Graphic Abstract

Lipid Labeling from Acetate or Glycerol in Cultured Embryos of *Camelina sativa* Seeds: A Tale of Two Substrates

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Use of acetate and glycerol substrates for prolonged ¹³C-labeling of lipids by cultured developing embryos of *Camelina sativa* was validated and quantified. Acyl fluxes at biosynthetic

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Dedicated to the memory of the late Professor Paul K. Stumpf, who inspired Mike Pollard's interest in plant lipid research.

Abstract

Studies on the metabolism of lipids in seeds frequently use radiolabeled acetate and glycerol supplied to excised developing seeds to track the biosynthesis of acyl and lipid head groups, respectively. Such experiments are generally restricted to shorter time periods and the results may not be quantitatively reflected for *in planta* rates. These limitations can be removed by using cultured embryos, provided they mimic growth and lipid deposition observed for embryos *in planta*. Mid-maturation embryos from *Camelina sativa* were cultured *in vitro* to assess the use of sufficient acetate or glycerol concentrations and labeling periods for stable isotope labeling and mass spectrometric detection. Maximum incorporation of exogenous acetate into fatty acids occurred at 1 mM and above. This provides about 5% of the total carbon flux entering fatty acids, enough for ¹³C isotopomer analysis while maintaining normal biosynthetic rates for over 24h. Labeling analysis indicates that acetate reports lipid metabolism uniformly across the embryo. At higher acetate concentrations with longer incubations, the rate of fatty acid synthesis is reduced and the composition of newly synthesized fatty acids changes. While the mole fractions of oleate that undergo $\Delta 12$ -desaturation or elongation are independent of biosynthetic flux, $\Delta 15$ -desaturation shows a bimodal dependence. These observations are consistent with changes occurring *in planta* over seed development. Incorporation rates of the glyceryl moiety into lipids saturates at about 0.5 mM exogenous glycerol. At saturation, the exogenous glycerol almost completely replaces the endogenous supply of glycerol-3-phosphate without affecting net lipid accumulation or fatty acid composition. It is concluded that acetate and glycerol labeling of cultured *C. sativa* embryos can provide an accurate representation of lipid metabolism in embryos *in vivo*, and that in *Camelina* embryos glycerol-3-phosphate levels do not co-limit triacylglycerol synthesis.

Keywords: *Camelina sativa*; Brassicaceae; embryo culture; lipid labeling; acetate; glycerol; stable isotope labeling

1. Introduction

Camelina sativa is of growing interest as a model oilseed crop plant to study the effects of heterologous gene expression on seed oil content and composition of oilseeds [Kang et al., 2011; Liang et al., 2013; Liu et al., 2014; Lu and Kang, 2008; Mansour et al., 2014]. This makes the development of *in vitro* culturing and analysis methods for measuring lipid biosynthesis and turnover desirable. In a companion paper [Pollard et al., co-submitted], the development of such tools is described. Cultured wild-type *Camelina* embryos showed rates of lipid accumulation and fatty acid compositions similar to those of seeds developing *in planta*. The next step is to develop a quantitative lipid biosynthesis flux map for these cultured maturing embryos. Flux maps for central carbon metabolism in cultured developing embryos have been completed for rapeseed [Schwender et al., 2004, 2006], soybean [Sriram et al 2005; Allen et al., 2009], sunflower [Alonso et al., 2007], maize [Alonso et al., 2010] and *Arabidopsis* [Lonien and Schwender, 2009]. To generate these maps, mass balances between nutrients consumed and end-products accumulated in the growing embryo are quantified. Stable isotope-labeled substrates are then administered to the embryo under steady state growth and the isotopomer distributions in reporter molecules for central metabolism are measured [Ratcliffe and Shachar-Hill, 2006; O'Grady et al., 2012]. Although this methodology works well to reveal fluxes of central metabolism, it has limited value for tracking acyl group fluxes between lipid pools because of the lack of label rearrangement after acyl chains are formed. Instead, kinetic labeling studies must be used [Ratcliffe and Shachar-Hill, 2006; Rohwer, 2012]. This was previously carried out using cultured developing soybean embryos with ^{14}C -labeled substrates to quantify the substantial cyclic acyl fluxes through membrane lipids [Bates et al., 2009].

The labeling of lipids can employ generic substrates such as sugars, pyruvate, alanine or water; although water is problematic as large kinetic isotope effects associated with ^2H -C or ^3H -C bonds reduce desaturation [Behrouzian and Buist, 2003]. Substrates that more specifically target lipids are preferred. Since its first use [Smirnov, 1960], acetate has become the standard substrate to label fatty acids in plants. It has advantages that include low cost and the fact that it labels acyl groups, not head groups. Entry of carbon from exogenous acetate into central carbon metabolism is low in seeds. Glycerol labeling is useful for tracking lipid classes and is often used in conjunction with acetate in studies on oilseeds [Gurr et al., 1974; Slack et al., 1978]. Despite frequent use for tracer radio-labeling, quantitative descriptions of the ability of these substrates to label seed lipids appear to be lacking. For studies on stable isotope labeling with its associated isotopomer analysis of acylglycerols, the pre-requisites are longer duration labeling times and incorporation of a significant mole fraction of label into lipids.

Experiments with *Camelina* embryos show that acetate incorporation into lipids is linear for at least 48 hours and provides just sufficient mole fraction of labeling for fatty acid and lipid isotopomer analysis. However, if used at high concentrations and prolonged times, acetate induces metabolic changes in the embryos themselves, reducing biosynthetic fluxes and altering fatty acid compositions. A discussion of the possible causes of these effects is included. The sensitivity of cytosolic fatty acid desaturation rates to acyl fluxes through precursor pools is also reported. By contrast, addition of exogenous glycerol gives extensive labeling of the glyceryl moiety in lipids, ample for stable isotope analysis. Although exogenous glycerol provides an additional source for glycerol-3-phosphate (G3P), presumably mediated through glycerol kinase and out-competing the endogenous supply of G3P from dihydroxyacetone-phosphate (DHAP), no significant change in net lipid accumulation or fatty acid composition was observed. The results suggest that the glycerol-3-phosphate dehydrogenase (G3P DH) reaction *in vivo* is not close to equilibrium but is under G3P feedback regulation, and that G3P production exerts negligible control on the rate of lipid synthesis.

2. Results

2.1 Acetate incorporation is adequate for stable isotope labeling and reports metabolism uniformly across the embryo

In vivo experiments with rapidly expanding leaves have used acetate at concentrations up to 10mM [Preiss et al., 1993; Pollard and Ohlrogge, 1999]. At the higher concentrations, the mole fraction of exogenous acetate-derived carbon in fatty acids can be substantial, approaching 0.5. The concentration dependence (0.05-10.09 mM) for acetate incorporation into total lipids by *Camelina* embryos is reported in Figure 1 A, and provides for a much lower maximum fractional incorporation than seen in leaves. Maximum incorporation into total lipids occurred at ≥ 1 mM acetate, with a value in this particular experiment of 45 pmoles acetate/min/embryo. Figure 1B shows time courses for two experiments, run for 8 and 48 hours, respectively, using 2.6 mM acetate. The cultured embryos showed linear incorporation with rates of 30 and 33 pmoles acetate/embryo/min respectively. The initial lag phase was very short, as has been observed with leaf tissues [Pollard and Ohlrogge, 1999; Koo et al., 2004; Bates et al., 2007] and for soybean embryos [Bates et al., 2009]. Thus the embryos rapidly establish a balance between rates of uptake and utilization, and are in a kinetic steady state over the period of assay. In cultured embryos, the endogenous lipid deposition rate rises from 26 to 78 μg lipid/embryo/day with extended culture [Pollard et al., co-submitted]. This requires approximately 550 rising to 1650 pmoles acetyl units/embryo/min. Therefore, early on in the embryo culture period, exogenous acetate substrate can support a maximum of 5-8% of the total rate of fatty acid synthesis plus chain elongation. This maximum [^{13}C] acetate incorporation is adequate for the measurement of ($^{13}\text{C}_2$)_n isotopomer distributions in fatty acid methyl esters (FAMES) and triacylglycerols (TAGs) without significantly perturbing biosynthetic fluxes.

The [^{14}C]fatty acid product distributions from the experiment shown in Figure 1 A are reported in Supplement Table 1. They are consistent with the composition of *Camelina* seed oil and our knowledge of fatty acid biosynthesis in seeds. Significant C18 polyunsaturated fatty acid synthesis (21-28%) occurs in the 6 hour assay. Very long-chain (C20-C24) fatty acid (VLCFA) labeling is 33-35% at lower acetate concentrations, rising to 43-45% at acetate >2.5 mM. This small increase likely represents the differential competition and saturation kinetics for plastid and cytosolic acetyl-CoA synthesis to drive fatty acid synthesis and chain elongation, respectively. VLCFA constitute 20 mole % of fatty acids in mid-maturation embryos [Pollard et al., co-submitted]. It is known that exogenous acetate is preferentially utilized for cytosolic chain elongation over plastid fatty acid synthesis (FAS) [Ohlrogge et al., 1978; Pollard and Stumpf, 1980a, 1980b]. This preference is approximately 6-fold in *Camelina* embryos (a value similar to seeds from other plants), and leads to VLCFA being over represented by acetate labeling relative to quantification on a molar basis.

An intended use for ^{13}C -labeling with acetate or glycerol will be to track fatty acid and lipid fluxes. Such studies are underway in our laboratory. In the interim, an example of the novel perspectives ^{13}C -labeling can bring to an experiment traditionally conducted with ^{14}C substrate is shown in Figure 2. A criticism of the quantitative interpretation of tracer labeling experiments with [^{14}C]acetate is that it is unclear if the tissue is being uniformly labeled, or if there is a preferential labeling of a subset of cells. Such cells might be epidermal or vascular, or proximal to these cell types, and their disproportionate labeling may lead to a quantitative bias of the results. The low maximum incorporation shown in Figure 1A leaves this question unanswered. The "Control" in Figure 2 upper panel shows the molecular ion cluster for unlabeled methyl stearate obtained by GC-MS. Next to it ("Labeled") is a sample of methyl stearate obtained by

transmethylation of hydrogenated TAGs which have been labeled by incubating cultured *Camelina* embryos with 2.6 mM [$^{13}\text{C}_2$]acetate for 2 days. The isotopic enrichments at $m/z \geq (M+2)$ are evident. After correction for ^{13}C natural abundance levels, the distribution of the ^{13}C label from the substrate can be calculated ("Calcn 1"). There is about 10% (M+1) labeling compared to (M+2) labeling. The (M+1) isotopomer presumably represents a small flux of $^{13}\text{C}_2$ units moving through the C4 dicarboxylate pool to pyruvate with loss of one ^{13}C atom. The corrected intensity ratios from "Calcn 1" for (M+4)/(M+2) and (M+6)/(M+2) are 0.217 and 0.027 respectively. They provide the evidence for a uniform embryo labeling, as discussed below.

If exogenous [$^{13}\text{C}_2$]acetyl groups utilized for fatty acid synthesis mix randomly with the endogenous pool in the plastid at a specific ratio, the theoretical distribution of fatty acid isotopomers can be readily calculated. The results from such a calculation are shown in the middle panel of Figure 2 for a C18 fatty acid, for an enrichment of up to 20% [$^{13}\text{C}_2$]acetyl groups. The abundance ratios for (M+4)/(M+2) and (M+6)/(M+2) can therefore be calculated, and are shown in the lower panel of Figure 3. At 5% [$^{13}\text{C}_2$]acetyl enrichment, these values are 0.211 and 0.026 respectively. Thus the experimental ratio values for C18 fatty acids suggest that about 5% of FAS is from exogenous acetate. Direct comparisons of the rate of acetate uptake into lipids with the endogenous rate of fatty acid accumulation in cultured embryos produce values of 5-8% utilization of exogenous acetate for FAS plus chain elongation at saturating concentrations of acetate. Applying the correction for the acetate that is diverted into chain elongation to make LCFA, this figure is reduced by 19% (Supplement Table 1) to a 4-6.5% range for FAS alone. The fact that the estimate based on mass spectrometry data and the assumption of a uniform distribution falls in the middle of the range made by direct rate comparisons shows that the assumption of uniform distribution of acetate within the embryo is correct. If not so, then a smaller part of the tissue would experience a much higher fractional labeling and the distribution of (M+2n) values would move to higher values of n as shown in the middle panel of Figure 2.

2.2 Prolonged incubation with acetate reduce embryo growth and lipid deposition, especially at high acetate concentrations

Experimental design for stable isotope labeling requires higher acetate concentrations than used for short duration [^{14}C]acetate tracer experiments. Based on the data shown in Figure 1 the concentration should be > 1-1.5 mM, but since assays of several days duration with 5 embryos in 1.5 ml medium will deplete substrate, 2.6 mM (2.5 mM [$^{13}\text{C}_2$]acetate plus 0.1 mM [^{14}C]acetate) was used. As acetate is presumed to be a non-physiological substrate, it is important to assess whether acetate has any physiological impact on the embryos. Figure 3A shows the growth of cultured *Camelina* embryos over a four day period at 0-10 mM acetate. This period allows sufficient biomass accumulation for accurate comparative quantification of fresh weight, dry weight and total fatty acids. There is a discernable reduction in dry matter and lipid accumulation even at the lowest acetate concentration tested (0.75 mM). The reductions in fresh weight, dry weight and total fatty acid accumulations at 10 mM acetate were 84%, 75% and 83% respectively. In three separate experiments, the reduction in total fatty acid accumulation was $\geq 75\%$ at 5-6 mM acetate. The acetate inhibition also produced changes in the relative fatty acid accumulations (Figure 3B). With increasing acetate concentration, stearic acid (C18:0) and α -linolenic acid (C18:3) depositions were reduced prior to reductions in oleic acid (C18:1), eicosenoic acid (C20:1) and erucic acid (C22:1), while palmitic acid (C16:0) and linoleic acid (C18:2) were initially the most resistant to change.

To further characterize this inhibition, growth kinetics were tracked at 2.5 and 8 mM acetate concentrations, compared to 0 mM acetate control. One reason to do this was to reconcile the observation of a strong reduction in lipid accumulation rate from 1 to 10 mM exogenous acetate

over 4 days in culture with the experiment shown in Figure 1A, where for a 6 hour incubation at 1-10 mM acetate the incorporation rate into lipids remains the same. The data for fatty acid accumulation with time are shown in Figure 4. In fact, reductions in fresh and dry weights also track reductions in lipids closely and in an acetate concentration-dependent manner (Figure S1). Increasing the acetate concentration decreases both the rate of accumulation and the lag period before this rate reduction is reached. At 2.5 mM acetate, the dry weight and lipid accumulations were reduced to 45% and 56% of the control, respectively, after about 2 day lag period. At 8 mM acetate, the dry weight and lipid accumulations were reduced to 12% and 27% of the control, respectively, after about an 18 hour lag period. An important point is that acetate does not immediately change the rate of synthesis; instead, the rate change is progressive with both time and concentration. This means that the cause is unlikely to be an immediate metabolic effect. An additional control experiment compared acetate at either 0.1 mM or 2.6 mM concentrations for a two hour assay (Supplement Table 2). The labeled lipid class distributions are very similar, while the total [^{14}C] fatty acid methyl ester (FAME) distributions only show the small increase in VLCFA. Whatever happens at 0.1 mM also occurs at 2.5 mM. Thus if there is any immediate perturbation of physiology, it already is maximized at concentrations well below 0.1 mM acetate.

2.3 Acyl flux precursor-product correlations from modulation of lipid synthesis activity with acetate

The kinetic experiments shown in Figure 4 provided FAME compositions consistent with the experiment shown in Figure 3B. Taking FAME accumulations over a 4 day culture period (a measure of average flux) at different acetate concentrations, Figure 5 shows the relationship between total amount of the precursor fatty acid available and its metabolic products for acyl groups undergoing either $\Delta 12$ -desaturation (FAD2), $\Delta 15$ -desaturation (FAD3) or chain elongation (FAE1). The precursor-product relationships and thus the specific fatty acids that are included in either the precursor or product pool are defined in Figure 5A. Comparing the accumulated mass of C18:1 and its end products with the amount of those products that must undergo $\Delta 12$ -desaturation shows a strictly linear correlation whereby 0.72 of the mole fraction of C18:1 undergoes desaturation, independent of the flux over a ten-fold range (Figure 5B). Likewise, the mole fraction of oleoyl groups that are directed towards elongation is independent of the input flux, having a mole fraction value of approximately 0.15 (Figure 5B). The situation is different for C18:2 $\Delta 15$ -desaturation (Figure 5C). The mole fraction of C18:2 that is further desaturated is approximately 0.5 at lower fluxes, and then changes rather abruptly to about 0.9 at higher input fluxes. Because these acyl flux correlations originate from culturing embryos with a metabolite, the fatty acid composition data for *Camelina* seed development [Pollard et al., co-submitted] was reanalyzed to provide a similar *in planta*-derived data set (Supplement Figure 2). Inspection of this correlation shows the trends to be very similar to those seen in Figure 5. This implies that the phenomenon observed in culture has a direct parallel to actual seed development.

Of the best-fit linear correlations between substrate and product fluxes, that for C18:2 desaturation passes through the origin, while that for C18:1 desaturation has a negative intercept on the y-axis (-0.055 $\mu\text{moles/embryo}$) and that for C18:2 elongation has a positive intercept on the y-axis (+0.023 $\mu\text{moles/embryo}$). There are several potential interpretations for these different intercepts. The first point to note is that the zero point on the precursor axis is for net deposition. Both fatty acid synthesis and degradation could be occurring at zero net flux. Alternatively, at the initiation of the assay, some of the C18:1 already present has yet to be elongated, explaining both negative and positive intercepts. Finally, it may be that at very low fluxes, the flux relationships deviate from linearity and do in fact pass through the origin. If so, at

very low net flux elongation becomes the preferred metabolism, while C18:1 production needs to reach a set-point before it is channeled to the phosphatidylcholine (PC) pool for Δ 12-desaturation.

2.4 Glycerol is incorporated into lipids at high rates

Glycerol at low concentrations in MS medium (≤ 1 mM) has been reported to be an inhibitor of root growth in Arabidopsis seedlings (Eastmond, 2004). This inhibition was strongly repressed by 20 mM sucrose. Root growth of *gli1* mutant plants was unaffected by glycerol, and as GLI1 encodes the only known glycerol kinase enzyme in Arabidopsis phosphorylation to G3P appears required for the inhibitory effect. In assays with Camelina embryos, growth continues unabated at 3 mM glycerol, but as the medium contains high concentrations of sucrose (12 mM) and glucose (130 mM), inhibitory effects from glycerol were neither expected or observed (see section 2.5). In fact, glycerol proved to be quantitatively a very effective substrate for lipid labeling. Figure 6A shows a maximum measured rate for incorporation into the glyceryl backbone of lipids of 36.7 ± 1.1 pmoles glycerol/min/embryo, occurring at 1 mM glycerol concentration. When using [14 C]glycerol, a small percentage of the label in the lipids occurs as acyl groups rather than glycerol itself. In the experiment shown in Figure 6A, as the glycerol concentration increased from 0.02 to 1 mM, acyl labeling increased from 6.1 to 13.8%, as measured by radioactivity extracted into the hexane phase after transmethylation (Figure 6B). This acyl labeling provides some useful insights into G3P metabolism, discussed below. Although the incorporation of glycerol into the glyceryl backbone requires several steps (uptake, kinase activation and acyl transfer), the overall reaction shows a linear Lineweaver-Burke plot (Figure 6A, insert) from which estimates for $K_{1/2} = 0.25$ mM and $V_{\max} = 45$ pmoles glycerol/min/embryo can be made.

2.5 Glycerol does not perturb the rate of lipid accumulation or fatty acid composition

The data in section 2.4 show that high rates of glycerol incorporation into the glyceryl moiety of lipids are possible. Such rates enable the use of 13 C labeling strategies. However, glycerol has not been reported as a nutrient in the embryo sac fluid, and so is not expected to be a normal substrate for the embryo. The high rates raise the question as to whether this substrate could perturb metabolism. In order to check this issue, embryos were cultured with ± 3 mM [13 C₃]glycerol for periods of up to 6 days. The results are shown in Figure 7. Fresh and dry weight accumulations are shown in Figure 7A, and indicate no changes in growth. Lipid accumulation, as measured by total fatty acids, is shown in Figure 7B, and again there does not appear to be a statistical difference between control and 3 mM glycerol treatments. The fatty acid compositions for total lipids are also indistinguishable for controls and with inclusion of 3 mM glycerol (Supplement Table 3), with the same trends during culture as seen in previous experiments. From these experiments, it is clear that exogenous glycerol at a saturating concentration causes no measureable change in net lipid or fatty acid accumulation. A further control to assess the effect of low and high glycerol concentrations was to run assays for 3 hours. The results are shown in Supplement Table 4 and show insignificant differences in distributions between lipid classes for glycerol concentrations of 0.02 mM and 1.52 mM.

Hydrogenated triacylglycerols (TAGs) from embryos cultured in 3 mM [13 C₃]glycerol (Figure 7) were analyzed by ESI-MS. Figure 8A shows the ammonium adduct molecular ion clusters for a tripalmitin standard synthesized from [13 C₃]glycerol, together with unlabeled tripalmitin. The base peak is shifted by 3 a.m.u., and there are negligible (M-1) or (M-2) peaks relative to $m/z = 827.7$ that would indicate the presence of [13 C₂] or [13 C₁] isotopic impurities in the glycerol. Figure 8A also shows the TAG molecular species containing 54 acyl carbon atoms, for the control and

after 3 days cultured in 3 mM [$^{13}\text{C}_3$]glycerol. There are substantial increases in the (M+3) peak and its natural abundance isotopomers (M+4) through (M+6). In this particular sample, the fraction of $^{13}\text{C}_3$ labeling was 35%. A more detailed description of this isotopomer calculation is given in Supplement Figure 3. As TAGs are by far the dominant lipid product, the fractional labeling from TAGs can be applied to total lipids to generate Figure 8B, where the estimated rate of accumulation of [$^{13}\text{C}_3$]labeled TAGs is superimposed with the total fatty acid accumulation divided by three. From the rate of fatty acid accumulation the initial rate of glycerol utilization for lipid synthesis is estimated as 27 pmoles/min/embryo, rising to 60 pmoles/min/embryo by the fourth day in culture. The rate of incorporation of exogenous glycerol remains constant. It provides essentially all the G3P requirement initially. However, because glycerol is at a saturating concentration, as the rate of lipid synthesis climbs over 2-fold with time the endogenous G3P is increasingly required to supplement the exogenous substrate supply.

3. Discussion

One emphasis of this study and its companion paper [Pollard et al., co-submitted] is to assess the tools (substrates, embryo culture) which may provide quantitative flux descriptions of lipid biosynthesis in seeds. Embryo culture permits extended duration assays, thus allowing sufficient labeling for ^{13}C -isotopomer analyses. For kinetic measurements to be used to construct flux models, it is preferable that the cultured embryos have a constancy of metabolism over the period of measurement (kinetic steady state). Also, the embryos should have the same endogenous metabolism before and during labeling, and the label must reflect this metabolism. In this way, the labeling can be integrated with estimates of fluxes from endogenous lipid class mass and composition. Deviations from these ideals can be incorporated so as to produce correct models, but the deviations must first be known and quantified.

3.1 Use of acetate as a substrate to track lipid biosynthesis

For reasons described in the Introduction, acetate has been the most popular substrate in studies of oilseed metabolism even though it has uncertain physiological significance. As shown in Figure 1, incorporation into fatty acids saturates at ~1 mM acetate. To maintain a maximum and linear rate of incorporation over two days, 2.6 mM acetate was used. It is shown that acetate does indeed perturb embryo metabolism. Inhibition of lipid synthesis is progressive with acetate concentration and time (Figures 2 and 3), such that at 2-3 mM acetate by the end of the second day, the rate of lipid accumulation begins to decrease. Thus at 2.6 mM acetate concentration, 2 days is the maximum period before the steady state assumption begins to deviate from linearity. The concentration dependence for acetate incorporation into fatty acids and the sensitivity of cultured embryos to growth inhibition by acetate do not appear to have been reported previously for any oilseed. At saturating concentrations, acetate provides $\leq 8\%$ of the flux for acyl group biosynthesis. This is in one sense ideal, because as substrate it cannot itself greatly alter fluxes and thus can be considered metabolically almost non-perturbing. The constancy of the rate of incorporation between 1 to 10 mM is a function of the relatively short duration of the assay used to generate data presented in Figure 1. Over this period, the effects of acetate as a growth inhibitor have yet to appear. The 4 day assays shown in Figure 2 used [$^{13}\text{C}_2$]acetate. At 2.6, 6 and 10 mM, the rates of incorporation per assay fell from 8.7 to 4.6 to 3.5 $\mu\text{moles } ^{13}\text{C}$ atoms/assay. Thus net acetate uptake and utilization decline with reduced growth rate. This is consistent with other studies, such as the effect of light and dark, or herbicides, on seed lipid metabolism [Bao et al., 1998], or the case of the fatB mutant [Bonaventure et al., 2004], where acetate incorporation does reflect the induced metabolic

change. Although the decrease in the rate of fatty acid deposition in *Camelina* embryos was expected to reflect a much reduced rate of fatty acid and glycerolipid biosynthesis, a contribution from induction of β -oxidation to produce a rapid futile cycle between acyl synthesis and degradation currently cannot be ruled out [Eccleston and Ohlrogge, 1998].

3.2 Possible causes of growth inhibition by acetate

Since acetate inhibition of growth is not immediate but requires 1-2 days to become manifest, it is likely that acetate modulates the rate of lipid deposition through mechanisms that involve either gene transcription, signaling or protein post-transcriptional regulation. As acetyl-CoA synthetase is required for acetate incorporation into lipids, a simple explanation is to postulate that acetyl-CoA is responsible for the observed effects. Their onset may be progressive with time and concentration, but Figure 3 shows that a new steady state of lipid deposition is reached, rather than a continued exponential decline in activity.

Acetate is known to be a growth inhibitor for eukaryotic cells. However, acetate toxicity based on acidity is unlikely to be the cause. More pertinent is a study on the diauxic growth of suspension cells from carrot roots [Lee et al., 1999] in a medium (pH 5.8) containing 10 mM each of acetate and glucose. Acetate was the preferred substrate for growth, requiring an active glyoxylate cycle. Only after the acetate concentration had dropped to 1-3 mM was glucose used for growth. The researchers proposed that a major mechanism for this effect was interference by acetate uptake of the proton gradient across the plasma membrane that is required for glucose transport. But, for *Camelina* embryos, there are counter-arguments against such a mechanism. It seems unlikely that acetic acid import will cause sufficient alkalization of the apoplast to inhibit glucose uptake. Estimates of glucose uptake in developing *Camelina* embryos are 1-3 nmoles/min/embryo [Carey, L. and Shachar-Hill, Y., unpublished results], whereas the maximum acetate incorporation into lipids (Figure 1A) is 0.04 nmoles/min/embryo. Additional acetate assimilation into non-lipid pools is about half this value. Thus, the rate of apoplastic alkalization for glucose-proton co-transport will be at least 20-fold greater than any caused by acetate uptake. Furthermore a direct metabolic effect of acetate on the transmembrane pH gradient is expected to be rapid, whereas acetate incorporation into lipids from 1 to 10 mM is identical for at least 6 hours. Other explanations are needed.

Acetyl-CoA is a central metabolite that has profound effects on gene expression by virtue of its role as substrate for histone acetylation [Kaochar and Tu, 2012]. In addition to histones, there are many more proteins which undergo acetylation and deacetylation [Chen et al., 2012]. In animals, it is known that acetyl-CoA synthetases are themselves inactivated and activated by acetylation and SIRT-mediated deacetylation, respectively. The acetate concentrations in seeds remain unknown, although it can now be stated that exogenous acetate is a relatively poor substrate. Given the importance of acetylation and deacetylation cycles in regulating eukaryotic cell metabolism, the observation that acetate acts as an inhibitor to growth and lipid synthesis in seeds is intriguing and worthy of further investigation.

3.3 The relationships between fatty acid composition and flux as modulated by acetate

Although the mechanism by which acetate inhibits lipid synthesis is unknown, it is expected to be broadly based as it affects growth similarly. Increasing acetate concentrations titrate down net lipid synthesis allowing the construction of flux correlations shown in Figure 4. Across a wide range of fluxes, the mole fraction of oleate directed to elongation (0.15) or Δ 12-desaturation (0.72) remained invariant. This is not an obvious outcome as the pathways of fatty acid and lipid synthesis are fairly complex, involving competing acyl transferases, chain elongations and

desaturations, multiple diacylglycerol (DAG) and phospholipid pools, and cyclic fluxes in DAG-PC inter-conversions and acyl editing, as shown for soybean embryos [Bates et al., 2009]. The correlations are also apparent in seed development. When the fatty acid accumulations are analyzed in as incremental mass/day over seed maturation, the flux correlations (Supplement Figure 2) look remarkably similar to those in Figure 5. Thus it can be inferred that the correlations caused by acetate are not simply an artifact of *in vitro* culture conditions, but hold mechanistic relevance. Any model describing lipid metabolism must provide mechanisms for such constant molar flux ratios.

The most striking change in fatty acid composition over Camelina seed development is the mid- to late-stage accelerated accumulation of C18:3 relative to C18:2, followed by a very abrupt cessation of C18:3 deposition [Pollard et al., co-submitted]. The biosynthesis of linoleate requires the FAD2 desaturase [Okuley et al., 1994] whereas linolenate requires the sequential action of both FAD2 and FAD3 desaturases [Yadav et al., 1993]. While the flux correlation between C18:1 and its Δ 12-desaturated products is highly linear, the correlation between production of C18:2 and its consumption via Δ 15-desaturation, leading to C18:3 + C20:3 products, shows a biphasic dependence on the C18:2 flux. The mole fraction of C18:2 which is further desaturated is about 0.5 at lower fluxes, but then changes rather abruptly so that the flux becomes about 0.9 mole fraction desaturated. This flux description readily fits the changes in polyunsaturated fatty acid composition over Camelina seed development [Pollard et al., co-submitted]. The results suggest that a limitation in the desaturase electron transport chain is unlikely, not only because the mole fraction of C18:2 produced from C18:1 is unaffected by flux, but because at higher fluxes desaturation through FAD3 actually increases. Furthermore, one view of phospholipid desaturation is that the activity will depend on the total residence time of an acyl group in the PC pool available for desaturation. The prediction would be that as the flux of C18:1 increases, residence time in PC would decrease commensurately, and the proportion of desaturation product would decrease. This does not occur. Transcriptome data for Arabidopsis suggest that FAD2 expression precedes FAD3 expression slightly [Ruuska et al., 2002; Arabidopsis eFP Browser, Winter et al., 2007, <http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>], but by torpedo through green cotyledon stages the differences are small. Given interest in producing specialty unsaturated fatty acids in oilseeds, it would be worth understanding the mechanism(s) that lead to Δ 12- and Δ 15-desaturations increasing in unison at the higher flux.

3.4 Comments on the supply of glycerol-3-phosphate for lipid synthesis in oilseeds

Although glycerol is routinely used as a tracer to study lipid synthesis in oilseeds, glycerol kinase is expected to be a minor contributor to the synthesis of G3P *in vivo* as DHAP reduction by G3P DH (EC 1.1.1.8) will provide most of the G3P. In developing seeds, most (> 95%) glycerolipid synthesis is cytosolic so it is assumed that G3P synthesis would have the same localization. Indeed, G3P DH activity from extracts of developing castor bean endosperm purified as a single peak and was considered solely the cytosolic isoform [Finlayson and Dennis, 1980]. However, there are potentially confounding factors to this simple picture. First, in Arabidopsis there are two annotated genes for plastid-localized enzymes, At5g40610 [Wei et al., 2001] and At2g40690 (GLY1) [Chanda et al., 2011], two annotated genes for cytosolic-localized enzymes, At2g41540 and At3g07690, and a related enzyme (EC 1.1.5.3) associated with the mitochondrial outer envelope (At3g10370) [Shen et al., 2006; Quettier et al., 2008]. Further, numerous studies have shown chloroplasts can take up G3P for glycerolipid biosynthesis [Sauer and Heise, 1984; Andrews and Mudd, 1985], while a facile and reversible transport of G3P between plastids and cytosol can be expected [Fliege et al., 1978]. Given

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4 numerous G3P DH genes and potential G3P exchange between organelles, flexibility in G3P
5 supply to cytosolic G3P acyltransferases (GPATs) seems possible.
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8 A larger question is whether the first step of extra-plastidic glycerolipid synthesis can provide
9 some level of control over TAG synthesis. Acylation of G3P by acyl-CoAs requires GPAT
10 enzymes, which initiate membrane lipid and TAG biosynthesis through *sn*-1 acylation. The
11 provision of G3P to GPAT might be part of such regulation at this branch point in the metabolic
12 network, thus linking triose-phosphate (triose-P) metabolism directly to lipid synthesis at a point
13 other than the supply of FAS precursors. However, functional regulation would require a *bone*
14 *fide* feedback loop between acyl-CoA levels and FAS. G3P supply and GPAT have received
15 limited attention as possible control points for glycerolipid synthesis in seeds or other plant
16 tissues. Based on a labeling study co-injecting glycerol plus ^{14}C -substrates directly into
17 developing *Brassica napus* seeds on the plant, G3P levels have been proposed to “co-limit”
18 TAG synthesis [Vigeolas and Geigenberger, 2004]. In a follow-up study, expression of yeast
19 G3P DH gene under control of the napin promoter caused whole seed G3P levels to increase 3-
20 4 fold while mature seed lipid increased up to 40% [Vigeolas et al., 2007]. The former
21 phenotype was proposed to cause the latter. However, the fact that fatty acid levels in WT
22 controls were sub-optimal opens this conclusion to question.
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25 **3.5 Exogenous glycerol can supply most of the glycerol-3-phosphate required for lipid** 26 **synthesis but does not stimulate lipid synthesis** 27

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29 At saturating concentrations (≥ 0.5 mM), exogenous glycerol can supply the majority of G3P
30 required for glycerolipid synthesis in cultured *Camelina* embryos. This substantial incorporation
31 of glycerol into the glyceryl backbone of lipids was unexpected. By contrast, in soybean
32 embryos, it was reported that 0.5 mM glycerol provided only about 3% of the required G3P to
33 sustain lipid biosynthesis [Bates et al., 2009]. The reason(s) why soybean is less efficient is
34 unknown. It may be in part due to a diffusion limitation for glycerol in much larger embryos, or
35 perhaps there are restrictions imposed by glycerol transport and/or glycerol kinase.
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38 The reason to assert that exogenous glycerol provides the majority of G3P is as follows. Rates
39 of lipid accumulation were measured in cultured *Camelina* embryos. At mid-maturation the rate
40 was initially equivalent to 20 pmoles glycerol/min/embryo, but it climbed to a peak of about 60
41 pmoles glycerol/min/embryo over extended culture [Pollard et al., co-submitted] (Figure 7B). In
42 Figure 6, the maximum measured rate for incorporation of glycerol into glyceryl-labeled lipids
43 was 36.7 pmoles glycerol/min/embryo, 1.8-fold higher than the endogenous basal rate. In a
44 series of experiments performed at 0.5 mM glycerol, the average rate was 24.7 ± 5.9 ($n = 6$)
45 pmoles glycerol/min/embryo. Furthermore, extended culture provides a 2-fold or greater
46 increase in the net rate at which the embryos accumulate lipid, independent of whether glycerol
47 is present or absent (Figure 7B). Incorporation of 3 mM glycerol into lipids over this period is,
48 however, constant, providing almost all the G3P at the beginning of the period but only about
49 half by the end of the period (Figure 8B). Thus the rate comparisons provide strong evidence
50 that glycerol can be the major source for G3P in culture. The sole caveat to this conclusion
51 would be if glycerol induced a futile cycle of fatty acid and lipid synthesis and degradation (beta-
52 oxidation and glyoxylate cycle) akin to that seen in certain *UcFatB*-transgenes [Eccleston and
53 Ohlrogge, 1998]. This does not appear to be the case. Small contributions to the ^{13}C isotopomer
54 distribution of hydrogenated-TAGs labeled from $^{13}\text{C}_3$ glycerol can be observed that originate
55 from (M+2) and (M+5) biosynthetic species (Supplement Figure 3). These are isotopomers that
56 contain a single $^{13}\text{C}_2$ acyl group, either on its own or with $^{13}\text{C}_3$ glyceryl labeling respectively.
57 Contributions from (M+1) or (M+4) species that would report $^{13}\text{C}_1$ acyl groups were not
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observed. Such [$^{13}\text{C}_1$]acyl groups would arise from loss and randomization of label via futile cycling.

Because exogenous glycerol provides the majority of G3P for net TAG synthesis, at least initially during the time course (Figure 8), the inference is that G3P from glycerol kinase outcompetes G3P generated from the endogenous DHAP pool via the action of G3P DH. However, the availability of this additional source of G3P does not increase the net rates of growth and lipid accumulation. Thus, using *Camelina* embryos, the conclusion of other researchers [Vigeolas and Geigenberger, 2004; Vigeolas et al., 2007] that G3P levels “co-limit” TAG synthesis was not substantiated here. It may be that this discrepancy is a consequence of comparing rapeseed and *Camelina* embryos. However, using botanically diverse species, 1.5- to 4-fold enhanced rates of TAG accumulation produced by feeding exogenous decanoic acid to *Cuphea lanceolata*, *Ulmus carpinifolia* or *Ulmus parvifolia* developing embryos did not require, nor were further stimulated by, addition of 0.125 mM glycerol [Bao and Ohlrogge, 1999].

3.6 *In vivo* the glycerol-3-phosphate dehydrogenase reaction does not appear to be close to equilibrium but shows end-product inhibition

Labeling experiments with glycerol show a small but consistent fraction of the label directed into fatty acid synthesis (Figure 6B). This is also seen in pea leaf [Bates et al., 2007] and soybean embryos [Bates et al., 2009]. This acyl labeling is believed to occur as exogenous glycerol tracks through G3P and then the reverse reaction of G3P DH, thus entering the triose-P pool. It can be used to deduce some properties of G3P DH enzyme *in vivo*. Total cellular triose-Ps provide all the acetyl-CoA for plastid FAS. For triolein as end product, the ratio of triose-P net fluxes to G3P or FAS would be 1:27. If G3P DH was close to equilibrium *in vivo*, the introduction of exogenous glycerol to provide G3P would result in most of the label being utilized for FAS. This does not occur, so a high G3P $\leftrightarrow$ DHAP interconversion rate is absent. If most of the glycerol is passed through the glycerol cycle, namely via dihydroxyacetone to DHAP [Chen et al., 2012], acyl labeling would still be dominant. However, this is not the case. Thus the data supports the utilization of exogenous glycerol by a kinase, confirming the conclusion from analysis of the *gli1* mutant [Eastmond, 2004].

Flux maps for central carbon metabolism in developing seed embryos typically assume a unidirectional G3P flux to lipid synthesis originating from DHAP (or triose-Ps). The apparent equilibrium constant for the reaction $\text{G3P} + \text{NAD} \leftrightarrow \text{DHAP} + \text{NADH}$ at a cytosolic pH of 7.5 is $K' \approx 0.0002 = [\text{DHAP}]/[\text{G3P}] \cdot [\text{NADH}]/[\text{NAD}]$ [Goldberg et al., 1993]. Data for NAD, NADH, G3P and DHAP tissue concentrations are not available for *Camelina* embryos or for oilseeds more generally, but calling G3P DH by its other common name, DHAP reductase, seems a very reasonable description of the reaction. At the lowest glycerol concentration (21 μM) used here, 6% of the label is associated with acyl groups. As [$\text{U-}^{14}\text{C}$]glycerol is the substrate, one third of the label will be lost on conversion to acetyl-CoA. Correcting for this suggests that the maximum back reaction for G3P DH will be about 10% of the forward reaction. Any flux of exogenous glycerol through the putative glycerol cycle would reduce this percentage. However, triose-P flux must also include label mobilized for amino acid biosynthesis and respiration. Also, *Camelina* embryos have a low carbon efficiency caused by an unusually high oxidative pentose phosphate cycle activity [Carey, L., Clarke, T., Despande, R. and Shachar-Hill, Y., unpublished data] which will metabolize more ^{14}C label from glycerol. Thus it is anticipated the G3P DH back reaction is closer to 20-40% of the forward reaction.

As glycerol concentration in the medium increases, it can be inferred that G3P production via GLY1-like kinase predominates over and down regulates endogenous G3P production from

DHAP. G3P likely acts as an allosteric or competitive inhibitor of G3P DH. This is consistent with the finding that G3P acts as an inhibitor of the DHAP reduction reaction in all plant enzymes studied [Finlayson and Dennis, 1980; Gee et al., 1988; Sharma et al., 2001]. The provision of a second source of G3P does not increase net fatty acid accumulation. The simplest explanation for this observation is that lipid accumulation is limited by other factors, particularly those acting to enable FAS, while G3P pools merely respond accordingly and provide substrate for GPAT as demand requires. Under certain conditions, the embryo has the capacity to synthesize lipids at a significantly higher rate than can be sustained by exogenous glycerol. In this situation, provision of more acyl-CoA to GPAT would reduce the G3P concentration and partially relieve any G3P feedback inhibition on G3P DH. The reasons why glycerol kinase can so effectively provide G3P for lipid synthesis despite an active G3P DH remain to be explored, but it may indeed be fortuitous that in *Camelina* embryos maximum glycerol kinase activity matches glycerolipid synthesis so closely. When the *Euonymus alatus* DAcT gene is expressed in *Camelina* seeds, the production of substantial amounts of *sn*-3 acetyl TAGs requires up to 20% more G3P for TAG synthesis [Liu et al., 2015], again demonstrating the flexible capacity for G3P supply.

4. Conclusions

In this and the companion paper, the analytical tools required to use cultured developing embryos from *C. sativa* seeds for kinetic studies to produce quantitative lipid flux maps were established and characterized. Acetate and glycerol concentrations and assay times required for lipid labeling were assessed in order to give sufficient ^{13}C incorporation for mass spectrometry-based lipid isotopomer analysis. Some unexpected facets of using these substrates were explored. An acetate-catalyzed inhibition of embryo growth and lipid synthesis, whatever its relevance *in planta*, places limits on assay concentrations and times when using acetate as an *in vivo* reporter for acyl lipid metabolism. Lower acetate concentrations (1-2.5 mM) for 1-2 days allow steady state kinetic labeling before the onset of inhibitory effects become problematic. At saturation, acetate provides about 4-6% of the total carbon flux for fatty acid synthesis, sufficient for ^{13}C isotopomer analysis and allowing demonstration that acetate labeling is indeed uniform across the embryo. Using acetate to modulate biosynthetic rates, it was found that the competition between acyl transfer, elongation and $\Delta 12$ -desaturation of oleate remains remarkably constant over a wide flux range. By contrast, $\Delta 15$ -desaturation of linoleate is enhanced at higher fluxes. Glycerol is very efficiently incorporated into lipids at moderate concentrations and does not appear to perturb lipid metabolism in cultured embryos, either in terms of total TAG synthesis or fatty acid composition. Thus [$^{13}\text{C}_n$]glycerol will be ideal for stable isotope labeling studies. These experiments suggest that G3P acts as an effective inhibitor of G3P DH, that the DHAP reduction reaction is not close to equilibrium, and that no control of lipid synthesis resides with G3P DH. In conclusion, it is expected that the results from acetate or glycerol labeling to produce quantitative models of lipid biosynthesis in these cultured embryos will be readily transferable to the situation in the seed.

5. Experimental

5:1 Plant materials

Wild type *C. sativa* plants, var. Sunesson, were grown in a mixture of 3:1 potting soil (Sure-Mix, Michigan Grower's Products, Galesburg, MI) and vermiculite in a growth chamber at 60% relative humidity, 20°C, 16/8 hr day/night cycle, and 150-160 μE light intensity (at the chamber

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4 floor). Plants were alternately watered with deionized H₂O and half strength Hoagland's solution.
5 Plants started to bolt after three weeks, with first flowers appearing at day 35-40. Flowers were
6 tagged as petals opened. For embryo culture, siliques were harvested into 20% bleach then
7 rinsed thoroughly prior to seed removal and embryo dissection.
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9 10 **5:2 Embryo culture, incubations, growth measurement and lipid extraction**

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12 In this study, embryos at 15 days post anthesis unless otherwise stated, were aseptically
13 dissected from developing *C. sativa* seeds and transferred to 6 well culture plates. Each well
14 contained culture medium (1.0 ml, composition specified below), with 5 embryos per well. The
15 plate lid was attached with Parafilm and the plate incubated at 20°C with gentle rocking (0.2-0.3
16 Hz) in green light at 10-12 μ E. This light regime approximates that reaching the seed in the
17 silique. The culture medium reflects the composition of the embryo sac fluid (Cocuron, J.C. and
18 Shachar-Hill, Y., unpublished results). It contained alanine (4 mM), glutamine (8 mM), glucose
19 (130 mM) and sucrose (12 mM) as macronutrients. It also included Gamborg's vitamins, and
20 mineral micronutrients as used for *B. napus* embryo culture [Schwender and Ohlrogge, 2002]
21 but minus the ammonium and potassium nitrates, so that the only N source is from amino acids.
22 pH was maintained at 6.3 with 20 mM HEPES while 20% (w/v) polyethylene 4000 provided the
23 osmoticum.
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26 For embryo growth treatments or labeling, after one day in culture an additional 0.5 ml of
27 medium containing substrates was added per well. These aliquots contained either NaOAc or
28 [¹³C₂]NaOAc (Aldrich 282002, 99 atom% ¹³CH₃¹³COOH), or glycerol or [¹³C₃]glycerol (Aldrich
29 489476, 99 atom %), at concentrations specified in each experiment. When a radiotracer was
30 required, the added aliquot also included [1-¹⁴C]NaOAc (Perkin Elmer NEC084H001, 2.102
31 MBq/mol, 0.2775 MBq) or [U-¹⁴C]glycerol (Perkin Elmer NEC441X, 5.217 MBq/mol, 0.235 MBq).
32 For tracer labeling after the allotted incubation time, the embryos quickly washed with H₂O, and
33 heated at 80-85°C in *i*PrOH for 10-15 minutes to inactivate lipases. To measure embryo growth,
34 embryos were removed at defined times, rinsed in DI H₂O, blotted dry and transferred to pre-
35 weighed vials for weighing. After obtaining fresh weights, the embryos were lyophilized to give
36 dry weight. The freeze-dried embryos were then heated at 80-85°C in *i*PrOH for 10-15 minutes
37 to inactivate lipases prior to lipid extraction. Lipids were extracted with hexane-*i*PrOH according
38 to the method of Hara and Radin (1978). Tripentadecanoin was added during extraction as an
39 internal standard for fatty acid quantification. After extraction the total lipids were stored in
40 toluene at -20°C until aliquots were withdrawn for the variety of lipid analyses described below.
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43 44 **5:3 Lipid and Fatty Acid Analysis**

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46 Transmethylation of total lipids or lipid fractions from preparative TLC was accomplished using
47 the biphasic KOH-MeOH-heptane protocol of Ichihara et al. (1996). FAME composition and
48 content were analyzed by GC with FID detection (Agilent 6890N, split injection at 250°C, oven
49 temperature ramp from 140°C to 230°C at 5°C/min) on a DB-23 capillary column (30m x 0.25
50 mm id, 0.25 μ film thickness). Peak areas were corrected for C(H) response [Bannon et al.,
51 1986; Craske and Bannon, 1987].
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54 55 **5:4 ¹⁴C Lipid and Fatty Acid Analysis**

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57 Aliquots of the total lipid extract in toluene were assayed for radioactivity by liquid scintillation
58 counting. To analyze [¹⁴C]FAME composition, aliquots of total FAMEs were separated by both
59 AgNO₃ and reversed phase TLC. K6 TLC plates were impregnated with 7.5% (w/v) AgNO₃
60 solution in CH₃CN. After drying and sample application, they were developed halfway in
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4 toluene-CH₃CN (98:2), quickly air-dried and developed fully in toluene. KC18F reversed phase
5 TLC plates were developed using MeOH-CH₃CN-H₂O (65:35:0.5). TLC plates with ¹⁴C labeled
6 samples were subject to autoradiography using Kodak Phosphor Screens GP. After exposure
7 the screens were scanned using the Bio-Rad PMI FX phosphoimager and radioactivity bands
8 quantified using Bio-Rad Quantity One basic software. Neutral lipids were separated on silica
9 TLC plates developed with toluene-Et₂O (7:3); polar lipids were separated on silica TLC plates
10 developed with CHCl₃-MeOH-AcOH-H₂O (85:15:5:2).
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13 **5:5 ¹³C Lipid and Fatty Acid Analysis**

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15 For analysis of the ¹³C isotopomer distributions, TAGs were first purified by preparative TLC on
16 silica TLC plates developed with toluene-EtOAc (88:12). The recovered TAGs were recovered
17 by elution from the silica with CHCl₃-MeOH (2:1) and then hydrogenated by stirring in heptane-
18 EtOAc (3 ml, 9:1) at slightly greater than atmospheric pressure of H₂ in the presence of platinum
19 (VI) oxide catalyst (ca. 2 mg). Complete reaction was achieved within 2 hours at room
20 temperature, after which the catalyst was decanted. For samples from [¹³C₂]acetate labeling,
21 hydrogenated FAMES were recovered prepared from TAGs by the biphasic KOH-MeOH-
22 heptane transmethylation protocol, and analyzed by mass spectrometry using an Agilent 6850-
23 5975MSD GC-MS system. FAMES were separated on a DB-5 capillary column (30m x 0.25 mm
24 id, 0.25 film thickness) using splitless injection at 310°C and a temperature ramp from 120°C to
25 310°C at 10°C/min, with eluent analysis by electron impact mass spectrometry.
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28 The hydrogenated TAGs were analyzed by ESI-MS. TAG samples were dissolved in *i*PrOH,
29 typically at 10 µg/ml. ESI-MS in positive ion mode was performed by direct infusion with a
30 Shimadzu (Columbia, MD) SIL-5000 autosampler into a Waters (Milford, MA) Quattro micro
31 mass spectrometer. Sample solutions (10µl) were introduced to the electrospray source by flow
32 injection into a 97:3 *i*PrOH:10 mM aqueous NH₄OAc buffer flowing at 0.1 ml/min. The capillary,
33 extractor, and cone voltages were 3.2 kV, 2.0 V, and 25 V respectively. The source and
34 desolvation temperatures were 110 and 350 °C respectively; the desolvation gas flow rate was
35 400 l/hr. Mass spectra were collected for 2 min; the *m/z* range scanned in the MS
36 measurements was from 600 to 1100 (1 sec/scan). Mass spectra data were acquired with
37 MassLynx 4.0 software; [TAG-NH₄]⁺ ion peaks were smoothed and integrated using QuanLynx
38 software.
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44

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Figure Legends

Fig. 1: Incorporation of [$1\text{-}^{14}\text{C}$]acetate into labeled lipids by cultured developing embryos from *C. sativa*. Data for three individual assays (x) and the average value (■) shown. (A) Concentration dependence for 0.05-10.1 mM acetate, 6 hour assay. (B) Time course (6 hr). (C) Time course (2 day).

Fig. 2: Mass spectrometry of ^{13}C -labeled fatty acids to assess label penetration into *C. sativa* embryos. (A) Shows mass spectra for the molecular ion cluster (M) of methyl stearate as run by GC-MS, with $m/z = 298$ intensity set at 100%. From left to right are: unlabeled stearate (Control); labeled stearate from transmethylation of hydrogenated TAGs produced in a 48 hour assay of cultured embryos with 2.6 mM [$^{13}\text{C}_2$]acetate (average +SD, $n = 9$); and the control spectrum subtracted from the labeled spectrum, then the resulting difference spectrum corrected for natural abundances to reveal the biosynthetic distribution of ^{13}C -label present (Calcn). (B) and (C) describe a simulation whereby various mole fractions of [$^{13}\text{C}_2$]acetate units are incorporated randomly by fatty acid synthesis into C18 fatty acids. (B) shows the predicted biosynthetic isotopomer distribution for species with increasing [$^{13}\text{C}_2$] $_n$ labeling. Plots of the stimulated intensity ratios are shown in (C). The experimental intensity ratios determined from Calcn are $(M+4)/(M+2) = 0.217$ and $(M+6)/(M+2) = 0.027$ (dotted lines). At 5% acetate incorporation the intensity ratios from the simulation are 0.211 and 0.026 respectively. Thus the utilization of exogenous acetate is expected to be close to 5%.

Fig. 3: Effect of acetate concentration on lipid accumulation in cultured *C. sativa* embryos. Embryos were harvested at 15 days post anthesis and allowed to grow for 1 day in basal medium before supplementation with 0-10 mM acetate. Growth was measured over the ensuing 4 days. (A): Accumulation of dry weight (○,●, hatched line) and total fatty acids (□,■, solid line) over the 4 day period, with data for three individual assays (open symbols) and the average value (closed symbols) shown. (B): Accumulation of individual fatty acids (average + SD, $n = 3$).

Fig. 4: Kinetics of total fatty acid accumulation by cultured *C. sativa* embryos in the presence of acetate. Two sets of kinetic assays were run, one with 0 or 2.5 mM acetate, the second with 0 or 8 mM acetate. Data for three individual assays (open symbols) and the average value (solid symbols) are shown.

Fig. 5: Acyl flux precursor-product correlations for desaturation and elongation derived from *C. sativa* embryos cultured for 4 days with 0-10 mM acetate. Each data point represents the molar sum of a set of fatty acids specified in (A) which are accumulated by 10 embryos in 4 days of culture, and is the average ($n = 3$ assays) for a specific acetate concentration from a specific experiment. Data points in 5B and 5C are compiled from five independent culture experiments. (A) shows the biosynthetic precursor-product relations and the grouping of fatty acids used as either potential substrate or end product. (B) shows the relationships between oleate production and 12-desaturation ("FAD2") or chain elongation ("FAE1"). (C) shows the relationship between 12-desaturation ("linoleate") and subsequent 15-desaturation ("FAD3").

Fig. 6: Labeling of cultured *C. sativa* embryos with 0.02-2 mM glycerol for either 8 or 16 hours. Data for three individual assays (x) and the average value (■) shown. Panel (A) shows the concentration dependence of activity for incorporation into lipid glyceryl backbones, with the insert plotting $1/S$ versus $1/V$. Panel (B) shows the mole fraction of glycerol contributing to acyl group labeling relative to glycerol labeling of total lipids.

Fig. 7: Glycerol does not significantly affect *C. sativa* embryo growth rate or lipid deposition. (A) Fresh weight (□, 0 mM glycerol; ■, 3 mM glycerol) and dry weight (○, 0 mM glycerol; ●, 3 mM glycerol) accumulations. (B) Total fatty acid accumulations as determined by GC of FAME (○, 0 mM glycerol; □, 3 mM glycerol). Individual assays are represented by small symbols, averaged values (n = 3) by large symbols; dotted lines are for the control (0 mM glycerol), while solid lines are for embryo culture with 3 mM glycerol.

Fig. 8: Mass spectrometry of [$^{13}\text{C}_n$]glycerol-labeled TAGs, and accumulation of labeled TAGs with time. Panels A-D show the molecular ion clusters for [$^{13}\text{C}_3$ -glyceryl]tripalmitin and ^{13}C -labeled C54:0-TAG, obtained by hydrogenation, along with the unlabeled controls, analyzed as ammonium adducts by ESI-MS. Panel E shows the time course for accumulation of [$^{13}\text{C}_3$]TAG and total TAGs over the extended incubation of *Camelina* embryos with [$^{13}\text{C}_3$]glycerol. Further details of this analysis are to be found in Supplement Figure 3.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7: Glycerol does not significantly affect embryo growth rate or lipid deposition

Figure 8