Methods for Increasing Renewable Oil Productions

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(54) METHODS FOR INCREASING RENEWABLE OIL PRODUCTION

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Related U.S. Application Data
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Methods of increasing renewable oil production are provided and include transforming a plant cell with an isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyltransferase (VgDGAT) polypeptide, where the expression of the VgDGAT polypeptide increases an amount of renewable oil in the plant. Transgenic plant cells comprising an isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyltransferase 1 (VgDGAT1) polypeptide are further provided.

35 Claims, 6 Drawing Sheets
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OTHER PUBLICATIONS


* cited by examiner
FIG. 1

[Graph showing TAG synthesis for different samples]
FIG. 7
**FIG. 8**

Graph showing the seed protein content (%) for different seed genotypes: Vectot-ctr, SIEPX, VgDGAT1/SIEPX, and VgDGAT2/SIEPX.
METHODS FOR INCREASING RENEWABLE OIL PRODUCTION

RELATED APPLICATIONS

This application claims priority from U.S. Provisional Application Ser. No. 61/371,936, filed Aug. 9, 2010, the entire disclosure of which is incorporated herein by this reference.

TECHNICAL FIELD

The presently-disclosed subject matter relates to methods for increasing renewable oil production. In particular, the presently-disclosed subject matter relates to methods for increasing renewable oil production in a plant, where the expression of a Vernonia galamensis diacylglycerol acyltransferase (VgDGAT) polypeptide increases an amount of renewable oil in the plant.

BACKGROUND

Global plant oil production exceeded 120 metric tons (MT) in 2009, and continues to be dominated by four main oil crops: palm, soybeans, rapeseed or canola, and sunflowers (Wilson and Hildebrand, 2010). Indeed, world-wide palm and soybean production has increased rapidly in recent years, with rapeseed also showing steady increases, and it is expected that this trend will only continue as the projected global oil production in 2010 was expected to climb to over 170 million MT.

Of the oil being produced from the four main oil crops, palm oil production has been dominated by Malaysia and Indonesia, while the vast majority of soybeans are produced in the United States, Brazil, China and Argentina. Despite the production of different oils by different countries, however, global oilseed production has been consistently dominated by soybeans and has been followed by rapeseed as a distant second (Wilson and Hildebrand, 2010). Over 200 million MT of soybean seeds have been produced in recent years, and this dominance is believed to be because, among oilseeds, soybeans are low in oil and high in protein making soybeans the dominant global protein source. On average, soybeans consist of approximately 20% oil and 40% protein on a dry weight basis, whereas rapeseed is approximately 50% oil and palm fruit is close to 90% oil and includes both palm fruit oil and kernel oil.

Breeding for increased oilseed yield per unit land area has also continued to progress in recent years with steady soybean yield increases being a good example (Egli, 2008a; Egli, 2008b). This increased yield is often with little or no increased inputs, thus making renewable oil production from plants less expensive over time and, at the same time, more competitive with petroleum as an industrial chemical feedstock. Indeed, while most plant oil continues to be produced and used for food purposes, an increasing proportion of plant oil is being utilized for industrial uses, with the proportion of industrial versus food usage having increased from approximately 10% to approximately 20% in the last 10 years.

Because U.S. and global seed and oil production is extensive and important for the both the production of oils for human and animal consumption and for industrial purposes, the value of even a 3-5% increase in seed oil content is also significant and has been increasingly recognized. Accordingly, a method of increasing oil content by only a small percentage would be both desirable and beneficial. More specifically, a method of increasing renewable oil production in a plant that is not accompanied by a concomitant decrease in protein levels in the plant, would be highly desirable and beneficial not only for purposes of human consumption, but also from an industrial perspective.

SUMMARY

The presently-disclosed subject matter relates to methods for increasing renewable oil production. In particular, the presently-disclosed subject matter relates to methods for increasing renewable oil production in a plant, where the expression of a Vernonia galamensis diacylglycerol acyltransferase (VgDGAT) polypeptide increases an amount of renewable oil in the plant.

In some embodiments, expression of the VgDGAT polypeptide in a control plant increases the amount of renewable oil in the plant by at least about 2 or about 3 percent as compared to an amount of renewable oil in a control plant. In other embodiments, expression of the VgDGAT polypeptide increases the amount of renewable oil in the plant by at least about 5 percent as compared to an amount of renewable oil in a control plant.

In some embodiments of the methods for increasing renewable oil production in a plant, the plant is selected from: Arachis hypogaea, Borago officinalis, Brassica campestris, Brassica napus, Brassica rapa, Camelina salvia, Cannabis sativa, Carthamus tinctorius, Cocos nucifera, Crambe abyssinica, Cuphea species, Glycine max, Gossypium hirsutum, Gossypium barbadense, Gossypium herbaceum, Helianthus annuus, Linum usitatissimum, Oenothera biennis, Olea europea, Oriza sativa, Perilla frutescens, Ricinus communis, Salvia hispanica, Sesamum indicum, Sinapis alba, Theobroma cacao, Trifolium species, Zea mays, Juglans species, or Prunus dulcis. In some embodiments, increasing the amount of renewable oil in the plant comprises increasing the amount of renewable oil in a seed of the plant. In some embodiments, increasing the amount of renewable oil in the plant comprises increasing the amount of triacylglycerol (TAG) in the plant. In some embodiments, even though the amount of renewable oil found in the plant is increased, the protein levels in the plant are substantially unchanged as compared to a control plant. In some embodiments, both the amounts of renewable oil found in the plant and the amounts of protein found in the plant are increased.

In some embodiments of the presently-disclosed subject matter, the VgDGAT polypeptide expressed in the plant is a VgDGAT1a polypeptide. In some embodiments, the VgDGAT1a polypeptide is encoded by a nucleic acid mol-
subject matter are transgenic plant cells capable of producing an amino acid sequence of SEQ ID NO: 2.

In some embodiments of the presently-disclosed subject matter, the VgDGAT1b polypeptide is encoded by a nucleic acid molecule having the sequence of SEQ ID NO: 3. In some embodiments, the VgDGAT1b polypeptide comprises the amino acid sequence of SEQ ID NO: 4.

In some embodiments of the presently-disclosed subject matter are methods of producing triacylglycerols (TAGs). In some embodiments, a method of producing a triacylglycerol (TAG) is provided that comprises transforming a plant cell with an isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyltransferase 2 (VgDGAT2) polypeptide, where the expression of the VgDGAT1 polypeptide and the VgDGAT2 polypeptide increases an amount of renewable oil in the plant. In some embodiments, the co-expression of the VgDGAT1 polypeptide and the VgDGAT2 polypeptide increases the amount of renewable oil in the plant in a synergistic manner. In some embodiments, the VgDGAT2 polypeptide is encoded by a nucleic acid having the sequence of SEQ ID NO: 5. In some embodiments, the VgDGAT2 polypeptide comprises the amino acid sequence of SEQ ID NO: 6.

Further provided, in some embodiments of the presently-disclosed subject matter are methods of producing triacylglycerols (TAGs). In some embodiments, a method of producing a triacylglycerol (TAG) is provided that comprises transforming a cell with an isolated nucleic acid that encodes a Vernonia galamensis diacylglycerol acyltransferase 1 (VgDGAT1) polypeptide, wherein expression of the VgDGAT1 polypeptide increases an amount of TAG in the cell. In some embodiments, the cell is an animal cell, a plant cell, an algal cell, a fungal cell, or a yeast cell.

Still further provided, in some embodiments of the presently-disclosed subject matter, are methods for increasing renewable oil production in a plant that include transforming a plant cell with an isolated nucleic acid encoding an Arabidopsis thaliana DGAT1 polypeptide (AtDGAT1), a Glycine max DGAT1a polypeptide (GmDGAT1a), a Vernonia galamensis DGAT1a polypeptide (VgDGAT1a), or a Vernonia galamensis DGAT1b polypeptide (VgDGAT1b);

FIG. 2 is a schematic diagram showing the phylogenetic relationships between soybean (Gm), Vernonia (Vg), Euphorbia (E), and Arabidopsis (At) DGAT1s and also showing the association of those DGAT1s with other related DGAT1s;

FIG. 3 is a graph showing oil and protein levels of mature soybean seeds that were produced on a research farm from a line expressing a Vernonia galamensis DGAT1a (Li-67), a high-oil content line of soybeans from breeding (NC-381), and a control line (Jack);

FIG. 4 is a graph showing soybean oil levels of mature seeds expressing a Vernonia galamensis DGAT1a versus high oil lines from breeding and regular soybeans determined by single seed (SS) and bulk seed analyses;

FIG. 5 is a graph showing soybean oil and protein levels and calculated meal protein levels of mature seeds produced on a research farm from a line expressing a Vernonia galamensis VgDGAT1a (Li-67), a high oil line from breeding (NC-381), and a control line (Jack);

FIG. 6 is a graph showing the percentages of mean oil, protein, and oil and protein (O+P) in mature soybean seeds produced on a research farm over three years from a control line (Jack) and from various lines expressing VgDGAT1a (9648-2A; 9648-2D; 9652-A3; 9654-3E; 9627-4C; 9636-1A; 9642-5B; and 9652-A6);

FIG. 7 is a graph showing seed oil contents from a control soyline (Vector-ctr) and from transgenic soybean lines expressing either a Stokiesis laevis epoxygenase transgene (SIEPX), a VgDGAT1a transgene and a SIEPX transgene, or a VgDGAT2 transgene and a SIEPX transgene, where each data point represents seed oil content in whole seed samples from individual transgenic plants that were grown, and where horizontal bars indicate the mean for each dataset; and

FIG. 8 is a graph showing seed protein content from a control soyline (Vector-ctr) and from transgenic soybean lines expressing either a Stokiesis laevis epoxygenase transgene (SIEPX), a VgDGAT1a and a SIEPX transgene, or a VgDGAT2 and a SIEPX transgene, where each data point represents seed protein content in whole seed samples from individual transgenic plants that were grown, and where horizontal bars indicate the mean for each dataset.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO: 1 is a nucleic acid sequence of a diacylglycerol acyltransferase 1a cDNA obtained from Vernonia galamensis;

SEQ ID NO: 2 is an amino acid sequence of a diacylglycerol acyltransferase 1a polypeptide from Vernonia galamensis;

SEQ ID NO: 3 is a nucleic acid sequence of a diacylglycerol acyltransferase 1b cDNA obtained from Vernonia galamensis;

SEQ ID NO: 4 is an amino acid sequence of a diacylglycerol acyltransferase 1b polypeptide from Vernonia galamensis;

SEQ ID NO: 5 is a nucleic acid sequence of a diacylglycerol acyltransferase 2 cDNA obtained from Vernonia galamensis; and

SEQ ID NO: 6 is an amino acid sequence of a diacylglycerol acyltransferase 2 polypeptide from Vernonia galamensis.
SEQ ID NO: 7 is a nucleic acid sequence of a degenerate forward primer for amplifying diacylglycerol acyltransferase cDNA;
SEQ ID NO: 8 is a nucleic acid sequence of a degenerate reverse primer for amplifying diacylglycerol acyltransferase cDNA;
SEQ ID NO: 9 is an amino acid sequence of a conserved region in diacylglycerol acyltransferases from Arabidopsis thaliana and Mus musculus;
SEQ ID NO: 10 is another amino acid sequence of a conserved region in diacylglycerol acyltransferases from Arabidopsis thaliana and Mus musculus;
SEQ ID NO: 11 is a nucleic acid sequence of a forward primer for amplifying Vernonia galamensis diacylglycerol acyltransferase cDNA;
SEQ ID NO: 12 is a nucleic acid sequence of a reverse primer for amplifying Vernonia galamensis diacylglycerol acyltransferase cDNA;
SEQ ID NO: 13 is a nucleic acid sequence of a forward primer for amplifying Euphorbia tagascae diacylglycerol acyltransferase cDNA;
SEQ ID NO: 14 is a nucleic acid sequence of a reverse primer for amplifying Euphorbia tagascae diacylglycerol acyltransferase cDNA;
SEQ ID NO: 15 is a nucleic acid sequence of a forward primer for amplifying Glycine max diacylglycerol acyltransferase cDNA;
SEQ ID NO: 16 is a nucleic acid sequence of a reverse primer for amplifying Glycine max diacylglycerol acyltransferase cDNA;
SEQ ID NO: 17 is a nucleic acid sequence of another forward primer for amplifying Glycine max diacylglycerol acyltransferase cDNA;
SEQ ID NO: 18 is a nucleic acid sequence of another reverse primer for amplifying Glycine max diacylglycerol acyltransferase cDNA; and
SEQ ID NO: 19 is a nucleic acid sequence of an epoxyge-noase cDNA obtained from Stokesia laevis.

DESCRIPTION OF EXEMPLARY EMBODIMENTS

The details of one or more embodiments of the presently-disclosed subject matter are set forth in this document. Modifications to embodiments described in this document, and other embodiments, will be evident to those of ordinary skill in the art after a study of the information provided in this document. The information provided in this document, and particularly the specific details of the described exemplary embodiments, is provided primarily for clearness of understanding and no unnecessary limitations are to be understood therefrom. In case of conflict, the specification of this document, including definitions, will control.

Some of the polynucleotide and polypeptide sequences disclosed herein are cross-referenced to GENBANK® accession numbers. The sequences cross-referenced in the GENBANK® database are expressly incorporated by reference as are equivalent and related sequences present in GENBANK® or other public databases. Also expressly incorporated herein by reference are all annotations present in the GENBANK® database associated with the sequences disclosed herein. Unless otherwise indicated or apparent, the references to the GENBANK® database are references to the most recent version of the database as of the filing date of this Application.

The following terms are believed to be well understood by one of ordinary skill in the art, definitions are set forth to facilitate explanation of the presently-disclosed subject matter. 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 the presently-disclosed subject matter belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presently-disclosed subject matter, representative methods, devices, and materials are now described.

Following long-standing patent law convention, the terms "a", "an", and "the" refer to "one or more" when used in this application, including the claims. Thus, for example, reference to "a cell" includes a plurality of such cells, and so forth.

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about". Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently-disclosed subject matter.

As used herein, the term "about", when referring to a value or to an amount of mass, weight, time, volume, concentration or percentage is meant to encompass variations of in some embodiments ±20%, in some embodiments ±10%, in some embodiments ±5%, in some embodiments ±1%, in some embodiments ±0.5%, and in some embodiments ±0.1% from the specified amount, as such variations are appropriate to perform the disclosed method. In this regard, in some embodiments of the presently-disclosed subject matter, ranges can be expressed as from "about" one particular value, and/or to "about" another particular value. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

Plant seed oils represent a significant renewable resource, with most plant seed oils being predominately composed of triacylglycerols (TAGs) that are produced via the sequential incorporation of fatty acids. In plants, this sequential incorporation of fatty acids into TAG is commonly known as the Kennedy pathway, which consists of three successive acylation reactions of the hydroxyl groups of glycerol by three acyl-CoA-dependent acyltransferases, starting from glycerol-3-phosphate (G3P). Specifically, in the Kennedy pathway, lysophosphatidic acid (LPA) and phosphatidic acid (PA) are first formed through two acylations catalyzed by the acyltransferases glycerol-3-phosphate (G3P) and lyso-phosphatic acid acyltransferase (LPAAT). PA is then dephosphorylated by the action of phosphatidase phosphatase (PAP) to form sn-1,2-diacylglycerol (sn-1,2 DAG). The final acylation of sn-1,2 DAG is the transfer of a fatty acyl moiety, such as from acyl-CoA, to the sn-3 position of diacylglycerol by diacylglycerol acyltransferase (DGAT) to generate TAG.

It is thought that DGAT is one of the rate-limiting steps in plant storage lipid accumulation and plays a role in controlling both the quantitative and qualitative flux of fatty acids into storage TAGs. There are two distinct types of non-homologous DGAT gene families designated as DGAT1 and DGAT2, encoding proteins with DGAT activity in plants and animals. Furthermore, in certain species, such as soybean, Vernonia galamensis, and Euphorbia species, DGAT1 genes can further be divided into two distinct subclasses, designated DGAT1a and DGAT1b. Recently, the TAG biosynthetic activ-
ity of DGAT1s from a number of plant species, including soybean, Arabidopsis, and Euphorbia have been analyzed for their ability to increase the production of TAG in plants. However, for the commercial production of renewable oil in plants, the use of DGAT1 enzymes from those species have only proven to be modestly effective in increasing renewable oil production.

Disclosed herein are novel data demonstrating that certain DGAT1s have higher activity in TAG biosynthesis than other DGAT1s. As disclosed herein, DGAT1 proteins from Vernonia galamensis (Vg) were expressed in a number of cells and tissues, including, but not limited to, yeast cells, petunia leaves, soybean somatic embryos, and mature soybean seeds, and it was ascertained that the expression of VgDGAT1 enzymes greatly increased renewable oil production and, in particular, TAG biosynthesis and accumulation in the cells and tissues. To that end, the presently-disclosed subject matter includes methods of increasing renewable oil production in a plant, where the expression of a Vernonia galamensis diacylglycerol acyltransferase (VgDGAT1) polypeptide increases an amount of renewable oil in the plant.

In some embodiments of the presently-disclosed subject matter, a method of increasing renewable oil production in a plant is provided that comprises transforming a plant cell with an isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyltransferase 1 (VgDGAT1) polypeptide, where the expression of the VgDGAT1 polypeptide increases an amount of renewable oil in the plant.

Vernonia galamensis is a plant in the sunflower family of significant industrial value due to high levels of oil found within the seeds of the plant. In this regard, Vernonia galamensis is commonly grown as a source of oil that is used in a variety of industrial applications, such as the manufacture of plastics or paints. However, the large-scale farming of Vernonia galamensis is typically not economically feasible, particularly outside of equatorial regions, due to poor seed yield and poor seed retention, which thus makes the plants agronomically unsuit for the industrial scale growth and processing that would be required to make use of Vernonia galamensis plants as a viable source of renewable seed oil. Disclosed herein, however, are data indicating that the DGAT1 genes from Vernonia galamensis, including Vernonia galamensis DGAT1a and DGAT1b genes, can be inserted into a vector and then efficiently and economically used to produce VgDGAT1 polypeptides that are capable of significantly increasing the production of renewable oils in plants that can be grown on a commercial scale.

The term “renewable oil” as used herein in relation to plants refers to oils that include or are derived from TAG and are produced by or are derived from plants or portions thereof (e.g., the organs, tissues, cells, or propagation materials of a plant) such that the oils can be replaced or replenished by the growth of a new plant or by the initial plant that produced the oil. A number of plant oils are known to those of ordinary skill in the art and include, but are not limited to, oils derived from oil seeds (e.g., canola, peanut, corn, soybean, sunflower, cottonseed, and safflower) and fixed oils such as almond oil and castor oil. Regardless of the specific type of oil, however, and as noted above, most plant oils are predominantly composed of TAGs, the synthesis of which is catalyzed, at least in part, by the activity of DGATs. As such, in some embodiments of the presently-disclosed subject matter, a method of producing a TAG is further provided that includes transforming a cell with an isolated nucleic acid that encodes a VgDGAT1 polypeptide, where the expression of the VgDGAT1 polypeptide in the cell increases an amount of TAG in the cell. In some embodiments, the cell is an animal cell, a plant cell, an algal cell, a fungal cell, or a yeast cell. In some embodiments, the cell is an algal cell selected from Chlorella, Scenedesmus, Ankistrodesmus, Chlorella, Boekelovia Oscillatoria, Amphora, Euglena, and Synechococcus species.

The “amount” of a renewable oil, or TAG, in a cell can be determined by methods known to those of ordinary skill in the art. For example, gas chromatography-mass spectrometry, thin layer chromatography-gas chromatography, gas chromatography, near infrared (NIR) or nuclear magnetic resonance spectrophotometry, or gravimetric methods, such as Soxhlet, can be utilized to determine a total amount of renewable oil or a total amount of TAG in a sample obtained from a cell transformed with a nucleic acid encoding a VgDGAT1 polypeptide. An increase in the amount of renewable oil, or TAG, can then be measured relative to a control level of the oil, or TAG, such as an amount or range of amounts of the oil, or TAG, found in comparable samples in cells that have not been transformed with a nucleic acid encoding a VgDGAT polypeptide. In some embodiments, the increase in the amounts of renewable oils, or TAG, can be about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, or about 10% relative to the amounts in a control sample. In some embodiments, expression of the VgDGAT1 polypeptide increases the amount of renewable oil in the plant by at least about 2 or about 3 percent as compared to an amount of renewable oil in a control plant. In other embodiments, expression of the VgDGAT1 polypeptide increases the amount of renewable oil in the plant by at least about 5 percent as compared to an amount of renewable oil in a control plant.

In some embodiments, even though the amount of renewable oil found in the plant is increased, the protein levels in the plant are substantially unchanged as compared to a control plant. In attempts to increase amounts of oil biosynthesis in plants, any increases in amounts of renewable oils are frequently accompanied by a concomitant decrease in the levels of proteins in the plants themselves, which, in turn, decreases the value of the plant as a source of protein, such as for animal feed, for human consumption, and for many industrial applications. It has been ascertained, however, that by transforming a plant cell with a nucleic acid molecule encoding a VgDGAT1 polypeptide, plants can be produced that have increased seed oil content and little to no decrease in amount of proteins in the seeds of the plants. In some embodiments, plants are produced that have both increased seed oil content and increased amounts of proteins in the seeds of the plants, relative to control plants. In some embodiments, such an increase in the oil and protein levels in the seeds of the plants allows plant meal to be produced that is considerably higher in protein, which then allows for an increase in both the quality and value of the plant meal. Of course, any methods for measuring the protein content in a sample known to those of ordinary skill in the art, including, but not limited to, methods such as mass spectrometry, can be used to measure an amount of protein in accordance with the presently-disclosed subject matter.

In some embodiments of the presently-disclosed subject matter, the VgDGAT1 polypeptide expressed in the plant is a VgDGAT1 polypeptide (see, e.g., GENBANK® Accession No. EF653276.1, which is incorporated herein by this reference). In some embodiments, the VgDGAT1 polypeptide is encoded by a nucleic acid molecule having the sequence of SEQ ID NO: 1. In some embodiments, the VgDGAT1 polypeptide comprises the amino acid sequence of SEQ ID NO: 2.
VgDGAT1b polypeptide (see, e.g., GenBank® Accession No. EF653277, which is incorporated herein by this reference). In some of these embodiments, the VgDGAT1b polypeptide is encoded by a nucleic acid molecule having the sequence of SEQ ID NO: 3. In some embodiments, the VgDGAT1b polypeptide comprises the amino acid sequence of SEQ ID NO: 4.

In some embodiments of the presently-disclosed methods for increasing renewable oil production, the methods include the further step of transforming a plant cell with an isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyltransferase 2 (VgDGAT2) polypeptide (see, e.g., GenBank® Accession No. FJ652577, which is incorporated herein by this reference; see also U.S. patent application Publication Ser. No. 12/622,045, which is also incorporated herein by reference in its entirety), where the expression of the VgDGAT1 polypeptide and the VgDGAT2 polypeptide increases an amount of renewable oil in the plant. Without wishing to be bound by any particular theory, it has been discovered that, in some embodiments, the co-expression of the VgDGAT1 polypeptide and the VgDGAT2 polypeptide increases the amount of renewable oil in the plant in a synergistic manner. In some embodiments, the VgDGAT2 polypeptide is encoded by a nucleic acid molecule having the sequence of SEQ ID NO: 5. In some embodiments, the VgDGAT2 polypeptide comprises the amino acid sequence of SEQ ID NO: 6.

In yet further embodiments of the presently-disclosed methods for increasing renewable oil production in a plant, a method is provided that includes transforming a plant cell with nucleic acid encoding a VgDGAT polypeptide and with a nucleic acid encoding an epoxygenase polypeptide as it has also been surprisingly discovered that such a co-expression results in an increase in renewable oil content in a plant. It is appreciated that certain VgDGAT polypeptides are capable of increasing the amount of epoxy fatty acids such as vernolic acid; however, it was previously thought that the observed increase in vernolic acid came at the expense of linoleic acid, which then resulted in no increase in the amount of oil in a plant. It has now been determined that certain DGAT polypeptides, including VgDGAT1 and VgDGAT2 polypeptides, are able to effectively incorporate epoxy fatty acids into TAG, making a method of co-expressing a VgDGAT1 or VgDGAT2 polypeptide with an epoxygenase polypeptide a useful means to increase an amount of renewable oil in a plant.

In some embodiments of the presently-disclosed subject matter, a method of increasing an amount of renewable oil in a plant is provided that includes transforming a plant cell with a first isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyltransferase (VgDGAT) polypeptide and a second isolated nucleic acid encoding an epoxygenase polypeptide, where the expression of the VgDGAT polypeptide and the epoxygenase polypeptide increases an amount of renewable oil in the plant as compared to the amounts of renewable oil found in a control plant. In some embodiments, the co-expression of a VgDGAT polypeptide and an epoxygenase polypeptide allows for the production of a plant where the amount of renewable oil in the plant is increased, but where the amount of protein in the plant is substantially unchanged as compared to a control plant. In some embodiments, the amount of protein in the plant co-expressing the VgDGAT polypeptide and the epoxygenase polypeptide is increased as compared to a control plant.

In some embodiments of the presently-disclosed methods that include co-expressing a VgDGAT polypeptide and an epoxygenase polypeptide, the VgDGAT polypeptide can be a VgDGAT1a polypeptide, a VgDGAT1b polypeptide, or a VgDGAT2 polypeptide, such as those polypeptides described herein above. In some embodiments, the epoxygenase polypeptide is encoded by a nucleic acid sequence of SEQ ID NO: 19. In some embodiments, the epoxygenase polypeptide is a Stokedia laevis polypeptide, such as the epoxygenase described in U.S. Pat. No. 7,364,901, which is incorporated herein by reference (see also GenBank® Accession No. EAA922, which is incorporated herein by reference).

In some embodiments of the methods for increasing renewable oil production, transforming the plant cell with a first isolated nucleic acid and a second isolated nucleic acid comprises transforming the cell with a vector that includes the first isolated nucleic acid and a vector that includes the second isolated nucleic acid. For example, in some embodiments, a nucleic acid encoding a DGAT polypeptide can be inserted into an appropriate vector as described herein and a nucleic acid encoding an epoxygenase polypeptide can be inserted into another vector. In some embodiments, each of the vectors can then be electroporated into Agrobacterium tumefaciens cells, which can then be used to transform cells with the vectors according to agro-infiltration methods known to those of ordinary skill in the art.

The term “isolated,” when used in the context of an isolated nucleic acid or an isolated polypeptide, is a nucleic acid or polypeptide that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated nucleic acid or polypeptide can exist in a purified form or can exist in a non-native environment such as, for example, in a transgenic host cell.

The term “nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally-occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified or degenerate variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated.

The term “degenerate variant” refers to a nucleic acid having a residue sequence that differs from a reference nucleic acid by one or more degenerate codon substitutions. Degenerate codon substitutions can be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed base and/or deoxyribonucleotide residues (Batzer, et al. 1991; Ohitsuka, et al. 1985; Rosolini, et al. 1994).

The terms “polypeptide,” “protein,” and “peptide,” which are used interchangeably herein, refer to a polymer of the 20 protein amino acids, or amino acid analogs, regardless of its size or function. Although “protein” is often used in reference to relatively large polypeptides, and “peptide” is often used in reference to small polypeptides, usage of these terms in the art overlaps and varies. The term “polypeptide” as used herein refers to peptides, polypeptides, and proteins, unless otherwise noted. The terms “protein”, “polypeptide” and “peptide” are used interchangeably herein when referring to a gene product. Thus, exemplary polypeptides include gene products, naturally occurring proteins, homo logs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing.

The terms “polypeptide fragment” or “fragment,” when used in reference to a reference polypeptide, refers to a polypeptide in which amino acid residues are deleted as com-
pared to the reference polypeptide itself, but where the remaining amino acid sequence is usually identical to the corresponding positions in the reference polypeptide. Such deletions can occur at the amino-terminus or carboxy-terminus of the reference polypeptide, or alternatively both. A fragment can also be a “functional fragment,” in which case the fragment retains some or all of the activity of the reference polypeptide as described herein. For example, in some embodiments, a functional fragment of a VgDGAT1 polypeptide can retain some or all of the ability of the reference polypeptide to catalyze the final acylation step during TAG synthesis, such as what had been described for other DGAT1 polypeptides (see, e.g., Siloto et al., 2009; Siloto et al., 2009; and Xu et al. 2008).

The terms “modified amino acid,” “modified polypeptide,” and “variant” refer to an amino acid sequence that is different from the reference polypeptide by one or more amino acids, e.g., one or more amino acid substitutions. A variant of a reference polypeptide also refers to a variant of a fragment of the reference polypeptide, for example, a fragment wherein one or more amino acid substitutions have been made relative to the reference polypeptide. A variant can also be a “functional variant,” in which the variant retains some or all of the activity of the reference protein as described herein. For example, a functional variant of a DGAT1 polypeptide can retain some or all of the ability of the reference polypeptide to catalyze the final acylation step during TAG synthesis.

The term functional variant also includes a functional variant of a fragment of a reference polypeptide. The term functional variant further includes conservatively substituted variants. The term “conservatively substituted variant” refers to a peptide comprising an amino acid residue sequence that differs from a reference peptide by one or more conservative amino acid substitutions, and maintains some or all of the activity of the reference peptide as described herein. A “conservative amino acid substitution” is a substitution of an amino acid residue with a functionally similar residue. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another; the substitution of one charged or polar (hydrophilic) residue for another; the substitution of one basic residue such as lysine or arginine for another; or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another; or the substitution of one aromatic residue, such as phenylalanine, tyrosine, or tryptophan for another. The phrase “conservatively substituted variant” also includes peptides wherein a residue is replaced with a chemically-derivatized residue, provided that the resulting peptide maintains some or all of the activity of the reference peptide as described herein.

In some embodiments, the vectors of the presently-disclosed subject matter are plasmids, such as the plasmid pH121 or the pCAMBIA1301 plasmid.

In some embodiments, the isolated nucleic acid included in the vector is operably linked to an expression control sequence. The terms “associated with,” “operably linked,” and “operatively linked” refer to two nucleic acid sequences that are related physically or functionally. For example, a promoter or regulatory DNA sequence is said to be “associated with” a DNA sequence that encodes an RNA or a polypeptide if the two sequences are operatively linked, or situated such that the regulator DNA sequence will affect the expression level of the coding or structural DNA sequence.

The term “expression control sequence” is used interchangeably herein with the term “expression cassette” and is used to refer to a nucleic acid molecule capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operatively linked to the nucleotide sequence of interest which is operatively linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The coding region usually encodes a polypeptide of interest but can also encode a functional RNA of interest, for example antisense RNA or a non-translated RNA, in the sense or antisense direction. The expression control sequence comprising the nucleotide sequence of interest can be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette can also be one that is naturally occurring but has been obtained in a recombinant form useful for heterologous expression.

In some embodiments, an expression control sequence is provided that comprises a “constitutive promoter,” such as a 35S promoter, a figwort mosaic promoter, or the constitutive plant promoter of ubiquitin, that continually expresses a nucleic acid sequence of the presently-disclosed subject matter in all types of cells where it is inserted. For some applications, it is useful to direct the expression of a nucleic acid sequence of the presently-disclosed subject matter to different tissues of a plant. As such, in some embodiments, an expression control sequence is provided that comprises a “seed-specific promoter,” such as a phaseolin, glycinin, conglycinin, seed lectin, napin, eriferin, or other seed-specific promoter that expresses a nucleic acid sequence of the presently-disclosed subject matter only in seeds of a desired plant.

The presently-disclosed subject matter also provides transgenic plant cells or plants that have been transformed with one or more of the vectors disclosed herein. As used herein, the term “plant cell” is understood to mean any cell derived from a monocotyledonous or a dicotyledonous plant and capable of constituting undifferentiated tissues such as calli, differentiated tissues such as embryos, portions of monocotyledonous or dicotyledonous plants, monocotyledonous or dicotyledonous plants or seed. The term “plant” is understood to mean any differentiated multi-cellular organism capable of photosynthesis, including monocotyledons and dicotyledons. In some embodiments of the methods for increasing renewable oil production in a plant, the plant is selected from: *Arachis hypogaea*, *Borago officinalis*, *Brassica campestris*, *Brassica napus*, *Brassica rapa*, *Camelina sativa*, *Cannabis sativa*, *Carthamus tinctorius*, *Cocos nucifera*, *Cranbe abys­sista*, *Cuphea species*, *Glycine max*, *Gossypium hirsutum*, *Gossypium barbadense*, *Gossypium herbaceum*, *Helianthus annuus*, *Linnun uaitatisinnum*, *Oenothera biennis*, *Oleae europea*, *Oryza sativa*, *Perilla frutescens*, *Ricinus communis*, *Salvia hispanica*, *Sesamum indicum*, *Sinapis alba*, *Theo*
through, for example, the use of site-directed mutagenesis or transformation process, but also transgenic progeny thereof. The particular host cell or, if from the same source, is modified into an appropriate vector) of the presently-disclosed plant, into which a heterologous nucleic acid molecule has been expressed to yield exogenous polypeptides.

Introduction of a nucleic acid (e.g., a nucleic acid incorporated into an appropriate vector) of the presently-disclosed subject matter into a plant cell can be performed by a variety of methods known to those of ordinary skill in the art including, but not limited to, insertion of a nucleic acid sequence of interest into an Agrobacterium rhizogenes Ri or Agrobacterium tumefaciens Ti plasmid, microinjection, electroporation, or direct precipitation. By way of providing an example, specific methods are described below. Reaction mixtures were incubated in a thermocycler (Perkin Elmer, Waltham Mass., Model 2400) for 45 minutes at 48°C, followed by 2 minutes at 94°C and 40 cycles of 30 s at 94°C, 30 s at 50°C, and 1 minute at 72°C. The PCR primers used (DGATF, 5'-CGICCCYACWTTGTTGATTATSARC-3'; SEQ ID NO: 7, and DGATR, 5'-CCAYTTRTGRACGGSAATTTCAA-3'; SEQ ID NO: 8) represent two peptide sequences, [APTLCYE/Q] and [WNI/MPVHKW] which are the conserved regions in amino acid sequences of DGATs of Arabidopsis thaliana and Mus musculus. The amplified products of approximately 380 bp were fractionated on a 1% agarose gel, extracted from the gel using Quiquick Gel Extraction Kit (Biogen Inc., Valencia, Calif.) and subcloned into the pGEM-T Easy vector (Promega Co., Madison, Wis.) according to the manufacturer's instructions. The DNA insert was sequenced in both directions.

For additional guidance regarding methods of transforming and producing transgenic plant cells, see U.S. Pat. Nos. 4,459,355; 4,536,475; 5,464,763; 5,177,010; 5,187,073; 4,945,050; 5,036,006; 5,100,792; 5,371,014; 5,478,744; 5,179,022; 5,565,346; 5,484,956; 5,508,468; 5,538,877; 5,554,798; 5,489,520; 5,510,318; 5,204,253; 5,405,765; EP Nos. 267,159; 604,662; 672,752; 442,174; 486,233; 486,234; 539,563; 674,725; and, International Patent Application Publication Nos. WO 91/02071 and WO 95/06128, each of which is incorporated herein by this reference.


The presently-disclosed subject matter is further illustrated by the following specific but non-limiting examples.

EXAMPLES

Materials and Methods for Examples 1-2
cDNA Cloning.

Partial Vernonia galamensis and Euphorbia lagascae diacylglycerol acyltransferase (DGAT) cDNA fragments were obtained from RNA of developing embryos using an Access RT-PCR System (Promega Co., Madison Wis.). The PCR mixtures contained 1 μg of total RNA template, 0.2 mM dNTPs, 2.5 U of AMV reverse transcriptase, 2.5 U of Tfl polymerase and 1 μM each of two degenerate primers described below. Reaction mixtures were incubated in a thermocycler (Perkin Elmer, Waltham Mass., Model 2400) for 45 minutes at 48°C, followed by 2 minutes at 94°C and 40 cycles of 30 s at 94°C, 30 s at 50°C, and 1 minute at 72°C. The PCR primers used (DGATF, 5'-GCICCCYACWTTGTTGATTATSARC-3'; SEQ ID NO: 7, and DGATR, 5'-CCAYTTRTGRACGGSAATTTCAA-3'; SEQ ID NO: 8) represent two peptide sequences, [APTLCYE/Q] and [WNI/MPVHKW] which are the conserved regions in amino acid sequences of DGATs of Arabidopsis thaliana and Mus musculus. The amplified products of approximately 380 bp were fractionated on a 1% agarose gel, extracted from the gel using Quiquick Gel Extraction Kit (Qiagen Inc., Valencia, Calif.) and subcloned into the pGEM-T Easy vector (Promega Co., Madison, Wis.) according to the manufacturer's instructions. The DNA insert was sequenced in both directions.

For determination of the full-length cDNA sequence, a RACE (Rapid Amplification of cDNA Ends) strategy was applied. A cDNA was synthesized from poly(A)+RNA of developing seeds of S. laves using a Smart RACE cDNA
Amplification Kit (BD Biosciences Clontech, San Jose, Calif.). The two following primers were then designed from the sequence information of the partial cDNA fragment of *Vernonia galamensis* DGAT; VerDaF: 5'-TCCAAGAAAGGT-TGGTGTTAGCAGCACTG-3' (SEQ ID NO: 11), and VerDaR: 5'-CAGTGGCGTAACACCCAAACCTTTTCC-3' (SEQ ID NO: 12), and *Escherichia coli* DGAT; Ephu5: 5'-CACTTGCAAACTGGAGAACCACCC-3' (SEQ ID NO: 13), and Ephu3: 5'-GGGTGTTCCCGTAGTGTGGC-AACTGGTG-3' (SEQ ID NO: 14). The 5'-half and 3'-half of the cDNAs were amplified using the PCR conditions described in the user manual of the kit. Fractionation of the amplified fragments, cloning and sequencing were carried out as described above.

For soybean DGAT cloning, a BLAST search of the sequence database using the *Arabidopsis* protein sequence identified soybean EST (Gen-c 1036-7949). The EST was fully sequenced in both directions. Since the EST lacked the 5' end of the cDNA, it was obtained by 5' RACE with appropriate nested primers using a Smart RACE cDNA Amplification kit (BD Biosciences Clontech, San Jose, Calif.). A cDNA was synthesized from poly(A)+RNA of developing seeds of the soybean cultivar, Jack. The following two primers were then designed from the sequence information of the cDNA of the EST; SoyD5-1: 5'-GGTGAGTGTGACCTCCCTTGGAGGATGC-3' (SEQ ID NO: 15), and SoyD3-1: 5'-GTGGCCTTCATACATGTACCAGCCAG-3' (SEQ ID NO: 16). The 5'-half and 3'-half of the cDNAs were amplified using the PCR conditions described in the user manual of the kit. In order to obtain the second possible DGAT sequence, another set of primers were designed that were; SoyD5-2: 5'-GAAAAACCGCTCGGTCTCTC-3' (SEQ ID NO: 17), and SoyD3-2: 5'-TGCTAAITGCCAG-GAGGATGGT-3' (SEQ ID NO: 18). Fractionation of the amplified fragments (1.5 kb), cloning and sequencing were carried out as described above.

Expression in Insect Cells.

The expression in Sf9 cells was tested with the Bac-to-Bac expression system (Gibco BRL, Carlsbad, Calif.), and the recombinant baculovirus was prepared following their instruction manual. Sf9 cells were then infected by the baculovirus possessing *Vernonia* or *Euphorbia* DGAT and cultured for 4 days and the cells were collected. Another set of cultured cells was infected by the baculovirus without cloned genes as a control. Their lipids were extracted with chloroform:methanol (2:1, v/v) and subsequently sprayed with primulin in lanes of silica gel TLC plates 1 cm from the bottom of the plates. The plates were put in a chamber with chloroform:methanol:water (65:25:4, v/v)+0.0001% BHT for running until the first solvent reached 1/2 the plate (~10 cm). Then, the plate was moved into the second solvent, hexane:diethyl ether:acetic acid (100:100:2, v/v)+0.0001% BHT and developed until solvent was approximately 1 cm from the top. After development, the plate was dried, and subsequently sprayed with 0.05% primulin in 80% acetone, followed by visualizing under UV light and marking the bands of interest. The bands were scraped and transferred to a Pasteur pipette with a glass wool plug washed with CHCl₃:CH₃OH. The lipid samples were eluted with 0.5 mL of CHCl₃:CH₃OH+0.001% BHT twice. Finally, eluted lipid samples were analyzed by GC as described above.

Seed-Specific Expression Vector Construction and Soybean Somatic Embryo Transformation.

An expression vector for soybean transformation was constructed using the plant expression vector pCAMBIA1301 containing the hygromycin resistance gene as a selector and the GUS gene as a reporter (Cambia, ACT, Australia; GEN-BANK® No. AF234297). The coding sequences for VgDGAT1 and VgDGAT2 were amplified by a high fidelity polymerase (Invitrogen, Carlsbad, Calif.) using end-specific primers containing restriction sites. The amplification product was then subcloned into the respective sites of pPHI4752 vector containing a phaseolin promoter, which confers strong seed-specific expression of transgenes (Slightom et al., 1983). The phaseolin promoter cassette containing the coding region of each target gene was transferred into the corresponding sites of the binary pCAMBIA1301, T-DNA vector. These recombinant expression vectors were subsequently loaded on TLC plates and the radioactive bands were detected by phosphorimaging and scintillated. For identification of radioactive products, methylated fractions were analyzed by GC with a hexane:MTBE (methyl tert-butyl ether):acetic acid (85:15:1, v/v/v) solvent system.

Lipid Analysis.

Samples prepared as described above were frozen in liquid N₂ stored at ~80°C and then lyophilized. Weighed samples were transferred to glass test tubes and tri-heptadecanoin (tri-17:0) was added at 10 μg/mg tissue as a standard. The samples were finely ground, and 1-2 mL of chloroform and methanol (2:1) containing 0.001% butylated hydroxytoluene (BHT) was added and the samples were ground further. After a brief spin, the lower layer (CHCl₃ phase) was then transferred into a new glass tube, and the samples were divided into two aliquots. One was used for GC and the other directly for GC analysis.

For GC analysis, samples were dried with N₂. 0.5 mL of 0.5 M sodium methoxide (NaOCH₃) in methanol was added and incubated for at least 15 minutes with shaking at 22°C. 0.5 mL of isooctane containing 0.001% BHT was added to each tube and mixed well. Phase separation was obtained with centrifugation or adding aqueous 0.9% KCl if needed. The top layer was extracted and transferred into GC auto-sampler vials. The fatty acid methyl esters (FAMEs) were analyzed with gas chromatography on a Varian CP-3800 GC with a 24 mmx0.25 mm ID CP-Select CB for FAME analysis using a fused silica column with a 0.25 μm film thickness. The temperature program was 90°C for 1 min., then to 155°C at 20° C/min. with no hold, then to 175°C at 3.6° C/min. with no hold and finally to 250°C at 12° C/min. holding for one min.

For separation of individual lipid classes by TLC, the samples (CHCl₃ lipid extracts) were concentrated to about 50-100 μL. Ten μL of the sample was loaded in a narrow band in lanes of silica gel 60 (Whatman LK6D Silica gel 60A) TLC plates 1 cm from the bottom of the plates. The plates were put in a chamber with chloroform:methanol:water (65:25:4, v/v)+0.0001% BHT for running until the first solvent reached 1/2 the plate (~10 cm). Then, the plate was moved into the second solvent, hexane:diethyl ether:acetic acid (100:100:2, v/v)+0.0001% BHT and developed until solvent was approximately 1 cm from the top. After development, the plate was dried, and subsequently sprayed with 0.05% primulin in 80% acetone, followed by visualizing under UV light and marking the bands of interest. The bands were scraped and transferred to a Pasteur pipette with a glass wool plug washed with CHCl₃:CH₃OH. The lipid samples were eluted with 0.5 mL of CHCl₃:CH₃OH+0.001% BHT twice. Finally, eluted lipid samples were analyzed by GC as described above.
introduced into somatic embryos of soybean (cv. 'Jack') using the particle bombardment method of transformation.

Soybean somatic embryo induction and culture was carried out using a protocol modified from prior procedures (Collins et al., 1991; Finer and Nagasawa, 1988a; Finer and Nagasawa, 1988b; Samoylov et al., 1998; Trick et al., 1997). Briefly, immature soybean seeds at 3-5 mm length were dissected, and cotyledons were placed on D40 (40 mg/L 2,4-D in MS media) solid medium for one-month induction of somatic embryo induction. The induced embryos were transferred to D20 plates for proliferation. The globular embryogenic cultures from D20 (20 mg/L 2,4-D in MS media) plates were then moved into FN (Finer and Nagasawa, 1988a) liquid medium for one-month suspension culture. Small embryo clumps were selected for particle bombardment gene delivery.

Plasmid DNA/gold preparation for the particle bombardment was conducted according to standard protocols (Trick et al., 1997). A DuPont Biolistic PDS 1000 HE instrument (helium retrofit) was used for all transformations. After bombardment the embryo clumps were transferred into FN liquid medium containing 30 mg/L hygromycin for selective culture for four to five weeks. The positive transformed embryos obtained by hygromycin selection were then moved into fresh FN liquid medium for culture and simultaneously for GUS test and identification of the transgene presence by PCR. The PCR-positive transgenic embryo lines were transferred into maturation medium (SFMaM) (Schmidt et al., 2005) for three to five weeks. Matured individual embryos were desiccated for 4-7 days, and then were placed on ½ strength MS solid medium for germination. Germinated plantlets were transferred to closed sterile soil cups for growth in a culture room under 23:1 (light:dark) photoperiod cycle and 25°C. Once seedlings reached a proper height (approximately 13 cm), the seedlings were transferred to a greenhouse for flowering and seed set under a 16:8 (light:dark) cycle, 25/21°C. For the transgenic lines, one set of matured somatic embryos were sampled for lipid extraction and subsequent GC analysis. The rest of the matured somatic embryos were desiccated, germinated and grown to maturity in a greenhouse. Mature seeds were harvested from each regenerated soybean plant separately. Seed were chipped for genotyping by PCR and fatty acid analysis by GC. TAG levels of the calibration standards of known values and adjusting the TAG levels of the calibration samples determined by combustion for protein and Soxhlet for oil (AOAC, 1995; de Castro and Priego-Capote, 2010; Rotundo et al., 2011; Soxhlet, 1879). Protein levels were calculated as total nitrogenx6.25.

Expression of Diacylglycerol Acyltransferase 1a (VgDGAT1a) in Petunia Leaves and in Soybean Somatic Embryos

To further examine the ability of VgDGAT1a to increase renewable oil production, particularly in plants, VgDGAT1a was further expressed in petunia leaves and in soybean somatic embryos. Briefly, soybean somatic embryos expressing VgDGAT1a were regenerated, grown out in a greenhouse and mature T2 seeds were collected. The protein and oil levels of subsequent generations of progeny of lines selected for higher oil contents were determined in bulk by near infrared (NIR) spectroscopy using a This NIR seed analyzer (see, e.g., Yu et al., 2008; FIG. 2).
content of the mature seeds was then measured and many of the VgDGAT1a transformed soybean seeds showed 3-5% increases in oil content per seed dry weight, most without any decrease in protein levels. Seeds of these higher oil soybean lines were grown out in both greenhouse and field environments and progeny were again analyzed for protein and oil contents. Again 3-5% increases in oil content per seed dry weight were observed and little or no decrease in protein levels was seen (FIG. 3; Table 1).

These increases in oil and oil+protein were then corroborated by oil and protein analyses of individual seeds of plants from these lines by an independent set of analyses backed by standard wet chemical determination of protein and oil levels (FIG. 4). The estimated meal protein levels of such lines were also believed to be higher making meal more valuable for animal feed, food, and many industrial applications (FIG. 5).  

To further examine the lines, and their commercial feasibility, the lines highest in oil and oil+protein were grown out in the field following standard farming conditions, and again showed stable increases in oil and oil+protein with several lines consistently about 7% higher in oil+protein than the parental line or normal high yielding soybean cultivars (FIG. 6; Table 2), indicating that expression of a VgDGAT1 polypeptide is useful for increasing an amount of renewable oil production in a plant.

Example 3

Effect of Co-Expression of *Vernonia galamensis* Diacylglycerol Acyltransferase 1 and 2 and *Stokesia laevis* Epoxygenase on Oil Content of Transgenic Plants

To assess the effect of co-expressing *Vernonia galamensis* diacylglycerol acyltransferase 1 and 2 (VgDGAT1 & 2) and *Stokesia laevis* epoxygenase (SIEPX) polypeptide in transgenic plants, transgenic plant regeneration and identification of the transgenic expressions were first performed as described in detail previously (Li et al., 2010). Briefly, the expression vector for soybean transformation was constructed using the pCAMBIA1301 vector containing a hygromycin resistance gene and the GUS gene as a reporter (Cambridge ACT, Australia; GENBANK® No. AF2534297). The ORFs of *Stokesia epoxygenase* (SIEPX) and *Vernonia* DGATs (VgDGAT1a and VgDGAT2) were each driven by a seed-specific phaseolin promoter. The construct was introduced into soybean somatic embryo cultures using a particle delivery system (Gene Gun). Positive somatic embryos after hygromycin selection culture were confirmed by PCR, and then cultured to mature soybean somatic embryos. Matured embryos were subsequently germinated in germination media, and the seedlings were transferred to soil pots for growth and production of the transgenic seeds, which were subsequently grown on a research farm for two generations.

The soybean seeds were then collected for analysis from the soybean plants grown on the farm, and protein and oil levels were analyzed with a Pertem DAT200 NIR seed analyzer with extensive calibrations for soybean seeds. The NIR levels were verified by Soxhlet gravimetric determination of lipid levels and Kjeldahl analysis of nitrogen. Each replicate was measured by three times. A total of 6 replicates were measured for each line.

It has previously been observed that additional seed-specific expression of either VgDGAT1a or VgDGAT2 in SIEPX-transgenic soybeans results in vernolic acid accumulation up to 17% and 27.8% in the seeds and normal fatty acid profiles, with the exception of a decreased 18:2 level (Li et al., 2010). Upon analysis of the results from the present experiments, however, it was surprisingly found that co-expression of VgDGAT1a or VgDGAT2 with SIEPX was capable of increasing total seed oil content in the transgenic plants. As shown in FIG. 7, the reduced oil content observed in seeds expressing the SIEPX gene alone changed markedly when either VgDGAT1a or VgDGAT2 was co-expressed with SIEPX. In these double-transgenic plants, seed oil contents were returned to normal levels (20-21%), similar to levels in the non-transgenic and the vector control soybeans, regardless of the level of vernolic acid accumulation. Furthermore, although statistical analysis (t-test) showed that the difference of seed oil level was not significant (P<0.05) between the control and the double transgenic lines, a significant difference (P<0.05) was found between the controls and SIEPX-expressing lines and it was also observed that a number of the double transgenic lines produced higher seed oil levels (22.4%). Moreover, the VgDGAT1-mediated restoration of oil levels in SIEPX-transgenic soybean seeds showed stable inheritance in two subsequent generations examined so far under field conditions.

In soybean seeds, the oil content is usually inversely correlated with protein levels (Clemente and Cahoon, 2009). However, in conjunction with a reduction in oil content in the SIEPX-transgenic seeds, it was observed that seed protein levels showed dramatic changes, increasing in some transgenic lines and decreasing in others (FIG. 8). As in the case of total oil content, however, seed protein levels in the transgenic lines co-expressing SIEPX and either of the VgDGATs were restored to normal levels (approximately 40%) regardless of higher or lower protein level in soybean seeds only expressing SIEPX. Again, no statistical difference (P<0.05) in seed protein level was found between the control and the VgDGAT-SIEPX co-expressing lines, but significant differences (P<0.05) were detected between the control and SIEPX
expressing lines and a number of the double transgenic lines showed higher seed protein levels than what was observed in the control lines. These data indicate that VgDGATs are also able to overcome the unstable seed protein accumulation caused by SIEPX and/or vernolic acid accumulation in soybean seeds, further indicating that co-expression of VgDGAT and SIEPX polypeptides are useful for increasing an amount of renewable oil in a plant.

Throughout this document, various references are mentioned. All such references are incorporated herein by reference, including the references set forth in the following list:

REFERENCES


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It will be understood that various details of the presently disclosed subject matter can be changed without departing from the scope of the subject matter disclosed herein. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

US 9,133,469 B1

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SEQUENCE LISTING

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Ser Ser Asn Met Asp Tyr Phe Tyr Asp Val Asn Phe Lys Ser Leu Val

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What is claimed is:

1. A method of increasing renewable oil production in a plant, comprising transforming a plant cell with an isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyl transferase 1 (VgDGAT1) polypeptide, wherein expression of the VgDGAT1 polypeptide increases the total amount of renewable oil in the plant relative to a control plant that is not transformed with the isolated nucleic acid encoding the VgDGAT1 polypeptide.

2. The method of claim 1, wherein the increase in the total amount of renewable oil in the plant is at least about a 2 percent increase as compared to an amount of renewable oil in the control plant.

3. The method of claim 1, wherein the increase in the total amount of renewable oil in the plant is at least about a 5 percent increase as compared to an amount of renewable oil in the control plant.

4. The method of claim 1, wherein the plant is selected from the group consisting of Arachis hypogaea, Borago officinalis, Brassica campestris, Brassica napus, Brassica rapa, Camelina sativa, Cannabis sativa, Carthamus tinctorius, Cocos nucifera, Crambe abyssinica, Cuphea species, Glycine max, Gossypium hirsutum, Gossypium barbadense, Gossypium herbaceum, Helianthus annuus, Linum usitatissimum, Oenothera biennis, Olea europaea, Oryza sativa, Perilla frutescens, Ricinus communis, Salvia species, Sesamum indicum, Sinapis alba, Theobroma cacao, Triticum species, Zea mays, Juglans species, and Prunus dulcis.

5. The method of claim 1, wherein increasing the total amount of renewable oil in the plant relative to the control plant comprises increasing the total amount of renewable oil in a seed of the plant.

What is claimed is:

1. A method of increasing renewable oil production in a plant, comprising transforming a plant cell with an isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyl transferase 1 (VgDGAT1) polypeptide, wherein expression of the VgDGAT1 polypeptide increases the total amount of renewable oil in the plant relative to a control plant that is not transformed with the isolated nucleic acid encoding the VgDGAT1 polypeptide.

2. The method of claim 1, wherein the increase in the total amount of renewable oil in the plant is at least about a 2 percent increase as compared to an amount of renewable oil in the control plant.

3. The method of claim 1, wherein the increase in the total amount of renewable oil in the plant is at least about a 5 percent increase as compared to an amount of renewable oil in the control plant.

4. The method of claim 1, wherein the plant is selected from the group consisting of Arachis hypogaea, Borago officinalis, Brassica campestris, Brassica napus, Brassica rapa, Camelina sativa, Cannabis sativa, Carthamus tinctorius, Cocos nucifera, Crambe abyssinica, Cuphea species, Glycine max, Gossypium hirsutum, Gossypium barbadense, Gossypium herbaceum, Helianthus annuus, Linum usitatissimum, Oenothera biennis, Olea europaea, Oryza sativa, Perilla frutescens, Ricinus communis, Salvia species, Sesamum indicum, Sinapis alba, Theobroma cacao, Triticum species, Zea mays, Juglans species, and Prunus dulcis.

5. The method of claim 1, wherein increasing the total amount of renewable oil in the plant relative to the control plant comprises increasing the total amount of renewable oil in a seed of the plant.

What is claimed is:

1. A method of increasing renewable oil production in a plant, comprising transforming a plant cell with an isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyl transferase 1 (VgDGAT1) polypeptide, wherein expression of the VgDGAT1 polypeptide increases the total amount of renewable oil in the plant relative to a control plant that is not transformed with the isolated nucleic acid encoding the VgDGAT1 polypeptide.

2. The method of claim 1, wherein the increase in the total amount of renewable oil in the plant is at least about a 2 percent increase as compared to an amount of renewable oil in the control plant.

3. The method of claim 1, wherein the increase in the total amount of renewable oil in the plant is at least about a 5 percent increase as compared to an amount of renewable oil in the control plant.

4. The method of claim 1, wherein the plant is selected from the group consisting of Arachis hypogaea, Borago officinalis, Brassica campestris, Brassica napus, Brassica rapa, Camelina sativa, Cannabis sativa, Carthamus tinctorius, Cocos nucifera, Crambe abyssinica, Cuphea species, Glycine max, Gossypium hirsutum, Gossypium barbadense, Gossypium herbaceum, Helianthus annuus, Linum usitatissimum, Oenothera biennis, Olea europaea, Oryza sativa, Perilla frutescens, Ricinus communis, Salvia species, Sesamum indicum, Sinapis alba, Theobroma cacao, Triticum species, Zea mays, Juglans species, and Prunus dulcis.

5. The method of claim 1, wherein increasing the total amount of renewable oil in the plant relative to the control plant comprises increasing the total amount of renewable oil in a seed of the plant.
6. The method of claim 1, wherein increasing the total amount of renewable oil in the plant relative to the control plant comprises increasing the amount of triacylglycerol (TAG) in the plant.

7. The method of claim 1, wherein a total amount of protein in the plant is about equal to a total amount of protein in the control plant.

8. The method of claim 1, wherein a total amount of protein in the plant is increased relative to the control plant.

9. The method of claim 1, wherein the VgDGAT1 polypeptide is a VgDGAT1a polypeptide.

10. The method of claim 9, wherein the VgDGAT1a polypeptide is encoded by a nucleic acid molecule having the sequence of SEQ ID NO: 1.

11. The method of claim 9, wherein the VgDGAT1a polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

12. The method of claim 1, wherein the VgDGAT1 polypeptide is a VgDGAT1b polypeptide.

13. The method of claim 12, wherein the VgDGAT1b polypeptide is encoded by a nucleic acid molecule having the sequence of SEQ ID NO: 3.

14. The method of claim 12, wherein the VgDGAT1b polypeptide comprises the amino acid sequence of SEQ ID NO: 4.

15. The method of claim 1, further comprising transforming a plant cell with an isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyltransferase 2 (VgDGAT2) polypeptide, wherein expression of the VgDGAT1 polypeptide and the VgDGAT2 polypeptide increases the total amount of renewable oil in the plant relative to a control plant that is not transformed with the isolated nucleic acid encoding the VgDGAT2 polypeptide.

16. The method of claim 15, wherein the VgDGAT2 polypeptide is encoded by a nucleic acid molecule having the sequence of SEQ ID NO: 5.

17. The method of claim 15, wherein the VgDGAT2 polypeptide comprises the amino acid sequence of SEQ ID NO: 6.

18. A method of producing a triacylglycerol (TAG), comprising transforming a cell with an isolated nucleic acid that encodes a Vernonia galamensis diacylglycerol acyltransferase 1 (VgDGAT1) polypeptide, wherein expression of the VgDGAT1 polypeptide increases the total amount of TAG in the cell relative to a control cell that is not transformed with the isolated nucleic acid encoding the VgDGAT1 polypeptide.

19. The method of claim 18, wherein the transformed cell is an animal cell, a plant cell, an algal cell, a fungal cell, or a yeast cell.

20. A transgenic plant cell comprising an isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyltransferase 1 (VgDGAT1) polypeptide, wherein expression of the VgDGAT1 polypeptide increases the total amount of renewable oil in the plant cell relative to a control cell that is not transformed with the isolated nucleic acid encoding the VgDGAT1 polypeptide.

21. The transgenic plant cell of claim 20, wherein the isolated nucleic acid is operably linked to an expression control sequence.

22. The transgenic plant cell of claim 21, wherein the expression control sequence comprises a constitutive promoter or a seed-specific promoter.

23. A method of increasing renewable oil production in a plant, comprising transforming a plant cell with a first isolated nucleic acid encoding a Vernonia galamensis diacylglycerol acyltransferase (VgDGAT) polypeptide and a second isolated nucleic acid encoding an epoxygenase polypeptide, wherein expression of the VgDGAT polypeptide and the epoxygenase polypeptide increases an amount of renewable oil in the plant.

24. The method of claim 23, wherein the VgDGAT polypeptide is a VgDGAT1a polypeptide.

25. The method of claim 24, wherein the VgDGAT1a polypeptide is encoded by a nucleic acid molecule having the sequence of SEQ ID NO: 1.

26. The method of claim 24, wherein the VgDGAT1a polypeptide comprises the amino acid sequence of SEQ ID NO: 2.

27. The method of claim 23, wherein the VgDGAT polypeptide is a VgDGAT1b polypeptide.

28. The method of claim 27, wherein the VgDGAT1b polypeptide is encoded by a nucleic acid molecule having the sequence of SEQ ID NO: 3.

29. The method of claim 27, wherein the VgDGAT1b polypeptide comprises the amino acid sequence of SEQ ID NO: 4.

30. The method of claim 23, wherein the VgDGAT polypeptide is a VgDGAT2 polypeptide.

31. The method of claim 30, wherein the VgDGAT2 polypeptide is encoded by a nucleic acid molecule having the sequence of SEQ ID NO: 5.

32. The method of claim 30, wherein the VgDGAT2 polypeptide comprises the amino acid sequence of SEQ ID NO: 6.

33. The method of claim 23, wherein the epoxygenase polypeptide is encoded by a nucleic acid sequence comprising the sequence of SEQ ID NO: 19.

34. The method of claim 23, wherein an amount of protein in the plant is substantially unchanged as compared to a control plant.

35. The method of claim 23, wherein an amount of protein in the plant is increased as compared to a control plant.