Biochimica et Biophysica Acta 1838 (2014) 1488–1500

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Studies on the regulation of lipid biosynthesis in plants: application of control analysis to soybean☆

Irina A. Guschina [a], John D. Everard [b], Anthony J. Kinney [b], Patti A. Quant [c], John L. Harwood [a,*]

[a] School of Biosciences, Cardiff University, Cardiff CF10 3AX, UK
[b] DuPont Agricultural Biotechnology, P.O. Box 80353, Wilmington, DE 19880, USA
[c] Department of Biochemistry, University of Oxford, Oxford OX1 3QU, UK

ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form 3 February 2014
Accepted 11 February 2014
Available online 22 February 2014

Keywords:
Plant membrane lipid
Fatty acid
Metabolic regulation
Phosphatidylcholine formation
Metabolic control analysis

ABSTRACT

Although there is much knowledge of the enzymology (and genes coding the proteins) of lipid biosynthesis in higher plants, relatively little attention has been paid to regulation. We have demonstrated the important role for cholinephosphate cytidylyltransferase in the biosynthesis of the major extra-plastidic membrane lipid, phosphatidylcholine. We followed this work by applying control analysis to light-induced fatty acid synthesis. This was the first such application to lipid synthesis in any organism. The data showed that acetyl-CoA carboxylase was very important, exerting about half of the total control. We then applied metabolic control analysis to lipid accumulation in important oil crops — oilpalm, olive, and rapeseed. Recent data with soybean show that the block of fatty acid biosynthesis reactions exerts somewhat more control (63%) than lipid assembly although both are clearly very important. These results suggest that gene stacks, targeting both parts of the overall lipid synthesis pathway will be needed to increase significantly oil yields in soybean. This article is part of a Special Issue entitled: Membrane Structure and Function: Relevance in the Cell's Physiology, Pathology and Therapy.

Contents

Abbreviations: CPCT, cholinephosphate cytidylyltransferase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PDAT, phospholipid:diacylglycerol acyltransferase; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PUFA, polyunsaturated fatty acids; SQDG, sulphoquinovosyldiacylglycerol; TAG, triacylglycerol

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* Corresponding author at: School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3AX, Wales, UK. Tel.: +44 29 20874108; fax: +44 29 20874117.
E-mail address: Harwood@Cardiff.ac.uk (J.L. Harwood).

1. Introduction: a lipid primer for plants

Although plants share many characteristics in common with other eukaryotes, they have certain distinct features with regard to their lipid biochemistry. Plant lipids are located in three main regions — in the extra-cellular domains (wax, cuticle, suberin), in lipid stores (mainly as triacylglycerol) and in cellular membranes [1]. In this article, we will neglect the former but good reviews are available which describe surface coverings and their metabolism [2–6]. The economic importance of plant

http://dx.doi.org/10.1016/j.bbamem.2014.02.008
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lipids is huge. Each year oil crops produce about 115 million tonnes of oil and this is worth of the order of $116B [7,8]. A few crops (oilseed rape, palm, soybean and safflower) currently account for about 69% of the total plant production. Interestingly, genetically-modified oil crops already account for a significant output (i.e. 170 million ha in 2012 including nearly 90% of the soybean crop).

There is one commercial crop that produces a different type of stored lipid, jojoba (*Simmondsia chinensis*). In the fruits (nuts) from this species, the oil is a wax ester of 40–42 carbon atoms, which has its main uses in the cosmetic industry [7].

Plants contain phosphoglycerides of the same types as other eukaryotes. In the extra-plastidic membranes, phosphatidylcholine and phosphatidylethanolamine are major components with smaller amounts of phosphatidylinositol and phosphatidylserine [1] (Table 1). Phosphorylated derivatives of phosphatidylinositol which, as in animals, have signalling functions, are found in small amounts, mainly in the plasma membrane [9,10]. Diphosphatidylglycerol (cardiolipin) is confined to the inner mitochondrial membrane [11] where it is a major component (Table 1). Phosphatidylglycerol, in contrast to animal tissues, is a major constituent because it is the only significant phospholipid in chloroplast (plastid) thylakoids. Because of the dominance of chloroplasts in green tissues, phosphatidylglycerol is often present in comparable amounts to phosphatidylcholine (Table 1).

One aspect of membrane composition that sets plant and algae apart from other eukaryotes are their chloroplast thylakoids. The major lipids of these are three glycosylglycerides — monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulphoquinovosyldiacylglycerol (SQDG, the plant sulpholipid). The structures of MGDG, DGDG and SQDG are shown in Fig. 1. Interestingly, all oxygen-evolving photosynthetic organisms, including cyanobacteria, contain rather similar thylakoid membrane compositions with about 45% MGDG, 29% DGDG, 7% SQDG and 9% phosphatidylglycerol in different leaves (see Table 1). The reason for this is unclear although there have been some hypotheses put forward [12,13]. Because of its prevalence in thylakoids, MGDG is the most abundant membrane lipid on earth, although many textbooks fail to even acknowledge its existence!

Sphingolipids are found in plants but, again, with their own distinctiveness compared to animals and yeast. Of particular interest is their enrichment in membrane rafts [14]. Plant sphingolipids are summarised well in [15] and their metabolism is up-dated in [16]. There are also a number of novel lipids in plants which may be present in appreciable quantities in certain tissues. These would include molecules like *N*-acyl-phosphatidylethanolamine and sterol glycosides [17]. Plant sterols themselves are dominated by β-sitosterol and stigmasterol (rather than cholesterol in animals). However, a whole host of minor compounds, including cholesterol, are found widely [17,18].

Table 1
Acyl lipid composition of selected plant tissues.

% total lipids							
	PC	PE	PI	PG	MGDG	DGDG	SQDG
Leaf lipids	10	5	3	8	40	28	6
Mito. lipids — outer	68	24	5	2	n.d.	n.d.	n.d.
Mito. lipids — inner[a]	29	50	2	1	n.d.	n.d.	n.d.
PM lipids	32	46	19	tr.	n.d.	n.d.	n.d.
Thylakoid lipids	2	tr.	n.d.	10	48	31	8
Root microsomes	35	28	14	n.d.	n.d.	n.d.	n.d.
Cyanobacteria	n.d.	n.d.	n.d.	19	56	14	11

Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PM, plasma membrane; SQDG, sulphoquinovosyldiacylglycerol; n.d., none detected; tr., trace (<0.5); Mito., mitochondrial.

[a] Diphosphatidylglycerol (cardiolipin) is located to the inner mitochondrial membrane (14–20% total lipids). Microsomes included several other lipids such as cardiolipin. See [1] for further details.

Most membrane (and storage) lipids are acylated compounds. Saturated fatty acids themselves are made in plants, as in other organisms, by the combined action of (a multi-protein) acetyl-CoA carboxylase and (a Type II) fatty acid synthase. In most tissues, palmitate and stearate are produced in an approximately 1:5 ratio. Exceptions could be in cases where short chain fatty acids are produced as a result of the activity of a special thioesterase (e.g. in coconut) or where a higher percentage of palmitate is produced (e.g. in palm oil). More details of de novo fatty acid synthesis, which is localised in the plastid stroma, will be found in [19].

The long-chain (16 or 18C) saturated fatty acids are converted to unsaturated acids by the action of desaturases. While the desaturation of stearate to oleate is catalysed by a soluble stearoyl-ACP Δ9-desaturase (in the stroma) [20], other plant desaturases use complex lipid substrates. For such reactions both the desaturase proteins and the substrates are found in membranes. Given the prevalence of stearate as a product of plant fatty acid synthase, it is not surprising that the main unsaturated fatty acids in plants are the 18C molecules — oleate, linoleate, and α-linolenate (Table 2). Therefore, major reactions in fatty acid processing by higher plants are the three successive desaturations of stearate and its products at the Δ9, Δ12 and Δ15 positions. The latter two reactions are particularly important because they give rise to the "essential fatty acids", linoleate and α-linolenate, which are the simplest members of the n−6 and n−3 series of polyunsaturated fatty acids (PUFA), respectively. The n−6 and n−3 PUFA cannot be made by mammals and almost no animals (a few protozoa and insects are exceptions [7]) but are needed to produce very long chain (20C, 22C) PUFA that give rise to signalling molecules like eicosanoids, resolvins and protectins [21,22].

As mentioned above, linoleate and α-linolenate are produced by Δ12- and Δ15-desaturases, respectively, using complex lipid substrates. The idea that complex lipids could act as substrates for fatty acid desaturation came from work in Tony James' lab. at Unilever [23,24] and from Morris Kates' group in Ottawa [25]. We provided the first direct demonstration in higher plants [26] when we showed that linoleoyl-MGDG could be used by isolated chloroplasts to synthesise α-linolenate. At that time there was some controversy because Roughan and co-workers believed that phosphatidylcholine was the substrate for PUFA production [e.g. 27]. However, Roughan and co-workers were using developing oil crops and we were using leaves and this was the main difference for the data and their interpretation. In fact, identification of desaturase genes has allowed us to define the pathways for desaturation. These are shown in Fig. 2 where it will be seen that fatty acid desaturase genes for Δ12 and Δ15 desaturations are located in plastids (*fad 6*, *fad 7*/*8*) where they use mainly MGDG as a substrate, while the endoplasmic reticulum contains *fad 2* and *fad3*-encoded enzymes which perform the same reactions using mainly phosphatidylcholine (Fig. 2) [28]. In the diagram, which shows *Arabidopsis* pathways, it will be noted that there are also desaturations at the 16C level because this plant belongs to the group of "16:3-plants" that produce all *cis*-Δ7, 10, 13-hexadecatrienoic acids (n−3–16:3) (see Table 2 also).

So, it turned out that, despite controversy, our finding that MGDG was the substrate for α-linolenate production, first suggested by radiolabelling studies [29], turned out to be fully validated. Moreover, we and others suggested a cooperation of the membranes of the endoplasmic reticulum and plastids in producing the world's most prevalent fatty acid, α-linolenate [see 30].

1.1. Assembling acyl lipids

As noted above, the most abundant complex lipids in plants are acyl lipids such as the storage lipid triacylglycerol and the membrane lipids (glycosylglycerides, phosphoglycerides). The major route for their synthesis is based on the glycerol 3-phosphate pathway first elucidated by Eugene Kennedy in the late 50s [31]. The first two reactions involve

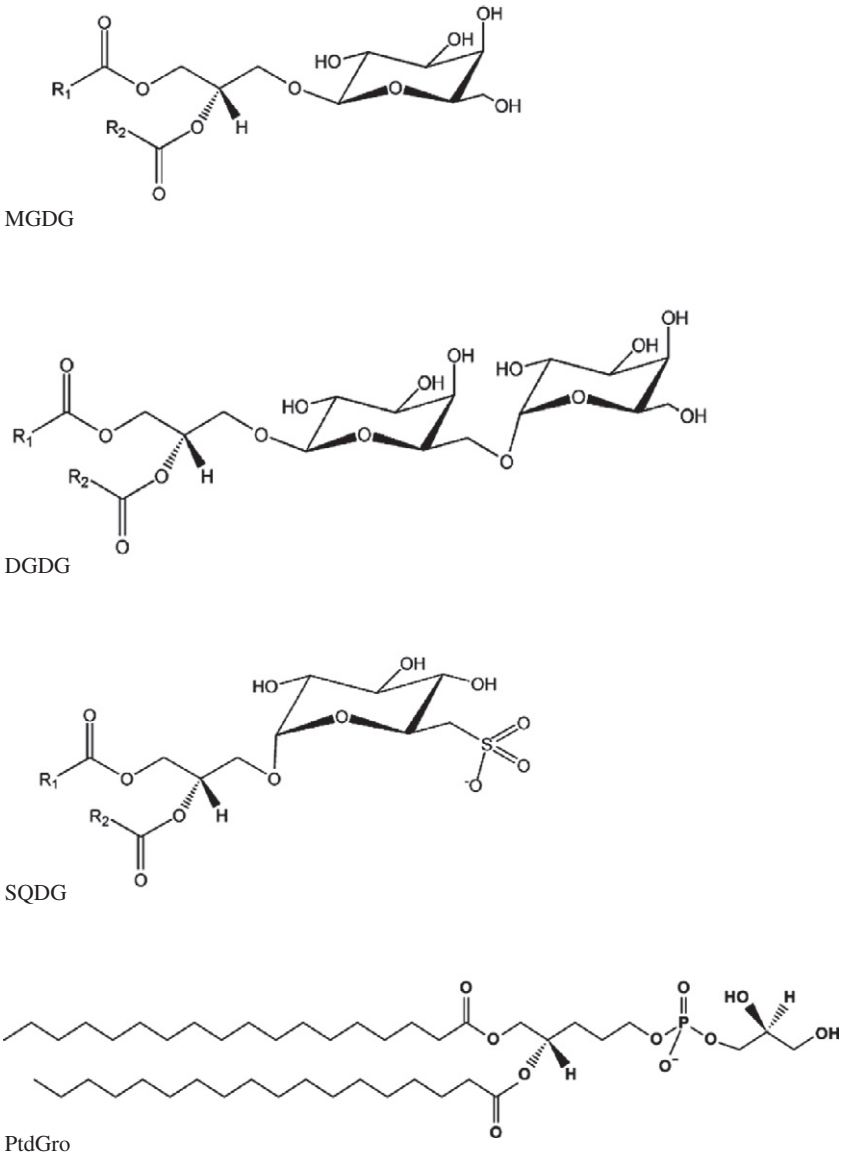

Fig. 1. Structures of chloroplast membrane glycerolipids. Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PtdGro, phosphatidylglycerol; SQDG, sulphoquinovosyldiacylglycerol.

successive acylation of glycerol 3-phosphate at the *sn*-1 and *sn*-2 positions (Fig. 3) and were discovered originally by Kornberg and Pricer [32]. These reactions will be familiar to scientists working with mammalian or yeast systems. In plants, the third intermediate in the pathway, diacylglycerol, is also used for the production of the three glycosylglycerides, MGDG, DGDG and SQDG (Fig. 3). Further details of the production of these three lipids will be found in [33,34].

Because we will be discussing some detailed experiments on the regulation of phosphatidylcholine and triacylglycerol production in plants, more detailed description of pathways for their formation are shown in Figs. 4 and 5, respectively. For more general discussion of phosphoglyceride, galactosylglyceride and triacylglycerol formation in plants refer to [7,33,35].

The major extra-chloroplastidic phosphoglyceride in plants is phosphatidylcholine (PtdCho). In fact, in most plant tissues PtdCho is the major phospholipid (see Table 1). It is made mainly by the CDP-choline (Kennedy) pathway although smaller amounts can be produced by phosphatidylethanolamine (PtdEtn) methylation, as demonstrated by Morris Kates' group in plants [36]. However, the activity and, indeed the presence [33], of this pathway in different tissues varies considerably. In fact, the methylation pathways to PtdCho in plants appear quite complex [37,38].

For triacylglycerol production, membranes and membrane-located enzymes are intimately involved (Fig. 5). While the Kennedy pathway (Fig. 3) forms the core reactions for TAG assembly there is significant flux through phosphatidylcholine (Fig. 5). This is mainly because

Table 2
Fatty acid composition of some representative plant tissues.

	Fatty acids (% of total)						
	16:0	16:1	16:3	18:0	18:1	18:2	18:3
Pea leaf	12	3	n.d.	1	2	25	53
Spinach leaf	13	3	5	tr.	7	16	56
Oilseed rape (canola) oil	4	n.d	n.d	2	62	22	10
Sunflower seed oil	6	n.d.	n.d.	4	24	64	tr.
Soybean seed oil	11	n.d.	n.d.	4	23	54	8
Palm mesocarp (fruit) oil	44	tr.	n.d.	4	39	11	tr.
Palm kernel (seed) oil[a]	9	n.d.	n.d.	2	15	2	n.d.

tr. = trace (<0.5%), n.d. = none detected.
Values indicate average values. For more details and specific examples see [7]. 16:1 shows all isomers. 16:3 is *cis*Δ7, 10, 13–16:3.
[a] 4% decanoic, 48% dodecanoic, 16% tetradecanoic acids.

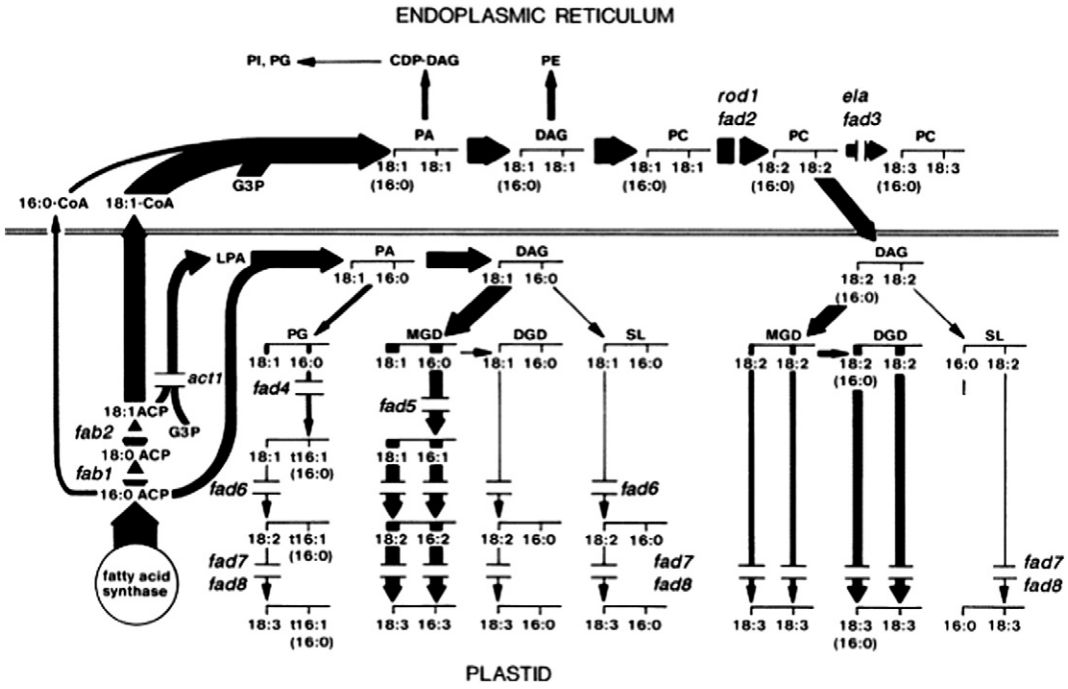

Fig. 2. Plant membrane glycerolipid synthesis (taken from ref. [28] with permission from Elsevier). Widths of the lines show the relative fluxes through different reactions in *Arabidopsis* leaves. Breaks indicate the putative enzyme differences in various mutants [see 28]. Abbreviations: DGD, digalactosyldiacylglycerol; MGD, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SL, sulphoquinovosyldiacylglycerol.

PtdCho is a substrate for PUFA production in the endoplasmic reticulum (Fig. 2) and also because most important crops contain significant amounts of PUFAs (see Table 2 [7]). The flux of carbon through PtdCho and back into TAG has been referred to as "acyl editing" by Philip Bates, John Ohlrogge and their associates [39,40]. In part, this is facilitated by the enzyme phospholipid:diacylglycerol acyltransferase (PDAT), first demonstrated in plants by Sten Stymne's group in Sweden [41].

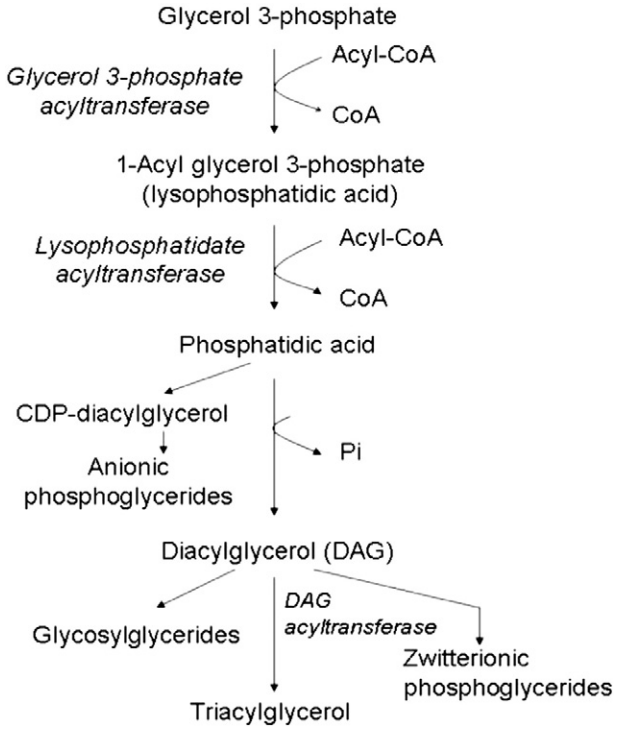

Fig. 3. The basic Kennedy pathway for glycerolipid biosynthesis.

However, as noted in Fig. 5 there are other ancillary reactions, such as lysophosphatidylcholine acyltransferase which are involved.

For the final step of the Kennedy pathway, diacylglycerol acyl–CoA acyltransferase (DGAT) is used. There are two main isoforms (DGAT 1, DGAT 2) in plants [42]. In general DGAT 1 seems to be used in oil crops producing "usual" TAGs containing the major abundant fatty acids (Table 2). For those species producing TAGs with unusual fatty acids, then DGAT 2 becomes more important. These aspects are discussed more fully in [42,43].

2. Materials and methods

2.1. Materials

Soybean (*Glycine max* c.v. Jack) somatic embryo cultures were initiated from immature zygotic embryos and were maintained in a proliferative state by regular subculture [44,45]. Experiments were performed on somatic embryos developed by dropping an approximately 5 mm diameter piece of proliferative tissue into flasks containing Soy Histodifferentiation and Maturation Media (SHaM) [46] and culturing them for 14 days at 24 °C on a 16:8 h (L:D) cycle (PAR = 35.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$) on a table shaker (110 rpm). Embryos were harvested by filtration prior to labelling studies.

2.2. Incubations

Cultures were suspended in 20 ml of fresh medium containing 3 μCi of radiolabel (1-[^{14}C]acetate or U-[^{14}C]glycerol, sp. activity = 1.85–2.29 $GBq \cdot mmol^{-1}$). Oleic acid (400 μM) was prepared as an emulsion in Tween-20 (Sigma) and used to pre-incubate the cultures for 1 h followed by a further incubation with radioisotopes for 4 h. Control cultures were treated with Tween-20 only. The final concentration of Tween-20 was 1%.

Where appropriate, metabolic inhibitors were added to the media throughout the incubation which was at 24 °C for 4 h. The effect of bromooctanoate was studied at 20 μM. As three independent

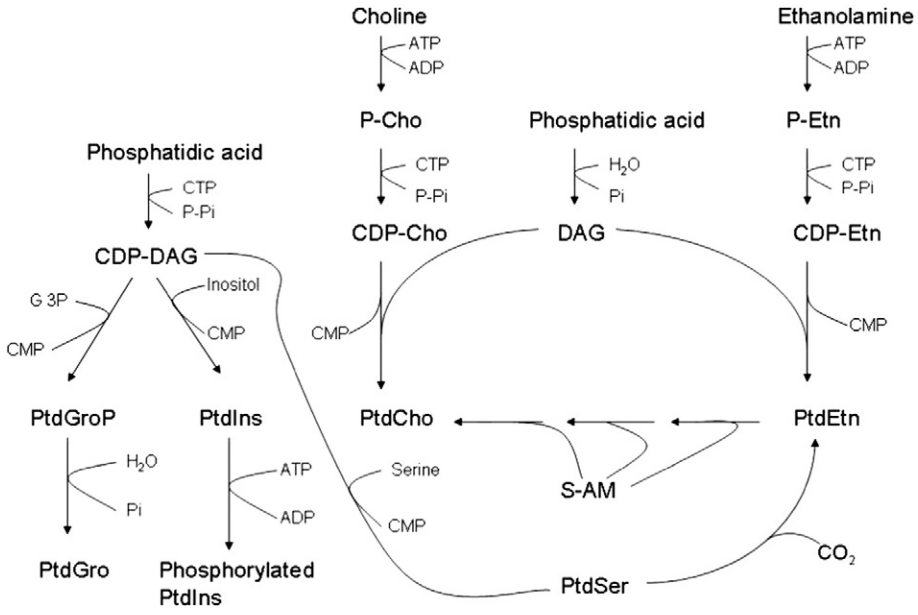

Fig. 4. Pathways for phosphoglyceride synthesis in plants. Additional abbreviations: CDP-DAG, CDP-diacylglycerol; PtdCho, phosphorylcholine; PtdEtn, phosphorylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; S-AM, S-adenosylmethionine.

experiments showed, this inhibitor (at concentrations of 10, 15 and 20 μM) did not affect the total lipid labelling from radioactive acetate, but decreased the labelling of that from radioactive glycerol. Cerulenin was used at a concentration of 55 μM where it inhibited fatty acid synthase.

Embryos were harvested by filtration, washed once with distilled water, and total lipids were pre-extracted using hot isopropanol to inactivate endogenous lipases. The isopropanol extracts were then dried under nitrogen gas and lipid extraction used a two-phase system based on Bligh and Dyer that gives quantitative extraction of all major acyl lipids [47]. Phospholipids and glycosylglycerides were separated into lipid classes using two-dimensional TLC on silica gel G plates using chloroform/methanol/water (65:25:4, by vol.) in the first dimension and then chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by vol.) in the second. Non-polar lipids were separated using one-dimensional TLC with hexane/diethyl ether/acetic acid (80:20:1, by vol.) as solvent. After

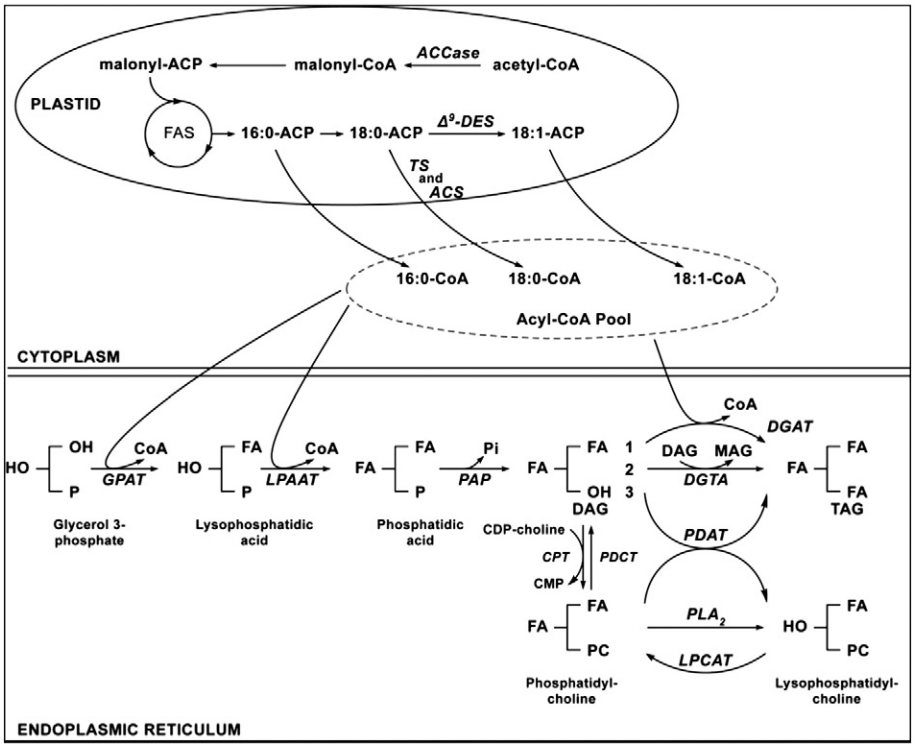

Fig. 5. Simplified scheme of TAG biosynthesis in plants. ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; ACS, acyl-CoA synthase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; Δ^9-DES, Δ^9-desaturase; DGAT, DAG acyltransferase; DGTA, diacylglycerol transacylase; FAS, fatty acid synthase; GPAT, glycerol 3-phosphate acyltransferase; LPAAT, lysophosphatidate acyltransferase; LPCAT, lysophosphatidylcholine acyltransferase; PAP, phosphatidate phosphohydrolase; PDAT, phospholipid:diacylglycerol acyltransferase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; PLA_2, phospholipase A_2; TS, acyl-ACP thioesterase.
Taken from [102] with permission from Elsevier.

drying, the plates were sprayed with a 0.05% solution of 8-anilino-4-naphthosulphonic acid in methanol and viewed under UV light to reveal lipids. Identification was made routinely by reference to authentic standards as described before [48]. To measure the incorporation of radioactivity into individual lipid classes, the lipids were transferred into scintillation vials to which 10 ml of OptiFluor (PerkinElmer Inc., Waltham, MA, USA) scintillant was added. The samples were counted in a Perkin-Elmer Tri-Carb 2800 TR liquid scintillation counter [48]. Quench correction was made automatically by the external-standard channels-ratio method.

Experimental data were obtained using, at least, triplicate independent samples for analysis.

2.3. Flux control calculations

The basic flux control system was simplified to two blocks of reactions (fatty acid biosynthesis and lipid assembly) (Fig. 6). The theory behind the assumptions and calculations has been discussed thoroughly in [49–51]. Because blocks of reactions are analysed then these include not only the major pathways (e.g. Kennedy pathway) but also all the ancillary reactions (e.g. PDAT). Taken together, the control coefficients for the two blocks will total 1.0. So any value above 0.5 indicates a majority of control in that part of the pathway. As before, we used single manipulation (oleate addition) and double manipulation with two separate reagents (inhibitors) of top down control analysis [50,51].

3. Regulation of phosphatidylcholine formation

Early work of phosphatidylcholine synthesis owes much to experiments in Morris Kates' lab. By using radiolabelled precursors he was able to demonstrate the Kennedy pathway reactions in plants [see 52,53]. For PtdCho we showed that the latter reactions were much more important than PtdEtn methylation in soybean [56]. We followed this up by purifying and characterising the choline [54] and ethanolamine kinases [55] as well as studying other features of the Kennedy pathway [56].

At that time it was known from animal work that the second step in the pathway – the cholinephosphate cytidylyltransferase (CPCT) – exerted a strong flux control [57]. We decided to test the situation in plants and needed a system where PtdCho was suddenly increased. The tissue we chose was elongating pea stems. These can be stimulated by auxins to elongate at impressive rates — up to 0.3 mm/h. Under these conditions we were able to measure increases in phosphoglyceride radiolabelling, especially PtdCho [58]. These increases in total PtdCho formation coincided with a marked change in the activity of CPCT but not of the other enzymes in the pathway [59]. Furthermore, of the intermediates in the pathway, the pool of CDP-choline was much smaller than the others [59] — usually indicative that its availability could control overall PtdCho production. Further studies on the regulation of CPCT in peas (see Table 3) showed that its activity could be changed by a number of factors including association with membranes, oleate and nucleotide concentrations [60] as well as by the amount of enzyme protein [61]. Later on, we isolated a gene for the pea enzyme [62]. In other plants, multiple genes for CPCT have been found, with four in rape [63] and two in *Arabidopsis* [64].

4. The first application of flux analysis to lipid synthesis

We were intrigued by regulation. Therefore, we searched for systems where lipid biosynthesis was changed markedly. One such instance is when leaves go from dark growth to light exposure. Under these conditions, fatty acid biosynthesis and, hence, lipid formation increases about 20-fold. The question was how and which enzyme(s) was responsible? We already had clues because in other eukaryotes, acetyl-CoA carboxylase was key [35] and, moreover, it had already been shown that the pool sizes of acetyl/malonyl thioesters were changed during light-stimulated fatty acid formation in a way that indicated regulation via acetyl-CoA carboxylase [65].

At that time, we had specific compounds available which were inhibitory towards acetyl-CoA carboxylase in grasses — the so-called graminicides which were (and still are) used extensively in crops (Fig. 7). With these specific inhibitors we were able to quantify the effect that acetyl-CoA carboxylase could have over flux control. We used two different graminaceous plants (barley, wheat) and two different types of herbicides (representative of the aryloxyphenoxypropionates and cyclohexanediones). The data showed quite clearly [66] (Table 4) how important acetyl-CoA carboxylase was. The experiments gave flux control coefficients of 0.45–0.61 for the enzyme showing that, under the experimental conditions, up to 60% of the total control of lipid synthesis was at the level of this one enzyme. Considering that over twenty major enzymes are involved in lipid synthesis in leaves, this was a remarkable finding.

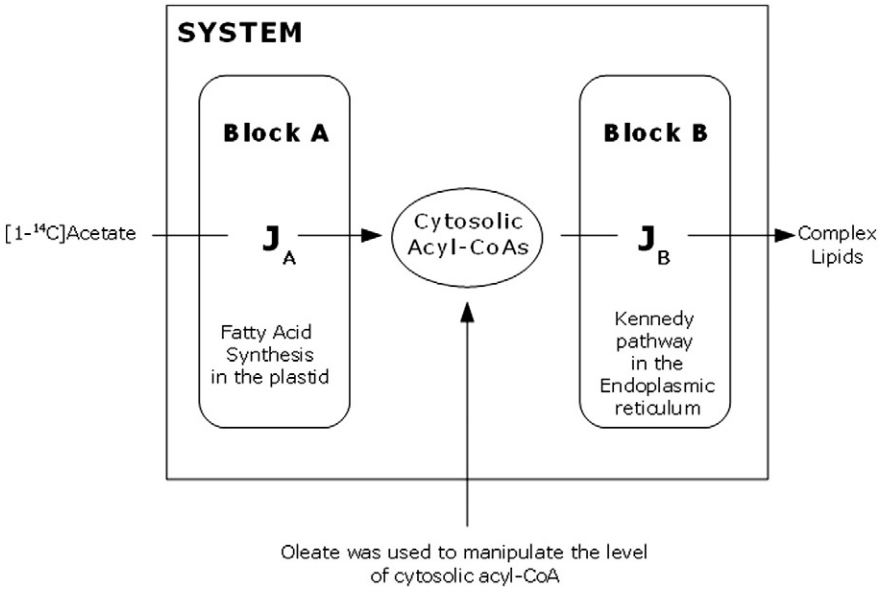

Fig. 6. Pathway reactions in lipid biosynthesis grouped together to allow the conceptually simplified system for metabolic control analysis.

Table 3
Detailed changes caused by an auxin on phosphatidylcholine formation by the Kennedy pathway in pea stem tissue.

	% control		
Changes in pool sizes	Choline 100%	Choline-P 159%	CDP-choline 78%
	% control		
Changes in enzyme activity	CK 100%	CPCT 63%	Phosphotrans. 280%
Regulation of CPCT	2 mM oleate ↓ activity		
	1 mM IAA ↓ protein ↓ activity		
	ATP ↓ (uncompetitive v. choline-P; mixed v. CTP)		
	CMP ↓ (uncompetitive v. choline-P; competitive v. CTP)		

Abbreviations: CK, choline kinase; CPCT, cholinephosphate cytidylyltransferase; IAA, indole-3-acetic acid; Phosphotrans., cholinephosphotransferase. See references [59–61] for details.

5. Applying flux control analysis to oil crops

As we have pointed out before [67], there are a number of ways in which one can study the regulation of metabolism. Particular methods which have been productive for oil crops including breeding methods to identify quantitative [7] and qualitative factors [68] and quantitative

Table 4
Flux control coefficients for acetyl-CoA carboxylase in leaves. Data from [66].

Plant leaf	Herbicide used	Control coeff.
Barley	Fluazifop	0.61 ± 0.05
	Sethoxydim	0.54 ± 0.17
Maize	Fluazifop	0.59 ± 0.05
	Sethoxydim	0.45 ± 0.13

Table 5
Group flux control values for different oil crops.

Crop	Single manipulation		Double manipulation	
	Block A	Block B	Block A	Block B
Oil palm	0.64 ± 0.05	0.36 ± 0.05	0.61 ± 0.03	0.39 ± 0.03
Olive	0.57 ± 0.10	0.43 ± 0.10	–	–
Oilseed rape	0.31 ± 0.03	0.69 ± 0.03	0.27 ± 0.06	0.73 ± 0.06

Block A reactions are those for fatty acid biosynthesis and Block B reactions are those of lipid assembly.

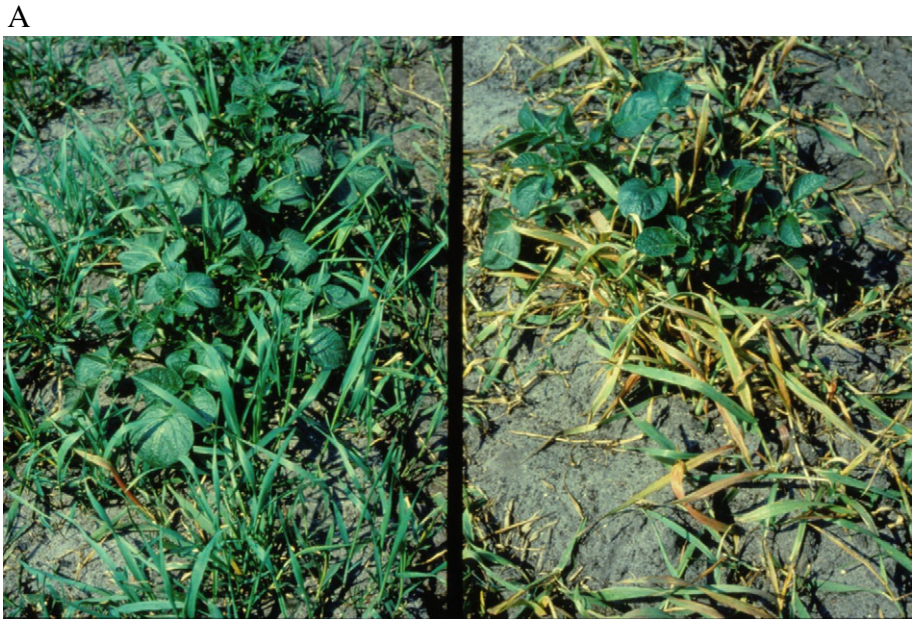

Fig. 7. Action of a graminicide in a crop. (A) Left plate shows an untreated soybean field. Right plate shows the crop after treatment with fluazifop with grass weeds killed. (B) Shows the structures of representative examples from the two main types of graminicide: aryloxyphenoxypropionates (fluazifop) and cyclohexanediones (sethoxydim).

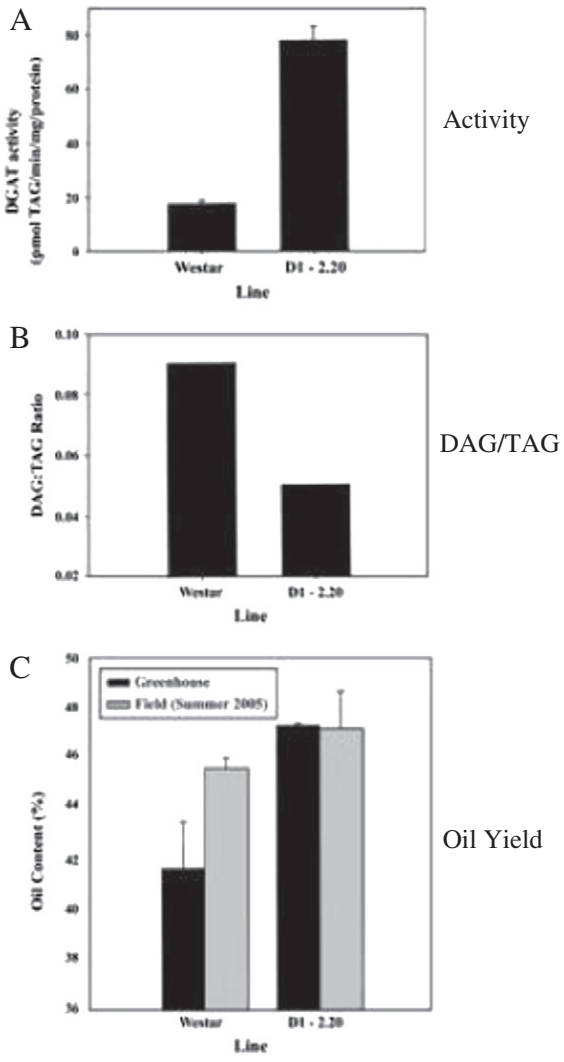

Fig. 8. Characteristics of transgenic canola seeds. Data from [84] where DGAT 1 activity was increased by overexpression. This led to raised oil yields in the field as well as in the greenhouse.

trait loci mapping [69,70]. Transcription factors, particularly WRI-1, have also been identified to be important [43,71–73] and classic biochemistry [43,74] or gene manipulation [75,76] can give important information.

However, flux analysis is particularly attractive for two reasons. First, it can be applied without detailed knowledge of the pathway concerned and, second, it gives quantitative data [77]. There are two basic methods, metabolic flux analysis (MFA) [78] and metabolic control analysis (MCA). MFA has been used a little in oil crops [79,80] and can give a detailed understanding of observed flux values. MCA, on the other hand, examines the influences giving rise to measured flux values [77]. Important aspects of the application of flux analysis for the study of metabolic regulation have been summarised [81].

We have applied MCA to oil crops and used both single-manipulation and double-manipulation methods. In all cases these two methods gave very similar results.

Despite the complications of TAG biosynthesis in plants (Fig. 5), we were able to simplify the process into the two blocks of reactions — Block A (fatty acid synthesis) and Block B (lipid assembly) (Fig. 6). The use of top-down control analysis [77], allowed us to measure the amount of control in each Block and, hence, be able to predict which Block might be worth up-regulating initially in order to increase oil production. Clearly, this was a much better situation than predicting (guessing) which enzyme activity might be worth targeting. In fact, the latter approach has seldom paid dividends in plant genetic manipulation [82].

We have applied control analysis to four important crop plants — olive, oilseed rape, oil palm and soybean. Together these crops alone currently account for about 70% of the world production of plant oils [7]. Data for the first three species have been reported [48–51,83,84] and are summarised in Table 5.

There are four important points to make for interpretation. First, the measurements apply to the reaction conditions and the system used. Although we tried to mimic *in planta* conditions (and produced evidence for this), it is an important caveat that applies to most flux control measurements. Second, control is shared between the Blocks. Indeed, it should be appreciated that control is shared by all enzymes involved in a pathway. Although some enzymes will exert more flux control than others, there is no such thing as *the* rate-controlling enzyme (despite what textbooks often tell us!). Third, different inhibitors were useful for double-manipulation in the different crops i.e. extrapolation from one species to another can be risky. Finally, the flux control exerted by the two Blocks was similar in olive and oil palm but different in oilseed rape.

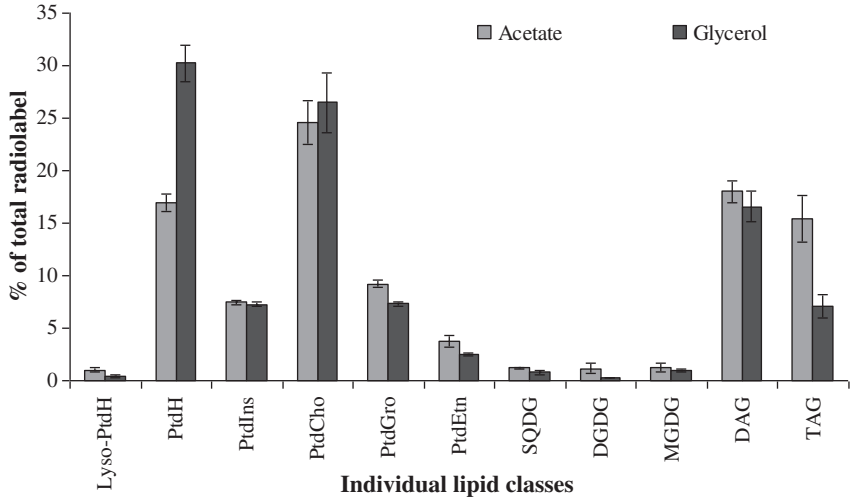

Fig. 9. Labelling of individual polar lipids in soybean cultures from [1-^{14}C]acetate and [U-^{14}C]glycerol. Abbreviation: PtdH, phosphatidic acid.

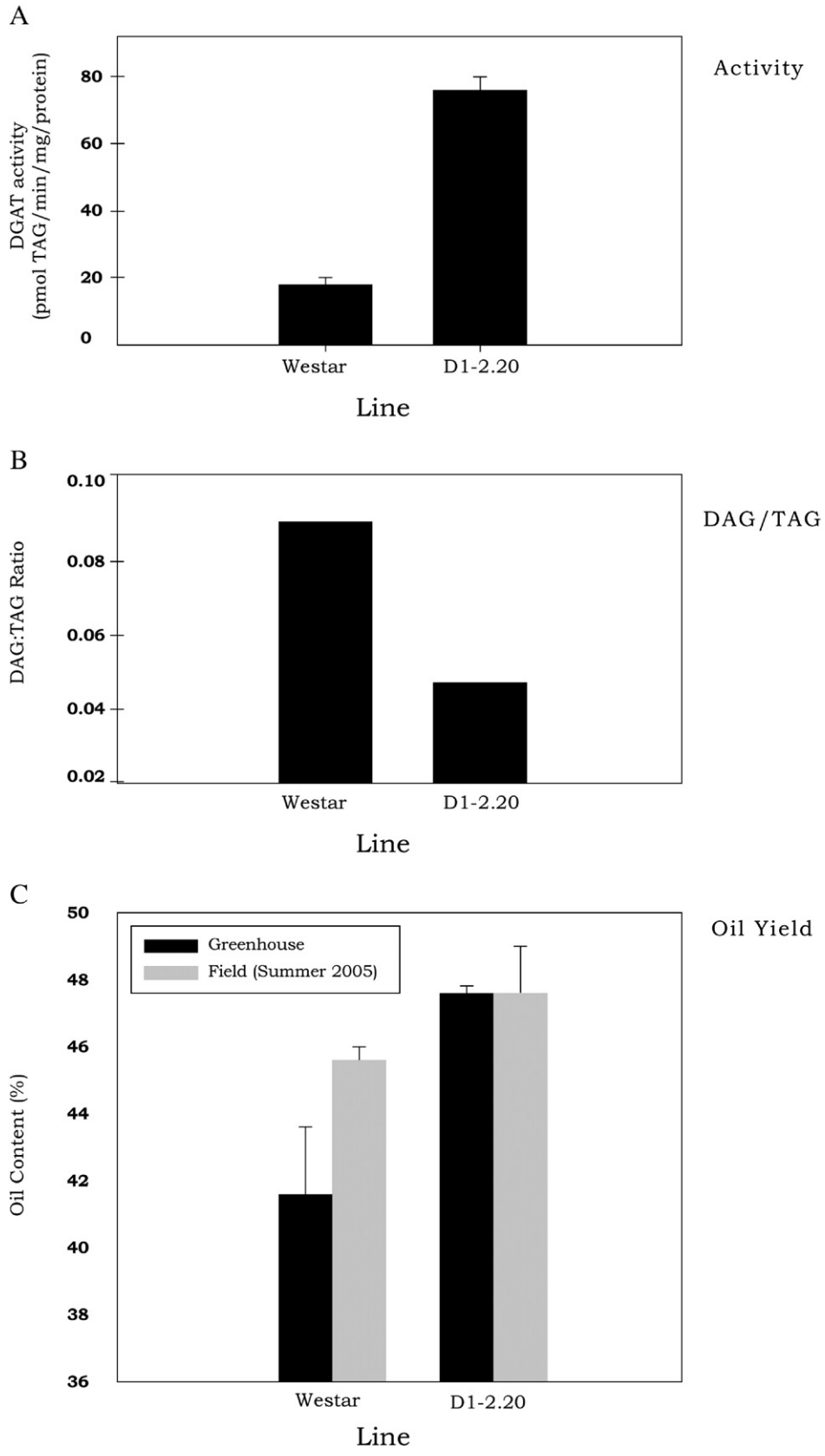

Fig. 10. Single manipulation top-down control analysis (TDCA) of soybean cultures using exogenous oleate. Fatty acid synthesis was measured using [1-^{14}C]acetate (A) and lipid assembly using [U-^{14}C]glycerol (B). Data as means ± S.D. (n = 3) for three separate experiments.

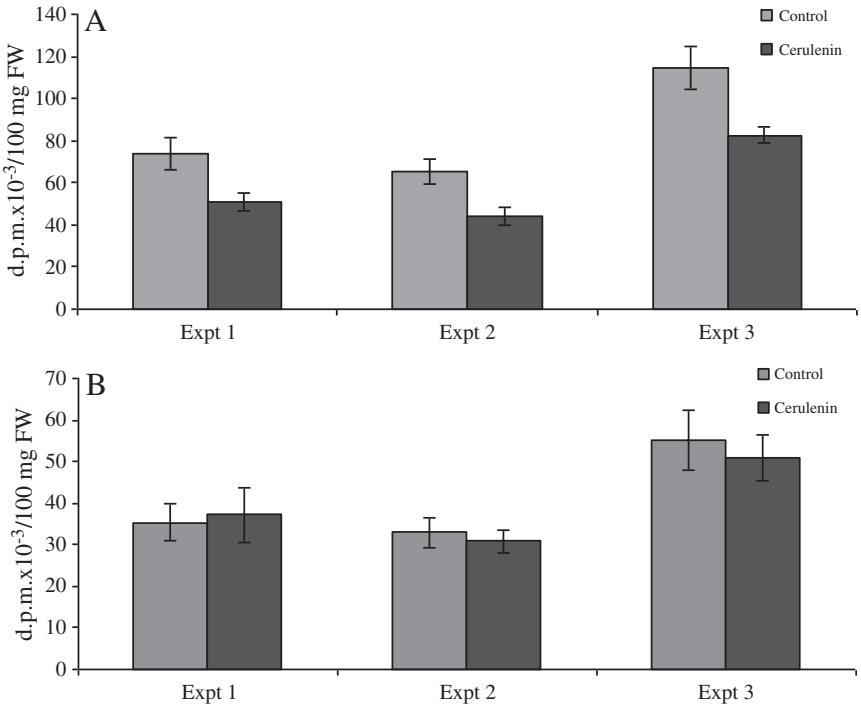

Fig. 11. Specificity of inhibitor (cerulenin) action in double manipulation TDCA of soybean cultures. Fatty acid synthesis was measured using [1-^{14}C]acetate (A) and lipid assembly using [U-^{14}C]glycerol (B). Data as means ± S.D. (n = 3) for independent experiments. Cerulenin was used at 55 μM.

The case of oilseed rape, where most control was in the lipid assembly block of reactions, begged the question — which enzyme might be the most important? Some twenty years ago, we had results from radiolabelling experiments that indicated that DGAT activity might be limiting carbon flux during periods of rapid oil accumulation [85] and this was followed up by measuring pool sizes of intermediates [86]. Again this indicated that DGAT was important. Accordingly, in conjunction with the Canadian groups of Taylor and of Weselake, we increased DGAT activity by overexpression and showed how this changed the distribution of control so that Block A now became more important [84] (Fig. 8). Lines with significantly enhanced DGAT activity produced elevated oil yields in field trials in successive years [87]. These results showed clearly how data from metabolic flux control experiments can be used for informed genetic manipulation.

Although the flux control experiments summarised in Table 5 indicate which Block exerts the most control in each plant, significant control is still exerted by the other Block. Thus, efforts to remove a perceived constraint in one part of the overall pathway (by increasing enzyme activity) will immediately cause more control to be exerted elsewhere. This means that manipulation of multiple genes will, almost certainly, be needed to achieve large increases in oil yield. In that regard, experiments with the transcription factor WRI 1 (which up-regulates a number of enzymes involved in fatty acid formation) [73] and gene cassettes for several genes are of interest. In the latter case, results have been reported for experiments up-regulating WRI 1 together with DGAT in vegetative tissue [88,89]. Such experiments are likely to become normal in the future for oil yield increases as well as for modifying oil quality [e.g. 90–92].

Table 6
Calculated block control coefficients for cultured soybean (somatic) embryos.

	Block A	Block B
Single manipulation (oleate addition)	0.70 ± 0.08	0.30 ± 0.08
Double manipulation (cerulenin/bromooctanoate)	0.56 ± 0.05	0.44 ± 0.05
Mean values	0.63	0.37

See Materials and methods for details.

6. Flux control studies in soybean cultures

Using the methods outlined in Section 5 and with precise details in Section 2, we have examined lipid accumulation in soybean somatic embryos. Up until two years ago, soybean produced more oil than any other crop plant, despite the fact that it is grown primarily for protein. Even today, soybean accounts for about 22% of the world production of oils and fats [7,8]. Therefore, it was important to study oil accumulation in this crop. The soybean embryos used in this study have been shown to be an excellent system for studying transgenic and physiological influences on resource partitioning and have proven to be a very predictive model for seeds [93].

In the flux control experiments we used [1-^{14}C]acetate to label fatty acids and [U-^{14}C]glycerol for incorporation into the backbone of complex lipids during assembly (Block B reactions). These two precursors are virtually specific for each type of incorporation (>96%), as demonstrated for other plant oil tissues [48,51]. The distribution of radioactivity into lipid classes during the linear period of incorporation (4 h) is shown in Fig. 9. Of the non-polar lipids, only TAG and diacylglycerol (DAG) were significantly labelled while phosphatidylcholine (PtdCho) contained the bulk of radiolabel amongst the polar lipids. The latter is indicative of a cycling of carbon flux between DAG and PtdCho as expected from the high activity of the "acyl editing" reactions in soybean [94]. Since, the soybean cultures are non-photosynthetic and mimic developing seed metabolism of chloroplast lipids such as MGDG and, consequently, their labelling was minor (Fig. 9). The relatively small accumulation of radioactivity in the Kennedy pathway intermediates, phosphatidate and, especially, lysophosphatidate, compared to DAG attests to the important control exerted by the final enzyme of the Kennedy pathway, DGAT, in soybean.

Single manipulation used the addition of oleate. We felt that was entirely appropriate for soybean which accumulates this fatty acid as a major component of its oil (~25%) and uses oleate to produce the

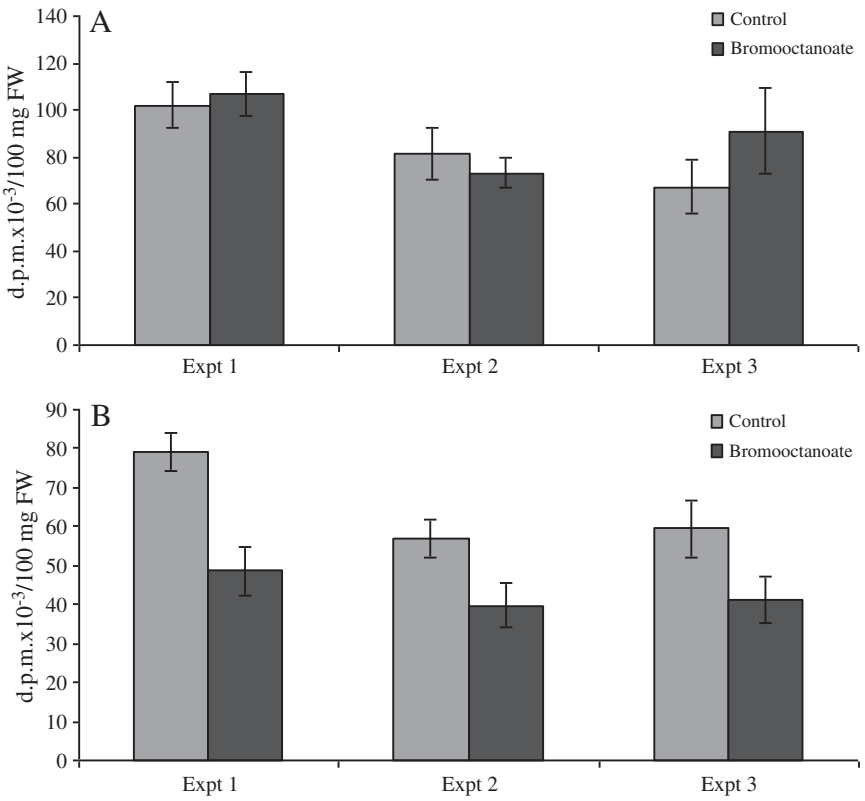

Fig. 12. Specificity of inhibitor (bromooctanoate) action in double manipulation TDCA of soybean cultures. Fatty acid synthesis was measured using [1-^{14}C]acetate (A) and lipid assembly using [U-^{14}C]glycerol (B). Data as means ± S.D. (n = 3) for independent experiments. Bromooctanoate was used at 20 μM.

main fatty acid, linoleate (~50%). Calculation of changes induced by the addition of oleate gave group flux controls for Block A and Block B of 0.63 and 0.37, respectively. In fact, the data showed that oleate reduced labelling of fatty acids from [1-^{14}C]acetate (Fig. 10A) and enhanced that of lipids from [U-^{14}C]glycerol (Fig. 10B). This is most simply interpreted as product inhibition (by oleate) of the fatty acid biosynthesis block while constraints caused by limitation in fatty acid supply are alleviated by oleate addition. Product inhibition may be similar to the reduction of acetyl-CoA carboxylase activity by oleoyl-ACP observed in oilseed rape seeds [95].

For double manipulation, we needed inhibitors which were specific for either Block A or Block B. After testing a number of potential candidates, we used cerulenin as an inhibitor of Block A and bromooctanoate as an inhibitor of Block B. Cerulenin is a known inhibitor of β-ketoacyl-ACP synthase [96] while bromooctanoate has been reported as an inhibitor of DGAT in some tissues [97], including the oil fruits olive and oil palm [83].

Cerulenin inhibited the labelling of fatty acids from [1-^{14}C]acetate but had no effect on the radiolabelling of lipids from [U-^{14}C]glycerol (Fig. 11). This showed that it could be utilised as a specific inhibitor of Block A reactions. In contrast, bromooctanoate only inhibited radiolabelling from [U-^{14}C]glycerol, showing that it was appropriate for inhibiting Block B reactions (Fig. 12). The two inhibitors were then used to modulate radiolabelling and block control coefficients were calculated (Table 6) using the techniques and controls discussed from our previous experiments with other oil crops [49–51]. The data showed that, as for olive and oil palm [49], the bulk of the control of carbon flux into TAG lay in Block A (fatty acid biosynthesis) reactions. This also agreed with biochemical experiments [98,99] showing that fatty acid supply was an important regulatory factor for oil accumulation in *Cuphea* or elm and in castor bean, respectively. Furthermore, the data from Bates and co-workers for developing soybean embryos [94] indicated that TAG formation involved a major flux of newly synthesised fatty acids through PtdCho (via lysoPtdCho and PDAT) as well as the presence of several DAG pools. Also, it should not be forgotten that the alternative technique, flux variability analysis in relation to metabolic flux analysis, is yielding useful data with regard to seed oil production [e.g. 79].

Our experiments with soybean show that fatty acid synthesis is important in controlling the overall flux of carbon to TAG. However, lipid assembly still exerts a significant control and, although approaches to increase fatty acid formation should be the first genes targeted, further manipulations using gene stacks would be advisable. Of the lipid assembly reactions, accumulation of label in the DAG pool confirms that DGAT is another useful target. The validity of each of these biochemically based predictions is fully supported by experiments with transgenic soybeans. The regulated expression of transcription factors (including WRI 1) known to up-regulate fatty acid synthesis leads to significant increases in oil content [100], as does the expression of heterologous DGAT genes [101]. Thus, these results confirm the observation that transgenic approaches to increase Block A (WRI 1) and Block B (DGAT) fluxes are viable approaches for increasing the oil content of soy seeds. Further, in soy the co-expression of transcription factors and DGAT was shown to be additive and led to oil content increases that were greater than when either element was expressed alone [100].

The recent data that we and others have obtained on metabolic flux control analysis have shown how useful system control experiments can be, not only for basic science, but also for industry. Where flux control analysis has been used to inform genetic modification [e.g. 84,87], the results have been significant. Perhaps, lipid regulation has now come of age?

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