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(54) Title: ENHANCED ACYLTRANSFERASE POLYNUCLEOTIDES, POLYPEPTIDES, AND METHODS OF USE

(57) Abstract: The invention provides modified DGAT1 proteins that are modified in the N-terminal region upstream of the acyl-Co A binding site. The modified DGAT proteins show enhanced activity, without reduced protein accumulation when expressed in cells. The modified DGAT1 proteins of the invention can be expressed in cells to increase cellular lipid accumulation and/or modify the cellular lipid profile. The invention also provides polynucleotides encoding the modified DGAT1 proteins, cells and compositions comprising the polynucleotides or modified DGAT proteins, and methods using the modified DGAT1 proteins to produce oil.

Enhanced acyltransferase polynucleotides, polypeptides, and methods of use

TECHNICAL FIELD

The invention relates to compositions and methods for the manipulation of cellular lipid production and/or cellular lipid profile.

BACKGROUND

Plant oil is an economically important product not only due to its broad utilization in the food industry and as a component of feed ingredients but it also has a wide range of applications as biofuels or in the manufacture of various nutraceutical and industrial products. Within the plant itself, oil is essential to carry out a number of metabolic processes which are vital to growth and development particularly during seed germination and early plant growth stages. Considering its value, there is a growing research interest within the biotechnology field to improve plant oil production and make the supply more sustainable.

The major component of plant oil is triacylglyceride (TAG). It is the main form of storage lipid in oil seeds and the primary source of energy for seed germination and seedling development. TAG biosynthesis via the Kennedy pathway involves sequential acylation steps starting from the precursor *sn*-glycerol-3-phosphate (G3P). Firstly, G3P is esterified by an acyl-CoA to form *lyso*phosphatidic acid (LPA) in a reaction catalyzed by glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15). This is followed by a second acylation step catalyzed by *lyso*phosphatidic acid acyltransferase (LPAT; EC 2.3.1.51) forming phosphatidic acid (PA), a key intermediate in the biosynthesis of glycerolipids. The PA is then dephosphorylated by the enzyme phosphatidic acid phosphatase (PAP; EC3.1.3.4) to release the immediate precursor for TAG, the *sn*- 1,2-diacylglycerol (DAG). Finally, DAG is acylated in the *sn-3* position by the enzyme diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) to form TAG.

Since this last catalytic action is the only unique step in TAG biosynthesis, DGAT is termed as the committed triacylglycerol-forming enzyme. As DAG is located at the branch point between TAG and membrane phospholipid biosyntheses, DGAT potentially plays a decisive role in regulating the formation of TAG in the glycerolipid synthesis pathway (Lung and Weselake, 2006, Lipids. Dec 2006;41(12):1073-88). There are two different families of DGAT proteins. The first family of DGAT proteins ("DGAT1") is related to the acyl-coenzyme A:cholesterol acyltransferase ("ACAT") and has been desbried in the U.A. at. 6,100,077 and 6,344,548. A

second family of DGAT proteins ("DGAT2") is unrelated to the DGAT1 family and is described in PCT Patention Publication WO 2004/011671 published Feb. 5, 2004. Other references to DGAT genes and their use in plants include PCT Publication Nos. WO2004/011,671, WO1998/055,631, and WO2000/001,713, and US Patent Publication No. 20030115632.

DGAT1 is typically the major TAG synthesising enzyme in both the seed and senescing leaf (Kaup *et al.*, 2002, Plant Physiol. 129(4):1616-26; for reviews see Lung and Weselake 2006, Lipids. Dec 2006;41(12):1073-88; Cahoon *et al.*, 2007, Current Opinion in Plant Biology. 10:236-244; and Li *et al.*, 2010, Lipids. 45:145-157).

Raising the yield of oilseed crops (canola, sunflower, safflower, soybean, corn, cotton, linseed, flax etc) has been a major target for the agricultural industry for decades. Many approaches (including traditional and mutational breeding as well as genetic engineering) have been tried, typically with modest success (Xu *et al.*, 2008, Plant Biotechnol J., 6:799-818 and references therein).

Although liquid biofuels offer considerable promise the reality of utilising biological material is tempered by competing uses and the quantities available. Consequently, engineering plants and microorganisms to address this is the focus of multiple research groups; in particular the accumulation of triacylglcerol (TAG) in vegetative tissues and oleaginous yeasts and bacteria (Fortman *et al.*, 2008, Trends Biotechnol 26, 375-381; Ohlrogge *et al.*, 2009, Science 324, 1019-1020). TAG is a neutral lipid with twice the energy density of cellulose and can be used to generate biodiesel a high energy density desirable biofuel with one of the simplest and most efficient manufacturing processes. Engineering TAG accumulation in leaves has so far resulted in a 5-20 fold increase over WT utilising a variety of strategies which includes: the over-expression of seed development transcription factors (LEC1, LEC2 and WRI1); silencing of APS (a key gene involved in starch biosynthesis); mutation of CGI-58 (a regulator of neutral lipid accumulation); and upregulation of the TAG synthesising enzyme DGAT (diacylglycerol O acyltransferase, EC 2.3.1.20) in plants and also in yeast (Andrianov *et al.*, 2009, Plant Biotech J 8, 1-11; Mu *et al.*, 2008, Plant Physiol 148, 1042-1054; Sanjaya *et al.*, 2011, Plant Biotech J 9, 874-883; Santos-Mendoza *et al.*, 2008, Plant J 54, 608-620; James *et al.*, 2010, Proc Natl Acad Sci U S A 107, 17833–17838; Beopoulos *et al.*, 2011, Appl Microbiol Biotechnol 90, 1193-1206; Bouvier-Navé *et al.*, 2000, Eur J Biochem 267, 85-96; Durrett *et al.*, 2008, Plant J 54, 593-607. However, it has been acknowledged that to achieve further increases in TAG, preventing its catabolism may

be crucial within non oleaginous tissues and over a range of developmental stages (Yang and Ohlrogge, 2009, Plant Physiol 150, 1981–1989).

Positively manipulating the yield and quality of triacylglycderides (TAG) in eukaryotes is difficult to achieve. The enzyme diacylglycerol-*O*-acyltransferase (DGAT) has the lowest specific activity of the Kennedy pathway enzymes and is regarded as a 'bottleneck' in TAG synthesis.

Attempts have been made previously to improve DGAT1 by biotechnological methods, with limited success. For example Nykiforuk et al., (2002, Biochimica et Biophysica Acta 1580:95-109) reported N-terminal truncation of the *Brassica napus* DGAT1 but reported approximately 50% lower activity. McFie *et al.*, (2010, JBC., 285:37377-37387) reported that N-terminal truncation of the mouse DGAT1 resulted in increased specific activity of the enzyme, but also reported a large decline in the level of protein that accumulated.

Xu *et al.*, (2008, Plant Biotechnology Journal, 6:799-818) recently identified a consensus sequence (X-Leu-X-Lys-X-X-Ser-X-X-X-Val) within *Tropaeolum majus* (garden nasturtium) DGAT1 (TmDGAT1) sequences as a targeting motif typical of members of the SNF1-related protein kinase-1 (SnRK1) with Ser being the residue for phosphorylation. The SnRK1 proteins are a class of Ser/Thr protein kinases that have been increasingly implicated in the global regulation of carbon metabolism in plants, e.g. the inactivation of sucrose phosphate synthase by phosphorylation (Halford & Hardie 1998, Plant Mol Biol. 37:735-48. Review). Xu *et al.*, (2008, Plant Biotechnology Journal, 6:799-818) performed site-directed mutagenesis on six putative functional regions/motifs of the TmDGAT1 enzyme. Mutagenesis of a serine residue (S197) in a putative SnRK1 target site resulted in a 38%–80% increase in DGAT1 activity, and over-expression of the mutated TmDGAT1 in Arabidopsis resulted in a 20%–50% increase in oil content on a per seed basis.

It would be beneficial to provide improved forms of DGAT1, which overcome one or more of the deficiencies in the prior art, and which can be used to increase cellular oil production.

It is an object of the invention to provide modified and enhanced DGAT1 proteins and methods for their use to increase cellular lipid production and/or at least to provide the public with a useful choice.

SUMMARY OF THE INVENTION

The inventors have shown that it is possible to modify the N-terminal region of DGAT1 proteins upstream of the acyl-CoA binding site, to produce a modified DGAT1 proteins with increase activity, without reduced protein accumulation seen in some modified DGAT1 proteins the prior art. The modified DGAT1 proteins of the invention can be expressed in cells to increase cellular lipid accumulation.

Polynucleotide encoding a polypeptide

In the first aspect the invention provides an isolated polynucleotide encoding a modified DGAT1 protein that is modified in the N-terminal region of the protein upstream of the acyl-CoA binding site.

In one embodiment the modified DGAT1 protein has at least one of:

i) increased DGAT1 activity
ii) increased stability
iii) altered oligomerisation properties
iv) substantially normal cellular protein accumulation properties
v) substantially normal cellular targeting properties

relative to the unmodified DGAT1.

In one embodiment the N-terminal region is at least least 3 amino acids upstream of the conserved motif ESPLSS (Glu-Ser-Pro-Leu-Ser-Ser) in the acyl-CoA binding site.

In a further embodiment the N-terminal region is at least 4, preferably at least 5, preferably at least 6, preferably at least 7, preferably at least 8, preferably at least 9, preferably at least 10, preferably at least 11, preferably at least 12, preferably at least 13, preferably at least 14, preferably at least 15, preferably at least 16 amino acids upstream of the conserved motif ESPLSS in the acyl-CoA binding site.

In a preferred embodiment the modified DGAT1 has an intact acyl-CoA binding site.

Modification

In one embodiment the modification is at least one of:

a) a deletion,

b) a substitution, and

c) an addition

of at least amino acid.

In a further embodiment the modification is of at least 2, more preferably at least 3, more preferably at least 4, more preferably at least 5, more preferably at least 10, more preferably at least 20, more preferably at least 30, more preferably at least 40, more preferably at least 50, more preferably at least 60, more preferably at least 70, more preferably at least 80, more preferably at least 90, more preferably at least 100, more preferably at least 110, more preferably at least 120 amino acids in the N-terminal region.

In a preferred embodiment the modification is a deletion.

Modification is a truncation

In one embodiment the modification is truncation of one or more amino acids from the N-terminal end of the N-terminal region.

In a further embodiment the truncation is of at least 2, more preferably at least 3, more preferably at least 4, more preferably at least 5, more preferably at least 10, more preferably at least 20, more preferably at least 30, more preferably at least 40, more preferably at least 50, more preferably at least 60, more preferably at least 70, more preferably at least 80, more preferably at least 90, more preferably at least 100, more preferably at least 110, more preferably at least 120 amino acids from the N-terminal end of the N-terminal region.

In one embodiment the modification is truncation of all of the N-terminal region.

Modification is a truncation with a methionine added to the truncated N-terminus

In a further embodiment an M (Met) residue is added to the truncated N-terminus.

The modified DGAT1 protein of this embodiment may also be regarded as having an internal deletion of one of more amino acids downstream of the N-terminal M (Met) of the unmodified DGAT1, but upstream of the acyl-CoA binding site.

In a further embodiment a flexible peptide linker is added to the truncated N-terminus.

In a preferred embodiment the flexible peptide linker is soluble.

In one embodiment the flexible peptide linker comprises the sequence (GGGS)n or (Gly-Gly-Gly-Ser)n. In one embodiment n is a number between 1 and 5.

In a further embodiment the sequence MGGGS (Met-Gly-Gly-Gly-Ser) is added to the truncated N-terminus.

In a further embodiment the polypeptide of the invention, when expressed in the cell, has altered substrate specificity relative to the unmodified DGAT1.

Constructs

In a further embodiment the invention provides a genetic construct comprising a polynucleotide of the invention.

Cells

In a further embodiment the invention provides a cell comprising a polynucleotide of the invention.

In a further embodiment the invention provides a cell comprising a genetic construct of the invention.

In a preferred embodiment the cell expresses the modified DGAT1.

In one embodiment the modified DGAT1 protein, when expressed in the cell, has at least one of:

i) increased DGAT1 activity,

ii) increased stability,

iii) altered oligomerisation properties,

iv) substantially normal cellular protein accumulation properties, and

v) substantially normal subcellular targeting properties

relative to the unmodified DGAT1 when expressed in a cell.

In a further embodiment the cell produces more lipid than does a control cell.

In one embodiment the cell produces at least 5% more, preferably at least 10% more, preferably at least 15% more, preferably at least 20% more, preferably at least 25% more, preferably at least 30% more, preferably at least 35% more, preferably at least 40% more, preferably at least 45% more, preferably at least 50% more, preferably at least 55% more, preferably at least 60% more, preferably at least 65% more, preferably at least 70% more, preferably at least 75% more, preferably at least 80% more, preferably at least 85% more, preferably at least 90% more, preferably at least 95% more preferably at least 100% more, preferably at least 105% more, preferably at least 110% more, preferably at least 115% more, preferably at least 120% more, preferably at least 125% more, preferably at least 130% more, preferably at least 135% more, preferably at least 140% more, preferably at least 145% more, preferably at least 150% more lipid than does a control cell.

In a further embodiment the cell has an altered lipid profile relative to a control cell.

In one embodiment the proportion of 16:0 in the triacylglycerol is altered relative to that in a control cell.

In one embodiment the proportion of 16:0 in the triacylglycerol is altered by at least 1%, preferably at least 2%, more preferably at least 3%, more preferably at least 4%, more preferably at least 5%, more preferably at least 6%, more preferably at least 7%, more preferably at least 8%, more preferably at least 9%, more preferably at least 10%, more preferably at least 11%, more preferably at least 12%, more preferably at least 13%, more preferably at least 14%, more preferably at least 15%, more preferably at least 16%, more preferably at least 17%, more preferably at least 18%, more preferably at least 19%, more preferably at least 20%, relative to that in a control cell.

In a further embodiment the proportion of 16:0 in the triacylglycerol is altered relative to that in a control cell. In one embodiment the altered lipid profile has a proportion of 16:0 in the triacylglycerol in the range 6% to 16%. In this embodiment the proportion of 16:0 in the triacylglycerol is altered within the range 6% to 16%.

In a further embodiment the proportion of 18:0 in the triacylglycerol is altered relative to that in a control cell.

In one embodiment the proportion of 18:0 in the triacylglycerol is altered by at least 1%, preferably at least 2%, more preferably at least 3%, more preferably at least 4%, more preferably at least 5%, more preferably at least 6%, more preferably at least 7%, more preferably at least 8%, more preferably at least 9%, more preferably at least 10%, more preferably at least 11%, more preferably at least 12%, more preferably at least 13%, more preferably at least 14%, more preferably at least 15%, more preferably at least 16%, more preferably at least 17%, more preferably at least 18%, more preferably at least 19%, more preferably at least 20%, relative to that in a control cell.

In a further embodiment the altered lipid profile has a proportion of 18:0 in the triacylglycerol in the range 7% to 15%. In this embodiment the proportion of 18:0 in the triacylglycerol is altered within the range 7% to 15%.

In a further embodiment the proportion of 18:1 in the triacylglycerol is altered relative to that in a control cell.

In one embodiment the proportion of 18:1 in the triacylglycerol is altered by at least 1%, preferably at least 2%, more preferably at least 3%, more preferably at least 4%, more preferably at least 5%, more preferably at least 6%, more preferably at least 7%, more preferably at least 8%, more preferably at least 9%, more preferably at least 10%, more preferably at least 11%, more preferably at least 12%, more preferably at least 13%, more preferably at least 14%, more preferably at least 15%, more preferably at least 16%, more preferably at least 17%, more preferably at least 18%, more preferably at least 19%, more preferably at least 20%, relative to that in a control cell.

In one embodiment the altered lipid profile has a proportion of 18:1 in the triacylglycerol in the range 39% to 55%. In this embodiment the proportion of 18:1 in the triacylglycerol is altered within the range 39% to 55%.

The control cell may be any cell of the same type that is not transformed with the polynucleotide, or construct, of the invention to express the modified DGAT1.

In one embodiment the control cell is an untransformed cell. In a further embodiment the control cell is transformed cell to express the unmodified DGAT1.

Cells also transformed to express an oleosin

In one embodiment the cell is also transformed to express at least one of: an oleosin, steroleosin, caloleosin, polyoleosin, and an oleosin including at least one artificially introduced cysteine (WO2011/053169).

Plant

In a further embodiment the invention provides a plant comprising a polynucleotide of the invention.

In a further embodiment the invention provides a plant comprising a genetic construct of the invention.

In a preferred embodiment the plant expresses the modified DGAT1.

In one embodiment the modified DGAT1 protein when expressed in the plant has at least one of:

i) increased DGAT1 activity,
ii) increased stability,
iii) altered oligomerisation properties,
iv) substantially normal cellular protein accumulation properties, and
v) substantially normal subcellular targeting properties

relative to the unmodified DGAT1.

In a further embodiment the plant produces more lipid, in at least one of its tissues or parts, than does the equivalent tissue or part in a control plant.

In one embodiment the plant produces at least 5% more, preferably at least 10% more, preferably at least 15% more, preferably at least 20% more, preferably at least 25% more, preferably at least 30% more, preferably at least 35% more, preferably at least 40% more, preferably at least 45% more, preferably at least 50% more, preferably at least 55% more, preferably at least 60% more, preferably at least 65% more, preferably at least 70% more, preferably at least 75% more, preferably at least 80% more, preferably at least 85% more, preferably at least 90% more, preferably at least 95% more preferably at least 100% more, preferably at least 105% more, preferably at least 110% more, preferably at least 115% more, preferably at least 120% more, preferably at least 125% more, preferably at least 130% more, preferably at least 135% more, preferably at least 140% more, preferably at least 145% more, preferably at least 150% more lipid than does a control plant.

In one embodiment the tissue is a vegetative tissue. In one embodiment the part is a leaf. In a further embodiment the part is a root. In a further embodiment the part is a tuber. In a further embodiment the part is a corm. In a further embodiment the part is a stalk. In a further embodiment the part is a stalk of a monoct plant. In a further embodiment the part is a stovum (stalk and leaf blade).

In a preferred embodiment the tissue is seed tissue. In a preferred embodiment the part is a seed. In a preferred embodiment the tissue is endosperm tissue.

In a further embodiment the plant as a whole produces more lipid than does the control plant as a whole.

In a further embodiment the plant has an altered lipid profile, in at least one of its tissues or parts, relative to a control plant.

In one embodiment the proportion of 16:0 in the triacylglycerol is altered relative to that in a control plant.

In one embodiment the proportion of 16:0 in the triacylglycerol is altered by at least 1%, preferably at least 2%, more preferably at least 3%, more preferably at least 4%, more preferably at least 5%, more preferably at least 6%, more preferably at least 7%, more preferably at least 8%, more preferably at least 9%, more preferably at least 10%, relative to that in a control plant.

In a further embodiment the altered lipid profile has a proportion of 16:0 in the triacylglycerol in the range 6% to 16%. In this embodiment the proportion of 16:0 in the triacylglycerol is altered within the range 6% to 16%.

In a further embodiment the proportion of 18:0 in the triacylglycerol is altered relative to that in a control plant.

In one embodiment the proportion of 18:0 in the triacylglycerol is altered by at least 1%, preferably at least 2%, more preferably at least 3%, more preferably at least 4%, more preferably at least 5%, more preferably at least 6%, more preferably at least 7%, more preferably at least 8%, more preferably at least 9%, more preferably at least 10%, more preferably at least 11%, more preferably at least 12%, more preferably at least 13%, more preferably at least 14%, more preferably at least 15%, more preferably at least 16%, more preferably at least 17%, more preferably at least 18%, more preferably at least 19%, more preferably at least 20%, relative to that in a control cell.

In a further embodiment the altered lipid profile has a proportion of 18:0 in the triacylglycerol in the range 7% to 15%. In this embodiment the proportion of 18:0 in the triacylglycerol is altered within the range 7% to 15%.

In a further embodiment the proportion of 18:1 in the triacylglycerol is altered relative to that in a control plant.

In one embodiment the proportion of 18:1 in the triacylglycerol is altered by at least 1%, preferably at least 2%, more preferably at least 3%, more preferably at least 4%, more preferably at least 5%, more preferably at least 6%, more preferably at least 7%, more preferably at least 8%, more preferably at least 9%, more preferably at least 10%, more preferably at least 11%, more preferably at least 12%, more preferably at least 13%, more preferably at least 14%, more preferably at least 15%, more preferably at least 16%, more preferably at least 17%, more

preferably at least 18%, more preferably at least 19%, more preferably at least 20%, relative to that in a control cell.

In a further embodiment the altered lipid profile has a proportion of 18:1 in the triacylglycerol in the range 39% to 55%. In this embodiment the proportion of 18:1 in the triacylglycerol is altered within the range 39% to 55%.

In one embodiment the tissue is a vegetative tissue. In one embodiment the part is a leaf. In a further embodiment the part is a root. In a further embodiment the part is a tuber. In a further embodiment the part is a corm. In a further embodiment the part is a stalk. In a further embodiment the part is a stalk of a monoct plant. In a further embodiment the part is a stovum (stalk and leaf blade).

In a preferred embodiment the tissue is seed tissue. In a preferred embodiment the part is a seed. In a preferred embodiment the tissue is endosperm tissue.

In a further embodiment the plant as a whole has an altered lipid profile relative to the control plant as a whole.

The control plant may be any plant of the same type that is not transformed with the polynucleotide, or construct, of the invention to express the modified DGAT1.

In one embodiment the control plant is an untransformed plant. In a further embodiment the control plant is transformed plant to express the unmodified DGAT1.

Plant also transformed to express an oleosin

In one embodiment the plant is also transformed to express at least one of: an oleosin, steroleosin, caloleosin, polyoleosin, and an oleosin including at least one artificially introduced cysteine (WO 2011/053169).

Polypeptide

In a further aspect the invention provides a modified DGAT1 protein that is modified in the N-terminal region of the protein upstream of the acyl-CoA binding site.

In one embodiment the modified DGAT1 protein has at least one of:

i) increased DGAT1 activity
ii) increased stability
iii) altered oligomerisation properties
iv) substantially normal cellular protein accumulation properties
v) substantially normal cellular targeting properties

relative to the unmodified DGAT1.

In one embodiment the N-terminal region is at least least 3 amino acids upstream of the conserved motif ESPLSS (Glu-Ser-Pro-Leu-Ser-Ser) in the acyl-CoA binding site.

In a further embodiment the N-terminal region is at least 4, preferably at least 5, preferably at least 6, preferably at least 7, preferably at least 8, preferably at least 9, preferably at least 10, preferably at least 11, preferably at least 12, preferably at least 13, preferably at least 14, preferably at least 15, preferably at least 16 amino acids upstream of the conserved motif ESPLSS in the acyl-CoA binding site.

In a preferred embodiment the modified DGAT1 has an intact acyl-CoA binding site.

Modification

In one embodiment the modification is at least one of:

a) a deletion,
b) a substitution, and
c) an addition

of at least amino acid.

In a further embodiment the modification is of at least 2, more preferably at least 3, more preferably at least 4, more preferably at least 5, more preferably at least 10, more preferably at least 20, more preferably at least 30, more preferably at least 40, more preferably at least 50, more preferably at least 60, more preferably at least 70, more preferably at least 80, more

preferably at least 90, more preferably at least 100, more preferably at least 110, more preferably at least 120 amino acids

In a preferred embodiment the modification is a deletion.

Modification is a truncation

In one embodiment the modification is truncation of one or more amino acids from the N-terminal end of the N-terminal region.

In a further embodiment the truncation is of at least 2, more preferably at least 3, more preferably at least 4, more preferably at least 5, more preferably at least 10, more preferably at least 20, more preferably at least 30, more preferably at least 40, more preferably at least 50, more preferably at least 60, more preferably at least 70, more preferably at least 80, more preferably at least 90, more preferably at least 100, more preferably at least 110, more preferably at least 120 amino acids from the N-terminal end of the N-terminal region.

In one embodiment the modification is truncation of all of the N-terminal region.

Modification is a truncation with a methionine added to the truncated N-terminus

In a further embodiment an M (Met) residue is added to the truncated N-terminus.

The modified DGAT1 protein of this embodiment may also be regarded as having an internal deletion of one of more amino acids downstream of the N-terminal M (Met) of the unmodified DGAT1, but upstream of the acyl-CoA binding site.

In a further embodiment a flexible peptide linker is added to the truncated N-terminus.

In a preferred embodiment the flexible peptide linker is soluble.

In one embodiment the flexible peptide linker comprises the sequence (GGGS)n or (Gly-Gly-Gly-Ser)n. In one embodiment n is a number between 1 and 5.

In a further embodiment the sequence MGGGS (Met-Gly-Gly-Gly-Ser) is added to the truncated N-terminus.

Method for producing an enhanced DGAT1

In a further aspect the invention provides a method for producing an enhanced DGAT1, the method comprising modifying the N-terminal region of the protein upstream of the acyl-CoA binding site.

In one embodiment the modified DGAT1 protein has at least one of:

i) increased DGAT1 activity
ii) increased stability
iii) altered oligomerisation properties
iv) substantially normal cellular protein accumulation properties
v) substantially normal cellular targeting properties

relative to the unmodified DGAT1.

In one embodiment the N-terminal region is at least least 3 amino acids upstream of the conserved motif ESPLSS (Glu-Ser-Pro-Leu-Ser-Ser) in the acyl-CoA binding site.

In a further embodiment the N-terminal region is at least 4, preferably at least 5, preferably at least 6, preferably at least 7, preferably at least 8, preferably at least 9, preferably at least 10, preferably at least 11, preferably at least 12, preferably at least 13, preferably at least 14, preferably at least 15, preferably at least 16 amino acids upstream of the conserved motif ESPLSS in the acyl-CoA binding site.

In a preferred embodiment the modified DGAT1 has an intact acyl-CoA binding site.

Modification

In one embodiment the modification is at least one of:

a) a deletion,
b) a substitution, and
c) an addition

of at least amino acid.

In a further embodiment the modification is of at least 2, more preferably at least 3, more preferably at least 4, more preferably at least 5, more preferably at least 10, more preferably at least 20, more preferably at least 30, more preferably at least 40, more preferably at least 50, more preferably at least 60, more preferably at least 70, more preferably at least 80, more preferably at least 90, more preferably at least 100, more preferably at least 110, more preferably at least 120 amino acids

In a preferred embodiment the modification is a deletion.

Modification is a truncation

In one embodiment the modification is truncation of one or more amino acids from the N-terminal end of the N-terminal region.

In a further embodiment the truncation is of at least 2, more preferably at least 3, more preferably at least 4, more preferably at least 5, more preferably at least 10, more preferably at least 20, more preferably at least 30, more preferably at least 40, more preferably at least 50, more preferably at least 60, more preferably at least 70, more preferably at least 80, more preferably at least 90, more preferably at least 100, more preferably at least 110, more preferably at least 120 amino acids from the N-terminal end of the N-terminal region.

In one embodiment the modification is truncation of all of the N-terminal end of the N-terminal region.

Modification is a truncation with a methionine added to the truncated N-terminus

In a further embodiment an M (Met) residue is added to the truncated N-terminus.

The modified DGAT1 protein of this embodiment may also be regarded as having an internal deletion of one of more amino acids downstream of the N-terminal M (met) of the unmodified DGAT1, but upstream of the acyl-CoA binding site.

In a further embodiment a flexible peptide linker is added to the truncated N-terminus.

In a preferred embodiment the flexible peptide linker is soluble.

In one embodiment the flexible peptide linker comprises the sequence (GGGS)n or (Gly-Gly-Gly-Ser)n. In one embodiment n is a number between 1 and 5.

In a further embodiment the sequence MGGGS (Met-Gly-Gly-Gly-Ser) is added to the truncated N-terminus.

In a further embodiment the method comprises testing at least one of the

i) activity
ii) stability
iii) oligomerisation properties
iv) cellular protein accumulation properties
v) cellular targeting properties

of the modified DGAT1 protein.

In a further embodiment method comprises the step of selecting a modified DGAT1 protein that has at least one of:

i) increased DGAT1 activity
ii) increased stability
iii) altered oligomerisation properties
iv) substantially normal cellular protein accumulation properties
v) substantially normal cellular targeting properties

relative to the unmodified DGAT1 protein.

Plant parts

In a further embodiment the invention provides a part, propagule or progeny of a plant of the invention.

In a preferred embodiment the part, propagule or progeny comprises at least one of a polynucleotide, construct or polypeptide of the invention.

In a preferred embodiment the part, propagule or progeny expressees at least one of a polynucleotide, construct or polypeptide of the invention.

In a preferred embodiment the part, propagule or progeny expresses a modified DGAT1 of the invention.

In a further embodiment the part, propagule or progeny produces more lipid than does a control part, propagule or progeny, or part, propagule or progeny of a control plant.

In one embodiment the part, propagule or progeny produces at least 5% more, preferably at least 10% more, preferably at least 15% more, preferably at least 20% more, preferably at least 25% more, preferably at least 30% more, preferably at least 35% more, preferably at least 40% more, preferably at least 45% more, preferably at least 50% more, preferably at least 55% more, preferably at least 60% more, preferably at least 65% more, preferably at least 70% more, preferably at least 75% more, preferably at least 80% more, preferably at least 85% more, preferably at least 90% more, preferably at least 95% more preferably at least 100% more, preferably at least 105% more, preferably at least 110% more, preferably at least 115% more, preferably at least 120% more, preferably at least 125% more, preferably at least 130% more, preferably at least 135% more, preferably at least 140% more, preferably at least 145% more, preferably at least 150% more lipid than does a control part, propagule or progeny, or part, propagule or progeny of a control plant.

In a further embodiment the part, propagule or progeny has an altered lipid profile relative to a control part, propagule or progeny, or part, propagule or progeny of a control plant.

In one embodiment the proportion of 16:0 in the triacylglycerol is altered relative to that in a control part, propagule or progeny, or part, propagule or progeny of a control plant.

In one embodiment the proportion of 16:0 in the triacylglycerol is altered by at least 1%, preferably at least 2%, more preferably at least 3%, more preferably at least 4%, more preferably at least 5%, more preferably at least 6%, more preferably at least 7%, more preferably at least

8%, more preferably at least 9%, more preferably at least 10%, more preferably at least 11%, more preferably at least 12%, more preferably at least 13%, more preferably at least 14%, more preferably at least 15%, more preferably at least 16%, more preferably at least 17%, more preferably at least 18%, more preferably at least 19%, more preferably at least 20%, relative to that in a control part, propagule or progeny, or part, propagule or progeny of a control plant.

In a further embodiment the altered lipid profile has a proportion of 16:0 in the triacylglycerol in the range 6% to 16%. In this embodiment the proportion of 16:0 in the triacylglycerol is altered within the range 6% to 16%.

In a further embodiment the proportion of 18:0 in the triacylglycerol is altered relative to that in a control part, propagule or progeny, or part, propagule or progeny of a control plant.

In one embodiment the proportion of 18:0 in the triacylglycerol is altered by at least 1%, preferably at least 2%, more preferably at least 3%, more preferably at least 4%, more preferably at least 5%, more preferably at least 6%, more preferably at least 7%, more preferably at least 8%, more preferably at least 9%, more preferably at least 10%, more preferably at least 11%, more preferably at least 12%, more preferably at least 13%, more preferably at least 14%, more preferably at least 15%, more preferably at least 16%, more preferably at least 17%, more preferably at least 18%, more preferably at least 19%, more preferably at least 20%, relative to that in control part, propagule or progeny, or part, propagule or progeny of a control plant.

In a further embodiment the altered lipid profile has a proportion of 18:0 in the triacylglycerol in the range 7% to 15%. In this embodiment the proportion of 18:0 in the triacylglycerol is altered within the range 7% to 15%.

In a further embodiment the proportion of 18:1 in the triacylglycerol is altered relative to that in a control part, propagule or progeny, or part, propagule or progeny of a control plant.

In one embodiment the proportion of 18:1 in the triacylglycerol is altered by at least 1%, preferably at least 2%, more preferably at least 3%, more preferably at least 4%, more preferably at least 5%, more preferably at least 6%, more preferably at least 7%, more preferably at least 8%, more preferably at least 9%, more preferably at least 10%, more preferably at least 11%, more preferably at least 12%, more preferably at least 13%, more preferably at least 14%, more

preferably at least 15%, more preferably at least 16%, more preferably at least 17%, more preferably at least 18%, more preferably at least 19%, more preferably at least 20%, relative to that in a control part, propagule or progeny, or part, propagule or progeny of a control plant.

In a further embodiment the altered lipid profile has a proportion of 18:1 in the triacylglycerol in the range 39% to 55%. In this embodiment the proportion of 18:1 in the triacylglycerol is altered within the range 39% to 55%.

The control plant may be any plant of the same type that is not transformed with the polynucleotide, or construct, of the invention to express the modified DGAT1.

In one embodiment the control plant is an untransformed plant. In a further embodiment the control plant is transformed plant to express the unmodified DGAT1.

Preferably the control the part, propagule or progeny is from a control plant as described above.

In one embodiment the part is from a vegetative tissue. In one embodiment the part is a leaf. In a further embodiment the part is a root. In a further embodiment the part is a tuber. In a further embodiment the part is a corm. In a further embodiment the part is a stalk. In a further embodiment the part is a stalk of a monocot plant. In a further embodiment the part is a stovum (stalk and leaf blade).

In a further embodiment the part is from a reproductive tissue. In a further embodiment the part is a seed. In a preferred embodiment the part is from or includes endosperm tissue.

Animal feed

In a further aspect the invention provides an animal feedstock comprising at least one of a polynucleotide, construct, modified DGAT1 protein, cell, plant cell, plant part, propagule and progeny of the invention.

Biofuel feedstock

In a further aspect the invention provides a biofuel feedstock comprising at least one of a polynucleotide, construct, modified DGAT1 protein, cell, plant cell, plant part, propagule and progeny of the invention.

Lipid

In one embodiment the lipid is an oil. In a further embodiment the lipid is triacylglycerol (TAG)

Methods for producing lipid

In a further aspect the invention provides a method for producing lipid, the method comprising expressing a modified DGAT1 protein of the invention in a cell, plant cell or plant.

In a preferred embodiment expressing the modified DGAT1 protein of the invention in the plant leads production of the lipid in the cell, plant cell or plant.

In one embodiment the method includes the step of transforming a cell, plant cell or plant with a polynucleotide of the invention encoding the modified DGAT1 protein.

In a further embodiment the method includes the step of extracting the lipid from the cell, plant cell, or plant, or from a part, propagule or progeny of the plant.

In one embodiment the lipid is an oil. In a further embodiment the lipid is triacylglycerol (TAG)

In a further embodiment the lipid is processed into at least one of:

a) a fuel,
b) an oleochemical,
c) a nutritional oil,
d) a cosmetic oil,
e) a polyunsaturated fatty acid (PUFA), and
f) a combination of any of a) to e).

In a further aspect the invention provides a method for producing lipid, the method comprising extracting lipid from at least one of a cell, plant cell, plant, plant part, propagule and progeny of the invention.

In one embodiment the lipid is an oil. In a further embodiment the lipid is triacylglycerol (TAG)

In a further embodiment the lipid is processed into at least one of:

a) a fuel,
b) an oleochemical,
c) a nutritional oil,
d) a cosmetic oil,
e) a polyunsaturated fatty acid (PUFA), and
f) a combination of any of a) to e).

DETAILED DESCRIPTION OF THE INVENTION

In this specification where reference has been made to patent specifications, other external documents, or other sources of information, this is generally for the purpose of providing a context for discussing the features of the invention. Unless specifically stated otherwise, reference to such external documents is not to be construed as an admission that such documents, or such sources of information, in any jurisdiction, are prior art, or form part of the common general knowledge in the art.

The term "comprising" as used in this specification means "consisting at least in part of". When interpreting each statement in this specification that includes the term "comprising", features other than that or those prefaced by the term may also be present. Related terms such as "comprise" and "comprises" are to be interpreted in the same manner. In some embodiments, the term "comprising" (and related terms such as "comprise and "comprises") can be replaced by "consisting of" (and related terms "consist" and "consists").

Definitions

The term "DGAT1" as used herein means acyl CoA: diacylglycerol acyltransferase (EC 2.3.1.20)

DGAT1 is typically the major TAG synthesising enzyme in both the seed and senescing leaf (Kaup *et al.*, 2002, Plant Physiol. 129(4):1616-26; for reviews see Lung and Weselake 2006, Lipids. Dec 2006;41(12):1073-88; Cahoon *et al.*, 2007, Current Opinion in Plant Biology. 10:236-244; and Li *et al.*, 2010, Lipids. 45:145-157).

DGAT1 contains approximately 500 amino acids and has 10 predicted transmembrane domains whereas DGAT2 has only 320 amino acids and is predicted to contain only two transmembrane domains; both proteins were also predicted to have their N- and C-termini located in the cytoplasm (Shockey et al., 2006, Plant Cell 18:2294-2313). Both *DGAT1* and *DGAT2* have orthologues in animals and fungi and are transmembrane proteins located in the ER.

In most dicotyledonous plants *DGAT1* & *DGAT2* appear to be single copy genes whereas there are typically two versions of each in the grasses which presumably arose during the duplication of the grass genome (Salse *et al.*, 2008, Plant Cell, 20:11-24).

The term "unmodified DGAT1" as used herein typically means a naturally occurring or native DGAT1. In some cases the DGAT1 sequence may have been assembled from sequences in the genome, but may not be expressed in plants.

In one embodiment the unmodified DGAT1 polypeptide sequences have the sequence of any one of SEQ ID NO: 1 to 29 or a variant thereof. Preferably the variant has at least 70% identity to any one of SEQ ID NO: 1 to 29. In a further embodiment the unmodified DGAT1 sequences have the sequence of any one of SEQ ID NO: 1 to 29.

In one embodiment the unmodified DGAT1 polynucleotide sequences have the sequence of any one of SEQ ID NO: 30 to 58 or a variant thereof. Preferably the variant has at least 70% identity to any one of SEQ ID NO: 30 to 58. In a further embodiment the unmodified DGAT1 sequences have the sequence of any one of SEQ ID NO: 30 to 58.

The term "modified DGAT1" as used herein refers to the DGAT1 of the invention that is modified upstream of the acyl-CoA binding site, relative to an unmodified DGAT1.

In one embodiment the modified DGAT1 sequences have the sequence of any SEQ ID NO: 59 and 62 to 66 or a variant thereof. Preferably the variant has at least 70% identity to any one

of SEQ ID NO: 59 and 62 to 66. In a further embodiment the modified DGAT1 sequences the sequence of any one of SEQ ID NO: 59 and 62 to 66.

In a further embodiment the modified DGAT1 polypeptide sequences have the sequence of any SEQ ID NO: 59 and 66 or a variant thereof. Preferably the variant has at least 70% identity to any one of SEQ ID NO: 59 and 66. In a further embodiment the modified DGAT1 sequences have the sequence of any one of SEQ ID NO: 59 and 66.

Although not preferred, the modified DGAT1 of the invention may include modifications additional to those upstream of the acyl -CoA binding site. Preferably the modified DGAT1 of the invention includes an intact acyl -CoA binding site.

The terms upstream and downstream are according to normal convention to mean towards the N-terminus of a polypeptide, and towards the C-terminus of a polypeptide, respectively.

Acyl-CoA binding site

The position of the acycl-CoA binding site in a number of DGAT1 sequences is shown if Figure 3.

Conserved motif ESPLSS

In a preferred embodiment the acycl-CoA binding site comprises the conserved motif ESPLSS

Acyl-CoA binding site general formulae

In a preferred embodiment the acyl-CoA binding site has the formula:
XXXESPLSSXXIFXXXHA,
where X is any amino acid.

In a preferred embodiment the acyl-CoA binding site has the formula:
XXXESPLSSXXIFXXSHA,
where X is any amino acid.

In a preferred embodiment the acyl-CoA binding site has the formula:
$X_1X_2X_3ESPLSSX_4X_5IFX_6X_7X_8HA$,
where X_1 = R, K, V, T, A, S or G; X_2 = A, T, V, I, N, R, S or L; X_3 = R or K; X_4 = D or G; X_5 = A, T, N, or L; X_6 = K or R; X_7 = Q or H; and X_8 = S or is absent.

In a preferred embodiment the acyl-CoA binding site has the formula:
$X_1X_2X_3ESPLSSX_4X_5IFX_6X_7SHA$,
where X_1 = R, K, V, T, A, S or G; X_2 = A, T, V, I, N, R, S or L; X_3 = R or K; X_4 = D or G; X_5 = A, T, N, or L; X_6 = K or R; and X_7 = Q or H.

Methods for modifying DGAT1

Methods for modifying the sequence of proteins, or the polynucleotide sequences encoding them, are well known to those skilled in the art. The sequence of a protein may be conveniently be modified by altering/modifying the sequence encoding the protein and expressing the modified protein. Approaches such as site-directed mutagenesis may be applied to modify existing polynucleotide sequences. Altered polynucleotide sequences may also be conveniently synthesised in its modified form.

The phrase "increased DGAT1 activity" means increased specific activity relative to that of the unmodified DGAT1.

An art skilled worker would know how to test the "specific activity" of the chimeric DGAT1. This may typically be done by isolating, enriching and quantifying the recombinant DGAT1 then using this material to determine either the rate of triaclyglyceride formation and/or the disappearance of precursor substrates (including various forms of acyl-CoA and DAG) as per Xu et al., (2008), Plant Biotechnology Journal. 6:799-818.

The phrase "increased stability" means that the modified DGAT1 protein is more stable, when expressed in a cell, than the unmodified DGAT1. This may lead to increased accumulation of active modified DGAT1 when it is expressed in cells, releative to when unmodified DGAT1 is expressed in cells.

Those skilled in the art know how to test the "stability" of the modified DGAT1. This would typically involve expressing the chimeric DGAT1 in a cell, or cells, and expressing the first or second DGAT1 in a separate cell, or cells of the same type. Accumulation of chimeric and the first or second DGAT1 protein in the respective cells can then be measured, for example by immunoblot and/or ELISA. A higher level of accumulation of the chimeric DGAT1 relative to the first or second DGAT1, at the same time point, indicates that the chimeric DGAT1 has increased stability. Alternatively, stability may also be determined by the formation of quaternary structure which can also be determined by immunoblot analysis.

The phrase "altered oligomerisation properties" means that the way in which, or the extent to which modified DGAT1 forms oligomers is altered relative to unmodified DGAT1.

Those skilled in the art know know how to test the " oligomerisation properties" of the modified DGAT1. This may typically be done by immunoblot analysis or size exclusion chromatography.

The phrase "substantially normal cellular protein accumulation properties" means that the modified DGAT1 of the invention retains substantially the same protein accumulation when expressed in a cell, as does the unmodified DGAT1. That is there is no less accumulation of modified DGAT1 than there is accumulation of unmodified DGAT1, when either are separately expressed in the same cell type.

An art skilled worker would know how to test the "cellular protein accumulation properties" of the modified DGAT1. This would typically involve expressing the modified DGAT1 in a cell, or cells, and expressing the unmodified DGAT1 in a separate cell, or cells of the same type. Accumulation of modified and unmodified DGAT1 protein in the respective cells can then be measured, for example by ELISA or immunoblot. A higher level of accumulation of the modified DGAT1 relative to the unmodified DGAT1, at the same time point, indicates that the modified DGAT1 has increased "cellular protein accumulation properties".

The phrase "substantially normal subcellular targetting properties" means that the modified DGAT1 of the invention retains substantially the same subcellular targetting when expressed in a cell, as does the unmodified DGAT1. That is the modified DGAT1 is targeted to the same subcellular compartment/s as the unmodified DGAT1, when either are separately expressed in the same cell type.

An art skilled worker would know how to test the "subcellular targetting properties " of the chimeric DGAT1. This would typically involve expressing the chimeric DGAT1 in a cell, or cells, and expressing the first or second DGAT1 in a separate cell, or cells of the same type. Subcellular targetting of chimeric and the first or second DGAT1 protein in the respective cells can then be assessed, for example by using ultracentrifugation to separate and isolating individual subcellular fractions then determining the level of DGAT1 in each fraction. Substantially similar " subcellular targeting" of the chimeric DGAT1 relative to the the first or second DGAT1, at the same time point, indicates that the chimeric DGAT1 has increased "substantially normal cellular protein has "substantially normal subcellular targetting properties".

Lipid

In one embodiment the lipid is an oil. In a further embodiment the oil is triacylglycerol (TAG)

Lipid production

In certain embodiments the cell, cells, tissues, plants and plant parts of the invention produces more lipid than control cells, tissues, plants and plant parts.

Those skilled in the art are well aware of methods for measuring lipid production. This may typically be done by quantitative fatty acid methyl ester gas chromatography mass spectral analysis (FAMES GC-MS). Suitable methods are also described in the examples section of this specification.

Substrate specificity

In certain embodiments, the polypeptides of the invention have altered substrate specificity relative to other DGAT1 proteins. Plant DGAT1 proteins are relatively promiscuous in terms of the fatty acid substrates and DAG species they are capable of utilisting to generate TAG. As such they can be considered to have relatively low substrate specificity. However, this can be modified such that certain fatty acids become a preferred substrate over others. This leads to an increase in the proportions of the preferred fatty acids in the TAG and decreases in the proportions of the non preferred fatty acid species. Substrate specificity can be determined by

in vitro quantitiative analysis of TAG production following the addition of specific and known quantities of purified substrates to known quantities of recombinant DGAT, as per Xu et al., (2008), Plant Biotechnology Journal. 6:799-818.

Lipid profile

In a further embodiment the cell, cells, tissues, plants and plant parts of the invention have an altered lipid profile relative to the control cells, tissues, plants and plant parts.

Those skilled in the art are well aware of methods for assessing lipid profile. This may involve assessing the proportion or percentage of at least one of the 16:0, 16:1, 18:0, 18:1c9 fatty acid species present in the lipid. This may typically be done by fatty acid methyl ester (FAME) analysis (Browse *et al.*, 1986, Anal. Biochem. 152, 141-145). Suitable methods are also described in the examples section of this specification.

Cells

The modified DGAT1 of the invention, or as used in the methods of the invention, may be expressed in any cell type.

In one embodiment the cell is a prokaryotic cell. In a further embodiment the cell is a eukaryotic cell. In one embodiment the cell is selected from a bacterial cell, a yeast cell, a fungal cell, an insect cell, algal cell, and a plant cell. In one embodiment the cell is a bacterial cell. In a further embodiment the cell is a yeast cell. In one embodiment the yeast cell is a *S. ceriviseae* cell. In further embodiment the cell is a fungal cell. In further embodiment the cell is an insect cell. In further embodiment the cell is an algal cell. In a further embodiment the cell is a plant cell.

In one embodiment the cell is a non-plant cell. In one embodiment the non-plant is selected from *E. coli, P. pastoris, S. ceriviseae, D. salina, C. reinhardtii.* In a further embodiment the non-plant is selected from *P. pastoris, S. ceriviseae, D. salina, C. reinhardtii.*

In one embodiment the cell is a microbial cell. In another embodiment, the microbial cell is an algal cell of the division of Chlorophyta (green algae), Rhodophyta (red algae), Phaeophyceae (brown algae), Bacillariophycaeae (diatoms), or Dinoflagellata (dinoflagellates). In another embodiment, the microbial cell is an algal cell of the species *Chlamydomonas, Dunaliella, Botrycoccus,*

Chlorella, Crypthecodinium, Gracilaria, Sargassum, Pleurochrysis, Porphyridium, *Phaeodactylum*, *Haematococcus*, *Isochrysis*, *Scenedesmus*, *Monodus*, *Cyclotella*, *Nitzschia*, or *Parietochloris.* In another embodiment, the algal cell is *Chlamydomonas reinhardtii.* In yet another embodiment, the cell is from the genus *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Trichosporon, Lipomyces, Pythium, Schizochytrium*, *Thraustochytrium*, or *Ulkenia.* In yet another embodiment, the cell is a bacterium of the genus *Rhodococcus*, *Escherichia*, or a cyanobacterium. In yet another embodiment, the cell is a yeast cell. In yet another embodiment, the cell is a synthetic cell.

Plants

The unmodified DGAT1 sequences, from which the modified DGAT1 sequences are produced, may be naturally-occurring DGAT1sequences. Preferably the unmodified DGAT1 sequences are from plants. In certain embodiments the cells into which the modified DGAT1 proteins are expressed are from plants. In other embodiments the modified DGAT1 proteins are expressed in plants.

The plant cells, from which the modified DGAT1 proteins are derived, the plants from which the plant cells are derived, and the plants in which the modified DGAT1 proteins are expressed may be from any plant species.

In one embodiment the plant cell or plant, is derived from a gymnosperm plant species.

In a further embodiment the plant cell or plant, is derived from an angiosperm plant species.

In a further embodiment the plant cell or plant, is derived from a from dicotyledonous plant species.

In a further embodiment the plant cell or plant, is derived from a monocotyledonous plant species.

Other preferred plants are forage plant species from a group comprising but not limited to the following genera: Zea, *Lolium, Hordium, Miscanthus, Saccharum, Festuca*, *Dactylis*, *Bromus*, *Thinopyrum*, *Trifolium*, *Medicago*, *Pheleum*, *Phalaris*, *Holcus*, *Glycine*, *Lotus*, *Plantago* and *Cichorium.*

Other preferred plants are leguminous plants. The leguminous plant or part thereof may encompass any plant in the plant family Leguminosae or Fabaceae. For example, the plants may be selected from forage legumes including, alfalfa, clover; leucaena; grain legumes including,

beans, lentils, lupins, peas, peanuts, soy bean; bloom legumes including lupin, pharmaceutical or industrial legumes; and fallow or green manure legume species.

A particularly preferred genus is *Trifolium*. Preferred *Trifolium* species include *Trifolium repens*; *Trifolium arvense*; *Trifolium affine*; and *Trifolium occidentale*. A particularly preferred *Trifolium* species is *Trifolium repens*.

Another preferred genus is *Medicago*. Preferred *Medicago* species include *Medicago sativa* and *Medicago truncatula*. A particularly preferred *Medicago* species is *Medicago sativa*, commonly known as alfalfa.

Another preferred genus is *Glycine*. Preferred *Glycine* species include *Glycine max* and *Glycine wightii* (also known as *Neonotonia wightii*). A particularly preferred *Glycine* species is *Glycine max*, commonly known as soy bean. A particularly preferred *Glycine* species is *Glycine wightii*, commonly known as perennial soybean.

Another preferred genus is *Vigna*. A particularly preferred *Vigna* species is *Vigna unguiculata* commonly known as cowpea.

Another preferred genus is *Mucana*. Preferred *Mucana* species include *Mucana pruniens*. A particularly preferred *Mucana* species is *Mucana pruniens* commonly known as velvetbean.

Another preferred genus is *Arachis*. A particularly preferred *Arachis* species is *Arachis glabrata* commonly known as perennial peanut.

Another preferred genus is *Pisum*. A preferred *Pisum* species is *Pisum sativum* commonly known as pea.

Another preferred genus is *Lotus*. Preferred Lotus species include *Lotus corniculatus*, *Lotus pedunculatus*, *Lotus glabar*, *Lotus tenuis* and *Lotus uliginosus*. A preferred *Lotus* species is *Lotus corniculatus* commonly known as Birdsfoot Trefoil. Another preferred *Lotus* species is *Lotus glabar* commonly known as Narrow-leaf Birdsfoot Trefoil. Another preferred preferred *Lotus* species is *Lotus pedunculatus* commonly known as Big trefoil. Another preferred *Lotus* species is *Lotus tenuis* commonly known as Slender trefoil.

Another preferred genus is *Brassica*. A preferred *Brassica* species is *Brassica oleracea*, commonly known as forage kale and cabbage. A preferred *Brassica* genus is *Camelina*. A preferred *Camelina* species is *Camelina sativa*.

Other preferred species are oil seed crops including but not limited to the following genera: *Brassica*, *Carthumus*, *Helianthus*, *Zea* and *Sesamum*.

A preferred oil seed genera is *Brassica*. A preferred oil seed species is *Brassica napus*.

A preferred oil seed genera is *Brassica*. A preferred oil seed species is *Brassica oleraceae*.

A preferred oil seed genera is *Carthamus*. A preferred oil seed species is *Carthamus tinctorius*.

A preferred oil seed genera is *Helianthus*. A preferred oil seed species is *Helianthus annuus*.

A preferred oil seed genera is *Zea*. A preferred oil seed species is *Zea mays*.

A preferred oil seed genera is *Sesamum*. A preferred oil seed species is *Sesamum indicum*.

A preferred silage genera is *Zea*. A preferred silage species is *Zea mays*.

A preferred grain producing genera is *Hordeum*. A preferred grain producing species is *Hordeum vulgare*.

A preferred grazing genera is *Lolium*. A preferred grazing species is *Lolium perenne*.

A preferred grazing genera is *Lolium*. A preferred grazing species is *Lolium arundinaceum*.

A preferred grazing genera is *Trifolium*. A preferred grazing species is *Trifolium repens*.

A preferred grazing genera is *Hordeum*. A preferred grazing species is *Hordeum vulgare*.

Preferred plants also include forage, or animal feedstock plants. Such plants include but are not limited to the following genera: *Miscanthus*, *Saccharum*, *Panicum*.

A preferred biofuel genera is *Miscanthus*. A preferred biofuel species is *Miscanthus giganteus*.

A preferred biofuel genera is *Saccharum*. A preferred biofuel species is *Saccharum officinarum*.

A preferred biofuel genera is *Panicum*. A preferred biofuel speices is *Panicum virgatum*.

Plant parts, propagues and progeny

The term "plant" is intended to include a whole plant, any part of a plant, a seed, a fruit, propagules and progeny of a plant.

The term 'propagule' means any part of a plant that may be used in reproduction or propagation, either sexual or asexual, including seeds and cuttings.

The plants of the invention may be grown and either self-ed or crossed with a different plant strain and the resulting progeny, comprising the polynucleotides or constructs of the invention, and/or expressing the modified DGAT1 sequences of the invention, also form an part of the present invention.

Preferably the plants, plant parts, propagules and progeny comprise a polynucleotide or construct of the invention, and/or express a modified DGAT1 sequence of the invention.

Polynucleotides and fragments

The term "polynucleotide(s)," as used herein, means a single or double-stranded deoxyribonucleotide or ribonucleotide polymer of any length but preferably at least 15 nucleotides, and include as non-limiting examples, coding and non-coding sequences of a gene, sense and antisense sequences complements, exons, introns, genomic DNA, cDNA, pre-mRNA, mRNA, rRNA, siRNA, miRNA, tRNA, ribozymes, recombinant polypeptides, isolated and purified naturally occurring DNA or RNA sequences, synthetic RNA and DNA sequences, nucleic acid probes, primers and fragments.

A "fragment" of a polynucleotide sequence provided herein is a subsequence of contiguous nucleotides.

The term "primer" refers to a short polynucleotide, usually having a free 3'OH group, that is hybridized to a template and used for priming polymerization of a polynucleotide complementary to the target.

The term "probe" refers to a short polynucleotide that is used to detect a polynucleotide sequence that is complementary to the probe, in a hybridization-based assay. The probe may consist of a "fragment" of a polynucleotide as defined herein.

Polypeptides and fragments

The term "polypeptide", as used herein, encompasses amino acid chains of any length but preferably at least 5 amino acids, including full-length proteins, in which amino acid residues are linked by covalent peptide bonds. Polypeptides of the present invention, or used in the methods of the invention, may be purified natural products, or may be produced partially or wholly using recombinant or synthetic techniques.

A "fragment" of a polypeptide is a subsequence of the polypeptide that preferably performs a function of and/or provides three dimensional structure of the polypeptide. The term may refer to a polypeptide, an aggregate of a polypeptide such as a dimer or other multimer, a fusion polypeptide, a polypeptide fragment, a polypeptide variant, or derivative thereof capable of performing the above enzymatic activity.

The term "isolated" as applied to the polynucleotide or polypeptide sequences disclosed herein is used to refer to sequences that are removed from their natural cellular environment. An isolated molecule may be obtained by any method or combination of methods including biochemical, recombinant, and synthetic techniques.

The term "recombinant" refers to a polynucleotide sequence that is removed from sequences that surround it in its natural context and/or is recombined with sequences that are not present in its natural context.

A "recombinant" polypeptide sequence is produced by translation from a "recombinant" polynucleotide sequence.

The term "derived from" with respect to polynucleotides or polypeptides of the invention being derived from a particular genera or species, means that the polynucleotide or polypeptide has the same sequence as a polynucleotide or polypeptide found naturally in that genera or species. The polynucleotide or polypeptide, derived from a particular genera or species, may therefore be produced synthetically or recombinantly.

Variants

As used herein, the term "variant" refers to polynucleotide or polypeptide sequences different from the specifically identified sequences, wherein one or more nucleotides or amino acid residues is deleted, substituted, or added. Variants may be naturally occurring allelic variants, or non-naturally occurring variants. Variants may be from the same or from other species and may encompass homologues, paralogues and orthologues. In certain embodiments, variants of the

inventive polypeptides and polypeptides possess biological activities that are the same or similar to those of the inventive polypeptides or polypeptides. The term "variant" with reference to polypeptides and polypeptides encompasses all forms of polypeptides and polypeptides as defined herein.

Polynucleotide variants

Variant polynucleotide sequences preferably exhibit at least 50%, more preferably at least 51%, more preferably at least 52%, more preferably at least 53%, more preferably at least 54%, more preferably at least 55%, more preferably at least 56%, more preferably at least 57%, more preferably at least 58%, more preferably at least 59%, more preferably at least 60%, more preferably at least 61%, more preferably at least 62%, more preferably at least 63%, more preferably at least 64%, more preferably at least 65%, more preferably at least 66%, more preferably at least 67%, more preferably at least 68%, more preferably at least 69%, more preferably at least 70%, more preferably at least 71%, more preferably at least 72%, more preferably at least 73%, more preferably at least 74%, more preferably at least 75%, more preferably at least 76%, more preferably at least 77%, more preferably at least 78%, more preferably at least 79%, more preferably at least 80%, more preferably at least 81%, more preferably at least 82%, more preferably at least 83%, more preferably at least 84%, more preferably at least 85%, more preferably at least 86%, more preferably at least 87%, more preferably at least 88%, more preferably at least 89%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, and most preferably at least 99% identity to a sequence of the present invention. Identity is found over a comparison window of at least 20 nucleotide positions, preferably at least 50 nucleotide positions, more preferably at least 100 nucleotide positions, and most preferably over the entire length of a polynucleotide of the invention.

Polynucleotide sequence identity can be determined in the following manner. The subject polynucleotide sequence is compared to a candidate polynucleotide sequence using BLASTN (from the BLAST suite of programs, version 2.2.5 [Nov 2002]) in bl2seq (Tatiana A. Tatusova, Thomas L. Madden (1999), "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250), which is publicly available from the NCBI website on the World Wide Web at ftp://ftp.ncbi.nih.gov/blast/. The default parameters of bl2seq are utilized except that filtering of low complexity parts should be turned off.

The identity of polynucleotide sequences may be examined using the following unix command line parameters:

bl2seq –i nucleotideseq1 –j nucleotideseq2 –F F –p blastn

The parameter –F F turns off filtering of low complexity sections. The parameter –p selects the appropriate algorithm for the pair of sequences. The bl2seq program reports sequence identity as both the number and percentage of identical nucleotides in a line "Identities = ".

Polynucleotide sequence identity may also be calculated over the entire length of the overlap between a candidate and subject polynucleotide sequences using global sequence alignment programs (e.g. Needleman, S. B. and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443-453). A full implementation of the Needleman-Wunsch global alignment algorithm is found in the needle program in the EMBOSS package (Rice,P. Longden,I. and Bleasby,A. EMBOSS: The European Molecular Biology Open Software Suite, Trends in Genetics June 2000, vol 16, No 6. pp.276-277) which can be obtained from the world wide web at http://www.hgmp.mrc.ac.uk/Software/EMBOSS/. The European Bioinformatics Institute server also provides the facility to perform EMBOSS-needle global alignments between two sequences on line at http:/www.ebi.ac.uk/emboss/align/.

Alternatively the GAP program may be used which computes an optimal global alignment of two sequences without penalizing terminal gaps. GAP is described in the following paper: Huang, X. (1994) On Global Sequence Alignment. Computer Applications in the Biosciences 10, 227-235.

A preferred method for calculating polynucleotide % sequence identity is based on aligning sequences to be compared using Clustal X (Jeanmougin et al., 1998, Trends Biochem. Sci. 23, 403-5.)

Polynucleotide variants of the present invention also encompass those which exhibit a similarity to one or more of the specifically identified sequences that is likely to preserve the functional equivalence of those sequences and which could not reasonably be expected to have occurred by random chance. Such sequence similarity with respect to polypeptides may be determined using the publicly available bl2seq program from the BLAST suite of programs (version 2.2.5 [Nov 2002]) from the NCBI website on the World Wide Web at ftp://ftp.ncbi.nih.gov/blast/.

The similarity of polynucleotide sequences may be examined using the following unix command line parameters:

bl2seq –i nucleotideseq1 –j nucleotideseq2 –F F –p tblastx

The parameter –F F turns off filtering of low complexity sections. The parameter –p selects the appropriate algorithm for the pair of sequences. This program finds regions of similarity between the sequences and for each such region reports an "E value" which is the expected number of times one could expect to see such a match by chance in a database of a fixed reference size containing random sequences. The size of this database is set by default in the bl2seq program. For small E values, much less than one, the E value is approximately the probability of such a random match.

Variant polynucleotide sequences preferably exhibit an E value of less than 1 x 10 -6 more preferably less than 1 x 10 -9, more preferably less than 1 x 10 -12, more preferably less than 1 x 10 -15, more preferably less than 1 x 10 -18, more preferably less than 1 x 10 -21, more preferably less than 1 x 10 -30, more preferably less than 1 x 10 -40, more preferably less than 1 x 10 -50, more preferably less than 1 x 10 -60, more preferably less than 1 x 10 -70, more preferably less than 1 x 10 -80, more preferably less than 1 x 10 -90 and most preferably less than 1 x 10-100 when compared with any one of the specifically identified sequences.

Alternatively, variant polynucleotides of the present invention, or used in the methods of the invention, hybridize to the specified polynucleotide sequences, or complements thereof under stringent conditions.

The term "hybridize under stringent conditions", and grammatical equivalents thereof, refers to the ability of a polynucleotide molecule to hybridize to a target polynucleotide molecule (such as a target polynucleotide molecule immobilized on a DNA or RNA blot, such as a Southern blot or Northern blot) under defined conditions of temperature and salt concentration. The ability to hybridize under stringent hybridization conditions can be determined by initially hybridizing under less stringent conditions then increasing the stringency to the desired stringency.

With respect to polynucleotide molecules greater than about 100 bases in length, typical stringent hybridization conditions are no more than 25 to 30° C (for example, 10° C) below the melting temperature (Tm) of the native duplex (see generally, Sambrook *et al.,* Eds, 1987, Molecular Cloning, A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press; Ausubel *et al.*, 1987, Current Protocols in Molecular Biology, Greene Publishing,). Tm for polynucleotide molecules greater

than about 100 bases can be calculated by the formula Tm = 81. 5 + 0. 41% (G + C-log (Na+). (Sambrook *et al.,* Eds, 1987, Molecular Cloning, A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press; Bolton and McCarthy, 1962, PNAS 84:1390). Typical stringent conditions for polynucleotide of greater than 100 bases in length would be hybridization conditions such as prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65°C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65° C and two washes of 30 minutes each in 0.2X SSC, 0.1% SDS at 65°C.

With respect to polynucleotide molecules having a length less than 100 bases, exemplary stringent hybridization conditions are 5 to 10° C below Tm. On average, the Tm of a polynucleotide molecule of length less than 100 bp is reduced by approximately (500/oligonucleotide length)° C.

With respect to the DNA mimics known as peptide nucleic acids (PNAs) (Nielsen *et al.*, Science. 1991 Dec 6;254(5037):1497-500) Tm values are higher than those for DNA-DNA or DNA-RNA hybrids, and can be calculated using the formula described in Giesen *et al.*, Nucleic Acids Res. 1998 Nov 1;26(21):5004-6. Exemplary stringent hybridization conditions for a DNA-PNA hybrid having a length less than 100 bases are 5 to 10° C below the Tm.

Variant polynucleotides of the present invention, or used in the methods of the invention, also encompasses polynucleotides that differ from the sequences of the invention but that, as a consequence of the degeneracy of the genetic code, encode a polypeptide having similar activity to a polypeptide encoded by a polynucleotide of the present invention. A sequence alteration that does not change the amino acid sequence of the polypeptide is a "silent variation". Except for ATG (methionine) and TGG (tryptophan), other codons for the same amino acid may be changed by art recognized techniques, e.g., to optimize codon expression in a particular host organism.

Polynucleotide sequence alterations resulting in conservative substitutions of one or several amino acids in the encoded polypeptide sequence without significantly altering its biological activity are also included in the invention. A skilled artisan will be aware of methods for making phenotypically silent amino acid substitutions (see, e.g., Bowie *et al.*, 1990, Science 247, 1306).

Variant polynucleotides due to silent variations and conservative substitutions in the encoded polypeptide sequence may be determined using the publicly available bl2seq program from the

BLAST suite of programs (version 2.2.5 [Nov 2002]) from the NCBI website on the World Wide Web at ftp://ftp.ncbi.nih.gov/blast/ via the tblastx algorithm as previously described.

Polypeptide variants

The term "variant" with reference to polypeptides encompasses naturally occurring, recombinantly and synthetically produced polypeptides. Variant polypeptide sequences preferably exhibit at least 50%, more preferably at least 51%, more preferably at least 52%, more preferably at least 53%, more preferably at least 54%, more preferably at least 55%, more preferably at least 56%, more preferably at least 57%, more preferably at least 58%, more preferably at least 59%, more preferably at least 60%, more preferably at least 61%, more preferably at least 62%, more preferably at least 63%, more preferably at least 64%, more preferably at least 65%, more preferably at least 66%, more preferably at least 67%, more preferably at least 68%, more preferably at least 69%, more preferably at least 70%, more preferably at least 71%, more preferably at least 72%, more preferably at least 73%, more preferably at least 74%, more preferably at least 75%, more preferably at least 76%, more preferably at least 77%, more preferably at least 78%, more preferably at least 79%, more preferably at least 80%, more preferably at least 81%, more preferably at least 82%, more preferably at least 83%, more preferably at least 84%, more preferably at least 85%, more preferably at least 86%, more preferably at least 87%, more preferably at least 88%, more preferably at least 89%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, and most preferably at least 99% identity to a sequences of the present invention. Identity is found over a comparison window of at least 20 amino acid positions, preferably at least 50 amino acid positions, more preferably at least 100 amino acid positions, and most preferably over the entire length of a polypeptide of the invention.

Polypeptide sequence identity can be determined in the following manner. The subject polypeptide sequence is compared to a candidate polypeptide sequence using BLASTP (from the BLAST suite of programs, version 2.2.5 [Nov 2002]) in bl2seq, which is publicly available from the NCBI website on the World Wide Web at ftp://ftp.ncbi.nih.gov/blast/. The default parameters of bl2seq are utilized except that filtering of low complexity regions should be turned off.

Polypeptide sequence identity may also be calculated over the entire length of the overlap between a candidate and subject polynucleotide sequences using global sequence alignment programs. EMBOSS-needle (available at http:/www.ebi.ac.uk/emboss/align/) and GAP (Huang, X. (1994) On Global Sequence Alignment. Computer Applications in the Biosciences 10, 227-235.) as discussed above are also suitable global sequence alignment programs for calculating polypeptide sequence identity.

A preferred method for calculating polypeptide % sequence identity is based on aligning sequences to be compared using Clustal X (Jeanmougin et al., 1998, Trends Biochem. Sci. 23, 403-5.)

Polypeptide variants of the present invention, or used in the methods of the invention, also encompass those which exhibit a similarity to one or more of the specifically identified sequences that is likely to preserve the functional equivalence of those sequences and which could not reasonably be expected to have occurred by random chance. Such sequence similarity with respect to polypeptides may be determined using the publicly available bl2seq program from the BLAST suite of programs (version 2.2.5 [Nov 2002]) from the NCBI website on the World Wide Web at ftp://ftp.ncbi.nih.gov/blast/. The similarity of polypeptide sequences may be examined using the following unix command line parameters:

bl2seq –i peptideseq1 –j peptideseq2 -F F –p blastp

Variant polypeptide sequences preferably exhibit an E value of less than 1 x 10 -6 more preferably less than 1 x 10 -9, more preferably less than 1 x 10 -12, more preferably less than 1 x 10 -15, more preferably less than 1 x 10 -18, more preferably less than 1 x 10 -21, more preferably less than 1 x 10 -30, more preferably less than 1 x 10 -40, more preferably less than 1 x 10 -50, more preferably less than 1 x 10 -60, more preferably less than 1 x 10 -70, more preferably less than 1 x 10 -80, more preferably less than 1 x 10 -90 and most preferably 1x10-100 when compared with any one of the specifically identified sequences.

The parameter –F F turns off filtering of low complexity sections. The parameter –p selects the appropriate algorithm for the pair of sequences. This program finds regions of similarity between the sequences and for each such region reports an "E value" which is the expected number of times one could expect to see such a match by chance in a database of a fixed reference size containing random sequences. For small E values, much less than one, this is approximately the probability of such a random match.

Conservative substitutions of one or several amino acids of a described polypeptide sequence without significantly altering its biological activity are also included in the invention. A skilled artisan will be aware of methods for making phenotypically silent amino acid substitutions (see, e.g., Bowie et al., 1990, Science 247, 1306).

Constructs, vectors and components thereof

The term "genetic construct" refers to a polynucleotide molecule, usually double-stranded DNA, which may have inserted into it another polynucleotide molecule (the insert polynucleotide molecule) such as, but not limited to, a cDNA molecule. A genetic construct may contain the necessary elements that permit transcribing the insert polynucleotide molecule, and, optionally, translating the transcript into a polypeptide. The insert polynucleotide molecule may be derived from the host cell, or may be derived from a different cell or organism and/or may be a recombinant polynucleotide. Once inside the host cell the genetic construct may become integrated in the host chromosomal DNA. The genetic construct may be linked to a vector.

The term "vector" refers to a polynucleotide molecule, usually double stranded DNA, which is used to transport the genetic construct into a host cell. The vector may be capable of replication in at least one additional host system, such as *E. coli.*

The term "expression construct" refers to a genetic construct that includes the necessary elements that permit transcribing the insert polynucleotide molecule, and, optionally, translating the transcript into a polypeptide. An expression construct typically comprises in a 5' to 3' direction:

a) a promoter functional in the host cell into which the construct will be transformed,
b) the polynucleotide to be expressed, and
c) a terminator functional in the host cell into which the construct will be transformed.

The term "coding region" or "open reading frame" (ORF) refers to the sense strand of a genomic DNA sequence or a cDNA sequence that is capable of producing a transcription product and/or a polypeptide under the control of appropriate regulatory sequences. The coding sequence may, in some cases, identified by the presence of a 5' translation start codon and a 3' translation stop codon. When inserted into a genetic construct, a "coding sequence" is capable of being expressed when it is operably linked to promoter and terminator sequences.

"Operably-linked" means that the sequenced to be expressed is placed under the control of regulatory elements that include promoters, tissue-specific regulatory elements, temporal regulatory elements, enhancers, repressors and terminators.

The term "noncoding region" refers to untranslated sequences that are upstream of the translational start site and downstream of the translational stop site. These sequences are also referred to respectively as the 5' UTR and the 3' UTR. These regions include elements required for transcription initiation and termination, mRNA stability, and for regulation of translation efficiency.

Terminators are sequences, which terminate transcription, and are found in the 3' untranslated ends of genes downstream of the translated sequence. Terminators are important determinants of mRNA stability and in some cases have been found to have spatial regulatory functions.

The term "promoter" refers to nontranscribed cis-regulatory elements upstream of the coding region that regulate gene transcription. Promoters comprise cis-initiator elements which specify the transcription initiation site and conserved boxes such as the TATA box, and motifs that are bound by transcription factors. Introns within coding sequences can also regulate transcription and influence post-transcriptional processing (including splicing, capping and polyadenylation).

A promoter may be homologous with respect to the polynucleotide to be expressed. This means that the promoter and polynucleotide are found operably linked in nature.

Alternatively the promoter may be heterologous with respect to the polynucleotide to be expressed. This means that the promoter and the polynucleotide are not found operably linked in nature.

In certain embodiments the modified DGAT1 polynucleotides/polypeptides of the invention may be andvantageously expessed under the contol of selected promoter sequences as described below.

Vegetative tissue specific promoters

An example of a vegetative specific promoter is found in US 6,229,067; and US 7,629,454; and US 7,153,953; and US 6,228,643.

Pollen specific promoters

An example of a pollen specific promoter is found in US 7,141,424; and US 5,545,546; and US 5,412,085; and US 5,086,169; and US 7,667,097.

Seed specific promoters

An example of a seed specific promoter is found in US 6,342,657; and US 7,081,565; and US 7,405,345; and US 7,642,346; and US 7,371,928. A preferred seed specific promoter is the napin promoter of *Brassica napus* (Josefsson et al., 1987, J Biol Chem. 262(25):12196-201; Ellerström et al., 1996, Plant Molecular Biology, Volume 32, Issue 6, pp 1019-1027).

Fruit specific promoters

An example of a fruit specific promoter is found in US 5,536,653; and US 6,127,179; and US 5,608,150; and US 4,943,674.

Non-photosynthetic tissue preferred promoters

Non-photosynthetic tissue preferred promoters include those preferentially expressed in non-photosynthetic tissues/organs of the plant.

Non-photosynthetic tissue preferred promoters may also include light repressed promoters.

Light repressed promoters

An example of a light repressed promoter is found in US 5,639,952 and in US 5,656,496.

Root specific promoters

An example of a root specific promoter is found in US 5,837,848; and US 2004/0067506 and US 2001/0047525.

Tuber specific promoters

An example of a tuber specific promoter is found in US 6,184,443.

Bulb specific promoters

An example of a bulb specific promoter is found in Smeets *et al.*, (1997) Plant Physiol. 113:765-771.

Rhizome preferred promoters

An example of a rhizome preferred promoter is found Seong Jang *et al.*, (2006) Plant Physiol. 142:1148-1159.

Endosperm specific promoters

An example of an endosperm specific promoter is found in US 7,745,697.

Corm promoters

An example of a promoter capable of driving expression in a corm is found in Schenk *et al.*, (2001) Plant Molecular Biology, 47:399-412.

Photosythetic tissue preferred promoters

Photosythetic tissue preferred promoters include those that are preferrentially expressed in photosynthetic tissues of the plants. Photosynthetic tissues of the plant include leaves, stems, shoots and above ground parts of the plant. Photosythetic tissue preferred promoters include light regulated promoters.

Light regulated promoters

Numerous light regulated promoters are known to those skilled in the art and include for example chlorophyll a/b (Cab) binding protein promoters and Rubisco Small Subunit (SSU) promoters. An example of a light regulated promoter is found in US 5,750,385. Light regulated in this context means light inducible or light induced.

A "transgene" is a polynucleotide that is taken from one organism and introduced into a different organism by transformation. The transgene may be derived from the same species or from a different species as the species of the organism into which the transgene is introduced.

Host cells

Host cells may be derived from, for example, bacterial, fungal, yeast, insect, mammalian, algal or plant organisms. Host cells may also be synthetic cells. Preferred host cells are eukaryotic cells. A particularly preferred host cell is a plant cell, particularly a plant cell in a vegetative tissue of a plant.

A "transgenic plant" refers to a plant which contains new genetic material as a result of genetic manipulation or transformation. The new genetic material may be derived from a plant of the same species as the resulting transgenic plant or from a different species.

Methods for isolating or producing polynucleotides

The polynucleotide molecules of the invention can be isolated by using a variety of techniques known to those of ordinary skill in the art. By way of example, such polypeptides can be isolated through use of the polymerase chain reaction (PCR) described in Mullis et al., Eds. 1994 The Polymerase Chain Reaction, Birkhauser, incorporated herein by reference. The polypeptides of the invention can be amplified using primers, as defined herein, derived from the polynucleotide sequences of the invention.

Further methods for isolating polynucleotides of the invention include use of all, or portions of, the polypeptides having the sequence set forth herein as hybridization probes. The technique of hybridizing labelled polynucleotide probes to polynucleotides immobilized on solid supports such as nitrocellulose filters or nylon membranes, can be used to screen the genomic or cDNA libraries. Exemplary hybridization and wash conditions are: hybridization for 20 hours at 65°C in 5. 0 X SSC, 0. 5% sodium dodecyl sulfate, 1 X Denhardt's solution; washing (three washes of twenty minutes each at 55°C) in 1. 0 X SSC, 1% (w/v) sodium dodecyl sulfate, and optionally one wash (for twenty minutes) in 0. 5 X SSC, 1% (w/v) sodium dodecyl sulfate, at 60°C. An optional further wash (for twenty minutes) can be conducted under conditions of 0.1 X SSC, 1% (w/v) sodium dodecyl sulfate, at 60°C.

The polynucleotide fragments of the invention may be produced by techniques well-known in the art such as restriction endonuclease digestion, oligonucleotide synthesis and PCR amplification.

A partial polynucleotide sequence may be used, in methods well-known in the art to identify the corresponding full length polynucleotide sequence. Such methods include PCR-based methods, 5'RACE (Frohman MA, 1993, Methods Enzymol. 218: 340-56) and hybridization- based method, computer/database –based methods. Further, by way of example, inverse PCR permits acquisition of unknown sequences, flanking the polynucleotide sequences disclosed herein, starting with primers based on a known region (Triglia et al., 1998, Nucleic Acids Res 16, 8186, incorporated herein by reference). The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by

intramolecular ligation and used as a PCR template. Divergent primers are designed from the known region. In order to physically assemble full-length clones, standard molecular biology approaches can be utilized (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press, 1987).

It may be beneficial, when producing a transgenic plant from a particular species, to transform such a plant with a sequence or sequences derived from that species. The benefit may be to alleviate public concerns regarding cross-species transformation in generating transgenic organisms. For these reasons among others, it is desirable to be able to identify and isolate orthologues of a particular gene in several different plant species.

Variants (including orthologues) may be identified by the methods described.

Methods for identifying variants

Physical methods

Variant polypeptides may be identified using PCR-based methods (Mullis *et al.*, Eds. 1994 The Polymerase Chain Reaction, Birkhauser). Typically, the polynucleotide sequence of a primer, useful to amplify variants of polynucleotide molecules of the invention by PCR, may be based on a sequence encoding a conserved region of the corresponding amino acid sequence.

Alternatively library screening methods, well known to those skilled in the art, may be employed (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press, 1987). When identifying variants of the probe sequence, hybridization and/or wash stringency will typically be reduced relatively to when exact sequence matches are sought.

Polypeptide variants may also be identified by physical methods, for example by screening expression libraries using antibodies raised against polypeptides of the invention (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press, 1987) or by identifying polypeptides from natural sources with the aid of such antibodies.

Computer based methods

The variant sequences of the invention, including both polynucleotide and polypeptide variants, may also be identified by computer-based methods well-known to those skilled in the art, using public domain sequence alignment algorithms and sequence similarity search tools to search sequence databases (public domain databases include Genbank, EMBL, Swiss-Prot, PIR and

others). See, e.g., Nucleic Acids Res. 29: 1-10 and 11-16, 2001 for examples of online resources. Similarity searches retrieve and align target sequences for comparison with a sequence to be analyzed (i.e., a query sequence). Sequence comparison algorithms use scoring matrices to assign an overall score to each of the alignments.

An exemplary family of programs useful for identifying variants in sequence databases is the BLAST suite of programs (version 2.2.5 [Nov 2002]) including BLASTN, BLASTP, BLASTX, tBLASTN and tBLASTX, which are publicly available from (ftp://ftp.ncbi.nih.gov/blast/) or from the National Center for Biotechnology Information (NCBI), National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894 USA. The NCBI server also provides the facility to use the programs to screen a number of publicly available sequence databases. BLASTN compares a nucleotide query sequence against a nucleotide sequence database. BLASTP compares an amino acid query sequence against a protein sequence database. BLASTX compares a nucleotide query sequence translated in all reading frames against a protein sequence database. tBLASTN compares a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames. tBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database. The BLAST programs may be used with default parameters or the parameters may be altered as required to refine the screen.

The use of the BLAST family of algorithms, including BLASTN, BLASTP, and BLASTX, is described in the publication of Altschul et al., Nucleic Acids Res. 25: 3389-3402, 1997.

The "hits" to one or more database sequences by a queried sequence produced by BLASTN, BLASTP, BLASTX, tBLASTN, tBLASTX, or a similar algorithm, align and identify similar portions of sequences. The hits are arranged in order of the degree of similarity and the length of sequence overlap. Hits to a database sequence generally represent an overlap over only a fraction of the sequence length of the queried sequence.

The BLASTN, BLASTP, BLASTX, tBLASTN and tBLASTX algorithms also produce "Expect" values for alignments. The Expect value (E) indicates the number of hits one can "expect" to see by chance when searching a database of the same size containing random contiguous sequences. The Expect value is used as a significance threshold for determining whether the hit to a database indicates true similarity. For example, an E value of 0.1 assigned to a polynucleotide hit is interpreted as meaning that in a database of the size of the database screened, one might expect to see 0.1 matches over the aligned portion of the sequence with a similar score simply by

chance. For sequences having an E value of 0.01 or less over aligned and matched portions, the probability of finding a match by chance in that database is 1% or less using the BLASTN, BLASTP, BLASTX, tBLASTN or tBLASTX algorithm.

Multiple sequence alignments of a group of related sequences can be carried out with CLUSTALW (Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680, http://www-igbmc.u-strasbg.fr/BioInfo/ClustalW/Top.html) or T-COFFEE (Cedric Notredame, Desmond G. Higgins, Jaap Heringa, T-Coffee: A novel method for fast and accurate multiple sequence alignment, J. Mol. Biol. (2000) 302: 205-217)) or PILEUP, which uses progressive, pairwise alignments. (Feng and Doolittle, 1987, J. Mol. Evol. 25, 351).

Pattern recognition software applications are available for finding motifs or signature sequences. For example, MEME (Multiple Em for Motif Elicitation) finds motifs and signature sequences in a set of sequences, and MAST (Motif Alignment and Search Tool) uses these motifs to identify similar or the same motifs in query sequences. The MAST results are provided as a series of alignments with appropriate statistical data and a visual overview of the motifs found. MEME and MAST were developed at the University of California, San Diego.

PROSITE (Bairoch and Bucher, 1994, Nucleic Acids Res. 22, 3583; Hofmann *et al.*, 1999, Nucleic Acids Res. 27, 215) is a method of identifying the functions of uncharacterized proteins translated from genomic or cDNA sequences. The PROSITE database (www.expasy.org/prosite) contains biologically significant patterns and profiles and is designed so that it can be used with appropriate computational tools to assign a new sequence to a known family of proteins or to determine which known domain(s) are present in the sequence (Falquet *et al.*, 2002, Nucleic Acids Res. 30, 235). Prosearch is a tool that can search SWISS-PROT and EMBL databases with a given sequence pattern or signature.

Methods for isolating polypeptides

The polypeptides of the invention, or used in the methods of the invention, including variant polypeptides, may be prepared using peptide synthesis methods well known in the art such as direct peptide synthesis using solid phase techniques (e.g. Stewart et al., 1969, in Solid-Phase Peptide Synthesis, WH Freeman Co, San Francisco California, or automated synthesis, for

example using an Applied Biosystems 431A Peptide Synthesizer (Foster City, California). Mutated forms of the polypeptides may also be produced during such syntheses.

The polypeptides and variant polypeptides of the invention, or used in the methods of the invention, may also be purified from natural sources using a variety of techniques that are well known in the art (e.g. Deutscher, 1990, Ed, Methods in Enzymology, Vol. 182, Guide to Protein Purification,).

Alternatively the polypeptides and variant polypeptides of the invention, or used in the methods of the invention, may be expressed recombinantly in suitable host cells and separated from the cells as discussed below.

Methods for producing constructs and vectors

The genetic constructs of the present invention comprise one or more polynucleotide sequences of the invention and/or polynucleotides encoding polypeptides of the invention, and may be useful for transforming, for example, bacterial, fungal, insect, mammalian or plant organisms. The genetic constructs of the invention are intended to include expression constructs as herein defined.

Methods for producing and using genetic constructs and vectors are well known in the art and are described generally in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press, 1987 ; Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publishing, 1987).

Methods for producing host cells comprising polynucleotides, constructs or vectors

The invention provides a host cell which comprises a genetic construct or vector of the invention.

Host cells comprising genetic constructs, such as expression constructs, of the invention are useful in methods well known in the art (e.g. Sambrook *et al.*, Molecular Cloning : A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press, 1987 ; Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publishing, 1987) for recombinant production of polypeptides of the invention. Such methods may involve the culture of host cells in an appropriate medium in conditions suitable for or conducive to expression of a polypeptide of the invention. The expressed recombinant polypeptide, which may optionally be secreted into the culture, may then be

separated from the medium, host cells or culture medium by methods well known in the art (e.g. Deutscher, Ed, 1990, Methods in Enzymology, Vol 182, Guide to Protein Purification).

Methods for producing plant cells and plants comprising constructs and vectors

The invention further provides plant cells which comprise a genetic construct of the invention, and plant cells modified to alter expression of a polynucleotide or polypeptide of the invention, or used in the methods of the invention. Plants comprising such cells also form an aspect of the invention.

Methods for transforming plant cells, plants and portions thereof with polypeptides are described in Draper *et al.*, 1988, Plant Genetic Transformation and Gene Expression. A Laboratory Manual. Blackwell Sci. Pub. Oxford, p. 365; Potrykus and Spangenburg, 1995, Gene Transfer to Plants. Springer-Verlag, Berlin.; and Gelvin *et al.*, 1993, Plant Molecular Biol. Manual. Kluwer Acad. Pub. Dordrecht. A review of transgenic plants, including transformation techniques, is provided in Galun and Breiman, 1997, Transgenic Plants. Imperial College Press, London.

Methods for genetic manipulation of plants

A number of plant transformation strategies are available (e.g. Birch, 1997, Ann Rev Plant Phys Plant Mol Biol, 48, 297; Hellens *et al.*, 2000, Plant Mol Biol 42: 819-32; Hellens *et al.*, Plant Meth 1: 13). For example, strategies may be designed to increase expression of a polynucleotide/polypeptide in a plant cell, organ and/or at a particular developmental stage where/when it is normally expressed or to ectopically express a polynucleotide/polypeptide in a cell, tissue, organ and/or at a particular developmental stage which/when it is not normally expressed. The expressed polynucleotide/polypeptide may be derived from the plant species to be transformed or may be derived from a different plant species.

Transformation strategies may be designed to reduce expression of a polynucleotide/polypeptide in a plant cell, tissue, organ or at a particular developmental stage which/when it is normally expressed. Such strategies are known as gene silencing strategies.

Genetic constructs for expression of genes in transgenic plants typically include promoters for driving the expression of one or more cloned polynucleotide, terminators and selectable marker sequences to detect presence of the genetic construct in the transformed plant.

The promoters suitable for use in the constructs of this invention are functional in a cell, tissue or organ of a monocot or dicot plant and include cell-, tissue- and organ-specific promoters, cell cycle specific promoters, temporal promoters, inducible promoters, constitutive promoters that are active in most plant tissues, and recombinant promoters. Choice of promoter will depend upon the temporal and spatial expression of the cloned polynucleotide, so desired. The promoters may be those normally associated with a transgene of interest, or promoters which are derived from genes of other plants, viruses, and plant pathogenic bacteria and fungi. Those skilled in the art will, without undue experimentation, be able to select promoters that are suitable for use in modifying and modulating plant traits using genetic constructs comprising the polynucleotide sequences of the invention. Examples of constitutive plant promoters include the CaMV 35S promoter, the nopaline synthase promoter and the octopine synthase promoter, and the Ubi 1 promoter from maize. Plant promoters which are active in specific tissues respond to internal developmental signals or external abiotic or biotic stresses are described in the scientific literature. Exemplary promoters are described, e.g., in WO 02/00894 and WO2011/053169, which is herein incorporated by reference.

Exemplary terminators that are commonly used in plant transformation genetic construct include, e.g., the cauliflower mosaic virus (CaMV) 35S terminator, the *Agrobacterium tumefaciens* nopaline synthase or octopine synthase terminators, the *Zea mays* zein gene terminator, the *Oryza sativa* ADP-glucose pyrophosphorylase terminator and the *Solanum tuberosum* PI-II terminator.

Selectable markers commonly used in plant transformation include the neomycin phophotransferase II gene (NPT II) which confers kanamycin resistance, the aadA gene, which confers spectinomycin and streptomycin resistance, the phosphinothricin acetyl transferase (*bar* gene) for Ignite (AgrEvo) and Basta (Hoechst) resistance, and the hygromycin phosphotransferase gene (hpt) for hygromycin resistance.

Use of genetic constructs comprising reporter genes (coding sequences which express an activity that is foreign to the host, usually an enzymatic activity and/or a visible signal (e.g., luciferase, GUS, GFP) which may be used for promoter expression analysis in plants and plant tissues are also contemplated. The reporter gene literature is reviewed in Herrera-Estrella *et al.*, 1993, Nature 303, 209, and Schrott, 1995, In: Gene Transfer to Plants (Potrykus, T., Spangenberg. Eds) Springer Verlag. Berline, pp. 325-336.

The following are representative publications disclosing genetic transformation protocols that can be used to genetically transform the following plant species: Rice (Alam *et al.*, 1999, Plant Cell Rep. 18, 572); apple (Yao *et al.*, 1995, Plant Cell Reports 14, 407-412); maize (US Patent Serial Nos. 5, 177, 010 and 5, 981, 840); wheat (Ortiz *et al.*, 1996, Plant Cell Rep. 15, 1996, 877); tomato (US Patent Serial No. 5, 159, 135); potato (Kumar *et al.*, 1996 Plant J. 9, : 821); cassava (Li *et al.*, 1996 Nat. Biotechnology 14, 736); lettuce (Michelmore *et al.*, 1987, Plant Cell Rep. 6, 439); tobacco (Horsch *et al.*, 1985, Science 227, 1229); cotton (US Patent Serial Nos. 5, 846, 797 and 5, 004, 863); grasses (US Patent Nos. 5, 187, 073 and 6. 020, 539); peppermint (Niu *et al.*, 1998, Plant Cell Rep. 17, 165); citrus plants (Pena *et al.*, 1995, Plant Sci.104, 183); caraway (Krens *et al.*, 1997, Plant Cell Rep, 17, 39); banana (US Patent Serial No. 5, 792, 935); soybean (US Patent Nos. 5, 416, 011 ; 5, 569, 834 ; 5, 824, 877 ; 5, 563, 04455 and 5, 968, 830); pineapple (US Patent Serial No. 5, 952, 543); poplar (US Patent No. 4, 795, 855); monocots in general (US Patent Nos. 5, 591, 616 and 6, 037, 522); brassica (US Patent Nos. 5, 188, 958 ; 5, 463, 174 and 5, 750, 871); cereals (US Patent No. 6, 074, 877); pear (Matsuda *et al.*, 2005, Plant Cell Rep. 24(1):45-51); Prunus (Ramesh *et al.*, 2006 Plant Cell Rep. 25(8):821-8; Song and Sink 2005 Plant Cell Rep. 2006 ;25(2):117-23; Gonzalez Padilla *et al.*, 2003 Plant Cell Rep.22(1):38-45); strawberry (Oosumi *et al.*, 2006 Planta. 223(6):1219-30; Folta *et al.*, 2006 Planta Apr 14; PMID: 16614818), rose (Li *et al.*, 2003), Rubus (Graham *et al.*, 1995 Methods Mol Biol. 1995;44:129-33), tomato (Dan *et al.*, 2006, Plant Cell Reports V25:432-441), apple (Yao *et al.*, 1995, *Plant Cell Rep.* **14**, 407–412), Canola (Brassica napus L.).(Cardoza and Stewart, 2006 Methods Mol Biol. 343:257-66), safflower (Orlikowska *et al*, 1995, Plant Cell Tissue and Organ Culture 40:85-91), ryegrass (Altpeter *et al.*, 2004 Developments in Plant Breeding 11(7):255-250), rice (Christou *et al.*, 1991 Nature Biotech. 9:957-962), maize (Wang *et al.,* 2009 In: Handbook of Maize pp. 609-639) and *Actinidia eriantha* (Wang *et al.*, 2006, Plant Cell Rep. 25,5: 425-31). Transformation of other species is also contemplated by the invention. Suitable methods and protocols are available in the scientific literature.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleic acid sequence and three frame translation of the *Arabidopsis thaliana* DGAT1 transcribed region (SEQ ID NO:81). Exon coding sequences are shown in bold face, underlined, grey blocks.

Figure 2 shows the nucleic acid sequence and three frame translation of the *Zea mays* short

DGAT1 transcribed region (SEQ ID NO:82). This genomic sequence has F469 deleted and Q67 added compared to the cDNA (EU039830) and peptide (ABV91586) sequences actually used in this patent. Exon coding sequences are shown in bold face, underlined, grey blocks.

Figure 3 shows the peptide sequence of the N-terminal cytoplasmic region of a number of plant DGAT1s including both long and short versions from the grasses as well as examples from dicotyledonous species. Left hand box represents acyl-CoA binding site (Nykiforuk *et al.*, 2002, Biochimica et Biophysica Acta 1580:95-109). Right hand box represents first transmembrane region (McFie *et al.*, 2010, JBC., 285:37377-37387). Left hand arrow represents boundary between exon 1 and exon 2. Right hand arrow represents boundary between exon 2 and exon 3. The sequences are AtDGAT1 (SEQ ID NO:83), BjDGAT1 (SEQ ID NO:84), BnDGAT1-AF (SEQ ID NO:85), BjDGAT1 (SEQ ID NO:86), TmajusDGAT1 (SEQ ID NO:87), EpDGAT1 (SEQ ID NO:88), VgDGAT1 (SEQ ID NO:89), NtDGAT1 (SEQ ID NO:90), PfDGAT1 (SEQ ID NO:91), ZmL (SEQ ID NO:92), SbDGAT1 (SEQ ID NO:93), OsL (SEQ ID NO:94), OsS (SEQ ID NO:95), SbDGAT1 (SEQ ID NO:96), ZmS (SEQ ID NO:97), PpDGAT1 (SEQ ID NO:98), SmDGAT1 (SEQ ID NO:99), EaDGAT1 (SEQ ID NO:100), VvDGAT1 (SEQ ID NO:101), GmDGAT1 (SEQ ID NO:102), GmDGAT1 (SEQ ID NO:103), LjDGAT1 (SEQ ID NO:104), MtDGAT1 (SEQ ID NO:105), JcDGAT1 (SEQ ID NO:106), VfDGAT1 (SEQ ID NO:107), RcDGAT1 (SEQ ID NO:108), PtDGAT1 (SEQ ID NO:109), Pt DGAT1 (SEQ ID NO:110).

EXAMPLES

Example 1: Plant DGAT1 sequence selection and splice site prediction

The majority of nucleic acid sequences and peptide sequences for the plant type 1 DGATs can be found by accession number in public domain libraries (Table 1). For creating initial alignments we used ClustalW (Thompson *et al.*, 1994, Nucleic Acids Res., 22, 4673-4680); these were manually edited and used to create the models to search the DGAT sequences, using the HMMER2 package (HMMER 2.3.2 (Oct 2003) Copyright © 1992-2003 HHMI/Washington University School of Medicine, available from the World Wide Web at http://hmmer.org). Initial matching of protein sequences against genomic DNA with splice prediction was performed with the GeneWise package (Birney *et al.*, 2004, Genome Res. 14: 988-995). Some of the sequences retrieved appeared to have errors; in particular incorrectly predicted splice sites which would result in internal deletions that would likely result in non-functional proteins. While both dicotyledonous and monocotyledonous type 1 DGATs have 16 exons there are some differences in the position of the splicing. Exon 8 in the dicotylendonous DGAT1 gene corresponds to exons 8 and 9 in the monocotyledonous DGAT1 gene, while exon 14 in the monocotyledonous gene corresponds to exons 13 and 14 in the dicotyledonous gene. We have found that the most accurate method for determing the likely genuine coding sequence from genomic data has been to use Vector NTI Advance (TM) 11.0 (© 2008 Invitrogen Corporation) to translate the genome in the three forward reading frames and align these with demonstrated functional DGAT1s from dicotyledonous or monocotyledous species as appropriate (for example *A. thaliana* cDNA NM_127503, protein NP_179535 and *Z. mays* cDNA EU039830, protein ABV91586). The genomic sequence and corresponding exon/intron boundary positions for *Arabidopsis thaliana* encoding NP_179535 and *Zea mays* encoding ABV91586 that can be used as a template for determining other plant DGAT coding regions are shown in Figure 1 and Figures 2, respectivlely. An example of this template use is shown for the determination of *Z. mays* DGAT1 SEQ ID NO: 10 and SEQ ID NO: 39.

Table 1

DGAT1 Species Source	DNA accession #s & BAC #	SEQ ID NO:	PROTEIN accession #s & BAC #	SEQ ID NO:
A. thaliana	NM_127503	1	NP_179535	30
B. juncea	AF164434	2	AAY40784	31
B. napus	AF164434_1	3	AAD45536.1	32
B. juncea	DQ016107	4	AAY40785	33
T. majus	AY084052	5	AAM03340	34
E. pitardii	FJ226588	6	ACO55635	35
V. galamensis	EF653276	7	ABV21945	36
N. tabacum	AF129003_1	8	AAF19345.1	37
P. frutescens	AF298815_1	9	AAG23696.1	38
Z. mays	From: CHORI-201 Maize B73 BAC	10	From: CHORI-201 Maize B73 BAC	39
S. bicolor	XM_002439374	11	XP_002439419	40
O. sativa	Os05g0196800	12	NP_001054869	41
O. sativa	From: AP003714.1	13	From: AP003714.1	42
S. bicolor	XM_002437120.1	14	XP_002437165	43
Z. mays	EU039830	15	ABV91586	44
P. patens	XM_001770877.1	16	XP_001770929	45
S. moellendorffii	XM_002964119	17	XP_002964165	46
E. alatus	AY751297	18	AAV31083	47
V. vinifera	XM_002279309	19	XP_002279345	48
G. max	AY496439	20	AAS78662	49
G. max	AB257590	21	BAE93461	50
L. japonicus	AY859489	22	AAW51456	51
M. truncatula	AC174465.2	23	ABN09107	52
J. curcas	DQ278448.1	24	ABB84383	53
V. fordii	DQ356680.1	25	ABC94472	54
V. galamensis	EF653276.1	26	ABV21945	55
R. communis	XM_002514086.1	27	XP_002514132	56
P. trichocarpa	XM_002308242.1	28	XP_002308278	57
P. trichocarpa	XM_002330474.1	29	XP_002330510	58

Example 2: Modification of DGAT1 proteins in the region upstream of the Acyl CoA binding site.

Figure 3 shows alignment of a number of DGAT1 sequences from plants. The left box shows the position of the Acyl-CoA binding site.

As a starting point for their experiments the applicants used the DGAT1 sequences of SEQ ID NO: 30, 34, 39, 41, 42 and 44 as summarised in the Table 2 below. These DGAT1s were modified by replacing the sequence 13 residues upstream of the beginning of the *N*-terminal acyl-CoA binding region (Weselake *et al.* 2006) with Met-Gly-Gly-Gly-Ser (MGGGS) (Table 2, Region 1 specific modifications/truncation constructs for expression in *Saccharomyces cerevisiae*). This meant their truncated *N*-termini were approximately 9 residues longer than the native *N*-terminus of the *Selaginella moellendorffii* native DGAT1 (SEQ ID NO: 46). Furthermore this placed the *N*-terminal truncations 18 residues upstream of the 84 amino acid truncation performed by McFie *et al.*, (2010, JBC., 285:37377-37387) on the mouse DGAT1 which resulted in a large increase in activity but substantial drop in both accumulation of recombinant DGAT1 and its ability to oligomerise. Thus the *N*-terminal truncations shown in SEQ ID NO: 59, 60, 62, 63, 64 and 65, left 32 residues of the original *N*-terminal putative cytoplasmic domains intact. In addition we generated a number of other truncated forms of AtDGAT1 in which repeat residues from OsL-DGAT1 were added (Table 2).

Sequences with modifications were synthesised either by GENEART AG (Germany) or GeneScript (USA). Sequences were optimised for expression in *Saccharomyces cerevisiae* and flanked with appropriate restriction sites to enable the cloning into the pYES2.1 vector (Invitrogen).

Table 2

Starting Sequence SEQ ID NO:	Species	N-teminal modification	Mutation #	Modified Sequence SEQ ID NO:
30	*A. thaliana*	MGGGS		59
30	*A. thaliana*	MAPPPGGGSPQQQQGGGSQQQQGGGS		60
30	*A. thaliana*	Multiple individual additions within *N*-terminus		61
34	*T. majus*	MGGGS		62

42	*O. sativa*-S	MGGGS	63
41	*O. sativa*-L	MGGGS	64
44	*Z. mays*-S	MGGGS	65
39	*Z. mays*-L	MGGGS	66

Example 3: Expression of modified DGAT1 sequences in cells

Expression of constructs in *S. cerevisiae*

The parent DGAT1 constructs and modified DGAT1 contstructs were placed into the galactose-inducible yeast expression vector pYES2.1/V5-His TOPO® (Invitrogen). This resulted in the addition of an inframe *C*-terminal V5 epitope and 6xhistidine tag. The names of the modified constructs, and the number of their corresponding peptide sequences, are shown in Table 3.

The *Saccharomyces cerevisiae* quadruple mutant (H1246) in which all four neutral lipid biosynthesis genes have been disrupted (Sandager *et al.*, 2002, The Journal of Biological Chemistry, 277:6478-6482) was transformed as per Elble (1992, BioTechniques 13, 18-20) and selected by the ability to grow in the absence of uracil. Routinely, yeast cells were grown aerobically overnight in a synthetic medium with 0.67% YNB, without uracil (SC-U) and containing 2% glucose. Cells from overnight culture were used to inoculate 200 mL of induction medium (SC-U containing 2% galactose and 1% raffinose) to an initial OD_{600} of 0.4. Cells were allowed to further grow at 30°C, with shaking at 200 rpm until late stationary phase, normally 48 h. Cells were harvested by centrifugation at 1500 x *g* for 5 min, then cell pellets were washed with distilled water and either used immediately for subsequent analysis or kept in -80°C until required. Cell pellets for neutral lipid extraction were freeze-dried for 48 h and stored in -20°C freezer until required.

Lipid analysis of *S. cerevisiae*

Approximately 10 mg of freeze-dried yeast cell material was accurately weighed then disrupted using glass beads by vortexing for 1 minute. This lysate was extracted in hot methanolic HCL for fatty acid methyl ester (FAME) analysis (Browse *et al.*, 1986, Anal. Biochem. 152, 141-145).

For FA profile analysis approximately 50 mg freeze dried yeast was placed in a 13-mm screw cap tube, and an equal volume of glass beads added before vortexing at high speed in 3x 1 min bursts. Following addition of 50 µg of 19:0 TAG internal standard, 2.4 mL of 0.17 M NaCl in MeOH was added and the mixture vortexed for 15 sec followed by the addition of then 4.8 mL of heptane and the entire contents mixed.

The solution was then incubated in 80°C water bath for 2 h without shaking. After incubation, the solution was cooled to room temperature. After cooling, the upper phase (lipidic phase) was transferred to fresh screw-cap tube and evaporated to dryness under stream of nitrogen gas. The dried residue was then dissolved in 1 mL heptane and mixed thoroughly for TAG SPE separation using Strata Si-1 Silica column (Phenomenwx, 8B-S012-EAK).

After predonditioning with methanol and equilibrating the Silica column with heptanes the 1 mL TAG extract (including 50 µg 17:0 TAG Internal Standard was passed through the pre-equilibrated column, followed by 1.2 mL of heptane and then 2 mL of chloroform:heptane (1:9 v/v/) and the eluate collected.The total eluate collected was evaporated to dryness under the stream of N gas and the residue used for FAMEs extraction.

FAMEs of extracted TAG

To the TAG residue above 10 µL of internal standard 15:0 FA (4 mg/mL dissolved in heptane) and 1 mL of methanolic HCl (1N) reagent containing 5% of 2,2-dimeethoxypropane (as water scavenger) were added.

The tube was then flushed with N gas, then sealed immediately with Teflon-lined cap, and heated at 80°C in a water bath for 1 h. After cooling down, 0.6 mL heptane and 1.0 mL of 0.9% (w/v) NaCl was added, the mixture vortexed then spun at 500 rpm for 1 min.

From the top heptane layer, 100 µL was collected and transfered to a flat-bottom glass insert fitted into a vial for FAMES GC/MS analysis.

Protein extraction and Trypsin digestion

Yeast cell pellets were washed with lysis buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol, 1 mM PMSF) then resuspended in 500 µL lysis buffer, glass beads were added and cells disrupted by vortexing 2x at medium speed for 30 seconds. Cell debris was pelleted by centrifugation at 1000 x *g* for 5 min, the supernatant transferred to fresh tubes and total cellular membranes pelleted by ultracentrifugation at 100,000 x *g* for 1 h. Membrane proteins were resuspended in lysis buffer with or without detergent (1% Dodecyl maltoside) and quantified in a Qubit Fluorometer using the Qubit IT Quantitation Kit.

Trypsin was added to give a final concentration of 25 µg/mL to 50 µL of protein extract and the mixture incubated at 30°C for 30 min. The reaction was terminated by addition of Trypsin inhibitor from *Glycine max* (Sigma-Aldrich catalogue # T6414) to a final concentration of 0.4 µg/µL. After addition of trypsin inhibitor, 4x SDS loading dye and 10x reducing agent (Invitrogen) were added, and the protein incubated at 70°C for 10 min prior to SDS-PAGE followed by immunoblotting. The blot was probed with either Anti V5-HRP antibody (Cat #R96125, Invitrogen) at 1:2500 dilution, or anti Kar2 (y-115) antibody produced in rabbit (SC-33630, Santa Cruz Biotechnology) at 1:200 dilution. Anti Kar2 was used to detect the yeast protein Kar2, an ER luminaly-located protein (Rose *et al*, 1989) which serves as a control to demonstrate the presence of intact microsomes.

Example 4: Truncation of the *N*-terminal cytoplasmic region – Region 1 of plant DGAT1s- enhances lipid production in *Saccharomyces cerevisiae*

The *N*-terminal cytoplasmic region can be truncated to raise the lipid yield. Table 3 shows the lipid yields of a variety of DGAT1s in which the *N*-terminal cytoplasmic region has been truncated. The lipid yields are presented both as grams of lipid produced per litre of (which therefore compensates for any differences in growth rate) as well as normalised as a percentage of the lipid yield of the corresponding unmodified parent DGAT1.

A comparison of *A. thaliana*, *T. majus*, *O. sativa*-S, *O. sativa*-L, *Z. mays*-S and *Z. mays*-L and their *N*-terminal cytoplasmic region truncated counterparts are shown in Table 3. The lipid yields are presented as grams of lipid per litre of culture at 32 and 48 hous of culture as well as a percentage of the lipid yield obtained with the corresponding native (non-truncated) DGAT1 parent isolated at the same time.

Table 3

Construct Description	SEQ ID NO:	Lipid yield @ 32 hr (g FA/L)	Lipid yield as % of native parent @ 32 hr	Lipid yield @ 48 hr (g FA/L)	Lipid yield as % of native parent @ 48 hr
native *A. thaliana*	67	0.25	100	0.25	100
N-truncated *A. thaliana*	59	0.36	147.55	0.37	148.91
Native *T. majus*	68	0.34	100	0.40	100
N-truncated *T. majus*	62	0.32	95.95	0.37	93.81
Native *O. sativa*-S	69	0.40	100	0.47	100
Truncated *O. sativa*-S	63	0.32	79.72	0.35	74.24
Native *O. sativa*-L	70	0.44	100	0.52	100
Truncated *O. sativa*-L	64	0.54	122.01	0.53	101.79
Native *Z. mays*-S	71	0.30	100	0.31	100
Truncated *Z. mays*-S	65	0.25	80.53	0.25	81.27
Native *Z. mays*-L	72	0.50	100	0.54	100
Truncated *Z. mays*-L	66	0.54	108.44	0.68	125.60

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Example 5: Expression of modified DGAT1 in *Brassica napus*

The strategy above can also be used to generate a variety of modified DGAT1 constructs for expression in the seeds of *Brassica napus*. The parent DGATs and their modified forms can be transferred into the Gateway®-compatible binary vector pMD107 (courtesy of Dr Mark Smith, NRC Saskatoon, SK, Canada, S7N 0W9) to place them under the control of the seed- specific napin promoter (Josefsson *et al.*, 1987, J Biol Chem. 262(25):12196-201; Ellerström *et al.*, 1996, Plant Molecular Biology, Volume 32, Issue 6, pp 1019-1027).

Plant transformation

B. napus (cv. DH12075) can be transformed via *Agrobacterium tumefaciens* (GV3101) using the cotyledon co-cultivation method (adapted from that of Maloney *et al.*, 1989, Plant Cell Reports Vol 8, No 4, pg 238-241). Control lines may contain an empty-vector, and when identified, null sibling lines may be subsequently used as true controls.

Approximately 200 T_0 transformed lines may be produced and their corresponding T_1 selfed seeds may be analysed for oil content by GC. Approximately 50 individual transgenic lines (including control lines) may be selected for the next generation (10 plants/line) based on their oil content, or seed weight (8 lines).

A total of approximately T_1 plants may be grown and screened by PCR for copy number and identification of null sibing lines. T_2 seeds may be analysed in triplicate for oil content by NMR.

Example 6: Expression of modified DGAT1 in *Camelina sativa*

The strategy above can also be used to generate a variety of modified DGAT1 constructs for expression in the seeds of *Camilina sativa* and other plants

Sequences with modifications were synthesised either by GENEART AG (Germany) or GeneScript (USA). Sequences were optimised for expression in *Brassica species* and included an intron (SEQ ID NO:73) from *Arabidopsis thaliana* DGAT1 – intron 3. Each sequence was flanked with appropriate attL recombination sites sites to enable the cloning Gateway® adapted vectors.

Table 3

Starting seq ID #	Species	N-terminal modification	C-terminal modification	Additional information	Type of sequence	Modified SEQ ID NO
39	Z. mays-L	none	V5-His tag	+ intron	NUCLEIC	74
39	Z. mays-L	none	V5-His tag	ORF only	NUCLEIC	75
39	Z. mays-L	none	V5-His tag		PEPTIDE	76
39	Z. mays-L	MGGGS	V5-His tag	+ intron	NUCLEIC	77
39	Z. mays-L	MGGGS	V5-His tag	ORF only	NUCLEIC	78
39	Z. mays-L	MGGGS	V5-His tag		PEPTIDE	79

The parent DGATs and their modified forms were transferred into the Gateway®-compatible binary pRSh1 Gateway adapted binary vector (Winichayakul *et al.*, 2009, Biotechnol. Appl. Biochem. 53, 111–122) modified by replacement of the CaMV35S promoter replaced with the *Brassica napus* Napin promoter (SEQ ID NO:80).

***Camelina sativa* transformation**

C. sativa (cf. Calena) were transformed via *Agrobacterium tumefaciens* (GV3101) using the floral dip method (adapted from that of Clough and Bent, 1998, Plant J. 16(6):735-745). Essentially seeds were sown in potting mix in 10 cm pots in a controlled environment, approximately 6 weeks after planting the flowers were dipped for 5-14 minutes under vacuum (70-80 inch Hg) in an overnight culture of appropriated Agrobacterium GV3101 cells re-suspended in a floral dip buffer. After vacuum-transformation, plants were kept for 24 h under low light conditions by partly covering with a black plastic sheet. Vacuum transformations can be repeated three times at

approximately 10-12 days intervals, corresponding to the flowering duration. Plants were grown in potting mix in a controlled environment (16-h day length, 21-24 °C, 65-70 % relative humidity).

The T_1 seeds produced can be collected and screened for transformants by germinating and growing seedlings at 22 °C with continuous light on a half-strength MS medium (pH 5.6) selection plate containing 1 %(w/v) sucrose, 300 mg/L Timentin, and 25 mg/L DL-phosphinothricin to select for herbicide resistance. T_2 selfed seed populations can also be screened by immuno blot for the presence of the V5 eptiope.

T_2 selfed seeds may be analysed for oil content by GC. Approximately 50 individual transgenic lines (including control lines) may be selected for the next generation (10 plants/line) based on their oil content, or seed weight. T_2 plants may be grown and screened by PCR for copy number and identification of null sibing lines. T_2 seeds may be analysed in triplicate for oil content by NMR or GC/MS.

CLAIMS

1. An isolated polynucleotide encoding a modified DGAT1 protein that is modified in the N-terminal region of the protein upstream of the acyl- CoA binding site.

2. The polynucleotide of claim 1 wherein the modified DGAT1 protein has at least one of:

i) increased DGAT1 activity
ii) increased stability
iii) altered oligomerisation properties
iv) substantially normal cellular protein accumulation properties
v) substantially normal cellular targeting properties

relative to the unmodified DGAT1.

3. The polynucleotide of claim 1 or 2 wherein the N-terminal region is at least least 3 amino acids upstream of the conserved motif ESPLSS (Glu-Ser-Pro-Leu-Ser-Ser) in the acyl-CoA binding site.

4. The polynucleotide of any one of claims 1 to 3 wherein the modified DGAT1 has an intact acyl-CoA binding site.

5. The polynucleotide of any one of claims 1 to 4 wherein the modification is at least one of:

a) a deletion,
b) a substitution, and
c) an addition

of at least amino acid.

6. The polynucleotide of any one of claims 1 to 4 wherein the modification is a deletion.

7. The polynucleotide of any one of claims 1 to 4 wherein the modification is truncation of one or more amino acids from the N-terminal end of the N-terminal region.

8. The polynucleotide of any one of claims 1 to 4 wherein the modification is truncation of all of the N-terminal region.

9. The polynucleotide of claim 7 or 8 wherein an M (Met) residue is added to the truncated N-terminus.

10. The polynucleotide of claim 7 or 8 wherein a flexible peptide linker is added to the truncated N-terminus.

11. The polynucleotide of any one of claims 1 to 10 wherein when the modified DGAT1 protein is expressed in a cell, it has altered substrate specificity relative to the unmodified DGAT1.

12. A genetic construct comprising a polynucleotide of any one of claims 1 to 11.

13. A cell comprising a polynucleotide of any one of claims 1 to 11.

14. The cell of claim 13 that expresses the modified DGAT1.

15. The cell of claim 14 wherein the modified DGAT1 protein has at least one of:

i) increased DGAT1 activity,

ii) increased stability,

iii) altered oligomerisation properties,

iv) substantially normal cellular protein accumulation properties, and

v) substantially normal subcellular targeting properties,

relative to the unmodified DGAT1 when expressed in a cell.

16. The cell of any one of claims 13 to 15 which produces more lipid than does a control cell.

17. The cell of any one of claims 13 to 15 which has an altered lipid profile relative to a control cell.

18. The cell of any one of claims 13 to 17 which is also transformed to express at least one of: an oleosin, steroleosin, caloleosin, polyoleosin, and an oleosin including at least one artificially introduced cysteine.

19. A plant comprising the polynucleotide of of any one of claims 1 to 11.

20. The plant of claim 19 that expresses the modified DGAT1.

21. The plant of claim 19 or 20 wherein the modified DGAT1 protein when expressed in the plant that has at least one of:

i) increased DGAT1 activity,
ii) increased stability,
iii) altered oligomerisation properties,
iv) substantially normal cellular protein accumulation properties, and
v) substantially normal subcellular targeting properties

relative to the unmodified DGAT1.

22. The plant of any one of claims 19 to 21 that produces more lipid, in at least one of its tissues or parts, or as a whole, than does a control plant.

23. The plant of any one of claims 19 to 22 that has an altered lipid profile, in at least one of its tissues or parts, or as a whole, relative to a control plant.

24. The plant of any one of claims 19 to 22 that is also transformed to express at least one of: an oleosin, steroleosin, caloleosin, polyoleosin, and an oleosin including at least one artificially introduced cysteine.

25. A modified DGAT1 protein that is modified in the N-terminal region of the protein upstream of the acyl-CoA binding site.

26. The modified DGAT1 protein of claim 25 that has at least one of:

i) increased DGAT1 activity
ii) increased stability
iii) altered oligomerisation properties
iv) substantially normal cellular protein accumulation properties

v) substantially normal cellular targeting properties

relative to the unmodified DGAT1.

27. The modified DGAT1 protein of claim 25 or 26 wherein the N-terminal region is at least least 3 amino acids upstream of the conserved motif ESPLSS (Glu-Ser-Pro-Leu-Ser-Ser) in the acyl-CoA binding site.

28. The modified DGAT1 protein of claim 25 or 27 that has an intact acyl-CoA binding site.

29. The modified DGAT1 protein of claim 25 or 28 that is modified as described in any one of claims 5-11.

30. A method for producing an enhanced DGAT1, the method comprising modifying the N-terminal region of the protein upstream of the acyl-CoA binding site.

31. The method of claim 30 wherein the modified DGAT1 protein has at least one of:

i) increased DGAT1 activity

ii) increased stability

iii) altered oligomerisation properties

iv) substantially normal cellular protein accumulation properties

v) substantially normal cellular targeting properties

relative to the unmodified DGAT1.

32. The method of claim 30 or 31 wherein the N-terminal region is at least least 3 amino acids upstream of the conserved motif ESPLSS (Glu-Ser-Pro-Leu-Ser-Ser) in the acyl-CoA binding site.

33. The method of any one of claims 30 to 32 wherein the modified DGAT1 has an intact acyl-CoA binding site.

34. The method of any one of claims 30 to 33 wherein the modification is as described in any one of claims 5-11

35. The method of any one of claims 30 to 34 that comprises a step of testing at least one of the:

i) activity,

ii) stability,

iii) oligomerisation properties,

iv) cellular protein accumulation properties, and

v) cellular targeting properties

of the modified DGAT1 protein.

36. The method of any one of claims 30 to 35 that comprises the step of selecting a modified DGAT1 protein that has at least one of:

i) increased DGAT1 activity,

ii) increased stability,

iii) altered oligomerisation properties,

iv) substantially normal cellular protein accumulation properties, and

v) substantially normal cellular targeting properties

relative to the unmodified DGAT1 protein.

37. A part, propagule or progeny of the plant of any one of claims 19 to 24.

38. The part, propagule or progeny of claim 37 that comprises at least one of the polynucleotide of any one of claims 1 to 11 or the modified DGAT1 protein of any one of claims 25 to 28.

39. The part, propagule or progeny of claim 37 or 38 that produces more lipid than does a control part, propagule or progeny, or part, propagule or progeny of a control plant.

40. The part, propagule or progeny of any one of claims 37 to 39 that has an altered lipid profile relative to a control part, propagule or progeny, or part, propagule or progeny of a control plant.

41. An animal feedstock comprising at least one of a polynucleotide, construct, modified DGAT1 protein, cell, plant cell, plant part, propagule and progeny of any one of claims 1-29 and 37 to 40.

42. A biofuel feedstock comprising at least one of a polynucleotide, construct, modified DGAT1 protein, cell, plant cell, plant part, propagule and progeny of any one of claims 1-29 and 37 to 40.

43. A method for producing lipid, the method comprising expressing a modified DGAT1 protein of any one of claims 25 to 29 in a cell, plant cell or plant.

44. The method of claim 43 wherein expressing the modified DGAT1 protein of the invention in a plant leads to production of the lipid in the cell, plant cell or plant.

45. The method of claim 43 or 44 wherein the method includes the step of transforming a cell, plant cell or plant with a polynucleotide of any one of claims 1 to 11 encoding the modified DGAT1 protein.

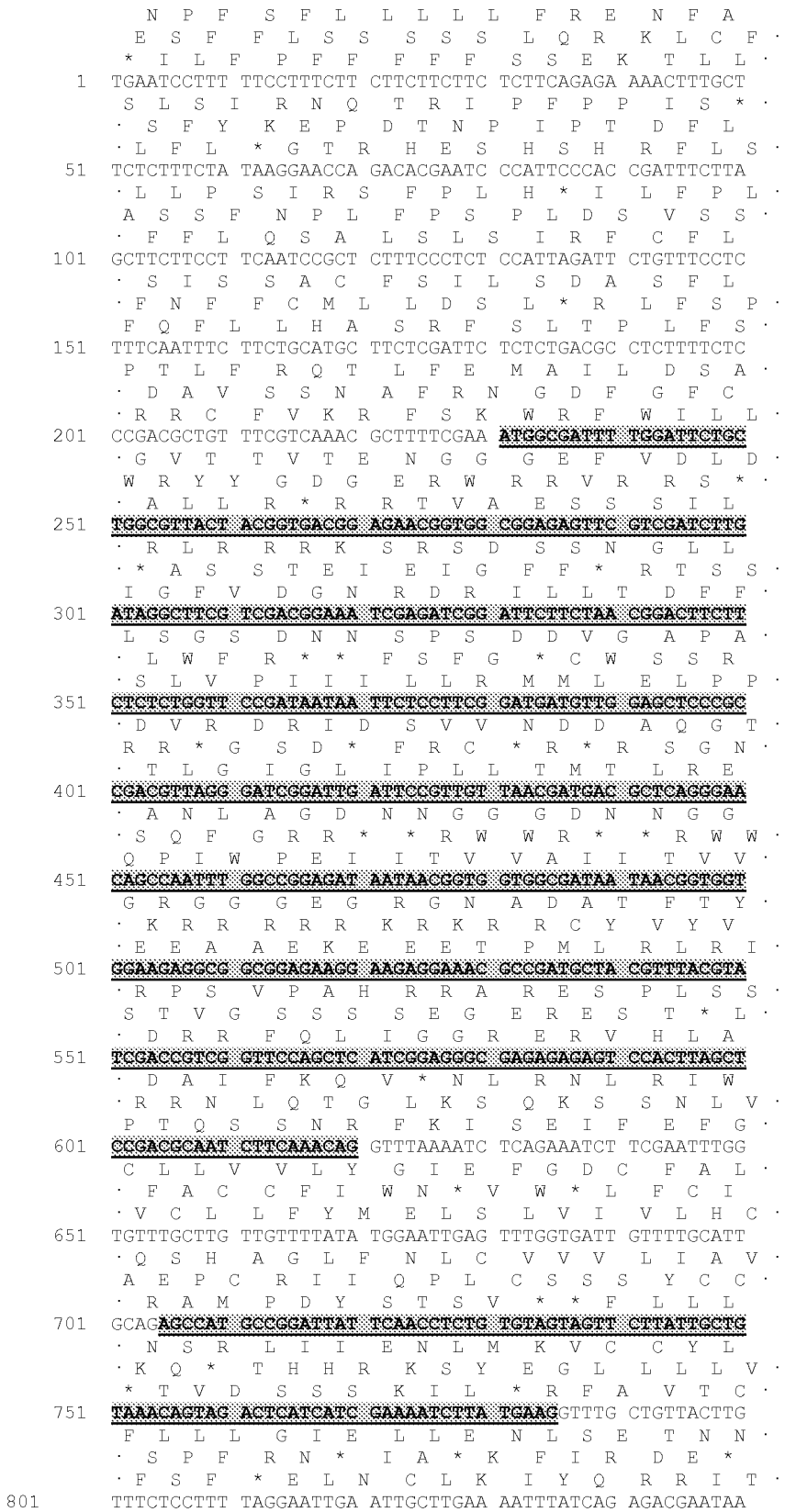
46. The method of any one of claims 43 to 45 which includes the step of extracting the lipid from the cell, plant cell, or plant, or from a part, propagule or progeny of the plant.

47. A method for producing lipid, the method comprising extracting lipid from at least one of a cell, plant cell, plant, plant part, propagule and progeny of any one of claims 13 to 24 and 37 to 40.

48. The method of any one of claims 43 to 47 wherein the lipid is processed into at least one of:

a) a fuel,
b) an oleochemical,
c) a nutritional oil,
d) a cosmetic oil,
e) a polyunsaturated fatty acid (PUFA), and
f) a combination of any of a) to e).

Figure 1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* I L F P F F F F F S S E K T L L
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S F Y K E P D T N P I P T D F L
L F L * G T R H E S H S H R F L S
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L L P S I R S F P L H * I L F P L
A S S F N P L F P S P L D S V S S
F F L Q S A L S L S I R F C F L
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F Q F L L H A S R F S L T P L F S
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P T L F R Q T L F E M A I L D S A
D A V S S N A F R N G D F G F C
R R C F V K R F S K W R F W I L L
201 CCGACGCTGT TTCGTCAAAC GCTTTTCGAA ATGGCGATTT TGGATTCTGC
G V T T V T E N G G G E F V D L D
W R Y Y G D G E R W R R V R R S *
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L S G S D N N S P S D D V G A P A
L W F R * * F S F G * C W S S R
S L V P I I I L L R M M L E L P P
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D V R D R I D S V V N D D A Q G T
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Q S H A G L F N L C V V V L I A V
A E P C R I I Q P L C S S S Y C C
R A M P D Y S T S V * * F L L L
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K Q * T H H R K S Y E G L L L L V
* T V D S S S K I L * R F A V T C
751 TAAACAGTAG ACTCATCATC GAAAATCTTA TGAAGGTTTG CTGTTACTTG
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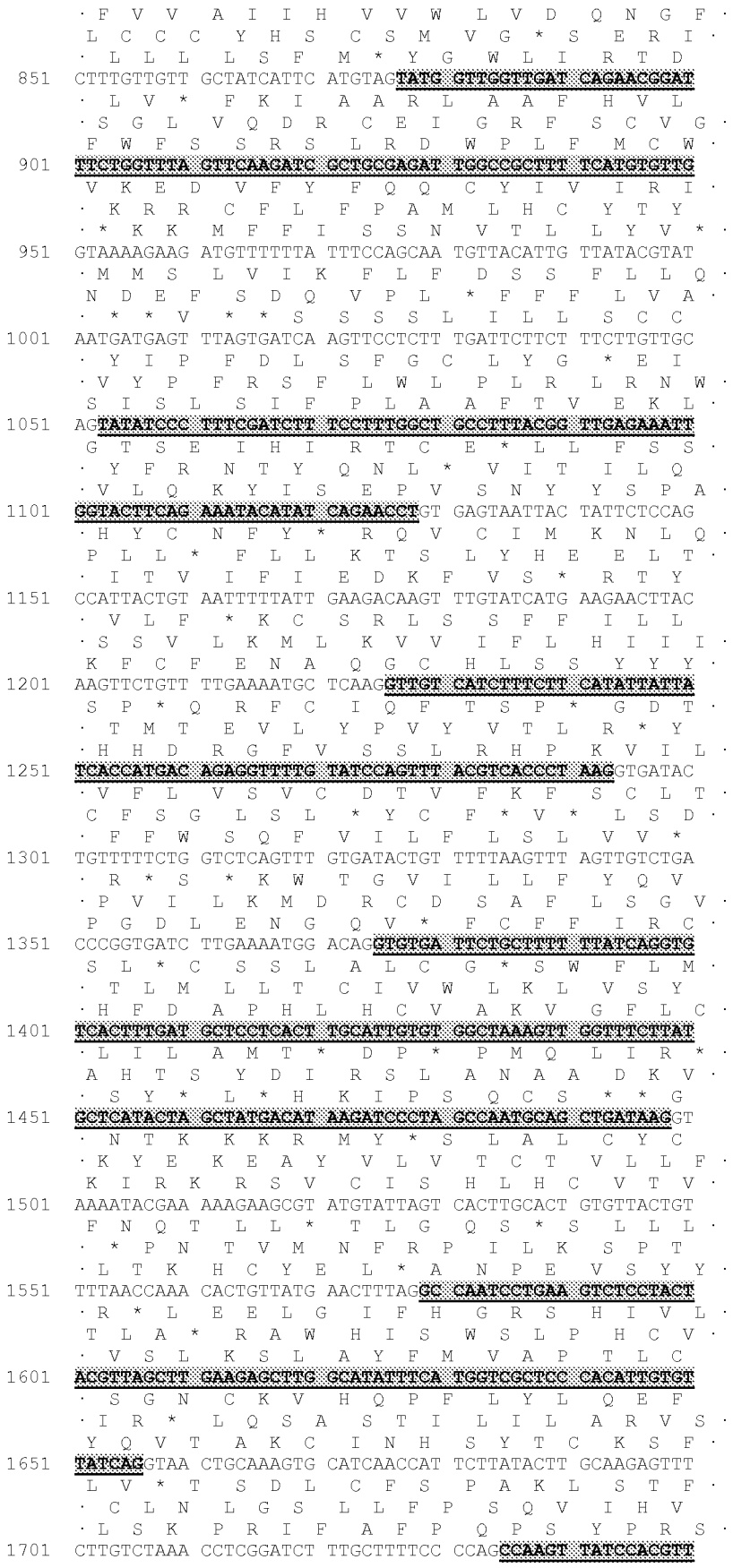

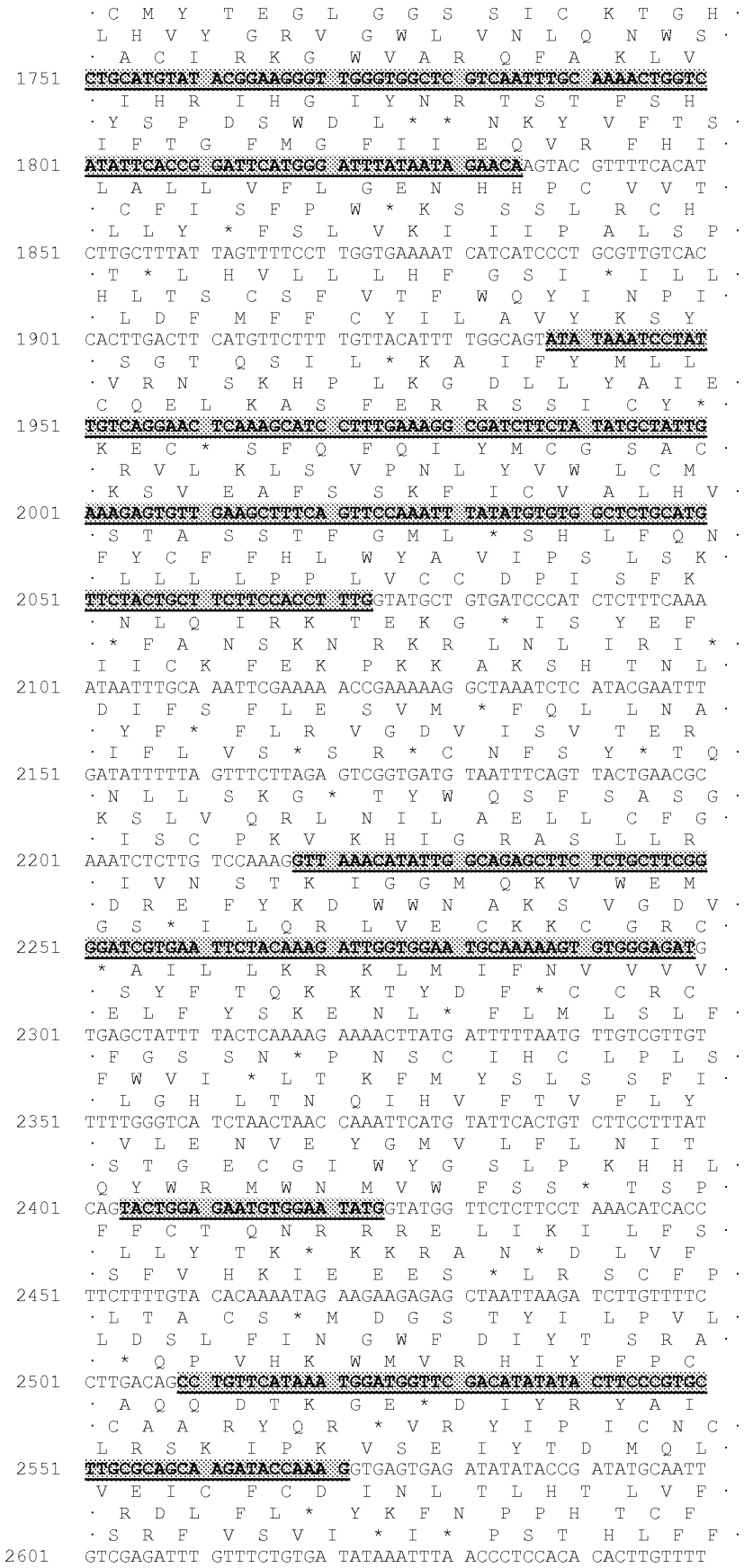

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      · R  H  S  P  L  S  L  L  S  *  S  L  Q  S  F  M
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      · G  I  H  T  F  Y  I  A  L  S  L  D  A  *  T  H  A ·
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      · S  E  R  N  A  N  I  Q  S  I  V  F  T  *  R  S
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       L  I  I  K  V  I  C  F  G  M  G  S  Y  Y  *  V  G ·
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3101  TTAATAATAA AGGTGATCTG TTTTGGAATG GGATCATATT ATTAGGTGGG
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      · A  S  L  L  P  R  P  D  E  P  K  R  I  D  V  M  K ·
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       S  P  L  M  L  W  W  F  *  C  *  N  D  K  *  C  Y ·
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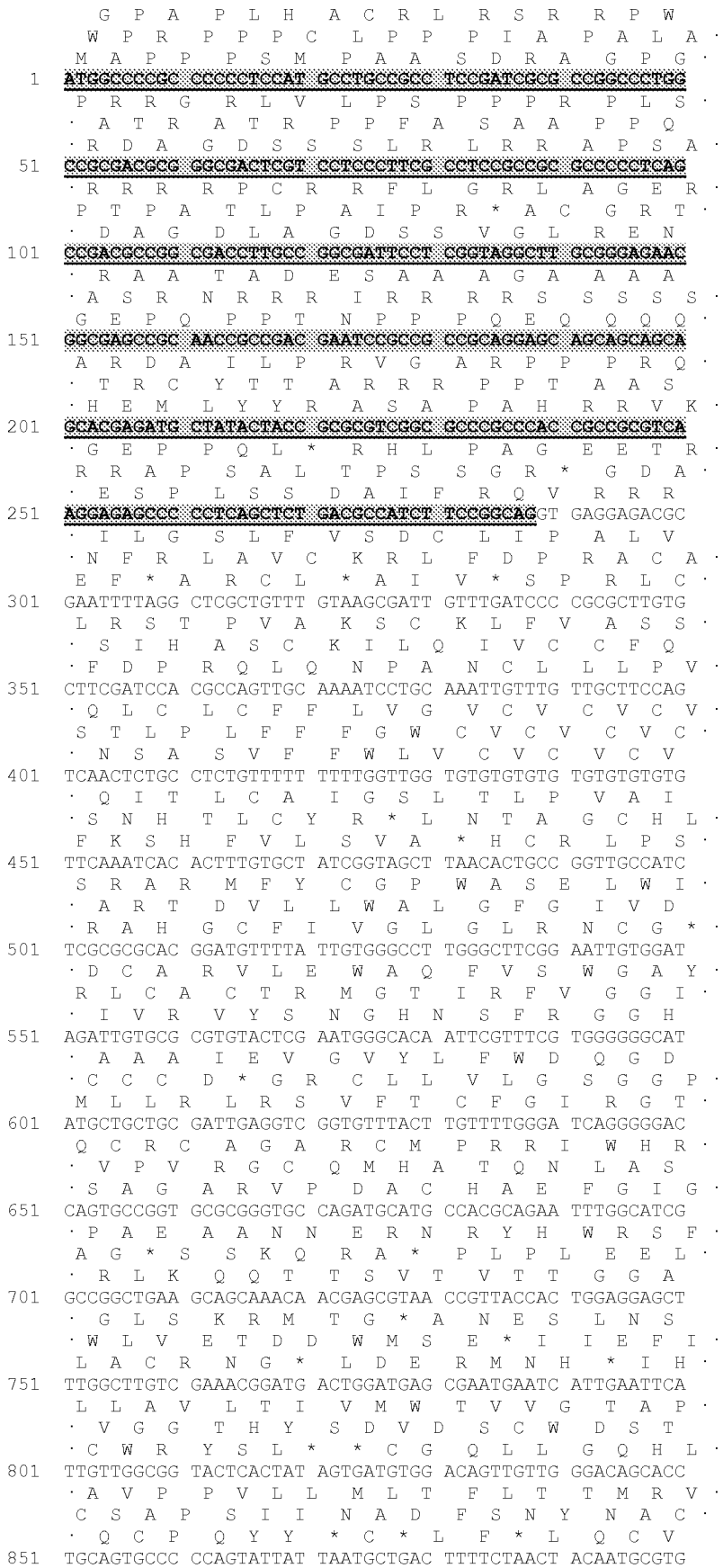
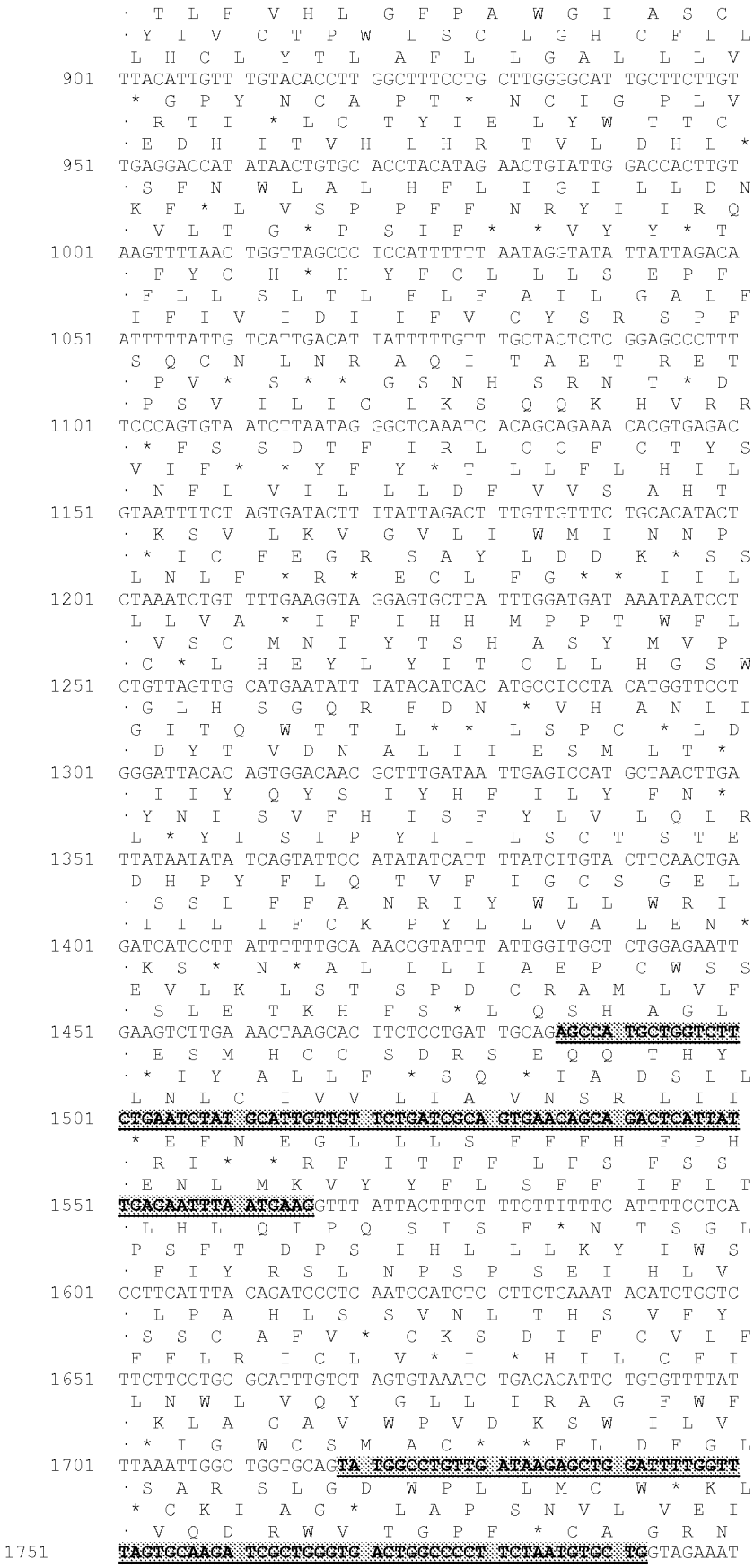
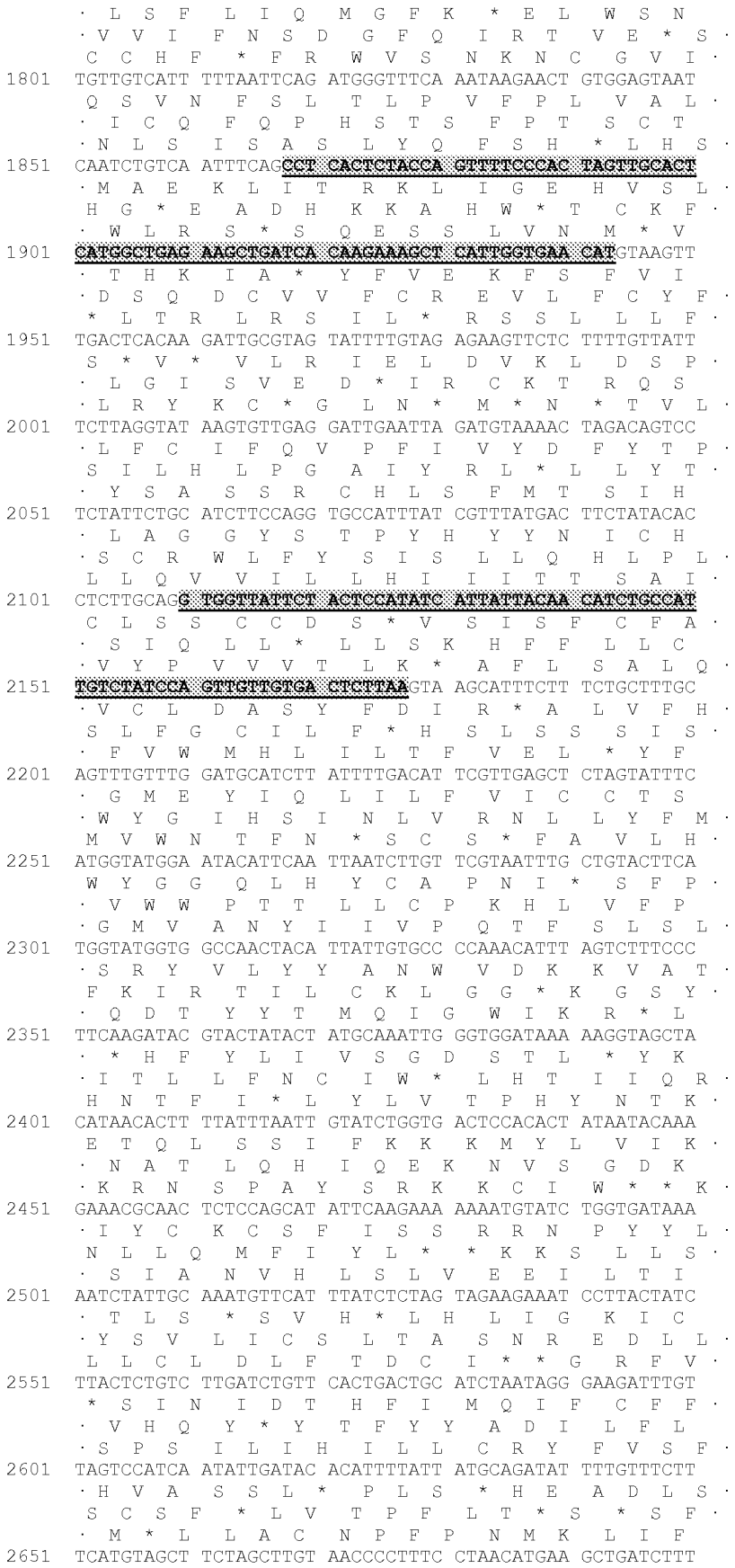
Figure 2







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      · L L E  K I L  S H A S  I M D  Y Q F
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      · * W A  L V H  L T L L  Y Q C  I P N
      ·V M G  L G P P  N P V  I S M  H S Q P ·
3501  TGTAATGGGC CTTGGTCCAC CTAACCCTGT TATATCAATG CATTCCCAAC
      ·L I R  V R V S  L I L  T S G  N G S I ·
       P N * G  * G F  P H S  N F R Q  R * H ·
      · * L G  L G F  P S F *  L Q A  T V A
3551  CCTAATTAGG GTTAGGGTTT CCCTCATTCT AACTTCAGGC AACGGTAGCA
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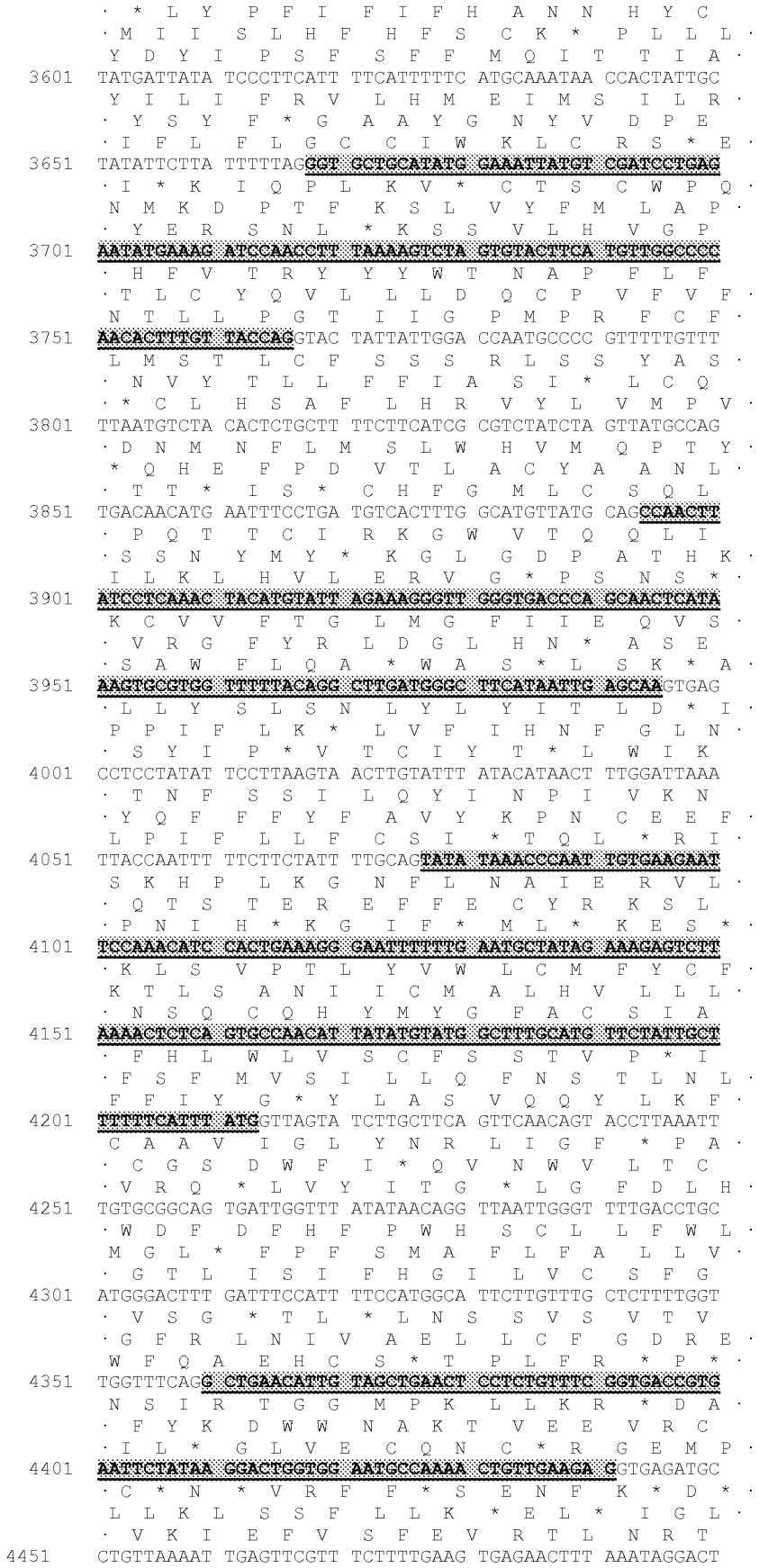

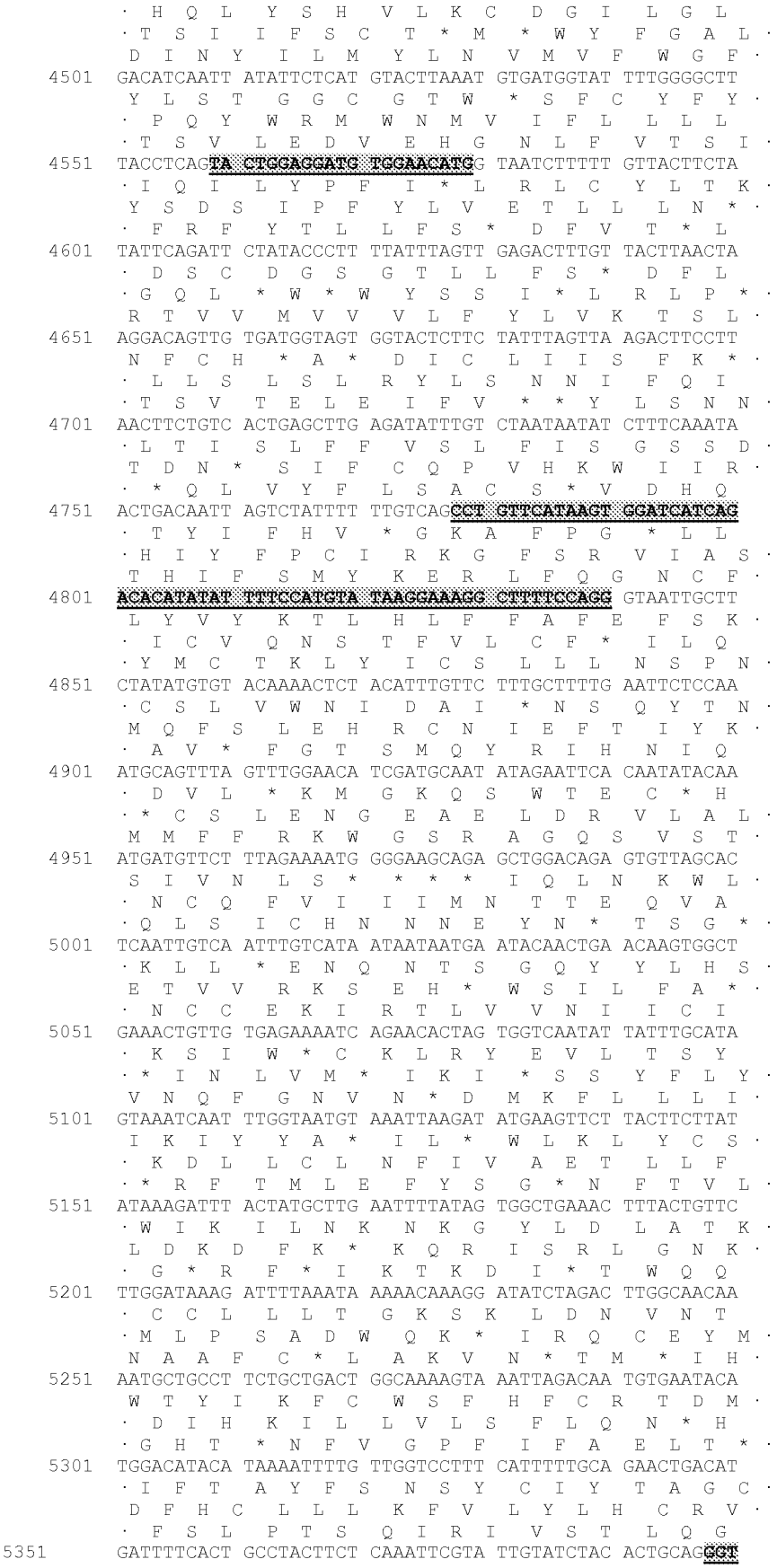

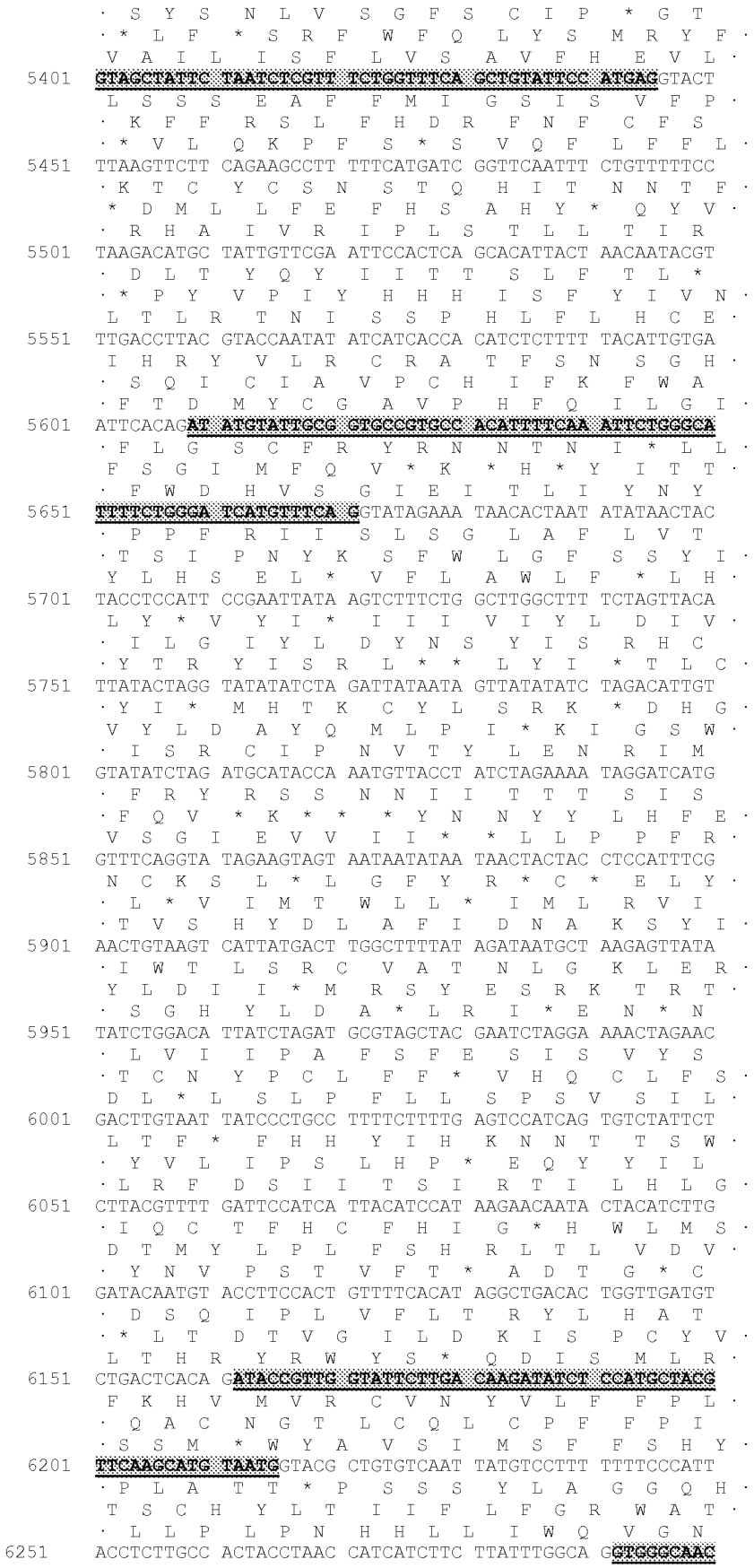

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      · D I L  V L Q  Y S R T  A D V  C P S
      · * Y F  G S S  V * S D  S R C  V S F  Y ·
       M I F W  F F S  I V G  Q P M C  V L L ·
6301  ATGATATTTT GGTTCTTCAG TATAGTCGGA CAGCCGATGT GTGTCCTTCT
       I L P *  R H E  Q A G  P G K *  I
      · T T M  T S *  T G R P  R Q V  D
      · Y Y H  D V M N  R Q A  Q A S  R *
6351  ATACTACCAT GACGTCATGA ACAGGCAGGC CCAGGCAAGT AGATAG(SEQ ID NO: 82)
```

Figure 3