(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 September 2013 (06.09.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/130062 A1

(51) International Patent Classification:
C07H 21/04 (2006.01) *C12N 15/82* (2006.01)

(21) International Application Number:
PCT/US2012/027091

(22) International Filing Date:
29 February 2012 (29.02.2012)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant *(for all designated States except US)*: **EXXONMOBIL RESEARCH AND ENGINEERING COMPANY** [US/US]; 1545 Route 22 East, P. O. Box 900, Annandale, NJ 08801-0900 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **HOLTZAPPLE, Erik** [US/US]; 2625 1/2 E Street, San Diego, CA 92102 (US). **VERRUTO, John, H.** [US/US]; 4065 Georgia St., San Diego, CA 92103 (US).

(74) Agent: **RAKERS, Leanne, M.**; Harness, Dickey & Pierce, P.L.C., 7700 Bonhomme Avenue, Suite 400, St. Louis, MO 63105 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*
— *with amended claims (Art. 19(1))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2013/130062 A1

(54) Title: FOUR-GENE PATHWAY FOR WAX ESTER SYNTHESIS

(57) Abstract: The invention relates to methods for producing a wax ester in recombinant host cells engineered to express a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and a wax ester synthase. The methods of the invention may take place in photosynthetic microorganisms, and particularly in cyanobacteria. Isolated nucleotide molecules and vectors expressing the thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and wax ester synthase, recombinant host cells expressing the thioesterase, acyl-CoA synthetase, alcohol- forming fatty acyl reductase, and wax ester synthase, and systems for producing a wax ester via a pathway using these four enzymes, are also provided.

FOUR-GENE PATHWAY FOR WAX ESTER SYNTHESIS

REFERENCE TO A SEQUENCE LISTING

[0001] This application contains references to amino acid sequences and/or nucleic acid sequences which have been submitted concurrently herewith as the sequence listing text file "60930821.1.txt", file size 251 KiloBytes (KB), created on 23 February 2012. The aforementioned sequence listing is hereby incorporated by reference in its entirety pursuant to 37 C.F.R. §1.52(e)(5).

FIELD OF THE INVENTION

[0002] The present invention relates to the fields of bioengineering, metabolic biochemistry, and molecular biology. In particular, the invention relates to the production in recombinant microorganisms of lipids such as wax esters that can be used for producing fuels and chemicals.

BACKGROUND

[0003] The ever-increasing global demand for energy has led to depletion of fossil fuels, which are buried combustible geologic deposits of organic materials that have been converted to crude oil, coal, natural gas, or heavy oils. Because fossil fuels are formed by exposure to heat and pressure in the earth's crust over hundreds of millions of years, they are a finite, non-renewable resource. Further, the burning of fossil fuels is thought to play a key role in global warming. Accordingly, there is a need for non-fossil fuel energy sources.

[0004] Hydrocarbons from biological sources represent a cleaner, sustainable alternative energy source. Further, many industries, including plastics and chemical manufacturers, rely heavily on the availability of hydrocarbons for manufacturing processes. Currently, energy-rich lipids and fatty acids ("nature's petroleum") are isolated from plant and animal oils to produce diverse products such as fuels and oleochemicals. Recent efforts have focused on the microbial production of fatty acids and fatty acid derivatives by cost-effective bioprocesses. Methods of producing fatty acids and/or fatty acid derivatives in microbial hosts are described in, *e.g.*, PCT Publication Nos. WO 2007/136762, WO 2008/119082, WO 2009/009391, WO 2009/076559,

WO 2009/111513, WO 2010/006312,WO 2010/044960, WO 2010/118410, WO 2010/126891, WO 2011/008535 and WO 2011/019858 and in Schirmer *et al.*, *Science* 329(5991):559-562 (2010).

[0005] Free fatty acids are known to cause damage to cellular membranes and are thus difficult to produce in amounts sufficient for large scale production. The reduction of fatty acids to more neutral lipids such as wax esters may help to circumvent free fatty acid toxicity. Wax esters possess high energy density relative to shorter-chain biofuel products such as ethanol, and can be produced in cultured cells via a series of enzymatic processes. Wax esters have numerous commercial applications in, *e.g.*, the medical, cosmetic and dietetic industries. For example, wax esters may be used to produce candles, cosmetics, lubricants, printing inks, solvents and fuels.

SUMMARY OF THE INVENTION

[0006] The present invention provides improved nucleic acid molecules, recombinant microorganisms, and methods for producing fatty acid esters, such as but not limited to wax esters, using a four-enzyme pathway engineered into a recombinant microorganism. The enzymes of the pathway can be encoded by genes that are co-regulated, for example, two, three, or all four genes of the pathway can be configured as a single transcriptional unit, in which the genes of the transcriptional unit can be regulated by the same promoter that can optionally be a promoter endogenous to the recombinant host microorganism. The first enzyme in the pathway is a thioesterase capable of converting acyl thioesters (*e.g.*, acyl-ACPs) into free fatty acids. The second enzyme is an acyl-CoA synthetase that is capable of using the free fatty acids as a substrate to produce acyl-CoA. The third enzyme is an alcohol-forming fatty acyl reductase that is capable of using acyl-CoA as a substrate to produce fatty alcohols. The fourth enzyme is a wax ester synthase capable of using acyl-CoA and fatty alcohols as substrates to produce wax esters. Introduction of genes encoding these four enzymes into a recombinant host cell (*e.g.*, a prokaryotic and/or photosynthetic host cell) thus allows for production of wax esters from an acyl thioester produced by the fatty acid biosynthesis pathway, such as acyl-ACP. Additionally, using a photosynthetic host cell, which is able to use carbon dioxide as a carbon source, can allow for more efficient and cost-effective wax ester synthesis methods than using a host cell that depends on reduced and/or longer chain carbon sources. For example, demonstrated herein is

expression of a non-native gene encoding an acyl-ACP thioesterase, a non-native gene encoding an acyl-CoA ligase/synthase, a non-native gene encoding an acyl-CoA reductase, and a non-native gene encoding a wax ester synthase together in a cyanobacterium (which is unable to naturally synthesize acyl-CoA, fatty alcohols, or wax esters), resulting in wax ester production.

[0007] In one aspect, the invention provides a nucleic acid molecule comprising nucleic acid sequences encoding at least one of: a) a thioesterase that releases fatty acids from an acyl thioester substrate (*e.g.*, an acyl-ACP thioesterase), b) an acyl-CoA synthetase, c) an alcohol-forming fatty acyl reductase (*e.g.*, an alcohol-forming acyl-CoA reductase), and d) a wax ester synthase. The nucleic acid sequence encoding d) a wax ester synthase and at least one of the nucleic acid sequences encoding a) a thioesterase, b) an acyl-CoA synthetase, and c) an alcohol-forming fatty acyl reductase may be configured as a single transcriptional unit. The nucleic acid molecule may comprise, for example, nucleic acid sequences encoding a) a thioesterase (*e.g.*, an acyl-ACP thioesterase), b) an acyl-CoA synthetase, c) an alcohol-forming fatty acyl reductase (*e.g.*, an alcohol-forming acyl-CoA reductase), and d) a wax ester synthase, in which all of the nucleic acid sequences are configured as a single transcriptional unit. The transcriptional unit of the isolated or recombinant nucleic acid molecule can in some examples be promoterless. Alternatively, a transcriptional unit of the nucleic acid molecule can be configured as an operon that includes a promoter, in which the promoter can be any promoter that can direct expression of the genes of the transcriptional unit / operon, and can be, for example, a promoter that is heterologous with respect to the genes of the transcriptional unit, a promoter heterologous or homologous with respect to the host recombinant microorganism, and/or a synthetic promoter, and can additionally be a constitutive promoter or a regulatable promoter, for example, an inducible promoter.

[0008] The nucleic acid molecule may further comprise at least one additional nucleic acid sequence of at least 50 nucleotides, at least 100 nucleotides, at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, at least 900 nucleotides, or at least 1,000 nucleotides derived from a prokaryotic and/or photosynthetic microorganism. Additionally, the nucleotide sequence derived from a prokaryotic and/or photosynthetic microorganism may mediate recombination of the transcriptional unit into a host genome. Additionally but optionally, an additional nucleic acid sequence derived from a prokaryotic and/or photosynthetic

microorganism may comprise a nucleic acid sequence derived from the 5' region of a gene, and can, for example, optionally include at least a portion of a promoter. Additionally or alternatively, the nucleic acid molecule may comprise two or more nucleic acid sequences of at least 50 nucleotides, at least 100 nucleotides, at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, at least 900 nucleotides, or at least 1,000 nucleotides derived from a prokaryotic and/or photosynthetic microorganism. In some examples the transcriptional unit that comprises the genes of the wax ester synthesis pathway can be flanked by nucleic acid sequences derived from a prokaryotic and/or photosynthetic microorganism, for example a first nucleotide sequence derived from a prokaryotic and/or photosynthetic microorganism can be 5' of the transcriptional unit and a second nucleotide sequence derived from a prokaryotic and/or photosynthetic microorganism can be 3' of the transcriptional unit or operon. Additionally the nucleic acid sequences flanking the transcriptional unit or operon may mediate recombination of the transcriptional unit into the host genome. A nucleic acid sequence derived from a prokaryotic and/or photosynthetic microorganism positioned 5' of the transcriptional unit that includes two or more enzymes of the wax synthesis pathway may optionally comprise a promoter. Alternatively, the nucleic acid molecule can include a sequence of at least 50 nucleotides of a sequence derived from the genome of a prokaryotic and/or photosynthetic microorganism, but the genomic sequence may not comprise a promoter operably linked to any of the nucleic acid sequences of the transcriptional unit that includes two or more genes of the wax ester synthesis pathway.

[0009] The nucleic acid sequences encoding enzymes of the wax ester synthesis pathway provided as a transcriptional unit may each comprise an initiation codon, wherein one or more, and optionally all, of the nucleic acid sequences may additionally comprise a heterologous translational regulatory sequence upstream of the initiation codon. For example, the nucleic acid sequence encoding the acyl-CoA synthetase, the nucleic acid sequence encoding the alcohol-forming fatty acyl reductase, and the nucleic acid sequence encoding the wax ester may each comprise a heterologous translational regulatory sequence upstream of the initation codon.

[0010] Nonlimiting examples of the genes that can be included in the transcriptional unit include genes encoding polypeptides having wax ester synthase activity such as for example, prokaryotic wax ester synthase derived from prokaryotic species including, without limitation,

Acinetobacter and *Marinobacter* species, as well as wax synthases from algal, plant, and animal species.

[0011] In particular examples, the nucleic acid sequence encoding the thioesterase (*e.g.*, an acyl-ACP thioesterase) can encode a thioesterase having sequence identity of, *e.g.*, at least 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% to the amino acid sequence of any one of SEQ ID NOS: 1-9, or to a functional fragment thereof. The nucleic acid sequence encoding the acyl-CoA synthetase can encode an acyl-CoA synthetase having sequence identity of, *e.g.*, at least 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% to the amino acid sequence of any one of SEQ ID NOS: 10-14, or to a functional fragment thereof. The nucleic acid sequence encoding the alcohol-forming fatty acyl reductase (*e.g.*, an alcohol-forming acyl-CoA reductase) can encode an alcohol-forming acyl-CoA reductase having sequence identity of, *e.g.*, at least 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% to the amino acid sequence of any one of SEQ ID NOS: 15-21, or to a functional fragment thereof. The nucleic acid sequence encoding the wax ester synthase may encode a wax ester synthase having sequence identity of, *e.g.*, at least 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% to the amino acid sequence of any one of SEQ ID NOS: 22-30, or to a functional fragment thereof.

[0012] Additionally or alternatively to any of the aforementioned aspects, the nucleic acid sequence(s) encoding a thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and/or wax ester synthase can be integrated into a chromosome of the recombinant host cell, can be present on an autonomously replicating episome in the recombinant host cell, and/or can be present in a vector in the recombinant host cell. The nucleic acid sequence(s) encoding the thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and/or wax ester synthase can be operably linked to a promoter and/or enhancer. The promoter in various aspects can be heterologous with respect to the host organism or homologous to the host organism, can be regulatable, and/or can be inducible.

[0013] The invention also provides vectors and recombinant host cells comprising the nucleic acid molecules described herein. The vectors may be, *e.g.*, cloning vectors, and may comprise an origin of replication for propagation in a cloning strain (*e.g.*, yeast or *E. coli*). The vectors may additionally or alternatively comprise at least one selectable marker, for example, a gene

encoding a protein that confers resistance to an antibiotic or herbicide. Additionally, a vector may comprise sequences for integration into the genome of a prokaryotic and/or photosynthetic microorganism.

[0014] In some aspects, the vectors do not comprise a promoter operably linked to the transcriptional unit comprising genes of the wax ester synthase pathway. The nucleic acid molecules and vectors can be designed for integration of the transcriptional unit that encodes two or more enzymes of the wax ester synthesis pathway into a host genome, such as the genome of a prokaryotic and/or a photosynthetic microorganism. For example, in some aspects, the vectors comprise two nucleic acid sequences derived from the genome of a prokaryotic and/or a photosynthetic microorganism, where the nucleic acid sequences flank the transcriptional unit and can mediate recombination of the transcriptional unit into the genome of a prokaryotic and/or a photosynthetic microorganism. Additionally, the nucleic acid sequence derived from the genome of a prokaryotic and/or a photosynthetic microorganism that is positioned upstream of the transcriptional unit can optionally include sequences from the 5' region of a gene of a prokaryotic and/or a photosynthetic microorganism, where the genomic sequences may or may not comprise a promoter. For example, a transcriptional unit can be positioned between two nucleic acid sequences that can mediate homologous recombination with a host genome, such that the transcriptional unit can become integrated into the host genome. In some examples, the transcriptional unit can be configured as an operon that comprises a promoter, which can be, in various examples, a heterologous promoter (with respect to the genes of the transcriptional unit), an inducible promoter, a constitutive promoter, a synthetic promoter, a promoter heterologous with respect to the intended host microorganism (*i.e.*, from a different species), and/or a promoter a promoter from the same species as the intended host microorganism. A transcriptional unit operably linked to a promoter can be configured as a two, three, or four gene operon. Alternatively, the transcriptional unit can be promoterless, where the nucleic acid molecule and/or vector is designed such that integration into the genomic site of the recombinant host microorganism positions the transcriptional unit 3' of a promoter endogenous to the recombinant host microorganism, where the endogenous promoter of the host genome becomes operably linked to the transcriptional unit. In particular examples, the promoter endogenous to the intended host microorganism can be provided in the sequences derived from the genome of a

prokaryotic and/or a photosynthetic microorganism that are positioned 5' of the transcriptional unit in the nucleic acid molecule or vector.

[0015] The invention provides a recombinant microorganism, which may be, *e.g.*, a prokaryotic and/or a photosynthetic microorganism, genetically engineered for the production of wax esters, wherein the recombinant host cell contains non-native nucleic acid sequences encoding a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and a wax ester synthase. One or more of the nucleic acid sequences (*e.g.*, all of the nucleic acid sequences) may be non-native with respect to the recombinant host cell. The nucleic acid sequence(s) encoding the thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and/or wax ester synthase can be heterologous with respect to the recombinant host cell, and optionally the nucleic acid sequence(s) encoding the thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and/or wax ester synthase can be codon optimized for expression in the host cell, which can be, for example, a prokaryotic host cell or photosynthetic host cell, e.g., a photosynthetic microorganism such as a cyanobacterium or microalga.

[0016] For example, the recombinant host cell can be of an *Agmenellum, Anabaena, Anabaenopsis, Anacystis, Aphanizomenon, Arthrospira, Asterocapsa, Borzia, Calothrix, Chamaesiphon, Chlorogloeopsis, Chroococcidiopsis, Chroococcus, Crinalium, Cyanobacterium, Cyanobium, Cyanocystis, Cyanospira, Cyanothece, Cylindrospermopsis, Cylindrospermum, Dactylococcopsis, Dermocarpella, Fischerella, Fremyella, Geitleria, Geitlerinema, Gloeobacter, Gloeocapsa, Gloeothece, Halospirulina, Iyengariella, Leptolyngbya, Limnothrix, Lyngbya, Microcoleus, Microcystis, Myxosarcina, Nodularia, Nostoc, Nostochopsis, Oscillatoria, Phormidium, Planktothrix, Pleurocapsa, Prochlorococcus, Prochloron, Prochlorothrix, Pseudanabaena, Rivularia, Schizothrix, Scytonema, Spirulina, Stanieria, Starria, Stigonema, Symploca, Synechococcus, Synechocystis, Thermosynechococcus, Tolypothrix, Trichodesmium, Tychonema,* or *Xenococcus* species.

[0017] The recombinant host cell can comprise, for example, a non-native nucleic acid molecule comprising a non-native nucleic acid sequence encoding a thioesterase, a non-native nucleic acid sequence encoding an acyl-CoA synthetase, a non-native nucleic acid sequence encoding an alcohol-forming fatty acyl reductase, and a non-native nucleic acid sequence encoding a wax ester synthase, wherein two or more of the nucleic acid sequences may optionally be present in the same transcriptional unit, for example, as a single operon.

Additionally, the non-native genes encoding a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and a wax ester synthase may be configured as a single operon in the host microorganism. The operon may comprise a promoter heterologous with respect to the nucleic acid sequences, and may be heterologous or homologous with respect to the host cell and may be constitutive, or alternatively may be regulatable (*e.g.*, inducible). Additionally or alternatively, the nucleic acid molecule may be integrated into a genomic site of the recombinant host cell, which may be, *e.g.*, a cyanobacterium, such that the transcriptional unit comprising two to four genes of the wax ester synthesis pathway becomes operably linked to a promoter endogenous to the host cell. The genomic insertion site may be, for example, within or adjacent to the 5' region of a gene endogenous to the host cell. The genomic insertion site may in some examples include at least a portion of the protein coding region of a gene regulated to the promoter operably linked to the transcriptional unit, and in particular examples, the gene at the insertion site may be attenuated or disrupted, *e.g.*, integration of the nucleic acid molecule that comprises the wax synthesis transcription unit into the genomic site may inactivate or reduce expression of one or more endogenous genes. Nonlimiting examples of genes that may be at or near the locus of insertion include oxidoreductase or dehydrogenase genes (*e.g.*, the slr0338 gene of *Synechocystis* (*e.g.*, *Synechocystis* sp. PCC 6803) or an ortholog thereof in another cyanobacterial species), or genes encoding enzymes that participate in glycogen biosynthesis *e.g.*, a glycogen synthase gene (*e.g.*, *glgA*), a glycogen branching enzyme gene (*e.g.*, *glgB*) gene, or a glucose-1-phosphate adenyltransferase gene (*e.g.*, *glgC*). In a particular aspect, the recombinant host cell may be *Synechocystis* sp. 6803, and the nucleic acid sequences may be integrated at the RS1 site and be operably linked to an endogenous promoter of the RS1 site, such as, for example, the promoter.

[0018] In some aspects, the recombinant host cell is a microorganism that does not endogenously produce acyl-CoA, and/or does not include an endogenous gene encoding an acyl-CoA synthetase, *e.g.*, the recombinant host microorganism is a cyanobacterium. Alternatively, the recombinant host microorganism may endogenously produce acyl-CoA, and expression of a non-native gene encoding an acyl-CoA synthetase (and, in many cases, an acyl-ACP thioesterase to provide the fatty acid substrate for the acyl-CoA synthetase) causes acyl-CoA to be produced in higher amounts than occur in the absence of acyl-CoA synthetase (and acyl-ACP thioesterase, if present) overexpression.

[0019] The invention also provides methods for producing a wax ester, comprising the steps of culturing a recombinant host cell that includes non-native nucleic acid sequences encoding a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and a wax ester synthase in a suitable culture medium (which optionally does not comprise an alcohol or a fatty acid, and additionally or alternatively does not comprise a substantial amount of a reduced carbon source) and allowing expression of the non-native nucleic acid sequences to produce one or more wax esters. The methods can be used to produce wax esters in microorganisms that lack acyl-CoA (such as cyanobacteria). The recombinant host cell may comprise a nucleic acid molecule comprising the nucleic acid sequences encoding a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and a wax ester synthase. The recombinant host cell may produce an increased level of the wax ester relative to a control host cell identical to the recombinant host cell in all respects except that it lacks one or more (*e.g.*, all) of the non-native nucleic acid sequences encoding the thioesterase, the acyl-CoA synthetase, the alcohol-forming fatty acyl reductase, and the wax ester synthase. For example, the recombinant host cell may produce at least 50% more of the wax ester relative to a control host cell lacking the non-native nucleic acid sequences encoding the thioesterase, the acyl-CoA synthetase, the alcohol-forming fatty acyl reductase, and the wax ester synthase. The recombinant host cell may produce, *e.g.*, at least 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, or 1000% more of the wax ester relative to a control host cell lacking the non-native nucleic acid sequences encoding the thioesterase, the acyl-CoA synthetase, the alcohol-forming fatty acyl reductase, and the wax ester synthase. Additionally or alternatively, the recombinant host cell may produce at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 mg/L of wax ester in a culture period of from about one to about thirty days, such as from about one to about fifteen days, from about one to about ten days, from about one to about five days, or per day. Additionally or alternatively, the recombinant host cell may produce less than about 100 g/L, 50 g/L, 20 g/L, 10 g/L, 5 g/L, 2 g/L, 1 g/L, 500 mg/L, 200 mg/L, 100 mg/L, or 50 mg/L of the wax ester in a culture period of from about one to about thirty days, such as from about one to about fifteen days, from about one to about ten days, from about one to about five days, or per day, and/or can produce more than about 1 g/L, 500 mg/L, 200 mg/L, 100 mg/L, 50 mg/L, 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 9 mg/L, 8 mg/L, 7 mg/L, 6 mg/L, 5 mg/L, 4 mg/L, 3 mg/L, 2 mg/L, or 1 mg/L of the wax ester in a culture period of from about one to about thirty

days, such as from about one to about fifteen days, from about one to about ten days, from about one to about five days, or per day. As one example, the recombinant microorganism may produce at least 1-5 mg/L of wax ester over a period of seven days.

[0020] The methods provided herein include producing at least one wax ester molecule wherein both the A chain derived from a fatty alcohol and the B chain derived from an acyl substrate (*e.g.*, acyl-ACP) can have chain lengths of C8-C24. For example, at least one wax ester molecule produced by a method disclosed herein can have both an A chain and a B chain of C12-C18. Additionally, at least a portion of the wax ester produced by any of the methods described herein may be secreted by the host cell. The methods may optionally further include the step of isolating a wax ester or wax ester derivative.

[0021] The invention also provides methods for producing a wax ester using a photosynthetic host cell, *e.g.*, a photosynthetic microorganism. Photosynthetic host cells are able to use carbon dioxide as a carbon source, and may thus provide a more efficient and cost-effective method of wax ester production than host cells that wholly depend on reduced and/or longer chain carbon sources. For example, the methods of the invention are carried out in a photosynthetic microorganism, *e.g.*, a cyanobacterium. In certain aspects, the photosynthetic microorganism does not endogenously produce acyl-CoA. The methods of the invention may be advantageously carried out in cyanobacterial host cells, for example. Cyanobacteria synthesize acyl-ACP, but do not naturally make acyl-CoA, fatty alcohols or wax esters. Therefore, cyanobacterial host cells can be engineered to produce wax esters by introducing nucleic acid sequences encoding a) a thioesterase, b) an acyl-CoA synthetase, c) an alcohol-forming fatty acyl reductase, and d) a wax ester synthase. Because cyanobacteria are photosynthetic microorganisms that can utilize inorganic (non-reduced) carbon sources, such as CO_2, compared to, *e.g.*, heterotrophic cells that depend on organic carbon sources such as sugars that must be added to the media, cyanobacteria transformed with nucleic acid sequences encoding a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and a wax ester synthase may provide a more streamlined and energy-efficient biological system for producing wax esters.

[0022] Additionally, wax ester production may be enhanced by upregulating acyl-ACP production in the recombinant host cell, for example, by expression or overexpression of one or more exogenous or endogenous polypeptides such as, for example, a beta-ketoacyl synthetase, an acetyl-CoA carboxylase, a malonyl CoA:ACP transacylase, an acyl-ACP synthetase, or an acyl

carrier protein. Additionally or alternatively, the recombinant host cell can express or overexpress one or more exogenous or endogenous polypeptides that increase carbon fixation or photosynthetic light harvesting efficiency, or promote secretion of the wax ester product, such as, for example, ribulose 1,5-bisphosphate carboxylase, a phycobiliprotein, or a transmembrane transporter. Additionally or alternatively, the recombinant host cell can have attenuated expression of one or more of glycerol-3-phosphate dehydrogenase, acetaldehyde CoA dehydrogenase, pyruvate dehydrogenase, or acetate kinase. Any enzymes that function in the wax ester biosynthesis pathway may optionally be introduced and/or overexpressed.

[0023] The invention also provides a system for producing a wax ester that includes a recombinant photosynthetic microorganism (*e.g.*, a recombinant microorganism such as a cyanobacterium) having non-native nucleic acid sequences encoding a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and a wax ester synthase cultured in a medium that does not include a substantial amount of an organic (*e.g.*, reduced) carbon source, wherein the photosynthetic microorganism is exposed to light for at least a portion of the production period. Optionally, the system can further include an inorganic (*e.g.*, non-reduced) carbon source, such as, for example, CO_2. The inorganic carbon source may provide the carbon for the synthesis of a wax ester product. The system for producing a wax ester may comprise any of the nucleic acid molecules, vectors, or recombinant host cells described herein, and may perform any of the methods described herein.

[0024] The invention also provides a composition that includes a wax ester. The wax ester is produced by the methods provided herein, and can include one or more wax esters having both an A chain and a B chain with chain lengths of C8-C24. The composition may comprise, for example, at least one wax ester molecule produced by a method disclosed herein that has both an A chain and a B chain of C12-C18, or of C12-C16, or of C14-C16, of C14, or of C16. Additionally or alternatively, a wax ester composition of the invention may, according to certain aspects, be identifiable as having been produced according to a method of the invention by detection of one or more nucleci acid molecules as a minor component which my be detected for example, by polymerase chain reaction (PCR) or by an alternative sequence-specific nucleic acid amplification detection method, where the nucelcie acid molecules may comprise one or more sequences derived from a recombinant nucleic acid molecule as disclosed herein, for example, a recombinant nucleic acid molecule comprising a non-native gene encoding a wax ester synthase,

and, preferably, one or more of a a non-native gene encoding a thioesterase, a non-native gene encoding an alcohol forming acyl CoA reductase, and a non-native gene encoding an acyl-CoA synthetase.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] **FIGURE 1** shows the amino acid sequences of exemplary thioesterases for use in the wax ester synthesis pathways of the invention (SEQ ID NOS: 1-9).

[0026] **FIGURE 2** shows the amino acid sequences of exemplary acyl-CoA synthetases for use in the wax ester synthesis pathways of the invention (SEQ ID NOS: 10-14).

[0027] **FIGURE 3** shows the amino acid sequences of exemplary fatty acyl reductases for use in the wax ester synthesis pathways of the invention (SEQ ID NOS: 15-21).

[0028] **FIGURE 4** shows the amino acid sequences of exemplary wax ester synthases for use in the wax ester synthesis pathways of the invention (SEQ ID NOS: 22-30).

[0029] **FIGURE 5** is a schematic representation of fatty acid derivative metabolic pathways.

[0030] **FIGURE 6** is a schematic representation of a four-step metabolic pathway for producing wax esters from acyl-ACP.

[0031] **FIGURE 7** shows a plasmid map (pSGE05109; SEQ ID NO: 70) of an integration vector that includes four wax ester synthesis pathway genes in the order: cc1FatB1, Faa2p, Maqu_2220, and petunia WS, operably linked to a Prbc promoter. RS1-down and RS1-up refer to integration sites on the chromosome of *Synechocystis* sp. PCC 6803.

[0032] **FIGURE 8** shows a plasmid map (pSGE05110; SEQ ID NO: 71) of an integration vector that includes four wax ester synthesis pathway genes in the order: cc1FatB1, Faa2p, Maqu_2220, and Maqu0168 WS1, operably linked to a TrcE promoter. RS1-down and RS1-up refer to integration sites on the chromosome of *Synechocystis* sp. PCC 6803.

[0033] **FIGURE 9** shows a plasmid map (pSGE05023; SEQ ID NO: 72) of an integration vector that includes four wax ester synthesis pathway genes in the order: cc1FatB1, Faa2p, Maqu_2220, and MELB17 WS. The vector does not contain an isolated promoter sequence cloned 5' of these genes. RS1-down and RS1-up refer to integration sites on the chromosome of *Synechocystis* sp. PCC 6803.

[0034] **FIGURE 10** shows a plasmid map (pSGE05062; SEQ ID NO: 73) of an integration vector that includes four wax ester synthesis pathway genes in the order: Faa2p, MELB17 WS, cc1FatB1, and Maqu_2220. The vector does not contain an isolated promoter sequence cloned 5' to these genes. RS1-down and RS1-up refer to integration sites on the chromosome of *Synechocystis* sp. PCC 6803.

DETAILED DESCRIPTION OF THE INVENTION

[0035] The invention provides methods of producing a wax ester in recombinant host cells via a four-gene pathway, as well as isolated nucleotide molecules, vectors, and recombinant host cells and systems for producing a wax ester via a four-gene pathway.

[0036] Elements of the aspects described herein can be combined to form additional aspects not specifically described that are also within the scope of the invention. Headings within the application are solely for the convenience of the reader, and do not limit in any way the scope of the invention or its aspects.

[0037] All publications and patent applications cited in this specification are incorporated herein by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Definitions

[0038] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention is related.

[0039] Throughout this specification and embodiments, the word "comprise," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated entity, item, or group of items but not the exclusion of any other entity, item, or group of items.

[0040] Singular articles "a," "an" and "the" include plural references unless the context clearly dictates otherwise. A reference to a cell, for example, includes a plurality of cells.

[0041] The term "and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B", "A or B", "A", and "B".

[0042] A "fatty alcohol" is a primary alcohol having the formula ROH, in which R is an aliphatic group, preferably an alkyl group. R can comprise between about 6 and about 24 carbon atoms. The aliphatic chain can be saturated, monounsaturated, or polyunsaturated. "One or more fatty alcohols" refers to one or more fatty alcohols of different chain length and/or saturation pattern, for example, a C16:1 fatty alcohol, a C18:2 fatty alcohol, and a C14 fatty alcohol are particular fatty alcohols.

[0043] A "short chain alcohol" is an alcohol having from 1 to 5 carbon atoms. A short chain alcohol can be linear or branched. Nonlimiting examples of short chain alcohols include methanol, ethanol, propanol, butanol, isobutanol, 2-methylbutanol, and 3-methylbutanol.

[0044] A "wax ester" is an ester of a fatty acid and a long chain aliphatic alcohol. Wax esters have an A chain, derived from a fatty alcohol, of at least 8 carbons, and a B chain, derived from an acyl-thioester, of at least 8 carbons. The number of carbons in the A and B chains of a wax ester can vary idependently.

[0045] A "fatty acid ester" is an ester of a fatty acid and an alcohol. The carbon chain originating from an alcohol is referred to as the A chain and the carbon chain originating from a fatty acid (the fatty acid moiety can be provided by an acyl thioester) is referred to as the B chain. A fatty acid ester can have an A side of any length, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, or more than 24 carbons in length. A fatty acid ester can have a B side of any length, for example, 4, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, or more than 24 carbons in length. The lengths of the A and B chains of a fatty acid ester can vary independently. For example, condenstation of methanol (C1) and an acyl chain (fatty acid or acyl-thioester) of C4 or greater can result in a fatty acid methyl ester ("FAME") and condenstation of ethanol and an acyl chain can result in a fatty acid ethyl ester ("FAEE"). Condensation of a fatty alcohol (C8 or above) with an acyl thioester (C8 or greater) produces a wax ester.

[0046] The term "acyl-ACP thioesterase" refers to a protein that is able to convert acyl-ACP into free fatty acids. The term "acyl-CoA thioesterase" refers to a protein that is able to convert acyl-CoA into free fatty acids. Acyl-ACP thioesterases include thioesterases that use only acyl-ACP as the acyl-thioester substrate and acyl-ACP thioesterases able to use other acyl-thioester substrates (e.g., acyl-CoA) in addition to acyl-ACP.

[0047] The term "alcohol-forming acyl-CoA reductase" refers to a protein that is able to convert acyl-CoA (and optionally other acyl-thioester substrates) to fatty alcohol. The term "alcohol-forming acyl-ACP reductase" refers to a protein that is able to convert acyl-ACP (and optionally other acyl-thioester substrates) to fatty alcohol. "Alcohol-forming fatty acyl reductase" refers to enzymes that can convert either acyl-ACP or acy-CoA to fatty alcohols, and includes "promiscuous alcohol-forming fatty acyl reductases" that are able to use both acyl-ACP and acyl-CoA as substrates for the production of fatty alcohols.

[0048] As used herein, the term "wax ester synthase" or "wax synthase" refers to a protein that is able to transfer an acyl chain from an acyl substrate such as acyl-CoA to a fatty alcohol to form a wax ester. The wax ester synthases used in the methods of the invention can condense a fatty alcohol (*e.g.*, a C6, C7, C8, C10, C12, C14, C16, C18, C20, C22, C24, or longer chain alcohol) with an acyl-thioester substrate to produce a wax ester. A wax ester synthase can also condense a short chain alcohol (*e.g.*, a C1, C2, C3, C4, or C5 alcohol) with an acyl-thioester substrate such as acyl-CoA to form a fatty acid ester such as a fatty acid methyl ester or fatty acid ethyl ester. The alcohol condensed with the acyl-thioester substrate can be produced by the transgenic host cell or supplied to the transgenic host cell, for example, in the culture medium. Various polypeptides identified or characterized as acyltransferases, including fatty acyl transferases, alcohol acyltransferases (AATs, EC 2.3.1.84) and alcohol synthase/acyl-CoA:diacylglycerol acyltransferases, O-acyltransferases (*e.g.*, long-chain-alcohol O-fatty-acyltransacylases (EC 2.3.1.75) or acyl-CoA:alchol acyltransferases, diacylglycerol O-acyltransferases, membrane bound O-acyltranferases (MBOATs)), diacylglycerol acyltransferases (DGATs), acyl-coA wax alcohol acyltransferases, and bifunctional wax ester synthase/acy 1-CoA acyltransferases, have been found to have wax ester synthase activity. The term "wax synthase" or "wax ester synthase" without limitation includes enzymes that have wax ester synthase activity regardless of the name formally or informally given to the enzyme or its class. Thus "wax ester synthase" and "polypeptide having wax ester synthase activity" are used interchangeably herein.

[0049] As used herein, a "wax ester synthesis pathway" refers to the pathway of four enzymes (or their encoding genes) that convert acyl-ACP to a wax ester: a thioesterase that can use acyl-ACP as a substrate, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and a wax ester synthase.

[0050] The terms "peptide," "polypeptide" and "protein" are used interchangeably herein, although "peptide," in some instances, may be used to refer to a polypeptide having no more than about 100 amino acids, or no more than about 60 amino acids.

[0051] The term "functional fragment" refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, where the remaining amino acid sequence has at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the corresponding positions in the reference sequence, and that retains about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the activity of the full-length polypeptide. Functional fragments may comprise, *e.g.*, 90% or less, 80% or less, 70% or less, 60% or less, 50% or less, 40% or less, 30% or less, or 20% or less of the full-length polypeptide, and can include, for example, up to about 20%, 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the full-length polypeptide.

[0052] This application discloses and refers to nucleic acids and polypeptides by identifiers used in long-established and extensively referenced databases maintained by the National Center for Biotechnology Information (NCBI). Accession numbers are unique identifiers for a sequence record publicly available at the National Center for Biotechnology Information website (ncbi.nlm.nih.gov) maintained by the United States National Institutes of Health. The "GenInfo Identifier" (GI) sequence identification number is specific to a nucleotide or amino acid sequence. If a sequence changes in any way, a new GI number is assigned. A Sequence Revision History tool is available to track the various GI numbers, version numbers, and update dates for sequences that appear in a specific GenBank record. Searching and obtaining nucleic acid or gene sequences or protein sequences based on Accession numbers and GI numbers is well known in the arts of, *e.g.*, cell biology, biochemistry, molecular biology, and molecular genetics.

[0053] Percent identity or homology with respect to amino acid or nucleotide sequences is defined herein as the percentage of amino acid or nucleotide residues in the candidate sequence that are identical with the known polypeptides, after aligning the sequences for maximum percent identity and introducing gaps, if necessary, to achieve the maximum percent homology. Homology or identity at the nucleotide or amino acid sequence level may be determined using methods known in the art, including but not limited to BLAST (Basic Local Alignment Search

Tool) analysis using the algorithms employed by the programs blastp, blastn, blastx, tblastn and tblastx (Altschul (1997), Nucleic Acids Res. 25, 3389-3402, and Karlin (1990), Proc. Natl. Acad. Sci. USA 87, 2264-2268), which are tailored for sequence similarity searching.

[0054] "Pfam" is a large collection of protein domains and protein families maintained by the Pfam Consortium and is available at several sponsored world wide web sites, including: pfam.sanger.ac.uk/ (Welcome Trust, Sanger Institute); pfam.sbc.su.se/ (Stockholm Bioinformatics Center); pfam.janelia.org/ (Janelia Farm, Howard Hughes Medical Institute); pfam.jouy.inra.fr/ (Institut national de la Recherche Agronomique); and pfam.ccbb.re.kr/. The latest release of Pfam is Pfam 26.0 (November 2011, 13,672 families) based on the UniProt protein database release 2020_05. Pfam domains and families are identified using multiple sequence alignments and hidden Markov models (HMMs). Pfam-A families, which are based on high quality assignments, are generated by a curated seed alignment using representative members of a protein family and profile hidden Markov models based on the seed alignment. (Unless otherwise specified, matches of a queried protein to a Pfam are Pfam-A matches.) All identified sequences belonging to the family are then used to automatically generate a full alignment for the familiy (Sonnhammer *et al.* (1998) Nucleic Acids Research 26: 320-322; Bateman *et al.* (2000) Nucleic Acids Research 26: 263-266; Bateman *et al.* (2004) Nucleic Acids Research 32, Database Issue: D138-D141; Finn *et al.* (2006) Nucleic Acids Research Database Issue 34: D247-251; Finn *et al.* (2010) Nucleic Acids Research Database Issue 38: D211-222). By accessing the pfam database (for example, using any of the above-reference websites), protein sequences can be queried against the HMMs using HMMER homology search software (*e.g.*, HMMER3, hmmer.janelia.org/). Significant matches that identify a queried protein as being in a pfam family (or as having a particular pfam domain) are those in which the bit score is greater than or equal to the gathering threshold for the Pfam domain. Expectation values (e values) can also be used as a criterion for inclusion of a queried protein in a pfam or for determining whether a queried protein has a particular pfam domain, where low e values (much less than 1.0, for example less than 0.1, or less than or equal to 0.01) represent low probabilies that a match is due to chance.

[0055] A "conservative variant" of a polypeptide is a polypeptide having one or more conservative amino acid substitutions with respect to the reference polypeptide, in which the

activity (*e.g.* effect on transcription), affinity for co-regulators or ligands, or DNA-binding affinity of the polypeptide does not substantially differ from that of the reference polypeptide.

[0056] The term "conservative amino acid substitution" or "conservative mutation" refers to the replacement of one amino acid by another amino acid with a common property. A functional way to define common properties between individual amino acids is to analyze the normalized frequencies of amino acid changes between corresponding proteins of homologous organisms (Schulz (1979) Principles of Protein Structure, Springer-Verlag). According to such analyses, groups of amino acids can be defined where amino acids within a group exchange preferentially with each other, and therefore resemble each other most in their impact on the overall protein structure (Schulz (1979) Principles of Protein Structure, Springer-Verlag). Examples of amino acid groups defined in this manner can include: a "charged/polar group" including Glu, Asp, Asn, Gln, Lys, Arg and His; an "aromatic or cyclic group" including Pro, Phe, Tyr and Trp; and an "aliphatic group" including Gly, Ala, Val, Leu, Ile, Met, Ser, Thr and Cys. Within each group, subgroups can also be identified. For example, the group of charged/polar amino acids can be sub-divided into sub-groups including: the "positively-charged sub-group" comprising Lys, Arg and His; the "negatively-charged sub-group" comprising Glu and Asp; and the "polar sub-group" comprising Asn and Gln. In another example, the aromatic or cyclic group can be sub-divided into sub-groups including: the "nitrogen ring sub-group" comprising Pro, His, and Trp; and the "phenyl sub-group" comprising Phe and Tyr. In another further example, the aliphatic group can be sub-divided into sub-groups including: the "large aliphatic non-polar sub-group" comprising Val, Leu and Ile; the "aliphatic slightly-polar sub-group"comprising Met, Ser, Thr and Cys; and the "small-residue sub-group" comprising Gly and Ala. Examples of conservative mutations include amino acid substitutions of amino acids within the sub-groups above, such as, but not limited to: Lys for Arg or vice versa, such that a positive charge can be maintained; Glu for Asp or vice versa, such that a negative charge can be maintained; Ser for Thr or vice versa, such that a free -OH can be maintained; and Gln for Asn or vice versa, such that a free $-NH_2$ can be maintained.

[0057] The term "gene" is used broadly to refer to any segment of nucleic acid molecule (typically DNA, but optionally RNA) encoding a protein or expressed RNA. Thus, genes include sequences encoding expressed RNA (which can include polypeptide coding sequences). Genes may further comprise the regulatory sequences required for their expression. Genes can

be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

[0058] The "5' region of a gene" as used herein, is a term that refers to nucleic acid sequence of any length, but preferably from 10 to 2,000 nucleotides, or more preferably from 15 to 1,000 nucleotides, or from 20 to 1,000 nucleotides, that includes sequences of the genome of an organism that are upstream, or 5' to, the translational start site of open reading frame of the gene (or, if the gene encodes a functional RNA, are upstream of the transcriptional start site of the functional RNA). The 5' region of a gene includes one or more of the following: a promoter, the 5' untranslated region of the gene or a portion thereof, or at least one codon encoding the N-terminal amino acid(s) of the gene.

[0059] The term "nucleic acid" or "nucleic acid molecule" refers to, *e.g.*, DNA or RNA (*e.g.*, mRNA). The nucleic acid molecules can be double-stranded or single-stranded; single stranded RNA or DNA can be the coding (sense) strand or the non-coding (antisense) strand.

[0060] The nucleic acid molecules of the present invention may be isolated or purified. As used herein, an "isolated" nucleic acid molecule or nucleotide sequence refers to a nucleic acid molecule or nucleotide sequence that is not flanked by nucleotide sequences normally flanking the gene or nucleotide sequence (as in genomic sequences), and therefore can be a recombinant nucleic acid molecule or sequence, and/or has been completely or partially removed from its native environment (*e.g.* a cell, tissue). For example, nucleic acid molecules that have been removed or purified from cells are considered isolated. In some instances, the isolated material will form part of a composition (for example, a crude extract containing other substances), buffer system or reagent mix. In some circumstances, the nucleic acid molecules may be purified to near homogeneity, for example as determined by PAGE or column chromatography such as HPLC. An isolated nucleic acid molecule or nucleotide sequence can includes a nucleic acid molecule or nucleotide sequence that is chemically synthesized, using recombinant DNA technology or using any other suitable method. A nucleic acid contained in a vector would also be included in the definition of "isolated" as used herein. Both *in vivo* and *in vitro* RNA transcripts of an isolated DNA molecule of the present invention are also encompassed by "isolated" nucleotide sequences.

[0061] The term "codon optimized" refers to changes in the codons of a nucleotide sequence encoding a protein to those preferentially used in a particular organism such that the encoded protein is efficiently expressed in the organism of interest. In some aspects, a nucleotide sequence encoding a protein may be codon optimized for optimal production of the protein from a host organism. As used in the context of the invention, a "codon-optimized" gene or nucleic acid molecule of the invention need not have every codon altered to conform to the codon preference of the intended host organism, nor is it required that altered codons of a "codon-optimized" gene or nucleic acid molecule be changed to the most prevalent codon used by the organism of interest. For example, a codon-optimized gene may have one or more codons changed to codons that are used more frequently than the original codon(s), whether or not they are used most frequently in the organism to encode a particular amino acid.

[0062] The terms "expression vector" and "expression construct" refer to a nucleic acid molecule that has been generated via human intervention, including by recombinant means and/or direct chemical synthesis, with a series of specified nucleic acid "expression control elements" that permit transcription and/or translation of a particular nucleic acid in a host cell. The expression vector can be a plasmid, a part of a plasmid, a viral construct, a nucleic acid fragment, or the like, or a combination thereof.

[0063] An "expression cassette" or "nucleic acid cassette," as used herein, refers to a nucleotide sequence encoding a protein or functional RNA (*e.g.* a tRNA, a short hairpin RNA, one or more microRNAs, a ribosomal RNA, *etc.*) operably linked to expression control elements, such as a promoter, and optionally, any or a combination of other nucleic acid sequences that affect the transcription or translation of the gene, such as, but not limited to, a transcriptional terminator, a ribosome binding site (rbs), a splice site or splicing recognition sequence, an intron, an enhancer, a polyadenylation signal, an internal ribosome entry site, *etc.* "Operable linkage" or "operably linked" refers to a functional linkage between two nucleic acid sequences, such as a control sequence (such as a promoter) and the linked sequence (such as a sequence that encodes a protein and/or functional RNA). A promoter is in operable linkage with a nucleic acid sequence if it can mediate transcription of the gene. A nucleic acid sequence derived from the genome of a host microorganism can be operably linked to a nucleic acid sequence exogenous to the host microorganism, wherein the genome-derived sequence can promote homologous recombination resulting in the insertion of the exogenous nucleic acid sequence into the genome

of the host microorganism. For example, a nucleic acid molecule of the invention can include a nucleic acid sequence exogenous to the host microorganism that encodes a protein of interest, wherein the exogenous nucleic acid sequence is operably linked to sequences (for example, flanked by sequences) derived from the host microorganism that allow recombination of the exogenous nucleic acid sequence into the host genome.

[0064] The term "transcriptional unit", as used herein, refers to a unit of one or more genes configured such that when placed under the control of a single promoter (*e.g.*, a single promoter 5' of the 5' most gene of the transcriptional unit), the genes transcribed together to produce a single transcript (RNA molecule). Thus, a transcriptional unit does not comprise promoters or transcriptional terminators between genes of the transcriptional unit. A transcriptional unit may or may not be an operon that comprises a promoter at the 5' end of the transcriptional unit.

[0065] The term "operon", as used herein, refers to a unit of more than one gene under the control of a single regulatory signal or promoter. The genes may be transcribed, *e.g.*, into a single RNA molecule. The genes may then be translated together as a single RNA strand, or the transcribed RNA molecule may undergo trans-splicing to produce monocistronic RNAs that may be translated separately, *etc.*

[0066] "Stringency conditions" for hybridization of nucleotide sequences refer to the incubation and wash conditions, *e.g.* conditions of temperature and buffer concentration, which permit hybridization of a particular nucleic acid to a second nucleic acid; the first nucleic acid may be perfectly (*i.e.*, 100%) complementary to the second, or the first and second may share some degree of complementarity, which is less than perfect, *e.g.*, 60%, 75%, 85%, 95% or more. For example, certain high stringency conditions can be used which distinguish perfectly complementary nucleic acids from those of less complementarity.

[0067] "High stringency conditions," "moderate stringency conditions" and "low stringency conditions" for nucleic acid hybridizations are explained in Current Protocols in Molecular Biology (2011) John Wiley & Sons). The exact conditions which determine the stringency of hybridization depend not only on ionic strength (*e.g.* 0.2× SSC, 0.1× SSC, *etc.*) of the wash buffers, temperature (*e.g.*, 23°C, 42°C, 68°C, *etc.*) and the concentration of destabilizing agents such as formamide or denaturing agents such as SDS, but also on factors such as the length of the nucleic acid sequence, base composition, percent mismatch between hybridizing sequences

and the frequency of occurrence of subsets of that sequence within other non-identical sequences. Thus, high, moderate or low stringency conditions may be determined empirically.

[0068] By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, conditions which will allow a given sequence to hybridize with the most similar sequences in the sample can be determined.

[0069] Exemplary hybridization conditions are described in Krause (1991) *Methods in Enzymology*, 200, 546-556. Washing is the step in which conditions are usually set so as to determine a minimum level of complementarity of the hybrids. Generally, starting from the lowest temperature at which only homologous hybridization occurs, each degree (°C) by which the final wash temperature is reduced, while holding SSC concentration constant, allows an increase by 1% in the maximum extent of mismatching among the sequences that hybridize. Generally, doubling the concentration of SSC results in an increase in Tm. Using these guidelines, the washing temperature can be determined empirically for high, moderate or low stringency, depending on the level of mismatch sought. Exemplary high stringency conditions include, but are not limited to, hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1× SSC at 60°C. Example of progressively higher stringency conditions include, after hybridization, washing with 0.2× SSC and 0.1% SDS at about room temperature (low stringency conditions); washing with 0.2× SSC and 0.1% SDS at about 42°C (moderate stringency conditions); and washing with 0.1× SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.* high stringency conditions, washing may encompass two or more of the stringency conditions in order of increasing stringency. Optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

[0070] Equivalent conditions can be determined by varying one or more of the parameters given as an example, as known in the art, while maintaining a similar degree of identity or similarity between the target nucleic acid molecule and the primer or probe used. Hybridizable nucleotide sequences are useful as probes and primers for identification of organisms comprising a nucleic acid of the invention and/or to isolate a nucleic acid of the invention, for example.

[0071] A "purified" nucleic acid molecule or nucleotide sequence, or protein or polypeptide sequence, is substantially free of cellular material and cellular components. The purified nucleic

acid molecule or protein may be free of chemicals beyond buffer or solvent, for example. "Substantially free" is not intended to mean that other components beyond the novel nucleic acid molecules are undetectable.

[0072] A "recombinant" or "engineered" nucleic acid molecule is a nucleic acid molecule that has been altered through human manipulation. As non-limiting examples, a recombinant nucleic acid molecule: (1) has been synthesized or modified *in vitro*, for example, using chemical or enzymatic techniques (for example, by use of chemical nucleic acid synthesis, or by use of enzymes for the replication, polymerization, digestion (exonucleolytic or endonucleolytic), ligation, reverse transcription, transcription, base modification (including, *e.g.*, methylation), or recombination (including homologous and site-specific recombination)) of nucleic acid molecules; (2) includes conjoined nucleotide sequences that are not conjoined in nature, (d) has been engineered using molecular cloning techniques such that it possesses and/or lacks one or more nucleotides with respect to the naturally occurring nucleic acid molecule sequence, or (4) has been manipulated using molecular cloning techniques such that it has one or more sequence changes or rearrangements with respect to the naturally occurring nucleic acid sequence. As non-limiting examples, a cDNA is a recombinant DNA molecule, as is any nucleic acid molecule that has been generated by in vitro polymerase reaction(s), or to which linkers have been attached, or that has been integrated into a vector, such as a cloning vector or expression vector.

[0073] When applied to organisms, the term "recombinant," "engineered," or "genetically engineered" refers to organisms that have been manipulated by introduction of a heterologous or recombinant nucleic acid sequence into the organism, and includes gene knockouts, targeted mutations and gene replacement, promoter replacement, deletion, or insertion, as well as introduction of transgenes into the organism. The heterologous or recombinant nucleic acid molecule can be integrated into the recombinant/genetically engineered organism's genome or in other instances are not integrated into the recombinant/genetically engineered organism's genome.

[0074] Similarly, the term "recombinant protein" as used herein may refer to a protein altered through human manipulation, *e.g.*, produced by genetic engineering.

[0075] The terms "naturally-occurring" and "wild-type" refer to a form found in nature. For example, a naturally occurring or wild-type nucleic acid molecule, nucleotide sequence or

protein may be present in and isolated from a natural source, and is not intentionally modified by human manipulation.

[0076] As used herein "attenuated" means reduced in amount, degree, intensity, or strength. Attenuated gene expression may refer to a significantly reduced amount and/or rate of transcription of the gene in question, or of translation, folding, or assembly of the encoded protein. For example, an attenuated gene can be a disrupted or deleted gene that results in no detectable production of the encoded protein.

[0077] "Exogenous nucleic acid molecule" or "exogenous gene" refers to a nucleic acid molecule or gene that has been introduced ("transformed") into a cell. A transformed cell may be referred to as a recombinant cell, into which additional exogenous gene(s) may be introduced. A descendent of a cell transformed with a nucleic acid molecule is also referred to as "transformed" if it has inherited the exogenous nucleic acid molecule. The exogenous gene may be from a different species (and so "heterologous"), or from the same species (and so "homologous"), relative to the cell being transformed. An "endogenous" nucleic acid molecule, gene or protein is a native nucleic acid molecule, gene or protein as it occurs in, or is naturally produced by, the host.

[0078] The term "heterologous" is used broadly in this aspect to refer to nucleic acid molecules or proteins introduced into a host cell, wherein the nucleic acid molecules or proteins are derived from a different strain/organism. A heterologous gene may have an equivalent in the transformed host, *i.e.*, a gene which normally performs the same or a similar function, or the exogenous heterologous gene may encode a protein that does not have an endogenous homolog in the host strain/organism. When referring to a gene regulatory sequence or to an auxiliary nucleic acid sequence used for maintaining or manipulating a gene sequence (*e.g.* a 5' untranslated region, 3' untranslated region, poly A addition sequence, intron sequence, splice site, ribosome binding site, internal ribosome entry sequence, genome homology region, recombination site, *etc.*), "heterologous" means that the regulatory sequence or auxiliary sequence is from a different source than the gene with which the regulatory or auxiliary nucleic acid sequence is juxtaposed in a construct, genome, chromosome or episome. Thus, a promoter operably linked to a gene to which it is not operably linked to in its natural state (*i.e.* in the genome of a non-genetically engineered organism) is referred to herein as a "heterologous

promoter," even though the promoter may be derived from the same species (or, in some cases, the same organism) as the gene to which it is linked.

[0079] The term "native" is used herein to refer to nucleic acid sequences or amino acid sequences as they naturally occur in the host. The term "non-native" is used herein to refer to nucleic acid sequences or amino acid sequences that do not occur naturally in the host. A nucleic acid sequence or amino acid sequence that has been removed from a host cell, subjected to laboratory manipulation, and introduced or reintroduced into a host cell is considered "non-native." Synthetic or partially synthetic genes introduced into a host cell are "non-native." Non-native genes further include genes endogenous to the host microorganism operably linked to one or more heterologous regulatory sequences that have been recombined into the host genome.

[0080] The term "wax ester composition" refers to a composition that comprises at least one wax ester molecule. Wax esters include, *e.g.*, compositions comprising only wax ester molecules (*i.e.*, a composition which does not contain a fatty acid derivative other than wax ester molecules) and compositions comprising wax esters and at least one other type of fatty acid derivative selected from, *e.g.*, alcohols, aldehydes, alkenes, alkynes and alkanes. Wax esters may comprise only one type of wax ester molecule or more than one type of wax ester molecule.

[0081] The terms "releasing" and "secreting," as used herein, are used interchangeably to refer to active and/or passive mechanisms to transport substances across the cell membrane. Examples of such transport mechanisms include, but are not limited to, passive diffusion, gradient diffusion, facilitated diffusion, active transport, and combinations thereof.

[0082] The terms "recombinant," "engineered" or "genetically engineered," when applied to host cells, refer to cells that have been manipulated by introduction of a non-native (*e.g.*, heterologous or recombinant) nucleic acid sequence into the host cell, or deletion of a native nucleic acid sequence from the host cell, and include, *e.g.*, gene knockouts; targeted mutations and gene replacement; promoter replacement, deletion or insertion; as well as introduction of transgenes into the host cell. In some aspects, an introduced non-native nucleic acid molecule is integrated into the genome of the recombinant/genetically engineered host. In other aspects, an introduced non-native nucleic acid molecule is not integrated into the genome of the recombinant/genetically engineered host.

[0083] The terms "transformation," "transfection," "conjugation" and "transduction," as used in the present context, are intended to comprise a multiplicity of methods known to those skilled

in the art for the introduction of foreign nucleic acids (for example, exogenous DNA) into a host cell, including calcium phosphate and/or calcium chloride coprecipitation, DEAE-dextran-mediated transfection, lipofection, natural competence, chemically mediated transfer, electroporation, particle bombardment, or the like, or combinations thereof. Transfection may be transient or stable (*e.g.*, genomic integration). Examples of suitable methods for the transformation and/or transfection of host cells, *e.g.* can be found in Molecular Cloning - A Laboratory Manual (2010), Cold Spring Harbor Laboratory Press.

[0084] The term "culturing" refers to the intentional fostering of growth (*e.g.* increases in cell size, cellular contents and/or cellular activity such as production of biomolecules) and/or propagation (*e.g.* increases in cell numbers via mitosis) of one or more cells by use of selected and/or controlled conditions. The combination of both growth and propagation may be termed proliferation. Nonlimiting examples of selected and/or controlled conditions can include the use of a defined medium (with known characteristics such as pH, ionic strength and/or carbon source), specified temperature, oxygen tension, carbon dioxide levels, growth in a bioreactor, or the like, or combinations thereof.

[0085] The term "bioreactor" refers to an enclosure or partial enclosure in which cells (*e.g.*, microalgal cells) are cultured, optionally in suspension and, when suspended, preferably in an aqueous liquid. The bioreactor can be used to culture cells through the various phases of their physiological cycle.

Metabolic Pathways

[0086] The fatty acid biosynthesis pathway is highly conserved in prokaryotes and in the chloroplasts of eukaryotic algae and higher plants. Fatty acid biosynthesis is initiated by the conversion of acetyl-CoA to malonyl-CoA, catalyzed by acetyl-CoA carboxylase (ACCase). Malonyl-CoA is then converted to malonyl-ACP, catalyzed by malonyl-CoA-ACP transacylase (FabD). Finally, malonyl-ACP is converted to acyl-ACP, catalyzed by the enzyme complex fatty acid synthase (FAS). The fatty acid synthase complex initiates the elongation cycle by first condensing malonyl-ACP with acetyl-ACP, catalyzed by a beta-ketoacyl-ACP synthase III (*e.g.*, FabH). The β-ketoacyl-ACP (3-ketoacyl-ACP) formed by the FabH reaction is reduced to a β-hydroxyacyl-ACP (3-hydroxyacyl-ACP) by 3-ketoacyl-ACP reductase (*e.g.* FabG). The β-hydroxyacyl-ACP is then acted on by a β-hydroxyacyl-ACP dehydratase (*e.g.* FabA, FabZ) to

form trans-2-enoyl-ACP, which in turn is reduced by enoyl-ACP reductase (*e.g.* Fab I, Fab K, FabL) to form the 2 carbon-elongated acyl-ACP product. Subsequent cycles are initiated by a beta-ketoacyl-ACP synthase I or II (*e.g.*, FabB or FabF) catalyzed condensation of malonyl-ACP with acyl-ACP. The cycles of condensation, reduction, dehydration, and reduction are repeated, with each cycle adding two carbons from malonyl-ACP, until the acyl chain is cleaved from ACP by a thioesterase, such as FatA or FatB in chloroplasts, to form free fatty acid or transferred to another molecule (*e.g.* glycerol 3-phosphate) by a transacylase. In certain bacteria, the free fatty acids are then converted to acyl-CoA, a precursor for fatty acid derivative biosynthesis.

[0087] Unlike plant chloroplasts, cyanobacteria do not produce free fatty acids, and unlike *E. coli* and other heterotrophic bacteria, cyanobacteria do not produce acyl-CoA. After fatty acid elongation with the acyl chain covalently bound to acyl carrier protein, acyl transferases can transfer the acyl chain to a glycerol backbone to produce membrane lipids, or the acyl-ACP can be converted into fatty alkanes. Accordingly, to produce fatty acid derivatives in cyanobacteria, it is typically considered necessary to introduce several exogenous genes encoding enzymes for producing acyl-CoA and converting the acyl-CoA to the desired end product (*e.g.*, an alcohol, aldehyde, alkane, alkene, fatty acid ester or wax ester). As illustrated in Fig. 5, a thioesterase or a gene encoding a thioesterase (*e.g.*, acyl-ACP thioesterase, 3.1.2.20) can be introduced to hydrolyze the acyl-ACP thioester, thus liberating free fatty acid. An acyl-CoA synthetase (*e.g.*, 6.2.1.3) or a gene encoding one can be introduced to convert free fatty acids to acyl-CoA.

[0088] If fatty alcohols and/or wax esters are the desired end product, an alcohol-forming fatty acyl reductase or a gene encoding an alcohol-forming fatty acyl reductase (*e.g.*, alcohol-forming acyl-CoA reductase, 1.2.1.50) may be introduced. Further, a fatty aldehyde reductase or a gene encoding one may be introduced to reduce fatty aldehydes to fatty alcohols.

[0089] Wax esters may be formed by introducing a wax ester synthase or a gene encoding a wax ester synthase to catalyze condensation of a fatty alcohol with a fatty acyl thioester. As demonstrated herein, a combination of four enzymes is able to produce wax esters in prokaryotic and/or photosynthetic microorganisms such as cyanobacteria, where two, three, or four of the genes of the wax ester synthase pathway may be coregulated by the same promoter. The promoter can be a promoter endogenous to the host microorganism, and the transcriptional unit comprising genes of the pathway can optionally be integrated into the host microorganism

genome at a locus adjacent to the endogenous promoter for co-regulation of the genes of the transcriptional unit.

Thioesterases

[0090] As used herein, the term "thioesterase" is intended to include hydrolases capable of acting on a thioester bond. Such enzymes can correspond to, *e.g.*, Enzyme Commission Number 3.1.2.2, 3.1.2.14, 3.1.2.18, 3.1.2.19, 3.2.1.20, 3.1.2.22, 3.1.2.23, or 3.1.2.27. A thioesterase encoded by nucleic acid sequences of a nucleic acid molecule as disclosed herein, or expressed in a recombinant microorganism or host cell of the invention can be, for example, an acyl-ACP thioesterase, an acyl-CoA thioesterase, or a hydroxylbenzoyl thioesterase. In some aspects, a protein known or suspected of having thioesterase activity against a particular substrate, *e.g.*, a acyl-CoA substrate or hydroxybenzoyl substrate, can also exhibit acyl-ACP thioesterase activity.

[0091] A recombinant microorganism or host cell can in some examples be transformed with a gene encoding an exogenous acyl-ACP thioesterase, such as a gene encoding a polypeptide that when queried against the Pfam database, provides a match with Pfam PF01643 having a bit score of less than or equal to 20.3 (the gathering cut-off for PF01643). The acyl-ACP thioesterase gene can encode an acyl-ACP thioesterase from a higher plant species. Genes encoding acyl-ACP thioesterases derived from higher plants can include, without limitation, genes encoding acyl-ACP thioesterases from *Cuphea* species (*e.g. Cuphea carthagenensis*, *Cuphea wrightii* (*e.g.*, GenBank Accession AAC49784), *Cuphea lanceolata* (*e.g.*, GenBank Accession CAA54060), *Cuphea palustris*, (*e.g.*, GenBank Accessions AAC49783; AAC49179); *Cuphea hookeriana* (*e.g.*, GenBank Accessions AAC72882; AAC49269; AAC72881; AAC72883), *Cuphea calophylla* (*e.g.*, GenBank Accession ABB71580) or genes of various *Cuphea* species disclosed in United States patent application publication US 2011/0020883, incorporated by reference herein) or genes from other higher plant species. In further examples, a microorganism used in the methods and cultures disclosed herein can include a gene encoding an acyl-ACP thioesterase from species such as but not limited to, *Arabidopsis* (GenBank Accessions XP_002885681; NP_172327); *Arachis hypogaea* (*e.g.*, GenBank Accession ABO38556); *Brassica* species (*e.g.*, GenBank Accession CAA52069.1), *Camellia oleifera* (*e.g.*, GenBank Accession ACQ57189); *Cinnamonum camphorum* (*e.g.*, GenBank Accession AAC49151); *Cocos nucifera* (*e.g.*, GenBank Accessions AEM72519; AEM72520; AEM72521); *Glycine max* (*e.g.*, GenBank Accession ABD91726); *Garcinia mangostana* (*e.g.*, GenBank Accession AAB51525);

Gossypium hirsutum (*e.g.*, GenBank Accession AAD01982); *Helianthus annuus* (*e.g.*, GenBank Accession AAQ08226); *Jatropha curcas* (*e.g.*, GenBank Accession ABU96744); *Macadamia tetraphylla* (*e.g.*, GenBank Accession ADA79524); *Elaeis oleifera* (*e.g.*, GenBank Accession AAM09524); *Elaeis guineensis* (*e.g.*, GenBank Accession AAD42220); *Oryza sativa* (*e.g.*, GenBank Accession BAA83582); *Populus tomentosa* (*e.g.*, GenBank Accession ABC47311); *Umbellularia californica* (*e.g.*, GenBank Accession AAC49001); *Ulmus Americana* (*e.g.*, GenBank Accession AAB71731); and *Zea mays* (GenBank Accession ACG41291), or any of those disclosed in U.S. Patent No. 5,455,167; U.S. Patent No. 5,654,495; and U.S. Patent No. 5,455,167; and in U.S. Patent Appl. Pub. Nos. 2009/0298143 and 2011/0020883; all incorporated by reference herein in their entireties. Further included are acyl-ACP thioesterases from mosses (Bryophyta), such as, for example, *Physcomitrella patens*, (*e.g.*, GenBank Accession XP 001770108). These examples are not limiting with regard to the types or specific examples of acyl-ACP thioesterase genes that can be used.

[0092] Further included are acyl-ACP thioesterase genes from prokaryotic organisms. Illustrative examples of prokaryotic acyl-ACP thioesterases that may be expressed by a microorganism useful in the methods and cultures provided herein include, but are not limited to acyl-ACP thioesterases from *Desulfovibrio desulfuricans* (*e.g.*, GenBank Accession Q312L1); *Elusimicrobium minutum* (*e.g.*, GenBank Accession ACC98705); *Carboxydothermus hydrogenoformans* (*e.g.*, GenBank Accession YP_359670); *Clostridium thermocellum* (*e.g.*, GenBank Accession YP_001039461); *Moorella thermoacetica* (*e.g.*, GenBank Accession YP_431036); *Geobacter metallireducens* (*e.g.*, GenBank Accession YP_384688); *Salinibacter ruber* (*e.g.*, GenBank Accession YP_444210); *Microscilla marina* (*e.g.*, GenBank Accession EAY28464); *Parabacteroides distasonis* (*e.g.*, GenBank Accession YP_001303423); *Enterococcus faecalis* (*e.g.*, GenBank Accession ZP_03949391); *Lactobacillus plantarum* (*e.g.*, GenBank Accession YP_003062170); *Leuconostoc mesenteroides* (*e.g.*, GenBank Accession YP_817783); *Oenococcus oeni* (*e.g.*, GenBank Accession ZP_01544069); *Mycobacterium smegmatis* (*e.g.*, GenBank Accession ABK74560); *Mycobacterium vanbaalenii* (*e.g.*, GenBank Accession ABM11638); *Rhodococcus erythropolis* (*e.g.*, GenBank Accession ZP_04385507; *Rhodococcus opacus* (*e.g.*, GenBank Accession YP_002778825), or any of those disclosed in the co-pending, commonly-assigned U.S. patent application No. 13/324,623 entitled "Prokaryotic

Acyl-ACP Thioesterases for Producing Fatty Acids in Genetically Engineered Microorganisms", filed on December 13, 2011, and which is incorporated herein by reference in its entirety.

[0093] In additional examples, a gene encoding an acyl-CoA thioesterase can be introduced into a microorganism or host cell and can be from a plant, animal, or microbial source. For example, a gene encoding the TesA or TesB thioesterase of *E. coli*, an ortholog thereof in another species, or a variant thereof, for example, an acyl-CoA thioesterase such as, but not limited to, a variant as disclosed in WO 2010/075483, incorporated by reference herein in its entirety, can be introduced into a microorganism or host cell. Also included are genes encoding proteins that when queried against the Pfam database of protein families are identified as members of Pfam PF02551 (acyl-CoA thioesterase), where the bit score is equal to or greater than the gathering cut off (20.7).

[0094] Alternately or in addition, the microorganism or host cell of the invention can include one or more genes encoding a hydroxybenzoyl thioesterase, for example an exogenous 4-hydroxybenzoate thioesterase or 4-chlorobenzoate thioesterase. Genes encoding hydroxybenzoyl thioesterases that may be useful in a recombinant microorganism for producing free fatty acids can include, for example, those disclosed in the commonly-assigned U.S. patent application No. 13/324,607 entitled "Genetically Engineered Microorganisms Comprising 4-Hydroxybenzoyl-CoA Thioesterases and Methods of Using Same for Producing Free Fatty Acids and Fatty Acid Derivatives", filed on December 13, 2011, and which is incorporated herein by reference in its entirety; 4-hydroxybenzoyl thioesterases from *Bacillus* species and *Geobacillus* species; as well as 4-hydroxybenzoyl thioesterases of *Acidiphilium, Bartonella, Rhodopseudomonas, Magnetospirillum, Burkholderia, Granulibacter, Rhizobium*, and *Labrenzia* species, or the like, or combinations thereof.

[0095] Acyl-ACP thioesterases typically can be active to some degree on acyl-ACP substrates having a plurality of different acyl chain lengths, but can have higher activity on (*e.g.*, have a substrate preference for) one or more acyl-ACP substrates having particular acyl chain lengths than on other chain length substrates. In some examples, an enzyme referred to as having a substrate preference for particular acyl chain length substrates produces at least twice as much product of the preferred substrate or substrates as it does from a non-preferred substrate, and for example can produce at least three times, at least four times, or at least five times as much product from a preferred substrate as from a non-preferred substrate. For example, an acyl-ACP

thioesterase may have a substrate preference for one or more of acyl-ACP substrates having acyl chain lengths of 8, 10, 12, 14, 16, 18, 20, 22, and/or 24 carbons. Additionally or alternately, the acyl-ACP thioesterase can hydrolyze one or more acyl-ACP substrates having an acyl chain length from 8 to 18 carbons, for example from 12 to 18 carbons. For example, the acyl-ACP thioesterase can hydrolyze one or more acyl-ACP substrates having an acyl chain length from 12 to 16 carbons. Further additionally or alternately, an acyl-ACP thioesterases of the present invention can, in some embodiments, have its highest level of activity on an acyl-ACP substrate having an acyl chain length of 12, 14, and/or 16 carbons.

[0096] In some aspects, a nucleic acid sequence encoding a thioesterase useful in the wax ester synthesis constructs disclosed herein can encode an acyl-ACP thioesterase that has at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or has 100% amino acid sequence identity to a corresponding thioesterase from a plant species such as a *Cuphea* or *Elaeis* species, *e.g.*, *Cuphea carthagenensis*, *Cuphea decandra*, *Cuphea paucipetala*, *Cuphea lanceolata*, *Elaeis oleifera,*or *Elaeis guineensis.* In some aspects, a thioesterase useful in the wax synthesis constructs disclosed herein has amino acid sequence identity of at least, *e.g.*, 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or 100% sequence identity to the amino acid sequence of a thioesterase selected from *Cuphea carthagenensis* cc1FatB1 ("Cc1FatB1"; SEQ ID NO:1 or SEQ ID NO:2), *Cuphea decandra* Cd1FatB1 ("Cd1FatB1"; SEQ ID NO:3 or SEQ ID NO:4), *Cuphea paucipetala* Cp1FatB1 ("Cp1FatB1"; SEQ ID NO:5 or SEQ ID NO:6), and *Elaeis guineensis* thioesterase ("oil palm thioesterase"; SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:9). See, *e.g.*, Fig. 1. For example, a nucleic acid molecule as disclosed herein can comprise a sequence encoding a thioesterase having at least 85% identity to the amino acid sequence of any one of SEQ ID NOS: 1-9 or a functional fragment thereof, or having at least 90% identity to the amino acid sequence of any one of SEQ ID NOS: 1-9 or a functional fragment thereof, for example, at least 95% identity to the amino acid sequence of any one of SEQ ID NOS: 1-9. Biochemical assays for demonstrating and measuring the activity of a thioesterase are well known (see, for example, US Patent No. 5,298,421, incorporated herein by reference in its entirety), as are biological assays that measure levels of free fatty acid production such as demonstrated in the examples herein, using, *e.g.*, gas chromatography, gas chromatography-mass

spectrometry, liquid chromatography-mass spectrometry, ion chromatography-mass spectrometry, *etc.*).

[0097] In some cases, certain eukaryotic acyl-ACP thioesterase genes (*e.g.*, from *Cuphea, Elaeis*, or other plant species) encode N-terminal transit peptides for transport of the thioesterase into plastids. As the transit peptides are not be necessary for the activity of these thioesterases, in certain aspects, the wax ester synthesis reagents and methods of the invention utilize mature forms of the thioesterases described herein, wherein all or a portion of the transit peptide has been deleted. Further, in some cases, certain thioesterase genes encode N-terminal segments that are not necessary for thioesterase activity, and, in certain aspects, the wax ester synthesis reagents and methods of the invention utilize forms of the thioesterases described herein that lack N-terminal segments that are not necessary for thioesterase activity.

[0098] Accordingly, the amino acid sequence of a thioesterase used in the wax ester synthesis reagents and methods of the invention can in some examples be a deletion mutant of any of the thioesterases described herein, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 residues are deleted from the N-terminus of the reference thioesterase. Preferably, the deletion mutant retains at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%, or retains 100% of the thioesterase activity of the reference thioesterase. For example, any eukaryotic thioesterase (*e.g.*, any eukaryotic acyl-ACP thioesterase) used in the wax ester synthesis reagents and methods of the invention may be truncated to remove a transit peptide and/or an N-terminal segment unnecessary for thioesterase activity.

[0099] For example, a gene encoding a thioesterase having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%, or 100% amino acid sequence identity to an oil palm thioesterase can be used in the wax ester synthesis constructs of the invention, for example, a gene encoding a thioesterase having at least 70% identity, for example, at least 85% or at least 95% identity to the amino acid sequence of SEQ ID NO: 7, which may be truncated at the N-terminus to remove transit peptide residues 1-93 of the amino acid sequence of SEQ ID NO: 7 (SEQ ID NO: 8). Alternatively, the oil palm thioesterase having at least 70%, at least 85% or at least 95% identity to the amino acid sequence of SEQ ID NO:7 may be truncated at the N-

terminus to remove residues 1-118 of the amino acid sequence of SEQ ID NO: 7 (SEQ ID NO: 9). Alternatively, the polypeptide of SEQ ID NO: 8 or 9 may replace the polypeptide of SEQ ID NO: 7 in any of the methods or systems for producing a wax ester as described herein. In some aspects, a nucleotide sequence encoding the polypeptide of SEQ ID NO: 8 or a nucleotide sequence encoding the polypeptide of SEQ ID NO: 9 may replace a nucleotide sequence encoding the polypeptide of SEQ ID NO: 7 in any of the nucleic acid molecules, constructs, cassettes, vectors, or recombinant host cells described herein.

[00100] Any of the thioesterases described herein may be used in any of the wax ester synthesis constructs, host microorganisms, and methods of the invention.

Acyl-CoA Synthetases

[00101] A recombinant or isoloated nucleic acid molecule used in the microorganisms and methods of the invention can comprise a nucleic acid sequence encoding an acyl-CoA synthetase. The acyl-CoA synthetase can be, for example, a prokaryotic acyl-CoA synthetase, for example, such as FadD (GenBank Accession NP_416319) or FadK of *E. coli* (GenBank Accession NP_416216), or their homologs in other bacterial species, including, as nonlimiting examples, the acyl-CoA synthetase of *Vibrio splendidus* (GenBank Accession EGU44230) or *Marinobacter adhaerens* HP15 (GenBank Accession ADP96803). Additional nonlimiting examples of prokaryotic proteins known to have or suspected of having acyl-CoA synthetase activity include, but are not limited to, *Acinetobacter* sp. ADP1 fadD (GenBank Accession YP_045024), *Haemophilus influenza* RdKW20 fadD (GenBank Accession NP_438551), *Bacillus halodurans* C-125 BH3103 (GenBank Accession NP_243969), *Bacillus subtilis* yhFl (GenBank Accession NP_388908), *Pseudomonas fluorescens* Pfo-1 Pfl-4354 (GenBank Accession YP_350082), *Comamonas testosteroni* KF-1 EAV15023 (GenBank Accession ZP_01520072), *Pseudomonas aeruginosa* fadD1 (GenBank Accession NP_251989), *Pseudomonas aeurginosa* PAO1 fadD2 (GenBank Accession NP_251990), *Rhizobium etli* CFN42 fadD (GenBank Accession YP_468026), *Rhodopseudomo nas palustris* Bis B18 RPC_4074 (GenBank Accession YP_533919), *Rasltonia Solanacearum* GM1 1000 fadD1 (GenBank Accession NP_520978), *Mycobacterium tuberculosis* H37Rv fadDD35 (GenBank Accession NP_217021), *Mycobacterium tuberculosis* H37Rv fadDD22 (GenBank Accession

NP_217464), and *Stenotrophomon as Maltophilia* R551-3 PRK0059 (GenBank Accession ZP_01644857).

[00102] Alternatively, the nucleic acid sequence encoding an acyl-CoA synthetase can encode an acyl-CoA synthetase derived from a fungal species, such as, for example, a *Saccharomyces cerevisiae* acyl-CoA synthetase (*e.g.*, the medium chain fatty acyl-CoA synthetase Faa2p (GenBank Accession NP_010931) or the SCRG_04483 acyl-CoA synthetase (GenBank Accession EDV08843) or a *Yarrowia lipolytica* acyl-CoA synthetase (*e.g.*, GenBank Accession CAG77892). Additional acyl-CoA synthetase genes that may be used in the constructs and microorganisms disclosed herein include acyl-CoA synthetases of plants, such as, for example, the long chain acyl-CoA synthetase of *Brassica napus* (GenBank Accession CAC19877) or the long chain acyl-CoA synthetase of *Arabidopsis thaliana* (GenBank Accession AEE74324), or the Yng-I-like acyl-CoA synthetase of *Glycine max* (GenBank Accession XP_003524920), and acyl-CoA synthetases of algal species, such as, for example, the long chain acyl-CoA synthetase of *Chlamydomonas reinhardtii* (GenBank Accession XP_001693692), or acyl-CoA synthetases of *Nannochloropsis oculata* (*e.g.*, GenBank Accession ADP09391), or *Chlorella variabilis* (*e.g.*, GenBank Accession EFN56588). Further considered are acyl-CoA synthetases of animal species, including insects (e.g., *Apis mellifera*, for example, the acyl-CoA synthetase family member 2, mitochondrial precursor, GenBank Accession NP_001193902) and mammals such as *Mus musculus* (*e.g.*, the "MACS" acyl-CoA synthetase, GenBank Accession EDL17174).

[00103] Specifically included for use in the constructs and microorganisms disclosed herein for making wax esters are nucleic acid sequences that encode polypeptides having at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, amino acid sequence identity to known or suspected acyl-CoA synthetases, including but not limited to the examples above, where the encoded polypeptides have acyl-CoA synthetase activity. For example, a nucleic acid sequence that encodes an acyl-CoA synthetase can have at least 85%, at least 90%, or at least 95% to an identified acyl-CoA synthetase, including but not limited to those disclosed herein.

[00104] For example, an acyl-CoA synthetase useful in the wax ester synthesis pathways and methods of the invention can have sequence identity of at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or or can have sequence identity of 100%, to the amino acid sequence

of an acyl-CoA synthetase selected from *Saccharomyces cerevisiae* "Faa2p" (SEQ ID NO: 10), *Saccharomyces cerevisiae* SCRG_04483 (SEQ ID NO: 11), *E. coli* FadD (SEQ ID NO: 12), *E. coli* FadK (SEQ ID NO: 13), and *Mus musculus* MACS (SEQ ID NO: 14). See, *e.g.*, Fig. 2. For example, the acyl-CoA synthetase encoded by a nucleic acid sequence of a construct of the invention may be or comprise a polypeptide having at least 85% identity to the amino acid sequence of any one of SEQ ID NOS: 10-14 or a functional fragment thereof, or may be or comprise a polypeptide having at least 90% identity to the amino acid sequence of any one of SEQ ID NOS: 10-14 or a functional fragment thereof. For example, the acyl-CoA synthetase can be or comprise a polypeptide having at least 95% identity to the amino acid sequence of any of SEQ ID NOS: 10-14.

[00105] Any of the acyl-CoA synthetases described herein may be used in any of the wax ester synthesis reagents and methods of the invention.

Alcohol-Forming Fatty Acyl Reductases

[00106] For production of a fatty alcohol that can be used as a substrate by a wax ester synthase, a nucleic acid molecule as provided herein that includes a sequence encoding a wax ester synthase can further include a sequence encoding a fatty alcohol-forming acyl reductase or "FAR" that can reduce acyl-CoA to a fatty alcohol. FARs have been identified in, *e.g.*, *Euglena* (see, *e.g.*, Teerawanichpan *et al.*, *Lipids* 45:263-273 (2010)), *Arabidopsis* (see, *e.g.*, Rowland *et al.*, *Plant Physiol.* 142:866-877 (2006), Doan *et al.*, *J. Plant Physiol.* 166:787-796 (2009) and Domergue *et al.*, *Plant Physiol.* 153:1539-1554 (2010)), *Artemisia* (see, *e.g.*, Maes *et al.*, *New Phytol.* 189:176-189 (2011)), jojoba (see, *e.g.*, Metz *et al.*, *Plant Physiol.* 122:635-644 (2000)), moth (see, *e.g.*, Lienard *et al.*, *Proc. Natl. Acad. Sci.* 107:10955-10960 (2010)), bee (see, *e.g.*, Teerawanichpan *et al.*, *Insect Biochemistry and Molecular Biology* 40:641-649 (2010)) and mammals (see, *e.g.*, Honsho *et al.*, *J. Biol. Chem.* 285:8537-8542 (2010)). An alcohol-forming fatty acyl reductase useful in the wax ester synthesis reagents and methods of the invention can be any alcohol-forming reductase that has activity in the host microorganism.

[00107] For example, an alcohol-forming fatty acyl reductase useful in the wax ester synthesis pathways and methods of the invention can be a prokaryotic alcohol-forming acyl-CoA reductase and can have at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity, or can be 100%

identical, to the amino acid sequence of a prokaryotic alcohol-forming reductase such as *Marinobacter aquaeolei* VT8 Maqu_2220 (SEQ ID NO: 15, GenBank Accession YP_959486), *Marinobacter algicola* DG893 (GenBank Accession ZP_01892457); *Hahella chejuensis* KCTC 2396 HCH_05075; SEQ ID NO: 20, GenBank Accession YP_436183); *Oceanobacter sp.* RED65 (GenBank Accession ZP_01305629), or *Marinobacter aquaeoli* VT8 2220 Maqu_2507 gene (SEQ ID NO:19, GenBank Accession ABM19582).

[00108] Nonlimiting examples of other alcohol-forming fatty acyl reductases that can be used in the wax ester synthesis constructs, microorganisms, and methods of the invention may include, but are not limited to, alcohol-forming fatty acyl reductases that have at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity, or have 100% identity to the amino acid sequence of an identified alcohol-forming reductase such as but not limited to bfar from *Bombyx mmori* (GenBank Accession BAC79426), jjfar *from Simmondsia chinensis* (SEQ ID NO: 21, GenBank Accession AAD38039), an acyl-CoA reductase from *Triticum aestivum* (GenBank Accession CAD30694 or CAD30692), mfarl from *Mus musculus* (GenBank Accession NP_081655), mfar2 from *Mus musculus* (GenBank Accession NP_848912), hfar from *H. sapiens* (GenBank Accession NP_115604), FARXIII from *Ostrinia scapulalis* (SEQ ID NO: 18, GenBank Accession ACJ06520), MS2 from *Z. mays* (GenBank Accession NP_001151388 or EU970865), or MS2 (GenBank Accession NP_187805), FAR4 (GenBank Accession NP_001030809 or NP_190040), FAR6 (SEQ ID NO: 16, SEQ ID NO: 17, GenBank Accession 67633703), CER4 (GenBank Accession NP_567936) or Ath (GenBank Accession NP567936) from *Arabidopsis thaliana*, *Yev*-pgFAR from *Yponomeuta evonymellus* (GenBank Accession GQ907231-GQ907233), *Yro*-pgFAR from *Yponomeuta rorellus* (GenBank Accession GQ907234), *Ypa*-pgFAR from *Yponomeuta padellus* (GenBank Accession GQ907235), OnuE from *Ostrinia nubilalis* (GenBank Accession FJ807735), Has from *Homo sapiens* (GenBank Accession AAT42129), *etc.*

[00109] Specifically included for use in the constructs and microorganisms disclosed herein for making wax esters are nucleic acid sequences that encode polypeptides having at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, amino acid sequence identity to known or suspected alcohol-forming fatty acyl reductases, including but not limited to the examples above,

where the encoded polypeptides have alcohol-forming fatty acyl reductases activity. For example, a nucleic acid sequence that encodes an alcohol-forming fatty acyl reductases can have at least 85%, at least 90%, or at least 95% to an identified alcohol-forming fatty acyl reductases, including but not limited to those disclosed herein.

[00110] For example, an alcohol-forming reductase useful in the wax ester synthesis pathways and methods of the invention can have sequence identity of at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or or can have sequence identity of 100%, to the amino acid sequence of an alcohol-forming reductase selected from *Arabidopsis thaliana* FAR6 (GenBank Accession AEE79553) with or without a transit peptide (SEQ ID NO: 16 or 17, respectively), *Ostrinia scapulalis* FARXIII (GenBank Accession ACJ06520; SEQ ID NO: 18), *M. aquaeolei* VT8 Maqu_2507 (GenBank Accession ABM19582, SEQ ID NO: 19), *Hahella chejuensis* Hch_05075 (GenBank Accession YP_436183; SEQ ID NO: 20), and *Simmondsia chinensis* jjfar (GenBank Accession AAD38039; SEQ ID NO: 21). See, *e.g.*, Fig. 3. For example, the alcohol-forming fatty acyl reductase may be or comprise a polypeptide having at least 85% identity to the amino acid sequence of any one of SEQ ID NOS: 15-21 or a functional fragment thereof, or may be or comprise a polypeptide having at least 90% identity to the amino acid sequence of any one of SEQ ID NOS: 15-21 or a functional fragment thereof. For example, the alcohol-forming fatty acyl reductase can be or comprise a polypeptide having at least 95% identity to the amino acid sequence of any of SEQ ID NOS: 15-21. Methods of demonstrating and measuring the activity of an alcohol-forming fatty acyl reductase are well known (*e.g.*, biochemical assays (Reiser and Somerville (1997) *J. Biol. Chem.* 79: 2969-2975) as well as measuring rates/levels of fatty aclohol production using, *e.g.*, gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry, thin layer chromatography, *etc.*).

[00111] In some aspects, an alcohol-forming fatty acyl reductase useful in the wax ester synthesis reagents and methods of the invention is encoded by a nucleic acid sequence that has at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a corresponding alcohol-forming fatty acyl reductase-encoding nucleic acid sequence from a plant species such as an *Arabidopsis* or *Simmondsia* species, *e.g.*, *Arabidopsis thaliana* (*e.g.*, GenBank Accession 67633703) or *Simmondsia chinensis* (*e.g.*, GenBank Accession AAD38039).

[00112] In some aspects, an alcohol-forming fatty acyl reductase useful in the wax ester synthesis reagents and methods of the invention is encoded by a nucleic acid sequence that has at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a corresponding alcohol-forming fatty acyl reductase-encoding nucleic acid sequence from a moth species such as an *Ostrinia* species (for example, *Ostrinia scapulalis*, *e.g.*, Accession No. ACJ06520).

[00113] In some cases, certain eukaryotic fatty acyl reductase genes (*e.g.*, from plants) encode N-terminal transit peptides. As the transit peptides may not be necessary for the activity of these fatty acyl reductases, in certain aspects, the wax ester synthesis reagents and methods of the invention may utilize mature forms of the fatty acyl reductases described herein, wherein all or a portion of the transit peptide not necessary for fatty acyl reductase activity has been deleted.

[00114] Accordingly, in some aspects, the amino acid sequence of an alcohol-forming fatty acyl reductase used in the wax ester synthesis reagents and methods of the invention is a deletion mutant of any of the alcohol-forming fatty acyl reductases described herein, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 residues are deleted from the N-terminus of the reference alcohol-forming fatty acyl reductase. In some aspects, the deletion mutant retains at least 95%, 96%, 97%, 98%, 99%, or 100% of the thioesterase activity of the reference alcohol-forming fatty acyl reductase. In some aspects, any eukaryotic fatty acyl reductase (*e.g.*, any eukaryotic alcohol-forming fatty acyl-CoA reductase) used in the wax ester synthesis reagents and methods of the invention may be truncated to remove a transit peptide unnecessary for fatty acyl reductase activity.

[00115] For example, in some aspects, a FAR6 reductase used in the wax ester synthesis reagents and methods of the invention, having the amino acid sequence of SEQ ID NO: 16, is truncated at the N-terminus to remove transit peptide residues 1-47 of the amino acid sequence of SEQ ID NO: 16 (SEQ ID NO: 17). In some aspects, the polypeptide of SEQ ID NO: 17 may replace the polypeptide of SEQ ID NO: 16 in any of the reagents and methods for producing a wax ester as described herein. In some aspects, a nucleotide sequence encoding the polypeptide

of SEQ ID NO: 16 lacking the nucleotide residues encoding the transit peptide (*e.g.*, SEQ ID NO: 48) may replace a nucleotide sequence encoding the polypeptide of SEQ ID NO: 16 (*e.g.*, SEQ ID NO: 47) in any of the nucleic acid molecules, constructs, cassettes, vectors, or recombinant host cells described herein.

[00116] In some aspects, the conversion of acyl-CoA to fatty alcohol may occur via synthesis of a fatty aldehyde, wherein a fatty aldehyde reductase (*e.g.*, an aldehyde-forming acyl-CoA reductase) expressed in the host cell first reduces acyl-CoA to a fatty aldehyde. In certain aspects, the host cell can be engineered to overexpress an endogenous fatty aldehyde reductase (*e.g.*, by inserting promoter and/or enhancer transcriptional control elements near the fatty aldehyde reductase gene). In other aspects, the host cell may be engineered to express an exogenous fatty aldehyde reductase.

[00117] Any of the fatty acyl reductases described herein may be used in any of the wax ester synthesis reagents and methods of the invention.

Wax Ester Synthases

[00118] Wax esters are the product of a condensation reaction between a fatty acyl-thioester substrate and a fatty alcohol, catalyzed by a wax ester synthase. Polypeptides having wax ester synthase activity may be polypeptides identified as wax synthases, O-acyltransferases, including membrane-bound O-acyltransferases (MBOATs), diacylglycerol O-acyltransferases, alcohol acyltransferases (AATs, EC 2.3.1.84), or alcohol synthase/acyl-CoA:diacylglycerol acyltransferases. Some polypeptides identified as diacylglycerol acyltransferases (DGATs) may also be found to have wax ester synthase activity. Wax ester synthases have been identified in, *e.g.*, *Acinetobacter* (Ishige *et al.*, *Appl. Environ. Microbiol.* 68:1192-1195 (2002); Kalscheuer and Steinbuchel, *J. Biol. Chem.* 278:8075-8082 (2003); Kalscheuer *et al.*, *Appl. Environ. Microbiol.* 72:1373-1379 (2006)), *Marinobacter* (Holtzapple and Schmidt-Dannert, *J. Bacteriol.* 189:3804-3812 (2007)), *Arabidopsis* (Li *et al.*, *Plant Physiol.* 148:97-107 (2008)), petunia (King *et al.*, *Planta* 226:381-394 (2007)), jojoba (Lardizabal *et al.*, *Plant Physiol.* 122:645-655 (2000), and mammalian species (Cheng and Russell, *J. Biol. Chem.* 279:37798-37807 (2004); Yen *et al.*, *J. Lipid Res.* 46:2388-2397 (2005)).

[00119] Wax ester synthases may be identified using methods known in the art, (*e.g.*, Hidden Markov Models ("HMMs") based on pattern similarity to a set of known wax ester

synthase/DGAT sequences, respectively. As nonlimiting examples, a gene that encodes a polypeptide that recruits to Pfam PF03007 (wax ester synthase like acyl-CoA acyltransferase domain) with a bit score greater than the gathering cutoff of 20.6 and an E value of 0.01 or less or recruits to Pfam PF13813 ("MBOAT_2") with a bit score greater than the gathering cutoff of 25.0 and an E value of 0.01 or less can be selected for use in the nucleic acid molecules and microorganisms provided herein.

[00120] Wax ester synthesis proteins encoded by nucleic acid molecules provided herein can include, but are not limited to: acyltransferases or wax synthases, fatty acyl transferases, diacylglycerol acyltransferases, acyl-coA wax alcohol acyltransferases, and bifunctional wax ester synthase/acy 1-CoA: diacylglycerol acy transferase selected from a multienzyme complex from *Simmondsia chinensis*, *Acinetobacter* sp. strain ADPl (formerly *Acinetobacter calcoaceticus* ADPl), *Pseudomonas aeruginosa*, *Fundibacter (Alcanivorax) jadensis*, *Arabidopsis thaliana*, or *Alkaligenes eutrophus*. Wax synthases can also be from a multienzyme complex from *Alkaligenes eutrophus* and other organisms known in the literature to produce wax and fatty acid esters.

[00121] Proteins known or suspected of having wax ester synthase activity that are considered for use in the nucleic acid molecules and transgenic microorganisms provided herein include wax synthases from prokaryotic species, such as but not limited to, *Marinobacter hydrocarbonoclasticus* WS1 (GenBank Accession ABO21020), *M. hydrocarbonoclasticus* DSM 8798 WS2 (GenBank Accession ABO21021), *M.* sp. ELB 17 (GenBank Accession EBA00388), *M. aquaeolei* Maqu_0168 WS (GenBank Accession YP_957462), *M. adhaerens* HP15 WS (ADP99639), Hahella chejuensis KCTC 2396 (GenBank Accession YP_432512), *Acinetobacter baumannii* wax ester synthase (GenBank Accession EGJ63408), *A. calcoaceticus* WS/DGAT (GenBank Accession ZP_06058985) *Acinetobacter baylyi* ADP1 wax ester synthase (GenBank Accession AAO17391 or Q8GGG1), *Bradyrhizobium japonicum* USDA 110 (GenBank Accession NP_769520), *Erythrobacter litoralis* HTCC 2594 (GenBank Accession YP_457389), *Rhodococcus* opacus wax ester synthase (GenBank Accession BAH53702), *Mycobacterium tuberculosis* wax ester synthase (GenBank Accession NP_334638), *M. smegmatis* wax ester synthase (GenBank Accession ABK74273), the "WS/DGAT/MGAT" subfamily proteins of *Alcanivorax* species (GenBank Accessions CAL17252; EDX90960; EDX89052; ZP_05043539; ZP_05041631), wsadpl from *Nocardia farcinica* IFM 10152 (GenBank Accession YP_117375),

Photobacterium profundum SS9 (GenBank Accession YP_130413), *Rhodoferax ferrireducens* DSM 15236 (GenBank Accession ZP_00691704), and *Salinibacter ruber* DSM 13855 (GenBank Accession YP_446603).

[00122] Examples of eukaryotic polypeptides that may be useful as wax synthases include, without limitation, jojoba wax ester synthase JjWS (GenBank Accession AF149919), *Euglena gracilis* wax ester synthase (GenBank Accession ADI60058), *Arabidiopsis thaliana* WSD1 O-acyltransferase (GenBank Accession NP_568547), *Arabidiopsis thaliana* GPAT acyltransferase (GenBank Accession NP_174499), the putative long-chain-alcohol O-fatty-acyltransferase 4 of *Arabidiopsis thaliana* (GenBank Accession NP_200346) *Murraya koenigii* wax ester synthase, acyl-CoA wax alcohol acyltransferase 2 from *H. sapiens* (GenBank Accession NP_001002254), mWS from *Mus musculus* (GenBank Accession Q6E1M8), SAAT from *Fragaria xananas* (GenBank Accession AAG13130), the membrane bound O-acyltransferase (MBOAT) of *Zea mays* (GenBank Accession NP_001131179), mdAAT2 from *Malus x domestica* (GenBank Accession AAS79797), as well as insect wax ester synthases, *etc.*

[00123] In some illustrative examples, a wax ester synthase useful in the wax ester synthesis nucleic acid molecules, production hosts, and methods of the invention can have a sequence identity of at least, *e.g.*, 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or 100% sequence identity to the amino acid sequence of a wax ester synthase selected from *Marinobacter* sp. strain ELB17 MELB17 WS ("MELB17 WS"; SEQ ID NO: 22), *Marinobacter aquaeolei* 0168 WS ("Maqu_0168 WS"; SEQ ID NO: 23), *Marinobacter adhaerens* HP15 wax ester synthase ("HP15 WS"; SEQ ID NO: 24), *Marinobacter hydrocarbonoclasticus* DSM 8798 WS1 ("DSM 8798 WS1"; SEQ ID NO: 25), *Marinobacter hydrocarbonoclasticus* DSM 8798 WS2 ("DSM 8798 WS2"; SEQ ID NO: 26), *Petunia hybrida* wax ester synthase ("petunia WS"; SEQ ID NO: 27), *Mus musculus* WS ("*Mus musculus* WS"; SEQ ID NO: 28), *Simmondsia chinensis* wax ester synthase ("jojoba WS"; SEQ ID NO: 29), and *Acinetobacter* sp. ADP1 wax ester synthase ("ADP1 WS"; SEQ ID NO: 30). See, *e.g.*, Fig. 4. For example, the wax ester synthase encoded by a nucleic acid sequence used in a nucleic acid molecule of the invention may be or comprise a polypeptide having at least 85% identity to the amino acid sequence of any one of SEQ ID NOS: 22-30 or a functional fragment thereof, or may be or comprise a polypeptide having at least 85% identity, at least 90%, or at least 95% identity to the amino acid sequence of any one of SEQ ID

NOS: 22-30 or a functional fragment thereof. For example, the wax ester synthase can be or comprise the amino acid sequence of any of SEQ ID NOS: 22-30.

[00124] Methods of demonstrating and measuring the activity of a wax ester synthase are well known (*e.g.*, measuring rates/levels of wax ester production using, *e.g.*, gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry, thin layer chromatography, *etc.*). Methods to identify wax synthase activity are also provided in, *e.g.*, U.S. Patent No. 7,118,896, which is incorporated herein by reference in its entirety.

[00125] For example, a gene useful in the nucleic acid molecules, recombinant microorganisms, and methods of the invention can be encoded by a nucleic acid sequence that has at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity, or 100% sequence identity, to a corresponding wax ester synthase-encoding nucleic acid sequence from a bacterium such as *Acinetobacter calcoaceticus* (*e.g.*, GenBank Accession YP_045555.1).

[00126] For example, a wax ester synthase useful in the wax ester synthesis reagents and methods of the invention is encoded by a nucleic acid sequence that has at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity, or 100% sequence identity, to a corresponding wax ester synthase-encoding nucleic acid sequence from a plant, *e.g.*, a petunia or jojoba species. In some examples, the wax ester synthase is from *Petunia hybrida* (*e.g.*, GenBank Accession AAZ08051) or *Simmondsia chinensis* (*e.g.*, GenBank Accession AF149919).

[00127] For example, a wax ester synthase useful in the nucleic acid molecules and methods provided herein can be encoded by a nucleic acid sequence that has at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a corresponding wax ester synthase-encoding nucleic acid sequence from a mammal, *e.g.*, *Mus musculus* (*e.g.*, GenBank Accession 49854217).

[00128] Any of the wax ester synthases described herein may be used in any of the wax ester synthesis reagents and methods of the invention.

Nucleic Acid Molecules

[00129] The present invention provides recombinant nucleic acid molecules comprising nucleic acid sequences that encode a) thioesterases, b) acyl-CoA synthetases, c) alcohol-forming fatty acyl reductases, and/or d) wax ester synthases (*e.g.*, any of the thioesterases, acyl-CoA synthetases, alcohol-forming fatty acyl reductases, and/or wax ester synthases disclosed herein) or functional fragments thereof. A nucleic acid molecule of the invention can be isolated and/or purified. The nucleic acid molecules described herein can be used in any of the methods of the invention, and may be included in any of the vectors or host cells of the invention.

[00130] A recombinant nucleic acid molecule as provided herein can encode a polypeptide having wax ester synthase activity and one or more of a) a thioesterase, b) an acyl-CoA synthetase, and c) an alcohol-forming fatty acyl reductase. For example, a recombinant nucleic acid molecule as provided herein can include a sequence that encodes a wax ester synthase and a sequence that encodes a thioesterase; or can include a sequence that encodes a wax ester synthase and a sequence that encodes an acyl-CoA synthetase; or can include a sequence that encodes a wax ester synthase and a sequence that encodes an alcohol-forming fatty acyl reductase. In further examples, a recombinant nucleic acid molecule as provided herein can include a sequence that encodes a wax ester synthase, a sequence that encodes a thioesterase, and a sequence that encodes an acyl-CoA synthetase; or can include a sequence that encodes a wax ester synthase, a sequence that encodes a thioesterase, and a sequence that encodes an alcohol-forming fatty acyl reductase; or can include a sequence that encodes a wax ester synthase, a sequence that encodes an acyl-CoA synthetase, and a sequence that encodes an alcohol-forming fatty acyl reductase. In another example, the nucleic acid molecule encodes a) an acyl-ACP thioesterase, b) an acyl-CoA synthetase, c) an alcohol-forming acyl-CoA reductase, and d) a polypeptide having wax ester synthase activity.

[00131] A host cell, e.g., a prokaryotic microorganism such as a cyanobacterium, transformed with a nucleic acid molecule that includes a sequence encoding a wax ester synthase and one or more additional genes of the wax ester synthesis pathway (*e.g.*, all four genes of the wax ester synthesis pathway as provided herein) can produce a greater amount of a wax ester than does a control host cell, where the control host cell is cultured under the same conditions and is substantially identical to the host cell expressing the isolated nucleic acid molecule(s) in all

respects, with the exception that the host cell does not include or does not express the isolated nucleic acid molecule.

[00132] The nucleic acid sequence encoding a thioesterase can encode any thioesterase that is capable of releasing a free fatty acid from any acyl-ACP substrate and can be, for example, of the thioesterases disclosed herein, including an acyl-CoA thioesterase, a hydroxylbenzoyl thioesterase, or an acyl-ACP thioesterase, which can be an acyl-ACP thioesterase from a prokaryotic or eukaryotic species. In some examples the thioesterase encoded by a nucleic acid molecule provided herein has a substrate preference of C8-C24, for example, C12-C18. As nonlimiting examples, a thioesterase encoded by a nucleic acid sequence of a nucleic acid molecule as provided herein can be derived from acyl-ACP thioesterases from a plant species, e.g., a *Cuphea* or *Elaeis* species, and in illustrative and nonlimiting examples can comprise an amino acid sequence having at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the amino acid sequence of any of SEQ ID NOS: 1-9; or to a functional fragment of the polypeptide having thioesterase activity.

[00133] The nucleic acid sequence encoding an acyl-CoA synthetase can encode any acyl-CoA synthetase, such as but not limited to any disclosed herein, and in illustrative and nonlimiting examples can comprise an amino acid sequence having at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the amino acid sequence of any of SEQ ID NOS: 10-14, or to a functional fragment of the polypeptide having acyl-CoA synthetase activity.

[00134] The nucleic acid sequence encoding an alcohol-forming fatty acyl reductase can encode any alcohol-forming fatty acyl reductase, such as but not limited to any disclosed herein, and in illustrative and nonlimiting examples can comprise an amino acid sequence having at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the amino acid sequence of any of SEQ ID NOS: 15-21; or to a functional fragment of the polypeptide having alcohol-forming fatty acyl reductase activity.

[00135] The nucleic acid sequence encoding a wax ester synthase can encode any polypeptide having wax ester synthase activity, such as but not limited to any disclosed herein, and in

illustrative and nonlimiting examples can comprise an amino acid sequence having at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the amino acid sequence of any of SEQ ID NOS: 22-30; or to a functional fragment of the polypeptide having wax ester synthase activity.

[00136] Additionally, the invention encompasses isolated nucleic acid molecules comprising nucleic acid sequences encoding one or more of a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, or a wax ester synthase, wherein one or more of the polypeptides is a deletion mutant in which one or more amino acids have been deleted from the original polypeptide (*e.g.*, the reference thioesterase, acyl-CoA synthetase, alcohol-forming acyl reductase, or polypeptide having wax ester synthase activity). In some aspects, the deletion mutant lacks at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids from the N- and/or C-terminus and has an amino acid sequence at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical, or 100% identical, to the corresponding amino acid sequence of the original polypeptide. In certain aspects, the deletion mutant retains at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the activity of the original polypeptide when expressed in a recombinant host cell.

[00137] Further, the invention provides variants of thioesterases, acyl-CoA synthetases, alcohol-forming fatty acyl reductases, and wax ester synthases for use in the wax ester synthesis pathways described herein. Variants may be naturally occurring, or non-naturally-occurring, such as those induced by various mutagens and mutagenic processes. In some aspects, at least one amino acid residue has been inserted N- and/or C-terminal to, and/or within, the reference sequence. In some aspects, at least one amino acid residue has been deleted N- and/or C-terminal to, and/or within, the reference sequence. In some aspects, variants may be sequences containing predetermined mutations by, *e.g.*, homologous recombination or site-directed or PCR mutagenesis; corresponding proteins of other species; alleles or other naturally occurring variants; and/or derivatives wherein the protein has been covalently modified by chemical, enzymatic or other appropriate means with a moiety other than a naturally occurring amino acid.

[00138] A substitution, insertion or deletion may adversely affect the protein when the altered sequence substantially inhibits a biological function associated with the protein. For example, a

variant of a thioesterase used in the methods of the invention may have activity that is reduced by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, or 70% in comparison to the activity of the thioesterase from which the variant is derived. For example, a variant of an acyl-CoA synthetase used in the methods of the invention may have activity that is reduced by not more than about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, or 70% in comparison to the activity of the acyl-CoA synthetase from which the variant is derived. For example, a variant of an alcohol-forming fatty acyl reductase used in the methods of the invention may have activity that is reduced by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, or 70% in comparison to the activity of the alcohol-forming fatty acyl reductase from which the variant is derived. For example, a variant of a wax ester synthase used in the methods of the invention may have activity that is reduced by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, or 70% in comparison to the activity of the wax ester synthase from which the variant is derived. For example, the amount of wax ester produced by a host cell expressing a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and/or a wax ester synthase, where at least one of the enzymes is a variant of a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and/or a wax ester synthase disclosed herein, can be not less than about 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 85%, 80% or 75% of the amount of wax ester produced by a control host cell expressing the enzyme(s) from which the variant(s) are derived.

[00139] The invention also encompasses fragments and variants of any of a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and/or a polypeptide having wax ester synthase activity that has increased activity in comparison to the reference polypeptide. For example, a thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, or wax ester synthase fragment or variant may have activity that is increased by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, or 1000% in comparison to the activity of the enzyme from which the variant is derived. For example, the amount of wax ester produced by a host cell expressing a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and/or a wax ester synthase, where at least one of the enzymes is a fragment or variant of a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and/or a wax ester synthase disclosed herein, can be at least

70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% of the amount of wax ester produced by a host cell expressing the protein(s) from which the fragment(s) or variant(s) are derived.

[00140] The invention also encompasses deletion mutants of a thioesterase in which one or more amino acids have been deleted from a thioesterase but where the thioesterase activity of the deletion mutant can be reduced by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, or 70% in comparison with the reference thioesterase. For example, a thioesterase deletion mutant can lack at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids from the N- and/or C-terminus and can have an amino acid sequence at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of the reference thioesterase (*e.g.*, any of SEQ ID NOS: 1-9). For example, the invention encompasses deletion mutants of an acyl-CoA synthetase in which one or more amino acids have been deleted from an acyl-CoA synthetase as disclosed herein but where the acyl-CoA synthetase activity of the deletion mutant is reduced by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, or 70% in comparison with the reference acyl-CoA synthetase. For example, the acyl-CoA synthetase deletion mutant lacks at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids from the N- and/or C-terminus and has an amino acid sequence at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the corresponding amino acid sequence of the reference acyl-CoA synthetase (*e.g.*, any of SEQ ID NOS: 10-14). For example, the invention encompasses deletion mutants of an alcohol-forming fatty acyl reductase in which one or more amino acids have been deleted from an alcohol-forming fatty acyl reductase described herein but where the alcohol-forming fatty acyl reductase activity of the deletion mutant is reduced by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, or 70% in comparison with the reference alcohol-forming fatty acyl reductase. For example, the alcohol-forming fatty acyl reductase deletion mutant can lack at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids from the N- and/or C-terminus and has an amino acid sequence at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the corresponding amino acid sequence of the reference alcohol-forming fatty acyl reductase (*e.g.*,

any of SEQ ID NOS: 15-21). For example, the invention encompasses deletion mutants of a wax ester synthase in which one or more amino acids have been deleted from a wax ester synthase described herein but where the wax ester synthase activity of the deletion mutant is reduced by not more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, or 70% in comparison with the reference wax ester synthase. For example, the wax ester synthase deletion mutant lacks at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids from the N- and/or C-terminus and has an amino acid sequence at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the corresponding amino acid sequence of the reference wax ester synthase (*e.g.*, any of SEQ ID NOS: 22-30).

[00141] The invention also provides nucleic acid molecules that hybridize under high stringency hybridization conditions, such as selective hybridization conditions, to the nucleotide sequences described herein. Hybridization probes include synthetic oligonucleotides or oligonucleotide analogs (including peptide nucleic acids, as described in Nielsen (1991) *Science*, 254, 1497-1500) which bind in a base-specific manner to a complementary strand of nucleic acid or nucleic acid molecules comprising sequences that encode at least a portion of a thioesterase, an acyl-CoA synthetase, an alcohol-forming acyl reductase, or a polypeptide having wax ester synthase activity. For example, further nucleic acid sequences for use in the nucleic acid constructs, transgenic microorgansism, and methods of the invention can be detected and/or isolated by specific hybridization, *e.g.*, under high stringency conditions, with nucleic acid sequences encoding the enzymatic activities as disclosed herein or obtained from sequence databases (*e.g.*, NCBI "GenBank", Benson et al. (2011) *Nucleic Acids Res.* 39 (Database Issue): D32-7).

[00142] Any of the nucleic acid molecules described herein can further comprise one or more additional nucleic acid sequences of at least 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, or 1500 nucleotides from a prokaryotic and/or photosynthetic organism, *e.g.*, a cyanobacterium. For example a nucleic acid molecule can have two sequences of at least 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, or 1500 nucleotides from a prokaryotic and/or photosynthetic organism that can mediate recombination of at least a portion of the nucleic acid molecule into the genome of the prokaryotic and/or photosynthetic organism.

[00143] Any of the nucleic acid molecules described herein can further comprise one or more regulatory sequences including, for example, a promoter, an enhancer, a transcriptional terminator, one or more ribosome binding sites, *etc.*

Other Modifications

[00144] The nucleic acid molecules provided herein can include further variants of the nucleotide sequences that may encode fragments or variants of the polypeptides described herein. The nucleotide sequence variants can be naturally-occurring, non-naturally-occurring, including, *e.g.*, variants induced by various mutagens and mutagenic processes, or a combination of naturally- and non-naturally-occuring. A given nucleic acid sequence may be modified, for example, according to standard mutagenesis or artificial evolution or domain swapping methods to produce modified sequences. Accelerated evolution methods are described, *e.g.* by Stemmer (1994) *Nature* 370, 389-391, and Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91, 10747-10751. Additionally, chemical or enzymatic alteration of expressed nucleic acids and polypeptides can be performed by standard methods. For example, a sequence can be modified by addition of phosphate groups, methyl groups, lipids, sugars, peptides or organic or inorganic compounds, by the inclusion of modified nucleotides or amino acids, or the like.

[00145] For optimal expression of a recombinant protein, in certain instances it may be beneficial to employ coding sequences that produce mRNA with codons preferentially used by the host cell to be transformed ("codon optimization"). Thus, additional to any of the above features, for enhanced expression of transgenes, the codon usage of a transgene (*e.g.*, a sequence encoding an enzyme of the wax ester synthesis pathway) can be altered to increase the number of codons most frequently used by the organism in which the transgene is desired to be expressed. Methods of recoding genes for expression in microalgae are described in, *e.g.*, U.S. Patent No. 7,135,290. The precise mechanisms underlying this effect are believed to be many, but can include the proper balancing of available aminoacylated tRNA pools with proteins being synthesized in the cell, coupled with more efficient translation of the transgenic messenger RNA (mRNA) when this need is met. The coding sequences may be codon optimized for optimal production of a desired product in the host organism selected for expression. For example, the nucleic acid sequences encoding any of a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and/or a polypeptide having wax ester synthase activity can be

codon optimized for expression in a photosynthetic microorganism, *e.g.*, a cyanobacterium. All or any portion of the codons of a gene may be changed to reflect a preferred codon usage of a host microorganism. One or more codons may be additionally but optionally changed to codons that are not necessarily the most preferred codon of the host microorganism encoding a particular amino acid. Additional information for codon optimization is available, *e.g.* at the codon usage database of GenBank.

[00146] Additionally but optionally, the nucleic acid molecules of the invention may encode fusion proteins that comprise a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and/or a wax ester synthase. For example, the nucleic acids of the invention may comprise polynucleotide sequences that encode glutathione-S-transferase (GST) or a portion thereof, thioredoxin or a portion thereof, maltose binding protein or a portion thereof, poly-histidine (*e.g.* His_6), poly-HN, poly-lysine, a hemagglutinin tag sequence, HSV-Tag and/or at least a portion of HIV-Tat fused to the thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and/or wax ester synthase sequence.

Transcription Units and Operons

[00147] A recombinant or isolated nucleic acid molecule of the invention can comprise a transcriptional unit comprising a nucleic acid sequence encoding a polypeptide having wax ester synthase activity and one or more of a) a nucleic acid sequence encoding a thioesterase, b) a nucleic acid sequence encoding an acyl-CoA synthetase, and c) a nucleic acid encoding an alcohol-forming fatty acyl reductase. The nucleic acid sequences of the transcriptional unit are organized such that the two or more sequences encoding separate polypeptides, when operably linked to a promoter, can be transcribed together, as a single RNA transcript. For example, the transcriptional unit does not include a transcriptional termination sequence upstream of any of the genes of the transcriptional unit (*e.g.*, between any of the distinct polypeptide-encoding sequences) and typically does not include any functional promoters between the genes of the transcriptional unit (*i.e.*, downstream of a first protein coding region and upstream of a second, third, or fourth protein coding region).

[00148] The nucleic acid sequences encoding the wax ester synthase and one or more of a thioesterase, acyl-CoA synthetase, and alcohol-forming fatty acyl reductase may be any of the nucleic acid sequences described herein. In some examples, the transcriptional unit comprises a)

a nucleic acid sequence encoding an acyl-ACP thioesterase, b) a nucleic acid sequence encoding an acyl-CoA synthetase, c) a nucleic acid sequence encoding an alcohol-forming acyl-CoA reductase, and d) a nucleic acid sequence encoding a polypeptide having wax ester synthase activity. In some illustrative examples, the transcriptional unit comprises nucleic acid sequences encoding (a) a polypeptide selected from polypeptides comprising amino acid sequences having at least 85% identity to SEQ ID NOS: 1-9, (b) a polypeptide selected from polypeptides comprising amino acid sequences having at least 85% identity to SEQ ID NOS: 10-14, (c) a polypeptide selected from polypeptides comprising amino acid sequences having at least 85% identity to SEQ ID NOS: 15-21, and a polypeptide selected from polypeptides comprising amino acid sequences having at least 85% identity to SEQ ID NOS: 22-30.

[00149] Optionally, a nucleic acid molecule as provided herein can be configured as an operon, *i.e.*, a transcriptional unit (two or more tandemly arranged genes that can be transcribed as a single transcript) operably linked to a promoter. The promoter of the operon is typically positioned upstream of the 5'-most gene of the transcriptional unit. The operon comprises a nucleic acid sequence encoding a polypeptide having wax ester synthase activity and any combination of one or more of a nucleic acid sequence encoding a thioesterase (*e.g.*, an acyl-ACP thioesterase), a nucleic acid sequence encoding an acyl-CoA synthetase, and a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase (*e.g.*, an alcohol-forming acyl-CoA reductase). For example, a nucleic acid sequence encoding a polypeptide having wax ester synthase activity, a nucleic acid sequence encoding a thioesterase, a nucleic acid sequence encoding an acyl-CoA synthetase, and a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase can all be operably linked to the same promoter in an operon.

[00150] A promoter operably linked to a nucleic acid sequence encoding a polypeptide having wax ester synthase activity and a nucleic acid sequence encoding a thioesterase, a nucleic acid sequence encoding an acyl-CoA synthetase, and/or a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase may be a promoter that is heterologous with respect to the nucleic acid sequence encoding the wax ester synthase, thioesterase, acyl-CoA synthetase, and/or alcohol-forming fatty acyl reductase, and may be heterologous (from a different species) or homologous (from the same species) with respect to a recombinant host cell. The promoter sequence can be from any organism, provided that it is functional in the host organism. The promoter can be a constitutive promoter or alternatively, the promoter may be a conditional

and/or regulatable, for example, the promoter may be inducible, *i.e.*, a promoter that mediates transcription of an operably linked gene in response to a particular stimulus. Regulatable promoters may be advantageous, *e.g.*, to minimize any deleterious effects on the growth of the host cell and/or to maximize production of the wax ester. An inducible promoter can be responsive to, *e.g.*, light or dark or high or low temperature, and/or can be responsive to specific compounds. Nonlimiting examples of inducible promoters include an ara promoter, a lac promoter, a tet promoter (*e.g.* U.S. Patent No. 5,851,796), a trp promoter or a hybrid promoter that includes one or more portions of an ara, tet, trp and/or lac promoter. An inducible promoters may be formed by fusing one or more portions or domains from a known inducible promoter to at least a portion of a different promoter that can operate in the host cell, *e.g.* to confer inducibility on a promoter that operates in the host species.

[00151] A nucleic acid molecule that includes genes of a wax ester synthesis pathway can include, for example, a promoter that functions in prokaryotes, such as cyanobacteria, including, but not limited to, the lac, tac and trc promoters, as well as derivatives such as but not limited to the *trcE* and *trcY* promoters that are inducible by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG), promoters that are naturally associated with transposon- or bacterial chromosome-borne antibiotic resistance genes (*e.g.* neomycin phosphotransferase, chloramphenicol acetyltransferase, spectinomycin adenyltransferase, *etc.*, or combinations thereof), promoters associated with various heterologous bacterial and native cyanobacterial genes, promoters from viruses and phages, synthetic promoters or combinations thereof. Promoters useful in the constructs that include a wax ester synthase gene and one or more additional genes for producing wax esters may be isolated or derived from cyanobacteria, *e.g.*, secA (secretion; controlled by the redox state of the cell), Prbc (Rubisco promoter), psaAB (PS I reaction center proteins; light regulated), NtcA or glnA promoter and psbA (Dl protein of PSII; light-inducible). The cyanobacterial promoter can be a promoter from upstream of the *Synechocystis* sp. PCC 6803 genomic RS1 insertion site, e.g., a promoter of the slr0338 gene of *Synechocystis* or an ortholog of the *Synechocystis* slr0338 gene of another cyanobacterial species.

[00152] Promoters may be regulated by nitrogen compounds, such as, for example, nar, ntc, nir or nrt promoters, or may be regulated by phosphate levels (*e.g.*, pho or pst promoters) or nickel (*e.g.*, nrs promoter). Promoters for use in cyanobacteria can also be or be modified from

naturally-occurring promoters, and include combinations of naturally-occurring promoters, including, but not limited to, the promoters disclosed herein. Promoter(s) can be selected from prokaryotic promoters from a range of species, including eubacterial and cyanobacterial species, such as, for example, an araC or pBAD promoter, a rha promoter, a Pm promoter, a xylS promoter, a nir promoter, a nar promoter, a pho promoter, a tet promoter, a cys promoter, a metallothionien promoter, an ftf promoter, a gln promoter, a heat shock promoter, a cold-inducible promoter or a viral promoter. The foregoing promoters are exemplary and are not limiting.

[00153] The nucleic acid sequences of a four gene transcriptional unit or operon may be can be arranged in the 5' to 3' direction in any of the following orders:

- thioesterase - acyl-CoA synthetase - alcohol-forming fatty acyl reductase - wax ester synthase;
- thioesterase - acyl-CoA synthetase - wax ester synthase - alcohol-forming fatty acyl reductase;
- thioesterase - alcohol-forming fatty acyl reductase - acyl-CoA synthetase - wax ester synthase;
- thioesterase - wax ester synthase - acyl-CoA synthetase - alcohol-forming fatty acyl reductase;
- thioesterase - alcohol-forming fatty acyl reductase - wax ester synthase - acyl-CoA synthetase;
- thioesterase - wax ester synthase - alcohol-forming fatty acyl reductase - acyl-CoA synthetase;
- wax ester synthase - thioesterase - acyl-CoA synthetase - alcohol-forming fatty acyl reductase;
- wax ester synthase - thioesterase - alcohol-forming fatty acyl reductase - acyl-CoA synthetase;
- wax ester synthase - acyl-CoA synthetase - thioesterase - alcohol-forming fatty acyl reductase;
- wax ester synthase - alcohol-forming fatty acyl reductase - thioesterase - acyl-CoA synthetase;
- wax ester synthase - acyl-CoA synthetase - alcohol-forming fatty acyl reductase - thioesterase;
- wax ester synthase - alcohol-forming fatty acyl reductase - acyl-CoA synthetase - thioesterase;
- alcohol-forming fatty acyl reductase - wax ester synthase - thioesterase - acyl-CoA synthetase;
- alcohol-forming fatty acyl reductase - wax ester synthase - acyl-CoA synthetase - thioesterase;
- alcohol-forming fatty acyl reductase - thioesterase - wax ester synthase - acyl-CoA synthetase;
- alcohol-forming fatty acyl reductase - acyl-CoA synthetase - wax ester synthase - thioesterase;
- alcohol-forming fatty acyl reductase - thioesterase - acyl-CoA synthetase - wax ester synthase;
- alcohol-forming fatty acyl reductase - acyl-CoA synthetase - thioesterase - wax ester synthase;
- acyl-CoA synthetase - alcohol-forming fatty acyl reductase - wax ester synthase - thioesterase;
- acyl-CoA synthetase - alcohol-forming fatty acyl reductase - thioesterase - wax ester synthase;
- acyl-CoA synthetase - wax ester synthase - alcohol-forming fatty acyl reductase - thioesterase;

- acyl-CoA synthetase - thioesterase - alcohol-forming fatty acyl reductase - wax ester synthase;
- acyl-CoA synthetase - wax ester synthase - thioesterase - alcohol-forming fatty acyl reductase; or
- acyl-CoA synthetase - thioesterase - wax ester synthase - alcohol-forming fatty acyl reductase.

For example, the transcriptional unit or operon can comprise the four nucleic acid sequences arranged in the 5' to 3' direction in the order of thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and wax ester synthase. In another example, the transcriptional unit or operon comprises the four nucleic acid sequences arranged in the 5' to 3' direction in the order of acyl-CoA synthetase, wax ester synthase, thioesterase, and alcohol-forming fatty acyl reductase. The four nucleic acid sequences in the transcriptional unit or operon can be arranged in the order that results in optimal wax ester production, as determined empririically.

[00154] A nucleic acid molecule of the invention can comprise a transcriptional unit comprising a nucleic acid sequence encoding a polypeptide having wax ester synthase activity (*e.g.*, a wax ester synthase) and any combination of a nucleic acid sequence encoding a thioesterase (*e.g.*, an acyl-ACP thioesterase), a nucleic acid sequence encoding an acyl-CoA synthetase, and/or a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase (*e.g.*, an alcohol-forming acyl-CoA reductase) arranged in tandem, where the nucleic acid molecule optionally does not include a promoter sequence that operates in the intended host cell upstream of the genes of the transcriptional unit. For example, a promoterless transcriptional unit can preferably be designed for integration (*e.g.*, homologous recombination) into a site of the host genome that includes a promoter sequence, such that the nucleic acid sequences in the operon or cassette can be transcriptionally regulated by a promoter in the genome of the host cell (which may be, *e.g*,. a prokaryotic and/or photosynthetic microorganism, for example, a cyanobacterium). In some examples, a prmoter endogenous to the intended host cell can be included in a genomic sequence of a prokaryotic and/or photosynthetic microorganism that is positioned upstream of a transcriptional unit in a nucleic acid molecule.

[00155] Additionally to any of the above features, a nucleic acid molecule comprising an operon or transcriptional unit that includes a gene encoding a polypeptide having wax ester synthase activity and one or more of a thioesterase gene, an acyl-CoA synthetase gene, and an alcohol-forming fatty acyl reductase gene, can optionally include one or more additional regulatory sequences can be included in the operon or transcriptional unit, for example, a sequence for enhancing translation (*e.g.*, a ribosome binding site; rbs) can be included upstream

of any of the polypeptide-encoding sequences, and/or, an intergenic region (*e.g.*, the *S. elongatus* KaiBC intergenic region) may be included between any two genes in the transcriptional unit or operon, or between all of the genes of the transcriptional unit or operon, and/or, a transcription terminator sequence can be positioned 3' of the 3'-most gene of the transcriptional unit or operon. A wide variety of transcriptional terminators can be used in any of the vectors of the invention. Examples of possible terminators can include, but are not limited to, psbA, psaAB, rbc, secA, T7 coat protein, rrnB, and the like, and combinations thereof.

[00156] In addition to the nucleic acid sequence encoding the wax ester synthase and a nucleic acid sequence encoding a thioesterase, a nucleic acid sequence encoding an acyl-CoA synthetase, and/or a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase, and, one or more additional nucleic acid sequences can optionally be included in an operon or transcriptional unit as provided herein, where the one or more additional genes may include, for example, one or more nucleic acid sequences encoding enzymes or proteins that may enhance wax ester synthesis, one or more nucleic acid sequences that may enhance photosynthesis or carbon-fixation, one or more nucleic acid sequences that may enhance wax ester transport, and/or one or more reporter genes or selectable markers. For example, any of the nucleic acid molecules, operons, nucleic acid cassettes, and constructs referred to herein may optionally comprise a nucleic acid sequence encoding a wax transporter (*e.g.*, any of the wax transporters described herein). A transcriptional unit or operon for wax synthesis can optionally include five genes, for example, comprising nucleic acid sequences that encode a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, a wax ester synthase, and a wax transporter.

Nucleic Acid Constructs

[00157] The invention also provides nucleic acid constructs comprising a nucleic acid sequence encoding a polypeptide having wax ester synthase activity in a transcriptional unit with one or more of: a nucleic acid sequence encoding a thioesterase (*e.g.*, an acyl-ACP thioesterase), a nucleic acid sequence encoding an acyl-CoA synthetase, and a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase (*e.g.*, an alcohol-forming acyl-CoA reductase). For example, the nucleic acid construct can comprise a transcriptional unit that includes all four nucleic acid sequences encoding polypeptides of the wax ester synthesis pathway.

[00158] The nucleic acid construct can comprise any transcriptional unit or operon as described herein. A nucleic acid construct of the invention may comprise a nucleic acid sequence encoding a polypeptide having wax ester synthase activity and a nucleic acid sequence encoding a thioesterase, an acyl-CoA synthetase, and/or an alcohol-forming fatty acyl reductase, as described herein, and can optionally further include sequences that regulate or mediate transcription, translation, or integration of nucleotide sequences into a host genome. For example, as described herein, a nucleic acid molecule can comprise an operon in which a promoter can be operably linked to the nucleic acid sequences encoding two or more genes of the wax ester pathway. The promoter can be any promoter active in a host cell, and can be, for example, any disclosed herein. For example, a construct can comprise an operon as disclosed hereinabove comprising a nucleic acid sequence encoding a thioesterase, a nucleic acid sequence encoding an acyl-CoA synthetase, a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase, and a nucleic acid sequence encoding a wax ester synthase operably linked to a promoter. In additional examples, a nucleic acid construct does not contain a promoter in operable linkage with the transcriptional unit that includes a nucleic acid sequence encoding a polypeptide having wax ester synthase activity and one or more of a nucleic acid sequence encoding a thioesterase, a nucleic acid sequence encoding an acyl-CoA synthetase, and a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase. For example, the nucleic acid construct can be designed to be transformed into the host cell such that the transcriptional unit becomes operably linked to an endogenous promoter by, *e.g.*, homologous recombination, site specific integration, and/or vector integration.

[00159] A nucleic acid construct that comprises a wax synthesis transcriptional unit or operon may additionally comprise at least one nucleic acid sequence of at least 50 nucleotides, at least 100 nucleotides, at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, at least 900 nucleotides, at least 1000 nucleotides, or at least 1500 nucleotides derived from a prokaryotic and/or photosynthetic microorganism, where the nucleotide sequence derived from a prokaryotic and/or photosynthetic microorganism can mediate recombination of the transcriptional unit or operon into a host genome. The nucleic acid construct may, for example, comprise two or more nucleic acid sequences of at least 50 nucleotides, at least 100 nucleotides, at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500

nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, at least 900 nucleotides, at least 1000, or at least 1500 nucleotides derived from a prokaryotic and/or photosynthetic microorganism. For example, the transcriptional unit or operon that includes a gene encoding a polypeptide having wax ester synthase activity and at least one additional gene of a wax ester synthesis pathway can be flanked by the sequences derived from the genome of a prokaryotic and/or photosynthetic microorganism. For example, a first nucleotide sequence derived from a prokaryotic and/or photosynthetic microorganism can be 5' of the transcriptional unit and a second nucleotide sequence derived from a prokaryotic and/or photosynthetic microorganism can be 3' of the transcriptional unit.

[00160] A nucleic acid sequence derived from the genome of a prokaryotic and/or photosynthetic microorganism may optionally comprise a nucleic acid sequence derived from the 5' region of a gene. For example, a nucleic acid construct can be designed such that the genomic nucleic acid sequences mediate recombination into a site proximal to and downstream of a promoter in the genome of the host organism, such that the transcriptional unit can be regulated by a promoter of the host microorganism. Further additionally, a genomic nucleic acid sequence of the construct can include at least a portion of a promoter which is endogenous to the intended host microorganism. For example, the transgene(s) of the construct can be operably linked to a promoter that is endogenous to the production host, and the promoter endogenous to the production host can be within or part of a sequence that mediates homologous recombination into the host genome. Optionally, the genomic nucleic acid sequence can include a promoter that may be active in the production microorganism, but may be inactive or have very low activity in a cloning organism, such as *E. coli* or yeast, such that the cloning microorganism does not produce wax esters or wax ester intermdietes or is not impaired by production of wax esters or wax ester intermediates such as fatty acids or fatty alcohols. Alternatively, the nucleic acid molecule can include a sequence of at least 50 nucleotides of a sequence derived from the genome of a prokaryotic and/or photosynthetic microorganism, but the genomic sequence may not comprise a promoter operably linked to any of the nucleic acid sequences of the transcriptional unit that includes two or more genes of the wax ester synthesis pathway.

[00161] The nucleic acid construct comprising a nucleic acid sequence encoding a thioesterase, a nucleic acid sequence encoding an acyl-CoA synthetase, a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase, and/or a nucleic acid sequence encoding a wax

ester synthase can be designed for transformation into cyanobacteria, *e.g.*, *Synechocystis* sp. PCC 6803. For example, the vector can be designed to permit homologous recombination of nucleic acid sequences of the vector with the cyanobacterial genome, such that the enzyme-encoding sequences become integrated into a locus of the genome. For example, sequences of the RS1 site or RS2 site, or genomic sequence that can include or are proximal to sequence of glgA, glgB, or glgC genes can be operably linked to a transcriptional unit or operon that includes genes of the wax ester synthesis pathway.

[00162] The nucleic acid sequences encoding enzymes of the wax ester synthesis pathway may each comprise an initiation codon, wherein one or more, and optionally all, of the nucleic acid sequences may additionally comprise a heterologous translational regulatory sequence upstream of the initiation codon. For example, the nucleic acid sequence encoding the acyl-CoA synthetase, the nucleic acid sequence encoding the alcohol-forming fatty acyl reductase, and the nucleic acid sequence encoding the wax ester may each comprise a heterologous translational regulatory sequence upstream of the initation codon.

[00163] A nucleic acid construct of the present invention can include any of the sequences disclosed herein that encode one or more of a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and a wax ester synthase in a vector, such as, but not limited to, an expression vector. A vector can include, for example, one or more of: 1) an origin of replication for propagation of the nucleic acid sequences in one or more hosts (which may or may not include the production host); 2) one or more selectable markers; 3) one or more reporter genes; 4) one or more expression control sequences, such as, but not limited to, promoter sequences, enhancer sequences, terminator sequences, sequence for enhancing translation, *etc.*; and/or 5) one or more sequences for promoting integration of the nucleic acid sequences into a host genome, for example, one or more sequences having homology with one or more nucleotide sequences of the host microorganism.

[00164] The transformation vectors can include a selectable marker, such as but not limited to a drug resistance gene, an herbicide resistance gene, a metabolic enzyme and/or factor required for survival of the host (for example, an auxotrophic marker), or the like, or a combination thereof. Transformed cells can optionally be selected based upon the ability to grow in the presence of the antibiotic and/or other selectable marker under conditions in which cells lacking the resistance cassette or auxotrophic marker could not grow. Additionally or alternatively, a

non-selectable marker (*e.g.*, a reporter gene) may be present on a vector, such as a gene encoding a fluorescent protein or an enzyme that generates a detectable reaction product.

[00165] The transformation vector may be an integration vector that includes one or more sequences that promote integration of a nucleic acid sequence or nucleic acid cassette into the genome of the host cell. For example, an integration vector used to transform a host cell can include at least one sequence of at least 50, at least 100, at least 200, at least 300, at least 400, at least 500, at least 600, at least 700, at least 800, at least 900, at least 1000, at least 1200, or at least 1500 nucleotides with homology to a sequence in the genome of the host cell to allow integration of the nucleic acid sequence or nucleic acid cassette into the genome of the host cell via homologous recombination. In some examples, the nucleic acid sequence or nucleic acid cassette is flanked by sequences homologous to a region of the host chromosome to promote integration of the gene of interest into the host chromosome. Additionally or alternatively, an integration vector can include one or more sequences that promote site-specific recombination or random integration such as, but not limited to, sequences recognized by recombinases, integrases or transposases. The integration vector can optionally further include a gene encoding a recombinase, integrase or transposase. The integration vector can be designed to promote integration of a nucleic acid sequence encoding a thioesterase, a nucleic acid sequence encoding an acyl-CoA synthetase, a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase, and/or a nucleic acid sequence encoding a wax ester synthase (*e.g.*, all four nucleic acid sequences) into cyanobacteria. In particular examples, the vector promotes integration at the RS1 site or RS2 site of, *e.g.*, *Synechocystis* sp. PCC 6803, where the vector can include RS1 or RS2 sequences.

[00166] Vectors can be introduced into host cells (*e.g.*, any of the host cells described herein) via conventional transformation and/or transfection techniques. Cyanobacteria, for example, can be transformed by any suitable methods, including, *e.g.*, natural DNA uptake (Zang (2007) *J. Microbiol.* 45, 241-245), conjugation, transduction, glass bead transformation (Feng (2009) *Mol. Biol. Rep.* 36, 1433-9), silicon carbide whisker transformation (Dunahay (1997) *Methods Mol. Biol.* 62, 503-9), biolistics (Kroth (2007) *Methods Mol. Biol.* 390, 257-267), electroporation (Ludwig (2008) *Appl. Microbiol. Biotechnol.* 78, 729-35), laser-mediated transformation (WO2009/140701), incubation with DNA in the presence of or after pre-treatment with any of poly(amidoamine) dendrimers (Pasupathy (2008) *Biotechnol. J.* 3, 1078-82), polyethylene glycol

(Ohnuma (2008) *Plant Cell Physiol.* 49, 117-120), cationic lipids (Muradawa (2008) *J. Biosci. Bioeng.* 105, 77-80), dextran, calcium phosphate and/or calcium chloride (Mendez-Alvarez (1994) *J. Bacteriol.* 176, 7395-7397), optionally after treatment of the cells with cell wall-degrading enzymes (Perrone (1998) *Mol. Biol. Cell* 9, 3351-3365), or the like, or combinations thereof. Agrobacterium-mediated transformation can additionally or alternatively be performed on algal cells, for example after removing or wounding the algal cell wall (Kumar (2004) *Plant Sci.* 166, 731-738).

[00167] The above-described vectors may be used in any of the recombinant host cells or methods for producing a wax ester as described herein.

Recombinant Host Cells

[00168] The invention also provides a recombinant host cell comprising a non-native nucleic acid sequence encoding a wax ester synthase, and one or more of (a) a non-native nucleic acid sequence encoding a thioesterase; (b) a non-native nucleic acid sequence encoding an acyl-CoA synthetase; and (c) a non-native nucleic acid sequence encoding an alcohol-forming fatty acyl reductase; in which the non-native nucleic acid sequence encoding a wax ester synthase and at least one of (a) a non-native nucleic acid sequence encoding a thioesterase; (b) a non-native nucleic acid sequence encoding an acyl-CoA synthetase; and (c) a non-native nucleic acid sequence encoding an alcohol-forming fatty acyl reductase are in an operon. The operon comprising two or more genes of the wax synthesis pathway (for example, comprising any transcriptional unit as described herein) comprises a promoter that can be heterologous or homologous with respect to the host microorganism, and can be endogenous to the host microorganism. For example, the recombinant host cell can include a non-native nucleic acid molecule that comprises an operon that includes a promoter operably linked to a transcriptional unit that includes each of (a) a non-native nucleic acid sequence encoding an acyl-ACP thioesterase; (b) a non-native nucleic acid sequence encoding an acyl-CoA synthetase; (c) a non-native nucleic acid sequence encoding an alcohol-forming fatty acyl reductase; and (d) a non-native nucleic acid sequence encoding a wax ester synthase. The recombinant host cell can be genetically engineered for the production of wax esters, in which expression of the non-native nucleic acid sequences encoding a wax ester synthase and a thioesterase, an acyl-CoA synthetase, and an alcohol-forming fatty acyl reductase results in production of a wax ester. The

recombinant host cell may comprise, *e.g.*, non-native nucleic acid sequences encoding any of the thioesterases, acyl-CoA synthetases, alcohol-forming fatty acyl reductases, and/or wax ester synthases described herein, and/or can comprises any of the constructs, operons, and/or transcriptional units as described herein.

[00169] The recombinant host cell can be a prokaryotic and/or photosynthetic microorganism. The recombinant host cell can be without limitation, a eubacterium, archaebacterium, green nonsulfur bacterium, purple nonsulfur bacterium, or cyanobacterium. The recombinant host cell can be, in particular examples, a host cell that does not endogenously produce acyl-CoA, for example a cyanobacterium. For example, the recombinant photosynthetic microorganism can be a cyanobacterium of a genus selected from, *e.g.*, *Agmenellum, Anabaena, Anabaenopsis, Anacystis, Aphanizomenon, Arthrospira, Asterocapsa, Borzia, Calothrix, Chamaesiphon, Chlorogloeopsis, Chroococcidiopsis, Chroococcus, Crinalium, Cyanobacterium, Cyanobium, Cyanocystis, Cyanospira, Cyanothece, Cylindrospermopsis, Cylindrospermum, Dactylococcopsis, Dermocarpella, Fischerella, Fremyella, Geitleria, Geitlerinema, Gloeobacter, Gloeocapsa, Gloeothece, Halospirulina, Iyengariella, Leptolyngbya, Limnothrix, Lyngbya, Microcoleus, Microcystis, Myxosarcina, Nodularia, Nostoc, Nostochopsis, Oscillatoria, Phormidium, Planktothrix, Pleurocapsa, Prochlorococcus, Prochloron, Prochlorothrix, Pseudanabaena, Rivularia, Schizothrix, Scytonema, Spirulina, Stanieria, Starria, Stigonema, Symploca, Synechococcus, Synechocystis, Thermosynechococcus, Tolypothrix, Trichodesmium, Tychonema* or *Xenococcus*. A number of cyanobacterial species have genomes that have been completely sequenced. Cyanobacterial strains such as but not limited to *Synechocystis sp.* PCC 6803 and *Synechococcus elongates* PCC 7942 have been manipulated using molecular biological techniques. In some aspects, the cyanobacterial host organism is a *Synechococcus*, *Synechocystis*, or *Thermosynechococcus* species, and in an illustrative example is *Synechocystis* sp. PCC 6803. Alternatively, the recombinant photosynthetic microorganism can be a *Cyanobium*, *Cyanothece*, or *Cyanobacterium* species, or further alternatively, the recombinant photosynthetic microorganism can be a *Gloeobacter*, *Lyngbya*, or *Leptolyngba* species.

[00170] A nucleic acid sequence provided in the host recombinant microorganism that encodes a thioesterase can encode any thioesterase, including any disclosed herein. A nucleic acid sequence provided in the host recombinant microorganism that encodes an acyl-CoA

synthetase can encode any acyl-CoA synthetase, including any disclosed herein. A nucleic acid sequence provided in the host recombinant microorganism that encodes an acyl-CoA fatty acyl reductase can encode any an acyl-CoA fatty acyl reductase, including any disclosed herein. A nucleic acid sequence provided in the host recombinant microorganism that encodes a wax ester synthase can encode any wax ester synthase, including any disclosed herein. A recombinant host cell expressing a thioesterase (*e.g.*, an acyl-ACP thioesterase), an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase (*e.g.*, an alcohol-forming acyl-CoA reductase), and a polypeptide having wax ester synthase activity can produce a greater amount of a wax ester than a control host cell that does not express these four enzymes. For example, the amount of wax ester produced by a culture of the recombinant host cell expressing the thioesterase, the acyl-CoA synthetase, the alcohol-forming fatty acyl reductase, and/or the wax ester synthase can be at least 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 65%, 70%, 75%, 80%, 90%, 95%, 100%, 125%, 150%, 175%, 200%, 225%, 250%, 275%, 300%, 325%, 350%, 375%, 400%, 425%, 450%, 475%, 500%, 525%, 550%, 575%, 600%, 625%, 650%, 675%, 700%, 725%, 750%, 775%, 800%, 825%, 850%, 875%, 900%, 925%, 950%, 975%, or 1000% greater than the amount of wax ester produced by a control host cell that does not express the thioesterase, the acyl-CoA synthetase, the alcohol-forming fatty acyl reductase, and the wax ester synthase.

[00171] In illustrative examples, the recombinant host cell can comprise: (1) a nucleic acid sequence encoding a polypeptide having at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the polypeptide of any of SEQ ID NOS: 1-9; or to a functional fragment of the polypeptide having thioesterase activity, (2) a nucleic acid sequence encoding a polypeptide having at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the polypeptide of any of SEQ ID NOS: 10-14, or to a functional fragment of the polypeptide having acyl-CoA synthetase activity, (3) a nucleic acid sequence encoding a polypeptide having at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the polypeptide of any of SEQ ID NOS: 15-21; or to a functional fragment of the polypeptide having alcohol-forming fatty acyl reductase activity, and (4) a nucleic acid sequence encoding a polypeptide having at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%,

89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the polypeptide of any of SEQ ID NOS: 22-30; or to a functional fragment of the polypeptide having wax ester synthase activity.

[00172] The nucleic acid sequence encoding a wax ester synthase and one or more of a nucleic acid sequence encoding a thioesterase, a nucleic acid sequence encoding an acyl-CoA synthetase, and a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase, can be organized in an operon in the recombinant host cell. The operon further includes a promoter that can be heterologous or homologous with respect to the recombinant host cell, and can be, for example, an endogenous promoter of the host cell. For example, the recombinant host cell can comprise a nucleic acid sequence encoding a thioesterase, a nucleic acid sequence encoding an acyl-CoA synthetase, a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase, and a nucleic acid sequence encoding a wax ester synthase, where all of the nucleic acid sequences are operably linked to a promoter. A promoter operably linked to a wax ester synthesis operon can be any promoter operable in the host microorganism, including but not limited to any disclosed herein, and can be constitutive or conditional, and can be for example, regulatable, for example inducible by one or more conditions or compounds. In particular examples, the promoter is endogenous to the recombinant host cell.

[00173] The nucleic acid sequences encoding the thioesterase, the acyl-CoA synthetase, the alcohol-forming fatty acyl reductase, and the wax ester synthase can be operably linked to a promoter that is endogenous to a photosynthetic host cell, *e.g.*, a photosynthetic microorganism. The photosynthetic microorganism in some examples can be a cyanobacterium, *e.g.*, a species of *Synechocystis*, and the nucleic acid sequences encoding the thioesterase, the acyl-CoA synthetase, the alcohol-forming fatty acyl reductase, and the wax ester synthase can be integrated at the RS1 or RS2 site, or within or proximal to a *glgA*, *glgB*, or *glgC* gene, and may be operably linked to an endogenous promoter upstream of the integration site.

[00174] For example, a transcriptional unit that comprises a non-native nucleic acid sequence encoding a wax ester synthase and one or more of a non-native nucleic acid sequence encoding a thioesterase, non-native nucleic acid sequence encoding an acyl-CoA synthetase, and a non-native nucleic acid sequence encoding an alcohol-forming acyl-CoA reductase can be inserted into the genome of the host microorganism such that the transcriptional unit becomes juxtaposed with, and operably linked to, an endogenous promoter of the host microorganism. The

endogenous promoter can be any endogenous promoter of the host microorganism, and in some examples is a promoter of a gene encoding an oxidoreductase or dehydrogenase, e.g., the slr0338 gene of *Synechocystis* (*e.g.*, *Synechocystis* sp. PCC6803) or an ortholog thereof in another cyanobacterial species. In further nonlimiting examples the endogenous promoter can be a promoter of a glgA (glycogen synthase) gene (*e.g.*, sll0945, protein coding region at nucleotides 2265213-2266646 of the *Synechocystis* sp. PCC6803 genome (GenBank Accession NC_000911) or sll1393, protein coding region at nucleotides 57340-58815 of the *Synechocystis* sp. PCC6803 genome (GenBank Accession NC_000911)), a promoter of a glgB (glycogen branching enzyme) gene (*e.g.*, sll0158, protein coding region at nucleotides 2331505-2333817 of the *Synechocystis* sp. PCC6803 genome (GenBank Accession NC_000911)), or a glg C (glucose-1-phosphate adenyltransferase) gene (*e.g.*, sll1176, protein coding region at nucleotides 3516539-3517858 of the *Synechocystis* sp. PCC6803 genome (GenBank Accession NC_000911)). Insertion of the wax ester synthesis transcriptional unit can in some instances attenuate or disrupt the gene that is naturally regulated by the endogenous promoter. For example, insertion of a wax synthesis transcriptional unit into the RS1 site of *Synechocystis* can disrupt the slr0338 gene. In other examples, the sll0945, sll1393, sll0158, or sll1176 gene of *Synechocystis* or an ortholog of any of these genes in another cyanobacterial host can be disrupted by integration of the wax ester synthesis transcriptional unit.

[00175] Alternatively, a transcriptional unit encoding two or more genes of a wax synthesis pathway can be operably linked to a promoter that is homologous with respect to the host microorganism, but the operon that includes the host-homologous promoter and wax synthesis transcriptional unit can be present in a vector in the host microorganism or integrated at a site in the genome of the host microorganism that is not the natural site of the host-homologuos promoter. For example, where the host microorganism is a cyanobacterium, a cyanobacterial promoter as provided hereinabove (e.g., Prbc, nrs, nir, nar, pho, secA, *etc.*) can regulate transcription of the operon. Further alternatively, a transcriptional unit encoding two or more genes of a wax synthesis pathway can be operably linked to a promoter that is heterologous with respect to the host microorganism, but the operon that includes the heterologous promoter and wax synthesis transcriptional unit can be present in a vector in the host microorganism or integrated at a site in the genome of the host microorganism. For example, where the host microorganism is a cyanobacterium, a promoter of another cyanobacterial or prokaryotic species,

or a synthetic promoter, as provided hereinabove (e.g., Trc, TrcE, TrcY, lac, ara, *etc.*) can regulate transcription of the operon.

[00176] Additionally to any of the above features, a recombinant host cell expressing a non-native gene encoding a thioesterase, a non-native gene encoding an acyl-CoA synthetase, a non-native gene encoding an alcohol-forming fatty acyl reductase, and/or a non-native gene encoding a wax ester synthase can optionally expresses at least one additional recombinant or exogenous gene, or can overexpresses an endogenous gene, that functions in the wax ester biosynthesis pathway. The additional gene may be encoded by a nucleic acid molecule that is the same as the nucleic acid molecule that encodes any of the thioesterase, the acyl-CoA synthetase, the alcohol-forming fatty acyl reductase, and the wax ester synthase, or the additional gene may be encoded by a separate nucleic acid molecule. Where two or more genes are encoded by the same nucleic acid molecule (*e.g.*, on the same expression vector), the expression of each gene may optionally be independently regulated by a same or a different promoter and/or enhancer. An additional gene may increase the rate and/or level of wax ester production. Additionally and/or alternatively, an additional gene may, *e.g.*, increase the concentration of wax ester precursors such as acyl-ACP, free fatty acids, acyl-CoA, and fatty alcohol; decrease the amount of acyl-ACP, fatty alcohol or wax ester conversion to other products (such as, for example, other fatty acid derivatives, or fatty alcohol or wax ester breakdown products); or lower fatty acid, fatty alcohol, and/or wax ester toxicity to the cell. For example, the polypeptide encoded by the additional gene can be selected from, *e.g.*, one or more enzymes of the fatty acid synthase complex (*e.g.*, a beta-ketoacyl-ACP synthase, a 3-ketoacyl-ACP reductase, a β-hydroxyacyl-ACP dehydratase, an enoyl-ACP reductase, *etc.*), an acetyl-CoA carboxylase, a malonyl-CoA:ACP transacylase, an acyl carrier protein, or an acyl-ACP synthetase. Additionally or alternatively, the recombinant host cell can express a ribulose 1,5-bisphophate carboxylase, a phycobiliprotein (*e.g.*, phycocyanin), an acyl carrier protein, and/or a transmembrane transporter (*e.g.*, an ATP-binding cassette, or ABC, transporter, an RND pump, multi-drug efflux protein, *etc.*) to facilitate wax ester secretion. For example, a transporter can be encoded by at least one gene selected from a group including, but not limited to, ABC transporters such as *A. thaliaia* Atlg51500 (AY734542), CER5, WBC11, AtMRPS, AmiS2 and AtPGP1 or fatty acid transporter (FATP) genes from *Saccharomyces*, *Drosophila* (*e.g.*, CG7400-PA, isoform A, at locus NP_524723), *C. elegans*, mycobacterial species, or mammalian species. Additional transporter

proteins that can be encoded by a non-native gene in a host microorganism of the invention include, but are not limited to, *Rhodococcus erythopolis* ansP (AAN73268), multi-drug efflux protein *E. coli* acrAB (NP_414996.1, NP_414995.1) or AcrEF (NP_417731.1, NP_417732.1), efflux protein *E. coli* tolC (NP_417507.2), *T. elongatus* BP-I tlll618, (NP_682408.1), *T. elongatus* BP-I tlll619 (NP_682409), *T. elongatus* BP-I tllO139 (NP 680930), and transporters from *Acinetobacter* sp. HOl-N. The polypeptide encoded by the additional gene may be exogenous or endogenous; if endogenous, the recombinant host cell may be engineered to overexpress or overproduce the endogenous polypeptide. If exogenous, the gene encoding the polypeptide may be located on a vector or may be integrated into the host genome, may be is under the control of a promoter (*e.g.*, a regulatable promoter such as an inducible promoter).

[00177] Additionally but optionally, the recombinant host cell can be engineered to attenuate or eliminate the expression of one or more beta-oxidation pathway enzymes. For example, the recombinant host cell may be engineered to attenuate or eliminate expression of at least one of glycerol-3-phosphate dehydrogenase, acetaldehyde-CoA dehydrogenase, pyruvate dehydrogenase and acetate kinase. The recombinant host cell may be engineered to attenuate or eliminate any gene that functions to shunt substrates from the wax ester production pathway, to convert produced wax esters or fatty alcohols to a non-wax ester product, or to increase fatty alcohol or wax ester toxicity to the cell.

[00178] Mutations to attenuate or eliminate expression of known genes can be introduced either by recombinant or non-recombinant methods. The genes may be targeted specifically by disruption, deletion, replacement, or generation of antisense sequences, *e.g.*, by use of micro RNAs or shRNA constructs, generation of ribozymes and/or other recombinant approaches known to the practitioner. Inactivation of the genes can additionally or alternatively be accomplished by random mutation techniques such as exposure to UV and/or chemical mutagens followed by screening of the cells for successful mutants. Additionally or alternatively, the proteins encoded by the genes can be inhibited by intracellular generation of appropriate antibodies, intracellular generation of peptide inhibitors, or the like, or some combination thereof.

[00179] The above-described recombinant host cells may be used in any of the methods or systems of producing a wax ester described herein.

Methods of Producing a Wax Ester

[00180] The invention provides methods of producing a wax ester using a recombinant host cell as disclosed herein, such as a recombinant microorganism expressing non-native genes encoding a thioesterase, an acyl-CoA synthetase, an alcohol-forming fatty acyl reductase, and a wax ester synthase, *e.g.*, any of the recombinant host cells described herein. The genes can encode any thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and wax ester synthase as disclosed herein. For example, the thioesterase can be an acyl-ACP thioesterase and/or the alcohol-forming fatty acyl reductase can be an alcohol-forming acyl-CoA reductase.

[00181] In a particular illustrative examples, the thioesterase encoded by the non-native nucleic acid sequence of the host microorganism is or comprises the polypeptide of SEQ ID NO: 1, the acyl-CoA synthetase encoded by the non-native nucleic acid sequence of the host microorganism is or comprises the polypeptide of SEQ ID NO: 10, the alcohol-forming fatty acyl reductase encoded by the non-native nucleic acid sequence of the host microorganism is or comprises the polypeptide of SEQ ID NO: 15, and the wax ester synthase encoded by the non-native nucleic acid sequence of the host microorganism is or comprises the polypeptide of SEQ ID NO: 22. The nucleic acid sequences encoding the wax ester synthase and one or more of the thioesterase, the acyl-CoA synthetase, and the alcohol-forming fatty acyl reductase, can be provided in an operon, where the operon can include a promoter heterologous or homologous with respect to the host microorganism, and where a homologous promoter can optionally be a promoter endogenous to the host microorganism and the transcriptional unit that includes the nucleic acid sequences encoding the wax ester synthase and one or more of the thioesterase, the acyl-CoA synthetase, and the alcohol-forming fatty acyl reductase can be intergrated into the host genome downstream of the endogenous promoter.

[00182] The method comprises the steps of: (1) culturing a recombinant host cell that comprises a non-native nucleic acid sequence that encodes a polypeptide having wax ester synthase activity and one or more more of a non-native nucleic acid sequences that encode a thioesterase, a non-native nucleic acid sequence that encodes an acyl-CoA synthetase, and a non-native nucleic acid sequence that encodes an alcohol-forming acyl-CoA reductase in a suitable culture medium; and (2) allowing expression of the non-native nucleic acid sequences, wherein the expression results in the production of a wax ester. Preferably, one or more fatty acids or

alcohols (e.g., one or more fatty alcohols) are not provided in the culture medium. In some examples, the recombinant host cell is a photosynthetic microorganism and the method comprises the steps of: (1) culturing a recombinant host cell in a suitable culture medium, wherein the recombinant host cell is a photosynthetic microorganism that comprises a non-native nucleic acid sequence encoding an acyl-ACP thioesterase, a non-native nucleic acid sequence encoding an acyl-CoA synthetase, a non-native nucleic acid sequence encoding an alcohol-forming acyl-CoA reductase, and a non-native nucleic acid sequence encoding a polypeptide having wax ester synthase activity, and (2) allowing expression of the non-native nucleic acid sequences,wherein the expression results in the production of a wax ester.

[00183] In various examples, the recombinant photosynthetic microorganism may be cultured photoautotrophically. The photosynthetic microorganism can be, for example, a cyanobacterium. The nucleic acid sequences encoding any of the thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and/or wax ester synthase may optionally be codon-optimized for expression in the recombinant host cell (*e.g.*, a photosynthetic microorganism such as a cyanobacterium).

[00184] The non-native nucleic acid sequences encoding the thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and/or wax ester synthase can be heterologous with respect to the recombinant photosynthetic microorganism and can be any as described herein. In various aspects, one or more (*e.g.*, all) of the nucleic acid sequence(s) can be integrated into a chromosome of the recombinant photosynthetic microorganism, and the nucleic acid sequence encoding a wax ester synthase and one or more of the nucleic acid sequences encoding a thioesterase, an acyl-CoA synthetase, and an alcohol-forming fatty acyl reductase may be organized in an operon in which the sequences are operably linked to a promoter, which may be *e.g.*, a promoter endogenous to the recombinant photosynthetic microorganism, or can be, for example, a heterologous promoter, and which in some examples may be regulatable. For example, the promoter (whether endogenous to the host or exogenous, whether heterologous or homologous with respect to the host species) can be inducible, and the method may further comprise the step of inducing expression of the non-native genes encoding the thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and/or wax ester synthase. Optionally, all of the nucleic acid sequences encoding the thioesterase, acyl-CoA synthetase, alcohol-forming fatty acyl reductase, and wax ester synthase may be configured in the same operon.

[00185] The recombinant photosynthetic microorganism can be grown mixotrophically, using both light and a reduced carbon source, or can be cultured phototrophically. When cultured phototrophically, the photosynthetic microorganism can advantageously use light as an energy source. An "inorganic" or non-reduced carbon source can be used for synthesis of biomolecules by the photosynthetic microorganism. Typically a "non-reduced carbon source" can be in the form of CO_2 (carbon dioxide), carbonic acid, bicarbonate salts, carbonate salts, hydrogen carbonate salts, or the like, or combinations thereof, which cannot be further oxidized for sustainable energy nor used as a source of reducing power by host cells. In some examples, inorganic carbon can be substantially the only carbon source present in the culture medium. In these examples, if an organic (reduced) carbon source or compound is present in the culture medium of a host cell grown phototrophically, it generally cannot be taken up and/or metabolized by the cell for energy or as a carbon source for the synthesis of biomolecules, and/or is not present in an amount sufficient to provide sustainable energy for the growth of the cell culture or production or organic molecules.

[00186] Microorganisms that can be useful as host cells in accordance with the methods of the present invention can be found in various locations and environments throughout the world. Without being bound by theory, it is observed that, perhaps as a consequence of their isolation from other species and/or their evolutionary divergence, the particular growth medium for optimal growth and generation of lipid and/or hydrocarbon constituents can vary. In some cases, certain strains of microorganisms may be unable to grow in a particular growth medium because of the presence of some inhibitory component or the absence of some essential nutritional requirement required by the particular strain of microorganism.

[00187] Solid and liquid growth media are generally available from a wide variety of sources, as are instructions for the preparation of particular media suitable for a wide variety of host cell types. For example, various fresh water and salt water media are well known in the art, *e.g.*, those described in Barsanti (2005) Algae: Anatomy, Biochemistry & Biotechnology, CRC Press for media and methods for culturing algae. The growth medium used in exemplary methods is not supplemented with either of a fatty acid or a fatty alcohol.

[00188] The culture methods can include inducing expression of a particular gene described herein for the production of wax esters (*e.g.*, a thioesterase gene, an acyl-CoA synthetase gene, an alcohol-forming fatty acyl reductase gene, and/or a wax ester synthase gene), and/or for

regulating metabolic pathways in the microorganism. Inducing expression can include adding a nutrient or compound to the culture, removing one or more components from the culture medium, increasing or decreasing light and/or temperature, and/or other manipulations that promote expression of the gene of interest. Such manipulations can largely depend on the nature of the promoter operably linked to the gene of interest.

[00189] In some aspects of the methods, the recombinant host cells can be cultured in a bioreactor. Bioreactors can offer many advantages for use in heterotrophic growth and propagation methods. To produce biomass for use in food, microorganisms are preferably fermented in large quantities in liquid, such as, *e.g.*, in suspension cultures. Bioreactors such as steel fermentors can accommodate very large culture volumes (40,000 liter and greater capacity bioreactors can be used in various aspects of the invention). Bioreactors can also typically allow for the control of one or more culture conditions such as temperature, pH, oxygen tension, carbon dioxide levels, and the like, as well as combinations thereof. Bioreactors can typically be configurable, for example, using ports attached to tubing, to allow gaseous components, such as CO_2, CO_2-enriched air, oxygen and/or nitrogen, to be contacted with (*e.g.*, bubbled through) a liquid culture. Other culture parameters, such as the pH of the culture media, the identity and/or concentration of trace elements and/or nutrients, the identity and/or concentration of other media constituents, or the like, or combinations thereof, can typically be more readily manipulated using a bioreactor.

[00190] In some aspects, the cells (*e.g.*, photosynthetic microorganisms) can be cultured in a bioreactor equipped with a natural or artificial light source (a "photobioreactor"), and/or can have one or more walls that is transparent enough to light, including sunlight, to enable, facilitate and/or maintain acceptable microorganism growth. For production of wax esters, the recombinant host cells can additionally or alternatively be cultured in shake flasks, test tubes, vials, microtiter dishes, petri dishes, or the like, or combinations thereof.

[00191] Genetically engineered photosynthetic microorganisms may also be grown in, *e.g.*, ponds, canals, trenches, raceways, channels, or the like, or combinations thereof. As with standard bioreactors, a source of inorganic carbon including, but not limited to, air, CO_2-enriched air, flue gas, *etc.*, or combinations thereof, can be supplied to the culture. When supplying flue gas and/or other sources of inorganic carbon that may contain CO in addition to CO_2, it may be necessary to pre-treat such sources such that the CO level introduced into the (photo)bioreactor

does not constitute a dangerous and/or lethal dose vis-à-vis the growth and/or survival of the microorganisms. In some aspects, the carbon source is a non-reduced carbon source, *e.g.*, (such as, but not limited to, CO_2, bicarbonate, carbonate salts, and the like). In some aspects, the carbon source does not provide a source of energy in the production of a wax ester.

[00192] In some aspects, the wax ester produced by a system of the invention can be secreted into the culture medium by the recombinant host cell. Additionally or alternatively, the wax ester may be extracted from the recombinant host cell. In some aspects, the wax ester may be isolated using a method described herein.

[00193] The recombinant host cell can optionally secrete at least a portion of the produced wax ester into the growth media. For example, the ratio of the amount of wax ester produced to the amount of wax ester secreted can be less than about 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1 or 1:1. For example, the ratio of the amount of wax ester produced to the amount of wax ester secreted can be less than about 5:1, 4:1, 3:1, 2:1 or 1:1. The recombinant host cell may express an exogenous transmembrane transporter such as, but not limited to, those disclosed herein (*e.g*, an ATP-binding cassette (ABC) transporter, multidrug efflux protein, or an RND pump) to facilitate wax ester secretion. Expression of a transporter protein may increase the amount of a wax ester released from the recombinant host cell and/or may increase production of a wax ester by the recombinant host cell. Secretion of the wax ester may in some examples be regulatable, and in some examples secretion of the wax ester may be inducible.

[00194] The method can further comprise the step of isolating the produced wax ester. For example, wax esters can be recovered from the culture medium by recovery means known to those of ordinary skill in the art, such as by whole culture extraction, *e.g.*, using immiscible (*e.g.*, organic) solvents. Additionally or alternatively, particulate adsorbents can be employed. These may include, *e.g.*, lipophilic particulates and/or ion exchange resins, depending on the design of the recovery method. The particulate absorbents may circulate in the separated medium and then undergo collection, and/or the medium may be passed over a fixed bed column, for example a chromatographic column, containing the particulates. The wax esters can then be eluted from the particulate adsorbents, *e.g.* by the use of an appropriate solvent. The solvent may then be evaporated, followed by further processing of the isolated lipids to yield chemicals and/or fuels that can be used for a variety of purposes. Isolation of the wax ester may in some examples

occur simultaneously with wax ester production. In some examples, isolation of the wax ester may be continuous.

[00195] Alternatively or in addition, recovery of wax esters may be enhanced by homogenization of the host cells (via, *e.g.*, heat, treatment with an acid or base, treatment with enzymes, osmotic shock, mechanical disruption, sonication, freeze-thaw, *etc.*). In some examples, material containing cells or cell fractions can be treated with proteases to degrade contaminating proteins. After digestion, the lipids may be purified from residual proteins, peptide fragments and amino acids, *e.g.*, by centrifugation and/or filtration. The recovery method can be adapted to efficiently recover only the released wax esters, only the wax esters produced and stored within the cells, or both the stored and released wax esters.

[00196] The methods of the invention may result in the production of at least 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 mg/L of one or more wax esters over a culture period of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days. For example, the recombinant host cell can produce at least 1, 2, 5 or 10 mg/L of wax ester. In some examples, the methods of the invention can produce at least 1-5 mg/L of wax ester over a seven day culture period. Alternatively or in addition, the recombinant host cell comprising nucleic acid sequences encoding a) a thioesterase, b) an acyl-CoA synthetase, c) an alcohol-forming fatty acyl reductase, and d) a wax ester synthase can produce an increased level (*e.g.*, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% more) of wax ester relative to a control host cell lacking the nucleic acid sequences. For example, the recombinant host cell comprising nucleic acid sequences encoding a) an acyl-ACP thioesterase, b) an acyl-CoA synthetase, c) an alcohol-forming acyl-CoA reductase, and d) a wax ester synthase can produce an increased level (*e.g.*, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% more) of wax ester relative to a control host cell lacking the nucleic acid sequences.

[00197] Alternatively in addition to any of the above, the recombinant host cell comprising nucleic acid sequences encoding a) a thioesterase, b) an acyl-CoA synthetase, c) an alcohol-forming fatty acyl reductase, and d) a wax ester synthase can produce at least 0.5 milligrams per liter of wax esters in a period of seven days, for example, at least 1 mg/L, 2 mg/L, 5 mg/L or 10 mg/L of wax esters in a period of seven days, or an average of at least 0.1 mg/L, 0.2 mg/L, 0.5

mg/L, 1 mg/L or 2 mg/L of wax esters per day for a culture period of from about one day to about thirty days, or between about 0.5 milligrams per liter and about 500 milligrams per liter, or between about 1 mg/L and about 250 mg/L, or between about 1 mg/L and about 100 mg/L, or between about 2 mg/L and about 200 mg/L, or between about 2 mg/L and about 25 mg/L, or between about 5 mg/L and about 100 mg/L, or between about 2 mg/L and about 50 mg/L, or between about 2 mg/L and about 25 mg/L, or between about 5 mg/L and about 25 mg/L, or between about 5 mg/L and about 50 mg/L, or between about 10 mg/L and about 50 mg/L, or between about 10 mg/L and about 100 mg/L of wax esters per day for a culture period of from about one day to about thirty days.

[00198] Wax esters typically comprise an A chain derived from a fatty alcohol and a B chain derived from acyl-CoA (see, *e.g.*, Fig. 6). Using the four-gene wax ester synthesis reagents and methods of the invention, both the A chain and the B chain of a wax ester can be produced without the addition of substrate fatty acids or fatty acid derivatives (e.g., fatty alcohols).

[00199] The methods of the invention can produce wax esters comprising at least one wax ester molecule wherein both the A chain and the B chain have chain lengths of C8-C24. For example, both the A chain and the B chain can have chain lengths of C12-C18. The A and/or B chain of a wax ester in the wax ester can comprise, *e.g.*, C6, C8, C10, C12, C14, C16, C18, C20, C22 or C24 fatty alcohol molecules, in any combination. For example, at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 97% or at least 99% by weight of the total produced wax esters are wax esters comprising C8 to C24 A and/or B chains. For example, at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 97% or at least 99% by weight of the total produced wax esters can comprise C12 to C20 A and/or B chains. For example, at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 97% or at least 99% by weight of the total produced wax esters can comprise C12 to C18 A and/or B chains. In preferred examples, both the A and B chains of a wax ester produced by the methods of the invention have chain lengths of C8-C24. In further preferred examples, both the A and B chains of a wax ester produced by the methods of the invention have chain lengths of C12-C18.

[00200] The A and B chains of the wax esters produced by the methods of the invention may comprise straight chain, branched chain and/or cyclic chains, and may comprise saturated, monounsaturated and/or polyunsaturated chains. It is understood that a reference to a "Cx fatty acid" includes both saturated and unsaturated fatty acids having "x" carbon atoms, and that a

reference to a "Cx fatty alcohol" includes both saturated and unsaturated fatty alcohols having "x" carbon atoms.

[00201] The invention also provides a composition comprising a wax ester isolated according to the methods of the invention. Wax esters as described herein can be used as components of fuel compositions. Wax esters are produced by the methods provided herein can include one or more wax esters having both an A chain and a B chain with chain lengths of C8-C24. For example, a wax ester composition can comprise at least one wax ester molecule produced by a method disclosed herein that has both an A chain and a B chain of C12-C18. Compositions of the invention may, according to certain aspects, comprise a mixture of different wax esters where the mixture comprises different wax esters in similar poportions (for example, within +/- 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20%) to those produced by a recombinant host cell of the invention. Additionally or alternatively, a wax ester composition of the invention may, according to certain aspects, be identifiable as having been produced according to a method of the invention by detection of a minor impurity in the composition which identifies its source from a recombinant host cell of the invention. For example, the composition may contain one or more nucleci acid molecules as a minor component which my be detected for example, by polymerase chain reaction (PCR) or by an alternative sequence-specific nucleic acid amplification detection method, where the nucelcie acid molecules may comprise a sequence encoding a portion of one or more sequences corresponding to any of SEQ ID NOS: 1-30.

[00202] Methods of the invention as described herein may be carried out using a variety of nucleic acid molecules, vectors, polypeptides, host cells, and/or systems (*e.g.*, those described herein).

Systems

[00203] The invention also provides a system for producing a wax ester, *e.g.*, in a four-gene wax ester synthesis pathway. The system can comprise a recombinant host cell that comprises a nucleic acid sequence encoding a) a thioesterase, b) a nucleic acid sequence encoding an acyl-CoA synthetase, c) a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase, and/or d) a nucleic acid sequence encoding a wax ester synthase, wherein any of the sequences may optionally be non-native. The system can comprise a prokaryotic and/or photosynthetic host microorganism.

[00204] The recombinant host cell may be, *e.g.*, any of the recombinant host cells described herein and may comprise any of the nucleic acid sequences described herein. For example, the recombinant host cell can be a recombinant photosynthetic microorganism and can be cultured in a medium that does not include a substantial amount of a reduced carbon source. In such examples, the recombinant photosynthetic microorganism is preferably exposed to light for at least a portion of the production period.

[00205] In some aspects, the systems of the invention can produce at least 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 mg/L of wax ester over a culture period of up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days by culturing recombinant host cells described herein. In some aspects, the systems of the invention can produce at least 1-100, 1-50, 1-20, 1-10, or 1-5 mg/L of wax ester over a culture period of up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days by culturing recombinant host cells as described herein.

[00206] Systems of the invention as described herein may use a variety of nucleic acid molecules, vectors, polypeptides and/or host cells. In some aspects, the systems use one or more nucleic acid molecules, vectors, polypeptides and/or host cells described herein. Further, the systems may be used to perform any of the methods for producing a wax ester described herein.

[00207] Additionally or alternatively, the present invention can include one or more of the following embodiments.

Embodiments

[00208] Embodiment 1: An isolated or recombinant nucleic acid molecule comprising a nucleic acid sequence encoding a wax ester synthase and at least one of (a) a nucleic acid sequence encoding an acyl-ACP thioesterase, an acyl-CoA thioesterase, or a hydroxybenzoylthioesterase; (b) a nucleic acid sequence encoding an acyl-CoA synthetase; and (c) a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase; wherein the nucleic acid sequence encoding a wax ester synthase and at least one of the nucleic acid sequences encoding a thioesterase, an acyl-CoA synthetase, or an alcohol-forming fatty acyl reductase are configured as a single transcriptional unit; and optionally wherein the nucleic acid molecule further comprises at least one nucleic acid sequence of at least 50, 100, 200, 300, 400,

500, 600, 700, 800, 900, 1000, or 1500 nucleotides derived from the genome of a prokaryotic and/or photosynthetic microorganism.

[00209] Embodiment 2: The isolated or recombinant nucleic acid molecule according to embodiment 1, comprising: (a) a nucleic acid sequence encoding an acyl-ACP thioesterase; (b) a nucleic acid sequence encoding an acyl-CoA synthetase; (c) a nucleic acid sequence encoding an alcohol-forming acyl-CoA reductase; and (d) a nucleic acid sequence encoding a wax ester synthase wherein (a), (b), (c), and (d) are configured as a single transcriptional unit.

Embodiment 3: The isolated or recombinant nucleic acid molecule according to embodiment 1 or 2, wherein: (a) is a nucleic acid sequence encoding an acyl-ACP thioesterase derived from a plant species, optionally a *Cuphea* species or an *Eleais* species, and/or is selected from the group consisting of an acyl-ACP thioesterase of: *Cuphea wrightii* (GenBank Accession AAC49784), *Cuphea lanceolata* (GenBank Accession CAA54060), *Cuphea palustris*, (GenBank Accessions AAC49783; AAC49179); *Cuphea hookeriana* (GenBank Accessions AAC72882; AAC49269; AAC72881; AAC72883), *Cuphea calophylla* (GenBank Accession ABB71580), *Arabidopsis* (GenBank Accessions XP_002885681; NP_172327); *Arachis hypogaea* (GenBank Accession ABO38556); *Brassica* (GenBank Accession CAA52069.1), *Camellia oleifera* (GenBank Accession ACQ57189); *Cinnamonum camphorum* (GenBank Accession AAC49151); *Cocos nucifera* (GenBank Accessions AEM72519; AEM72520; AEM72521); *Glycine max* (GenBank Accession ABD91726); *Garcinia mangostana* (GenBank Accession AAB51525); *Gossypium hirsutum* (GenBank Accession AAD01982); *Helianthus annuus* (GenBank Accession AAQ08226); *Jatropha curcas* (GenBank Accession ABU96744); *Macadamia tetraphylla* (GenBank Accession ADA79524); *Elaeis oleifera* (GenBank Accession AAM09524); *Elaeis guineensis* (GenBank Accession AAD42220); *Oryza sativa* (GenBank Accession BAA83582); *Populus tomentosa* (GenBank Accession ABC47311); *Umbellularia californica* (GenBank Accession AAC49001); *Ulmus Americana* (GenBank Accession AAB71731); *Zea mays* (GenBank Accession ACG41291), *Cuphea carthagenensis* cc1FatB1 (SEQ ID NO:1 or SEQ ID NO:2), *Cuphea decandra* Cd1FatB1 (SEQ ID NO:3 or SEQ ID NO:4), *Cuphea paucipetala* Cp1FatB1 (SEQ ID NO:5 or SEQ ID NO:6), *Elaeis guineensis* thioesterase (SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:9) and *Physcomitrella patens* (GenBank Accession XP 001770108); wherein (b) is a nucleic acid sequence encoding an acyl-CoA synthetase selected from the group consisting of: *E. coli* FadD (GenBank Accession NP_416319), *E. coli* FadK

(GenBank Accession NP_416216), or the acyl-CoA synthetase of *Vibrio splendidus* (GenBank Accession EGU44230), *Marinobacter adhaerens* HP15 (GenBank Accession ADP96803), *Acinetobacter* sp. ADP1 (fadD, GenBank Accession YP_045024), *Haemophilus influenza* RdKW20 (fadD, GenBank Accession NP_438551), *Bacillus halodurans* C-125 BH3103 (GenBank Accession NP_243969), *Bacillus subtilis* yhFl (GenBank Accession NP_388908), *Pseudomonas fluorescens* Pfo-1 Pfl-4354 (GenBank Accession YP_350082), *Comamonas testosteroni* KF-1 EAV15023 (GenBank Accession ZP_01520072), *Pseudomonas aeruginosa* fadD1 (GenBank Accession NP_251989), *Pseudomonas aeurginosa* PAO1 fadD2 (GenBank Accession NP_251990), *Rhizobium etli* CFN42 fadD (GenBank Accession YP_468026), *Rhodopseudomo nas palustris* Bis B18 RPC_4074 (GenBank Accession YP_533919), *Rasltonia Solanacearum* GM1 1000 fadD1 (GenBank Accession NP_520978), *Mycobacterium tuberculosis* H37Rv fadDD35 (GenBank Accession NP_217021), *Mycobacterium tuberculosis* H37Rv fadDD22 (GenBank Accession NP_217464), *Stenotrophomon as Maltophilia* R551-3 PRK0059 (GenBank Accession ZP_01644857), *Saccharomyces cerevisiae* (Faa2p, GenBank Accession NP_010931), *Saccharomyces cerevisiae* (SCRG_04483, GenBank Accession EDV08843), *Yarrowia lipolytica* (GenBank Accession CAG77892), *Brassica napus* (GenBank Accession CAC19877), *Arabidopsis thaliana* (GenBank Accession AEE74324), *Glycine max* (GenBank Accession XP_003524920), *Chlamydomonas reinhardtii* (GenBank Accession XP_001693692), *Nannochloropsis oculata* (*e.g.*, GenBank Accession ADP09391), or *Chlorella variabilis* (*e.g.*, GenBank Accession EFN56588). *Apis mellifera*, (GenBank Accession NP_001193902) and *Mus musculus* (GenBank Accession EDL17174); wherein (c) is a nucleic acid sequence encoding an alcohol-forming acyl-CoA reductase selected from the group consisting of: *Marinobacter aquaeolei* VT8 Maqu_2220 (SEQ ID NO: 15, GenBank Accession YP_959486), *Marinobacter algicola* DG893 (GenBank Accession ZP_01892457); *Hahella chejuensis* KCTC 2396 HCH_05075; SEQ ID NO: 20, GenBank Accession YP_436183); *Oceanobacter sp.* RED65 (GenBank Accession ZP_01305629), *Marinobacter aquaeoli* VT8 2220 Maqu_2507 gene (SEQ ID NO:19, GenBank Accession ABM19582), *Bombyx mmori* (GenBank Accession BAC79426), *Simmondsia chinensis* (SEQ ID NO: 21, GenBank Accession AAD38039), *Triticum aestivum* (GenBank Accession CAD30694 or CAD30692), *Mus musculus* (GenBank Accession NP_081655), *Mus musculus* (GenBank Accession NP_848912), *H. sapiens* (GenBank Accession NP_115604), *Ostrinia scapulalis* (SEQ ID NO: 18, GenBank Accession

ACJ06520), *Z. mays* (GenBank Accession NP_001151388 or EU970865), *Arabidopsis thaliana* (GenBank Accession NP_187805), *Arabidopsis thaliana* FAR4 (GenBank Accession NP_001030809 or NP_190040), *Arabidopsis thaliana* FAR6 (SEQ ID NO: 16, SEQ ID NO: 17, GenBank Accession 67633703), *Arabidopsis thaliana* CER4 (GenBank Accession NP_567936) or *Arabidopsis thaliana* (GenBank Accession NP567936) *Yponomeuta evonymellus* (GenBank Accession GQ907231-GQ907233), *Yponomeuta rorellus* (GenBank Accession GQ907234), *Yponomeuta padellus* (GenBank Accession GQ907235), *Ostrinia nubilalis* (GenBank Accession FJ807735), and *Homo sapiens* (GenBank Accession AAT42129); and/or wherein (d) is a nucleic acid sequence encoding a wax ester synthase selected from the group consisting of: *Marinobacter hydrocarbonoclasticus* WS1 (GenBank Accession ABO21020), *M. hydrocarbonoclasticus* DSM 8798 WS2 (GenBank Accession ABO21021), *M.* sp. ELB 17 (GenBank Accession EBA00388), *M. aquaeolei* Maqu_0168 WS (GenBank Accession YP_957462), *M. adhaerens* HP15 WS (ADP99639), Hahella chejuensis KCTC 2396 (GenBank Accession YP_432512), *Acinetobacter baumannii* wax ester synthase (GenBank Accession EGJ63408), *A. calcoaceticus* WS/DGAT (GenBank Accession ZP_06058985) *Acinetobacter baylyi* ADP1 wax ester synthase (GenBank Accession AAO17391 or Q8GGG1), *Bradyrhizobium japonicum* USDA 110 (GenBank Accession NP_769520), *Erythrobacter litoralis* HTCC 2594 (GenBank Accession YP_457389), *Rhodococcus* opacus wax ester synthase (GenBank Accession BAH53702), *Mycobacterium tuberculosis* wax ester synthase (GenBank Accession NP_334638), *M. smegmatis* wax ester synthase (GenBank Accession ABK74273), the "WS/DGAT/MGAT" subfamily proteins of *Alcanivorax* species (GenBank Accessions CAL17252; EDX90960; EDX89052; ZP_05043539; ZP_05041631), wsadpl from *Nocardia farcinica* IFM 10152 (GenBank Accession YP_117375), *Photobacterium profundum* SS9 (GenBank Accession YP_130413), *Rhodoferax ferrireducens* DSM 15236 (GenBank Accession ZP_00691704), and *Salinibacter ruber* DSM 13855 (GenBank Accession YP_446603), JjWS (GenBank Accession AF149919), *Euglena gracilis* wax ester synthase (GenBank Accession ADI60058), *Arabidiopsis thaliana* WSD1 O-acyltransferase (GenBank Accession NP_568547), *Arabidiopsis thaliana* GPAT acyltransferase (GenBank Accession NP_174499), the putative long-chain-alcohol O-fatty-acyltransferase 4 of *Arabidiopsis thaliana* (GenBank Accession NP_200346) *Murraya koenigii* wax ester synthase, acyl-CoA wax alcohol acyltransferase 2 from *H. sapiens* (GenBank Accession NP_001002254), mWS from *Mus*

musculus (GenBank Accession Q6E1M8), SAAT from *Fragaria xananas* (GenBank Accession AAG13130), the membrane bound O-acyltransferase (MBOAT) of *Zea mays* (GenBank Accession NP_001131179), and mdAAT2 from *Malus x domestica* (GenBank Accession AAS79797).

[00210] Embodiment 4: The nucleic acid molecule according to any of claims 1-3, wherein: (a) the nucleic acid sequence encoding an acyl-ACP thioesterase encodes an acyl-ACP thioesterase having sequence identity of at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or or sequence identity of 100% to the amino acid sequence of any one of SEQ ID NOS: 1-9, or to a functional fragment thereof; (b) the nucleic acid sequence encoding an acyl-CoA synthetase encodes an acyl-CoA synthetase having a sequence identity of at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or sequence identity of 100% to the amino acid sequence of any one of SEQ ID NOS: 10-14, or to a functional fragment thereof; (c) the nucleic acid sequence encoding an alcohol-forming acyl-CoA reductase encodes an alcohol-forming acyl-CoA reductase having sequence identity of at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or sequence identity of 100% to the amino acid sequence of any one of 15-21, or to a functional fragment thereof; and (d) the nucleic acid sequence encoding a wax ester synthase encodes a wax ester synthase having sequence identity of at least 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% to the nucleotide sequence of any one of SEQ ID NOS: 22-30, or to a functional fragment thereof.

[00211] Embodiment 5: The nucleic acid molecule according to any one of embodiments 2-4, wherein the nucleic acid sequences are in an order selected from:

(a) acyl-ACP thioesterase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - wax ester synthase;

(b) acyl-ACP thioesterase - acyl-CoA synthetase - wax ester synthase - alcohol-forming acyl-CoA reductase;

(c) acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - wax ester synthase;

(d) acyl-ACP thioesterase - wax ester synthase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase;

(e) acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-CoA synthetase;

(f) acyl-ACP thioesterase - wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase;

(g) wax ester synthase - acyl-ACP thioesterase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase;

(h) wax ester synthase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase;

(i) wax ester synthase - acyl-CoA synthetase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase;

(j) wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - acyl-CoA synthetase;

(k) wax ester synthase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase;

(l) wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - acyl-ACP thioesterase;

(m) alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-ACP thioesterase - acyl-CoA synthetase;

(n) alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-CoA synthetase - acyl-ACP thioesterase;

(o) alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - wax ester synthase - acyl-CoA synthetase;

(p) alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - wax ester synthase - acyl-ACP thioesterase;

(q) alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - acyl-CoA synthetase - wax ester synthase;

(r) alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - acyl-ACP thioesterase - wax ester synthase;

(s) acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-ACP thioesterase;

(t) acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - wax ester synthase;

(u) acyl-CoA synthetase - wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase;

(v) acyl-CoA synthetase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - wax ester synthase;

(w) acyl-CoA synthetase - wax ester synthase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase; or

(x) acyl-CoA synthetase - acyl-ACP thioesterase - wax ester synthase - alcohol-forming acyl-CoA reductase.

[00212] Embodiment 6: The nucleic acid molecule according to any of the previous embodiments, wherein one or more, and optionally all, of the nucleic acid sequences encoding the thioesterase, the nucleic acid sequence encoding the acyl-CoA synthetase, the nucleic acid sequence encoding the alcohol-forming fatty acyl reductase, and the nucleic acid sequence encoding the wax ester synthase comprise an initiation codon, and

further optionally wherein one or more, and optionally all, of the nucleic acid sequences encoding the thioesterase, the nucleic acid sequence encoding the acyl-CoA synthetase, the nucleic acid sequence encoding the alcohol-forming fatty acyl reductase, and the nucleic acid sequence encoding the wax ester synthase comprise a heterologous translational regulatory sequence upstream of the initiation codon.

[00213] Embodiment 7: The nucleic acid molecule according to any of embodiments 1-6, wherein the nucleic acid sequence encoding the wax ester synthase and one or more of the nucleic acid sequence encoding the thioesterase, the nucleic acid sequence encoding acyl-CoA synthetase, and the nucleic acid sequence encoding the alcohol-forming fatty acyl reductase, are in an operon, optionally wherein the nucleic acid sequences are operably linked to a promoter derived from a prokaryotic and/or photosynthetic microorganism.

[00214] Embodiment 8: The nucleic acid molecule according to any of embodiments 1-7, wherein the nucleic acid molecule comprises a nucleic acid sequence derived from the genome of a prokaryotic and/or photosynthetic microorganism comprising a sequence derived from the 5'

region of a gene, optionally wherein the sequence derived from the the 5' region of a gene comprises at least a portion of a promoter; preferably further wherein the nucleic acid sequence derived from the 5' region of a gene of a prokaryotic and/or photosynthetic microorganism is positioned 5' of the transcriptional unit; and further optionally wherein nucleic acid sequence (d) and one or more of nucleic acid sequences (a), (b), and (c), and preferably all of nucleic acid sequences (a), (b), (c), and (d), are configured as an operon, optionally wherein the 5' region of the gene of a prokaryotic and/or photosynthetic microorganism comprises the promoter of the operon.

[00215] Embodiment 9: The nucleic acid molecule according to any of embodiments 1-6, wherein the nucleic acid molecule does not comprise a promoter sequence operably linked to any of the nucleic acid sequences.

[00216] Embodiment 10: A vector comprising the nucleic acid molecule of any of the previous embodiments, wherein the vector optionally comprises: (a) one or more of a selectable marker, optionally a selectable marker conferring resistance to an antibiotic or herbicide; and/or (b) an origin of replication for propagation in a cloning strain, optionally wherein the cloning strain, optionally wherein the cloning strain is yeast or *E.coli*.

[00217] Embodiment 11: A recombinant host cell comprising any of the nucleic acid molecules or vectors of embodiments 1-10.

Embodiment 12: A recombinant host cell according to embodiment 11, wherein the nucleic acid molecule is integrated into the genome of the recombinant host cell, optionally wherein the nucleic acid molecule is integrated into a genomic site within or adjacent to the 5' region of a gene endogenous to the recombinant host cell, further optionally wherein the 5' region includes a promoter operably linked to one or more, and preferably all, of nucleic acid sequences a) – d) of the nucleic acid molecule, and further optionally wherein the nucleic acid molecule inactivates the endogenous gene, for example, a gene that encodes a oxidoreductase or a polypeptide that participates in carbohydrate, starch, or polyhydroxyalkanoate synthesis.

[00218] Embodiment 13: A recombinant host cell according to embodiment 11 or 12, wherein the recombinant host cell is a prokaryote, a photosynthetic microorganism, or a cyanobacterium, or an *Agmenellum, Anabaena, Anabaenopsis, Anacystis, Aphanizomenon, Arthrospira, Asterocapsa, Borzia, Calothrix, Chamaesiphon, Chlorogloeopsis, Chroococcidiopsis, Chroococcus, Crinalium, Cyanobacterium, Cyanobium, Cyanocystis,*

Cyanospira, Cyanothece, Cylindrospermopsis, Cylindrospermum, Dactylococcopsis, Dermocarpella, Fischerella, Fremyella, Geitleria, Geitlerinema, Gloeobacter, Gloeocapsa, Gloeothece, Halospirulina, Iyengariella, Leptolyngbya, Limnothrix, Lyngbya, Microcoleus, Microcystis, Myxosarcina, Nodularia, Nostoc, Nostochopsis, Oscillatoria, Phormidium, Planktothrix, Pleurocapsa, Prochlorococcus, Prochloron, Prochlorothrix, Pseudanabaena, Rivularia, Schizothrix, Scytonema, Spirulina, Stanieria, Starria, Stigonema, Symploca, Synechococcus, Synechocystis, Thermosynechococcus, Tolypothrix, Trichodesmium, Tychonema, or *Xenococcus* species.

[00219] Embodiment 14: A recombinant host cell according to embodiment 13, wherein any of the following are satisfied: the transcriptional unit is operably linked to the 5' region of an oxidoreductase gene, a dehydrogenase gene, a glycogen synthase gene, a glucose-1-phosphate adenylyltransferase gene, a glycogen branching enzyme gene; the transcriptional unit is operably linked to a promoter endogenous to the recombinant host cell; the recombinant host cell is a species of *Synechocystis* or *Synechococcus (*optionally wherein any or any combination of the following are satisfied: the recombinant host cell is *Synechocystis* sp. PCC 6803, the nucleic acid molecule is integrated at the RS1 site; the transcriptional unit is operably linked to the 5' region of the slr0338 (NCBI protein accession number BAA10046; gi:1001423) gene, the transcriptional unit is operably linked to the promoter of the slr0338 gene).

[00220] Embodiment 15: A recombinant host cell according to any of embodiments 11-14, wherein any of the following are satisfied: the recombinant host cell produces an increased level of wax ester relative to a control host cell lacking one or more of the nucleic acid sequences a)-d); the recombinant host cell produces an increased level of wax ester relative to a control host cell lacking (d) a nucleic acid sequence encoding a wax ester synthase; the recombinant host cell produces at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mg/L of wax ester over a period of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days; the one or more wax esters comprise at least one wax ester molecule having an A chain derived from a fatty alcohol and a B chain derived from acyl-CoA, wherein both the A chain and the B chain have chain lengths of C8-C24, or chain lengths of C12-C18; and wherein at least a portion of the produced wax ester is secreted by the host cell.

[00221] Embodiment 16: The recombinant host cell according to any of embodiments 11-15, wherein any of the following are satisfied: acyl-ACP production is upregulated in the recombinant host cell; the recombinant host cell expresses or produces at least one exogenous

polypeptide; the recombinant host cell overexpresses or overproduces at least one endogenous polypeptide, optionally selected from the group consisting of a beta-ketoacyl synthetase, an acetyl-CoA carboxylase, a malonyl CoA:ACP transacylase, an acyl-ACP synthetase, ribulose 1,5-bisphosphate carboxylase, a phycobiliprotein, acyl carrier protein, and a transmembrane transporter.

[00222] Embodiment 17: A method for producing one or more wax esters, comprising the steps of: a)culturing a recombinant host cell according to any of embodiments 13-19 in a suitable culture medium; and b) allowing expression of the nucleic acid sequences encoding the thioesterase (*e.g.*, an acyl-ACP thioesterase), the acyl-CoA synthetase, the alcohol-forming fatty acyl reductase (*e.g.*, an alcohol-forming acyl-CoA reductase), and the wax ester synthase, wherein the expression of the nucleic acid sequences results in the production of one or more wax esters; optionally wherein the suitable culture medium does not comprise a substantial amount of a reduced carbon source and/or does not include an alcohol or a fatty acid.

[00223] Embodiment 18: The method according to embodiment 17, wherein one or any combination of the following is satisfied: the recombinant host cell produces an increased level of wax ester relative to a control host cell lacking one or more of the nucleic acid sequences; the recombinant host cell produces an increased level of wax ester relative to a control host cell lacking (d) a nucleic acid sequence encoding a wax ester synthase; the recombinant host cell produces at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mg/L of wax ester over a period of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days; the recombinant host cell produces at least 1-5 mg/L of wax ester over a period of 7 days; wherein the one or more wax esters comprise at least one wax ester molecule having an A chain derived from a fatty alcohol and a B chain derived from acyl-CoA, wherein both the A chain and the B chain have chain lengths of C8-C24; wherein the one or more wax esters comprise at least one wax ester molecule having an A chain derived from a fatty alcohol and a B chain derived from acyl-CoA, wherein both the A chain and the B chain have chain lengths of C12-C18; wherein at least a portion of the produced wax ester is secreted by the host cell.

[00224] Embodiment 19: The method according to embodiment 17 or 18, wherein any of the following are satisfied: acyl-ACP production is upregulated in the recombinant host cell; the recombinant host cell expresses or produces at least one exogenous polypeptide, or overexpresses or overproduces at least one endogenous polypeptide, selected from a beta-

ketoacyl synthetase, an acetyl-CoA carboxylase, a malonyl CoA:ACP transacylase, an acyl-ACP synthetase, ribulose 1,5-bisphosphate carboxylase, a phycobiliprotein, acyl carrier protein, and a transmembrane transporter.

[00225] Embodiment 20: The method according to any of embodiments 17-19, further comprising the step of isolating the produced wax ester.

[00226] Embodiment 21: A composition comprising the wax ester isolated according to the method of embodiment 22, optionally wherein at least 70%, at least 80%, at least 85%, at least 90%, or at least 95% of the wax esters have both A chain and the B chain chain lengths of C8-C24 or both A chain and the B chain chain lengths of C12-C18; optionally wherein the composition comprises at least one detectable nucleic acid molecule derived from the recombinant host microorganism; optionally wherein the composition comprises at least a detectable amount of a nucleic acid molecule or vector of any of claims 1-10.

Embodiment 24: A system for producing one or more wax esters, comprising a recombinant host cell according to any of embodiments 11-16 cultured in a medium that does not include a reduced carbon source, and wherein the recombinant host cell is exposed to light for at least a portion of the production period, optionally further comprising a non-reduced carbon source, which is preferably CO_2 and/or carbonate.

EXAMPLES

Example 1: Synthesis of Wax Ester Pathway Operons

[00227] To engineer *Synechocystis* for the production of wax esters, the following genes were chemically synthesized by DNA 2.0 in cloning vectors: the *Cuphea carthagenensis* Cc1FatB1 thioesterase gene, codon-optimized for expression in *Synechocystis* (SEQ ID NO: 31); the *Saccharomyces cerevisiae* acyl-CoA synthetase Faa2p gene (SEQ ID NO: 40), the *Marinobacter aquaeolei* 0168 WS gene ("Maqu_0168"; SEQ ID NO: 55), the *Marinobacter* sp. ELB17 MELB17 WS gene ("MELB17_04692"; SEQ ID NO: 54), the *Marinobacter* sp. ELB17 MELB17 WS gene codon-optimized for expression in *Synechocystis* (SEQ ID NO: 53), and the petunia wax synthase gene (SEQ ID NO: 59).

[00228] The genes were then used to create constructs in which the *Cuphea carthagenensis* Cc1FatB1 thioesterase gene (SEQ ID NO: 31), the *Saccharomyces* Faa2p acyl-CoA synthetase gene (SEQ ID NO: 40), and the Maqu_2220 alcohol forming acyl reductase gene (SEQ ID NO:

45) were arranged in tandem along with a wax synthase gene, which was either the petunia wax synthase (WS) gene (SEQ ID NO: 59), *Marinobacter aquaeolei* Maqu_0168 gene (SEQ ID NO: 55), the *Marinobacter* sp. ELB17 MELB17 WS gene (SEQ ID NO: 54), or the *Marinobacter* sp. ELB17 MELB17 WS gene codon-optimized for expression in *Synechocystis* (SEQ ID NO: 53). As controls, constructs were made with only the Cc1FatB1 acyl-ACP thioesterase gene (SEQ ID NO: 31) (single gene construct); with the Cc1FatB1 acyl-ACP thioesterase gene (SEQ ID NO: 31) and the Faa2p acyl-CoA synthetase gene (SEQ ID NO: 40) (two gene operon construct); and with the Cc1FatB1 acyl-ACP thioesterase gene (SEQ ID NO: 31), the Faa2p acyl-CoA synthetase gene (SEQ ID NO: 40), and the Maqu 2220 alcohol-forming reductase gene (SEQ ID NO: 45) (three gene operon construct). The genes were amplified using high temperature polymerases (PCR) with compatible ends to create the constructs of **Table 1**.

[00229] The four gene operon constructs were engineered such that the "rbs" ribosome binding site derived from the TrcE promoter (SEQ ID NO: 68) preceded each of the genes of the operon.

[00230] The genes were cloned without the addition of a promoter in a cloning vector that included "RS1" sites for recombination into the *Synechocystis* genome (see Figs. 7-10) to create constructs 5020, 5021, 5022, 5059, 5060, 5084, 5023, and 5062 (see Table 1). The genes were cloned between the "RS1-up" (SEQ ID NO: 63) and "RS1-down" (SEQ ID NO: 64) *Synechocystis* genomic DNA sequences of the vector. The RS1 landing region of the *Synechocystis* genome, spanning sequences 2298515 to 2300500 (genome sequence Accession number AP012205.1; GI:339272262) and used for homologous recombination, includes the slr0338 gene of the oxidoreductase family (NAD-binding Rossman fold; NCBI protein accession number BAA10046; gi:1001423) and is proximal to slr0168 (hypothetical open reading frame; NCBI protein accession number BAA10047; gi:1001424). The "RS1-up" sequence includes approximately 830 nucleotides of the 5' region of the slr0338, that is, sequence upstream of the coding region of the slr0338 gene, as well as approximately 158 nucleotides of the 5' end of the slr0338 gene. Cloning of a gene downstream of this sequence (as depicted in Figs. 9 and 10 for constructs 5023 (SEQ ID NO: 72) and 5062 (SEQ ID NO: 73)) may allow gene expression sequences from the "RS1-up" genomic sequence to mediate transcription of the transgenes.

[00231] Additionally, constructs were made in which the TrcE promoter (SEQ ID NO: 67) or the Prbc (large subunit ribulose bisphosphate carboxylase) promoter of *Synechococcus elongatus*

(SEQ ID NO: 69) were cloned 5' of the first gene of the operon (see, for example, Fig. 7 depicting the 5109 construct (SEQ ID NO: 70) and Fig. 8 depicting the 5110 construct (SEQ ID NO: 71)).

Table 1. Constructs for Wax Ester Synthesis

Construct	Promoter	Genes, in operon order
5020	(RS1)	cc1FatB1
5021	(RS1)	cc1FatB1 - Faa2p
5022	(RS1)	cc1FatB1 - Faa2p- Maqu2220
5059	(RS1)	cc1FatB1 - Faa2p - Maqu2220 - petuniaWS
5108	trcE/lacIQmut	cc1FatB1 - Faa2p - Maqu2220 - petuniaWS
5109 (SEQ ID NO: 70)	Prbc/sc7942	cc1FatB1 - Faa2p - Maqu2220 - petuniaWS
5060	(RS1)	cc1FatB1 - Faa2p - Maqu2220 - Maqu0168WS
5110 (SEQ ID NO: 71)	trcE/lacIQmut	cc1FatB1 - Faa2p - Maqu2220 - Maqu0168WS
5114	Prbc/sc7942	cc1FatB1 - Faa2p - Maqu2220 - Maqu0168WS
5084	(RS1)	cc1FatB1 - Faa2p - Maqu2220 - Melb17WS
5023 (SEQ ID NO: 72)	(RS1)	cc1FatB1 - Faa2p - Maqu2220 - Melb17WSopt
5062 (SEQ ID NO: 73)	(RS1)	Faa2p - Melb17WSopt - cc1FatB1 - Maqu2220
5064	Prbc/sc7942	cc1FatB1 - Faa2p - Maqu2220 - Melb17WSopt
5065	trcE- no lacIQ	cc1FatB1- Faa2p - Maqu2220 - Melb17WSopt
5066	trcE/lacIQ	cc1FatB1- Faa2p- Maqu2220- Melb17WSopt

[00232] To introduce the one, two, three, and four gene operon constructs into cyanobacteria, *Synechocystis* sp. PCC 6803 cells were cultured in BG-11 media to an OD (730 nm) of about 0.7-0.9. About 10 mL of the culture was spun down at approximately 2000 g for 15 minutes, then the cell pellet was resuspended in 1 mL fresh BG-11 media. An aliquot of 300 µL of cells was transformed with about 100 ng of integration vector. The cells were incubated under lights (80 µE) for about 6 hours, then spread onto Minipore filters and placed on top of BG-11 agar plates containing no antibiotics. The plates were incubated at about 30°C under about 80 µE of light for about 24 hours. The filters were then transferred onto fresh BG-11 1.5% agar plates with 20 µg/mL kanamycin and cultured for 7 days. Colonies of *Synechocystis* sp. PCC 6803 were picked and patched onto new agar plates.

Table 2: ATCC 616 Medium BG-11 for Cyanobacteria

$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 * 7H_2O$	0.075 g
$CaCl_2 * 2H_2O$	0.036 g
Citric acid	6.0 mg
Ferric ammonium citrate	6.0 mg
EDTA	1.0 mg
Na_2CO_3	0.02 g
Trace Metal Mix A5[#]	1.0 ml
Agar (if needed)	(up to) 10.0 g
Distilled water	1.0 L
Trace Metal Mix A5	H_3BO_3 .....2.86 g $MnCl_2 * 4H_2O$ .....1.81 g $ZnSO_4 * 7H_2O$ .....0.22 g $Na_2MoO_4 * 2H_2O$.....0.39 g $CuSO_4 * 5H_2O$ .....0.080 g $Co(NO_3)_2 * 6H_2O$.....49.4 mg Distilled water……..….to 1.0L

Example 2: Lipid Production by *Synechocystis* sp. PCC 6803 strains expressing wax synthesis pathway genes

[00233] Cultures of *Synechocystis* sp. PCC 6803 transformed with the constructs of Table 1 were grown for testing free fatty acid, fatty alcohol, and wax ester production. Three different colony patches for each clone were inoculated into 20 mL glass scintillation vials containing 10 mL of BG-11 liquid media with 50 µg/ml kanamycin. BG-11 medium, which does not include a substantial amount of a reduced carbon source, supports photoautotrophic growth of *Synechocystis*. Cultures were covered with filter floss tape. The scintillation vials were incubated at about 30°C with about 5% ambient CO_2 and continuously shaken at about 200 rpm under about 70 µE of light for 7 days. 5 mL of each culture was then spun down at

approximately 5000 rpm and resuspended in 0.4 mL of water, then extracted by a hexane/sulfuric acid solvent system to extract neutral lipids.

[00234] Approximately 0.5 mL of glass beads were added to the resuspended cells, along with 50 µl of 50% H_2SO4 and 100 µl of 5M NaCl. The cells were subjected to bead beating for 5 min at 1000 rpm, after which 2 mL of hexane were added. The vials were then capped, placed in white ACME racks, and bead-beaten again for 5 min at 1000 rpm. The samples were then shaken on a multi-tube vortexer for 30 min at 1000 rpm, followed by 30 sec at 2500 rpm. The samples were then centrifuged for 4 min at 2000 rpm. Approximately 0.5 ml of the upper hexane layer was transferred to an HPLC vial. Fifty microliters of an internal standards stock was then added to the hexane extract to a final concentration of 100 µg per ml. The internal standards were oleoyl oleate, oleic acid, cholesterol, 1,2-diolein, 1-monolein, 1-octadeconal, and n-eicosane. The vials were capped and vortexed prior to analysis.

[00235] For analysis of fatty acids and wax esters, an Agilent 1200 series HPLC equipped with a binary pump and an ES Industries Chormegasphere SI-60 150 mm x 4.6 mm, 10 µm pore column was used with the following solvent system: [eluent A: hexanes; eluent B: hexanes/isopropanol/ethyl acetate/10% formic acid in isopropanol in a 80:10:10:1 ratio]. A 20 µL injection was used, the flow rate was set to 2 mL/min, the column compartment set to 40°C, and the solvent gradient started at 98% eluent A, 2% eluent B and ramped up to 2% eluent A, 98% eluent B over an 11 minute run. ELSD was set at 30°C, 3.5 bar N2, and a gain of 5 was used. Analytes were quantified via an 8-point calibration curve from 1.5-100 µg/mL.

Example 3: Gas chromatography of *Synechocystis* sp. PCC 6803 expressing wax synthesis operons

[00236] *The same samples* were analyzed by gas chromatography for fatty alcohols. For this analysis, 0.5 mL of the hexanes (upper) layer of the extract were transferred to a 2.0 mL GC vial and 50 µL of internal standard (1 mg/mL 1-pentadecanol in CH_2Cl_2) were added for a final concentration of internal standard of 100 µg/mL. The vials were then vortexed and analyzed by GC/MS-SCAN/SIM. The GC run conditions were as follows: 1.4 mL/min H_2 with an oven temperature of 100°C for 0.5 min, then ramped at 20°C/min to 270°C and held for 1 min. The solvent delay was set at 4.3 min. A 1 µL injection was made on an inlet set at 280°C utilizing a

3:1 split and containing a deactivated single gooseneck liner w/ glass wool. The GC column was an Agilent HP-5MS, 30 m x 0.25 mm x 0.25 µm. The mass spectrometer scan range was set for m/z of 35-275, the SIM ions monitored were 55.0 and 41.0, and a 10 ms dwell time was used. Analytes were quantified via a 5-point calibration curve from 2-200 µg/mL.

[00237] As shown in **Table 3**, expression of all the operons with four gene combinations (acyl-ACP thioesterase, acyl-CoA synthetase, alcohol-forming reductase, and wax synthase) resulted in the production of fatty alcohol and wax esters. The wax ester productivity was not noticeably affected by the specific wax synthase gene. Petunia WS (SEQ ID NO: 55), *M. aquaeolei* Maqu_0168 WS (SEQ ID NO: 55), and the *M.* species ELB17 WS (SEQ ID NO: 54), and codon-optimized *M.* species ELB17 WS (SEQ ID NO: 53) all demonstrated comparable productivity when expressed as part of a *Synechocystis* operon. By contrast, expression of the one, two, and three gene operons (all of which lacked a wax synthase gene) did not produce wax esters (rows 1-3 of Table 3). The particular promoter used to direct expression of the four gene operon (RS1 endogenous promoter, TrcE, or Prbc) also did not affect the amount of wax ester produced, although the stronger promoters (*e.g.*, TrcE, Prbc) did result in a higher level of fatty alcohol, a wax ester precursor, possibly indicating that the activity of the wax synthase gene was limiting. Changing the gene order within the operon did not noticeably improve wax ester production (see plasmid, for example Table 3, construct 5062).

Table 3: Production of lipids by *Synechocystis* strains containing wax ester synthesis operons

Construct	Promoter	genes, in operon order	FFA mg/L	FOH mg/L	WE mg/L
5020	RS1	cc1FatB1	-	-	-
5021	RS1	cc1FatB1-Faa2p	-	-	-
5022	RS1	cc1FatB1-Faa2p-Maqu2220	-	-	-
5059	RS1	cc1FatB1-Faa2p-Maqu2220-petuniawtWS	-	5-10	1-5
5108	trcE/ laclQmut	cc1FatB1-Faa2p-Maqu2220-petuniawtWS	-	10-15	1-5
5109	Prbc/ sc7942	cc1FatB1-Faa2p-Maqu2220-petuniawtWS	1-5	10-15	1-5
5060	RS1	cc1FatB1-Faa2p-Maqu2220-Maqu0168WS	-	5-10	1-5
5110	trcE/ laclQmut	cc1FatB1-Faa2p-Maqu2220- Maqu0168WS	-	15-20	1-5
5114	Prbc/ sc7942	cc1FatB1-Faa2p-Maqu2220- Maqu0168WS	-	10-15	1-5

5084	RS1	cc1FatB1-Faa2p-Maqu2220-Melb17WSwt	-	5-10	1-5
5023	RS1	cc1FatB1-Faa2p-MAqu2220-Melb17WS	-	-	-
5062	RS1	Faa2p-Melb17WS-cc1FatB1-Maqu2220	-	1-5	1-5
5064	Prbc/ sc7942	cc1FatB1-Faa2p-MAqu2220-Melb17WS	-	10-15	1-5
5065	trcE-nolacIQ	cc1FatB1-Faa2p-MAqu2220-Melb17WS	-	10-15	1-5
5066	trcE/ lacIQ	cc1FatB1-Faa2p-MAqu2220-Melb17WS	-	5-10	1-5

Claims

What is claimed is:

1. A nucleic acid molecule comprising:
   a) a nucleic acid sequence encoding a thioesterase;
   b) a nucleic acid sequence encoding an acyl-CoA synthetase;
   c) a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase; and
   d) a nucleic acid sequence encoding a wax ester synthase;
   wherein the nucleic acid sequence encoding a wax ester synthase and at least one of the nucleic acid sequences encoding a thioesterase, an acyl-CoA synthetase, and an alcohol-forming fatty acyl reductase are configured as a transcriptional unit.

2. A nucleic acid molecule according to claim 1, wherein the nucleic acid sequence encoding a wax ester synthase, the nucleic acid sequence encoding a thioesterase, an the nucleic acid sequence encoding acyl-CoA synthetase, and the nucleic acid sequence encoding an alcohol-forming fatty acyl reductase are configured as a transcriptional unit.

3. A nucleic acid molecule according to claim 1, wherein the nucleic acid molecule further comprises an additional nucleic acid sequence of at least 50 nucleotides from a photosynthetic microorganism.

4. A nucleic acid molecule according to claim 3, wherein the additional nucleic acid sequence comprises at least 100 nucleotides from a photosynthetic microorganism.

5. A nucleic acid molecule according to claim 3, wherein the additional nucleic acid sequence from a photosynthetic microorganism comprises at least a portion of a promoter.

6. A nucleic acid molecule according to claim 1, wherein the nucleic acid sequences are, respectively:
   a) a nucleic acid sequence encoding an acyl-ACP thioesterase;
   b) a nucleic acid sequence encoding an acyl-CoA synthetase;
   c) a nucleic acid sequence encoding an alcohol-forming acyl-CoA reductase; and
   d) a nucleic acid sequence encoding a wax ester synthase.

7. A nucleic acid molecule according to claim 6, wherein the nucleic acid sequence of a) encodes an acyl-ACP thioesterase having at least 70% sequence identity to any one of SEQ ID NOS: 1-9, or to a functional fragment thereof.

8. A nucleic acid molecule according to claim 6, wherein the nucleic acid sequence of b) encodes an acyl-CoA synthetase having at least 70% sequence identity to any one of SEQ ID NOS: 10-14, or to a functional fragment thereof.

9. A nucleic acid molecule according to claim 6, wherein the nucleic acid sequence of c) encodes an alcohol-forming acyl-CoA reductase having at least 70% sequence identity to any one of SEQ ID NOS: 15-21, or to a functional fragment thereof.

10. A nucleic acid molecule according to claim 6, wherein the nucleic acid sequence of d) encodes a wax ester synthase having at least 70% sequence identity to any one of SEQ ID NOS: 15-21, or to a functional fragment thereof.

11. A nucleic acid molecule according to claim 6, wherein the nucleic acid sequences respectively comprise the nucleotide sequences of SEQ ID NOS: 31, 40, 45, and 53.

12. A nucleic acid molecule according to claim 6, wherein the nucleic acid sequences of a) – d) are in an order selected from:
   (a) acyl-ACP thioesterase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - wax ester synthase;

(b) acyl-ACP thioesterase - acyl-CoA synthetase - wax ester synthase - alcohol-forming acyl-CoA reductase;

(c) acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - wax ester synthase;

(d) acyl-ACP thioesterase - wax ester synthase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase;

(e) acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-CoA synthetase;

(f) acyl-ACP thioesterase - wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase;

(g) wax ester synthase - acyl-ACP thioesterase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase;

(h) wax ester synthase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase;

(i) wax ester synthase - acyl-CoA synthetase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase;

(j) wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - acyl-CoA synthetase;

(k) wax ester synthase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase;

(l) wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - acyl-ACP thioesterase;

(m) alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-ACP thioesterase - acyl-CoA synthetase;

(n) alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-CoA synthetase - acyl-ACP thioesterase;

(o) alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - wax ester synthase - acyl-CoA synthetase;

(p) alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - wax ester synthase - acyl-ACP thioesterase;

(q) alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - acyl-CoA synthetase - wax ester synthase;

(r) alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - acyl-ACP thioesterase - wax ester synthase;

(s) acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-ACP thioesterase;

(t) acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - wax ester synthase;

(u) acyl-CoA synthetase - wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase;

(v) acyl-CoA synthetase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - wax ester synthase;

(w) acyl-CoA synthetase - wax ester synthase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase; and

(x) acyl-CoA synthetase - acyl-ACP thioesterase - wax ester synthase - alcohol-forming acyl-CoA reductase.

13. A nucleic acid molecule according to claim 2, wherein the nucleic acid molecule does not comprise a promoter operably linked to any of the nucleic acid sequences of a) – d).

14. A nucleic acid molecule according to claim 2, wherein the nucleic acid sequences a) – d) each comprise an initiation codon, further wherein each of nucleic acid sequences b) – d) comprise a heterologous translational regulatory sequence upstream of the initiation codon.

15. A nucleic acid molecule according to claim 3, wherein the additional nucleic acid sequences promote integration of nucleic acid sequences a) – d) into the genome of a photosynthetic microorganism.

16. A nucleic acid molecule according to claim 15, wherein the additional nucleic acid sequences comprise genomic sequences that in the photosynthetic microorganism are positioned immediately upstream of the coding region of a gene, wherein the genomic sequences are positioned immediately upstream of sequences a) – d) of the nucleic acid molecule.

17. A vector comprising a nucleic acid molecule according to claim 2.

18. A vector according to claim 17, wherein the vector includes at least one selectable marker.

19. A vector according to claim 17, wherein the vector does not comprise a promoter operably linked to any of nucleic acid sequences a) – d).

20. A recombinant photosynthetic microorganism that comprises a nucleic acid molecule according to claim 1, wherein the recombinant photosynthetic microorganism produces a wax ester.

21. A recombinant microorganism according to claim 20, wherein the nucleic acid sequence encoding a wax ester synthase, the nucleic acid sequence encoding a thioesterase, the nucleic acid sequence encoding acyl-CoA synthetase, and the nucleic acid sequence encoding an alcohol-forming fatty acyl reductase are configured as a transcriptional unit.

22. The recombinant microorganism according to claim 21, wherein the nucleic acid molecule is integrated into a genomic site of the recombinant microorganism.

23. The recombinant microorganism according to claim 22, wherein integration of the nucleic acid molecule into the genomic site inactivates an endogenous gene.

24. The recombinant microorganism according to claim 23, wherein the genomic site is adjacent to the 5' region of a gene endogenous to the host microorganism

25. The recombinant microorganism according to claim 24, wherein the 5' region includes a promoter, wherein insertion of the nucleic acid molecule results in the promoter becoming operably linked to the transcriptional unit comprising nucleic acid sequences of a) – d).

26. The recombinant microorganism according to claim 20, wherein the recombinant photosynthetic microorganism is a cyanobacterium.

27. The recombinant microorganism according to claim 26, wherein the cyanobacterium is of an *Agmenellum, Anabaena, Anabaenopsis, Anacystis, Aphanizomenon, Arthrospira, Asterocapsa, Borzia, Calothrix, Chamaesiphon, Chlorogloeopsis, Chroococcidiopsis, Chroococcus, Crinalium, Cyanobacterium, Cyanobium, Cyanocystis, Cyanospira, Cyanothece, Cylindrospermopsis, Cylindrospermum, Dactylococcopsis, Dermocarpella, Fischerella, Fremyella, Geitleria, Geitlerinema, Gloeobacter, Gloeocapsa, Gloeothece, Halospirulina, Iyengariella, Leptolyngbya, Limnothrix, Lyngbya, Microcoleus, Microcystis, Myxosarcina, Nodularia, Nostoc, Nostochopsis, Oscillatoria, Phormidium, Planktothrix, Pleurocapsa, Prochlorococcus, Prochloron, Prochlorothrix, Pseudanabaena, Rivularia, Schizothrix, Scytonema, Spirulina, Stanieria, Starria, Stigonema, Symploca, Synechococcus, Synechocystis, Thermosynechococcus, Tolypothrix, Trichodesmium, Tychonema,* or *Xenococcus* species.

28. The recombinant microorganism according to claim 20, wherein the recombinant photosynthetic microorganism is *Synechocystis* sp. PCC 6803.

29. A method for producing one or more wax esters, comprising the steps of:

a) culturing a recombinant photosynthetic microorganism according to claim 22 in a suitable culture medium; and

b) allowing expression of the non-native nucleic acid sequences encoding the acyl-ACP thioesterase, the acyl-CoA synthetase, the alcohol-forming acyl-CoA reductase, and the wax ester synthase,

wherein expression of the non-native nucleic acid sequences results in the production of one or more wax esters.

30. The method according to claim 29, wherein the culture medium does not comprise an alcohol or a fatty acid.

31. The method according to claim 29, wherein the culture medium does not comprise a substantial amount of a reduced carbon source.

32. A method according to claim 29, wherein the recombinant microorganism produces an increased amount of a wax ester relative to a control host cell lacking the non-native nucleic acid sequences.

33. A method according to claim 29, wherein the one or more wax esters comprise at least one wax ester molecule having an A chain derived from a fatty alcohol and a B chain derived from acyl-CoA, wherein both the A chain and the B chain have chain lengths of C12-C18.

34. A method according to claim 29, further comprising the step of isolating a wax ester from the cells, the culture medium, or both.

AMENDED CLAIMS
received by the International Bureau on 6 August 2012 (06.08.2012)

What is claimed is:

1. A recombinant photosynthetic microorganism that comprises a non-native nucleic acid molecule comprising:

a) a nucleic acid sequence encoding a thioesterase;

b) a nucleic acid sequence encoding an acyl-CoA synthetase;

c) a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase; and

d) a nucleic acid sequence encoding a wax ester synthase;

wherein the nucleic acid sequence encoding a wax ester synthase and at least one of the nucleic acid sequence encoding a thioesterase, the nucleic acid sequence encoding an acyl-CoA synthetase, and the nucleic acid sequence encoding an alcohol-forming fatty acyl reductase are configured as a transcriptional unit;

wherein the nucleic acid molecule is integrated into a genomic site of the recombinant microorganism;

further wherein integration of the nucleic acid molecule into the genomic site results in the transcriptional unit becoming operably linked to a promoter endogenous to the photosynthetic microorganism;

wherein the recombinant photosynthetic microorganism produces a wax ester.

2. A recombinant photosynthetic microorganism according to claim 1, wherein integration of the nucleic acid molecule into the genomic site inactivates an endogenous gene.

3. A recombinant photosynthetic microorganism according to claim 2, wherein the endogenous gene encodes an oxidoreductase, a dehydrogenase, a glycogen synthase, or a glucose-1-phosphate adenyltransferase.

4. A recombinant photosynthetic microorganism according to claim 1, wherein the nucleic acid sequence encoding a wax ester synthase, the nucleic acid sequence encoding a thioesterase, the nucleic acid sequence encoding an acyl-CoA synthetase, and the nucleic

acid sequence encoding an alcohol-forming fatty acyl reductase are configured as a transcriptional unit.

5. A recombinant photosynthetic microorganism according to claim 1, wherein the thioesterase is an acyl-ACP thioesterase, an acyl-CoA thioesterase, or a hydroxybenzoyl thioesterase.

6. A recombinant photosynthetic microorganism according to claim 5, wherein the thioesterase is an acyl-ACP thioesterase.

7. A recombinant photosynthetic microorganism according to claim 1, wherein the alcohol-forming fatty acyl reductase is a prokaryotic alcohol-forming fatty acyl reductase.

8. A recombinant photosynthetic microorganism according to claim 1, wherein the wax synthase is a prokaryotic wax synthase.

9. A recombinant photosynthetic microorganism according to claim 1, wherein the recombinant photosynthetic microorganism is a cyanobacterium

10. A recombinant photosynthetic microorganism according to claim 9, wherein the cyanobacterium is of an *Agmenellum, Anabaena, Anabaenopsis, Anacystis, Aphanizomenon, Arthrospira, Asterocapsa, Borzia, Calothrix, Chamaesiphon, Chlorogloeopsis, Chroococcidiopsis, Chroococcus, Crinalium, Cyanobacterium, Cyanobium, Cyanocystis, Cyanospira, Cyanothece, Cylindrospermopsis, Cylindrospermum, Dactylococcopsis, Dermocarpella, Fischerella, Fremyella, Geitleria, Geitlerinema, Gloeobacter, Gloeocapsa, Gloeothece, Halospirulina, Iyengariella, Leptolyngbya, Limnothrix, Lyngbya, Microcoleus, Microcystis, Myxosarcina, Nodularia, Nostoc, Nostochopsis, Oscillatoria, Phormidium, Planktothrix, Pleurocapsa, Prochlorococcus, Prochloron, Prochlorothrix, Pseudanabaena, Rivularia, Schizothrix, Scytonema, Spirulina, Stanieria, Starria, Stigonema, Symploca, Synechococcus, Synechocystis, Thermosynechococcus, Tolypothrix, Trichodesmium, Tychonema,* or *Xenococcus* species.

11. A method for producing one or more wax esters, comprising the steps of:
    a) culturing a recombinant photosynthetic microorganism according to claim 1 in a suitable culture medium; and
    b) allowing expression of the non-native nucleic acid sequences encoding the thioesterase, the acyl-CoA synthetase, the alcohol-forming acyl-CoA reductase, and the wax ester synthase,

wherein expression of the non-native nucleic acid sequences results in the production of one or more wax esters.

12. A method according to claim 11, wherein integration of the nucleic acid molecule into the genomic site of the recombinant photosynthetic microorganism inactivates an endogenous gene.

13. A method according to claim 12, wherein the endogenous gene encodes an oxidoreductase, a dehydrogenase, a glycogen synthase, or a glucose-1-phosphate adenyltransferase.

14. A method according to claim 11, wherein the nucleic acid sequence encoding a wax ester synthase, the nucleic acid sequence encoding a thioesterase, the nucleic acid sequence encoding an acyl-CoA synthetase, and the nucleic acid sequence encoding an alcohol-forming fatty acyl reductase are configured as a transcriptional unit.

15. The method according to claim 11, wherein the culture medium does not comprise an alcohol or a fatty acid.

16. The method according to claim 11, wherein the culture medium does not comprise a substantial amount of a reduced carbon source.

17. A method according to claim 11, wherein the recombinant microorganism produces an increased amount of a wax ester relative to a control host cell lacking the non-native nucleic acid sequences.

18. A method according to claim 11, wherein the one or more wax esters comprise at least one wax ester molecule having an A chain derived from a fatty alcohol and a B chain derived from acyl-CoA, wherein both the A chain and the B chain have chain lengths of C12-C18.

19. A method according to claim 11, further comprising the step of isolating a wax ester from the cells, the culture medium, or both.

20. A nucleic acid molecule comprising:

a) a nucleic acid sequence encoding a thioesterase;

b) a nucleic acid sequence encoding an acyl-CoA synthetase;

c) a nucleic acid sequence encoding an alcohol-forming fatty acyl reductase; and

d) a nucleic acid sequence encoding a wax ester synthase;

wherein the nucleic acid sequence encoding a wax ester synthase and at least one of the nucleic acid sequence encoding a thioesterase, the nucleic acid sequence encoding an acyl-CoA synthetase, and the nucleic acid sequence encoding an alcohol-forming fatty acyl reductase are configured as a transcriptional unit; and further wherein the nucleic acid molecule comprises at least one additional nucleic acid sequence of at least 50 nucleotides derived from a 5' region of a gene from a photosynthetic microorganism.

21. A nucleic acid molecule according to claim 20, wherein the at least one additional nucleic acid sequence comprises at least 100 nucleotides.

22. A nucleic acid molecule according to claim 20, wherein the at least one additional nucleic acid sequence comprises at least a portion of a promoter.

23. A nucleic acid molecule according to claim 20, wherein the nucleic acid sequences are, respectively:

a) a nucleic acid sequence encoding an acyl-ACP thioesterase;

b) a nucleic acid sequence encoding an acyl-CoA synthetase;

c) a nucleic acid sequence encoding an alcohol-forming acyl-CoA reductase; and

d) a nucleic acid sequence encoding a wax ester synthase.

24. A nucleic acid molecule according to claim 23, wherein the nucleic acid sequence of a) encodes an acyl-ACP thioesterase having at least 70% sequence identity to any one of SEQ ID NOS: 1-9, or to a functional fragment thereof.

25. A nucleic acid molecule according to claim 23, wherein the nucleic acid sequence of b) encodes an acyl-CoA synthetase having at least 70% sequence identity to any one of SEQ ID NOS: 10-14, or to a functional fragment thereof.

26. A nucleic acid molecule according to claim 23, wherein the nucleic acid sequence of c) encodes an alcohol-forming acyl-CoA reductase having at least 70% sequence identity to any one of SEQ ID NOS: 15-21, or to a functional fragment thereof.

27. A nucleic acid molecule according to claim 23, wherein the nucleic acid sequence of d) encodes a wax ester synthase having at least 70% sequence identity to any one of SEQ ID NOS: 22-30, or to a functional fragment thereof.

28. A nucleic acid molecule according to claim 23, wherein the nucleic acid sequences respectively comprise the nucleotide sequences of SEQ ID NOS: 31, 40, 45, and 53.

29. A nucleic acid molecule according to claim 23, wherein the nucleic acid sequences of a) – d) are in an order selected from:

(a) acyl-ACP thioesterase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - wax ester synthase;

(b) acyl-ACP thioesterase - acyl-CoA synthetase - wax ester synthase - alcohol-forming acyl-CoA reductase;

(c) acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - wax ester synthase;

(d) acyl-ACP thioesterase - wax ester synthase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase;

(e) acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-CoA synthetase;

(f) acyl-ACP thioesterase - wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase;

(g) wax ester synthase - acyl-ACP thioesterase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase;

(h) wax ester synthase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase;

(i) wax ester synthase - acyl-CoA synthetase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase;

(j) wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - acyl-CoA synthetase;

(k) wax ester synthase - acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase;

(l) wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - acyl-ACP thioesterase;

(m) alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-ACP thioesterase - acyl-CoA synthetase;

(n) alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-CoA synthetase - acyl-ACP thioesterase;

(o) alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - wax ester synthase - acyl-CoA synthetase;

(p) alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - wax ester synthase - acyl-ACP thioesterase;

(q) alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - acyl-CoA synthetase - wax ester synthase;

(r) alcohol-forming acyl-CoA reductase - acyl-CoA synthetase - acyl-ACP thioesterase - wax ester synthase;

(s) acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - wax ester synthase - acyl-ACP thioesterase;

(t) acyl-CoA synthetase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase - wax ester synthase;

(u) acyl-CoA synthetase - wax ester synthase - alcohol-forming acyl-CoA reductase - acyl-ACP thioesterase;

(v) acyl-CoA synthetase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase - wax ester synthase;

(w) acyl-CoA synthetase - wax ester synthase - acyl-ACP thioesterase - alcohol-forming acyl-CoA reductase; and

(x) acyl-CoA synthetase - acyl-ACP thioesterase - wax ester synthase - alcohol-forming acyl-CoA reductase.

30. A nucleic acid molecule according to claim 20, wherein the nucleic acid molecule does not comprise a promoter operably linked to any of the nucleic acid sequences of a) – d).

31. A nucleic acid molecule according to claim 20, wherein the nucleic acid sequences a) – d) each comprise an initiation codon, further wherein each of nucleic acid sequences b) – d) comprise a heterologous translational regulatory sequence upstream of the initiation codon.

32. A nucleic acid molecule according to claim 20, wherein the at least one additional nucleic acid sequence promotes integration of nucleic acid sequences a) – d) into the genome of a photosynthetic microorganism.

33. A nucleic acid molecule according to claim 20, wherein the at least one additional nucleic acid sequence comprises genomic sequences that in the photosynthetic microorganism are positioned immediately upstream of the coding region of a gene.

34. A vector comprising a nucleic acid molecule according to claim 20.

35. A vector according to claim 34, wherein the vector includes at least one selectable marker.

36. A vector according to claim 34, wherein the vector does not comprise a promoter operably linked to any of nucleic acid sequences a) – d).

Thioesterases	
Cc1FatB1: construct sequence	SEQ ID NO: 1
MANGSAVSLKSGSLNTQEDTSSSPPPRAFINQLPDWSMLLTAITTVFVAAEKQWTMLDRKSKRSDMLVDS FGMERIVQDGLVFRQSFSIRSYEIGADRRASIETLMNHLQETSLNHCKSIRLLNEGFGRTPEMCKRDLIW VVTRMHIMVNRYPTWGDTVEINTWVSQSGKNGMGRDWLISDCNTGEILIRATSAWAMMNQKTRRLSKLPY EVSQEIAPHFVDSPPVIEDGDRKLHKFDVKTGDSIRKGLTPRWNDLDVNQHVNNVKYIGWILESMPTEVL ETHELCFLTLEYRRECGRDSVLESVTAMDPSNEGGRSHYQHLLRLEDGTDIVKGRTEWRPKNARNIGAIS TGKTSNGNPAS	
Cc1FatB1: coding sequence from genomic DNA	SEQ ID NO: 2
AASSAFFPVTTPGTSRKPGKFGNWLSSLSPPFRPKSIPSGGFQVKANASAHPKANGSAVSLKSGSLNTQE DTSSSPPPRAFINQLPDWSMLLTAITTVFVAAEKQWTMLDRKSKRSDMLVDSFGMERIVQDGLVFRQSFS IRSYEIGADRRASIETLMNHLQETSLNHCKSIRLLNEGFGRTPEMCKRDLIWVVTRMHIMVNRYPTWGDT VEINTWVSQSGKNGMGRDWLISDCNTGEILIRATSAWAMMNQKTRRLSKLPYEVSQEIAPHFVDSPPVIE DGDRKLHKFDVKTGDSIRKGLTPRWNDLDVNQHVNNVKYIGWILESMPTEVLETHELCFLTLEYRRECGR DSVLESVTAMDPSNEGGRSHYQHLLRLEDGTDIVKGRTEWRPKNARNIGAISTGKTSNGNPAS	
Cd1FatB1: construct sequence	SEQ ID NO: 3
MGINGSSVGLKSGSLKTQEDTPSSPPPRTFINQLPDWSMLLAAITTVFLAAEKQWMMLDWKPKRPDMLVD PFGLGRIVQDGLVFRQNFSIRSYEIGADRTASIETLMNHLQETALNHVKSAGLLNDGFGRTPEMYKRDLI WVVAKMQVMVNRYPTWGDTVEVNTWVAKSGKNGMRRDWLISDCNTGEILTRASSVWVMMNQKTRRLSKIP DEVRHEIEPHFVDSPPVIEDDDRKLPKLDEKTADSIRKGLTPRWNDLDVNQHVNNVKYIGWILESTPQEV LETQELCSLTLEYRRECGRDSVLESLTAVDHSGKGSGSNFQHLLRLEDGGEIVKGRTEWRPKNAVINGAV APGETSPGNSVS	
Cd1FatB1: coding sequence from genomic DNA	SEQ ID NO: 4
AASSACFPVPSPDASRKPGKHGNGASSLSPFKPKSIPSGGLQVQANASAPPKINGSSVGLKSGSLKTQED TPSSPPPRTFINQLPDWSMLLAAITTVFLAAEKQWMMLDWKPKRPDMLVDPFGLGRIVQDGLVFRQNFSI RSYEIGADRTASIETLMNHLQETALNHVKSAGLLNDGFGRTPEMYKRDLIWVVAKMQVMVNRYPTWGDTV EVNTWVAKSGKNGMRRDWLISDCNTGEILTRASSVWVMMNQKTRRLSKIPDEVRHEIEPHFVDSPPVIED DDRKLPKLDEKTADSIRKGLTPRWNDLDVNQHVNNVKYIGWILESTPQEVLETQELCSLTLEYRRECGRD SVLESLTAVDHSGKGSGSNFQHLLRLEDGGEIVKGRTEWRPKNAVINGAVAPGETSPGNSVS	
Cp1FatB1: construct sequence	SEQ ID NO: 5
MANGSAVSLKDGSLETQEGTSSSSHPPRTFINQLPDWSMLLSAITTVFVAAEKQWTMLDRKSKRPDMLVE PFVQDGVSFRQSFSIRSYEIGADRTASIETLMNIFQETSLNHCKSLGLLNDGFGRTPEMCKRDLIWVVTK MQIEVNRYPTWGDTIEVTTWVSESGKNGMSRDWLISDCHTGEILIRATSVWAMMNQKTRRLSKIPDEVRQ EIVPYFVDSAPVIEDDRKLHKLDVKTGDSIRNGLTPRWNDLDVNQHVNNVKYIGWILKSVPTEVFVTQEL CGLTLEYRRECRRDSVLESVTAMDPSKEGDRSLYQHLLRLENGADIALGRTEWRPKNAGTNGAISTTKTS PGNSVS	
Cp1FatB1: coding sequence from genomic DNA	SEQ ID NO: 6
AASSAFFSFPAPGTSLKPGKFGNWPSNLSVPFNPKANHNGGFHVKANTSAHPKANGSAVSLKDGSLETQE GTSSSSHPPRTFINQLPDWSMLLSAITTVFVAAEKQWTMLDRKSKRPDMLVEPFVQDGVSFRQSFSIRSY EIGADRTASIETLMNIFQETSLNHCKSLGLLNDGFGRTPEMCKRDLIWVVTKMQIEVNRYPTWGDTIEVT TWVSESGKNGMSRDWLISDCHTGEILIRATSVWAMMNQKTRRLSKIPDEVRQEIVPYFVDSAPVIEDDRK LHKLDVKTGDSIRNGLTPRWNDLDVNQHVNNVKYIGWILKSVPTEVFVTQELCGLTLEYRRECRRDSVLE SVTAMDPSKEGDRSLYQHLLRLENGADIALGRTEWRPKT	

Figure 1

Thioesterases (cont.)	
oil palm thioesterase	SEQ ID NO: 7
MVASIVAWAFFPTPSFSPTASAKASKTIGEGSENLNVRGIIAKPTSSSAAKQGKVMAQAVPKINGAKVGL KAESQKAEEDAAPSSAPRTFYNQLPDWSVLLAAVTTIFLAAEKQWTLLDWKPRRPDMLTGAFSLGKIVQD GLVFRQNFSIRSYEIGADRTASIETLMNHLQETALNHVRNAGLLGDGFGATPEMSKRNLIWVVTKMQVLI EHYPSWGDVVEVDTWVGASGKNGMRRDWHVRDYRTGQTILRATSIWVMMDKHTRKLSKMPEEVRAEIGPY FMEHAAIVDEDSRKLPKLDDDTADYIKWGLTPRWSDLDVNQHVNNVKYIGWILESAPISILENHELASMT LEYRRECGRDSVLQSLTAVANDCTGGLPEASIECQHLLQLECGAEIVRGRTQWRPRRASGPTSAGSA	
oil palm thioesterase without transit peptide	SEQ ID NO: 8
LPDWSVLLAAVTTIFLAAEKQWTLLDWKPRRPDMLTGAFSLGKIVQDGLVFRQNFSIRSYEIGADRTASI ETLMNHLQETALNHVRNAGLLGDGFGATPEMSKRNLIWVVTKMQVLIEHYPSWGDVVEVDTWVGASGKNG MRRDWHVRDYRTGQTILRATSIWVMMDKHTRKLSKMPEEVRAEIGPYFMEHAAIVDEDSRKLPKLDDDTA DYIKWGLTPRWSDLDVNQHVNNVKYIGWILESAPISILENHELASMTLEYRRECGRDSVLQSLTAVANDC TGGLPEASIECQHLLQLECGAEIVRGRTQWRPRRASGPTSAGSA	
oil palm thioesterase without residues 1-118	SEQ ID NO: 9
DWKPRRPDMLTGAFSLGKIVQDGLVFRQNFSIRSYEIGADRTASIETLMNHLQETALNHVRNAGLLGDGF GATPEMSKRNLIWVVTKMQVLIEHYPSWGDVVEVDTWVGASGKNGMRRDWHVRDYRTGQTILRATSIWVM MDKHTRKLSKMPEEVRAEIGPYFMEHAAIVDEDSRKLPKLDDDTADYIKWGLTPRWSDLDVNQHVNNVKY IGWILESAPISILENHELASMTLEYRRECGRDSVLQSLTAVANDCTGGLPEASIECQHLLQLECGAEIVR GRTQWRPRRASGPTSAGSA	

Figure 1 (cont.)

Acyl-CoA Synthetases	
Faa2p	SEQ ID NO: 10
MAAPDYALTDLIESDPRFESLKTRLAGYTKGSDEYIEELYSQLPLTSYPRYKTFLKKQAVAISNPDNEAG FSSIYRSSLSSENLVSCVDKNLRTAYDHFMFSARRWPQRDCLGSRPIDKATGTWEETFRFESYSTVSKRC HNIGSGILSLVNTKRKRPLEANDFVVAILSHNNPEWILTDLACQAYSLTNTALYETLGPNTSEYILNLTE APILIFAKSNMYHVLKMVPDMKFVNTLVCMDELTHDELRMLNESLLPVKCNSLNEKITFFSLEQVEQVGC FNKIPAIPPTPDSLYTISFTSGTTGLPKGVEMSHRNIASGIAFAFSTFRIPPDKRNQQLYDMCFLPLAHI FERMVIAYDLAIGFGIGFLHKPDPTVLVEDLKILKPYAVALVPRILTRFEAGIKNALDKSTVQRNVANTI LDSKSARFTARGGPDKSIMNFLVYHRVLIDKIRDSLGLSNNSFIITGSAPISKDTLLFLRSALDIGIRQG YGLTETFAGVCLSEPFEKDVGSCGAIGISAECRLKSVPEMGYHADKDLKGELQIRGPQVFERYFKNPNET SKAVDQDGWFSTGDVAFIDGKGRISVIDRVKNFFKLAHGEYIAPEKIENIYLSSCPYITQIFVFGDPLKT FLVGIVGVDVDAAQPILAAKHPEVKTWTKEVLVENLNRNKKLRKEFLNKINKCTDGLQGFEKLHNIKVGL EPLTLEDDVVTPTFKIKRAKASKFFKDTLDQLYAEGSLVKTEKL	
SCRG_04483	SEQ ID NO: 11
MAAPDYALTDLIESDPRFESLKTRLAGYTKGSDEYIEELYSQLPLTSYPRYKTFLKKQAVAISNPDNEAG FSSIYRSSLSSENLVSCVDKNLRTAYDHFMFSARRWPQRDCLGSRPIDKATGTWEETFRFESYSTVSKRC HNIGSGILSLVNTKRKRPLEANDFVVAILSHNNPEWILTDLACQAYSLTNTALYETLGPNTSEYILNLTE APILIFAKSNMYHVLKMVPDMKFVNTLVCMDELTHDELRMLNESLLPVKCNSLNEKITFFSLEQVEQVGC FNKIPAIPPTPDSLYTISFTSGTTGLPKGVEMSHRNIASGIAFAFSTFRIPPDKRNQQLYDMCFLPLAHI FERMVIAYDLAIGFGIGFLHKPDPTVLVEDLKILKPYAVALVPRILTRFEAGIKNALDKSTVQRNVANTI LDSKSARFTARGGPDKSIMNFLVYHRVLIDKIRDSLGLSNNSFIITGSAPISKDTLLFLRSALDIGIRQG YGLTETFAGVCLSEPFEKDVGSCGAIGISAECRLKSVPEMGYHADKYLKGELQIRGPQVFERYFKNPNET SKAVDQDGWFSTGDVAFIDGKGRISVIDRVKNFFKLAHGEYIAPEKIENIYLSSCPYITQIFVFGDPLKT FLVGIVGVDVDAAQPILAAKHPEVKTWTKEVLVENLNRNKKLRKEFLNKINKCTDGLQGFEKLHNIKVGL EPLTLEDDVVTPTFKIKRAKASKFFKDTLDQLYAEGSLVKTEKL	
FadD	SEQ ID NO: 12
MKKVWLNRYPADVPTEINPDRYQSLVDMFEQSVARYADQPAFVNMGEVMTFRKLEERSRAFAAYLQQGLG LKKGDRVALMMPNLLQYPVALFGILRAGMIVVNVNPLYTPRELEHQLNDSGASAIVIVSNFAHTLEKVVD KTAVQHVILTRMGDQLSTAKGTVVNFVVKYIKRLVPKYHLPDAISFRSALHNGYRMQYVKPELVPEDLAF LQYTGGTTGVAKGAMLTHRNMLANLEQVNATYGPLLHPGKELVVTALPLYHIFALTINCLLFIELGGQNL LITNPRDIPGLVKELAKYPFTAITGVNTLFNALLNNKEFQQLDFSSLHLSAGGGMPVQQVVAERWVKLTG QYLLEGYGLTECAPLVSVNPYDIDYHSGSIGLPVPSTEAKLVDDDDNEVPPGQPGELCVKGPQVMLGYWQ RPDATDEIIKNGWLHTGDIAVMDEEGFLRIVDRKKDMILVSGFNVYPNEIEDVVMQHPGVQEVAAVGVPS GSSGEAVKIFVVKKDPSLTEESLVTFCRRQLTGYKVPKLVEFRDELPKSNVGKILRRELRDEARGKVDNK A	

Figure 2

Acyl-CoA Synthetases (cont.)	
FadK	SEQ ID NO: 13
MKVTLTFNEQRRAAYRQQGLWGDASLADYWQQTARAMPDKIAVVDNHGASYTYSALDHAASCLANWMLAK GIESGDRIAFQLPGWCEFTVIYLACLKIGAVSVPLLPSWREAELVWVLNKCQAKMFFAPTLFKQTRPVDL ILPLQNQLPQLQQIVGVDKLAPATSSLSLSQIIADNTSLTTAITTHGDELAAVLFTSGTEGLPKGVMLTH NNILASERAYCARLNLTWQDVFMMPAPLGHATGFLHGVTAPFLIGARSVLLDIFTPDACLALLEQQRCTC MLGATPFVYDLLNVLEKQPADLSALRFFLCGGTTIPKKVARECQQRGIKLLSVYGSTESSPHAVVNLDDP LSRFMHTDGYAAAGVEIKVVDDARKTLPPGCEGEEASRGPNVFMGYFDEPELTARALDEEGWYYSGDLCR MDEAGYIKITGRKKDIIVRGGENISSREVEDILLQHPKIHDACVVAMSDERLGERSCAYVVLKAPHHSLS LEEVVAFFSRKRVAKYKYPEHIVVIEKLPRTTSGKIQKFLLRKDIMRRLTQDVCEEIE	
MACS	SEQ ID NO: 14
MQWLKSFQICKVLQGFSLSPTQLHRRLFSRVGAPRWNDHDSPEEFNFASDVLDYWAQMEEEGKRGPSPAF WWVNGQGDEIKWSFRKLRDLTCRTANVFEQICGLQQGDHLALILPRVPEWWLVTVGCMRTGIIFMPGTTQ LKAKDILYRIQISRAKAIVTTASLVPEVESVASECPDLKTKLVVSDHSHEGWLDFCSLIKSASPDHTCIK SKMKDPMAIFFTSGTTGYPKMAKHNQGLAFRSYIPSCRKLLKLKTSDILWCMSDPGWILATVGCLIEPWT SGCTVFIHHLPQFDPKVIVEVLFKYPITQCLAAPGVYRMVLQQKTSNLRFPTLEHCTTGGESLLPEEYEQ WKQRTGLSIHEVYGQSETGISSATLREMKIKRGSIGKAILPFDLQIIDEKGNILPPNTEGYIGIRIKPTR PLGLFMEYENSPESTSEVECGDFYNSGDRATIDEEGYIWFLGRGDDVINASGYRIGPAEVENALAEHPAV AESAVVSSPDKDRGEVVKAFIVLNPEFLSHDQEQLIKELQHHVKSVTAPYKYPRKVEFVSELPKTVTGKI KRKELRNKEFGQL	

Figure 2 (cont.)

Alcohol-Forming Fatty Acyl Reductases	
Maqu_2220	SEQ ID NO: 15
MAIQQVHHADTSSSKVLGQLRGKRVLITGTTGFLGKVVLERLIRAVPDIGAIYLLIRGNKRHPDARSRFL EEIATSSVFDRLREADSEGFDAFLEERIHCVTGEVTEAGFGIGQEDYRKLATELDAVINSAASVNFREEL DKALAINTLCLRNIAGMVDLNPKLAVLQVSTCYVNGMNSGQVTESVIKPAGEAVPRSPDGFYEIEELVRL LQDKIEDVQARYSGKVLERKLVDLGIREANRYGWSDTYTFTKWLGEQLLMKALNGRTLTILRPSIIESAL EEPAPGWIEGVKVADAIILAYAREKVTLFPGKRSGIIDVIPVDLVANSIILSLAEALGEPGRRRIYQCCS GGGNPISLGEFIDHLMAESKANYAAYDHLFYRQPSKPFLAVNRALFDLVISGVRLPLSLTDRVLKLLGNS RDLKMLRNLDTTQSLATIFGFYTAPDYIFRNDELMALANRMGEVDKGLFPVDARLIDWELYLRKIHLAGL NRYALKERKVYSLKTARQRKKAA	
FAR6	SEQ ID NO: 16
MATTNVLATSHAFKLNGVSYFSSFPRKPNHYMPRRRLSHTTRRVQTSCFYGETSFEAVTSLVTPKTETSR NSDGIGIVRFLEGKSYLVTGATGFLAKVLIEKLLRESLEIGKIFLLMRSKDQESANKRLYDEIISSDLFK LLKQMHGSSYEAFMKRKLIPVIGDIEEDNLGIKSEIANMISEEIDVIISCGGRTTFDDRYDSALSVNALG PAYVTGKREGTVLETPLCIGENITSDLNIKSELKLASEAVRKFRGREEIKKLKELGFERAQHYGWENSYT FTKAIGEAVIHSKRGNLPVVIIRPSIIESSYNEPFPGWIQGTRMADPIILAYAKGQISDFWADPQSLMDI IPVDMVANAAIAAMAKHGCGVPEFKVYNLTSSSHVNPMRAGKLIDLSHQHLCDFPLEETVIDLEHMKIHS SLEGFTSALSNTIIKQERVIDNEGGGLSTKGKRKLNYFVSLAKTYEPYTFFQARFDNTNTTSLIQEMSME EKKTFGFDIKGIDWEHYIVNVHLPGLKKEFLSKKKTE	
FAR6 without transit peptide	SEQ ID NO: 17
CFYGETSFEAVTSLVTPKTETSRNSDGIGIVRFLEGKSYLVTGATGFLAKVLIEKLLRESLEIGKIFLLM RSKDQESANKRLYDEIISSDLFKLLKQMHGSSYEAFMKRKLIPVIGDIEEDNLGIKSEIANMISEEIDVI ISCGGRTTFDDRYDSALSVNALGPAYVTGKREGTVLETPLCIGENITSDLNIKSELKLASEAVRKFRGRE EIKKLKELGFERAQHYGWENSYTFTKAIGEAVIHSKRGNLPVVIIRPSIIESSYNEPFPGWIQGTRMADP IILAYAKGQISDFWADPQSLMDIIPVDMVANAAIAAMAKHGCGVPEFKVYNLTSSSHVNPMRAGKLIDLS HQHLCDFPLEETVIDLEHMKIHSSLEGFTSALSNTIIKQERVIDNEGGGLSTKGKRKLNYFVSLAKTYEP YTFFQARFDNTNTTSLIQEMSMEEKKTFGFDIKGIDWEHYIVNVHLPGLKKEFLSKKKTE	
FARXIII	SEQ ID NO: 18
MSANTMETDEQFTDNSPIVNFYSGKSVFVTGATGFLGTVLVEKLLFSCKGINNIYILIKQTEDLTIEARI LNYLNSKAFHRVKNTNPELMKKIIPICGNLEDKNLGISDSDMKTLLEEVSIVFHVAAKLLFKMSLTAAVN INTKPTEQLIAICKKMRRNPIFIYVSSAYSNVNEQIIDEKVYNTGVPLETIYDTLDTENTRITDIFLDKR PNTYTYSKALAEVVVEKEFDESAAIVRPSIIVSSIREPIPGWLSGSHGFPRVVGAACKGLLLRWHGDGTV VCDLIPVDHVANLIIAAAWESNERRLMGNKGVKVYNCCSSLRNPIDVITVVKTCIKYRKYFGTRTMSIFT PRFIMKKNYFIYKLLYFTCHTIPAAIIDGFFWLTGRTPIMLKTLDKLSKISSVLEYFTHHQFIFLDSNVR GLLRRMEGTDRQTFNFDVTEIEWEPYLQNFVRGIANNYDYSM	

Figure 3

Alcohol-Forming Fatty Acyl Reductases (cont.)	
Maqu_2507	SEQ ID NO: 19
MNYFLTGGTGFIGRFLVEKLLARGGTVYVLVREQSQDKLERLRERWGADDKQVKAVIGDLTSKNLGIDAK TLKSLKGNIDHVFHLAAVYDMGADEEAQAATNIEGTRAAVQAAEAMGAKHFHHVSSIAAAGLFKGIFRED MFEEAEKLDHPYLRTKHESEKVVREECKVPFRIYRPGMVIGHSETGEMDKVDGPYYFFKMIQKIRHALPQ WVPTIGIEGGRLNIVPVDFVVDALDHIAHLEGEDGNCFHLVDSDPYKVGEILNIFCEAGHAPRMGMRIDS RMFGFIPPFIRQSIKNLPPVKRITGALLDDMGIPPSVMSFINYPTRFDTRELERVLKGTDIEVPRLPSYA PVIWDYWERNLDPDLFKDRTLKGTVEGKVCVVTGATSGIGLATAEKLAEAGAILVIGARTKETLDEVAAS LEAKGGNVHAYQCDFSDMDDCDRFVKTVLDNHGHVDVLVNNAGRSIRRSLALSFDRFHDFERTMQLNYFG SVRLIMGFAPAMLERRRGHVVNISSIGVLTNAPRFSAYVSSKSALDAFSRCAAAEWSDRNVTFTTINMPL VKTPMIAPTKIYDSVPTLTPDEAAQMVADAIVYRPKRIATRLGVFAQVLHALAPKMGEIIMNTGYRMFPD SPAAAGSKSGEKPKVSTEQVAFAAIMRGIYW	
Hch_05075	SEQ ID NO: 20
MKQSLTLTAFANKNVLITGTTGFVGKVVLEKLLRSVPTIGKIYLLIRGNSKNPTARKRFQNEIATSSIFD TLKASQGSRFEELCETRIHCVTGEVTEPLFGLSEKDFTDLAADIDVIINSAASVNFREALDQALTINTLC LKNIIELSRRAADCPVVQVSTCYVNGFNQGVMEEEIVSPAGERIERSERGYYEVEPLIARLLQDVEQVSA AAADDHSREKDLIDLGIKEANKYGWNDTYTFTKWMGEQLLMKELYGKTLTILRPSIVESTLLGPAPGWIE GVKVADAIILAYAREKVSLFPGKKNAVIDIIPADLVANSIILSATEALLDSGAHRIYQCCSSEVNPIRIR EVIGHVQQEAEHNYQTHDKLFYRKPKKPFVMIPGAVFHALMAISFHMLKWSSRLQSLFGRKASGRKLSNM ETTMKLSKVFSFYTSPSYTFSNRRLQELSTRLGEYDQSEFPVNAGMYDWAHYLREVHVAGLNKYALRPKV VKMNPPAAKPRSRAA	
jjfar	SEQ ID NO: 21
MEEMGSILEFLDNKAILVTGATGSLAKIFVEKVLRSQPNVKKLYLLLRATDDETAALRLQNEVFGKELFK VLKQNLGANFYSFVSEKVTVVPGDITGEDLCLKDVNLKEEMWREIDVVVNLAATINFIERYDVSLLINTY GAKYVLDFAKKCNKLKIFVHVSTAYVSGEKNGLILEKPYYMGESLNGRLGLDINVEKKLVEAKINELQAA GATEKSIKSTMKDMGIERARHWGWPNVYVFTKALGEMLLMQYKGDIPLTIIRPTIITSTFKEPFPGWVEG VRTIDNVPVYYGKGRLRCMLCGPSTIIDLIPADMVVNATIVAMVAHANQRYVEPVTYHVGSSAANPMKLS ALPEMAHRYFTKNPWINPDRNPVHVGRAMVFSSFSTFHLYLTLNFLLPLKVLEIANTIFCQWFKGKYMDL KRKTRLLLRLVDIYKPYLFFQGIFDDMNTEKLRIAAKESIVEADMFYFDPRAINWEDYFLKTHFPGVVEH VLN	

Figure 3 (cont.)

Wax Ester Synthases	
M.ELB17 WS	SEQ ID NO: 22
MKRLATLDASWLAVESDDTPMHVGNLQIFSLPDNAPSTFAGDLVKSMKQAGNVELPWGCKLVWPGFLGRV LAPTWKHDKHIDLDYHVRHSALPKPGGERELGELVSRLHSNPLDLSRPLWECHMIEGLEHNRFALYTKMH HCMIDGISGVRLMQRVLSKSPDERDMLPPWSVRPESTRGKKTDSEASVPGAISQAMEALKLQLGLAPRLW QASNRLIHSVRHPEDGLTAPFTGPVSKINHRVTGQRRFATQQYQLEDMKAMARASGSSMNDIVLYLCGTA LRRFLLEQDDLPEISLTAGIPVNIRPADDEGTGTQISFMIAALATNQPDPLTRLKCIKESSCKAKEHLQK LPKKALTQYTMMLMSPYILQLMSGLGGRMRPVFNVTISNVPGPTEDLYYEGAKLEAMYPVSLITHGGALN ITCLSYAGSLNFGFTGCRDTLPSMQKLAVYTGEALEELRTLLLPPKKKPSPRKPRTAAKKKPAVNSNAS	
DSM8798 WS1	SEQ ID NO: 23
MTPLNPTDQLFLWLEKRQQPMHVGGLQLFSFPEGAPDDYVAQLADQLRQKTEVTAPFNQRLSYRLGQPVW VEDEHLDLEHHFRFEALPTPGRIRELLSFVSAEHSHLMDRERPMWEVHLIEGLKDRQFALYTKVHHSLVD GVSAMRMATRMLSENPDEHGMPPIWDLPCLSRDRGESDGHSLWRSVTHLLGLSGRQLGTIPTVAKELLKT INQARKDPAYDSIFHAPRCMLNQKITGSRRFAAQSWCLKRIRAVCEAYGTTVNDVVTAMCAAALRTYLMN QDALPEKPLVAFVPVSLRRDDSSGGNQVGVILASLHTDVQEAGERLLKIHHGMEEAKQRYRHMSPEEIVN YTALTLAPAAFHLLTGLAPKWQTFNVVISNVPGPSRPLYWNGAKLEGMYPVSIDMDRLALNMTLTSYNDQ VEFGLIGCRRTLPSLQRMLDYLEQGLAELELNAGL	
DSM8798 WS2	SEQ ID NO: 24
MKRLGTLDASWLAVESEDTPMHVGTLQIFSLPEGAPETFLRDMVTRMKEAGDVAPPWGYKLAWSGFLGRVIA PAWKVDKDIDLDYHVRHSALPRPGGERELGILVSRLHSNPLDFSRPLWECHVIEGLENNRFALYTKMHHSMI DGISGVRLMQRVLTTDPERCNMPPPWTVRPHQRRGAKTDKEASVPAAVSQAMDALKLQADMAPRLWQAGNRL VHSVRHPEDGLTAPFTGPVSVLNHRVTAQRRFATQHYQLDRLKNLAHASGGSLNDIVLYLCGTALRRFLAEQ NNLPDTPLTAGIPVNIRPADDEGTGTQISFMIASLATDEADPLNRLQQIKTSTRRAKEHLQKLPKSALTQYT MLLMSPYILQLMSGLGGRMRPVFNVTISNVPGPEGTLYYEGARLEAMYPVSLIAHGGALNITCLSYAGSLNF GFTGCRDTLPSMQKLAVYTGEALDELESLILPPKKRARTRK	
petunia WS	SEQ ID NO: 25
MKSLATELRNRSSEPCLKPIETKRKTIEEYETVAVEEEPLSPTARLFHDANFNVHVVVIIALDTRISPQP IKDKLVHTLLKHPRFTSLMVVDEENLADMKWVQTKIDLDQHIIVPEVDETQLESPDKFVEDYIYNLTKTS LDRTKPLWDLHLVNVKTRDAEAVALLRVHHSLGDGTSLISLLLACTRQTADELKLPTIPTKKRRPTPSGY STKEESFKLWHYLAVIWLFIRMIGNTLVDVLMFIITVIFLKDTKTPINTVPDSESRVRRIVHRIIDLDDL KLVKNAMNMTINDVALGITQAGLSKYLNRRYAVDEEDKGDTERNNNLPKNIRLRSCLVINLRPSAGIEDL ADMMEKGPKEKRGWGNWFGYVLLPFKIALRDDPLDYVKEAKATVDRKKRSFEALYTLIMAEVLIKIFGIK VATAVTVRVFSNATVCFSNVVGPQEEIGFCGHPISYLAPSIYGQPSALMINFQSYIDKMIIVVAVDEGAI PDPQQLLDDFENSLHLIKEAVLERGLVKNLK	
Mus musculus WS	SEQ ID NO: 26
MFWPTKKDLKTAMEVFALFQWALSALVIVTTVIIVNLYLVVFTSYWPVTVLMLTWLAFDWKTPERGGRRFTC VRKWRLWKHYSDYFPLKMVKTKDISPDRNYILVCHPHGLMAHSCFGHFATDTTGFSKTFPGITPYMLTLGAF FWVPFLRDYVMSTGSCSVSRSSMDFLLTQKGTGNMLVVVVGGLAECRYSTPGSTTLFLKKRQGFVRTALKHG VSLIPAYAFGETDLYDQHIFTPGGFVNRFQKWFQKMVHIYPCAFYGRGLTKNSWGLLPYSQPVTTVVGEPLP LPKIENPSEEIVAKYHTLYIDALRKLFDQHKTKFGISETQELVIV	

Figure 4

Wax Ester Synthases (cont.)	
jojoba WS	SEQ ID NO: 27
MEVEKELKTFSEVWISAIAAACYCRFVPAVAPHGGALRLLLLLPVVLLFIFLPLRLSSFHLGGPTALYLV WLANFKLLLFAFHLGPLSNPSLSLLHFISTTLLPIKFRDDPSNDHEKNKRTLSFEWRKVVLFVAKLVFFA GILKIYEFRKDLPHFVISVLYCFHFYLGTEITLAASAVIARATLGLDLYPQFNEPYLATSLQDFWGRRWN LMVSDILGLTTYQPVRRVLSRWVRLRWEVAGAMLVAFTVSGLMHEVFFFYLTRARPSWEVTGFFVLHGVC TAVEMVVKKAVSGKVRLRREVSGALTVGFVMVTGGWLFLPQLVRHGVDLKTIDEYPVMFNYTQKKLMGLL GW	
ADP1 WS	SEQ ID NO: 28
MRPLHPIDFIFLSLEKRQQPMHVGGLFLFQIPDNAPDTFIQDLVNDIRISKSIPVPPFNNKLNGLFWDED EEFDLDHHFRHIALPHPGRIRELLIYISQEHSTLLDRAKPLWTCNIIEGIEGNRFAMYFKIHHAMVDGVA GMRLIEKSLSHDVTEKSIVPPWCVEGKRAKRLREPKTGKIKKIMSGIKSQLQATPTVIQELSQTVFKDIG RNPDHVSSFQAPCSILNQRVSSSRRFAAQSFDLDRFRNIAKSLNVTINDVVLAVCSGALRAYLMSHNSLP SKPLIAMVPASIRNDDSDVSNRITMILANLATHKDDPLQRLEIIRRSVQNSKQRFKRMTSDQILNYSAVV YGPAGLNIISGMMPKRQAFNLVISNVPGPREPLYWNGAKLDALYPASIVLDGQALNITMTSYLDKLEVGL IACRNALPRMQNLLTHLEEEIQLFEGVIAKQEDIKTAN	

Figure 4 (cont.)

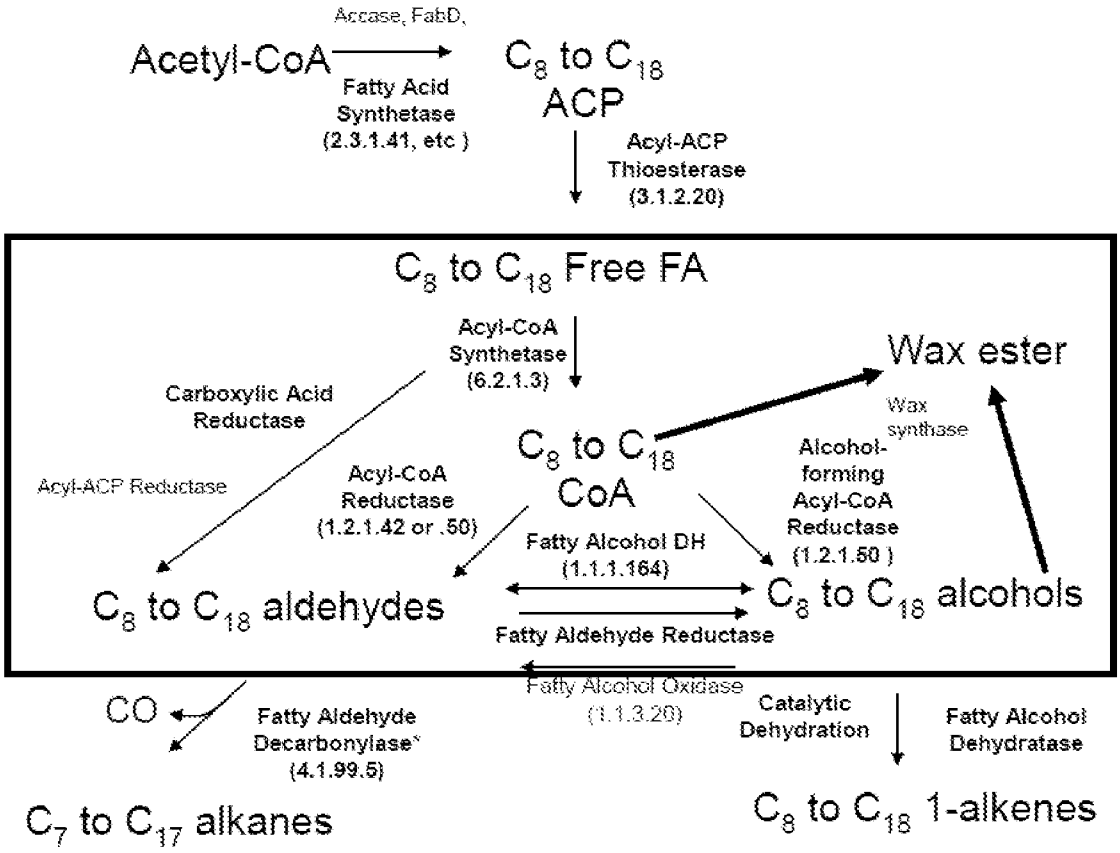

Accase, FabD,
Acetyl-CoA
Fatty Acid Synthetase (2.3.1.41, etc)
C8 to C18 ACP
Acyl-ACP Thioesterase (3.1.2.20)
C8 to C18 Free FA
Acyl-CoA Synthetase (6.2.1.3)
Wax ester
Wax synthase
Carboxylic Acid Reductase
Acyl-ACP Reductase
Acyl-CoA Reductase (1.2.1.42 or .50)
C8 to C18 CoA
Alcohol-forming Acyl-CoA Reductase (1.2.1.50)
Fatty Alcohol DH (1.1.1.164)
C8 to C18 aldehydes
C8 to C18 alcohols
Fatty Aldehyde Reductase
Fatty Alcohol Oxidase (1.1.3.20)
CO
Fatty Aldehyde Decarbonylase (4.1.99.5)
Catalytic Dehydration
Fatty Alcohol Dehydratase
C7 to C17 alkanes
C8 to C18 1-alkenes

Figure 5

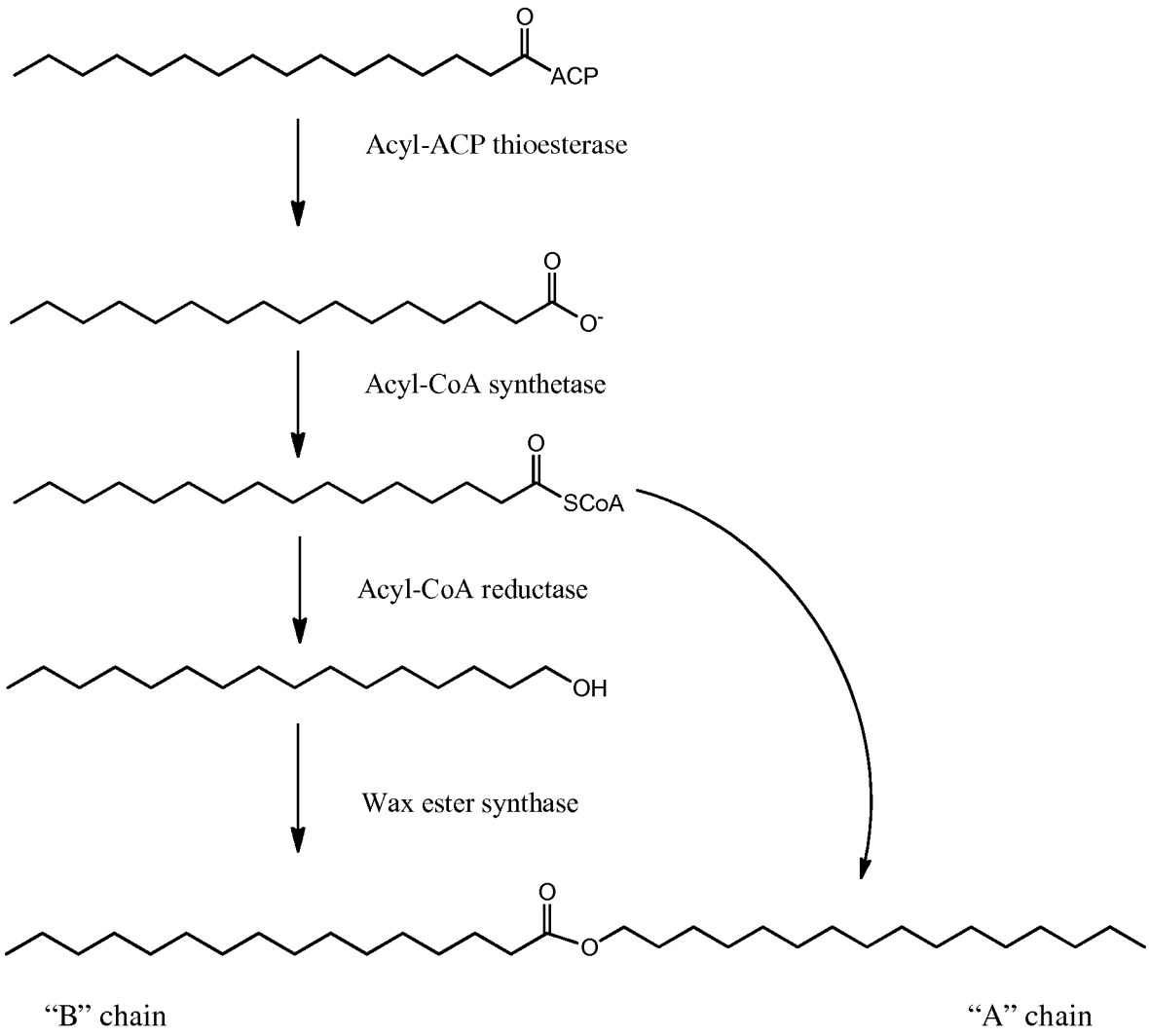

O
ACP
Acyl-ACP thioesterase
O
O-
Acyl-CoA synthetase
O
SCoA
Acyl-CoA reductase
OH
Wax ester synthase
O
O
"B" chain
"A" chain

Figure 6

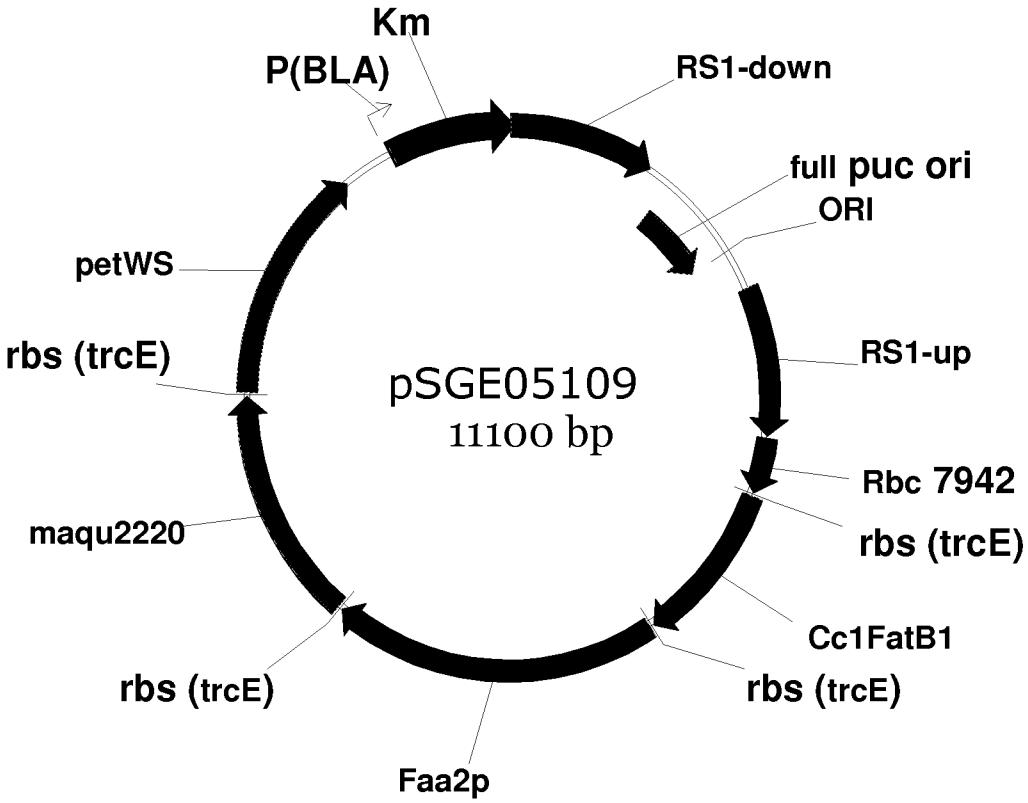

Km
P(BLA)
RS1-down
full puc ori
ORI
petWS
RS1-up
rbs (trcE)
pSGE05109
11100 bp
Rbc 7942
rbs (trcE)
maqu2220
Cc1FatB1
rbs (trcE)
rbs (trcE)
Faa2p

Figure 7

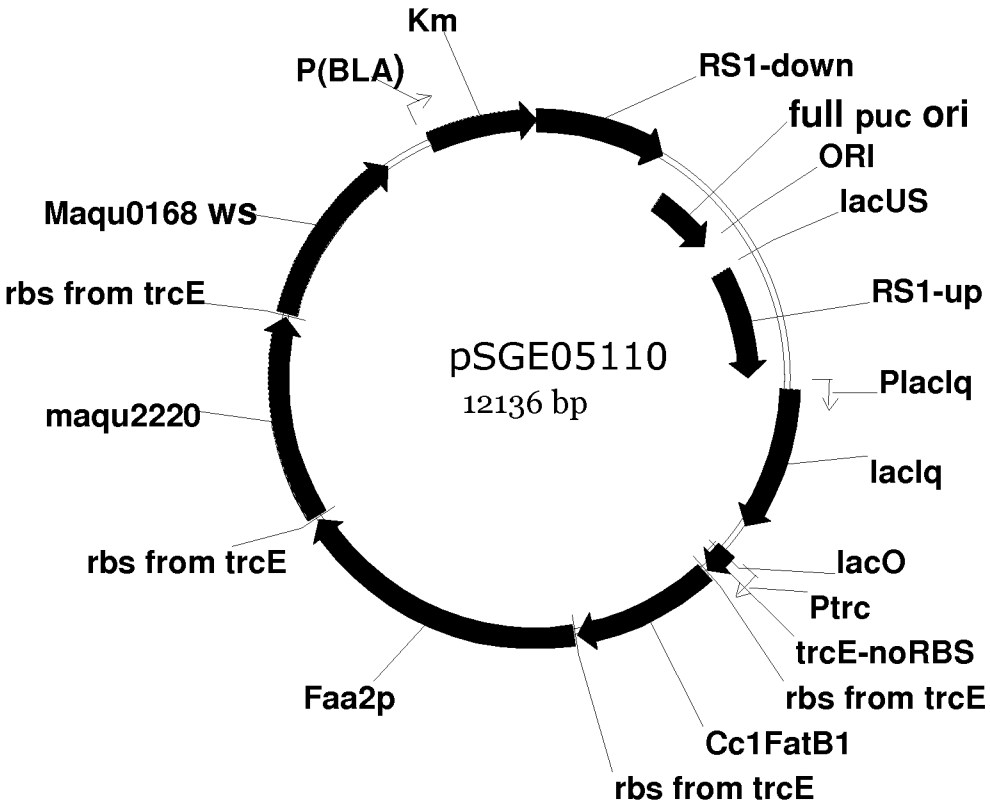

Km
P(BLA)
RS1-down
full puc ori
ORI
lacUS
RS1-up
PlacIq
lacIq
lacO
Ptrc
trcE-noRBS
rbs from trcE
Cc1FatB1
rbs from trcE
Faa2p
rbs from trcE
maqu2220
rbs from trcE
Maqu0168 WS
pSGE05110
12136 bp

Figure 8

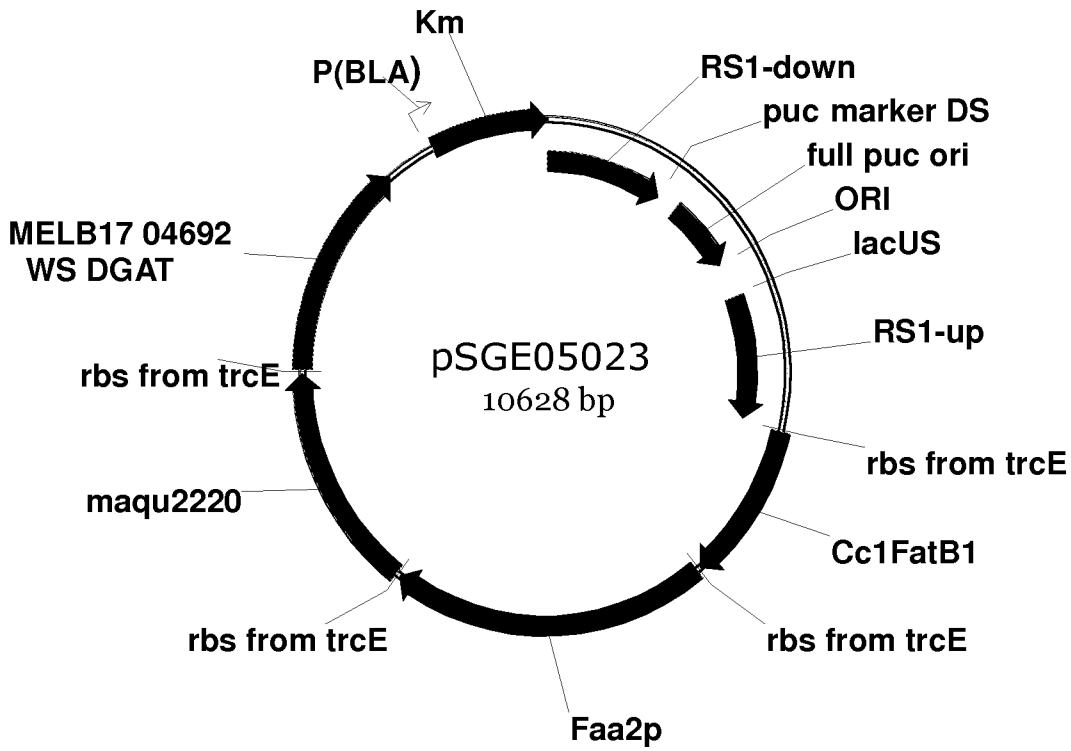

Km
P(BLA)
RS1-down
puc marker DS
full puc ori
ORI
lacUS
RS1-up
rbs from trcE
Cc1FatB1
rbs from trcE
Faa2p
rbs from trcE
maqu2220
rbs from trcE
MELB17 04692
WS DGAT
pSGE05023
10628 bp

Figure 9

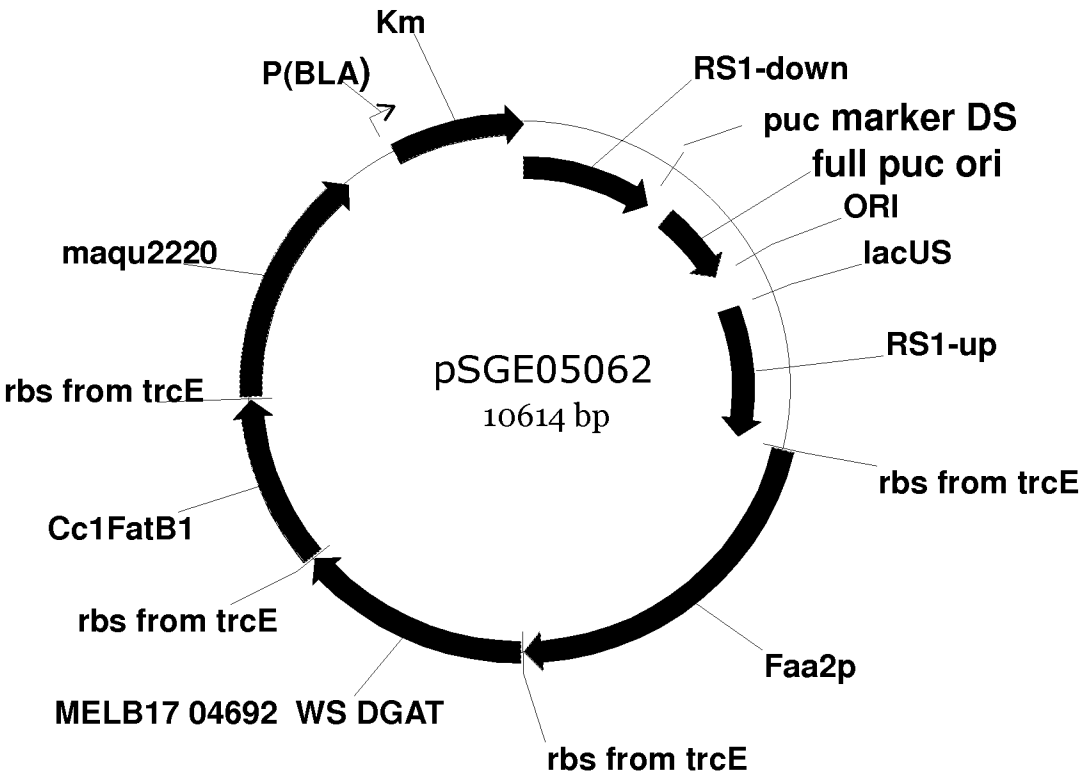

Km
P(BLA)
RS1-down
puc marker DS
full puc ori
ORI
lacUS
RS1-up
rbs from trcE
Faa2p
rbs from trcE
MELB17 04692 WS DGAT
rbs from trcE
Cc1FatB1
rbs from trcE
maqu2220
pSGE05062
10614 bp

Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/27091

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07H 21/04; C12N 15/82 (2012.01)
USPC - 536/23.6; 800/281
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C07H 21/04; C12N 15/82 (2012.01)
USPC: 536/23.6; 800/281

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 536/231.; 435/320.1; 800/278

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB,USPT,EPAB,JPAB); Google Scholar; esp@cenet; GenCore 6.3: wax ester, fatty alcohol, acyl CoA, cyanobacteria, agmenellum, anabaena, borzia, calothrix, cyanospira, dermocarpella, spirulina, starria, rivularia, integrate, genome, inactivate, deactivate, upstream, vector, regulatory, optional, promoter, acyl thioesterase, acyl synthetase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y — A	US 2011/0195469 A1 (ROESSLER et al.) 11 August 2011 (11.08.2011), abstract; para [0032], [0036], [0037], [0086], [0087], [0088], [0089], [0091], [0093], [0097], [0126], [0127], [0144], [0152], [0160], [0173]; SEQ ID NO: 2; SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 14; SEQ ID NO: 15	1-10, 12-34 — 11
Y — A	US 2011/0111470 A1 (BERRY et al.) 12 May 2011 (12.05.2011), abstract; para [0011], [0044], [0052], [0083], [0155], [0191]; SEQ ID NO: 2	1-10, 12-34 — 11
Y — A	US 2009/0117629 A1 (SCHMIDT-DANNERT et al.) 7 May 2009 (07.05.2009), abstract; para [0023], [0070]; [0083], [0084], [0089], [0101], [0152], SEQ ID NO: 1	10, 13, 14, 19, 32 — 11
Y	US 6,492,509 B1 (LARDIZABAL et al.) 10 December 2002 (10.12.2002), abstract; col 5, ln 35-50; col 8, ln 35-36; col 13, ln 25-31; col 13, ln 25-41; col 14, ln 5-11; col 16, ln 19-28.	16, 24, 25, 33
A	WO 2011/157848 A1 (PHADNAVIS) 22 December 2011 (22.12.2011), SEQ ID NO: 1.	11
A	HOLTZAPPLE et al., Biosynthesis of Isoprenoid Wax Ester in Marinobacter hydrocarbonoclasticus DSM 8798: Identification and Characterization of Isoprenoid Coenzyme A Synthetase and Wax Ester Synthases. Journal of Bacteriology. May 2007, Vol 189, No 10, Pages 3804-3812, especially abstract.	1

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 June 2012 (07.06.2012)	21 JUN 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

2012/027091 21.06.2012
International application No.
PCT/US 12/27091

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☒ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

GenCore 6.3; SEQ ID NOs: 1-31, 40, 45, 53