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Expression of Lauroyl–Acyl Carrier Protein Thioesterase in *Brassica napus* Seeds Induces Pathways for Both Fatty Acid Oxidation and Biosynthesis and Implies a Set Point for Triacylglycerol Accumulation

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Expression of a California bay lauroyl–acyl carrier protein thioesterase (MCTE) in developing seeds of transgenic oilseed rape alters the fatty acid composition of the mature seed, resulting in up to 60 mol% of laurate in triacylglycerols. In this study, we examined the metabolism of lauric acid and ^{14}C-acetate in developing seeds of oilseed rape that express high levels of MCTE. Lauroyl–CoA oxidase activity but not palmitoyl–CoA oxidase activity was increased several-fold in developing seeds expressing MCTE. In addition, isocitrate lyase and malate synthase activities were six- and 30-fold higher, respectively, in high-laurate developing seeds. Control seeds incorporated ^{14}C-acetate almost entirely into fatty acids, whereas in seeds expressing MCTE, only 50% of the label was recovered in lipids and the remainder was in a range of water-soluble components, including sucrose and malate. Together, these results indicate that the pathways for β-oxidation and the glyoxylate cycle have been induced in seeds expressing high levels of MCTE. Although a substantial portion of the fatty acid produced in these seeds is recycled to acetyl–CoA and sucrose through the β-oxidation and glyoxylate cycle pathways, total seed oil is not reduced. How is oil content maintained if lauric acid is inefficiently converted to triacylglycerol? The levels of acyl carrier protein and several enzymes of fatty acid synthesis were increased two- to threefold at midstage development in high-laurate seeds. These results indicate that a coordinate induction of the fatty acid synthesis pathway occurs, presumably to compensate for the lauric acid lost through β-oxidation or for a shortage of long-chain fatty acids.

INTRODUCTION

More than 90% of the fatty acids produced in wild-type oilseed rape (*Brassica napus*) seeds have a chain length of 16 carbons or longer. However, this fatty acid composition can be radically altered in seed oils of transgenic oilseed rape plants by expression of a California bay lauroyl–acyl carrier protein (ACP) thioesterase (MCTE) (Voelker et al., 1996). In some MCTE transformants, laurate (12:0) constitutes up to 60 mol% of acyl chains stored in triacylglycerols.

Voelker et al. (1996) examined >100 independent oilseed rape transformants and observed that laurate content in mature seeds of different transformation events ranged from 0 to 59 mol%. This wide range of laurate production was correlated with different levels of expression of the MCTE and in turn could be related to copy number and position effects. The relationship between the activity of extractable MCTE and the laurate content of the seed was linear up to a seed content of 30 to 35 mol% laurate. However, linearity in response to MCTE expression levels was lost in those transformants with very high thioesterase expression in which proportionately small changes in laurate accumulation were achieved. For example, an eight- to 10-fold increase in MCTE activity resulted in only a twofold increase in mol% laurate (from 30 to 59 mol%).

A partial explanation for the nonlinear increase in laurate was obtained after examining the distribution of this fatty acid among the three positions of triacylglycerol. Seeds containing 52% laurate distributed essentially all of this fatty acid into the *sn*-1 and *sn*-3 positions, with only 5% at the *sn*-2 position of the triacylglycerol (Voelker et al., 1996). These data suggest that laurate is excluded from *sn*-2 because of the strict substrate preference of the *sn*-2 acyltransferase but can be efficiently incorporated into the *sn*-1 and *sn*-3 positions of triacylglycerol. We reasoned that in those transformants expressing very high levels of MCTE, there may be an excess production of laurate that cannot be used in triacylglycerol synthesis because of its low efficiency of transfer into the *sn*-2 position. This putative surplus of laurate in the seed tissue of some MCTE transformants may be comparable to the production of laurate in leaf tissue of plants transformed with MCTE under the control of the cauliflower mosaic virus 35S promoter (Eccleston et al.,

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1996). Although these plants express high levels of MCTE in leaves and isolated chloroplasts produce up to 34% laurate, the leaves do not accumulate laurate. It has been hypothesized that this lack of accumulation of laurate in leaves could be due to rapid turnover of the unusual fatty acid in the leaves (Eccleston et al., 1996).

In this study, we have examined how transgenic oilseed rape plants respond to high levels of MCTE expression and to the production of lauric acid. We report a coordinate induction of pathways for both fatty acid β-oxidation and biosynthesis in these developing seeds. Currently, there is interest in the metabolic engineering of oilseeds to alter their composition toward higher value oil products. These results help to define how transgenic plants respond to major changes in fatty acid metabolism.

RESULTS

Accumulation of Laurate Is Limited in Seed Tissue Expressing Lauroyl–ACP Thioesterase

The observation that very high levels of MCTE expression do not yield corresponding increases in medium-chain fatty acid content of seeds (Voelker et al., 1996) prompted us to examine the limitations on laurate production in developing seeds of transgenic oilseed rape plants. Control wild-type and four transgenic lines expressing MCTE under the control of the napin or 35S promoter were examined, and their characteristics are described in Table 1. As previously reported by Eccleston et al. (1996), expression of MCTE under the control of the 35S promoter (transformation lines 8 and 11) results in high levels of MCTE activity in leaves but essentially no accumulation of lauric acid in leaf lipids. Although these same plants express lower levels of MCTE in seeds, this tissue accumulates 2 and 7% lauric acid. Transgenic plants expressing MCTE under the control of the napin promoter (lines 18 and 198) expressed much higher MCTE levels in seeds and accumulated ~40 and 60 mol% lauric acid, respectively (Table 1). Based on Figure 4 of Voelker et al. (1996), line 18 plants (40% laurate) are at the transition, and line 198 plants (60% laurate) are well beyond the linear relationship between MCTE expression and laurate accumulation.

High-Level Expression of Lauroyl–ACP Thioesterase Induces β-Oxidation Activity

Voelker and Davies (1994) observed that β-oxidation mutants but not control *Escherichia coli* secrete large amounts of laurate when expressing California bay MCTE. This result, together with our earlier study on MCTE expression in leaves (Eccleston et al., 1996), suggests that β-oxidation may limit the amount of laurate produced in plant cells. The activity of acyl–CoA oxidase is a specific indicator of peroxisomal β-oxidation capacity. In maize, acyl–CoA oxidase isozymes with short, medium, or long acyl chain length specificity have been described (Hooks et al., 1995). In our study, oxidation of lauroyl–CoA and palmitoyl–CoA was examined in developing seeds, etiolated seedlings, and leaf tissue of transformant lines expressing MCTE activity. Compared with controls, there were three- to 10-fold increases in lauroyl–CoA oxidase activity in plants expressing MCTE (Figure 1A). In addition, within each tissue and with either the 35S or napin promoter, the rates of lauroyl–CoA oxidation were roughly proportional to the level of MCTE such that plants with the highest thioesterase activity also expressed the highest lauroyl–CoA oxidase activity. In con-

Table 1. Summary of Laurate Content and Thioesterase Activities of Transformants and the Wild Type

	Leaf			Seed		
		Thioesterase Activity[b,c]			Thioesterase Activity[b,c]	
Plant	12:0 mol%[a,b]	12:0-ACP	18:1-ACP	12:0 mol%[a,b]	12:0-ACP	18:1-ACP
Control	ND[d]	5.4 (0.4)	65.4 (6.6)	ND	3.1 (0.5)	38.1 (4.9)
35S–MCTE						
Line 8	0.1 (0.1)	63.0 (10.3)	96.9 (6.9)	1.8 (0.2)	12.2 (2.9)	50.4 (9.2)
Line 11	0.1 (0.1)	90.7 (13.6)	104.5 (3.9)	7.0 (0.5)	7.1 (1.2)	47.5 (5.5)
Napin–MCTE						
Line 18	NA[d]	NA	NA	42.4 (1)	124.1 (6)	84.2 (4.9)
Line 198	NA	NA	NA	59.6 (3)	257.7 (21)	90.1 (3.2)

[a] Lauric acid content of tissue.
[b] Numbers in parentheses indicate the standard error ($n = 3$).
[c] Expressed as picomoles per minute per milligram of protein.
[d] ND, not detected; NA, not analyzed.

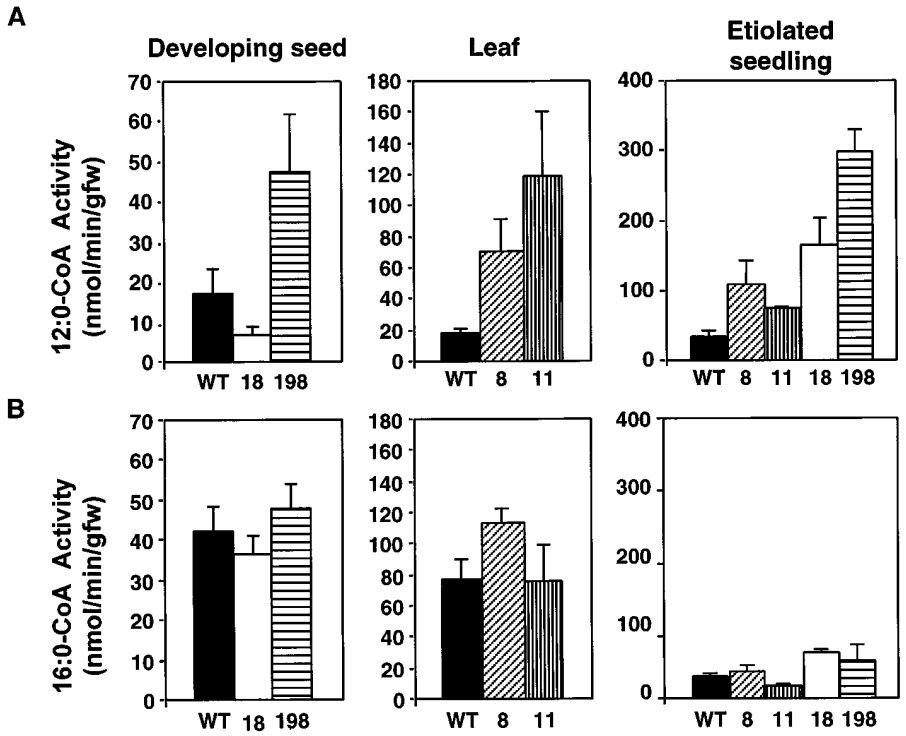

Figure 1. Acyl–CoA Oxidase Activities in Transgenic Plants Expressing Lauroyl–ACP Thioesterase.

(A) Lauroyl–CoA oxidase activity.

(B) Palmitoyl–CoA oxidase activity.

Enzyme activity was determined in developing seed (28 days after flowering [DAF]), leaves, and etiolated seedlings. Results are expressed as the mean of three determinations, with error bars indicating the standard error of the mean. gfw, gram fresh weight; WT, wild type.

trast, the oxidation of palmitoyl–CoA was not altered by MCTE expression (Figure 1B).

This specific increase in lauroyl–CoA oxidase activity without alteration of palmitoyl–CoA activity indicates that the induction of β-oxidation results from the production of laurate in the tissue and is not the result of a nonspecific or an indirect effect introduced by transformation. The observed increase in lauroyl–CoA oxidase in etiolated seedlings in which the napin promoter is not active suggests that release of laurate from triacylglycerol during seed germination also leads to a specific increase of the medium-chain acyl–CoA oxidase needed for its metabolism.

Glyoxylate Cycle Enzymes Are Induced in Lauroyl–ACP Thioesterase Transformants

In plants, acetyl–CoA produced by β-oxidation activity often is metabolized further by the glyoxylate cycle. Therefore, we examined the activities of isocitrate lyase and malate synthase, which are two enzymes unique to this pathway. As indicated in Table 2, malate synthase activity in developing seed extracts of napin MCTE plants (line 198) was increased 30-fold compared with that of wild-type controls at the same stage of development. Similarly, isocitrate lyase activity was 6.6-fold higher in developing seeds of high MCTE transformants. This increased activity of both marker enzymes of the glyoxylate cycle in MCTE transformant lines strongly suggests that high production of laurate can stimulate peroxisome/glyoxysome interconversion in oilseed rape. We also observed less dramatic increases in activities of malate synthase and isocitrate lyase in etiolated seedlings of both 35S and napin MCTE transformants (Table 2).

Acetate Is Metabolized to Sucrose and Malate in Developing Seeds of MCTE Transformants

When ^{14}C-acetate was provided to developing seeds of controls, almost all of the radiolabel was incorporated into lipids within 4 to 6 hr (Figure 2A). In contrast, much less carbon-14 was recovered in lipids of seeds expressing high levels of MCTE (Figure 2B). The amount of radiolabel remaining in

Table 2. Glyoxylate Cycle Enzyme Activities in Developing Seeds and Etiolated Seedlings

	Isocitrate Lyase[a]		Malate Synthase[a]	
Plant	Developing Seed	Etiolated Seedling	Developing Seed	Etiolated Seedling
Control	10 (3.4)	23 (6.3)	1.4 (1.4)	29 (1.9)
35S–MCTE				
Line 8	NA[b]	52 (2.2)	NA	199 (3.6)
Line 11	NA	36 (2.3)	NA	119 (5.8)
Napin–MCTE				
Line 18	NA	45 (7.2)	NA	200 (1.8)
Line 198	66 (5.9)	86 (11)	44.2 (5.9)	466 (6.6)

[a] Expressed as nanomoles per minute per gram fresh weight of tissue. Numbers in parentheses indicate the standard error ($n > 3$).
[b] NA, not analyzed.

the aqueous phase after lipid extraction was five- to 10-fold higher for MCTE seeds than for control seeds. This radiolabel was not unincorporated acetate because it was not volatile under acidic conditions. Analysis of the aqueous phase by cellulose thin-layer chromatography (TLC) (Figure 3) indicated carbon-14 incorporation into a range of products, with the two major species comigrating with malate and sucrose. These are the same products observed from ^{14}C-acetate incubations of germinating seeds active in the glyoxylate cycle (Canvin and Beevers, 1961). The formation of ^{14}C-sucrose from ^{14}C-acetate was further confirmed by the disappearance of this material after treatment with invertase (Figure 3). The production of sucrose from acetate is not expected in developing seeds and provides evidence in vivo for the induction of the glyoxylate cycle in these transgenic seeds.

Lauroyl–ACP Thioesterase Expression Alters the Acyl–ACP Profile

Very high MCTE expression in transformant lines diverts carbon flux away from long-chain fatty acid synthesis and toward laurate. The intermediates of fatty acid biosynthesis are acyl chains attached to the ACP. The profile of free ACP and acyl–ACPs reflects the relative rates of synthesis and turnover of acyl groups (Ohlrogge et al., 1995). To evaluate how MCTE expression impacts these pools, ACPs were separated by urea–PAGE and examined by immunoblotting with antibodies raised against ACP. Oilseed rape seeds express a family of ACP isoforms, and analysis of the acyl–ACP profiles in developing seeds is complicated by the large number of bands representing the different acyl chains and different ACP isoforms (Figure 4). However, based on scanning densitometry, we observed a twofold increase in the total amount of ACP plus acyl–ACP in extracts from seeds expressing high levels of MCTE. In addition, in control plants, the long-chain acyl–ACP pool made up a greater proportion of the total acyl–ACP than it did in the MCTE transformant (Figure 4), confirming that the diversion of carbon flux into medium-chain fatty acids resulted in a decrease in the long-chain acyl–ACP pool in situ. These results are similar to experiments with *E. coli* in which MCTE expression resulted in increased rates of fatty acid synthesis and depletion of the saturated long-chain acyl–ACP pool (Ohlrogge et al., 1995).

Lauroyl–ACP Thioesterase Activity Results in Increased Abundance of Several Fatty Acid Synthesis Proteins

The data presented above suggest that a portion of the laurate produced in MCTE seeds is β-oxidized. Nevertheless, no major reduction in oil content of the seeds occurs (Voelker et al., 1996). This raised the question of how these seeds maintain a high oil content, despite the loss of fatty acid products to β-oxidation. Alterations in the free-ACP and acyl–ACP pools as a result of MCTE expression (Figure 4) suggested that the activity or expression of other proteins synthesizing fatty acids also might have increased in the

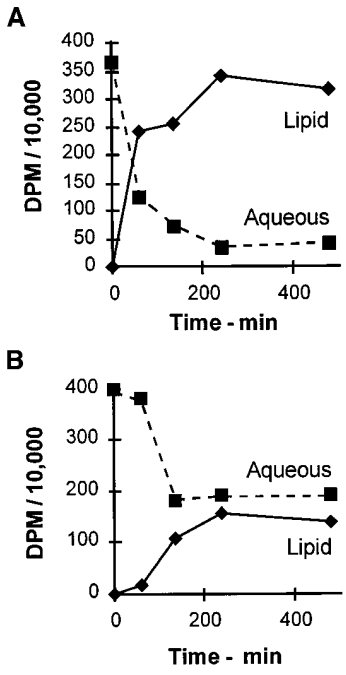

Figure 2. ^{14}C-Acetate Is Metabolized Differently by Seeds Expressing MCTE.

Developing seeds were incubated with ^{14}C-acetate for the times indicated. After extraction, radioactivity incorporated into lipid and aqueous fractions by oilseed rape seeds was determined.
(A) Wild-type seeds.
(B) Transgenic (line 198) oilseed rape seeds.

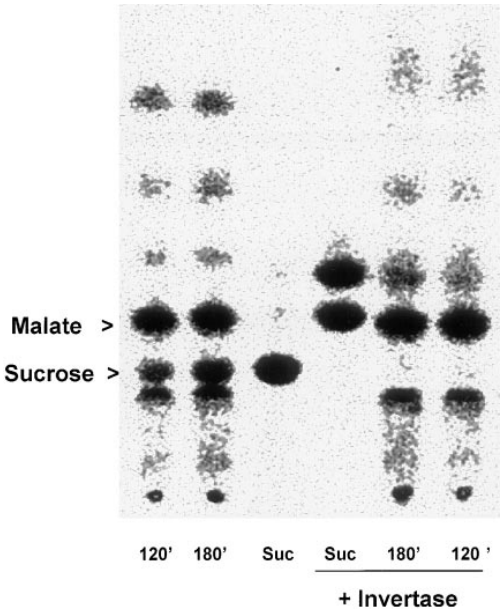

Figure 3. Acetate Is Metabolized to Sucrose and Malate by Developing Seeds of MCTE Transformants.

Developing seeds from line 198 were incubated with ^{14}C-acetate for 120 and 180 min, as given in Figure 2B. After lipid extraction, the aqueous phase was separated by cellulose TLC. The three lanes at right contain samples and a ^{14}C-sucrose (Suc) standard that were treated with invertase (+ Invertase) before TLC. Malate was identified by comigration with a ^{14}C-malate standard (data not shown). The prime signs indicate minutes.

MCTE transformants. Therefore, we examined the influence of MCTE expression on several enzymes involved in fatty acid synthesis. The abundance of two different subunits of plastidial acetyl–CoA carboxylase (BCCP and biotin carboxylase) was monitored by quantitative immunoblot analysis (Figure 5). Densitometry of immunoreactive protein bands indicated a threefold increase in BCCP during the midstage of seed development in the line 198 MCTE transformant line (Figure 5A). Similarly, a twofold increase over control levels of the biotin carboxylase subunit of acetyl–CoA carboxylase was observed in midstage MCTE seeds. This pattern of enzyme increase was also observed for stearoyl–ACP desaturase and 3-ketoacyl–ACP synthase III when control and transformant seed extracts were compared (Figure 5A). In addition, comparison of line 18 and line 198 transformants, which express different levels of MCTE activity, revealed an approximate correlation between MCTE activity and the increases in BCCP, biotin carboxylase, stearoyl–ACP desaturase, and 3-ketoacyl–ACP synthase III protein levels at midstage development (Figure 5A). At earlier and later stages of development, there was less increase or no difference between control and MCTE transformant levels of BCCP, biotin carboxylase, and stearoyl–ACP desaturase (Figure 5B).

In a previous study, Eccleston et al. (1996) noted that higher levels of oleoyl–ACP thioesterase activity are associated with MCTE expression in leaves. A similar increase was also observed in seeds of napin MCTE transformant lines (Table 1), with the highest expressing transformants having the largest increase in oleoyl–ACP thioesterase activity. Thus, based on immunoblot analysis (Figures 4 and 5) and enzyme assays (Table 1), the expression and/or activity of at least six proteins of the fatty acid synthesis pathway is increased. Several lines of evidence indicate that these differences in expression of the fatty acid synthesis pathway are due to MCTE expression rather than nonspecific factors. At 21 days after flowering (DAF), before major activity of the napin promoter (Kridl et al., 1991), there was little or no increase in fatty acid synthesis protein abundance (Figure 5B). Second, the increase in fatty acid synthesis proteins was more pronounced in the line 198 transformant expressing higher MCTE than in line 18 (Figure 5A). Finally, fatty acid synthesis proteins remained at control levels in a transformant line that did not express MCTE activity (data not shown).

High expression of MCTE activity caused an increase in the abundance of several fatty acid synthesis proteins (Figure 5). To determine whether MCTE expression also alters specific mRNA levels, we subjected total RNA extracts from control and MCTE transformant seed to RNA gel blot analysis.

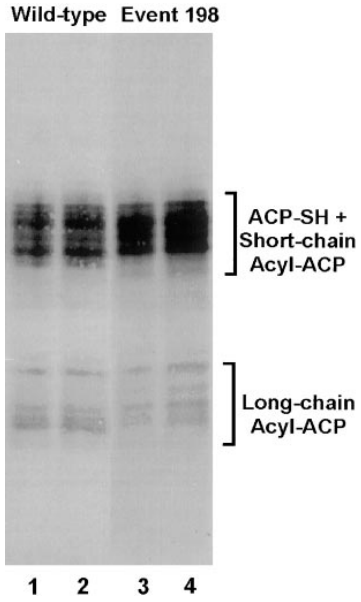

Figure 4. Analysis of ACP and Acyl–ACP in Wild-Type and Line 198 Seeds.

Proteins extracted from developing seeds were electrophoresed on a 2.0 M urea–polyacrylamide gel, blotted to nitrocellulose, and probed with antibodies raised against ACP. Lanes 1 to 4 were loaded with extracts from 7.5, 10, 5, and 7.5 mg fresh weight of tissues, respectively. ACP-SH, free, unesterifed ACP.

^{32}P-probes were synthesized using cDNA templates for oilseed rape ACP and BCCP and Arabidopsis biotin carboxylase. The RNA gel blot signals differed less than twofold for these three mRNAs (data not shown), suggesting that the increases in their protein expression (Figure 5) may have resulted from post-transcriptional controls.

DISCUSSION

In most plant tissues, the major fatty acids are metabolically stable and have long turnover times (Roughan, 1970). Thus, fatty acid breakdown and the glyoxylate cycle are quantitatively minor pathways, which may be involved primarily in lipid turnover or remodeling (Gerhardt, 1992). However, upon germination of oil-storing seeds, β-oxidation activity is induced many-fold, and within a few days, a massive breakdown of stored fats occurs (Beevers, 1980). Most of the acetyl–CoA produced from this β-oxidation enters the glyoxylate cycle, where it is converted to malate and succinate and eventually produces sugars or amino acids for new seedling growth. The expression of the glyoxylate cycle enzymes has been studied in some detail. Very low activities of isocitrate lyase and malate synthase are found in most vegetative tissues and in developing seeds until the final stages of maturation. When seeds germinate, expression of both enzymes increases markedly (Ettinger and Harada, 1990). The promoters of isocitrate lyase and malate synthase are considered to have at least two *cis*-acting elements, one that participates in specific expression during seed germination and a second that is responsive to metabolite levels (Sarah et al., 1996).

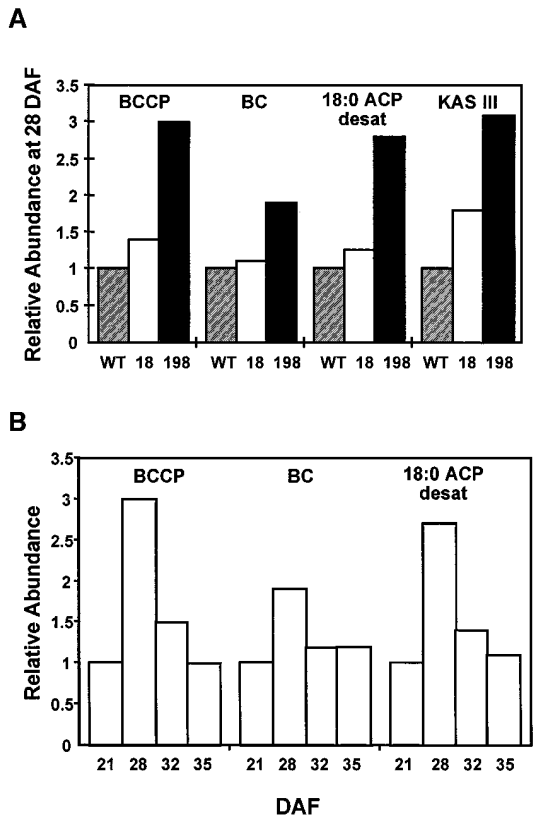

Figure 5. MCTE Expression Induces Several Enzymes of the Fatty Acid Synthesis Pathway.

(A) Immunoblot analysis of fatty acid synthase protein levels at 28 DAF in the wild type and in line 18 and line 198 transformants.

(B) Immunoblot analysis of BCCP, biotin carboxylase, and stearoyl–ACP desaturase protein levels at four stages of seed development in line 198 transformants.

Densitometry signals from immunoblots were corrected for differences in protein loading (as determined by Coomassie blue staining of equivalent gel lanes) and normalized against values for the control plant. BC, biotin carboxylase; desat, desaturase; KAS III, 3-ketoacyl–ACP synthase; WT, wild type.

In this study, we have found that plants engineered to produce high levels of medium-chain fatty acids show increased expression of both β-oxidation (Figure 1) and glyoxylate cycle enzymes (Table 2). These observations of fatty acid oxidation during seed development seem at first paradoxical because developing seeds are metabolically programmed for fatty acid synthesis rather than fatty acid breakdown. However, it is likely that cells must maintain mechanisms for disposal of excess fatty acids to prevent their interference with other enzymes or disruption of membrane integrity. In the case of oilseed rape expressing MCTE, our results indicate that these mechanisms include the induction of β-oxidation and glyoxylate cycle enzymes even in tissues that normally have very low activity of these enzymes. Thus, we conclude that the metabolic demand for these pathways, induced by high medium-chain fatty acid production, can override the developmental controls that normally lead to little or no expression of the enzymes in developing seeds.

In a recent study using maize seedlings, it was proposed that β-oxidation could be regulated by either coordinate or differential expression of the different acyl–CoA oxidases in response to developmental or metabolic signals (Hooks et al., 1995). Candidates for metabolite signals potentially involved in regulation of β-oxidation activity are free fatty acids, acetyl–CoA, and acyl–CoAs. Indeed, metabolite changes resulting from the use of lipids instead of carbohydrates as the major respiratory substrates in glucose-starved root tips of maize also cause an increase in β-oxidation activity (Dieuaide et al., 1992). The results from our study indicate that lauroyl–CoA oxidase activity is specifically induced, whereas palmitoyl–CoA oxidase activity is not. Thus, rather than a global induction of all β-oxidation enzymes, medium-chain acyl–CoA oxidase and long-chain acyl–CoA oxidase are under differential control, which is responsive to the chain length of the acyl substrate.

Both the acetate incorporation into sucrose/malate (Figure 3) and the induction of β-oxidation/glyoxylate cycle en-

zymes (Figure 1 and Table 2) suggest that a fraction of the fatty acid produced by developing seeds of line 198 plants is turned over in a futile cycle of synthesis and breakdown of lauric acid. It is not possible, using these data, to directly determine the exact proportion of laurate that is oxidized. However, the incorporation of 50% of added ^{14}C-acetate into non-lipid intermediates suggests that this amount may be as high as 50% of total fatty acid synthesis. Surprisingly, neither oil yield nor seed production is reduced in these transgenic plants (Voelker et al., 1996). How is oil content maintained if a significant amount of the lauric acid produced by fatty acid synthesis is turned over in a futile cycle? One explanation for this result is our observation that high levels of MCTE also resulted in increased levels of at least six other fatty acid synthetic proteins (Figures 4 and 5, and Table 1).

These results have several implications. First, a coordinate induction of the enzymes of the fatty acid synthetic pathway occurred, presumably to compensate for the lauric acid lost through β-oxidation or the shortage of long-chain fatty acids in these seeds. This suggests that the genes for the entire fatty acid synthesis pathway may be subject to a system of global regulation similar to that for lipid biosynthesis genes of yeast (Chirala, 1992; Schuller et al., 1992). Second, these results indicate that although oilseed rape seeds are relatively high in oil content (~40%), the expression of the fatty acid synthesis enzymes is not at a maximum and can be induced a further two- to threefold over the levels found at midstage of seed development. Finally, maintenance of an oil content that is the same as that of the wild type suggests that seeds might be preprogrammed to produce a particular amount of oil with levels of lipid biosynthetic enzyme expression adjusted to meet the prescribed demand for triacylglycerol synthesis.

What signals lead to the increased expression of fatty acid synthesis enzymes? The major products of fatty acid synthesis in oilseed rape plastids are long-chain acyl–ACPs, which are rapidly converted into acyl–CoA esters on transport to the cytoplasm. In this study, expression of MCTE results in hydrolysis of lauroyl–ACP and consequently reduction of the long-chain acyl–ACP pool (Figure 4). These changes in the acyl–ACP pools were accompanied by increases in at least six different fatty acid synthesis proteins, including two different subunits of acetyl–CoA carboxylase. This suggests that the cell detects and responds to the levels of specific metabolite signals (such as acyl–ACP or acyl–CoA intermediates) that then trigger the induction of a suite of fatty acid synthesis enzymes. However, this induction does not appear to respond to the total amount of fatty acid produced but rather is sensitive to the chain length of the products. Because the major end product of fatty acid metabolism in the seed is triacylglycerol, and the total amount of triacylglycerol was not altered in transformants producing high lauric acid (Voelker et al., 1996), the increase in fatty acid synthesis enzyme expression that we observed is unlikely to be a general response to changes in fatty acid production but rather may be a specific response to depletion of long-chain fatty acid biosynthesis. Thus, we propose that the developing seeds (either directly or indirectly) sense and respond to a shortage of long-chain fatty acids, even though total fatty acid synthesis is not reduced. Although it is tempting to speculate that this control system involves specific transcriptional regulators, we found no evidence for increased mRNA levels for three of the fatty acid synthesis proteins that were examined.

The information generated by this study also has implications for the future development of transgenic oil crops. As demonstrated by MCTE transformants, despite expression of very high levels of thioesterase activity, limitations on metabolism of laurate into triacylglycerol appear to confine laurate content to ~60% of the seed fatty acids. It seems likely that the metabolic regulation of β-oxidation will become an important consideration in the design of engineered oilseed crops, particularly in the production of high-purity oils with a single predominant fatty acid component.

METHODS

Plant Materials

Seeds of *Brassica napus* (cv 212/86) wild-type plants and plants transformed with the plasmid pCGN3831 (cauliflower mosaic virus 35S lauroyl–acyl carrier protein thioesterase [MCTE]; Voelker et al., 1992) or the plasmid pCGN3828 (napin MCTE; Voelker et al., 1996) were provided by Calgene (Davis, CA). The lines used in this study are derived from transformation events 8 and 11 from pCGN3831 and events 18 and 23 to 198 from pCGN3828. Plants were grown in growth chambers at 22°C with 16 hr of illumination. Flowers were tagged when petals were first opened, and developing seeds were harvested and immediately frozen in liquid nitrogen before enzyme assays. Fatty acids of mature seed embryos and leaf tissue were analyzed using gas chromatography of their methyl ester derivatives, as described previously (Eccleston et al., 1996). Etiolated seedlings were obtained by germinating seeds on wet filter paper in foil-wrapped Petri dishes for 5 days before harvest. The appearances of the etiolated seedlings (wild-type and transformed lines) were identical in height, color, and proportion of germination.

Labeling with ^{14}C-Acetate and Analysis of Products

Seed coats were removed, and eight embryos (26 to 30 days after flowering [DAF]) were incubated at 25°C for 1 to 8 hr with gentle shaking and illumination in 0.2 mL of 30 mM Mes, pH 5, containing 2.2 μCi of ^{14}C-acetate (55 μCi/μmol). Incubations were stopped by adding 0.75 mL of 0.15 M acetic acid, 2.5 mL of methanol, and 1.25 mL of chloroform and homogenizing. After mixing for 2 hr, 1 mL of water and 1.25 mL of chloroform were added and mixed, and the phases were separated by centrifugation. The lower lipid–containing phase was removed, the aqueous phase was reextracted with 3 mL of hexane, and the combined organic phases were dried under nitrogen. Incorporation of carbon-14 into lipids and aqueous soluble components was determined by liquid scintillation counting, and lipids were further characterized by thin-layer chromatography (TLC) on

silica gel plates developed with ether–petroleum ether–acetic acid (50:50:1). The carbon-14 components of the aqueous phase were examined by TLC on cellulose plates, as described by Canvin and Beevers (1961), with standards of ^{14}C-sucrose and ^{14}C-malate. Some aqueous extracts were concentrated, adjusted to pH 6, and incubated with 25 units of invertase at 55°C for 3 min before TLC, as given above. A ^{14}C-sucrose standard treated at the same time served as control and TLC standard.

Enzyme Assays

Acyl–acyl carrier protein (ACP) thioesterases were assayed in crude homogenates of leaf or developing seed tissue prepared in 20 mM Tris-HCl, pH 8.5, containing 0.5 mM DTT and 5% glycerol (by volume), and clarified with 10 min of centrifugation at full speed in a benchtop microcentrifuge at 4°C. Protein was determined using the method of Bradford (1976) with BSA as the standard. Lauroyl–ACP thioesterase activity was measured in tissue extracts equivalent to 5 mg of total protein by following the hydrolysis of 1-^{14}C-12:0–ACP (53 μCi/μmol; 10,000 dpm per incubation) in 50 μL of homogenization buffer for a 10-min period at room temperature. Reactions were terminated by addition of 50 μL of 1 M acetic acid in isopropanol containing 1 mM carrier free fatty acid. Free fatty acids were extracted in two aliquots of petroleum ether saturated with 50% isopropanol (300 μL), and radioactivity was determined by scintillation counting. Oleoyl–ACP thioesterase activity was assayed as described above, except that 1-^{14}C-oleoyl–ACP was used as the substrate.

Acyl–CoA oxidase was assayed in developing seed, leaf, or etiolated seedling tissue homogenized in 150 mM Tris-HCl, pH 7.5, containing 10 mM potassium chloride, 10 mM flavin adenine dinucleotide, 1 mM EDTA, and 10% glycerol (by volume) (0.3 g of tissue in 500 μL). After centrifugation (at full speed in a benchtop microcentrifuge for 10 min at 4°C), the pellet was reextracted in homogenization buffer (250 μL). The combined supernatant was assayed for protein and then precipitated with 40% ammonium sulfate. The corresponding pellet was resuspended in homogenization buffer (50 μL). Aliquots (10 μL) were assayed for acyl–CoA oxidase by using the peroxidase-coupled reaction with *p*-hydroxybenzoic acid, originally described by Hyrb and Hogg (1979), and using 50 μM acyl–CoA substrate, as described by Hooks et al. (1995).

Isocitrate lyase and malate synthase were assayed spectrophotometrically on aliquots of soluble protein samples equivalent to 100 mg of total protein by using phenylhydrazine or dithiodinitrobenzene as the chromophoric substrates, as described by Ettinger and Harada (1990) and as adapted originally from Cooper and Beevers (1969).

Immunoblot Analysis

Total soluble protein extracts were prepared from developing seed samples, as described above for acyl–CoA oxidase assays. Proteins were fractionated by 10% SDS-PAGE and transferred to nitrocellulose (0.45-μm membrane). Antisera raised against castor biotin carboxylase (Roesler et al., 1996), avocado stearoyl–ACP desaturase (Shanklin and Somerville, 1991), and spinach 3-ketoacyl–ACP synthase III (Tai and Jaworski, 1993) were prepared as previously described. Anti-biotin antibody was purchased from Sigma. Antibody bound proteins were detected using alkaline phosphatase–conjugated anti-rabbit secondary antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD). After colorimetric development, protein bands were quantitated by a computing densitometer (Molecular Dynamics, Sunnyvale, CA). Densitometer signals of blots were corrected for differences in protein loading (as determined by Coomassie Brilliant Blue R 250 staining of equivalent gel lanes) and normalized against values for the control plant.

For acyl–ACP analysis, seed embryos were quick frozen in liquid nitrogen and homogenized in trichloroacetic acid (5% w/v) (1 mL per 0.5 g frozen weight) and incubated on ice for 10 min. Acyl–ACPs and cell debris were pelleted by centrifugation at 10,000*g* for 20 min. Pellets were washed in cold trichloroacetic acid (1% w/v) and resuspended in ~5 volumes of Mops (50 mM), pH 7.5. Acyl–ACP extracts were clarified by centrifugation to remove debris, and fresh weight equivalents were separated by 2.0 M urea/13% native PAGE. *N*-Ethylmaleimide (100 mM) was added to all samples to a final concentration of 10 mM before PAGE separation. Acyl–ACPs were detected on immunoblots by using antibodies raised against spinach ACP, as previously described (Post-Beittenmiller et al., 1991).

RNA Isolation and Blotting

Total RNA was isolated from seed tissues by minor modification of the method of Wilkins and Smart (1996). Aliquots (10 μg) of total RNA were separated using 1.1% formaldehyde gel electrophoresis and transferred to Zeta probe membranes (Bio-Rad). RNA gel blots were prehybridized for 4 to 8 hr at 42°C, and hybridizations were performed at 42°C for 12 to 24 hr using a random-primed ^{32}P-radiolabeled DNA probe, as described previously (Bao et al., 1997). ^{32}P-radiolabeled random DNA probes were synthesized using cDNA templates for oilseed rape ACP (EcoRI and HindIII fragments from pBN45 [Safford et al., 1988] and oilseed rape BCCP [gift of Q. Sun, Monsanto, St. Louis, MO]) and Arabidopsis biotin carboxylase (Bao et al., 1997).

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