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Producing Wax Esters in Transgenic Plants by Expression of Genes Derived from Jojoba

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Jojoba [*Simmondsia chinensis*, Simmondsiaceae, (Link) Schnieder], a native of deserts of the American Southwest, is unusual and perhaps unique amongst higher plants in that its seed storage lipids are liquid waxes rather than triglycerides. These waxes are esters of long chain (mostly; C20, C22, and C24), monounsaturated, fatty acids and alcohols. The waxes are produced in developing embryos during seed formation. Jojoba oil is primarily used as an ingredient in cosmetics. The stability of the oil under high temperatures and pressures suggests it has potential as a component of industrial lubricants, however, the high price of jojoba oil has served as a barrier to its widespread use for these types of applications. The production of liquid waxes in an oilseed crop more suited to large scale agriculture could allow the production of wax esters at a price more acceptable to industrial users.

As in other plants, jojoba fatty acids up to 18 carbons in length are synthesized in plastids. Oleic acid (18:1) is exported from plastids, and its CoA esters serve as substrates for microsomal membrane enzymes involved in wax formation. Three enzyme activities are involved in the formation of waxes (Fig.1, Pollard et al. 1979):

1. Fatty acid elongase (FAE) is a complex of four enzymes that converts 18:1 to eicosenoic acid (20:1), erucic acid (22:1), and nervonic acid (24:1).
2. An acyl-CoA reductase catalyzes the formation of fatty alcohols from 20:1-, 22:1-, and 24:1-CoAs.
3. Wax synthase is an acyltransferase that forms wax esters from fatty alcohol and acyl-CoA substrates.

In this manuscript we describe the isolation of genes encoding the enzymes involved in wax synthesis and the use of these genes to produce wax esters in transgenic arabidopsis plants.

FATTY ACID ELONGASE

The membrane-associated nature of the elongation enzymes has hindered investigation of their biochemistry. As in animal systems (Bernert and Sprecher 1979), fatty acid elongation in plants is believed to occur by way of a four-step mechanism that is similar to fatty acid synthesis, except that CoA, rather than ACP, is the acyl carrier (Cassagne et al. 1994b; Fehling and Mukherjee 1991; Stumpf and Pollard 1983). The first step in the cycle involves condensation of malonyl-CoA with a long-chain acyl-CoA to yield carbon dioxide and a ß-ketoacyl-CoA in which the acyl moiety has been elongated by two carbons. The enzyme that catalyzes this reaction is ß-ketoacyl-CoA synthase (KCS). Subsequent reactions are: reduction to ß-hydroxyacyl-CoA, dehydration to an enoyl-CoA, and a second reduction to yield the elongated acyl-CoA. In both mammalian and plant systems where 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). Thus, the overexpression of KCS can increase the quantity of very long chain fatty acids and increase the substrates available for wax formation.

We previously described the isolation of a jojoba gene encoding the KCS associated with fatty acid elongation in developing seeds, and showed that expression of this gene complimented the canola (*Brassica napus*) mutation which knocked out the production of very long chain fatty acids in the seed oil. Additionally, the influence of the jojoba KCS substrate preferences was evident from the appearance of up to 5% nervonic acid in the transgenic oil—nervonic acid is present in jojoba oil but is found at very low levels in rapeseed oil (Lassner et al. 1996). More recently, we isolated the corresponding gene from seeds of *Lunaria annua*, Brassicaceae. *Lunaria* seed oil is rich in nervonic acid. Expression of the *Lunaria* KCS in transgenic *Brassica* resulted in rapeseed oils with greater than 25% nervonic acid (Lassner 1997; M.W. Lassner unpubl. data).

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ACYL-COA REDUCTASE

The jojoba fatty acyl- CoA reductase is also associated with membranes in developing embryos. Based on both the wax composition and in vitro assays, the enzyme has a strong preference for very long chain acyl-CoA substrates (24:1 > 22:1 > 20:1) and has very little activity towards 18:1-CoA. The enzyme was purified from a microsomal membrane fraction isolated from developing embryos. We used the detergent, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), to solubilize the reductase. Although the presence of high levels of CHAPS in assay solutions resulted in an apparent loss of enzyme activity, this inhibition could be completely reversed by dilution of the detergent to below its CMC. We routinely diluted CHAPS to 0.3% (w/v) in our assays.

The reductase enzyme was purified using three chromatography steps. Blue A agarose chromatography was used to separate the reductase activity from a majority of the proteins. Size exclusion chromatography on Sephacryl S-100 HR did not result in an increase in specific activity, but did improve subsequent chromatography. The final purification step was affinity chromatography in which the reductase was bound to palmitoyl-CoA agarose and eluted using NADPH. Two polypeptides with apparent molecular masses of 54 and 56 kDa were present in the NADPH eluted fractions from the palmitoyl-CoA column. Several peptides, generated using either trypsin or cyanogen bromide, were isolated from the two proteins and N-terminal sequences were determined. All of the sequences obtained from peptides of the 54 kD protein were found in peptides isolated from the 56 kD polypeptide. Subsequent immunoblot analysis of a jojoba embryo cell free extract showed that only the 56 kD polypeptide was present. Thus, the smaller 54 kDa polypeptide appeared to be an artifact of protein purification.

The peptide sequences were used to design oligonucleotide primers, and this enabled PCR amplification of a partial cDNA from jojoba embryo tissue. The PCR product was used to isolate a full-length cDNA clone. The cDNA clone was expressed in *E. coli*. Extracts from the transformed *E. coli* gained acyl-CoA reductase enzyme activity when compared to the control extracts, and analysis of the lipids revealed that the transformed *E. coli* had increased levels of fatty alcohols (Fig.2). This demonstrated that we had indeed isolated the gene encoding the acyl-CoA reductase responsible for fatty alcohol production.

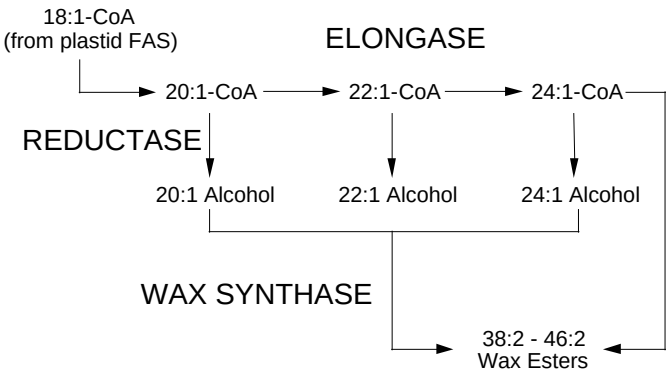

Fig. 1. The wax biosynthetic pathway in jojoba. Three enzyme activities compose the wax synthesis pathway: fatty acid elongase, acyl-CoA reductase, and wax synthase. The relative activities of the three enzymes determines the chain lengths of the waxes found in jojoba oil.

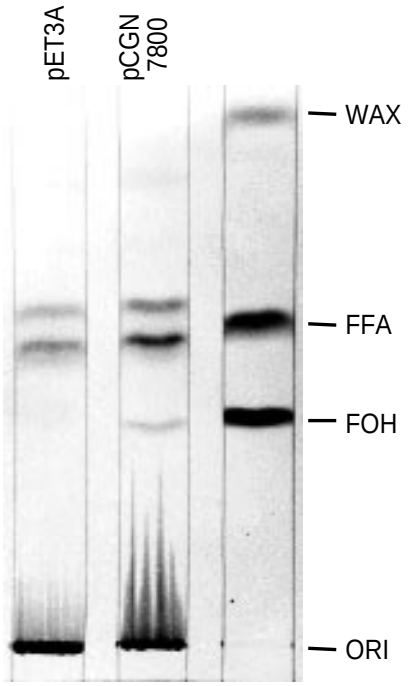

Fig. 2. TLC analysis of *E. Coli* expressing the jojoba acyl-CoA reductase. Lipids were extracted from *E. coli* in hexane:isopropanol (3:2) and separated by normal phase TLC. The plasmid pCGN7800 contains the acyl-CoA reductase under control of a T7 polymerase promoter. Plasmid pET3A is the vector used for expression of the reductase. The lipids extracted from *E. coli* containing pCGN8500 contain fatty alcohols not found in the lipids from *E. coli* containing the empty vector. Abreviations: FFA, free fatty acid; FOH, fatty alcohol; and ORI, origin.

The reductase cDNA was placed under the control of oleosin regulatory sequences. Expression of the acyl-CoA reductase gene in transgenic high erucic acid rapeseed (HEAR) directed the synthesis of low levels of fatty alcohol in the seed oil. The best transgenic line had 0.4% alcohol. Analysis of the nucleotide composition of the reductase gene showed that the A+T content of the gene exceeded 60% in many portions of the gene (determined by examining the A+T content of windows of 25 nucleotides). The gene was resynthesized to reduce the A+T composition of the gene to between 40% and 50% in all 25 nucleotide segments. Seed oils extracted from individual seeds from HEAR transformed with the resynthesized reductase gene now contained up to 4.4% (by weight) fatty alcohol. Interestingly, a portion of these fatty alcohols were esterified to fatty acids, forming wax esters. The wax content of the seed oil ranged up to 8.5% weight percent of the seed oil. Apparently an endogenous wax synthase in the *Brassica* embryos could convert some of the alcohol to wax esters. Enzyme assays showed that low levels of wax synthase activity could be detected in cell free extracts of *Brassica* embryos. The wax content was variable and ranged from less than the alcohol content to twice the alcohol content (full conversion of alcohol to wax). Seeds with the highest alcohol content germinated poorly. Since high concentrations of fatty alcohols are likely to be toxic to cells, we postulated that the addition of an efficient wax synthase activity would allow the generation of transgenic plants with higher alcohol and wax content.

WAX SYNTHASE AND PRODUCTION OF WAX ESTERS IN TRANSGENIC *ARABIDOPSIS*

Initial attempts to solubilize the jojoba embryo wax synthase using conditions developed for the fatty acyl-CoA reductase were unsuccessful. CHAPS was used as the detergent, however a higher detergent to protein ratio was required to achieve solubilization and higher concentrations of CHAPS in the chromatography buffers was needed to prevent aggregation. Additionally, after treatment with the detergent, enzyme ac-

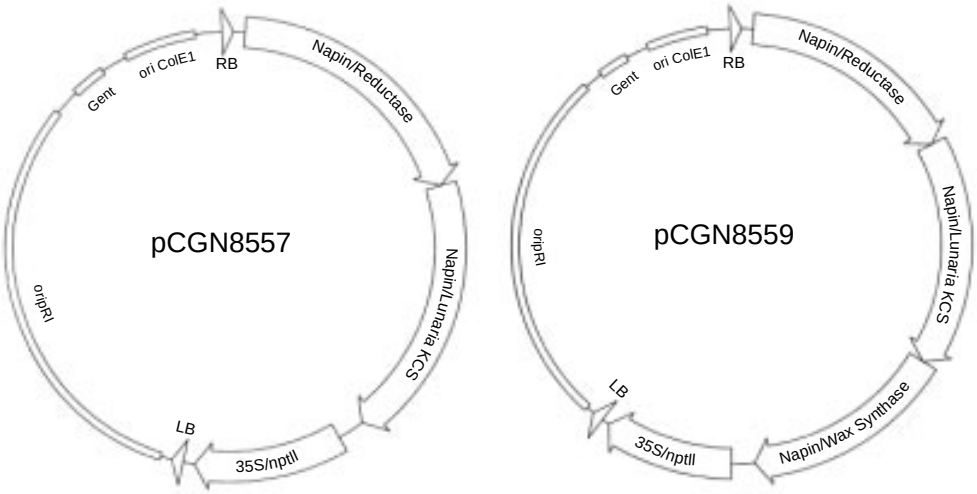

Fig. 3. Plasmids used to express wax ester biosynthetic genes into *Arabidopsis*. The plasmids pCGN8557 and pCGN8559 were introduced into *Agrobacterium tumefaciens*. These *Agrobacterium* strains were used to transform *Arabidopsis thaliana*.

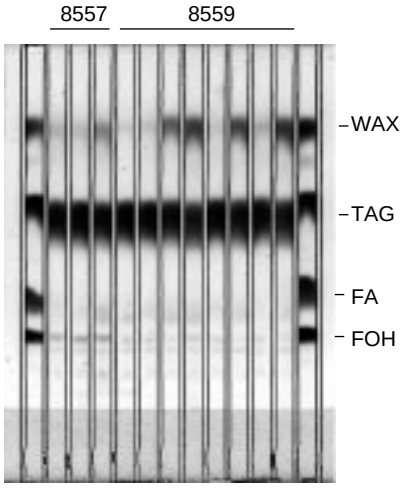

Fig. 4. TLC analysis of *Arabidopsis* seed oil from plants transformed with jojoba wax synthesis genes. Lipids were extracted from transgenic *Arabidopsis* seeds in hexane and separated by normal phase TLC. Plasmid pCGN8557, the control plasmid, contains KCS and acyl-CoA reductase genes. Plasmid pCGN8559 contains the same two genes with the addition of a wax synthase gene. Seed oils from plants transformed with pCGN8559 contain higher quantities of wax esters than. untransformed plants and plants transformed with pCGN8557. Abreviations: TAG, triglyceride, FA, free fatty acid; and FOH, fatty alcohol.

tivity was dependent upon reconstitution into phospholipid vesicles. The wax synthase was partially purified by chromatography on Blue A agarose, size exclusion, and hydroxyapatite columns. A 33 kDA protein was identified whose abundance correlated with the amount of enzyme activity present in fractions from multiple chromatographic separations. This protein was isolated by SDS-PAGE, and digested with trypsin. The sequences of several peptides were determined. The peptide sequences were used to design oligonucleotide primers, and a PCR product representing a partial cDNA was isolated. DNA sequence of the PCR product enabled the cloning of the 5' and 3' ends of the cDNA via RACE (Frohman et al. 1988).

Two plasmids were constructed for plant transformation (Fig. 3). One construct, pCGN8557, contained the *Lunaria* KCS and the jojoba acyl-CoA reductase under control of napin regulatory sequences. The second construct contained the *Lunaria* KCS, the jojoba acyl-CoA reductase and wax synthase candidate under control of napin regulatory sequences. Napin is a *Brassica* seed storage protein and the napin regulatory sequences drive high level expression of the associated transgenes in embryos of transgenic plants (Kridl et al. 1991). The two plasmids were introduced in *Agrobacterium tumefaciens*, and subsequently transformed into *Arabidopsis thaliana*.

Immature seeds were dissected from the transgenic plants, and wax synthase enzyme assays were performed. Seed extracts from most of the plants transformed with pCGN8559 exhibited wax synthase activity, while very little activity was detected in the seed extracts from pCGN8557 plants. This demonstrated that we had isolated the gene encoding wax synthase.

TLC analysis showed much higher levels of wax esters in the oil derived from pCGN8559 plants than present in oil derived from pCGN8557 plants (Fig. 4). Gas chromatography analysis of transmethylated oil showed that up to 16 weight percent of the oil from the transgenic pCGN8559 plants was fatty alcohols. If the wax synthase was efficient at converting fatty alcohol to wax esters, this fatty alcohol composition data suggested that the oil was comprised of 30 weight percent wax. Another method of analyzing the wax content is ^{13}C NMR. Fig. 5 shows the region of the NMR spectrum that indicates the glycerol backbone carbons of triglyceride and the #1 carbon of the alcohol moiety of wax esters. NMR analysis suggested that the oil was comprised of approximately 50 mole percent (approximately 40% by weight) of wax.

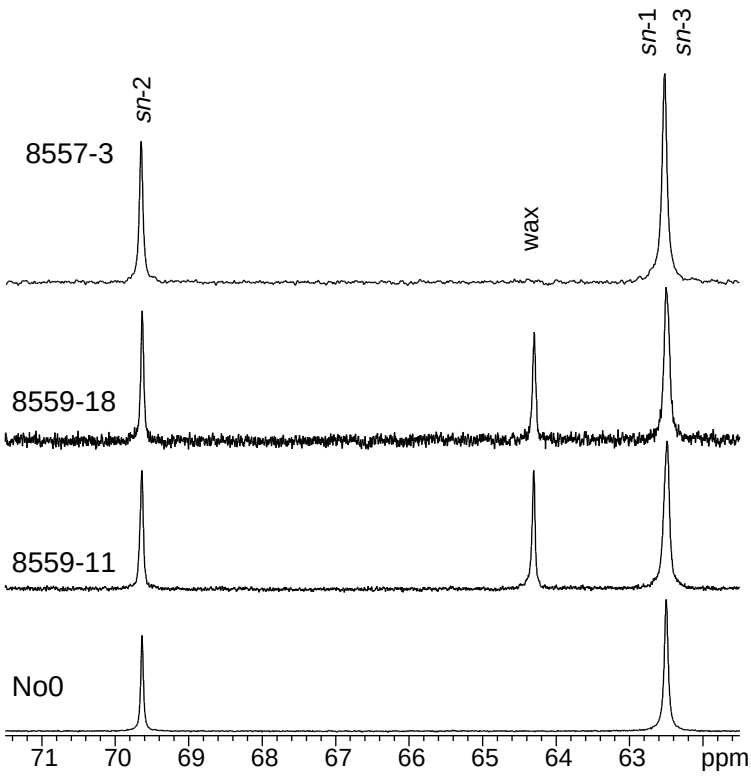

Fig. 5. ^{13}C NMR analysis of seeds from *Arabidopsis* plants transformed with jojoba wax synthesis genes. NMR analysis was performed on whole seeds from *Arabidopsis* plants. The peak at 62.4 ppm represents carbons at the *sn*-1 and *sn*-3 positions of the glycerol backbone of triglycerides, while the peak at 69.6 ppm represents carbons in the sn-2 position of the glycerol backbone. The peak at 64.3 ppm represents the #1 carbon of the alcohol moiety of wax esters. Since each triglyceride has one *sn*-2 glycerol carbon, and each wax molecule has one alcohol molecule, the relative areas of the 64.3 and 69.6 ppm peaks provide an estimate of the molar ratios of wax esters and triglycerides, respectively, in the oil samples. The chromatograms labeled 8559-11 and 8559-18 show the analysis of seeds from two independent pCGN8559 transformants, the chromatogram labeled 8557 shows the analysis of seeds from a pCGN8557 plant, and the chromatogram labeled NoO shows the analysis of seeds from an untransformed control plant.

CONCLUSIONS

The work described in this manuscript shows that we have isolated a number of key genes involved in jojoba wax biosynthesis. Data from plant genome projects which unveil orthologous genes in other plant species may lead to understanding how jojoba evolved to use wax esters as their seed storage lipids. The levels of wax achieved in transgenic *Arabidopsis* are quite promising—50 mole % of the seed oil is wax in the transgenic pCGN8559 plant with the best phenoytpe. The analyzed seeds of this plant were segregating for the presence of the transgene and the wax content of homozygous plants would be expected to be higher. Currently, the transgenes are being introduced into high erucic acid varieties of rapeseed. The development of transgenic rapeseed varieties could provide a new source of wax esters with a lower price than the liquid wax produced in jojoba.

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