



(86) Date de dépôt PCT/PCT Filing Date: 1999/12/03
(87) Date publication PCT/PCT Publication Date: 2000/06/22
(85) Entrée phase nationale/National Entry: 2001/05/28
(86) N° demande PCT/PCT Application No.: US 99/28678
(87) N° publication PCT/PCT Publication No.: WO 00/36095
(30) Priorité/Priority: 1998/12/04 (09/205,815) US

(51) Cl.Int.⁷/Int.Cl.⁷ C12N 9/10, C12N 15/82, C12P 7/64,
C12P 21/00, C12N 15/54, C12N 15/29, C12N 1/21,
C12N 5/10

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(54) Titre : ACYLE GRAS-CoA: ALCOOL GRAS ACYLTRANSFERASES
(54) Title: FATTY ACYL-CoA: FATTY ALCOHOL ACYLTRANSFERASES

(57) **Abrégé/Abstract:**

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.



**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 9/10, 15/82, C12P 7/64, C12N 15/29, 15/54, 1/21, 5/10, C12P 21/00	A3	(11) International Publication Number: WO 00/36095 (43) International Publication Date: 22 June 2000 (22.06.00)
(21) International Application Number: PCT/US99/28678 (22) International Filing Date: 3 December 1999 (03.12.99) (30) Priority Data: 09/205,815 4 December 1998 (04.12.98) US (71) Applicant: CALGENE LLC [US/US]; Patent Department Central, Monsanto G.D. Searle, P.O. Box 5110, Chicago, IL 60680-5110 (US). (72) Inventors: LARDIZABAL, Kathryn, Dennis; 1546 Owens Valley Road, Woodland, CA 95695 (US). METZ, James, George; 2803 Belhaven Place, Davis, CA 95616 (US). LASSNER, Michael, W.; 721 Falcon Avenue, Davis, CA 95616 (US). (74) Agent: SCHWEDLER, Carl, J.; Patent Department Central, Monsanto/G.D. Searle, P.O. Box 5110, Chicago, IL 60680-5110 (US).		(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). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 3 August 2000 (03.08.00)
(54) Title: FATTY ACYL-CoA: FATTY ALCOHOL ACYLTRANSFERASES (57) Abstract 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.		

FATTY ACYL-CoA: FATTY ALCOHOL ACYLTRANSFERASES

5 This application is a continuation in part of U.S. Pat. Application No. 09/205,815, which is a continuation in part of U.S. Patent Application No. 09/092,562, which is a continuation in part of U.S. Pat. Application No. 60/048,651 and a continuation of U.S. Patent No. 5,679,881.

10 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.

15 INTRODUCTION

Background

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.

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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.

5 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-Coenzyme A 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
10 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.

15 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
20 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.

25

SUMMARY OF THE INVENTION

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
30 substrates. This 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, although a given wax synthase may show preference

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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. The wax synthases are preferably obtained from plant sources.

Preferably, the wax synthase of this invention has activity towards at least those
5 acyl and alcohol substrates having a chain length of from 8 to 30 carbons, more preferably from 8 to 28 carbons. 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.

Thus, the present invention is directed to fatty acyl-CoA: fatty alcohol *O*-
10 acyltransferase (also referred to herein as wax synthase), and in particular to wax synthase polynucleotides. The polynucleotides of the present invention include those derived from plant sources.

One aspect of the present invention relates to oligonucleotides which include partial or complete wax synthase encoding sequences.

15 It is also an aspect of the present invention to provide recombinant DNA constructs which can be used for transcription or transcription and translation (expression) of wax synthase. In particular, constructs are provided which are capable of transcription or transcription and translation in host cells. Particularly preferred constructs are those capable of transcription or transcription and translation in plant cells.

20 In another aspect of the present invention, methods are provided for production of wax synthase in a host cell or progeny thereof. In particular, host cells are transformed or transfected with a DNA construct which can be used for transcription or transcription and translation of wax synthase. The recombinant cells which contain wax synthase are also part of the present invention.

25 In a further aspect, the present invention relates to methods of producing a wax ester in a host cell or progeny thereof. In particular, host cells are transformed or transfected with a DNA construct which can be used for transcription or transcription and translation of wax synthase in a host cell having fatty alcohol and fatty acid substrates. The wax ester so produced find use in a variety of applications including pharmaceuticals,
30 cosmetics, detergents, plastics, food applications and lubricants. In particular, the wax esters can be used in food applications as a low calorie fat.

In addition, the wax esters can be used as sources for the production of fatty alcohols and fatty acyl products. The wax esters can be subjected to hydrolysis conditions

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to produce fatty alcohols and fatty acyl products for use in a variety of applications, including, detergents, lubricants, corrosion inhibition, plasticizers, cosmetics, emulsifiers, and for use in pharmaceutical compositions.

5 In yet a further aspect of the present invention, methods of using polynucleotide and polypeptide sequences of the present invention to alter the oil content of a host cell are provided. In particular the methods involve growing a host cell having a recombinant nucleic acid construct comprising a promoter and a nucleic acid sequence which encodes an acyltransferase active in the formation of a wax ester. Such methods can be used in the production of host cells with modified oil/protein ratios.

10 The modified plants, seeds and oils obtained by the expression or suppression of the wax synthase proteins are also considered part of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 A calculation of the contribution of wax synthase to the production of very long chain fatty acids (VLCFAs).

Figure 2 Results of wax synthase activity assay using various alcohol substrates and 1 ug 16:0-CoA substrate.

20 Figure 3 Results of wax synthase activity assay using various alcohol substrates and 10 ug 16:0-CoA substrate.

Figure 4 Results of wax synthase activity assay using various alcohol substrates and 1 ug 24:1-CoA substrate.

Figure 5 Results of wax synthase activity assay using various alcohol substrates and 10 ug 24:1-CoA substrate.

25 Figure 6 provides the results of fatty acid methyl ester (FAME) analysis for oils obtained from *Arabidopsis* plants transformed with ATWS1 (lines 9708) and ATWS2 (lines 9712).

DETAILED DESCRIPTION OF THE INVENTION

30 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 (Ohlrogge *et al.* (*Lipids* (1978) 13:203-210), as well as *Murraya koenigii* (Karth, (1969) *Chemistry and Industry* 4:1342-1343 and Karth, *et al.* (1972) *Chemistry and Industry* 891-892), produce a seed storage wax. 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,

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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.

5 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.

10 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
15 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.

20 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*
25 *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
30 was obtained with good recovery of enzyme activity associated with fatty acylreductase, 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.

5 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
10 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
15 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-
20 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
25 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
30 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

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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.

- 5 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.

- 10 *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.

- 15 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
20 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
25 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
30 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

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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
5 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
10 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
15 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
20 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
25 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
30 leader or signal peptide of a precursor wax synthase protein that may be required for insertion or retention into the endoplasmic reticulum membrane, which may or may not be 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
 5 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.

Thus, another aspect of the present invention relates to isolated wax synthase polynucleotides. The polynucleotide sequences of the present invention include isolated
 10 polynucleotides that encode the polypeptides of the invention having a deduced amino acid sequence selected from the group of sequences set forth in the Sequence Listing and to other polynucleotide sequences closely related to such sequences and variants thereof.

The invention provides a polynucleotide sequence identical over its entire length to each coding sequence as set forth in the Sequence Listing. The invention also provides the
 15 coding sequence for the mature polypeptide or a fragment thereof, as well as the coding sequence for the mature polypeptide or a fragment thereof in a reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, pro-, or prepro- protein sequence. The polynucleotide can also include non-coding sequences, including for example, but not limited to, non-coding 5' and 3' sequences, such as the
 20 transcribed, untranslated sequences, termination signals, ribosome binding sites, sequences that stabilize mRNA, introns, polyadenylation signals, and additional coding sequence that encodes additional amino acids. For example, a marker sequence can be included to facilitate the purification of the fused polypeptide. Polynucleotides of the present invention also include polynucleotides comprising a structural gene and the naturally
 25 associated sequences that control gene expression.

The invention also includes polynucleotides of the formula:



wherein, at the 5' end, X is hydrogen, and at the 3' end, Y is hydrogen or a metal, R_1 and R_3 are any nucleic acid residue, n is an integer between 1 and 3000, preferably between 1
 30 and 1000 and R_2 is a nucleic acid sequence of the invention, particularly a nucleic acid sequence selected from the group set forth in the Sequence Listing and preferably SEQ ID NOs: 1,3,5,7,9,11,13,15, and 19. In the formula, R_2 is oriented so that its 5' end residue is at the left, bound to R_1 , and its 3' end residue is at the right, bound to R_3 . Any stretch of

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nucleic acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

The invention also relates to variants of the polynucleotides described herein that encode for variants of the polypeptides of the invention. Variants that are fragments of the polynucleotides of the invention can be used to synthesize full-length polynucleotides of the invention. Preferred embodiments are polynucleotides encoding polypeptide variants wherein 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues of a polypeptide sequence of the invention are substituted, added or deleted, in any combination. Particularly preferred are substitutions, additions, and deletions that are silent such that they do not alter the properties or activities of the polynucleotide or polypeptide.

Further preferred embodiments of the invention that are at least 50%, 60%, or 70% identical over their entire length to a polynucleotide encoding a polypeptide of the invention, and polynucleotides that are complementary to such polynucleotides. More preferable are polynucleotides that comprise a region that is at least 80% identical over its entire length to a polynucleotide encoding a polypeptide of the invention and polynucleotides that are complementary thereto. In this regard, polynucleotides at least 90% identical over their entire length are particularly preferred, those at least 95% identical are especially preferred. Further, those with at least 97% identity are highly preferred and those with at least 98% and 99% identity are particularly highly preferred, with those at least 99% being the most highly preferred.

Preferred embodiments are polynucleotides that encode polypeptides that retain substantially the same biological function or activity as the mature polypeptides encoded by the polynucleotides set forth in the Sequence Listing.

The invention further relates to polynucleotides that hybridize to the above-described sequences. In particular, the invention relates to polynucleotides that hybridize under stringent conditions to the above-described polynucleotides. As used herein, the terms "stringent conditions" and "stringent hybridization conditions" mean that hybridization will generally occur if there is at least 95% and preferably at least 97% identity between the sequences. An example of stringent hybridization conditions is overnight incubation at 42°C in a solution comprising 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 micrograms/milliliter denatured, sheared salmon sperm DNA, followed by washing the hybridization support in 0.1x SSC at approximately

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65°C. Other hybridization and wash conditions are well known and are exemplified in Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly Chapter 11.

The invention also provides a polynucleotide consisting essentially of a
5 polynucleotide sequence obtainable by screening an appropriate library containing the complete gene for a polynucleotide sequence set forth in the Sequence Listing under stringent hybridization conditions with a probe having the sequence of said polynucleotide sequence or a fragment thereof; and isolating said polynucleotide sequence. Fragments useful for obtaining such a polynucleotide include, for example, probes and primers as
10 described herein.

As discussed herein regarding polynucleotide assays of the invention, for example, polynucleotides of the invention can be used as a hybridization probe for RNA, cDNA, or genomic DNA to isolate full length cDNAs or genomic clones encoding a polypeptide and to isolate cDNA or genomic clones of other genes that have a high sequence similarity to a
15 polynucleotide set forth in the Sequence Listing. Such probes will generally comprise at least 15 bases. Preferably such probes will have at least 30 bases and can have at least 50 bases. Particularly preferred probes will have between 30 bases and 50 bases, inclusive.

The coding region of each gene that comprises or is comprised by a polynucleotide sequence set forth in the Sequence Listing may be isolated by screening using a DNA
20 sequence provided in the Sequence Listing to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the invention is then used to screen a library of cDNA, genomic DNA or mRNA to identify members of the library which hybridize to the probe. For example, synthetic oligonucleotides can be prepared which correspond to the wax synthase EST sequences.
25 The oligonucleotides can be used as primers in polymerase chain reaction (PCR) techniques to obtain 5' and 3' terminal sequence of wax synthase genes. Alternatively, where oligonucleotides of low degeneracy can be prepared from particular wax synthase peptides, such probes may be used directly to screen gene libraries for wax synthase gene sequences. In particular, screening of cDNA libraries in phage vectors is useful in such
30 methods due to lower levels of background hybridization.

Typically, a wax synthase sequence obtainable from the use of nucleic acid probes will show 60-70% sequence identity between the target wax synthase sequence and the encoding sequence used as a probe. However, lengthy sequences with as little as 50-60%

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sequence identity can also be obtained. The nucleic acid probes may be a lengthy 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

5 have 20-50% deviation (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 nucleotides. A higher degree of sequence identity is desired when shorter regions are used as opposed to longer regions. It

10 may thus be desirable to identify regions of highly conserved amino acid sequence to design oligonucleotide probes for detecting and recovering other related wax synthase genes. Shorter probes are often particularly useful for polymerase chain reactions (PCR), especially when highly conserved sequences can be identified. (See, Gould, *et al.*, *PNAS USA* (1989) 86:1934-1938.).

15 Another aspect of the present invention relates to wax synthase polypeptides. Such polypeptides include isolated polypeptides set forth in the Sequence Listing, as well as polypeptides and fragments thereof, particularly those polypeptides which exhibit wax synthase activity and also those polypeptides which have at least 50%, 60% or 70% identity, preferably at least 80% identity, more preferably at least 90% identity, and most

20 preferably at least 95% identity to a polypeptide sequence selected from the group of sequences set forth in the Sequence Listing, and also include portions of such polypeptides, wherein such portion of the polypeptide preferably includes at least 30 amino acids and more preferably includes at least 50 amino acids.

“Identity”, as is well understood in the art, is a relationship between two or more

25 polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, “identity” also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as determined by the match between strings of such sequences. “Identity” can be readily calculated by known methods including, but not limited to, those described in *Computational Molecular Biology*, Lesk,

30 A.M., ed., Oxford University Press, New York (1988); *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M. and Griffin, H.G., eds., Humana Press, New Jersey (1994); *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press (1987);

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Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., Stockton Press, New York (1991); and Carillo, H., and Lipman, D., *SIAM J Applied Math*, 48:1073 (1988).

Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly

5 available programs. Computer programs which can be used to determine identity between two sequences include, but are not limited to, GCG (Devereux, J., et al., *Nucleic Acids Research* 12(1):387 (1984); suite of five BLAST programs, three designed for nucleotide sequences queries (BLASTN, BLASTX, and TBLASTX) and two designed for protein sequence queries (BLASTP and TBLASTN) (Coulson, *Trends in Biotechnology*, 12: 76-10 80 (1994); Birren, et al., *Genome Analysis*, 1: 543-559 (1997)). The BLAST X program is publicly available from NCBI and other sources (*BLAST Manual*, Altschul, S., et al., NCBI NLM NIH, Bethesda, MD 20894; Altschul, S., et al., *J. Mol. Biol.*, 215:403-410 (1990)). The well known Smith Waterman algorithm can also be used to determine identity.

15 Parameters for polypeptide sequence comparison typically include the following:

Algorithm: Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970)

Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, *Proc. Natl. Acad. Sci USA* 89:10915-10919 (1992)

Gap Penalty: 12

20 Gap Length Penalty: 4

A program which can be used with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison Wisconsin. The above parameters along with no penalty for end gap are the default parameters for peptide comparisons.

25 Parameters for polynucleotide sequence comparison include the following:

Algorithm: Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970)

Comparison matrix: matches = +10; mismatches = 0

Gap Penalty: 50

Gap Length Penalty: 3

30 A program which can be used with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison Wisconsin. The above parameters are the default parameters for nucleic acid comparisons.

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The invention also includes polypeptides of the formula:



wherein, at the amino terminus, X is hydrogen, and at the carboxyl terminus, Y is hydrogen or a metal, R_1 and R_3 are any amino acid residue, n is an integer between 1 and 1000, and R_2 is an amino acid sequence of the invention, particularly an amino acid sequence selected from the group set forth in the Sequence Listing and preferably SEQ ID NOs: 2,4, 6, 8, 10, 12, 14, and 16. In the formula, R_2 is oriented so that its amino terminal residue is at the left, bound to R_1 , and its carboxy terminal residue is at the right, bound to R_3 . Any stretch of amino acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

Polypeptides of the present invention include isolated polypeptides encoded by a polynucleotide comprising a sequence selected from the group of a sequence contained in the Sequence Listing set forth herein .

The polypeptides of the present invention can be mature protein or can be part of a fusion protein.

Fragments and variants of the polypeptides are also considered to be a part of the invention. A fragment is a variant polypeptide which has an amino acid sequence that is entirely the same as part but not all of the amino acid sequence of the previously described polypeptides. The fragments can be "free-standing" or comprised within a larger polypeptide of which the fragment forms a part or a region, most preferably as a single continuous region. Preferred fragments are biologically active fragments which are those fragments that mediate activities of the polypeptides of the invention, including those with similar activity or improved activity or with a decreased activity. Also included are those fragments that antigenic or immunogenic in an animal, particularly a human.

Variants of the polypeptide also include polypeptides that vary from the sequences set forth in the Sequence Listing by conservative amino acid substitutions, substitution of a residue by another with like characteristics. In general, such substitutions are among Ala, Val, Leu and Ile; between Ser and Thr; between Asp and Glu; between Asn and Gln; between Lys and Arg; or between Phe and Tyr. Particularly preferred are variants in which 5 to 10; 1 to 5; 1 to 3 or one amino acid(s) are substituted, deleted, or added, in any combination.

Variants that are fragments of the polypeptides of the invention can be used to produce the corresponding full length polypeptide by peptide synthesis. Therefore, these

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variants can be used as intermediates for producing the full-length polypeptides of the invention.

The polynucleotides and polypeptides of the invention can be used, for example, in the transformation of host cells, such as plant host cells, as further discussed herein.

5 The invention also provides polynucleotides that encode a polypeptide that is a mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids within the mature polypeptide (for example, when the mature form of the protein has more than one polypeptide chain). Such sequences can, for example, play a role in the processing of a protein from a precursor to a mature form, allow protein transport, shorten
10 or lengthen protein half-life, or facilitate manipulation of the protein in assays or production. It is contemplated that cellular enzymes can be used to remove any additional amino acids from the mature protein.

A precursor protein, having the mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. The inactive precursors
15 generally are activated when the prosequences are removed. Some or all of the prosequences may be removed prior to activation. Such precursor protein are generally called proproteins.

For immunological screening, antibodies to the jojoba wax synthase can be prepared by injecting rabbits or mice with the purified protein. Methods of preparing
20 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 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
25 with the antibodies to the jojoba wax synthase. This 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, including radiolabeling of the antibody and second antibody/enzyme conjugate systems. Some of the available
30 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 isolated by screening expression libraries representing the desired

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plant species. Expression libraries can be constructed in a variety of commercially available vectors, including lambda gt11, as described in Maniatis, *et al.* (*supra*).

5 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 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.

10 Alternatively, databases containing nucleic acid and amino acid sequences from various organisms may be searched with the sequences of the present invention to identify similar sequences. Surprisingly, using the jojoba wax synthase protein sequence to search a database containing DNA sequences from *Arabidopsis*, an approximately 12 Kb sequence containing at least seven repeats of an open reading frame with high similarity to the jojoba sequence are identified. The deduced amino acid sequences demonstrate a high level of homology to the jojoba wax synthase amino acid sequence. For example, amino acid sequence comparisons between jojoba wax synthase and the sequences obtained from the *Arabidopsis* homologues reveals an identity ranging from about 36% to about 44% between the amino acids. Thus, as shown herein, homologous similarity (identity + similarity) of at least 52% is shown in protein sequence comparisons between the jojoba and *Arabidopsis* sequences.

20 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 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 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 invention.

30 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 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

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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
5 the codons used most frequently in the proteins expressed in a desired host species.

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 may contain the sequence which encodes the entire wax
10 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 necessary for a desired wax synthase activity may thus be prepared.

Useful systems for expression of the wax synthase sequences of this invention
15 include prokaryotic cells, such as *E. coli*, yeast cells, and plant cells, both vascular and 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 protein.

20 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 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
25 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
30 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,

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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 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 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 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 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 (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,

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i.e., having transcription initiation regions derived from one 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
5 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
10 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
15 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
20 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
25 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
30 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

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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
5 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
10 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
15 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
20 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
25 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
30 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

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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.

Where expression of the wax synthase, as well as other genes involved in wax synthesis, is to be directed in other plant tissues, additional promoters may find use in the constructs of the present invention. For example, where preferential expression of the genes in the pollen is desired, promoter regions, for example, Rop1At (Li, *et al.* (1998) *Plant Physiol.* 118:407-417) and others described by Hamilton, *et al.* (1998) *Plant Mol Biol.* 38(4):663-669 may be employed. Where preferential expression in the epidermis is desired, promoter regions such as Ntltp1 (Canevascini, *et al.* (1996) *Plant Physiol.* 112:513-524) may be employed.

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 targeting 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.

5 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,
10 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
15 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
20 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
25 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. The constructs may also provide for the expression of a third gene encoding, for example β -ketoacyl-CoA synthase (KCS).
30 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

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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
5 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
10 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.

In addition, by engineering wax synthesis systems into plants which normally do not produce wax, it may be possible to increase the production of novel fatty acids. For
15 example, there may be intrinsic limitations in oilseed triacylglyceride and phospholipid synthesis which prefer specific fatty acids for a given position on the glycerol backbone. Therefore, plants engineered to produce a given "exotic" fatty acid may be limited in the amount of that fatty acid produced due to the limitations of positions on the glycerol backbone. Thus, by introducing a wax synthesis system into a plant, it may be possible to
20 increase the amount of an "exotic" fatty acid produced by providing an alternative fatty acid sink. Furthermore, it is also possible to increase the amount of an "exotic" fatty acid using a wax synthesis system derived from a source which accumulates wax esters which are composed of such fatty acids. For example, by utilizing a wax synthase from *Euglena*, it is possible to increase the amount of C12 fatty acids.

25 Furthermore, the introduction of a wax synthesis system into oil producing plants may allow for a further increase in the amount of oil produced as a component of the host cell. By expressing a wax synthesis system in an oil producing plant tissue, such as an oil seed, it is possible to further increase the amount of oil produced in such a tissue, by utilizing the native oil production, such as through triacylglycerol synthesis, and the
30 introduced wax synthesis.

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

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many industries, including pharmaceuticals, cosmetics, detergents, plastics, food applications 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
5 having saturated carbon chain components, may be solid at room temperature, especially if the saturated carbon chains are longer carbon chains.

The wax esters so produced can be used as a source for obtaining fatty alcohols for use in a variety of applications. Methods for the production of fatty alcohols from wax esters are known in the art, and are described for example by Duncan, *et al.* (1974) *J. Am.*
10 *Oil Chem. Soc.* 51(12):534-536. Fatty alcohols find use in a variety of applications known to the skilled artisan, and include but are not limited to detergents, lubricants, corrosion inhibition, plasticizers, cosmetics, emulsifiers, and for use in pharmaceutical compositions. The fatty alcohols produced can be saturated or unsaturated, and preferably include short chain fatty alcohols, medium chain fatty alcohols and long chain fatty
15 alcohols, most preferred are medium and long chain fatty alcohols, most especially preferred are long chain fatty alcohols.

Furthermore, production of transgenic plants which produce wax esters in the epidermal cell layer provides for enhanced tolerance to various environmental stresses, such as drought tolerance, as well as pathogen and insect tolerances. Such roles for
20 epicuticular waxes are known in the art, and are reviewed, for example, by Post-Beittenmiller (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47:405-430 and Jeffree (1986) *Insects and the Plant Surface* (Southwood and Juniper, eds.) 23-64 (Edward Arnold).

The method of transformation is not critical to the instant invention; various
25 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
30 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.

5

EXAMPLES

Example 1 - Wax synthase Assays

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

A. Radiolabeled Material

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-docosenoic acid and 15-cis-tetracosenoic acid are prepared by the reaction of potassium [1-¹⁴C]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 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 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. [1-¹⁴C]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 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-
5 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 accommodate the preference of the acyl-CoA reductase enzyme. Membrane preparations were later changed to the second
10 buffer system to accommodate 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
15 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.

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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- ^{14} C-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 sodium chloride. 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.

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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.

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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 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%.

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.

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 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 construction were thus harvested

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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.

B. Microsomal Membrane Preparation

5 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 pounding in a steel mortar and pestle at liquid nitrogen temperature. In a typical experiment, 70g of
10 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 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
15 embryos in the buffer with a tissue 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 supernatant fraction is collected and dialyzed overnight (with
20 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 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
25 is estimated at 34% of the original activity in the cell free homogenate. Wax synthase 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
30 above to determine the range of substrates recognized by the jojoba wax synthase.

Acyl-CoA and alcohol substrates having varying carbon chain lengths and degrees of unsaturation were added to microsomal membrane fractions prepared as described in Example 3A to determine the range of substrates recognized by the jojoba wax synthase.

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Assays were performed as described in Example 1B using the Tricine buffer system with the following change, both acyl-CoA and alcohol concentrations were 40 μ M instead of the 200 μ M alcohol concentration normally used. Acyl-CoA's were prepared as 2.5 mM stocks (in 1.25 mM Na Acetate buffer pH 4.8 and 1.5% CHAPS) and 4 μ l of these stocks were used in a 250 μ l assay making the final CHAPS concentration 0.024%. Without the addition of detergent, the long-chain saturated acyl-CoA's would not dissolve in the buffer. Alcohols were prepared as 25 mM stocks in 2-methoxyethanol and 0.4 μ l of the stock was used in a 250 μ l assay. To evaluate the acyl-CoA specificity, 1-¹⁴C-hexadecanol (10.3 mCi/mmol, Sigma 31,326-2) was used as substrate. The purchased 1-¹⁴C-hexadecanol was only 62% pure and had to be further purified by thin layer chromatography prior to use. The product was spotted onto a glass silica gel TLC plate and migrated in hexane:diethyl ether:acetic acid (70:30:2). Unlabeled alcohol was spotted in outside lanes and used to identify the migration level of the radiolabeled product. The TLC plate was briefly exposed to iodine vapors to identify the location of the alcohol. The 1-¹⁴C-hexadecanol spot was scraped from the TLC plate and transferred to a new vial. The product was eluted from the silica with hexane/isopropanol and the organic extract was filtered to remove silica. The filtered solvent was transferred to a new vial where the solvent was evaporated to dryness. The final product was resuspended in 2-methoxyethanol at a concentration of 0.15 μ Ci/ μ l. The final product appeared to be 100% pure by TLC in the solvent system above. The 1-¹⁴C-16:0-CoA was as described in Example 1. Results of these experiments are presented in Table 1 below.

Table 1

Structure	Acyl Group	pmol/min/mg
		Alcohol Group
8:0	147.5	2656.3
10:0	197.8	2396.8
12:0	345.5	5663.4
14:0	1584.6	4919.1
16:0	1533.8	5250.6
18:0	1693.8	2557.9
20:0	1373.2	1666.4
22:0	1196.6	1555.9
24:0	1308.3	2582.0
18:1 9-c	821.9	12623.8
18:1 9-t	nd	12600.2
18:1 11-c	nd	12147.6
18:1 11-t	nd	13739.4
18:2 9-c,12-c	198.8	11344.2
18:3 9-2,12-c,15-c	516.1	nd
20:1 11-c	3880.1	6172.4
22:1 13-c	916.8	2783.0
22:1 13-t	nd	1862.0
24:1 15-c	1794.0	1576.3

For comparison purposes it is desirable to evaluate acyl-CoA's and alcohol's at equivalent concentrations, however, in practicality there is an endogenous pool of alcohol present in jojoba microsomal fractions of unknown concentration. This pool dilutes the ¹⁴C labeled hexadecanol used to evaluate the acyl-CoA's rendering some of the waxes formed undetectable. The result is an underestimation of the specific activity for all of the acyl-CoA's relative to the specific activities of the alcohols.

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

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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 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,

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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.

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.

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, 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

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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) 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

5

D. SDS PAGE Analysis

Samples from the column fractions were diluted in SDS PAGE sample buffer (1x buffer = 2% SDS, 250 mM β -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.* (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% 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. 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

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not appear as a major component of the 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.

5 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 cell fitted with a YM 30 membrane (Amicon, Inc., Beverly, MA). The sample was further
10 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
15 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.

20 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 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
25 at 4 C) to pellet the precipitated protein. The supernatants were removed and the pellets washed twice with 0.6 ml ice cold 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 volume of 60 μ l for each
30 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 was stained with Coomassie Blue and destained using Gel-Clear (Novex, San

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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% 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 sample concentrated by ultrafiltration and three gel slices from the samples concentrated by precipitation were pooled for in-gel tryptic digestion.

10 **Example 5 - Determination of Amino Acid Sequence**

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. 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.

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

Amino Acid Sequence of Jojoba Wax Synthase Tryptic Peptides

25	WSpep29	FVPAVAPHGGALR (SEQ ID NO:20)
	WSpep33	TIDEYPVMFNVTQK (SEQ ID NO:21)

Example 6 - Purification of Additional Wax Synthases and Reductases

30 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
5 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
10 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
15 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.

20 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
25 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.

30 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

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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.

5

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.

20 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-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 (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 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 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 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. 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% 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 200µl 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

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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.

- 5 Alternatively, the enzyme may be composed of subunits which are dissociated during SDS-PAGE.

Example 7 - Isolation of Wax Synthase Nucleic Acid

10 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
- 15 cDNA or genomic DNA library by radiolabeling the oligonucleotides or previously isolated sequences for use as probes.

A. Construction of Jojoba cDNA Libraries

RNA may be isolated using the methods described by Cathala, *et al.* (1983) *DNA*, 3:329-335. RNA is isolated from jojoba embryos collected at 80-90 days post-anthesis
 5 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
 10 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%
 15 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
 20 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
 25 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 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
 30 ethanol. After several hours at -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 4°C. The aqueous phase is

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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 previously described, to yield the polyribosomal RNA.

5 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 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
10 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 *Bam*HI, treatment with mung bean
15 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
20 "*Sac*I" 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.

25 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
30 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-

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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
5 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/Eco*RI (Stratagene, San Diego, CA). The library is
10 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.

15 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
20 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
25 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
30 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

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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

5 WSPEP33.

5' TTY GTN CCN GCN GTN GCN CCN CAY GGNGGN GCN YTN MGN 3' (SEQ ID NO:22)

F V P A V A P H G G A L R (SEQ ID NO:20)

10 3' AAR CAN GGN CGN CAN CGN GGN GTR CCNCCN CGN RAN KCN 5' (SEQ ID NO:23)

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

15 WSPEP29.

5' ACN ATH GAY GAR TAY CCN GTN ATG TTY AAY TAY ACN CAR AAR 3' (SEQ ID NO:24)

T I D E Y P V M F N Y T Q K (SEQ ID NO:21)

20 3' TGN TAD CTR CTY ATR GGN CAN TAC AAR TTR ATR TGN GTY TTY 5' (SEQ ID NO:25)

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

25 WSPEP14.

5' TTY MGN GAYGAY CCN WSN AAY GAY CAY 3' (SEQ ID NO:27)

F R D D P S N D H (SEQ ID NO:26)

3' AAR KCN CTRCTR GGN WSN TTR CTR GTR 5' (SEQ ID NO:28)

30

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

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name designates a PCR forward reaction primer. The letter "R" designates a PCR reverse reaction primer.

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	WSPEP29-F1	5' TTYGTNCCNGCNGTNGC 3' (SEQ ID NO:29)
	WSPEP29-F2	5' GCNCCNCA YGGNGGNGC 3' (SEQ ID NO:30)
	WSPEP29-R1	5' GCNCCNCCRTGNGGNGC 3' (SEQ ID NO:31)
5	WSPEP29-R2	5' GCNACNGCNGGNACRAA 3' (SEQ ID NO:32)
	WSPEP33-F1	5' ACNATHGAYGARTAYCCNGT 3' (SEQ ID NO:33)
	WSPEP33-F2	5' CCNGTNATGTTYAAAYTAYAC 3' (SEQ ID NO:34)
	WSPEP33-R1	5' TTYTGNGTRTARTTRAACAT 3' (SEQ ID NO:35)
	WSPEP33-R2	5' AACATNACNGGRTAYTCRTC 3' (SEQ ID NO:36)
10	WSPEP14-F1	5' GAYGAYCCNWSNAA YGAYCA (SEQ ID NO:37)
	WSPEP14-R1	5' TGRTCRTTNSWNGGRTCRTC (SEQ ID NO:38)

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

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

25

C. PCR Reactions

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
30 Marathon cDNA Amplification Kit (Clontech Laboratories Inc according to the manufacturer's directions. The jojoba cDNA is used in PCR reactions 1-16 set forth below.

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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 reaction and optimization reagents are used according to manufacturer's specifications

5

	<u>Reaction</u>	<u>Forward Primer</u>	<u>Reverse Primer</u>
	1	WSPEP14-F1	WSPEP29-R1
	2	WSPEP14-F1	WSPEP29-R2
	3	WSPEP14-F1	WSPEP33-R1
10	4	WSPEP14-F1	WSPEP33-R2
	5	WSPEP29-F1	WSPEP14-R1
	6	WSPEP29-F1	WSPEP33-R1
	7	WSPEP29-F1	WSPEP33-R2
	8	WSPEP29-F2	WSPEP14-R1
15	9	WSPEP29-F2	WSPEP33-R1
	10	WSPEP29-F2	WSPEP33-R2
	11	WSPEP33-F1	WSPEP14-R1
	12	WSPEP33-F1	WSPEP29-R1
	13	WSPEP33-F1	WSPEP29-R2
20	14	WSPEP33-F2	WSPEP14-R1
	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

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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 SEQ ID NO:17.

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:

10	WSRACEF1 (SEQ ID NO:39)	GATTTGCCTCATTTTGTGATCTCGGTGCT
	WSRACEF2 (SEQ ID NO:40)	GACCTATACCCCCAGTTCAACGAGCCATAC
	WSRACEF3 (SEQ ID NO:41)	TTCAACGAGCCATACTTAGCCACCTCGCTG
15	WSRACER1 (SEQ ID NO:42)	AACAACCACCCTCCAGTCACCATCACGAAC
	WSRACER2 (SEQ ID NO:61)	TTGCCTGAAACCGCCTTCTTCACCACCATC
20	WSRACER3 (SEQ ID NO:43)	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.

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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

5 WAXSYNFOR is GGATCCGTCGACACAATGGAGGTGGAGAAGGAGCTAAAG (SEQ ID NO:44), and the sequence of WASXYNREV is GCATGCAGATCTCACCACCCCAACAAACCCATC (SEQ ID NO:45). 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

10 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 manufacturers protocol (Invitrogen Corp.). The resulting plasmid was designated pCGN8538. The nucleic acid sequence and the derived amino acid sequence of the jojoba wax synthase is determine and provided in SEQ ID NO:1 and SEQ ID NO:2, respectively.

15

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

20 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

25 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

30 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-O

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according to the vacuum infiltration protocol of Bent et al. (1994, Science 265:1856-1860).

Example 9 Analysis of Developing *Arabidopsis* Seed

5 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
10 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
15 a band which comigrated with a wax standard in 5 of the 7 plants analyzed. 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.
20 A positive control for wax activity was the jojoba (DP2) membrane fraction.

Example 10 Analysis of the seed oil

A. Thin-layer chromatographic analysis

Seeds from *Arabidopsis* plants were homogenized in hexane using a mortar and
25 pestle. The hexane was transferred to a new vial and evaporated under nitrogen gas and the oil was resuspended in hexane at a concentration of 10mg/ml. 10 μ l of the oil solution was spotted on a silica gel-G TLC plate and the chromatogram was developed in hexane:diethyl ether:acetic acid (80:20:1). The lipids were stained with iodine. Elevated levels of wax were seen in the oil extracted from plants expressing pCGN8559 that also
30 tested positive for wax synthase activity by assay. Intermediate levels of wax were detected in seeds from pCGN8557 which contained only reductase/KCS and low, background levels of wax were detected in *Arabidopsis* No-O controls.

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B. Gas Chromatographic Analysis

The oil in about 10mg of *Arabidopsis* seed was derivatized to form fatty acid methyl esters (FAME) and free alcohol. The extractable lipid was analyzed for FAME and alcohol content by gas chromatography. Internal standard 17:0 TAG was spiked into the lipid fraction and derivatization was carried out at 90° C in the presence of acidic methanol and toluene. Lipid products were extracted by adding hexane and 1M aqueous NaCl. The fatty acid methyl esters (FAME) and alcohols were separated on a 0.25mm x 15m Supelcowax-10 column. Percent composition of the products was determined by their weight contribution to the sample (Table 3A). A correction factor for the Flame Ionization Detector (FID) response, determined experimentally to be 1.155, was used to adjust the quantitation of the alcohol peak relative to the 17:0 methyl ester standard. The weight composition was used to determine the percentage of very long chain carbon groups (>18C) present (Table 3B).

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A															Fatty Acyl Groups										Fatty Alcohols				B	
Sample	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	22:2	24:0	24:1	OH18:1	OH20:1	OH22:1	OH24:1	VLCFA wt %											
8559-3	6.1	0.3	2.3	14.7	21.5	19.3	1.1	12.2	1.0	0.8	9.8	0.1	1.0	3.4	0.1	0.5	3.3	2.4	35.7											
8559-4	6.1	0.3	2.8	17.5	25.1	18.9	2.2	22.1	1.9	0.3	2.4	0.1	0.1	0.2	0.0	0.0	0.0	0.0	29.4											
8559-5	6.1	0.3	2.8	17.2	25.4	18.9	2.3	22.0	1.9	0.3	2.4	0.1	0.1	0.2	0.0	0.0	0.0	0.0	29.3											
8559-6	6.0	0.3	2.7	17.9	25.4	18.9	2.2	21.7	1.9	0.3	2.4	0.1	0.1	0.2	0.0	0.0	0.0	0.0	28.8											
8559-7	6.0	0.3	2.6	17.0	25.2	19.6	2.2	22.1	1.9	0.3	2.4	0.1	0.1	0.2	0.0	0.0	0.0	0.0	29.4											
8559-8	5.9	0.3	2.2	14.9	21.4	18.7	1.1	13.5	1.1	0.7	8.7	0.2	0.6	2.0	0.1	1.0	5.3	2.2	36.5											
8559-9	5.9	0.4	2.0	13.3	21.7	14.8	1.2	12.9	1.1	0.7	8.8	0.2	1.7	6.5	0.1	0.4	3.8	4.4	42.0											
8559-10	5.7	0.4	1.9	13.5	20.6	19.0	1.0	10.7	0.9	0.8	10.1	0.2	1.3	5.5	0.0	0.5	4.2	3.6	38.9											
8559-11	5.5	0.2	1.6	10.9	17.5	17.8	0.7	8.9	0.7	0.9	11.3	0.2	1.3	5.3	0.2	1.1	9.3	6.5	46.5											
8559-13	6.1	0.3	2.8	16.8	24.3	18.8	2.1	19.4	1.6	0.5	5.3	0.1	0.4	1.3	0.0	0.1	0.1	0.1	31.0											
8559-14	5.6	0.3	2.7	18.3	24.4	18.5	2.2	22.5	2.0	0.3	2.8	0.1	0.1	0.2	0.0	0.0	0.0	0.0	30.2											
8559-15	6.1	0.5	1.8	12.6	20.8	16.4	1.0	11.8	1.0	0.8	9.4	0.2	1.1	4.5	0.2	0.8	6.7	4.3	41.9											
8559-16	5.9	0.3	2.8	17.9	24.4	18.5	2.3	22.6	1.9	0.3	2.6	0.1	0.2	0.2	0.0	0.0	0.0	0.0	30.2											
8559-17	5.8	0.3	2.5	16.7	23.2	18.9	1.7	17.2	1.4	0.6	7.3	0.1	0.5	2.3	0.0	0.2	0.7	0.5	32.5											
8559-18	5.7	0.4	1.7	10.9	18.5	16.4	1.0	12.4	1.0	0.8	8.3	0.2	1.2	4.3	0.0	1.3	9.9	5.9	46.4											
8557-1	5.6	0.3	2.4	16.6	22.5	19.6	1.5	15.4	1.3	0.6	8.0	0.2	0.5	2.7	0.0	0.1	1.1	1.5	33.0											
8557-3	5.9	0.3	2.6	16.6	22.3	21.1	2.1	21.4	2.1	0.3	2.8	0.1	0.1	0.2	0.0	1.8	0.2	0.0	31.1											
8557-4	6.4	0.4	2.5	14.1	23.0	17.7	1.4	12.8	1.3	0.8	8.8	0.2	0.8	2.5	0.0	0.3	2.9	4.2	35.9											
8557-5	5.6	0.2	2.3	13.6	21.9	21.8	1.1	11.3	1.3	0.8	10.9	0.2	0.7	3.2	0.0	0.2	2.1	2.8	34.6											
8557-6	5.8	0.4	2.6	14.4	23.1	19.6	1.4	14.3	1.4	0.7	8.8	0.2	0.7	3.1	0.0	0.1	1.3	2.1	34.1											
8557-7	5.5	0.3	2.4	13.7	23.2	21.0	1.6	17.4	1.7	0.6	8.9	0.2	0.5	2.8	0.0	0.0	0.1	0.2	34.0											
8557-8	6.0	0.4	2.3	12.6	21.5	20.9	0.9	8.7	1.0	0.9	12.3	0.3	0.9	4.3	0.0	0.2	2.6	4.0	36.2											
8557-9	5.6	0.3	2.4	14.4	22.5	21.7	1.2	12.6	1.3	0.7	10.5	0.2	0.6	3.1	0.0	0.1	1.2	1.4	33.1											
8557-10	6.0	0.3	2.5	14.3	22.6	21.1	1.2	11.7	1.3	0.8	10.1	0.2	0.7	3.0	0.0	0.2	1.9	2.4	33.3											

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8557-11	6.0	0.4	2.5	13.2	22.4	19.5	1.2	11.8	1.2	0.8	10.1	0.2	0.9	3.8	0.0	0.2	2.2	3.6	36.0
8557-12	5.7	0.3	2.8	16.3	24.5	19.4	2.1	23.4	2.1	0.3	2.6	0.1	0.1	0.2	0.0	0.0	0.0	0.0	30.9
8557-13	6.1	0.3	2.5	13.3	22.3	21.2	1.1	11.2	1.2	0.8	10.6	0.2	0.7	3.0	0.0	0.2	2.1	3.0	34.2
8557-14	5.9	0.2	2.5	13.4	23.0	19.4	1.1	11.4	1.3	0.8	10.9	0.2	0.7	2.9	0.0	0.2	2.7	3.2	35.5
No-O-1	6.5	0.4	3.1	15.6	26.3	17.7	2.7	22.1	2.1	0.4	2.6	0.2	0.2	0.2	0.0	0.0	0.0	0.0	30.5
No-O-2	6.4	0.4	3.1	15.6	26.2	17.9	2.7	22.0	2.1	0.4	2.6	0.2	0.2	0.2	0.0	0.0	0.0	0.0	30.4
No-O-3	6.4	0.4	3.1	15.6	26.3	17.6	2.7	22.1	2.1	0.4	2.6	0.2	0.2	0.2	0.0	0.0	0.0	0.0	30.6

C. ^{13}C -NMR analysis of Intact Arabidopsis seed

TLC analysis showed that plants expressing pCGN8559 did not contain any free alcohol therefore the alcohol peak present in the transmethylated samples was likely due to the derivatization of wax present in the seed oil. Based on this evidence it is estimated the amount of wax in plants expressing pCGN8559 at twice the weight of the alcohol detected by gas chromatography (Table 4A). This was not the case for plants expressing pCGN8557 where both alcohol and wax spots were visible by TLC after exposure to iodine vapor. In order to find a more direct measure of the amount of wax present, ^{13}C -NMR spectra is obtained for intact mature Arabidopsis seed to determine the molar ratio of wax and TAG. A region of the spectra between 60-70 ppm was identified as containing unique carbon shifts for the *sn*-2 carbon of the glycerol backbone at 69.5 ppm, representing the molar amount of TAG, and the first carbon on the alcohol side of the wax ester linkage at 64.2 ppm, representing the molar amount of wax. Ratios of these two unique carbons were determined. From these ratios the mole percent wax was calculated (Table 4B). The mole percent data was converted to weight percent data using the following conversion:

$$\text{Wt\%} = 100 * \frac{(\text{mole\%wax} * \text{molecular weight wax})}{(\text{mole\%wax} * \text{MW wax}) + [(100 - \text{mole\%wax}) * \text{MW TAG}]}$$

The molecular weight of wax and TAG was assigned as 20:1-20:1 wax and tri 20:1 TAG.

The weight percent of wax calculated by the GC method was in agreement with the weight percent wax determined by the nondestructive measurement using ^{13}C -NMR. Using this method a determination of the mole percent wax present in the plants expressing pCGN8557 is obtained. A calculation of the contribution of wax synthase to the production of very long chain fatty acids (VLCFAs) is shown in Figure 1.

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Table 4

Sample	GC wt% OH	GC wt% wax	NMR wt% wax	Wax mole% wax
8559-3	6.2	12.4	12.8	19.4
8559-4	0.0	0.0	0.0	0.0
8559-5	0.0	0.0	0.0	0.0
8559-6	0.0	0.0	0.0	0.0
8559-7	0.0	0.0	0.0	0.0
8559-8	8.6	17.1	17.2	25.4
8559-9	8.7	17.5	nd	nd
8559-10	8.4	16.7	15.5	23.1
8559-11	17.1	34.1	37.2	49.2
8559-13	0.3	0.5	nd	nd
8559-14	0.0	0.0	nd	nd
8559-15	12.0	24.1	24.4	34.6
8559-16	0.0	0.0	0.0	0.0
8559-17	1.4	2.8	1.9	3.0
8559-18	17.2	34.3	34.9	46.8
8557-1	2.7	---	nd	nd
8557-3	2.0	---	1.2	2.0
8557-4	7.4	---	9.9	15.3
8557-5	5.1	---	5.8	9.1
8557-6	3.6	---	3.8	6.0
8557-7	0.2	---	0.0	0.0
8557-8	6.8	---	10.4	16.0
8557-9	2.8	---	2.4	3.8
8557-10	4.4	---	6.3	9.9
8557-11	6.0	---	9.9	15.3
8557-12	0.0	---	0.0	0.0
8557-13	5.4	---	4.4	7.0

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8557-14	6.1	---	7.4	11.5
No-O-1	0.0	---	0.0	
No-O-2	0.0	---	0.0	
No-O-3	0.0	---	nd	

Example 11 Analysis of Transgenic Arabidopsis Leaves

5 Rosette leaves from plants transformed with construct pCGN 8593, expressing wax synthase under the control of the 35S promoter, were analyzed for wax synthase activity. Leaves were homogenized in 200 μ l homogenization buffer (100 mM Tricine/NaOH, pH 7.8, 280 mM NaCl, 10 % glycerol, and protease inhibitors 0.1 uM Aprotinin, 1 μ M Leupeptin, and 100 μ M Pefabloc) and the solids were pelleted by centrifugation at 16,000 x g for 10 min and 4° C. The
10 supernatant was removed and the pellet resuspended in 200 μ l homogenization buffer. This fraction was referred to as the P1 fraction. Five control leaves harvested from Arabidopsis cultivar No-O were assayed as controls. The P1 fractions were assayed for wax synthase activity as described in Example 1B. Wax synthase activity in the plants expressing pCGN8593 was compared with the average activity found in the No-O controls. Eleven of the 24 plants analyzed
15 demonstrated activity more than twice the average background level found in No-O. One plant demonstrated nearly a 10-fold increase in activity.

Example 12 Identification of Additional Wax Synthase Sequences

20 The protein sequence of the jojoba wax synthase (SEQ ID NO:2) is used to query the Arabidopsis DNA sequence database (<http://genome-www.stanford.edu/Arabidopsis/>). One of the accessions, P1 clone MTE17(Genbank accession AB015479), contains 7 repeats of open reading frames with similarity to the jojoba wax synthase. The open reading frames have been designated ATWS1 to ATWS7 (SEQ ID NOs: 3, 5, 7, 9, 11, 13,
25 and 15, respectively). They are found between nucleotides 23670 and 11479 of MTE17 using the numbering system of the Genbank entry. The inferred protein sequences, SEQ ID NOs: 4, 6, 8, 10,12, 14, and 16, respectively. The conserved peptide sequences

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between the amino acid sequences (Table 5). The percent identities and similarities are also determined, and are presented in table 6.

Table 5

	SEQUENCE	SEQ ID NO:
1	LXLF(A/S)(F/L)XX(G/E)	46
2	PYL(A/S)TSL(Q/H)(D/E)FW(G/S)(R/H)RWNL(M/I)V	47
3	FX(V/T)SGXXHEXX(F/Y)FYX(I/T)R	48
4	P(S/T)(W/G)EV(T/A)XFF(V/L)LHG	49

5

Table 6

SEQUENCES COMPARED	PERCENT IDENTITY	PERCENT SIMILARITY
JoWS vs ATWS1	41%	17%
JoWS vs ATWS2	37%	19%
JoWS vs ATWS3	42%	15%
JoWS vs ATWS4	42%	16%
JoWS vs ATWS5	44%	13%
JoWS vs ATWS6	41%	17%
JoWS vs ATWS7	36%	16%

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ATWS3 vs ATWS6	59%	14%
ATWS3 vs ATWS1	49%	17%
ATWS3 vs ATWS4	71%	10%
ATWS3 vs ATWS7	55%	15%
ATWS3 vs ATWS2	49%	21%
ATWS3 vs ATWS5	64%	14%
ATWS6 vs ATWS1	47%	18%
ATWS6 vs ATWS4	57%	15%
ATWS6 vs ATWS7	51%	17%
ATWS6 vs ATWS2	45%	18%
ATWS6 vs ATWS5	57%	15%
ATWS1 vs ATWS4	52%	17%
ATWS1 vs ATWS7	46%	19%
ATWS1 vs ATWS2	65%	12%
ATWS1 vs ATWS5	49%	17%
ATWS4 vs ATWS7	58%	13%
ATWS4 vs ATWS2	50%	19%
ATWS4 vs ATWS5	65%	13%
ATWS7 vs ATWS2	45%	18%
ATWS7 vs ATWS5	55%	16%
ATWS2 vs ATWS5	49%	17%

Complementary DNA (cDNA) is constructed from Arabidopsis RNA isolated from immature seeds, whole seedlings (vegetative tissue), and inflorescences (flowers and flower stalks) using the SMART PCR cDNA Library construction kit according to the manufacturer's protocol (Clontech). SMART cDNA is also constructed from RNA from Brassica napus leaves, and immature seeds harvested at 15 days after pollination (DAP), 18 DAP, and 30 DAP. The SMART cDNAs are used for virtual Northern analysis, according to the protocol in the SMART cDNA manual from Clontech, of expression of the Arabidopsis ATWS cDNAs. ATWS2 is most highly expressed in Arabidopsis immature seeds and Brassica 30 DAP seeds. Expression is not detected in Brassica leaves

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or *Arabidopsis* seedlings. This is an expression pattern consistent with that expected for DAGAT, since triglycerides are primarily formed in developing seeds of these plants.

In addition, an expressed sequence tag (EST) sequence is identified from databases containing protein and nucleic acid sequences obtained from soybean (*Glycine*
5 *max*). This sequence is provided in SEQ ID NO:19.

Example 13 Expression Constructs for AT-WS Homologues

13A. Expression in Insect Cell Cultures

Constructs are prepared to direct the expression of the *Arabidopsis* wax synthase
10 homologous sequence in cultured insect cells. The entire coding region of the *Arabidopsis* wax synthase sequences, ATWS1 (SEQ ID NO:3) and ATWS2, (SEQ ID NO:5) and ATWS3 (SEQ ID NO:7) are amplified in a polymerase chain reaction (PCR) and subcloned into pCR2.1 (Invitrogen) to produce pCGN9705, pCGN9706 and pCGN9707, respectively. Double stranded DNA sequence was obtained to verify that no errors were
15 introduced by PCR amplification.

The fragment containing the entire coding region of the *Arabidopsis* ATWS1 and ATWS2 sequences were subcloned into baculovirus expression vector pFastBac1 (Gibco-BRL, Gaithersburg, MD).

A baculovirus expression construct containing the ATWS1 and ATWS2 sequences
20 are transformed and expressed using the BAC-to-BAC Baculovirus Expression System (Gibco-BRL, Gaithersburg, MD) according to the manufacturers directions, except harvesting of recombinant viruses was done 5 days post-transfection. The supernatant from the transfection mixture is used for generating virus stock which in turn is used for infecting Sf9 cells for use in the assay.

25 The transformed insect cells are assayed for wax synthase activity using methods described herein. Insect cells are centrifuged and the resulting pelleted cells were resuspended in Medium I (0.25 M sucrose and 1 mM EDTA in 10 mM Tris-Cl, pH 7.4) and homogenized on ice. The homogenate is centrifuged at 105,000 x g for 1 at 4° C. Total membranes are resuspended in Medium I.

30 Wax synthase activity is determined using two different acyl-CoA substrates, 16:0-CoA, and 24:1-CoA, and five different alcohol substrates, 16:0-OH, 18:0-OH, 18:1-OH, 20:1-OH, 22:1-OH, and 24:1-OH. Wax synthase activity is assayed in a 0.25 ml reaction mixture containing 50 mM Tricine, pH 7.8, 2 mM β -mercaptoethanol, 250 mM sodium

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chloride, 200 μ M respective alcohol (40 μ M 18:1-OH) and, 20 μ M 1- ^{14} C palmitoyl-CoA, or 1- ^{14} C nervonoyl-CoA. The reactions were run at 25°C for 10 minutes.

The results of the wax synthase activity assay demonstrates that the protein encoded by the ATWS1 sequence has wax synthase activity for all acyl-CoA and alcohol substrates tested (Figures 2, 3, 4, and 5). The wax synthase particularly has greater activity for longer chain substrates, particularly C20 and greater substrates.

13B. Plant Expression

To characterize the *Arabidopsis* sequence which is preferentially expressed in the developing seeds, constructs are prepared to direct the expression of the sequence in host plant cells.

A plasmid containing the napin cassette derived from pCGN3223 (described in USPN 5,639,790, the entirety of which is incorporated herein by reference) was modified to make it more useful for cloning large DNA fragments containing multiple restriction sites, and to allow the cloning of multiple napin fusion genes into plant binary transformation vectors. An adapter comprised of the self annealed oligonucleotide of sequence 5'-CGCGATTAAATGGCGCGCCCTGCAGGCGGCCGCCTGCAGGG CGCGCCATTAAAT (SEQ ID NO:50) was ligated into the cloning vector pBC SK+ (Stratagene) after digestion with the restriction endonuclease BssHII to construct vector pCGN7765. Plasmids pCGN3223 and pCGN7765 were digested with NotI and ligated together. The resultant vector, pCGN7770, contains the pCGN7765 backbone with the napin seed specific expression cassette from pCGN3223.

The cloning cassette, pCGN7787, essentially the same regulatory elements as pCGN7770, with the exception of the napin regulatory regions of pCGN7770 have been replaced with the double CAMV 35S promoter and the tml polyadenylation and transcriptional termination region.

A binary vector for plant transformation, pCGN5139, was constructed from pCGN1558 (McBride and Summerfelt, (1990) Plant Molecular Biology, 14:269-276). The polylinker of pCGN1558 was replaced as a HindIII/Asp718 fragment with a polylinker containing unique restriction endonuclease sites, AscI, PacI, XbaI, SmaI, BamHI, and NotI. The Asp718 and HindIII restriction endonuclease sites are retained in pCGN5139.

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A series of turbo binary vectors are constructed to allow for the rapid cloning of DNA sequences into binary vectors containing transcriptional initiation regions (promoters) and transcriptional termination regions.

The plasmid pCGN8618 was constructed by ligating oligonucleotides 5'-
 5 TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:51) and 5'-
 TCGACCTGCAGGAAGCTTGCGGCCGCGGATCC-3' (SEQ ID NO:52) into
 SalI/XhoI-digested pCGN7770. A fragment containing the napin promoter, polylinker and
 napin 3' region was excised from pCGN8618 by digestion with Asp718I; the fragment was
 blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into
 10 pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in
 the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that
 the napin promoter was closest to the blunted Asp718I site of pCGN5139 and the napin 3'
 was closest to the blunted HindIII site was subjected to sequence analysis to confirm both
 the insert orientation and the integrity of cloning junctions. The resulting plasmid was
 15 designated pCGN8622.

The plasmid pCGN8619 was constructed by ligating oligonucleotides 5'-
 TCGACCTGCAGGAAGCTTGCGGCCGCGGATCC -3' (SEQ ID NO:53) and 5'-
 TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:54) into
 SalI/XhoI-digested pCGN7770. A fragment containing the napin promoter, polylinker and
 20 napin 3' region was removed from pCGN8619 by digestion with Asp718I; the fragment
 was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into
 pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in
 the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that
 the napin promoter was closest to the blunted Asp718I site of pCGN5139 and the napin 3'
 25 was closest to the blunted HindIII site was subjected to sequence analysis to confirm both
 the insert orientation and the integrity of cloning junctions. The resulting plasmid was
 designated pCGN8623.

The plasmid pCGN8620 was constructed by ligating oligonucleotides 5'-
 TCGAGGATCCGCGGCCGCAAGCTTCCTGCAGGAGCT -3' (SEQ ID NO:55) and 5'-
 30 CCTGCAGGAAGCTTGCGGCCGCGGATCC-3' (SEQ ID NO:56) into SalI/SacI-
 digested pCGN7787. A fragment containing the d35S promoter, polylinker and tml 3'
 region was removed from pCGN8620 by complete digestion with Asp718I and partial
 digestion with NotI. The fragment was blunt-ended by filling in the 5' overhangs with

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Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid containing the insert oriented so that the d35S promoter was closest to the blunted Asp718I site of pCGN5139 and the tml 3' was closest to the blunted HindIII site was
 5 subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8624.

The plasmid pCGN8621 was constructed by ligating oligonucleotides 5'-TCGACCTGCAGGAAGCTTGCGGCCGCGGATCCAGCT -3' (SEQ ID NO:57) and 5'-GGATCCGCGGCCGCAAGCTTCCTGCAGG-3' (SEQ ID NO:58) into SalI/SacI-
 10 digested pCGN7787. A fragment containing the d35S promoter, polylinker and tml 3' region was removed from pCGN8621 by complete digestion with Asp718I and partial digestion with NotI. The fragment was blunt-ended by filling in the 5' overhangs with Klenow fragment then ligated into pCGN5139 that had been digested with Asp718I and HindIII and blunt-ended by filling in the 5' overhangs with Klenow fragment. A plasmid
 15 containing the insert oriented so that the d35S promoter was closest to the blunted Asp718I site of pCGN5139 and the tml 3' was closest to the blunted HindIII site was subjected to sequence analysis to confirm both the insert orientation and the integrity of cloning junctions. The resulting plasmid was designated pCGN8625.

The open reading frame of AT-WS2 was PCR amplified from Arabidopsis
 20 genomic DNA using the primers 5'-GGATCCGCGGCC GCATTATGAAACAGTTAGCAACCAACAGA-3' (SEQ ID NO:59) and 5'-GGATCCCCTGCAGGTACAT TAAAATACAGACAACGTGCC-3' (SEQ ID NO:60). The PCR product is cloned into plasmid pCR 2.1 according to the manufacturer's protocol (Clontech) to generate the plasmid pCGN9706. To direct transcription of a sense
 25 transcript under control of a napin expression cassette in transgenic plants, plasmid pCGN9712 is constructed by cloning the NotI/Sse8387I fragment from pCGN9706 into NotI/PstI digested binary vector pCGN8622. To direct transcription of an antisense transcript under control of a napin expression cassette in transgenic plants, plasmid pCGN9713 was constructed by cloning the NotI/Sse8387I fragment from pCGN9706 into
 30 NotI/PstI digested binary vector pCGN8623. To direct transcription of a sense transcript under control of a double 35S expression cassette in transgenic plants, plasmid pCGN9714 was constructed by cloning the NotI/Sse8387I fragment from pCGN9706 into NotI/PstI digested binary vector pCGN8624. Plasmids pCGN9712, pCGN9713, and pCGN9714

were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation, and the resultant *Agrobacterium* strains were used to transform *Arabidopsis thaliana* plants by vacuum infiltration.

The open reading frame of the ATWS1 sequence was amplified using PCR and
 5 cloned into pCGN8622, pCGN8623, and pCGN8624 to create the plant expression constructs pCGN9708, pCGN9709, and pCGN9710, respectively.

Example 14 Plant Transformation Methods and Analyses

A variety of methods have been developed to insert a DNA sequence of interest
 10 into the genome of a plant host to obtain the transcription or transcription and translation of the sequence to effect phenotypic changes.

High erucic acid varieties, such as cultivar Reston, or Canola-type varieties of *Brassica napus* may be transformed using *Agrobacterium* mediated transformation methods as described by Radke *et al.* (*Theor. Appl. Genet.* (1988) 75:685-694; *Plant Cell Reports* (1992) 11:499-505). Transgenic *Arabidopsis thaliana* plants may be obtained by
 15 *Agrobacterium*-mediated transformation as described by Valverkens *et al.*, (*Proc. Nat. Acad. Sci.* (1988) 85:5536-5540), or as described by Bent *et al.* ((1994), *Science* 265:1856-1860), or Bechtold *et al.* ((1993), *C.R.Acad.Sci, Life Sciences* 316:1194-1199). Other plant species may be similarly transformed using related techniques.

20 Alternatively, microprojectile bombardment methods, such as described by Klein *et al.* (*Bio/Technology* 10:286-291) may also be used to obtain transformed plants comprising the reductase and wax synthase expression constructs described herein.

Seed oils obtained from transgenic *Arabidopsis* plants expressing ATWS1 (pCGN9708) or ATWS2 (pCGN9712) from the napin promoter are analyzed for
 25 triglyceride production by thin layer chromatography (TLC) and fatty acid methyl ester (FAME) analysis using methods described above.

Results of the analyses demonstrate that lines expressing the ATWS1 sequence have a reduced amount of triglycerides as well as a reduced amount of total fatty acids. Lines 9708-AT001-7 and 9708-AT001-23 contained 27.34% and 24.85% FAME by dry
 30 weight compared to non-transformed *Arabidopsis* seed, which contain approximately 34% FAME. In addition, lines expressing ATWS2 have about 31% FAME (Figure 6).

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
5 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 acylreductase protein
10 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.
15

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
20 or scope of the appended claims.

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SEQUENCE LISTING

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Acyltransferases

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<150> 09/205,815

<151> 1998-12-04

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His	Gly	Gly	Ala	Leu	Arg	Leu	Leu	Leu	Leu	Leu	Pro	Val	Val	Leu	Leu	40	45	50	55
Phe	Ile	Phe	Leu	Pro	Leu	Arg	Leu	Ser	Ser	Phe	His	Leu	Gly	Gly	Pro	60	65	70	75
Thr	Ala	Leu	Tyr	Leu	Val	Trp	Leu	Ala	Asn	Phe	Lys	Leu	Leu	Leu	Phe	80	85	90	95
Ala	Phe	His	Leu	Gly	Pro	Leu	Ser	Asn	Pro	Ser	Leu	Ser	Leu	Leu	His	100	105	110	115
Phe	Ile	Ser	Thr	Thr	Leu	Leu	Pro	Ile	Lys	Phe	Arg	Asp	Asp	Pro	Ser	120	125	130	135
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Val	Val	Leu	Phe	Val	Ala	Lys	Leu	Val	Phe	Phe	Ala	Gly	Ile	Leu	Lys	160	165	170	175
Ile	Tyr	Glu	Phe	Arg	Lys	Asp	Leu	Pro	His	Phe	Val	Ile	Ser	Val	Leu	180	185	190	195
Tyr	Cys	Phe	His	Phe	Tyr	Leu	Gly	Thr	Glu	Ile	Thr	Leu	Ala	Ala	Ser	200	205	210	215
Ala	Val	Ile	Ala	Arg	Ala	Thr	Leu	Gly	Leu	Asp	Leu	Tyr	Pro	Gln	Phe	220	225	230	235
Asn	Glu	Pro	Tyr	Leu	Ala	Thr	Ser	Leu	Gln	Asp	Phe	Trp	Gly	Arg	Arg	240	245	250	255
Trp	Asn	Leu	Met	Val	Ser	Asp	Ile	Leu	Gly	Leu	Thr	Thr	Tyr	Gln	Pro	260	265	270	275
Val	Arg	Arg	Val	Leu	Ser	Arg	Trp	Val	Arg	Leu	Arg	Trp	Glu	Val	Ala	280	285	290	295
Gly	Ala	Met	Leu	Val	Ala	Phe	Thr	Val	Ser	Gly	Leu	Met	His	Glu	Val	300	305	310	315
Phe	Phe	Phe	Tyr	Leu	Thr	Arg	Ala	Arg	Pro	Ser	Trp	Glu	Val	Thr	Gly	320	325	330	335
Phe	Phe	Val	Leu	His	Gly	Val	Cys	Thr	Ala	Val	Glu	Met	Val	Val	Lys	340	345	350	
Lys	Ala	Val	Ser	Gly	Lys	Val	Arg	Leu	Arg	Arg	Glu	Val	Ser	Gly	Ala				
Leu	Thr	Val	Gly	Phe	Val	Met	Val	Thr	Gly	Gly	Trp	Leu	Phe	Leu	Pro				
Gln	Leu	Val	Arg	His	Gly	Val	Asp	Leu	Lys	Thr	Ile	Asp	Glu	Tyr	Pro				
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<212> DNA

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<400> 3

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aacatcgagc cgggtgttcaa tgagccctac ctacgtaccc cctacaaga cttctggagc      600
cgcagatgga acctcatggt ttcagccgtc ctacgtcaca ccgttcacat tccgggttcag      660
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<211> 341

<212> PRT

<213> Arabidopsis sp

<400> 4

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Val Leu Arg Leu Leu Ser Ile Leu Pro Val Cys Ile Leu Phe Leu Val
          35          40          45
Leu Pro Leu Phe Leu Ser Cys Val His Phe Cys Ala Ile Ser Val Leu
          50          55          60
Phe Leu Ser Trp Leu Ala Asn Phe Lys Leu Leu Leu Phe Ala Phe Asp
65          70          75          80
Glu Gly Pro Leu Phe Pro Leu Pro Pro Lys Leu Ser Arg Phe Ile Cys
          85          90          95
Phe Ala Cys Leu Pro Ile Lys Ile Arg Gln Asp Pro Ser Pro Asn Ala
          100          105          110
Ile Pro Asn Leu His Pro Lys Pro Met Pro Lys Trp Val Leu Ala Val
          115          120          125
Lys Ile Leu Val Leu Gly Val Leu Leu His Val Tyr Glu Tyr Arg Asp
          130          135          140
Gly Leu Pro Arg Phe Val Val Leu Ala Leu Tyr Cys Leu His Ile Tyr
145          150          155          160
Leu Glu Val Glu Leu Val Leu Val Phe Val Gly Ala Val Val Ser Thr
          165          170          175
Leu Leu Gly Cys Asn Ile Glu Pro Val Phe Asn Glu Pro Tyr Leu Ala
          180          185          190
Thr Ser Leu Gln Asp Phe Trp Ser Arg Arg Trp Asn Leu Met Val Ser
          195          200          205
Ala Val Leu Arg Ser Thr Val His Ile Pro Val Gln Arg Phe Phe Lys
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Arg Ile Leu Ser Pro Asp Gly Ala Met Phe Ala Gly Val Met Ala Ser
225          230          235          240

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			20					25					30		
Ser	Tyr	Cys	Tyr	Tyr	Ile	Ser	Ser	Lys	Ile	Ser	Lys	Gly	Val	Leu	Arg
		35					40					45			
Leu	Leu	Ser	Leu	Leu	Pro	Ile	Phe	Ile	Ile	Phe	Leu	Leu	Leu	Pro	Leu
	50					55					60				
Phe	Phe	Ser	Ser	Val	His	Phe	Cys	Val	Ile	Ser	Gly	Phe	Phe	Phe	Thr

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65	Trp	Leu	Ala	Asn	Phe	70	Lys	Leu	Phe	Leu	Phe	75	Ala	Phe	Asp	Gln	80	Glu	Pro
					85							90					95		
	Leu	Ser	Pro	Leu	Pro	Ser	Asn	Leu	Thr	Arg	Phe	Phe	Cys	Phe	Ala	Cys			
			100						105										
	Phe	Pro	Ile	Lys	Ile	Asn	Lys	Asn	Pro	Ser	Ser	Asn	Arg	Ile	His	Asn			
			115					120											
	Lys	Pro	Met	Ser	Lys	Trp	Val	Leu	Ala	Phe	Lys	Leu	Leu	Ile	Phe	Ser			
		130					135					140							
	Phe	Leu	Leu	His	Val	Tyr	Arg	Asn	Asn	Tyr	Asp	Ser	Gly	Leu	Ser	Arg			
145						150					155								
	Phe	Ala	Phe	Leu	Ala	Leu	Phe	Thr	Ile	His	Val	Tyr	Leu	Glu	Ala	Glu			
				165						170									
	Leu	Ile	Leu	Val	Phe	Val	Gly	Ala	Leu	Met	Ser	Met	Leu	Leu	Gly	Cys			
			180						185										
	Glu	Met	Glu	Pro	Val	Phe	Asn	Asp	Pro	Tyr	Leu	Ala	Thr	Ser	Leu	Gln			
		195						200											
	Glu	Phe	Trp	Ser	Arg	Arg	Trp	Asn	Leu	Met	Val	Pro	Ala	Val	Leu	Arg			
		210					215					220							
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225						230					235								
	Leu	His	Arg	Ala	Phe	Tyr	Ala	Gly	Met	Leu	Ala	Thr	Phe	Ile	Val	Ser			
				245						250									
	Gly	Leu	Met	His	Glu	Leu	Ile	Tyr	Phe	Tyr	Val	Ile	Arg	Lys	Ser	Pro			
			260						265										
	Thr	Trp	Glu	Val	Thr	Cys	Phe	Phe	Leu	Leu	His	Gly	Val	Val	Thr	Cys			
		275						280											
	Leu	Glu	Ile	Ala	Met	Lys	Arg	Met	Arg	Trp	Leu	Pro	Thr	Pro	Arg	Arg			
		290					295					300							
	Ala	Val	Ser	Gly	Leu	Ala	Ile	Thr	Val	Phe	Leu	Leu	Val	Thr	Ala	Gly			
305						310					315								
	Trp	Leu	Phe	Tyr	Pro	Gln	Met	Leu	Arg	Asn	Asp	Val	His	Lys	Arg	Val			
				325						330									
	Ile	Ser	Glu	Cys	Leu	Leu	Val	Ile	Asp	Val	Val	Lys	Arg	His	Val	Val			
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	Cys	Ile	Leu	Met															
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	cccatcaagc	ctcaacaaaa	ccctaataat	caaaattata	aaatccccat	atggcttttc	360
	gccattaaag	ttgtcatctt	tgttgtcttg	ttacaaatgt	atgaatacaa	acaatatctg	420
	tctccggctt	tattattggg	ttttaattct	ctacatatat	tcttggagct	tgagattgtc	480
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	aatgaaccat	acttagccac	ttctcttcaa	gacttctggg	gtcgtcgggtg	gaacctcatg	600
	gtccccggcga	ttctccggcc	ggctgtctac	ctccccggcga	gacgaatggc	ctgtcggaaa	660

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<211> 342

<212> PRT

<213> Arabidopsis sp

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			20					25					30			
Val	Phe	Arg	Leu	Leu	Ser	Val	Leu	Pro	Val	Cys	Ala	Leu	Phe	Leu	Val	
		35					40					45				
Phe	Pro	Leu	Phe	Phe	Ser	Tyr	Val	His	Phe	Ser	Gly	Cys	Met	Ala	Phe	
	50					55					60					
Phe	Leu	Ser	Trp	Leu	Ala	Asn	Phe	Lys	Leu	Ile	Leu	Phe	Ser	Phe	Asp	
65					70					75					80	
Gln	Gly	Pro	Leu	Ser	Pro	Leu	Pro	Arg	Thr	Leu	Ser	Arg	Phe	Ile	Cys	
				85					90					95		
Ile	Thr	Cys	Phe	Pro	Ile	Lys	Pro	Gln	Gln	Asn	Pro	Asn	Ile	Gln	Asn	
			100					105					110			
Tyr	Lys	Ile	Pro	Ile	Trp	Leu	Phe	Ala	Ile	Lys	Val	Val	Ile	Phe	Val	
		115					120					125				
Val	Leu	Leu	Gln	Met	Tyr	Glu	Tyr	Lys	Gln	Tyr	Leu	Ser	Pro	Ala	Leu	
	130					135					140					
Leu	Leu	Val	Phe	Asn	Ser	Leu	His	Ile	Phe	Leu	Glu	Leu	Glu	Ile	Val	
145					150					155					160	
Phe	Met	Leu	Val	Lys	Ala	Leu	Val	Phe	Ile	Thr	Leu	Gly	Cys	Asp	Leu	
				165					170					175		
Glu	Pro	Gln	Ser	Asn	Glu	Pro	Tyr	Leu	Ala	Thr	Ser	Leu	Gln	Asp	Phe	
			180					185					190			
Trp	Gly	Arg	Arg	Trp	Asn	Leu	Met	Val	Pro	Ala	Ile	Leu	Arg	Pro	Ala	
		195					200					205				
Val	Tyr	Leu	Pro	Ala	Arg	Arg	Met	Ala	Cys	Arg	Lys	Val	Asn	Ser	Asp	
	210					215					220					
Gln	Ala	Met	Phe	Leu	Gly	Val	Phe	Ala	Ala	Phe	Leu	Val	Ser	Gly	Ala	
225					230					235					240	
Val	His	Glu	Met	Leu	Phe	Phe	Tyr	Leu	Thr	Arg	Glu	Val	Pro	Thr	Gly	
				245					250					255		
Glu	Val	Thr	Trp	Phe	Phe	Leu	Leu	His	Gly	Val	Cys	Thr	Val	Ala	Glu	
			260					265					270			
Val	Ala	Val	Lys	Lys	Ser	Thr	Phe	Val	Arg	Arg	Trp	Trp	Arg	Val	Ser	
		275					280									
Pro	Thr	Val	Ser	Arg	Leu	Leu	Thr	Val	Gly	Phe	Val	Val	Val	Thr	Ser	
	290					295					300					
Gly	Trp	Phe	Phe	Phe	Pro	Leu	Ile	Arg	Ser	Gly	Ile	Ile	Glu	Arg	Leu	
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Ala	Ser	Glu	Ala	Leu	Met	Cys	Ile	Asp	Phe	Val	Lys	His	Lys	Phe	Leu	

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330

335

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<211> 1038
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<213> Arabidopsis sp

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caagggtccac	ttttcccagt	tccctcaa	ctctcccgat	tcgtctgctt	cacttgcttc	300
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			20					25					30		
Val	Phe	Arg	Leu	Leu	Ser	Val	Leu	Pro	Val	Cys	Val	Leu	Phe	Leu	Val
		35					40					45			
Leu	Pro	Leu	Phe	Val	Ser	Ser	Val	His	Phe	Ser	Gly	Ser	Thr	Ala	Phe
	50					55					60				
Phe	Leu	Ser	Trp	Leu	Ala	Asn	Phe	Lys	Leu	Ile	Leu	Phe	Ser	Phe	Asp
65				70					75					80	
Gln	Gly	Pro	Leu	Phe	Pro	Val	Pro	Ser	Asn	Leu	Ser	Arg	Phe	Val	Cys
			85					90						95	
Phe	Thr	Cys	Phe	Pro	Ile	Lys	Leu	Gln	Gln	Asn	Pro	Lys	Pro	Gln	Asn
			100					105						110	
Gln	Met	Pro	Lys	Trp	Gly	Phe	Ala	Val	Lys	Leu	Ala	Phe	Phe	Gly	Val
		115					120					125			
Leu	Leu	His	Met	Tyr	Glu	Tyr	Lys	Gln	His	Met	Ser	Pro	Thr	Val	Leu
	130					135					140				
Leu	Val	Leu	Tyr	Ser	Leu	His	Ile	Tyr	Leu	Glu	Tyr	Glu	Ile	Leu	Leu
145					150					155					160

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			180					185					190		
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	210					215					220				
Ala	Leu	Phe	Leu	Gly	Val	Phe	Ala	Ser	Phe	Leu	Val	Ser	Gly	Val	Val
225				230						235					240
His	Glu	Leu	Ile	Phe	Phe	Tyr	Phe	Thr	Arg	Glu	Ser	Pro	Thr	Gly	Glu
			245					250						255	
Val	Thr	Leu	Phe	Phe	Val	Leu	His	Gly	Val	Cys	Thr	Ala	Ala	Glu	Cys
			260					265					270		
Ala	Ala	Lys	Arg	Thr	Arg	Leu	Val	Arg	Arg	Trp	Lys	Val	Ser	Gln	Met
		275				280						285			
Val	Ser	Arg	Leu	Leu	Thr	Val	Gly	Phe	Val	Val	Met	Thr	Gly	Gly	Trp
	290					295					300				
Leu	Phe	Phe	Pro	His	Leu	Ala	Arg	Ser	Gly	Met	Ile	Glu	Arg	Leu	Ala
305					310					315					320
Asp	Glu	Ala	Phe	Leu	Phe	Ile	Gly	Phe	Val	Lys	His	Lys	Phe	Phe	Tyr
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<211> 1002

<212> DNA

<213> Arabidopsis sp

<400> 11

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<211> 333

<212> PRT

<213> Arabidopsis sp

<400> 12

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 35 40 45
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 50 55 60
 Phe Leu Thr Trp Leu Ala Asn Phe Lys Leu Ile Leu Phe Ser Phe Asp
 65 70 75 80
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 85 90 95
 Phe Thr Cys Phe Pro Ile Lys Val Gln Gln Asn Pro Lys Ser Gln Asn
 100 105 110
 His Leu Pro Lys Leu Val Phe Ala Ile Lys Leu Ala Ile Phe Ala Val
 115 120 125
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 130 135 140
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 145 150 155 160
 Thr Phe Val Lys Val Val Phe Ile Ser Leu Gly Cys Asp Leu Glu
 165 170 175
 Pro Gln Ser Asn Lys Pro Tyr Leu Ala Thr Ser Leu Gln Asp Phe Trp
 180 185 190
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 195 200 205
 Tyr Ala Pro Met Arg Arg Val Ser Glu Arg Lys Met Ser Ser Gly Trp
 210 215 220
 Ala Leu Phe Pro Gly Ile Leu Ala Ala Phe Ile Val Ser Gly Leu Val
 225 230 235 240
 His Glu Leu Leu Phe Phe Tyr Leu Ile Arg Glu Met Pro Thr Gly Glu
 245 250 255
 Val Thr Leu Phe Phe Val Leu His Gly Val Cys Thr Ala Val Glu Leu
 260 265 270
 Ala Val Lys Lys Lys Thr Thr Val Ala Gln Arg Trp Arg Leu Ser Pro
 275 280 285
 Gly Val Ser Arg Val Leu Thr Val Gly Phe Val Phe Val Thr Gly Gly
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 <212> DNA
 <213> Arabidopsis sp

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 actattgcat ttttctcttc aggtcttgca gttccaaaac tcatcctctt tgcattagaa 240
 aaaggctctc tttttccact tctcctaata ctccctcatt tcgtctgctt tgcttgcttc 300
 cccatcaagc ttcaaaaaaa acctaaccct gaaaatacta accatttccc caaatgggtt 360

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tttgcctga aagttttcat ctttggtgcc ttgttactac aagcgtatca ttacaaacaa 420
tttctatcta cgaattttct attgggtctc tatgctctgc atatatactt ggagcttgag 480
atttccttaa ccttgataaa atttctcgtc agtatcactc ttgggtgtga cctcgagcca 540
caattcaacg aaccatactt agccacctct ctacatgact tctggggtca cccgatggaac 600
ctcatggtct cgaagattct ctggctcgca gtgtacaacc ccatacggca atggcgagcc 660
aagagctccg agtgggatcg gttcttcgcg attttcgcca cgttcctcgt ctctggtgtg 720
gctcaccgaga ttctctactt ctatttgaca cgtgagaagc ctacatggga ggtgacttgg 780
ttctttgtgt tacatggggt ttgcatggcg gctgaagtgg cactgaagag gaagacgaag 840
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tttgtgactg gtgtttggct attttcccc cagcctatta ggcacggctt gatggagagg 960
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<210> 14

<211> 346

<212> PRT

<213> Arabidopsis sp

<400> 14

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35      40      45
Ile Pro Ile Phe Phe Ser Thr Val His Ser Ser Phe Thr Ile Ala Phe
50      55      60
Phe Leu Ser Gly Leu Ala Val Pro Lys Leu Ile Leu Phe Ala Leu Glu
65      70      75      80
Lys Gly Pro Leu Phe Pro Leu Pro Pro Asn Leu Pro His Phe Val Cys
85      90      95
Phe Ala Cys Phe Pro Ile Lys Leu Gln Lys Lys Pro Asn Pro Glu Asn
100     105     110
Thr Asn His Phe Pro Lys Trp Val Phe Ala Leu Lys Val Phe Ile Phe
115     120     125
Gly Ala Leu Leu Leu Gln Ala Tyr His Tyr Lys Gln Phe Leu Ser Thr
130     135     140
Asn Phe Leu Leu Gly Leu Tyr Ala Leu His Ile Tyr Leu Glu Leu Glu
145     150     155     160
Ile Ser Leu Thr Leu Ile Lys Phe Leu Val Ser Ile Thr Leu Gly Cys
165     170     175
Asp Leu Glu Pro Gln Phe Asn Glu Pro Tyr Leu Ala Thr Ser Leu His
180     185     190
Asp Phe Trp Gly His Arg Trp Asn Leu Met Val Ser Lys Ile Leu Trp
195     200     205
Leu Ala Val Tyr Asn Pro Ile Arg Gln Trp Arg Ala Lys Ser Ser Glu
210     215     220
Trp Asp Arg Phe Phe Ala Ile Phe Ala Thr Phe Leu Val Ser Gly Val
225     230     235     240
Ala His Glu Ile Leu Tyr Phe Tyr Leu Thr Arg Glu Lys Pro Thr Trp
245     250     255
Glu Val Thr Trp Phe Phe Val Leu His Gly Phe Cys Met Ala Ala Glu
260     265     270
Val Ala Leu Lys Arg Lys Thr Lys Leu Val Gln Arg Trp Pro Val Asn
275     280     285

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Pro Ala Val Ser Arg Leu Leu Thr Val Gly Phe Val Phe Val Thr Gly
 290 295 300
 Val Trp Leu Phe Ser Pro Gln Pro Ile Arg His Gly Leu Met Glu Arg
 305 310 315 320
 Phe Ile Asn Glu Asp Leu Phe Leu Ile Asp Phe Phe Asn Arg Lys Leu
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 cgtacttttc tattgtgtct ccatccactg tatgtatat tgttacttga gattctctta 480
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 <211> 339
 <212> PRT
 <213> Arabidopsis sp

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 35 40 45
 Leu Pro Leu Phe Phe Ser Phe Ser Ile Phe Thr Ser Thr Thr Ala Phe
 50 55 60
 Phe Leu Ser Ala Ile Ala Asn Ser Arg Leu Ile Leu Phe Ser Phe Asp
 65 70 75 80
 Gln Gly Pro Leu Phe Pro Leu Pro Ser Asn Leu Phe Arg Phe Thr Cys
 85 90 95
 Phe Thr Cys Phe Pro Ile Gln Arg Gln Gln Asn Pro Lys Ser Gln Asp
 100 105 110
 His Leu Ser Thr Tyr Val Phe Pro Val Lys Ile Ala Ile Phe Val Val
 115 120 125

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Leu Leu Tyr Val His Asn Asp Ile Gln Asn Leu Pro Arg Thr Phe Leu
 130 135 140
 Leu Cys Leu His Pro Leu Tyr Val Tyr Leu Leu Leu Glu Ile Leu Leu
 145 150 155 160
 Thr Leu Leu Arg Ile Leu Met Thr Ile Ile Leu Gly Cys Asp Leu Glu
 165 170 175
 Pro His Phe His Glu Pro Tyr Leu Ala Thr Ser Leu Gln Asp Phe Trp
 180 185 190
 Gly Arg Arg Trp Asn Leu Ile Val Ser Ala Ser Leu Arg Ala Ile Val
 195 200 205
 Tyr Thr Pro Val Arg Arg Val Cys Gln Arg Val Met Ser Ser Asp Tyr
 210 215 220
 Ala Met Leu Ile Gly Val Phe Ala Thr Phe Val Thr Ser Gly Val Ala
 225 230 235 240
 His Glu Val Val Phe Phe Tyr Ile Thr Arg Ala Met Pro Thr Gly Glu
 245 250 255
 Val Ala Leu Phe Phe Leu Leu His Gly Val Cys Thr Val Ala Glu Val
 260 265 270
 Ala Ala Lys Arg Thr Ala Phe Val Arg Arg Trp Pro Val Arg Pro Val
 275 280 285
 Val Ser Trp Met Phe Thr Ile Ala Phe Val Asn Val Thr Ala Gly Trp
 290 295 300
 Leu Phe Phe Pro Gln Leu Ile Arg Asn Asn Leu Gly Glu Arg Cys Ser
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<210> 17
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 <213> *Simmondsia chinensis*

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 aaggcgggtt caggcaaggt gcggctgcgc cgggaggtgt caggggcgct gacggtgggg 600
 ttcgtgatgg tgactggagg gtggttgttt ttgccgcagc tgggtgaggca tggggtagat 660
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<210> 18
 <211> 1335

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<212> DNA

<213> *Simmondsia chinensis*

<400> 18

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ctccgcctcc	tctctectct	ccccgtcgtc	ctcctcttca	ttttcctccc	cctccgcctc	180
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aaaaacaaga	gaactctgag	ttttgagtg	cgtaaagtgt	ttctttttgt	tgctaagttg	420
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gaaaaatgca	gatcaracgg	caagacaggt	cgggaaaaaa	aaatgatcaa	tttttcctta	1200
agtagccggc	ctgccaccct	gtccgattgt	ggcatttttg	tggtcacttt	ttcatatcgt	1260
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<210> 19

<211> 436

<212> DNA

<213> *Glycine sp*

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cgtcacacgt	gtagtccca	cgtgggaggt	cacgtgcttc	tttttgctcc	atgggggtgtg	180
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<210> 20

<211> 13

<212> PRT

<213> Unknown

<220>

<223> WSpép-29 peptide

<400> 20

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 <212> PRT
 <213> Unknown

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 <223> WSpép-33 peptide

<400> 21
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<210> 22
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<221> misc_feature
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39

<210> 23
 <211> 39
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<220>
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<221> misc_feature
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 <223> n = A,T,C or G

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<220>
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<221> misc_feature
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<400> 24

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42

<210> 25

<211> 42

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<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<221> misc_feature

<222> (1)...(42)

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<400> 25

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42

<210> 26

<211> 9

<212> PRT

<213> unknown

<220>

<223> WSpép-14 peptide

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Phe Arg Asp Asp Pro Ser Asn Asp His

1

5

<210> 27

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<221> misc_feature

<222> (1)...(27)

<223> n = A,T,C or G

<400> 27

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27

<210> 28

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<221> misc_feature

<222> (1)...(27)

<223> n = A,T,C or G

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<400> 28
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27

<210> 29
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<220>
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<221> misc_feature
<222> (1)...(17)
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17

<210> 30
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<220>
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<221> misc_feature
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<223> n = A,T,C or G

<400> 30
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17

<210> 31
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<220>
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<221> misc_feature
<222> (1)...(17)
<223> n = A,T,C or G

<400> 31
gcncncrcrt gnggngc

17

<210> 32
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<223> Synthetic Oligonucleotide

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17

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<211> 20

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<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<221> misc_feature

<222> (1)...(20)

<223> n = A,T,C or G

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20

<210> 34

<211> 20

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<213> Artificial Sequence

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<221> misc_feature

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<210> 35

<211> 20

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<223> n = A,T,C or G

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20

<210> 36

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<221> misc_feature
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 <223> n = A,T,C or G

<400> 36
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20

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<221> misc_feature
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20

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<220>
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<400> 39
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29

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<210> 40
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<220>
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30

<210> 41
<211> 30
<212> DNA
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<220>
<223> Synthetic Oligonucleotide

<400> 41
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30

<210> 42
<211> 30
<212> DNA
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<220>
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30

<210> 43
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<400> 43
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30

<210> 44
<211> 39
<212> DNA
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39

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<210> 45
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<220>
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<400> 45
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33

<210> 46
 <211> 9
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Conserved peptide

<221> VARIANT
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<210> 47
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 <212> PRT
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<220>
 <223> Conserved peptide

<221> VARIANT
 <222> (1)...(19)
 <223> Xaa = Any Amino Acid

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 Leu Xaa Val

<210> 48
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 <212> PRT
 <213> Artificial Sequence

<220>
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<221> VARIANT
 <222> (1)...(17)

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<223> Xaa = Any Amino Acid

<400> 48

Phe Xaa Xaa Ser Gly Xaa Xaa His Glu Xaa Xaa Xaa Phe Tyr Xaa Xaa
 1 5 10 15
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<210> 49

<211> 13

<212> PRT

<213> Artificial Sequence

<220>

<223> Conserved peptide

<221> VARIANT

<222> (1)...(13)

<223> Xaa = Any Amino Acid

<400> 49

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<210> 50

<211> 56

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

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<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic Oligonucleotide

<400> 51

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<210> 52

<211> 32

<212> DNA

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<223> Synthetic Oligonucleotide

<400> 52

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<400> 53
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<400> 54
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<210> 55
<211> 36
<212> DNA
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<220>
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<400> 55
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<210> 56
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic Oligonucleotide

<400> 56
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<210> 57
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic Oligonucleotide

<400> 57

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tcgacctgca ggaagcttgc ggccgcggat ccagct 36

<210> 58
<211> 28
<212> DNA
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<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic Oligonucleotide

<400> 61
ttgcctgaaa ccgccttctt caccaccatc 30

CLAIMS

What is claimed is:

1. An isolated polynucleotide selected from the group consisting of:
 - 5 a) an isolated polynucleotide comprising a nucleotide sequence encoding the polypeptide of SEQ ID NO: 2;
 - b) an isolated polynucleotide comprising SEQ ID NO: 1;
 - c) an isolated polynucleotide comprising a nucleotide sequence which has at least 70% identity to that of SEQ ID NO: 1 over the entire length of SEQ ID NO: 1;
 - 10 d) an isolated polynucleotide comprising a nucleotide sequence which has at least 80% identity to that of SEQ ID NO: 1 over the entire length of SEQ ID NO: 1;
 - e) an isolated polynucleotide comprising a nucleotide sequence which has at least 90% identity to that of SEQ ID NO: 1 over the entire length of SEQ ID NO: 1;
 - f) an isolated polynucleotide comprising a nucleotide sequence which has at least 95% identity to that of SEQ ID NO: 1 over the entire length of SEQ ID NO: 1;
 - 15 g) an isolated polynucleotide that hybridizes, under stringent conditions, to SEQ ID NO: 1 or a fragment thereof; and
 - h) an isolated polynucleotide complementary to the polynucleotide sequence of (a), (b), (c), (d), (e), (f), or (g).
- 20 2. An isolated polynucleotide selected from the group consisting of:
 - a) an isolated polynucleotide comprising a nucleotide sequence encoding the polypeptide of SEQ ID NO: 4;
 - b) an isolated polynucleotide comprising SEQ ID NO: 3;
 - 25 c) an isolated polynucleotide comprising a nucleotide sequence which has at least 70% identity to that of SEQ ID NO: 3 over the entire length of SEQ ID NO: 3;
 - d) an isolated polynucleotide comprising a nucleotide sequence which has at least 80% identity to that of SEQ ID NO: 3 over the entire length of SEQ ID NO: 3;
 - e) an isolated polynucleotide comprising a nucleotide sequence which has at least 90% identity to that of SEQ ID NO: 3 over the entire length of SEQ ID NO: 3;
 - 30 f) an isolated polynucleotide comprising a nucleotide sequence which has at least 95% identity to that of SEQ ID NO: 3 over the entire length of SEQ ID NO: 3;

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- g) an isolated polynucleotide that hybridizes, under stringent conditions, to SEQ ID NO: 3 or a fragment thereof; and
- h) an isolated polynucleotide complementary to the polynucleotide sequence of (a), (b), (c), (d), (e), (f) or (g).

5

3. An isolated polynucleotide selected from the group consisting of SEQ ID Nos: 5, 7, 9, 11, 13, and 15.

4. A method of producing wax synthase in a host cell comprising the steps of growing a host cell having a recombinant DNA construct comprising a promoter functional in a host cell, and a nucleic acid sequence encoding an acyltransferase active in the formation of a wax ester from fatty alcohol and fatty acyl substrates.

5. The method according to Claim 4, wherein said host cell is selected from the group consisting of a bacterial cell, an insect cell, and a plant cell.

6. The method according to Claim 4, wherein said nucleic acid sequence is selected from the group consisting of the sequence of Claim 1 and the sequence of Claim 2.

20

7. A method of producing a wax ester in a host cell comprising the steps of growing a host cell having a recombinant construct comprising a promoter functional in a host cell, and a nucleic acid sequence which encodes at least a portion of an acyltransferase active in the formation of a wax ester, under conditions which will cause the expression of said acyltransferase,

wherein said host cell comprises a fatty alcohol substrate of said acyltransferase, whereby wax ester is produced in said host cell.

8. The method according to Claim 7 wherein said wax ester is produced from a fatty alcohol and a fatty-CoA substrate.

9. The method of Claim 7 wherein said host cell is selected from the group consisting of a bacterial cell, an insect cell, and a plant cell.

10. The method of Claim 7 wherein said promoter is functional in a plant cell.

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11. The method according to Claim 10 wherein said promoter is preferentially expressed in a specific plant tissue.
12. The method according to Claim 10 wherein said promoter is preferentially expressed in a plant seed cell.
- 5 13. A method according to Claim 7 further comprising a second construct comprising a nucleic acid sequence encoding for reductase under the regulatory control of promoters functional in said host cell.
14. A method according to Claim 13 further comprising a third construct comprising a β -ketoacyl-CoA synthase encoding sequence under the regulatory control of
10 a promoter functional in said host cell.
15. The method of Claim 7, wherein wax ester is produced results in an increase in oil as a component of said host cell.
16. A host cell comprising a wax ester produced according to the method of Claim 7.
- 15 17. A wax ester obtained from a cell according to Claim 16.
18. A low calorie food composition comprising a wax ester according to Claim
17. 17.
19. A method for altering the oil content of a host cell comprising,
growing a host cell having a recombinant construct comprising a promoter
20 functional in a host cell, and a nucleic acid sequence which encodes at least a portion of an acyltransferase active in the formation of a wax ester, under conditions which will cause the expression of said acyltransferase.
20. The method according to Claim 19, wherein said host cell is selected from the group consisting of a bacterial cell, an insect cell and a plant cell.
- 25 21. The method according to Claim 19, wherein said triglyceride content is reduced.
22. The method according to Claim 19, wherein the oil content is reduced.
23. The method according to Claim 22, whereby the protein composition of said cell is increased.
- 30 24. A method of producing a fatty alcohol comprising the steps of
growing a host cell having a recombinant construct comprising a promoter
functional in a host cell, and a nucleic acid sequence which encodes an acyltransferase
active in the formation of a wax ester, under conditions which will cause the expression of

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said acyltransferase, wherein said host cell comprises a fatty alcohol substrate of said acyltransferase, whereby wax ester is produced in said host cell,

isolating said wax ester from said host cell, and

subjecting said wax ester to conditions which fatty alcohols are produced.

5

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Correlation of Wax with VLCFA

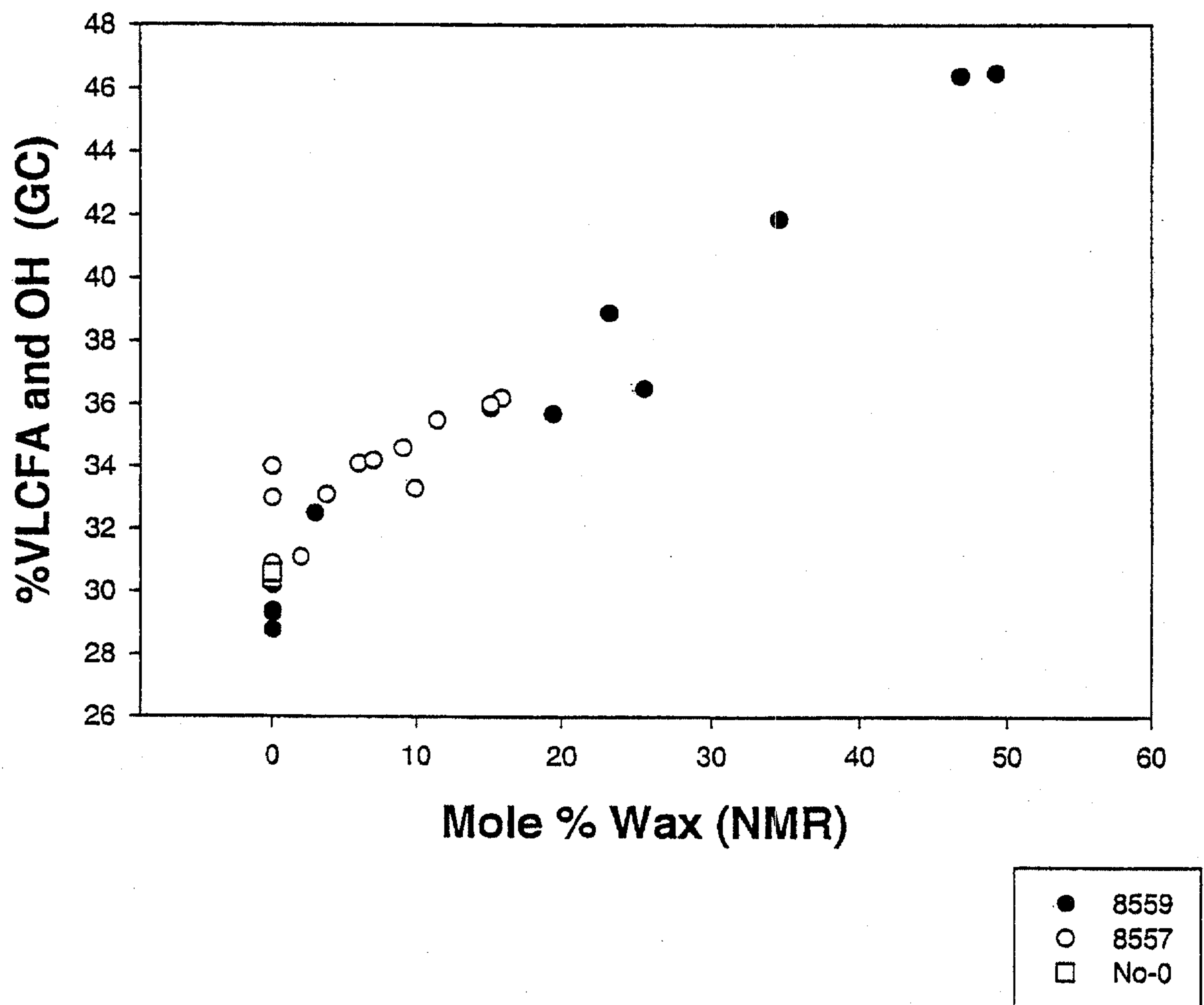


Figure 1

**Wax Synthase Assays of
BVIC/Insect Cell membranes 052599
(16:0-acyl, varying OH, 1 ug protein)**

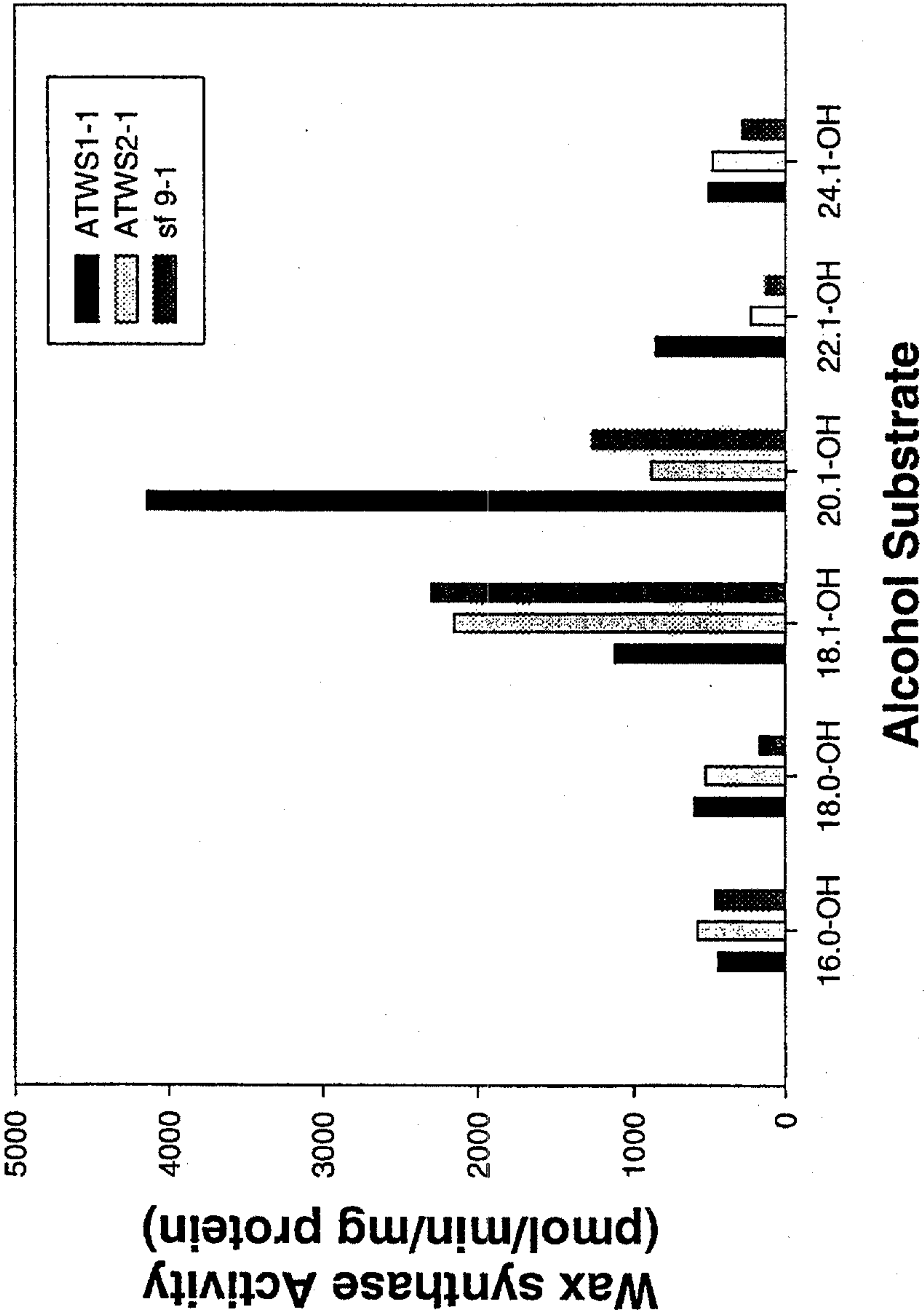


FIGURE 2

**Wax Synthase Assays of
BVIC/Insect Cell Membranes 052599**
(16:0-acyl, varying alcohol, 10 ug protein)

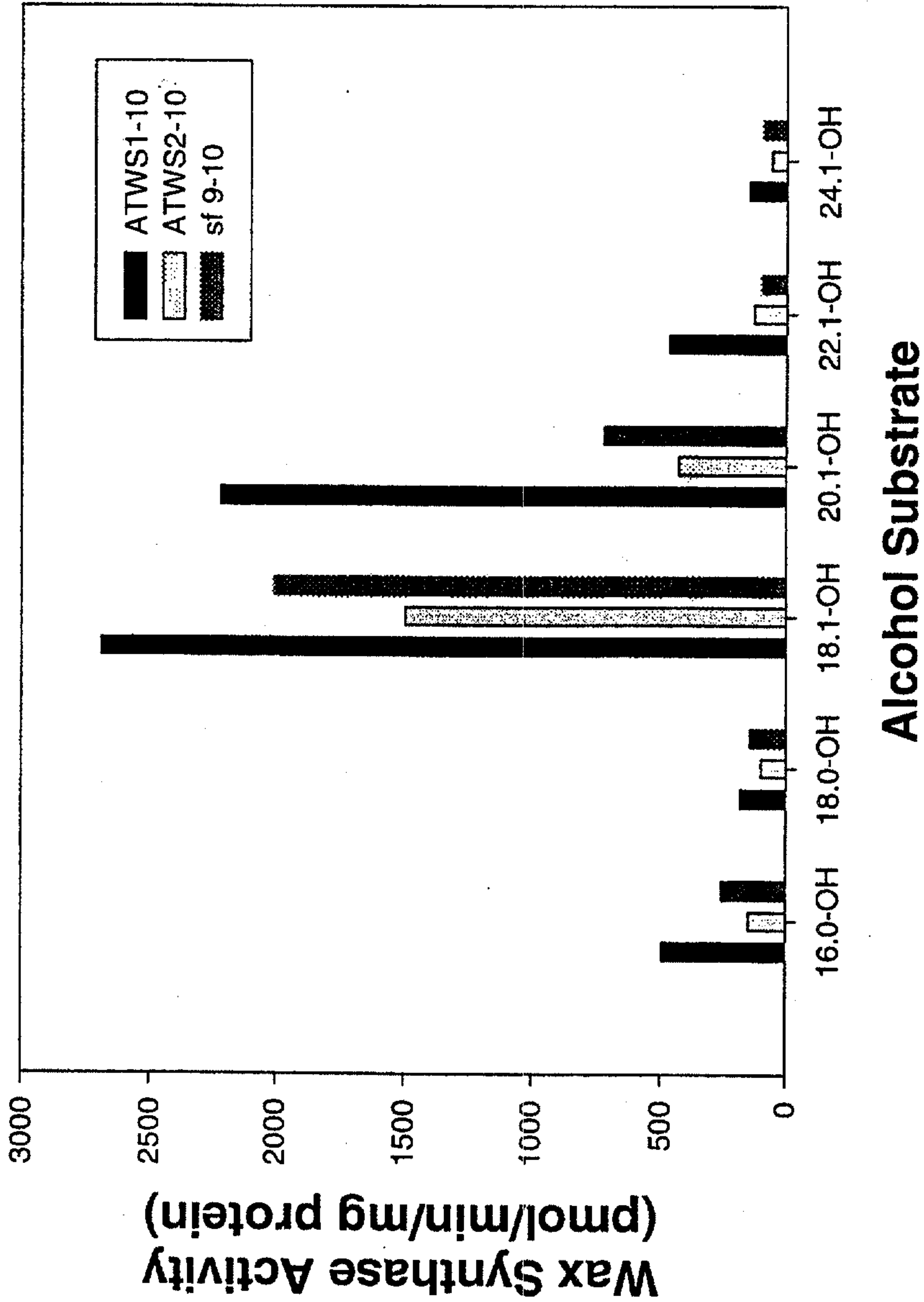
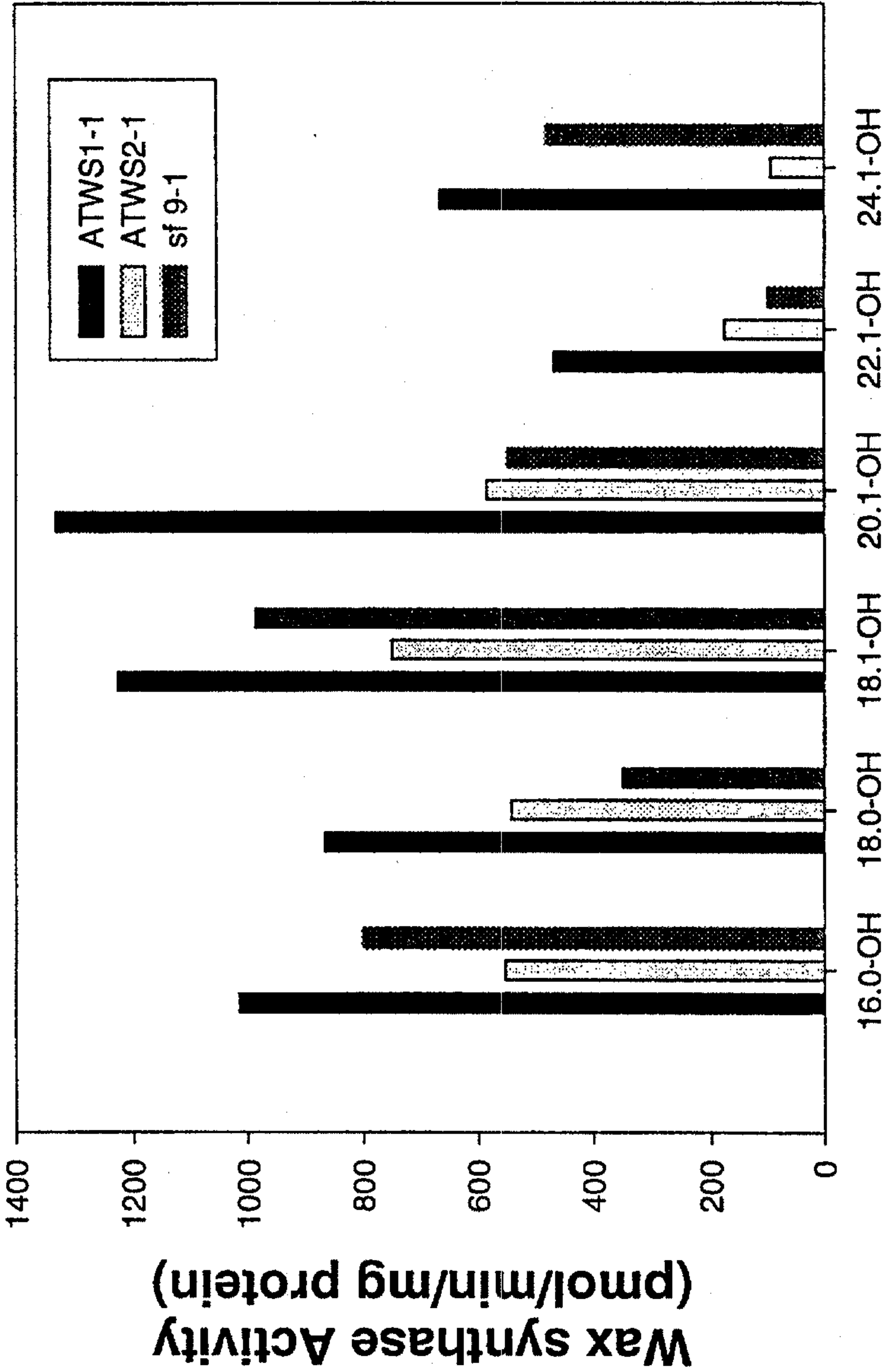


FIGURE 3

**Wax Synthase Assays of
BVIC/Insect Cell membranes 052599
(24:1-acyl, varying OH, 1 ug protein)**



Alcohol Substrate

FIGURE 4

**Wax Synthase Assays of
BVIC/Insect Cell Membranes 052599**
(24:1-acyl, varying alcohol, 10 ug protein)

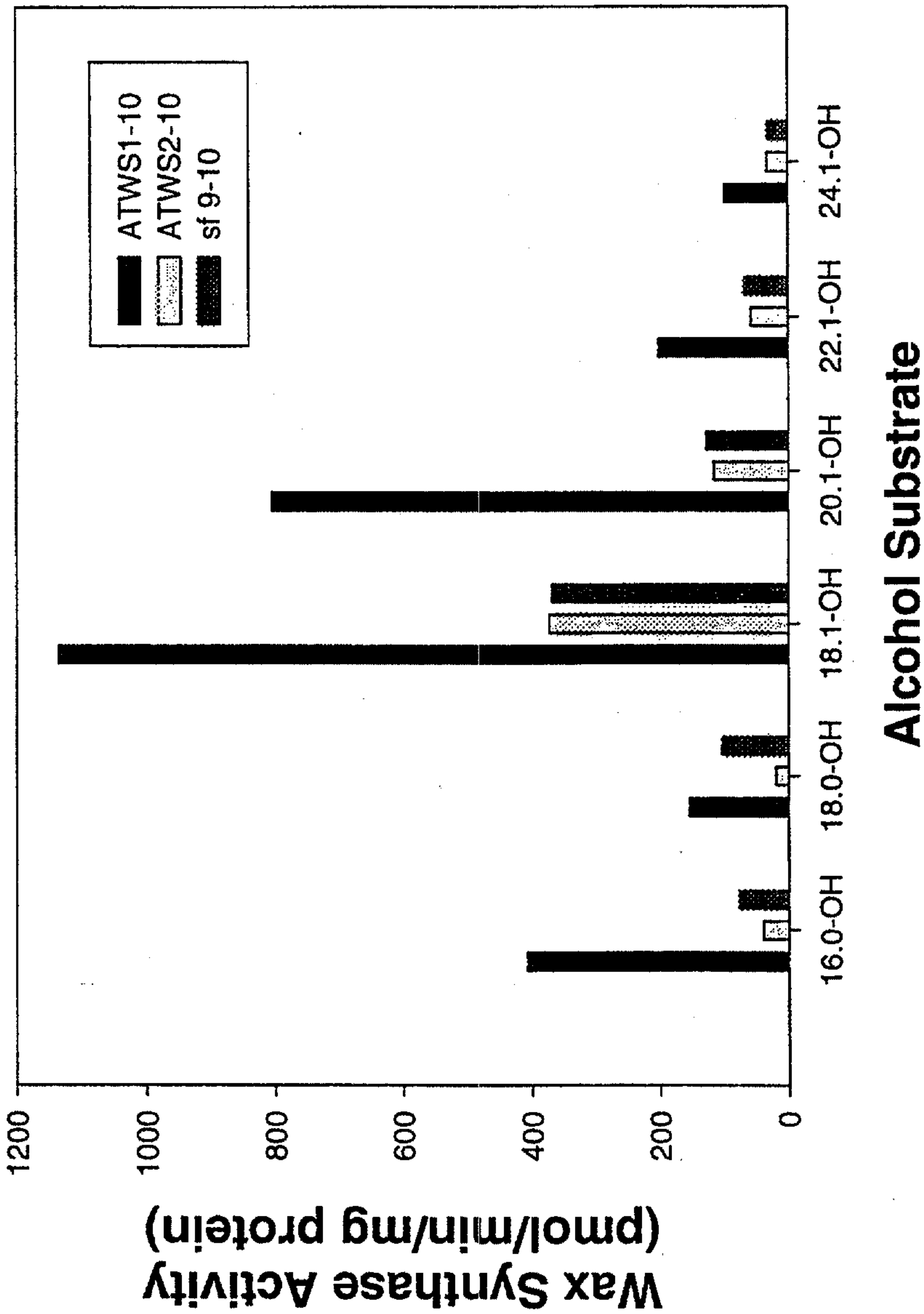


FIGURE 5

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STRAIN ID	%FAME		STRAIN ID	%FAME
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9708-03	33.5%		9712-18	30.6%
9708-04	34.1%		9712-16	31.1%
9708-05	35.1%		9712-24	31.5%
9708-06	36.1%		9712-22	31.5%
9708-07	27.3%		9712-26	31.8%
9708-08	34.1%		9712-20	32.6%
9708-09	28.9%		9712-17	32.7%
9708-10	34.3%		9712-28	33.6%
9708-11	31.4%		9712-33	34.1%
9708-12	31.0%		9712-29	34.1%
9708-13	29.6%		9712-07	34.2%
9708-14	30.0%		9712-10	34.2%
9708-15	32.9%		9712-23	34.2%
9708-16	31.3%		9712-06	34.6%
9708-17	26.8%		9712-32	35.4%
9708-18	36.2%		9712-05	35.4%
9708-19	28.7%		9712-19	35.8%
9708-20	29.6%		9712-27	36.0%
9708-20	29.8%		9712-31	36.1%
9708-21	34.4%		9712-21	36.3%
9708-23	24.9%		9712-04	37.1%
9708-24	27.1%		9712-34	37.1%
9708-26	29.5%		9712-02	37.3%
9708-27	34.3%		9712-25	37.4%
			9712-08	37.8%
			9712-15	38.0%
			9712-03	38.3%
			9712-11	38.8%
Control	34.8%		9712-12	38.9%
Control	35.4%		9712-14	41.8%

Figure 6