

Bottlenecks in erucic acid accumulation in genetically engineered ultrahigh erucic acid *Crambe abyssinica*

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Summary

Erucic acid is a valuable industrial fatty acid with many applications. The main producers of this acid are today high erucic rapeseed (*Brassica napus*) and mustard (*Brassica juncea*), which have 45%–50% of erucic acid in their seed oils. *Crambe abyssinica* is an alternative promising producer of this acid as it has 55%–60% of erucic acid in its oil. Through genetic modification (GM) of three genes, we have previously increased the level of erucic acid to 71% (68 mol%) in *Crambe* seed oil. In this study, we further investigated different aspects of oil biosynthesis in the developing GM *Crambe* seeds in comparison with wild-type (Wt) *Crambe*, rapeseed and safflower (*Carthamus tinctorius*). We show that *Crambe* seeds have very low phosphatidylcholine-diacylglycerol interconversion, suggesting it to be the main reason why erucic acid is limited in the membrane lipids during oil biosynthesis. We further show that GM *Crambe* seeds have slower seed development than Wt, accompanied by slower oil accumulation during the first 20 days after flowering (DAF). Despite low accumulation of erucic acid during early stages of GM seed development, nearly 86 mol% of all fatty acids accumulated between 27 and 50 DAF was erucic acid, when 40% of the total oil is laid down. Likely bottlenecks in the accumulation of erucic acid during early stages of GM *Crambe* seed development are discussed.

Keywords: erucic acid, seed oil, *Crambe abyssinica*, biosynthesis, acylation, enzymes.

Introduction

There is an increasing utilization of seed oils as renewable resources in industrial feedstocks, especially those accumulating unusual fatty acids (FAs) that may vary in carbon chain length, number and position of unsaturated bonds or contain specialized functional groups (Dyer *et al.*, 2008; Carlsson *et al.*, 2011). Erucic acid (*cis*-13-docosenoic acid, 22:1), a very long-chain FA commonly found in seed oils of Brassicaceae, is an industrially important feedstock (Temple-Heald, 2004). High purity of desirable FAs is essential for the industrial application of target plant oils (Carlsson *et al.*, 2011). Therefore, it is necessary to understand in detail the synthesis and metabolism of uncommon FAs, such as erucic acid in developing seeds in order to further increase its content by using gene technology and other plant breeding methods.

In developing oilseeds, triacylglycerols (TAG) biosynthesis is initiated with *de novo* FA synthesis in the plastid. In most oil seeds, the major forms of FAs exported from the plastid is oleoyl-CoA (18:1-CoA), palmitoyl-CoA (16:0-CoA) and stearoyl-CoA (18:0-CoA) (Bates *et al.*, 2013). Erucic acid (22:1) is synthesized outside the plastid by the malonyl-CoA-dependent elongation of 18:1 catalysed by the condensing enzyme fatty acid elongase (FAE), a 3-keto reductase, a 3-hydroxy hydratase and an enoyl reductase. It has been suggested that part of the 18:1 exported from the plastid is diluted with a pre-existing oleate pool in the cytosol before elongation to erucic acid (Hlousek-Radojic *et al.*, 1995; Bao *et al.*, 1998). From studies of pea leaves and soybean embryos, it has been concluded that there is major acyl flux of 18:1 from the plastid into phosphatidylcholine (PC) where further modification like desaturation and hydroxylation occurs before the acyl groups are used for *de novo* glycerol lipid synthesis (Bates

et al., 2007, 2009; Bates and Browse, 2011; Bates *et al.*, 2012, 2013).

The direct lipid precursor for TAG assembly is diacylglycerol (DAG) (Bates *et al.*, 2013), which represents an important branch point between TAG and membrane lipids synthesis (Li-Beisson *et al.*, 2013). Isotope labelling studies have shown that there are kinetically distinct DAG pools in developing soybeans used for PC and TAG synthesis (Bates *et al.*, 2009; Bates and Browse, 2012).

De novo DAG synthesis is formed by the sequential esterification of glycerol-3-phosphate (G-3-P) at *sn*-1 and *sn*-2 position and forming phosphatidic acid (PA) which is then dephosphorylated. This process, known as Kennedy pathway (Kennedy, 1961), involves three key enzymes: glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT) and phosphatidic acid phosphatase (PAP). Specialized LPAATs have been cloned from coconut endosperm and *Limnanthes douglasii* seeds showing high specificities for lauroyl-CoA and 22:1-CoA, respectively (Hanke *et al.*, 1995; Knutzon *et al.*, 1999). Despite that plants in the Brassicaceae family accumulate high amounts of 22:1 in their seed oils, LPAATs from these species have been found incapable of acylating 22:1-CoA to lysophosphatidic acid (LPA) (Frentzen, 1993), and thus, this FA is virtually absent from the *sn*-2 position of their seed TAG. This block might partly explain why no *Brassica* species have more than two-thirds of the total oil as erucic acid.

Apart from *de novo* synthesis, DAG can also be derived from PC by interconversion between these two molecules catalysed by a phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT) (Lu *et al.*, 2009; Hu *et al.*, 2012). Such an interconversion reaction has also been proposed to be catalysed by the reverse reaction of CDP-choline: diacylglycerol cholinephospho-

transferase (CPT), the enzyme responsible for the net synthesis of PC (Slack *et al.*, 1983, 1985). The polyunsaturated FAs (PUFA), linoleic and linolenic acids, are produced when the precursor FAs are esterified in PC (Bates *et al.*, 2013). These acids could enter DAG by the interconversion between DAG and PC or by the transfer of acyl groups from PC to the acyl-CoA pool that is used in the G-3-P pathway. The latter transfer has been suggested to be catalysed by the reverse action of acyl-CoA: lysophosphatidylcholine acyltransferase (LPCAT) (Stymne and Stobart, 1984) or by phospholipase A (PLA) and subsequent activation to acyl-CoA by acyl-CoA synthetases (Bates *et al.*, 2013). PC-DAG interconversion and LPCAT-mediated acyl-editing mechanism have been suggested to control the two-thirds of acyl fluxes of PUFA from PC to TAG in Arabidopsis (Bates *et al.*, 2012).

The esterification of *sn*-3 position of DAG to form TAG is catalysed by diacylglycerol acyltransferase (DGAT) (Stymne and Stobart, 1987) or the acyl-CoA-independent phospholipid/diacylglycerol acyltransferase (PDAT) (Ståhl *et al.*, 2004). Two different classes of DGATs have been identified and suggested to possess different substrate specificities, and their contribution to TAG biosynthesis may vary among different oilseed species (Bates *et al.*, 2013). DGAT1 and PDAT1 have been found to have compensating functions in TAG production in Arabidopsis (Zhang *et al.*, 2009). Castor bean PDAT has been shown to have high activity towards hydroxylated FAs (Dahlqvist *et al.*, 2000), and its expression increased the accumulation of hydroxy FA in the seed oil of transgenic Arabidopsis expressing castor bean hydroxylase (Van Erp *et al.*, 2011).

Although the FA compositions in PC and TAGs of the common five acyl groups, 16:0, 18:0, 18:1, 18:2 and 18:3, reflect each other in most oil seeds, this is not the case for many unusual FAs (Dyer *et al.*, 2008). They could occur in very high amounts in TAG, whereas their contents are very low in PC. These unusual TAG-specific FAs can be divided into two groups. One group consists of those FAs that are synthesized while being esterified to PC, catalysed by divergent FAD2-like enzymes. These include hydroxylated, acetylenic, epoxygenated and conjugated FAs (Dyer *et al.*, 2008). In these cases, there must be mechanisms of removing these FAs from PC with high selectivity and channelling them into TAG. The other group consists of unusual FAs that are not produced on PC, such as medium chain and very long-chain FAs. Erucic acid belongs to the second group as it is synthesized by elongation of 18:1-CoA to 22:1-CoA. The exclusion of medium chain FAs from PC has been studied in some details in *Cuphea lanceolata*, a species having TAG with about 85% carpic (10:0) FAs (Bafar *et al.*, 1990). One of the mechanisms preventing medium chain fatty acid to enter PC from DAG in *Cuphea* seeds was suggested to be very low PC-DAG interconversion. Here, we report on the metabolism of erucoyl groups through analysing the enzymatic activities in microsomal fractions from developing seeds of wild-type (Wt) *Crambe abyssinica*, a species accumulating high amount of erucic acid in its seed oil, and a genetic modified (GM) high erucic acid *Crambe* line with over 70% of erucic acid in its seed oil. These results are compared with the metabolism in microsomal fractions from developing seeds of safflower and rapeseed. Further, *in vivo* [^{14}C]glycerol-labelling data in rapeseed and *Crambe* as well as the FA amount and composition in PC, DAG and TAG at different stages of *Crambe* seed development are presented. Based on these results, we discuss possible mechanisms in the exclusion of erucoyl groups from PC

in *Crambe* as well as limiting factors in producing oils with even higher amount of erucic acid in GM *Crambe*.

Results

Acylation of 18:1-CoA to glycerol backbone in microsomes from developing oil seeds

Microsomal preparation from developing seeds of *Crambe* and rapeseed (20 days after flowering, DAF) and safflower (18 days after pollination, DAP, Stobart *et al.*, 1997) were incubated with [^{14}C]G-3-P and nonradioactive 18:1-CoA for 20, 40 and 80 min, and the radioactivity in the different lipids was determined. We used microsomes from both Wt *Crambe* and a GM *Crambe* line expressing a *LdLPAAT* gene, a *CaFAD2-RNAi* and a *BnFAE1* with increased erucic acid content (Li *et al.*, 2012). Wt *Crambe*, GM *Crambe* and rapeseed microsomes all incorporated radioactivity in a very similar manner with PA being the dominant labelled lipid at all the time points. DAGs and TAGs showed a time-dependent increase but only very small amounts of radioactivity accumulated in PC (Figure 1). A similar incorporation pattern was also seen in microsomes prepared from developing seeds of an erucic acid producing turnip rape (*Brassica rapa*), except that a significant higher amount of label was found in PC (Figure S1). In contrast, safflower microsomes showed quite different labelling pattern of the lipids. Radioactive PA was efficiently converted to DAG from which about equal amount of radioactivity was transferred to PC and TAG (Figure 1). A similar incorporation pattern for the glycerol backbone from [^{14}C]G-3-P and 18:1-CoA has previously been reported in microsomal preparation of safflower seeds (Griffiths *et al.*, 1985).

The inefficient conversion of PA to DAG in *Crambe*, rapeseed and turnip rape microsomes (Figures 1 and S1) indicates that the phosphatidic acid phosphohydrolase (PAP) has a significant control of flux of acyl groups into TAG. However, the PAP is only partially bound to the membranes, at least in safflower (Ichiyama *et al.*, 1990), and thus a substantial part of this activity might have been lost during preparation of the microsomal fractions.

The high proportion of radioactivity in DAG and TAG compared with PC in Wt *Crambe* and rapeseed indicates much lower DAG-PC interconversion rates in these microsomes than in safflower and, presumably, low PDCT activities, the enzyme catalysing such interconversion (Lu *et al.*, 2009).

We incubated the same microsomal fractions with glycerol-labelled [^{14}C]18:1-LPA and nonradioactive 18:1-CoA (Figure 2). PA was rapidly formed in assays with all membranes and was very similar metabolized as in incubation with [^{14}C]G-3-P and 18:1-CoA. We then compared the acylation of glycerol-labelled 18:1-LPA with incubations with glycerol-labelled [^{14}C] 22:1-LPA with nonradioactive 18:1-CoA (Figure 3). Contrary to the metabolism of 18:1-LPA, the 22:1-LPA was acylated much slower to PA in microsomes for all the species (Figure 3). The higher conversion into PA with the GM *Crambe* membranes than with the Wt *Crambe* and rapeseed indicates that the introduced *Limnanthes* LPAAT accepts 22:1-LPA somewhat better than the endogenous LPAATs in this species. However, it could also be due to general higher LPAAT activity, as indicated by more complete acylation of 18:1-LPA by the membranes of GM *Crambe* than Wt *Crambe* (Figure 2). PC was hardly labelled except in safflower membranes, where it was the major metabolite after 80 min, indicating that the PDCT did not exclude DAG species with erucoyl groups.

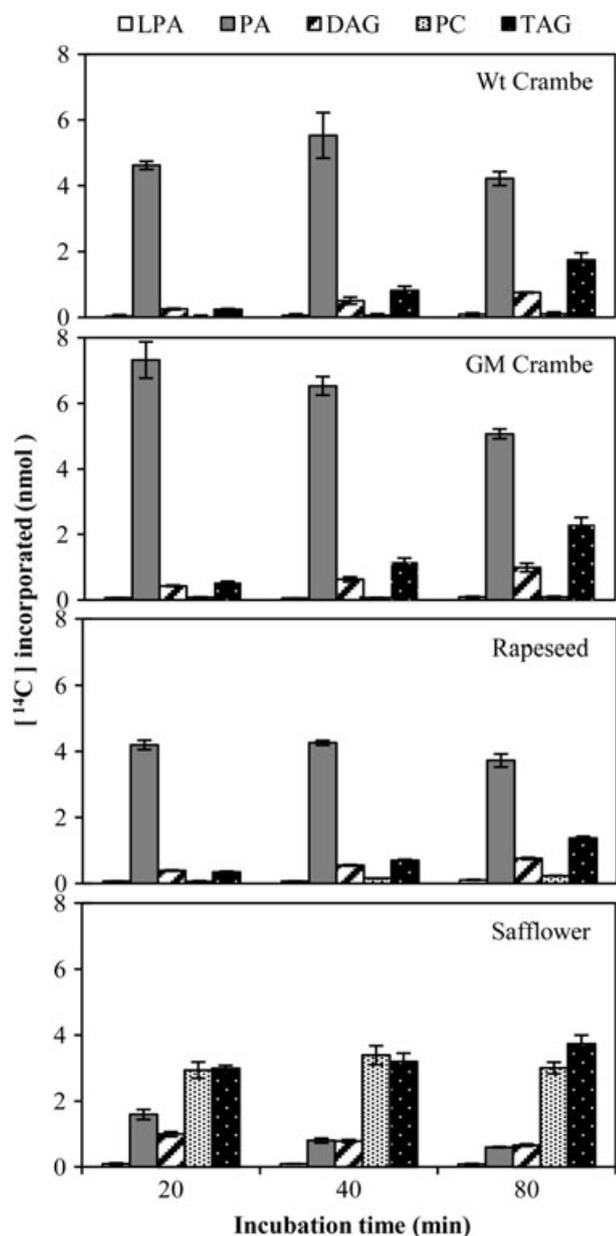


Figure 1 Time course incorporation of radioactivity into various lipids in microsomal preparations from developing seeds of different species, incubated with [^{14}C]glycerol 3-phosphate and nonradioactive 18:1-CoA. Wt, wild type; GM, genetically modified; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerols. Results are shown from triplicate samples \pm SD.

Acylation of 22:1-CoA to the glycerol backbone in microsomes from developing oil seeds

Incubations of Wt and GM Crambe and safflower microsomes with [^{14}C]G-3-P and 22:1-CoA yielded only 2.5%–3.5% of the radioactivity seen in complex lipids with 18:1-CoA and [^{14}C]G-3-P substrates (compare Figures 1 and 4). In Wt Crambe, the LPA substrate remained the most labelled lipid at all incubation times. The next highly labelled lipid was comigrating with monoacylglycerol (MAG) according to the authentic standard. The most likely origin of this compound is dephosphorylation of the formed

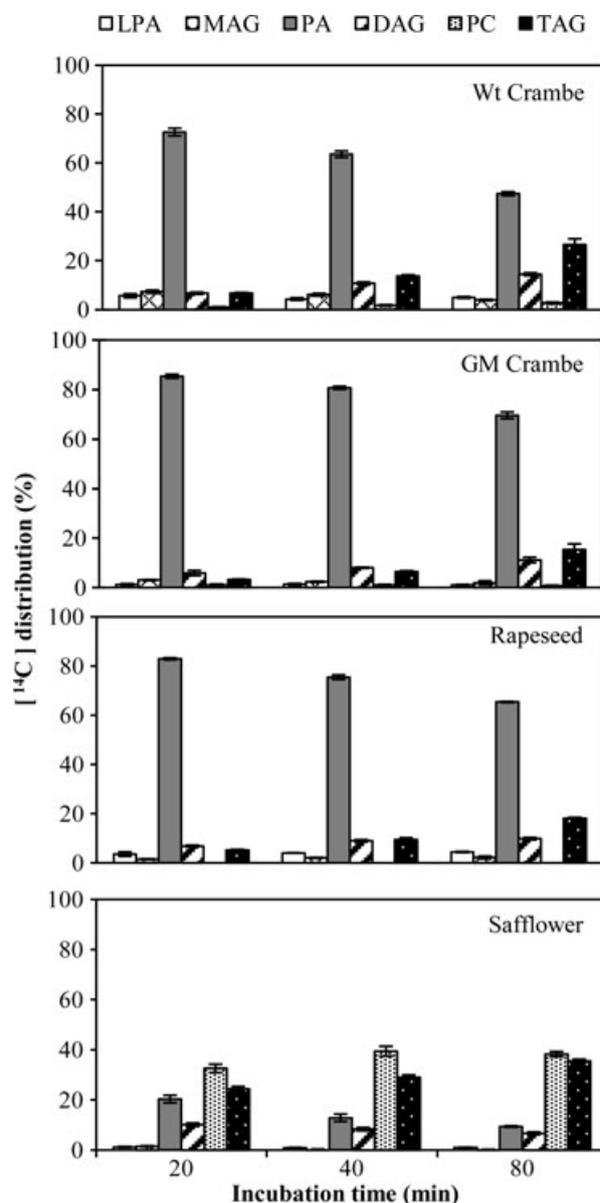


Figure 2 Time course incorporation of radioactivity into various lipids in microsomal preparations from developing seeds of different species, incubated with glycerol-labelled [^{14}C]18:1-LPA and nonradioactive 18:1-CoA. Wt, wild type; GM, genetically modified; LPA, lysophosphatidic acid; MAG, monoacylglycerol; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerols. Results are shown from triplicate samples \pm SD.

lyso-PA by a phospholipase of C type. Only a very small amount of PA was produced. In GM Crambe, a substantial amount of the LPA was converted to PA, demonstrating that the introduced *Limnanthes* LPAAT was able to acylate 22:1-CoA to 22:1-LPA. No labelled MAG could be detected in the assays with the GM Crambe lines, indicating that the *Limnanthes* LPAAT efficiently outcompeted the enzyme responsible for the formation MAG in Wt membranes. The safflower microsomes could acylate 22:1-CoA to G-3-P to produce 22:1-LPA, of which a substantial part was converted to MAG, but, interestingly, a large proportion was also found in PA and even higher in PC. We could not detect

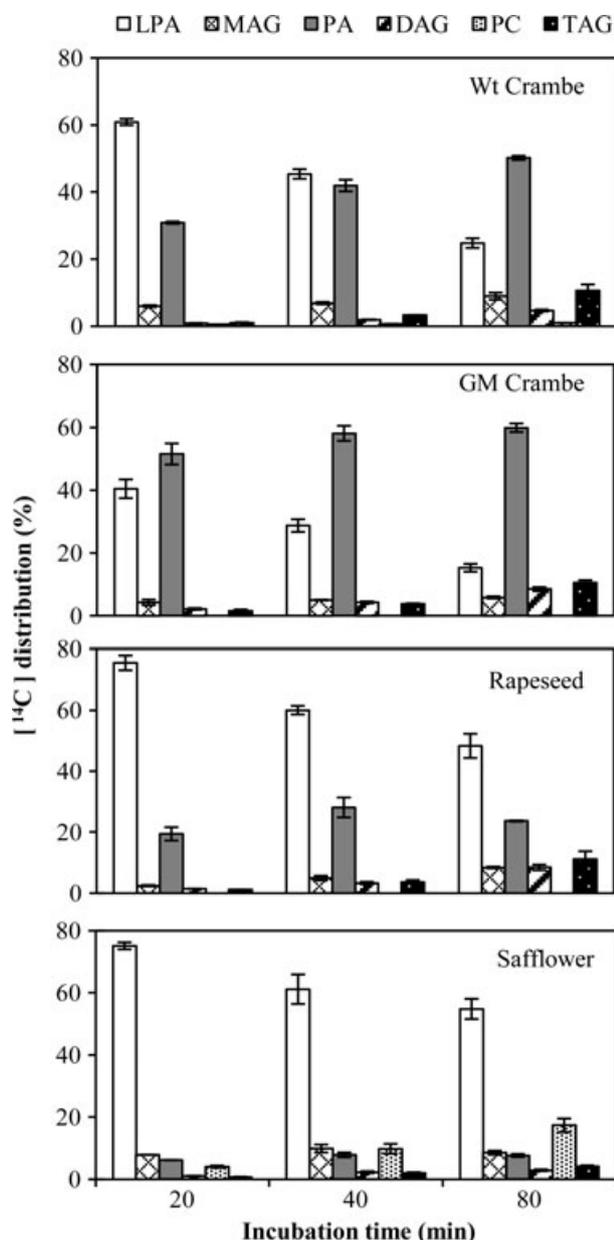


Figure 3 Time course incorporation of radioactivity into various lipids in microsomal preparations from developing seeds of different species, incubated with glycerol-labelled [^{14}C]22:1-LPA and nonradioactive 18:1-CoA. Wt, wild type; GM, genetically modified; LPA, lysophosphatidic acid; MAG, monoacylglycerol; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerols. Results are shown from triplicate samples \pm SD.

any labelled DAG or TAG in any of these incubations for all the species (Figure 4). However, it should be noted that the total amount of ^{14}C incorporation into complex lipids was very low and the label in these lipids might be below the detection limit.

Incubations with glycerol-labelled [^{14}C]18:1-LPA and nonradioactive 22:1-CoA demonstrated a dramatic difference in the metabolism between GM and Wt Crambe (Figure 5). Virtually, all LPA was acylated with 22:1 within 20 min with the membranes of GM Crambe, further confirming the functional role of introduced *Limnanthes* LPAAT gene in the acylation of 22:1 at the *sn*-2 position. In contrast, very little labelled PA was detected

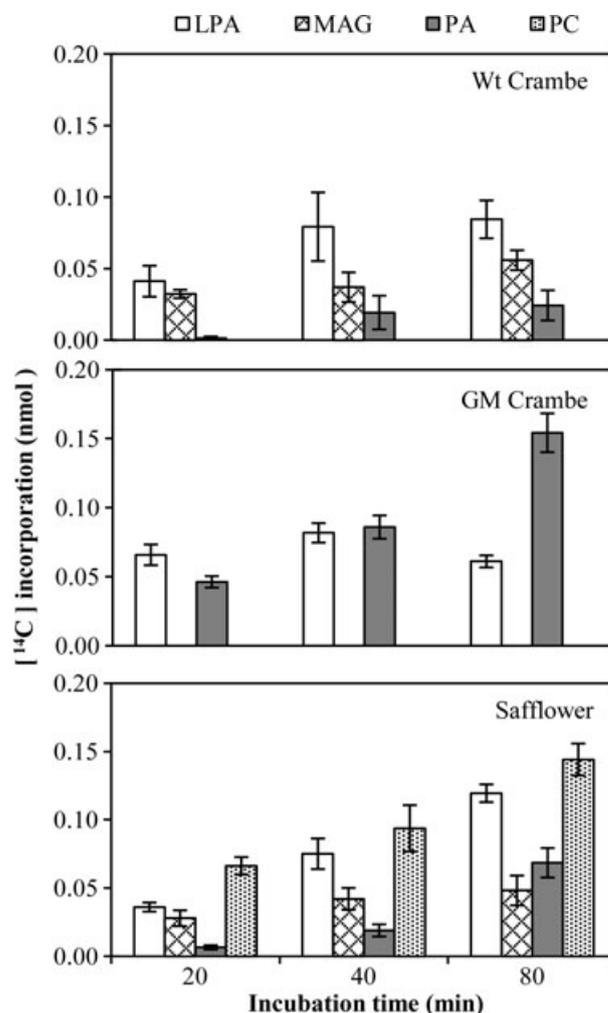


Figure 4 Time course incorporation of radioactivity into various lipids in microsomal preparations from developing seeds of different species, incubated with [^{14}C]glycerol 3-phosphate and nonradioactive 22:1-CoA. Wt, wild type; GM, genetically modified; LPA, lysophosphatidic acid; MAG, monoacylglycerol; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerols. Results are shown from triplicate samples \pm SD.

in Wt Crambe where a substantial amount of labelled MAG was formed. The rapeseed had somewhat better ability to acylate 22:1 than the Wt Crambe. Surprisingly, a substantial amount of 22:1 was acylated to 18:1-LPA in safflower microsomes and PA was efficiently converted to DAG and further to PC and TAG, again demonstrating no constraints in DAG-PC interconversion due to erucyl groups in DAG in these microsomes.

The next set of incubations tested were Wt Crambe, GM Crambe and safflower membranes with glycerol-labelled [^{14}C] 22:1-LPA and 22:1-CoA (Figure 6). Very little PA was formed with these combination of substrates in Wt Crambe where MAG was the dominating metabolite. In contrast, the GM Crambe efficiently acylated 22:1 to this acyl acceptor, however, at lower rate than with 18:1-LPA (compare Figures 5 and 6). The 22:1-LPA was also to some extent acylated with 22:1 in safflower membranes but with much lower efficiency than 18:1-LPA. Again, glycerol label was found in PC in safflower microsomes and was the major labelled metabolite after 80 min incubation.

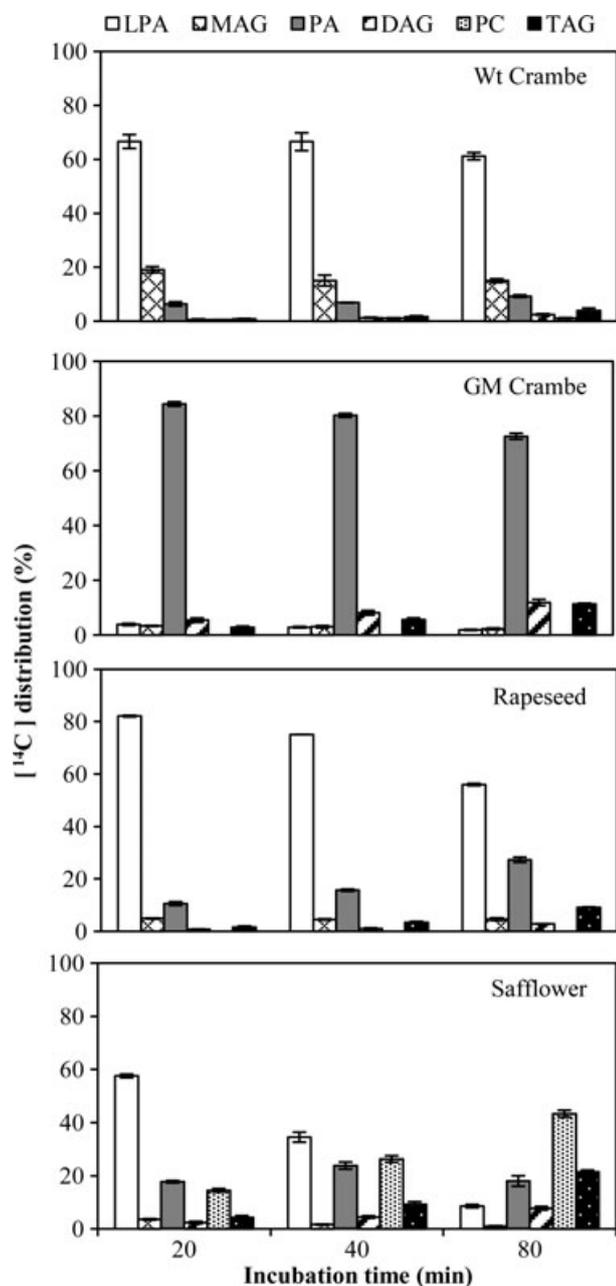


Figure 5 Time course incorporation of radioactivity into various lipids in microsomal preparations from developing seeds (from species indicated in the figure) incubated with glycerol-labelled [^{14}C]18:1-LPA and nonradioactive 22:1-CoA. Wt, wild type; GM, genetically modified; LPA, lysophosphatidic acid; MAG, monoacylglycerol; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerols. Results are shown from triplicate samples \pm SD.

In vivo [^{14}C]glycerol labelling

To correlate the detected *in vitro* activities of the flow of glycerol backbone towards TAGs in the microsomal membranes of the developing seeds with *in vivo* activities, we fed [^{14}C]glycerol to detached developing embryos of rapeseed and Wt Crambe collected 20 DAF and determined the amount of radioactivity in the complex lipid at different time intervals. Only four lipids, PA,

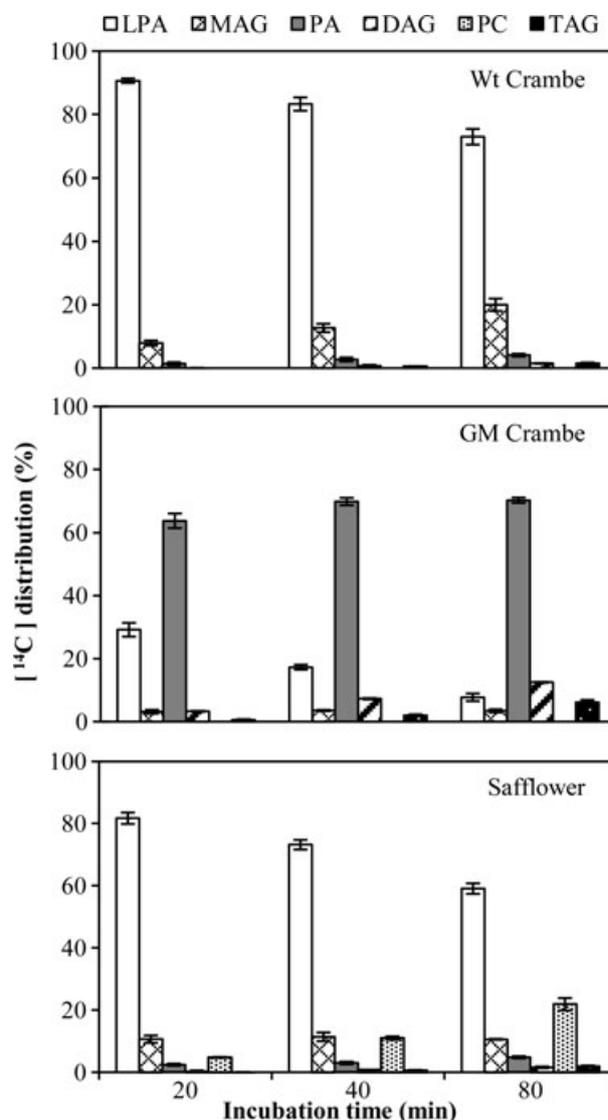


Figure 6 Time course incorporation of radioactivity into various lipids in microsomal preparations from developing seeds (from species indicated in the figure) incubated with glycerol-labelled [^{14}C]22:1-LPA and nonradioactive 22:1-CoA. Wt, wild type; GM, genetically modified; LPA, lysophosphatidic acid; MAG, monoacylglycerol; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerols. Results are shown from triplicate samples \pm SD.

DAG, PC and TAG, contained a significant amount of radioactivity, of which PA accounted only for a very small portion of the total labelled lipids (Figure 7). DAG and TAG were the main labelled lipids at all the time points in Crambe seeds, while the radioactivity in PC was two to three times less than DAG or TAG. In rapeseed, DAG was the highest labelled lipid, while TAG showed about half of the radioactivity compared with PC.

Fatty acid composition of PC, DAG and TAG in developing Crambe seeds

Analysis of total FA composition and amount in the Wt and GM Crambe seeds as well as acyl composition of TAG, DAG and PC were conducted at 20, 27, 34, 41 and 50 DAF, and the complete analyses are presented in Supporting Information Tables S1 and

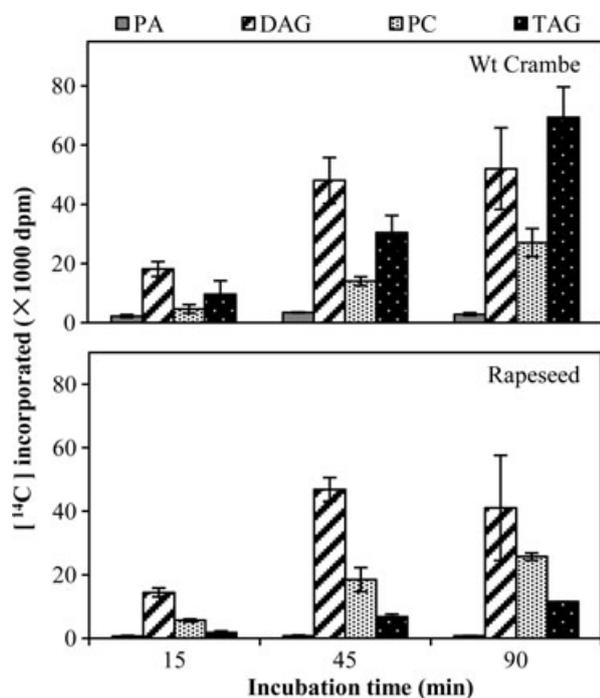


Figure 7 Time course incorporation of radioactivity into various lipids by detached developing wild-type Crambe and rapeseed embryos after incubation with [¹⁴C]glycerol. PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerols. Results are shown from triplicate samples ± SD.

S2. Some of these data are extracted in graphic form in Figure 8. During the first 20 DAF, the rate of oil accumulation was about one-third in GM Crambe compared with Wt Crambe (Figure 8a). Between 20 and 27 DAF, the rate of oil accumulation increased and was somewhat higher than in Wt during that period (Table 1), thereafter the oil content continued to increase at a decreasing pace up to 50 DAF. The Wt seeds accumulated oil in a near linear fashion between 20 DAF and 34 DAF, where it had maximal amount of oil accumulation and with a slight decrease afterwards. It has been shown that oil content in rape seeds decline upon maturation and that a TAG lipase is involved in this process (Kelly *et al.*, 2013). The Wt seeds lost chlorophyll between 20 and 27 DAF, whereas the GM seeds peaked in chlorophyll content at 27 DAF (Figure 8a). At 50 DAF, both lines had completely lost all their chlorophyll and were regarded as fully matured. Thus, the GM seeds had a delayed seed development as well as a much longer period of oil accumulation than the Wt seeds. The mature GM seeds had 21% lower amount of oil per seed than the Wt, but were 16% less in weight, thus giving similar oil content per seed weight as the Wt seeds.

Erucic acid amounted to about 40 mol% of all acyl groups in both Wt and GM Crambe seeds at 20 DAF. At 34 DAF, GM and Wt seeds had about 68 and 53 mol% erucic acid, respectively, and this proportion remained quite stable during the rest of the seed development (Figure 8b). It should be noted that the 68 mol % 22:1 in GM corresponds to about 71% by weight, as previously reported in this line (Li *et al.*, 2012).

The nmol of total acyl groups and the nmol erucic acid per seed accumulated between 20, 27 and 50 DAF are shown in Table 1. It can be concluded that of all FAs accumulated between 20 and 27 DAF, erucic acid accounted for 63 mol% in Wt and 68 mol% in

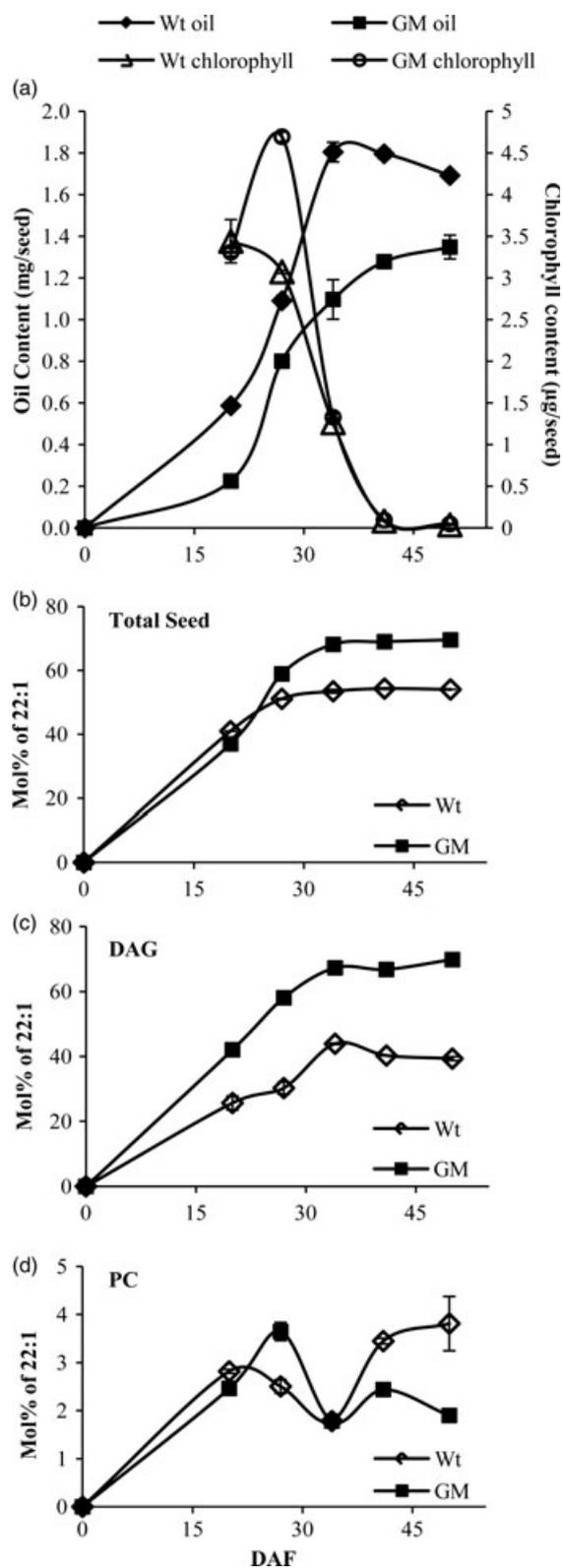


Figure 8 Oil accumulation and fatty acid composition of wild-type (Wt) and genetically modified (GM) Crambe seeds at different days after flowering (DAF). (a) Oil content and chlorophyll content; (b) Total seed fatty acid composition; (c) Fatty acid composition of diacylglycerols (DAG); (d) Fatty acid composition of phosphatidylcholine (PC). The data are mean ± SD of duplicate analyses presented in Supporting Information Tables S1 and S2.

Table 1 Total fatty acid (FA) and erucic acid (22:1) content of wild-type (Wt) and genetically modified (GM) *Crambe* seeds at various days after flowering (DAF). The data (means \pm SD) are duplicate analyses presented in Supporting Information Table S1

DAF	Wt		GM	
	FA nmol/seed	22:1 nmol/seed	FA nmol/seed	22:1 nmol/seed
20	1822 \pm 9	746 \pm 5	704 \pm 10	260 \pm 4
27	3320 \pm 74	1696 \pm 45	2396 \pm 37	1411 \pm 22
50	5125 \pm 32	2768 \pm 17	3960 \pm 168	2755 \pm 118
nmol of FA accumulated between 20 and 27 DAF	1498	950	1692	1151
mol% of 22:1 accumulated between 20 and 27 DAF	63.4%		68.0%	
nmol of FA accumulated between 27 and 50 DAF	1805	1072	1564	1344
mol% of 22:1 accumulated between 27 and 50 DAF	59.4%		85.9%	

GM. Between 27 and 50 DAF, 59 and 86 mol% of the accumulated FAs were erucic acid in Wt and GM lines, respectively, assuming that there is no conversion of oleic acid to erucic acid occurring on the acyl groups that had already accumulated at the first 27 DAF. The mol% of erucic acid in DAG at the different stages of seed development is presented in Figure 8c. The proportion of erucic acid at 27 and 50 DAF amounted to 30 and 39 mol% in Wt and 58 and 70 mol% in GM, respectively (Figure 8c). The much higher proportion of 22:1 in DAG also at 20 DAF in the GM line (Figure 8c) indicates that the introduced *Limnanthes* LPAAT enzyme has high activity already at this early stage of seed development. It was previously reported that mature seeds of the high erucic GM *Crambe* line had half the amount of erucic acid in PC compared with Wt seeds (Li *et al.*, 2012). The amount of erucic acid found in PC at the different stages of seed development is depicted in Figure 8d. Although the level of 22:1 in GM seeds was half of that found in Wt seeds at 50 DAF, the levels fluctuated during the seed development and it was 40% higher than in Wt seeds at 27 DAF. It is particular noteworthy that the levels of erucic acid in PC doubled in PC in Wt seeds between 34 and 50 DAF, despite that there was no net synthesis of TAG during this period (compare Figure 8a,d).

Discussion

GPAT and LPAAT activities towards erucoyl and oleoyl substrates

Crambe abyssinica is a species having 55%–60% of erucic acid in its seed oil (TAG). We have developed GM *Crambe* lines with over 70% of erucic acid by coexpressing a *BnFAE1*, a *LdLPAAT* and a

CaFAD2-RNAi (Li *et al.*, 2012). When measuring the activity of the acylation enzymes in the G-3-P pathway leading to TAG synthesis in microsomal preparations from developing *Crambe* seeds, we can conclude that 22:1-CoA is utilized much less efficient (2.5%–3.5%) than 18:1-CoA in the acylation of G-3-P by GPAT(s) and is not better utilized than by the safflower GPAT enzyme. This is quite unexpected in the view of the high amount of erucic acid (>85%) found in *sn*-1 + *sn*-3 positions of Wt *Crambe* TAGs (Li *et al.*, 2012). It raises a question if there is a GPAT with high activity for erucoyl-CoA in *Crambe* that has been lost or inactivated during membrane preparations. It should be pointed out here that no plant gene for GPAT involved in either seed TAG or membrane lipid synthesis has with certainty been identified (Bates *et al.*, 2013).

The very poor acylation of LPA with 22:1-CoA by Wt *Crambe* microsomal enzymes is expected as this acyl group is only found at low levels (8%) at position *sn*-2 in *Crambe* seed TAGs (Li *et al.*, 2012). The enzymes in the membranes prepared from GM *Crambe* expressing the *Limnanthes* LPAAT gene, effectively acylated this acyl group to 18:1-LPA. The *Limnanthes* enzyme has previously been shown to efficiently utilize this acyl-CoA species (Hanke *et al.*, 1995). Our results showed that 22:1-LPA was a much poorer substrate than 18:1-LPA in the acylation of 18:1-CoA in all membranes tested. In membranes of GM *Crambe*, 22:1-LPA was also a markedly poorer substrate than 18:1-LPA in acylation with 22:1-CoA. This indicates a bottleneck in the formation of PA with two 22:1 acyl groups, even with the *Limnanthes* LPAAT expressed. When studying the glycerol acylation enzymes in rapeseed, we obtained very similar results as with Wt *Crambe*. Moreover, we included microsomal preparations from developing safflower seeds in our assays. The lipid metabolizing enzymes in safflower microsomal membranes have been extensively studied (Stymne *et al.*, 1983; Stymne and Stobart, 1984; Griffiths *et al.*, 1985; Stobart and Stymne, 1985) and have significantly contributed to our present understanding of TAG biosynthesis in seeds (Stymne and Stobart, 1987; Bates *et al.*, 2013). Unexpectedly, the safflower LPAAT(s) had much higher capacity to acylate 22:1 to 18:1-LPA than Wt *Crambe* and rapeseed enzymes although safflower does not produce 22:1 in its seed oil. Among the species accumulating high amounts of very long-chain FAs in their seed oil, it is only the *Limnanthes* species and *Tropoleum majus* that have been reported to have 22:1 in high amounts at the *sn*-2 position (Löhden and Frentzen, 1992). Our studies show that the safflower LPAAT had poor capacity to acylate 22:1 when 22:1-LPA was acyl acceptor compared with the introduced *Limnanthes* LPAAT in GM *Crambe* seeds.

The metabolism of DAG in microsomal membranes

As reported previously (Stobart and Stymne, 1985), microsomal preparations from safflower seeds effectively channelled DAG produced from PA into both TAG and PC. With 18:1 containing substrates, about equal proportions of the label entered these two lipids. The main enzyme responsible for the channelling of DAG into PC is believed to be PDCT, interconverting the two molecules by transferring a phosphocholine group to DAG (Lu *et al.*, 2009). It should be noted that when the 22:1 substrates were used, the safflower enzymes readily incorporated DAG with one or two erucoyl groups into PC, indicating that safflower PDCT does not discriminate against these DAG species.

The amount of label entering PC in *in vitro* assays was very low in membranes of both Wt *Crambe* and rapeseed, indicating that

the PDCT activity in these membranes is much lower compared with that in safflower. It is likely that the low DAG-PC interconversion is limiting the amount of erucoyl groups entering the membrane lipids in Crambe. The low flow of glycerol backbone into PC in Wt Crambe was confirmed by *in vivo* labelling of developing Crambe seeds with [¹⁴C]glycerol. The relative incorporation [¹⁴C]glycerol into DAG, PC and TAG in the Crambe is very similar to that reported for [¹⁴C]glycerol-labelling experiment with developing seeds from the *rod1* (*PDCT*) mutant of Arabidopsis (Lu *et al.*, 2009) and radically different from Wt Arabidopsis (Lu *et al.*, 2009; Bates and Browse, 2012). The developing rapeseed incorporated relatively more [¹⁴C]glycerol label in PC than in TAG compared with Crambe but significantly less than Wt Arabidopsis (Lu *et al.*, 2009; Bates and Browse, 2012), suggesting that rapeseeds has intermediate DAG-PC interconversion activity compared with Arabidopsis and Crambe.

Apart from PDCT, there is also a net synthesis of PC from DAG catalysed by DAG/CDP-choline choline phosphotransferase (CPT) (Li-Beisson *et al.*, 2013). It has been shown that when a *FAE1* is expressed constitutively in Arabidopsis, up to 20% of very long-chain FAs are accumulated in the leaf PC despite the very low amount of very long-chain FAs in seed PC, indicating that CPT does, to some extent, accept very long chain containing DAG in Arabidopsis (Millar *et al.*, 1998). In studies of acyl fluxes in detached developing soybeans, Bates *et al.* (2009) showed that there were at least two kinetically distinct DAG pools. The high amount of erucic acid detected in total DAG in Wt Crambe seeds at different developmental stages might therefore not reflect the DAG pool used by CPT in the net synthesis of PC. Thus, it is likely that Crambe has at least two distinct DAG pools, one with low amounts of erucic acid used for net synthesis of PC and one highly enriched in erucic acid, used for TAG synthesis.

It is clear from the lipid analyses of the developing Crambe seeds in this study that some erucic acid is accumulating in PC, but the level oscillates in a way that is difficult to explain with present knowledge. The most remarkable difference between the Wt and GM lines in this respect is over doubling of the mol% of erucic acid in PC in Wt between 34 and 50 DAF, when no net synthesis of TAG is taken place, whereas there are only small changes in the erucic acid levels in PC in the GM line during the same period. Because the GM seeds, unlike the Wt seeds, continue to accumulate oil during this period, it can be speculated that there is a mechanism editing out erucic acid from PC operating only during active oil deposition, keeping the erucic acid level low close to maturity in the GM line. Editing out mechanisms for medium chain and oxygenated acyl groups from PC have been suggested to be catalysed by specific phospholipases (Ståhl *et al.*, 1995), but proofs of their *in vivo* functions are still lacking.

Oil accumulation during seed development

In GM Crambe seeds, the oil accumulation occurred at a much slower rate during the first 20 DAF and proceeded over longer period than in Wt seeds. The fact that the chlorophyll content of the GM seeds started to decline at least 7 days later than in the Wt seeds indicates that the whole seed development was delayed. The proportion of erucic acid was about half of that of mature seeds in the GM line at 20 DAF and about 30% lower than in mature seeds in the Wt seeds. It has previously been reported that the proportion of erucic acid increase with seed development and low at the onset on TAG accumulation in erucic acid producing rapeseed (Bhardwaj and Hamama, 2003). It is clear from the biochemical data on LPAAT activity and from the

much higher proportion of erucic acid in DAG in GM seeds than in Wt at 20 DAF that the introduced *Limnanthes* LPAAT has already good activity at this early stage. All the three genes, *BnFAE1*, *LdLPAAT* and *CaFAD2-RNAi*, introduced into GM Crambe, are under control of the seed-specific napin promoter and can be anticipated to be simultaneously expressed. It is therefore likely that the enzyme BnFAE1 is active at a stage where endogenous FAE enzyme is not fully expressed. Because the elongation of oleic acid to erucic acid needs four enzymes, that is, the condensing enzyme FAE, a 3-keto reductase, a 3-hydroxy hydratase and an enoyl reductase, these enzymes can be assumed to be coordinately produced in the Wt Crambe. It is therefore tempting to speculate that the much slower initial oil accumulation in GM seeds compared with Wt is due to an imbalanced activity of the FAE activity compared with the reducing enzymes, leading to a release of a 3-keto and/or 3-hydroxy intermediates that will be shunted to beta-oxidation and thereby creating a futile cycle. However, the transgenes could also have pleiotropic effects, *for example*, by disturbing basal membrane lipid metabolism at early stages of seed development. Regardless of the reason for the initial slower oil accumulation in the GM seeds, it is clear that the bottleneck in achieving even higher amount than 72% of erucic acid is probably due to a lower accumulation of erucic acid during the first 27 DAF when about 60% of the oil is laid down. Between 20 and 27 DAF, when the endogenous enzymes involved in erucic acid synthesis can be anticipated to be fully expressed, the amount of erucic acid accounted to 68 mol% of all the FAs accumulated in GM seeds but between 27 and 50 DAF 86 mol% of all FAs accumulated was erucic acid. In contrast, the proportion of erucic acid of all FAs accumulated between 20 and 50 DAF remained rather constant in Wt seeds (59%–63%). This suggests that there is a limiting factor in erucic acid synthesis between 20 and 27 DAF in the GM seeds. As the FA synthesis slows down after 27 days, the proportion of erucic acid produced sharply increases. Because such bottleneck(s) does not impair oil accumulation, they can be expected to be different from the bottlenecks occurred during the first 20 DAF. Such later factors might be inadequate supply of cytosolic malonyl-CoA and/or not enough activity of the introduced genes. If the bottleneck(s) in the production of erucic acid during the first 27 DAF can be solved, it should be possible to develop Crambe lines with over 80% mol% of erucic acid in the oil. It should be noted that single GM Crambe seeds could accumulate up to 77% of erucic acid (Li *et al.*, 2012), demonstrating a certain plasticity in the maximal amount of 22:1 that could be accumulated in the seeds in the studied line. However, seeds from subsequent generations raised by half-seed techniques from seeds with over 73% of 22:1 did show the same distribution of erucic acid among the seeds with a mean of 71–73% (Li *et al.* unpublished results).

Concluding remarks

We show that Crambe seeds have very low DAG-PC interconversion and this can be assumed to be the major reason of the low amount of erucic acid found in PC. Our results also indicate that the GPAT acylating erucic acid to G-3-P is a different enzyme than the GPAT acylating oleic acid that is lost or inactivated in the microsomal membranes from the developing seeds. We further show that GM Crambe seeds, expressing three genes for increased erucic acid synthesis, have a slower seed development than Wt with a slower oil accumulation during the first 20 DAF. Erucic acid accounted for 86 mol% of all FAs accumulated in the

GM between 27 and 50 DAF when about 40% of the final oil is laid down. In order to further increase in erucic acid above the 68 mol% achieved in the GM lines, the bottlenecks in the production of erucic acid at the first 27 DAF must be identified and alleviated. Future comparative transcriptomic, lipidomic and *in vivo* isotope labelling studies on developing GM and Wt seeds are likely to confirm or disapprove this hypothesis.

Experimental procedures

Plant materials

Wt *Crambe* (*Crambe abyssinica* Hochst. cv. Galactica), the transgenic high erucic acid *Crambe* line 3G7-6-13 (Li *et al.*, 2012), rapeseed (*Brassica napus* L. cv. Mosaik), turnip (*Brassica rapa* L. cv. Bele) and safflower (*Carthamus tinctorius* L. cv. Centennial) were used in this study. All the plant materials were grown in a controlled growth chamber with photoperiod of 16 h (250 $\mu\text{mol}/\text{m}^2/\text{s}$), temperature of 23/15 °C (day/night) and 60% humidity.

Chemicals

[U- ^{14}C] Glycerol-*sn*-3-phosphate and [U- ^{14}C]glycerol were products from Perkin-Elmer. Nonradioactive glycerol-3-phosphate, bovine serum albumin (BSA, essentially fatty acid free) and catalase were products from Sigma Chemical Company. Coenzyme A, oleic acid and erucic acid were products from Larodan (Malmö, Sweden). Acyl-CoAs (18:1-CoA, 22:1-CoA) were prepared according to the method described by Sánchez *et al.* (1973). [^{14}C]Glycerol-labelled oleoyl-lysophosphatidic acid (18:1-LPA) and erucoyl-lysophosphatidic acid (22:1-LPA) were synthesized by acylating [^{14}C] glycerol-3-phosphate with oleic acid and erucic acid mixed anhydride, respectively, according to the study described by Kanda and Wells (1981) and isolated from the reaction mixture by separation on TLC plates (Silica 60, Merck). The LPAs were eluted from the gel with methanol/chloroform (2:1, v/v) and left at room temperature for 24 h to allow for isomerization of the *sn*-2 isomer to the stable *sn*-1 form. The LPAs were then extracted into chloroform, evaporated and dissolved in water before used in assays. LPA concentrations were measured by quantification of the acyl groups relative to methylheptadecanoic acid by GLC analysis after methylation. Specific radioactivity of the substrate was calculated by measuring the radioactivity by scintillation counting and the amount of acyl groups as determined by GLC.

Microsomal preparations

Developing cotyledons were harvested at 20 days after flowering (DAF), except for safflower seeds that was harvested 18 days after pollination. Embryos with cotyledons were excised from the seed coat and collected in ice-cold 0.1 M potassium phosphate buffer before homogenized, and all the manipulation was carried out at 4 °C. The tissues were ground in 0.1 M potassium phosphate buffer, pH 7.2, containing 1% BSA, 1000 units of catalase/mL and 0.33 M sucrose. The supernatant was filtered through a double layer of Miracloth[®] and centrifuged at 20 000 **g** for 10 min. The supernatant was filtered through a single layer of Miracloth[®] and centrifuged at 105 000 **g** for 90 min. The resulting microsomal pellets were resuspended in a small volume of potassium phosphate buffer with 1000 units of catalase/mL, flash frozen in liquid nitrogen and stored at -80 °C until used. Protein content of the microsomal preparations was measured with BCA[®] reagents (Pierce Chemical Company) with BSA as standard.

Enzyme assays

Microsomal preparations were incubated at 30 °C with gentle shaking in 0.1 M Tris buffer pH 7.2, containing 4 mM MgCl₂, 1 mg BSA, 25 nmol 18:1-CoA or 22:1-CoA, 25 nmol of [^{14}C] glycerol-3-phosphate or 9 nmol [^{14}C]glycerol-labelled 18:1-LPA or 22:1-LPA in a final assay volume of 100 μL . The assays were initiated by addition of microsomal preparations (equivalent to 100 μg microsomal protein) into the reaction buffer, and terminated by addition of 100 μL 0.15 M acetic acid and 500 μL of methanol/chloroform (2:1, by vol.), followed by extraction of the lipids into a chloroform phase according to the method described by Bligh and Dyer (1959). Assays were performed in triplicate and the values shown in Figures and Tables are mean values \pm standard deviation (SD).

Analysis of radio-labelled lipids

After counting an aliquot of the lipid containing chloroform extract from the assays by liquid scintillation, the rest of the lipids were separated on TLC (Silica 60, Merck). The plates were first developed to 3/4th of their height in chloroform/methanol/acetic acid/water, (90/15/10/3, by vol.) to separate the polar lipids. After drying, the plates were redeveloped to the top in n-hexane/diethyl ether/acetic acid, (70/30/1, by vol.) to separate the neutral lipids. The radioactive spots on the TLC plates were visualized by electronic autoradiography Instant Imager[®] (Canberra Packard Instruments) and identified by migration of authentic radioactive standard lipid and the relative radioactivity in each lipid was calculated by the Instant Imager[®] software. The absolute amount of radioactivity in each lipid was calculated based on its relative amount on the plate and the total amount of ^{14}C -activity in the chloroform phase.

In vivo [^{14}C] glycerol labelling

Developing seeds at 20 DAF were harvested and kept on ice. Ten dissected embryos were pooled for each incubation in 300 μL prechilled medium (pH 5.8, 2% sucrose, 0.5x Murashige and Skoog medium). After 20 min preincubation under light at room temperature, labelling was initiated by removing the media and replacing with 100 μL fresh media containing 10⁶ dpm [^{14}C] glycerol. At time intervals, the incubations were terminated by first removing the media and washing with fresh media twice, and then adding 0.15 M acetic acid/(50 mM EDTA + 0.1 M glycerol) (1:5, by vol.). The total lipids were then rapidly extracted from the embryos into a chloroform phase by homogenizing the tissues with Ultra-Turrax[®] according to the method described by Bligh and Dyer (1959). The radio-labelled lipid analysis was carried out as described above. All assays were performed in triplicate.

Fatty acid composition and chlorophyll determination

A hundred developing *Crambe* seeds including the pod (each seed is surrounded by a pod) were collected from both the Wt and the GM *Crambe* lines, at 20, 27, 34, 41 and 50 DAF. The total lipids were then rapidly extracted from the seeds into a chloroform phase by homogenizing the seeds with Ultra-Turrax[®] as described above. The chloroform phase was evaporated under nitrogen, and the lipids were redissolved in 2 mL chloroform. In order to determine the total amount of FAs and FA composition in the total lipids, 20 μL of extract was redissolved in 2 mL methylation solution (2% H₂SO₄ in water-free methanol) and methylated at 90 °C for 1 h. Heptadecanoic acid (17:0) was added as the internal standard. After methylation, 2 mL water and 2 mL hexane were added, followed by brief vortexing and centrifugation. The hexane phase

containing FA methyl esters (FAMES) was analysed by gas chromatography according to the method described by Li *et al.* (2012). Two hundred μL and 100 μL of each total chloroform phase were loaded on TLC and developed in chloroform/methanol/acetic acid/water, (90/15/10/3, by vol.) for polar lipids and in n-hexane/diethyl ether/acetic acid, (70/30/1, by vol.) for neutral lipids, respectively. PC, DAG and TAG spots were identified under UV light after staining with 0.05% primuline in acetone/water (80/20, by vol.), by migration relative to standard 18:1-PC, di-18:1-*sn*-1,2-DAG and 18:1-TAG. Each lipid spot corresponding to PC, DAG and TAG was scraped from TLC and methylated *in situ* on the gel as described above. All analyses were carried out in duplicate from the same chloroform extract.

For chlorophyll measurements, duplicate samples of 40 μL of chloroform extracts were dried under nitrogen and redissolved in 1 mL 96% ethanol. The chlorophyll content was determined in replicate by the UV/VIS spectrophotometer (Multiskan GO, Thermo Scientific) according to the equation (Arnon, 1949).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Time course incorporation of radioactivity into various lipids in microsomal preparations from developing turnip incubated with [¹⁴C]glycerol 3-phosphate and nonradioactive 18:1-CoA.

Table S1 Total fatty acid (FA) composition and content in wild-type (Wt) and genetically modified (GM) *Crambe* seeds at different days after flowering (DAF).

Table S2 Fatty acid (FA) composition of diacylglycerols (DAG), triacylglycerols (TAG) and phosphatidylcholine (PC) in wild-type (Wt) and genetically modified (GM) *Crambe* seeds at different days after flowering (DAF).