New Phytologist

Metabolic control analysis of developing oilseed rape (*Brassica napus* cv Westar) embryos shows that lipid assembly exerts significant control over oil accumulation

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Received: *23 April 2012*
Accepted: *1 July 2012*

New Phytologist (2012) **196**: 414–426
doi: 10.1111/j.1469-8137.2012.04262.x

Key words: lipid biosynthesis, oilseed rape (*Brassica napus*), seed oil accumulation, top-down control analysis, triacylglycerol formation.

Summary

- Metabolic control analysis allows the study of metabolic regulation. We applied both single- and double-manipulation top-down control analysis to examine the control of lipid accumulation in developing oilseed rape (*Brassica napus*) embryos.
- The biosynthetic pathway was conceptually divided into two blocks of reactions (fatty acid biosynthesis (Block A), lipid assembly (Block B)) connected by a single system intermediate, the acyl-coenzyme A (acyl-CoA) pool. Single manipulation used exogenous oleate. Triclosan was used to inhibit specifically Block A, whereas diazepam selectively manipulated flux through Block B.
- Exogenous oleate inhibited the radiolabelling of fatty acids from [1-^{14}C]acetate, but stimulated that from [U-^{14}C]glycerol into acyl lipids. The calculation of group flux control coefficients showed that *c.* 70% of the metabolic control was in the lipid assembly block of reactions. Monte Carlo simulations gave an estimation of the error of the resulting group flux control coefficients as 0.27 ± 0.06 for Block A and 0.73 ± 0.06 for Block B.
- The two methods of control analysis gave very similar results and showed that Block B reactions were more important under our conditions. This contrasts notably with data from oil palm or olive fruit cultures and is important for efforts to increase oilseed rape lipid yields.

Abbreviations: ACP, acyl carrier protein; DAF, days after flowering; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; ER, endoplasmic reticulum; FAME, fatty acid methyl ester; FFA, free (non-esterified) fatty acid; lyso-PA, lysophosphatidate; MCA, metabolic control analysis; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; TAG, triacylglycerol; TDCA, top-down control analysis.

Introduction

Plant oils are of great importance for the agrochemical industry (Gunstone *et al.*, 2007). Not only do they serve as edible oils but, increasingly, interest has focused on their (potential) use as renewable sources of industrial chemicals (Murphy, 1994; Jaworski & Cahoon, 2003; Durrett *et al.*, 2008). In the last decade, there have been numerous attempts to engineer oil crops genetically in order to improve oil yield or to generate oils with improved properties (Abbadi *et al.*, 2004; Guschina & Harwood, 2006). However, many difficulties have been encountered in producing crops of utility for the agricultural industry. One reason for some of the disappointing results has been that our knowledge of the regulation of lipid biosynthesis is inadequate, so that new problems are often being encountered (see Bates & Browse, 2011). Thus, although we have a considerable knowledge of individual enzymes and of genes encoding their activities (Harwood, 1996; Murphy, 2005), our understanding of the control of synthesis is poor (Ohlrogge & Jaworski, 1997; Voelker & Kinney, 2001). This has meant that the manipulation of particular

enzymes to change yield is usually unimpressive, not only for oil crops (Ohlrogge & Jaworski, 1997), but for many genetically engineered plants (Stitt & Sonnewald, 1995).

One way to examine the regulation of pathways is to use metabolic control analysis (MCA). The theory of MCA was developed and applied by Kacser & Burns (1973) and Heinrich & Rapoport (1974). With MCA, it is possible to measure quantitatively the importance of particular parts of a metabolic pathway in controlling fluxes under defined conditions (Fell, 1997). Moreover, MCA recognizes that for an enzyme even to approach becoming rate limiting is extremely rare, and that is why many efforts to manipulate individual steps often give disappointing results (Stitt & Sonnewald, 1995).

There are two important variants of control analysis: top-down (or modular; TDCA) and bottom-up. We applied single-manipulation TDCA to oil synthesis in oleaginous fruits (Ramli *et al.*, 2002a,b) and, later, double-manipulation methods (Ramli *et al.*, 2009). In these papers, there is detailed discussion of the techniques used (see Fig. 1). Here, we apply TDCA to developing embryos of oilseed rape, one of the major oil crops (Gunstone *et al.*, 2007).

Metabolic fluxes have been examined in *Brassica napus* by NMR methods (Schwender & Ohlrogge, 2002) and extended further by flux variability analysis (Hay & Schwender, 2011a,b). Further studies in oilseed rape (or the related Arabidopsis) that have shed light on metabolic regulation include quantitative trait locus (QTL) mapping (Burns *et al.*, 2003; Hobbs *et al.*, 2004), the use of mutants (Katavic *et al.*, 1995; Zou *et al.*, 1999; Ruuska *et al.*, 2002), the development of transgenic varieties (Roesler *et al.*, 1997; Stahl *et al.*, 2004; Mhaske *et al.*, 2005; Zhang *et al.*, 2009; Bates & Browse, 2011) and the examination of transcription factors (Baud & Lepiniec, 2010; Tan *et al.*, 2011). A recent review of the control of oil accumulation in seeds has been given by Weselake *et al.* (2009).

Previously, we used information from MCA to inform genetic manipulation in oilseed rape (Weselake *et al.*, 2008). In this article, we compare single-manipulation with double-anipulation TDCA in detail and show that the two methods give very similar results. An advantage of TDCA is that it provides an immediate overview of the distribution of flux over a complex metabolic pathway (Quant, 1993) and can be refined to allow further detailed analysis of each block. Our data for oilseed rape contrast noticeably with the results for oil palm and olive where *c.* 60% of the regulation is provided by fatty acid synthesis.

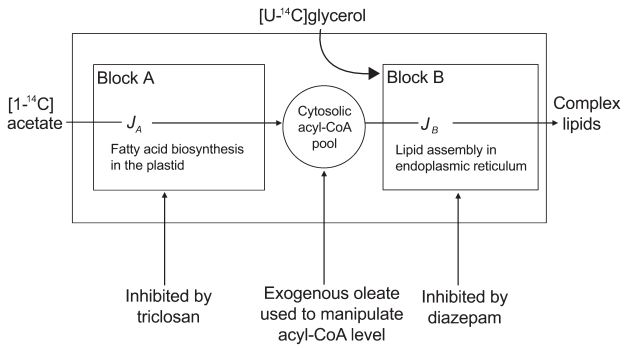

Fig. 1 The simplified lipid biosynthetic system used for analysis. Carbon flux from [1-^{14}C]acetate enters Block A to produce fatty acids. Block A contains a number of enzymes involved in [1-^{14}C]acetate metabolism, including acetyl-CoA synthase, acetyl-CoA carboxylase, the Type II fatty acid synthase reactions, stearoyl-ACP desaturase, acyl-ACP thioesterases and acyl-CoA synthase. The cytosolic acyl-CoA pool is the defined system intermediate and its level could be increased with exogenous oleate in single-manipulation experiments. In contrast with radioactivity from [1-^{14}C]acetate, which entered Block B reactions (Kennedy pathway conversions and other reactions for lipid assembly) via the acyl-CoA pool, [U-^{14}C]glycerol labelled acyl lipids via the Kennedy pathway reactions directly. Block B reactions not only include the Kennedy pathway, but also other enzymes, such as phospholipid:diacylglycerol acyltransferase (PDAT) and phosphatidylcholine:diacylglycerol cholinephosphotransferase that are associated with the flux of carbon during lipid assembly. Block A (fatty acid biosynthesis) could be inhibited selectively by triclosan and Block B by diazepam in double-manipulation experiments. Please see Ramli *et al.* (2002b) for further discussion of the theory behind the flux control experiments and Weselake *et al.* (2009) for the reactions involved in lipid synthesis.

Materials and Methods

Plant material

Oilseed rape (*Brassica napus* L. cv Westar) seeds were a kind gift from Dr R. Weselake (University of Alberta, Edmonton, AB, Canada). They were germinated in Petri dishes on well-wetted filter paper at 20°C and then transferred to seed trays filled with multipurpose compost (John Innes No. 1) for 10 d. After this period, individual plants were placed in 8-inch pots filled with multipurpose compost. Growth was under a 16-h light (25°C) and 8-h dark (20°C) cycle with a light intensity of 250 μmol m^{-2} s^{-1}. Flowers were hand pollinated and the pods were tagged. These conditions are typical of previous studies with oilseed rape embryos (e.g. O'Hara *et al.*, 2007).

For use in experiments, pods were harvested, opened and embryos dissected quickly and placed in cold 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 M sorbitol before use.

Chemicals

Sodium [1-^{14}C]acetate (specific radioactivity, 2.11 GBq $mmol^{-1}$) and [U-^{14}C]glycerol (specific radioactivity, 5.51 GBq $mmol^{-1}$) were from Amersham International (Amersham, Little Chalfont, UK). Thin layer chromatography (TLC) used silica-gel G plates (Merck Ltd, Lutterworth, UK). Phospholipids and fatty acid methyl ester (FAME) standards were obtained from Sigma (Poole, UK) and NuChek (Elysian, MN, USA), respectively. Sep-Pak C_{18} cartridges were from Waters (Milford, MA, USA). The inhibitors used were bromolaurate (Lancaster Synthesis Ltd, Morecambe, UK), bromooctanoate (Sigma-Aldrich, Gillingham, UK), bromopalmitate (Sigma), quercetin (dehydrate) (Sigma), diazepam (Sigma) and triclosan (a kind gift from Professor A. D. Russell, School of Pharmacy, Cardiff University, Cardiff, UK).

All other chemicals and solvents used were from Sigma or BDH (Poole, UK), and were the best available grades.

Radiolabelling of embryos

Seeds were selected for uniformity of mass and appearance. Dissected embryos were pooled and incubations were carried out with three to six replicates, each containing 10 embryos for seeds at 21–24 DAF (days after flowering and hand pollination) and five embryos for seeds at 27–35 DAF. Incubations were in 1 ml of 0.1 M sorbitol, 0.1 M potassium phosphate buffer (pH 7.0) containing 1 μCi of [1-^{14}C]acetate or 1 μCi of [U-^{14}C]glycerol with gentle shaking at 20°C for up to 24 h. In order to preserve labelling rates and to avoid upsetting endogenous metabolite levels, these radiolabelled precursors were carrier free. At the end of the incubations, samples were washed three times in unlabelled medium and treated with 1.25 ml of isopropanol at 70°C for 30 min to inactivate endogenous lipases.

For single-manipulation TDCA, embryos from pods at 27 DAF were used. These were pre-incubated with 1 mM oleic acid dissolved in 1 mM tetramethylammonium hydroxide in 0.1 M sorbitol for 2 h with gentle shaking at 20°C. After rinsing the embryos with 0.1 M sorbitol–0.1 M potassium phosphate (pH 7.0), they were incubated with radioactivity as already described. Control samples (no oleate treatment) were pre-incubated for 2 h with 1 mM tetramethylammonium hydroxide in 0.1 M sorbitol. Likewise, chemicals for double manipulation were added to the medium and were present for the whole of the incubation. The standard incubation time for all TDCA experiments was 6 h at 20°C (see the Results section).

Lipid extraction and analysis

Lipids were extracted from embryos at 4°C using a two-phase method that gave quantitative extraction for plant tissues (Smith *et al.*, 1982). Aliquots were taken of the upper aqueous and lower chloroform phases for radioactivity counting. The lower (lipid) phase was separated by TLC (Ramli *et al.*, 2002a). In addition, two-dimensional TLC used a first solvent of chloroform : methanol : water (65 : 25 : 4, by volume) and a second solvent of chloroform/acetone : methanol : water : acetic acid (50 : 20 : 10 : 10 : 5, by volume). Individual lipids were routinely revealed with UV light after spraying with 0.2% (w/v) 8-anilino-1-naphthalenesulphonic acid in anhydrous methanol. Further identification of individual lipids was with authentic markers and spraying with colour reagents (Kates, 1986).

The preparation of FAMEs, their separation and quantification of radioactivity were performed as described previously (Ramli *et al.*, 2002a), except that a Unicam ProGC was used. If the amount of radioactivity in FAME samples was insufficient for radio-gas–liquid chromatography (radio-GLC), silver nitrate TLC was utilized (Christie, 2003).

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

Acyl-CoAs and acyl-ACPs were extracted from the aqueous phase obtained from the total lipid extraction by rapid reverse-phase partition chromatography (Stymne & Glad, 1981). A full discussion of the method and control experiments is given in Ramli *et al.* (2002a). The control value of the acyl-CoA concentration for 27-DAF embryos was 7.1 ± 2.2 μM.

Quantification of glycerol 3-phosphate

Glycerol 3-phosphate was quantified by two enzymatic methods (Lang, 1984; Ramli *et al.*, 2002a). No change in the glycerol 3-phosphate levels could be detected following the addition of oleate, triclosan or diazepam in the TDCA experiments (Supporting Information Table S1). Therefore, alterations in the radioactive labelling of lipids from [U-^{14}C]glycerol were not a result of changes in the glycerol 3-phosphate pool.

Evaluation of alternative pathways for triacylglycerol (TAG) radiolabelling

In addition to the Kennedy pathway using diacylglycerol acyltransferase (DGAT), alternative methods for TAG synthesis have been reported in plants. Phospholipid: diacylglycerol (DAG) acyltransferase (PDAT) and DG: DAG acyltransferase activities were assessed using microsomal fractions and [1-^{14}C]dioleoylphosphatidylcholine and [^{14}C]dioleoylglycerol substrates, respectively (Stahl *et al.*, 2004). DGAT was measured as described previously (Ramli *et al.*, 2005).

Determination of radioactivity

Radioactivity was determined using an LKB Wallac 1209 Rackbeta (Wallac Oy, Turku, Finland) liquid scintillation counter. All samples were evaporated to dryness and counted in Optifluor scintillant (Canberra Packard, Pangbourne, UK). Quench correction was made by the external-standard channels-ratio method.

Top-down control analysis

A full discussion of the theory and application of TDCA to lipid biosynthesis and the derivation of the mathematical equations can be found in Ramli *et al.* (2002b). Briefly, the pathway was divided into two blocks. The reactions of fatty acid biosynthesis (Block A) are in the plastid, whereas lipid assembly reactions (Block B) are in the endoplasmic reticulum (ER). The cytosolic acyl-CoA pool is the system intermediate (Fig. 1). Block A reactions were measured with [1-^{14}C]acetate, whereas [U-^{14}C] glycerol measured Block B reactions independently. In oilseed rape, these precursors radiolabel fatty acids or the polar part of complex lipids, respectively (Table S2). For single manipulation, exogenous oleate was added to change the level of the system intermediate, acyl-CoA, and flux alterations were monitored by the incorporation of radioactivity from the precursors. Use of the acyl-CoA pool as an intermediate also had the advantage that this was the form in which carbon is exported from the plastids for lipid assembly, thus connecting the two, specially separate Blocks

A and B. The equations for calculating both the group elasticity and group flux control coefficients are detailed in Ramli *et al.* (2002b).

To analyse the experimental system by double manipulation, we carried out two separate manipulation experiments using inhibitors specific for each block of reactions, that is, triclosan for Block A (fatty acid synthesis) and diazepam for Block B (lipid assembly). For analysis, we manipulated the activity of Block A reactions using triclosan and measured the response of Block B reactions, by measuring changes in [1-^{14}C]acetate incorporation into total lipids, to the resulting changes in the cytosolic acyl-CoA pool (X). This allowed us to calculate the group elasticity of the enzymes in Block B in relation to X ($^{*}\varepsilon_X^B$). The second step involved the manipulation of enzyme activity within the complex lipid assembly pathway using diazepam. By similar methods, this manipulation allowed us to calculate the group elasticity of Block A in relation to X ($^{*}\varepsilon_X^A$). From these two sets of group elasticities, we calculated the two group flux control coefficients for Block A and Block B, respectively.

Monte Carlo simulations

Crystal Ball 7.2 (Academic Edition), by Decisioneering (Denver, CO, USA), was used with Microsoft Excel (Office XP) running under Microsoft Windows 2000 Professional to generate the simulated values (Ramli *et al.*, 2009).

As in Ainscow & Brand (1998), the simulations were generated assuming that each experimental value was normally distributed, with a mean equal to the mean of the experimental repeats, and that the standard deviation was equal to the standard error of the mean (SEM) of the experimental repeats.

For each data point, 1000 observations of the standardized normal distribution were generated and converted to simulated observations of the data using the formula:

$$X = \chi\sigma + \mu$$

(X, simulated data point; σ, observed SEM; μ, experimental mean; χ, observation from the standard distribution) (Ainscow & Brand, 1998). Therefore, we improved our confidence in the accuracy of our results by generating 1000 sets of the control coefficients.

When setting up our simulations, we assumed that the initial experimental error was normally distributed and that the individual data points were independent of each other (see Ainscow & Brand, 1998).

We determined the mean and the median of the final calculated coefficients and compared these with the averaged values of the experimental data as a test for the normality of the distribution of the calculated results. Further, for a normal distribution, the pseudo-standard deviation (calculated as the limits of the calculated results that encompass 68% of the results around the median, divided by two) and the standard deviation should be the same (data not shown).

Probability density curves were plotted against the relevant median values to visualize, characterize and compare the error distribution associated with each set of coefficients calculated from the Monte Carlo simulations (data not shown).

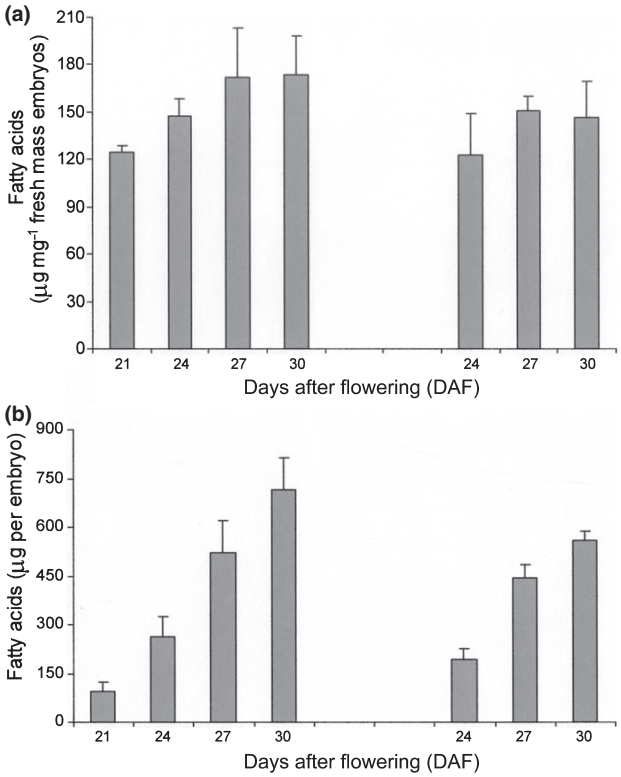

Fig. 2 Effect of maturation on lipid accumulation in oilseed rape (*Brassica napus*) embryos. Lipids were extracted from embryos of different maturity, methylated and analysed as in the Materials and Methods section. Results are means + SD (n = 3) using five embryos each time for 27-DAF (days after flowering) or 30-DAF samples and 10 embryos each time for 21-DAF and 24-DAF samples. (a) Shows lipid on a fresh wt. basis and (b) per embryo.

Results

Optimization of the experimental system

In order to decide on a suitable maturation stage in which to study regulation, we examined lipid formation in the period 21–35 DAF, which represents the period of rapid oil accumulation (Turnham & Northcote, 1983). In Fig. 2, the accumulation of lipid is shown on a fresh weight or per embryo basis for the period up to 30 DAF. On the former basis, accumulation appeared to be maximal by *c.* 27 DAF, whereas it still increased (although more slowly) on a per embryo basis beyond this time. This agreed excellently with the data for cv Haplona (Turnham & Northcote, 1983).

When lipid labelling was tested using [1-^{14}C]acetate as precursor, incorporation was maximal at *c.* 27 DAF on a fresh mass basis (Fig. 3). As > 95% of the label from [1-^{14}C]acetate is present in acyl chains (Table S2), this incorporation represents fatty acid synthesis and the subsequent incorporation of these fatty acids into complex lipids. For embryos from older seeds, incorporation was reduced considerably, being halved

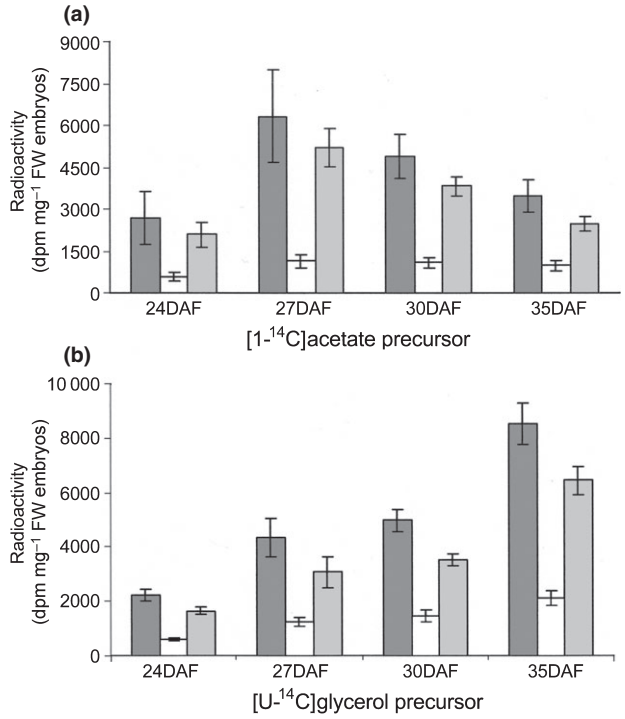

Fig. 3 Lipid labelling in oilseed rape (*Brassica napus*) embryos of different maturation (days after flowering, DAF). Embryos were incubated with [1-^{14}C]acetate (a) or [U-^{14}C]glycerol (b) for 8 h, and the incorporated radioactivity was analysed as described in the Materials and Methods section. Results are means ±SD (n = 3). White columns, polar lipids; light grey columns, non-polar lipids; dark grey columns, total lipids.

Table 1 Effect of oilseed rape (*Brassica napus*) embryo maturity on the labelling of major lipid classes

	Labelling (% total lipids)			
[1-^{14}C]acetate precursor	Polar	DAG	TAG	Other non-polar lipids
21 DAF	20.8 ± 0.3	29.3 ± 1.6	34.2 ± 3.8	15.7 ± 2.3
24 DAF	18.8 ± 2.4	30.9 ± 7.0	42.0 ± 8.4	8.3 ± 1.1
27 DAF	18.2 ± 1.8	30.4 ± 2.5	44.0 ± 1.4	7.4 ± 0.6
30 DAF	23.6 ± 0.5	31.1 ± 1.0	33.3 ± 1.6	12.1 ± 0.3
35 DAF	31.1 ± 1.0	32.6 ± 3.0	27.2 ± 1.5	8.9 ± 1.0
	Labelling (% total lipids)			
[U-^{14}C]glycerol precursor	Polar	DAG	TAG	Other non-polar lipids
21 DAF	22.1 ± 0.5	57.6 ± 2.4	18.3 ± 1.8	2.1 ± 0.8
24 DAF	27.1 ± 1.9	54.4 ± 2.9	17.3 ± 1.2	1.2 ± 0.3
27 DAF	29.2 ± 1.5	53.6 ± 0.7	15.4 ± 1.2	1.9 ± 0.6
30 DAF	36.3 ± 0.9	49.8 ± 2.2	11.1 ± 1.8	2.7 ± 0.2
35 DAF	35.3 ± 1.8	56.3 ± 1.8	5.9 ± 0.6	2.4 ± 0.4

Embryos were incubated with [1-^{14}C]acetate or [U-^{14}C]glycerol for 8 h under standard conditions and lipid classes were separated by thin layer chromatography (TLC) (see the Materials and Methods section). Results show means ± SD (n = 3). DAF, days after flowering; DAG, diacylglycerol; TAG, triacylglycerol.

by 35 DAF. Throughout the period examined, the incorporation of radioactivity from either precursor was higher into non-polar rather than polar lipids (Fig. 3), as expected for an oilseed.

Assuming equilibration of the labelled fatty acids with the total acyl lipids, one can calculate an approximate rate for lipid accumulation in the embryos. This is *c.* 40 μg lipid per embryo per 24 h which is comparable with oil accumulation *in vivo* (Turnham & Northcote, 1983). Moreover, lipid labelling did not show any marked decrease with incubation time. These results show the viability of the embryos and indicate that they mimic *in vivo* rates well.

The rate of incorporation of radioactivity from [U-^{14}C] glycerol showed a different pattern during seed maturation. Increasing values, on a fresh weight basis, were obtained throughout the period examined, with 35-DAF embryos giving the highest incorporation rates (Fig. 3).

We separated the lipid classes by TLC so as to evaluate the labelling of TAG from either precursor in embryos of different ages, as TAG represents the final product of lipid accumulation in seeds. The data (Table 1) showed that, after 27 DAF, a significant reduction in the label percentage in TAG was found with both precursors. It was also noteworthy that, during the incubation period, DAGs were well labelled, especially from ^{14}C-glycerol.

Another factor taken into consideration was the pattern of radiolabelling seen for the two precursors. To evaluate oil accumulation, we needed to be able to monitor appreciable TAG production and, in addition, to be on the linear part of the incorporation curve. Figure 4 shows that the incorporation of radioactivity from either [1-^{14}C]acetate or [U-^{14}C]glycerol was approximately linear for *c.* 8 h and was well maintained for over 24 h for embryos in the period 24–30 DAF, showing that the embryos remained viable and were able to maintain oil accumulation despite no additional exogenous carbon source being present. The initial 8-h period was repeated another three times for 27-DAF embryos to confirm the linearity of incorporation from both precursors (see insets in Fig. 4).

In all cases, there was a significant proportion of radioactivity in the two key non-polar lipids, DAG and TAG (Table S3), allowing us to examine oil production during the linear incubation period. Based on the above data, we chose to use an incubation period of 6 h and 27-DAF embryos for further experiments.

Single manipulation with exogenous oleate

In our TDCA experiments with cultures of olive or oil palm, we added exogenous oleate in Tween-20 (Ramli *et al.*, 2002b). However, Tween-20 caused a reduction in radioactive incorporation, presumably because of physiological damage to the embryos. Of the other solvents tested, only tetramethylammonia was found to be non-damaging (Table S4). In an initial experiment, 1 mM oleate was used with [1-^{14}C]acetate precursor (Table S5). Oleate caused a reduction in total lipid labelling but, unexpectedly, had no effect on the labelling of the intermediate pool, acyl-ACPs. Labelling of the acyl-CoA pool was reduced in

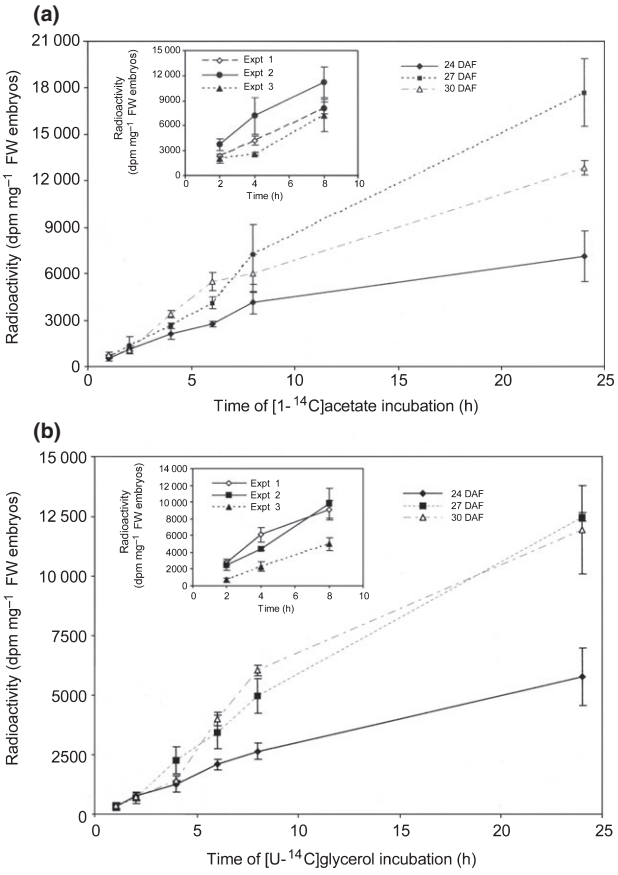

Fig. 4 Time course of radioactivity incorporation into lipids in developing oilseed rape (*Brassica napus*) embryos of different ages. For incubation and analysis, see the Materials and Methods section. Incubations were with [1-^{14}C]acetate (a) or [U-^{14}C]glycerol (b). The insets show further experiments with 27-DAF (days after flowering) embryos incubated for up to 8 h. Results are means ± SD (n = 3).

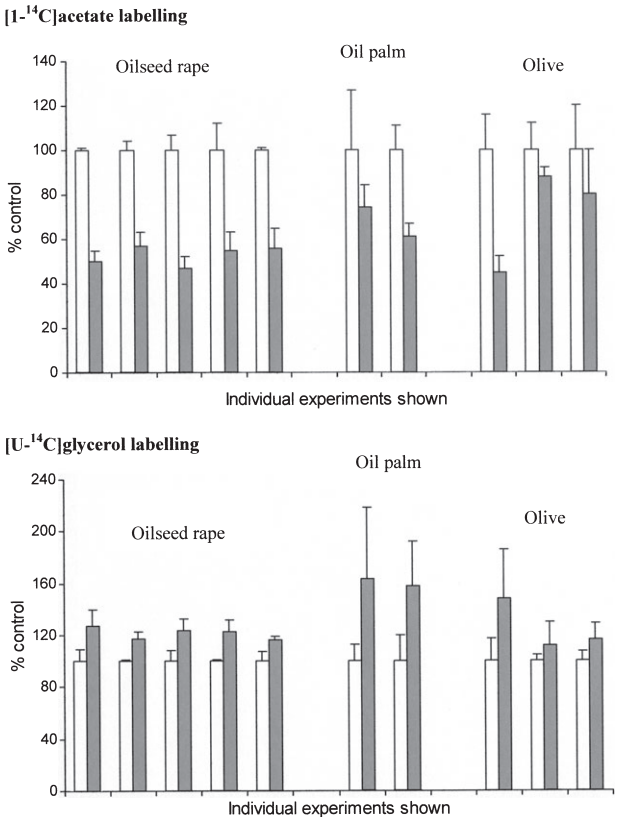

Fig. 5 Single-manipulation experiments with developing oilseed rape (*Brassica napus*) embryos compared with data for oil fruits. Experiments were carried out as detailed in the Materials and Methods section. Data show means + SD. Mean values (% of control, following oleate addition) for the labelling of Block A (from [1-^{14}C]acetate) and Block B (from [U-^{14}C]glycerol) were 54 ± 5 and 121 ± 5, respectively. Data for oil palm and olive calli are re-drawn from Ramli *et al.* (2002b). White bars, control; grey bars, plus oleate (1mM).

proportion to that of total lipids. The low labelling of acyl-CoAs probably reflects the small size of their pool in oilseed rape (Larson & Graham, 2001), as well as their rapid utilization for lipid assembly.

Having established that 1 mM exogenous oleate addition was able to reduce fatty acid synthesis, we carried out experiments for quantitative calculations of flux control. The data in Fig. 5 show that the addition of oleate, as evaluated in five separate experiments, caused around a 46% decrease in fatty acid labelling from [1-^{14}C]acetate, whereas lipid labelling from [U-^{14}C]glycerol was increased by *c.* 21% (Fig. 5). These data are compared with those from similar experiments using oil palm or olive (Ramli *et al.*, 2002b) in the figure.

In oilseed rape embryos, either precursor resulted in the labelling mainly of phosphatidylcholine (PC), DAG and TAG (Table S6). However, some other lipids contained appreciable radioactivity. Not surprisingly, phosphatidylglycerol (PG) contained up to 10% label when [U-^{14}C]glycerol was the precursor used. Following exogenous oleate addition, there was a general reduction in the proportion of membrane lipid labelling from [U-^{14}C]glycerol, whereas that in DAG and TAG was increased.

Group flux control coefficients

We measured changes in the steady-state cytosolic acyl-CoA levels (by two methods (Lang, 1984; Ramli *et al.*, 2002b) as a function of radiolabel incorporation and of absolute concentration) and in steady-state fluxes through Blocks A and B before and following manipulation of the system intermediate by the addition of exogenous oleate. The absolute acyl-CoA

Table 2 Group flux control coefficients (${}^{*}C^{J_{TL}}_{BlkA}$; ${}^{*}C^{J_{TL}}_{BlkB}$) for fatty acid synthesis and lipid assembly over lipid biosynthesis in developing oilseed rape (*Brassica napus*) embryos

	${}^{*}C^{J_{TL}}_{BlkA}$	${}^{*}C^{J_{TL}}_{BlkB}$
Expt 1 (n = 6)	0.34 ± 0.11	0.67 ± 0.11
Expt 2 (n = 5)	0.28 ± 0.07	0.72 ± 0.07
Expt 3 (n = 4)	0.31 ± 0.08	0.69 ± 0.08
Expt 4 (n = 5)	0.34 ± 0.10	0.67 ± 0.10
Expt 5 (n = 4)	0.29 ± 0.02	0.71 ± 0.02
Mean ± SD	0.31 ± 0.03	0.69 ± 0.03
Mean ± SEM	0.31 ± 0.01	0.69 ± 0.01

Values were calculated as outlined briefly in the Materials and Methods section and detailed previously in Ramli *et al.* (2002b) from experimental data summarized in Fig. 5.

concentrations in the oilseed rape embryos were 7 ± 2 and 19 ± 3 μM in the control samples and following oleate addition, respectively. It was necessary to take these measurements to confirm that the unique primary target of exogenous oleate is to change the absolute steady-state cytosolic acyl-CoA concentrations. However, once appropriate changes in the intermediate concentrations had been observed, we used a method to calculate the absolute group flux control coefficients, as a unique function of relative changes in system fluxes (Quant, 1993; New *et al.*, 1999), without the need to include the data for the relative or absolute changes in the system intermediate or to calculate the absolute elasticities. This method obviated problems with achieving accurate measurements of changes in the low levels of the acyl-CoA pool and gave us values (± SD; (± SEM)) of 0.31 (± 0.03; (± 0.01)) and 0.69 (± 0.03; (± 0.01)) for the coefficients for fatty acid biosynthesis (Block A) and lipid assembly (Block B) reactions, respectively (Table 2).

Double manipulation: identification of appropriate inhibitors

In order to confirm the important observation (above) that lipid assembly exerts strong flux control for oil accumulation in rape, we used a second independent method of quantification of flux control – double manipulation.

To perform a valid analysis by double-manipulation TDCA, it is important that any changes in carbon flux through the fatty acid biosynthesis reactions (Block A) do not affect flux through the lipid assembly reactions (Block B) other than through the chosen intermediate (the cytosolic acyl-CoA pool). Similarly, an inhibitor affecting Block B should not affect directly the reactions of Block A.

In oil palm callus (Ramli *et al.*, 2009), we used diflufenican to inhibit Block A and bromooctanoate for Block B (Ramli *et al.*, 2005), but these had no activity in oilseed rape. After trying various potential inhibitors (Table S7), we found that triclosan could be used to inhibit fatty acid synthesis, whereas diazepam was utilized to inhibit Block B (lipid assembly).

Table 3 Triclosan inhibits fatty acid labelling from [1-^{14}C]acetate, but not lipid labelling from [U-^{14}C]glycerol in *Brassica napus*

	[1-^{14}C]Acetate labelling		[U-^{14}C]Glycerol labelling	
	Control	+triclosan	Control	+triclosan
Expt 1	100 ± 8	81 ± 6 ($P < 0.025$)	100 ± 7	104 ± 4 (n.s.)
Expt 2	100 ± 5	70 ± 12 ($P < 0.005$)	100 ± 8	89 ± 11 (n.s.)
Expt 3	100 ± 7	71 ± 7 ($P < 0.005$)	100 ± 10	88 ± 5 (n.s.)
Expt 4	100 ± 16	66 ± 12 ($P < 0.05$)	100 ± 8	98 ± 6 (n.s.)
Expt 5	100 ± 6	57 ± 11 ($P < 0.005$)	100 ± 11	95 ± 11 (n.s.)
Expt 6	100 ± 9	66 ± 2 ($P < 0.001$)	100 ± 11	88 ± 7 (n.s.)

Samples were incubated as described in the Materials and Methods section ± triclosan. Data show means ± SD with five replicates for each experiment. Numbers in parentheses show the statistical significance by Student's *t* test (ns, not significant at the 5% level). The concentration of triclosan used was 40 μM, except for Expt 1, when it was 30 μM.

Effect of triclosan on fatty acid synthesis

Triclosan is an inhibitor of the enoyl-ACP reductase reaction of the Type II fatty acid synthases, such as in *Escherichia coli* (Heath *et al.*, 2001), *Plasmodium falciparum* (Kapoor *et al.*, 2004) and plants (Levy *et al.*, 1999). It was effective in developing oilseed rape embryos at concentrations of 10 μM and above. For MCA, we needed to inhibit Block A by triclosan by *c.* 30% so that the whole system was not unnecessarily perturbed; 30–40 μM triclosan was found to produce such inhibition.

We checked the selectivity of inhibition for Block A. Six independent experiments are shown in Table 3, each of which gave significant inhibition of fatty acid labelling from [1-^{14}C]acetate, but no effect on lipid assembly from [U-^{14}C]glycerol. Inhibition was in the range 20–42%, which was appropriate for control analysis.

We extracted the complex lipids labelled from the radioactive fatty acids derived from [1-^{14}C]acetate and separated them by TLC. Triclosan inhibited the labelling of all lipid classes to about an equal extent, providing further proof that its effect was on fatty acid biosynthesis and not, additionally, on any enzyme used for lipid assembly. Triclosan also had no effect on the pattern of complex lipids labelled from [U-^{14}C]glycerol (Fig. S1).

Diazepam as an inhibitor of lipid assembly

Diazepam has been reported as an inhibitor of lipid synthesis in a number of studies (see Yanese *et al.*, 2002). Its mechanism of action is unclear, but may be a result of its interaction with acyl-CoA binding protein (Knudsen *et al.*, 1993) and, hence, regulation of enzymes of TAG synthesis and other acyltransferases (Kerkhoff *et al.*, 1997). We tested diazepam in the range 10–100 μM, and 40–100 μM diazepam was found to provide adequate inhibition of lipid assembly.

Results from a series of independent experiments are shown in Table 4. Statistically significant inhibition of labelling from [U-^{14}C]glycerol was obtained in the range 12–26% using 100 μM diazepam. No effect was found on fatty acid labelling from

Table 4 Diazepam inhibits lipid assembly from [U-^{14}C]glycerol, but does not affect fatty acid labelling from [1-^{14}C]acetate in *Brassica napus*

	[1-^{14}C]Acetate labelling		[U-^{14}C]Glycerol labelling	
	Control	+Diazepam	Control	+Diazepam
Expt 1	100 ± 2	100 ± 4	100 ± 5	74 ± 6 ($P < 0.005$)
Expt 2	100 ± 7	106 ± 20	100 ± 10	82 ± 6 ($P < 0.05$)
Expt 3	100 ± 20	104 ± 14	100 ± 3	88 ± 5 ($P < 0.02$)
Expt 4	100 ± 17	98 ± 9	100 ± 3	76 ± 7 ($P < 0.005$)
Expt 5	100 ± 2	103 ± 5	100 ± 3	83 ± 3 ($P < 0.01$)
Expt 6	100 ± 16	103 ± 13	100 ± 8	85 ± 9 ($P < 0.10$)
Expt 7	100 ± 11	116 ± 14	100 ± 13	75 ± 9 ($P < 0.05$)
Expt 8	100 ± 11	100 ± 12	100 ± 8	87 ± 9 ($P < 0.10$)

Incubations were carried out as described in the Materials and Methods section. Diazepam was used at 100 μM. Results show means ± SD for each experiment with five replicates in each case, expressed as a percentage of the mean control value. Statistical significance was estimated using Student's *t* test.

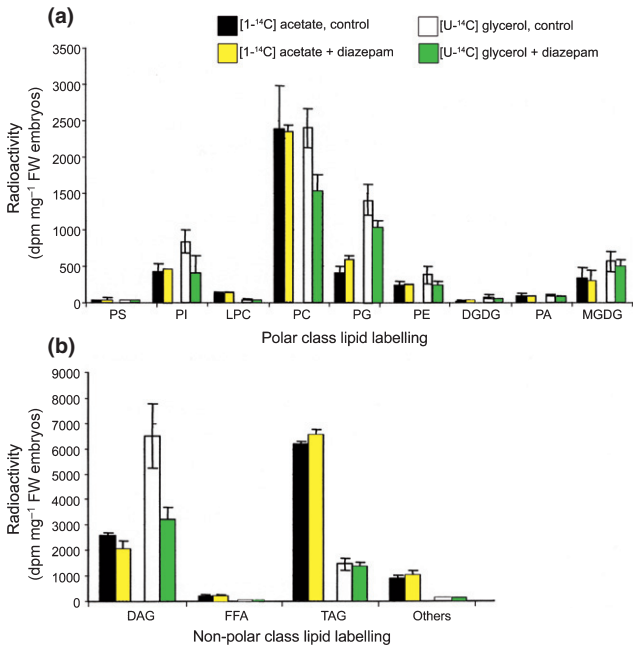

Fig. 6 Effect of diazepam on the radiolabelling of individual lipid classes in *Brassica napus*: (a) major polar lipid classes; (b) non-polar lipid classes. Incubations were carried out as detailed in the Materials and Methods section with diazepam at 100 μM. Means ± SD (n = 4) are shown. DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; FFA, free (non-esterified) fatty acids; LPC, lysophosphatidylcholine; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol.

[1-^{14}C]acetate (Table 4). Therefore, diazepam was shown to be an appropriate inhibitor for Block B reactions.

Total lipids were extracted, separated into classes by TLC and examined for the effect of diazepam on their labelling from [U-^{14}C]glycerol. The data (Fig. 6) showed that diazepam had minimal effects on labelling of individual lipids from [1-^{14}C]acetate, whereas from [U-^{14}C]glycerol most of the major labelled classes were inhibited, although the PC and DAG fractions, which were major labelled components, were reduced particularly (Fig. 6).

Double manipulation of TDCA

Labelling of the acyl-CoA pool from [1-^{14}C]acetate was reduced by triclosan, secondary to its direct action on the reactions of Block A, whereas diazepam appeared to increase slightly and indirectly the labelling of the acyl-CoA pool by its effects on Block B. However, this effect was only statistically significant in Experiment 3 (Table 5).

The group flux control coefficients shown in Table 6(a) were calculated using the data reported in Table 5. These were 0.27 ± 0.03 (± SEM) for Block A and 0.73 ± 0.03 (± SEM) for Block B. (The method for calculating the group elasticity and flux control coefficients from fractional changes in system fluxes and intermediate levels is detailed fully in Ramli *et al.* (2009).) Following 1000 Monte Carlo simulations of each of the three independent datasets (resulting in 1000 flux control coefficients for each separate experiment) and subsequent, additional, 1000 Monte Carlo

Table 5 Analysis of lipid (J) and acyl-CoA (X) pool labelling from double-manipulation flux control experiments with developing oilseed rape (*Brassica napus*) embryos

Labelling (dpm mg^{-1} FW embryo)			
Manipulation with triclosan (± 40 μM)			
Data used to calculate:	Expt 1	Expt 2	Expt 3
$J_{BlkA(-Tric)}$	4391 ± 388	4995 ± 266	5448 ± 337
$J_{BlkA(+Tric)}$	2799 ± 235 (64%) (P < 0.005)	2940 ± 328 (59%) (P < 0.001)	4139 ± 277 (76%) (P < 0.005)
$X_{(-Tric)}$	97 ± 6	99 ± 9	103 ± 7
$X_{(-Tric)}$	48 ± 5 (50%) (P < 0.001)	63 ± 8 (64%) (P < 0.005)	72 ± 5 (70%) (P < 0.005)
Manipulation with diazepam (± 100 μM)			
Data used to calculate	Expt 1	Expt 2	Expt 3
$J_{BlkB(-Diaz)}$	1809 ± 157	2208 ± 67	1371 ± 150
$J_{BlkB(+Diaz)}$	1456 ± 245 (80%) (P < 0.05)	1751 ± 181 (79%) (P < 0.005)	1060 ± 119 (77%) (P < 0.02)
$X_{(-Diaz)}$	106 ± 5 (106%) (n.s.)	95 ± 9 (108%) (n.s.)	73 ± 3 (110%) (P < 0.025)
$X_{(+Diaz)}$	1809 ± 157	2208 ± 67	1371 ± 150

Incubations were carried out as described in the Materials and Methods section. [1-^{14}C]Acetate was used to measure the acyl-CoA pool in both cases and the labelled lipids with triclosan. [U-^{14}C]Glycerol was used to label lipids in the diazepam experiments. Data show means ± SD (n = 5) and were used for calculation (via fractional changes in system fluxes and intermediates) of group flux coefficients. Numbers in parentheses are percentage of control. Significance was estimated by Student's t test. ns, not significant (P > 0.05).

Table 6 Group flux control coefficients ($^{*}C^{J_{TL}}_{BlkA}$; $^{*}C^{J_{TL}}_{BlkB}$) for fatty acid synthesis and lipid assembly over lipid biosynthesis from top-down control analyses (TDCAs) in developing oilseed rape (*Brassica napus*) embryos

	$^{*}C^{J_{TL}}_{BlkA}$	$^{*}C^{J_{TL}}_{BlkB}$
(a) Double-manipulation TDCA		
Expt 1	0.21	0.79
Expt 2	0.32	0.68
Expt 3	0.28	0.72
$\overline{X} \pm$ SD/ $\pm$ SEM	0.27 ± 0.06/ ±0.03	0.73 ± 0.06/ ±0.03
$\overline{X} \pm$ SD ± MCS	0.27 ± 0.06	0.73 ± 0.06
(b) Single-manipulation TDCA		
Expt 1	0.34 ± 0.11	0.67 ± 0.11
Expt 2	0.28 ± 0.07	0.72 ± 0.07
Expt 3	0.31 ± 0.08	0.69 ± 0.08
Expt 4	0.34 ± 0.10	0.67 ± 0.10
Expt 5	0.29 ± 0.02	0.71 ± 0.02
$\overline{X} \pm$ SD/ $\pm$ SEM	0.31 ± 0.03/ ±0.01 (n = 5)	0.69 ± 0.03/ ±0.01 (n = 5)
$\overline{X} \pm$ SD ± MCS	0.31 ± 0.03	0.69 ± 0.03
(c) Mean of experiments		
$\overline{X} \pm$ SD/ $\pm$ SEM	0.30 ± 0.04/ ±0.02 (n = 8)	0.70 ± 0.04/ ±0.02 (n = 8)
(d) Monte-Carlo analysis of data from (a) and (b) above		
Mean	0.30	0.70
Median	0.30	0.70
$\overline{X} \pm$ SD ± MCS	0.30 ± 0.02 (n = 8)	0.70 ± 0.02 (n = 8)

Calculations (see the Materials and Methods section) utilized data for individual experiments summarized in Fig. 5 and Table 5 or Monte-Carlo simulations.

simulation trials of the meaned results, we calculated ± SD and ± SEM on the meaned pair of group flux control coefficients to be ± 0.06 (Table 6a).

For completeness, we also provide (Table 6b) the final meaned pair (±SD) resulting from equivalent sets of Monte Carlo simulations (performed firstly on the five independent experimental datasets, and subsequently on the meaned pair) from our single-manipulation TDCA (Table 2).

Finally, in Table 6(d), we provide the pair of group flux control coefficients with errors calculated from 1000 Monte Carlo simulations performed on the meaned pair of each of the eight independent datasets (from both single- and double-manipulation TDCAs) that had previously each been independently subjected to 1000 Monte Carlo simulations.

Discussion

This study sought to use TDCA to determine the distribution of flux control between the fatty acid synthesis (Block A) and lipid assembly (Block B) reactions in developing embryos of oilseed rape, using both single- and double-manipulation approaches. First, we discuss the validity of the labelling approach and confirm that the major destination for synthesized fatty acids, in embryos, is storage lipids via the Kennedy pathway and associated reactions. Second, we discuss the group flux control coefficients for Block A and Block B reactions following manipulation of the cystosolic acyl-CoA pool and, separately, the inhibition of each block of reactions. Finally, we discuss the implications of this study for investigations into increasing oil yields in oilseed rape.

Synthesis of storage lipids in oilseed rape embryos

Newly synthesized fatty acids in the plastid have two major destinations: retention in that organelle for plastid lipid synthesis or export to the cytosol for lipid assembly on the ER. Oilseed rape is termed a '16 : 3 plant', where its monogalactosyldiacylglycerol (MGDG) contains appreciable hexadecatrienoic acid (see Heinz & Roughan, 1983; Browse & Somerville, 1991). This means that not only are sulpholipid and PG labelled directly from plastid-derived fatty acids, but also the galactosylglycerides will contain fatty acids obtained from the plastid 'prokaryotic' as well as the extraplastidial 'eukaryotic' pathways. However, in developing embryos, it is clear that the 'prokaryotic' pathway is much less important than lipid formation in the cytosolic compartment. Labelling of PG from [1-^{14}C]acetate was 3–5% of total lipids (Table S6), whereas sulpholipid was < 2%. MGDG labelling was 2–5% but, in '16 : 3-plants', some of this lipid's labelling will still come from the cytosolic acyl-CoA pool (Heinz & Roughan, 1983). This labelling agrees with the small mass contribution made by plastid lipids in oilseed rape (Zadernowski & Sosulski, 1978) Thus, although up to 10% of the newly synthesized fatty acids could be used from the acyl-ACP pool for plastid lipid synthesis, the vast majority (> 90%) are likely to be directed to the acyl-CoA pool and, hence, destined for the cytosolic compartment. In addition, non-esterified fatty acids never accounted for more than *c.* 1% of the total radioactive products. Therefore, from our experiments with [1-^{14}C]acetate, we conclude that fatty acids synthesized in the plastids are mainly (> 90%) channelled into the extraplastidic compartment for (storage) lipid assembly.

The rate of incorporation of [1-^{14}C]acetate was maximal at 27 DAF, whereas the rate from [U-^{14}C]glycerol increased throughout the period and was highest at 35 DAF. Glycerol taken up by embryos must be activated to glycerol 3-phosphate, and this metabolite can then be used as a starting material for the Kennedy pathway or for the head groups of PG and cardiolipin (Dormann, 2005). The increased proportional labelling of PG from [U-^{14}C]glycerol compared with [1-^{14}C]acetate (Table S6) suggests that some radio-incorporation into this lipid occurs via its head group.

The increased incorporation rates for [U-^{14}C]glycerol may be a result of reductions in the glycerol 3-phosphate pool at 30 DAF, as reported in *B. napus* cv Drakkar (Vigeolas & Geigenberger, 2004). We confirmed their data with cv Westar with a very similar proportional decrease in the glycerol 3-phosphate pool (from a maximum at 26 DAF of 183 ± 15 nmol g^{-1} fresh weight to 132 ± 10 nmol g^{-1} fresh weight at 35 DAF). Clearly, incorporation of radioactivity from [U-^{14}C]glycerol is dependent not only on its activation (Sadava & Moore, 1987), but also on the extent of its dilution with endogenous glycerol 3-phosphate produced mainly from dihydroxyacetone phosphate (Sharma *et al.*, 2001).

From [1-^{14}C]acetate, the major labelled classes were TAG, DAG and, to a lesser extent, PC. With time, there was a

reduction in PC labelling and an increase in that of DAG and TAG. These changes in the patterns of labelling were also seen from [U-^{14}C]glycerol. The reciprocal changes in labelling of TAG and PC with time suggest that interconversion takes place during TAG accumulation in oilseed rape, as documented previously in other oilseeds (e.g. Slack *et al.*, 1985; Bates *et al.*, 2007, 2009; Lu *et al.*, 2009).

With [U-^{14}C]glycerol, DAG was much better labelled than TAG (see Table S3), part of which may be explained simply by the difference in the mode of TAG labelling which, from [1-^{14}C]acetate, will be via the incorporation of three [^{14}C]acyl groups. This also fits well with DGAT exerting significant flux control in oilseed rape (Perry & Harwood, 1993a,b; Weselake *et al.*, 2008).

TAG synthesis can occur though a number of alternative enzymes, including DGAT, phospholipid:diacylglycerol acyltransferase (PDAT) and a DAG:DAG transacylase. The low activities of PDAT and DAG:DAG transacylase (Table S8) in oilseed rape agreed with experiments using Arabidopsis, in which knockouts for the PDAT gene showed no significant changes in oil accumulation (Stahl *et al.*, 2004; Mhaske *et al.*, 2005). Nevertheless, Zhang *et al.* (2009) have shown recently that PDAT has a complementary function to DGAT in Arabidopsis. These two enzymes appear to be the only ones that contribute significantly to TAG accumulation in Arabidopsis. Because the products of PDAT are lysophosphatidylcholine (LPC), in addition to TAG, an LPC acyltransferase could also be important for overall oil accumulation (Zhang *et al.*, 2009). Given the known differences between oilseed rape and Arabidopsis lipid biochemistry (e.g. Li *et al.*, 2006), it would be interesting to determine the effect of manipulating the above genes on oil synthesis in *B. napus*.

Although DGAT is a major source of TAG, there is clearly significant flux through PC. Apart from PDAT activity, this can be through systems for the desaturation of fatty acids which involve the equilibration of DAG with PC via cholinephosphotransferase (Stymne & Stobart, 1987) or the newly discovered PC:DAG cholinephosphotransferase (Lu *et al.*, 2009) or, generally, through acyl editing (Bates *et al.*, 2007, 2009). Although this is a complication of the Kennedy pathway, all of these reactions (including PDAT) are included within the overall enzymes of lipid assembly in Block B.

TDCA of lipid synthesis in oilseed rape embryos

A discussion of the validity of equations for the connectivity theorem as applied to the two-block TDCA system of the lipid biosynthesis pathway has been made previously (Ramli *et al.*, 2002b). The connectivity theorem, which holds for TDCA, expresses the relationship between flux control coefficients and elasticity coefficients, and it is essential that the two blocks of reactions only interact through the system intermediate, the acyl-CoA pool (Brown *et al.*, 1990). This is why we carried out appropriate control experiments (as in Ramli *et al.*, 2002a,b) to elucidate any interactions between the plastid and ER, and to evaluate complications in the use of the Kennedy pathway for oil accumulation.

Single-manipulation experiments

Exogenous oleate addition caused a reduction in fatty acid synthesis from [1-^{14}C]acetate and an increase in lipid synthesis from [U-^{14}C]glycerol. These results agreed, in general, with data obtained previously using oil palm and olive calli (Ramli *et al.*, 2002b). They were also in keeping with other results, where fatty acid addition caused a reduction in seed fatty acid synthesis (Shintani & Ohlrogge, 1995) or an increase in complex lipid formation (Bafor *et al.*, 1990). Moreover, Bao & Ohlrogge (1999) concluded that, for embryos from some oilseeds, fatty acid supply can be one limiting factor for TAG accumulation.

The observed increase in DAG labelling could be explained if DGAT exerted significant flux control in *B. napus*, as suggested previously in biochemical experiments (Perry & Harwood, 1993a,b) and confirmed recently by genetic manipulation (Weselake *et al.*, 2008; Taylor *et al.*, 2009). An important role for DGAT was also suggested in the analogous model plant, Arabidopsis, when the enzyme was reduced (Katavic *et al.*, 1995) or overexpressed (Jako *et al.*, 2001) to cause a corresponding change in TAG accumulation.

Calculation of the absolute group flux control coefficients indicates that, in developing oilseed rape embryos, as in other plant systems (Ramli *et al.*, 2002a,b), flux control for lipid accumulation is shared between both the fatty acid synthesis and lipid assembly blocks of reactions. However, unlike our experiments with tissue cultures of oil fruits where Block A exerted *c.* 60% control (Ramli *et al.*, 2002b, 2005, 2009), developing rape embryos showed greater control within the lipid assembly group of reactions – at least from embryos at 27 DAF when lipid accumulation was very high (Turnham & Northcote, 1983). Of relevance to the high control exerted by Block B reactions are the observations by Vigeolas, Geigenberger and colleagues (Vigeolas & Geigenberger, 2004; Vigeolas *et al.*, 2007) that the supply of substrates, including glycerol 3-phosphate, may influence overall oil accumulation in *B. napus*. This adds a further complication to finding simple ways of manipulating oilseed rape for increased lipid yields. Nevertheless, because oleate addition was able to increase lipid assembly (Fig. 5), the supply of glycerol 3-phosphate for the Kennedy pathway was not absolutely limiting.

Double-manipulation experiments

TDCA assumes that, in a conceptually simplified linear system, the fluxes to and from the unique intermediate will be equal in rate, but opposite in sign (Brand, 1996). Therefore, by inhibiting the Block A reactions and, hence, indirectly reducing the levels of the intermediate, and then measuring the new steady-state system flux in the presence of the inhibitor, TDCA gives a measure of the change in Block B flux as a result of the indirect inhibition of *X*. This allows the calculation of the fractional change in system flux B for a fractional change in *X*. Similarly, the inhibition of Block B permits the calculation of the fractional change in Block A flux for a fractional change in the levels of acyl-CoA, induced by the effects of inhibition of Block B.

Triclosan was found to be an appropriate inhibitor of Block A reactions and diazepam inhibited Block B reactions. The nature of inhibition by diazepam is not known, but may be a result of its interaction with acyl-CoA binding protein (Knudsen *et al.*, 1993); in our experiments, it had minimal effects on the labelling of individual lipids from [1-^{14}C]acetate, but most of the major lipid classes were inhibited when labelled with [U-^{14}C]glycerol. This may imply that an initial reaction of the Kennedy pathway (e.g. glycerol 3-phosphate acyltransferase) was inhibited. Although the supply of glycerol 3-phosphate has a significant effect on TAG formation in oilseed rape (Vigeolas & Geigenberger, 2004), we did not detect any change in its concentration following diazepam treatment. Thus, any effect of diazepam on this enzyme would not appear to be through alterations in substrate concentrations.

In addition, it is noteworthy that diazepam reduces significantly not only the radiolabelling of DAG, but that of PC. This result is consistent with quantitatively important DAG–PC interconversions in *B. napus*, as noted in other oilseeds (e.g. Bates *et al.*, 2009; Lu *et al.*, 2009).

Calculation of the group flux control coefficients (Table 6) for the double-manipulation experiments confirmed that the reactions in Block B (lipid assembly) exerted more control (73%) than those of Block A in oilseed rape. This value agreed very well with the 69% value obtained for the single-manipulation experiments using exogenous oleate. Such consistent results emphasize the validity of using single manipulation when selective inhibitors or other specific methods (e.g. gene manipulation) are not available for the modification of enzyme activity.

We subjected the experimental data from both the single- (n = 5) and double-manipulation (n = 3) TDCAs to rigorous scrutiny, using Monte Carlo simulations, to achieve a more accurate assessment of the errors in each case. The errors (±SD values) after Monte Carlo simulations, on both meaned pairs of flux control coefficients, would have been expected to be approximately equivalent to those for ± SEM of the experimental data, for the reasons outlined in an earlier paper (Ramli *et al.*, 2009) and in Ainscow & Brand (1998), because the calculations use the same equations and are essentially the same in approach. However, this was not observed, except for the result from all the collated data (n = 8, Table 6d). These results can be explained because the Monte Carlo simulation error values in Table 6(a,b) carry through the accumulated errors on the elasticities as well as the errors involved in averaging the final individual flux control coefficients, whereas, those in Table 6(d) are simply a facet of the errors on the final eight pairs of flux control coefficients.

Conclusions

The results reported here reveal a clear difference between oilseed rape and the two oleaginous fruit tissues (olive and oil palm) examined by control analysis in detail previously (Ramli *et al.*, 2009). In olive, we found that Block A reactions (fatty acid synthesis) exerted 57% of the total control (Ramli *et al.*, 2002b), whereas, in oil palm, they exerted 61–65% of the total control (Ramli *et al.*, 2009). Our data for oilseed rape are also consistent with the work of Vigeolas *et al.* (2007), who showed that an increased supply of glycerol 3-phosphate, and hence stimulation of complex lipid assembly, had an important controlling role for TAG accumulation in this crop. It also means that detailed examination of acyl fluxes through pathways of TAG formation, such as carried out for soybean (Bates *et al.*, 2009), should be revealing.

Arabidopsis, like oilseed rape, is a member of the Brassicaceae. Although these two plants show some differences in lipid metabolism (Li *et al.*, 2006), parallels are often drawn between them. Thus, it is noteworthy that the manipulation of enzymes that form TAG in Arabidopsis shows that both DGAT and PDAT are important for oil accumulation (Zhang *et al.*, 2009). This also confirms previous experiments showing the significance of DGAT (Katavic *et al.*, 1995; Jako *et al.*, 2001) in Arabidopsis. In view of the experimental data reported in this article, it would be interesting to perform equivalent experiments to those of Zhang *et al.* (2009), but in oilseed rape embryos.

Because we have demonstrated the relative importance of lipid assembly in controlling flux to TAG in oilseed rape, it is suggested that initial attempts at genetic manipulation could be profitably aimed at these reactions. In keeping with this suggestion, overexpression of a lysophosphatidate acyltransferase in *Brassica* resulted in increases of up to 48% in seed oil levels (Zou *et al.*, 1997), and increased supply of glycerol 3-phosphate for lipid assembly can also raise oil accumulation significantly (Vigeolas & Geigenberger, 2004; Vigeolas *et al.*, 2007). In addition, biochemical experiments with oilseed rape have suggested that DGAT is an important enzyme exerting significant control over lipid synthesis (Perry & Harwood, 1993a,b; Perry *et al.*, 1999). Thus, it is also especially noteworthy that experiments to manipulate DGAT have already shown promise for increasing oil yields in both glasshouse and field trials (Weselake *et al.*, 2008; Taylor *et al.*, 2009). Such data reveal how flux control experiments, as described here, can be utilized well for biotechnological exploitation.

Acknowledgements

We are grateful to Professor R. J. Weselake (University of Alberta, Canada) for the supply of *B. napus* (cv Westar) seeds. The work was supported by a grant (72/P16420) from the Biotechnology and Biological Sciences Research Council (BBSRC).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Triclosan does not affect the relative labelling of different lipid classes.

Table S1 Addition of reagents for single- or double-manipulation experiments does not affect endogenous glycerol 3-phosphate levels

Table S2 Labelling of lipids by radiolabelled precursors

Table S3 Percentage distribution of radioactivity in the main labelled lipid classes at different incubation times

Table S4 Solvent-dependent effects on labelling of oilseed rape (*Brassica napus*) embryos

Table S5 Effect of exogenous oleate on the distribution of radioactivity incorporated from [1-^{14}C]acetate into embryos of rapeseeds at 27 d after flowering (DAF)

Table S6 Effect of exogenous oleate on the relative incorporation of radioactivity into the main labelled lipid classes of embryos of oilseed rape (*Brassica napus*) at 27 d after flowering (DAF)

Table S7 Evaluation of potential inhibitors for double-manipulation experiments

Table S8 *In vitro* activities of enzymes involved in triacylglycerol production

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