



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : C12N 15/54, 15/82, C12P 7/64</p>	<p>A1</p>	<p>(11) International Publication Number: WO 98/55632</p> <p>(43) International Publication Date: 10 December 1998 (10.12.98)</p>
<p>(21) International Application Number: PCT/US98/11590</p> <p>(22) International Filing Date: 5 June 1998 (05.06.98)</p> <p>(30) Priority Data: 60/048,651 5 June 1997 (05.06.97) US</p> <p>(71) Applicant: CALGENE LLC [US/US]; 1920 Fifth Street, Davis, CA 95616 (US).</p> <p>(72) Inventors: LARDIZABAL, Kathryn, Dennis; 1546 Owens Valley Drive, Woodland, CA 95776 (US). METZ, James, George; 2803 Belhaven Place, Davis, CA 95616 (US). LASSNER, Michael, W.; 721 Falcon Avenue, Davis, CA 95616 (US).</p> <p>(74) Agent: SCHWEDLER, Carl, J.; Calgene LLC, 1920 Fifth Street, Davis, CA 95616 (US).</p>	<p>(81) Designated States: CA, CN, JP, KR, MX, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: FATTY ACYL-CoA: FATTY ALCOHOL ACYLTRANSFERASES</p>		
<p>(57) Abstract</p> <p>By this invention, nucleic acid sequences encoding for fatty acyl-CoA: fatty alcohol acyltransferase (wax synthase) are provided, wherein said wax synthase is active in the formation of a wax ester from fatty alcohol and fatty acyl-CoA substrates. Of special interest are nucleic acid sequences obtainable from a jojoba embryo wax synthase having an apparent molecular mass of approximately 33kD. Also considered are amino acid and nucleic acid sequences obtainable from wax synthase proteins and the use of such sequences to provide transgenic host cells capable of producing wax esters.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

FATTY ACYL-CoA: FATTY ALCOHOL ACYLTRANSFERASES5 Technical Field

The present invention is directed to enzymes, methods to purify, and obtain such enzymes, amino acid and nucleic acid sequences related thereto, and methods of use for such compositions in genetic engineering applications.

10

INTRODUCTIONBackground

Through the development of plant genetic engineering techniques, it is possible to transform and regenerate a variety of plant species to provide plants which have novel and desirable characteristics. One area of interest for such plant genetic engineering techniques is the production of valuable products in plant tissues. Such applications require the use of various DNA constructs and nucleic acid sequences for use in transformation events to generate plants which produce the desired product. For example, plant functional promoters are required for appropriate expression of gene sequences, such expression being either in the whole plant or in selected plant tissues. In addition, selective marker sequences are often used to identify the transformed plant material. Such plant promoters and selectable markers provide valuable tools which are useful in obtaining the novel plants.

A desirable goal which involves such genetic engineering techniques, is the ability to provide crop plants having a convenient source of wax esters. Wax esters are required in a variety of industrial applications, including pharmaceuticals, cosmetics, detergents, plastics, and lubricants. Such products, especially long chain wax esters have previously been available from the sperm whale, an endangered species, or more recently, from the desert shrub, jojoba. Neither of these sources provides a convenient supply of wax esters. Thus, in order to obtain a reliable source of such compounds, transformation of crop plants,

which are easily manipulated in terms of growth, harvest and extraction of products, is desirable.

In order to obtain such transformed plants, however, the genes responsible for the biosynthesis of the desired wax ester products must first be obtained. Wax ester production results from the action of at least two enzymatic activities, fatty acyl reductase and fatty acyl:fatty alcohol acyltransferase, or wax synthase. In addition, a β -ketoacyl-ACP synthase may also be involved in wax biosynthesis by providing very long chain fatty acyl-CoA substrates for the reductase and wax synthase enzymatic reaction. Preliminary studies with such enzymes and extensive analysis and purification of a fatty acyl reductase, indicate that these proteins are associated with membranes, however the enzyme responsible for the fatty acyl:fatty alcohol ligation reaction in wax biosynthesis has not been well characterized. Thus, further study and ultimately, purification of this enzyme is needed so that the gene sequences which encode the enzymatic activity may be obtained.

It is desirable, therefore, to devise a purification protocol whereby the wax synthase protein may be obtained and the amino acid sequence determined and/or antibodies specific for the wax synthase obtained. In this manner, library screening, polymerase chain reaction (PCR) or immunological techniques may be used to identify clones expressing a wax synthase protein. Clones obtained in this manner can be analyzed so that the nucleic acid sequences corresponding to wax synthase activity are identified. The wax synthase nucleic acid sequences may then be utilized in conjunction with fatty acyl reductase proteins, either native to the transgenic host cells or supplied by recombinant techniques, for production of wax esters in host cells.

Relevant Literature

Cell-free homogenates from developing jojoba embryos were reported to have acyl-CoA fatty alcohol acyl transferase activity. The activity was associated with a floating wax

pad which formed upon differential centrifugation (Pollard *et al.* (1979) *supra*; Wu *et al.* (1981) *supra*).

Solubilization of a multienzyme complex from *Euglena gracilis* having fatty acyl-SCoA transacylase activity is reported by Wildner and Hallick (Abstract from *The Southwest Consortium Fifth Annual Meeting*, April 22-24, 1990, Las Cruces, NM.).

Ten-fold purification of jojoba acyl-CoA: alcohol transacylase protein is reported by Pushnik *et al.* (Abstract from *The Southwest Consortium Fourth Annual Meeting*, February 7, 1989, Riverside, Ca.).

An assay for jojoba acyl-CoA:alcohol transacylase activity was reported by Garver *et al.* (*Analytical Biochemistry* (1992) 207:335-340).

WO 93/10241 is directed to plant fatty acyl-CoA:fatty alcohol 0-acyltransferases. A jojoba 57kD protein is identified as the jojoba fatty acyl-CoA:fatty alcohol 0-acyltransferase (wax synthase). The present inventors later reported that the 57kD protein from jojoba is a β -ketoacyl-CoA synthase involved in the biosynthesis of very long chain fatty acids (Lassner *et al.* (*The Plant Cell* (1996) 8:281-292).

Photoaffinity labeling of a 57 kD jojoba seed polypeptide postulated to be an acyl-CoA:fatty alcohol acyltransferase was also reported by Shockey *et al.* (*Plant Phys.* (1995) 107:155-160).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 presents results of analysis of wax synthase activity in column fractions from a first wax synthase purification protocol. Figure 1A provides results of Blue A agarose chromatography. Figure 1B provides results of ceramic hydroxyapatite chromatography. Figure 1C provides results of sephracryl S-100 size exclusion chromatography. Figure 1A provides results of hydroxyapatite chromatography.

Figure 2 presents results of analysis of wax synthase activity in column fractions from a second wax synthase

purification protocol. Figure 2A provides results of Blue A agarose chromatography. Figure 2B provides results of hydroxyapatite chromatography. Figure 2C provides results of Superdex 75 size exclusion chromatography.

5 Figure 3 provides the nucleotide sequence of the PCR product from primers WSPEP14-F1 and WSPEP33-R2 (Figure 3A) and the complete nucleotide sequence of a fatty acyl-CoA:fatty alcohol O-acyltransferase from jojoba inferred from 5' and 3' RACE products (Figure 3B).

10 Figure 4 provides the Radioimage of TLC plate showing incorporation of 1-¹⁴C 16:0 CoA into wax in assays of the pellet fractions prepared from developing seeds of arabidopsis transformed with pCGN8559. A membrane fraction from developing jojoba seed is the positive control. Background activity is
15 illustrated in the assays of arabidopsis plants 8612-3 and 8613-2.

SUMMARY OF THE INVENTION

20 By this invention, nucleic acid sequences encoding fatty acyl-CoA: fatty alcohol O-acyltransferase protein (fatty alcohol acyltransferase, E.C.2.3.1.75), are provided, wherein said protein is active in the formation of wax esters from fatty alcohol and fatty acyl substrates. This
25 fatty acyl-CoA: fatty alcohol O-acyltransferase is also referred to herein as "wax synthase". The wax synthase of this invention may be active with a variety of fatty acyl and fatty alcohol substrates, including acyl-CoAs and acyl-ACPs. The carbon chain length of these substrates may vary,
30 although a given wax synthase may show preference for acyl and alcohol substrates having a specific chain length or may be active with acyl and alcohol substrates having a wide range with respect to carbon chain length.

In general, the wax synthase of this invention has
35 activity towards at least those acyl and alcohol substrates having a chain length of from 8 to 26 carbons, although other acyl or alcohol substrates may be tested and further

activities discovered. In addition, having obtained the wax synthase protein of this invention, further manipulations are now possible as described in further detail below.

These manipulations may lead to production or discovery of other related wax synthases.

In one important aspect of this invention, nucleic acid sequences are provided which encode for wax synthase.

Methods are described whereby these sequences may be identified and obtained from the amino acid sequences of the wax synthase proteins of this invention. Uses of the structural gene sequences for isolation of other wax synthase sequences, as well as in recombinant constructs for transcription of wax synthase nucleic acid sequences and/or expression of wax synthase proteins in host cells are described. Uses of other nucleic acid sequences associated with wax synthase protein are also considered, such as the use of 5' and 3' noncoding regions.

Thus, this invention encompasses plant wax synthase nucleic acid sequences and the corresponding amino acid sequences, and the use of these nucleic acid sequences in the preparation of oligonucleotides containing wax synthase encoding sequences for analysis and recovery of plant wax synthase gene sequences. The plant wax synthase encoding sequence may encode a complete or partial sequence depending upon the intended use. All or a portion of the genomic sequence, or cDNA sequence, is intended.

Of special interest are recombinant DNA constructs which provide for transcription or transcription and translation (expression) of the plant wax synthase sequences. In particular, constructs which are capable of transcription or transcription and translation in plant host cells are preferred. For some applications a reduction in plant wax synthase may be desired. Thus, recombinant constructs may be designed having the plant wax synthase sequences in a reverse orientation for expression of an anti-sense sequence or use of co-suppression, also known as "transwitch", constructs may be useful. Such constructs may

contain a variety of regulatory regions including transcriptional initiation regions obtained from genes preferentially expressed in plant seed tissue. For some uses, it may be desired to use the transcriptional and translational initiation regions of the wax synthase gene either with the wax synthase encoding sequence or to direct the transcription and translation of a heterologous sequence.

In yet a different aspect, this invention relates to a method for producing a wax synthase in a host cell or progeny thereof via the expression of a construct in the cell. Cells containing a wax synthase as a result of the production of the plant wax synthase encoding sequence are also contemplated herein. Such constructs may employ other nucleic acid sequences which encode for proteins involved in the production of wax esters and/or various fatty acyl species.

Further, it may be recognized that the wax synthases of this invention may find application in the production of wax esters in such host cells which contain fatty acyl and fatty alcohol substrates of the wax synthase. Such host cells may exist in nature or be obtained by transformation with nucleic acid constructs which encode a fatty acyl reductase. Fatty acyl reductase, or "reductase", is active in catalyzing the reduction of a fatty acyl group to the corresponding alcohol. Co-pending US patent applications 07/659,975 (filed 2/22/91), 07/767,251 (filed 9/27/91) and 07/920,430 (filed 7/31/92), which are hereby incorporated by reference, are directed to such reductase proteins. This information is also provided in published PCT patent application WO 92/14816. In addition, other sources of wax synthase proteins are described herein which are also desirable sources of reductase proteins.

Especially considered in this aspect of the invention, are plant cells which contain the preferred alcohol substrates of a jojoba wax synthase described herein. A method of providing plant cells with such alcohol substrates

is considered wherein said cells are transformed with recombinant nucleic acid constructs which encode a fatty acyl reductase nucleic acid sequence. Thus, plant hosts which do not normally contain significant amounts of the alcohol substrates utilized by wax synthase, may be transformed with a reductase construct such that the alcohols are produced. In this manner, the fatty acyl groups present in the host cell will also provide the source of fatty alcohol substrate utilized by wax synthase in the synthesis of wax esters. Depending on the specificities of the wax synthase and reductase proteins, one recognizes that in this manner, plant cells may be obtained which produce a variety of desirable wax ester products. Such products will have different properties depending on the chain length and degree of saturation of the fatty alcohol and fatty acyl groups. Thus, the wax ester products produced according to the methods herein may be recovered from the host cells and are also considered in this invention.

Also considered in this invention are the modified plants, seeds and wax esters obtained by expression of the plant wax synthase sequences and proteins of this invention.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the subject invention, nucleic acid sequences are provided which encode for amino acids, such as a protein, polypeptide or peptide fragment, which are active in catalyzing the esterification of a fatty alcohol by a fatty acyl group to produce a wax ester. Such proteins are known as fatty acyl-CoA: fatty alcohol acyltransferase (E.C.2.3.1.75). The acyl-CoA: alcohol acyltransferase of this invention is also referred to hereafter as "wax synthase".

Although typically referred to as an acyl-CoA: alcohol acyltransferase, the wax synthases of this invention may demonstrate activity towards a variety of acyl substrates, including fatty acyl-CoA and fatty acyl-ACP molecules. In addition, both the acyl and alcohol substrates acted upon by

the wax synthase may have varying carbon chain lengths and degrees of saturation, although the wax synthase may demonstrate preferential activity towards certain molecules.

Many different organisms produce wax esters from alcohol and acyl substrates and are desirable sources of a wax synthase protein of this invention. For example, plants produce epidermal, or cuticular wax (Kolattukudy (1980) in *The Biochemistry of Plants* (Stumpf, P.K. and Conn, E.E., eds.) Vol.4, p. 571-645), and the desert shrub, jojoba, produces a seed storage wax (Ohlrogge *et al.* (*Lipids* (1978) 13:203-210). Wax synthesis has also been observed in various species of bacteria, such as *Acinetobacter* (Fixter *et al.* (1986) *J. Gen. Microbiol.* 132:3147-3157) and *Micrococcus* (Lloyd (1987) *Microbios* 52:29-37), and by the unicellular organism, *Euglena* (Khan and Kolattukudy (1975) *Arch. Biochem. Biophys.* 170:400-408). In addition, wax production and wax synthase activity have been reported in microsomal preparations from bovine meibomian glands (Kolattukudy *et al.* (1986) *J. Lipid Res.* 27:404-411), avian uropygial glands, and various insect and marine organisms. Consequently, many different wax esters which will have various properties may be produced by the wax synthases of this invention, and the activity of the enzyme and type of wax ester produced may depend upon the available substrate or the substrate specificity of the particular wax synthase of interest.

To obtain a reliable source of a wax synthase protein for use in esterification reactions, it is desirable to isolate nucleic acid sequences associated with the wax synthase such that these sequences may be cloned into host cells for the production of the wax synthase enzyme. For example, one may clone nucleic acid sequences encoding a wax synthase protein into vectors for expression in *E. coli* cells to provide a ready source of the wax synthase protein. The wax synthase protein so produced may also be used to raise antibodies against wax synthase proteins for use in identification and purification of related wax synthase

proteins from various sources, especially from plants. In addition, further study of the wax synthase protein may lead to site-specific mutagenesis reactions to further characterize and improve its catalytic properties or to alter its fatty alcohol or fatty acyl substrate specificity. A wax synthase with altered substrate specificity may find application in conjunction with other FAS enzymes.

Prior to the instant invention, nucleic acid and amino acid sequences of wax synthase proteins were not known.

Thus, in order to obtain the nucleic acid sequences associated with wax synthase, it was necessary to first purify the protein from an available source and determine at least partial amino acid sequence so that appropriate probes useful for isolation of wax synthase nucleic acid sequences could be prepared.

The desert shrub, *Simmondsia chinensis* (jojoba) was identified as a source of a candidate wax synthase protein. Initial studies reveal that the jojoba wax synthase is an integral membrane protein and hydrophobic in nature. In general, membrane associated proteins are difficult to purify as they tend to lose enzymatic activity when they are solubilized, i.e. separated from the membrane environment in which they normally function. Techniques that have been used to solubilize integral membrane proteins include addition of detergents or organic solvents to a preparation of a suitable membrane fraction. Further conventional purification techniques, such as precipitation, ion-exchange, gel-filtration and affinity chromatography may then be utilized, assuming the desired protein still retains functional activity that can be measured using a specific enzymatic assay.

Typically, as a first step towards obtaining a solubilized membrane protein, a microsomal membrane preparation which comprises wax synthase activity is desired. Standard microsomal membrane preparations utilize differential centrifugation of a cell-free homogenate (CFH) to yield a membrane fraction which is free of whole cells,

nuclei and soluble protein. (See, for example Mooré *et al.* (1987) *Biological Membranes: A Practical Approach*, pp. 37-72, eds. Finalay and Evans.) With oilseeds, initial centrifugation steps typically yield a pellet, supernatant and a floating fat pad, and microsomal membranes may then be recovered by further centrifugation of the supernatant.

A protocol is described in USPN 5,403,918, whereby a jojoba membrane fraction was obtained with good recovery of enzyme activity associated with fatty acyl reductase, another enzyme involved in the formation of wax esters in jojoba. The method also provides membrane fractions having wax synthase activity as described in detail in the examples which follow. In addition, microsomal membrane preparations from jojoba are also described in Lassner *et al.* (*supra*). Other procedures are known to those in the art and may be utilized to obtain similar membrane preparations. In addition, methods to assay for wax synthase activity in such preparations are described in Example 1.

A critical stage for further enzyme characterization and purification is that of obtaining solubilized wax synthase protein that is separated from its native lipid bilayer membrane environment, but retains substantial amounts of measurable wax synthase enzymatic activity. The removal of integral membrane proteins from the lipid bilayer is typically accomplished using amphiphilic detergents in aqueous solution, although organic solvents have also been used in a few cases. Many different detergents and methods of solubilization of membrane proteins are known to those skilled in the art, and are also reviewed by Neugebauer (*Methods Enzymol.* (1990) 182:239-253) and Hjelmiland (*Methods Enzymol.* (1990) 182:253-264).

Often, detergents which are used to solubilize membrane proteins are found to inhibit the enzymatic activity of a desired protein. Several detergents were tested for solubilization of jojoba wax synthase, including CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), which was demonstrated in USPN 5,403,918 to be useful in

purification of a fatty acyl reductase from jojoba. All were found to inhibit wax synthase enzymatic activity. Although strong inhibition by CHAPS was observed at concentrations above the CMC, it was found that addition of phospholipids, such as L-phosphatidyl choline, and adjustment of the CHAPS concentration from 1.0% to 0.2%, i.e. to below the CMC, results in reconstitution of a portion of the wax synthase activity. The primary requirement for reconstitution of wax synthase activity is the presence of phospholipids during the removal or dilution of the detergent, so that the wax synthase protein is incorporated into phospholipid vesicles. This differs from the protocol developed for reconstitution of jojoba reductase activity, which does not require addition of phospholipids. Thus, if phospholipids are present in a wax synthase preparation, such as that from a microsomal membrane fraction, activity may be detected simply by removal or dilution of detergent. However, in further purified wax synthase preparations, phospholipids must be added to detect activity. Optimum activity recovery is obtained when a ratio of CHAPS to PL is 2.8/1 (w/w) in the assay. A method to reconstitute and assay wax synthase activity in solubilized wax synthase preparations is described in Example 1.

Having obtained solubilized wax synthase protein, it can be seen that further experiments to characterize the enzyme as to substrate specificity, cofactor requirements and possible activity inhibiting agents may now be conducted. For example, it has been found that the jojoba wax synthase of this invention has a broad range of acyl substrates, including acyl-ACP and acyl-CoA molecules. In addition, the acyl and fatty alcohol substrates may have a broad size range with respect to carbon chain length. For example, activity was tested using substrates having carbon chain lengths of from C12 to C24, and all were shown to be utilized by the enzyme. In addition, activity was shown with fatty acyl and fatty alcohols having varying degrees of unsaturation.

Chromatography techniques may be utilized to provide enriched preparations of plant wax synthase. One such purification step involves chromatography over an immobilized reactive dye matrix, such as the Cibacron Blue F3GA (Blue A) used in this invention. The jojoba wax synthase activity binds to such a column when loaded in a buffer containing approximately 0.3M NaCl, while greater than approximately 85% of other protein passes through or is removed in subsequent washes. As described in USPN 5,403,918, reductase activity is also bound to the Blue A column under such conditions. It is demonstrated herein that approximately 70% of the wax synthase activity loaded to a Blue A column can be recovered by elution with a 2.0M NaCl buffer wash. The jojoba reductase and β -ketoacyl-CoA synthase (KCS) proteins are also present in this Blue A eluate.

Further purification of the Blue A eluate is obtained by loading the sample onto a crystalline hydroxyapatite (HA) column. Wax synthase activity does not bind to the column and is found in the flow through and wash. The majority of the reductase and KCS activities bind to the column, as does the majority of the protein in the sample. The HA fraction enriched in wax synthase activity can be used for size exclusion chromatography, and using a Superdex 75 size exclusion column, the jojoba wax synthase protein is estimated to have a molecular weight of 48kD.

Using such purification techniques, the jojoba wax synthase protein can be recovered as a substantially purified protein preparation and the amino acid sequence can be obtained. Similarly, due to the hydrophobic nature of the fatty alcohol substrates of wax synthase enzymes, other wax synthases would also be predicted to be associated with membranes in their native cells, and thus purification techniques described herein for jojoba wax synthase, may also be useful in recovery of purified preparation of other wax synthase proteins.

For example, *Euglena gracilis* produces waxes through the enzymatic actions of a fatty acyl-CoA reductase and a fatty acyl-CoA alcohol transacylase, or wax synthase. Typically, waxes having carbon chain lengths ranging from 24-32 are detected in this organism. As described above for jojoba, the *Euglena* wax synthase enzyme may be solubilized using a CHAPS/NaCl solution, and a partially purified wax synthase preparation is obtained by dye-ligand, HA and size exclusion chromatography.

Acinetobacter species are also known to produce wax ester compositions, although the mechanism is not well defined. As described herein a fatty acyl-CoA alcohol transacylase, or wax synthase activity is detected in *Acinetobacter* species. The wax synthase activity is solubilized in CHAPS/NaCl, enriched by Blue A column chromatography and may be further purified using such techniques as size exclusion chromatography.

In order to obtain nucleic acid sequences encoding the wax synthase of the present invention, the band containing the purified protein is cut out of an SDS gel to use in amino acid sequencing reactions. In gel digestion was used as opposed to more convenient methods, such as transfer of the protein to nitrocellulose or polyvinylidenedifluoride (PVDF) membranes due to the fact that conditions under which the jojoba wax synthase protein could be blotted and bound to such membranes have not been discovered. A commercial laboratory, W.M. Keck Foundation/Yale University, was provided with gel slices containing purified jojoba wax synthase protein for use in determining amino acid sequences of the jojoba protein by in-gel digest and subsequent protein sequencing. The peptide sequences generated in this manner may be used in PCR gene isolation techniques and cDNA library screening as described in more detail in the following examples.

Further experiments to confirm the identity of the wax synthase may also be desirable, such as expression of the protein in *E. coli*. The wax synthase may then act on fatty

acyl and fatty alcohol substrates in such cells to produce wax esters which may be detected by various analytical methods. If the host cells do not contain the alcohol substrate of the wax synthase, activity may be verified by assaying cell extracts. Alternatively, wax synthase protein may be prepared by *in vitro* translation using wax synthase nucleic acid sequences and commercially available translation kits. Addition of microsomal membrane preparations to the *in vitro* translation sample may be necessary to obtain active wax synthase protein if membrane insertion is critical to activity. Other testing may include immunological assays, whereby antibodies specific for the candidate protein are prepared and found to inhibit wax synthase activity in protein preparations.

Thus, as described in more detail in the examples below, nucleic acid sequences are isolated using amino acid sequences determined for the proteins associated with wax synthase activity, both to confirm the identity of a wax synthase protein and to provide for transcription of the sequences and/or expression of the protein in host cells, either prokaryotic or eukaryotic.

As the wax synthase is a membrane bound protein, it may be desirable to express a candidate protein in a plant cell in order to verify the activity. Electroporation or bombardment of plant tissue for transient expression may be useful for this purpose. Ultimately, stable plant expression in a plant which produces substrates recognized by this enzyme is desired. If a plant targeted for transformation with wax synthase sequences does not naturally contain the fatty alcohol and fatty acyl ester substrates of this enzyme, a plant extract may be prepared and assayed for wax synthase activity by adding substrates of the wax synthase to the extract. Constructs and methods for transformation of plant hosts with wax synthase sequences are discussed in more detail below.

The wax synthase nucleic acids of this invention may be genomic or cDNA and may be isolated from cDNA or genomic

libraries or directly from isolated plant DNA. As described in more detail in the examples below, a method for obtaining nucleic acid sequence for the jojoba wax synthase by PCR from primers specific for the disclosed jojoba wax synthase peptides is provided herein.

Wax synthase nucleic acid sequences of this invention include those corresponding to the jojoba wax synthase protein, as well as sequences obtainable from the jojoba protein or nucleic acid sequences. By "corresponding" is meant nucleic acid sequences, either DNA or RNA, including those which encode jojoba wax synthase protein or a portion thereof, regulatory sequences found 5' or 3' to said encoding sequences which direct the transcription or transcription and translation (expression) of the wax synthase in jojoba embryos, intron sequences not present in the cDNA, as well as sequences encoding any leader or signal peptide of a precursor wax synthase protein that may be required for insertion into the endoplasmic reticulum membrane, but is not found in the mature wax synthase enzyme.

By sequences "obtainable" from the jojoba sequence or protein, is intended any nucleic acid sequences associated with a desired wax synthase protein that may be synthesized from the jojoba wax synthase amino acid sequence, or alternatively identified in a different organism, and isolated using as probes jojoba wax synthase nucleic acid sequences or antibodies prepared against the jojoba wax synthase protein. In this manner, it can be seen that sequences of these other wax synthases may similarly be used to isolate nucleic acid sequences associated with wax synthase proteins from additional sources.

For isolation of nucleic acid sequences, cDNA or genomic libraries may be prepared using plasmid or viral vectors and techniques well known to those skilled in the art. Useful nucleic acid hybridization and immunological methods that may be used to screen for the desired sequences are also well known to those in the art and are provided,

for example in Maniatis, *et al.* (*Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

Typically, a sequence obtainable from the use of
5 nucleic acid probes will show 60-70% sequence identity
between the target sequence and the given sequence encoding
a wax synthase enzyme of interest. However, lengthy
sequences with as little as 50-60% sequence identity may
also be obtained. The nucleic acid probes may be a lengthy
10 fragment of the nucleic acid sequence, or may also be a
shorter, oligonucleotide probe. When longer nucleic acid
fragments are employed as probes (greater than about 100
bp), one may screen at lower stringencies in order to obtain
sequences from the target sample which have 20-50% deviation
15 (i.e., 50-80 sequence homology) from the sequences used as
probe. Oligonucleotide probes can be considerably shorter
than the entire nucleic acid sequence encoding a wax
synthase enzyme, but should be at least about 10, preferably
at least about 15, and more preferably at least about 20
20 nucleotides. A higher degree of sequence identity is
desired when shorter regions are used as opposed to longer
regions. It may thus be desirable to identify enzyme active
sites where amino acid sequence identity is high to design
oligonucleotide probes for detecting homologous genes.

25 To determine if a related gene may be isolated by
hybridization with a given sequence, the sequence is labeled
to allow detection, typically using radioactivity, although
other methods are available. The labeled probe is added to
a hybridization solution, and incubated with filters
30 containing the desired nucleic acids, either Northern or
Southern blots (to screen desired sources for homology), or
the filters containing cDNA or genomic clones to be
screened. Hybridization and washing conditions may be
varied to optimize the hybridization of the probe to the
35 sequences of interest. Lower temperatures and higher salt
concentrations allow for hybridization of more distantly
related sequences (low stringency). If background

hybridization is a problem under low stringency conditions, the temperature can be raised either in the hybridization or washing steps and/or salt content lowered to improve detection of the specific hybridizing sequence.

5 Hybridization and washing temperatures can be adjusted based on the estimated melting temperature of the probe as discussed in Beltz, *et al.* (*Methods in Enzymology* (1983) 100:266-285).

10 A useful probe and appropriate hybridization and washing conditions having been identified as described above, cDNA or genomic libraries are screened using the labeled sequences and optimized conditions. The libraries are first plated onto a solid agar medium, and the DNA
15 lifted to an appropriate membrane, usually nitrocellulose or nylon filters. These filters are then hybridized with the labeled probe and washed as discussed above to identify clones containing the related sequences.

For immunological screening, antibodies to the jojoba wax synthase can be prepared by injecting rabbits or mice
20 with the purified protein. Methods of preparing antibodies are well known to those in the art, and companies which specialize in antibody production are also available. Either monoclonal or polyclonal antibodies can be produced, although typically polyclonal antibodies are more useful for
25 gene isolation.

To screen desired plant species, Western analysis is conducted to determine that a related protein is present in a crude extract of the desired plant species, that cross-reacts with the antibodies to the jojoba wax synthase. This
30 is accomplished by immobilization of the plant extract proteins on a membrane, usually nitrocellulose, following electrophoresis, and incubation with the antibody. Many different systems for detection of the antibody/protein complex on the nitrocellulose filters are available,
35 including radiolabeling of the antibody and second antibody/enzyme conjugate systems. Some of the available systems have been described by Oberfelder (*Focus* (1989)

BRL/Life Technologies, Inc. 11:1-5). If initial experiments fail to detect a related protein, other detection systems and blocking agents may be utilized. When cross-reactivity is observed, genes encoding the related proteins can be
5 isolated by screening expression libraries representing the desired plant species. Expression libraries can be constructed in a variety of commercially available vectors, including lambda gt11, as described in Maniatis, *et al.* (*supra*).

10 The clones identified as described above using DNA hybridization or immunological screening techniques are then purified and the DNA isolated and analyzed using known techniques. In this manner, it is verified that the clones
15 encode a related wax synthase protein. Other wax synthases may be obtained through the use of the "new" wax synthase in the same manner as the jojoba wax synthase was used.

It will be recognized by one of ordinary skill in the art that wax synthase nucleic acid sequences of this invention may be modified using standard techniques of site
20 specific mutation or PCR, or modification of the sequence may be accomplished in producing a synthetic nucleic acid sequence. These modified sequences are also considered wax synthase nucleic acid sequence of this invention. For example, wobble positions in codons may be changed such that
25 the nucleic acid sequence encodes the same amino acid sequence, or alternatively, codons can be altered such that conservative amino acid substitutions result. In either case, the peptide or protein maintains the desired enzymatic activity and is thus considered part of the instant
30 invention.

A nucleic acid sequence of a wax synthase enzyme of this invention may be a DNA or RNA sequence, derived from genomic DNA, cDNA, mRNA, or may be synthesized in whole or
35 in part. The gene sequences may be cloned, for example, by isolating genomic DNA from an appropriate source, and amplifying and cloning the sequence of interest using a polymerase chain reaction (PCR). Alternatively, the gene

sequences may be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences. Thus, all or a portion of the desired structural gene (that portion of the gene which encodes the wax synthase protein) may be synthesized using codons preferred by a selected host. Host-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a desired host species.

10 The nucleic acid sequences associated with wax synthase proteins will find many uses. For example, recombinant constructs can be prepared which can be used as probes or will provide for expression of the wax synthase protein in host cells. Depending upon the intended use, the constructs
15 may contain the sequence which encodes the entire wax synthase, or a portion thereof. For example, critical regions of the wax synthase, such as an active site may be identified. Further constructs containing only a portion of the wax synthase sequence which encodes the amino acids
20 necessary for a desired wax synthase activity may thus be prepared.

Useful systems for expression of the wax synthase sequences of this invention include prokaryotic cells, such as *E. coli*, yeast cells, and plant cells, both vascular and
25 nonvascular plant cells being desired hosts. In this manner, the wax synthase protein may be produced to allow further studies, such as site-specific mutagenesis of encoding sequences to analyze the effects of specific mutations on reactive properties of the wax synthase
30 protein.

The DNA sequence encoding a wax synthase of this invention may be combined with foreign DNA sequences in a variety of ways. By "foreign" DNA sequences is meant any DNA sequence which is not naturally found joined to the wax
35 synthase sequence, including DNA sequences from the same organism which are not naturally found joined to wax synthase sequences. Both sense and antisense constructs

utilizing wax synthase encoding sequences are considered, wherein sense sequence may be used for expression of wax synthase in a host cell, and antisense sequences may be used to decrease the endogenous levels of a homologous wax synthase protein naturally produced by a target organism. In addition, the wax synthase gene sequences of this invention may be employed in a foreign host in conjunction with all or part of the sequences normally associated with the wax synthase, such as regulatory or membrane targeting sequences.

In its component parts, a DNA sequence encoding wax synthase is combined in a recombinant construct having, in the 5' to 3' direction of transcription, a transcription initiation control region capable of promoting transcription and translation in a host cell, the nucleic acid sequence encoding wax synthase and a transcription termination region. Depending upon the host, the regulatory regions will vary, and may include regions from viral, plasmid or chromosomal genes, or the like. For expression in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or regulatable promoters may be employed. Expression in a microorganism can provide a ready source of the plant enzyme. Among transcriptional initiation regions which have been described are regions from bacterial and yeast hosts, such as *E. coli*, *B. subtilis*, *Saccharomyces cerevisiae*, including genes such as beta-galactosidase, T7 polymerase, tryptophan E and the like.

For the most part, the recombinant constructs will involve regulatory regions functional in plants which provide for expression of the wax synthase gene to produce functional wax synthase protein. The open reading frame, coding for the plant wax synthase or a functional fragment thereof will be joined at its 5' end to a transcription initiation regulatory region such as the wild-type sequence naturally found 5' upstream to the wax synthase structural gene. Numerous other promoter regions from native plant

genes are available which provide for a wide variety of constitutive or regulatable expression of structural gene sequences.

In addition to sequences from native plant genes, other
5 sequences can provide for constitutive gene expression in plants, such as regulatory regions associated with *Agrobacterium* genes, including regions associated with nopaline synthase (*Nos*), mannopine synthase (*Mas*), or octopine synthase (*Ocs*) genes. Also useful are regions
10 which control expression of viral genes, such as the 35S and 19S regions of cauliflower mosaic virus (CaMV). The term constitutive as used herein does not necessarily indicate that a gene is expressed at the same level in all cell types, but that the gene is expressed in a wide range of
15 cell types, although some variation in abundance is often detectable. Other useful transcriptional initiation regions preferentially provide for transcription in certain tissues or under certain growth conditions, such as those from napin, seed or leaf ACP, the small subunit of RUBISCO, and
20 the like.

In embodiments wherein the expression of the wax synthase protein is desired in a plant host, the use of all or part of the complete plant wax synthase gene may be desired, namely the 5' upstream non-coding regions
25 (promoter) together with the structural gene sequence and 3' downstream non-coding regions may be employed. If a different promoter is desired, such as a promoter native to the plant host of interest or a modified promoter, i.e., having transcription initiation regions derived from one
30 gene source and translation initiation regions derived from a different gene source or enhanced promoters, such as double 35S CaMV promoters, the sequences may be joined together using standard techniques. Additionally, 5' untranslated regions from highly expressed plant genes may
35 be useful to provide for increased expression of the wax synthase proteins described herein.

The DNA constructs which provide for wax synthase expression in plants may be employed with a wide variety of plant life, particularly, plants which produce the fatty acyl-CoA substrates of the wax synthase enzyme, such as *Brassica*. Other plants of interest produce desirable fatty acyl substrates, such as medium or long chain fatty acyl molecules, and include but are not limited to rapeseed (Canola varieties), sunflower, safflower, cotton, *Cuphea*, soybean, peanut, coconut and oil palms, and corn. Of particular interest is the use of such constructs in high erucic acid varieties of rapeseed *Brassica* (HEAR) for production of long-chain liquid waxes. Further uses envisioned for HEAR plants includes the production of varieties containing substantially increased levels of erucic acid as the result of providing an additional wax "sink" for the erucic acid, which is normally stored in the seed TAG.

As to the fatty alcohol substrate of the wax synthase enzyme, other than jojoba, seed plants are not known to produce large quantities of fatty alcohols, although small amounts of this substrate may be available to the wax synthase enzyme. Therefore, in conjunction with the wax synthase constructs of this invention, it is desirable to provide the target host cell with the capability to produce fatty alcohols from the fatty acyl molecules present in the host cells. For example, a plant fatty acyl reductase and methods to provide for expression of the reductase enzymes in plant cells are described in USPN 5,370,996. The nucleic acid sequence and translated amino acid sequence of the jojoba reductase is provided in Figure 1 of that patent. Thus, by providing both the wax synthase and reductase proteins to the host plant cell, wax esters may be produced from the fatty alcohol and fatty acyl substrates. Furthermore, expression of β -ketoacyl-CoA synthase in conjunction with expression of wax synthase and reductase proteins is considered in the present invention. In this manner, the production of very long chain fatty acid

substrates of these enzymes may be increased in the target plant species.

In addition to the jojoba reductase, reductase enzymes from other organisms may be useful in conjunction with the wax synthases of this invention. Other potential sources of reductase enzymes include *Euglena*, *Acinetobacter*, *Micrococcus*, certain insects and marine organisms, and specialized mammalian or avian tissues which are known to contain wax esters, such as bovine meibomian glands or ovian uropygial glands. Other potential sources of reductase proteins may be identified by their ability to produce fatty alcohols or, if wax synthase is also present, wax esters.

The wax synthase and reductase sequences may be provided during the same transformation event, or alternatively, two different transgenic plant lines, one having wax synthase constructs and the other having reductase constructs may be produced by transformation with the various constructs. These plant lines may then be crossed using known plant breeding techniques to provide wax synthase and reductase containing plants for production of wax ester products.

Furthermore, other nucleic acid sequences encoding for enzymes involved in the formation of very long chain fatty acids may also find use in the DNA constructs of the present invention for the production of wax esters in a plant host. Such nucleic acid sequences are known in the art and are as described in U.S. Patent Number 5,679,881. For example, as described in the examples below, the wax synthase of the present invention is used in plant expression constructs in conjunction with nucleic acid sequences encoding for a fatty acid elongase (described in USPN 5,679,881, the entirety of which is incorporated herein by reference) and an acyl-CoA reductase (described in USPN 5,403,918, the entirety of which is incorporated herein by reference). Such plant expression constructs provide for the production of wax esters in transgenic *Arabidopsis thaliana* plants.

For applications leading to wax ester production, 5' upstream non-coding regions obtained from genes regulated during seed maturation are desired, especially those preferentially expressed in plant embryo tissue, such as regions derived from ACP, oleosin (Lee and Huang (1991) *Plant Physiol.* 96:1395-1397) and napin regulatory regions. Transcription initiation regions which provide for preferential expression in seed tissue, i.e., which are undetectable in other plant parts, are considered desirable for wax ester production in order to minimize any disruptive or adverse effects of the gene product in other plant parts. Further, the seeds of such plants may be harvested and the lipid reserves of these seeds recovered to provide a ready source of wax esters. Thus, a novel seed product may be produced in oilseed plants which, absent transformation with wax synthase constructs as described herein, are not known to produce wax esters as a component of their seed lipid reserves.

Such "seed-specific promoters" may be obtained and used in accordance with the teachings of USPN 5,420,034, and USPN 5,430,194. In addition, where plant genes, such as the jojoba reductase and wax synthases are expressed, it may be desirable to use the entire plant gene, including 5' and 3' regulatory regions and any introns that are present in the encoding sequence, for expression of the jojoba genes in a transformed plant species, such as *Arabidopsis* or *Brassica*.

Regulatory transcription termination regions may be provided in recombinant constructs of this invention as well. Transcription termination regions may be provided by the DNA sequence encoding the plant wax synthase or a convenient transcription termination region derived from a different gene source, especially the transcription termination region which is naturally associated with the transcription initiation region. The transcript termination region will contain at least about 0.5kb, preferably about 1-3kb of sequence 3' to the structural gene from which the termination region is derived.

Additional plant gene regions may be used to optimize expression of wax synthase and reductase genes in plant tissues. For example, 5' untranslated regions of highly expressed genes, such as that of the small subunit (SSU) of RuBP-carboxylase, inserted 5' to DNA encoding sequences may provide for enhanced translation efficiency. Portions of the SSU leader protein encoding region (such as that encoding the first 6 amino acids) may also be used in such constructs. In addition, for applications where targetting to plant plastid organelles is desirable, transit peptide encoding sequences from SSU or other nuclear-encoded chloroplast proteins may be used in conjunction with wax synthase and reductase sequences.

Depending on the method for introducing the DNA expression constructs into the host cell, other DNA sequences may be required. Importantly, this invention is applicable to dicotyledons and monocotyledons species alike and will be readily applicable to new and/or improved transformation and regeneration techniques.

In developing the recombinant construct, the various components of the construct or fragments thereof will normally be inserted into a convenient cloning vector which is capable of replication in a bacterial host, e.g., *E. coli*. Numerous vectors exist that have been described in the literature. After each cloning, the plasmid may be isolated and subjected to further manipulation, such as restriction, insertion of new fragments, ligation, deletion, insertion, resection, etc., so as to tailor the components of the desired sequence. Once the construct has been completed, it may then be transferred to an appropriate vector for further manipulation in accordance with the manner of transformation of the host cell.

Normally, included with the recombinant construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin,

etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Similarly, genes encoding enzymes providing for production of a compound identifiable by color change, such as GUS, or luminescence, such as luciferase are useful. Depending upon the number of different host species the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

In addition to the sequences providing for transcription of wax synthase sequences, the DNA constructs of this invention may also provide for expression of an additional gene or genes, whose protein product may act in conjunction with the wax synthase to produce a valuable end product. For example, as discussed above, DNA constructs which provide for expression of wax synthase and a fatty acyl reductase so that wax esters may produced in transformed hosts, are considered in this invention. Furthermore, production of different wax esters having varying carbon chain lengths and degrees of saturation is desired and may be provided by transforming host plants having fatty alcohol or fatty acyl substrates of varying chain lengths. Such plants may be provided, for example, by methods described in the published international patent application number PCT WO 91/16421, which describes various thioesterase genes and methods of using such genes to produce fatty acyl substrates having varying chain lengths in transformed plant hosts.

Furthermore, to optimize the production of wax esters in oilseed plant hosts, one may wish to decrease the production of the triacylglyceride oils that are normally produced in the seeds of such plants. One method to accomplish this is to antisense a gene critical to this process, but not necessary for the production of wax esters. Such gene targets include diacylglycerol acyltransferase, and other enzymes which catalyse the synthesis of triacylglycerol. Additionally, it may be desirable to

provide the oilseed plants with enzymes which may be used to degrade wax esters as a nutrient source, such as may be isolated from jojoba or various other wax producing organisms. In this manner, maximal production of wax esters in seed plant hosts may be achieved.

The wax esters produced in the methods described herein may be harvested using techniques for wax extraction from jojoba or by various production methods used to obtain oil products from various oilseed crops. The waxes thus obtained will find application in many industries, including pharmaceuticals, cosmetics, detergents, plastics, and lubricants. Applications will vary depending on the chain length and degree of saturation of the wax ester components. For example, long chain waxes having a double bond in each of the carbon chains are liquid at room temperature, whereas waxes having saturated carbon chain components, may be solid at room temperature, especially if the saturated carbon chains are longer carbon chains.

The method of transformation is not critical to the instant invention; various methods of plant transformation are currently available. As newer methods are available to transform crops, they may be directly applied hereunder. For example, many plant species naturally susceptible to *Agrobacterium* infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium* mediated transformation. Other sequences useful in providing for transfer of nucleic acid sequences to host plant cells may be derived from plant pathogenic viruses or plant transposable elements. In addition, techniques of microinjection, DNA particle bombardment, electroporation have been developed which allow for the transformation of various monocot and dicot plant species.

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included for purposes of illustration only and are not intended to limit the invention unless so stated.

EXAMPLES**Example 1 - Wax synthase Assays**

5 Methods to assay for wax synthase activity in
microsomal membrane preparations or solubilized protein
preparations are described.

A. Radiolabeled Material

10 The substrate generally used in the wax synthase
assays, [1-¹⁴C]palmitoyl-CoA, is purchased from Amersham
(Arlington Heights, IL). Other chain length substrates were
synthesized in order to perform chain length specification
studies. Long chain [1-¹⁴C] fatty acids (specific activity
51-56 Ci/mole), namely 11-cis-eicosenoic acid, 13-cis-
15 docosenoic acid and 15-cis-tetracosenoic acid are prepared
by the reaction of potassium [¹⁴C]cyanide with the
corresponding alcohol mesylate, followed by the base
hydrolysis of the alcohol nitrile to the free fatty acid.
The free fatty acids are converted to their methyl esters
20 with ethereal diazomethane, and purified by preparative
silver nitrate thin layer chromatography (TLC). The fatty
acid methyl esters are hydrolyzed back to the free fatty
acids. Radiochemical purity is assessed by three TLC
methods: normal phase silica TLC, silver nitrate TLC, and
25 C18 reversed phase TLC. Radiochemical purity as measured by
these methods was 92-98%. Long chain [1-¹⁴C] acyl-CoAs are
prepared from the corresponding [1-¹⁴C] free fatty acids by
the method of Young and Lynen (*J. Bio. Chem.* (1969)
244:377), to a specific activity of 10Ci/mole. [1-
30 ¹⁴C]hexadecanal is prepared by the dichromate oxidation of
[1-¹⁴C]hexadecan-1-ol, according to a micro-scale
modification of the method of Pletcher and Tate (*Tet. Lett.*
(1978) 1601-1602). The product is purified by preparative
silica TLC, and stored as a hexane solution at -70°C until
35 use.

**B. Assay for Wax synthase Activity in a Microsomal
Membrane**

Preparation

Wax synthase activity in a microsomal membrane preparation is measured by incubation of 40 μ M [1-¹⁴C]acyl-CoA (usually palmitoyl-CoA, sp. act. 5.1-5.6 mCi/mmol) and 200mM oleyl alcohol with the sample to be assayed in a total volume of 0.25ml. The incubation mixture also contains either 25 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethane-sulfonic acid), pH 7.5, as the buffering agent with 20% w/v glycerol, 1mM DTT, 0.5M NaCl or 25 mM Tricine-NaOH, pH 7.8, as the buffering agent with 0.28M NaCl, 10% glycerol, and 2mM β -mercaptoethanol. Initial studies were performed with the first buffer system, when the pH was chosen to accomodate the preference of the acyl-CoA reductase enzyme. Membrane preparations were later changed to the second buffer system to accomodate the higher pH optimum of wax synthase.

A substrate mixture is prepared in a glass vial, with oleyl alcohol being added immediately before use, and is added to samples. Incubation is carried out at 30°C for up to one hour. The assay is terminated by placing the assay tube on ice and immediately adding 0.25ml isopropanol:acetic acid (4:1 v/v). Unlabeled wax esters (0.1mg) and oleyl alcohol (0.1mg) are added as carriers. The [¹⁴C] lipids are extracted by the scaled-down protocol of Hara and Radin (Anal. Biochem. (1978) 90:420). Two ml of hexane/isopropanol (3:2, v/v) is added to the terminated assay. The sample is vortexed, 1ml of aqueous sodium sulphate solution (6.6% w/v) is added, and the sample is again vortexed.

30 C. Assay for Solubilized Wax synthase Activity

Solubilized wax synthase is assayed using up to 50 μ l sample in a 250 μ l assay that contains 40 μ M 1-¹⁴C-16:0 CoA (5 Ci/mol), 200 μ M 18:1-OH, 0.07% soybean phospholipid (Sigma, P-3644), 0.2 %CHAPS, 280 mM NaCl, 25 mM Tricine-NaOH, pH 7.8, 2mM β -ME and 5.6% glycerol. Phospholipid (50mg/ml in 0.5% CHAPS) is added directly to the sample, which is in 1% CHAPS, then diluted by a cocktail containing the remaining

assay components. Reconstitution of activity is presumed to be based on the incorporation of wax synthase into the phospholipid vesicles. Wax synthase is sensitive to detergent and requires the amount of phospholipid (PL) and detergent (CHAPS) to be balanced at 2.8/1 (CHAPS/PL, w/w) in the assay for maximal activity. Assays for wax synthase activity in samples concentrated by ultra-filtration require a readjustment of the sample volume assayed because of the concentration of CHAPS. Introducing too much CHAPS into the assay results in inhibition of activity. If samples are concentrated by ultrafiltration, the optimum volume of sample to be assayed may be reestablished by performing a concentration curve of %CHAPS in the assay using a small amount of sample and assaying at a fixed concentration of phospholipid and NaCl. Wax synthase is less sensitive to changes in PL concentration than it is to changes in CHAPS concentration.

D. Analysis of Assay Products

For analyzing the products of either the microsomal membrane preparation wax synthase assay or the solubilized wax synthase assay, two protocols have been developed. One protocol, described below as "extensive assay" is more time-consuming, but yields more highly quantitative results. The other protocol, described below as "quick assay" also provides a measure of wax synthase activity, but is faster, more convenient and less quantitative.

1. *Extensive Analysis:* Following addition of the sodium sulphate and vortexing the sample, the upper organic phase is removed and the lower aqueous phase is washed with 4ml hexane/isopropanol (7:2 v/v). The organic phases are pooled and evaporated to dryness under nitrogen. The lipid residue is resuspended in a small volume of hexane, and an aliquot is assayed for radioactivity by liquid scintillation counting. The remainder of the sample can be used for TLC analysis of the labeled classes and thereby give a measure of total wax produced.

For lipid class analysis the sample is applied to a silica TLC plate, and the plate is developed in hexane/diethyl ether/acetic acid (80:20:1 or 70:30:2 v/v/v). The distribution of radioactivity between the lipid classes, largely wax esters, free fatty acids, fatty alcohols, and polar lipids at the origin, is measured using an AMBIS radioanalytic imaging system (AMBIS Systems Inc., San Diego, CA). If necessary the individual lipid classes can be recovered from the TLC plate for further analysis. Reversed-phase TLC systems using C18 plates developed in methanol have also been used for the analysis.

2. *Quick Analysis:* Following addition of the sodium sulfate and vortexing the sample, a known percentage of the organic phase is removed and counted via liquid scintillation counting. This calculation is used to estimate the total counts in the organic phase. Another portion of the organic phase is then removed, dried down under nitrogen, redissolved in hexane and spotted on TLC

plates and developed and scanned as described for the detailed assay. In this manner the percentage of the total counts which are incorporated into wax is determined.

5 **Example 2 - Further Studies to Characterize Wax**

Synthase Activity

A. Seed Development and Wax Synthase Activity Profiles

Embryo development was tracked over two summers on five plants in Davis, CA. Embryo fresh and dry weights were
10 found to increase at a fairly steady rate from about day 80 to about day 130. Lipid extractions reveal that when the embryo fresh weight reaches about 300mg (about day 80), the ratio of lipid weight to dry weight reaches the maximum level of 50%.

15 Wax synthase activity was measured in developing embryos as described in Example 1B. As the jojoba seed coats were determined to be the source of an inhibiting factor(s), the seed coats were removed prior to freezing the embryos in liquid nitrogen for storage at -70°C.

20 Development profiles for wax synthase activities as measured in either a cell free homogenate or a membrane fraction, indicate a large induction in activity which peaks at approximately 110-115 days after anthesis. Embryos for enzymology studies were thus harvested between about 90 to
25 110 days postanthesis, a period when the wax synthase activity is high, lipid deposition has not reached maximum levels, and the seed coat is easily removed. The highest rate of increase of wax synthase activity is seen between days 80 and 90 postanthesis. Embryos for cDNA library
30 construction were thus harvested between about 80 to 90 days postanthesis when presumably the rate of synthase of wax synthase protein would be maximal. Correspondingly, the level of mRNA encoding wax synthase would be presumed to be maximal at this stage.

35 B. Microsomal Membrane Preparation

Jojoba embryos are harvested at approximately 90-110 days after flowering, as estimated by measuring water

content of the embryos (45-70%). The outer shells and seed coats are removed and the cotyledons quickly frozen in liquid nitrogen and stored at -70°C for future use. For initial protein preparation, frozen embryos are powdered by 5 pounding in a steel mortar and pestle at liquid nitrogen temperature. In a typical experiment, 70g of embryos are processed.

The powder is added, at a ratio of 280ml of solution per 70g of embryos, to the following high salt solution: 3M 10 NaCl, 0.3M sucrose, 100mM HEPES, 2mM DTT, and the protease inhibitors, 1mM EDTA, 0.7mg/ml leupeptin, 0.5mg/ml pepstatin and 17mg/ml PMSF. A cell free homogenate (CFH) is formed by dispersing the powdered embryos in the buffer with a tissue 15 homogenizer (Kinematica, Switzerland; model PT10/35) for approximately 30 sec. and then filtering through three layers of Miracloth (CalBioChem, LaJolla, CA). The filtrate is centrifuged at 100,000 x g for one hour.

The resulting sample consists of a pellet, supernatant and a floating fat pad. The fat pad is removed and the 20 supernatant fraction is collected and dialyzed overnight (with three changes of the buffering solution) versus a solution containing 1M NaCl, 100mM HEPES, 2mM DTT and 0.5M EDTA. The dialyzate is centrifuged at 200,000 x g for 1 1/2 hour to yield a pellet, DP2. The pellet is suspended in 25mM 25 HEPES and 10% glycerol, at 1/20 of the original CFH volume, to yield the microsomal membrane preparation.

Activity is assayed as described in Example 1. Recovery of wax synthase activity is estimated at 34% of the original activity in the cell free homogenate. Wax synthase 30 activity in this preparation is stable when stored at -70°C.

C. Substrate Specificity

Acyl-CoA and alcohol substrates having varying carbon chain lengths and degrees of unsaturation were added to microsomal membrane fractions prepared as described above to 35 determine the range of substrates recognized by the jojoba wax synthase. Wax synthase activity was measured as described in Example 1B, with acyl specificity measured

using 80mM of acyl-CoA substrate and 100mM of radiolabeled oleyl alcohol. Alcohol specificity was measured using 100mM of alcohol substrate and 40mM of radiolabeled eicosenoyl-CoA. Results of these experiments are presented in Table 1 below.

Table 1
Acyl and Alcohol Substrate Specificity of
Jojoba Wax Synthase

	Substrate		Wax synthase Activity (pmoles/min)	
	<u>Structure</u>	<u>Acyl Group</u>	<u>Alcohol Group</u>	
	12:0	12	100	
15	14:0	95	145	
	16:0	81	107	
	18:0	51	56	
	20:0	49	21	
	22:0	46	17	
20	18:1	22	110	
	18:2	7	123	
	20:1	122	72	
	22:1	39	41	
	24:1	35	24	

25 The above results demonstrate that the jojoba wax synthase utilizes a broad range of fatty acyl-CoA and fatty alcohol substrates.

30 In addition, wax synthase activity towards various acyl-thioester substrates was similarly tested using palmitoyl-CoA, palmitoyl-ACP and N-acetyl-S-palmitoyl cysteamine as acyl substrates. The greatest activity was observed with the acyl-CoA substrate. Significant activity (~10% of that with acyl-CoA) was observed with acyl-ACP, but
35 no activity was detectable with the N-acetyl-S-palmitoyl cysteamine substrate.

D. Effectors of Activity

Various sulphhydryl agents were screened for their effect on wax synthase activity. Organomercurial compounds were shown to strongly inhibit activity. Iodoacetamide and N-ethylmaleamide were much less effective. Inhibition by para-hydroxymercuribenzoate was observed, but this inhibition could be reversed by subsequent addition of DTT. These results demonstrate that inhibition by para-hydroxymercuribenzoate involves blocking of an essential sulphhydryl group.

Example 3 - Purification of Jojoba Wax Synthase

Methods are described which may be used for isolation of a jojoba membrane preparation having wax synthase activity, solubilization of wax synthase activity, and further purification of the wax synthase protein.

A. Microsomal Membrane Preparation

The following modification of the method described in Example 2 is employed and provides an improved membrane fraction useful for purification of wax synthase from solubilized membranes.

Typically, 100 g of jojoba embryos are 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. All subsequent steps are performed at 4°C. The blended material is filtered through Miracloth (CalBioChem). Centrifugation (20,000 x g; 20 min.) of the filtrate yielded a floating wax layer, a turbid supernatant fraction and a dark green pellet. The supernatant fraction is collected and centrifuged (100,000 x g; 2 h) to obtain membrane pellets which are then 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 is distributed into four SW28 centrifuge tubes (Beckman) and each is overlaid with 10 ml Buffer A containing 20% sucrose and then with 13 ml Buffer A. After centrifugation (28,000 rpm; 2 h), a membrane fraction is

collected from the 20%/50% sucrose interface, diluted with four volumes Buffer A and collected by centrifugation (200,000 x g; 1 h). The membranes are then homogenized in 10 ml 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 via this protocol is typically between 7 and 9 mg/ml. Protein concentrations are estimated as described (Bradford, 1976) using BSA as the protein standard.

10 B. Solubilization of Wax synthase Protein

The membrane suspension is adjusted to approximately 0.83mg of protein per ml by dilution with storage buffer (25mM Tricine-NaOH, pH 7.8, 1M NaCl, 10% glycerol, 5 mM β -mercaptoethanol). Solid 3-([3-cholamidopropyl]dimethyl-ammonio)-1-propanesulfate (CHAPS) is 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 is centrifuged (200,000g for 1 hr), and the supernatant fraction collected.

20 C. Purification of Wax Synthase Activity

The 200,000g supernatant fraction is diluted (with 0.57% CHAPS, 25 mM Tricine-NaOH, pH 7.8, 20% glycerol) to yield final concentrations of NaCl and CHAPS of 0.3M and 1%, respectively. The sample is loaded onto a Blue A-agarose (Amicon, Inc., Beverly, MA) column that has been equilibrated with buffer B (25 mM Tricine-NaOH, pH 7.8, 1% CHAPS, 20% glycerol,) containing 0.3M NaCl. After washing with equilibration buffer, wax synthase activity is eluted with buffer B containing 2M NaCl. Active fractions eluted from the Blue A column are pooled (Blue Pool) and used for further chromatography.

Two purification protocols were used for band identification and further purification of the wax synthase protein. In Protocol 1 (Figure 1), the Blue Pool was concentrated 5.4 fold by ultrafiltration in a pressure cell fitted with a YM 30 membrane (Amicon, Inc., Beverly, MA). One-half of the concentrate was applied to a Ceramic

Hydroxyapatite (CHT) column (Bio-Scale CHT-2; Bio-Rad, Hercules, CA) equilibrated in buffer B containing 2M NaCl. The column was washed with 6 column volumes of equilibration buffer and bound proteins were eluted with buffer B containing 0.1M dipotassium phosphate and 2M NaCl. After reequilibration of the CHT column, the second half of the Blue Pool concentrate was chromatographed in the same manner. In order to detect activity, wax synthase was assayed according to the protocol for samples concentrated by ultrafiltration. Wax synthase activity, measured on CHT-Run 1, was found in the flow through and wash. Protein profiles of the two CHT runs were identical so the CHT-run 2 was not assayed. Active fractions from the two CHT runs were pooled and concentrated 10 fold and applied to a Sephacryl S100 HR column (2.5 x 90cm) equilibrated in buffer B with 1.0 M NaCl. Protein and activity determinations were made and active fractions were selected from the retained portion of the run which maximized activity and minimized protein. The S100 pool (fractions 64-70) was applied to a crystalline hydroxylapatite (HA) column (Bio-Gel HT; Bio-Rad, Hercules, CA, 1 x 19.3cm) equilibrated in buffer B with 1 M NaCl. Again, the majority of the wax synthase activity was present in the flow through and wash. Bound proteins were eluted in buffer B with 0.1M dipotassium phosphate, and 1M NaCl. Fractions from the final HA run were examined by SDS-PAGE. A single protein migrating at 33 kD on SDS-PAGE was correlated with the presence of wax synthase activity.

In a second preparation (Protocol 2, Figure 2) the Blue Pool was applied directly to a crystalline HA column (1 x 11.7 cm), equilibrated in buffer B with 1M NaCl, without concentration. Two fractions were selected for further purification by size exclusion chromatography on a Superdex 75 HR 10/30 column (Bio-Rad, Hercules, CA; sizing range:5000 - 75,000 daltons) equilibrated with 25 mM Tricine-NaOH, pH 7.8, 1% CHAPS, 20% glycerol, 1M NaCl. Wax synthase activity was measured according to the protocol described for solubilized samples in Example 1C. One fraction eluted

early in the flow through of the HA column (fraction 31) and the other eluted in the wash (fraction 67). The protein profiles of the two fractions were different based on SDS-PAGE analysis. Both Superdex 75 runs were examined by gradient SDS-PAGE and a protein of approximately 33 kD was identified that chromatographed with activity. A calibration curve was generated using molecular mass standards chromatographed under the same buffer and column conditions. Comparison of the elution volume of the peak of Wax Synthase activity to this standard curve yielded a value of 48 kDa for the molecular mass of the solubilized enzyme.

A chart representing the purification of wax synthase from Protocol 1 (Table 2) shows a 150 fold purification of the enzyme from the solubilized protein fraction.

Table 2

Purification of Jojoba Wax Synthase

Purification Step	Enzyme Activity (nmol/min)	Yield %	Protein (mg)	Specific Activity (nmol/min/mg)	Purification (fold)
Solubilized Fraction	274.4	100	415	0.7	1
Blue A Agarose	214.7	78.2	15	14.3	22
Ceramic Hydroxyapatite	176.6	64.3	6.4	27.6	42
Sephacryl S-100 (sizing)	41.3	15.1	1.2	33.1	50
Hydroxyapatite					

(crystalline) 18.8 6.9 0.2 99.2 150

D. SDS PAGE Analysis

Samples from the column fractions were diluted in SDS PAGE sample buffer (1x buffer = 2% SDS, 250 mM β -
5 mercaptoethanol, 0.0025% bromphenol blue) and analyzed by electrophoresis. Polyacrylamide gradient gel electrophoresis (10-13%) was carried out according to the method of Laemmli (*Nature* (1970) 227:680-685) with some of the modifications of Delepelaire (*Proc. Nat. Acad. Sci.*
10 (1979) 76:111-115). Sodium dodecyl sulfate was used in the upper reservoir buffer at 0.1% but was omitted from the lower reservoir buffer, stacking and resolving gels. The stacking gel contained 5% of a 30% acrylamide stock (29.2% acrylamide, 0.8% N,N'-bis-methyleneacrylamide, w/v), 0.06%
15 ammonium persulfate (w/v) and 0.1% TEMED (v/v). The resolving gel contained a 10-13% linear gradient of acrylamide stock stabilized by a 0-10% linear gradient of sucrose. Electrophoresis was carried out at room temperature at 150V, constant voltage, for 9-10 hours.
20 Proteins were visualized by staining with silver according to the method of Blum *et al.* (*Electrophoresis* (1987) 8:93-99 or with Coomassie Blue (0.1% Coomassie Blue R-250, 50% methanol, 10% acetic acid). The 33 kDa protein identified as wax synthase does not appear as a major component of the
25 active fraction until purification through the hydroxyapatite column. Following purification Protocol 1 (Example 3C) the only protein that correlates with activity on the final column is one at 33 kDa.

30 **Example 4 - Preparation of Protein for In-Gel Digestion**

A. Preparation of Samples for SDS-PAGE by Concentration

Odd numbered fractions from the flow through/wash of the final HA column (Protocol 1) were pooled and concentrated three fold by ultrafiltration in a pressure
35 cell fitted with a YM 30 membrane (Amicon, Inc., Beverly, MA). The sample was further concentrated using two

Centricon-30 units (Amicon, Inc., Beverly, MA) to volumes of approximately 50 μ l. Each sample was treated with 6 μ l SDS Cocktail (4 μ l 20%SDS, 1 μ l 14.3M β -metcaptoethanol, and 1 μ l 0.1% Bromophenol Blue). After sitting at room temperature for 15 minutes, the samples were applied to a 10-13% acrylamide gradient gel (Example 3D) (16 x 16 cm x 1mm thick) and proteins were resolved by electrophoresis at 150V, constant voltage, for 9.5 hours. The gel was stained with 0.1% Coomassie Blue in 50% methanol, 10% acetic acid for 15 minutes then destained in 50% methanol, 10% acetic acid for 2 x 20 minutes. The 33 kD Wax Synthase band was excised from the gel and destained in 50% ethanol for 3 x 20 minutes. One lane contained a streak of protein and was not used in the final digestion.

B. Preparation of Samples for SDS-PAGE by Precipitation

Aliquots (0.8 ml) of the even numbered fractions from the final HA column (Protocol 1) were pooled in groups of three over the column profile. The pools were divided
5 equally into three, 1.5 ml vials. Protein was precipitated by the addition of 0.2ml 40% TCA. After 30 minutes on ice the samples were centrifuged (12,000 x g, 15 minutes at 4 C) to pellet the precipitated protein. The supernatants were removed and the pellets washed twice with 0.6 ml ice cold
10 acetone. The final three pellets for each pooled set of samples were resuspended with the same 50 μ l of SDS sample buffer by transferring the buffer from one vial to the next. The emptied vials, that had already been resuspended, were washed with 10 μ l of sample buffer for a total resuspended
15 volume of 60 μ l for each pooled sample. The samples were applied to a 12% acrylamide Tris/Glycine mini-gel (Novex, San Diego, CA, 1.5mm x 10 well) and proteins were resolved by electrophoresis at 150 V, constant voltage, for 20 minutes beyond the elution of dye from the foot of the gel. The gel
20 was stained with Coomassie Blue and destained using Gel-Clear (Novex, San Diego, CA). Wax Synthase was excised from three non-equivalent lanes on the gel representing the peak and tailing fractions from the column. The gel slices were placed in 1.5 ml vials and destained with 1 ml of 50%
25 methanol, 10% acetic acid for 2 hours. The destain solution was removed and the gel slices were frozen in liquid nitrogen and sent on dry ice, overnight, to the W M Keck Foundation Biotechnology Resource Laboratory at Yale University for in-gel-digestion. One gel slice from the
30 sample concentrated by ultrafiltration and three gel slices from the samples concentrated by precipitation were pooled for in-gel tryptic digestion.

Example 5 - Determination of Amino Acid Sequence

35 Protein sequencing was performed at the W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University. Procedures include amino acid analysis of a

portion (10-15%) of the gel slice for quantitation and amino acid composition, digestion of the protein with one of the proteolytic enzymes (trypsin or lysyl endopeptidase), and fractionation of the products by reverse phase HPLC.

5 Absorbance peaks are selected from the HPLC run and subjected to laser desorption mass spectrometry to determine the presence, amount, and mass of the peptide prior to protein sequencing. The longest peptides are selected for microsequencing.

10 Amino acid sequences of jojoba wax synthase peptides obtained by trypsin digestion are presented in Table 3 below using the one letter code.

Table 3

15 Amino Acid Sequence of Jojoba Wax Synthase Tryptic Peptides

WSpep29	FVPAVAPHGGALR
WSpep33	TIDEYPVMFNQTQK

20 **Example 6 - Purification of Additional Wax Synthases
and Reductases**

A. Adaptation of jojoba wax synthase solubilization and purification methods to obtain partially purified preparations of wax synthase from other organisms are described.

Acinetobacter

Cells of *Acinetobacter calcoaceticus* strain BD413 (ATCC #33305) are grown on ECLB (*E. coli* luria broth), collected during the logarithmic growth phase and washed in a buffer containing either HEPES-NaOH, pH 7.5, or Tricine-NaOH pH 7.8, in 0.1M NaCl, 1mM DTT and protease inhibitors. Washed cells were resuspended in fresh buffer and ruptured by passage through a French pressure cell (two passes at ~16,000p.s.i.). Unbroken cells are removed by centrifugation at 5000 x g for 10 minutes, and membranes are collected by centrifugation at 100,000 x g for 1 hour. The membrane pellet is homogenized in storage buffer (25mM

HEPES-NaOH, pH 7.5, or 25mM Tricine-NaOH, pH 7.8, in 10% (w/v) glycerol, 100mM NaCl). Wax synthase activity is detected in these membranes using assay conditions described for the jojoba enzyme in Example 1B, using [1-¹⁴C] palmitoyl-CoA and 18:1 alcohol as the substrates.

Wax synthase activity is solubilized by incubation of the membranes with 2% CHAPS in the presence of 0.5M NaCl, at a detergent to protein ratio of 5:1. Solubilization of the activity is demonstrated by the detection of wax synthase enzyme activity in the supernatant fraction after centrifugation at 200,000g for 1 hour and by size exclusion chromatography (i.e. the activity elutes from the column in the retained fractions as a symmetrical peak). The activity of the solubilized enzyme is detected by simple dilution of the CHAPS concentration to ~0.3% (i.e. to below its CMC). Incorporation of the enzyme into phospholipid vesicles is not required to detect solubilized activity.

For purification, the solubilized *Acinetobacter* wax synthase activity is subjected to chromatographic procedures similar to those described for the jojoba wax synthase. In one protocol, the soluble protein preparation is loaded to a Blue A agarose column under low salt conditions (100 mM NaCl in a column buffer containing 0.75% CHAPS, 10% glycerol, 25 mM HEPES-NaOH, pH 7.5) and eluted from the column using 1.0M NaCl in the column buffer.

Size exclusion chromatography on Superose 12 (Pharmacia; Piscataway, NJ) medium is used to obtain an estimate of the size of the native enzyme. Comparison to molecular mass standards chromatographed under identical conditions yields an apparent mass of ~40 kDa for the solubilized wax synthase.

In another protocol, solubilized protein is loaded onto a Blue A column equilibrated with 25 mM Tricine-NaOH, pH 7.8, 1% CHAPS, 20% glycerol containing 0.1M NaCl and eluted in the same buffer containing 1.0M NaCl. The eluted material is then loaded onto a hydroxyapatite column equilibrated with column buffer containing 1.0 M NaCl and

unlike the jojoba wax synthase, the acinetobacter wax synthase activity binds the column and is eluted in a gradient of 1-100 mM dipotassium phosphate. When examined by SDS-PAGE, several protein candidates can be correlated with wax synthase activity.

Euglena

Euglena gracilis, strain Z (ATCC No. 12716) is grown heterotrophically in the dark (Tani et al. (1987) *Agric. Biol. Chem.* 51:225-230) at ~26°C with moderate shaking. Cells are collected and washed in buffer containing 25mM Bis-Tris-Propane, pH 7.0, 0.25M NaCl and 1mM EDTA. Washed cells are resuspended in fresh buffer and ruptured by passage through a French pressure cell (two passes at ~16,000 p.s.i.). Unbroken cells, cell debris and nuclei are removed by centrifugation at 20,000 x g for 20 minutes, and microsomal membranes are collected by centrifugation at 200,000 x g for 1 hour. The membrane pellet is homogenized in storage buffer (25mM Bis-Tris-Propane, pH 7.0, 0.25M NaCl, 10% (w/v) glycerol and 1mM EDTA). Wax synthase activity is detected in these membranes using assay conditions as described for the jojoba enzyme. The radiolabelled substrate is the same as for the jojoba example (i.e. [1-¹⁴C] palmitoyl-CoA), however, 16:0 rather than 18:1 is used as the alcohol acceptor, and Bis-Tris-Propane buffer at pH 7.0 is utilized.

The *Euglena* wax synthase activity is solubilized by incubation of the membranes with 2% CHAPS in the presence of 0.5M NaCl. Solubilization of the protein is demonstrated by the detection of enzyme activity in the supernatant fraction after centrifugation at 200,000 x g for 1 hour. The activity of the solubilized enzyme is detected by dilution of the CHAPS concentration to ~0.3% (i.e. to below its CMC). It is not necessary to incorporate the enzyme into phospholipid vesicles as was the case for the solubilized jojoba wax synthase.

For partial purification, the solubilized *Euglena* wax synthase activity is subjected to chromatographic separation on Blue A agarose medium. The column is equilibrated with 0.1M NaCl in a column buffer containing; 25mM Bis-Tris-
5 Propane, pH 7.0, 20% (w/v) glycerol, 0.75% CHAPS and 1mM EDTA. The sample containing solubilized wax synthase activity is diluted to 0.1M NaCl and loaded onto a 1 x 7cm column (5.5ml bed volume). The column is washed with equilibration buffer and subjected to a linear NaCl gradient
10 (0.1M to 1.0M NaCl) in column buffer. Wax synthase activity is eluted as a broad peak in the last half of the salt gradient.

SDS-PAGE analysis of column fractions reveals that the polypeptide complexity of the activity eluted from the
15 column is greatly reduced relative to the loaded material. A polypeptide with an apparent molecular mass of ~41kD was observed to track with wax synthase activity in the column fractions. Further purification techniques, such as described for jojoba and *Acinetobacter* are conducted to
20 verify the association of wax synthase activity with the ~41kD peptide.

For further analysis of wax synthase activity in *Euglena*, size exclusion chromatography was conducted as follows. A microsomal membrane preparation was obtained
25 from *Euglena* cells grown on liquid, heterotrophic, medium (Tani et al., supra) in the dark. Wax synthase activity was solubilized by treating the membranes with 2% (w/v) CHAPS and 500mM NaCl in a buffered solution (25mM Bis-Tris, pH 7.0, 1mM EDTA and 10% (w/v) glycerol) for 1 hour on ice.
30 After dilution of the CHAPS to 0.75% and the NaCl to 200mM by addition of a dilution buffer, the sample was centrifuged at ~200,000 x g for 1.5 hours. The supernatant fraction was loaded onto a Blue A dye column pre-equilibrated with Column Buffer (25mM Bis-Tris pH 7.0, 1mM EDTA, 10% glycerol, 0.75%
35 CHAPS) which also contained 200mM NaCl. The column was washed with Column Buffer containing 200mM NaCl until the A280 of the effluent returned to the preload value. Wax

synthase activity which had bound to the column was released by increasing the NaCl concentration in the Column Buffer to 1.5M. The fractions from the Blue A column containing wax synthase activity released by the 1.5M NaCl (~20ml combined volume) were pooled and concentrated approximately 30-fold via ultrafiltration (Amicon pressure cell fitted with a YM 30 membrane). The concentrated material from the Blue A column was used as the sample for a separation via size exclusion chromatography on Superose 12 medium (Pharmacia).

Approximately 200 μ l of the sample was loaded onto a Superose 12 column (HR 10/30), pre-equilibrated with Column Buffer containing 0.5M NaCl, and developed at a flow rate of 0.1ml/min. The wax synthase activity eluted from the column as a smooth peak. Comparison of the elution volume of the wax synthase activity with the elution profiles of molecular mass standard proteins yielded an estimate of 166kD for the apparent molecular mass of the enzyme. Fractions which contained wax synthase activity were analyzed via SDS-polyacrylamide gel electrophoresis followed by silver staining. A preliminary analysis of the polypeptide profiles of the various fractions did not reveal any proteins with molecular masses of 100kD or greater whose staining intensity appeared to match the activity profile. The wax synthase polypeptide may be present as a minor component in the sample mixture that is not readily detectable on the silver-stained gel. Alternatively, the enzyme may be composed of subunits which are dissociated during SDS-PAGE.

B. In addition to jojoba reductase, such as that encoded by the sequence provided in Figure 1, reductase proteins from other sources are also desirable for use in conjunction with the wax synthase proteins of this invention. Such proteins may be identified and obtained from organisms known to produce wax esters from alcohol and acyl substrates.

For example, an NADH-dependent fatty acyl-CoA reductase activity can be obtained from microsomal membranes isolated

from *Euglena gracilis*. Methods which may be used to isolate microsomal membranes are described, for example in the published PCT patent application WO 92/14816 (application number PCT/US92/03164, filed February 21, 1992). The reductase activity is solubilized from these membranes using the same approaches as used for jojoba reductase and wax synthase. Membranes are incubated on ice for one hour with various amounts of the detergent, CHAPS, in a buffering solution consisting of 25mM BisTris, pH 6.9, 250mM NaCl, 10% glycerol and 1 mM EDTA. The sample is then centrifuged at 200,000 x g for one hour, and the supernatant and pellet fractions assayed for NADH-dependent reductase activity using radiolabeled palmitoyl-CoA and NADH as substrates. A convenient assay for reductase activity is described in PCT patent application WO 92/14816. Incubation of the membranes with 0.3, 0.5 or 0.7 %(w/v) CHAPS results in retention of reductase activity in the supernatant fractions, indicative of solubilization of the enzyme. If CHAPS is omitted during the incubation and centrifugation, all of the reductase activity is found in the pellet fraction. All of the samples are diluted ten-fold in this same buffer solution prior to assaying in order to dilute the CHAPS present during the incubation. The presence of CHAPS in the assay at levels above the CMC (approximately 0.5%(w/v) results in inhibition of enzyme activity. Stability of the reductase activity in up to 2% CHAPS may be improved by increasing the glycerol concentration in the buffering solution to 20%. Reductase activity is recovered by dilution of the CHAPS to below the CMC.

Example 7 - Isolation of Wax Synthase Nucleic Acid Sequences

DNA sequences encoding wax synthase peptides are obtained from jojoba using synthetic oligonucleotides designed from wax synthase peptide sequences. The wax synthase nucleic acid sequences may be obtained by amplification of DNA by polymerase chain reaction (PCR)

using oligonucleotides as primers, or alternatively, by screening a cDNA or genomic DNA library by radiolabeling the oligonucleotides or previously isolated sequences for use as probes.

5

A. Construction of Jojoba cDNA Libraries

RNA is isolated from jojoba embryos collected at 80-90 days post-anthesis using a polyribosome isolation method, initially described by Jackson and Larkins (*Plant Physiol.* (1976) 57:5-10), as modified by Goldberg *et al.* (*Developmental Biol.* (1981) 83:201-217). In this procedure all steps, unless specifically stated, are carried out at 4°C. 10gm of tissue are ground in liquid nitrogen in a Waring blender until the tissue becomes a fine powder. After the liquid nitrogen has evaporated, 170ml of extraction buffer (200mM Tris pH 9.0, 160mM KCl, 25mM EGTA, 70mM MgCl₂, 1% Triton X-100, 05% sodium deoxycholate, 1mM spermidine, 10mM β-mercaptoethanol, and 500mM sucrose) is added and the tissue is homogenized for about 2 minutes. The homogenate is filtered through sterile miracloth and centrifuged at 12,000 x g for 20 minutes. The supernatant is decanted into a 500ml sterile flask, and 1/19 volume of a 20% detergent solution (20% Brij 35, 20% Tween 40, 20% Noidet p-40 w/v) is added at room temperature. The solution is stirred at 4°C for 30 minutes at a moderate speed and the supernatant is then centrifuged at 12,000 x g for 30 minutes.

About 30ml of supernatant is aliquoted into sterile Ti 60 centrifuge tubes and underlaid with 7ml of a solution containing 40mM Tris pH 9.0, 5mM EGTA, 200mM KCl, 30mM MgCl₂, 1.8M sucrose, 5mM β-mercaptoethanol. The tubes are filled to the top with extraction buffer, and spun at 60,000 rpm for 4 hours at 4°C in a Ti60 rotor. Following centrifugation, the supernatant is aspirated off and 0.5ml of resuspension buffer (40mM Tris pH 9.0, 5mM EGTA, 200mM KCl, 30mM MgCl₂, 5mM β-mercaptoethanol) is added to each tube. The tubes are placed on ice for 10 minutes, after

which the pellets are thoroughly resuspended and pooled. The supernatant is then centrifuged at 120 x g for 10 minutes to remove insoluble material. One volume of self-digested 1mg/ml proteinase K in 20mM Tris pH 7.6, 200mM
5 EDTA, 2% N-lauryl-sarcosinate is added to the supernatant and the mixture incubated at room temperature for 30 minutes.

RNA is precipitated by adding 1/10 volume of sodium acetate and 2 volumes of ethanol. After several hours at
10 -20°C RNA is pelleted by centrifugation at 12,000 x g at 4°C for 30 minutes. The pellet is resuspended in 10ml of TE buffer (10mM Tris, 1mM EDTA) and extracted with an equal volume of Tris pH 7.5 saturated phenol. The phases are separated by centrifuging at 10,000 x g for 20 minutes at
15 4°C. The aqueous phase is removed and the organic phase is re-extracted with one volume of TE buffer. The aqueous phases are then pooled and extracted with one volume of chloroform. The phases are again separated by centrifugation and the aqueous phase ethanol precipitated as
20 previously described, to yield the polyribosomal RNA.

Polysaccharide contaminants in the polyribosomal RNA preparation are removed by running the RNA over a cellulose column (Sigma-cell 50) in high salt buffer (0.5M NaCl, 20mM Tris pH 7.5, 1mM EDTA, 0.1% SDS). The contaminant binds to
25 the column and the RNA is collected in the eluant. The eluant fractions are pooled and the RNA is ethanol precipitated. The precipitated total RNA is then resuspended in a smaller volume and applied to an oligo d(T) cellulose column to isolate the polyadenylated RNA.

Polyadenylated RNA is used to construct a cDNA library in the plasmid cloning vector pCGN1703, derived from the commercial cloning vector Bluescribe M13- (Stratagene Cloning Systems; San Diego, CA), and made as follows. The polylinker of Bluescribe M13- is altered by digestion with
35 *Bam*HI, treatment with mung bean endonuclease, and blunt-end ligation to create a *Bam*HI-deleted plasmid, pCGN1700. pCGN1700 is digested with *Eco*RI and *Sst*I (adjacent

restriction sites) and annealed with a synthetic linker having restriction sites for *Bam*HI, *Pst*I, *Xba*I, *Apa*I and *Sma*I, a 5' overhang of AATT, and a 3' overhang of TCGA. The insertion of the linker into pCGN1700 eliminates the *Eco*RI site, recreates the *Sst*I (also, sometimes referred to as "SacI" herein) site found in Bluescribe, and adds the new restriction sites contained on the linker. The resulting plasmid pCGN1702, is digested with *Hind*III and blunt-ended with Klenow enzyme; the linear DNA is partially digested with *Pvu*II and ligated with T4 DNA wax synthase in dilute solution. A transformant having the *lac* promoter region deleted is selected (pCGN1703) and is used as the plasmid cloning vector.

Briefly, the cloning method for cDNA synthesis is as follows. The plasmid cloning vector is digested with *Sst*I and homopolymer T-tails are generated on the resulting 3'-overhang stick-ends using terminal deoxynucleotidyl transferase. The tailed plasmid is separated from undigested or un-tailed plasmid by oligo(dA)-cellulose chromatography. The resultant vector serves as the primer for synthesis of cDNA first strands covalently attached to either end of the vector plasmid. The cDNA-mRNA-vector complexes are treated with terminal transferase in the presence of deoxyguanosine triphosphate, generating G-tails at the ends of the cDNA strands. The extra cDNA-mRNA complex, adjacent to the *Bam*HI site, is removed by *Bam*HI digestion, leaving a cDNA-mRNA-vector complex with a *Bam*HI stick-end at one end and a G-tail at the other. This complex is cyclized using an annealed synthetic cyclizing linker which has a 5' *Bam*HI sticky-end, recognition sequences for restriction enzymes *Not*I, *Eco*RI and *Sst*I, and a 3' C-tail end. Following ligation and repair the circular complexes are transformed into *E. coli* strain DH5a (BRL, Gaithersburg, MD) to generate the cDNA library. The jojoba embryo cDNA bank contains between approximately 1.5×10^6 clones with an average cDNA insert size of approximately 500 base pairs.

Additionally, jojoba polyadenylated RNA is also used to construct a cDNA library in the cloning vector lZAPII/EcoRI (Stratagene, San Diego, CA). The library is constructed using protocols, DNA and bacterial strains as supplied by the manufacturer. Clones are packaged using Gigapack Gold packaging extracts (Stratagene), also according to manufacturer's recommendations. The cDNA library constructed in this manner contains approximately 1×10^6 clones with an average cDNA insert size of approximately 400 base pairs.

B. Synthetic Oligonucleotides

In general, for use as PCR primers from single stranded DNA template reverse-transcribed from mRNA, oligonucleotides containing the sense orientation sequence corresponding to wax synthase peptide encoding sequences are prepared. These oligonucleotides are used as primers for the "forward" amplification reaction to produce sense strand DNA.

For the "reverse" reaction for amplification of the non-coding DNA strand, an oligonucleotide may be designed to be identical to a portion of a primer used to prepare DNA template for PCR. Alternatively, oligonucleotides which contain sequence complementary to wax synthase peptide encoding sequences may be used in combination with a "forward" wax synthase oligonucleotide primer as described above.

Where the wax synthase peptide sequences contain amino acids which may be encoded by a number of different codons, the forward or reverse primers may be "degenerate" oligonucleotides, i.e. containing a mixture of all or some of the possible encoding sequences for a particular peptide region. To reduce the number of different oligonucleotides present in such a mixture, it is preferable to select peptide regions which have the least number of possible encoding sequences when preparing the synthetic oligonucleotide for PCR primers. Similarly, where the synthetic oligonucleotide is to be used to directly screen a

library for wax synthase sequences, lower degeneracy oligonucleotides are preferred.

Following is an example of the sequence of peptide WSPEP33 (center line) and the forward (top line) and reverse (bottom line) DNA sequences that encode the peptide WSPEP33.

```
5' TTY GTN CCN GCN GTN GCN CCN CAY GGN GGN GCN YTN MGN 3'
    F  V  P  A  V  A  P  H  G  G  A  L  R
3' AAR CAN GGN CGN CAN CGN GGN GTR CCN CCN CGN RAN KCN 5'
```

10

Following is an example of the sequence of peptide WSPEP29 (center line) and the forward (top line) and reverse (bottom line) DNA sequences that encode the peptide WSPEP29.

```
15 5' ACN ATH GAY GAR TAY CCN GTN ATG TTY AAY TAY ACN CAR AAR
    3'
        T  I  D  E  Y  P  V  M  F  N  Y  T  Q  K
3' TGN TAD CTR CTY ATR GGN CAN TAC AAR TTR ATR TGN GTY TTY
    5'
```

20

Following is an example of the sequence of peptide WSPEP14 (center line) and the forward (top line) and reverse (bottom line) DNA sequences that encode the peptide WSPEP14.

```
25 5' TTY MGN GAY GAY CCN WSN AAY GAY CAY 3'
    F  R  D  D  P  S  N  D  H
3' AAR KCN CTR CTR GGN WSN TTR CTR GTR 5'
```

Following are sequences of synthetic oligonucleotides which may be used to obtain wax synthase sequences. The oligonucleotide names reflect the particular wax synthase peptide fragment numbers as listed in Example 5. The letter "F" in the oligonucleotide name designates a PCR forward reaction primer. The letter "R" designates a PCR reverse reaction primer.

35

```
WSPEP29-F1      5' TTYGTNCCNGCNGTNGC 3'
```

WSPEP29-F2 5' GCNCCNCAYGGNGGNGC 3'
 WSPEP29-R1 5' GCNCCNCCRTGNGGNGC 3'
 WSPEP29-R2 5' GCNACNGCNGGNACRAA 3'
 WSPEP33-F1 5' ACNATHGAYGARTAYCCNGT 3'
 5 WSPEP33-F2 5' CCNGTNATGTTYAAYTAYAC 3'
 WSPEP33-R1 5' TTYTGNGTRTARTTRAACAT 3'
 WSPEP33-R2 5' AACATNACNGGRTAYTCRTC 3'
 WSPEP14-F1 5' GAYGAYCCNWSNAAYGAYCA
 10 WSPEP14-R1 5' TGRTCRTTNSWNGGRTCRC

The nucleotide base codes for the above oligonucleotides are as follows:

A = adenine T = thymine Y = cytosine or thymine
 C = cytosine U = uracil R = adenine or guanine
 15 G = guanine I = inosine O = inosine or cytosine
 H = adenine, cytosine or thymine
 N = adenine, cytosine, guanine or thymine
 W = adenine or thymine
 S = guanine or cytosine
 20 B = guanine, cytosine or thymine
 K = guanine or thymine
 M = adenine or cytosine

C. PCR Reactions

25 Poly(A)+ RNA is isolated from total RNA prepared from
 jojoba tissue as described above. cDNA is prepared from
 poly(A)+ or total RNA by reverse transcription using the
 Marathon cDNA Amplification Kit (Clontech Laboratories Inc
 30 according to the manufacturer's directions. The jojoba cDNA
 is used in PCR reactions 1-16 set forth below.

PCR is conducted in a Perkin Elmer Cetus GeneAmp PCR
 System 9600 PCR machine using reverse transcribed single-
 stranded cDNA as template. Commercially available PCR
 35 reaction and optimization reagents are used according to
 manufacturer's specifications

Reaction Forward Primer Reverse Primer

	1	WSPEP14-F1	WSPEP29-R1
	2	WSPEP14-F1	WSPEP29-R2
	3	WSPEP14-F1	WSPEP33-R1
	4	WSPEP14-F1	WSPEP33-R2
5	5	WSPEP29-F1	WSPEP14-R1
	6	WSPEP29-F1	WSPEP33-R1
	7	WSPEP29-F1	WSPEP33-R2
	8	WSPEP29-F2	WSPEP14-R1
	9	WSPEP29-F2	WSPEP33-R1
10	10	WSPEP29-F2	WSPEP33-R2
	11	WSPEP33-F1	WSPEP14-R1
	12	WSPEP33-F1	WSPEP29-R1
	13	WSPEP33-F1	WSPEP29-R2
	14	WSPEP33-F2	WSPEP14-R1
15	15	WSPEP33-F2	WSPEP29-R1
	16	WSPEP33-F2	WSPEP29-R2

The temperature program used for PCR amplification is as follows: 1 cycle of 95 degrees C for 2 minutes; 4 cycles of 95 degrees C for 30 seconds, 60 degrees C for 1 minute, and 72 degrees C for 4 minutes; 4 cycles of 95 degrees C for 30 seconds, 57 degrees C for 1 minute, and 72 degrees C for 4 minutes; 4 cycles of 95 degrees C for 30 seconds, 54 degrees C for 1 minute, and 72 degrees C for 4 minutes; 4 cycles of 95 degrees C for 30 seconds, 51 degrees C for 1 minute, and 72 degrees C for 4 minutes; and 25 cycles of 95 degrees C for 30 seconds, 48 degrees C for 1 minute, and 72 degrees C for 4 minutes.

From reactions 3 and 4, a PCR product approximately 700 nucleotides in length was detected. The PCR product was purified using gel electrophoresis and cloned into pCR2.1 using a Topo TA cloning kit (Invitrogen Corp.). The DNA sequence of the cloned PCR product was determined and was 708 nucleotides long (Figure 3).

The entire cDNA can be amplified using 5' and 3' RACE (Frohman et al., 1988) using the Marathon cDNA Amplification Kit (Clontech Laboratories Inc.) according to the manufacturers instructions. From the sequence of the 708 nucleotide PCR fragment derived using primers WSPEP14-F1 and WSPEP33-R2 the following primers were synthesized:

WSRACEF1	GATTTGCCTCATTGTTGTGATCTCGGTGCT
WSRACEF2	GACCTATACCCCCAGTTCAACGAGCCATAC
WSRACEF3	TTCAACGAGCCATACTTAGCCACCTCGCTG
WSRACER1	AACAACCACCCTCCAGTCACCATCACGAAC
WSRACER2	TTGCCTGAAACCGCCTTCTTCACCACCATC
WSRACER3	AAGATGTCTGACACCATGAGGTTCCACCTG

3'RACE reactions were set up using primers WSRACEF1, WSRACEF2, and WSRACEF3. 5'RACE reactions were set up using primers WSRACER1, WSRACER2, and WSRACER3. PCR reactions were performed according to the manufacturer's protocol (Clontech Laboratories Inc.). All 6 PCR reactions gave visible PCR products ranging in size from approximately 700 nucleotides to 1000 nucleotides. The PCR products were gel purified and cloned into pCR2.1 according to the manufacturer's protocol (Invitrogen Corp.). The DNA sequence of several clones from both the 5' and 3' RACE reactions and the previous PCR product derived from primers WSPEP14-F1 and WSPEP33-R2 were assembled using Sequencher software (Gene Codes Corp.). The assembled sequence of all the PCR products contains the coding region of the cDNA sequence.

To isolate a gene fragment suitable for cloning the wax synthase gene into expression cassettes for plant lipid modification, the coding region of the gene can be amplified from cDNA using the primers WAXSYNFOR and WASXYNREV. The sequence of WAXSYNFOR is

GGATCCGTCGACACAATGGAGGTGGAGAAGGAGCTAAAG, and the sequence of WASXYNREV is GCATGCAGATCTCACCACCCCAACAAACCCATC. The PCR reaction is performed using the Marathon CDNA (Clontech

Laboratories Inc.) according to the manufacturer's instructions. The PCR program consists of 30 cycles of 94 degrees C for 15 seconds, 60 degrees C for 1 minute, 72 degrees C for 2 minutes. . The PCR products were cloned into pCR2.1 according to the manufacturer's protocol (Invitrogen Corp.). The resulting plasmid was designated pCGN8538.

Example 8 Generation of transgenic plants containing the wax synthase cDNA.

Two plant binary vectors were constructed. Plasmid pCGN8559 contains 3 genes necessary for wax biosynthesis: the condensing enzyme involved in fatty acid elongation to chain lengths greater than 18 carbons (KCS), the acyl-CoA reductase involved in formation of fatty alcohols, and the wax synthase. A control plasmid, pCGN8557, contains the KCS and acyl-CoA reductase genes. The Asp718 fragment of pCGN7698, which contains the jojoba acyl-CoA reductase under control of napin regulatory sequences, was cloned into the Asp718 site of binary vector pCGN5139 to form pCGN8555. The NotI fragment of pCGN7844, which contains the Lunaria KCS under control of napin regulatory sequences, was cloned into the NotI site of pCGN8555 to form pCGN 8557. The SalI-BglII fragment from pCGN8538 which contains the coding region of the jojoba wax synthase gene, was cloned into the napin expression cassette of pCGN7770 digested with the same two restriction endonucleases to form pCGN8553. The Sse8387 fragment of pCGN8553, which contains the jojoba wax synthase under control of napin regulatory sequences, was cloned into the Sse8387 site of pCGN8557 to form pCGN8559. The binary vectors were introduced into *Agrobacterium tumefaciens* EHA105 via electroporation. The vectors were used to transform *Arabidopsis thaliana* ecotype No-0 according to the vacuum infiltration protocol of Bent et al. (1994, *Science* 265:1856-1860).

Example 9 Analysis of the seed oil

Siliques were harvested from seven arabidopsis plants transformed with pCGN8559 which were in various stages of development. Developing seed was removed from ten siliques collected from each plant and homogenized in 275 μ l of buffer (100 mM HEPES/NaOH pH 7.5, 250 mM NaCl). A portion of the homogenate (200 μ l) was centrifuged at 16000 x g for 20 minutes at 4°C resulting in a supernatant and pellet. The pellet was resuspended in 200 μ l of the same buffer. The homogenate and the two fractions were assayed for wax synthase activity according to the protocol detailed in Example 1B. 25 μ l of sample were used per assay in a final volume of 250 μ l. The assay buffer contained 40 μ M 1-¹⁴C 16:0-CoA (specific activity 5 μ Ci/ μ mol), 200 μ M 18:1 alcohol, 50 mM HEPES/NaOH pH 7.5, 250 mM NaCl and 2 mM β -mercaptoethanol. TLC analysis showed the incorporation of radiolabel from 1-¹⁴C 16:0-CoA into a band which comigrated with a wax standard in 5 of the 7 plants analyzed (Figure 4). This activity was detected in the homogenate and pellet fractions but not in the supernatant fraction. The wax synthase activity detected in these samples is several orders of magnitude greater than an endogenous wax synthase activity previously shown to be present in developing arabidopsis seed. The activity detected in 8612-3 and 8613-2 is indicative of this endogenous "background" activity. A positive control for wax activity was the jojoba (DP2) membrane fraction.

The above results demonstrate nucleic acid sequences obtained from partially purified wax synthase proteins are active in the formation of wax esters from fatty alcohol and fatty acyl substrates. Methods to obtain the wax synthase proteins and amino acid sequences thereof are provided. Such nucleic acid sequences may be manipulated to provide for transcription of the sequences and/or expression of wax synthase proteins in host cells, which proteins may be used for a variety of applications. Such applications include the production of wax ester compounds when the wax synthase

is used in host cells having a source of fatty alcohol substrates, which substrates may be native to the host cells or supplied by use of recombinant constructs encoding a fatty acyl reductase protein which is active in the formation of alcohols from fatty acyl substrates.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be readily apparent to those of ordinary skill in the art in light of the teaching of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A recombinant DNA construct comprising a nucleic acid sequence which encodes at least a portion of an acyltransferase active in the formation of a wax ester, and
5 a heterologous DNA sequence not naturally associated with said acyltransferase encoding sequence.
2. The construct of Claim 1 wherein said wax ester is formed from a fatty alcohol and a fatty acyl-CoA substrate.
3. The construct of Claim 1 wherein said
10 acyltransferase is active toward a fatty acyl substrate having a carbon chain of the formula C_{2x} wherein X is selected from the group 6-12.
4. The construct of Claim 1 wherein said acyltransferase is active toward a fatty alcohol substrate
15 having a carbon chain of the formula C_{2x} wherein X is selected from the group 6-12.
5. The construct of Claim 1 wherein said acyltransferase encoding sequence is from a seed plant.
6. The construct of Claim 1 wherein said
20 acyltransferase encoding sequence is from jojoba.
7. The construct of Claim 1 further comprising a promoter which provides for at least transcription of said acyltransferase encoding sequence in a host cell.
8. The construct of Claim 7 wherein said promoter
25 provides for expression of said acyltransferase encoding sequence in a plant cell.
9. The construct of Claim 8 wherein said plant cell is a plant embryo seed cell.

10. The construct of of Claim 7 wherein said promoter provides for expression of said acyltransferase encoding sequence in a bacterial cell.

11. The construct of Claim 8 wherein said promoter is
5 from a gene preferentially expressed in a plant seed embryo cell.

12. A cell comprising a construct according to Claim 1.

13. A plant cell comprising a construct according to
10 Claim 1.

14. A host cell comprising a recombinant construct comprising a nucleic acid sequence which encodes a fatty acyl reductase protein; and

a recombinant construct comprising a nucleic acid
15 sequence which encodes a acyltransferase protein active in the formation of a wax ester, wherein said acyltransferase encoding sequence is heterologous to said host cell, and wherein said reductase and acyltransferase encoding sequences are under the regulatory control of promoters
20 functional in said host cell.

15. The host cell of Claim 14, wherein said host cell is a plant cell.

16. The plant cell of Claim 15, wherein said plant cell is a *Brassica* plant cell.

25 17. A *Brassica* plant cell comprising a construct according to Claim 1.

18. A procaryotic cell comprising a seed-plant acyltransferase active in the formation of a wax ester.

19. A method of producing a wax ester in a host cell comprising the steps of

growing a host cell comprising a recombinant construct, said construct comprising a nucleic acid sequence which
5 encodes at least a portion of an acyltransferase active in the formation of a wax ester from a fatty alcohol and a fatty acyl-CoA substrate, wherein said acyltransferase encoding sequence is under the control of regulatory elements functional in said cell,

10 under conditions which will cause the expression of said acyltransferase,

wherein said host cell comprises a fatty alcohol substrate of said acyltransferase.

20 20. The method of Claim 19 wherein said host cell is a procaryote.

21. The method of Claim 19 wherein said host cell is a seed plant embryo cell.

22. The method of Claim 21 wherein said seed plant is *Brassica*.

20 23. The method of Claim 19 wherein said acyltransferase encoding sequence is from a seed plant.

24. The method of Claim 19, wherein said fatty alcohol substrate of said wax synthase is produced in said host cell as the result of expression of a fatty acyl reductase
25 encoding sequence from a heterologous recombinant construct.

25. A host cell comprising a wax ester produced according to the method of Claim 19.

26. A *Brassica* seed cell, wherein the internal lipid reserves of said seed cell comprise wax esters.

27. A *Brassica* seed cell of Claim 26, wherein said wax esters are long chain wax esters.

5 28. A wax ester obtained from a cell according to Claim

29. The construct of of Claim 1 wherein said acyltransferase comprises the peptide sequence TIDEYPVMFNVTQK.

10 30. The acyltransferase of Claim 1 wherein said acyltransferase comprises the peptide sequence FVPAVAPHGGALR.

Blue A - Agarose Chromatography

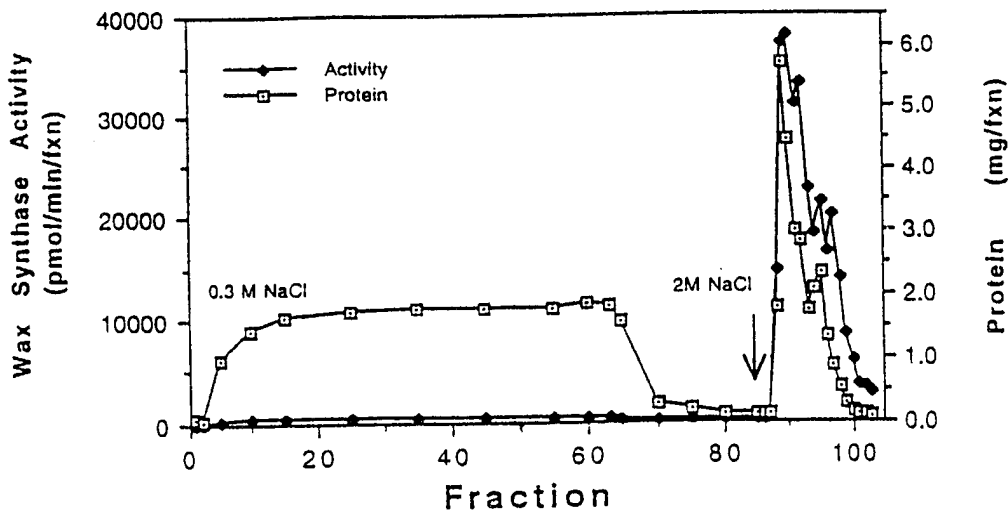


Figure 1A

Ceramic Hydroxyapatite Chromatography

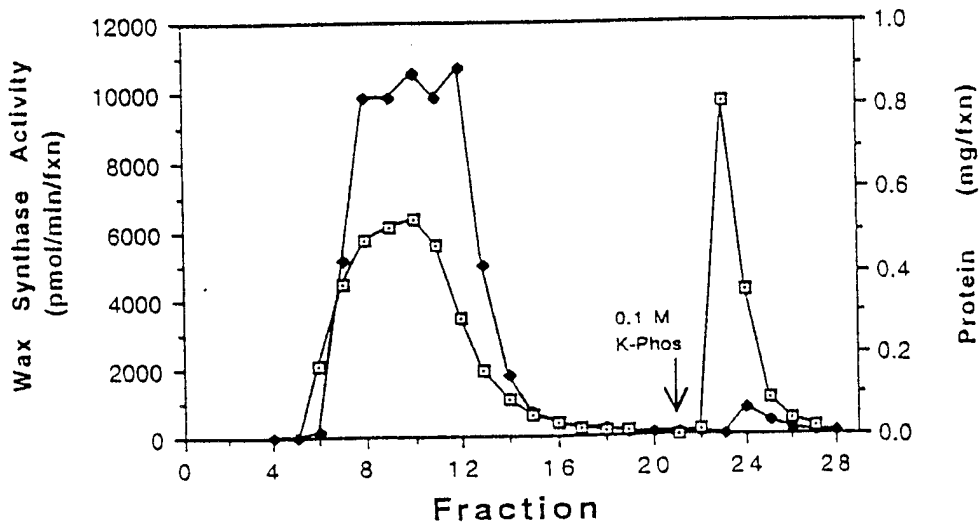


Figure 1B

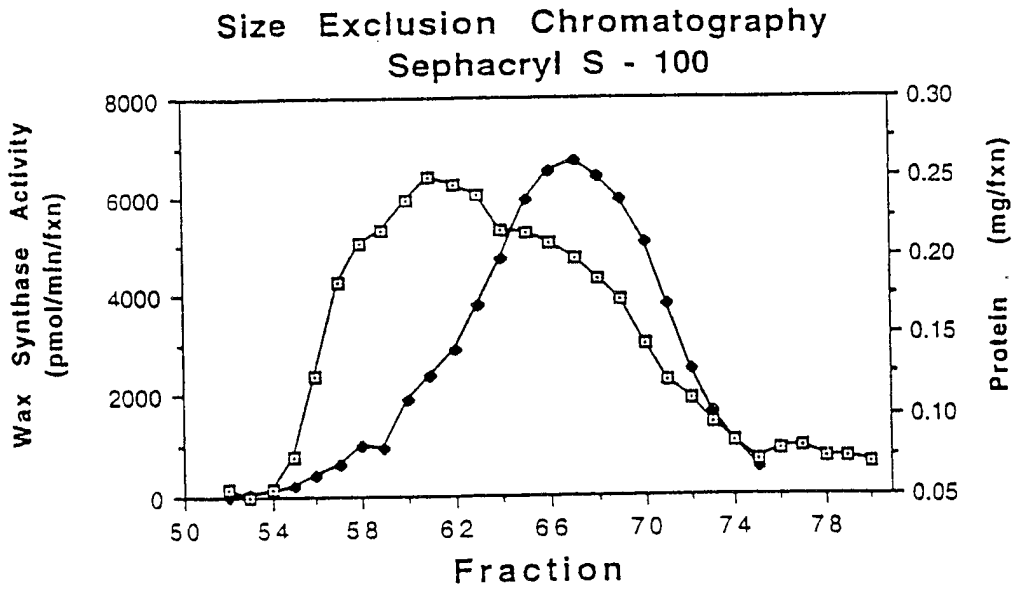


Figure 1C

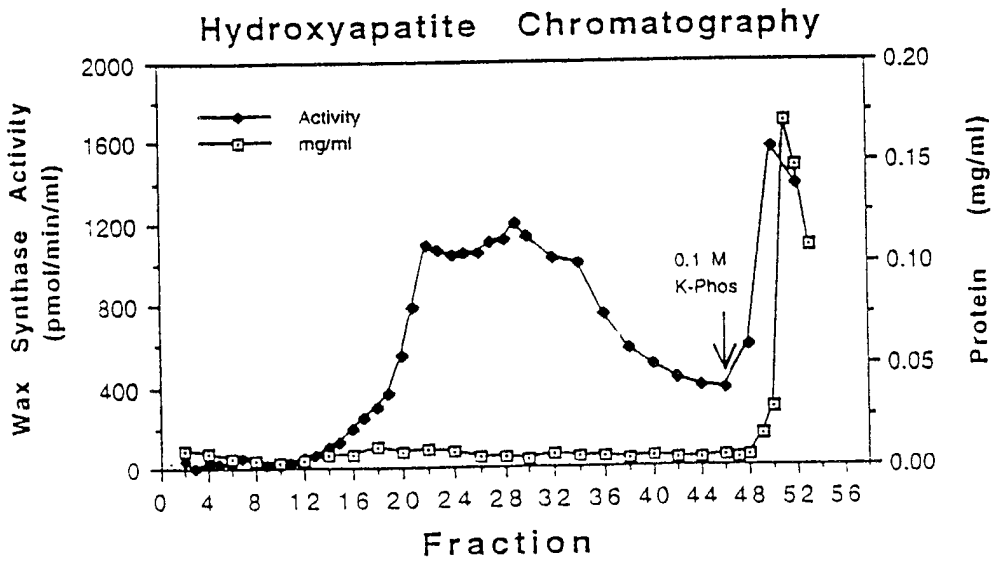


Figure 1D

3/5

Blue A - Agarose Chromatography

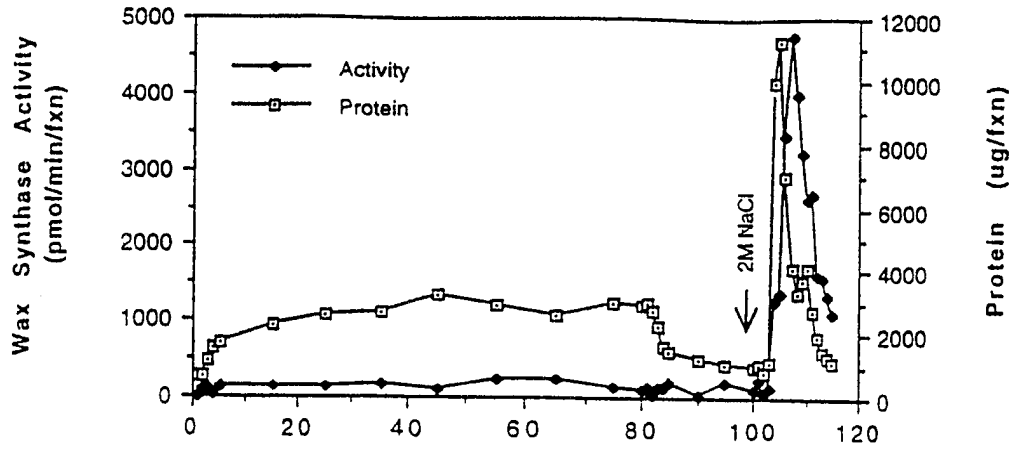


Figure 2A

Hydroxyapatite Chromatography

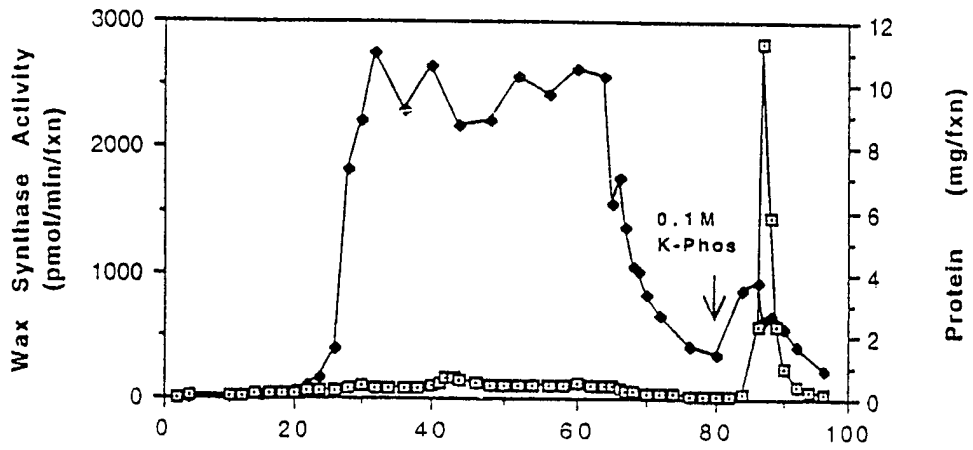


Figure 2B

Size Exclusion Chromatography Superdex 75

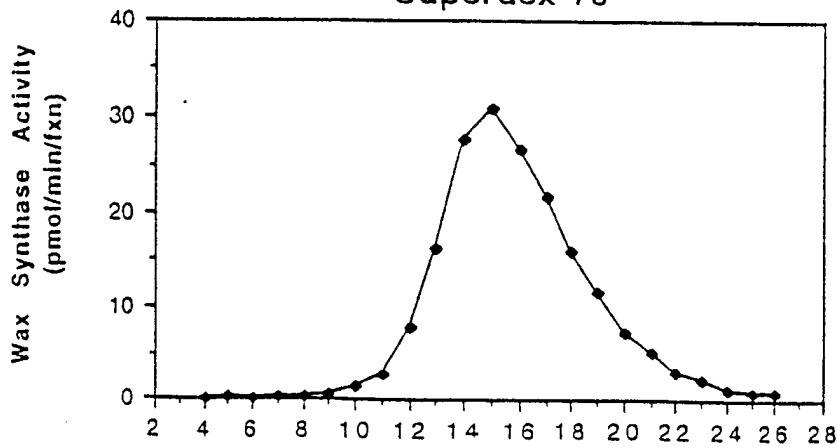


Figure 2C

Figure 3A: DNA sequence of the PCR product from primers WSPEP14-F1 and WSPEP33-R2

GATGACCCAWSNAAYGACCATGAGAAAAACAAGAGAACTCTGAGTTTTGAGTGGCGT
 AAAGTTGTTCTTTTTGTTGCTAAGTTGGTGTTTTTTTCGGGTATTTTAAAGATTTAT
 GAGTTTAGAAAAGATTTGCCTCATTGTTGATCTCGGTGCTTTACTGTTTTCACTTC
 TATCTCGGGACGGAGATCACCTTAGCAGCAAGCGCAGTCATAGCTCGAGCCACGCTA
 GGGTTAGACCTATACCCCCAGTTCAACGAGCCATACTTAGCCACCTCGCTGCAAGAC
 TTCTGGGGGCGCAGGTGGAACCTCATGGTGTGACACATCTTGGGGTTGACAACATAC
 CAGCCTGTCCGGCGTGTCTCTCGAGGTGGGTGAGGCTGCGGTGGGAGGTGCGCCGC
 GCAATGTTGGTGGCGTTCACGGTGTGCGGGCTAATGCATGAAGTGTTTTTCTTNTAC
 TTAACTCGCGCAGGCCCTCGTGGGAGGTGACGGGGTCTTTGTBTTGCATGGGGTT
 TGCACAGCCGTGGAGATGGTGGTGAAGAAGGCGGTTTCAGGCAAGGTGCGGCTGCGC
 CGGGAGGTGTGAGGGGCGCTGACGGTGGGGTTCGTGATGGTGAAGGTTGGTGGT
 TTTTTCGCCGAGCTGGTGAGGCATGGGGTAGATTTGAAGACCATTGATGAGTATCCT
 GTCATGTTAAAYTAYACCCAGAAA

Figure 3B: DNA sequence of the WAX cDNA inferred from 5' and 3' RACE products.

GTCTCCATTACAATGGAGGTGGAGAAGGAGCTAAAGACCTTCTCAGAGGTATGGATC
 TCCGCCATAGCCGCCGCTGCTACTGCCGCTTCGTCCCCGCCGTTGCCCTCACGGC
 GCGCTCTCCGCTCCTCCTCCTCCTCCCCGTCGTCTCCTCTTCATTTTCTCCCC
 CTCCGCTCTCCTCCTTCCACCTCGGCGGGCCACCAGCCTTGTATCTCGTCTGGCTT
 GCCAACTTCAAGCTCCTTCTCTTCGCCTTTCATCTTGGCCCTTATCTAACCCTCT
 CTCTCTCCTTCACTTCATCTCCACCACCCTCCTCCCCATCAAGTTCAGAGATGAC
 CCATCTAATGATCATGAGAAAAACAAGAGAACTCTGAGTTTTGAGTGGCGTAAAGTT
 GTTCTTTTTGTTGCTAAGTTGGTGTTTTTTTCGGGTATTTTAAAGATTTATGAGTTT
 AGAAAAGATTTGCCTCATTTTGTGATCTCGGTGCTTTACTGTTTTCACTTCTATCTC
 GGGACGGAGATCACCTTAGCAGCAAGCGCAGTCATAGCTCGAGCCACGCTAGGGTTA
 GACCTATACCCCCAGTTCAACGAGCCATACTTAGCCACCTCGCTGCAAGACTTCTGG
 GGGCGCAGGTGGAACCTCATGGTGTGACACATCTTGGGGTTGACAACATACCAGCCT
 TCCCGGCGTGTCTCTCGAGGTGGGTGAGGCTGCGGTGGGAGGTGCGCCGGCGCAATG
 TTGGTGGCGTTCACGGTGTGCGGGCTAATGCATGAAGTGTTTTTCTTCTACTTAACT
 CGCGCAGGCCCTCGTGGGAGGTGACGGGGTCTTTGTGTTGCATGGGGTTGCACA
 GCCGTGGAGATGGTGGTGAAGAAGGCGGTTTCAGGCAAGGTGCGGCTGCGCCGGGAG
 GTGTCAGGGGCGCTGACGGTGGGGTTCGTGATGGTGAAGTGGTGGTGGTGGTGGTGGT
 CCGCAGCTGGTGAAGCATGGGGTAGATTTGAAGACCATTGATGAGTATCCTGTCATG
 TTTAATTATACTCAGAAGAAATTGATGGGTTTGTGGGGTGGTGAATGATGAGA
 TGATGATCATGCATCTTCTTTTTTCGAGATCGGTTGTACGTCACGAGGAGAACCCAT
 GAAAAATGCAGATCARACGGCAAGACAGGTCGGGAAAAAAAATGATCAATTTTTTCC
 TTAAGTAGCCGCCCTGCCACCCTGTCCGATTTGTGGCATTTTTGTGGTCACTTTTTCA
 TATCGTGTAGTATTTTTGGTTTTTTTTGTTTTTAATGTTTTCTATGAATTTTGAATAAT
 TTGTGCTTCATGAAAATTTTTTTT

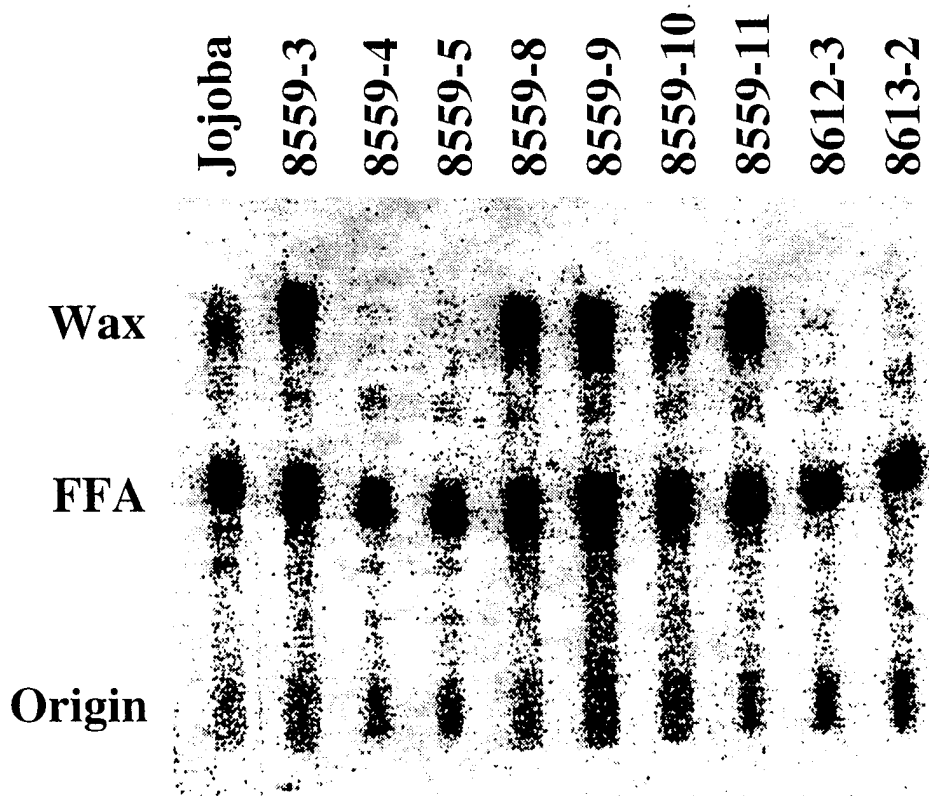


FIGURE 4

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/11590

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/54 C12N15/82 C12P7/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 10241 A (CALGENE INC) 27 May 1993 cited in the application see the whole document ---	1-25
X	WO 92 14816 A (CALGENE INC) 3 September 1992 cited in the application see the whole document ---	26-28
X	WO 95 33055 A (METZ JAMES GEORGE ;CALGENE INC (US); LASSNER MICHAEL (US)) 7 December 1995 see the whole document ---	26-28
	-/--	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

16 October 1998

Date of mailing of the international search report

30/10/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Maddox, A

INTERNATIONAL SEARCH REPORT

Inte. Application No

PCT/US 98/11590

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FENG, J., ET AL.: "F16P7-T7 IGF Arabidopsis thaliana genomic clone F19P7" EMBL SEQUENCE ACCESSION NO. B09476, 15 May 1997, XP002081021 see the whole document ---	1
A	DATABASE DISSABS AN 91:33230, 1991 GARVER, W.: "Partial purification and characterization of acyl-CoA: alcohol transacylase in subcellular organelles from developing jojoba cotyledons (Simmondsia chinensis)" XP002081026 see abstract & PH.D THESIS FROM NEW MEXICO STATE UNIVERSITY, AVAILABLE FROM UMI ORDER NO. AAR9214695, ---	1-13
A	DATABASE DISSABS AN 96:62529, 1996 SHOCKEY, J.: "Identification and characterization of lipid biosynthetic enzymes in developing seed of jojoba (Simmondsia chinensis, link) (Liquid wax)" XP002081027 see abstract & PH.D THESIS FROM NEW MEXICO STATE UNIVERSITY, AVAILABLE FROM UMI ORDER NO. AA9635003, ---	1-13
A	WILDNER, G.F., ET AL.: "Wax ester biosynthesis in Euglena gracilis" ABSTRACTS FROM THE SOUTHWEST CONSORTIUM ON PLANT GENETICS AND WATER RESOURCES, 5TH ANNUAL MEETING, April 1990, XP002081022 see the whole document ---	1-13
A	SHOCKEY, J.M., ET AL.: "Photoaffinity labeling of developing jojoba seed microsomal membranes with a photoreactive analog of acyl-coenzyme A (acyl-CoA)" PLANT PHYSIOLOGY, vol. 107, 1995, pages 155-160, XP002081023 cited in the application see the whole document ---	1-13
A	POLLARD, M.R., ET AL.: "Studies on biosynthesis of waxes by developing jojoba seed. II. The demonstration of wax biosynthesis by cell-free homogenates" LIPIDS, vol. 14, 1979, pages 651-662, XP002081024 see the whole document ---	1-13
	-/--	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/11590

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 15387 A (CALGENE INC ;METZ JAMES GEORGE (US); LARDIZABAL KATHRYN DENNIS (US) 8 June 1995 see the whole document -----	1-28
A	LASSNER, M.W., ET AL.: "A jojoba beta-ketoacyl-CoA synthase cDNA complements the canola fatty acid elongation mutation in transgenic plants" THE PLANT CELL, vol. 8, 1996, pages 281-292, XP002081025 cited in the application see the whole document -----	1-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/11590

Patent document cited in search report	A	Publication date	Patent family member(s)	Publication date
WO 9310241	A	27-05-1993	CA 2079113	23-08-1992
			CA 2123893	27-05-1993
			CN 1064312	09-09-1992
			CN 1078492	17-11-1993
			EP 0529048	03-03-1993
			EP 0572603	08-12-1993
			JP 7501221	09-02-1995
			US 5445947	29-08-1995
			WO 9214816	03-09-1992
			US 5723747	03-03-1998
			US 5679881	21-10-1997
			US 5370996	06-12-1994
WO 9214816	A	03-09-1992	US 5403918	04-04-1995
			CA 2079113	23-08-1992
			CN 1064312	09-09-1992
			EP 0529048	03-03-1993
			US 5411879	02-05-1995
			US 5723747	03-03-1998
			US 5370996	06-12-1994
			CA 2123893	27-05-1993
			CN 1078492	17-11-1993
			EP 0572603	08-12-1993
			JP 7501221	09-02-1995
			US 5445947	29-08-1995
			WO 9310241	27-05-1993
			US 5679881	21-10-1997
WO 9533055	A	07-12-1995	US 5723747	03-03-1998
			CA 2168042	07-12-1995
			CN 1129014	14-08-1996
			EP 0711351	15-05-1996
			JP 9501325	10-02-1997
WO 9515387	A	08-06-1995	US 5679881	21-10-1997
			CA 2177598	08-06-1995
			EP 0731840	18-09-1996
			JP 9505739	10-06-1997