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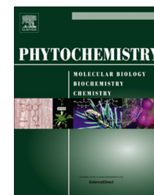

Lipid analysis of developing *Camelina sativa* seeds and cultured embryos

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Dedicated to Professor Frank D. Gunstone, who was Mike Pollard's PhD advisor.

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ABSTRACT

Camelina sativa is a cultivated oilseed rich in triacylglycerols containing oleic, linoleic, α-linolenic and eicosenoic acids. As it holds promise as a model species, its lipid synthesis was characterized *in vivo* and in culture. Lipid accumulates at a maximum rate of about 26 μg/day/seed (11.5 mg lipid/day/g fresh seed weight), a rate comparable with other oilseeds. Noteworthy is a late stage surge in α-linolenic acid accumulation. Small amounts of unusual epoxy and hydroxy fatty acids are also present in the triacylglycerols. These include 15,16-epoxy- and 15-hydroxy-octadecadienoic acids and homologous series of ω7-hydroxy-alk-ω9-enoic and ω9/10-hydroxy-alkanoic acids. Mid-maturation embryos cultured *in vitro* have growth and lipid deposition rates and fatty acid compositions that closely match that of seeds, but extended culture periods allow these rates to rise and surpass those observed *in planta*. Optimized thin layer chromatography systems for characterization of labeled products from acetate or glycerol labeling are described. Glycerol label is only found in acylglycerols, largely as the intact glyceryl backbone, but acetate can label acyl groups and sterols, the latter to a much higher relative specific activity. This presumably occurs because mevalonic acid precursor is derived from the non-plastid pool of acetyl-CoA that is also the source for malonyl-CoA to drive FAE1-dependent chain elongation. Particular attention has been paid to the separation of sterols and diacylglycerols, and to hydrogenation of triacylglycerols to simplify their analysis. These improved methods will allow more accurate analyses of the fluxes of lipid metabolism in cultured plant embryos.

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1. Introduction

Camelina sativa has a long history as an Old World seed crop. It has recently been introduced into the US where it is regarded as a new industrial oilseed crop and is grown largely in the Pacific Northwest for the potential purpose of producing oil to be converted into biofuel (methyl and/or ethyl fatty acid esters) (Frohlich and Rice, 2005; Moser and Vaughn, 2010). It fits the need for lower inputs and intercropping in dry land farming. *C. sativa* has also become a useful model to study oil biosynthesis (Horn et al., 2013; Kang et al., 2011; Liang et al., 2013; Mansour et al., 2014; Mudalkar et al., 2014). A member of the *Brassicaceae*, it has several advantages over *Arabidopsis thaliana*. Like Arabidopsis, genetic transformation is facile (Lu and Kang, 2008), and several generations can be grown in a year. However, it produces seeds that are about 50 times larger than those of Arabidopsis, making it preferable for biochemical studies. Thus Camelina is also attractive for oilseed engineering experiments because promising lines can be readily scaled up to produce significant quantities of test oils (Liu et al., 2015). Given these advantages, it is timely to develop a quantitative flux map for lipid biosynthesis in developing Camelina embryos.

The pathways of fatty acid and triacylglycerol (TAG) synthesis in developing seeds have been extensively investigated, with biochemical and genetic studies providing information for genetic engineering of oilseed crops to produce more oil, to modulate fatty acid composition, or to produce novel oils, usually containing uncommon fatty acids. Enzymes responsible for unique facets of fatty acid metabolism, including downstream acyl transferases, have been identified and the corresponding genes cloned and expressed, with various levels of success in optimizing yields of novel oil products (Cahoon et al., 2007; Clemente and Cahoon, 2010; Napier et al., 2014). However, understanding the lipid metabolism of seeds requires not just a pathway and an accounting of genes, from transcription factors to enzymes, but an understanding of the fluxes and their regulation, particularly if maximizing a particular composition is desired. Unlike central metabolism, where isotopomer imprinting of reporter molecules and mass balances are used to determine fluxes, lipid flux measurements require kinetic labeling. Previously such a study was carried out on cultured embryos from developing soybeans using short term

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(⩽30 min) labeling with ^{14}C-substrates, to quantify the substantial cyclic fluxes of acyl groups through membrane lipids (Bates et al., 2009).

To extend such studies to stable isotope labeling with its associated isotopomer analysis of acylglycerols, one pre-requisite is longer duration labeling times. Longer assay times will also provide a very complementary kinetic data set for modeling when added to that from initial rate studies. In this paper, lipid deposition by cultured zygotic embryos from developing *C. sativa* seeds is compared with seed development in siliques on the plant. The cultured embryos closely mirror lipid deposition *in planta*, both in terms of their rates of lipid accumulation and their fatty acid composition. In addition, minor epoxy- and hydroxy-fatty acids were identified. The presence of these acids is accentuated 3-fold by their distinctive TAG molecular species. Separation methods have been customized to allow better analyses of labeled fatty acids and lipids specifically from Camelina embryos, with particular attention paid to the TLC separation of sterols and diacylglycerols, and to hydrogenation of triacylglycerols to simplify their analysis. In a companion paper (Pollard et al., 2015), aspects of long term acetate and glycerol labeling at concentrations sufficient to permit stable isotope isotopomer analysis of acylglycerol products are described. These two papers provide the baseline information required to use cultured embryos for the quantitative analysis of lipid biosynthesis in developing *C. sativa* seeds.

2. Results and discussion

2.1. Fatty acid and lipid accumulation in *C. sativa* seeds during development

The embryo is the main storage tissue of *C. sativa* seeds. Knowledge of embryo development, and that of the entire seed, is a foundation for understanding the biochemistry and genetics of lipid deposition. This data was used to define the best age to harvest embryos for culture. Since dissection of embryos from seeds is difficult beyond mid-maturation, seed development was tracked from 5 to 50 days post anthesis (DPA) (Fig. 1). At 5 DPA, embryos are at "torpedo" stage or entering "linear cotyledon" stage. They pass through the "green cotyledon" stage at mid-maturation (14–20 DPA), a period where the rate of oil synthesis is maximal, after which the embryos slowly turn yellow and desiccate.

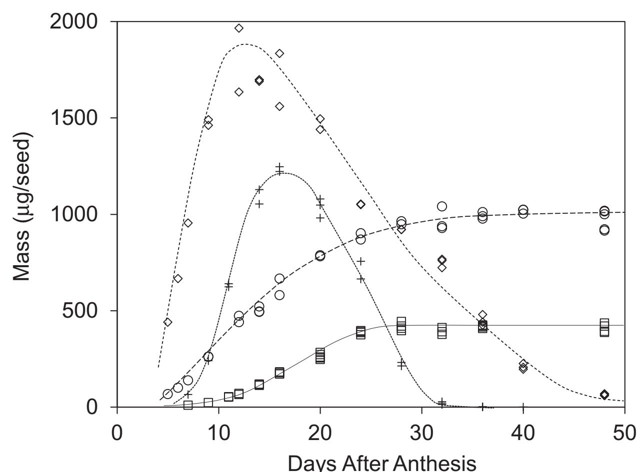

Fig. 1. Lipid (□), dry matter (○), moisture (◇) and chlorophyll (+) accumulation by *C. sativa* seeds during development. Individual data points are shown, with each data point representing 150–300 seed sample harvested from multiple plants. Chlorophyll is expressed as ng/seed rather than µg/seed.

Chlorophyll levels peak at 16 DPA, at about 1 mg/g fresh wt. in the embryos. This peak chlorophyll concentration on a fresh weight basis is about half of that found in *C. sativa* leaves, and indicates a substantial development of photosynthetic machinery in the embryo at mid-maturation. The mid-point for oil deposition occurs at 17–18 DPA. At mid-maturation, the oil accumulation rate is 26 µg lipid/seed/day (that is, about 11.5 mg lipid/g fresh wt/day). Most of the oil deposition is associated with the embryo and the product is TAG. For comparison, a number of oilseed species grown in the greenhouse had oil deposition rates from 2.75 (soybean) to 40 mg lipid/g fresh wt/day (*Cuphea lutea*), with safflower and rapeseed at 15.5 and 12.5 mg lipid/g fresh wt/day respectively (Singh et al., 1986). Data presented on Arabidopsis seed development (Baud et al., 2002) allows the Arabidopsis lipid accumulation rate at mid-maturation to be estimated at 19 mg lipid/g fresh wt/day. At maturity, the oil content (dry weight basis) of *C. sativa* seeds from growth chamber plants was 42.1 ± 1.6% (n = 12), which compares with reported values of 29.9–38.3% for 13 accessions of field grown plants (Budin et al., 1995) and 15.5–41.7% for 40 accessions of field grown plants (Rodriguez-Rodriguez et al., 2013). In the latter study, seed development was also tracked for a line grown in the growth-chamber. The data are broadly similar to those reported here, but the maximum rate was lower (15 µg lipid/seed/day) while the midpoint for oil deposition occurred three days later (21 DPA). These differences are likely to be attributed to different cultivars and growth conditions between the studies.

The seed oil of mature, wild type Camelina seeds is rich in polyunsaturated fatty acids, with α-linolenic (C18:3) acid being the most abundant fatty acid (Budin et al., 1995; Zubr and Matthus, 2002; Abramovic and Abram, 2005; Rodriguez-Rodriguez et al., 2013). In common with other members of the Brassicaceae, *C. sativa* seeds contain significant amounts of C20–C24 very long chain fatty acids (VLCFA), primarily eicos-11-*cis*-enoic acid (C20:1). The accumulations of the individual fatty acids are shown in Fig. 2A. Eicos-11-*cis*-enoic acid is first detected at 11 DPA. It and the other VLCFA are presumably synthesized by an elongase homologous to *AtFAE1* (Millar and Kunst, 1997). *FAE1* expression can serve as a marker for seed-specific lipid synthesis (Girke et al., 2000; Ruuska et al., 2002), so the appearance of C20:1 provides a useful determinant for the onset of the seed maturation. The deposition of minor VLCFAs, such as eicosanoic acid (C20:0) and erucic acid (C22:1), closely parallels that of C20:1. The rates of accumulation of individual fatty acids are shown in Fig. 2B. At no time are the rates negative for any fatty acid, which would indicate removal of acyl groups from TAG for further desaturation, elongation or turnover. Of particular note is the accelerating accumulation of C18:3, such that by 22 DPA it constitutes about 60% of the rate of total fatty acid accumulation. Equally noteworthy is the abrupt drop in the deposition rate of C18:3 as the maturation phase draws to a close. The rate of oleic acid (C18:1) deposition shows a strong dip during mid-maturation consistent with its role as precursor for linoleic acid (C18:2), C18:3 and C20:1 biosynthesis. Also, in late seed maturation, stearic acid (C18:0) accumulation is greatly reduced relative to palmitic acid (C16:0).

The fatty acid content and composition of the embryo major lipid classes at 16 DPA are shown in Supplement Table 1. This age was chosen as it is the starting age for embryos in the kinetic experiments (see below, and (Pollard et al., 2015)). The data can be used to calculate limiting net flux values for acyl groups moving between lipid classes. Total lipids contained 383 ± 24 (SD, n = 3) nmoles of fatty acids/embryo, while 326 ± 17, 3.4 ± 0.1 and 28.0 ± 1.4 nmoles/embryo respectively were recovered in TAG, diacylglycerols (DAG) and polar lipids (PL) (i.e. mainly membrane lipids). The sum of TAG + DAG + PL = 357.1 nmoles/embryo, which is ~93% recovery from total lipid acyl groups, but when epoxy-

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