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Changes in Kennedy pathway intermediates associated with increased triacylglycerol synthesis in oil-seed rape

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Abstract

The rates of triacylglycerol synthesis in maturing oil-seed rape (*Brassica napus* cv Shiralee) were manipulated by light/dark treatments. Under conditions of high lipid accumulation the amounts of the Kennedy pathway intermediates, phosphatidate and particularly, diacylglycerol were increased significantly. At the same time there were no significant changes in the activities of the four pathway enzymes, of which diacylglycerol acyltransferase had the lowest detectable activity. The alteration in carbon flux was accompanied by some changes in the acyl quantity of the diacylglycerol pool but not in that of other intermediates. The results provide additional evidence for our proposal that diacylglycerol acyltransferase can exert significant flux control at times of high lipid accumulation in oil-seed rape.

Keywords: Oil-seed rape; Kennedy pathway; Oil accumulation; Flux control; Diacylglycerol acyltransferase

1. Introduction

The production of storage oils by plants is of great importance for the agricultural industry (Harwood, 1998; Murphy, 1994). Such oils are used not only for edible purposes but can also act as raw materials for chemical processes (Murphy, 1994) as lubricants and for other industrial purposes (Luhs & Friedt, 1994). World production of plant oils and fats amounts to over 70 million tonnes (Gunstone, Harwood & Padley, 1994) and there is considerable interest in the possible manipulation of plants either to increase production or to alter the quantity of the accumulated triacylglycerols (Harwood, 1998; Murphy, 1994; Ohlrogge, 1994). Indeed, some transgenic crops with altered storage lipid patterns have been produced already (e.g. Kinney, 1998; Knutzon & Knauf, 1998).

Plant triacylglycerols are produced by the classic Kennedy pathway (Harwood & Page, 1994) which involves two acylations of glycerol 3-phosphate to produce phosphatidate, a dephosphorylation and a final acylation of diacylglycerol. The individual enzymes have been examined in various tissues and some partial purifications reported (see Harwood, 1998; Stobart, Stymne, Shewry & Napier, 1998). The substrate selectivity of the acyltransferases has been studied in a number of cases and shown to play a strong influence in the quality of the triacylglycerols accumulated (e.g. Frentzen & Wolter, 1998; Knutzon & Knauf, 1998). In contrast, little is known about the regulation of carbon flux down the pathway (Harwood et al., 1999; Ohlrogge & Jaworski, 1997). Clearly, before attempting to produce transgenic plants with a greater capacity to accumulate oils, it is desirable to identify any possible constraints on the overall biosynthetic rates.

We have been studying oil-seed rape, which is the most important oil crop in Northern Europe. It con-

Abbreviations: G3PAT: glycerol 3-phosphate acyltransferase; LPAAT: lysophosphatidate (1-acyl glycerol 3-phosphate) acyltransferase; PAPase: phosphatidate phosphohydrolase; DAGAT: diacylglycerol acyltransferase.

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Table 1
Endogenous levels of Kennedy pathway intermediates in oil-seed rape embryos exposed to light and dark conditions[a]

	mg/g fresh wt.			
	Glycerol 3-phosphate (G3-P)	Lysophosphatidate (lyso PA)	Phosphatidate (PA)	Diacylglycerol (DAG)
Dark	0.0215	0.34 ± 0.06	0.66 ± 0.04	3.4 ± 0.2
Light	0.0387	0.27 ± 0.09	$0.81 \pm 0.02^*$	$4.0 \pm 0.1^*$

[a] Data as means $\pm$ SD ($n = 3$) except for G3-P which was analysed by NMR. Statistical significance by Student's t-test: $^* = P < 0.01$.

tributes about 15% of the total world production of fats and oils (Gunstone et al., 1994; Harwood, 1998). From the accumulation of Kennedy pathway intermediates (Perry, Bligny, Gait, Douce & Harwood, 1992) and various radio-labelling experiments, we suggested that diacylglycerol acyltransferase (DAGAT) could exhibit significant flux control at times of high lipid production (Perry & Harwood, 1993a, 1993b). Extra evidence in favour of this proposal was obtained with EMS — mutated *Arabidopsis* where lowering of DAGAT activity was accompanied by less oil accumulation (Katavic et al., 1995). We now report the results of experiments in which we deliberately changed the carbon flux towards triacylglycerols. The data, which have been partly reported in a preliminary form (Perry et al., 1992; Perry & Harwood, 1994), show that DAGAT and, possibly, phosphatidate phosphohydrolase may limit triacylglycerol formation when lipid accumulation rates are high.

2. Results and discussion

It is well-known that light stimulates fatty acid biosynthesis in plants (Harwood, 1996; Ohlrogge & Jaworski, 1997) and we used this environmental factor in order to manipulate the overall rate of lipid synthesis in oilseed rape embryos. If embryos were prelabelled with [1–^{14}C]acetate (which we have found previously to be a good precursor of total lipids) (Perry & Harwood, 1993a) and then incubated further in the light or dark, we found that total lipid labelling in the light was an average $127 \pm 5\%$ of that in the dark for three experiments (data not shown). Therefore, we had manipulated the flux through the Kennedy pathway and could use our test system in order to examine any possible changes for the individual enzymatic steps.

2.1. Changes in intermediates as lipid synthesis is altered

The endogenous levels of Kennedy pathway intermediates were measured and the results are shown in Table 1. The overall pool sizes for both dark- or light-treated embryos increased down the Kennedy pathway with glycerol 3-phosphate being the smallest and diacylglycerol the largest. When flux through the pathway was elevated by light-treatment there were increases in the pool sizes for all intermediates except lysoPA. The increases in phosphatidate and diacylglycerol were statistically significant. Because glycerol 3-phosphate was analysed by NMR we only had a single value and, therefore, could do no statistics. However, the glycerol 3-phosphate pool size appeared to be increased markedly.

In view of their sizes, perhaps the most significant changes in intermediate pool sizes were the increases in phosphatidate and, especially, in diacylglycerol. A general conclusion for flux change experiments is that, when an intermediate increases, it points to a significant measure of control by the enzyme utilizing that intermediate (see Ohlrogge & Jaworski, 1997). Thus the increase in diacylglycerol pointed to an important role for DAGAT in oilseed rape, as proposed pre-

Table 2
Distribution of glycerol 3-phosphate acyltransferase (G3PAT) and phosphatidate phosphohydrolase (PAPase) activities between membrane and soluble fractions[a]

	Specific activity (nmol/min/mg protein)		
	Post-mitochondrial supernatant	Microsomal fraction	Particle-free supernatant
G3PAT	0.34 ± 0.02	1.80 ± 0.11	nd
	(11.2 ± 0.5)	(8.4 ± 0.4)	nd
PAPase	2.0 ± 0.4	2.2 ± 0.2	1.8 ± 0.2
	(79.1 ± 6.3)	(13.3 ± 1.4)	(64.2 ± 8.2)

[a] Means $\pm$ SD ($n = 3$). nd = none detected. Figures in brackets show total activity (nmol/min/g fresh wt. embryo).

Table 3
Effect of light exposure of rape seed embryos on the activities of Kennedy pathway enzymes in the microsomal fraction[a]

Enzyme	Specific activity (nmol/min/mg protein)		Total activity[b] (nmol/min/g FWt)	
	Dark	Light	Dark	Light
Glycerol 3-phosphate acyltransferase (G3PAT)	1.4 ± 0.1	1.6 ± 0.1	10.6 ± 0.7	10.0 ± 0.5
Lysophosphatidate acyltransferase (LPAAT)	18.6 ± 5.0	16.9 ± 0.5	104.1 ± 27.9	95.0 ± 2.8
Phosphatidate phosphohydrolase (PAPase)	2.2 ± 0.2	2.2 ± 0.2	71.7 ± 4.1[b]	77.5 ± 5.9[b]
Diacylglycerol acyltransferase (DAGAT)	0.43 ± 0.04	0.53 ± 0.20*	3.5 ± 0.3	3.8 ± 0.2

[a] Means $\pm$ SD ($n = 3$). Statistical significance by Student's t-test: $^*P < 0.02$.
[b] Total activity for all enzymes was estimated from values in the microsomal and particle-free supernatant but only PAPase had detectable activity in the latter. The relative distribution of this enzyme between the two fractions was not affected by light treatment.

viously by us (Perry & Harwood, 1993a, 1993b) and confirmed by others (Katavic et al., 1995). The rather small size of the glycerol 3-phosphate pool in comparison to the other intermediates suggests that availability of this substrate could exert a limit to the overall rate of triacylglycerol accumulation. Given the K_m for another oil-rich tissue's G3PAT, that from avocado (Eccleston & Harwood, 1995), glycerol 3-phosphate is probably used as quickly as it is formed in oilseed rape and this would account for its low level (Table 1).

2.2. Kennedy pathway enzyme measurements

In order to see whether enzyme activities in the oil-seed rape embryos correlated with labelling patterns of intermediates (Perry & Harwood, 1993a, 1993b), and also, whether there was further evidence that DAGAT could be important for flux control, we assayed the four enzymes of the Kennedy pathway. While LPAAT and DAGAT are known to be endoplasmic reticulum-localised (Harwood & Page, 1994) there are reports in some tissues that G3PAT and PAPase can also be soluble (e.g. Ichihara, Murota & Fujii, 1990). Therefore, we examined the distribution of these two enzymes in subcellular fractions from embryos (Table 2). For G3PAT, assayed under optimal conditions, we could detect activity in the microsomal fractions but not in the soluble fraction. Recovery of activity from the post-mitochondrial supernatant was 75% (see Table 2), which given the unstable nature of G3PAT activity (see Eccleston & Harwood, 1995), was reasonable and indicated that in oil-seed rape this enzyme was associated with microsomal membranes — most probably the endoplasmic reticulum. For PAPase, activity was distributed between the microsomal and soluble fractions. In fact, given the size of the latter, most of the total recovered activity (83%) was found there. However, the specific activity of PAPase in the two fractions was rather similar (Table 2). These results show that, in oil-seed rape as in safflower (Ichihara et al., 1990), PAPase has a dual localisation in cells – as has also been reported for animals (see Brindley, 1988).

We then assayed the four enzymes of the Kennedy pathway in fractions prepared from embryos that had been given light or dark treatments. The results (Table 3) show firstly, that LPAAT had by far the highest activity and that DAGAT was the lowest. Secondly, on exposure to light there were no significant changes in total enzyme activity on a fresh weight (i.e. embryo) basis. However, there was a small increase in the specific activity of DAGAT, which presumably reflected a qualitative change in the microsomal membranes.

Because the enzymes were assayed under optimal conditions in vitro, which do not necessarily reflet their activity in vivo, one has to be cautious in interpreting the results. However, the high relative activity of LPAAT agrees not only with the general consensus that this enzyme has much higher activity than other acyltransferases (Frentzen & Wolter, 1998; Harwood & Page, 1994; Knutzon & Knauf, 1998) but also with the lack of accumulation of endogenous lysophosphatidate in vivo (Harwood & Page, 1994; Table 1) or radioactivity in this intermediate in vitro (Perry & Harwood, 1993a, 1993b). In contrast, the lowest activity was found for DAGAT in keeping with diacylglycerol being the largest endogenous pool size. Furthermore, the increase in diacylglycerol on incubating embryos in the light (Table 1) was not due to any decrease in the total activity of DAGAT (Table 3). These results add further support to the proposal that DAGAT can exert significant flux control at times of high lipid accumulation in oil-seed rape (Perry & Harwood, 1993a, 1993b). Similar suggestions have been made for olive (Ramli, Quant & Harwood, 1998; Rutter, Sanchez & Harwood, 1997) but not for oil palm (Ramli et al., 1998).

2.3. Fatty acid patterns of intermediates

We also examined fatty acid patterns for the three intermediates (and total lipids) under the two incu-

Table 4
Comparison of the acyl compositions of lipids in oil-seed rape embryos after dark or light treatment[a]

		Percentage distribution					Total (mg/g FWt)					
		16:0	18:0	18:1	18:2	18:3	Others	16:0	18:0	18:1	18:2	18:3	Others	Total
Total lipids	Dark	6 ± tr	2 ± tr	61 ± 1	20 ± 1	10 ± 1	1 ± tr	10 ± tr	4 ± tr	111 ± 7	36 ± 2	19 ± 1	1 ± tr	181 ± 9
	Light	6 ± tr	3 ± tr	62 ± tr	19 ± tr	10 ± tr	tr	10 ± tr	5 ± tr	113 ± 8	36 ± 3	19 ± 2	1 ± tr	184 ± 13
LysoPA	Dark	29 ± 7	11 ± 1	17 ± 2	23 ± 2	21 ± 3	nd	0.10 ± 0.02	0.03 ± 0.01	0.06 ± 0.02	0.08 ± 0.02	0.07 ± 0.01	nd	0.34 ± 0.06
	Light	36 ± 14	15 ± 8	15 ± 7	18 ± 7	17 ± 7	nd	0.09 ± 0.01	0.04 ± 0.01	0.04 ± 0.03	0.05 ± 0.03	0.05 ± 0.03	nd	0.27 ± 0.09
PA	Dark	15 ± 3	4 ± 1	22 ± 2	27 ± 2	33 ± 3	nd	0.10 ± 0.02	0.02 ± 0.01	0.14 ± 0.01	0.18 ± 0.02	0.22 ± 0.02	nd	0.66 ± 0.04
	Light	16 ± 1	4 ± 1	20 ± 1	30 ± 1	31 ± 1	nd	0.13 ± 0.02	0.03 ± tr	+0.16 ± tr	*0.24 ± 0.02	0.25 ± 0.01	nd	**0.81 ± 0.02
DAG	Dark	6 ± tr	2 ± tr	45 ± 1	30 ± 1	16 ± tr	1 ± tr	0.21 ± 0.01	0.07 ± tr	1.53 ± 0.07	1.02 ± 0.02	0.53 ± 0.02	0.04 ± 0.01	3.40 ± 0.18
	Light	5 ± 1	2 ± tr	51 ± 1**	27 ± 1	13 ± tr**	1 ± tr	0.21 ± 0.04	0.08 ± tr	**2.07 ± 0.11	*1.09 ± 0.03	0.53 ± 0.01	0.05 ± tr	**4.03 ± 0.14

[a] Means ± SD ($n = 3$). Statistical significance by Student's t-test: $^{**}P < 0.01$, $^{*}P < 0.05$. tr = trace; nd = none detected.

bation conditions in order to evaluate any qualitative changes in their acyl composition (Table 4). The total lipids, as expected, were dominated by oleic acid which is the main constituent of rape seed oil (Gunstone et al., 1994). Naturally, during the short incubation period, there was no change in the overall lipid composition. By contrast, lysophosphatidate contained much higher levels of saturated fatty acids (palmitate, stearate) and α-linolenate. This composition reflected the fatty acid preference of G3PAT (Harwood & Page, 1994) and, also, the distribution of fatty acids in the molecular species of oil-seed rape triacylglycerol (Gunstone et al., 1994). There was no significant change in the fatty acid composition of lysophosphatidate between the two incubation conditions (Table 4). Compared to lysophosphatidate, phosphatidate contained higher levels of unsaturated fatty acids. This was expected partly because of the normal selectivity of LPAAT for such acyl-CoAs. However, in view of the high level of oleate in oil-seed rape triacylglycerol especially at the *sn*-2 position (Gunstone et al., 1994), it was surprising that phosphatidate did not contain very high levels. It may be that the phosphatidate pool showed enrichment in those molecular species that were poorly utilised further for triacylglycerol formation. Again, there were no changes in the phosphatidate pool quality induced by light incubation (Table 4).

On the other hand, the diacylglycerol pool showed some changes induced by light incubations with an increase in oleate percentage at the expense of the polyunsaturates, linoleate and α-linolenate (Table 4). This could be explained by a preferential channelling of diacylglycerol into storage (rather than membrane) lipid formation so that the high oleate level of triacylglycerol was reflected in the diacylglycerol pool.

For all three intermediates of the Kennedy pathway, pool sizes were relatively small compared with the total lipid in embryos at this stage of development. Even for diacylglycerol, the largest intermediate pool, the level was only about 2% of the total lipid level (Table 4). However, in terms of the amount of triacylglycerol synthesised during the period of incubation, the pool sizes would be relatively large.

2.4. Summary remarks

The data reported here suggest that DAGAT is the most likely enzyme of the Kennedy pathway to limit overall flux in oil-seed rape. This agrees with the observation that when the carbon flux through the pathway is increased, the diacylglycerol pool increases significantly. These data support previous proposals that DAGAT may be limiting for storage limit accumulation when this occurs at high rates (Perry & Harwood, 1993a, 1993b). Together with the obser-

vation that changes in DAGAT activity in mutated *Arabidopsis* correlate with alterations in lipid accumulation (Katavic et al., 1995), our experiments emphasise the importance of DAGAT as a potential target for manipulation in transgenic crops.

3. Experimental

3.1. Seeds

Plants of the spring variety of oil-seed rape, *Brassica napus* L. cv Shiralee, were raised singly in 12 cm pots containing John Innes No. 1 compost and kept in a growth room for a 12-h photoperiod at 20° and a 12-h dark period at 17°. Light intensity at flower height was *ca* 280 μmol m^{-2} s^{-1} (photosynthetically active wavelengths). Flowers were hand-pollinated to ensure good seed-set and date-tagged to record the age of the pods. Seeds were harvested at 30 days after pollination either in the dark or 4 h into the light period. For lipid analysis they were steam-killed and the embryos rapidly dissected into *iso*-PrOH and heated for 30 min at 70°. For NMR studies the seeds were immersed in liquid N_2 and stored at −70° until needed. Embryos were then dissected out a few at a time, under a dissecting microscope, and re-immersed immediately in liquid N_2, after which they too were stored at −70°.

3.2. NMR

In the ^{31}P-NMR analysis for glycerol 3-phosphate, neutralised perchloric acid extracts (PCA) were prepared from 4 g frozen embryos as previously described (Bligny, Gardestrom, Roby & Douce, 1990).

^{31}P-NMR spectra for neutralised PCA were recorded on an AMX 400 spectrometer (Bruker) equipped with a 10 mm probe tuned at 162 Mhz. Acquisition conditions were: 70° radio frequency pulses (15 μs) at 3.6 s intervals; spectral width 8200 Hz; 1024 scans; and Waltz-16 ^{1}H decoupling sequence, 1 w during acquisition time, 0.5 w during delay.

3.3. Lipid extraction, separation and analysis

These were carried out as before (Perry & Harwood, 1993a).

3.4. Subcellular fractionation

Embryos were homogenised in isolation buffer (100 mM Tris-HCl, pH 7.6, 0.5 m sucrose, 1 mM EDTA, 1 mM DTT) (10 μl/mg Fwt) at 4° and centrifuged at 450 × *g* for 10 min to remove cell debris. The supernatant was recentrifuged at 8000 × *g* for 10 min and the resulting post-mitochondrial supernatant was recentrifuged at 105,000 × *g* for 1 h to yield a microsomal fraction and a particle-free supernatant. The 8000 × *g* and 105,000 × *g* pellets were resuspended in buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM DTT). Protein was determined using the Bradford method (Bradford, 1976). Subcellular fractions were assayed immediately.

3.5. Enzyme assays

All incubations were carried out at 25° in a shaking H_2O bath.

3.5.1. Glycerol 3-phosphate acyltransferase (G3PAT; EC 2.3.1.15)

Incubations, which were for 60 min, used 100 mM K–Pi buffer, pH 7.0, with 150 nmol G3P (including 0.05 μCi [U-^{14}C]G3P), 80 nmol palmitoyl CoA, 100 nmol oleoyl CoA, 10 mM $MgCl_2$. 5 mg BSA and 100 μg protein in a final volume of 0.5 ml. The reaction was stopped by the addition of 0.5 ml 0.155 M acetic acid followed by 3.75 ml of $CHCl_3$–MeOH (1:2, v/v). Lipids were extracted and separated as above except that each plate was run first in the polar solvent system, $CHCl_3$–MeOH–HOAc–H_2O (170:30:20:7, by vol.), to 4 cm from the top, dried for 10 min under N_2, and then developed to the top of the plate in the non-polar solvent system, petrol (60–80° b.p.)–Et_2O–HOAc (80:20:2, v/v/v). Bands were identified and determined for radioactivity as before (Perry and Harwood, 1993b).

3.5.2. 1-Acylglycerol 3-phosphate acyltransferase (LPAAT; EC 2.3.1.51)

Incubations used 80 mM Tris-HCl buffer, pH 7.9, with 20 nmol oleoyl CoA (including 0.02 μCi [1–^{14}C] oleoyl CoA), 100 nmol 1-oleoylglycerol 3-phosphate (added as a sonicated dispersion using a bath sonicator), 5 mM $MgCl_2$ and 100 μg protein in a final volume of 0.5 ml. After 5 min the reaction was stopped and the products analysed as for G3PAT.

3.5.3. 1,2-Diacylglycerol acyltransferase (DAGAT; EC 2.3.1.20)

Incubations used 80 mM K–Pi buffer, pH 7.4, with 20 nmol oleoyl CoA (including 0.02 mCi [1–^{14}C] oleoyl CoA), 100 nmol dioleoylglycerol (added as a sonicated dispersion as above), 5 mM $MgCl_2$ and 100 μg protein in a final volume of 0.5 ml. After 10 min the reaction was stopped and the products analysed as for G3PAT.

3.5.4. Phosphatidate phosphatase (3-sn-phosphatidate phosphohydrolase, PAPase; EC 3.1.3.4)

Incubations used 100 mM Tric-HCl buffer, pH 7.0, with 1 mg sodium phosphatidate (dioleoyl), 1 mg egg yolk phosphatidylchline, 0.1 mM EDTA, 2 mM

$MgCl_2$, 2 mg BSA and 100 μg protein in a final volume of 1 ml. After 60 min the reaction was stopped with 1 mol 10% (w/v) TCA and the tube vortexed and centrifuged, and Pi determined (Chen, Toribara & Warner, 1956), as used by Ichihara et al. (1990).

All these conditions were found to be optimal and the reactions shown to be dependent on protein during the incubation periods.

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