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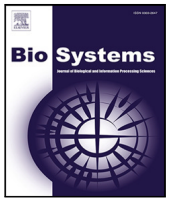

Metabolic Control Analysis of triacylglycerol accumulation in oilseed rape

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ABSTRACT

The increasing global demand for vegetable oils will only be met if there are significant improvements in the productivity of the major oil crops, such as oilseed rape. Metabolic engineering offers the prospect of further gains in yield beyond that already achieved by breeding and selection but requires guidance as to the changes that need to be made. Metabolic Control Analysis, through measurement and estimation of flux control coefficients, can indicate which enzymes have the most influence on a desired flux. Some experiments have previously reported flux control coefficients for oil accumulation in the seeds of oilseed rape, and others have measured control coefficient distributions for multi-enzyme segments of oil synthesis in seed embryo metabolism measured *in vitro*. In addition, other reported manipulations of oil accumulation contain results that are exploited further here to calculate previously unknown flux control coefficients. These results are then assembled within a framework that allows an integrated interpretation of the controls on oil accumulation from the assimilation of CO_2 to deposition of oil in the seed. The analysis shows that the control is distributed to an extent that the gains from amplifying any single target are necessarily limited, but there are candidates for joint amplification that are likely to act synergistically to produce much more significant gains.

1. Introduction

Vegetable oils are a valuable agricultural commodity and global production for 2020/2021 was about 210 million metric tons (Statista, 2022). Most of the oil is used for food and feed with some utilised for the production of industrial bio-products and biofuels (Taylor et al., 2011). Since demand has been rising steadily at around 5% per year for the last five decades (Gunstone et al., 2007) and agricultural land is limited (or even reduced by climate change), there is an urgent need to increase existing production (McKeon et al., 2016) to feed growing global populations. One way to augment oil yields is to target proteins involved in the biosynthesis of storage oils (Weselake et al., 2017).

Four major crops account for over 85% of the total global oil production. Of these, oilseed rape *Brassica napus* is grown widely in Northern Europe, Canada, China and India (Woodfield and Harwood, 2017) and is the third most important crop worldwide, producing about 15% of the total vegetable oils (Weselake et al., 2017). Oilseed rape is an allotetrapoid crop, resulting from interspecies breeding of varieties of *B. oleracea* and *B. rapa* (An et al., 2019).

Historically, *B. napus* oil was high in erucic acid and the meal produced from the seed was high in glucosinolates (McVetty et al., 2016; Woodfield and Harwood, 2017). These compounds can potentially result in nutritional problems for humans and livestock, respectively (Woodfield and Harwood, 2017). Cultivars were produced by conventional breeding in Canada that were varieties 'double-low' in both erucate and glucosinolates and were given the trade name 'Canola'. In Europe, such varieties are termed LEAR (low erucic acid rape) in contrast to HEAR (high erucic acid rape) varieties of *B. napus* which are still used for industrial purposes (McVetty et al., 2016; Woodfield and Harwood, 2017).

In oil crops the accumulating lipid is largely triacylglycerol (TAG). The biosynthetic pathway for TAG begins in the plastid, where fatty acids are formed by the concerted action of acetyl-CoA carboxylase (ACCase) and enzymes of the fatty acid synthase complex (Ohlrogge and Browse, 1995). The normal products will be palmitoyl–ACP and stearoyl–ACP (roughly in a 1:4 ratio). The latter is desaturated in the plastid to form oleoyl-ACP and, after cleavage by thioesterases, the fatty acids are exported to the cytosol where they are re-esterified to coenzyme A by the catalytic action of acyl-CoA synthetase and transported to the endoplasmic reticulum (Weselake et al., 2009; Chen et al., 2015; Bates, 2016), likely bound to acyl-CoA binding proteins (Xiao and Chye, 2011; Du et al., 2016). In terms of fatty acid export from the

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plastid, fatty acid exporter 1 is a membrane-bound protein involved in transporting fatty acids across the inner membrane of plastid (Li et al., 2015a). In turn, the cytoplasmic acyl-CoAs formed, following fatty acid export, can be utilised by three endoplasmic reticulum (ER)-bound acyl-CoA-dependent acyltransferases that are three of the four reactions of the Kennedy pathway (Kennedy, 1961) leading to TAG. Diacylglycerol acyltransferase (DGAT) catalyses the final reaction in this process (Chen et al. 2022). In some plants but not in *B. napus* (Woodfield et al., 2018), significant TAG can also be synthesised by an acyl-CoA independent final step catalysed by PDAT (phospholipid: diacylglycerol acyltransferase) (Dahlqvist et al., 2000; Fenyk et al., 2022). Additional reactions permit the modification of the fatty acyl chains by e.g. the elongase complex, desaturases or hydroxylases (Weselake et al., 2009; Chen et al., 2015; Bates, 2016) which are then routed into TAG.

Given the goal of improving oil yields in a crop that has already been subject to artificial selection to this end, new approaches are needed. Metabolic engineering of plants by direct manipulation of enzyme levels is one such method, but given the large number of enzymes involved from carbon assimilation to oil deposition, it is of primary importance to have a means to determine which enzymes are most useful to manipulate. Metabolic Control Analysis (MCA) (Kacser and Burns, 1973; Heinrich and Rapoport, 1973, 1974) provides a framework for this through the definition and evaluation of the flux control coefficient, C_E^J, whose value quantifies the effect that altering the activity of an enzyme or group of enzymes, E, has on the flux, J, in a metabolic pathway. Methods and examples of how MCA has been applied in metabolic engineering have been reviewed in Fell (2021). From the measurement of the response of a metabolic flux to a known change in an enzyme activity, the flux control coefficient can be determined (Kacser and Burns, 1973; Heinrich and Rapoport, 1974; Fell, 1992). Once this is known, the response of the metabolic flux to different degrees of change in the enzyme activity can be estimated (Small and Kacser, 1993).

There are two major approaches to the application of MCA to whole metabolic pathways: top-down (TDCA) and bottom-up (BUCA) control analyses (Fell, 1992, 1997). For TDCA (Brown et al., 1990; Quant et al., 1993), an overall modular view of control of a pathway is obtained and does not necessarily need specific ways of manipulating the activity of individual enzymes to known degrees. In contrast, BUCA gradually builds up a picture from the response of each individual enzyme in a pathway by using specific inhibitors or selective changes in enzyme protein levels introduced via genetic engineering (Kacser and Burns, 1973; Fell, 1992).

We applied MCA to seed oil biosynthesis when we used TDCA to examine oilseed rape embryos *in vitro* and showed that the lipid assembly block of reactions had more control over TAG formation than the fatty acid synthesis reactions (Weselake et al., 2008; Tang et al., 2012). Indeed, earlier research using developing zygotic embryos of *B. napus* suggested that the DGAT-catalysed reaction had a substantial effect on the flow of carbon into TAG (Perry and Harwood, 1993; Perry et al., 1999). Over-expression of one of four highly-homologous *DGAT1* genes, *BnaA.DGAT1.b* (Greer et al., 2016; Chen et al., 2022) was shown to cause a change in the control exerted by the TAG assembly block operating in the ER (Weselake et al. 2008). Substantial increases in the TAG content of transgenic *B. napus* seed over-expressing DGAT1s were observed under both greenhouse and field conditions (Weselake et al., 2008; Taylor et al., 2009). We also used TDCA to examine oil biosynthesis *in vitro* in other important crops such as oil palm (*Elaeis guineensis*), olive (*Olea europaea*) (Ramli et al., 2002, 2005, 2009) and soybean (*Glycine max*) (Guschina et al., 2014) where the fatty acid biosynthetic block was more important. More recently we have used BUCA by over-production of lysophosphatidic acid acyltransferase (LPAAT) and PDAT by genetic engineering in oilseed rape to determine their flux control coefficients *in vivo* in the whole pathway from assimilation to storage (Woodfield et al., 2019; Fenyk et al., 2022).

Here we propose that TDCA provides a framework to integrate these various results into a modular analysis of the control of TAG accumulation *in vivo*, similar to source-sink analysis in plants, but more detailed. Further, we note that the methodology developed in our LPAAT studies to estimate flux control coefficients can be applied to experimental results in the literature that have not been analysed previously in an MCA framework but that can be incorporated in this modular analysis. Given that the experiments have been carried out at different times, in different laboratories, and often with different cultivars, the overview obtained is indicative rather than definitive. However, it illustrates that a more fine-grained analysis of control, and identification of sites for metabolic engineering interventions, could be carried out through a purpose-designed study,

2. Methods

The estimates of the distribution of the control of TAG accumulation presented here depend on the MCA relationships below. The flux control coefficient for an enzyme E on a flux J can be defined (Fell, 1997) as:

$$C_E^J = \frac{\partial \ln J}{\partial \ln E}. \tag{1}$$

Hence the flux control coefficient can be determined as the slope of a graph of $\ln J$ against $\ln E$, or, for two levels of enzyme activity close enough that the flux control coefficient does not change as:

$$C_E^J \simeq \frac{\Delta \ln J}{\Delta \ln E}. \tag{2}$$

However, this will be less accurate for the large changes in enzyme activity associated with over-expression of genes encoding enzymes. Instead, the following finite difference equation (Neuhaus et al., 1989; Kruckeberg et al., 1989; Small and Kacser, 1993) can be used for a pair of flux-enzyme measurements where, as is often the case, the flux shows a hyperbolic response to enzyme activity (e.g. Kacser and Burns, 1973; Fell, 1997):

$$C_{E_1}^{J_1} = \frac{(J_2 - J_1)E_2}{(E_2 - E_1)J_2}. \tag{3}$$

Writing any planned fold-change in expression as $r = E_2/E_1$, the expected fold-change in flux, $f = J_2/J_1$, is (Small and Kacser, 1993):

$$f = \frac{1}{1 - \frac{r-1}{r} C_{E_1}^{J_1}}. \tag{4}$$

In the *in vitro* TDCA experiments, flux to TAG was measured directly. However, in the case of increased production of enzymes in plants by genetic engineering, changes in the flux to TAG are not easily measurable by equivalent techniques and, in any case, the more relevant factor is the change in the final yield of TAG in the seeds, which is not directly proportional to the change in flux. This is because, as shown in Woodfield et al. (2019, Supplementary Information), the main phase of TAG deposition in oilseed rape follows an exponential curve, at least under greenhouse conditions. Fortescue and Turner (2007) have shown that the expansion of the embryo follows a sigmoidal logistic curve between around 10 to 35 days after flowering (DAF), contemporaneous with the main period of oil synthesis and deposition.

Given that the process is exponential, the rate of change of the mass M of TAG in the embryo is given by:

$$\frac{dM}{dt} = kM \tag{5}$$

where k is the specific rate constant of TAG deposition, and kM is the instantaneous flux to TAG. Fell (2018) showed that k is equivalent to J in terms of the MCA Eqs. (1)–(4) given above. The integrated form of Eq. (5) from the start of the exponential phase ($t = 0$) to the end of the exponential phase at $t = t$ is:

$$M_1 = M_0 \exp kt \tag{6}$$

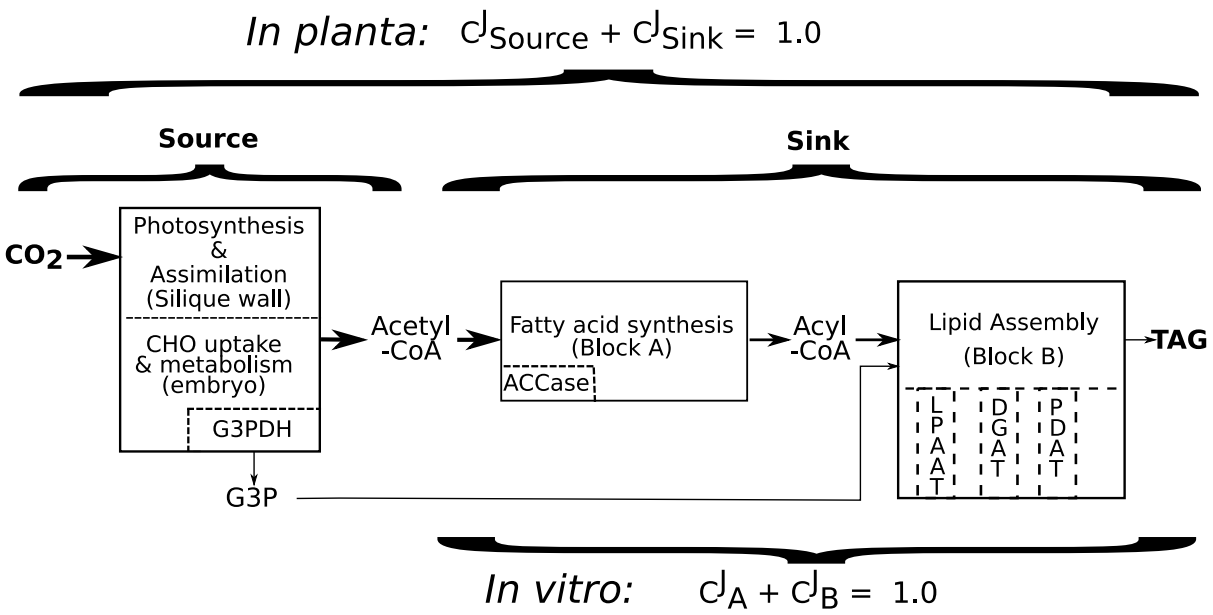

Fig. 1. The modular relationships between *in planta* MCA and the *in vitro* TDCA. Only those enzymes whose flux control coefficients are discussed here are shown within their appropriate module: G3PDH, glycerol-3-phosphate dehydrogenase (NAD^+); ACCase, acetyl-CoA carboxylase; LPAAT, lysophosphatidic acid acyltransferase; DGAT, diacylglycerol acyltransferase, and PDAT, phospholipid: diacylglycerol acyltransferase (which uses phosphatidylcholine as acyl donor). G3P: *sn*-glycerol 3-phosphate.

where M_0 is the weight of TAG in the embryo at the start and M_1 the final weight, closely approximated by the TAG content of the mature seed. M_0 was estimated as 0.034 mg per seed (Woodfield et al., 2019), which allows estimation of kt as $\ln M_1 - \ln M_0$. For an over-expressor with a flux that is f–fold higher, i.e:

$$M_2 = M_0 \exp f\,kt \tag{7}$$

$f\,kt$ can similarly be extracted, so essentially the final TAG weights in the control and over-expressor give f and this can be used in either Eq. (3) or Eq. (4) to calculate the flux control coefficient *in vivo* for the whole pathway from initial photosynthetic assimilation of the CO_2 used in embryo metabolism to TAG storage. Alternatively, given a known flux control coefficient, calculation of f from Eq. (4) for a degree of over-expression of r allows prediction of the expected increase in yield.

The relationship between such measurements and the results from the modular TDCA *in vitro* (described in the Introduction) is illustrated in Fig. 1. An important point is that the flux summation theorem of Kacser and Burns (1973) – that the sum of all the flux control coefficients of processes contributing to a pathway flux is 1.0 – spans different ranges in the two cases, and the embryo metabolism studied *in vitro* by TDCA is a subsystem of the whole plant metabolism. However, if the experimental environment of the embryos is sufficiently close to *in planta* conditions, so that the relative distribution of control within the embryos is unchanged, the flux control coefficients measured *in vitro* will be scaled down proportionally *in planta* so as to add up to that fraction of the control residing in the embryos from acetyl-CoA onwards (the 'sink'). In addition to the flow of acetyl-CoA from the embryo central metabolism block through Block A via acyl-CoA to Block B, there is a flow of *sn*-glycerol 3-phosphate (G3P) from the embryo block to the lipid assembly, Block B. Were the latter a large and independent flux, it would complicate the analysis presented here, but to the degree of accuracy we can achieve, it is unlikely to do so. One reason is that the fluxes are stoichiometrically coupled by joining together at Block B for TAG synthesis so they cannot vary independently to any great extent. Secondly, to make a molecule of the main oil constituent, triolein, one glycerol 3-phosphate is used for every twenty seven acetyl-CoAs (and, also, for every three oleoyl-CoAs). So its flux contribution is quantitatively less important.

Data points for some calculations reported herein were recovered from diagrams in published papers by digitisation using g3data (Frantz 2000) on Linux. Non-linear functions were fitted to data with Gnuplot.

Table 1
Over-expression of *B. napus DGAT1* in D1-2.20 relative to Westar controls. Data from Tables 3 & 4, pp. 49 and 51 of Weselake et al. (2005) for weights, and from Figs. 12 and 1a of Weselake et al. (2005, 2008) for DGAT activity.

Line	seed wt mg	% TAG	SE	TAG wt mg	DGAT pmol TAG $min.^{-1}$ mg^{-1} protein	SE
Westar 1	3.07					
Westar 2	2.62					
Westar 3	3.87					
Westar 4	3.12					
Mean	3.17	41.65	1.65	1.32	17.59	1.19
D1-2.20	3.51	47.25		1.66	77.95	5.21

3. Data analysis

3.1. DGAT results

Weselake et al. (2008) describe the over-expression of a *DGAT1* (gene form *BnaA.DGAT1.b*) in *B. napus* cv Westar and compared control plants with transformant line D1-2.20. Further details of these experiments are given in Weselake et al. (2005). Both sources (Fig. 1A and Fig. 12 respectively) show the increase in measured DGAT activity as 4.43 fold (numbers recovered from electronic versions of the figures, Table 1).

From the weight and oil analyses, the final TAG weights were 1.32 and 1.66 mg per seed for Westar and D1-2.20 respectively (a 26% relative increase). Using the calculation methods given in Section 2 yields a flux control coefficient *in planta* $C^{J_p}_{DGAT}$ of 0.076 for the Westar control, falling to 0.018 upon *DGAT1* over-expression in D1-2.20.

Taylor et al. (2009) reported expression of a *DGAT1* gene from *Arabidopsis thaliana* (Arabidopsis) under the control of a seed-specific *napin* promoter in the *B. napus* canola cultivar 'Quantum' developed at the University of Alberta. The data extracted from the paper on six transgenic lines is given in Table 2 and was used to calculate the change in TAG flux relative to the control line. Eq. (4) was then fitted to the flux and enzyme activities to give an *in planta* flux control coefficient of 0.036 ± 0.003 (SE), as shown in Fig. 2. This value of the coefficient is half that reported above. The difference might be due to variations

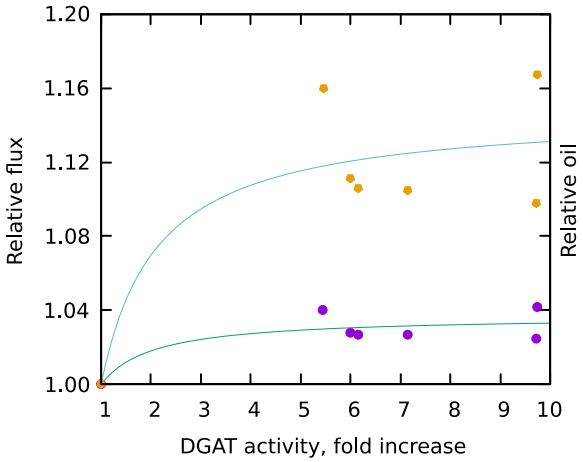

Fig. 2. Dependence of relative flux to TAG and relative TAG yield on DGAT1 activity. Data from Table 2. Relative flux, purple; relative yield, orange. The green curve is derived from non-linear fitting of Eq. (4) to the relative flux values, yielding a flux control coefficient of 0.036 ± 0.003 (SE). The blue curve for yield is calculated by inserting the fitted values of relative flux into Eq. (7).

Table 2
DGAT1 activity and flux to TAG in *B. napus* cv Quantum control (Q-con) and six transgenic *DGAT1* over-expressers. Data taken from Taylor et al. (2009, Figs. 2 and 3). TAG weight per seed was calculated based on a seed weight of 3.35 mg and an oil content of 43.75%.

Line	Relative DGAT activity, r	Relative oil yield, %	TAG weight, mg seed^{-1}	Relative flux, f
Q-con	1.0	100	1.47	1.000
7a1-1	5.45	116	1.70	1.040
9a-1-4	6.16	111	1.62	1.027
13a-2-4	6.00	111	1.63	1.028
17a-3-3	9.73	110	1.61	1.025
21a-2-3	9.75	117	1.71	1.042
23a-1-5	7.14	111	1.62	1.027

in control between different cultivars of *B. napus* or a consequence of using an Arabidopsis gene rather than a native one.

The other data reported on the Westar/D1-2.20 transgenic plants was a TDCA (with additional details of the Westar controls, but not the transgenics, in Tang et al. (2012)). The results were that the Block B (i.e. containing DGAT, Fig. 1) control coefficient, $C_B^{J_{embryo}}$ was 0.69 ± 0.02 in the controls, decreasing to 0.51 ± 0.05 in D1-2.20. These results cannot be directly compared with the *in planta* results because the coefficient refers to the whole of the TAG assembly block, containing other enzymes as well as DGAT, and is calculated for a subsection of the full pathway. However, the transfer of control of $0.69-0.51=0.18$ from Block B to A clearly reflects to some degree the drop in control exerted by DGAT upon 4.43-fold over-expression. Unfortunately, the relative flux to TAG in the embryo experiments for the controls and transgenics is no longer available so the DGAT control coefficient *in vitro* cannot be determined. It is possible to derive an estimate assuming that the relative change in flux would be the same as derived from the *in planta* experiments where the DGAT flux control coefficient fell by 0.057 from its initial value of 0.076. If we assume that the control coefficients in Blocks A and B are scaled-up versions of the control coefficients in the sink block of the plant (as in Fig. 1), then an approximate measure of the scaling factor is given by $0.18 : 0.057 = 1.0 : 0.319$. Applying this scaling factor to the *in planta* control coefficients for DGAT suggests that the minimum values for the control coefficients in the embryo experiments are 0.237 and 0.057 for the controls and transgenics respectively.

Why are the values for the control coefficients of DGAT *in vitro* termed minimum values? It is because the calculation assumed that the whole of the drop in the control coefficient upon *DGAT1* over-expression was reflected in a loss of control in Block B and a transfer to Block A. However, some of the control lost by DGAT could have been transferred to other enzymes in Block B, in which case the amount of control lost from DGAT could be greater than 0.18. If Block B were a simple linear pathway, some transfer of control to other enzymes in the block would be likely, but as it and other enzymes use acyl-CoAs as one of their substrates, and acyl-CoA is the common intermediate in the top-down experimental design, Block B has some characteristics of a set of parallel pathways, and there might not necessarily be strong cross-talk between them. Although we cannot take this calculation further immediately, analysis of other data (below) allows an estimate of the upper end of the range for these control coefficients.

For the same reasons as in the previous paragraph, the scaling factor of 0.319 between the *in vitro* and *in planta* control coefficients represents a maximum value. It also represents the fraction of the control in the sink, leaving a fraction of 0.68 as the control in the source. This distribution essentially corresponds to source *versus* sink strength for developing seeds, at least if 'source' is defined somewhat broadly as extending from photosynthetic generation of assimilate as far as the supply of acetyl-CoA and G3P to lipid metabolism of the embryos. Later we can examine how credible this figure is in relation to other estimates of source *vs*. sink strength for TAG accumulation. However, it also reflects the total amount of control available on TAG accumulation within embryo metabolism from acetyl-CoA onwards and puts an upper limit on the flux control coefficients of any other enzymes within this metabolic chain, bearing in mind that after the 0.076 assigned to DGAT, of the 0.319 flux control assigned to the sink, only 0.24 (= 0.319−0.076) remains to be accounted for. Nevertheless, it shows that *DGAT1* over-expression has not exhausted the potential scope to increase TAG by manipulation of other enzymes between acetyl-CoA and TAG.

As no equivalent TDCA experiments were performed on the Quantum transgenics, we cannot make equivalent calculations to determine whether the lower flux control coefficient in this cultivar represents a different distribution of control within the TAG assembly block or a lower fraction of control in the sink reactions as a whole.

It may seem surprising that the final enzyme in the Kennedy pathway should have some positive flux control, especially as it follows a relatively irreversible enzyme, phosphatidate phosphatase, that would tend to isolate it from kinetic influences from higher up the pathway. However, DGAT has been shown to be activated by the LPAAT product phosphatidate, thus creating a feed-forward link from LPAAT that bypasses phosphatidate phosphatase and that interacts with the strong positive cooperativity towards DGAT's second substrate acyl-CoA, which in turn sensitises it directly to the input into the lipid assembly block (Caldo et al., 2018).

3.2. LPAAT results

The *in planta* control coefficient of LPAAT was measured by over-expression of nasturtium (*Tropaeolum majus*) *LPAAT* in *B. napus* cv DH12075 (Woodfield et al., 2019). From the 24%–29% increase in TAG weight in the over-expressors relative to null segregants (i.e. siblings of the over-expressors not inheriting the transgene), we calculated the flux control coefficient of LPAAT as 0.14–0.17 in the null segregant controls, falling to 0.07–0.12 in the over-expressors. Using the scaling factor between the *in vitro* and *in planta* control coefficients derived above implies that the latter are, as minimum estimates, 0.49 and 0.30 *in vitro*, respectively, since the ratio could be larger in the plant to embryo direction. Adding the *in vitro* LPAAT (0.49) and DGAT (0.24) flux control coefficients together gives 0.73 for controls without any enzyme over-expression. Since the flux control coefficient measured for Block B in the control plants was 0.69 ± 0.02, it appears that LPAAT and DGAT between them account for the positive flux control within the block, unless there is some process within Block B that has negative control (meaning that its knockdown or attenuation would increase TAG deposition), in which case there could be some further compensating positive control to be found.

3.3. PDAT results

The result above implies that another acyltransferase in Block B, PDAT, would have little control over total TAG accumulation, which is consistent with studies in Arabidopsis (Mhaske et al., 2005) and *Camelina sativa* (Marmon et al., 2017) that suggested it made little contribution to total oil weight in those plant seeds, and the conclusions reached in Woodfield et al. (2018). Interestingly, PDAT has been shown to influence the amount of TAG in plants that incorporate larger proportions of hydroxy fatty acids (e.g. Kim et al., 2011). When we expressed Arabidopsis *PDAT1* sequence *At5g13640* in oilseed rape (Fenyk et al., 2022), we found that its *in planta* flux control coefficient was -0.028 ± 0.004 as over-expression of *PDAT1* decreased TAG deposition. This would scale, as above, to −0.088, which potentially (though uncertainly) allows for additional positive control in the TAG assembly block B, allbeit much less than accounted for by LPAAT and DGAT. Nevertheless, within experimental error, these estimates of the flux control coefficients for LPAAT and DGAT are not far from their feasible maximum values. In turn, that implies that the 1:0.32 ratio for the (source + sink):sink strength is a consistent estimate.

3.4. Glycerol-3-phosphate dehydrogenase and acetyl-CoA carboxylase

Vigeolas and Geigenberger (2004) showed that increasing the G3P supply in oilseed rape embryos enhanced the rate of formation of TAG. They followed this up by over-expressing yeast *GPD1*, a glycerol-3-phosphate dehydrogenase (NAD^+) (G3PDH), in the embryos of *B. napus* (cv Westar) (Vigeolas et al., 2007). Three separate over-expressing transformants were studied, and the final fatty acid content per seed and the enzymic activities were reported. Analysis of the reported data for the T4 generation plants, using Eqs. (6), (7) and the finite difference relationship Eq. (3), gave a mean *in planta* flux control coefficient for the wild type of 0.16 ± 0.01 (SE). A second set of data for the T3 plants in the supplementary information of Vigeolas et al. (2007) gave a control coefficient of 0.27 ± 0.02. The difference is accounted for by higher relative enzyme production in the T4 plants (2.1 fold) compared to the T3 plants (1.49), as the final fatty acid contents per seed reported, and hence the fold increase in rate of deposition, were comparable. As the cause of the divergence is unknown, it is safer to say the flux control coefficient of G3PDH is about 0.21 with a range ± 0.05. Note that as this is a BUCA estimate of the *in planta* coefficient, it is independent of the block structure and flux pattern used for the TDCA.

The T4 transformants also showed a smaller fold increase of 1.5 in ACCase activity over wild-type. This however, is unlikely to make a significant difference to the calculation. Roesler et al. (1997) over-expressed a cDNA encoding a homomeric ACCase from Arabidopsis in the plastids of *B. napus* embryos giving an average 7.6-fold increase in measured activity but only a 6% increase in fatty acid content per seed compared with the 34% increase generated by the 2.1-fold increase of G3PDH. The figures for ACCase, inserted in Eq. (3), yield a flux control coefficient of 0.03. There is substantial uncertainty in this value as the 8 different transformant lines had widely varying ACCase activities, but there was no consistent relationship between the measured activities and the final yield of fatty acid, other than that the transformants were all higher than the controls. The authors attribute the variation to the difficulty of reliably assaying the native plastid ACCase activity, and suggest the fold increase might be as low as 2, though this still only gives a flux control coefficient of 0.05. Small though the flux control coefficient is, it is likely to be an over-estimate as the Arabidopsis homomeric enzyme is not feedback-inhibited as the plastid enzyme is Andre et al. (2012). As originally demonstrated by Kacser and Burns (1973), feedback inhibition on an enzyme lowers its flux control coefficient relative to the value without the inhibition, so the native heterologous enzyme is likely to exhibit a lower control coefficient.

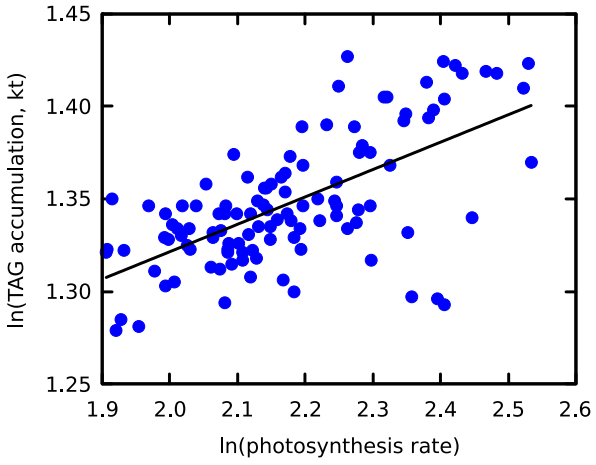

Fig. 3. Flux control coefficient determination for silique wall photosynthetic rate on rate of oil accumulation. Data taken from (Hua et al., 2012, Fig. 4, and supplementary material). The flux control coefficient is the slope of the best-fit line (shown): 0.15 ± 0.02.

3.5. Source:sink strength data

A number of lines of evidence suggest that photosynthesis in the silique wall is the major source of assimilate for the oil deposition phase of seed development in *B. napus*, such as the limited impact of phloem girdling and leaf detachment on oil content compared to severe reduction caused by silique darkening (Hua et al., 2012; Tan et al., 2015). Ruuska et al. (2004) demonstrated that there was sufficient photosynthetic capacity in the green seeds themselves potentially to refix a significant fraction of the CO_2 evolved during the formation of acetyl-CoA, but this represents prevention of carbon loss rather than net photosynthetic gain. Hua et al. (2012) also showed that two rapeseed lines of differing oil content had different ribulose-bisphosphate carboxylase (Rubisco) contents and photosynthetic activity in the silique wall, but no differences in the leaves. They examined the photosynthetic activity in the silique walls of 112 progeny of crosses between the two lines, as well as the resulting percentage oil content and showed a significant positive correlation between the measurements. Such use of genetic variation of enzymic activity on metabolic flux is one of the classic techniques for determination of flux control coefficients (Fell, 1992, 1997). By digitising the graph of these results (Hua et al., 2012, Fig. 4d) and converting the percent oil to a final oil weight using a mean weight for the seeds, the exponential rate constants for oil deposition in the progeny can be computed as described in Methods. (The seed weights of the parent lines are given in their Fig. 2d and only differ by 4%.) Plotting the log of the rate constant against the rate of silique wall photosynthesis and fitting a line (Fig. 3) gives an estimate of its flux control coefficient on oil deposition: 0.15 ± 0.02.

In Hua et al. (2012, Supplementary Table 5), values were given for silique wall Rubisco activity and final seed oil for the two parent lines of differing oil content and eight crosses. Treating these results in the same way as above gives an estimate for the flux control coefficient of Rubisco on TAG deposition rate of 0.10 ± 0.02 (Fig. 4).

Though it is encouraging that these different experiments pointed to a flux control coefficient of 0.10 to 0.15 for photosynthesis on oil accumulation, the precise assignment of the control coefficient to underlying cellular processes is difficult. A comparison of the transcriptome of the silique walls of the two parent lines showed that the major areas of photosynthesis, Calvin cycle and carbohydrate metabolism were up-regulated in parallel in the more active parent. It therefore seems likely that the flux control coefficients represent group control coefficients that may extend beyond the light reactions and Calvin cycle to include some contribution from metabolism beyond (such as sucrose

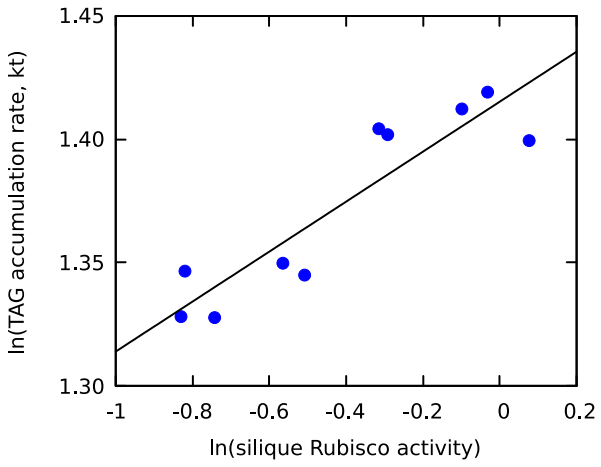

Fig. 4. Estimate of the flux control coefficient of silique wall Rubisco on the rate of oil deposition. Data taken from Hua et al. (2012, Supplementary Table 5) for measured Rubisco activities in high and low oil-yielding lines and crosses between them. The flux control coefficient is the slope of the best-fit line (shown): 0.10 ± 0.02.

synthesis and export), and as such represent the control coefficient of the source as physiologically defined. However, the calculated flux control coefficients for photosynthetic rate and Rubisco activity may not fully subsume the control coefficients of the other processes, so there may be some additional control to assign. The 'source block' shown in Fig. 1 extends beyond this to include phloem unloading by the embryos and carbohydrate metabolism to generate acetyl-CoA. If the total control by the source block is 0.68, as deduced in Section 3.1, then possibly as much as 0.5 of the control of flux could reside within these parts of embryo metabolism.

4. Discussion

4.1. Distribution of control of oil accumulation

The results presented above have exploited experiments on various cultivars of oilseed rape carried out by many independent research groups around the world who would not have planned the studies in anticipation of their being subject to the MCA methodologies applied to them here. Nevertheless, together they can be assembled into an overall MCA perspective on the distribution of the control of TAG accumulation in oilseed rape, with the understanding that the outcome is indicative rather than precise.

The results are summarised in the flux control coefficient values shown in Fig. 5. It can be seen that control is distributed in small increments over the whole length of the pathway. The only possible place for a significant element of control to be found is in embryo metabolism supplying acetyl-CoA, G3P, ATP and NADPH to fatty acid synthesis and TAG assembly. It is inherent in the modular top-down approach that the details of this internal network are hidden. For example, metabolic flux analysis of oilseed rape embryos showed that the oxidative pentose phosphate pathway supplied at most 44% of the NADPH requirement for TAG synthesis (Schwender et al., 2003), though this was subsequently shown to be somewhat plastic in cultivars with different TAG yields (Schwender et al., 2015). Given the demonstrated capacity for photosynthesis in the embryos (Ruuska et al., 2004), this presumably supplies the balance of the NADPH, the contribution from malate metabolism appearing very minor (Schwender et al., 2015), unlike some other seeds. These multiple routes to NADPH will make it difficult to explore the flux control contribution of their enzymes on TAG accumulation. Nevertheless, even though we lack experiment results to attribute the control distribution within this module's enzymes, apart from G3PDH, the total control available (around 0.5, less the 0.2 attributed to G3DPH) limits the likelihood that there is a step possessing highly significant control. This, in itself, is no longer a surprising result; ever since the development of MCA established the principle that the flux in a metabolic pathway is potentially influenced by all the steps as a systemic property, experiments have demonstrated distributed control in many pathways (see Fell (1997) for examples) and there are few instances where a single enzyme has a major fraction of the flux control.

However, there is potentially an additional reason why control over oil accumulation is broadly distributed in oilseed rape. The development of MCA has always been linked to theories of the genetics and evolution of metabolism. One area of investigation has been the consequences of evolutionary selection for maximisation of metabolic flux given a fixed level of investment in enzyme protein for a pathway, or equivalently, maintaining a desired level of flux whilst minimising the amount of enzyme protein committed. Evidently, for a non-optimised pathway, a way to obtain an increase in flux at constant total protein would be to reduce the protein level of an enzyme with a near-zero flux control coefficient. This would have a negligible impact on the flux, and would allow that protein to be re-assigned to increase the amount (and activity) of the enzyme with the largest flux control coefficient, thereby increasing the pathway flux. The inevitable consequence of increasing the amount of an enzyme with a large flux control coefficient is that its flux control coefficient will decrease. Hence, in the next round of optimisation, an equivalent redistribution will have less effect. Furthermore, the control lost by this enzyme is redistributed over other enzymes in the pathway, including the ones that originally had the lowest control. It can be shown that this results in the numerical spread of the control coefficients becoming smaller and the enzyme distribution approaching an optimum (Hartl et al., 1985; Brown, 1991; Klipp and Heinrich, 1999). Differences in kinetic properties of the enzymes and the equilibrium constants of the different reactions prevent reaching a state where all the control coefficients are identical, but the variance in their values is minimised at the optimum.

The significance for oilseed rape of this tendency towards distributing the control of flux is that it is an agricultural crop plant that has recently been, and still is, subject to evolution by artificial selection for high oil yield (amongst other properties). We have shown that high oil yield is a reflection of increased flux in lipid synthesis, so the selection has been acting directly on pathway flux. In fact, as the response of the oil yield with respect to variation in an enzyme activity is the enzymes' flux control coefficient times kt (Fell, 2018), and the value of kt for oilseed rape is typically 3.6–3.8 (Woodfield et al., 2019, Supplementary information), selection for yield is more intense than selection for flux to oil. Hence it is likely that the very distributed control of this flux has been accentuated by the selection process, and that a related plant that has not been subjected to selection for oil yield in its seeds, such as Arabidopsis, may have a different distribution of control that represents the outcome of selection for a number of different objectives. We have already mentioned (Section 3.5) the results of Hua et al. (2012) showing that enzyme activities differed between oilseed rape cultivars of low and high oil yield.

Further evidence of the metabolic effects of selection for yield has been obtained by assaying enzyme activities in embryos derived from two near isogenic lines that had 36% and 46% seed oil content (Li et al., 2006). Three enzymes, plastidial pyruvate kinase, sucrose synthase and ATP citrate lyase were found to have significantly higher activity in the higher yielding line, whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aldolase and invertase were unchanged. Schwender et al. (2015) assayed a panel of 26 enzyme activities in nine different cultivars, seven of which came from a panel of 63 accessions varying in embryo biomass composition and growth rate, as well as a transgenic expressing Arabidopsis *DGAT1* and its reference control. Three of the enzymes were in common with those measured by Li et al. (2006) – plastidial pyruvate kinase, GAPDH and aldolase – and the results coincided, with the first correlating significantly with

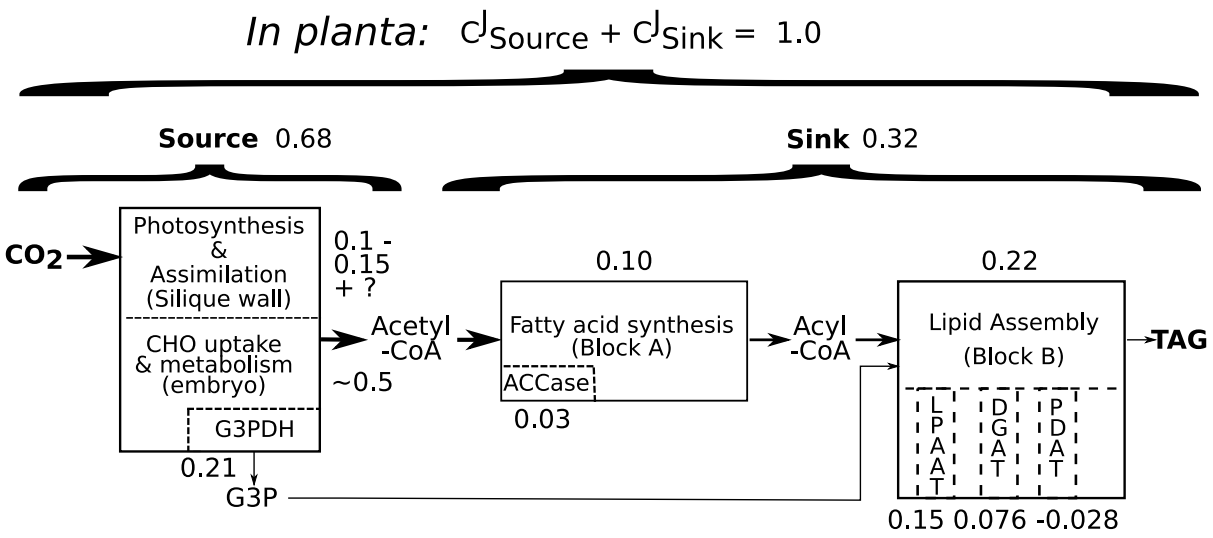

Fig. 5. The assignment of control of TAG accumulation *in planta*. The estimated flux control coefficient values presented in the text are superimposed on Fig. 1.

lipid content and the other two not. Schwender et al. (2015) also identified another four enzyme activities with significant correlations to lipid content: pyrophosphate-dependent phosphofructokinase, phospho*enol*pyruvate carboxylase, phospho*enol*pyruvate carboxykinase and aspartate aminotransferase. On the assumption that the seven enzymes showing correlations with oil yield across the two studies are those most responsive to selection, then we have potential candidates that between them could account for the unattributed control in embryo metabolism from sucrose uptake to acetyl-CoA, but as this totals about 0.3 after allowing for the control assigned to G3PDH, they may each have flux control coefficients of only 0.1 or less. Note that none of these additional candidates ostensibly points to changes in NADPH production, unless the phospho*enol*pyruvate metabolising enzymes are pointing to an increased involvement of malate metabolism (*c.f.* this section, above).

4.2. Joint over-expression

Low values of an enzyme's flux control coefficient place a limit on the flux increase that can be obtained even by high degrees of over-expression of its activity. Qualitatively, this can be seen because the low value indicates that the typically hyperbolic response of the flux to enzyme activity is already beginning to level off. Quantitatively it is expressed by the fold-change equation, Eq. (4): given a flux control coefficient of 0.2, even for a relative increase in activity, r, that is large enough for $(r-1)/r$ to approach 1, the maximum fold change in flux, f, is limited to 1.25. Within MCA, strategies have been considered that could deliver larger flux increases. Small and Kacser (1993) showed, in the derivation of Eq. (4), that it could also predict the effects of changing several enzymes simultaneously in a linear pathway. If the enzyme activities were all changed by the same factor, then the sum of their control coefficients could be used in the equation. A modified version allowed for different degrees of amplification within the group. However, an important conclusion was that the total effect of amplifying the group was always greater than might be expected from either adding or multiplying the flux changes obtained with each enzyme in isolation. Such synergism was shown experimentally in a five enzyme segment of the tryptophan synthesis pathway in yeast (Niederberger et al., 1992). Simultaneous over-expression of up to three of the genes gave up to double the flux, but all five together gave an 8 to 9-fold increase.

Synergism between enzymes has been observed for stimulation of TAG accumulation in oilseed rape. Liu et al. (2015) over-expressed *GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE*, (*GPAT*), *LPAAT*, *DGAT*, and *G3PDH* in *B. napus* ZS6 and got a greater increase in oil yield (relative increases of 12.6–14.5% over wild type) than for each gene over-expressed individually, but gave no measurements of enzyme activity. However, their yield increase is less than obtained with single over-expression of either *DGAT1* or *LPAAT* alone in the experiments reviewed in Sections 3.1 and 3.2. Using a relative yield increase of 1.135, application of Eqs. (6) and (7) suggest that a relative flux increase, f, of 1.035 would suffice to account for their results. The sum of the *in planta* control coefficients for LPAAT and DGAT are 0.23 (= 0.15 + 0.076), and, according to Eq. (4), an over-expression of these two enzymes each by a factor of 1.17 would achieve the flux change reported by Liu et al. (2015) as can be seen in Fig. 6. However, this calculation of the synergy between LPAAT and DGAT manipulation may be uncertain as the kinetic interactions between the two enzymes and their metabolites, mentioned in Section 3.1 (e.g. the LPAAT product phosphatidate, acting as a positive allosteric regulator of DGAT), are more complex than assumed in the derivation of the equations by Small and Kacser (1993).

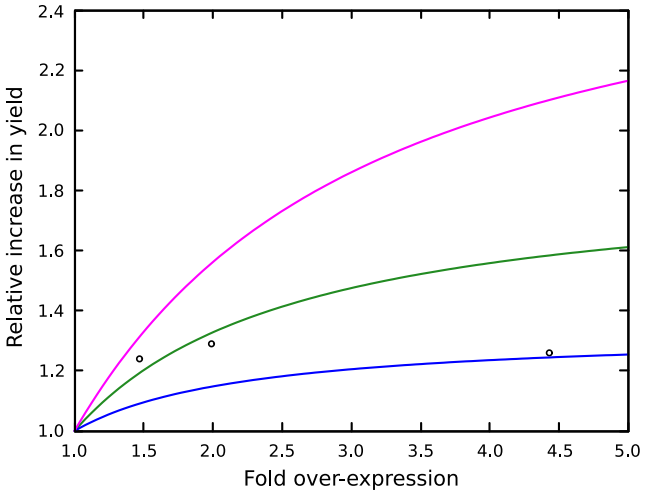

Fig. 6. Predicted relative increase in yield for joint over-expression of LPAAT and DGAT enzymes. The upper curve is for the two enzymes, with the curve below for LPAAT alone and the lowest curve for DGAT alone. The curves were calculated using Eqs. (4) and (7) using an average value of k_I of 3.5 and control coefficients for LPAAT and DGAT of 0.15 and 0.076 respectively. The circles mark the experimental yield/over-expression values for LPAAT and DGAT reported in Sections 3.2 and 3.1.

There is another potential benefit of metabolic engineering designs that involve over-production of multiple enzymes. Over-producing a single enzyme to increase flux is likely to have much larger impacts (positive and negative) on metabolite concentrations (Fell, 1997), which can have unwanted impacts if the metabolites are effectors of other pathways or are toxic. However, in a linear pathway, if all the enzymes are increased in activity by the same factor, the flux will increase by that factor but the intermediate metabolites will remain at the same concentration (Kacser and Burns, 1973). In a complex cellular metabolic network, it is, in principle, possible to design a set of over-productions by specific degrees that confine the flux changes to the targeted part of metabolism leaving fluxes elsewhere and the metabolite concentrations unchanged (Kacser and Acerenza, 1993).

The practical difficulties of implementing this, through accumulation of the required set of precise genetic changes in a single organism, are enormous, as can be seen in the experiments of Liu et al. (2015) cited above. On the other hand, cells and organisms achieve similar outcomes through structures and mechanisms that implement *multisite modulation* (Fell and Thomas, 1995; Fell, 1997), which include operons and regulons as means for coordinating the expression of sets of genes for particular metabolic functions. Hence a different approach might be to exploit an organism's existing mechanisms of control and regulation rather than trying to over-ride them. There are examples of this in the engineering of increased TAG accumulation (see below).

Focks and Benning (1998) reported a mutation in Arabidopsis, *wrinkled1*, that led to greatly reduced oil content in seeds and reduced glycolytic activity. The mutation was then found to affect gene expression, particularly in glycolysis and fatty acid synthesis (Ruuska et al., 2002). Cernac and Benning (2004) identified the *WRI1* locus and gene sequence, which included DNA binding domains typical of plant transcription factors. Expression of *WRI1* restored TAG accumulation in seeds of the *wri1* mutant and increased it in wild-type Arabidopsis. Homologues of *WRI1* have been found in other plants, including oilseed rape (Li et al., 2015b). Over-expression of *BnWRI1* in *B. napus* cv. Westar increased TAG synthesis in seeds and leaves and the transcription of genes in glycolysis, fatty acid synthesis and *DGAT1*, and, to a lesser extent *GPAT9* and *LPAT2*, of the genes measured (Li et al., 2015b). This shows parallel up-regulation of enzymes in three of the blocks having some control over TAG accumulation as shown in Fig. 5, but it is not possible to pursue any further quantitative analysis within an MCA framework as no enzyme activity measurements were reported.

Further evidence of the synergistic effects of multiple gene overexpression has been given by Van Erp et al. (2014) who over-expressed *WRI1* and *DGAT1* in Arabidopsis and suppressed the expression of the *SUGARDEPENDENT1* gene (which encodes a triacylglycerol lipase involved in TAG turnover) via RNA interference. Plants expressing all three constructs had significantly greater oil content than wild-type, or plants expressing the genes singly. Similarly, Vanhercke et al. (2013) transiently co-expressed Arabidopsis *WRI1* and *DGAT1* in *Nicotiana benthamiana* leaves and observed a 22-fold increase over the controls in their TAG content, which was over two-fold higher than the sum of the individual gene effects.

Overall, it seems that multiple gene over-expression could increase the seed TAG content of oilseed rape by a greater amount than any single gene manipulation has achieved. A significant difficulty in achieving a rational design, however, is a lack of sufficient quantitative data on the distribution of control and the baseline enzyme activities.

5. Conclusions

In addition to the two previously measured flux control coefficients on TAG deposition in oilseed rape, LPAAT and PDAT (Woodfield et al., 2019; Fenyk et al., 2022), we have been able to infer estimates for those of silique wall Rubisco with associated photosynthetic metabolism, G3PDH, ACCase and DGAT. Furthermore, by comparison of the results from TDCA of isolated embryo metabolism and the *in planta* control coefficients of LPAAT and DGAT, we can infer an approximate distribution of control between three blocks of metabolism: silique wall photosynthetic assimilation with seed embryo uptake of photosynthate and central carbon metabolism; fatty acid synthesis from acetyl-CoA in the embryos, and assembly of G3P and acyl-CoAs into TAG. Given the summation theorem (Kacser and Burns, 1973), that the sum of the *in planta* flux control coefficients is 1.0, this allowed us to conclude that the control is distributed throughout these three blocks. None of the control coefficients we have measured or estimated are large enough to indicate a dominant controlling step. Further, the control that we have accounted for leaves relatively little to be assigned to the steps that have not yet been measured. For example, in the first large, source block, photosynthetic assimilation and G3PDH appear to account for around half of the 0.68 share of control attributed to it. The fatty acid synthesis block as a whole has about 0.1 of the control which is currently unassigned, except that ACCase is unlikely to account for it. In the TAG assembly block, LPAAT and DGAT between them seem to account for the control assigned to it, leaving essentially nothing for the other two enzymes. The largest amount of unassigned control, around 0.35, is in the embryo's assimilate uptake and central carbon metabolism, which encompasses many enzymes and transporters. The most likely candidates for possessing some of this control are the enzymes identified by Li et al. (2006) and Schwender et al. (2015) as positively correlated with higher oil yields and the encoded enzymes whose transcription is promoted by *WRI1*.

The finite change theory (Small and Kacser, 1993) implies that the increase in flux to TAG cannot be increased by a large factor even by substantial over-expression of any of the enzymes whose control coefficients we have measured. However, the theory also shows that joint over-expression of a number of enzymes can have synergistic effects, as we have illustrated in Fig. 6. From our results, a combination of G3PDH, LPAAT and DGAT would be suitable candidates. In fact, these were included in the study by Liu et al. (2015) and did show synergy, though the improvement in yield was not as great as we observed with LPAAT alone (Woodfield et al., 2019). However, it is not known from the experiments whether the enzyme activities were increased sufficiently. Along with these three enzymes, candidates from central embryo carbon metabolism need to be identified. The methods outlined in this paper could be applied to measure the flux control coefficients of the enzymes whose activity correlates with high yield, though an alternative would be to over-express *WRI1* as that acts on several targets in the block and would involve just one additional over-expression.

CRediT authorship contribution statement

David A. Fell: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **David C. Taylor:** Writing – review & editing. **Randall J. Weselake:** Writing – review & editing. **John L. Harwood:** Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.biosystems.2023.104905.

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