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Lipid labeling from acetate or glycerol in cultured embryos of *Camelina sativa* seeds: A tale of two substrates

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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.

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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 quantitatively reflect *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 ^{13}C isotopomer analysis while maintaining normal biosynthetic rates for over 24 h. 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 Δ12-desaturation or elongation are independent of biosynthetic flux, Δ15-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.

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., 2015; 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., 2015), 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., 2004; 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).

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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 ^{2}H–C or ^{3}H–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 h 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 10 mM (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 Fig. 1A, and provides for a much lower maximum fractional incorporation than seen in leaves. Maximum incorporation into total lipids occurred at $\geqslant$1 mM acetate, with a value in this particular experiment of 45 pmol acetate/min/embryo. Fig. 1B shows time courses for two experiments, run for 8 and 48 h, respectively, using 2.6 mM acetate. The cultured embryos showed linear incorporation with rates of 30 and 33 pmol 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., 2015). This requires approximately 550 rising to 1650 pmol acetyl

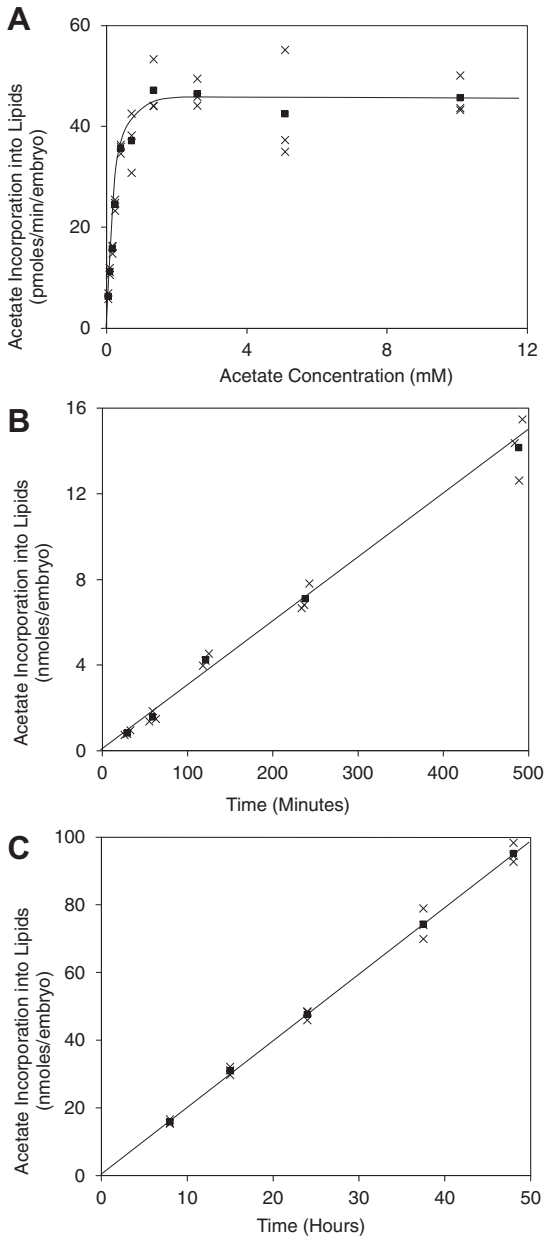

Fig. 1. Incorporation of [1-^{14}C]acetate into labeled lipids by cultured developing embryos from *C. sativa*. Data for three individual assays (×) and the average value (■) shown. (A) Concentration dependence for 0.05–10.1 mM acetate, 6 h assay. (B) Time course (8 h). (C) Time course (2 day).

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 $(^{13}C_2)_n$ 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 Fig. 1A 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 h 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

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