	7 ± 1	1 ± tr.
Expt. 3						
No addition		34 ± 1	8 ± 1	50 ± 2	6 ± 1	2 ± tr.
+ 800 μM oleate		37 ± 1	13 ± tr.	44 ± 1	5 ± 1	1 ± tr.
P (Student's paired *t*-test) ...		< 0.05	< 0.01	< 0.05	< 0.05	< 0.01

(b)

		Radioactivity (% of total)				
Treatment	Fatty acid ...	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Expt. 1						
No addition		42 ± 2	4 ± 1	50 ± 3	2 ± tr.	2 ± tr.
+ 400 μM oleate		47 ± 2	6 ± 1	42 ± 3	3 ± tr	2 ± tr.
Expt. 2						
No addition		45 ± 1	6 ± 2	45 ± 2	3 ± tr.	1 ± tr.
+ 400 μM oleate		48 ± 5	6 ± tr.	40 ± 2	4 ± tr.	2 ± 1
Expt. 3*						
No addition		38 ± 3	3 ± tr.	55 ± 2	2 ± tr.	2 ± tr.
+ 800 μM oleate		40 ± 3	5 ± tr.	49 ± 1	3 ± tr.	3 ± tr.
P (Student's paired *t*-test) ...		> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

use oleic acid as the exogenous fatty acid, since this is a major metabolite of both olive and oil palm [37] and because its melting properties made it convenient to use *in vitro*. Owing to the insolubility of oleic acid in aqueous systems [38], it was first made up in Tween-20 for use in experiments with final concentrations of 100 mM sorbitol and 1% Tween-20 as utilized in tobacco cultures (see [29]). Consequently control calli were pre-incubated with 1% Tween-20 in 100 mM sorbitol but without exogenous oleic acid. Tween-20, at 1% concentrations, did not cause any change in penetration of radiolabelled precursors, in total lipid labelling or in the pattern of products compared with control calli with no addition (results not shown).

Results (Tables 1a and 1b) indicated that exogenous oleate decreased incorporation of radioactivity from [1-^{14}C]acetate in both of the calli. Variations from experiment to experiment presumably reflected the metabolic and/or developmental state of the preparations. These experiments showed that it is possible to manipulate fatty acid synthesis (J_A) by exogenous oleate in cultures of both oil palm and olive, as had been previously reported for tobacco [29].

As one might anticipate, an overall decrease in fatty acid synthesis was observed, due to a negative-feedback effect of newly formed acyl-CoAs following the addition and activation of unlabelled exogenous oleate to oleoyl-CoA. Hence, in both tissues, there was a decrease in the labelling of the acyl-CoA pool (Tables 1a and 1b) as the concentration of the intermediate increased to new steady-state levels.

Effect of exogenous oleate on the incorporation of radioactivity from [1-^{14}C]acetate into fatty acids

In addition to the inhibitory effect of exogenous fatty acids on the incorporation of radioactivity from [1-^{14}C]acetate into fatty acids, the calli responded towards the externally imposed imbalance in fatty acids by modulating the quality of fatty acids synthesized *de novo*. Thus, in oil palm, the results (Table 2a) showed that there was an increase, particularly, in the proportion of stearate that was labelled. This build-up of radioactivity counts in stearate indicated a constraint of carbon flux at the level of the Δ^9-desaturase, as might be expected, since oleate is the product of this enzyme. Treatment of the calli with exogenous oleate resulted in some reduction in the labelling of polyunsaturated fatty acids, particularly for α-linolenate, in olive compared with the results with oil palm. In any case, the polyunsaturates were less labelled in olive than in oil palm (Table 2b). Broadly, addition of exogenous oleate resulted in small changes in the proportion of radioactivity in individual fatty acids in olive. In contrast, there were increases in both linoleate and α-linolenate in the mass traces, which presumably reflects the

Table 3 Effect of exogenous oleate on the proportion of total fatty acids in oil-palm calli (a) and olive calli (b)

Incubations were carried out as detailed in Table 1.Fatty acid methyl esters were produced from total lipid extracts and analysed by radio-GLC. Mass traces were analysed by comparing peak areas of individual fatty acids with a pentadecanoate standard [24]. For fatty acid abbreviations, see Table 2.Data are means ± S.D. ($n = 3$, except *, where $n = 4$). tr. = < 0.5%.

(a)

		Proportion (% of total mass)				
Treatment	Fatty acid ...	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Expt. 1						
No addition		43 ± 3	3 ± tr.	40 ± 2	5 ± tr.	9 ± 2
+ 400 μM oleate		37 ± 2	2 ± tr.	43 ± 4	6 ± tr	12 ± 1
Expt. 2						
No addition		39 ± 2	2 ± tr.	40 ± 3	5 ± tr.	14 ± 5
+ 400 μM oleate		29 ± 2	3 ± tr.	43 ± 4	7 ± 1	18 ± 4
Expt. 3						
No addition		37 ± 3	3 ± tr.	38 ± 1	12 ± 2	10 ± 2
+ 800 μM oleate		26 ± 4	4 ± tr.	40 ± 4	17 ± tr.	13 ± 3
P (Student's paired *t*-test) ...		> 0.01	> 0.05	< 0.05	> 0.05	> 0.05

(b)

		Proportion (% of total mass)				
Treatment	Fatty acid ...	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Expt. 1						
No addition		41 ± 1	5 ± tr.	47 ± 2	3 ± tr.	4 ± 1
+ 400 μM oleate		38 ± 3	4 ± tr.	50 ± 1	4 ± tr.	4 ± tr.
Expt. 2						
No addition		39 ± 2	3 ± tr.	44 ± 2	3 ± tr.	11 ± 1
+ 400 μM oleate		36 ± 1	3 ± tr.	50 ± 2	2 ± tr.	9 ± 1
Expt. 3*						
No addition		32 ± 2	4 ± tr.	40 ± 2	6 ± tr.	18 ± 2
+ 400 μM oleate		30 ± 2	3 ± tr.	46 ± 1	6 ± tr.	15 ± 1
P (Student's paired *t*-test) ...		> 0.05	> 0.05	< 0.05	< 0.05	> 0.05

appreciable usage of exogenous oleate for further desaturation by the cells. Thus, although oleate actually enhanced total unsaturation (Tables 3a and 3b) because the exogenous acid was being used itself, it diluted the specific radioactivity of the substrate oleate pool and, hence, reduced radiolabel in the polyunsaturated fatty acids (Tables 2a and 2b).

Effect of exogenous fatty acids on the incorporation of [U-^{14}C]glycerol into lipids

While the addition of oleate caused a decrease in radioactivity incorporation from [1-^{14}C]acetate into fatty acids, the acid had a stimulatory effect on lipid labelling from [U-^{14}C]glycerol. Thus, for oil palm, oleate addition was able to increase total lipid labelling (Table 4a) about 60%. This agreed with the results of other experiments using plant tissues where exogenous fatty acids were able to stimulate lipid accumulation in oil crops [31]. As noted above, [U-^{14}C]glycerol only labelled Kennedy-pathway intermediates, and, furthermore, there was negligible radioactivity in the aqueous phase of the Garbus extraction system [34], showing that, after glycerol was taken up by the callus, it was selectively incorporated into lipids. Again, as seen with the [1-^{14}C]acetate labelling, olive calli seemed to be less susceptible to exogenous oleate than oil-palm calli, and there was an average 25% increase in the total lipid radioactivity from [U-^{14}C]glycerol (Table 4b). In both tissues (Tables 4a and 4b), exogenous oleate stimulated not only total lipid labelling but also the proportion in TAG. For olive but not oil palm the increased labelling of TAG was accompanied by a build-up of radioactivity in DAG. These data agree with those from our previous experiments [24] and suggest the DAG acyltransferase may exert significant flux control within the Kennedy pathway for olive but only weak control for oil palm. The conclusion for olive is in agreement with the results of Rutter et al. [39], where it was suggested that the enzyme might exert strong limitation over overall carbon flux at higher rates of lipid biosynthesis. Since there was no accumulation of radioactivity in lysophosphatidate (lysoPtdOH) or phosphatidic acid (PtdOH) for either callus type (Tables 4a, 4b), lysoPtdOH acyltransferase and phosphatidate phosphohydrolase did not seem to exert significant flux control, as concluded in similar studies with embryos [31]. In oilseed rape (*Brassica napus*), expression of yeast lysoPtdOH acyltransferase increased oil yields [40], though this has been suggested to be due to a stronger 'sink effect' in pulling carbon through the pathway [31]. Clearly, to quantify the possible control effects of individual steps in the Kennedy pathway requires ways of manipulating the enzymes' activities. In the absence of selective inhibitors, the use of genetically modified plants with altered activity seems to be an alternative approach for further definitive quantitative analysis.

Group flux-control coefficients

It is important to mention that the connectivity theorem used (eqn 5) is only valid for our two-block TDCA system of the lipid-

Table 4 Effect of exogenous oleate on the incorporation of radioactivity from [U-^{14}C]glycerol into total lipids and lipid classes of oil-palm calli (a) and olive calli (b)

Oil-palm and olive calli were pre-incubated with 400 μM oleate for 1 h prior to a further 8 h incubation with [U-^{14}C]glycerol at 30 °C. Lipids were extracted and analysed as described in the Experimental section. Only the four lipid classes shown contained significant radioactivity. Data are means $\pm$ S.D. ($n = 3$) *Significantly different from untreated controls (Student's paired *t*-test, $P < 0.05$). Values in parentheses are percentages of untreated-control values.

(a)

Treatment	10^{-4} × Radioactivity in total lipids (d.p.m./g fresh wt. of callus)	Lipid class ...	Labelling (% of total) LysoPtdOH	PtdOH	DAG	TAG*
Expt. 1						
No addition	3.8 $\pm$ 0.5		31 $\pm$ 6	32 $\pm$ 4	17 $\pm$ 3	20 $\pm$ 3
+ 400 μM oleate	6.2 $\pm$ 2.3 (163%)		27 $\pm$ 2	29 $\pm$ 1	15 $\pm$ 2	28 $\pm$ 2
Expt. 2						
No addition	4.3 $\pm$ 0.9		20 $\pm$ 2	46 $\pm$ 4	19 $\pm$ 5	15 $\pm$ 1
+ 400 μM oleate	6.8 $\pm$ 1.5 (158%)		21 $\pm$ 1	43 $\pm$ 1	15 $\pm$ 1	21 $\pm$ 1

(b)

Treatment	10^{-4} × Radioactivity in total lipids (d.p.m./g fresh wt. of callus)	Lipid class ...	Labelling (% of total) LysoPtdOH	PtdOH	DAG	TAG
Expt. 1						
No addition	2.3 $\pm$ 0.4		22 $\pm$ 2	30 $\pm$ 3	28 $\pm$ 4	20 $\pm$ 2
+ 400 μM oleate	3.4 $\pm$ 0.9 (148%)		14 $\pm$ 3	26 $\pm$ 2	34 $\pm$ 3	26 $\pm$ 1
Expt. 2						
No addition	5.8 $\pm$ 0.3		23 $\pm$ 1	37 $\pm$ 3	28 $\pm$ 2	12 $\pm$ 1
+ 400 μM oleate	6.5 $\pm$ 1.1 (112%)		17 $\pm$ 2	31 $\pm$ 1	34 $\pm$ 3	18 $\pm$ 2
Expt. 3						
No addition	3.8 $\pm$ 0.3		22 $\pm$ 2	35 $\pm$ 1	27 $\pm$ 2	16 $\pm$ 2
+ 400 μM oleate	4.4 $\pm$ 0.5 (116%)		16 $\pm$ 2	31 $\pm$ 2	32 $\pm$ 3	21 $\pm$ 2
	P (Student's paired *t*-test) ...		< 0.01	< 0.05	< 0.05	< 0.01

biosynthesis pathway (Scheme 1) if none of the intermediates in either subsystem interacts with the other subsystem, except through the system intermediate acyl-CoA [26]. In our analysis several control experiments were initially carried out to ensure there was no intermixing process between plastidial and endoplasmic lipid pools [24,41,42].

First, we determined the major destination of fatty acids formed by *de novo* synthesis in the plastid. This was achieved by monitoring the labelling of lipids made by the plastid-localized pathway such as phosphatidylglycerol and sulpholipid. In such a case, the fatty acids produced by *de novo* synthesis would be utilized directly for plastidic lipid membrane formation at the level of acyl-ACPs instead of being directed towards the acyl-CoA pool i.e. the 'prokaryotic' pathway. We found only low labelling rates in respect of these lipids ($< 2\%$ of total lipid labelling), showing that almost all the newly synthesized fatty acids were directed to the acyl-CoA pool, and, therefore, destined for the cytosolic compartment. In addition, thioesterase activity could deplete the acyl-CoA pool directly by giving rise to non-esterified fatty acids. These products could also be formed by acyl-ACP hydrolysis, which could reduce the quantity of acyl-CoA labelling indirectly. However, such reactions were found to account only for about 3% of the total radioactive products. Therefore, from these experiments with [1-^{14}C]acetate, we concluded that fatty acids synthesized in the plastids of both olive and oil-palm cultures are mainly ($> 95\%$) channelled into the extraplastidic compartment for (storage) lipid assembly.

Furthermore, radiolabelling of lipids from [U-^{14}C]glycerol revealed that only the Kennedy-pathway intermediates (lyso-PtdOH, PtdOH, DAG and TAG) were labelled, so that side reactions (such as conversion of DAG into PtdCho) were not a complication when using this precursor. These data showed that a simplistic view of Block A as representing *de novo* fatty acid synthesis in plastids and Block B as the Kennedy pathway in the endoplasmic reticulum (Scheme 2) was justified.

We measured changes in the steady-state cytosolic acyl-CoA levels (by two methods: as a function of radiolabel incorporation and of absolute concentration) and fluxes through Blocks A and B (J_A, J_B) following manipulation of the system intermediate (X) by exogenous oleate. The absolute cytosolic acyl-CoA concentrations in oil palm were 53 $\pm$ 16 μM and 130 $\pm$ 36 μM in the absence and presence of oleate respectively. This allowed us to derive absolute elasticity and, hence, flux control coefficients for the two blocks of reactions (Scheme 2) over lipid biosynthesis. Our analyses [using cytosolic acyl-CoA data expressed as d.p.m. (Tables 1a and 1b)] gave values of 0.64 $\pm$ 0.05 versus 0.36 $\pm$ 0.05 for the group flux control coefficients of fatty-acid-synthetic and the Kennedy-pathway blocks in oil palm respectively (Table 5a). The same coefficients were also calculated to be 0.67 and 0.33 in oil palm using the appropriate equations and the cytosolic acyl-CoA concentration data given above. Parallel studies in olive gave group flux control coefficients of 0.57 $\pm$ 0.10 for Block A and 0.43 $\pm$ 0.10 for Block B (Table 5b). In the presence of higher concentrations of oleic acid (800 μM), the sum of the fluxes and intermediates were slightly different, but their ratios were similar.

In the accompanying paper [24] we briefly describe a method detailed with a worked example and justified in two previous papers [27,43]. This method permits the calculation of absolute

Table 5 Group elasticity coefficients ($^{*}\epsilon_{\chi}^{BlkA}$; ϵ_{χ}^{BlkB}) and group flux control coefficients ($^{*}C_{BlkA}^{J_{TL}}$; $^{*}C_{BlkB}^{J_{TL}}$) for lipid biosynthesis in oil-palm calli (a) and olive calli (b)

Flux control coefficients for Block A and Block B were calculated using eqns. (3) and (4). Whether flux control coefficients are calculated from the data with 400 μM or 800 μM oleate, the values are similar and, therefore, all were used to obtain mean values for the experiments.

(a)

Expt.	[Oleate] (μM)	$^{*}\epsilon_{\chi}^{BlkA}$	$^{*}\epsilon_{\chi}^{BlkB}$	$^{*}C_{BlkB}^{J_{TL}}$	$^{*}C_{BlkA}^{J_{TL}}$
1	400	1.87	−4.54	0.70	0.30
2	400	1.67	−2.48	0.60	0.40
3	800	0.61	−0.99	0.62	0.38
			Mean ± S.D. ($n = 3$) ...	0.64 ± 0.05	0.36 ± 0.05

(b)

Expt.	[Oleate] (μM)	$^{*}\epsilon_{\chi}^{BlkA}$	$^{*}\epsilon_{\chi}^{BlkB}$	$^{*}C_{BlkA}^{J_{TL}}$	$^{*}C_{BlkB}^{J_{TL}}$
1	400	1.89	−1.65	0.47	0.53
2	400	0.40	−0.95	0.70	0.30
3	400	1.18	−1.76	0.60	0.40
4	800	1.25	−1.25	0.50	0.50
			Mean ± S.D. ($n = 4$) ...	0.57 ± 0.10	0.43 ± 0.10

group flux control coefficients for simple linear systems as a unique function of relative changes in the system fluxes, without the need to measure or assess the relative or absolute changes in a complex system intermediate. Because we had some experimental concerns associated with potential errors in intermediate measurements, we also calculated the group flux control coefficients using this alternative method (full calculations not shown). Regardless of the method used for calculating the coefficients, our answers were essentially the same, e.g. the meaned result for experiments 1 and 2 in oil palm, using the second method, gave us the following numerical values for the flux control coefficients: 0.65 for Block A and 0.35 for Block B.

These data and our previous results showed that, in both tissues, approx. 60% of the control over lipid biosynthesis resided in the fatty acid synthesis block. These values were also comparable with those obtained using double manipulation of flux (a third method of calculating flux control), where specific inhibitors were used to alter Block A or Block B [44]. Since double-manipulation TDCA does not change the acyl-CoA pool directly, these experiments gave us added confidence that the present experiments using oleate to manipulate the acyl-CoA pool were giving meaningful results. Moreover, we have shown that the overall control structure for overall lipid biosynthesis was not significantly different in olive and oil-palm cultures.

Our results agree with those from other studies where it has been suggested that fatty acid supply is more important than the activity of lipid assembly enzymes for TAG accumulation in oil crops [30,31]. The preliminary experiments that we carried out by temperature manipulation [24] also intimated that Block A was more important in olive and oil-palm calli. Even so, it is noteworthy that about 40% of the overall control is exerted by the steps comprising Block B. This would suggest that increasing expression of genes for enzyme steps in Block A would not, necessarily, increase product yields and could explain the disappointing results obtained in many transgenic crops [14].

Conclusions

We have shown clearly that, in our experimental systems (using two commercially important oil crops), control of lipid biosynthesis is distributed between both parts of the overall pathway. Although there is more control invested in the fatty-acid-synthetic block, significant control is exerted by both blocks of reactions in both tissues. This means, as in the study of metabolite production in yeast [21], manipulation of a single step in the overall pathway will have little effect on product yield.

We have also shown that manipulation of lipid synthesis in conjunction with TDCA can serve as an effective way of studying the regulation and control of this primary pathway of metabolism. Because of the commercial interest in manipulating oil production in plants [20] and the often poor results that have been obtained after specific genetic manipulations [6,14], knowledge of the control structure of the pathway is obviously vital. Our experiments represent the first use of flux control analysis to examine the overall pathway and, thus, provide a foundation for further, much needed, experiments.

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