Feedback regulation of plastidic acetyl-CoA carboxylase by 18:1-acyl carrier protein in *Brassica napus*

Carl Andre[a,1], Richard P. Haslam[b], and John Shanklin[a,2]

[a]Biology Department, Brookhaven National Laboratory, Upton, NY 11973; and [b]Department of Biological Chemistry, Center for Crop Genetic Improvement, Rothamsted Research, West Common, Harpenden, Herfordshire AL5 2JQ, United Kingdom

Edited* by George H. Lorimer, University of Maryland, College Park, MD, and approved May 8, 2012 (received for review March 20, 2012)

Plant seed oil represents a major renewable source of reduced carbon, but little is known about the biochemical regulation of its synthesis. The goal of this research was to identify potential feedback regulation of fatty acid biosynthesis in *Brassica napus* embryo-derived cell cultures and to characterize both the feedback signals and enzymatic targets of the inhibition. Fatty acids delivered via Tween esters rapidly reduced the rate of fatty acid synthesis in a dose-dependent and reversible manner, demonstrating the existence of feedback inhibition in an oil-accumulating tissue. Tween feeding did not affect fatty acid elongation in the cytosol or the incorporation of radiolabeled malonate into nascent fatty acids, which together pinpoint plastidic acetyl-CoA carboxylase (ACCase) as the enzymatic target of feedback inhibition. To identify the signal responsible for feedback, a variety of Tween esters were tested for their effects on the rate of fatty acid synthesis. Maximum inhibition was achieved upon feeding oleic acid (18:1) Tween esters that resulted in the intracellular accumulation of 18:1 free fatty acid, 18:1-CoA, and 18:1-acyl-carrier protein (ACP). Direct, saturable inhibition of ACCase enzyme activity was observed in culture extracts and in extracts of developing canola seeds supplemented with 18:1-ACP at physiological concentrations. A mechanism for feedback inhibition is proposed in which reduced demand for de novo fatty acids results in the accumulation of 18:1-ACP, which directly inhibits plastidic ACCase, leading to reduced fatty acid synthesis. Defining this mechanism presents an opportunity for mitigating feedback inhibition of fatty acid synthesis in crop plants to increase oil yield.

allosteric regulation | enzyme regulation | oil synthesis

Feedback inhibition of biosynthetic pathways occurs when a downstream metabolite accumulates and causes inhibition of an enzyme controlling an early step in its synthesis. Plant fatty acid biosynthesis is feedback-regulated by an unknown mechanism (1–3) and a basic understanding of this process is vital for the successful engineering of this pathway to increase oil yield. A variety of mechanisms contribute to feedback inhibition of fatty acid synthesis in animals, fungi, and bacteria. In rats and yeast, palmitoyl-CoA, an intermediate of fatty acid synthesis, binds to and inhibits acetyl-CoA carboxylase (ACCase) (4). Also in yeast, ACCase and fatty acid synthase (FAS) gene expression are repressed in an acyl-CoA–dependent manner (5). In *Escherichia coli*, ACCase and the β-ketoacyl acyl carrier protein (ACP) synthase subunit of FAS are inhibited by long-chain (C16-C18) acyl-ACP, an intermediate of fatty acid synthesis (6, 7). Repression of bacterial fatty acid biosynthetic genes (including ACCase) by interaction of long-chain acyl-ACP or acyl-CoA with transcription factors has also been observed (8). Thus, a picture has emerged in which decreased demand for de novo fatty acids is signaled by a variety of signals, including acyl-ACP and acyl-CoA. These metabolites allosterically inhibit fatty acid biosynthetic enzymes and can thereby rapidly decrease the production of malonyl-CoA for use in fatty acid synthesis. When the levels of acyl-ACP and acyl-CoA are not sufficiently reduced, gene expression for the entire biosynthetic pathway can also be repressed.

Tween esters are effective agents for the delivery of fatty acids and have been shown to elicit feedback inhibition in various plant cell culture systems (1–3). Shintani and Ohlrogge (2) hypothesized that feedback possibly occurs through biochemical or posttranslational modification of ACCase and FAS. Purified monomeric ACCases from maize and diatom were inhibited by palmitoyl-CoA (9, 10), but long-chain (C16-C18) acyl-ACPs failed to inhibit partially purified ACCases from castor and pea (11). Decanoyl-ACP did, however, inhibit ketoacyl-ACP synthase activity in crude extracts of canola and spinach (12). The relevance of these results to feedback inhibition in oil-accumulating tissues is unclear because changes in the steady-state pools of acyl-CoA or acyl-ACP during feedback were not measured. The situation is further complicated in dicotyledonous plants because of the presence of structurally distinct heteromeric and monomeric ACCase and FAS systems in the plastid and cytosol, responsible for fatty acid synthesis and elongation, respectively. Whether or not the cytosolic fatty acid elongation pathway is subject to feedback inhibition is unknown. It is also not known if feedback inhibition occurs in oil-seed tissues, which have higher rates of fatty acid synthesis than in vegetative tissues.

To characterize feedback inhibition of fatty acid synthesis in oil seeds and to elucidate its molecular mechanism, we have used a *Brassica napus* cv Jet Neuf cell-suspension culture created from microspore-derived embryos. This culture has been used for studies on the synthesis of storage lipids and for purification of native enzymes (13, 14). In contrast to soybean and tobacco cell cultures (1, 2), oil (triacylglycerol or TAG) is present, which contains very-long-chain fatty acids generally found only in seed tissues. With this system we have characterized feedback inhibition in oil-accumulating tissue, and have systematically dissected the inhibition of fatty acid synthesis and present a model to explain its mechanism.

Results

Plastidic ACCase Is Reversibly Inhibited in Response to Tween 80. Tween 80, containing predominately oleic acid (18:1) (Table 1), was tested for its effects on the rate of fatty acid synthesis in the *B. napus* suspension cells described above. The rate of fatty acid synthesis was determined by in vivo labeling of cells with ^{14}C-acetate for 15 min, which fell within the linear range of ^{14}C incorporation (Fig. S1*A*). Fig. 1*A* shows that as soon as 3 h after

Author contributions: C.A. and J.S. designed research; C.A. and R.P.H. performed research; C.A. contributed new reagents/analytic tools; C.A. and J.S. analyzed data; and C.A. and J.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

[1]Present address: BASF Plant Science, Research Triangle Park, NC 27709.

[2]To whom correspondence should be addressed. E-mail: shanklin@bnl.gov.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1204604109/-/DCSupplemental.

Table 1. Inhibition of ^{14}C-acetate labeling of lipids by various Tweens

	Composition of Tween (mol %)					Relative ^{14}C-acetate incorporation (% control)
	14:0	16:0	16:1	18:0	18:1	
Tween 40	2.3	94.0	ND	3.7	ND	84.4 ± 3.4
Tween 60	2.8	45.4	ND	51.8	ND	85.9 ± 3.4
Tween 80	1.9	7.3	2.4	2.1	86.4	50.9 ± 7.0
Tween 85	2.7	5.4	6.7	2.2	83.0	48.8 ± 5.9
Tween 18:1	ND	ND	ND	ND	100	51.5 ± 6.7

Values are the mean of three repeats ± SD where included. Fatty acid composition was determined by preparing FAMEs of various commercial and custom (Tween 18:1) Tweens. ^{14}C-acetate labeling is relative to a control sample without any Tweens. All values for relative ^{14}C-incorporation are significantly different from the control as determined by Student's *t* test (all $P < 0.05$). ND, not detected.

the addition of 10 mM Tween 80 to the medium, the rate of ^{14}C-acetate incorporation was reduced by 40%, indicating feedback inhibition of fatty acid synthesis. The degree of inhibition was found to be dose-dependent on the concentration of Tween 80 in the medium (Fig. 1*B*) and was completely reversible after its removal (Fig. 1*C*). These results are consistent with a biochemical mode of action for feedback inhibition. To focus on the early events leading to biochemical feedback, all subsequent experiments in this study used 3-h Tween feedings.

Plants contain ACCase and FAS enzyme systems in both the plastid and cytosol, which are capable of incorporating ^{14}C-acetate into fatty acids. Comparing the distribution of label in individual fatty acid species can therefore provide information on the relative contribution of these pathways. Tween 80 feeding, although reduces the rate of incorporation of ^{14}C-acetate into all fatty acids (Fig. 2*A*), had no effect on the relative distribution of ^{14}C-label (Fig. 2*B*). This finding is consistent with reduced de novo fatty acid synthesis inside the plastid rather than reduced cytosolic elongation, which would have resulted in a lower proportion of label in very-long-chain fatty acids (C20-24). ^{14}C-acetate labeling was also conducted in the presence of haloxyfop, an inhibitor of the cytosolic ACCase involved in fatty acid elongation (Fig. S2). The degree to which ^{14}C-acetate incorporation is inhibited by haloxyfop is proportional to cytosolic elongation activity, whereas haloxyfop-resistant incorporation is from the de novo pathway within the plastid. Fig. 2*C* shows that haloxyfop inhibited ^{14}C-acetate incorporation by the same amount in cultures with or without Tween 80 (i.e., the inhibition curves parallel one another). However, haloxyfop-resistant incorporation (represented by the area below the curves) was reduced by

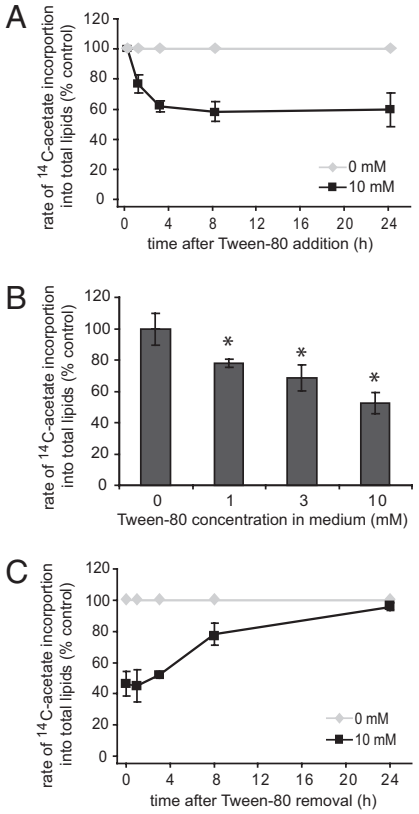

Fig. 1. Inhibition of ^{14}C-acetate labeling of lipids by *B. napus* cells in the presence of Tween 80. (*A*) Incorporation of ^{14}C-acetate into total lipids by intact cells in the presence or absence of 10 mM Tween 80. (*B*) ^{14}C-actetate labeling of total lipids by intact cells after 3 h of exposure to various concentrations of Tween 80. *$P < 0.05$ determined by Dunnett's test. (*C*) ^{14}C-actetate labeling of total lipids after removal of Tween 80 following a 3-h exposure to 10 mM Tween 80. All data are the mean ± SD ($n = 3$).

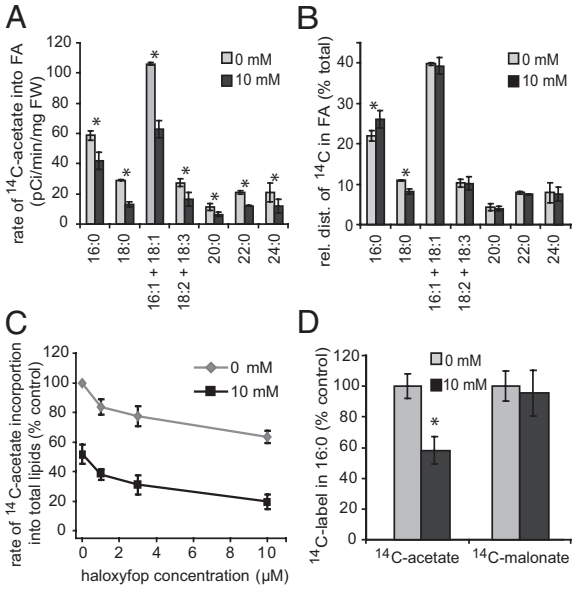

Fig. 2. Specific inhibition of plastidic ACCase in *B. napus* cells after 3 h of Tween 80 feeding. (*A*) Rate of ^{14}C-acetate incorporation into fatty acids after 3 h of Tween 80 feeding. (*B*) Relative distribution of ^{14}C-acetate in fatty acids after 3 h of 10 mM Tween 80 feeding. (*C*) ^{14}C-acetate incorporation into total lipids by cells fed various concentrations of haloxyfop for 30 min after 3 h of 10 mM Tween 80 feeding. (*D*) Incorporation of label from ^{14}C-malonate and ^{14}C-acetate into 16:0 fatty acid after 3 h of 10 mM Tween 80 feeding. All data are the mean ± SD ($n = 3$). *$P < 0.05$ determined by Student's *t* test.

approximately half in cultures with 10 mM Tween 80, showing that Tween 80 feeding specifically causes inhibition of de novo fatty acid synthesis in the plastid. Fatty acid synthesis requires acetyl-CoA and ATP, both of which are also used for sterol synthesis. Therefore, if fatty acid synthesis is inhibited because of substrate limitation, this should also be observed for sterol biosynthesis. After 3 h of Tween 80 feeding, ^{14}C-acetate incorporation into sterols was 102 ± 4.4% relative to a control, but incorporation into free fatty acids (FFA) was 55.8 ± 5.0%, confirming that the observed inhibition was specific to fatty acid synthesis.

The data indicate that ACCase or FAS in the plastid is inhibited upon the addition of Tween 80. In an attempt to distinguish between these possibilities, we exploited the fact that exogenous malonate can be used by FAS, bypassing the ACCase reaction (15). If ACCase is the only point of inhibition, then the rate of ^{14}C-malonate labeling of de novo fatty acids should be the same in cultures with or without Tween 80. Cells were labeled with ^{14}C-malonate for 15 min (same as for ^{14}C-acetate), which falls within the linear range of ^{14}C-incorporation into lipids (Fig. S1*B*). The incorporation of ^{14}C-malonate into nascent fatty acids (e.g., 16:0 in Fig. 2*D* and a full panel of C16-C24 fatty acids in Fig. S1*C*) was not inhibited by Tween 80, in contrast to ^{14}C-acetate controls. The malonate incorporation data supports the hypothesis that plastidic ACCase is the target for Tween 80 induced feedback inhibition.

Signal of 18:1-ACP Mediates Feedback Inhibition of Plastidic ACCase. Commercial Tween 80 contains a mixture of fatty acids. To dissect the effects of the individual components, a variety of Tween esters were tested for their effect on fatty acid synthesis. The compositions of individual Tweens are listed in Table 1, along with results from ^{14}C-acetate labeling experiments. Tween 40 and Tween 60 containing only saturated fatty acids did not inhibit acetate labeling of lipids to the same extent as Tween 80 or 85, containing primarily 18:1. Custom synthesized Tween 18:1 also produced maximum inhibition. Fatty acids from Tween are activated by esterification to free CoA or ACP before being deposited into cellular lipids. Both acyl-CoA and acyl-ACP are inhibitors of ACCase in other organisms, so steady-state pools of these metabolites as well as FFA were measured in cells fed Tween 80. After 3 h of feeding, 18:1 FFA appeared where there was none detected in untreated cells (Fig. 3*A*). Similarly, both 18:1-ACP and 18:1-CoA doubled in amount upon Tween 80 feeding but most other molecular species went down (Fig. 3 *B* and *C*). In a separate experiment designed to distinguish the incorporation of fatty acids from Tween from secondary effects of feedback inhibition, FFA and acyl-ACPs were analyzed in cells fed ^{13}C-18:1-Tween. The results showed that at 3 h, 99.1 ± 2.2% of 18:1 FFA and 46.2 ± 4.2% of 18:1-ACP contained ^{13}C-oleic acid (Fig. 3), meaning that the increases observed in these metabolites result directly from the uptake of fatty acids from Tween.

Because in other systems 18:1-ACP and 18:1-CoA have been shown to act as allosteric regulators (4, 6, 7), we tested their direct effects on ACCase specific activity. First, the in vivo concentrations of these intermediates were calculated based on the values in Fig. 3 and the water content (fresh weight/dry weight, FW/DW) of the cells. The intracellular concentration of 18:1 FFA was estimated to be 0–100 μM. The estimated concentration of 18:1-ACP was 0.6–1.2 μM. However, when taken into account that acyl-ACPs are restricted to the plastid, the volume of which in developing embryos was reported to be 5–20% of the cell (16), the estimate increased to 3.0–24 μM. Total cellular 18:1-CoA was estimated to be 1–3 μM and assuming that the cytosol occupies 10–45% of the cell, this value increased to 2.2–30 μM. These values established ranges of concentrations to test in vitro. As crude cell-culture extracts contained significant FATA thioesterase activity (see *SI Materials and Methods*) care was taken to ensure minimal degradation of 18:1-CoA or 18:1-ACP during enzyme assays. FFA had no effect on ACCase activity. Inhibition of ACCase was observed when CoA or ACP esterified to 18:1, but not saturated fatty acids, were added at 10 μM concentration (Fig. 4*A*). When included together in an assay, 18:1-CoA and 18:1-ACP did not cause increased inhibition compared with when each was added alone (Fig. S3). Inhibition with 18:1-CoA and 18:1-ACP was then tested over a range of concentrations and was found to be saturable (Fig. 4*B*). We note that a highly expressed ACP isoform from *B. napus* seeds (17) was used for all experiments. The specificity of inhibition to 18:1 acyl moieties is consistent with 18:1-containing Tweens causing maximum feedback. We also tested ACCase inhibition in crude extracts prepared from 25-d-after-flowering canola seeds dissected from siliques. Because of its exclusion from the chloroplast, 18:1-CoA was not tested with this tissue. ACCase from developing seeds was inhibited by 18:1-ACP to a similar extent as ACCase from cell cultures (Fig. 4*B*).

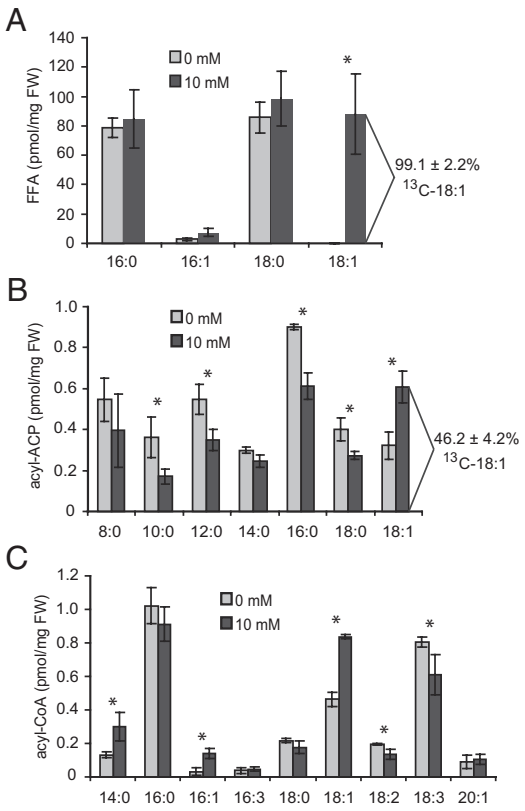

Fig. 3. Quantification of lipid intermediates in *B. napus* cells after 3 h of Tween 80 feeding. (*A*) FFA, (*B*) acyl-ACP, and (*C*) acyl-CoA content in cells after 3 h of 10 mM Tween 80 feeding. Where present, numbers represent the percent of 18:1 that was ^{13}C-labeled after 3 h of 10 mM ^{13}C-oleoyl-Tween feeding. All data are the mean ± SD (n = 3). *P < 0.05 determined by Student's t test.

To preclude transcriptional and posttranslational contributions to Tween 80–dependent feedback inhibition, enzyme assays were performed. ACCase-specific activity was unchanged relative to a control in crude extracts from cells fed Tween 80 for 3 h (Fig. 5*A*). ACCase can be inactivated by dephosphorylation (18) or by interaction with PII protein in a 2-oxoglutarate–dependent manner (19). ACCase assays following phosphatase treatment of crude extracts or in the presence of 5 mM 2-oxoglutarate revealed no differences between control and Tween 80–fed cultures (Fig. 5*A*). Treatment with haloxyfop did not cause a significant reduction in specific activity, indicating that assays with crude extracts are largely specific for plastidic ACCase (Fig. 5*B*). Tween

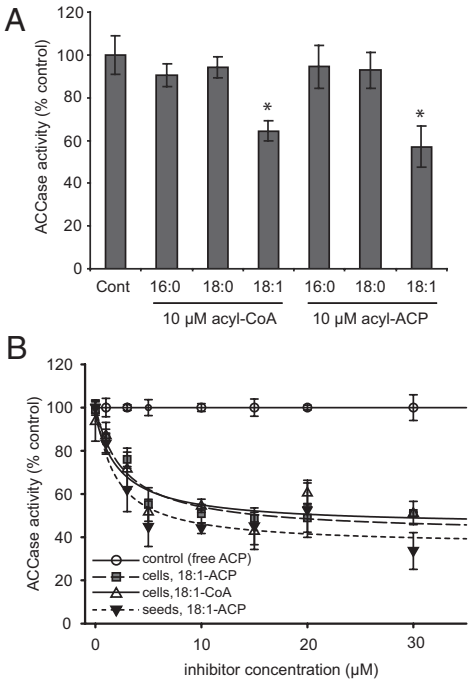

Fig. 4. Effects of lipid intermediates on ACCase activity in crude *B. napus* cell extracts and in developing seeds. (*A*) Effects of 10 μM acyl-CoAs and acyl-ACPs on ACCase activity in crude cell-culture extracts. $*P < 0.01$ determined by Dunnett's test. All other $P > 0.10$. (*B*) Inhibition of ACCase from cell cultures (cells) or 25-d-after-flowering seeds (seeds) caused by various concentrations of 18:1-CoA or 18:1-ACP. Values are normalized to the control, which is free ACP. Data are the mean ± SE ($n = 3$).

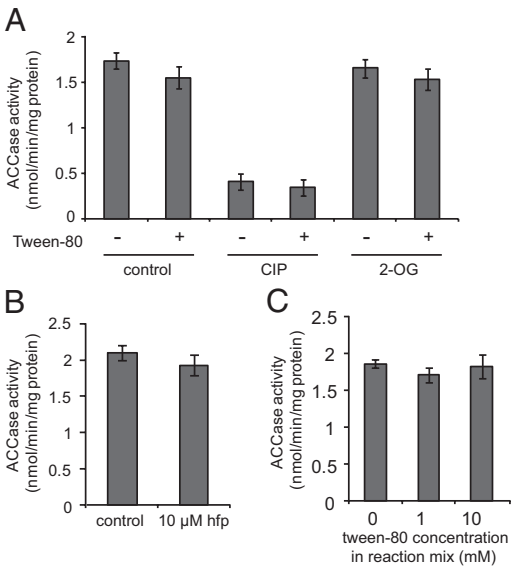

Fig. 5. ACCase specific activity in crude cell extracts. (*A*) ACCase activity in control, phosphatase treated (CIP), and 2-oxoglutarate (2-OG) supplemented crude extracts from cells grown with or without Tween 80 for 3 h. (*B*) Effect of 10 μM haloxyfop on ACCase activity when added to crude extracts. (*C*) Effect of Tween 80 on ACCase activity when added directly to enzyme assays. Samples from *B* and *C* were grown without Tween in the culture medium. Data are the mean ± SD ($n = 3$). Significance between + and − Tween samples (*A*), and between controls and haloxyfop (*B*) or Tween 80 (*C*) treated samples was calculated using Student's *t*-test and all $P > 0.05$.

80, when added directly to in vitro enzyme assays, did not inhibit ACCase (Fig. 5*C*). Taken together, these results suggest that transcriptional or known posttranslational mechanisms leading to a reduction in plastidic ACCase specific activity do not account for the observed feedback inhibition.

Discussion

Data from work presented here identifies 18:1-ACP as the signal molecule and plastidic ACCase as the target for biochemical feedback inhibition of fatty acid biosynthesis in an oleogenic plant tissue. That 18:1-ACP is the signal responsible for rapid and reversible feedback inhibition is supported by the observations that: (*i*) feeding experiments with 18:1-containing Tween esters cause maximal inhibition; (*ii*) 18:1-ACP accumulates in vivo during feedback inhibition; (*iii*) ACCase enzyme activity displays saturable in vitro inhibition upon supplementation of 18:1-ACP at the physiological concentrations directly measured in this study; and (*iv*) although we demonstrate the in vitro inhibitory effects of both 18:1-ACP and 18:1-CoA on ACCase, the involvement of 18:1-CoA in feedback inhibition is discounted because its measured level in this study is insufficient to cause significant inhibition and because long-chain acyl-CoAs have not been detected in plastids (20). That the plastidic multimeric isoform of ACCase is the specific target of in vivo feedback inhibition is supported by the observations that: (*i*) feedback inhibition affects de novo fatty acid synthesis in the plastid, and not fatty acid elongation in the cytosol; (*ii*) experiments showing that haloxyfop, an inhibitor of cytosolic ACCase, causes an additive effect together with 18:1-Tween inhibition; (*iii*) that acetate, but not malonate, incorporation into fatty acids is inhibited by Tween feeding, thus ruling out FAS as the target of inhibition; (*iv*) the level of inhibition of ACCase by 18:1-ACP observed in vitro was equivalent to that measured in vivo; and (*v*) the plastidial homomeric isoform of ACCase is not expressed in oilseed embryos (21). Moreover, we extended our analysis to developing canola seeds and also observed inhibition of ACCase by 18:1-ACP. Based on these results, we propose a mechanism in which the concentration of 18:1-ACP controls plant fatty acid synthesis through its biochemical inhibition of plastidic ACCase (Fig. 6). This proposed biochemical mechanism is also consistent with the observations that the levels of enzymatic activities measured in extracts from inhibited cultures were equivalent to untreated controls, demonstrating that transcriptional and posttranslational

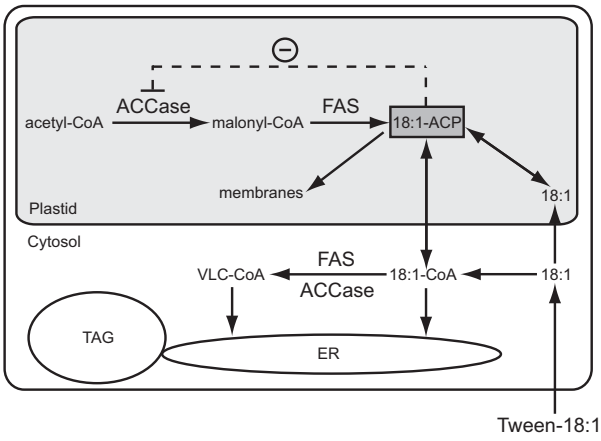

Fig. 6. Model for proposed mechanism of feedback inhibition of fatty acid synthesis. Plastidic ACCase is inhibited by 18:1-ACP. This metabolite is a product of de novo fatty acid synthesis, or can be synthesized from exogenous fatty acids provided by Tween 18:1. Reactions that can produce or consume, and therefore participate in the regulation of 18:1-ACP concentration, are indicated with arrows.

mechanisms are not responsible for the observed feedback inhibition. Inhibition of the multimeric plant ACCase by 18:1-ACP is consistent with the biochemical feedback mechanism of *E. coli* (6) and the bacterial lineage of plastidic ACCase.

A previous study of feedback inhibition in tobacco vegetative tissue reported the rate of production of intermediates of fatty acid synthesis, but not their steady-state pool size (2). Reduced synthesis of long-chain ACPs upon Tween feeding led the authors to conclude that they were unlikely to be involved in feedback inhibition. We measured steady-state levels of metabolites and found that FFAs, 18:1-ACP, and 18:1-CoA concentrations actually increased as a result of incorporation of fatty acids from Tween. The FFAs that accumulated were exclusively from Tween 80 and are likely the result of its hydrolysis upon entering the cells. A plastid localized acyl-ACP synthetase can esterify exogenous fatty acids to ACP (22). Although the amount of acyl-ACP reported in this work is higher than that of spinach leaves, the composition of individual molecular species is similar (23). This quantitative difference may be attributable to the embryo-like identity and associated higher rate of fatty acid synthesis of the cells used here. In addition, long-chain (C16-C18) acyl-ACP concentration in *E. coli* is as high as 63 μM (7) and the tissue used here is estimated to have 7.5–60 μM. Therefore, the acyl-ACP concentrations reported here fall within the range of previously published values. Total acyl-ACP concentration was reduced by feedback inhibition, which is consistent with the previously reported reduced rate of acyl-ACP synthesis (2). This finding is likely the result of limited substrate availability for the synthesis of acyl-ACPs resulting from reduced ACCase activity. The biochemical mechanism of ACCase inhibition by acyl-ACP is unknown, even for *E. coli* ACCase (6). Inhibition of ACCase by acyl-ACP only occurred when (*i*) species specific ACP isoforms are used and (*ii*) DTT, which is an activator of ACCase (23), is included in the assay buffer (6, 11, and present study). Moreover, the apparent IC_{50} values for 18:1-CoA and 18:1-ACP were similar and we did not observe increased inhibition by the addition of both 18:1-ACP and 18:1-CoA, implying a common mode of action that is dependent on both the identity of the fatty acid and the presence of a thioester bond.

The reduced rate of fatty acid synthesis during Tween feeding could result from a shortage of free ACP and CoA because of thioesterification of exogenous fatty acids. If this were the case, reduced incorporation of both ^{14}C-acetate and ^{14}C-malonate into fatty acids would be observed. However, only the incorporation of ^{14}C-acetate was reduced. ATP is required by ACCase and its availability could influence enzyme activity. Exogenous long-chain acyl-CoAs have been shown to reduce fatty acid synthesis in isolated plastids by inhibition of ATP import (24). However, for the experiments presented here, the combined amount of long-chain acyl-CoAs was unaffected by Tween feeding, making it unlikely that ATP import was inhibited, as well as supporting the notion that CoA was not limiting. In addition, sterol biosynthesis and growth rate of the cells, both of which would be adversely affected by limited ATP supply, were unaffected by Tween feeding. Plastidic ACCase is regulated by a variety of factors in vivo (25) and the identification of plastidic ACCase as the target of feedback inhibition is consistent with its regulatory role in chloroplast fatty acid synthesis (26). Inhibition of FAS would be predicted to result in the accumulation of malonyl-CoA. In the absence of FAS activity, malonyl-CoA would be a dead-end product in the plastid, and therefore inhibition of ACCase can be considered to be more efficient than that of FAS. In the cytosol, malonyl-CoA is required for flavonoid biosynthesis and loss of cytosolic ACCase activity is lethal (27). This function of cytosolic ACCase may explain its evident immunity to the effects of feedback.

Our observations of the seed-like cell line used herein demonstrates that feedback inhibition of ACCase can occur in tissues where fatty acid synthesis is a primary metabolic function and may explain why overexpression of ACCase resulted in marginal increases in fatty acid production in seeds (28). Paradoxically, it also implies that oil seeds may have evolved means of reducing feedback to achieve high oil content. The metabolic predisposition of seeds to synthesize TAG could explain why higher concentrations of Tween 80 were needed to induce feedback in *B. napus* cells than was required for tobacco (2). Feedback inhibition is relieved in *E. coli* by overexpression of a thioesterase (29) and in *B. napus* indirect reduction in 18:1-ACP by overexpression of a medium-chain thioesterase resulted in higher rates of fatty acid synthesis (30). Conversely, in *Arabidopsis* reduction of FATA thioesterase gene expression causes reduced fatty acid accumulation in developing seeds (31). Feedback inhibition may also explain the shared control of oil accumulation between synthesis and assembly in *B. napus* (3). Diacylglycerol acyltransferase (DGAT), which consumes acyl-CoA in the cytosol, exerts control over oil accumulation (32) and overexpression of this enzyme in *Arabidopsis* results in enhanced oil content (33). Increased fatty acid synthesis is a logical prerequisite for elevated oil content. Conversely, the *Arabidopsis asi1*(*tag1*) mutant deficient in DGAT has less oil than wild-type and reduced incorporation of ^{14}C-acetate into lipids, indicating reduced fatty acid synthesis (34). Premised on these results and the present study, DGAT might exert control over oil accumulation by consuming acyl-CoA in the cytosol, thus driving vectorial export of de novo fatty acids from the plastid and thus attenuating feedback inhibition.

This study was designed to address early events in biochemical feedback inhibition. However, feedback is persistent during prolonged Tween feeding, and by analogy with other systems may involve several distinct mechanisms capable of rapid and then persistent response to oversupply of fatty acids. Nevertheless, the mechanism of biochemical feedback inhibition presented here provides a potential target for designing strategies for increasing fatty acid synthesis in plants. Precedent for a strategy involving defeating the biochemical inhibition of a single regulatory enzyme comes from the expression of a feedback-insensitive ADP-Glc pyrophosphorylase in potatoes, which resulted in increased starch content (35).

Materials and Methods

Cell Culture Growth. *B. napus* cv Jet Neuf suspension cell cultures were grown in NLN medium, as previously described (36). Explicit details can be found in *SI Materials and Methods*.

Lipid Extraction and Quantification. Total lipids were extracted from ~100 mg FW of frozen cells by homogenizing twice in 500 μL of methanol:chloroform:formic acid (20:10:1 vol/vol). The organic solvent was extracted with 500 μL of 1 M KCl, 0.2 M H_3PO_4. The organic phase was dried under N_2 and suspended in hexane. Lipids were separated by TLC with hexane:diethylether:acetic acid (80:20:1, vol/vol) or with acetone:toluene:water (91:30:7 vol/vol) and 0.15 M $(NH_4)_2SO_4$ impregnated plates. Fatty acid methyl esters (FAMEs) were prepared with 1 mL of 12% (wt/wt) BCl_3 in methanol for 1 h at 85 °C, and were extracted with 1 mL of water and 2 mL hexane. FAMEs were quantified by GC-MS, as previously described (37). ^{13}C-fatty acids were detected and quantified as previously described (38). Heptadecanoic acid was routinely used for internal standard.

^{14}C and ^{13}C Labeling. All radioisotopes were obtained from American Radiolabeled Chemicals. Labeling was done on cells 5 d after subculturing with a cell density of ~20 mg FW mL^{-1} with medium refreshed 16 h prior. Typically, 1-mL aliquots of cells were labeled with either 0.2 μCi of 1,2-^{14}C-acetate (50-60 mCi·mmol^{-1}) or 2-^{14}C-malonate (40-60 mCi·mmol^{-1}) for 15 min at 25 °C with shaking. Haloxyfop (Sigma) was dissolved in DMSO and added to cultures 30 min before labeling. Lipids were extracted and separated by TLC as described above. Radioactivity was detected and quantified by phosphorimaging. Incorporation of label into fatty acids species was determined as previously described (22). The 1-^{13}C-oleoyl-Tween synthesis is described in *SI Materials and Methods*.

FFA, Acyl-CoA, and Acyl-ACP Extraction. FFAs were extracted from tissue by quenching ~300 mg of frozen cells in 2 mL boiling isopropanol for 5 min. Once cooled, 2 mL of 0.9% NaCl was added and lipids were extracted twice with 4 mL of hexane. FFA were separated by neutral lipid TLC, made into FAMEs, and analyzed as described above. Acyl-CoAs were extracted from ~15 mg FW of cells and quantified as previously described (39). Acyl-ACPs were extracted and quantified as previously described (23), with modifications: (*i*) TCA precipitated proteins were dissolved with three extractions of Mops buffer, and (*ii*) internal standards used were 11:0-CoA (Sigma) and 17:0-ACP made from spinach ACP as described in *SI Materials and Methods*.

Enzyme Assays. ACCase activity was measured as the acetyl-CoA dependent incorporation of ^{14}C-$NaHCO_3$ into acid-stable products as previously described (40). Details of ACCase assays, acyl-ACP synthesis, and thioesterase assays can be found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Jacob Hooker for his input into the synthesis of custom Tween esters, and Prof. R. Weselake for the generous gift of the *Brassica napus* somatic embryo culture. This work was supported by the Office of Basic Energy Sciences of the US Department of Energy and Bayer Crop Science.

1. Terzaghi WB (1986) Metabolism of Tween-fatty acid esters by cultured soybean cells: Kinetics of incorporation into lipids, subsequent turnover, and associated changes in endogenous fatty acid synthesis. *Plant Physiol* 82:780–786.
2. Shintani DK, Ohlrogge JB (1995) Feedback inhibition of fatty-acid synthesis in tobacco suspension cells. *Plant J* 7:577–587.
3. Ramli US, Baker DS, Quant PA, Harwood JL (2002) Control analysis of lipid biosynthesis in tissue cultures from oil crops shows that flux control is shared between fatty acid synthesis and lipid assembly. *Biochem J* 364:393–401.
4. Ogiwara H, Tanabe T, Nikawa J, Numa S (1978) Inhibition of rat-liver acetyl-coenzyme-A carboxylase by palmitoyl-coenzyme A. Formation of equimolar enzyme-inhibitor complex. *Eur J Biochem* 89:33–41.
5. Feddersen S, Neergaard TB, Knudsen J, Faergeman NJ (2007) Transcriptional regulation of phospholipid biosynthesis is linked to fatty acid metabolism by an acyl-CoA-binding-protein-dependent mechanism in *Saccharomyces cerevisiae*. *Biochem J* 407: 219–230.
6. Davis MS, Cronan JE, Jr. (2001) Inhibition of *Escherichia coli* acetyl coenzyme A carboxylase by acyl-acyl carrier protein. *J Bacteriol* 183:1499–1503.
7. Heath RJ, Rock CO (1995) Regulation of malonyl-CoA metabolism by acyl-acyl carrier protein and beta-ketoacyl-acyl carrier protein synthases in *Escherichia coli*. *J Biol Chem* 270:15531–15538.
8. Zhang YM, Rock CO (2009) Transcriptional regulation in bacterial membrane lipid synthesis. *J Lipid Res* 50(Suppl):S115–S119.
9. Roessler PG (1990) Purification and characterization of acetyl-CoA carboxylase from the diatom *Cyclotella cryptica*. *Plant Physiol* 92:73–78.
10. Nikolau BJ, Hawke JC (1984) Purification and characterization of maize leaf acetyl-coenzyme A carboxylase. *Arch Biochem Biophys* 228:86–96.
11. Roesler KR, Savage LJ, Shintani DK, Shorrosh BS, Ohlrogge JB (1996) Co-purification, co-immunoprecipitation, and coordinate expression of acetyl-coenzyme A carboxylase activity, biotin carboxylase, and biotin carboxyl carrier protein of higher plants. *Planta* 198:517–525.
12. Bruck FM, Brummel H, Schuch R, Spener F (1996) In-vitro evidence for feed-back regulation of beta-ketoacyl-acyl carrier protein synthase III in medium-chain fatty acid biosynthesis. *Planta* 198:271–278.
13. Weselake RJ, Taylor DC (1999) The study of storage lipid biosynthesis using microspore-derived cultures of oil seed rape. *Prog Lipid Res* 38:401–460.
14. Smith CR, Knowles VL, Plaxton WC (2000) Purification and characterization of cytosolic pyruvate kinase from *Brassica napus* (rapeseed) suspension cell cultures: Implications for the integration of glycolysis with nitrogen assimilation. *Eur J Biochem* 267:4477–4485.
15. Kannangara CG, Jacobson BS, Stumpf PK (1973) Fat metabolism in higher plants: LVII. A comparison of fatty acid-synthesizing enzymes in chloroplasts isolated from mature and immature leaves of spinach. *Plant Physiol* 52:156–161.
16. Mansfield SG, Briarty LG (1992) Cotyledon cell-development in *Arabidopsis thaliana* during reserve deposition. *Can J Bot* 70:151–164.
17. Safford R, et al. (1988) Plastid-localised seed acyl-carrier protein of *Brassica napus* is encoded by a distinct, nuclear multigene family. *Eur J Biochem* 174:287–295.
18. Savage LJ, Ohlrogge JB (1999) Phosphorylation of pea chloroplast acetyl-CoA carboxylase. *Plant J* 18:521–527.
19. Feria Bourrellier AB, et al. (2010) Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit. *Proc Natl Acad Sci USA* 107:502–507.
20. Post-Beittenmiller D, Jaworski JG, Ohlrogge JB (1991) In vivo pools of free and acylated acyl carrier proteins in spinach. Evidence for sites of regulation of fatty acid biosynthesis. *J Biol Chem* 266:1858–1865.
21. Troncoso-Ponce MA, et al. (2011) Comparative deep transcriptional profiling of four developing oilseeds. *Plant J* 68:1014–1027.
22. Koo AJ, Fulda M, Browse J, Ohlrogge JB (2005) Identification of a plastid acyl-acyl carrier protein synthetase in *Arabidopsis* and its role in the activation and elongation of exogenous fatty acids. *Plant J* 44:620–632.
23. Kopka J, Ohlrogge JB, Jaworski JG (1995) Analysis of in vivo levels of acyl-thioesters with gas chromatography/mass spectrometry of the butylamide derivative. *Anal Biochem* 224:51–60.
24. Fox SR, Rawsthorne S, Hills MJ (2001) Fatty acid synthesis in pea root plastids is inhibited by the action of long-chain acyl- coenzyme as on metabolite transporters. *Plant Physiol* 126:1259–1265.
25. Sasaki Y, Nagano Y (2004) Plant acetyl-CoA carboxylase: Structure, biosynthesis, regulation, and gene manipulation for plant breeding. *Biosci Biotechnol Biochem* 68: 1175–1184.
26. Post-Beittenmiller D, Roughan G, Ohlrogge JB (1992) Regulation of plant fatty acid biosynthesis: Analysis of acyl-coenzyme a and acyl-acyl carrier protein substrate pools in spinach and pea chloroplasts. *Plant Physiol* 100:923–930.
27. Baud S, et al. (2003) Multifunctional acetyl-CoA carboxylase 1 is essential for very long chain fatty acid elongation and embryo development in *Arabidopsis*. *Plant J* 33:75–86.
28. Roesler K, Shintani D, Savage L, Boddupalli S, Ohlrogge J (1997) Targeting of the *Arabidopsis* homomeric acetyl-coenzyme A carboxylase to plastids of rapeseeds. *Plant Physiol* 113:75–81.
29. Jiang P, Cronan JE, Jr. (1994) Inhibition of fatty acid synthesis in *Escherichia coli* in the absence of phospholipid synthesis and release of inhibition by thioesterase action. *J Bacteriol* 176:2814–2821.
30. Eccleston VS, Ohlrogge JB (1998) 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. *Plant Cell* 10: 613–622.
31. Moreno-Perez AJ, et al. (2012) Reduced expression of FatA thioesterases in *Arabidopsis* affects the oil content and fatty acid composition of the seeds. *Planta* 235: 629–639.
32. Weselake RJ, et al. (2008) Metabolic control analysis is helpful for informed genetic manipulation of oilseed rape (*Brassica napus*) to increase seed oil content. *J Exp Bot* 59:3543–3549.
33. Jako C, et al. (2001) Seed-specific over-expression of an *Arabidopsis* cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. *Plant Physiol* 126:861–874.
34. Katavic V, et al. (1995) Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in *Arabidopsis thaliana* affecting diacylglycerol acyltransferase activity. *Plant Physiol* 108:399–409.
35. Stark DM, Timmerman KP, Barry GF, Preiss J, Kishore GM (1992) Regulation of the amount of starch in plant tissues by ADP glucose pyrophosphorylase. *Science* 258: 287–292.
36. Shi YZ, et al. (2008) Microspore-derived cell suspension cultures of oilseed rape as a system for studying gene expression. *Plant Cell Tiss Org* 92:131–139.
37. Nguyen HT, et al. (2010) Metabolic engineering of seeds can achieve levels of omega-7 fatty acids comparable with the highest levels found in natural plant sources. *Plant Physiol* 154:1897–1904.
38. Schwender J, Ohlrogge JB, Shachar-Hill Y (2003) A flux model of glycolysis and the oxidative pentosephosphate pathway in developing *Brassica napus* embryos. *J Biol Chem* 278:29442–29453.
39. Larson TR, Graham IA (2001) Technical advance: A novel technique for the sensitive quantification of acyl CoA esters from plant tissues. *Plant J* 25:115–125.
40. Thelen JJ, Ohlrogge JB (2002) The multisubunit acetyl-CoA carboxylase is strongly associated with the chloroplast envelope through non-ionic interactions to the carboxyltransferase subunits. *Arch Biochem Biophys* 400:245–257.