The Plant Cell, Vol. 8, 281–292, February 1996

A Jojoba β-Ketoacyl-CoA Synthase cDNA Complements the Canola Fatty Acid Elongation Mutation in Transgenic Plants

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β-Ketoacyl–coenzyme A (CoA) synthase (KCS) catalyzes the condensation of malonyl-CoA with long-chain acyl-CoA. This reaction is the initial step of the microsomal fatty acyl-CoA elongation pathway responsible for formation of very long chain fatty acids (VLCFAs, or fatty acids with chain lengths >18 carbons). Manipulation of this pathway is significant for agriculture, because it is the basis of conversion of high erucic acid rapeseed into canola. High erucic acid rapeseed oil, used as an industrial feedstock, is rich in VLCFAs, whereas the edible oil extracted from canola is essentially devoid of VLCFAs. Here, we report the cloning of a cDNA from developing jojoba embryos involved in microsomal fatty acid elongation. The jojoba cDNA is homologous to the recently cloned Arabidopsis *FATTY ACID ELONGATION1* (*FAE1*) gene that has been suggested to encode KCS. We characterize the jojoba enzyme and present biochemical data indicating that the jojoba cDNA does indeed encode KCS. Transformation of low erucic acid rapeseed with the jojoba cDNA restored KCS activity to developing embryos and altered the transgenic seed oil composition to contain high levels of VLCFAs. The data reveal the key role KCS plays in determining the chain lengths of fatty acids found in seed oils.

INTRODUCTION

Very long chain fatty acids (VLCFAs) have chain lengths >18 carbons and are widely distributed in nature. They are found in cuticular waxes of most plant species and in the seed oils of several plant genera, most notably *Arabidopsis*, *Brassica*, *Limnanthes*, *Tropaeolum*, and *Simmondsia*. In plants, de novo fatty acid synthesis (FAS) is localized in plastids and involves intermediates bound to acyl carrier proteins (ACPs) (reviewed in Stumpf, 1980; Browse and Somerville,1991; Slabas and Fawcett, 1992; Ohlrogge et al., 1993). Typically, this FAS system does not produce fatty acids with chain lengths >18 carbons. The products of the plastid FAS are exported from the plastid and converted to acyl–coenzyme A (acyl-CoA) derivatives that are thought to serve as substrates for a microsomal fatty acid elongation (FAE) system.

The membrane-associated nature of the elongation enzymes has hindered investigation of their biochemistry. As in animals (Bernert and Sprecher, 1979), FAE in plants is believed to be the result of a four-step mechanism similar to FAS, except that CoA, rather than ACP, is the acyl carrier (Stumpf and Pollard, 1983; Fehling and Mukherjee, 1991; Cassagne et al., 1994b). The first step in FAE involves condensation of malonyl-CoA with a long-chain acyl-CoA to yield carbon dioxide and a β-ketoacyl–CoA in which the acyl moiety has been elongated by two carbons. Subsequent reactions are reduction to β-hydroxyacyl–CoA, dehydration to an enoyl-CoA, and a second reduction to yield the elongated acyl-CoA. In both mammalian and plant systems in which the relative activities of the four enzymes have been studied, the initial condensation reaction is the rate-limiting step (Suneja et al., 1991; Cassagne et al., 1994a).

Wild-type rapeseed and most Brassica species contain eicosenoic (20:1) and erucic (22:1) acids as major components of their seed oils. Canola was derived from rapeseed by the introduction of recessive alleles at two loci that control the elongation of C18 fatty acids (Downey and Craig, 1964; Harvey and Downey, 1964). Rapeseed varieties can be classified as high erucic acid rapeseed (HEAR) or low erucic acid rapeseed (LEAR), based on the composition of the seed oil. Stumpf and Pollard (1983) demonstrated that extracts from developing HEAR embryos could elongate 18:1-CoA to 20:1 and 22:1, whereas LEAR extracts could not elongate the 18:1-CoA substrate. They did not investigate which of the four enzyme activities involved in FAE were defective in LEAR.

In Arabidopsis, mutation of the *FAE1* locus reduces the quantities of VLCFAs in seed oil (James and Dooner, 1990; Lemieux et al., 1990) and results in deficiency in elongation of both 18:1-CoA and 20:1-CoA (Kunst et al., 1992). Recently, this locus was cloned via transposon tagging (James et al., 1995). The homology of the *FAE1* gene product with other condensing enzymes was noted, and the authors speculated that *FAE1* encodes a condensing enzyme specifically involved in seed oil FAE.

The seed oil of jojoba is unusual in that it consists of waxes rather than the triacylglycerols constituting other seed oils. The waxes are esters of monounsaturated fatty acids and alcohols (Miwa, 1971). Acyl-CoAs are precursors of both the fatty acid

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and the fatty alcohol moieties of the wax esters (Pollard et al., 1979). More than 90% of these fatty acids and alcohols have chain lengths longer than 18 carbons, indicating the presence of an active acyl-CoA elongation system. As in rapeseed, malonyl-CoA and acyl-CoA serve as substrates for VLCFA synthesis (Pollard et al., 1979).

Two enzyme activities involved in wax synthesis in developing jojoba seeds were characterized by Pollard et al. (1979): an acyl-CoA reductase and an acyl-CoA:alcohol acyl transferase (referred to here as wax synthase). Previously, we identified a protein associated with the acyl-CoA reductase and cloned the corresponding cDNA (Metz and Lassner, 1994). As part of our study of wax synthase, we obtained a chromatographic fraction enriched in enzyme activity, and we selected proteins as wax synthase candidates. One of these proteins was further purified by electroelution from SDS gels, and the protein sequence was obtained. The sequence information was used to isolate a cDNA clone that is the subject of this report. Data base comparisons showed that the jojoba cDNA encodes a protein homologous to the acyl-CoA condensing enzymes chalcone synthase and resveratrol synthase. When expressed in maturing canola seed, the jojoba cDNA complemented the mutation in FAE. We present data demonstrating that the cDNA encodes a β-ketoacyl-CoA synthase (KCS) of the microsomal fatty acyl-CoA elongation pathway. In addition, our data establish the key role this enzyme plays in determining the chain lengths of seed oil fatty acids.

RESULTS

Cloning of a Jojoba Embryo cDNA That Complements the Canola *FAE* Mutations

Many of the enzymes associated with synthesis of seed oils are membrane bound. A wide range of detergents has been used, with variable results, in an effort to solubilize these enzymes so that they could be subjected to chromatographic enrichment. We have used the detergent 3-([3-cholamidopropyl] dimethylammonio)-1-propanesulfonate (CHAPS) to solubilize and characterize several plant oil synthesis enzymes (Metz and Lassner, 1994; Knutzon et al., 1995). As part of our effort to identify proteins associated with jojoba wax synthase, we treated microsomal membranes from jojoba with 2% CHAPS. Wax synthase activity was recovered in a 200,000*g* supernatant fraction, suggesting that solubilization of the enzyme had been achieved. This supernatant fraction was subjected to chromatography on the dye–ligand matrix Blue A–agarose. Wax synthase activity bound to the column and was eluted during a 1.5 M NaCl wash. Based on SDS-PAGE analysis of proteins present in various fractions from this column, several proteins were identified as wax synthase candidates. On SDS gels, one of these candidates had an apparent molecular mass of 57 kD. A sample enriched in this candidate was obtained by preparative SDS-PAGE. Tryptic and cyanogen bromide peptides were generated, purified, and subjected to microsequencing. A major component of the electroeluted fraction was a 56-kD protein that we had identified previously as an acyl-CoA reductase (Metz and Lassner, 1994). Because we had isolated and sequenced a cDNA encoding the acyl-CoA reductase, we excluded from use in this study any peptide sequences that matched its deduced protein sequence.

The peptide sequences were used to design degenerate oligonucleotides, and a combination of polymerase chain reaction (PCR) and cDNA library screening was used to characterize cDNA clones encoding the peptides. Figure 1 shows the predicted amino acid sequence of the protein encoded by these cDNAs. The deduced protein contains 521 amino acids with a predicted molecular mass of 58.6 kD and a pI of 9.64. Analysis with TopPred II (Claros and von Heijne, 1994) suggested that there are between four and seven transmembrane segments in the protein. A BLAST search of nucleotide and protein data banks (Altschul et al., 1990) showed that the protein has significant homology with the soluble enzymes chalcone and resveratrol synthases. These enzymes catalyze condensation

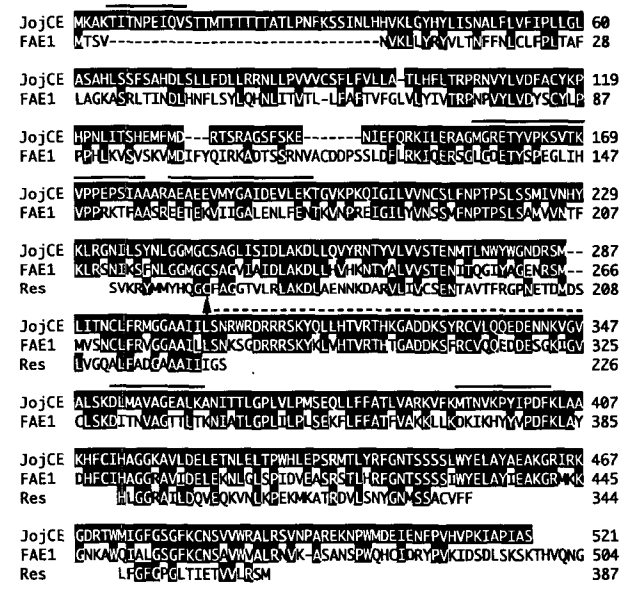

Figure 1. Amino Acid Sequence of the Jojoba KCS and Alignment with Related Proteins.

The amino acid sequence of the jojoba cDNA (JojCE; GenBank accession number U37088) was deduced from the cDNA nucleotide sequence. Solid lines over the sequence show tryptic and cyanogen bromide peptides that were sequenced before isolation of the cDNA. The dashed line over amino acids 304 to 347 indicates the hydrophilic region of the jojoba protein expressed in *E. coli* and used for antibody production. The sequence was aligned with a peanut resveratrol synthase sequence (Res; PIR data base accession number S003341) and the protein encoded by the Arabidopsis *FAE1* locus (James et al., 1995). Amino acids in black squares are identical to the jojoba KCS sequence. The arrowhead indicates the active site cysteine of resveratrol synthase (Lanz et al., 1991).

Table 1. Fatty Acyl Composition of Oilseeds[a]

Sample	Weight Percentage of Fatty Acids										
	18:1	18:2	18:3	20:1	20:2	22:0	22:1	22:2	24:0	24:1	>18[b]
Jojoba[c]	6.4	0.0	0.0	58.3	0.0	0.0	31.2	0.0	0.0	4.1	93.6
Control LEAR											
212/86	58.9	22.1	11.3	1.3	0.1	0.3	0.0	0.0	nd[d]	nd	1.7
Pooled T_2 transgenic LEAR											
7626-2	38.1	19.6	14.6	14.3	1.1	0.4	4.8	0.7	nd	nd	21.3
7626-3	46.7	18.8	14.9	9.8	0.7	0.3	1.7	0.2	nd	nd	12.7
Control HEAR											
Reston	13.7	18.1	12.3	6.0	0.8	0.5	40.6	0.8	0.0	0.1	48.7
Pooled T_2 transgenic HEAR											
7626-1	12.9	17.5	12.7	5.2	0.8	0.8	38.3	1.7	0.1	2.7	49.6
7626-17	14.9	16.5	10.9	6.3	0.8	0.8	39.1	1.6	0.0	2.5	51.1
Control Arabidopsis											
No-0	17.6	26.2	18.3	21.0	2.0	0.1	2.0	0.0	0.1	0.1	25.3
Pooled T_2 transgenic Arabidopsis											
7626-2	15.8	25.5	20.8	18.6	2.0	0.9	4.0	0.3	0.1	0.7	26.6
7626-10	15.3	25.6	20.6	15.7	1.8	1.3	5.7	0.7	1.1	1.6	27.8
Half-seed: T_3 transgenic LEAR and control HEAR											
7626-2-1-1	22.9	10.4	6.9	15.2	0.6	0.9	28.8	1.3	0.5	7.8	55.1
7626-2-1-9	21.5	11.4	9.0	13.5	0.6	0.8	33.5	1.1	0.2	3.9	53.6
Reston[e]	19.0	11.7	8.7	8.8	0.4	0.5	45.4	0.3	0.0	0.7	56.7

[a] The seed oil fatty acid composition of control plants and transgenic plants expressing the jojoba KCS is given. Numbers indicate the fatty acid compositions as weight percentages of total fatty acids. Palmitic (16:0), stearic (18:0), and behenic (20:0) acid compositions are not shown because there are no significant differences between control and transgenic plant oils.

[b] Total VLCFA composition of the seed oils.

[c] Jojoba values represent the sum of fatty acids and fatty alcohols.

[d] nd, not determined.

[e] The values for the Reston half-seed analysis represent an average of the values from the analysis of 10 individual half-seeds.

reactions whose products are β-ketoacyl-CoA thioesters incrementally elongated by two carbon atoms derived from malonyl-CoA. The data base accession with the highest BLAST score was the peanut resveratrol synthase protein (Schröder et al., 1988; Lanz et al., 1991), and Figure 1 shows the BLAST-generated alignments. The BLAST search also revealed homology between the jojoba protein and β-ketoacyl-ACP synthase III (KASIII) from *Escherichia coli* and plants. KASIII catalyzes the condensation of malonyl-ACP and acetyl-CoA (Tsay et al., 1992; Tai and Jaworski, 1993).

Based on the homology of the jojoba microsomal protein with known condensing enzymes, we hypothesized that the jojoba cDNA encodes a KCS involved in the formation of VLCFAs. The jojoba cDNA sequence was ligated adjacent to regulatory sequences derived from a *B. rapa* napin gene. The napin regulatory sequences mediate gene expression in maturing embryos of transgenic plants (Kridl et al., 1991). The napin–jojoba cDNA fusion was cloned into a binary plant transformation vector, transferred to Agrobacterium, and used to transform Arabidopsis, HEAR, and LEAR.

Pooled T_2 seed from the transgenic plants were analyzed to determine the fatty acyl composition of the seed oils. The majority of the transgenic plants had altered oil phenotypes. The data in Table 1 show that the change was a shift toward longer chain fatty acids. The most dramatic change occurred in the composition of transgenic LEAR oil. The seed oil of control LEAR (212/86) was, as expected for canola oil, low in VLCFA content. Of the 20 transgenic 212/86 plants analyzed, 16 had increased VLCFA content (data not shown), and the oil from one plant, 7626-2, was composed of >20% VLCFAs (Table 1). The transgene's effect on the composition of HEAR and Arabidopsis oil was less dramatic; however, these plants produce significant quantities of VLCFAs. The transgenic Arabidopsis oil had increased 22:1 and 24:1 content, with a concomitant decrease in 20:1 content (Table 1). The primary effect of the transgene on HEAR oil was to increase slightly its 24:1 composition. Control HEAR oil contained <1% of 24:1, whereas oil from the transgenic HEAR plants contained as much as 2.7% of 24:1 (Table 1). In transgenic canola plants, the jojoba cDNA complemented the mutation(s) that blocks formation of VLCFAs.

Recently, James et al. (1995) isolated the Arabidopsis *FAE1* locus. Alignment of the jojoba protein with the *FAE1* gene product shows that the proteins share 52% amino acid identity

(Figure 1). A BLAST search of the randomly sequenced cDNAs in the dBEST data base revealed multiple Arabidopsis and rice cDNA clones encoding proteins related to the jojoba cDNA and *FAE1*. The enzymes encoded by these cDNAs are not known.

Optimization of KCS Assay Conditions and Solubilization of the Jojoba Enzyme

Activity measurements of membrane-associated enzymes that utilize fatty acyl-CoA substrates can be problematic, due in part to the detergent-like properties of these molecules. The effective concentration of these amphiphilic substrates in assay mixtures is difficult to establish because they partition into biological membranes and can aggregate to form micelles (Juguelin et al., 1991). In addition, loss of enzyme activity as the substrate levels are increased has been noted (Moore and Snyder, 1982; Juguelin et al., 1991). We found this to be the case for the jojoba KCS. Figure 2 shows that as 18:1-CoA concentration is increased, there is an apparent inhibition of enzyme activity. The substrate concentration at which this inhibition occurs is dependent on the amount of membrane material (as measured by protein content) present in the assay. Inclusion of subcritical micelle concentration levels of CHAPS in the assay mixture has been observed to alleviate

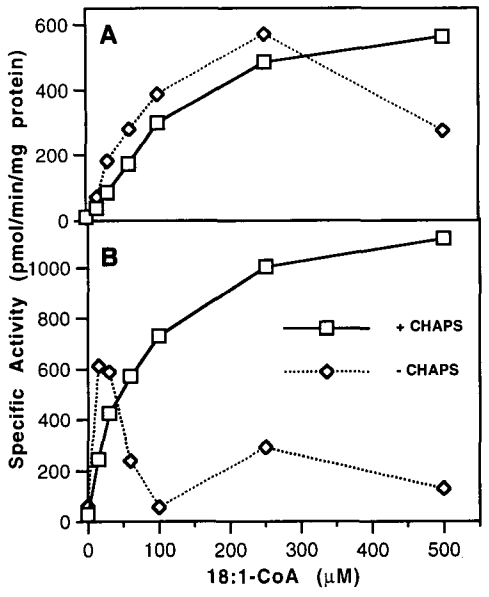

Figure 2. Effect of CHAPS on KCS Activity.

Jojoba microsomal membranes were assayed for KCS activity at various 18:1-CoA concentrations in the presence or absence of 0.375% CHAPS. The assay volume was 40 μL.

(A) High protein concentration. Sixty micrograms of jojoba membrane protein was assayed at each 18:1-CoA concentration.

(B) Low protein concentration. Five micrograms of jojoba membrane protein was assayed at each 18:1-CoA concentration.

the substrate inhibition of some enzyme activities (M.R. Pollard, personal communication). Figure 2 shows the effect of 0.375% CHAPS on KCS activity at two different protein concentrations. The highest specific activity was obtained by inclusion of CHAPS with a relatively low protein level. Based on these data, we routinely used 250 μM 18:1-CoA and 0.375% CHAPS in our enzyme assays. The data in Figure 2 demonstrate some of the variables associated with attempts to quantitate KCS activity in membrane fractions.

Jojoba embryo microsomal membranes were prepared and treated with CHAPS, using the protocol developed for the solubilization of wax synthase. After centrifugation for 2 hr at 200,000*g*, the supernatant fraction was assayed. We found that KCS activity could be detected in this fraction when CHAPS was diluted to below the subcritical micelle concentration in the assay. More than 70% of the activity detected in membranes before exposure to 2% CHAPS was recovered in the supernatant. Thus, the enzyme appeared to have been solubilized.

Partial Purification and Substrate Preference of Jojoba KCS

One criterion for solubilization of a membrane protein is retention of the enzyme activity in a supernatant fraction after high-speed centrifugation. Another is detection of the activity in the included volume after elution from large-pore gel filtration media. The 200,000*g* supernatant fraction described above was applied to a Superose 12 column that had been equilibrated with a buffer containing 1% CHAPS and 1 M NaCl. Figure 3A shows the elution profile of KCS activity detected in fractions from this column. Also shown is the elution pattern of molecular mass standards that were chromatographed using the same conditions. The enzyme activity eluted as a nearly symmetrical peak. Comparison with the protein standards yielded a mass estimate of 138 kD for KCS. For comparison, activities associated with the two other enzymes involved in wax synthesis in jojoba embryos, acyl-CoA reductase and wax synthase, were eluted from this column in volumes corresponding to molecular masses of 49 and 57 kD, respectively (data not shown).

Figure 3B shows an immunoblot analysis of fractions from the Superose 12 column, using antibodies raised against a portion of the protein encoded by the jojoba cDNA. The antibody reacted with a 57-kD protein. In addition, the intensity of the signal detected in these fractions correlated with the KCS activity detected in those same fractions. These data provided a direct link between the protein encoded by the cDNA and KCS. These data also suggested that KCS may have been solubilized in a multimeric state (see Discussion).

Figure 4 shows the enzyme activity profiles obtained from two columns that were used to purify KCS partially. Solubilized material was applied to a Blue A column. The elution profile is shown in Figure 4A. Although 84% of the applied protein flowed through the column in a buffer containing 0.3 M NaCl, KCS activity bound; ~50% of the applied activity was recovered when eluted with 2 M NaCl. The protein complexity

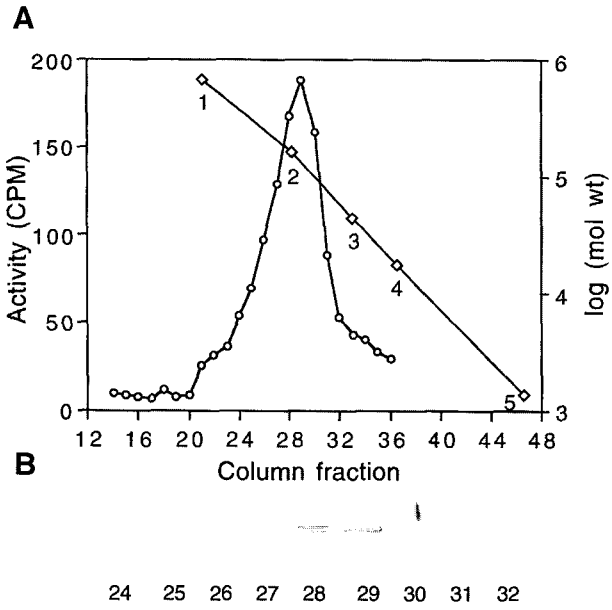

Figure 3. The 57-kD Jojoba Protein Chromatographs with KCS Activity on Superose 12.

(A) Superose 12 chromatography of solubilized KCS. Solubilized jojoba microsomal membranes were analyzed by size-exclusion chromatography. The circles indicate counts per minute incorporated into diol in the KCS assay. The diamonds represent elution positions of the molecular mass (mol wt) standards: point 1, thyroglobulin (670 kD); point 2, bovine γ-globulin (158 kD); point 3, chicken ovalbumin (44 kD); point 4, equine myoglobin (17 kD); and point 5, vitamin B-12 (1.35 kD). **(B)** Immunoblot analysis of Superose 12 column fractions. Proteins present in fractions from the Superose 12 column were separated by SDS-PAGE. After electroblotting, the membranes were incubated with antibody raised against a hydrophilic region of the 57-kD jojoba protein. The antibody did not react with any proteins in the column fractions that were devoid of KCS activity.

of the material eluted from the Blue A column was greatly simplified relative to the applied sample, and SDS-PAGE analysis revealed only a few major protein bands present in this fraction (Figure 5, lane 1).

Enzyme assays showed that the Blue A eluate was enriched in all three enzymes associated with wax synthesis in jojoba: KCS, acyl-CoA reductase, and wax synthase. Chromatography on S100 size exclusion media proved to be an effective means of separating the KCS from the other two enzyme activities. Figure 4B shows the KCS activity profile in fractions from the S100 column. As expected from the Superose 12 data, KCS activity was detected in the excluded volume. By contrast, the majority of wax synthase and reductase activities eluted in the included volume. Figure 5 shows the relative enrichment of the 57-kD band in the void fraction (lane 2). Several of the other proteins, including the 56-kD reductase protein, were enriched in the retained volume (Figure 5, lane 3). Immunoblot analysis of a duplicate gel showed that antibodies raised against the protein encoded by the cDNA recognize the 57-kD protein present in the load and in the S100 void fraction (data not shown).

Determinations of substrate preferences of membrane-associated enzymes can be complicated by a competition of other enzymes for those substrates (e.g., thioesterases and acyltransferases). Therefore, the partially purified sample from the void fractions of the S100 column was used for analysis of the substrate preferences of KCS. Figure 6 indicates the condensing activity of this preparation by using various fatty acyl-CoA substrates. KCS present in this sample showed

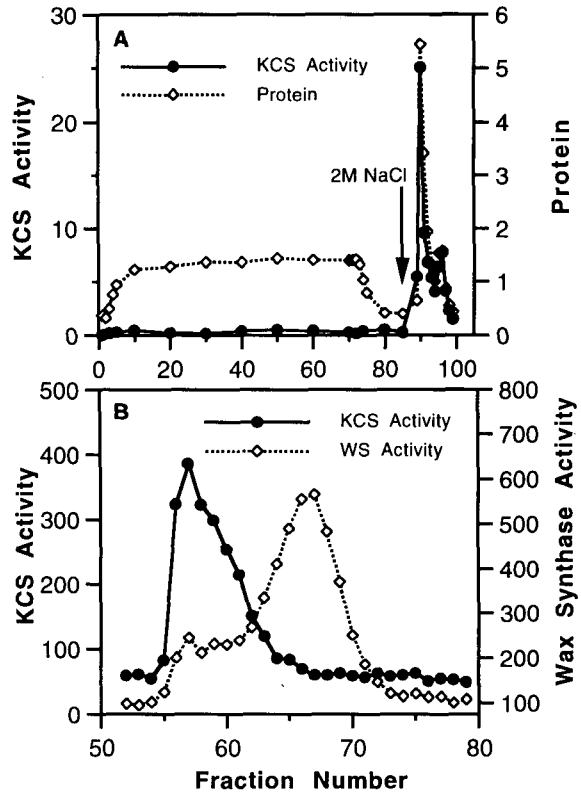

Figure 4. Chromatographic Enrichment of Jojoba KCS Activity.

(A) Blue A-agarose chromatography. Solubilized jojoba microsomal membranes were applied to a Blue A-agarose column in a buffer containing 0.3 M NaCl. The column was washed with the equilibration buffer, and KCS activity was eluted using a buffer containing 2 M NaCl. Protein content was determined according to the method of Bradford (1976) and is reported in milligrams of protein per 8.7-mL fraction. KCS activity is reported as nanomoles of diol formed per minute per fraction. **(B)** S100 chromatography. The 2 M NaCl eluate from the Blue A-agarose column was analyzed by Sephacryl S100 chromatography. The eluted fractions were assayed for KCS activity, wax synthase activity, and acyl-CoA reductase activity. KCS activity is reported as counts per minute incorporated into diol product per assay, and wax synthase (WS) activity is reported as counts per minute incorporated into wax ester per assay. The acyl-CoA reductase activity coeluted with wax synthase activity (data not shown).

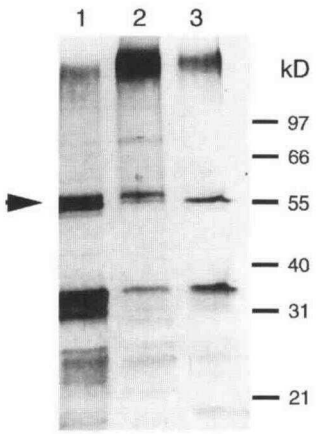

Figure 5. SDS-PAGE Showing the Partial Purification of Jojoba KCS.

Proteins were resolved on a 12% gel and stained with silver. Lane 1 shows the 2 M NaCl eluate from Blue A–agarose chromatography. Lane 2 is an excluded fraction from S100 chromatography of the sample shown in lane 1 (Figure 4B, fraction 57). Lane 3 contains an included fraction from the S100 chromatography (Figure 4B, fraction 67). The arrowhead indicates a 56- to 57-kD region that contains a doublet in lane 1. The excluded fraction (lane 2) is enriched in the 57-kD protein and KCS activity. The included fraction (lane 3) is enriched in the 56-kD protein, which we have identified as an acyl-CoA reductase (Metz and Lassner, 1994).

maximal activity with monounsaturated and saturated C18- and C20-CoA substrates. Also, the sample exhibited activity on C22 substrates. By contrast with its activity on the 18:0- and 18:1-CoAs, the jojoba preparation exhibited very low activity on the polyunsaturated 18:2- and 18:3-CoA substrates.

Expression of the Jojoba cDNA Restores KCS Activity to Canola Seed Extracts

Because the preliminary analysis of the seed oil was performed on T_2 seed segregating for the presence of the transgene, we anticipated that T_3 seed of the transgenic canola plant 7626-212/86-2 might exhibit a more dramatic phenotype and would provide superior material for biochemical characterization. Single cotyledons (half-seeds) of 100 T_2 seed were analyzed for fatty acyl composition, and the plants were propagated in growth chambers. None of the 100 progeny exhibited a LEAR phenotype, suggesting that the primary transformant contained at least three transgene loci (seed germination assays using the antibiotic G418 also indicated the plant 7626-212/86-2 contained three or more transgene loci; data not shown). Five of the T_2 seed had VLCFA content ranging between 42 and 52%. These plants were propagated for further study.

Immature T_3 seed from two of the above-mentioned T_2 plants were used to study KCS activity. Figure 7 shows that LEAR seed were deficient in the KCS activity found in HEAR seed and jojoba microsomal membranes. LEAR embryos lacked KCS activity on both 18:1-CoA and 20:1-CoA substrates. By contrast, extracts from transgenic T_3 seed elongated both 18:1-CoA and 20:1-CoA. Thus, both KCS activities were dependent on introduction of the jojoba cDNA.

Effect of Jojoba KCS Gene Dosage on Transgenic Oil Composition

Because the T_2 and T_3 seeds were segregating for at least three transgene loci, the seeds varied in transgene dosage and presumably in levels of KCS activity. Figure 8 shows the compositions of the seed oils of 132 individual T_2 and T_3 transgenic LEAR half-seeds plotted against total VLCFA content. VLCFA accumulation occurred at the expense of 18 carbon fatty acids (Figure 8A), with 18:1 being the most affected. Seed with <20% VLCFA accumulated primarily 20:1, and as VLCFA content increased, 22:1 predominated (Figure 8B). As VLCFA content increased above 35%, 22:1 and 24:1 increased at the expense of both 18:1 and 20:1. Table 1 shows the fatty acid compositions of half-seed of control HEAR and of two individual T_3 transgenic LEAR seed with the maximal observed VLCFA phenotype. The total VLCFA content of the transgenic seed was equivalent to that observed in HEAR oil; however, the proportions of the major VLCFAs—20:1, 22:1, and 24:1—differed.

DISCUSSION

We isolated a cDNA clone from jojoba involved in the formation of VLCFAs. Several lines of evidence suggested that the cDNA encodes KCS. The protein shares sequence similarity

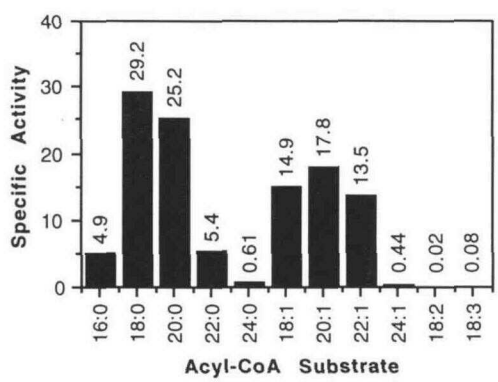

Figure 6. Substrate Preference of Partially Purified Jojoba KCS.

KCS-specific activity was determined using various acyl-CoA substrates supplied at a concentration of 250 μM. Each assay contained 0.375% CHAPS and 3 μg of protein from an S100 void fraction similar to that shown in Figure 5, lane 2. KCS-specific activity is reported as nanomoles of diol formed per min per mg of protein.

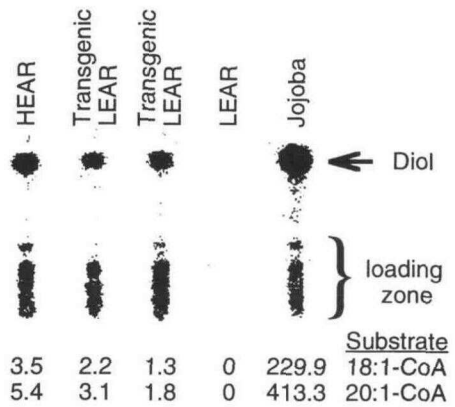

Figure 7. KCS Assays of Seed Extracts from Control and Transgenic Plants.

Extracts from immature seeds of HEAR, LEAR, *and two different transgenic* LEAR plants and from membranes of immature jojoba embryos were assayed for KCS activity. The reaction products were separated by TLC and visualized using a radioanalytic detector. Shown are the results of the TLC analysis performed using 18:1-CoA as a substrate; the image obtained using 20:1-CoA as a substrate was qualitatively similar. Numbers below indicate the specific activity of KCS in picomoles of diol formed per minute per milligram of protein by using 18:1-CoA and 20:1-CoA substrates. Radioactive material detected in the preadsorbent zone (loading zone) of the TLC plate was not identified; however, its presence is correlated with KCS activity, and it is likely to be elongated free acid (Garwin et al., 1980).

with other condensing enzymes involved in CoA thioester metabolism. These include resveratrol and chalcone synthase. *In addition, both immunoblot and enzyme assay analyses of* chromatographic separations showed the 57-kD polypeptide encoded by the jojoba cDNA to be associated with KCS activity. Finally, the transformation of LEAR with the jojoba cDNA resulted in the formation of VLCFAs in the seed oil and the restoration of KCS activity in maturing seed. *Taken together,* these data provide compelling evidence that the jojoba cDNA encodes KCS or one of its subunits. These data confirm the proposal by James et al. (1995) that the Arabidopsis *FAE1* locus encodes KCS.

We used the detergent CHAPS in the presence of salt to solubilize jojoba KCS. Biochemical characterization was performed by chromatography in the presence of the detergent, and subsequent enzyme assays were performed after dilution of the detergent below its critical micelle concentration. These techniques have proven useful for the isolation and characterization of several membrane proteins involved in lipid synthesis (Metz and Lassner, 1994; Knutzon et al., 1995) and may be of general utility for the characterization of membrane proteins. The enzymes associated with wax synthesis in jojoba (KCS, acyl-CoA reductase, and wax synthase) all utilize an acyl-CoA substrate. It is not surprising that conditions were found in which all three enzymes bound to a Blue A matrix. This matrix contains the dye Cibacron Blue F3G-A, which has an affinity for enzymes requiring adenylyl-containing cofactors. The step–elution protocol, utilized to maximize recovery of activity from this column, yielded a fraction enriched in all three enzymes. Subsequent chromatographic separation of these enzymes required individual optimization of conditions to prevent aggregation and to stabilize enzyme activity.

Our data suggest that KCS from jojoba may be active as a dimer. Based on its amino acid sequence, the size of the gene product is calculated to be 58.6 kD. The mobility of the KCS protein identified on SDS gels, both by protein staining and by immunoblot analysis, is 57 kD. Based on size exclusion chromatography, the solubilized enzyme has a mass of 138 kD. This estimate is consistent with the enzyme being composed of two 58.6-kD subunits. However, the precise nature of the solubilized complex has yet to be determined. The analogous soluble enzymes in plant and bacterial FAS, β-ketoacyl–ACP synthases, are thought to be active as dimers (MacKintosh et al., 1989; Siggard-Anderson et al., 1991). Chalcone and resveratrol synthases are also considered to be active as dimers (Kreuzaler et al., 1979; Schoppner and Kindl, 1984). The ability of the single jojoba cDNA to restore KCS activity

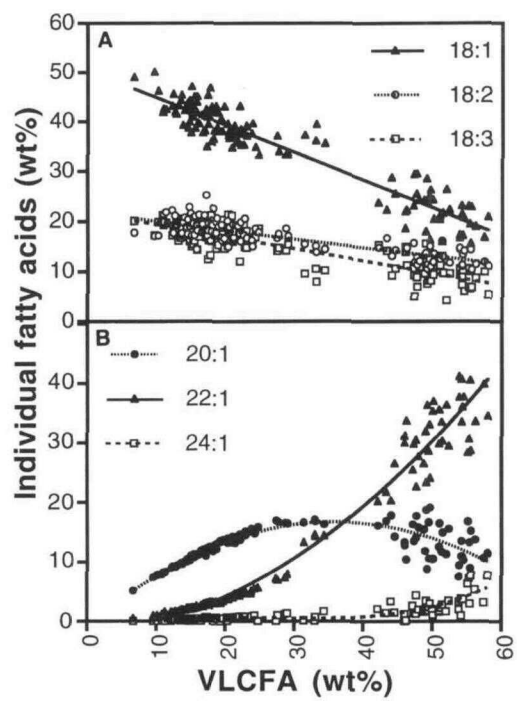

Figure 8. Fatty Acyl Compositions of Seed Oils from Transgenic LEAR Plants Expressing Jojoba KCS.

T_2 and T_3 half-seeds were analyzed by methanolysis and gas–liquid chromatography. The majority of the seeds with total VLCFA contents of >30% were T_3 seed, and the majority of the seeds with total VLCFA contents of <30% were T_2 seed.

(A) 18:1, 18:2, and 18:3 contents of 132 individual seeds plotted against their total VLCFA contents.

(B) 20:1, 22:1, and 24:1 contents of 132 individual seeds plotted against their total VLCFA contents.

suggests that if the enzyme does function as a dimer, it is likely to be composed of identical subunits.

Hydrophobicity analysis indicated that KCS is an integral membrane protein containing between four and seven transmembrane domains. One of the tryptic peptides we sequenced, TITPEIQV, lies near the N terminus of the open reading frame of the cDNA sequence (Figure 1). Thus, the protein does not have a cleaved signal peptide involved in targeting the protein to endoplasmic reticulum membranes (von Heijne, 1990). The jojoba KCS also lacks the KXKXX or KKXX (where X stands for any amino acid) sequences commonly found at the C terminus of proteins retained in endoplasmic reticulum membranes (Jackson et al., 1990). Consequently, this protein is likely to be targeted to endoplasmic reticulum membranes by its internal membrane-spanning domains (von Heijne, 1990).

Previous researchers have shown that LEAR, which does not contain VLCFAs in its seed oil, lacks FAE (Stumpf and Pollard, 1983). Our data suggested that the mutations that distinguish HEAR and LEAR cultivars reside in genes that specifically encode or regulate KCS. Cloning of the KCS genes from HEAR and LEAR should allow clarification of the basis of this agriculturally important mutation. In addition, our assays showed that LEAR is deficient in KCS activities elongating both 18:1-CoA and 20:1-CoA. The restoration of both KCS activities by the introduction of a single gene from jojoba indicates that one enzyme can catalyze both reactions. The seed oil composition of plants transformed with the jojoba gene also demonstrates that a single condensing enzyme can catalyze the formation of 20:1, 22:1, and 24:1 fatty acids.

The seed oil from the primary LEAR transformants (pooled T_2 seed) contained higher levels of 20:1 than of 22:1 fatty acids. This was also true for the majority of the T_2 half-seed analyzed from the 7626-212/86-2 plant. In contrast, the T_2 and T_3 half-seeds that exhibited the highest VLCFA content contained higher levels of 22:1 than of 20:1 (Figure 8). This suggests that as the enzyme activity increases in developing embryos, not only does the quantity of VLCFA increase, but the fatty acyl profile switches to the longer chain lengths. The increase in the amount of 24:1 in the oil of transgenic HEAR plants and the increase in the amount of 22:1 and 24:1 in transgenic Arabidopsis plants without a concomitant increase in the quantity of VLCFAs may reflect differences in substrate preferences of the jojoba, Arabidopsis, and Brassica enzymes (for 18:1-, 20:1-, and 22:1-CoA). The variation in fatty acid compositions of oils from seeds that differ only in gene dosage of the jojoba cDNA demonstrates the importance of KCS in controlling both the quantity and the composition of the VLCFAs in seed oil.

Enzyme assays of the partially purified jojoba KCS showed that the enzyme has little activity on the polyunsaturated substrates 18:2- and 18:3-CoA. These data are consistent with the oil composition of the transgenic rapeseed plants that have low levels of polyunsaturated VLCFAs. In contrast to its lack of activity on polyunsaturated acyl-CoAs, the jojoba KCS is more active on 18:0- and 20:0-CoAs than on 18:1- and 20:1-CoA substrates. The lack of saturated VLCFAs in jojoba oil reflects the high proportion of monounsaturated acyl-CoAs available for elongation. Saturated VLCFAs are found in the cuticular waxes of most plants. Thus, related enzymes may be involved in the formation of cuticular waxes. Homology searches of the dBEST data base showed multiple cDNAs in Arabidopsis with open reading frames that are homologous to the jojoba-condensing enzyme. Because some of these cDNAs were isolated from tissues that do not store lipids, we speculated that these clones may encode elongase-condensing enzymes involved in cuticular wax formation. Our research reported here may open new avenues to the study of these enzymes.

The jojoba cDNA may also be useful for biotechnology. For example, increased KCS activity may be needed to achieve high levels of 22:1 in transgenic rapeseed oil. Rapeseed excludes 22:1 from the *sn*-2 position of triacylglycerol because its lysophosphatidic acid acyl transferase (LPAAT) does not use 22:1-CoA as an acyl donor. Thus, the maximal VLCFA composition of rapeseed oil approaches 67%. We recently reported that the expression of meadowfoam LPAAT in transgenic rapeseed caused the incorporation of 22:1 into the *sn*-2 position of transgenic seed oil but did not increase the total 22:1 content of the seed oil (Lassner et al., 1995). Increase of the 22:1-CoA pools by transgenic expression of KCS in conjunction with meadowfoam LPAAT may provide the key to dramatic increases in the VLCFA composition of rapeseed oil.

METHODS

Plant Materials

Developing embryos of jojoba (*Simmondsia chinensis*) were harvested from plantations in Arizona. Seed were collected at 90 to 110 days postanthesis, when wax biosynthetic rates in the embryos were approaching their highest levels. The hulls and the seed coats were completely removed before the embryos were frozen in liquid nitrogen and stored at −70°C. Two *Brassica napus* rapeseed cultivars were used. Reston was the high erucic acid rapeseed (HEAR) cultivar, and 212/86 was the low erucic acid rapeseed (LEAR) cultivar. *Arabidopsis thaliana* race Nossen (No-0) was used.

Microsomal Preparation, Solubilization, and Chromatography

Typically, 100 g of jojoba embryos was added to 400 mL of extraction buffer (40 mM Tricine-NaOH, pH 7.8, 200 mM KCl, 10 mM EDTA, 5 mM β-mercaptoethanol), ground in a blender, and homogenized with a Polytron tissue disrupter (Brinkmann Instruments, Westbury, NY). All subsequent steps were performed at 4°C. The blended material was filtered through Miracloth (Calbiochem, La Jolla, CA). Centrifugation (20,000*g* for 20 min) of the filtrate yielded a floating wax layer, a turbid supernatant fraction, and a dark green pellet. The supernatant fraction was collected and centrifuged (100,000*g* for 2 hr) to obtain membrane pellets that were resuspended in 40 mL of buffer A (25 mM Tricine-NaOH, pH 7.8, 200 mM KCl, 5 mM EDTA, 5 mM β-mercaptoethanol) containing 50% (w/v) sucrose. This homogenate was distributed into four SW28 centrifuge tubes (Beckman Instruments), and each was overlaid with 10 mL of buffer A containing 20% sucrose and then with 13 mL of buffer A. After centrifugation (28,000 rpm for

2 hr), a membrane fraction was collected from the 20%/50% sucrose interface, diluted with four volumes of buffer A, and collected by centrifugation (200,000*g* for 1 hr). The membranes were then homogenized in 10 mL of storage buffer (25 mM Tricine-NaOH, pH 7.8, 1 M NaCl, 10% [w/v] glycerol, 5 mM β-mercaptoethanol). The protein concentration of membranes prepared by this protocol was typically between 7 and 9 mg/mL. Protein concentrations were estimated as described by Bradford (1976), using BSA as the protein standard.

The membrane suspension was adjusted to 0.83 mg of protein per mL by dilution with the storage buffer. Solid 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) was added to achieve a final concentration of 2% (w/v) and a detergent-to-protein ratio of 24:1. After incubation on ice for 1 hr, the sample was centrifuged (200,000*g* for 1 hr), and the supernatant fraction was collected.

The 200,000*g* supernatant fraction was diluted (with 0.57% CHAPS, 25 mM Tricine-NaOH, pH 7.8, 20% glycerol) to yield final concentrations of NaCl and CHAPS of 0.3 M and 1%, respectively. The sample was loaded onto a Blue A–agarose (Amicon, Inc., Beverly, MA) column that had been equilibrated with buffer B (25 mM Tricine-NaOH, pH 7.8, 20% glycerol, 1% CHAPS) containing 0.3 M NaCl. After washing with equilibration buffer, KCS activity was eluted with buffer B containing either 1.5 or 2 M NaCl. Fractions from the Blue A column were pooled and concentrated using ultrafiltration in a pressure cell fitted with a YM30 membrane (Amicon). The sample was applied to a Sephacryl S100 HR (Pharmacia) column (2.5 × 90 cm) that had been equilibrated with buffer B containing 1 M NaCl. The proteins were eluted with equilibration buffer. A Superose 12 column (Pharmacia) was used to estimate the molecular size of the solubilized β-ketoacyl–coenzyme A synthase (KCS). This column was also equilibrated and developed with buffer B containing 1 M NaCl. Molecular mass standards used to generate a calibration curve were chromatographed under the same buffer and column conditions.

57-kD Protein: Purification and Microsequencing

Fractions from Blue A columns that were enriched in a 57-kD protein were pooled and concentrated by ultrafiltration as described above. The NaCl concentration was reduced to 0.2 M by gel filtration chromatography (PD-10 columns; Pharmacia), and SDS was added to the sample to yield a final concentration of 2%. The sample was loaded onto a preparative SDS-PAGE apparatus (PrepCell; Bio-Rad) prepared with a 40-mL, 12% acrylamide separating gel and a 5-mL, 5% acrylamide stacking gel. Electrophoresis was performed according to recommendations of the manufacturer. Fractions from the PrepCell that were enriched in the 57-kD protein were pooled and concentrated via ultrafiltration (YM30 membrane). The sample was then diluted 20-fold with 2% CHAPS in 0.1 M $NaHCO_3$ and concentrated to a final volume of 100 μL. Peptides were generated by incubation of the sample with trypsin. The tryptic peptides were separated via reverse phase HPLC on a C18 column (Vydac, Hesperia, CA) by using a 0.1 mM sodium phosphate/acetonitrile gradient (Rosner and Robbins, 1982) and sequenced on an Applied Biosystems (Foster City, CA) 477A protein sequencer.

cDNA Isolation

RNA was isolated from developing jojoba seed as described by Cathala et al. (1983). Poly(A) RNA was purified by oligo(dT) chromatography, and a cDNA library was prepared by the method of Alexander (1987). Initially, a partial cDNA clone was isolated by reverse transcription of the jojoba mRNA and polymerase chain reaction (PCR) amplification, using the degenerate oligonucleotides 5′-ATGACNAAYGTNAARCC NTA-3′ and 5′-GCNGCDATNSWNGGYTC-3′, which encode the peptides MTNVKPY and EPSIAA. This fragment was used as a probe to screen the cDNA library. Because no full-length clones were isolated from the cDNA library, the 5′ end of the mRNA was characterized by 5′ rapid amplification of cDNA ends (RACE; Frohman et al., 1988), using the gene-specific primer 5′-CUACUACUACUAGGTCCATGAACATCTCG TGGG-3′ and a nonspecific amplification primer 5′-CAUCAUCAUC AUAAGCTTCTGCAGGAGCTC-3′. The first 12 nucleotides of these two primers include deoxyuridine (U), which allows the use of uracil DNA glycosylase to generate the 3′ protruding termini necessary for cloning of the RACE products into pAMP1 (Gibco BRL). The sequence of an mRNA has a GenBank accession number of U37088. Data base searches were performed at the National Center for Biotechnology Information using the BLAST network service (Altschul et al., 1990). MacVector (Eastman Kodak) was used for routine DNA and protein sequence analysis, Megalign (DNASTAR, Inc., Madison, WI) was used to generate multiple sequence alignments, and potential membrane-spanning domains were identified using TopPred II software (Claros and von Heijne, 1994).

To introduce a suitable cloning site upstream of the protein-coding region of the jojoba cDNA, the cDNA was PCR amplified using the primers 5′-CAUCAUCAUCAUGTCGACACAATGAAGGCCAAACAAT CAC-3′ and 5′-CUACUACUACUATTGCCCCAGTACCAATTAAG-3′, and the PCR products were cloned in pAMP1. The EcoRI-AccI fragment was then subcloned into a similarly digested cDNA clone to replace the 5′ end of the cDNA clone with the cloned PCR product. A BclI linker was introduced into an AflIII site downstream of the open reading frame, the cDNA was digested with SalI and BclI, and the chimeric gene was cloned into SalI-BglII–digested pCGN3223. Plasmid pCGN3223 contains 3 kb of napin regulatory sequences that direct high levels of gene expression in maturing embryos (Kridl et al., 1991). The Asp718-digested napin/KCS DNA fragment was cloned into a similarly digested binary plant transformation vector, pCGN1578 (McBride and Summerfelt, 1990), to yield pCGN7626.

Plant Transformation

The binary vector pCGN7626 was transferred to *Agrobacterium tumefaciens* and cocultivated with *Brassica napus* and Arabidopsis (Radke et al., 1987; Valvekens et al., 1988). The plants derived by transformation are designated T_1 plants (e.g., 7626-2), and seed harvested from these plants are T_2 seed (e.g., 7626-2-1). T_2 seed give rise to T_2 plants, and seed harvested from T_2 plants are designated T_3 seed (e.g., 7626-2-1-1).

Antibody Production

We were unable to express the entire jojoba KCS in *Escherichia coli* by using several standard expression systems. A hydrophilic region of the protein (amino acids 305 to 347, noted in Figure 1) was expressed as a fusion protein with glutathione *S*-transferase (GST; Frangioni and Neel, 1993). The oligonucleotides 5′-CAUCAUCAUCAUGGAT CCTCAAACCGCTGGCGTGATCGT-3′ and 5′-CUACUACUACUAGAATT CAACACCTACCTTGTTATTTTCATCTTC-3′ were used as PCR primers to amplify the corresponding region of the cDNA and to introduce appropriate cloning sites for subcloning into vector pGEX2T. The

GST–jojoba KCS fusion protein was produced and cleaved as described by Frangioni and Neel (1993). Sera were produced in rabbits (BABCO, Richmond, CA). The antibodies were immunoaffinity purified on Sephadex coupled to the GST–jojoba KCS fusion protein as described by Harlow and Lane (1988).

Enzyme Assays

Our assay of KCS activity was based on the β-ketoacyl–acyl carrier protein synthase assay described by Garwin et al. (1980). The assay involved incubating the enzyme with 100 μM 2-^{14}C-malonyl–CoA (Amersham) and 250 μM 18:1-CoA in a buffer containing 0.375% CHAPS, 375 mM NaCl, 25 mM Tricine (or Hepes)-NaOH, pH 7.8, and 2 mM β-mercaptoethanol at 30°C. The absence of reductant (NADH or NADPH) in the assay prevented the elongation reactions from proceeding beyond the condensation step. The β-ketoacyl–CoA product was reduced to a diol by $NaBH_4$ in the presence of tetrahydrofuran, and long-chain-neutral lipids were extracted with toluene. Radioactivity present in a portion of the organic phase was determined using liquid scintillation counting, whereas a second portion was used for thin layer chromatography (TLC) analysis (silica gel G with a preadsorbent zone developed with diethyl ether–ammonium hydroxide [100:1]). Because unreacted substrate partitioned into the aqueous phase, it was not applied to the TLC plate. After TLC analysis, the diol product was identified and quantified using a radioanalytic scanner (Scanalytics, Billerica, MA). For assays of the CHAPS solubilized enzyme, care was taken to reduce the detergent concentration to 0.375%.

Developing Brassica seed were harvested 30 days after pollination and frozen at −70°C. Twenty seeds of each sample were homogenized in 1 mL of extraction buffer (25 mM Hepes-NaOH, 250 mM NaCl, 2 mM EDTA) using a Dounce homogenizer (Kontes, Vineland, NJ). The homogenate was centrifuged (15,000*g* for 10 min), and the pellet was washed with the extraction buffer and resuspended in 500 μL of the extraction buffer containing 10% glycerol and 5 mM β-mercaptoethanol.

Wax synthase was assayed with 40 μM 1-^{14}C-16:0–CoA (5 Ci/mol), 200 μM 18:1-alcohol, 25 mM Tricine-NaOH, pH 7.8, 280 mM NaCl, 5.6% glycerol, 2 mM β-mercaptoethanol, 0.28% CHAPS, and 0.7 μg/μL soybean phospholipids (Sigma) in 250 μL. Activity was measured as the incorporation of radioactivity into wax ester.

Gel Electrophoresis and Immunoblots

SDS-PAGE was performed with commercially available gels (Novex, San Diego, CA), and proteins were visualized with silver (Blum et al., 1987). Immunoblots were prepared by electroblotting of proteins from polyacrylamide gels to polyvinylidene difluoride membranes. The membranes were briefly incubated in TBST (10 mM Tris-HCl, 1 M NaCl, 0.1% Tween 20, pH 8.0) containing 3% skim milk powder and incubated overnight with the purified antibody (diluted 1:10) in TBST. The immunoblots were visualized using alkaline phosphatase–conjugated goat anti–rabbit antisera and Western Blue stain (Promega), according to the supplier's protocol.

Oil Analysis

Oil fatty acyl composition was analyzed by gas–liquid chromatography of methyl esters (Browse et al., 1986). Nondestructive (half-seed) analysis of individual Brassica seed was performed after imbibition of seed with H_2O for ~24 hr. One cotyledon was used for composition analysis; the rest of the embryo was propagated.

ACKNOWLEDGMENTS

We thank Janice Bleibaum for help with protein sequencing, William Schreckengost for DNA sequencing, Joann Turner for generating the transgenic plants, and Thomas Hayes and Laura Torchin for analyzing the seed oil compositions. We also thank Maelor Davies, Toni Voelker, Gregory Thompson, and Ling Yuan for helpful discussion.

Received October 3, 1995; accepted December 7, 1995.

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