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QUANTITATIVE ANALYSIS OF FLUX CONTROL OVER LIPID BIOSYNTHESIS IN OIL PALM (*Elaeis guineensis*) MESOCARP

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ABSTRACT

*Plant storage oils are of major commercial importance, yet our understanding of the regulation and control of their synthesis is poor. The control of lipid biosynthesis fluxes in the mesocarp of oil palm (*Elaeis guineensis*) was studied using modular (top-down) metabolic control analysis (TDCA). This allowed us to determine the relative contribution of two groups of reactions, fatty acid formation (Block A) and lipid assembly (Block B), to the control structure of overall pathway to triacylglycerol. The pathway was manipulated in two ways. Single manipulation involved the addition of oleate which inhibited fatty acid formation in Block A and stimulated lipid assembly in Block B. In double manipulation experiments, cerulenin was used in inhibition of Block A and Block B was inhibited by bromooctanoate. Single manipulation-TDCA revealed that the group flux control coefficient for fatty acid synthesis was 0.65 and 0.35 for lipid assembly. Double manipulation-TDCA has a value of 0.6 for fatty acid synthesis and 0.4 for lipid assembly. Taken together, these data showed that under our experimental conditions, about 60%-65% of the total metabolic flux control lay in the fatty acid synthesis group of reactions. Nevertheless because both parts of the lipid biosynthesis pathway exert significant flux control, we suggest strongly that manipulation of single enzyme will not affect the product yield appreciably.*

Keywords: palm oil, metabolic control analysis, lipid biosynthesis pathway.

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INTRODUCTION

Palm oil being the world's major edible oil is of great importance for Malaysia which exports over 90% of its palm oil products (Ahmad, 2003). Recent attempts at genetic manipulation have proved relatively useful and it is probable that there will be many transgenic varieties of utility in the future (Kinney, 2002). Indeed, such transgenics are already proven useful in providing information about regulation of metabolism (Voelker and Kinney, 2001; Kinney, 2006). Despite the economic importance of oil crops, as well as the great advances in our knowledge of their basic biochemistry and molecular biology (Harwood, 1996; Murphy, 2005), we still know relatively little about the regulation and/or control of lipid synthesis and accumulation in any oil crop (Ohlrogge and Jaworski, 1997). In oil palm, the genetic engineering programme has successfully developed genetic modification technology that will enhance the productivity and value of the oil palm and its by-products (Parveez *et al*., 2005). Elucidating fluxes through the network of carbon metabolism is needed to provide a framework for rational metabolic engineering of oil crops such as oil palm. To partly address this deficiency, we have applied the principles of metabolic control analysis. Initial experiments were conducted with oil palm calli (Ramli *et al*., 2002a, b; 2005). In order to study the control of carbon flux in oil palm fruits, a similar flux analysis was extended to the oil palm mesocarp.

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The theory of metabolic control analysis has been discussed thoroughly by Fell (1997). Using this method, the degree of control exerted by a step in a pathway can be measured using two different approaches (i) 'bottom-up control analysis' (BUCA), and (ii) 'top-down control analysis' (TDCA). Our first experiments with oil palm calli allowed the development of appropriate mathematical equations to demonstrate the reliability of assays and to asses the importance of minor pathways compared to the overall biosynthesis of lipids (triacylglycerol). Through TDCA, the lipid biosynthesis pathway was divided into two blocks, the first of which is fatty acid biosynthesis [Block A) [which includes among the enzymes involved, acetyl-CoA synthase, acetyl-CoA carboxylase, the various enzymes of fatty acid synthases and desaturases] which takes place in the plastid (Harwood, 1998). The acyl-CoAs pool is the system intermediate, and the lipid assembly that involves all the reactions associated with the Kennedy pathway is the second block of reactions (Block B). The latter takes place in the endoplasmic reticulum and, therefore, is in a separate subcellular compartment to the fatty acid biosynthesis block of reactions. The simplified system used for TDCA and further explanation can be found in Ramli *et al*. (2002b).

Initially, single manipulation-TDCA was carried out using exogenous fatty acid (oleate) to manipulate the steady-state levels of the system intermediate, cytosolic acyl-CoAs. Using two independent assays (with radiolabel [1-^{14}C]acetate and [U-^{14}C]glycerol as precursors), we measured the effect of oleate addition on the system fluxes through Blocks A and B, respectively. The data showed that fatty acid formation exerted higher control than lipid assembly. In order to obtain additional evidence to support our findings and to allow elasticity analysis, the double manipulation-TDCA approach was used. For this, inhibitors were used to reduce the activity of enzyme(s) either in Block A or Block B. To perform a valid analysis for the double manipulation-TDCA, the method requires that any changes in the carbon flux through Block A do not affect the carbon flux through Block B other than through the chosen intermediate, acyl-CoAs. Similarly, any effect that an inhibitor of the activity of Block B, has on the fatty acid synthesis reactions must also only be via the effect on the system intermediate. In this experiment, cerulenin and bromooctanoate were used to manipulate the carbon fluxes through Blocks A and B independently. Cerulenin was reported to be a specific inhibitor of condensing enzymes while bromooctanoate is a specific inhibitor of diacylglycerol acyltransferase (Harwood 1998; Weselake *et al*., 1991). Here we discuss the results from our experiments using metabolic control analysis to examine the control structure over the lipid biosynthesis pathway in the oil palm mesocarp using single and double manipulation-TDCA.

MATERIALS AND METHODS

Materials

Oil palm (*Elaeis guineensis*) fruits at 18 weeks after anthesis were used.

Radiolabelling Studies

Oil palm mesocarp slices (0.5 g fresh weight) were first pre-incubated with cerulenin or bromooctanoate for 1 hr followed by a further incubation for 4 hr with 1 μCi [1-^{14}C]acetate or [U-^{14}C]glycerol at 30^{o}C, respectively. Control experiments were also carried out to ensure that the inhibitor treatment did not alter penetration of the radiolabelled precursors throughout the mesocarp tissues. After incubation, the tissues were rinsed briefly in 100 mM sorbitol and then inactivated by heating the tissues in 1.25 ml propan-2-ol for 30 min at 70^{o}C, and quantitative extraction of lipids was carried out as described by Ramli *et al*. (2002a).

Analytical Procedures

Lipid and acyl-CoAs analyses were carried out using the methods described in Ramli *et al*. (2002a).

Defining the System

The system used for the top-down analysis is shown and briefly discussed in *Figures 1a* and *b*. To analyse the system, we carried out two separate control analyses - single manipulation-TDCA and double manipulation-TDCA (Ramli, 1999).

RESULTS AND DISCUSSION

Single Manipulation-TDCA

Oil palm mesocarp slices were incubated with various concentrations of oleate in Tween-20 carrier to allow single manipulation experiments to be performed. Our results showed that sufficient manipulation for TDCA was obtained using a final concentration of 400 μM oleate. The effect of oleate on [1-^{14}C]acetate uptake was measured for both the fatty acid synthesis and lipid assembly blocks of reactions. A typical result using 400 μM oleate is shown in *Figure 2*. As judged by the incorporation of radiolabelled [1-^{14}C]acetate relative to the control values (without oleate addition), fatty acid synthesis was inhibited by 22% while lipid assembly was

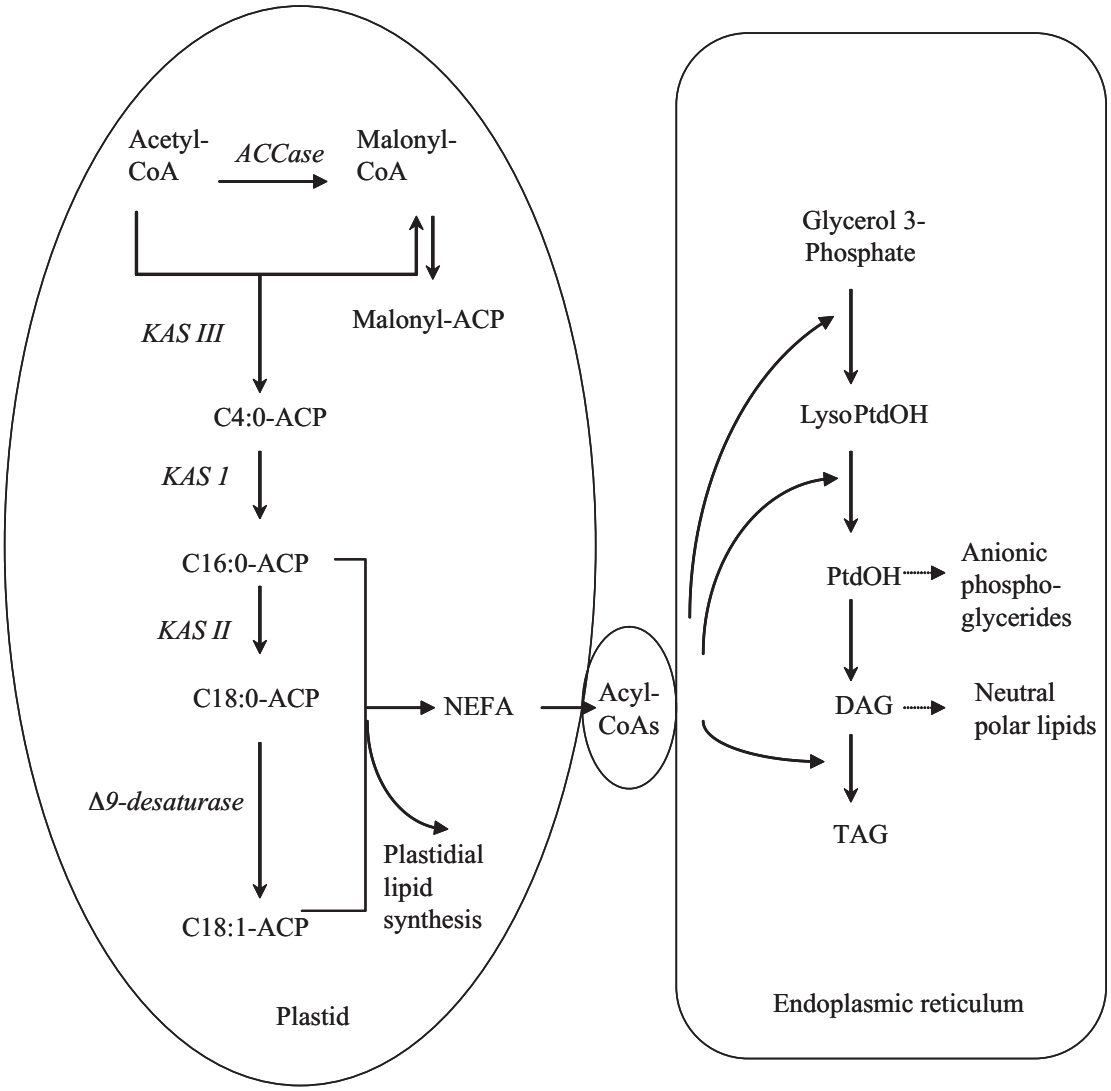

Figure 1a. Simplified scheme to show the co-operation of de novo *fatty acid synthesis in the plastid with triacylglycerol formation in the endoplasmic reticulum. Basically, three main areas in lipid biosynthesis have been recognized; (i) initiating step catalyzed by ACCase (ii)* de novo *fatty acid synthesis by fatty acid synthase together with desaturation reactions which determine the chain length and total saturated fatty acids, and (iii) assembly of complex lipids (*e.g. *triacylglycerol).*

Abbreviations: ACCase, acetyl CoA carboxylase; KAS, β-ketoacyl ACP synthase; NEFA, none esterified fatty acids; LysoPtdOH, lysophosphatidate; PtdOH, phosphatidate; DAG, diacylglycerol; TAG, triacylglycerol.

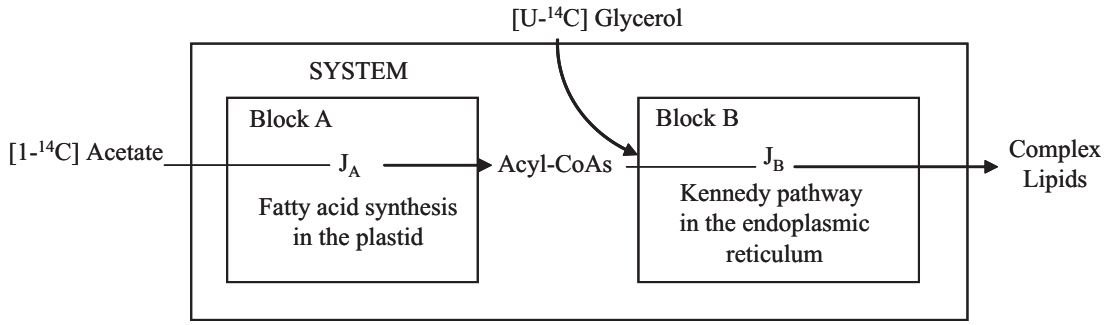

Figure 1b. Simplified lipid biosynthesis system for TDCA.

Notes: Carbon flux from substrate pool, acetate, enters Block A to produce fatty acids. These fatty acids are exported to the cytosol as acyl-CoA esters, which then serve as substrates for the endoplasmic reticulum activities during TAG synthesis on the endoplasmic reticulum. For the purpose of measuring the kinetics of each block (*i.e.* J_A and J_B) independently, [1-^{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.

stimulated by 28% using 400 μM oleate. Thus, this method caused a decrease in fatty acid synthesis (Block A reactions) and elevated lipid assembly (Block B reactions). By using data from a number of experiments and equations previously described (Ramli *et al*., 2002b), our result (summarized in *Table 1*) shows the group flux control coefficients to be about 0.65 for Block A and 0.35 for Block B. This indicated that, like the cultures of oil palm and olive (Ramli *et al*., 2002b) and, recently, of soya bean (Guschina *et al*., 2006), more control resided in the fatty acid synthesis than in lipid assembly. Therefore, measurement of the flux control coefficients for the mesocarp tissues using a different approach of TDCA was attempted to check these values.

TABLE 1. GROUP FLUX CONTROL COEFFICIENTS FOR LIPID BIOSYNTHESIS IN OIL PALM MESOCARP

Flux control coefficient (single manipulation-TDCA)	
Fatty acid synthesis (Block A)	Complex lipid assembly (Block B)
0.65	0.35

Double Manipulation-TDCA

For the double manipulation-TDCA, we used two specific inhibitors to manipulate fatty acid synthesis and lipid assembly, independently. Cerulenin is a known inhibitor of the condensing (KAS) reactions of fatty acid synthase, being particularly effective with KAS I (Jaworski, 1987). Examination of the pattern of fatty acid radiolabelling from [1-^{14}C]acetate revealed a relative increase in the 18C products formed, consistent with cerulenins's rather poor inhibition of KAS II (Harwood, 2005). We tested the effect of cerulenin on fatty acid synthesis. In oil palm mesocarp, cerulenin was found to be appropriate for TDCA at 10 μM with no significant effect on Block B (*Figure 3*). Bromooctanoate has been reported to be a specific inhibitor of diacylglycerol acyltransferase (DAGAT) from microspore-derived embryos of *Brassica napus* (Weselake *et al*., 1991). In previous experiments, addition of bromooctanoate to callus tissues resulted in the reduction in total lipid labelling from [U-^{14}C]glycerol but not from [1-^{14}C]acetate (Ramli *et al*., 2005). Thus, as shown in *Figure 3*, a reduced total labelling of lipids from

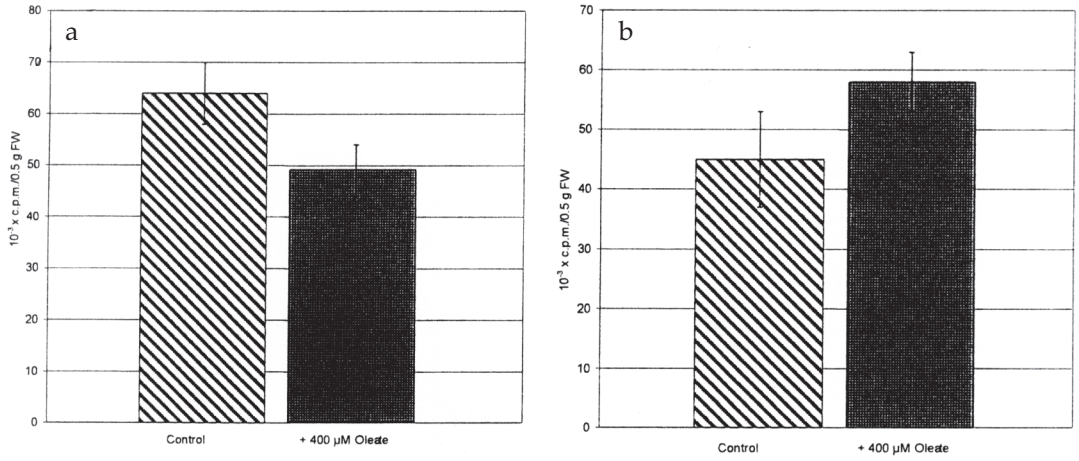

Figure 2. Single manipulation TDCA of oil palm mesocarp. Exogenous oleate was used to manipulate fatty acid synthesis and complex lipid assembly. Fatty acid synthesis was measured using [1-^{14}C]acetate (a) and lipid assembly using [U-^{14}C] glycerol (b). Results show means ± S.D.s (n=3).

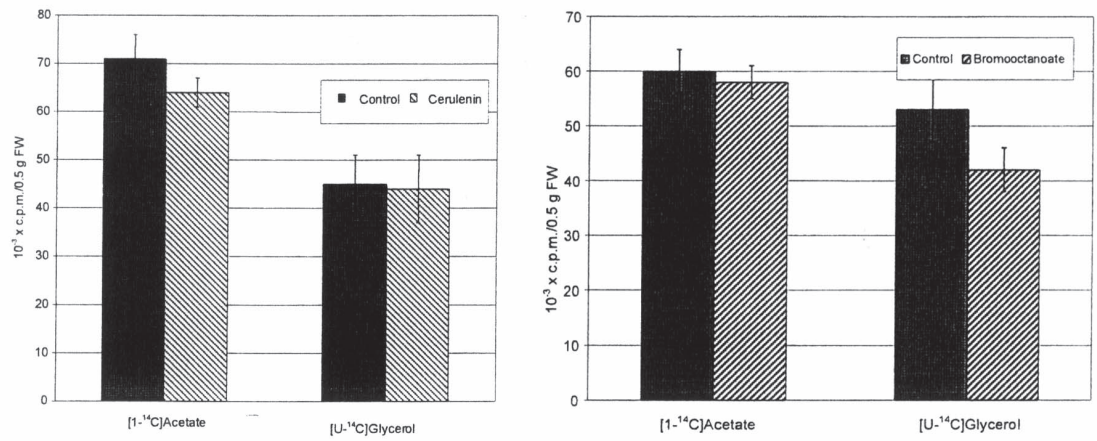

Figure 3. Specificity of inhibitor action in double manipulation TDCA. Cerulenin was used to inhibit fatty acid synthesis (Block A) and bromooctanoate to inhibit the endoplasmic reticulum activities (Block B). Results show means ± S.D.s (n=3).

[U-^{14}C]glycerol by 10%-15% was coordinately achieved with 0.2 μM bromooctanoate. At the same concentration, bromooctanoate had no significant effect on the labelling of fatty acids from [1-^{14}C]acetate.

Calculating the Flux Control Coefficients

The theory and equations used for calculating the elasticity and flux control coefficients have been described in detail (Ramli *et al*., 2002b) and are used here to derive the relationship between the changes in flux and intermediate concentrations (acyl-CoA) and the group flux control coefficients for each of the blocks over the pathway flux.

Our results has confirmed that the two inhibitors each targeted only one of the Blocks of reactions directly and could, therefore, be used for double manipulation experiments. We then carried out a series of such experiments using cerulenin and bromooctanoate at concentrations that only inhibited total labelling 10% – 20% (*Table 2*). The data were then used to construct a titration plot as shown in *Figure 4* which also gives the method to calculate the flux control coefficients. The theory used to derive the relationship between changes in flux and intermediate concentration and the group flux control coefficients for each block over pathway flux is elaborated in *Figure 5*. Our calculation for group flux control coefficients substituting the measurement of *a* and *b* (*Figure 4*) into Equations 4 and 5 (*Figure 5*) gave values of = 0.60 and 0.40 (*Table 3*). We also calculated group elasticity coefficients (*Table 2*) from the same data using the theory as

TABLE 2. VALUES FOR [1-^{14}C]ACETATE INCORPORATION INTO TOTAL LIPIDS AND ACYL-COAS FROM DOUBLE MANIPULATION-TDCA EXPERIMENTS WITH CERULENIN OR BROMOOCTANOATE IN OIL PALM MESOCARP AND GROUP ELASTICITY COEFFICIENTS

Expt.	[1-^{14}C]acetate ± 10 μM cerulenin			[1-^{14}C]acetate ± 0.2 mM bromooctanoate	
	Total lipids (c.p.m.)	Acyl-CoA (c.p.m.)		Total lipid (c.p.m.)	Acyl-CoA (c.p.m.)
Control	6 494 ± 110	1 188 ± 44	Control	6 415 ± 216	1 198 ± 54
+			+		
Cerulenin	6 163 ± 498	1 134 ± 108	Bromooctanoate	5 588 ± 243	1 394 ± 130
Group elasticity coeffients ($*\varepsilon_{\chi}^{BlkB}$,$*\varepsilon_{\chi}^{BlkA}$)					
$*\varepsilon_{\chi}^{BlkB}$(when cerulenin is added)			$*\varepsilon_{\chi}^{BlkA}$(when bromooctanoate is added)		
1.10			0.61		

Notes: Data as means ± S.D.s (n=6). Oil palm mesocarp was incubated with [1-^{14}C]acetate and with cerulenin or bromooctanoate. Total lipids were extracted and acyl-CoA was purified from the non-lipid phases as described previously (Ramli *et al*., 2002b). Group elasticity coefficients were calculated based on Equations 6 and 7 (*Figure 6*).

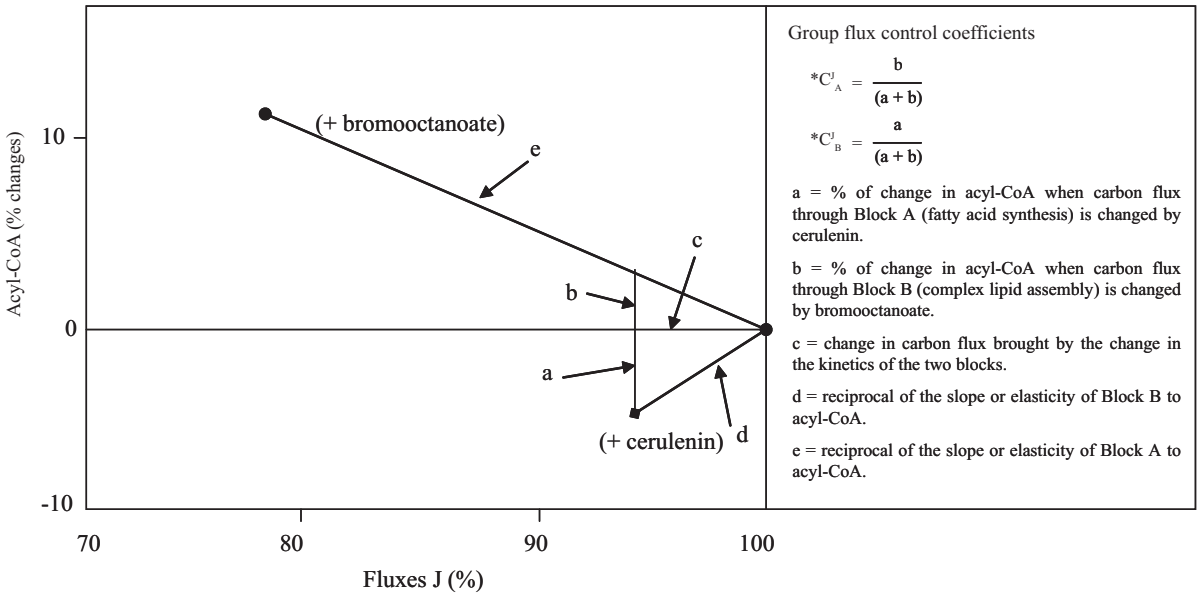

Figure 4. Geometric determination of flux control coefficients for lipid biosynthesis pathway in oil palm mesocarp.

described in *Figure 6* (Equations 6, 7). The values obtained were then used to calculate the group flux control coefficients derived from Equations 8 and 9 as given in *Table 3*. Our calculations gave values of 0.65 and 0.45 for Blocks A and B, respectively. Together with results from the single manipulation technique of top-down analysis (*Table 1*), we conclude that the fatty acid synthesis block of reactions (Block A) exerts about 60% of control over carbon flux through the lipid biosynthesis pathway while Block B exerts the remaining 40% of control in

TABLE 3. SUMMARY OF GROUP FLUX CONTROL COEFFICIENTS FOR FATTY ACIDS SYNTHESIS AND COMPLEX LIPID ASSEMBLY IN OIL PALM MESOCARP

Double-manipulation TDCA		
Method	$^{*}C^{J_{\pi}}_{BlkA}$	$^{*}C^{J_{\pi}}_{BlkB}$
Geometric Analysis (see *Figure 4*)	0.60	0.40
Elasticity coefficients (Equations 8 and 9, *Figure 6*)	0.65	0.35

From *Figure 4*, lines *d* and *e* represent changes in carbon flux with cerulenin (d) and 2-bromooctanoate (e), starting from a point with 100% of flux (no inhibition). The reciprocals of the slopes of *d* and *e* represent the group elasticity coefficients of Blocks B and A to changes in intermediate, acyl-CoA ().
Thus:

Slope d = $\frac{a}{c}$ and represents $\frac{(\delta\chi/\chi)}{(\delta J/J)}$ when cerulenin is added and, hence, equals $\frac{1}{^{*}\varepsilon^{BlkB}_{\chi}}$.

Therefore, $\frac{c}{a}$ represents $^{*}\varepsilon^{BlkB}_{\chi}$.

Slope e = $\frac{b}{c}$ and represents $\frac{(\delta\chi/\chi)}{(\delta J/J)}$ when 2-bromooctanoate is added and, hence, equals $\frac{1}{^{*}\varepsilon^{BlkA}_{\chi}}$.

Therefore, $\frac{c}{b}$ represents $^{*}\varepsilon^{BlkA}_{\chi}$.

From Connectivity Theorem : $\frac{^{*}C^{J_{\pi}}_{BlkB}}{^{*}C^{J_{\pi}}_{BlkA}} = \frac{^{*}\varepsilon^{BlkA}_{\chi}}{-^{*}\varepsilon^{BlkB}_{\chi}}$ **Eqn. 1**

Therefore, $\frac{^{*}C^{J_{\pi}}_{BlkB}}{^{*}C^{J_{\pi}}_{BlkA}} = \frac{c}{b}.\frac{a}{c}$ but, as *c* cancels, this gives $^{*}C^{J_{\pi}}_{BlkB} = \frac{a}{b}.^{*}C^{J_{\pi}}_{BlkA}$ **Eqn. 2**

From Summation Theorem : $^{*}C^{J_{\pi}}_{BlkB} + ^{*}C^{J_{\pi}}_{BlkA} = 1$ **Eqn. 3**

Hence, $^{*}C^{J_{\pi}}_{BlkA} = 1 - ^{*}C^{J_{\pi}}_{BlkB}$.

Substituting $(1 - ^{*}C^{J_{\pi}}_{BlkB})$ for $^{*}C^{J_{\pi}}_{BlkA}$ in Eqn. 6 gives:

$^{*}C^{J_{\pi}}_{BlkB} = \frac{a}{b}.(1 - ^{*}C^{J_{\pi}}_{BlkB})$ and, expanding the terms gives: $^{*}C^{J_{\pi}}_{BlkB} = \frac{a}{b} - \frac{a}{b}.^{*}C^{J_{\pi}}_{BlkB}$.

Therefore, $^{*}C^{J_{\pi}}_{BlkB} + \frac{a}{b}.^{*}C^{J_{\pi}}_{BlkB} = \frac{a}{b}$ or: $\left(\frac{b+a}{b+b}\right).^{*}C^{J_{\pi}}_{BlkB} = \frac{a}{b} = \left(\frac{b+a}{b}\right).^{*}C^{J_{\pi}}_{BlkB}$.

Thus, $^{*}C^{J_{\pi}}_{BlkB} = \frac{\left\{\frac{a}{b}\right\}}{\left\{\frac{(b+a)}{b}\right\}}$ and, therefore $\boxed{^{*}C^{J_{\pi}}_{BlkB} = \frac{a}{(a+b)}}$ **Eqn. 4**

Similarly, it can be shown that: $\boxed{^{*}C^{J_{\pi}}_{BlkA} = \frac{b}{(a+b)}}$ **Eqn. 5**

Figure 5. Derivation of equations for geometric calculation of group flux control coefficients.

The group elasticity coefficients (measures of the sensitivity of responsiveness of a block of reactions to small fractional changes in the intermediate, acyl-CoA, χ) are:

$$*\varepsilon_{\chi}^{BlkA} = \left(\frac{\delta J_A}{J_A}\right)\left(\frac{\chi}{\delta\chi}\right)$$ when bromooctanoate is added **Eqn. 6**

and

$$*\varepsilon_{\chi}^{BlkB} = \left(\frac{\delta J_B}{J_B}\right)\left(\frac{\chi}{\delta\chi}\right)$$ when cerulenin is added **Eqn. 7**

Calculation of the group flux control coefficients for Block A and Block B can be calculated using the same equations described previously (Ramli *et al*., 2002b).

$$*C_{BlkA}^{J_{TL}} = \frac{(*\varepsilon_{\chi}^{BlkB})}{(*\varepsilon_{\chi}^{BlkB}) - (*\varepsilon_{\chi}^{BlkA})}$$ **Eqn. 8**

and

$$*C_{BlkB}^{J_{TL}} = \frac{(*\varepsilon_{\chi}^{BlkA})}{(*\varepsilon_{\chi}^{BlkA}) - (*\varepsilon_{\chi}^{BlkB})}$$ **Eqn. 9**

Figure 6. Derivation of equations for calculation of group flux control coefficients from group elasticities.

oil palm. The results from both methods showed that under our experimental conditions, control over the flux is shared between the two blocks with Block A having a flux control coefficient of ~0.6. Thus, the reactions of the fatty acid biosynthesis block which comprise ACCase, acyl-ACP thioesterases, fatty acid synthase (FAS) together with desaturation reactions appear to have more control under the conditions studied.

CONCLUSION

Our experiments revealed that, under the conditions used, oil palm mesocarp exhibited more flux control in the fatty acid biosynthesis group of reactions rather than those for lipid assembly. Nevertheless, control is distributed between both parts of the overall pathway, and thus, it can be predicted that any effects to increase the rate of fatty acid synthesis would likely result in more flux control burden for lipid assembly. Our experiments in oil palm mesocarp represent the extension of previous work on cultures to examine the overall pathway of lipid biosynthesis. Although there is more control invested in the fatty acid synthetic block, considerable control is also exerted by the endoplasmic reticulum activities. Thus, we have shown that manipulation of lipid biosynthesis in conjunction with TDCA can serve as an effective way of studying the regulation and control of this primary pathway of metabolism.

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