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Targeting of the Arabidopsis Homomeric Acetyl-Coenzyme A Carboxylase to Plastids of Rapeseeds[1]

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Acetyl-coenzyme A carboxylase (ACCase) occurs in at least two forms in rapeseed (*Brassica napus*): a homomeric (HO) and presumably cytosolic isozyme and a heteromeric, plastidial isozyme. We investigated whether the HO-ACCase of Arabidopsis can be targeted to plastids of *B. napus* seeds. A chloroplast transit peptide and the napin promoter were fused to the Arabidopsis *ACC1* gene and transformed into *B. napus*, with the following results. (a) The small subunit transit peptide was sufficient to provide import of this very large protein into developing seed plastids. (b) HO-ACCase in isolated plastids was found to be biotinylated at a level comparable to extraplastidial HO-ACCase. (c) In vitro assays of HO-ACCase in isolated plastids from developing seeds indicate that it occurs as an enzymatically active form in the plastidial compartment. (d) ACCase activity in mature *B. napus* seeds is normally very low; however, plants expressing the *SSU/ACC1* gene had 10- to 20-fold higher ACCase activity in mature seeds, suggesting that plastid localization prevents the turnover of HO-ACCase. (e) ACCase overexpression altered seed fatty acid composition, with the largest effect being an increase in oleic acid. (f) The total oil content of seeds was increased approximately 5% by the expression of HO-ACCase in plastids.

The factors limiting fatty acid synthesis and oil content of oilseed crops are not well understood. Two enzymes previously suggested as possible limitations are ACCase and KAS III (reviewed by Ohlrogge et al., 1993). An *Escherichia coli* KAS III gene has now been overexpressed in transgenic rapeseed (*Brassica napus*), resulting in 3- to 4-fold higher KAS III activity (Verwoert et al., 1995). Although fatty acid composition was altered, indicating in vivo activity of the *E. coli* enzyme, total seed fatty acid content was not significantly changed. ACCase has not previously been overexpressed in plants, but considerable in vivo evidence suggests that this enzyme is involved in regulation of plant fatty acid synthesis. This evidence was obtained in studies with spinach leaves and chloroplasts (Post-Beittenmiller et al., 1991, 1992), barley and maize leaves (Page et al., 1994), and tobacco suspension cells (Shintani and Ohlrogge, 1995). However, similar in vivo evidence for a regulatory role of ACCase in oilseeds is not available.

There are two ACCase structural types in plants (reviewed by Sasaki et al., 1995), and each type presents challenges to designing overexpression experiments. The heteromeric ACCase type, also referred to as the multisubunit or prokaryotic type, is present in plastids of dicotyledons and of non-Gramineae monocotyledons (Konishi et al., 1996). This ACCase type is composed of biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase α and β subunits. Because each of these subunits is encoded by corresponding genes, overexpression of all four subunits of the heteromeric ACCase in transgenic plants may be complex. The HO-ACCase type, also referred to as the multifunctional or eukaryotic type, is probably present in the cytosol of all plant species and is composed of dimers of a >200-kD polypeptide. In principle, engineering of this form of ACCase may be simpler because of the requirement to overexpress only one gene. However, because de novo fatty acid synthesis occurs primarily in the plastids, a transit peptide must be added to the cytosolic ACCase to accomplish the appropriate subcellular localization. A major challenge in engineering such a large cytosolic protein to influence fatty acid synthesis would be efficiently targeting it to plastids, where de novo fatty acid synthesis occurs. The protein would not only have to be imported but would also have to fold correctly into an active structure and then function in a nonnative, subcellular location. Furthermore, the prosthetic group biotin must be attached to ACCase, and it is not certain where or when in the cell this addition takes place (Tissot et al., 1996).

The plastid ACCase is known to be tightly regulated by mechanisms, including feedback inhibition (Shintani and Ohlrogge, 1995). Previous efforts to engineer metabolic pathways have often had increased success when regulatory enzymes engineered or mutated to lack feedback inhibition have been overexpressed (Stark et al., 1992). Therefore, a further advantage of relocation of the cytosolic ACCase to plastids may be the avoidance of the feedback mechanisms known to operate on plastid ACCase.

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Abbreviations: ACCase, acetyl-CoA carboxylase; HO, homomeric; KAS III, 3-ketoacyl-ACP synthase III; PCCase, propionyl-CoA carboxylase; SSU, small subunit; X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

An Arabidopsis gene (*ACC1*) encoding a cytosolic, HO-ACCase isozyme was previously identified by three groups (Elborough et al., 1994; Roesler et al., 1994; Yanai et al., 1995). The gene encodes a >200-kD polypeptide and is expressed in all Arabidopsis tissues examined, including seed. In the present study, we describe efforts to target the *ACC1* gene product to plastids of developing rapeseed and we examine whether the relocated gene product is biotinylated and active in the plastid. We also report the effects of *ACC1* plastidial expression on rapeseed fatty acid metabolism.

MATERIALS AND METHODS

Construct Preparation

The full-length *ACC1* gene was assembled from partial-length genomic clones *ACC-2* and *ACC-7* of Roesler et al. (1994), plus an identical sibling of *ACC-2*, *ACC-4a*. All PCR-derived regions were sequenced to verify that no PCR-generated mistakes were present.

To create a unique *Asp718* site at the 3′ end of the *ACC1*-coding region, PCR was conducted with primers 5′-CTGGTTTCCTGATTCAG-3′ and 5′-AAGGTACCGA-TATCAGTCAACCCAAG-3′ with λ genomic clone *ACC-7* as the template. The PCR product was digested with *Sac*I (*ACC1* position 9142) and *Asp718*. A subclone of *ACC-7* was digested with *Sal*I (position 7467)/*Sac*I (position 9142). The entire *Sal*I-to-*Asp718* region of *ACC-7* was then inserted in pBluescript by three-way ligation.

To create a unique *Spe*I site on the 5′ end of the *ACC1*-coding region, PCR was with primers 5′-CAUCAUC-AUCAUACTAGTGACAATGGCTGGCTC-3′ and 5′-CUA-CUACUACUATGAACTCTACCGCTGGTTGG-3′ with a subclone of genomic clone *ACC-2* as the template. The PCR product was digested with *Spe*I/*Bam*HI and ligated into the same sites of the *ACC1* construct to give the entire coding region in the *Spe*I to *Asp718* sites of pBluescript.

To create a unique *Eag*I site and to add the region encoding a soybean Rubisco SSU transit peptide plus 15 amino acids of the pea mature Rubisco SSU, PCR was with primers 5′-CAUCAUCAUCAUCGGCCGTAAACAATG-GCTTCCTCAATG-3′ and 5′-CUACUACUACUAACTA-GTGTCTCAAACTTCTTCTTTCC-3′, using the soybean Rubisco SSU/pea mature SSU fusion of Lubben and Keegstra (1986) as the template. The PCR product was digested with *Eag*I/*Spe*I and ligated into the same sites of the construct to give the entire *ACC1*-coding region plus transit peptide in the *Eag*I to *Asp718* sites of pBluescript. A subclone of genomic clone *ACC-7* was digested with *Xho*I (position 6307) and *Mvr*II (position 7965). Genomic clone *ACC-4a* (an identical sibling of *ACC-2*) was digested with *Bam*HI (position 2136)/*Xho*I (position 6307). A three-way ligation yielded the entire coding region in pBluescript. The entire *ACC1* insert (including transit peptide) was excised from pBluescript and blunt-end-ligated into the filled-in *Xho*I site of the Calgene plasmid pCGN 3223, which contained the napin promoter and a 3′-nontranslated region (kindly provided by J. Kridl, Calgene, Davis, CA). The cassette including the napin promoter, Rubisco transit, *ACC1* gene, and napin 3′-nontranslated region was then subcloned into the *Asp718* site of the Calgene binary vector pCGN1557. *B. napus* variety Reston was transformed with this construct.

Plastid Isolation

Plastids were isolated from developing *Brassica napus* embryos harvested 3 to 4 weeks postanthesis using a modification of the procedure described by Kang and Rawsthorne (1994). Approximately 200 embryos were dissected from siliques and seed coats into 3 mL of ice-cold plastid isolation buffer, PIM (0.5 M sorbitol, 20 mM Hepes, pH 7.4, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, and 1% BSA). The dissected embryos were homogenized twice with 10-s bursts of a Polytron (Brinkmann, Westbury, NY) at the highest setting. The crude homogenate was then filtered through two prewetted layers of Miracloth (Calbiochem) and centrifuged for 5 min at 750*g*. The supernatant was decanted and the plastid-enriched pellet was washed with 3 mL of PIM. The washed plastids were collected by centrifugation for 5 min at 750*g*. The washed, plastid-enriched pellet was resuspended in 500 μL of PIM. Portions of the crude homogenate and the 750*g* supernatant and pellet fractions were saved for further analysis. The protein content of each fraction was determined as described by Bradford (1976) using bovine γ-globulin as a standard.

Thermolysin Treatment of Isolated Embryo Plastids

Approximately 20 μg of thermolysin (Sigma) was added to 100 μL of the embryo plastid-enriched fraction. The mixture was incubated on ice for 30 min, after which the thermolysin was inactivated by adding EDTA to 10 mM. The thermolysin-treated plastids were layered over 20% Percoll in 75% PIM and 10 mM EDTA and centrifuged for 5 min at 1500*g*. The plastid pellet was resuspended in 100 μL of PIM containing 10 mM EDTA.

Immunoblot Analysis of Biotinylated Proteins of *B. napus* Embryos

Protein extracts were separated on 6.5% SDS-polyacrylamide gels and blotted to PVDF filters. Biotinylated polypeptides were detected using a 1/1000 dilution of anti-biotin antibodies (Sigma) as described by Roesler et al. (1996). Relative levels of biotinylated proteins were estimated by densitometer scanning of the immunoblots.

Enzyme Assays

ACCase activity of mature seeds was assayed as described by Roesler et al. (1996). Propionyl-CoA carboxylase in the plastid-enriched fractions was assayed in triplicate essentially as described by Alban et al. (1994). Ten microliters of each fraction was assayed in 50-μL reactions containing 50 mM Tricine, pH 8.0, 40 mM KCl, 1 mM ATP, 0.05% Triton X-100, 0.5 mM propionyl-CoA, and 12 mM [^{14}C]$NaHCO_3$ (1 mCi/mM) for 10 min at 30°C. Reactions were stopped by adding 1 volume of 2 N HCl. Fifty micro-

liters of the stopped reaction mixture was transferred to scintillation vials and heated at 65°C for approximately 20 min. The amount of ^{14}C incorporated into acid-stable products was then determined by scintillation counting. Nonspecific carboxylation in each extract was determined by assays in the absence of propionyl-CoA. Glyceraldehyde-3-P dehydrogenase assays were performed as described by Kang and Rawsthorne (1994).

Fatty Acid Analysis

GC Analysis

Ten to 30 mature seeds from each plant were ground to a powder with a mortar and pestle. Triplicate weighed samples of approximately 30 mg were added to individual tubes containing 1.5 mg of heptadecanoic acid. Fatty acid methyl esters were directly transesterified from seed material by incubating samples in 1 mL of 10% (w/v) boron trichloride in methanol at 80°C for 90 min. The mixture was cooled and 1 mL of H_2O was added. Fatty acid methyl esters were then extracted with 2 mL of hexane and analyzed by GC (model 5890 gas chromatograph, Hewlett-Packard) using a 30- × 0.25-mm i.d. DB23 column (J&W Scientific, Rancho Cordova, CA) with an oven temperature programmed from 195°C (8-min hold) to 230°C at 12°C/min (5-min hold) with a column head pressure of 200 kPa.

NMR Analysis

Seeds (0.8–1.1 g) were baked at 130°C for 2 h and equilibrated to room temperature for 2 h prior to NMR imaging (model Newport 4000, Oxford Analytical Instruments, Oakridge, TN), using an R_F level of 20 μA. Samples were run in duplicate in Nessler cylinders of identical weight against 35, 45, and 55% oil controls.

RESULTS

Construct Design and Recovery of Transgenic Plants

The ACCase construct for these experiments was designed to target the product of the Arabidopsis extraplastidial ACCase gene *ACC1* (Roesler et al., 1994) to the plastids of rapeseed. The Rubisco SSU transit peptide was selected for plastid targeting and import because it is known to efficiently import heterologous proteins. Fifteen amino acids of the mature Rubisco SSU were included to help ensure proper cleavage of the transit peptide. The considerable heterogeneity at the N terminus of various cytosolic ACCases (apparent from sequence alignment) suggested that the N-terminal extension derived from the Rubisco polypeptide would likely be tolerated. To control expression, the promoter of the *B. napus* seed storage protein napin was chosen because it is seed-specific and therefore would minimize any deleterious effects of ACCase overexpression in the vegetative parts of the plant. Also, as a promoter for an abundant seed storage protein, the napin promoter was expected to give high expression levels.

The chimeric transit peptide/*ACC1* construct was introduced into *B. napus* by co-cultivation of hypocotyls with *Agrobacterium* containing the construct. Nineteen *ACC1* transformants and six untransformed control plants were regenerated from calli. Eight of the *SSU/ACC1* transformants and three controls that appeared normal and had good seed set were selected for further analysis. In some cases, seeds from *B. napus* plants transformed with the binary vector alone in a separate co-cultivation were used as controls. Characterization of these plants and their progeny is presented here.

Expression of the *SSU/ACC1* Chimeric Gene Resulted in Increased ACCase Protein and Activity in Mature Seeds

The only known plant biotinylated polypeptides with a molecular mass greater than 200 kD are the HO-ACCase isozymes (Wurtele and Nikolau 1990; Wurtele et al., 1992; Baldet et al., 1993; Duval et al., 1994). Therefore, anti-biotin immunoblots were used to assess the presence or absence of the *ACC1* gene product in mature rapeseed (Fig. 1). In control plants a biotin polypeptide with a high molecular mass was detected early in seed development only, not in mature seed, consistent with previous observations for wild-type rapeseed (Roesler et al., 1996). In contrast, a 220-kD biotin polypeptide was detected in mature seed of several plants transformed with the *SSU/ACC1* gene. The polypeptide appeared to be similar in size to the high-molecular-mass biotin polypeptide from developing wild-type seed. (The size difference due to the 15-amino acid N-terminal extension of the recombinant polypeptide would probably not be resolvable by a 6.5% polyacrylamide gel.) The results in Figure 1, therefore, indicated that the *SSU/ACC1* gene construct yielded a full-length polypeptide of the appropriate size, which, furthermore, was capable of being biotinylated.

To determine whether this transgene product was active, ACCase activity was determined with mature seed of control and *SSU/ACC1* plants (Table I). For the control plants, ACCase activity was barely detectable (mean of 0.11 nmol min^{-1} mg^{-1} protein). A range of values from 1.7 to 19 times the control values was observed in the *SSU/ACC1* plants, indicating that the *SSU/ACC1* gene construct was

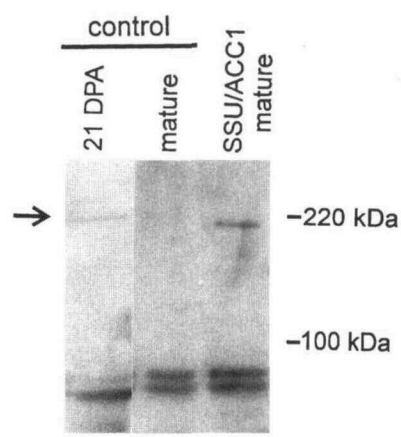

Figure 1. Anti-biotin blot of protein extracts from mature seeds transformed with *SSU/ACC1* construct and seed from nontransformed control plants (mature and 21 d postanthesis [DPA]). Proteins were separated by 6.5% SDS-PAGE, blotted to PVDF membranes, and probed with antibodies to biotin. The arrow indicates the HO-ACCase polypeptide.

Table I. *ACCase activity and fatty acid content and composition of mature T_1 B. napus seeds*

ACCase activity was determined on single-pooled 10-seed samples and fatty acid content/composition were determined with triplicates of pooled 10-seed samples. Values in parentheses are SDs. 20:0, 22:0, 24:0, and 24:1 totaled less than 3.0% and were used in total fatty acid calculations.

Plant	ACCase Activity	Total Fatty Acid	Fatty Acid						
			16:0	18:0	18:1	18:2	18:3	20:1	22:1
	nmol min^{-1} mg^{-1} protein	*mg/g dry wt*	*wt %*						
Untransformed controls									
A	0.09	373 (12)	3.5	0.9	13.5	17	8.7	7.6	46.9
B	0.14	396 (17)	3.6	1	17.2	14.9	8.1	9.8	43.7
C	0.09	382 (17)	3.5	0.9	15.5	15.7	9	8.8	44.9
SSU/AAC1 transformants									
1	1.13	408 (17)	3.4	1.1	21.4	13.1	6.5	11.1	41.9
2	2.09	405 (17)	3.6	1.1	20.5	13.9	7.1	11	41.4
3	0.63	424 (16)	3.6	1.1	19.5	14.1	7.3	10.5	42.2
4	0.19	422 (5)	3.5	1.1	19.8	12.9	7.5	10.6	43.1
5	0.9	395 (15)	3.5	1	19.5	14.3	7.7	9.9	42.5
6	0.77	401 (18)	3.6	1	17.1	15	8.6	9.5	43.6
7	0.57	397 (13)	3.9	1.3	22.3	13	6.1	10.8	40.9
8	0.34	414 (16)	3.6	1.1	20	13.4	7.2	10.4	42.9
Control mean	0.11 (0.03)	384 (12)	3.5 (0.1)	0.9 (0.1)	15.4 (1.9)	15.9 (1.1)	8.6 (0.5)	8.7 (1.1)	45.2 (1.6)
Transformed mean	0.83 (0.59)	408 (11)	3.6 (0.1)	1.1 (0.1)	20.0 (1.5)	13.7 (0.7)	7.3 (0.8)	10.5 (0.5)	42.3 (0.9)

yielding active enzyme. The presence of active ACCase in dry, mature seeds of *SSU/ACC1* plants and its absence in controls suggests that plastid targeting of this enzyme may prevent its turnover during later stages of seed development. In support of this conclusion, seeds overexpressing the *ACC1* gene without the additional transit peptide did not have increased ACCase activity in mature seeds (K. Roesler and J. Ohlrogge, unpublished observations).

Twenty to 50 seeds each of the *SSU/ACC1* transformants in Table I were germinated on media containing kanamycin. The progeny of lines 1, 3, and 4 segregated in an approximate 3:1 ratio of survivors:nonsurvivors and indicated either a single transgene insertion event or two closely linked events. These three lines displayed a wide range of ACCase activity, as evident in Table I, and were selected for further study. Line 2 was also selected because it had the highest ACCase activity. Forty of 42 line 2 progeny survived on kanamycin, indicating that this line probably had transgene insertions at two or more loci.

The Cytosolic ACCase Isozyme Was Imported into Plastids of Developing *B. napus* Embryos

To assess the subcellular localization of the *SSU/ACC1* gene product, extracts of developing embryos harvested 3 to 4 weeks postanthesis were prepared. Plastid-enriched fractions were obtained from the crude embryo homogenates by low-speed centrifugation and after washing were free of cytosolic contamination as shown by the absence of NADH-glyceraldehyde-3-P dehydrogenase. One hundred micrograms each of protein from the crude homogenate, supernatant, and plastid-enriched fractions were fractionated by SDS-PAGE and transferred to PVDF filters, and biotinylated polypeptides were detected by antibodies to biotin.

Biotinylated proteins corresponding to the high-molecular-mass HO-ACCase were detected in the crude homogenate, the low-speed supernatant, and the pellet fractions of developing embryos harvested from both control and *SSU/ACC1* plants. However, based on scanning densitometry, the levels of the 220-kD biotin protein were at least 3- to 4-fold more abundant in all embryo protein fractions of the *SSU/ACC1* transformants. Furthermore, in contrast to the control plants, the levels of the high-molecular-mass biotinylated polypeptide from *SSU/ACC1* embryos were greatly enriched in the washed-plastid fractions relative to the crude and supernatant fractions (Fig. 2). These results indicate that the *SSU/ACC1* construct resulted in targeting of the cytosolic ACCase to plastids. Furthermore, the level of the 220-kD ACCase was substantially increased.

To confirm that the protein was inside the plastids, rather than associated with the plastid envelope, the plastid-enriched, low-speed pellet fractions of *SSU/ACC1* and control embryos were treated with the protease thermolysin or left untreated. Treated plastids were then reisolated and subjected to immunoblot analysis with antibiotin antibodies. As shown in Figure 2B, the 220-kD biotinylated polypeptide was detected at approximately the same level in protease-treated and untreated plastid fractions of *SSU/ACC1* embryos, confirming localization of the *SSU/ACC1* gene product within the plastid. When identical blots were probed with antibodies to both biotin and to alfalfa HO-ACCase (gift of B. Shorrosh, Michigan State University, East Lansing, MI), densitometry indicated a similar relationship of band intensities with the two antibody probes between the *SSU/ACC1* and control plants (data not shown). These results indicated that the transgene product in the plastids had a level of biotinylation comparable to the endogenous cytosolic ACCase.

We also detected low levels of a 220-kD biotinylated polypeptide in the plastid-enriched, low-speed pellet of control plant embryos. Furthermore, as was observed for

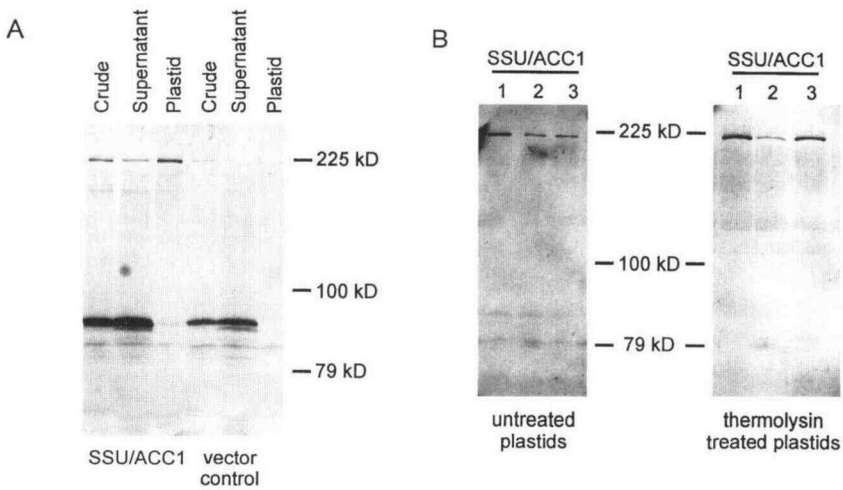

Figure 2. Anti-biotin blot of fractions from developing embryos. A, One hundred micrograms of protein from homogenate, supernatant, and washed-plastid pellets was separated by 7.5% SDS-PAGE, blotted to membranes, and probed with antibodies to biotin. B, Washed-plastid pellets either treated with the protease thermolysin and re-isolated or untreated were analyzed as in A. Lanes 1, 2, and 3 are different transformant lines as designated in the text.

the *SSU/ACC1* gene product, this protein was resistant to protease treatment (results not shown). This band may correspond to the product of the *B. napus* cDNA described by Schulte et al. (1994), which is an HO-ACCase with an N-terminal amino acid extension similar to plastid-targeting sequences.

The *SSU/ACC1* Transgene Product Is Active in the Plastids of Developing *B. napus* Embryos

Although the subcellular fractionation results (Fig. 2) indicated that the *SSU/ACC1* transgene product was correctly targeted to the plastids of developing rapeseed embryos, these results were not sufficient to demonstrate that the transgene product was correctly assembled and active after import. Therefore, the plastid-enriched fractions were assayed in vitro to test whether the transgene product was active. Because of the instability of the native heteromeric plastid ACCase, it is difficult to obtain consistent results from in vitro ACCase activity assays from rapeseed embryo extracts. This made it difficult to compare the contribution of the *SSU/ACC1* transgene product with the total plastid ACCase activity. However, the homomeric ACCase is also able to catalyze the carboxylation of propionyl-CoA at approximately one-sixth the rate of its ACCase activity (Alban et al., 1994). Therefore, it was possible to determine whether the *SSU/ACC1* transgene product was active after import using in vitro PCCase assays.

As shown in Figure 3, PCCase assays on the plastid-enriched, low-speed pellet fractions from *SSU/ACC1* and control embryos indicated that the *SSU/ACC1* transgene product was active in vitro. The plastid-enriched fractions from embryos of two *SSU/ACC1* transformants, lines 1 and 3, had PCCase specific activities of 225.9 and 121.7 pmol min^{-1} mg^{-1} protein, respectively, whereas activities in the plastid-enriched fractions of control embryos ranged from 18.5 to 23.0 pmol min^{-1} mg^{-1} protein. Thus, the PCCase activities measured in the plastid-enriched fractions of *SSU/ACC1* embryos were 6- to 10-fold higher than those in the plastid fractions of control embryos.

Fatty Acid and Oil Analysis of Mature Seed

Fatty acid content of seeds from the T_1 plants was determined (Table I). The mean seed fatty acid content of the *SSU/ACC1* plants was 408 mg/g dry weight, which was 6% higher than the control mean of 384 mg/g dry weight. The fatty acid contents of the two populations were statistically different at the 95% level as revealed by a Student's *t* test analysis. The *SSU/ACC1* transformants also had increased percentages of 18:1 and 20:1, and small decreases in the 18:2, 18:3, and 22:1 percentages. The largest effect was on 18:1, with the *SSU/ACC1* transformants averaging 20% 18:1 compared with the control mean of 15.4%. To assess the degree of correlation of transgene ACCase activity with the weight percentage of the five most abundant fatty acids, correlation coefficients were calculated using the ACCase activities determined for the T_1 mature seed (Table I). In both T_1 and T_2 generations there were significant positive correlations of transgene ACCase activity with 18:1 and 20:1 and significant negative correlations with 18:2, 18:3, and 22:1.

T_2 plants from *SSU/ACC1* transgenic lines and from binary vector control lines were grown to maturity in two locations. Mature seed fatty acid composition and oil con-

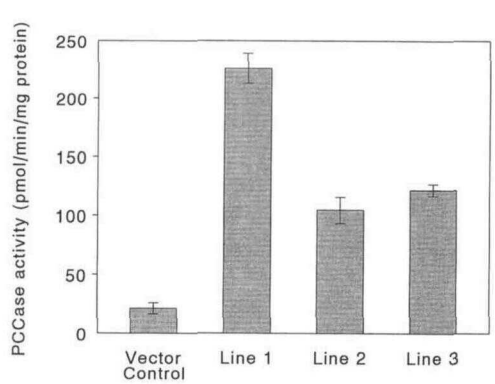

Figure 3. PCCase activity in plastid fractions isolated from developing embryos (4 weeks postanthesis) of *B. napus* transformed with vector alone or with *SSU/ACC1* construct.

tent were determined by GC and NMR analysis. The growth conditions at the two locations differed, resulting in different values for total fatty acid content. At Michigan State University, under growth conditions similar to those used for T_1 plants, significant increases in 18:1 and small decreases in 18:3 and 22:1 were again observed in the *SSU/ACC1* lines, substantiating the T_1 results. With plants grown in St. Louis in growth chambers with higher light, the compositional differences observed with T_1 seed were evident only in the highest expressing *SSU/ACC1* lines 1 and 2. As determined by GC, the mean fatty acid contents for the *SSU/ACC1* lines at the St. Louis and Michigan State University locations were 6.4 and 5.0% higher than the control means at these two locations, respectively. These increases were statistically significant for the St. Louis location but not for the Michigan State University experiment, which was based on far fewer plant numbers. NMR analysis was conducted on 1-g samples of the seeds produced in St. Louis. This analysis confirmed the increase in oil content observed by GC. Furthermore, as shown in Figure 4, the increase in oil content was correlated with the ACCase levels determined in mature seeds.

DISCUSSION

Different plant species vary greatly in their seed oil content, ranging from several percent to more than 60% of seed dry weight. An understanding of the factors that control oil content could be of considerable practical value for crops such as rapeseed, which are grown primarily for their oil. If we consider that world rapeseed oil production is approximately 10 billion tons, with a value of 4 to 5 billion dollars, even small increases in oil production, if not accompanied by losses in yield, could add considerable value to the crop. Targeting a cytosolic ACCase to the plastid to influence fatty acid synthesis seemed attractive in light of previous studies indicating a regulatory role for ACCase in leaf and suspension culture fatty acid synthesis. The plastid heteromeric

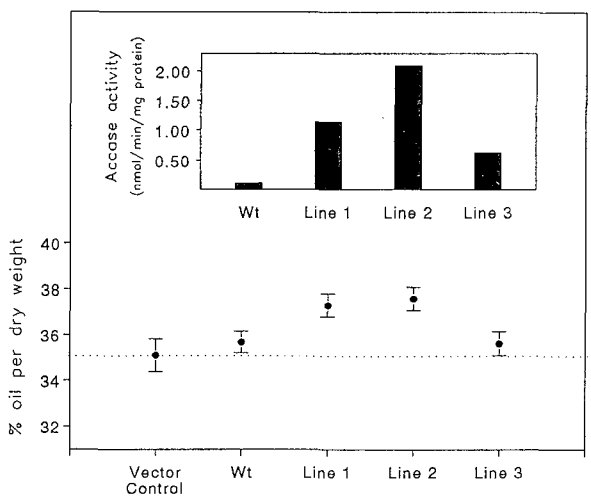

Figure 4. NMR analysis of T_2 seeds: total fatty acid per dry weight of mature *B. napus* seeds transformed with *SSU/ACC1* construct or with vector alone. Error bars indicate SEs. Inset, ACCase activity determined in mature seeds of each line. Wt, Wild type.

ACCase was the apparent site of feedback inhibition of fatty acid synthesis in tobacco suspension cells supplemented with exogenous fatty acids (Shintani and Ohlrogge, 1995). It seemed likely that the very different homomeric isozyme might not be regulated in the same manner and therefore might be more effective in influencing oil content than overexpression of the plastid ACCase. Also, the kinetic properties of the homomeric and heteromeric isozymes from pea differed, with the former having a much lower K_m with respect to acetyl-CoA (Dehaye et al., 1994). Although acetyl-CoA concentrations of oilseed plastids are not known, in chloroplasts the level of acetyl-CoA has been estimated to be 30 to 50 μM (Post-Beittenmiller et al., 1992). Therefore, it seemed possible that a higher affinity for this substrate might contribute to higher fatty acid synthesis rates at the normal plastid acetyl-CoA concentrations.

There were several questions regarding the approach used in this study. First, the transit peptide added to the Arabidopsis HO-ACCase normally imports only a 14-kD Rubisco SSU polypeptide, and there were no previous reports of a polypeptide as large as the *ACC1* gene product being imported with a heterologous transit peptide. Second, the Arabidopsis *ACC1* gene, not cDNA, was used. This meant that 30 introns in the transgene must be processed in *B. napus*, including one that did not fit the consensus sequence for exon/intron border junctions (Roesler et al., 1994). Third, there was a possibility that the cytosolic protein would be recognized as foreign and quickly degraded in the plastid, therefore not accumulating to detectable levels. Furthermore, biotinylation, proper folding, and assembly of the ACCase in a nonnative environment were also required for expression of enzyme activity. The immunoblots and activity assays reported here, however, clearly demonstrate that the use of this chimeric *SSU/ACC1* construct resulted in an active *ACC1* gene product in the desired plastidial location. Because the heteromeric ACCase easily dissociates into inactive subunits, its assay is often unreliable and it is difficult to determine the extent to which overall plastid ACCase activity has been increased in the *SSU/ACC1* transformants compared with the controls. However, based on both PCCase assays (Fig. 3) and ACCase assays of the plastids we estimate that the targeting of the cytosolic ACCase to plastids increased total ACCase activity in the plastids 1- to 2-fold.

Our efforts to assess the influence of the *SSU/ACC1* transgene on oil content were confounded by several sources of variability. Plant-to-plant and line-to-line variability (Table I) in seed fatty acid content make it difficult to assess small changes in oil content. Previous rapeseed studies have shown large impacts of growth conditions on seed fatty acid composition. In one study using the same cultivar used here, Reston, 22:1 content varied from 20 to 40%, depending on growth temperature (Wilmer et al., 1996). In experiments in which wild-type plants were included as additional controls, we noted a slight depression of oil content associated with plants transformed with vector alone. Similar alterations in fatty acid metabolism have been reported associated with tissue culture of *B. napus* (Craig and Millam, 1995) and with transformation of tobacco (Ghosh et al., 1994).

Furthermore, as previously observed, plants grown under different conditions have different oil contents (Canvin, 1965). All of these factors indicate that control of oil content is a complex trait subject to many perturbations. Nevertheless, in these experiments, overexpression and plastid targeting of the *ACC1* gene resulted in small increases in total fatty acid content in three sets of experiments in two locations. When all data are combined, a statistically significant increase is obtained. Furthermore, the increase in 18:1 content is consistent with results in maize, indicating a positive correlation between total seed oil content and 18:1 percentages (Alrefai et al., 1995).

If the increases in oil content are maintained in field trials with no losses in yield, they may prove of value to rapeseed oil production. These relatively small increases in oil are consistent with the notion that control of rapeseed oil synthesis is shared among several enzymes, such that when ACCase activity in the plastid is increased other enzymes may become limiting. If this interpretation is correct, a promising approach to further increase oil might be to cross *ACC1*-overexpressing lines with lines overexpressing other lipid biosynthetic enzymes that share control of oil synthesis with ACCase.

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