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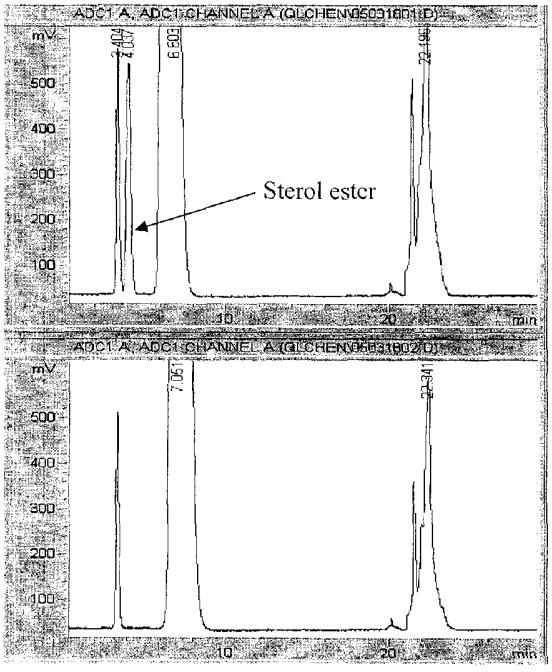

At3g51970 cDNA-transformed yeast

pYES2.1 empty vector transformed yeast

(57) Abstract: The present invention relates to the use of genetic engineering to produce sterol esters. In one embodiment, an isolated or recombinant nucleic acid molecule encoding a sterol acyltransferase is disclosed. In another embodiment, a cell transformed with the isolated or recombinant nucleic acid molecule encoding a sterol acyltransferase is disclosed. A process for producing sterol esters using the transformed cell is also disclosed. In a further embodiment; an isolated or recombinant sterol acyltransferase is disclosed.

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IDENTIFICATION OF A STEROL ACYLTRANSFERASE GENE

PRIOR APPLICATION INFORMATION

This application claims the benefit of US Provisional Application 60/666,250, filed March 30, 2005.

TECHNICAL FIELD

The present invention relates generally to biotechnology, and more particularly, to the use of genetic engineering to produce sterol esters.

BACKGROUND OF THE INVENTION

The ability of phytosterols to lower low density lipoprotein ("LDL") cholesterol in human subjects as part of a diet has been established in the medical field. Phytosterol-containing foods having therapeutic value have been approved for use by the Food and Drug Administration ("FDA") and are available on the market.

However, free phytosterols are difficult to incorporate into food stuffs due to the low solubility of the free phytosterols. Phytosterol esters can, on the other hand, be dissolved in oil at a concentration ten times higher than that of the free phytosterols. Thus, the current commercial production of phytosterol-containing foods requires a costly fatty acid acylation procedure.

Further, since phytosterol esterification processes *in planta* may represent one biochemical bottleneck that limits phytosterol biosynthesis and, hence, the amount of phytosterols produced, there exists a need for a more efficient process for the production of sterol-esters.

SUMMARY OF THE INVENTION

In one embodiment, a sterol acyltransferase gene is identified. The sterol acyltransferase gene may be expressed or overexpressed in a cell and used to enhance sterol-ester production in the cell. In another embodiment, the sterol acyltransferase gene is expressed or overexpressed *in planta* in order to enhance the production of sterol-esters in a crop. In an additional embodiment, a plant, plant seed or progeny thereof includes the sterol acyltransferase gene.

In another embodiment, the sterol acyltransferase gene may be

expressed or overexpressed in a cell and used to enhance the biosynthesis and accumulation of sterols in the cell. In as additional embodiment, the sterol acyltransferase gene is expressed or overexpressed *in planta* in order to increase the total content of sterols in a crop. In yet a further embodiment, a plant, plant seed or progeny thereof includes the sterol acyltransferase gene.

In another embodiment, a vector having a sterol acyltransferase gene is disclosed. The vector may be used to transform a cell, thus producing a recombinant cell having the sterol acyltransferase gene. The cell may comprise a bacterial cell, a yeast cell or a plant cell. In another embodiment, the cell expresses the sterol acyltransferase gene and produced a sterol acyltransferase peptide that may be isolated or purified from the cell. The isolated or purified sterol acyltransferase peptide may be used to generate antibodies having utility in diagnostics or further studies.

In yet a further embodiment, the nucleotide and deduced amino acid sequence associated with a sterol acyltransferase gene are disclosed. The sequence, or a portion of which, may be used to identify genes from other species that encodes a polypeptide with sterol acyltransferase activity. The nucleotide sequence may be used to transform a cell, thus producing a recombinant cell having the sterol acyltransferase gene. The cell may comprise a bacterial cell, a yeast cell or a plant cell.

In one embodiment, a process for producing sterol esters includes transforming a cell with a sterol acyltransferase gene. The transformed cell expresses the sterol acyltransferase gene and produces sterol esters. The sterol esters may be isolated or purified from the recombinant cell or culture media in which the cell grows, and subsequently incorporated into a composition as described herein.

In yet an additional embodiment, the sterol-esters produced with the process of the instant invention are administered in combination with the active ingredients of pharmaceutical or nutraceutical compositions such as, for example, cholesterol lowering agents. Non-limiting examples of cholesterol lowering agents include, without limitation, plant sterols, psyllium, beta glucan, niacin, guggul extract, red rice yeast extract, policosanol, garlic, fenugreek, rice bran oil, fish oil, flaxseed oil, borage oil, other omega-3-fatty acid containing oils, and combinations

of any thereof.

In an additional exemplary embodiment, the sterol-esters produced by the processes of the instant invention are incorporated in a food product, such as a beverage or a food, a multi-ingredient nutritional supplement or any combination thereof. Non-limiting examples of food products that the sterol-esters may be incorporated into include cholesterol lowering margarine, soy protein, nuts, flaxseed, olive oil, fish oil, any other oil, and combinations of any thereof.

The sterol-esters produced by the process of the instant invention can be used directly as food additives or admixed with a consumable carrier to a used as the food additive or food composition. One food additive of the invention includes the sterol-esters and a consumable carrier.

In another embodiment, an article of manufacture includes a container for .holding an amount of a composition comprising sterol-esters of the instant invention, and indicia associated with the container. The indicia are organized to guide a reader of the indicia to ingest an effective amount of the composition sufficient to help lower or reduce the cholesterol content of a subject that ingests the composition.

It is also contemplated that sterol-esters produced by the process of the instant invention may be prepared in capsule, tablet or liquid form for regular administration to help treat conditions involving high cholesterol.

In another embodiment, the sterol-esters produced by the process of the instant invention are administered as a pharmaceutical or nutraceutical composition to a subject. Such a composition includes the sterol-esters and a pharmaceutically acceptable carrier such as, for example, lactose, cellulose, or equivalent, or contained within a pharmaceutical dosage such as a capsule or tablet and may be used in combination with other pharmaceutical or nutraceutical active ingredients, or cosmetic ingredients that improve the appearance (a. e., color) of the sterol-esters.

According to a first aspect of the invention, there is provided an isolated or recombinant nucleic acid molecule encoding a plant sterol acyltransferase having at least 70% homology to SEQ ID No. 2.

According to a second aspect of the invention, there is provided a cell transformed with the isolated or recombinant nucleic acid molecule as described

above.

According to a third aspect of the invention, there is provided a process for increasing sterol ester production in a cell, the process comprising:

transforming a cell with a nucleic acid molecule encoding a sterol acyltransferase; and

growing the cell under conditions wherein said sterol acyltransferase is expressed.

According to a fourth aspect of the invention, there is provided an isolated plant sterol acyltransferase, comprising an amino acid sequence that is at least 70% homologous to SEQ ID NO: 3.

According to a fifth aspect of the invention, there is provided a plant haying a genomic knock-out of a sterol acyltransferase gene.

According to a sixth aspect of the invention, there is provided a method of identifying sterol acyltransferase genes comprising:

(a) transforming a yeast mutant deficient in sterol ester synthesis with a nucleic acid molecule comprising a plant cDNA suspected of encoding a sterol acyltransferase; and

(b) detecting sterol ester formation in said yeast, wherein presence of sterol esters indicates that said plant cDNA encodes a functional sterol acyltransferase.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a genetic map of pYES2.1/V5-His-TOPO®.

FIG. 2 is the nucleotide sequence of At3 g51970 (SEQ NO: 1).

FIG. 3 is the nucleotide sequence of the CDS of the plant sterol acyltransferase gene of *Arabidopsis* (SEQ ID NO: 2).

FIG. 4 is the predicted amino acid sequence of At3g51970 (SEQ ID NO: 3).

FIG. 5 illustrates HPLC chromatograms demonstrating the production of sterol-esters in cells transformed with At3g51970.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

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 invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

In one embodiment, a sterol acyltransferase gene is identified with a yeast complementation approach in combination with high performance liquid chromatography (HPLC) analysis of neutral lipids in a yeast extract. Specifically, the genomic sequence of the sterol acyltransferase gene is shown in Figure 2 (SEQ ID No. 1), the coding sequence of the acyltransferase gene is shown in Figure 3 (SEQ ID No. 2) and the amino acid sequence of the acyltransferase enzyme is shown in Figure 4 (SEQ ID No. 3).

A class of Membrane Binding O-Acyltransferase motif-containing genes may be found in *Arabidopsis* based on interrogation of a genomic database, combined with an assumption that phytosterol acyltransferase is a membrane-bound acyltransferase. Since not all of the cDNAs are expected to encode a functional sterol acyltransferase, a biochemical functional characterization of the gene products is performed in a yeast strain defective in sterol ester production to discover the sterol acyltransferase gene. The members of cDNAs of the genes found with the interrogation are obtained by RT- PCR using seedling and/or silique RNA as a template. The cDNAs are cloned into a yeast expression vector such as, for example, the readily and commercially available plasmid pYES2.1/V5-His-TOPO® (FIG. 1), and the plasmids having the cDNAs are introduced into a yeast mutant strain such as for example, *are1are2* that is deficient in sterol ester synthesis.

For selection, the transformed yeast is cultured in dropout SC medium that is *–his-leu-ura* at 28°C for 2 days. The yeast expression vector carries a URA gene and the double mutant yeast itself is able to synthesize histidine and leucine. The yeast transformants, upon being induced by galactose, are subjected to normal-phase HPLC analysis of neutral lipid extracts to detect the distinct peak that corresponds to the sterol ester. If a peak appears at the very retention time of sterol ester, the gene whose products possess a function of acylating sterol is identified to produce sterol esters. As will be appreciated by one of skill in the art,

other suitable mutants or combinations of mutants that are defective or deficient in sterol acylation may be used instead of are1are2.

In another aspect of the invention, there is provided a method of identifying sterol acyltransferase genes comprising:

(a) transforming a yeast mutant deficient in sterol ester synthesis with a nucleic acid molecule comprising a plant cDNA suspected of encoding a sterol acyltransferase; and

(b) detecting sterol ester formation in said yeast, wherein presence of increased sterol ester levels compared to a control indicates that said plant cDNA encodes a functional sterol acyltransferase.

As will be appreciated by one of skill in the art, the control may be an untransformed, mock transformed or vector transformed control and the control does not necessarily need to be repeated each time.

In those embodiments wherein the mutant is defective in sterol acylation, for example, are1are2, it is noted that simply the presence of sterol esters indicates that said plant cDNA encodes a functional sterol acyltransferase and no control is necessary.

In another embodiment, nucleotides that are at least 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94% or 95% identical over their entire length to the polynucleotide encoding sterol acyltransferase of instant invention, that is, to SEQ ID No. 1 or SEQ ID No. 2, and polynucleotides that are complementary to such polynucleotides are disclosed.

In other embodiments, polynucleotides that encode polypeptides having substantially the same function as the sterol acyltransferase disclosed herein are disclosed. Conservative substitutions are specifically included.

In yet other embodiments, purified or isolated plant sterol acyltransferases comprising an amino acid sequence that is at least 60%, 65%, 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90% or 95% homologous to SEQ ID No. 3 are disclosed. As will be appreciated by one of skill in the art, 'isolated' refers to polypeptides that have been 'isolated' from their native environment, in this case, from a plant cell, while 'purified' does not necessarily refer to absolute purity but rather refers to at least a

2, 3, 4 or 5 fold increase in purity. It is further of note that methods for identification of such plant sterol acyltransferase are described herein.

In yet a further embodiment, polynucleotides that hybridize to above disclosed sequences are disclosed. The hybridization conditions may be stringent in that hybridization will occur if there is at least a 90%, 95% or 97% identity with the polynucleotide that encodes the sterol acyltransferase of the instant invention. The stringent conditions may include those used for known Southern hybridizations such as, for example, incubation overnight at 42^0C in a solution having 50% formamide, 5xSSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5xDenhardt's solution, 10% dextran sulfate, and 20 micrograms/milliliter denatured, sheared salmon sperm DNA, following by washing the hybridization support in 0.1xSSC at about 65°C. Other known hybridization conditions are well known and are described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor, N.Y. (2001), incorporated.herein in its entirety by this reference. In another embodiment, the present invention is directed towards homologs of the sterol acyltransferase gene described herein obtained from other organisms such as, for example, plants. Such homologs of the sterol acyltransferase gene described herein may be obtained by screening appropriate libraries that include the homologs, wherein the screening is performed with the nucleotide sequence of the plant sterol acyltransferase gene of the instant invention or portions or probes thereof.

In yet an additional embodiment, the nucleotide sequence of the sterol acyltransferase gene (FIG. 2, SEQ ID No. 1), the coding region (FIG. 3, SEQ ID No. 2), or the predicted amino acid sequence (FIG. 4, SEQ ID No. 3) of the instant invention may be used to search for homologous sequences using computer programs designed to search for homologous sequences. For instance, readily commercially available computer programs that may be used for such searches include without limitation, BLASTN, BLASTX and TBLASTX which may be used to search for nucleotide sequences, and BLASTP and TBLASTN which may be used to search for amino acid sequences. Such computer programs are readily accessible at the web-site www.ncbi.nlm.nih.gov.

In yet a further embodiment, a nucleotide sequence that codes for a sterol acyltransferase is transformed into a plant. As known in the art, there are a

number of ways by which genes and gene constructs can be introduced into plants, and a combination of plant transformation and tissue culture techniques have been successfully integrated into effective strategies for creating transgenic crop plants. These methods, which can be used in the present invention, have been described elsewhere (Potrykus, 1991; Vasil, 1994; Walden and Wingender, 1995; Songstad et al., 1995), and are well known to persons skilled in the art. For example, one skilled in the art will certainly be aware that, in addition to *Agrobacterium*-mediated transformation of *Arabidopsis* by vacuum infiltration (Bechtold et at., 1993) or wound inoculation (Katavic et al., 1994), it is equally possible to transform other plant and crop species, using *Agrobacterium* Ti-plasmid-mediated transformation (*e.g.*, hypocotyl (DeBlock et al., 1989) or cotyledonary petiole (Moloney et al., 1989) wound infection), particle bombardment/biolistic methods (Sanford et al., 1987; Nehra. et al., 1994; Becker et al., 1994) or polyethylene glycol-assisted, protoplast transformation (Rhodes et al., 1988; Shimamoto et al., 1989) methods.

As will also be apparent to persons skilled in the art, and as described elsewhere (Meyer, 1995; Dada et al., 1997), it is possible to utilize plant promoters to direct any intended up- or down-regulation of transgene expression using constitutive promoters (*e.g.*, those based on CaMV35S), or by using promoters which can target gene expression to particular cells, tissues (*e.g.*, napin promoter for expression of transgenes in developing seed cotyledons), organs (*e.g.*, roots), to a particular developmental stage, or in response to a particular external stimulus (*e.g.*, heat shock).

Plants that may be modified or used for sterol ester production according to the instant invention include, without limitation, borage (*Borago* spp.), Canola, castor (*Ricinus communis*); cocoa bean (*Theobroma cacao*), corn (*Zea mays*), cotton (*Gossypium* spp), *Crambe* spp., *Cuphea* spp., flax (*Linum* spp.), *Lesquerella* and *Limnanthes* spp., *Linola*, nasturtium (*Tropaeolum* spp.), *Oeanothera* spp., olive (*Olea* spp.), palm (.*Elaeis* spp.), peanut (*Arachis* spp.), rapeseed, safflower (*Carthamus* spp.), soybean (*Glycine* and *Soja* spp.), sunflower (*Helianthus* spp.), tobacco (*Nicotiana* spp.), *Vernonia* spp,, wheat (*Triticum* spp.), barley (*Hordeum* spp.), rice (*Oryza* spp.), oat (*Avena* spp.) sorghum (*Sorghum* spp.), rye (*Secale* spp.) or other members of the *Gramineae*. It will further be

apparent by those of ordinary skill in the art that genomic or sequence libraries of each of these plants may be screened with the nucleotide or amino acid sequences of the instant invention for other sequences that encode or are homologous to sequences associated with the plant sterol acyltransferase of the instant invention.

As will be appreciated by one of skill in the art, the level of sterol-esters in plants are typically about 0.5% of total oil in seeds. As such, a transgenic plant comprising a non-native sterol acyltransferase gene as discussed herein will produce seeds having above 0.5% sterol-esters.

In an additional embodiment, knock-out mutants of plans are constructed. The plants are constructed by knocking-out the nucleotide sequence in the genome of the plants that codes for a sterol acyltransferase that is homologous to the sterol acyltransferase gene of the instant invention using known techniques.

In another embodiment; plants transformed with a nucleotide sequence of the instant invention that codes for a sterol acyltransferase and the knock-out mutants are studied for the impact of expressing the transformed nucleotide sequence that codes for the sterol acyltransferase or the lack of expression of the nucleotide sequence that codes for the sterol acyltransferase in knock-out mutants.

In yet a further embodiment, plants transformed with a nucleotide sequence of the instant invention that codes for a sterol acyltransferase are grown. Seeds of the transgenic plants are harvested and sterol-esters of the seeds are extracted. The extracted sterol esters are used for subsequent incorporation into a pharmaceutical composition, a nutraceutical composition or a food composition.

The invention will now be further explained and illustrated by way of examples; however, it is to be understood that the examples do not necessarily limit the invention and are primarily for illustrative purposes.

Example I.

The *Arabidopsis* cDNA corresponding to the gene encoding a sterol acyltransferase, *i.e.*, At3g51970 (FIG. 2), was cloned by RT-PCR using the commercial kit of *SuperScript First-Strand Synthesis System for RT-PCR,*

commercially available from Invitrogen. The cDNA was sequenced and inserted into the vector pYES2.1/V5-His-TOPO® (FIG. 1), commercially available from Invitrogen and used according to the manufacture's protocol. The vector having the inserted At3g51970 cDNA was transformed into the yeast *are1/are2* mutant using the established procedure of Small-Scale Yeast Transformation as described in the pYES2.1/V5-His-TOPO® manual from Invitrogen. Neutral lipid extracts of the yeast were subjected to normal-phase HPLC analysis to assay for the production of sterol esters in the yeast. The top panel of FIG. 5 illustrates the production of sterol esters in the yeast transformed with At3g51970, while the vector lacking At3g51970, *i.e.*, pYES2.1 served as a negative control which lacked the sterol esters as illustrated in the bottom panel of FIG. 5.

The *Arabidopsis* gene, At3g51970, is a novel plant sterol acyltransferase gene and the full-length coding sequence is shown in FIG. 3. The amino acid sequence of At3g51970 is shown in FIG. 4.

Example II Identification of a Brassica sterol acyltransferase gene.

The nucleotide and deduced. amino acid sequence information from *Arabidopsis* is used to search against Brassica genomic, cDNA and/or Expressed Sequence Tag information to identify a sterol acyltransferase gene from other *Brassica* species, *i.e.*, *Brassica napus*. In another embodiment, the gene and/or the cDNA of At3g51970 is us as a labeled probe to carry out nucleotide hybridization to identify genes encoding sterol acyltransferase, In yet another embodiment, the polypeptide that is produced or generated according to sequence information of At3g51970 is used to generate antibody that is used to s for cDNA library for a sterol acyltransferase cDNA.

Example III

Transformation of a plant with plant sterol acyltransferase gene.

The transformation protocol is adapted from that described by Bechtold et. al., (1993). Plants of *Arabidopsis thaliana* ecotype Columbia are grown in moist soil at a density of 10-12 plants per pot, in 4-inch square pots, and are covered with a nylon screen fixed in place with an elastic band. When the plants reach the stage at which bolts emerge, plants are watered, the bolts and

some of the leaves are clipped, and the plants are infiltrated in *Agrobacterium* suspension as outlined below.

Agrobacterium transformed with the sterol acyltransferase gene of the instant invention is grown in a 25 mL suspension in LB medium containing kanamycin at a concentration of 50 µg/mL. The *Agrobacterium* is cultured for two to three days. The day before infiltration, this "seed culture" is added to 400 mL of LB medium containing 50 µglmL kanamycin. When the absorbance at 600 nm is >2.0, the cells are harvested by centrifugation (5,000 times g, 10 min in a GSA rotor at room temperature) and are re-suspended in 3 volumes of infiltration medium (1/ .times Murashige and Skoog salts, 1 times. B5 vitamins, 5.0% sucrose, 0.044 µM benzylaminopurine) to an optical density at 600 nm of 0.8. The Agrobacterium suspension is poured into a beaker and the potted plants are inverted into the beaker so that the bolts and entire rosettes are submerged. The beaker is placed into a large Bell jar and a vacuum is drawn using a vacuum pump, until bubbles form on the leaf and stem surfaces and the solution starts to bubble a bit, and the vacuum is rapidly released. The necessary time and pressure vanes from one lab setup to the next; but good infiltration is visibly apparent as uniformly darkened, water-soaked tissue. Pots are removed from the beaker, are laid on their side in a plastic tray and are covered with a plastic dome, to maintain humidity. The following day, the plants are uncovered, set upright and are allowed to grow for approximately four weeks in a growth chamber under continuous light conditions as described by Katavic et al., (1995). When the siliques are mature and dry, seeds are harvested and selected for positive transformants.

Example IV Selection of Putative Transformants (Transgenic plants) and Growth and Analysis of Transgenic Plants.

Seeds are harvested from vacuum-infiltration transformation procedures, and are sterilized by treating for 1 min in ethanol and 5 min in. 50% bleach/0.05% Tween 20™ in sterile distilled water. The seeds are rinsed several times with sterile distilled water. Seeds are plated by re-suspending them in sterile 0.1% agarose at room temperature (about 1 mL agarose for every 500-1000 seeds), and applying a volume equivalent to about 2,000-4,000 seeds onto 150.times.15 mm selection plates (1/2.times.Murashige and Skoog salts, 0.8%

agar, autoclave, cool and add 1.times.B5 vitamins and kanamycin at a final concentration of 50 µg/mL). The plates are dried in a laminar flow hood until seed no longer flows when the plates are tipped. The plates are vernalized for two nights at 4°C in the dark, and are moved to a growth chamber (conditions as described by Katavic et al., 1995). After 7-10 days, transformants are clearly identifiable as dark green plants with healthy green secondary leaves and roots that extend over and into the selective medium.

Seedlings are transplanted to soil, plants are grown to maturity and mature seeds (T_2 generation as defined in Katavic et al., 1994) are collected and analyzed. T_2 seeds are propagated. The vegetative growth patterns are monitored by measuring shoot tissue dry weights, and/or by counting the number of rosette leaves present by the time plants began to enter the generative (flower initiation) stage. Floral initiation (beginning of generative phase of growth) is analyzed by recording, on a daily basis, the percentage of plants in which a flower bud first appears and/or the percentage of plants that are bolting (as described by Zhang et al. 1997). Data is reported in terms of percentage of plants flowering/bolting on a given day after planting (d.a.p.).

Example V. Analysis of sterol esters.

Cells or plants transformed with the sterol acyltransferase gene of the instant invention are grown to maturity and mature seeds are harvested. Neutral lipids are extracted from the cells or plants transformed with the sterol acyltransferase gene. The neutral lipids are subjected to normal-phase HPLC analysis to assay for the production of sterol esters in the transformed cells or plants.

While the preferred embodiments of the invention have been described above, it will be recognized and understood that various modifications may be made therein, and the appended claims are intended to cover all such modifications which may fall within the spirit and scope of the invention.

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CLAIMS

1. An isolated or recombinant nucleic acid molecule encoding a plant sterol acyltransferase having at least 70% homology to SEQ ID No. 2.

2. A vector comprising the isolated or recombinant nucleic acid molecule of claim 1.

3. A plant; plant seed or progeny thereof having a genome transformed with the isolated or recombinant nucleic acid molecule of claim 1 or the vector of claim 2.

4. The plant, plant seed or progeny thereof of claim 3, wherein the plant, plant seed or progeny thereof is of a species selected from the group consisting of borage (*Borago* spp.), Canola, castor (*Ricinus communis*); cocoa bean (*Theobroma cacao*), corn (*Zea mays*), cotton (*Gossypium* spp), *Crambe* spp., *Cuphea* spp., flax (*Linum* spp.), *Lesquerella* and *Limnanthes* spp., *Linola*, nasturtium (*Tropaeolum* spp.), *Oeanothera* spp., olive (*Olea* spp.), palm (.*Elaeis* spp.), peanut (*Arachis* spp.), rapeseed, safflower (*Carthamus* spp.), soybean (*Glycine* and *Soja* spp.), sunflower (*Helianthus* spp.), tobacco (*Nicotiana* spp.), *Vernonia* spp,, wheat (*Triticum* spp.), barley (*Hordeum* spp.), rice (*Oryza* spp.), oat (*Avena* spp.) sorghum (*Sorghum* spp.), rye (*Secale* spp.) or other members of the *Gramineae*.

5. The isolated or recombinant nucleic acid molecule of claim 1 wherein the nucleic acid molecule comprises SEQ ID NO: 1 or SEQ ID No. 2.

6. A cell transformed with the isolated or recombinant nucleic acid molecule of claim 1or 5.

7. The cell of claim 6, wherein the cell is a bacterial cell or a yeast cell.

8. A process for increasing sterol ester production in a cell, the process comprising:

transforming a cell with a nucleic acid molecule encoding a sterol acyltransferase; and

growing the cell under conditions wherein said sterol acyltransferase is expressed.

9. The process of claim 8, wherein the sterol acyltransferase comprises an amino acid sequence that is at least 70% homologous to the amino acid sequence of SEQ ID NO: 3.

10. The process of claim 8 or claim 9, wherein the nucleic acid molecule comprises a nucleotide sequence that is at lest 70% homologous to SEQ ID NO: 1 or 2.

11. The process of any one of claims 8-10, further comprising isolating or purifying sterol esters from the cell.

12. The process of claim 11, further comprising admixing the isolated or purified sterol esters with a food composition or a pharmaceutically acceptable carrier.

13. The process of claim. 11, further comprising admixing the isolated or purified sterol esters with a cholesterol lowering agent selected from the group consisting of plant sterols, psyllium, beta glucan, niacin, guggul extract, red rice yeast extract, policosanol, garlic, fenugreek, rice bran oil, fish oil, flaxseed oil, borage oil, other omega-3-fatty acid containing oils, and. combinations of any thereof.

14. The process of any one of claims 8-13, wherein the cell is a bacterial cell, a yeast cell or a plant cell.

15. The process of any one of claims 8-10, further comprising isolating the sterol acyltransferase.

16. An isolated plant sterol acyltransferase, comprising an amino acid sequence that is at least 70% homologous to SEQ ID NO: 3.

17. A plant haying a genomic knock-out of a sterol acyltransferase gene.

18. A a method of identifying sterol acyltransferase genes comprising:

(a) transforming a yeast mutant deficient in sterol ester synthesis with a nucleic acid molecule comprising a plant cDNA suspected of encoding a sterol acyltransferase; and

(b) detecting sterol ester formation in said yeast, wherein presence of sterol esters indicates that said plant cDNA encodes a functional sterol acyltransferase.

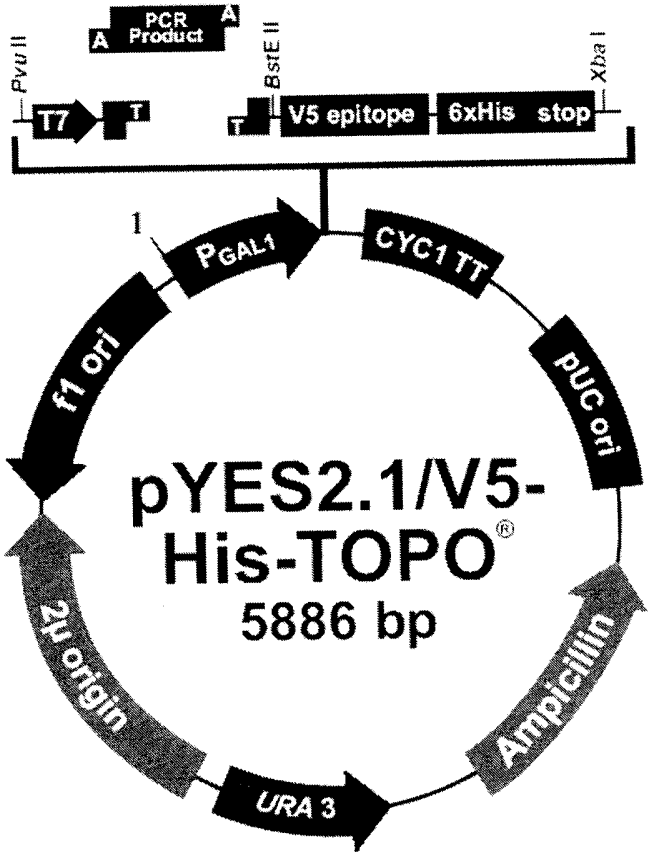

PCR Product
A
A
Pvu II
BstE II
Xba I
T7
T
T
V5 epitope
6xHis
stop
1
PGAL1
CYC1 TT
pUC ori
Ampicillin
URA 3
2µ origin
f1 ori
pYES2.1/V5-His-TOPO®
5886 bp

FIGURE 1

```
   1 ATGGCGAGTT TCATCAAGGC ATGGGGTTTA GTGATCATCT CACTGTGTTA
  51 CACTTTTTTC ATTGCCAAAT TGGTTCCAAA AGGAATCAAA AGGCTCATAC
 101 TATTTTTCCC TGTCTTCCTC ATTTTCTTCA TAGTACCTTT CTTGATATAT
 151 TCCTTACATT TACTCGGCAT CACGGCTTTC TTCATCGCTT GGCTAGCAAA
 201 TTTCAAGCTC TTATTATTTG CATTAGGGCG CGGTCCTCTC TCTTCAAACC
 251 ATAAACCCCT ATCTCTCCCT ATTTTCTTAG CTGTCTCTTG CTTGCCCATC
 301 AAGATTCAGC TGAGCCCAAA ACCTACAAAA ACTCACTCCC ATGAAGGATC
 351 CACAGAGGGT CCTTTGATTT ATACCATAAA GGCAGTTTTT GTGGTTCTCA
 401 TCATCAAAGC CTACGAATAC AGTACCAAAT TGCCTGAGAA AGTCGTGCTG
 451 ACTCTCTACG CGATCCACAT ATATTTCGCC CTTGAGATCA TCCTTGCCGC
 501 CACAGCTGCT GCGGTTCGAG CCATGTCGGA TCTTGAGCTC GAGCCACAGT
 551 TCAACAAGCC GTACCTAGCG ACATCACTTC AAGATTTCTG GGGGAGACGA
 601 TGGAACCTGA TGGTCACTGG AATCTTACGG CCAACCGTGT ACGAACCGTC
 651 ACTTCAACTG TTCTCGGTTT TGGGCCCGAA CTATTCCCAG ATTCTTGCAG
 701 CTTTCGGGAC GTTTGTTGTC TCTGGGATAA TGCACGAGCT CATCTTCTTC
 751 TACATGGGAC GGTTGAGGCC AGACTGGAAG ATGATGTGGT TCTTCCTCAT
 801 AAATGGATTT TGCACGACCG TGGAGATCGC CATCAAGAAA ACCATTAACG
 851 GTAGGTGGAG ATTCCCGAAA GCAATCAGTC AGGTTTTGAC ACTCACTTTT
 901 GTGATGGTGA CGGCATTGTG GCTGTTCTTG CCCGAATTTA ATCGGTGCAA
 951 CATAGTTGAG AAGGCTCTTG ATGAGTACGC AGCCATAGGC GCATTTGCAG
1001 TCGAGGTCAG GAGGAAACTG ACCGCATATC TTTTTTAACC CTGCCGTGTG
1051 ACAAAGCTTG AGTGATCTCT ATTTTAACTT ATTTTTTTCT AACACTGTAA
1101 AA
```

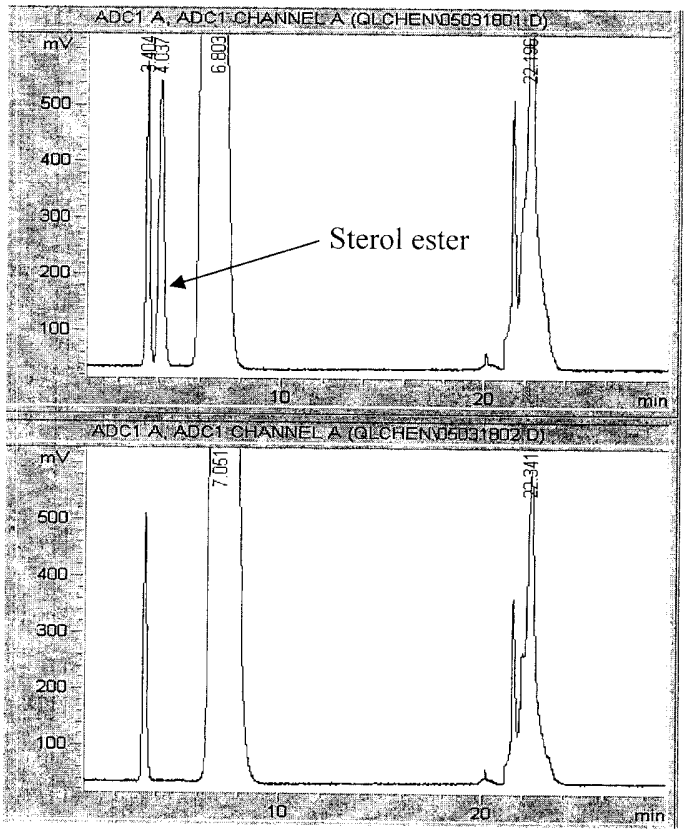
Figure 2 (SEQ ID No. 1)

```
   1 ATGGCGAGTT TCATCAAGGC ATGGGGTTTA GTGATCATCT CACTGTGTTA
  51 CACTTTTTTC ATTGCCAAAT TGGTTCCAAA AGGAATCAAA AGGCTCATAC
 101 TATTTTTCCC TGTCTTCCTC ATTTTCTTCA TAGTACCTTT CTTGATATAT
 151 TCCTTACATT TACTCGGCAT CACGGCTTTC TTCATCGCTT GGCTAGCAAA
 201 TTTCAAGCTC TTATTATTTG CATTAGGGCG CGGTCCTCTC TCTTCAAACC
 251 ATAAACCCCT ATCTCTCCCT ATTTTCTTAG CTGTCTCTTG CTTGCCCATC
 301 AAGATTCAGC TGAGCCCAAA ACCTACAAAA ACTCACTCCC ATGAAGGATC
 351 CACAGAGGGT CCTTTGATTT ATACCATAAA GGCAGTTTTT GTGGTTCTCA
 401 TCATCAAAGC CTACGAATAC AGTACCAAAT TGCCTGAGAA AGTCGTGCTG
 451 ACTCTCTACG CGATCCACAT ATATTTCGCC CTTGAGATCA TCCTTGCCGC
 501 CACAGCTGCT GCGGTTCGAG CCATGTCGGA TCTTGAGCTC GAGCCACAGT
 551 TCAACAAGCC GTACCTAGCG ACATCACTTC AAGATTTCTG GGGGAGACGA
 601 TGGAACCTGA TGGTCACTGG AATCTTACGG CCAACCGTGT ACGAACCGTC
 651 ACTTCAACTG TTCTCGGTTT TGGGCCCGAA CTATTCCCAG ATTCTTGCAG
 701 CTTTCGGGAC GTTTGTTGTC TCTGGGATAA TGCACGAGCT CATCTTCTTC
 751 TACATGGGAC GGTTGAGGCC AGACTGGAAG ATGATGTGGT TCTTCCTCAT
 801 AAATGGATTT TGCACGACCG TGGAGATCGC CATCAAGAAA ACCATTAACG
 851 GTAGGTGGAG ATTCCCGAAA GCAATCAGTC AGGTTTTGAC ACTCACTTTT
 901 GTGATGGTGA CGGCATTGTG GCTGTTCTTG CCCGAATTTA ATCGGTGCAA
 951 CATAGTTGAG AAGGCTCTTG ATGAGTACGC AGCCATAGGC GCATTTGCAG
1001 TCGAGGTCAG GAGGAAACTG ACCGCATATC TTTTTTAA
```

Figure 3 (SEQ ID No. 2)

```
  1 MASFIKAWGL VIISLCYTFF IAKLVPKGIK RLILFFPVFL IFFIVPFLIY
 51 SLHLLGITAF FIAWLANFKL LLFALGRGPL SSNHKPLSLP IFLAVSCLPI
101 KIQLSPKPTK THSHEGSTEG PLIYTIKAVF VVLIIKAYEY STKLPEKVVL
151 TLYAIHIYFA LEIILAATAA AVRAMSDLEL EPQFNKPYLA TSLQDFWGRR
201 WNLMVTGILR PTVYEPSLQL FSVLGPNYSQ ILAAFGTFVV SGIMHELIFF
251 YMGRLRPDWK MMWFFLINGF CTTVEIAIKK TINGRWRFPK AISQVLTLTF
301 VMVTALWLFL PEFNRCNIVE KALDEYAAIG AFAVEVRRKL TAYLF
```

Figure 4 (SEQ ID No. 3)



At3g51970 cDNA-transformed yeast

pYES2.1 empty vector transformed yeast

FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2006/000476

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C12N 15/54*** (2006.01) , ***A01H 5/10*** (2006.01) , ***A01H 5/00*** (2006.01) , ***C12N 1/15*** (2006.01) , ***C12N 1/21*** (2006.01) , ***C12N 15/63*** (2006.01)***C12N 15/82*** (2006.01) , ***C12N 9/10*** (2006.01) , ***C12P 7/02*** (2006.01) , ***C12Q 1/48*** (2006.01) , ***C12Q 1/68*** (2006.01)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***C12N 15/54*** (2006.01) , ***A01H 5/10*** (2006.01) , ***A01H 5/00*** (2006.01) , ***C12N 1/15*** (2006.01) , ***C12N 1/21*** (2006.01) , ***C12N 15/63*** (2006.01), ***C12N 15/82*** (2006.01) , ***C12N 9/10*** (2006.01) , ***C12P 7/02*** (2006.01) , ***C12Q 1/48*** (2006.01) , ***C12Q 1/68*** (2006.01)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
WEST; Delphion; STN (Biosis); Scopus; Canadian Patent Database; GenomeQuest; Keywords: sterol acyltransferase; plant; sterol ester(s);

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO02/10210 A2 (BAYER AKTIENGESELLSCHAFT (DE)) 7 February 2002	1-7, 16
A	- SEQ ID NO: 1840	8-15, 17, 18
X	TOWN, C.D. et al. Arabidopsis thaliana chromosome 3 genomic sequence. GenBank nucleotide sequence database. Bethesda, MD:	1-7, 16
A	National Center for Biotechnology Information, 16 September 2003 [retrieved on 29 June 2006]. Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=18409462>, accession number NM_115056.	8-15, 17, 18

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier 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
29 June 2006 (29-06-2006)	02 August 2006 (02-08-2006)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001(819)953-2476	Michael W. De Vouge (819) 997-2952

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TOTOKI, Y. et al. Large-scale analysis of RIKEN Arabidopsis full-length (RAFL) cDNAs. GenBank nucleotide sequence database. Bethesda, MD:	1-7, 16
A	National Center for Biotechnology Information, 9 September 2004 [retrieved on 15 June 2006]. Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=51968421>, accession number AK175140.	8-15, 17, 18
X	WO01/16308 A2 (MONSANTO TECHNOLOGY LLC (US)) 8 March 2001 - pages 29, 42; Examples 4, 6, 7, 9; claims 79-120	8, 11-15, 17
A		1-7, 9, 10, 16, 18
A	WO02/061072 A2 (MONSANTO TECHNOLOGY LLC (US)) 8 August 2002	1-18

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2006/000476

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO0210210 A2	07-02-2002	AU8984301 A	13-02-2002
WO0116308 A2	08-03-2001	AU7092900 A	26-03-2001
		BR0014154 A	07-05-2002
		CA2381901 A1	08-03-2001
		CN1372599 A	02-10-2002
		EP1210417 A2	05-06-2002
		JP2003508052T T	04-03-2003
		MXPA02002248 A	30-09-2002
		ZA200201410 A	06-06-2003
WO02061072 A2	08-08-2002	BR0206292 A	29-06-2004
		CA2433532 A1	08-08-2002
		EP1381267 A2	21-01-2004
		US6822142 B2	23-11-2004
		US2005086713 A1	21-04-2005

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2006/000476

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [ ] Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :

2. [ ] Claim Nos. :
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :
because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

- as listed on **Extra Sheet**

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

An *a posteriori* analysis has concluded that sterol acyltransferase genes are known in the art. For this reason, broadly recited sterol acyltransferase genes cannot represent the special technical feature that serves to unify the claims.

Consequently, the claims are directed to a plurality of inventive concepts as follows:

Invention I: claims 1-7, 9, 10, 11-15 (partial), 16;
plant sterol acyltransferase nucleic acid sequence having >70% "homology" to SEQ ID NO:1 or 2; plant sterol acyltransferase amino acid sequence having 70% "homology" to SEQ ID NO:3; process for increasing sterol production in a cell using gene of claim 1.

Invention II: claims 8, 11-15 (partial);
process for increasing sterol production in a cell.

Invention III: claim 17;
plant having genomic knock-out of a sterol acyltransferase gene.

Invention IV: claim 18;
method of identifying sterol acyltransferase genes.