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(54) Titre : DIMINUTION DE LA TENEUR EN ACIDE GRAS SATURÉ DE GRAINES DE PLANTE

(54) Title: LOWERING SATURATED FATTY ACID CONTENT OF PLANT SEEDS

(57) Abrégé/Abstract:

Compositions and methods include genetically encoding and expressing a novel delta-9 desaturase in plant cells. In some embodiments, methods of expressing nucleic acids in a plant cell to take advantage of the delta-9 desaturase enzyme's activity, such that the percent composition of saturated fatty acids in plant seeds is decreased and there is a concomitant increase in ω -7 fatty acids. In other embodiments, amino acid sequences have delta-9 desaturase activity. Methods can involve expression of delta-9 desaturase in plant cells, plant materials, and whole plants for the purpose of increasing the amount of unusual fatty acids in whole plants, plant seeds, and plant materials, for example, seeds.

ABSTRACT

Compositions and methods include genetically encoding and expressing a novel delta-9 desaturase in plant cells. In some embodiments, methods of expressing nucleic acids in a plant cell to take advantage of the delta-9 desaturase enzyme's activity, such that the percent composition of saturated fatty acids in plant seeds is decreased and there is a concomitant increase in ω -7 fatty acids. In other embodiments, amino acid sequences have delta-9 desaturase activity. Methods can involve expression of delta-9 desaturase in plant cells, plant materials, and whole plants for the purpose of increasing the amount of unusual fatty acids in whole plants, plant seeds, and plant materials, for example, seeds.

LOWERING SATURATED FATTY ACID CONTENT OF PLANT SEEDS

5 This application is a division of application 2,803,098 filed June 24, 2011.

10

TECHNICAL FIELD

Some embodiments generally relate to certain delta-9 desaturase enzymes,
15 nucleic acids encoding these enzymes, and methods of expressing the same in a plant
cell. Some embodiments relate to utilizing the activity of certain delta-9 desaturase
enzymes to decrease the percent composition of saturated fatty acids in plant materials
(*e.g.*, seed) and increasing the percent composition of ω -7 fatty acids. Also disclosed
herein are plants and plant materials produced by methods in particular embodiments.

20

BACKGROUND

Vegetable-derived oils have gradually replaced animal-derived oils and fats
as the major source of dietary fat intake. However, saturated fat intake in most
industrialized nations has remained at about 15% to 20% of total caloric
25 consumption. In efforts to promote healthier lifestyles, the United States
Department of Agriculture (USDA) has recently recommended that saturated fats
make up less than 10% of daily caloric intake. To facilitate consumer awareness,
current labeling guidelines issued by the USDA now require total saturated fatty
acid levels be less than 1.0 g per 14 g serving to receive the "low-sat" label and less
30 than 0.5 g per 14 g serving to receive the "no-sat" label. This means that the
saturated fatty acid content of plant oils needs to be less than 7% and 3.5% to
receive the "low-sat" or "no-sat" label, respectively. Since issuance of these
guidelines, there has been a surge in consumer demand for "low-sat" and "no-sat"

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oils. To date, this demand has been met principally with canola oil, and to a much lesser degree with sunflower and safflower oils.

While unsaturated fats (monounsaturated and polyunsaturated) are beneficial (especially when consumed in moderation), saturated and trans fats are not. Saturated fat and trans fat raise undesirable LDL cholesterol levels in the blood. Dietary cholesterol also raises LDL cholesterol and may contribute to heart disease even without raising LDL. Therefore, it is advisable to choose foods low in saturated fat, trans fat, and cholesterol as part of a healthful diet.

The characteristics of oils, whether of plant or animal origin, are determined predominately by the number of carbon and hydrogen atoms in the oil molecule, as well as the number and position of double bonds comprised in the fatty acid chain. Most oils derived from plants are composed of varying amounts of palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) fatty acids. Conventionally, palmitic and stearic acids are designated as "saturated," because their carbon chains are saturated with hydrogen atoms, and hence have no double bonds; they contain the maximal number of hydrogen atoms possible. However, oleic, linoleic, and linolenic acids are 18-carbon fatty acid chains having one, two, and three double bonds, respectively, therein. Oleic acid is typically considered a monounsaturated fatty acid, whereas linoleic and linolenic are considered to be polyunsaturated fatty acids. The U.S.D.A. definition of "no sat" oil products as those having less than 3.5% fatty acid content is calculated as the combined saturated fatty acid content by weight (as compared to the total amount of fatty acids).

Canola oil has the lowest level of saturated fatty acids of all vegetable oils. "Canola" refers to rapeseed (*Brassica*) which has an erucic acid (C22:1) content of at most 2% by weight, based on the total fatty acid content of a seed (preferably at most 0.5% by weight, and most preferably essentially 0% by weight), and which produces, after crushing, an air-dried meal containing less than 30 $\mu\text{mol/g}$ of defatted (oil-free) meal. These types of rapeseed are distinguished by their edibility in comparison to more traditional varieties of the species.

It is postulated that, in oilseeds, fatty acid synthesis occurs primarily in the plastid. The major product of fatty acid synthesis is palmitate (16:0), which appears

to be efficiently elongated to stearate (18:0). While still in the plastid, the saturated fatty acids may then be desaturated by an enzyme known as acyl-ACP delta-9 desaturase, to introduce one or more carbon-carbon double bonds. Specifically, stearate may be rapidly desaturated by a plastidial delta-9 desaturase enzyme to yield oleate (18:1). In fact, palmitate may also be desaturated to palmitoleate (16:1) by the plastidial delta-9 desaturase, but this fatty acid appears in only trace quantities (0-0.2%) in most vegetable oils. Thus, the major products of fatty acid synthesis in the plastid are palmitate, stearate, and oleate. In most oils, oleate is the major fatty acid synthesized, as the saturated fatty acids are present in much lower proportions.

10 Newly-synthesized fatty acids are exported from the plastid to the cytoplasm. Subsequent desaturation of plant fatty acids in the cytoplasm appears to be limited to oleate, which may be desaturated to linoleate (18:2) and linolenate (18:3) by microsomal desaturases acting on oleoyl or lineoleoyl substrates esterified to phosphatidyl choline (PC). In addition, depending on the plant, oleate may be
15 further modified by elongation (to 20:1, 22:1, and/or 24:1), or by the addition of functional groups. These fatty acids, along with the saturated fatty acids, palmitate and stearate, are then assembled into triglycerides in endoreticular membranes.

The plant acyl-ACP delta-9 desaturase enzyme is soluble. It is located in the plastid stroma, and uses newly-synthesized fatty acids esterified to ACP,
20 predominantly stearyl-ACP, as substrates. This is in contrast to the other delta-9 desaturase enzymes, which are located in the endoplasmic reticular membrane (ER, or microsomal), use fatty acids esterified to Co-A as substrates, and desaturate both the saturated fatty acids, palmitate and stearate. U.S. Patents 5,723,595 and 6,706,950 relate to a plant desaturase.

25 The yeast delta-9 desaturase gene has been isolated from *Saccharomyces cerevisiae*, cloned, and sequenced. Stuke *et al.* (1989) J. Biol. Chem. 264:16537-44; Stuke *et al.* (1990) J. Biol. Chem. 265:20144-9. This yeast gene has been introduced into tobacco leaf tissue (Polashock *et al.* (1991) FASEB J. 5:A1157; Polashock *et al.* (1992) Plant Physiol. 100:894-901), and was apparently expressed in
30 this tissue. Further, this yeast gene was expressed in tomato. See Wang *et al.* (1996) J. Agric. Food Chem. 44:3399-402; and Wang *et al.* (2001) Phytochemistry 58:227-32. While some increases in certain unsaturated fatty acids, and some decreases in

certain saturated fatty acids, were reported for both tobacco and tomato using this yeast delta-9 desaturase gene, tobacco and tomato are clearly not oil crops. This yeast gene was also introduced into *Brassica napus*. U.S. Patent 5,777,201.

A different fungal acyl-CoA delta-9 desaturase from *Aspergillus nidulans* has been introduced into canola, thereby achieving reduced saturated fatty acid levels in seed oil. U.S. Patent Application Publication US 2008/0260933 A1. The *A. nidulans* acyl-CoA delta-9 desaturase provided greater depletion of stearate (61-90%) than the more abundant palmitate fatty acids (36-49%) in the seed oil.

10

DISCLOSURE OF THE INVENTION

Disclosed herein are novel fungal delta-9 desaturase enzymes; nucleic acids comprising at least one nucleotide sequence encoding such a desaturase; and plants, plant materials (e.g., seed), plant parts, and plant commodity products comprising either of the foregoing. Aspects of some embodiments are exemplified by fungal delta-9 desaturase enzymes isolated from *Magnaporthe grisea*, *Leptosphaeria nodorum*, and *Helicoverpa zea*. Some examples include native and synthetic delta-9 desaturases that have a substrate preference for palmitic acid or stearic acid.

Some embodiments comprise an isolated nucleic acid molecule encoding a delta-9 desaturase enzyme comprising an amino acid sequence being at least 80% identical to a sequence selected from the group consisting of SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:72, and SEQ ID NO:73. In particular examples, the nucleic acid molecule comprises a sequence being at least 60% identical to a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:48, and SEQ ID NO:49. These and further embodiments may include an isolated delta-9 desaturase polypeptide comprising an amino acid sequence being at least 80% identical to a sequence selected from the group consisting of SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:72, and SEQ ID NO:73.

Also disclosed are methods of expressing at least one of the aforementioned nucleic acids and/or polypeptides in a plant cell. Particular embodiments take

advantage of a delta-9 desaturase enzyme's activity, such that the percent composition of saturated fatty acids may be decreased in a plant, plant material (*e.g.*, seed), and/or plant part comprising the plant cell, and/or a plant commodity product produced from any of the foregoing. In certain embodiments, ω -7 fatty acids may concomitantly be increased in the plant, plant material, plant part, and/or plant commodity product.

Some embodiments include a method for decreasing the amount of saturated fatty acids in a plant, plant material, plant part, and/or plant commodity product, the method comprising transforming a plant cell with a nucleic acid molecule encoding a delta-9 desaturase polypeptide of the invention, such that the amount of saturated fatty acids in the cell is decreased. Some embodiments include a method for creating a genetically engineered plant that comprises decreased amounts of saturated fatty acids in the plant compared to a wild-type plant of the same species. Such a method may comprise transforming a plant material (or plant cell) with a nucleic acid molecule encoding a delta-9 desaturase polypeptide of the invention, and culturing the transformed plant material (or plant cell) to obtain a plant. In particular examples, a plant cell and/or plant material from an *Arabidopsis* sp. may be transformed with a nucleic acid molecule encoding a delta-9 desaturase polypeptide of the invention.

The disclosure as claimed relates to:

- an isolated nucleic acid molecule comprising a polynucleotide operably linked to a promoter that is functional in a plant cell, wherein the polynucleotide encodes an acyl-CoA delta-9 desaturase enzyme comprising an amino acid sequence at least 80% identical over the full length of SEQ ID NO:14;

- a method for producing a genetically modified plant cell, the method comprising: transforming a plant cell with the nucleic acid molecule as described herein; and

- a cell comprising the nucleic acid molecule as described herein.

The foregoing and other features will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 includes a schematic phylogenetic analysis of various fungal desaturase protein sequences. The complete protein sequences of the depicted desaturases were aligned using ClustalX and displayed using MEGA.

5 **FIGs. 2(a-d)** include an alignment of fungal delta-9 desaturase gene sequences. Capital font represents conserved nucleotides in this alignment. Shaded font represents identical nucleotides in this alignment.

FIGs. 3(a-b) include an alignment of fungal delta-9 desaturase polypeptides.

10 **FIGs. 4-18** include plasmid maps of exemplary plasmids comprising fungal delta-9 desaturase polypeptide-encoding nucleotide sequences that may be useful in some embodiments. **FIG. 4** specifically includes plasmid maps of exemplary plasmids

comprising LnD9DS-2-encoding (FIG. 4a; pDAB110110) and HzD9DS-encoding (FIG. 4b; pDAB110112) nucleotide sequences that further comprise the PvPhas 5' UTR and PvPhas 3' UTR.

FIG. 19 includes data showing the total saturated fatty acid content (%FAMES) of exemplary T₂ *Arabidopsis* seed from plants transformed with certain exemplary fungal delta-9 desaturase gene sequences.

FIG. 20 includes data showing the palmitic acid (C16:0) content (%FAMES) of exemplary T₂ *Arabidopsis* seed from plants transformed with certain exemplary fungal delta-9 desaturase gene sequences.

FIG. 21 includes data showing the stearic acid (C18:0) content (%FAMES) of exemplary T₂ *Arabidopsis* seed from plants transformed with certain exemplary fungal delta-9 desaturase gene sequences.

FIG. 22 includes data showing the palmitoleic acid (C16:1) content (%FAMES) of exemplary T₂ *Arabidopsis* seed from plants transformed with certain exemplary fungal delta-9 desaturase gene sequences.

FIG. 23 includes a graphical representation of the accumulation of HzD9DS and LnD9DS-2 mRNA transcripts (relative to AnD9DS transcripts) in developing seeds from canola plants transformed with pDAB7319 (AnD9DS v3 and LnD9DS-2 v2) or pDAB7324 (AnD9DS v3 and HzD9DS v2). The qRT-PCR $\Delta\Delta C_t$ of each gene was determined relative to the actin transcript level, and the amount of transcript for HzD9DS and LnD9DS-2 then normalized to the level of AnD9DS transcript in each sample.

SEQUENCE LISTING

The nucleic acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, as defined in 37 C.F.R. § 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand. In the accompanying sequence listing:

SEQ ID NO:1 shows a forward primer used to PCR amplify a fragment of a *Magnaporthe grisea* acyl-CoA delta-9 desaturase gene (referred to in some places as MgD9DS).

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SEQ ID NO:2 shows a reverse primer used to PCR amplify a fragment of a *M. grisea* acyl-CoA delta-9 desaturase gene (referred to in some places as MgD9DS).

SEQ ID NO:3 shows an exemplary fragment of a *M. grisea* acyl-CoA delta-9 desaturase gene (referred to in some places as MgD9DS) that was amplified by PCR.

5 SEQ ID NO:4 shows an exemplary intronless MgD9DS clone.

SEQ ID NO:5 shows an exemplary nucleic acid sequence encoding a first *Leptosphaeria nodorum* acyl-CoA delta-9 desaturase, referred to in some places as LnD9DS-1.

10 SEQ ID NOs:6 and 7 show primer sequences that may be useful in some embodiments..

SEQ ID NO:8 shows an exemplary nucleic acid sequence encoding a second exemplary *L. nodorum* acyl-CoA delta-9 desaturase, referred to in some places as LnD9DS-2.

15 SEQ ID NO:9 shows a coding region from an exemplary native delta-9 desaturase gene from *M. grisea* (labeled as MgD9DS v1).

SEQ ID NO:10 shows a coding region from an exemplary native delta-9 desaturase gene from *Helicoverpa zea* (labeled as HzD9DS v1).

SEQ ID NO:11 shows a coding region from an exemplary native delta-9 desaturase (LnD9DS-2 v1) gene from *L. nodorum*.

20 SEQ ID NO:12 shows the amino acid sequence of an exemplary native delta-9 desaturase from *M. grisea* (MgD9DS).

SEQ ID NO:13 shows the amino acid sequence of an exemplary native delta-9 desaturase from *H. zea* (HzD9DS).

25 SEQ ID NO:14 shows the amino acid sequence of an exemplary native delta-9 desaturase from *L. nodorum* (LnD9DS-2).

SEQ ID NO:15 shows the sequence of an exemplary canola-optimized delta-9 desaturase gene from *M. grisea* (MgD9DS v2).

SEQ ID NO:16 shows the sequence of an exemplary canola-optimized delta-9 desaturase gene from *H. zea* (HzD9DS v2).

30 SEQ ID NO:17 shows the sequence of an exemplary canola-optimized delta-9 desaturase gene from *L. nodorum* (LnD9DS-2 v2).

SEQ ID NOs:18-39 show the sequence of primers and probes that may be useful in some embodiments.

SEQ ID NOs:40-43 show exemplary alternative Kozak sequences that may be used to increase expression in some embodiments.

5 SEQ ID NO:44 shows the sequence of a further exemplary canola-optimized delta-9 desaturase gene from *L. nodorum* (LnD9DS-2 v3).

SEQ ID NO:45 shows the sequence of a further exemplary canola-optimized delta-9 desaturase gene from *H. zea* (HzD9DS v3).

SEQ ID NO:46 shows the amino acid sequence of a Myc tag.

10 SEQ ID NO:47 shows the amino acid sequence of a HA tag.

SEQ ID NO:48 shows an exemplary nucleic acid sequence encoding an *Aspergillus nidulans* delta-9 desaturase, referred to in some places as AnD9DS v2.

SEQ ID NO:49 shows a second exemplary nucleic acid sequence encoding an *A. nidulans* delta-9 desaturase, referred to in some places as AnD9DS v3.

15 SEQ ID NO:50 shows the amino acid sequence encoded by nucleic acids as exemplified by SEQ ID NOs:48-49 (AnD9DS).

SEQ ID NO:51 shows the amino acid sequence of another exemplary AnD9DS desaturase.

20 SEQ ID NO:52 shows the amino acid sequence of an exemplary native delta-9 desaturase (ScOLE1) from *Saccharomyces cerevisiae*.

SEQ ID NOs:53-66 show plasmids that may be useful in some embodiments.

SEQ ID NOs:67-71 include several nucleic acid regulatory control elements that may be useful in some embodiments.

25 SEQ ID NO:72 shows the N-terminal 68 residues (1-68) of an exemplary AnD9DS desaturase.

SEQ ID NO:73 shows the C-terminal 175 residues (281-455) of an exemplary AnD9DS desaturase.

SEQ ID NO:74 shows a map of plasmid pDAB110110.

SEQ ID NO:75 shows a map of plasmid pDAB110112.

30 SEQ ID NO:76 shows an exemplary nucleic acid sequence encoding an exemplary *M. grisea* acyl-CoA delta-9 desaturase, referred to in some places as MgD9DS.

SEQ ID NO:77 shows an amino acid sequence comprised within the exemplary native delta-9 desaturase from *L. nodorum* of SEQ ID NO:14.

SEQ ID NO:78 shows an amino acid sequence comprised within the exemplary native delta-9 desaturase from *H. zea* of SEQ ID NO:13.

5

MODE(S) FOR CARRYING OUT THE INVENTION

I. Overview of several embodiments

We previously introduced a fungal acyl-CoA delta-9 desaturase from *Aspergillus nidulans* into canola, thereby achieving reduced saturated fatty acid levels in seed oil. U.S. Patent Application Publication US 2008/0260933 A1. The *A. nidulans* delta-9 desaturase provided greater depletion of stearate (61-90%) than the more abundant palmitate fatty acids (36-49%) in the seed oil. Therefore, co-introduction of a delta-9 desaturase that acts preferentially on palmitate saturates will achieve further reductions in total saturates by complementing the stearate-
10 preferring activity of the *A. nidulans* delta-9 desaturase. In some embodiments of the present invention, fungal delta-9 desaturase polypeptides having a range of substrate specificities are disclosed. Particular embodiments include a palmitate-
15 preferring delta-9 desaturase (*e.g.*, a native fungal enzyme as disclosed herein, or a functional equivalent thereof; and a synthetic polypeptide designed to have a preference for a palmitic acid substrate).
20

Disclosed herein are nucleic acid molecules encoding a delta-9 desaturase polypeptide comprising a nucleotide sequence being at least 60% identical to a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:15,
25 SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:48, and SEQ ID NO:49. In some embodiments, the nucleic acid molecule may further comprises a gene regulatory element operably linked to the delta-9 desaturase polypeptide-encoding sequence. In particular embodiments, a gene regulatory element may be a phaseolin promoter, a phaseolin 5' untranslated region, a phaseolin 3' untranslated region, an *Agrobacterium tumefaciens* ORF1 3' untranslated region, a
30 Cassava vein Mosaic Virus promoter, a *Nicotiana tabacum* RB7 Matrix Attachment Region, a T-strand border sequence, a LfKCS3 promoter, and FAE 1 promoter.

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Also disclosed are delta-9 desaturase polypeptides comprising an amino acid sequence being at least 80% identical to a sequence selected from the group consisting of SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:50, SEQ ID NO:52, SEQ ID-NO:72, and SEQ ID NO:73, as well as nucleic acid molecules encoding such, ... ,
 5 delta-9 desaturase polypeptides.

In some embodiments, nucleic acid molecules and delta-9 desaturase polypeptides may
 embodiments of the invention include methods for decreasing the amount of saturated
 fatty acids in the plant material, cell, tissue, or whole plant. Such methods may
 comprise transforming a plant material, cell, tissue, or whole plant with at least one of
 10 the aforementioned nucleic acid molecules, such that the amount of saturated fatty
 acids in the plant material, cell, tissue, or whole plant is decreased. Particular
 embodiments include methods for preferentially decreasing palmitic and/or stearic fatty
 acids in a plant material, cell, tissue, or whole plant.

Methods disclosed herein may be performed, for example, on plants, or plant
 15 materials derived from plants (*e.g.*, plants of the genus *Arabidopsis*, or canola). A
 particular embodiment is drawn to methods for creating or regenerating a genetically
 engineered plant comprising decreased amounts of saturated fatty acids in the plant
 compared to a wild-type plant of the same species, the method comprising
 transforming a plant cell or material with at least one of the aforementioned nucleic
 20 acid molecules; and culturing the transformed plant material to obtain a plant. Plants,
 plant materials, plant cells, and seeds obtained by any of the aforementioned methods
 are also disclosed.

II. Abbreviations

25	$x:y\Delta^z$	fatty acid containing x carbons and y double bonds in position z counting from the carboxyl end
	ACP	acyl carrier protein
	CoA	coenzyme A
30	FA	fatty acids
	FAM	fluorescein
	FAS	fatty acid synthase
	FAME	fatty acid methyl ester
	KASII	β -ketoacyl-ACP synthase II
35	MUFA	monounsaturated fatty acid
	WT	wild type

III. Terms

Fatty acid: As used herein, the term "fatty acid" refers to long chain aliphatic acids (alkanoic acids) of varying chain lengths, for example, from about C12 to C22, although both longer and shorter chain-length acids are known. The structure of a fatty acid is represented by the notation, $x:y\Delta^z$, where "x" is the total number of carbon (C) atoms in the particular fatty acid, and "y" is the number of double bonds in the carbon chain in the position "z," as counted from the carboxyl end of the acid.

Metabolic pathway: The term, "metabolic pathway," refers to a series of chemical reactions occurring within a cell, catalyzed by enzymes, to achieve either the formation of a metabolic product, or the initiation of another metabolic pathway. A metabolic pathway may involve several or many steps, and may compete with a different metabolic pathway for specific reaction substrates. Similarly, the product of one metabolic pathway may be a substrate for yet another metabolic pathway.

Metabolic engineering: For the purposes of the present invention, "metabolic engineering" refers to the rational design of strategies to alter one or more metabolic pathways in a cell, such that the step-by-step modification of an initial substance into a product having the exact chemical structure desired is achieved within the overall scheme of the total metabolic pathways operative in the cell.

Desaturase: As used herein, the term "desaturase" refers to a polypeptide that can desaturate (*i.e.*, introduce a double bond) in one or more fatty acids to produce a fatty acid or precursor of interest. A plant-soluble fatty acid desaturase enzyme may introduce a double bond regiospecifically into a saturated acyl-ACP substrate. Acyl-CoA desaturases introduce a double bond regiospecifically into a saturated fatty acyl-CoA substrate. The reaction involves activation of molecular oxygen by a two-electron reduced diiron center coordinated by a four-helix bundle that forms the core of the desaturase architecture. Of particular interest in some embodiments are acyl-CoA delta-9 desaturases.

The delta-9-18:0¹-ACP desaturase is required by all plants for the maintenance of membrane fluidity. While this enzyme primarily desaturates stearoyl-ACP, it is also active to a minor extent with palmitoyl-ACP.

Variant desaturase: As used herein, the term "variant desaturase" encompasses those desaturases that exhibit specific activity profiles consistent with a role in producing unusual fatty acids. A variant desaturase may be isolated from an organism, engineered *via* a directed evolution program, or engineered as a synthetic desaturase incorporating conserved amino acids from one or more characterized desaturase.

Progeny plant: For the purposes of the present invention, "progeny plant," refers to any plant, or plant material obtained therefrom, that may be obtained by plant breeding methods. Plant breeding methods are well-known in the art, and include natural breeding, artificial breeding, selective breeding involving DNA molecular marker analysis, transgenics, and commercial breeding.

Plant material: As used herein, the term "plant material" refers to any cell or tissue obtained from a plant.

Nucleic acid molecule: A polymeric form of nucleotides, which can include both sense and anti-sense strands of RNA, cDNA, genomic DNA, and synthetic forms and mixed polymers of the above. A nucleotide refers to a ribonucleotide, deoxynucleotide, or a modified form of either type of nucleotide. A "nucleic acid molecule" as used herein is synonymous with "nucleic acid" and "polynucleotide." The term includes single- and double-stranded forms of DNA. A nucleic acid molecule can include either or both naturally occurring and modified nucleotides linked together by naturally occurring and/or non-naturally occurring nucleotide linkages.

Nucleic acid molecules can be modified chemically or biochemically, or can contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those of ordinary skill in the art. Such modification include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications, such as uncharged linkages (for example, methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), charged linkages (for example, phosphorothioates, phosphorodithioates, etc.), pendent moieties (for example, peptides), intercalators (for example, acridine, psoralen, etc.), chelators, alkylators, and modified linkages (for example, alpha anomeric nucleic acids, etc.). The term "nucleic acid molecule" also includes any topological conformation,

including single-stranded, double-stranded, partially duplexed, triplexed, hairpinned, circular and padlocked conformations.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. When recombinantly produced, operably linked nucleic acid sequences are generally contiguous and, where necessary to join two protein-coding regions, in the same reading frame. However, nucleic acids need not be contiguous to be operably linked.

Regulatory element: As used herein, the term "regulatory element" refers to a nucleic acid molecule having gene regulatory activity; *i.e.*, one that has the ability to affect the transcription or translation of an operably-linked transcribable nucleic acid molecule. Regulatory elements such as promoters, leaders, introns, and transcription termination regions are non-coding nucleic acid molecules having gene regulatory activity which play an integral part in the overall expression of genes in living cells. Isolated regulatory elements that function in plants are therefore useful for modifying plant phenotypes through the techniques of molecular engineering. By "regulatory element," it is intended a series of nucleotides that determines if, when, and at what level a particular gene is expressed. The regulatory DNA sequences specifically interact with regulatory proteins or other proteins.

As used herein, the term "gene regulatory activity" refers to a nucleic acid molecule capable of affecting transcription or translation of an operably linked nucleic acid molecule. An isolated nucleic acid molecule having gene regulatory activity may provide temporal or spatial expression or modulate levels and rates of expression of the operably linked nucleic acid molecule. An isolated nucleic acid molecule having gene regulatory activity may comprise a promoter, intron, leader, or 3' transcriptional termination region.

Promoters: As used herein, the term "promoter" refers to a nucleic acid molecule that is involved in recognition and binding of RNA polymerase II or other proteins such as transcription factors (trans-acting protein factors that regulate transcription) to initiate transcription of an operably linked gene. Promoters may

themselves contain sub-elements such as cis-elements or enhancer domains that effect the transcription of operably linked genes. A "plant promoter" is a native or non-native promoter that is functional in plant cells. A plant promoter can be used as a 5' regulatory element for modulating expression of an operably linked gene or genes.

- 5 Plant promoters may be defined by their temporal, spatial, or developmental expression pattern. The nucleic acid molecules described herein may comprise nucleic acid sequences comprising promoters.

Sequence identity: The term "sequence identity" or "identity," as used herein in the context of two nucleic acid or polypeptide sequences, may refer to the residues in
10 the two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

When percentage of sequence identity is used in reference to proteins, it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino
15 acid residues with similar chemical properties (*e.g.*, charge, hydrophobicity, or steric effects), and therefore do not change the functional properties of the molecule.

Therefore, when sequences differ by conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution at the site of the non-identical residue. Sequences that differ by such
20 conservative substitutions are said to have "sequence similarity" or "similarity." Techniques for making this adjustment are well known to those of ordinary skill in the art. Typically, such techniques involve scoring a conservative substitution as a partial, rather than a full, mismatch, thereby increasing the percentage sequence identity. For example, where an identical amino acid is given a score between 0 and 1, and a non-
25 conservative substitution is given a score of 0, a conservative substitution is given a score between 0 and 1. The scoring of conservative substitutions may be calculated, for example, as implemented in the program PC/GENE (Intelligenetics, Mountain View, CA).

As used herein, the term "percentage of sequence identity" may refer to the
30 value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does

not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleotide or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the comparison window, and multiplying the result by 100 to yield the percentage of sequence identity.

Analogous position in an amino acid sequence: Nucleic acid and amino acid sequences may be aligned by the methods described in the following paragraphs. When aligned, a position in one sequence is in "an analogous position" with a position in the aligned sequence if the positions are identical within the consensus sequence.

Methods for aligning sequences for comparison are well-known in the art. Various programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. math.* 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins and Sharp, *Gene* 73:237-44, 1988; Higgins and Sharp, *CABIOS* 5:151-3, 1989; Corpet *et al.*, *Nucleic Acids Research* 16:10881-10890, 1988; Huang, *et al.*, *Computer Applications in the Biosciences* 8:155-65, 1992; Pearson *et al.*, *Methods in Molecular Biology* 24:307-31, 1994; Tatiana *et al.*, *FEMS Microbiol. Lett.*, 174:247-50, 1990. Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990 (detailed consideration of sequence-alignment methods and homology calculations).

The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) is available on the Internet (at blast.ncbi.nlm.nih.gov/Blast.cgi), for use in connection with sequence-analysis programs, for example, *blastp* and *blastn*. A description of how to determine sequence identity using this program is available on the Internet through NCBI.

For comparisons of amino acid sequences, the "Blast 2 sequences" function of the BLAST program (*bl2seq*) is employed using the default parameters. Specific parameters may be adjusted within the discretion of one of skill in the art, to for example, provide a penalty for a mismatch or reward for a match.

Transformed: As used herein, the term "transformed" refers to a cell, tissue, organ, or organism into which has been introduced a foreign nucleic acid molecule,

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such as a construct. The introduced nucleic acid molecule may be integrated into the genomic DNA of the recipient cell, tissue, organ, or organism such that the introduced polynucleotide molecule is inherited by subsequent progeny. A "transgenic" or "transformed" cell or organism also includes progeny of the cell or organism and
5 progeny produced from a breeding program employing such a transgenic plant as a parent in, for example, a cross and exhibiting an altered phenotype resulting from the presence of a foreign nucleic acid molecule.

10 *IV. Metabolic engineering approaches to decreasing saturated fatty acids in a host cell, tissue, or organism*

A. Overview

An embodiment of the invention includes introducing delta-9 desaturases with specific acyl-CoA preferences (for example, for palmitic or stearic acid) in plant seeds. The specific acyl-CoA preference of the delta-9 desaturase enables targeting of certain
15 specific saturated fatty acid pools (e.g., palmitate for conversion to monounsaturated products). Acyl-CoA delta-9 desaturases were selected for lowering the saturated fatty acid content in plants as they are not normally produced in plant systems to any appreciable extent.

B. Polypeptides

20 Polypeptides according to some embodiments of the present invention comprise an amino acid sequence showing increasing percentage identities when aligned with a sequence selected from the group consisting of SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:72, and SEQ ID NO:73. Specific amino acid sequences within these and other embodiments may
25 comprise sequences having, for example, at least about 70%, about 75%, about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity with the aforementioned sequences. In many embodiments, the amino acid sequence having the aforementioned sequence identity when aligned with the aforementioned sequences encode a peptide with enzymatic
30 delta-9-18:0-ACP desaturase activity, or part of a such a peptide.

C. Nucleic acids

Some embodiments include nucleic acid molecules encoding a polypeptide described above. For example, nucleic acid sequences in some embodiments show increasing percentage identities when aligned with a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:48, and SEQ ID NO:49. Specific nucleic acid sequences within these and other embodiments may comprise sequences having, for example, at least about 60%, about 65%, about 70%, about 75%, about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity with a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:48, and SEQ ID NO:49. It is understood by those of ordinary skill in the art that nucleic acid molecules may be modified without substantially changing the amino acid sequence of an encoded polypeptide, for example, by introducing permissible nucleotide substitutions according to codon degeneracy.

In some embodiments, nucleic acid molecules of the present invention comprise a gene regulatory element (*e.g.*, a promoter). Promoters may be selected on the basis of the cell type into which the vector construct will be inserted. Promoters which function in bacteria, yeast, and plants are well-known in the art. The promoters may also be selected on the basis of their regulatory features. Examples of such features include enhancement of transcriptional activity, inducibility, tissue-specificity, and developmental stage-specificity. In plants, promoters that are inducible, of viral or synthetic origin, constitutively active, temporally regulated, and spatially regulated have been described. *See, e.g.*, Poszkowski *et al.* (1989) EMBO J. 3:2719; Odell *et al.* (1985) Nature 313:810; and Chau *et al.* (1989) Science 244:174-81).

Useful inducible promoters include, for example, promoters induced by salicylic acid or polyacrylic acids induced by application of safeners (substituted benzenesulfonamide herbicides), heat-shock promoters, a nitrate-inducible promoter derived from the spinach nitrate reductase transcribable nucleic acid molecule

sequence, hormone-inducible promoters, and light-inducible promoters associated with the small subunit of RuBP carboxylase and LHCP families.

Examples of useful tissue-specific, developmentally-regulated promoters include the β -conglycinin 7S α -promoter and seed-specific promoters. Plant functional promoters useful for preferential expression in seed plastid include those from proteins involved in fatty acid biosynthesis in oilseeds and from plant storage proteins. Examples of such promoters include the 5' regulatory regions from such transcribable nucleic acid molecule sequences as phaseolin, napin, zein, soybean trypsin inhibitor, ACP, stearyl-ACP desaturase, and oleosin. Another exemplary tissue-specific promoter is the lectin promoter, which is specific for seed tissue.

Other useful promoters include the nopaline synthase, mannopine synthase, and octopine synthase promoters, which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*; the cauliflower mosaic virus (CaMV) 19S and 35S promoters; the enhanced CaMV 35S promoter; the Figwort Mosaic Virus 35S promoter; the light-inducible promoter from the small subunit of ribulose-1,5-bisphosphate carboxylase (ssRUBISCO); the EIF-4A promoter from tobacco (Mandel *et al.* (1995) Plant Mol. Biol. 29:995-1004); corn sucrose synthetase; corn alcohol dehydrogenase I; corn light harvesting complex; corn heat shock protein; the chitinase promoter from Arabidopsis; the LTP (Lipid Transfer Protein) promoters; petunia chalcone isomerase; bean glycine rich protein 1; potato patatin; the ubiquitin promoter; and the actin promoter. Useful promoters are preferably seed-selective, tissue selective, or inducible. Seed-specific regulation is discussed in, for example, EP 0 255 378.

To obtain higher expression of a heterologous gene(s), it may be preferred to reengineer the gene(s) so that it is more efficiently expressed in the expression host cell (e.g., a plant cell, for example, canola, rice, tobacco, maize, cotton, and soybean). Therefore, an optional additional step in the design of a gene encoding a delta-9 desaturase for plant expression (*i.e.*, in addition to the provision of one or more gene regulatory elements) is reengineering of a heterologous gene protein coding region for optimal expression. Particular embodiments include redesigned genes that have been optimized to increase the expression level (*i.e.* produce more protein) in a transgenic

canola plant cell or *Arabidopsis* plant cell than in a canola plant cell or *Arabidopsis* plant cell transformed with the naturally-occurring heterologous gene sequence.

Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (*i.e.*, some amino acids are specified by more than one codon), evolution of the
5 genomes in different organisms or classes of organisms has resulted in differential usage of synonymous codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms having genomes with relatively low G+C contents utilize more codons having A or T in the third position of
10 synonymous codons, whereas those having higher G+C contents utilize more codons having G or C in the third position. Further, it is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this reasoning is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons.
15 Therefore, mRNAs having high relative contents of minor codons in a particular expression host would have correspondingly low translation rates. This rate may be reflected by correspondingly low levels of the encoded protein.

In engineering optimized genes encoding a delta-9 desaturase for expression in canola or *Arabidopsis* (or other plants, such as rice, tobacco, maize, cotton or soybean),
20 it is helpful if the codon bias of the prospective host plant(s) has been determined. Multiple publicly-available DNA sequence databases exist wherein one may find information about the codon distribution of plant genomes or the protein coding regions of various plant genes.

The codon bias is the statistical distribution of codons that the expression host
25 (*e.g.*, a plant such as canola or *Arabidopsis*) uses for coding the amino acids of its proteins. The codon bias can be calculated as the frequency at which a single codon is used relative to the codons for all amino acids. Alternatively, the codon bias may be calculated as the frequency at which a single codon is used to encode a particular amino acid, relative to all the other codons for that amino acid (synonymous codons).

30 In designing optimized coding regions for plant expression of delta-9 desaturase genes, the primary ("first choice") codons preferred by the plant should be determined, as well as the second, third, fourth *etc.* choices of preferred codons when

multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the delta-9 desaturase gene, wherein the new DNA sequence differs from the native DNA sequence (encoding the desaturase) by the substitution of expression host-preferred (first preferred, second preferred, third preferred, or fourth preferred, *etc.*) codons to specify the amino acid at each position within the amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modifications. The identified putative restriction sites are further modified by replacing these codons with a next-preferred codon to remove the restriction site. Other sites in the sequence which may affect transcription or translation of heterologous sequence are exon:intron junctions (5' or 3'), poly-A addition signals, and/or RNA polymerase termination signals. The sequence may be further analyzed and modified to reduce the frequency of TA or CG doublets. In addition to these doublets, sequence blocks that have more than about six G or C nucleotides that are the same may also adversely affect transcription or translation of the sequence. Therefore, these blocks are advantageously modified by replacing the codons of first or second choice, *etc.* with the next-preferred codon of choice.

The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402. Thus, optimized synthetic genes that are functionally equivalent to desaturases/genes of some embodiments may be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Patent 5,380,831.

Once a plant-optimized DNA sequence has been designed on paper or *in silico*, actual DNA molecules can be synthesized in the laboratory to correspond in sequence precisely to the designed sequence. Such synthetic DNA molecules may be cloned and otherwise manipulated exactly as if they were derived from natural or native sources.

D. Methods for genetic transformation of plant material

Some embodiments are directed to a method of producing a transformed cell that comprises one or more nucleic acid molecule(s) comprising a nucleic acid sequence at least 60% identical to a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:9, SEQ ID

NO:10, SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:48, and SEQ ID NO:49. Such nucleic acid molecules may also comprise, for example, non-coding regulatory elements, such as promoters. Other sequences may also be introduced into the cell along with the non-coding regulatory elements and transcribable nucleic acid molecule sequences. These other sequences may include 3' transcriptional terminators, 3' poly-adenylation signals, other untranslated sequences, transit or targeting sequences, selectable markers, enhancers, and operators.

A method of transformation generally comprises the steps of selecting a suitable host cell, transforming the host cell with a recombinant vector, and obtaining the transformed host cell. Technology for introduction of DNA into cells is well-known to those of skill in the art. These methods can generally be classified into five categories: (1) chemical methods (Graham and Van der Eb (1973) *Virology* 54(2):536-9; Zatloukal *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:136-53); (2) physical methods such as microinjection (Capechi (1980) *Cell* 22(2):479-88), electroporation (Wong and Neumann (1982) *Biochim. Biophys. Res. Commun.* 107(2):584-7; Fromm *et al.* (1985) *Proc. Natl. Acad. Sci. USA* 82(17):5824-8; U.S. Patent 5,384,253), and particle acceleration (Johnston and Tang (1994) *Methods Cell Biol.* 43(A):353-65; Fynan *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(24):11478-82; (3) viral vectors (Clapp (1993) *Clin. Perinatol.* 20(1):155-68; Lu *et al.* (1993) *J. Exp. Med.* 178(6):2089-96; Eglitis and Anderson (1988) *Biotechniques* 6(7):608-14); (4) receptor-mediated mechanisms (Curiel *et al.* (1992) *Hum. Gen. Ther.* 3(2):147-54; Wagner *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89(13):6099-103); and (5) bacterial-mediated mechanisms, such as with *Agrobacterium*. Alternatively, nucleic acids may be directly introduced into pollen by directly injecting a plant's reproductive organs. Zhou *et al.* (1983) *Methods in Enzymology* 101:433; Hess (1987) *Intern. Rev. Cytol.* 107:367; Luo *et al.* (1988) *Plant Mol. Biol. Reporter* 6:165; Pena *et al.* (1987) *Nature* 325:274. Other transformation methods include, for example, protoplast transformation as illustrated in U.S. Patent 5,508,184. Nucleic acid molecules may also be injected into immature embryos. Neuhaus *et al.* (1987) *Theor. Appl. Genet.* 75:30.

The most commonly used methods for transformation of plant cells are: the *Agrobacterium*-mediated DNA transfer process (Fraley *et al.* (1983) *Proc. Natl. Acad.*

- Sci. USA 80:4803) (as illustrated in U.S. Patent 5,824,877; U.S. Patent 5,591,616; U.S. Patent 5,981,840; and U.S. Patent 6,384,301) and the biolistics or microprojectile bombardment-mediated process (*i.e.*, the gene gun) (such as described in U.S. Patent 5,550,318; U.S. Patent 5,538,880; U.S. Patent 6,160,208; U.S. Patent 6,399,861; and
5 U.S. Patent 6,403,865). Typically, nuclear transformation is desired, but where it is desirable to specifically transform plastids, such as chloroplasts or amyloplasts, plant plastids may be transformed utilizing a microprojectile-mediated delivery of the desired nucleic acid molecule in certain plant species, such as for example, *Arabidopsis*, tobacco, potato, and *Brassica* species.
- 10 *Agrobacterium*-mediated transformation is achieved through the use of a genetically engineered soil bacterium belonging to the genus *Agrobacterium*. Several *Agrobacterium* species mediate the transfer of a specific DNA known as "T-DNA," which can be genetically engineered to carry any desired piece of DNA into many plant species. The major events marking the process of T-DNA mediated pathogenesis
15 are: induction of virulence genes, and processing and transfer of T-DNA. This process is the subject of many reviews. *See, e.g.*, Ream (1989) *Ann. Rev. Phytopathol.* 27:583-618; Howard and Citovsky (1990) *Bioassays* 12:103-8; Kado (1991) *Crit. Rev. Plant Sci.* 10:1-32; Zambryski (1992) *Annual Rev. Plant Physiol. Plant Mol. Biol.* 43:465-90; Gelvin (1993) in Transgenic Plants, Kung and Wu eds., Academic Press, San
20 Diego, CA, pp. 49-87; Binns and Howitz (1994) In Bacterial Pathogenesis of Plants and Animals, Dang, ed., Berlin: Springer Verlag, pp. 119-38; Hooykaas and Beijersbergen (1994) *Ann. Rev. Phytopathol.* 32:157-79; Lessl and Lanka (1994) *Cell* 77:321-4; and Zupan and Zambryski (1995) *Annual Rev. Phytopathol.* 27:583-618.

- To select or score for transformed plant cells regardless of transformation
25 methodology, the DNA introduced into the cell may contain a gene that functions in a regenerable plant tissue to produce a compound that confers upon the plant tissue resistance to an otherwise toxic compound. Genes of interest for use as a selectable, screenable, or scorable marker include, but are not limited to, β -glucuronidase (GUS), green fluorescent protein (GFP), luciferase, and antibiotic or herbicide tolerance genes.
- 30 Examples of antibiotic resistance genes include genes conferring resistance to the penicillins, kanamycin (and neomycin, G418, bleomycin); methotrexate (and trimethoprim); chloramphenicol; and tetracycline. For example, glyphosate resistance

may be conferred by a herbicide resistance gene. Della-Cioppa *et al.* (1987) Bio/Technology 5:579-84. Other selection devices can also be implemented, including for example and without limitation, tolerance to phosphinothricin, bialaphos, and positive selection mechanisms (Joersbro *et al.* (1998) Mol. Breed. 4:111-7), and are considered within the scope of embodiments of the present invention.

The transformed cells, identified by selection or screening and cultured in an appropriate medium that supports regeneration, may then be allowed to mature into plants.

The presently disclosed methods may be used with any transformable plant cell or tissue. Transformable cells and tissues, as used herein, includes but is not limited to those cells or tissues that are capable of further propagation to give rise to a plant. Those of skill in the art recognize that a number of plant cells or tissues are transformable in which after insertion of exogenous DNA and appropriate culture conditions the plant cells or tissues can form into a differentiated plant. Tissue suitable for these purposes can include but is not limited to immature embryos, scutellar tissue, suspension cell cultures, immature inflorescence, shoot meristem, nodal explants, callus tissue, hypocotyl tissue, cotyledons, roots, and leaves.

The regeneration, development, and cultivation of plants from transformed plant protoplast or explants are known in the art. Weissbach and Weissbach (1988) Methods for Plant Molecular Biology, (Eds.) Academic Press, Inc., San Diego, CA; Horsch *et al.* (1985) Science 227:1229-31. This regeneration and growth process typically includes the steps of selecting transformed cells and culturing those cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. In this method, transformants are generally cultured in the presence of a selective media which selects for the successfully transformed cells and induces the regeneration of plant shoots. Fraley *et al.* (1993) Proc. Natl. Acad. Sci. USA 80:4803. These shoots are typically obtained within two to four months. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil. Cells that survive the exposure to a selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. The shoots may then be transferred to an appropriate root-inducing medium containing the selective

agent and an antibiotic to prevent bacterial growth. Many of the shoots will develop roots. These are then transplanted to soil or other media to allow the continued development of roots. The method, as outlined above, will generally vary depending on the particular plant strain employed, and particulars of the methodology are therefore within the discretion of one of skill in the art.

The regenerated transgenic plants may be self-pollinated to provide homozygous transgenic plants. Alternatively, pollen obtained from the regenerated transgenic plants may be crossed with non-transgenic plants, preferably inbred lines of agronomically important species. Conversely, pollen from non-transgenic plants may be used to pollinate the regenerated transgenic plants.

The transgenic plant may pass along the transformed nucleic acid sequence to its progeny. The transgenic plant is preferably homozygous for the transformed nucleic acid sequence and transmits that sequence to all of its offspring upon, and as a result of, sexual reproduction. Progeny may be grown from seeds produced by the transgenic plant. These additional plants may then be self-pollinated to generate a true breeding line of plants.

The progeny from these plants may be evaluated, among other things, for gene expression. The gene expression may be detected by several common methods such as western blotting, northern blotting, immunoprecipitation, and ELISA (Enzyme-Linked ImmunoSorbent Assay). The transformed plants may also be analyzed for the presence of the introduced DNA and the expression level and/or fatty acid profile conferred by the nucleic acid molecules and amino acid molecules of the present invention. Those of skill in the art are aware of the numerous methods available for the analysis of transformed plants. For example, methods for plant analysis include, but are not limited to, Southern blots or northern blots, PCR-based approaches, biochemical assays, phenotypic screening methods, field evaluations, and immunodiagnostic assays.

Methods for specifically transforming dicots are well-known to those skilled in the art. Transformation and plant regeneration using these methods have been described for a number of crops including, but not limited to, members of the genus *Arabidopsis*, cotton (*Gossypium hirsutum*), soybean (*Glycine max*), peanut (*Arachis hypogaea*), and members of the genus *Brassica*. Methods for transforming dicots, primarily by use of *Agrobacterium tumefaciens*, and obtaining transgenic plants have

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been published for cotton (U.S. Patent 5,004,863; U.S. Patent 5,159,135; U.S. Patent 5,518,908); soybean (U.S. Patent 5,569,834; U.S. Patent 5,416,011; McCabe *et al.* (1988) Biotechnology 6:923; Christou *et al.* (1988) Plant Physiol. 87:671-4); *Brassica* (U.S. Patent 5,463,174); peanut (Cheng *et al.* (1996) Plant Cell Rep. 15:653-7; McKently *et al.* (1995) Plant Cell Rep. 14:699-703); papaya; and pea (Grant *et al.* (1995) Plant Cell Rep. 15:254-8).

Methods for transforming monocots are also well-known in the art. Transformation and plant regeneration using these methods have been described for a number of crops including, but not limited to, barley (*Hordeum vulgare*); maize (*Zea mays*); oats (*Avena sativa*); orchard grass (*Dactylis glomerata*); rice (*Oryza sativa*, including indica and japonica varieties); sorghum (*Sorghum bicolor*); sugar cane (*Saccharum sp.*); tall fescue (*Festuca arundinacea*); turfgrass species (e.g., *Agrostis stolonifera*, *Poa pratensis*, *Stenotaphrum secundatum*); wheat (*Triticum aestivum*); and alfalfa (*Medicago sativa*). It is apparent to those of skill in the art that a number of transformation methodologies can be used and modified for production of stable transgenic plants for any number of target crops of interest.

Any plant may be chosen for use in the presently disclosed methods. Preferred plants for modification according to the present invention include, for example and without limitation, oilseed plants, *Arabidopsis thaliana*, borage (*Borago spp.*), canola (*Brassica spp.*), castor (*Ricinus communis*), cocoa bean (*Theobroma cacao*), corn (*Zea mays*), cotton (*Gossypium spp.*), *Crambe spp.*, *Cuphea spp.*, flax (*Linum spp.*), *Lesquerella* and *Limnanthes spp.*, Linola, nasturtium (*Tropaeolum spp.*), *Oenothera spp.*, olive (*Olea spp.*), palm (*Elaeis spp.*), peanut (*Arachis spp.*), rapeseed, safflower (*Carthamus spp.*), soybean (*Glycine* and *Soja spp.*), sunflower (*Helianthus spp.*), tobacco (*Nicotiana spp.*), *Vernonia spp.*, wheat (*Triticum spp.*), barley (*Hordeum spp.*), rice (*Oryza spp.*), oat (*Avena spp.*) sorghum (*Sorghum spp.*), and rye (*Secale spp.*) or other members of the *Gramineae*.

It is apparent to those of skill in the art that a number of transformation methodologies can be used and modified for production of stable transgenic plants from any number of target crops of interest.

E. Transgenic seeds

In some embodiments, a transgenic seed may comprise a delta-9 desaturase polypeptide comprising an amino acid sequence being at least 80% identical to a sequence selected from the group consisting of SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:72, and SEQ ID NO:73. In these and other embodiments, the transgenic seed may comprise a nucleic acid sequence being at least 60% identical to a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:48, and SEQ ID NO:49. In certain embodiments, a transgenic seed may exhibit decreased levels of saturated fatty acids (for example, palmitic fatty acids and/or stearic fatty acids). The seeds may be harvested from a fertile transgenic plant, and may be used to grow progeny generations of transformed plants, including hybrid plant lines comprising at least one nucleic acid sequence as set forth above, and optionally at least one additional gene or nucleic acid construct of interest.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the invention to the particular features or embodiments described.

20

EXAMPLES

Example I: Cloning of acyl-CoA delta-9 desaturases and functional characterization in *ole1*-deficient yeast

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Cloning of *Magnaporthe grisea* acyl-CoA delta-9 desaturases

The *Magnaporthe grisea* acyl-CoA delta-9 desaturase gene (MgD9DS) was isolated from genomic DNA using primers based on a published NCBI/Broad Institute sequence originally annotated as a "hypothetical protein," and having 55.4% identity at the nucleotide level to the *S. cerevisiae* acyl-CoA delta-9 desaturase (i.e., *OLE1*). Forward and reverse primers, each 41 base pairs in length, were designed. The forward primer, MgA9F (SEQ ID NO:1), included an *EcoRI* site at the 5' end. The reverse

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primer, Mg9AR (SEQ ID NO:2), contained stop codons in each of three reading frames and a terminal *XhoI* site.

The MgD9DS gene was PCR amplified using the Takara EZ Taq™ PCR kit (Takara Bio Inc., Otsu, Shiga, Japan) following the manufacturer's protocol. The amplification conditions were 94 °C for 1 minute, followed by 30 cycles of 94 °C for 30 sec, 60 °C for 60 seconds, and an extension at 72 °C for 90 seconds. A final extension step was performed at 72 °C for 10 minutes. The expected 1,425 base pair PCR product was excised from an agarose gel and purified using Montage spin columns per manufacturer's recommendations (Millipore, Billerica, MA). The purified fragment was cloned into the pCR®2.1 TOPO® cloning vector (Invitrogen, Carlsbad, CA). The TOPO reaction was transformed into chemically competent Top 10 *E. coli* cells per supplier conditions. Bacterial colonies containing the putative clone were isolated. Mini-plasmid preps were performed with a Macherey-Nagel Nucleospin DNA isolation kit (Macherey-Nagel, Neumann-Neander-Strasse, Düren, Germany), and DNA was digested with *EcoRI* and *XhoI* restriction enzymes. Positive clones containing the expected 1,425 bp MgD9DS gene fragment were identified. The nucleotide sequence was obtained *via* sequencing reactions. The sequence of the PCR amplified fragment is listed as SEQ ID NO:3.

Sequence analysis revealed a small (90 bp) intron located in the 5' end of the MgD9DS gene. The intron was removed using Splice Overlap Extension PCR. The resulting PCR amplicon was gel purified, cloned into the pCR®2.1 TOPO® cloning vector, and transformed into Top 10 *E. coli* cells. Several clones were identified *via* analysis of restriction enzyme digests of purified DNA from single transformant colonies. These clones were sequenced to confirm the presence of an intronless MgD9DS clone. The resulting sequence is listed as SEQ ID NO:4.

The MgD9DS genes, with and without the intron, were each subcloned as an *EcoRI/XhoI* fragment into a yeast expression vector. This yeast expression vector contains an *Aspergillus nidulans* delta-9 desaturase (AnD9DS) gene flanked by the *S. cerevisiae* delta-9 desaturase promoter and delta-9 desaturase 3'UTR/terminator. The *Aspergillus nidulans* delta-9 desaturase gene was excised on an *EcoRI/XhoI* fragment, which was replaced with either the MgD9DS gene-containing fragment or the intronless MgD9DS gene-containing fragment. Two clones containing the MgD9DS

gene (one with an intron, and one without an intron) were advanced for *S. cerevisiae* transformation.

A delta-9 desaturase deficient *S. cerevisiae* strain (OFY093), which is maintained on Yeast Peptone Dextrose (YPD) media with Tween[®] 80, was transformed using the Alkali-Cation Yeast Transformation Kit (Qbiogene, Montreal, Canada). Complemented strains were identified by growth on media that did not contain Tween[®] 80 (monounsaturated fatty acid supplement) or uracil (Dropout Base with Agar with SC-URA). Complemented strains were single colony purified on selective media three times. Complemented strains were further verified by PCR amplification of the delta-9 desaturase gene, and sequencing of the PCR product. In addition, strains containing the MgD9DS clone were reverted to fatty acid and uracil dependence by passing the strain at least three times on YPD + Tween 80[®] media, then patching strains to DOBA SC-URA minus Tween[®] 80 media.

Expression of the intron-containing MgD9DS coding sequence was unsuccessful, indicating that the intron was not spliced by the yeast machinery. The substrate specificity of the yeast strain containing the intronless MgD9DS coding sequence was further characterized by FAME analysis.

Cloning of *Leptosphaeria nodorum* acyl-CoA delta-9 desaturases

Two *Leptosphaeria nodorum* EST sequences (1,246 and 429 base pairs, respectively) were identified from a collection of *L. nodorum* ESTs by using a BlastN search as sharing high levels of sequence identity (54.0% and 54.2% respectively) with the *S. cerevisiae* acyl-CoA delta-9 desaturase (*OLE1*). When aligned, these sequences were 64.6% identical to one another, suggesting the presence of two distinct *Leptosphaeria nodorum* acyl-CoA delta-9 desaturases. An LnD9DS-1 gene (SEQ ID NO:5) was isolated by screening a *L. nodorum* cDNA library with the 1,246 bp gene probe. The sequence of this gene was obtained, and the coding sequence was isolated. The entire sequence of an LnD9DS-2 gene was isolated by first BLAST searching the published Broad Institute *Leptosphaeria nodorum* genome sequence with the 429 bp EST sequence. This search identified Supercontig Ln 1.4 as containing a gene with 100% homology to the 429 bp fragment, which gene was annotated as encoding a "hypothetical protein." Next, the LnD9DS-2 gene was cloned from a *Leptosphaeria nodorum* cDNA library using PCR primers based on the Ln1.4 supercontig sequence.

The primer sequences used were Lnd9FAD2F (SEQ ID NO:6) and Lnd9FAD2R (SEQ ID NO:7). The forward primer was designed with a 5' *Bam*HI site, and the reverse primer contained stop codons in three reading frames and a terminal *Nco*I site.

An aliquot of the *Leptosphaeria nodorum* cDNA library was diluted 1/10 to provide 400 ng of template DNA for the PCR reaction. PCR amplification was performed using a Takara EZ Taq™ PCR kit following the recommended amplification conditions of 94 °C for 1 minute, followed by 30 cycles of 94 °C for 30 seconds, 60 °C for 60 seconds, and extension at 72 °C for 90 seconds. A final extension step was performed at 72 °C for 10 minutes. The expected 1,370 base pair product was excised from an agarose gel, and purified using Montage spin columns per the manufacturer's recommendations. The purified fragment was cloned into the pCR®2.1 TOPO® cloning vector. The ligation reaction was transformed into chemically competent Top 10 *E. coli* cells according to the manufacturer's recommended protocol. Colonies containing a putative clone were isolated. Mini plasmid preps were performed with Macherey-Nagel Nucleospin columns, and DNA was digested with *Bam*HI and *Nco*I restriction enzymes. Putative Lnd9DS-2 clones were identified and sequenced.

Upon sequencing, a clone of Lnd9DS-2 (SEQ ID NO:8) was confirmed by comparison with the "hypothetical protein" sequence. A conservative change in the sequence of Lnd9DS-2 was identified. The codon TGC (cysteine) was changed to AGC (serine) by substitution of an adenine for a thymidine at base position 271, which codon is translated to amino acid 89 of the published sequence. This is a conservative change, and the cysteine is not found to be a highly conserved amino acid among multiple filamentous fungi, so no correction was attempted.

The Lnd9DS-1 and Lnd9DS-2 genes of SEQ ID NOs:5 and 8, respectively, were cloned into a yeast expression vector. Clones containing either of the Lnd9DS-1 and Lnd9DS-2 coding sequences were confirmed by restriction enzyme analysis and DNA sequencing.

A delta-9 desaturase deficient *S. cerevisiae* strain (OFY093), which is maintained on YPD media with Tween® 80, was transformed using the Alkali-Cation Yeast Transformation Kit from Qbiogene. Complemented strains were identified by growth on media that did not contain Tween® 80 (monounsaturated fatty acid

supplement) or uracil (DOBA sc-ura). The complemented strains were single colony purified on selective media three times. Complemented strains were further verified by PCR amplification of the delta-9 desaturase gene and sequencing of the PCR product. In addition, strains containing a LnD9DS-2 clone were reverted to fatty acid and uracil dependence by passing each strain at least three times on YPD + Tween[®] 80 media, then patching strains to DOBA SC-URA minus Tween[®] 80 media. The substrate specificities of the yeast strains containing either the LnD9DS-1 or LnD9DS-2 coding sequence were further characterized by FAME analysis.

Cloning and Transformation of Delta-9 Desaturase Deficient *S. cerevisiae* with

10 HzD9DS Gene

A plant-optimized synthetic gene encoding the *Helicoverpa zea* acyl-CoA delta-9 desaturase (HzD9DS) (identified as HzPGDS2 in Rosenfield *et al.* (2001) Insect Biochem. Mol. Biol. 31(10):949-64) was excised from DASPICO89 (described below) on a *Bam*HI/*Xho*I fragment and gel purified using Montage spin columns. This fragment was ligated into corresponding restriction enzyme sites of a yeast expression vector described previously and transformed into *E. coli* strain DH5 α using standard molecular biology techniques and supplier protocols (Invitrogen, Carlsbad, CA).

Following restriction analysis and DNA sequencing, a clone containing the HzD9DS gene was selected for transformation into the delta-9 desaturase deficient *S. cerevisiae* strain, OFY093. The OFY093 strain, which is maintained on YPD media with Tween[®] 80, was transformed using the Alkali-Cation Yeast Transformation Kit from Qbiogene. Complemented strains were identified by growth on media that did not contain Tween[®] 80 (fatty acid supplement) and uracil (DOBA SC-URA). Putative complemented strains were single colony purified on selective media three times. Complemented strains were further verified by: i) extraction of plasmid DNA, using the Qbiogene Yeast plasmid purification kit, followed by PCR amplification using HzD9DS gene-specific primers; ii) sequencing of the HzD9DS gene-specific PCR product; and iii) reversion of the strain to fatty acid and URA-3 dependence by passing the strain at least three times on YPD + Tween[®] 80 media, then patching strains to DOBA SC-URA minus Tween[®] 80 media. The substrate specificity of one verified complemented HzD9DS yeast strain was further characterized by FAME analysis.

Analysis of LnD9DS-1, LnD9DS-2, MgD9DS, and HzD9DS expressed in OLE1-deficient yeast strain

As set forth, *supra*, three exemplary acyl-CoA delta-9 desaturase (D9DS) genes were cloned from the plant pathogenic fungi, *Magnaporthe grisea* (MgD9DS) and
5 *Leptosphaeria nodorum* (LnD9DS-1 and LnD9DS-2). These genes and their encoded proteins have not been previously characterized. Acyl-CoA delta-9 desaturases catalyze the formation of a cis double bond between carbon atoms 9 and 10 of saturated 14-, 16-, and 18-carbon fatty acyl thioesters of Coenzyme A, resulting in production of myristoleic (14:1), palmitoleic (16:1), or oleic acid (18:1), respectively.
10 Effects related to organism-specific biology are eliminated by expressing the different fungal acyl-CoA delta-9 desaturase genes in the same biological context. Expression of the fungal acyl-CoA delta-9 desaturase genes was therefore driven using the endogenous *ole1* gene promoter within a palmitoyl-stearoyl CoA desaturase (OLE1)-deficient OFY093 yeast strain. Thus, observed differences in fatty acid substrate
15 specificity in this system are attributable to the specific fungal delta-9 desaturase expressed in the complemented *S. cerevisiae* strain.

The substrate specificities of the MgD9DS, LnD9DS-1 and LnD9DS-2 CoA desaturases expressed in the complemented OYF093 strains were characterized and compared to OFY093 complemented with the AnD9DS (*sdeA*) described in
20 WO/1999/050430. A yeast expression construct containing the AnD9DS gene, expression of which is driven by the *ole1* gene promoter, was transformed into the *S. cerevisiae* OFY093 strain and expressed using the protocol described above.

The complemented *S. cerevisiae* strains were grown in minimal media with no fatty acid supplementation at 30 °C for 24 hours. Quantitative FAME analysis was
25 performed on washed and lyophilized cell pellets. The results of this analysis are shown in Table 1. LnD9DS-2 promotes formation of C14:1 and C16:1, whereas LnD9DS-1 and MgD9DS have a preference for C18:0, as indicated by the ratio of C16:1/18:1 fatty acids in the yeast fatty acid compositional analyses.

30 **Table 1:** Comparison of fatty acid composition of *ole1*-deficient yeast expressing four different fungal desaturases

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Desaturase	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C16:1/ 16:0	C18:1/ 18:0	C16:1/ 18:1
LnD9DS-1	1.5	0.0	36.5	8.7	1.8	51.5	0.2	28.2	0.17
LnD9DS-2	1.0	0.1	26.6	38.1	6.3	27.9	1.4	4.4	1.37
AnD9DS	0.5	0.0	26.3	7.8	2.0	63.4	0.3	31.7	0.12
MgD9DS	0.5	0.0	22.7	9.1	1.8	65.9	0.4	37.0	0.14
wild type yeast	0.6	0.0	9.6	38.6	6.9	44.3			
ole1-null + Tween® 80	2.6	0.4	38.0	10.9	7.8	40.4			
Empty vector + Tween® 80	2.2	0.3	40.3	8.7	8.7	39.8			

The novel desaturases were further compared to the native *S. cerevisiae* stearoyl-CoA delta-9 desaturase (ole1) transferred into the same recombinant expression environment. A yeast expression vector containing the nucleotide sequence from *S. cerevisiae* described in WO/2000/011012 was constructed. The yeast expression construct containing the native *S. cerevisiae* stearoyl-CoA delta-9 desaturase was transformed into the *S. cerevisiae* OFY093 strain and expressed using the protocol described above. Another non-fungal acyl-CoA delta-9 desaturase from the insect species, *Helicoverpa zea* (HzD9DS), was also evaluated in these experiments.

Complemented *S. cerevisiae* strains containing one of the MgD9DS, LnD9DS-2 and HzD9DS genes were grown in Drop Out Broth SC-URA. A control strain, pDAB467EV-1 (pDAB467B/N transformed into OFY093 by previously described Yeast Transformation methodology), was grown in DOB SC-URA + Tween® 80, and the parent delta-9 desaturase-deficient *S. cerevisiae* strain, OFY093, was grown in DOB scAA + Tween® 80. Cultures were inoculated with a loop of cells from a fresh streak plate of the same media containing 1.5% agar. Strains were grown at 30 °C for 24 hours. Cultures were spun at 6,000 rpm for 10 minutes. Pellets were washed in water, spun again at 6,000 rpm for 10 minutes, and then frozen at -20 °C until FAME analysis was performed. Three sets of expression cultures were analyzed.

Freeze-dried yeast pellets were saponified in methanol containing 10% (w/v) NaOH. Nonsaponifiable lipid contaminants (sterols) were removed with hexane. The methanol fraction was acidified by addition of H₂SO₄, and the protonated fatty acids were extracted with hexane. The isolated hexane fraction was dried down, and fatty acids were methylated with 0.5 N MeOHCl at 80 °C for 30 minutes. The resulting

FAMES were extracted with hexane containing undecanoate methyl ester as an internal standard. The FAME extracts were analyzed with a HP6890 Gas Chromatograph-Flame Ionization Detector (Santa Clara, CA) equipped with a capillary column BPX 70 (15m x 0.25mm x 0.25µm) from SGE (Austin, TX). FAMES were separated in a temperature gradient using helium as the carrier gas. Each FAME species was identified by retention time, and quantified by the injection of a FAME rapeseed oil reference mix from Matreya, LLC (Pleasant Gap, PA), as the calibration standard.

Table 2 shows the fatty acid composition (as %FAMES) of *ole1*-deficient OFY093 yeast cells expressing various exemplary acyl Co-A delta-9 desaturases. All strains grew well and were fully-complemented by the introduced desaturases without any requirement for exogenous MUFAs (monounsaturated fatty acids).

Table 2: Fatty acid composition (as % Total FAMES) of *ole1*-deficient yeast strain OFY093 expressing acyl Co-A delta-9 desaturases. (Standard Deviation is in parentheses).

Desaturase	n	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1
LnD9DS-2	7	1.4 (0.7)	1.4 (1.0)	26.6 (4.5)	38.8 (2.8)	6.0 (1.3)	25.4 (4.4)
HxD9DS	6	2.6 (1.3)	0.9 (0.5)	34.7 (6.8)	37.5 (4.2)	6.0 (1.1)	18.4 (4.1)
<i>ole1</i>	6	1.1 (0.4)	0.6 (0.4)	14.4 (2.6)	49.2 (1.6)	5.6 (1.1)	24.0 (1.1)
AnD9DS	8	0.5 (0.3)	0.2 (0.2)	23.5 (2.2)	9.3 (3.0)	2.1 (0.5)	64.6 (3.2)
MgD9DS	2	0.9 (0.0)	0.1 (0.0)	21.2 (0.2)	12.1 (0.1)	1.6 (0.1)	64.2 (0.3)

These data show that the fatty acid composition of the complemented yeast strains varies according to the introduced gene. LnD9DS-2 produces relatively high amounts of C16:1, as does HxD9DS and *ole1*, whereas AnD9DS and MgD9DS produce relatively high amounts of C18:1.

The differential level of conversion based upon chain length can be further shown by calculating the proportion of MUFA relative to the total fatty acids for each fatty acid chain length; C14, C16, or C18. These data show the relatively high conversion to C16:1 for LnD9DS-2 and HxD9DS, and to C18:1 for AnD9DS and MgD9DS. Table 3. The bottom four rows represent control samples complemented

with added tergitol, unsaturated fatty acids, or Tween[®]. Samples with different letters are significantly different, as determined via the Tukey-Kramer Test performed in the JMP statistical software suite (SAS Institute Inc., Cary, NC).

- 5 **Table 3:** Proportion of MUFA of total fatty acids for each chain length (C_{xx}:1/(C_{xx}:0 + C_{xx}:1).

Desaturase	C14	C16*	C18
LnD9DS-2	0.49	0.60 (b)	0.81 (b)
HzD9DS	0.30	0.52 (b)	0.75 (c)
ole1	0.34	0.79 (a)	0.81 (b)
AnD9DS	0.25	0.28 (c)	0.97 (a)
MgD9DS	0.07	0.36 (c)	0.98 (a)
None + tergitol	0.06	0	0
None + tergitol + ricinoleic	0.07	0	0.01
None + tergitol + linoleic	0	0	0.04
None + tween	0.65	0.23	0.87

* C16 MUFA includes cis-vaccenic acid (C18:1 Δ11), as it is derived from elongation of palmitoleic acid (C16:1 Δ9).

10

Phylogeny of fungal Acyl-CoA Desaturases

Phylogenetic analysis of multiple fungal acyl-CoA delta-9 desaturase amino acid sequences suggests that LnD9DS-2 is distinct from the 18:0-preferring delta-9 desaturases. Thus, we hypothesized that characterization of other fungal delta-9 desaturases closely associated with either the 18:0-preferring delta-9 desaturases, or with LnD9DS-2, may identify desaturases with a range of 18:0 or 16:0 activities. Our hypothesis predicts that a fungal delta-9 desaturase that is more closely associated with LnD9DS-2 will have increased 16:0 activity.

A search of the public DNA sequence databases (Broad Institute, NCBI, etc.) did not identify any gene sequences specifically annotated as delta-9 desaturases in *Magnaporthe grisea* or *Leptosphaeria nodorum*. Pfam analysis of the Broad Institute sequences that were identified within this disclosure indicates that these proteins contain cytochrome B5 and desaturase motifs that are also found in other fungal acyl-CoA delta-9 desaturases. However, the proteins had not been previously identified as acyl-CoA delta-9 desaturases. We have demonstrated this function of these proteins by complementation in yeast, reversal studies, and DNA sequence analysis.

The relationships of several fungal desaturase gene sequences were analyzed phylogenetically using the neighbor-joining method *via* the MEGA software package. Tamura *et al.* (2007) Mol. Biol. and Evolution 24:1596-9. **FIG. 1** illustrates this phylogenetic analysis of the fungal desaturase sequences. These sequences were recovered by BlastN searches of the NCBI sequence database using the AnD9DS (sdeA) amino acid sequence. LnD9DS-1 and MgD9DS share higher levels of sequence identity with one another, as compared to LnD9DS-2. Additionally, a ClustalW alignment of LnD9DS-1, LnD9DS-2, and MgD9DS shows the divergence of LnD9DS-2 from LnD9DS-1 and MgD9DS. **FIG. 2.** The nucleotide sequences of LnD9DS-1 and MgD9DS share a higher number of base pairs in common.

Table 4 and **FIG. 3** further illustrate the phylogenetic relationship of newly-identified proteins, LnD9DS-1, LnD9DS-2, and MgD9DS, as well as AnD9DS and the yeast desaturase, ScOLE1. The LnD9DS-1, MgD9DS, and AnD9DS (sdeA) amino acids sequences share a greater percentage of identity with one another as compared to LnD9DS-2. The conservation of amino acid identity allows us to predict that the substrate specificity for 18:0 acyl-CoA is dependent upon the conserved sequence shared between LnD9DS-1, MgD9DS, and AnD9DS (sdeA). In comparison, the acyl-CoA substrate specificity of LnD9DS-2 is preferential for 16:0 as a result of its divergent amino acid sequence.

20

Table 4: Amino acid identity of various fungal desaturase sequences aligned using ClustalW.

	LnD9DS-1	LnD9DS-2	MgD9DS	Yeast OLE1
AnD9DS (sdeA)	81%	61%	75%	49%
LnD9DS-1	—	61%	75%	47%
LnD9DS-2	61%	--	62%	49%
MgD9DS	75%	62%	--	49%

25 Example 2: Design and synthesis of optimized delta-9 desaturase genes from

Magnaporthe grisea, *Helicoverpa zea*, and *Leptosphaeria nodorum*

To obtain higher expression of fungal delta-9 desaturase genes in canola, we engineered these genes so that they are more efficiently expressed in transgenic canola cells containing the heterologous gene. Extensive analysis of the DNA sequence of the

native *Magnaporthe grisea*, *Helicoverpa zea* and *Leptosphaeria nodorum* delta-9 desaturase coding regions disclosed herein as SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:11, respectively, revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition for such optimal plant expression. In order to design optimized genes encoding a delta-9 desaturase protein, we generated DNA sequences *in silico* that are more "plant-like" (and specifically, more "canola-like") in nature, in which the sequence modifications do not hinder translation or create mRNA instability.

To engineer plant-optimized genes encoding a delta-9 desaturase, DNA sequences were designed to encode the amino acid sequences of the protein desaturases, utilizing a redundant genetic code established from a codon bias table compiled from the protein coding sequences for the particular host plants (*i.e.*, canola). Preferred codon usages for canola are shown in Table 5. Columns C and G of Table 5 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the coding regions of *Brassica napus*. It is evident that some synonymous codons for some amino acids are found only rarely in plant genes (*e.g.*, CGG in canola). A codon was considered to be rarely used if it is represented at about 10% or less of the time to encode the relevant amino acid in genes of either plant type (indicated by "DNU" in Columns D and H of Table 5). To balance the distribution of the remaining codon choices for an amino acid, a Weighted Average representation for each codon was calculated, using the formula:

Weighted Average % of C1 = $1/(\%C1 + \%C2 + \%C3 + \text{etc.}) \times \%C1 \times 100$,
 where C1 is the codon in question and %C2, %C3, *etc.* represent the averages of the % values for *Brassica napus* of remaining synonymous codons (average % values for the relevant codons are taken from Columns C and G) of Table 5.

[0001] The Weighted Average % value for each codon is given in Columns D and H of Table 5.

Table 5: Synonymous codon representation in coding regions of canola (*B. napus*) genes (Columns C and G). Values for a balanced-biased codon representation set for a plant-optimized synthetic gene design are in Columns D and H.

A	B	C	D	E	F	G	H
Amino Acid	Codon	Canola %	Weighted Average	Amino Acid	Codon	Canola %	Weighted Average

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A	B	C	D	E	F	G	H
Amino Acid	Codon	Canola %	Weighted Average	Amino Acid	Codon	Canola %	Weighted Average
ALA (A)	GCA	23.3	23.3	LEU (L)	CTA	10.1	DNU
	GCC	21.2	21.2		CTC	22.8	28.5
	GCG	14.2	14.2		CTG	11.6	14.6
	GCT	41.3	41.3		CTT	25.2	31.6
ARG (R)	AGA	31.8	43.8	LYS (K)	TTA	10.1	DNU
	AGG	22.1	30.5		TTG	20.2	25.3
	CGA	9.9	DNU	MET (M)	AAA	44.6	44.6
	CGC	8.9	DNU		AAG	55.4	55.4
	CGG	8.6	DNU	PHE (F)	ATG	100.0	100.0
	CGT	18.6	25.7		TTC	58.6	58.6
ASN (N)	AAC	62.6	62.6	PRO (P)	TTT	41.4	41.4
	AAT	37.4	37.4		CCA	29.6	29.6
ASP (D)	GAC	42.5	42.5		CCC	14.6	14.6
	GAT	57.5	57.5		CCG	18.4	18.4
CYS (C)	TGC	49.2	49.2	SER (S)	CCT	37.3	37.3
	TGT	50.8	50.8		AGC	16.0	17.9
END	TAA	38.5	DNU		AGT	14.1	15.8
	TAG	22.1	DNU		TCA	18.2	20.4
	TGA	39.4	100.0		TCC	16.7	18.7
GLN (Q)	CAA	50.0	50.0		TCG	10.7	DNU
	CAG	50.0	50.0		TCT	24.3	27.2
GLU (E)	GAA	43.6	43.6	THR (T)	ACA	26.3	26.3
	GAG	56.4	56.4		ACC	26.9	26.9
GLY (G)	GGA	36.4	36.4		ACG	16.9	16.9
	GGC	16.2	16.2		ACT	30.0	30.0
	GGG	15.2	15.2	TRP (W)	TGG	100.0	100.0
	GGT	32.1	32.1		TAC	59.4	59.4
HIS (H)	CAC	49.6	49.6	TYR (Y)	TAT	40.6	40.6
	CAT	50.4	50.4		GTA	10.8	DNU
ILE (I)	ATA	21.1	21.1	VAL (V)	GTC	24.1	27.0
	ATC	42.7	42.7		GTG	28.3	31.7
	ATT	36.2	36.2		GTT	36.8	41.3

**NA = Not Applicable

***DNU = Do Not Use

- 5 New DNA sequences which encode essentially the amino acid sequence of the *Magnaporthe grisea*, *Helicoverpa zea*, and *Leptosphaeria nodorum* delta-9 desaturases of SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14, respectively, were designed for optimal expression in canola using a first and second choice codon

distribution of frequently used codons found in canola genes. The new DNA sequences differ from the native DNA sequences encoding the delta-9 desaturase proteins by the substitution of plant-preferred (*i.e.*, first preferred, second preferred, third preferred, or fourth preferred) codons to specify an appropriate amino acid at each position within the protein amino acid sequence.

Design of the plant-optimized DNA sequences were initiated by reverse-translation of the protein sequences of SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14, using a canola codon bias table constructed from **Table 5** Columns D and H. The initial sequences were then modified by compensating codon changes (while retaining overall weighted average codon representation) to remove restriction enzyme recognition sites, remove highly stable intrastrand secondary structures, and remove other sequences that might be detrimental to cloning manipulations or expression of the engineered gene in plants. The DNA sequences were then re-analyzed for restriction enzyme recognition sites that might have been created by the modifications. The identified sites were then further modified by replacing the relevant codons with first, second, third, or fourth choice preferred codons. The modified sequences were further analyzed and further modified to reduce the frequency of TA and CG doublets, and to increase the frequency of TG and CT doublets. In addition to these doublets, sequence blocks that have more than about six consecutive residues of [G+C] or [A+T] were modified by replacing the codons of first or second choice, *etc.* with other preferred codons of choice. Rarely used codons were not included to a substantial extent in the gene design, and were used only when necessary to accommodate a different design criterion than codon composition *per se* (*e.g.*, addition or deletion of restriction enzyme recognition sites). Exemplary synthetic canola-optimized desaturase DNA sequences designed by this process are listed in SEQ ID NO:15, SEQ ID NO:16, and SEQ ID NO:17.

The resulting DNA sequences, as represented by SEQ ID NOs:15-17, have a higher degree of codon diversity and a desirable base composition. Furthermore, these sequences contain strategically placed restriction enzyme recognition sites, and lack sequences that might interfere with transcription of the gene, or translation of the product mRNA. **Tables 6-8** present a comparison of the codon compositions of the coding regions for the delta-9 desaturase proteins as found in the native gene, and in

the plant-optimized versions, and compare both to the codon composition recommendations for a plant-optimized sequence as calculated from Table 5 Columns D and H.

5 **Table 6:** Codon compositions of coding regions for a MgD9DS protein. The native *M. grisea* desaturase coding region is compared to a Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd	Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd
ALA (A)	GCA	4	10.5	10	26.3	23.3	LEU (L)	CTA	0	0.0	0	0.0	0.0
	GCC	18	47.4	8	21.1	21.2		CTC	11	28.9	11	28.9	28.5
	GCG	3	7.9	4	10.5	14.2		CTG	11	28.9	5	13.2	14.6
	GCT	13	34.2	16	42.1	41.3		CTT	12	31.6	12	31.6	31.6
ARG (R)	AGA	2	9.5	10	47.6	43.8	LYS (K)	TTA	0	0.0	0	0.0	0.0
	AGG	1	4.8	6	28.6	30.5		TTG	4	10.5	10	26.3	25.3
	CGA	2	9.5	0	0.0	0.0	MET (M)	AAA	1	3.4	13	44.8	44.6
	CGC	12	57.1	0	0.0	0.0		AAG	28	96.6	16	55.2	55.4
	CGG	0	0.0	0	0.0	0.0	PHE (F)	ATG	7	100	7	100	100.0
ASN (N)	CGT	4	19.0	5	23.8	25.7		TTC	17	89.5	11	57.9	58.6
	AAC	23	100.0	14	60.9	62.6	PRO (P)	TTT	2	10.5	8	42.1	41.4
ASP (D)	AAT	0	0.0	9	39.1	37.4		CCA	0	0.0	6	28.6	29.6
	GAC	17	68.0	11	44.0	42.5	SER (S)	CCC	9	42.9	3	14.3	14.6
CYS (C)	GAT	8	32.0	14	56.0	57.5		CCG	5	23.8	4	19.0	18.4
	TGC	2	66.7	1	33.3	49.2		CCT	7	33.3	8	38.1	37.3
END	TGT	1	33.3	2	66.7	50.8	THR (T)	AGC	3	10.7	5	17.9	17.9
	TAA	0	0.0	0	0.0	0.0		AGT	0	0.0	4	14.3	15.8
	TAG	0	0.0	0	0.0	0.0		TCA	5	17.9	7	25.0	20.4
GLN (Q)	TGA	1	100.0	1	100.0	100.0	TRP (W)	TCC	9	32.1	4	14.3	18.7
	CAA	2	9.5	11	52.4	50.0		TCG	9	32.1	0	0.0	0.0
GLU (E)	CAG	19	90.5	10	47.6	50.0		TCT	2	7.1	8	28.6	27.2
	GAA	1	6.7	7	46.7	43.6	TYR (Y)	ACA	4	16.7	6	25.0	26.3
GLY (G)	GAG	14	93.3	8	53.3	56.4		ACC	15	62.5	7	29.2	26.9
	GGA	8	19.5	15	36.6	36.4		ACG	1	4.2	4	16.7	16.9
	GGC	13	31.7	7	17.1	16.2	VAL (V)	ACT	4	16.7	7	29.2	30.0
	GGG	1	2.4	6	14.6	15.2		TGG	21	100	21	100	100.0
HIS (H)	GGT	19	46.3	13	31.7	32.1	TYR (Y)	TAC	16	94.1	10	58.8	59.4
	CAC	19	95.0	10	50.0	49.6		TAT	1	5.9	7	41.2	40.6
ILE (I)	CAT	1	5.0	10	50.0	50.4	VAL (V)	GTA	1	2.5	0	0.0	0.0
	ATA	1	4.2	5	20.8	21.1		GTC	21	52.5	11	27.5	27.0
	ATC	15	62.5	10	41.7	42.7		GTG	4	10.0	13	32.5	31.7
Totals	ATT	8	33.3	9	37.5	36.2	Totals	GTT	14	35.0	16	40.0	41.3
		232		232					244		244		

10 **Table 7:** Codon compositions of coding regions for a HzD9DS protein. The native *H. zea* desaturase coding region is compared to a Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd	Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd
ALA (A)	GCA	4	11.4	9	25.7	23.3	LEU (L)	CTA	2	5.9	0	0.0	0.0
	GCC	7	20.0	7	20.0	21.2		CTC	8	23.5	10	29.4	28.5
	GCG	8	22.9	4	11.4	14.2		CTG	14	41.2	6	17.6	14.6
	GCT	16	45.7	15	42.9	41.3		CTT	6	17.5	10	29.4	31.6

Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd	Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd
ARG (R)	AGA	1	7.7	6	46.2	43.8	LYS (K)	TAA	2	5.9	0	0.0	0.0
	AGG	5	38.5	4	30.8	30.5		TAT	2	5.9	8	23.5	25.3
	CGA	2	15.4	0	0.0	0.0		AAA	11	44.0	10	40.0	44.6
	CGC	5	38.5	0	0.0	0.0		AAG	14	56.0	15	60.0	55.4
	CGG	0	0.0	0	0.0	0.0	MET (M)	ATG	8	100	8	100	100.0
	CGT	0	0.0	3	23.1	25.7		PHE (F)	TTC	20	83.3	14	58.3
ASN (N)	AAC	13	72.2	11	61.1	62.6	PRO (P)	TTT	4	16.7	10	41.7	41.4
AAT	5	27.8	7	38.9	37.4	CCA		1	6.3	5	31.3	29.6	
ASP (D)	GAC	16	64.0	12	48.0	42.5		CCC	5	31.3	3	18.8	14.6
GAT	9	36.0	13	52.0	57.5	CCG		2	12.5	2	12.5	18.4	
CYS (C)	TGC	1	100.0	0	0.0	49.2	SER (S)	CCY	8	50.0	6	37.5	37.3
	TGT	0	0.0	1	100.0	50.8		AGC	2	12.5	3	18.8	17.9
END	TAA	1	100.0	0	0.0	0.0		AGT	1	6.3	3	18.8	15.8
	TAG	0	0.0	0	0.0	0.0		TCA	1	6.3	3	18.8	20.4
	TGA	0	0.0	1	100.0	100.0	TCC	6	37.5	3	18.8	18.7	
GLN (Q)	CAA	2	33.3	3	50.0	50.0	THR (T)	TCG	3	18.8	0	0.0	0.0
	CAG	4	66.7	3	50.0	50.0		TCT	3	18.8	4	25.0	27.2
GLU (E)	GAA	7	63.6	5	45.5	43.6		ACA	3	16.7	5	27.8	26.3
	GAG	4	36.4	6	54.5	55.4		ACC	7	38.9	5	27.8	26.9
GLY (G)	GGA	8	40.0	9	45.0	36.4	TRP (W)	ACG	4	22.2	3	16.7	16.9
	GGC	6	30.0	4	20.0	16.2		ACT	4	22.2	5	27.8	30.0
	GGG	2	10.0	3	15.0	15.2		TGG	14	100	14	100	100.0
	GGT	4	20.0	4	20.0	32.1		TYR (Y)	TAC	12	80.0	9	60.0
HIS (H)	CAC	11	73.3	8	53.3	49.6	VAL (V)	TAT	3	20.0	6	40.0	40.6
	CAT	4	26.7	7	46.7	50.4		GTA	0	0.0	0	0.0	0.0
ILE (I)	ATA	3	15.0	4	20.0	21.1		GTC	5	26.3	5	26.3	27.0
	ATC	10	50.0	9	45.0	42.7		GTG	13	68.4	6	31.6	31.7
	ATT	7	35.0	7	35.0	36.2	GTT	1	5.3	8	42.1	41.3	
Totals		165		165			Totals		189		189		

Table 8: Codon compositions of coding regions for a LnD9DS-2 protein. The native *L. nodorum* desaturase coding region is compared to a Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd	Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd
ALA (A)	GCA	3	9.4	7	21.9	23.3	LEU (L)	CTA	7	15.6	0	0.0	0.0
	GCC	9	28.1	7	21.9	21.2		CTC	14	31.1	13	28.9	28.5
	GCG	12	37.5	5	15.6	14.2		CTG	7	15.6	7	15.6	14.6
	GCT	8	25.0	13	40.6	41.3		CTT	5	11.1	14	31.1	31.6
ARG (R)	AGA	4	13.8	13	44.8	43.8	TTA	3	6.7	0	0.0	0.0	
	AGG	3	10.3	9	31.0	30.5		TTG	9	20.0	11	24.4	25.3
	CGA	7	24.1	0	0.0	0.0	LYS (K)	AAA	9	45.0	9	45.0	44.6
	CGC	8	27.6	0	0.0	0.0		AAG	11	55.0	11	55.0	55.4
	CGG	5	17.2	0	0.0	0.0	MET (M)	ATG	9	100	9	100	100.0
	CGT	2	6.9	7	24.1	25.7		PHE (F)	TTC	16	80.0	12	60.0
ASN (N)	AAC	6	50.0	8	66.7	62.6	TTT		4	20.0	8	40.0	41.4
	AAT	6	50.0	4	33.3	37.4	PRO (P)	CCA	3	16.7	5	27.8	29.6
ASP (D)	GAC	16	66.7	10	41.7	42.5		CCC	8	44.4	3	16.7	14.6
	GAT	8	33.3	14	58.3	57.5		CCG	2	11.1	3	16.7	18.4
CYS (C)	TGC	4	80.0	2	40.0	49.2		CCT	5	27.8	7	38.9	37.3
	TGT	1	20.0	3	60.0	50.8	SER (S)	AGC	8	27.6	5	17.2	17.9

END	TAA	0	0.0	0	0.0	0.0	AGT	6	20.7	5	17.2	15.8
	TAG	1	100.0	0	0.0	0.0	TCA	1	3.4	6	20.7	20.4
	TGA	0	0.0	1	100.0	100.0	TCC	6	20.7	5	17.2	18.7
GLN (Q)	CAA	10	55.6	10	55.6	50.0	TCG	7	24.1	0	0.0	0.0
	CAG	8	44.4	8	44.4	50.0	TCT	1	3.4	8	27.6	27.2
GLU (E)	GAA	5	33.3	7	46.7	43.6	THR (T)	ACA	11	44.0	7	28.0
16	GAG	10	66.7	8	53.3	56.4		ACC	5	20.0	7	28.0
GLY (G)	GGA	13	34.2	14	36.8	35.4		ACG	7	28.0	4	16.0
	GGC	16	42.1	6	15.8	16.2		ACT	2	8.0	7	28.0
	GGG	6	15.8	6	15.8	15.2	TRP (W)	TGG	19	100	19	100
	GGT	3	7.9	12	31.6	32.1	TYR (Y)	TAC	11	64.7	10	58.8
HIS (H)	CAC	12	66.7	9	50.0	49.6		TAT	6	35.3	7	41.2
	CAT	6	33.3	9	50.0	50.4	VAL (V)	GTA	6	17.6	0	0.0
ILE (I)	ATA	4	18.2	5	22.7	21.1		GTC	10	29.4	9	26.5
	ATC	9	40.9	10	45.5	42.7		GIG	12	35.3	11	32.4
	ATT	9	40.9	7	31.8	36.2		GTT	6	17.6	14	41.2
Totals		214		214			Totals	236		236		

Syntheses of DNA fragments comprising SEQ ID NO:15, SEQ ID NO:16, and SEQ ID NO:17 were performed by commercial suppliers (PicoScript, Houston, TX and Blue Heron Biotechnology, Bothell, WA). These canola-optimized sequences were labeled as version 2 (v2). The synthetic DNA fragments were then cloned into expression vectors, and transformed into *Agrobacterium* and canola as described in the Examples below.

Example 3: Plasmid construction

The following plasmids were constructed using standard molecular biology techniques. Polynucleotide fragments containing plant transcription units (comprised of a promoter linked to a gene of interest, terminated by a 3'UTR), or "PTUs," were constructed and combined with additional plant transcription units within the T-strand region of a binary vector.

Description of pDAB7318: pDAB7318 (FIG. 6; SEQ ID NO:58) was constructed using standard molecular biology techniques. This plasmid contains two desaturase PTU sequences. The first desaturase PTU contains the *Phaseolus vulgaris* phaseolin promoter (PvPhas promoter v2 (SEQ ID NO:67); Genbank: J01263), *Phaseolus vulgaris* 5' untranslated region (PvPhas 5' UTR (SEQ ID NO:68); Genbank: J01263), AnD9DS v3 gene (SEQ ID NO:49), *Phaseolus vulgaris* 3' untranslated region (PvPhas 3' UTR v1 (SEQ ID NO:69); Genbank: J01263) and *Phaseolus vulgaris* matrix attachment region (PvPhas 3' MAR v2 (SEQ ID NO:70); Genbank: J01263). The second desaturase PTU contains the PvPhas promoter v2, PvPhas 5'

UTR, LnD9DS-2 v2 (SEQ ID NO:17), and *Agrobacterium tumefaciens* ORF23 3' untranslated region (AtuORF23 3' UTR (SEQ ID NO:71); Huang *et al.* (1990) J. Bacteriol. 172:1814-22).

The elements in the desaturase PTUs are connected by additional short
5 intervening sequences. The two desaturase PTU sequences are flanked by Invitrogen's Gateway® Recombination sites, which are used to facilitate the transfer of these PTU expression cassettes into the *Agrobacterium* transformation plasmid. Additionally, the plasmid contains an origin of replication, and a kanamycin selectable marker.

Description of pDAB7319: pDAB7319 (FIG. 7; SEQ ID NO:60) was
10 constructed *via* Gateway® recombination between pDAB7318 and pDAB7309 (FIG. 5; SEQ ID NO:53). This plasmid contains the two desaturase PTU sequences set forth in the preceding "Description of pDAB7318." These PTUs were orientated in a head-to-tail orientation within the T-strand DNA border regions of the plant transformation binary vector, pDAB7309. This binary vector contains the phosphinothricin acetyl
15 transferase PTU, which consists of the Cassava vein Mosaic Virus Promoter (CsVMV promoter v2; Verdaguer *et al.* (1996) Plant Mol. Biol. 31:1129-39); phosphinothricin acetyl transferase (PAT v5; Wohlleben *et al.* (1988) Gene 70:25-37); and *Agrobacterium tumefaciens* ORF1 3' untranslated region (AtuORF1 3'UTR v4; Huang *et al.* (1990), *supra*), in addition to other regulatory elements such as the *Nicotiana*
20 *tabacum* RB7 Matrix Attachment Region (RB7 MARv2; Genbank: U67919), Overdrive (Toro *et al.* (1988) Proc. Natl. Acad. Sci. U.S.A. 85(22):8558-62), and T-strand border sequences (T-DNA Border A and T-DNA Border B; Gardner *et al.* (1986) Science 231:725-7, and PCT International Patent Publication No. WO2001/025459A1). Plasmids containing the PTUs described above were isolated
25 and confirmed *via* restriction enzyme digestion and DNA sequencing.

Description of pDAB7320: pDAB7320 (FIG. 8; SEQ ID NO:55) was constructed using standard molecular biology techniques. This plasmid contains one desaturase PTU sequence. The desaturase PTU contains the PvPhas promoter v2, PvPhas 5' UTR, LnD9DS-2 v2 (SEQ ID NO:17), and the AtuORF23 3' UTR. The
30 elements in the desaturase PTUs are connected by additional short intervening sequences. The desaturase PTU sequence also is flanked by Invitrogen's Gateway® Recombination sites to facilitate its transfer into an *Agrobacterium* transformation

plasmid. Additionally, the plasmid contains an origin of replication and kanamycin selectable marker.

Description of pDAB7321: pDAB7321 (FIG. 9; SEQ ID NO:61) was constructed *via* Gateway[®] recombination between pDAB7320 and pDAB7309. This plasmid contains the desaturase PTU sequence set forth in the preceding "Description of pDAB7319." This PTU was orientated in a head-to-tail orientation within the T-strand DNA border regions of the plant transformation binary vector, pDAB7309. This binary vector contains the phosphinothricin acetyl transferase PTU: CsVMV promoter v2; PAT v5; and *AtuORF1* 3'UTR v4, in addition to other regulatory elements such as Overdrive and T-strand border sequences (T-DNA Border A and T-DNA Border B). Plasmids containing the PTU described above were isolated and confirmed *via* restriction enzyme digestion and DNA sequencing.

Description of pDAB7323: pDAB7323 (FIG. 10; SEQ ID NO:56) was constructed using standard molecular biology techniques. This plasmid contains two desaturase PTU sequences. The first desaturase PTU contains the *PvPhas* promoter v2, *PvPhas* 5' UTR, *AnD9DS* v3 (SEQ ID NO:47), *PvPhas* 3' UTR, and *PvPhas* 3' MAR v2. The second desaturase PTU contains the *PvPhas* promoter v2, *PvPhas* 5' UTR, *HxD9DS* v2 (SEQ ID NO:16), and *AtuORF23* 3' UTR. The elements in the desaturase PTUs are connected by additional short intervening sequences. The two desaturase PTU sequences are flanked by Invitrogen's Gateway[®] Recombination sites to facilitate their transfer into an *Agrobacterium* transformation plasmid. Additionally, the plasmid contains an origin of replication and kanamycin selectable marker.

Description of pDAB7324: pDAB7324 (FIG. 11; SEQ ID NO:52) was constructed *via* Gateway[®] recombination between pDAB7323 and pDAB7309. This plasmid contains the two desaturase PTU sequences set forth in the preceding "Description of pDAB7323." These PTUs were orientated in a head-to-tail orientation within the T-strand DNA border regions of the plant transformation binary vector, pDAB7309. This binary vector contains the phosphinothricin acetyl transferase PTU: CsVMV promoter v2; PAT v5; and *AtuORF1* 3'UTR v4, in addition to other regulatory elements such as Overdrive and T-stand border sequences (T-DNA Border A and T-DNA Border B). Plasmids containing the PTUs described above were isolated and confirmed *via* restriction enzyme digestion and DNA sequencing.

Description of pDAB7325: pDAB7325 (FIG. 12; SEQ ID NO:57) was constructed using standard molecular biology techniques. This plasmid contains one desaturase PTU sequence. This desaturase PTU contains the PvPhas promoter v2, PvPhas 5' UTR, HzD9DS v2 (SEQ ID NO:16), and AtuORF23 3' UTR. The elements in the desaturase PTU are connected by additional short intervening sequences, and the desaturase PTU sequence is flanked by Invitrogen's Gateway® Recombination sites to facilitate its transfer into an *Agrobacterium* transformation plasmid. Additionally, the plasmid contains an origin of replication and kanamycin selectable marker.

Description of pDAB7326: pDAB7326 (FIG. 13; SEQ ID NO:63) was constructed *via* Gateway® recombination between pDAB7325 and pDAB7309. This plasmid contains the desaturase PTU sequence set forth in the preceding "Description of pDAB7325." The PTU was orientated in a head-to-tail orientation within the T-strand DNA border regions of the plant transformation binary vector, pDAB7309. This binary vector contains the phosphinothricin acetyl transferase PTU: CsVMV promoter v2; PAT v5; and AtuORF1 3'UTR v4, in addition to other regulatory elements such as Overdrive and T-stand border sequences (T-DNA Border A and T-DNA Border B). Plasmids containing the PTU described above were isolated and confirmed *via* restriction enzyme digestion and DNA sequencing.

Description of pDAB7327: pDAB7327 (FIG. 14; SEQ ID NO:58) was constructed using standard molecular biology techniques. This plasmid contains one desaturase PTU sequence. The desaturase PTU contains the PvPhas promoter v2, PvPhas 5' UTR, AnD9DS v3 gene (SEQ ID NO:49), and AtuORF23 3' UTR. The elements in the desaturase PTU are connected by additional short intervening sequences. The desaturase PTU sequence also is flanked by Invitrogen's Gateway® Recombination sites to facilitate its transfer into an *Agrobacterium* transformation plasmid. Additionally, the plasmid contains an origin of replication and kanamycin selectable marker.

Description of pDAB7328: pDAB7328 (FIG. 15; SEQ ID NO:64) was constructed *via* Gateway® recombination between pDAB7327 and pDAB7309. This plasmid contains the desaturase PTU sequence set forth in the preceding "Description of pDAB7327." This PTU was orientated in a head-to-tail orientation within the T-strand DNA border regions of the plant transformation binary vector, pDAB7309. This

binary vector contains the phosphinothricin acetyl transferase PTU: CsVMV promoter v2; PAT v5; and AtuORF1 3'UTR v4, in addition to other regulatory elements such as Overdrive and T-stand border sequences (T-DNA Border A and T-DNA Border B). Plasmids containing the PTU described above were isolated and confirmed via restriction enzyme digestion and DNA sequencing.

Description of pDAB7329: pDAB7329 (FIG. 16; SEQ ID NO:59) was constructed using standard molecular biology techniques. This plasmid contains one desaturase PTU sequence, which contains the PvPhas promoter v2, PvPhas 5' UTR, MgD9DS v2 (SEQ ID NO:15), and AtuORF23 3' UTR. The elements in this desaturase PTU are connected by additional short intervening sequences. The desaturase PTU sequence is flanked by Invitrogen's Gateway® Recombination sites to facilitate its transfer into an *Agrobacterium* transformation plasmid. Additionally, the plasmid contains an origin of replication and kanamycin selectable marker.

Description of pDAB7330: pDAB7330 (FIG. 17; SEQ ID NO:65) was constructed via Gateway® recombination between pDAB7329 and pDAB7309. This plasmid contains the desaturase PTU sequence set forth in the preceding "Description of pDAB7325." This PTU was orientated in a head-to-tail orientation within the T-strand DNA border regions of the plant transformation binary vector, pDAB7309. This binary vector contains the phosphinothricin acetyl transferase PTU: CsVMV promoter v2; PAT v5; and AtuORF1 3'UTR v4, in addition to other regulatory elements such as Overdrive and T-stand border sequences (T-DNA Border A and T-DNA Border B). Plasmids containing the PTU described above were isolated and confirmed via restriction enzyme digestion and DNA sequencing.

Description of pDAB7331: In addition to the foregoing, a control plasmid that did not contain a desaturase PTU was constructed (SEQ ID NO:66). FIG. 18. This construct only contained the phosphinothricin acetyl transferase PTU, in addition to the other regulatory elements described in pDAB7309.

Example 4: *Agrobacterium* Transformation

Electro-competent *Agrobacterium tumefaciens* cells (Table 9) were prepared using a protocol from Weigel and Glazebrook (2002) "How to Transform *Arabidopsis*," Ch. 5, in *Arabidopsis*, Cold Spring Harbor Laboratory Press, Cold

Spring Harbor, NY. 50 μ L of competent *Agrobacterium* cells were thawed on ice, and were transformed using 300 to 400 ng of binary vector plasmid DNA. The cell mix was electroporated in the presence of the DNA, using pre-chilled electroporation cuvettes (0.2 cm), and a Bio-Rad Gene Pulser[®] electroporator (Hercules, CA) under the following conditions: Voltage: 2.5 kV, Pulse length: 5 msec, capacitance output 25 μ F, resistance 200 Ω . After electroporation, 1 mL of YEP broth (Yeast Extract (10 g/L), Peptone (10 g/L), and NaCl (5 g/L)) was added to each cuvette, and the cell-YEP suspension was transferred to a 15 mL culture tube. The cells were incubated at 28 °C with gentle agitation for 4 hours, after which the culture was plated on YEP + agar with the appropriate selection according to **Table 9**. The plates were incubated for 2-4 days at 28 °C, and colonies were selected and streaked onto fresh YEP + agar plates with antibiotic selection and incubated at 28 °C for 1-3 days. Colonies were verified as *Agrobacterium* using the Ketolactose test, and Ketolactose positive colonies were further isolated using two passages of single colony isolation. A final patch plate was made of colonies after single colony isolation was completed.

Table 9. *Agrobacterium* strains and antibiotic selection.

Strain	Genomic Selection	Ti Selection	Helper	Binary Vector Selection
Z707S	Streptomycin	Kanamycin		Spectinomycin
DA2569	Erythromycin	Kanamycin		Spectinomycin
EHA105	Streptomycin	None Available		Spectinomycin
DA2552	Erythromycin	None		Spectinomycin

***Agrobacterium* Colony Validation:** Restriction digestion analysis was used to verify the presence of the intact plasmid by using vector specific restriction digest enzymes. Macherey-Nagel NucleoBond[®] Plasmid DNA kits were used according to the manufacturer's recommended protocol to purify the plasmid DNA from selected transformed *Agrobacterium* colonies. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. Four separate digest reactions were run using 0.75-1 μ g of DNA. The reaction was allowed to run for 1-2 hrs, and was then analyzed by agarose gel electrophoresis and ethidium bromide staining. Colonies were selected for which the digests for all enzymes were identical to the plasmid control and matched the expected band sizes.

A. tumefaciens strain LBA404 (Invitrogen Carlsbad, California) was used for *Arabidopsis* transformation, and *A. tumefaciens* strain Z707S (Hepburn *et al.* (1985) J. Gen. Microbiol. 131:2961-9) was used for canola transformation.

5 **Example 5: *Agrobacterium*-mediated Transformation of *Arabidopsis thaliana***

Arabidopsis Transformation: *Arabidopsis* was transformed using a floral dip method based on the method of Clough and Bent (1998) Plant J. 16:735-743. A selected *Agrobacterium* colony was used to inoculate one or more 30 mL pre-cultures of YEP broth containing appropriate antibiotics for selection. The culture(s) were incubated overnight at 28 °C with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 mL cultures of YEP broth containing antibiotics for selection, and the cultures were incubated overnight at 28 °C with constant agitation. The cells were then plated at approx. 8700 g for 10 minutes at room temperature, and the resulting supernatant was discarded. The cell pellet was gently resuspended in 500 mL infiltration media containing: 1/2x Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044 µM benzylamino purine (10 µL/liter of 1 mg/mL stock in DMSO), and 300 µL/liter Silwet® L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, with care taken to submerge the newest inflorescence. The plants were then laid down on their sides, and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22 °C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, seeds were harvested from the plants.

Arabidopsis thaliana Growth Conditions: Freshly harvested seed was dried for 7 days at room temperature in the presence of a desiccant. After drying, seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4 °C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification). Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoaglan's solution until wet. The soil mix was drained for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days. Seeds were germinated, and plants were grown in a Conviron controller (models CMP4030 and CMP3244, Controlled

Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16-hours light/8-hours dark) at a light intensity of 120-150 $\mu\text{mol}/\text{m}^2\text{sec}$ under constant temperature (22 °C) and humidity (40-50%). Plants were initially watered with Hoaglan's solution, and subsequently with deionized water to keep the soil moist but not wet. Plants nearing seed harvest (1-2 weeks before harvest) were dried out.

5 Selection of T₁ Transformed Plants: T₁ seed was sown on 10.5" x 21" germination trays (T.O. Plastics Inc., Clearwater, MN) as described above and grown under the conditions outlined. The domes were removed 5-6 days post-sowing. 5 days post-sowing, and again 10 days post-sowing, seedlings were sprayed with a 0.20% solution of glufosinate herbicide (Liberty) in a spray volume of 10 mL/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g/ha glufosinate per application. 10 mL of the glufosinate herbicide solution was pipetted into a 20 mL scintillation vial for each tray to be sprayed. The spray was delivered using a horizontal and vertical application pattern. After each spray, a spray label with the herbicide name, application rate, and application date was added to each selection tray. 4 to 7 days after the second spray, herbicide-resistant plants were identified and transplanted into pots prepared with Sunshine mix LP5. Transplanted plants were placed in a greenhouse with the above mentioned growth conditions. Six to eight weeks after transplanting, the seed from each plant was harvested and stored separately with a unique identification number.

Example 7: *Agrobacterium*-mediated transformation of canola

Agrobacterium Preparation: *Agrobacterium* strains containing either pDAB7319, pDAB7321, pDAB7324, pDAB7326, pDAB7328, pDAB7330 or pDAB7331 were used to streak YEP (Bacto Peptone (20.0 g/L) and Yeast Extract (10.0 g/L)) plates containing streptomycin (100 mg/mL) and spectinomycin (50 mg/mL), and incubated for 2 days at 28 °C. A loop of the 2-day streak plate was inoculated into 150 mL modified YEP liquid with streptomycin (100 mg/mL) and spectinomycin (50 mg/mL) into sterile 500 mL baffled flask(s) and shaken at 200 rpm at 28 °C. The cultures were resuspended in M-medium (LS salts; 3% glucose; modified B5 vitamins; 1 μM kinetin; 1 μM 2,4-D; pH 5.8), and diluted to the appropriate density (50 Klett Units), prior to transformation of canola hypocotyls.

Canola Transformation:

Seed germination: Canola seeds (variety Nexera 710) were surface-sterilized in 10% Clorox for 10 minutes, and rinsed in steel strainers three times with sterile distilled water. Seeds were planted for germination on ½ MS Canola medium (1/2 MS, 2% sucrose, 0.8% Agar) contained in Phytatrays (25 seeds per Phytatray). The trays were placed in an environmental growth chamber (Percival Scientific, Inc., Perry, IA) with a growth regime set at 25 °C and a photoperiod of 16-hours light/8-hours dark, and germinated for 5 days.

Pre-treatment: On day 5, ~3 mm hypocotyl segments were aseptically excised, discarding the root and shoot sections (drying of hypocotyls was prevented by placing them into 10 mL of sterile milliQ water during the excision process). Hypocotyl segments were placed horizontally on sterile filter paper on callus induction medium, MSK1D1 (MS; 1 mg/L Kinetin; 1 mg/L 2,4-D; 3% sucrose; 0.7% Phytagar) for 3 days pre-treatment in an environmental growth chamber with a growth regime set at 22-23 °C and a photoperiod of 16-hours light/8-hours dark.

Co-cultivation with Agrobacterium: The day before *Agrobacterium* treatment, flasks of YEP medium containing the appropriate antibiotics were inoculated. Hypocotyl segments were transferred from filter paper to empty 100 x 25 mm petri dishes containing 10 mL of liquid M medium to prevent the hypocotyl segments from drying. A spatula was used at this stage to scoop the segments and transfer. The liquid M medium was removed with a pipette, and 40 mL of *Agrobacterium* suspension was added to the petri dish (500 segments with 40 mL of *Agrobacterium* solution). The segments were treated for 30 minutes with periodic swirling of the petri dish so that the hypocotyls stayed immersed in the *Agrobacterium* solution. At the end of the treatment period, the *Agrobacterium* solution was pipetted into a waste beaker, autoclaved, and discarded (the *Agrobacterium* solution was completely removed to prevent *Agrobacterium* overgrowth). The treated hypocotyls were transferred with forceps back to the original plates containing MSK1D1 with filter paper, with care taken to ensure that the segments did not dry. The hypocotyl segments, along with control segments, were returned to the an environmental growth chamber under reduced light intensity (by covering the plates with aluminum foil), and the treated hypocotyls were co-cultivated with *Agrobacterium* for 3 days.

Callus induction on selection medium: After 3 days of co-cultivation, the hypocotyl segments were transferred individually with forceps onto callus induction medium, MSK1D1H1 (MS; 1 mg/L Kinetin; 1 mg/L 2,4-D; 0.5 g/L MES; 5 mg/L AgNO₃; 300 mg/L Timentin; 200 mg/L Carbenicillin; 1 mg/L Herbiace; 3% sucrose; 0.7% Phytagar). The hypocotyl segments were anchored on the medium, but were not embedded in the medium.

Selection and shoot regeneration: After 7 days on callus induction medium, the callusing hypocotyl segments were transferred to Shoot Regeneration Medium 1 with selection, MSB3Z1H1 (MS; 3 mg/L BAP; 1 mg/L Zeatin; 0.5 g/L MES; 5 mg/L AgNO₃; 300 mg/L Timentin; 200 mg/L Carbenicillin; 1 mg/L Herbiace; 3% sucrose; 0.7% Phytagar). After 14 days, the hypocotyls with shoots were transferred to Regeneration Medium 2 with increased selection, MSB3Z1H3 (MS; 3 mg/L BAP; 1 mg/L Zeatin; 0.5 g/L MES; 5 mg/L AgNO₃; 300 mg/L Timentin; 200 mg/L Carbenicillin; 3 mg/L Herbiace[®]; 3% sucrose; 0.7% Phytagar).

Shoot elongation: After 14 days, the segments with shoots were transferred to shoot elongation medium, MSMESH5 (MS; 300 mg/L Timentin; 5 mg/L Herbiace[®]; 2% sucrose; 0.7% TC Agar). Shoots that were already elongated were isolated and transferred to MSMESH5. After 14 days, the remaining shoots that had not elongated in the first round were placed on MSMESH5, and transferred to fresh selection medium of the same composition. At this stage, all remaining hypocotyl segments were discarded.

Shoots that elongated on MSB3Z1H3 medium after 2 weeks were isolated and transferred to MSMESH5 medium. Remaining shoots that had not elongated in the first round on MSMESH5 were isolated, and transferred to fresh selection medium of the same composition. At this stage all remaining hypocotyl segments were discarded.

Root induction: After 14 days, the shoots were transferred to MSMEST medium (MS; 0.5 g/L MES; 300 mg/L Timentin; 2% sucrose; 0.7% TC Agar) for root induction. The shoots that did not root in the first transfer on MSMEST medium were transferred for a second or third cycle on MSMEST medium until rooted plants were obtained. The shoots that did not elongate / root in the first transfer on MSMEST medium were transferred for a second or third cycle on MSMEST medium until rooted plants were obtained. Plants that rooted on MSMESH5 or MSMEST and were PCR-

positive were sent for transplanting into soil. After hardening, the T₀ canola plants were further analyzed for events which contained the transgene PTU cassettes. Plants were then transferred to a greenhouse, grown to maturity, and the seed was harvested for additional analysis.

5

Example 8: DNA analysis of T₁ *Arabidopsis* leaf tissue and T₀ canola leaf tissue

T₀ canola plants and T₁ *Arabidopsis* plants were analyzed to identify plants which contained the PTU expression cassettes. Invader[®] assays were performed to initially screen samples of putatively transformed plants, and identify events which contained a single copy of the *pat* PTU. Events that were identified as single copy events were kept and further analyzed for the presence of the desaturase PTU(s) via PCR. Events that were PCR positive for the desaturase expression cassette PTU(s) were further analyzed via Southern blot analysis. Southern blot analysis was completed to confirm that the plants contained the gene expression cassette PTUs from the binary vector used to transform the plants. Single copy events containing all of the PTUs were selected for advancement.

DNA Isolation: Total genomic DNA (gDNA) was extracted from lyophilized leaf tissue using Qiagen's DNeasy[®] 96 Plant Kit (Qiagen, Valencia, CA). This gDNA was then diluted to 10 ng/μL (canola) or 0.7 ng/μL (*Arabidopsis*) for use in PCR and Invader[®] assays for copy number.

Invader[®] Analysis: Copy number analysis of the selectable marker, *pat*, was completed using the Invader[®] assay (Third Wave Technologies, Madison, WI). Genomic DNA was denatured at 95 °C for 10 minutes, chilled on ice, and mixed with a master mix of reagents containing oligonucleotide probes, dye molecules capable of fluorescence resonance energy transfer (FRET), and cleavase enzyme, according to the manufacturer's recommended protocol. The reactions contained probes for the internal reference genes. The *1-deoxyxylulose-5-phosphate reductoisomerase (DXRI)* gene was used as an internal reference gene for *Arabidopsis* Invader[®] assay reactions, and *high mobility group protein gene (HMGa)* was used as an internal reference gene for canola Invader[®] assay reactions. In addition, the plates contained 1 copy, 2 copy, and 4 copy standards, as well as wild-type control samples and blank wells containing no

sample. The whole reaction was overlaid with mineral oil before incubation in a thermocycler at 63 °C for 1.5 hrs. The resulting reaction was read on a fluorometric plate reader (Synergy™ 2, BioTek Instruments, Winooski, VT). Readings were collected for both FAM (λ 485-528 nm) and RED (λ 560-620 nm) channels. From these, the fold-over-zero (*i.e.*, background) for each channel was determined for each sample by dividing the sample raw signal by the no template raw signal. From this data, a standard curve was constructed, and the best fit was determined by linear regression analysis. Using the parameters identified from this fit, the apparent *pat* copy number was then determined for each sample.

- 10 PCR Analysis: PCR analysis was completed using primers which amplified each plant transcription unit. These primers were located in the promoter (Phaseolin) and the 3' UTR (Phaseolin or ORF23). These same primer sets were used for PCR analysis of both canola and *Arabidopsis*. For PCR analysis of pDAB7319 and pDAB7324 events, primers MAS414 (SEQ ID NO: 18) and MAS415 (SEQ ID NO:
- 15 19) were used to amplify the first PTU. This PTU consisted of the Phaseolin promoter, a functional equivalent of an acyl-CoA delta-9 desaturase gene from *Aspergillus nidulans* (AnD9DS v3; SEQ ID NO:49), and the Phaseolin 3'UTR terminator. For PCR amplification of the second PTU in construct pDAB7319, primers MAS415 and MAS413 (SEQ ID NO: 20) were used. This PTU consists of the Phaseolin promoter, a
- 20 functional equivalent of an acyl-CoA delta-9 desaturase gene from *Leptosphaeria nodorum* (LnD9DS-2 v2; SEQ ID NO:17), and the ORF23 3'UTR. The MAS415 and MAS413 primer pairs were also used to amplify the second PTU of events generated by transformation with pDAB7324 (Phaseolin promoter, *Helicoverpa zea* acyl-CoA delta-9 desaturase gene v2 (HzD9DS v2; SEQ ID NO:16), and ORF23 3'UTR). In
- 25 addition, MAS415 and MAS413 primer pairs were used to amplify the PTUs in constructs pDAB7321 and pDAB7326.

- The PCR reactions were carried out in 25 μ L volumes using 20 ng genomic DNA, 5 units Ex Taq (Takara), 1x reaction buffer, 0.2 μ M of each dNTP, and 0.8 μ M of each primer. The amplification reactions were performed in a DNA Engine Tetrad®
- 30 2 thermal cycler (BioRad, Hercules, CA). The following cycling conditions were used for primers MAS413 and MAS415: 3 minutes at 94 °C; followed by 35 cycles of 30 sec at 94 °C, 30 sec at 63 °C, and 3 min at 72 °C; and a final extension of 10 minutes at

72 °C. The cycling conditions used for primers MAS414 and MAS415 were the same with the sole difference that the annealing temperature was reduced from 63 °C to 60 °C. The reaction products were run on a 1% agarose gel, stained with ethidium bromide, and visualized on a Gel-Doc™.

- 5 Southern Blot Analysis: Southern blot analysis was used to establish the integration pattern of the canola events. These experiments generated data which demonstrated the integration and integrity of the desaturase transgene within the canola genome. Selected events were characterized as a full-length, simple integration event containing a single copy of the desaturase transgene from the binary vector used for
10 plant transformation.

Detailed Southern blot analysis was conducted using probes specific to the desaturase genes and descriptive restriction enzymes, which cleaved at sites located within the plasmid. These digests produced hybridizing fragments internal to the plasmid, or fragments that spanned the junction of the plasmid with canola genomic
15 DNA (border fragments). The molecular sizes indicated from the Southern hybridization for the combination of the restriction enzymes and the probes were unique for each event. These analyses also showed that the plasmid fragment had been inserted into canola genomic DNA without rearrangements of the T-strand DNA.

For Southern blot analysis, 100 mg of lyophilized canola leaf tissue was
20 extracted using the Plant Mini Kit (Qiagen). Five micrograms (5 µg) of gDNA per sample was digested simultaneously with *SpeI* and *PacI* restriction endonucleases (New England Biolabs, Ipswich, MA) to obtain fragments containing either the PTUs of interest, and/or the selectable marker (PAT), to determine copy number. The digested DNA was separated on a 0.8% agarose gel.

25 Briefly, following electrophoretic separation and visualization of the DNA fragments, the gels were depurinated with 0.25N HCl for approximately 20 minutes, and then exposed to a denaturing solution for approximately 30 minutes, followed by a neutralizing solution for at least 30 minutes. Southern transfer was performed
overnight onto nylon membranes (Millipore, Billerica, MA) using a wicking system
30 with 10× SSC. After transfer, the membranes were washed with a 2× SSC solution, and the DNA was bound to the membrane by UV crosslinking. This process produced Southern blot membranes ready for hybridization.

Probes were generated and PCR fragments were amplified from plasmid DNA and purified *via* gel extraction using the QIAquick® Gel Extraction kit (Qiagen). The primers used to create the LnD9DS probe were arw008 (SEQ ID NO:21) and arw009 (SEQ ID NO:22). The primers used to create the HzD9 probe were arw010 (SEQ ID NO:23) and arw011 (SEQ ID NO:24). PCR conditions for all three reactions consisted of 35 cycles with an annealing temperature of 63 °C and an extension time of 1 minute. The PCR fragments were labeled with ³²P using the Prime-It® RmT Random Primer Labeling kit (Stratagene, La Jolla, CA).

The hybridization step was conducted at approximately 65 °C overnight in the hybridization oven. The nylon membrane blots were rinsed, and the blot was exposed on a phosphor image screen overnight, and scanned on a Storm™ 860 Scanner (Molecular Dynamics, Sunnyvale, CA).

Example 9: Fatty acid composition of seeds from transgenic *Arabidopsis* containing an acyl-CoA delta-9 desaturase

Arabidopsis plants were transformed with *Agrobacterium* vectors containing genes for LnD9DS-2 v2 (pDAB7321; SEQ ID NO:61), HzD9DS v2 (pDAB7326; SEQ ID NO:63) or MgD9DS v2 (pDAB7330; SEQ ID NO:65). Plants were also transformed with a vector containing a AnD9DS gene (pDAB7328; SEQ ID NO:64). An empty vector containing only the selectable marker *pat* gene (pDAB7331; SEQ ID NO:66) was used as a negative control. Transformations were also performed using two desaturases in combination, to combine a stearoyl-preferring desaturase (AnD9DS) with a palmitoyl-preferring desaturase, either LnD9DS-2 (pDAB7319; SEQ ID NO:60), or HzD9DS (pDAB7324; SEQ ID NO:62). In all cases, the desaturase genes were driven by the seed-specific PvPhas promoter (U.S. Patent 5,504,200). Bulk T₂ seed was harvested from herbicide-resistant T₁ plants that were confirmed to contain the *pat* gene by Invader® assay analysis and the desaturase PTU by PCR analysis.

Seed samples were homogenized in heptane-containing triheptadecanoin (Nucleo Prep, Elysian, MN) as a surrogate using a steel ball and ball mill. Prior to homogenization, a solution of 0.25 M freshly-prepared MeONa (Sigma) in MeOH was added to the sample. The reaction was conducted under mild heat (40 °C) and constant shaking. The reaction was verified by the recovery of the methylated surrogate.

Extraction of FAMES was repeated three times, and all heptane layers were pooled prior to analysis. The completeness of the extraction was verified by checking for the presence of FAMES in a fourth extraction/derivatization. The resulting FAMES were analyzed by GC-FID using a capillary column BPX 70 from SGE (15 m x 0.25 mm x 0.25 μ m). Each FAME was identified by retention time, and quantified by the injection of a rapeseed oil reference mix from Matreya, LLC (Pleasant Gap, PA), as a calibration standard.

FAME analysis of T₂ seed from the transgenic events showed that expression of each of the desaturases had a significant effect on reducing the total saturated fatty acid content of the seeds, as determined from the mean saturated fatty acid content of each set of events. **Table 10** and **FIG. 19**. In this table and the following tables, the values not connected by the same letter are significantly different, as determined using the Tukey-Kramer HSD test performed in the JMP[®] statistical software package (SAS Institute Inc., Cary, NC). Combinations of AnD9DS with LnD9DS-2 or HzD9DS yielded the lowest mean total saturated fatty acid content.

Table 10: Total saturated fatty acid content of T₂ *Arabidopsis* seed

Gene	Number of T2 samples			Mean Total Saturated FAs
Control	204	A		13.49
WT	60	A		13.16
MgD9DS v2	42		B	10.26
LnD9DS-2 v2	49		B	10.00
HzD9DS v2	70		B	9.58
AnD9DS v3	32		C	8.73
AnD9DS v3 + HzD9DS v2	39		C	8.23
AnD9DS v3 + LnD9DS-2 v2	51		C	8.09

Although the desaturases all lowered the total saturated fatty acid content in *Arabidopsis* seeds, they had different effects on the palmitic and stearic acid fatty acid contents, as predicted from the yeast experiments. **Table 11** and **FIG. 20** show the mean palmitic acid content for each set of events. **Table 12** and **FIG. 21** show the mean stearic acid content of T₂ seed for each set of events.

Table 11: Palmitic acid content of T₂ *Arabidopsis* seed

Gene				Mean Palmitic acid
Control	A			7.72
WT	A			7.54
MgD9DS v2		B		7.19
AnD9DS v3			C	6.02
LnD9DS-2 v2			C	5.98
HxD9DS v2			D	5.57
AnD9DS v3 + LnD9DS-2 v2			D	5.54
AnD9DS v3 + HxD9DS v2			D	5.41

Table 12: Stearic acid content of T₂ *Arabidopsis* seed

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Gene				Mean Stearic acid
Control	A			2.96
WT	A			2.94
LnD9DS-2 v2		B		2.09
HxD9DS v2		B		2.04
MgD9DS v2			C	1.53
AnD9DS v3 + HxD9DS v2			C	1.42
AnD9DS v3			C	1.35
AnD9DS v3 + LnD9DS-2 v2			C	1.28

AnD9DS and MgD9DS had greater effects on the stearic acid content than LnD9DS-2 and HxD9DS. Conversely, LnD9DS-2 and HxD9DS had greater effects on the palmitic content than AnD9DS and MgD9DS. Combinations of the desaturases have the greatest effect on both fatty acids. These results were also observed in the effects of the desaturases on increasing the seed content of palmitoleic acid, which is the primary product of delta-9 desaturation of palmitic acid. Table 13 and FIG. 22.

Table 13: Palmitoleic acid content of T₂ *Arabidopsis* seed

Gene				Mean Palmitoleic Acid
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Gene					Mean Palmitoleic Acid
AnD9DS v3 + HzD9DS v2	A				3.32
AnD9DS v3 + LnD9DS-2 v2	A				2.93
HzD9DS v2		B			2.48
AnD9DS v3		B	C		2.10
LnD9DS-2 v2			C		1.91
MgD9DS v2				D	1.40
Control				E	0.31
WT				E	0.30

There was expected variation in the effect of the desaturases on saturated fatty acid content across the events analyzed, due to position and copy number effects. A comparison of the complete fatty acid profile of events with the lowest total saturated fatty acid content (average of the five lowest events) is shown in Table 14 alongside the profile of seed from wild-type and control-transformed plants.

Table 14: Fatty acid profile of T₂ transgenic *Arabidopsis* with lowest total saturated fatty acid content. Standard deviations are in parentheses.

	C14:0	C16:0	C16:1	C18:0	C18:1	Vacc.*
WT	0.08 (0.02)	7.54 (0.41)	0.31 (0.05)	2.94 (0.19)	14.91 (1.44)	1.47 (0.10)
Control	0.08 (0.02)	7.72 (0.05)	0.32 (0.04)	2.96 (0.34)	14.20 (2.04)	1.46 (0.11)
AnD9DS v3	0.07 (0.01)	5.10 (0.38)	2.92 (0.55)	0.72 (0.03)	20.52 (2.12)	1.72 (0.26)
HzD9DS v2	0.06 (0.00)	4.13 (0.23)	4.11 (0.47)	1.26 (0.08)	19.34 (1.01)	1.94 (0.25)
LnD9DS-2 v2	0.05 (0.00)	4.68 (0.30)	3.49 (0.69)	1.53 (0.12)	19.35 (0.81)	2.05 (0.21)
MgD9DS v2	0.08 (0.02)	6.64 (0.26)	1.60 (0.54)	1.05 (0.20)	18.01 (1.86)	1.60 (0.16)
AnD9DS v3 + LnD9DS-2 v2	0.06 (0.00)	4.41 (0.17)	3.71 (0.35)	0.97 (0.33)	19.60 (0.88)	2.03 (0.21)
AnD9DS v3 + HzD9DS v2	0.08 (0.02)	4.86 (0.35)	4.09 (0.65)	1.01 (0.22)	18.10 (2.40)	2.03 (0.31)

* Vacc. = cis-vaccenic acid (18:1 n-7)

	C18:2	C18:3	C20:0	C20:1	C20:2	C22:0	C22:1	C24:0
WT	28.72	17.85	2.06	20.11	1.78	0.34	1.68	0.21

	C18:2	C18:3	C20:0	C20:1	C20:2	C22:0	C22:1	C24:0
	(0.97)	(0.81)	(0.16)	(0.90)	(0.15)	(0.10)	(0.19)	(0.10)
Control	29.28 (1.29)	18.07 (1.35)	2.08 (0.16)	19.62 (1.23)	1.85 (0.17)	0.39 (0.13)	1.70 (0.04)	0.27 (0.14)
AnD9DS v3	29.64 (1.34)	17.59 (1.28)	0.44 (0.04)	18.26 (0.83)	1.42 (0.15)	0.24 (0.16)	1.26 (0.09)	0.10 (0.05)
HxD9DS v2	29.31 (0.94)	17.26 (0.39)	0.81 (0.06)	18.39 (0.66)	1.47 (0.10)	0.18 (0.05)	1.50 (0.04)	0.23 (0.03)
LnD9DS-2 v2	27.72 (0.18)	17.46 (0.55)	1.00 (0.11)	19.33 (0.46)	1.45 (0.11)	0.32 (0.14)	1.48 (0.10)	0.10 (0.09)
MgD9DS v2	29.76 (1.10)	17.98 (0.84)	0.63 (0.63)	19.19 (0.86)	1.60 (0.09)	0.26 (0.20)	1.44 (0.09)	0.16 (0.03)
AnD9DS v3 + LnD9DS-2 v2	29.17 (0.31)	18.84 (0.41)	0.59 (0.27)	17.65 (0.23)	1.40 (0.04)	0.39 (0.03)	1.13 (0.06)	0.03 (0.02)
AnD9DS v3 + HxD9DS v2	29.28 (1.78)	18.83 (1.69)	0.65 (0.21)	17.88 (1.90)	1.55 (0.20)	0.20 (0.12)	1.33 (0.24)	0.11 (0.08)

In addition to reducing the content of the saturated palmitic and stearic fatty acids, and increasing the monounsaturated fatty acid content (palmitoleic and oleic), the presence of the desaturases also lowered the amount of arachidic acid (C20:0) in the seeds. This is presumably because this fatty acid is derived from elongation of stearic and palmitic acids. There appeared to be no direct desaturation of C20:0 by the introduced desaturases, as there is no concomitant rise in eicosenoic acid (C20:1) as C20:1Δ9.

Example 10: Delta-9 desaturase antibody preparation

Diagnostic tools such as antibodies are desirable to characterize transgenic delta-9 desaturase protein expression in plants. Because acyl-CoA delta-9 desaturases are membrane-bound proteins, routine over-expression in *Escherichia coli* is difficult. However, antibodies were successfully generated by over-expression of a C-terminal fragment of each delta-9 desaturase protein that does not include any of the transmembrane domains of the protein.

Polymerase Chain Reactions: PCR primers were designed to amplify an equivalent C-terminal fragment for each desaturase. The 3' primer was designed to encode a protein fragment with a C-terminal 6x His tag. *NdeI* and *BamHI* restriction sites were incorporated into the 5' and 3' primers, respectively, to facilitate cloning. The primer sequences are given below in Table 15. The

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expected amplification products were 659 bp for LnD9DS-2, 683 bp for MgD9DS, and 335 bp for HzD9DS. PCR reactions were carried out using the Takara Ex Taq™ PCR kit (Clontech, Mountain View, CA) using supplier conditions. The total PCR reaction volume was 50 μ L. Each reaction contained 200 ng of plasmid DNA and 50 pmol of each primer. The DNA was denatured at 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 sec, 60 °C for 1 minute, and 72 °C for 30 sec. A final extension was carried out at 72 °C for 10 minutes. Each PCR product was run across two wells on a sterile 0.75% agarose gel, and DNA was gel purified using Montage spin columns and eluted in 15 μ L TE buffer.

10

Table 15: Sequences of the oligonucleotide primers used in the PCR amplifications of C- terminal fragments from LnD9DS-2, MgD9DS, and HzD9DS.

Primer	Sequence	Purpose
AntiLnD9DS2F	SEQ ID NO:25 CATATGTTTCGACGACAGACGCACGCCTCGAGAC	Forward primer for LnD9DS2 C-terminal
AntiLnD9DS2Rh	SEQ ID NO:26 GGATCCGCAGCCACAGCCCCCTCAACCAACCTCTC	Reverse primer for LnD9DS2 C-terminal
AntiMgD9DSF	SEQ ID NO:27 CATATGTTTCGACGATCGCAACTCGCCGCGTGATCAC	Forward primer for MgD9DS C-terminal
AntiMgD9DSRh	SEQ ID NO:28 GGATCCGCGGCCTGAGCACCCGGAACAGGCTG	Reverse primer for MgD9DS C-terminal
AntiHzD9DSF	SEQ ID NO:29 CATATGTATGACAAGTCCATCAAGCCTTCC	Forward primer for HzD9DS C-terminal
AntiHzD9DSRh	SEQ ID NO:30 GGATCCTCGTCTTTAGGGTTGATCCTAATGGCTGC	Reverse primer for HzD9DS C-terminal

15 TOPO cloning: The purified C-terminal fragments were TA cloned into TOPO® pCR®2.1 vectors (Invitrogen, Carlsbad, CA), and transformed into Top 10 *E. coli* cells following the manufacturer's protocol (Invitrogen).

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Transformations were selected, and plasmid DNA was purified using NucleoSpin® columns (Macherey-Nagel GmbH & Co, Duren, Germany). Three microliters (3 µL) of DNA was digested with *NdeI* and *BamHI* in a total volume of 20 µL for 90 minutes at 37 °C, and run on a 0.8% agarose gel. In each case, a gene-specific
5 fragment (plus a 3.9 kb TOPO® vector band) was visible. Three positive clones were chosen for each cloned gene and sequenced to confirm that the amplified PCR fragment was free of errors. Each of the MgD9DS clones contained a silent point mutation at base pair 45, indicating either a single nucleotide polymorphism between the published sequence and the PCR template, or a silent PCR error. Since the
10 mutation was silent, no correction was necessary, and one clone was chosen for subcloning.

Preparation of the delta-9 desaturase C-terminal fragment expression plasmids:

The PCR-amplified delta-9 desaturase fragments were digested with *NdeI* and *BamHI* restriction enzymes and ligated into corresponding restriction sites within
15 the pET30b(+) expression vector. The cloning step resulted in the addition of 15 C-terminal amino acids, constituting a C-terminal 6x His tag to facilitate full-length protein purification. These additional amino acids were not expected to affect protein expression. Positive clones were obtained and confirmed *via* restriction enzyme digestion and sequencing reactions.

20 Expression of delta-9 desaturase C-terminal peptide fragments in *E. coli*: The delta-9 desaturase/pET30b(+) expression plasmids were transformed into BL21(DE3) *E. coli* cells according to the manufacturer's recommended protocol (Novagen, Madison, WI). Cells were plated on LA plates containing kanamycin (50 µg/mL) and glucose (1.25 M). The plates were incubated
25 overnight at 37 °C. A full loop of cells was scraped from the plates, and inoculated into 500 mL flasks containing 250 mL LB and kanamycin (50 µg/mL) with isopropyl-P-D-thiogalactoside (0.75 mM) inducer. Three induction conditions were tested. Cultures were induced at different temperatures, and harvested at different times as follows: overnight (~18 hrs) at 28 °C; overnight
30 at 16 °C; or 4 hours at 37 °C. Cells were harvested by centrifugation in 250 mL bottles at 6,000 rpm for 15 minutes, and then frozen at -20 °C.

Protein purification of delta-9 desaturase C-terminal peptide fragments: Cell pellets from 250 mL cultures were thawed and resuspended in 50 mL cold Phosphate Buffered Saline (PBS) containing 10% glycerol and 0.5 mL of Protease Inhibitor Cocktail (Sigma, St. Louis, MO) using a hand-held homogenizer. The cells were disrupted on ice for approximately 10 minutes using a Branson Model 450 Sonifier (Danbury, CT). Inclusion bodies were pelleted by centrifugation at 10,000 x g for 15 minutes, and extracted 2-3 times with PBS containing 0.5% Triton X-100 until the protein concentration of the supernatant reached baseline, as measured by a Bradford protein assay. The recovered inclusion bodies were solubilized in a PBS solution containing 6 M Urea and 5 mM DTT at room temperature with stirring for about 1 hour. Solubilized proteins were separated from insoluble materials by centrifugation at 30,000 x g for 15 minutes, and the retained supernatant was applied onto a 5 mL Ni-affinity column (GE Healthcare, HiTrap Chelating, Piscataway, NJ). The histidine tags of the C-terminal delta-9 desaturase peptides bound to the metal resin, and each fragment was eluted with a 50-200 mM imidazole gradient using an Akta® Explorer 100 (GE Healthcare, Piscataway, NJ). Fractions (3 mL each) were collected, and eluted peaks were analyzed by SDS-PAGE. Fractions containing C-terminal delta-9 desaturase peptide were pooled and concentrated using an Amicon® Ultra 10,000 MWCO filter device (Millipore, Billerica, MA) to less than 5 mL volume. The protein sample was then injected onto a Hi Load™ XK16/60 Superdex™ 200 size exclusion column (GE Healthcare, Piscataway, NJ), and equilibrated with 6 M Urea in 20 mM Tris-HCl, 150 mM NaCl, and 1 mM DTT. The peak fractions (4 mL each) containing pure C-terminal delta-9 desaturase peptide were saved (after validation by SDS-PAGE analysis and other biochemical characterization) and used for antibody production. Peptides with the expected sizes of 27 kDa for LnD9DS-2 peptide, 15 kDa for HzD9DS peptide, and 28 kDa for MgD9DS peptide were produced. The induction conditions produced sufficient protein for visualization by Coomassie blue staining of SDS-PAGE gels.

Polyclonal Antibody Production: A contract service (Strategic BioSolutions, Newark, DE) produced rabbit antibodies against each of the three C-terminal delta-9 desaturase peptides. Following their standard procedures, high titer (validated

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by using ELISA) antisera for each of the three protein fragments was obtained. Each purified C-terminal delta-9 desaturase peptide was diluted with 20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT buffer, and with a final concentration of 2-3 M urea, to keep the protein in solution. Approximately 10 mg of protein was sent to Strategic
5 BioSolutions for generation of a polyclonal antibody. Two rabbits were chosen for each immunogen, and standard protocols (70 days immunization) were used. A new adjuvant called TiterMax[®] Gold was purchased for preparation of the emulsion. ELISA titration during immunization and at the end of protocol was also performed to ensure the success of antibody production. The antisera were delivered in two
10 separate time points; one from the standard 2 month procedure, and the other from exsanguination.

To isolate total IgG from the rabbit sera, approximately 20-30 mL of high-titer antisera were applied to a 5 mL alkali-tolerant Protein A column (GE Healthcare, HiTrap[™] MabSelect SuRe[™], cat#11-0034-94). Following a standard
15 wash with PBS buffer, bound IgG was eluted from the resin by short exposure to 0.1 M sodium citrate, 0.3 M NaCl, pH 3.3, and immediately neutralized by adding 1/10 volume of 2 M Tris-HCl, pH 9 buffer to each fraction. The affinity column was sanitized by treating with 0.5 N NaOH following standard cleaning-in-place (CIP) procedure to avoid cross contamination of the IgG. Final recovered IgG from
20 each sample was dialyzed against 50 volumes of PBS at 4 °C overnight, and protein concentration was determined by Bradford assay using BSA standard (Pierce, prod# 23208). One mL aliquots were transferred to individual tubes and stored at -80 °C.

These antibodies are diagnostic tools that were used to measure desaturase protein expression in transgenic plant material. The antibodies were used to
25 develop correlations between low saturated fatty acid oil phenotype changes and the level of expression of the delta-9 desaturase proteins.

Example 11: Levels of acyl-CoA delta-9 desaturase proteins in T₂ *Arabidopsis* seed

30

Delta-9 desaturase polypeptides were detected in mature transgenic seed samples by Western blot. Seed was prepared for analysis by cracking dry seeds with stainless steel beads in a Kleco[™] Bead Beater (Garcia Machine, Visalia, CA).

Extraction buffer was added (50 mM Tris, 10 mM EDTA, 2% SDS), and sample tubes were rocked gently for 30 minutes. Samples were centrifuged for 15 minutes at 3,000 rcf. Then, the supernatant was collected and used for analysis. The amount of total soluble protein in the seed extract was determined by Lowry assay (BioRad, Hercules, CA). Samples were normalized to 1.55 mg/mL total soluble protein and prepared in LDS sample buffer (Invitrogen, Carlsbad, CA) with 40 mM DTT, for a normalized load of 20 µg total soluble protein per lane. Samples were electrophoresed in 4-12% Bis-Tris gels (Invitrogen), and transferred to nitrocellulose membranes. Blots were blocked in blocking buffer, and probed with antibodies against four different delta-9 desaturase polypeptides (AnD9DS, LnD9DS-2, HzD9DS, and MgD9DS) (see Example 10).

In all cases, polyclonal antibody was developed in rabbits against a His-tag purified C-terminal peptide fragment of the individual desaturases as described above. The purified C-terminal fragments were used as reference antigens for quantitation of the Western blots. An anti-rabbit fluorescent labeled secondary antibody (Goat Anti-Rabbit AF 633; Invitrogen) was used for detection. Blots were visualized on a Typhoon™ Trio Plus fluorescence imager (GE Healthcare). Standard curves were generated with quadratic curve fitting, and linear regression was used to quantify expression.

SDS-PAGE Western blots of extracts from mature T₂ seed from *Arabidopsis* events showed bands at the appropriate size when probed with specific antisera. These bands were quantified against specific reference antigens. Quantitative Western blotting of *Arabidopsis* T₂ seed extracts with appropriate antiserum indicated that an average of 63 ng LnD9DS-2/mg total protein (tp) (max. 228 ng/mg tp) was detected in mature seeds, and for HzD9DS, an average of 34 ng/mg tp (max. 100 ng/mg tp) was detected. For MgD9DS, an average of 58 ng/mg tp (max. 1179 ng/mg tp) was detected in T₂ seed. For the AnD9DS events, an average of 625 ng/mg tp (max 1.5 µg/mg tp) was detected in mature T₂ seeds. Thus, there was 10-18-fold less of the palmitoyl-preferring desaturases, LnD9DS-2 and HzD9DS, expressed in the transgenic seed, relative to AnD9DS. Higher levels of expression of these desaturases would therefore drive further reductions in saturates, especially palmitic acid.

Example 12: Expression of delta-9 desaturase genes in canola

A series of transgenic canola events were obtained from transformations performed with pDAB7321 (SEQ ID NO:61) and pDAB7326 (SEQ ID NO:63) (containing LnD9DS-2 and HzD9DS genes, respectively, driven by the seed-specific PvPhas promoter). Thirty nine pDAB7321 events containing the LnD9DS-2 gene were identified by PCR analysis of genomic DNA, and were grown in the greenhouse to produce T₁ seed. Similarly, 80 pDAB7326 events were identified that contained the HzD9DS gene, and produced T₁ seed. Canola was also transformed with pDAB7319 (SEQ ID NO:60) or pDAB7324 (SEQ ID NO:62), which contain an AnD9DS gene coupled with the LnD9DS-2 or HzD9DS genes, all driven by the PvPhas promoter. 44 and 76 events were recovered, respectively, that were confirmed to contain both desaturase genes by PCR analysis, and were grown in the greenhouse to produce T₁ seed.

FAME analysis of T₁ seed samples from events transformed with pDAB7321 (LnD9DS-2 v2) or pDAB7326 (HzD9DS v2) did not show significant reduction in saturated fatty acid levels relative to untransformed canola plants or plants transformed with an empty vector control. Western blots of the T₁ seed did not show detectable levels of the delta-9 desaturase proteins. In addition, no detectable protein for LnD9DS-2 or HzD9DS was detected in T₁ seed from plants transformed with pDAB7319 (AnD9DS v3 and LnD9DS-2 v2) or pDAB7324 (AnD9DS v3 and HzD9DS v2), whereas the AnD9DS protein could be readily detected. In these events, a reduction of saturated fatty acids was observed relative to control plants, but this was attributable to expression of AnD9DS.

To evaluate the relative mRNA levels of the delta-9 desaturase genes, total RNA was extracted from developing canola seed from events transformed with double desaturase constructs (pDAB7319 and pDAB7324) and analyzed by quantitative real-time PCR. Seeds were harvested on dry ice at 20, 25, 29, 32, 39, or 41 days after pollination from several canola plants and stored at -80 °C. Total RNA was prepared from 50 mg of pooled frozen seeds using a Plant RNeasy[®] RNA extraction kit (Qiagen) according to the manufacturer's recommended protocol. Extracted RNA was used as a template for cDNA synthesis using the SuperScript[®] III First Strand Synthesis

Supremix for qRT-PCR (Invitrogen) according to the manufacturer's recommended protocol.

RT-PCR assays were designed against the desaturase targets using the Roche Assay Design Center (Roche Diagnostics, Indianapolis, IN). Primers used in the assay are described in Table 16. Target assays utilized FAM-labeled UPL probes (Roche Diagnostics). These assays were executed in duplex reactions with a Texas-Red-labeled canola *actin* reference assay synthesized by Integrated DNA Technologies.

Table 16: q-RT-PCR assay details

Target	Forward primer	Reverse Primer	Probe
AnD9Ds	SEQ ID NO:31 GGACTTCTCTACTCTCACCTTGGG	SEQ ID NO:32 TCCGATCCTCTTTGGGTCT	UPL #9
HxD9Ds	SEQ ID NO:33 GACCCACACAATGCAACG	SEQ ID NO:34 CCTAACAAGAAGCCAGCCAAT	UPL #143
LnD9Ds	SEQ ID NO:35 GTTCTGACTGCGTTGGTCAC	SEQ ID NO:36 CGGAACTCATGGTGAAGT	UPL #7
Actin	SEQ ID NO:37 CTACTGGTATTGTGCTCGACT	SEQ ID NO:38 CTCTCTCGGTGAGAATCTTCAT	SEQ ID NO:39 CACGCTATCCTCCGTCTCGATC

Target	Label
AnD9Ds	FAM
HxD9Ds	FAM
LnD9Ds	FAM
Actin	Tx-Red

RT-PCR reactions were run on a LightCycler® 480II real-time PCR thermal cycler (Roche). Data for target UPL assays was collected using a 533 nm emission filter and a 483 nm excitation signal. Data for the *actin* reference assay was collected using a 610 nm filter and a 558 nm excitation signal. Cycle time values and target to reference ratios were calculated automatically using the LC480II software's "Advanced Relative Quantification" analysis workflow. Relative accumulation of desaturase transcript levels within each sample was calculated using the standard $\Delta\Delta Ct$ method (Roche).

For each canola seed sample from pDAB7319 (AnD9DS v3 and LnD9DS-2 v2) and pDAB7324 (AnD9DS v3 and HxD9DS v2), transcript accumulation of HxD9DS or LnD9DS-2 transgenes was significantly lower than the transcript of

AnD9DS in the same events. The observed differences in transcript accumulation varied between 3- and 20-fold less. **FIG. 23.** Thus, insufficient expression of HzD9DS and LnD9DS-2 may account for the lack of detection of the polypeptide and absence of phenotype attributable to these genes.

5

Example 13: Expression of the delta-9 desaturase PTUs by alternative promoters

The use of additional transcriptional regulatory regions to express gene(s) encoding LnD9DS-2, HzD9DS, and MgD9DS proteins can further increase the content of these delta-9 desaturases within canola. Identification and use of transcriptional regulatory regions which express earlier in development, and for longer periods of time, can increase the levels of heterologous delta-9 desaturases within canola seed by promoting robust seed-specific transcription of a heterologous gene at earlier stages of seed development. Examples of such transcriptional regulatory regions include, but are not limited to, the LfKCS3 promoter (U.S. Patent 7,253,337) and FAE 1 promoter (U.S. Patent 6,784,342). These promoters are used singularly, or in combination, to drive the expression of LnD9DS-2, HzD9DS, and MgD9DS expression cassettes, for example, through operable linkage with genes such as those previously described in plasmids, pDAB7319; pDAB7321; pDAB7324; pDAB7326; pDAB7328; and pDAB7330. Methods to replace transcriptional regulatory regions within a plasmid are well-known within the art. As such, a polynucleotide fragment comprising the PvPhas promoter is removed from pDAB7319, pDAB7321, pDAB7324, pDAB7326, pDAB7328, or pDAB7330 (or the preceding plasmids used to build pDAB7319, pDAB7321, pDAB7324, pDAB7326, pDAB7328, or pDAB7330), and replaced with either a LfKCS3 or FAE 1 promoter region. The newly-constructed plasmids are used to stably transform canola plants, according to the procedures set forth in the previous examples. Transgenic canola plants are isolated and molecularly characterized. The resulting delta-9 desaturase accumulation is determined, and canola plants which robustly express delta-9 desaturase are identified.

Further modifications to the transcriptional regulatory regions for increased expression of a delta-9 desaturase include replacing the existing Kozak sequence with any of the sequences described in **Table 17.** The engineering of alternative Kozak sequences upstream of the start site of a delta-9 desaturase is completed using standard

molecular biology techniques. Synthetic polynucleotide fragments are synthesized and cloned upstream of a delta-9 desaturase coding sequence using techniques known within the art. The context of the start codon has a strong effect on the level of expression of a transgene. Modifying the Kozak sequence to one listed in Table 17 increases the levels of expression of the heterologous delta-9 desaturase.

Table 17: Kozak sequences which are incorporated upstream of a heterologous delta-9 desaturase gene to increase expression.

Kozak Sequence	SEQ ID NO:	Sequence
Kozak #1	SEQ ID NO:40	GGATCCAACAATG
Kozak #2	SEQ ID NO:41	ACAACCAAAAATG
Kozak #3	SEQ ID NO:42	ACAACCAACCTACCATGG
Kozak #4	SEQ ID NO:43	ACAACCAAAAATG

Example 14: Design And synthesis of delta-9 desaturase genes from *Helicoverpa zea* and *Leptosphaeria nodorum*

To obtain higher levels of expression of heterologous genes in plants, the codon optimization strategy described in Example 2 was modified, and the heterologous gene protein coding regions for HzD9DS and LnD9DS-2 were re-engineered using a new design protocol.

Codon selection was made using a table which had calculated the codon bias of the prospective host plant, which in this case was canola. In designing coding regions for plant expression of delta-9 desaturase genes, the primary ("first choice") codons preferred by the plant were determined, and used at about 95% of the time. "Second choice" codons were used sparingly, at a frequency of about 5%. Accordingly, a new DNA sequence was designed which encodes the amino sequence of each delta-9 desaturase, wherein the new DNA sequence differed from the native delta-9 desaturase gene by the substitution of plant first preferred and second preferred codons to specify an appropriate amino acid at each position within the amino acid sequence. The new sequence was then analyzed for restriction enzyme sites that might have been created by the modifications. The identified restriction enzyme sites were then removed by replacing the codons with first or second choice preferred codons. Other sites in the

sequence which could affect transcription or translation of the gene of interest, specifically highly stable stem loop structures, were also removed.

The selections of preferred codon choices (first and second choices) from the genetic code of canola were determined from a codon bias table compiled from the protein coding sequences for canola. In **Tables 18 and 19**, Columns labeled as "Native Gene %" present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the coding regions of *Brassica napus* (canola). New DNA sequences which encode essentially the amino acid sequence of the *M. grisea*, *H. zea* and *L. nodorum* delta-9 desaturases were designed for optimal expression in canola using the preferred codon distribution of first and second choice codons found in canola genes. Design of the plant-optimized DNA sequences were initiated by reverse-translation of the protein sequences of SEQ ID NO:12 (*M. grisea*), SEQ ID NO:13 (*H. zea*), and SEQ ID NO:14 (*L. nodorum*) using the canola codon bias table constructed. Columns labeled as "Plnt Opt Gene %" indicate the preferred codons and the frequency with which they were incorporated into the delta-9 desaturase gene design. SEQ ID NO:44 and SEQ ID NO:45 set forth the nucleotide sequences of the new canola-optimized LnD9DS-2 and HzD9DS desaturases, respectively. These new canola-optimized sequences were labeled as LnD9DS-2 v3 and HzD9DS v3.

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Table 18. Codon compositions of coding regions for the HzD9DS protein. The native *H. zea* desaturase coding region is compared to a Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd	Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd
ALA (A)	GCA	4	11.4	1	2.9	0.0	LEU (L)	CTA	2	5.9	0	0.0	0.0
	GCC	7	20.0	0	0.0	0.0		CTC	8	23.5	0	0.0	0.0
	GCG	8	22.9	0	0.0	0.0		CTG	14	41.2	0	0.0	0.0
	GCT	16	45.7	34	97.1	100.0		CTT	6	17.6	34	100.0	100.0
ARG (R)	AGA	1	7.7	0	0.0	0.0		TTA	2	5.9	0	0.0	0.0
	AGG	5	38.5	13	100.0	100.0	LYS (K)	TTG	2	5.9	0	0.0	0.0
	CGA	2	15.4	0	0.0	0.0		AAA	11	44.0	0	0.0	0.0
	CGC	5	38.5	0	0.0	0.0	MET (M)	AAG	14	56.0	25	100.0	100.0
	CGG	0	0.0	0	0.0	0.0		ATG	8	100	8	100	100.0
	CGT	0	0.0	0	0.0	0.0	PHE (F)	TTC	20	83.3	24	100.0	100.0
ASN (N)	AAC	13	72.2	18	100.0	100.0		TTT	4	16.7	0	0.0	0.0
	AAT	5	27.8	0	0.0	0.0	PRO (P)	CCA	1	6.3	16	100.0	100.0
ASP (D)	GAC	16	64.0	2	8.0	0.0		CCC	5	31.3	0	0.0	0.0
	GAT	9	36.0	23	92.0	100.0		CCG	2	12.5	0	0.0	0.0

Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd	Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd
CYS (C)	TGC	1	100.0	1	100.0	100.0	SER (S)	CCT	8	50.0	0	0.0	0.0
	TGT	0	0.0	0	0.0	0.0		AGC	2	12.5	0	0.0	0.0
END	TAA	1	100.0	0	0.0	0.0		AGT	1	6.3	0	0.0	0.0
	TAG	0	0.0	0	0.0	0.0		TCA	1	6.3	1	6.3	0.0
	TGA	0	0.0	1	100.0	100.0		TCC	6	37.5	0	0.0	0.0
GLN (Q)	CAA	2	33.3	6	100.0	100.0		TCG	3	18.8	0	0.0	0.0
	CAG	4	66.7	0	0.0	0.0		TCT	3	18.8	15	93.8	100.0
GLU (E) 16	GAA	7	63.6	0	0.0	0.0	THR (T)	ACA	3	16.7	0	0.0	0.0
	GAG	4	36.4	11	100.0	100.0		ACC	7	38.9	18	100.0	100.0
GLY (G)	GGA	8	40.0	20	100.0	100.0		ACG	4	22.2	0	0.0	0.0
	GGC	6	30.0	0	0.0	0.0		ACT	4	22.2	0	0.0	0.0
	GGG	2	10.0	0	0.0	0.0	TRP (W)	TGG	14	100	14	100	0.0
	GGT	4	20.0	0	0.0	0.0		TYR (Y)	TAC	12	80.0	15	100.0
HIS (H)	CAC	11	73.3	15	100.0	100.0	VAL (V)	TAT	3	20.0	0	0.0	0.0
	CAT	4	26.7	0	0.0	0.0		GTA	0	0.0	0	0.0	0.0
ILE (I)	ATA	3	15.0	1	5.0	0.0		GTC	5	26.3	0	0.0	0.0
	ATC	10	50.0	19	95.0	100.0		GTG	13	68.4	0	0.0	0.0
	ATT	7	35.0	0	0.0	0.0		GTT	1	5.3	19	100.0	100.0
Totals		165		165			Totals		189		189		

Table 19. Codon compositions of coding regions for the LnD9DS-2 protein. The native *L. nodorum* desaturase coding region is compared to a Plant-Optimized version.

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Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd	Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd
ALA (A)	GCA	3	9.4	0	0.0	0.0	LEU (L)	CTA	7	15.6	0	0.0	0.0
	GCC	9	28.1	0	0.0	0.0		CTC	14	31.1	0	0.0	0.0
	GCG	12	37.5	0	0.0	0.0		CTG	7	15.6	0	0.0	0.0
	GCT	8	25.0	32	100.0	100.0		CIT	5	11.1	45	100.0	100.0
ARG (R)	AGA	4	13.8	1	3.4	0.0		TTA	3	6.7	0	0.0	0.0
	AGG	3	10.3	28	96.6	100.0	LYS (K)	TTG	9	20.0	0	0.0	0.0
	CGA	7	24.1	0	0.0	0.0		AAA	9	45.0	0	0.0	0.0
	CGC	8	27.6	0	0.0	0.0	MET (M)	AAG	11	55.0	20	100.0	100.0
	CGG	5	17.2	0	0.0	0.0		ATG	9	100	9	100	100.0
	CGT	2	6.9	0	0.0	0.0	PHE (F)	TTC	16	80.0	20	100.0	100.0
ASN (N)	AAC	6	50.0	12	100.0	100.0		TTT	4	20.0	0	0.0	0.0
	AAT	6	50.0	0	0.0	0.0	PRO (P)	CCA	3	16.7	18	100.0	100.0
ASP (D)	GAC	16	66.7	2	8.3	0.0		CCC	8	44.4	0	0.0	0.0
	GAT	8	33.3	22	91.7	100.0		CCG	2	11.1	0	0.0	0.0
CYS (C)	TGC	4	80.0	5	100.0	100.0		CCT	5	27.8	0	0.0	0.0
	TGT	1	20.0	0	0.0	0.0	SER (S)	AGC	8	27.6	0	0.0	0.0
END	TAA	0	0.0	0	0.0	0.0		AGT	6	20.7	0	0.0	0.0
	TAG	1	100.0	0	0.0	0.0		TCA	1	3.4	1	3.4	0.0
	TGA	0	0.0	1	100.0	100.0		TCC	6	20.7	0	0.0	0.0
GLN (Q)	CAA	10	55.6	18	100.0	100.0		TCG	7	24.1	0	0.0	0.0
	CAG	8	44.4	0	0.0	0.0		TCT	1	3.4	28	96.6	100.0
GLU (E) 16	GAA	5	33.3	1	6.7	0.0	THR (T)	ACA	11	44.0	0	0.0	0.0
	GAG	10	66.7	14	93.3	100.0		ACC	5	20.0	25	100.0	100.0
GLY	GGA	13	34.2	38	100.0	100.0		ACG	7	28.0	0	0.0	0.0

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Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd	Amino Acid	Codon	Native Gene #	Native Gene %	Plnt Opt Gene #	Plnt Opt Gene %	Plnt Opt Recm'd
(G)	GGC	16	42.1	0	0.0	0.0	TRP (W)	ACT	2	8.0	0	0.0	0.0
	GGG	6	15.8	0	0.0	0.0		TGG	19	100	19	100	0.0
	GGT	3	7.9	0	0.0	0.0		TAC	11	64.7	17	100.0	100.0
								TAT	6	35.3	0	0.0	0.0
HIS (H)	CAC	12	66.7	18	100.0	100.0	VAL (V)	GTA	6	17.6	0	0.0	0.0
	CAT	6	33.3	0	0.0	0.0		GTC	10	29.4	0	0.0	0.0
ILE (I)	ATA	4	18.2	1	4.5	0.0		GTG	12	35.3	0	0.0	0.0
	ATC	9	40.9	21	95.5	100.0		GTT	6	17.6	34	100.0	100.0
	ATT	9	40.9	0	0.0	0.0							
Totals		214		214			Totals		236		236		

Syntheses of DNA fragments comprising SEQ ID NO:44 and SEQ ID NO:45 were performed by PicoScript and Blue Heron Biotechnology. The synthetic DNA was then cloned into expression vectors and transformed into canola substantially as described in the foregoing examples.

Example 15: Modification of N- and C-termini to increase the accumulation of acyl-CoA desaturase polypeptides in plants

The accumulation and stability of membrane-bound proteins in the endoplasmic reticulum (ER) can be influenced by amino acid sequence motifs and modifications at their N- and C-termini. Ravid and Hochstrasser (2008) Nat. Rev. Mol. Cell. Biol. 9:679-90. In particular, N- and C-terminal motifs and modifications have been shown to modulate the accumulation and stability of lipid desaturases in fungi and plants, as well as animals. McCartney *et al.* (2004) Plant J. 37:156-73; Mziaut *et al.* (2000) Proc. Natl. Acad. Sci. U.S.A. 97:8883-8.

The addition of either a Myc or the hemagglutinin (HA) epitope tag to the N-terminal of FAD2 or FAD3 significantly increases the steady state level of these enzymes within yeast. O'Quin *et al.* (2009) Appl Microbiol Biotechnol 83:117-25. Accordingly, the addition of these, or similar epitopes, to the N-terminus of a delta-9 desaturase of the present invention is utilized to increase the expression of the polypeptide in a plant. A polynucleotide linker that encodes a Myc tag (SEQ ID NO:46) or a HA tag (SEQ ID NO:47) is cloned within the 5' end of a delta-9 desaturase (*e.g.*, HzD9DS, MgD9DS, AnD9DS, LnD9DS-1, and LnD9DS-2) coding sequence as a contiguous open reading frame. The resulting coding sequence is cloned within a plant expression plasmid using the cloning strategy described in Example 3.

The newly-constructed plasmid is used to stably transform an *Arabidopsis* and/or canola plant cell, material, or tissue. Transgenic plants are regenerated from the transformed plant cell, material, or tissue. Transgenic plants are isolated and molecularly characterized. The resulting delta-9 desaturase accumulation in seeds of transgenic plants is determined, and plants which robustly express the delta-9 desaturase polypeptide are identified.

Evidence from expression of AnD9DS in *Arabidopsis* and canola (Examples 11 and 12) indicates a significantly higher level of expression of this particular desaturase enzyme, relative to HzD9DS and LnD9DS-2. Thus, all or parts of the N- and C-termini lying outside the core desaturase domain (containing the transmembrane segments and conserved catalytic histidine residues) of AnD9DS may be used to replace equivalent residues in the lower-expressing desaturases and increase expression thereof. Accordingly, all or part of N-terminal residues 1-68 and C-terminal residues 281-455 of AnD9DS (SEQ ID NO:72 and SEQ ID NO:73, respectively) are used to replace all or part of the 68 N-terminal residues (1-68) and 168 C-terminal residues (281-449) of LnD9DS-2 (SEQ ID NO:14) and/or the 76 N-terminal residues (1-76) and 60 C-terminal residues (293-353) of HzD9DS (SEQ ID NO:13). The resulting coding sequence is cloned within a plant expression plasmid using the cloning strategy described in Example 3. The newly-constructed plasmid is used to stably transform an *Arabidopsis* and/or canola plant cell, material, or tissue. Transgenic plants are regenerated from the transformed plant cell, material, or tissue. Transgenic plants are isolated and molecularly characterized. The resulting delta-9 desaturase accumulation in seeds of transgenic plants is determined, and plants which robustly express the modified HzD9DS or modified LnD9DS-2 polypeptide are identified.

25

Example 16: Modifications to enhance mRNA expression of acyl-CoA desaturase within plants

It is known within the art that the expression of mRNA can be enhanced by the incorporation of genetic elements that stabilize and increase mRNA accumulation. The incorporation of 5' and 3' untranslated regions (e.g., Tobacco Osmotin 5' and 3' UTR sequences (Liu *et al.* (2003) Nat. Biotechnol. 21:1222-8), and the Tobacco Mosaic Virus Ω sequence (Gallie *et al.* (1987) Nucleic Acids Res. 15:8693-711)) or introns

(Kozziel *et al.* (1996) *Plant Mol. Biol.* 32:393-405), within close proximity of a HzD9DS or LnD9DS-2 coding sequence, is used to increase the levels of expression of the transgene when compared to the expression of the same coding sequence lacking the aforementioned genetic elements. The addition one or more of these genetic elements within a desaturases PTU is performed according to methods well-known in the art. Polynucleotide fragments comprising the 5' untranslated region, 3' untranslated region, and/or intron are added to a plant expression plasmid (e.g., pDAB7319, pDAB7321, pDAB7324, pDAB7326, pDAB7328, pDAB7330, or the preceding plasmids used to build pDAB7319, pDAB7321, pDAB7324, pDAB7326, pDAB7328, or pDAB7330) via standard cloning methods. The newly-constructed plasmid is used to stably transform an *Arabidopsis* and/or canola plant cell, material, or tissue. Transgenic plants are regenerated from the transformed plant cell, material, or tissue. Transgenic plants are isolated and molecularly characterized. The resulting delta-9 desaturase accumulation in seeds of transgenic plants is determined, and plants which robustly express the HzD9DS or LnD9DS-2 polypeptide are identified.

Furthermore, it is known in the art that yeast desaturase genes such as *OLE1* are highly-regulated. The deletion of sequences that encode transmembrane regions and that are a part of the cytochrome b5 domain reduce the stability of the *OLE1* transcript. Vemula *et al.* (2003) *J. Biol. Chem.* 278(46):45269-79. The presence of these sequences within *OLE1* act as mRNA stabilizing sequences. Accordingly, incorporation of the *OLE1* sequences that encode the transmembrane region and cytochrome b5 domain into a LnD9DS-2 or HzD9DS coding sequence is utilized to increase stability of the mRNA transcript of the coding sequence, thereby resulting in higher levels of expression and a subsequent increase of LnD9DS-2 or HzD9DS polypeptide. A chimeric LnD9DS-2 or HzD9DS coding sequence that includes the *OLE1* transmembrane region and cytochrome b5 domain sequences is constructed using methods known in the art. The coding sequence produced thereby is incorporated into a plant expression plasmid (e.g., as described in the foregoing examples), and used to generate transgenic plants via *Agrobacterium*-mediated plant transformation. Transgenic plants are isolated and characterized. The resulting delta-9 desaturase accumulation is determined, and plants which robustly express the delta-9 desaturase are identified.

Example 17**Use of an alternative 3' untranslated region terminator
for stable expression of a delta-9 desaturase in a plant**

5 Due to a limited number of available 3' UTR-terminators, the *Agrobacterium*
ORF 23 3' UTR-terminator (AtuORF23 3' UTR) is typically used to terminate
transcription. It was recently shown that other 3' UTR-terminators are more effective
in terminating transcriptional read-through in *Arabidopsis thaliana*. Accordingly, the
Phaseolus vulgaris Phaseolin 3'UTR-terminator (SEQ ID NO:69) is used in
10 combination with the *Phaseolus vulgaris* Phaseolin promoter to reduce transcriptional
read-through of upstream genes, thereby reducing transcriptional interference.

The *Phaseolus vulgaris* Phaseolin 3'UTR-terminator (PvPhas 3'UTR v1) was
incorporated within an LnD9DS-2 v2 expression cassette, and within an HzD9DS v2
expression cassette, which were previously described in plasmid pDAB7321 and
15 pDAB7326. According to methods well-known to those of skill in the art, a
polynucleotide fragment comprising the PvPhas 3'UTR v1 was placed downstream of
a LnD9DS-2 v2 gene to create binary plasmid, pDAB110110 (**FIG. 4a**; SEQ ID
NO:74). A polynucleotide fragment comprising the PvPhas 3'UTR v1 was also placed
downstream of a HzD9DS v2 gene to create binary plasmid pDAB110112 (**FIG. 4b**;
20 SEQ ID NO:75).

The resulting binary plasmids were confirmed *via* restriction enzyme digestion
and sequencing. The newly-constructed plasmids are each used to stably transform an
Arabidopsis and/or canola plant cell, material, or tissue. Transgenic plants are
regenerated from the transformed plant cell, material, or tissue. Transgenic plants are
25 isolated and molecularly characterized. The resulting delta-9 desaturase accumulation
in seeds of the transgenic plants is determined, and plants which robustly express the
HzD9DS or LnD9DS-2 polypeptide are identified.

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a
sequence listing in electronic form in ASCII text format (file: 84103436 Seq 12-MAR-18 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian
Intellectual Property Office.

CLAIMS:

1. An isolated nucleic acid molecule comprising a polynucleotide operably linked to a promoter that is functional in a plant cell, wherein the polynucleotide encodes an acyl-CoA delta-9 desaturase enzyme comprising an amino acid sequence at least 80% identical over the full length of SEQ ID NO:14.
5
2. The isolated nucleic acid molecule of claim 1, wherein the polynucleotide encodes an acyl-CoA delta-9 desaturase enzyme comprising an amino acid sequence at least 90% identical over the full length of SEQ ID NO:14.
3. The isolated nucleic acid molecule of claim 1, wherein the polynucleotide encodes an acyl-CoA delta-9 desaturase enzyme comprising an amino acid sequence at least 95% identical over the full length of SEQ ID NO:14.
10
4. The isolated nucleic acid molecule of claim 1, wherein the polynucleotide encodes an acyl-CoA delta-9 desaturase enzyme comprising the amino acid sequence of SEQ ID NO:14.
5. The nucleic acid molecule of any one of claims 1 to 4, wherein the promoter is selected from the group consisting of the *ole1* gene promoter, the phaseolin promoter, the Cassava vein Mosaic Virus Promoter, the *LjKCS3* promoter, and the *FAE1* promoter.
15
6. The nucleic acid molecule of any one of claims 1 to 5, wherein the polynucleotide encoding the acyl-CoA delta-9 desaturase enzyme comprises a nucleotide sequence that is at least 60% identical over the full length of a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:17, and SEQ ID NO:44.
20
7. The nucleic acid molecule of any one of claims 1 to 5, wherein the polynucleotide encoding the acyl-CoA delta-9 desaturase enzyme comprises a nucleotide sequence that is at least 70% identical over the full length of a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:17, and SEQ ID NO:44.
25

8. The nucleic acid molecule of any one of claims 1 to 5, wherein the polynucleotide encoding the acyl-CoA delta-9 desaturase enzyme comprises a nucleotide sequence that is at least 80% identical over the full length of a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:17, and SEQ ID NO:44.

5 9. The nucleic acid molecule of any one of claims 1 to 5, wherein the polynucleotide encoding the acyl-CoA delta-9 desaturase enzyme comprises a nucleotide sequence that is at least 90% identical over the full length of a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:17, and SEQ ID NO:44.

10 10. The nucleic acid molecule of any one of claims 1 to 5, wherein the polynucleotide encoding the acyl-CoA delta-9 desaturase enzyme comprises a nucleotide sequence that is at least 95% identical over the full length of a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:17, and SEQ ID NO:44.

15 11. The nucleic acid molecule of claim 6, wherein the nucleotide sequence is selected from the group consisting of SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:17, and SEQ ID NO:44.

12. The nucleic acid molecule of any one of claims 1 to 11, comprising a further gene regulatory element operably linked to the polynucleotide.

20 13. The nucleic acid molecule of claim 12, wherein the further gene regulatory element is selected from the group consisting of the delta-9 desaturase 3'UTR/terminator, the *Phaseolus vulgaris* phaseolin 5' untranslated region, the *Phaseolus vulgaris* phaseolin 3' untranslated region, the *Phaseolus vulgaris* phaseolin matrix attachment region, the *Agrobacterium tumefaciens* ORF23 3' untranslated region, the *Agrobacterium tumefaciens* ORF1 3' untranslated region, the *Nicotiana tabacum* RB7 Matrix Attachment Region, Overdrive, T-strand border sequences, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, and
25 SEQ ID NO:43.

14. A method for producing a genetically modified plant cell, the method comprising:

transforming a plant cell with the nucleic acid molecule of any one of claims 1 to 13.

15. The method according to claim 14, wherein the plant cell is comprised in a
5 plant tissue, plant tissue culture, plant callus culture, plant part, or plant.

16. The method according to claim 14, wherein the plant cell is from a plant
selected from the group consisting of *Arabidopsis*, *Borago*, *Canola*, *Ricinus*, *Theobroma*, *Zea*,
Gossypium, *Crambe*, *Cuphea*, *Linum*, *Lesquerella*, *Limnanthes*, *Linola*, *Tropaeolum*,
Oenothera, *Olea*, *Elaeis*, *Arachis*, rapeseed, *Carthamus*, *Glycine*, *Soja*, *Helianthus*, *Nicotiana*,
10 *Vernonia*, *Triticum*, *Hordeum*, *Oryza*, *Avena*, *Sorghum*, *Secale*, and other members of the
Gramineae family.

17. A cell comprising the nucleic acid molecule of any one of claims 1 to 13.

18. The cell of claim 17, wherein the cell is a transgenic plant cell.

19. The cell of claim 17, wherein the cell is a transgenic plant seed cell.

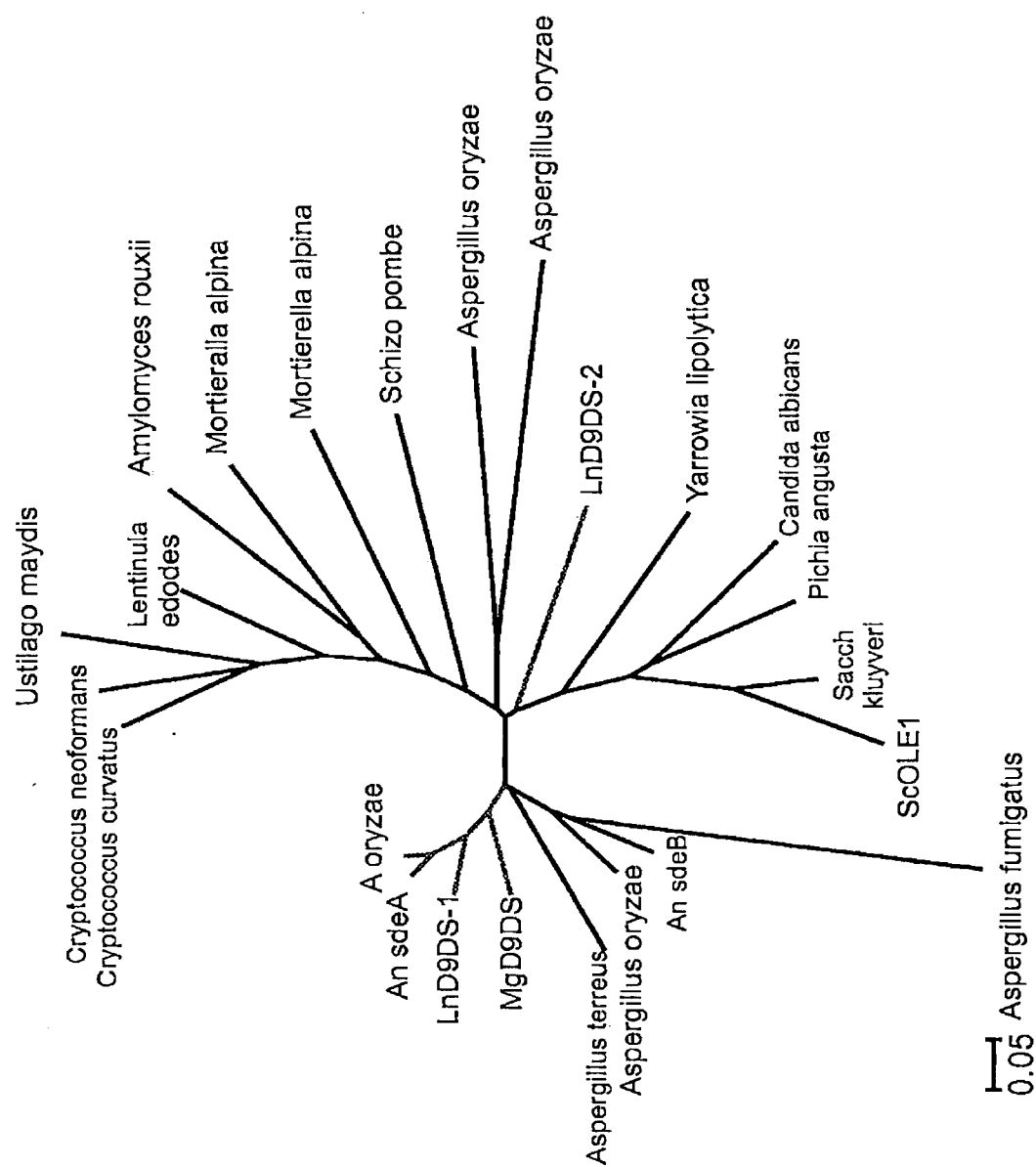


Fig. 1

SUBSTITUTE SHEET (RULE 26)

LnD9DS-1 (SEQ ID NO:5) (1) cccgattcattaatgcagctggcagcacaggtttcc
 LnD9DS-2 (SEQ ID NO:8) (1) -----
 MgD9DS (SEQ ID NO:76) (1) -----

(37) cgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcatta
 (1) -----
 (1) -----

(94) caggctttacactttatgcttcgggctcgtatgttggtggaattgtgagggcacc
 (1) -----
 (1) -----

(151) cggataacaattttcacacaggaaacagctatgaccatgattacgccaaagctcgaaat
 (1) -----
 (1) -----

(208) taaccctcactaaaggggaacaaaagctggagctccaccgcggtggcggccgctctag
 (1) -----
 (1) -----

(265) aactagtggatcccccgggctgcaggaattcggcacgagtatgccttcccaccAggc
 (1) -----
 (1) -----gAatt

(322) tgTtGCTggcatgcaggCCatCGacCCcGAGTTtGtcaaGCAGCcgTCTC--CTaTG
 (1) -----GgAtCCaTggCgGCctTG
 (6) caTgGCTtcgtcatcttCCtcCGtgCCgGAGTTgGc--tGCcGCctTCcCtGaTggc

(377) GCgAGCAcCTCgGAGCCCAAccGcAACTCCAAg---TACGAtcCtAAgAAGCCGCAC
 (19) GacAGCAttcCaGAGgatAAGgctAcCTC---G---T-----CgAAatcGaCtCAT
 (61) aCtAcCgaCTtcaAGCCCAAtGaGgAAcAcCAAGggcTACGAcgtcAgcAAGCCGCAC

(431) ATTaCAGAcAtgCCcATcACgCggtcaaAACTGGTAccAGCAtGTCAACTGGCTCAAC
 (64) ATTcaAtAtcaAgaagTaaCttTtcgGAACGGTATAAGaAgataaATGGCTCAAC
 (118) ATTtCcGAgAcACcTAIgACaCTcaaGAACGGcATAAGCacGTCAACTGGCTCAAC

(488) gtCAtCTTCAICaTcggCGTGCctCTCGCTGGCTgCGTCgCCgCCTtCTGGaCCcCT
 (121) ACgACgcTggTggTgcTCaTaCC---CGCT--CTTgGaCTctACCTAacacGCaCCa
 (175) ACCACCTTCAICtTgtTtGTGCCcCTgGCTGGtcTCaTaTCCAcTtACTGGGtCCCT

(545) CTGCAGTGGAA-GACCGCTGCg----TGGGCTGTcAtCTACTAtTTCTGgActGGCC
 (173) CgcCAcTtacAcGACctacGctcatcTGGtCcGTcCtTgTACTACTTCTGCACaGctt
 (232) CTGCAGTGGAA-GAcgGCTGta----TGGGCTGTcGtCTACTACTTcAaCacCGGCC

Fig. 2a

SUBSTITUTE SHEET (RULE 26)

(597) TCGGtATCAGcGCCGGATACCATCGtCTcTGGGCaCAaagTCaTACAACGCCgGTC
 (230) TCGGcATCAGcGCGGATatCATCGACTaTGGagTCatcGCagcTACTcCGGtcGTC
 (284) TgGGaATtACTcCGGStTACCACCGACTtTGGGCTCAGCTCgTACAAGGcCtgcG

(654) TTCCtCTgAGgATCTggCTcGCCGCCGTcGGCGGTGGTGGtGTtGAGGGTTCCATCC
 (287) TaCCGCTacGctTaTtCTaGCCttCacaGGCGcGGaGCCaTCCaAGGTagtgctC
 (341) TTCCGCTcAaaATCTaCTtGCCGCCGTtGGCGTGGTGGCGTCGAGGGcTCCATCa

(711) GcTGGTGGAGCcgtGAcCACC CGCGCCACCACCGcTACACCGACACCAaAAGGACC
 (344) GATGGTGGAGCcgaATCACC CGCGCCACCACCGATggACC GACACaATGAAGGACC
 (398) GATGGTGGtccaacGgTACC CGCGCaCACCACCGATACACCGatACCgAGAAGGACC

(768) CCTACagtGTCCGCAAGGGCCTtCTcTACagcCATcTCSGATGGATGGTcaTGAAGC
 (401) CCTACTCcGTtatgGcGGCCTatTaTtCTcGACATCGGATGGATGGTatTGAACA
 (455) CCTACTCaGTCCGCAAGGGtCTcCTgTACTCaCACATgGGATGGATGcTtCTGAAGC

(825) AGAACCCCAAGcgtAtcGGCCGCACCGACATCACCGACTTGAACGAGGACCCCGTGG
 (458) gcgACCCCAAggtcAAaGGCCGaACaGAGcGTCAgtGatCTcgACagcGACCCCGTcG
 (512) AGAACCCCAAGaagcAgGGCCGCACCGACATCACCGACCTGAACGAGGACCCCGTGG

(882) TCGICTGGCAGCACAGAActACaTCAAGgcCGTcgtCacCATGGgCTTGAICTTtC
 (515) TaGTCTGGCAGCACAGcACTACggCAAGTGCcTgcTgTTCgcccGcTgGATaTTC
 (569) TCGTtTGGCAGCACcgcAACTtCcTCAAGTGtGTtaTCTTCATGcCCcTgICTTC

(939) CCtCtgcCGTcGCCGStCTCatgTGGGgcGATTGGatGGGtGGCTTCATCTACGcTg
 (572) CCatgaTCGTaGcCGGCTCGGaTGGGgaGATTGGTGGGGAGGcTtgTCTACGcCG
 (626) CCACacTtGTgGcTGGCCTtGGcTGGGgtGAcTAcTGGGGAGgtTTCATCTACGgaG

(996) GTATCCTcCGTaTCTTCCTTCGTCCAGCAGGCACCTTCTGGGTCAACTCGCTTGGcC
 (629) GcATCaTtCGaGcgTgtTTCGTCCAGCAGGCgACaTtTTCGTgAACTCtCTcGGcC
 (683) GTATtCTgCGTGTCTTCCTTCGTCCAGCAGGCACCTTCTGGGTCAACTCGCTTGGcC

(1053) ACTGGCTCGGTGACcAGCCcTTCGACGACCGCAACTCtCCTCGTGAACACGTCAItA
 (686) AtTGGaTCCGcGAgTAGCCgTTCGACGACaGacgCaCGCCTCGaGACCACGTttTgA
 (740) ACTGGCTCGGTGACcAGCCcTTCGACGATCGCAACTCGCCgCGTGAatCACGTCAItA

(1110) CcGcTCTtGTCACTCTcGGAGAGGGcTACCACAActTCCACCACGAGTTCCcCTCCG
 (743) CAGCgtTGGTaACgaTgGGAGaAGGATatCataACTTCCACCACGAaTTCcCaagCG
 (797) CAGCcCTGGTCACcCTtGGAGAGGGATACCACAActTCCACCACGAGTTCCcCTCgG

(1167) ACTACCGCAACGCCcATCGAGTGGcACCAGTACGACCCTACCAAGTGGTCCATCTGGC
 (800) AtTATCGCAACGCCgATCatcTGGTACCAaTACGACCCTACCAaATGGctCATtTACC
 (854) ACTACCGCAACGCCcATtGAGTGGTACCAGTaTACCCcACCAAGTGGTCAATCTGGa

Fig. 2b

SUBSTITUTE SHEET (RULE 26)

(1224) TgTGA-----GCaAGCTCGGCCTCGGCTcCAACCTCAAGCAGTTCCGCTCCAACG
(857) TCTtctccctcgGCCcctTCccCTCGGcATAcTcgCTCAaaccTTCCGgTCCAAtG
(911) TCTGA-----agCAGCTtGGTcTtGCCcACAACCTgAAGCAGTTCCGCcaaAACG

(1275) AaATcGAGAAGGtCGtGTCCAGCAGGTCcAGAAGAAGaTtGACCAGAAGCGCGCA
(914) AGATTGAaAAaGGgCGgtTgCAaCAaCAaCAaAAagccCTgGACaAGAAGCGGtCag
(962) AGATTGAGAAGGCaCGcGTCCAGCAGGTCgAGAAGAAGCTcGACCAGAAGCGCGCA

(1332) AGCTcGActGGGgTgTcCCtCTCGACCAGCTgCCTGTcATAgaaTGGGACGACTaTG
(971) gaCTTGATTGGGccTaCCCTCttCCAaCTcCCTGTcATatcgTGGGACGACTTcc
(1019) AGCTTGATTGGGgTattCCCTTgGAgCAGCTtCCcGtTgTtagcTGGGAtGACTTTG

(1389) TcGAGCAG-GCCAAGAA-CgGcCGtGGT-CTcATcGCTGTcCGTcGGTGTcGtTCAtG
(1028) aaGcGCgttGCaAAGAgTCCGGCGAGaTgCTGgTTGCTGTcGCaGGTGTgATTcACG
(1076) TtGAGCAG-tCCAAGAA--CGGaaAGccttgGATTGCAgttGCcGGTGTcATcCAGC

(1443) ACGTTAcCGACTTCATCAAcGAgCACCCcGGTGGCAaGACgCTtATCAAGAGcGgCG
(1085) ACGTcAGCcAgTTtAttgAaGAtCACCTGGaGGCAGGAgTtTgATtcgGAGtGCGG
(1130) AtGTTgGtGACTTCATCAAgGACcACCTGGTGGCAGagCTCTcATCAActcgGCCa

(1500) TTGGCAAGGATGcCACCGCaATGTTCAACGGCGGTGTcTACttCLACTCCAACGgaG
(1142) TgGGCAAAaGATGggACaGggATGTTtAAtGgaGGcGtATatgAgCAcagtAATGCGG
(1187) TTGGCAAGGACcCaACCGCaATcTTCAACGGCGGTGTtTACaACCACTCCAACGCG

(1557) CcCACAACCTcCTtTCTACcATGAGGGTtGGTGTcATcCGcGGTGGCTGTGAaTTG
(1199) CgCAtAAtCTGtTgTCGACaATGAGGGTGGGAGTgcTtaGaGGTGGgcagGAGGTgG
(1244) CtCACAACCTGCTcTCGAGtATGcGTGIGGGTGTtTgCGtGGcGGCTGcGAGGTTG

(1614) AGATCTGGAAGCGCGctCAGcGTGAgAACAAAGGATGTcGGTcttGGTC--CTgGAcGA
(1256) AGgTgTGAAGaa-GCagAGaGTGg-----AtGtTtTaGGgAaGagCGaCaTttTGA
(1301) AGATCTGGAAGCGCGCcAGtccGaaAACAAAGGAcGTcTcaAccGTG--TTGATtc

(1669) cgCAGGcAACCCaATCaTcAGGGCTGGTaacCAGattACCAAGGTTGCGCAaCCaAT
(1307) gaCAGGTtACgCgggtGgagaGGtTGGTt--gAGGgGgCtgtGGcTGGtAGGtaAc
(1356) ttCgGGTAACGgcATCGTCcGcCGGGTgggCAaGcGACCAAGGTcGtcCAGGCTgT

(1726) TCAGaGTGCTagtGCaGCaTagattggatcttcatcttcacgagcgatgtatggcgt
(1362) TgAccaTg-----
(1413) TCcGgGtGCTcagGCcGCgTga-----

Fig. 2c

SUBSTITUTE SHEET (RULE 26)

(1783) ttggttgtctctcttccttggcggacagagtaattcaatttcttagcgatcgtta
(1371) -----
(1435) -----

(1840) gaaagcatcatggttacgatgctcagtcatgtagatggcgtatgtttgtagccttc
(1371) -----
(1435) -----

(1897) ctcgagtgattggstatgaaaagtagcctcacggcctagaccaagaatgaaaacatt
(1371) -----
(1435) -----

(1954) cacgatttcagaaaaaaaaaaaaaaaaaactcgagggggggcc
(1371) -----
(1435) -----

Fig. 2d

SUBSTITUTE SHEET (RULE 26)

LnD9DS-2 (SEQ ID NO:14) (1) -----
LnD9DS-1 (SEQ ID NO:49) (1) -----
AnD9DS (SEQ ID NO:50) (1) -----
MgD9DS (SEQ ID NO:12) (1) -----
ScOLE1 AAA34826 (SEQ ID NO:52) (1) MPTSGTTIELIDDQFPKDDAS

(1) -----MAALDSI
(1) -----MPSHQAVAGMQAIDPEFVKQSPMASTSEPN
(1) -----MSAPTADI
(1) -----MASSSSSVPELAAAFPDGTTDFKPM
(23) SGIVDEVDLTEANILATGLNKKAPRIVNGFGSLMGSKEMVSVEFDKKGNEKKSNDLRL

(8) PEDKA---TSSKSTHIQYQEVTFRNWYKKINWLNNTTLVVLIPALGLYLTRT--TPI
(32) RNSK----YDPKKPHITDMPITRSNWYQHVNWLNVIIFIIGVPLAGCVAAFW--TPEQ
(9) RARA----PEAKKVHIIADTAINRHNNYKHVNWLNVIIFIIGVPLAGCVAAFW--VPIQ
(26) RNTKG---YDVSKPHISETPMTLKNWVKHVNWLNNTTFILFVELAGLISTYW--VPEQ
(81) LEKDNQEKKEAKTKIHISEQPWTLNNWHQHNLNLMVLVCGMPEMIGWYFALSGKVELH

(60) RPTLIWSVLYYFCTAFGITGGYHRLWSHRSYSARLPLRLFLAFTGAGAIQGSARWWSA
(83) WKTAAWAVIYYFWTGLGITAGYHRLWAHKSYNAGLPLRIWLAAGVAGAVEGSIRWWSR
(60) LKTAIWAVIYYFFTGLGITAGYHRLWAHCSYSATLPLRIWLAAGVGGAVEGSIRWWAR
(78) WKTAVWAVVYFNTGLGITAGYHRLWAHSSYKASLPLKIYLAAGVAGAVEGSIRWWSN
(139) LNVFLFSVYYAVGGVSIITAGYHRLWSHRSYSAHWPLRLFYAIFGCASVEGSAKWWGH

(118) NERAHHRWTDTKDPYSVMRGLFSGHIGWVNLNSDPKVKGRTEVSDLDSDPVVVWQHK
(141) DHRAHHRWTDTKDPYSVRKGLLYSHLGWVMKQNPKRIGRTDITDLNEDPVVVWQHK
(118) DHRAHHRWTDTKDPYSVRKGLLYSHLGWVMKQNPKRIGRTDISDLNEDPVVVWQHR
(136) GHRAHHRWTDTEKDPYSVRKGLLYSEMGMWLLKQNPKKQGRTDITDLNEDPVVVWQHR
(197) SHRIHHRWTDTLRDPYDARRGLWYSHMGWMLLKPNPKYKARADITDMTDDWTIRFOHR

(176) HYGKCLLFAAWIFFMIVAGLGWGDWGGGLVYAGIIRACFVQOATFCVNSLAHWIGQPP
(199) NYIKAVVTMGLIFPSAVAGLMWGDWGGFIYAGILRIFFVQOATFCVNSLAHWIGDQP
(176) NYLKVVFTMGLAVTMLVAGLGWGDWGGFVYAGILRIFFVQOATFCVNSLAHWIGDQP
(194) NFLKCVIFMALVFPTLVAGLGWGDYWGFIYGGILRVFFVQOATFCVNSLAHWIGDQP
(255) HYILLMLLTAFVIETLICGYFFNDYMGGLIYAGFIRVFVQOATFCINSMAHYIGTOP

(234) FDDRRTPRDHVLTALVTMGEYHNFHHEFPSPDYRNAIEWHQYDPTKWSIWLWSK--LG
(257) FDDRNSPRDHVITALVTMGEYHNFHHEFPSPDYRNAIEWHQYDPTKWSIWLWSK--LG
(234) FDDRNSPRDHVITALVTMGEYHNFHHEFPSPDYRNAIEWHQYDPTKWSIWAQKQ--LG
(252) FDDRNSPRDHVITALVTMGEYHNFHHEFPSPDYRNAIEWHQYDPTKWSIWIWQK--LG
(313) FDDRRTPRDNWITAIVTFGEYHNFHHEFPSPDYRNAIEWHQYDPTKWSIYLTSL--VG

Fig. 3a

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(292) LAYSLKTFERSNETEKGRLOOQOKALDKKRSGLDWGLPLFQLEVISWDDFQARCKESGE
(313) LASNLKQERSNETEKGRVQOLOKKIDQKRAKLDWGVPLDQLEVIEWDDYVEQAK-NGR
(290) LAYDLKKFRANETEKGRVQOLOKKLDKIRATLDWGTPLDQLEVMWDDYVEQAK-NGR
(308) LAHNLKQFRONETEKGRVQOLOKKLDQKRAKLDWGIPLDQLEVVSWDDFVEQSK-NGK
(369) LAYDLKKFRSONALEEALIOQEQKKINKKKAKINWGPVITDLPMWDKQTFLLAKSK-ENK

(350) MLVAVAGVIHDVVSQFIEDHPGGRSLERSAVGKDGTMENGGVYEHSAAHNLLSTMRV
(370) GLIAGVGVHDVTDFFNEHPGGKTLIKSGVGKDATAMFNGGVYFHSNGAHNLLSTMRV
(347) GLVAGVGVHDVTDFFKDHPPGGKAMISSGIGKDATAMFNGGVYHSAAHNLLSTMRV
(365) AWIAGVGVHDVGDFFKDHPPGGRALINSAIGKDATAIFNGGVYNHSAAHNLLSTMRV
(426) GLVIISGIVHDVSGYIESEHPGGGETLIKALGKDATKAFSGGVYRHSNAAQNVLADMRV

(408) GVLRRGGQEVEVWKKQR-----VDVLGKSD----ILRQVTRVERLVEGAVAA
(428) GVIRGGCEVEIWKRAQRENKDVGLVLDDAGNPIIRAGNQITKVAQPIQSASAA
(405) GVIRGGCEVEIWKRAQKEN--VEYVRDGGSGQRVIRAGEOPTKIPEPIPTADAA
(423) GVLRRGGCEVEIWKRAQSENKDVSTVVDSSGNRIVRAGGQATKVVPVPGAQAA
(484) AVIK-----ESKN-S-----AIRMASKRGEIYETGKFF

Fig. 3b

SUBSTITUTE SHEET (RULE 26)

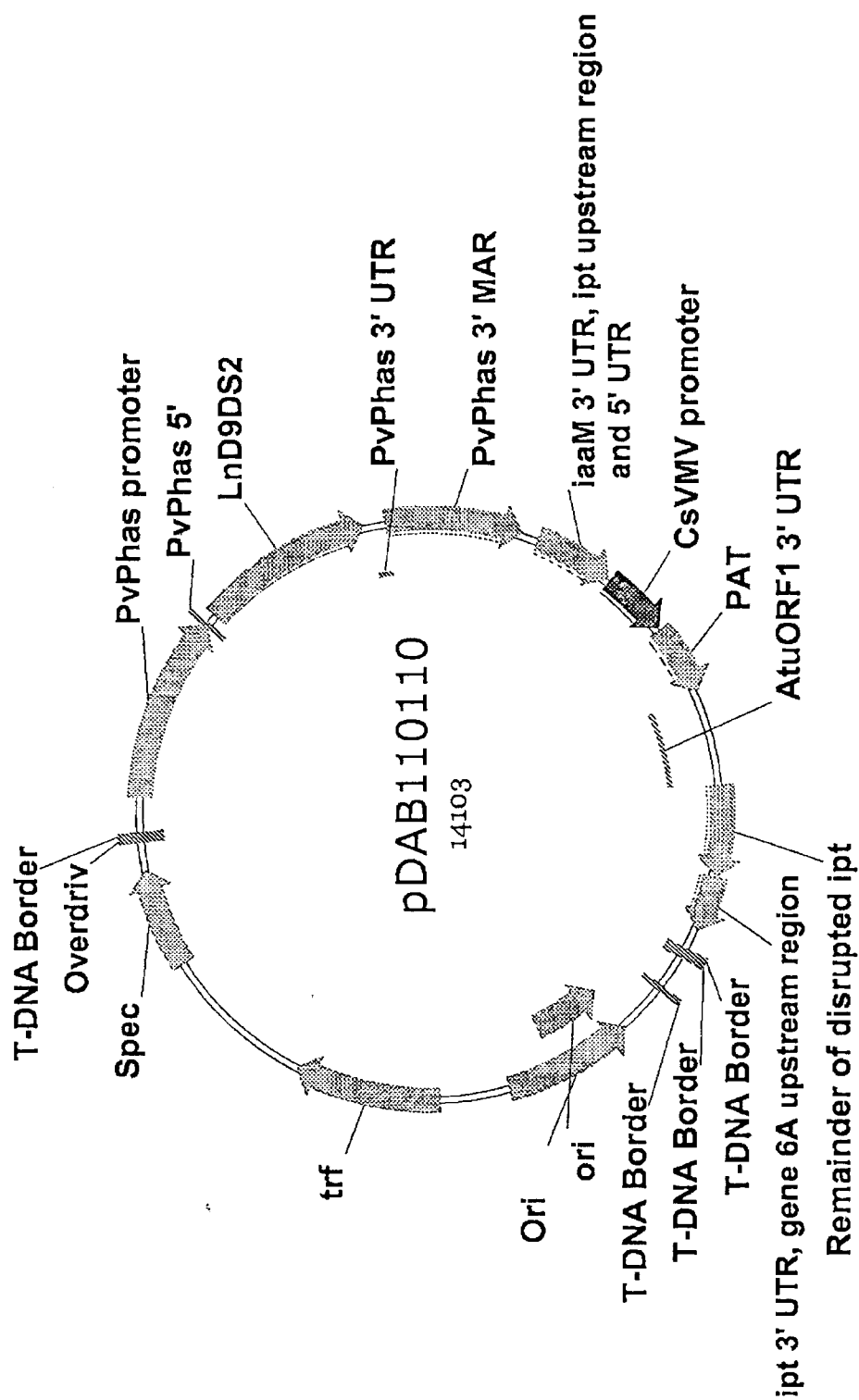


Fig. 4a

SUBSTITUTE SHEET (RULE 26)

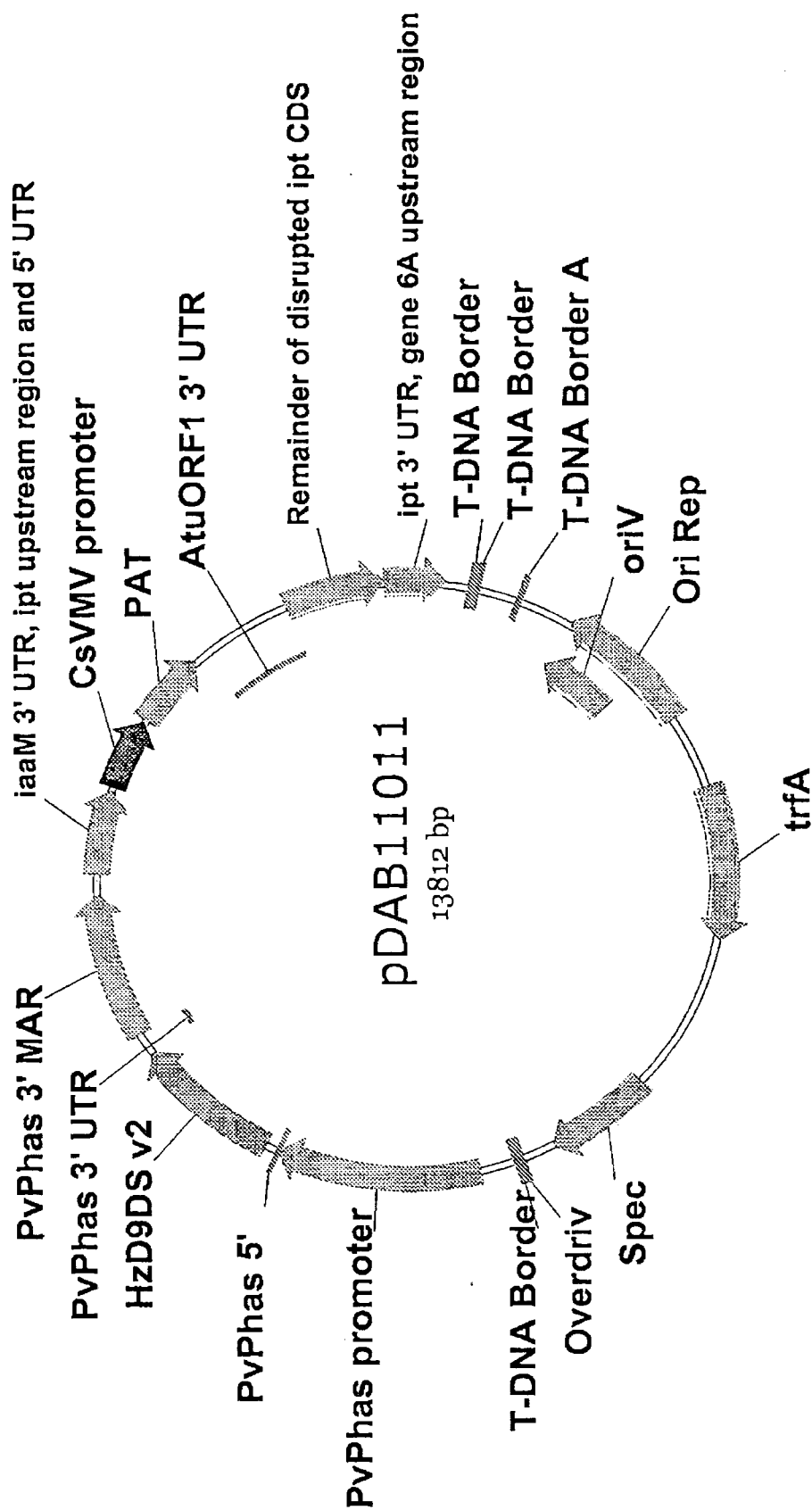


Fig. 4b

SUBSTITUTE SHEET (RULE 26)

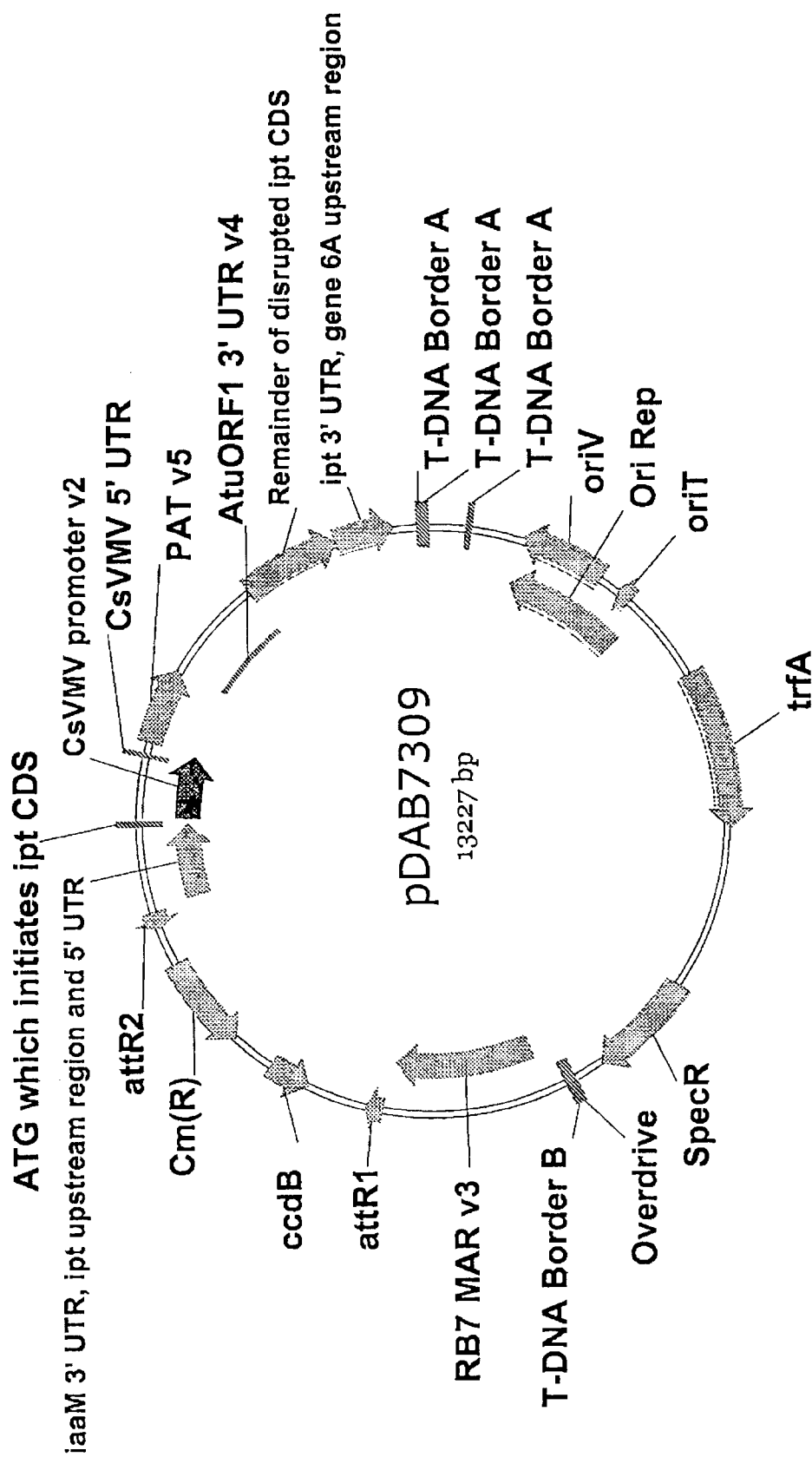


Fig. 5

SUBSTITUTE SHEET (RULE 26)

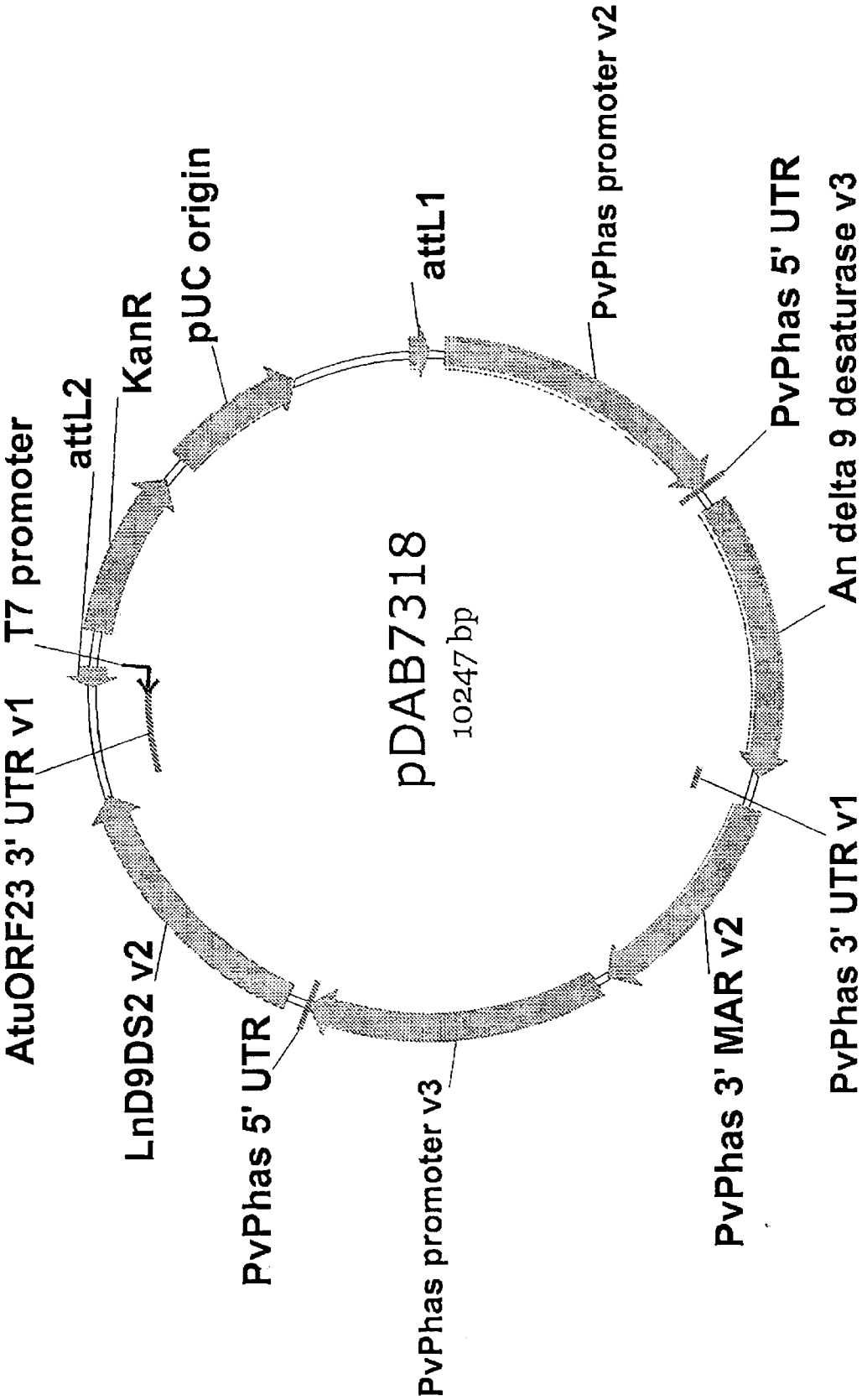


Fig. 6

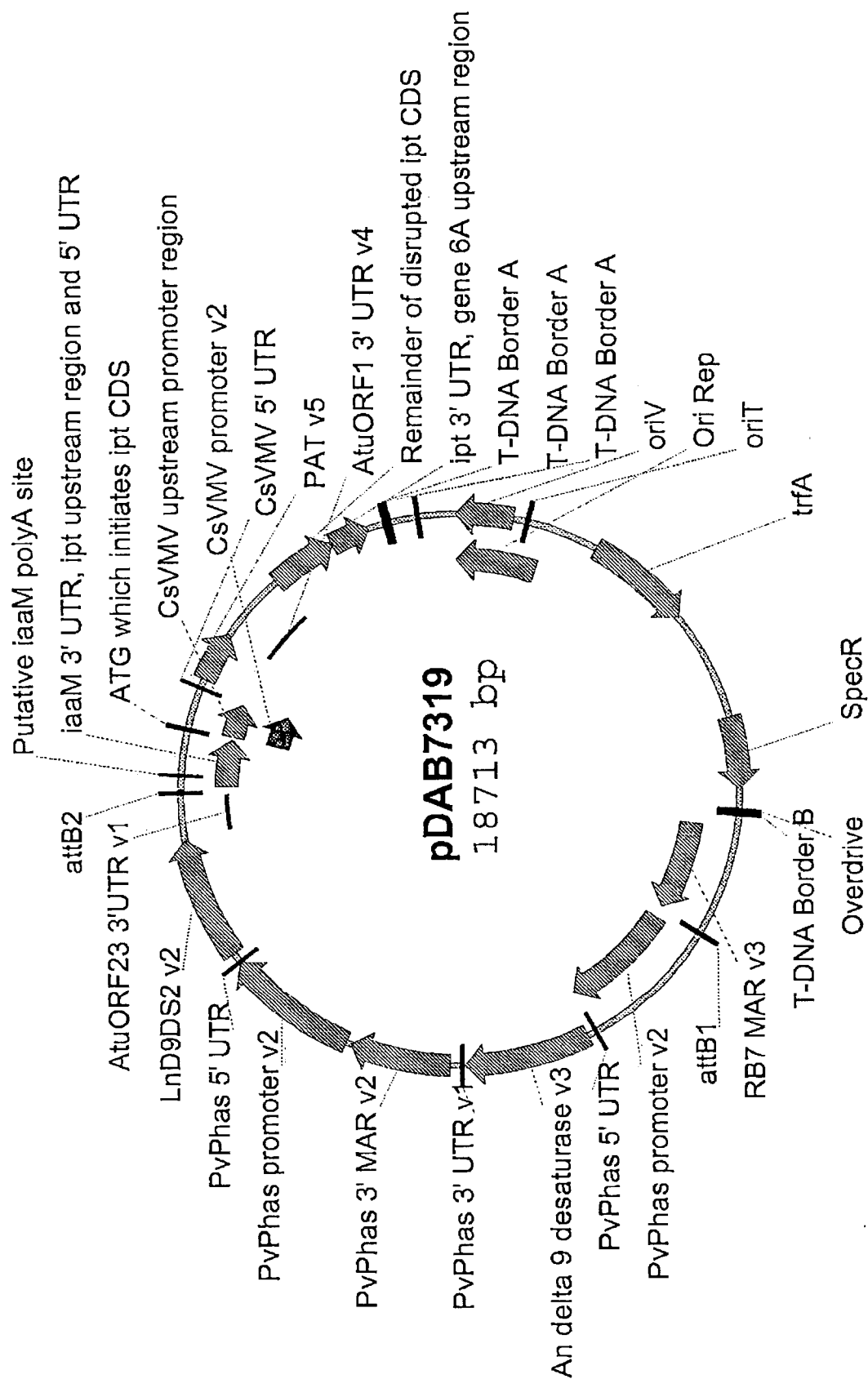


Fig. 7

SUBSTITUTE SHEET (RULE 26)

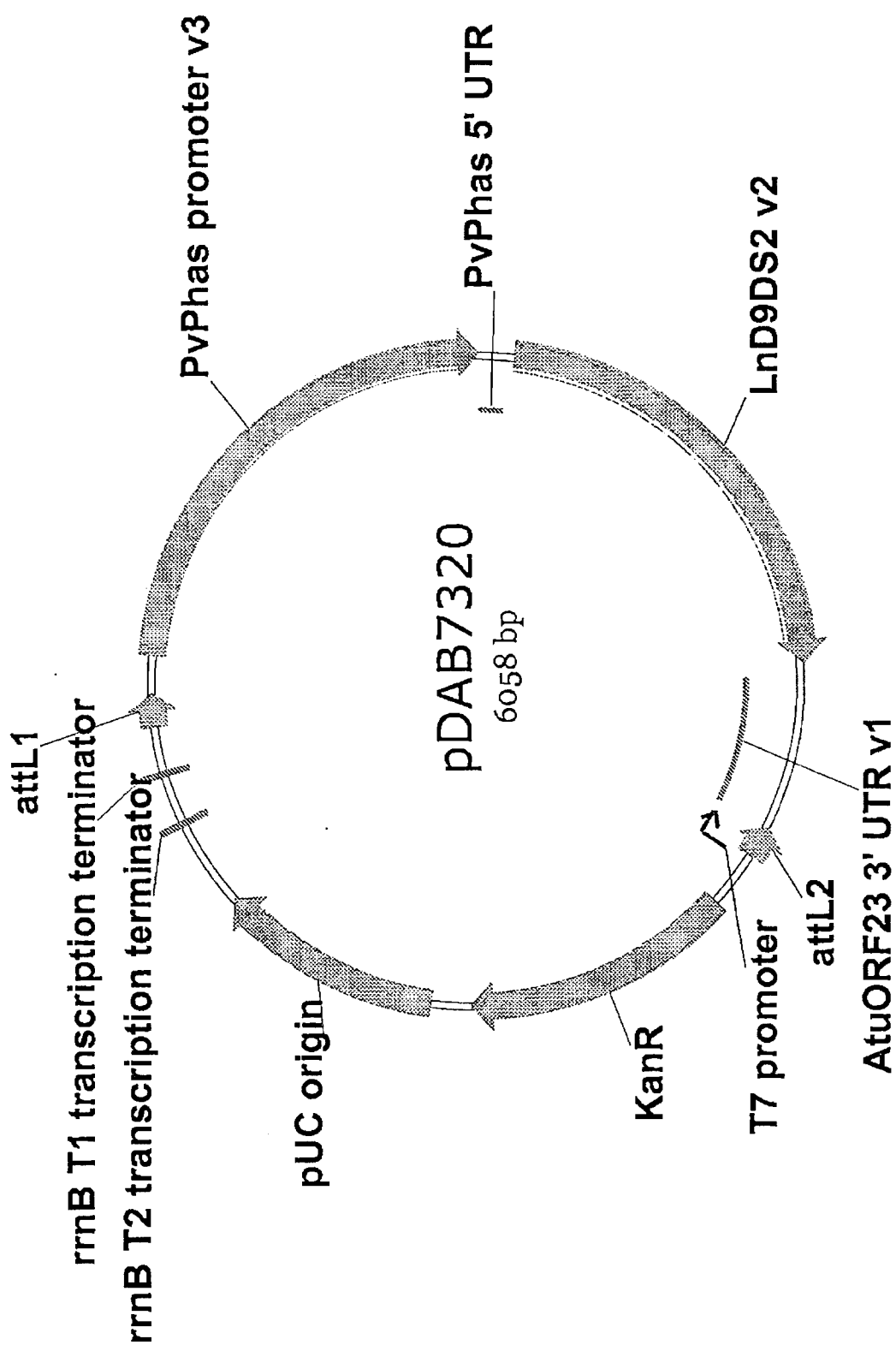


Fig. 8

SUBSTITUTE SHEET (RULE 26)

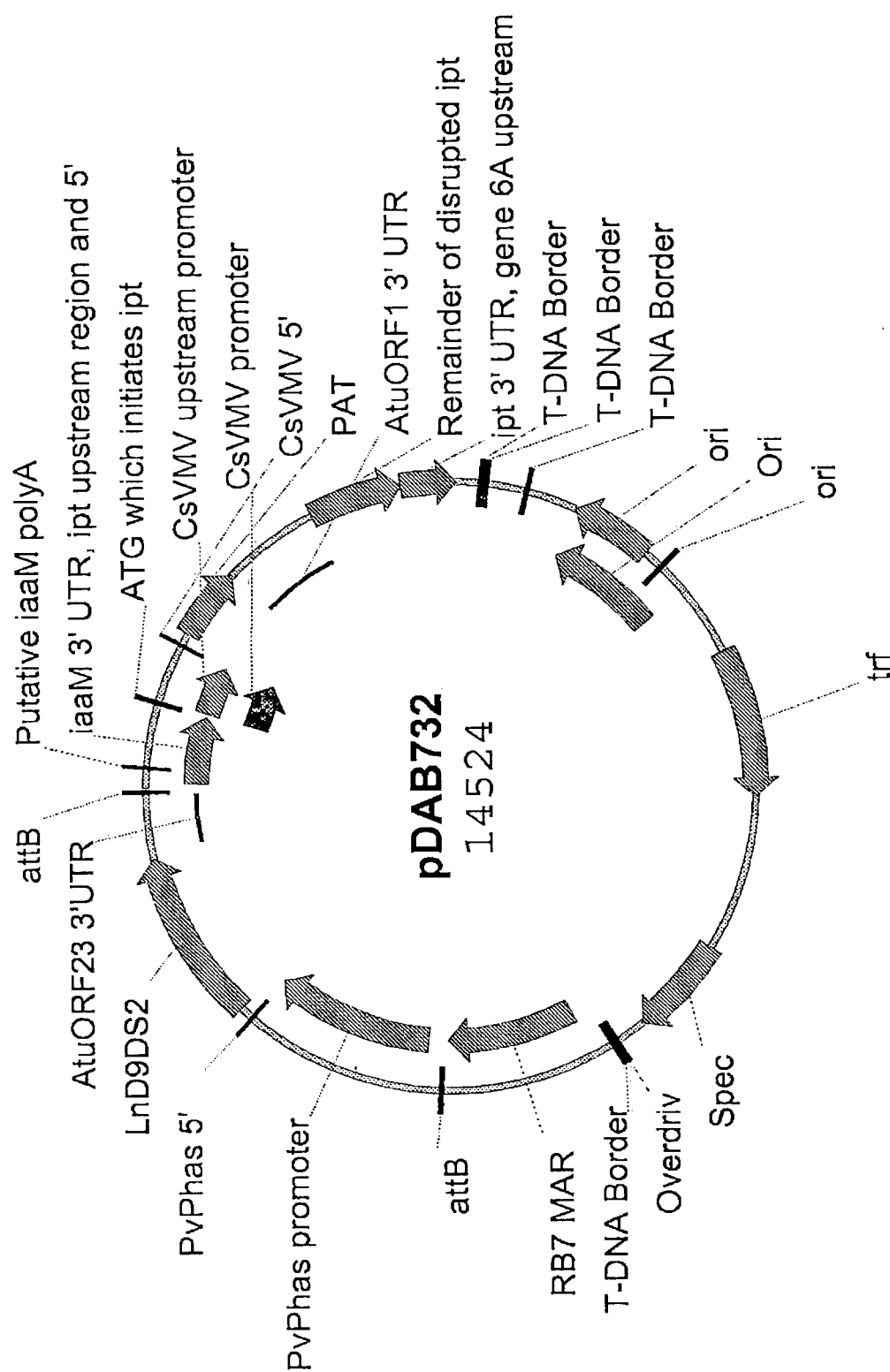


Fig. 9

SUBSTITUTE SHEET (RULE 26)

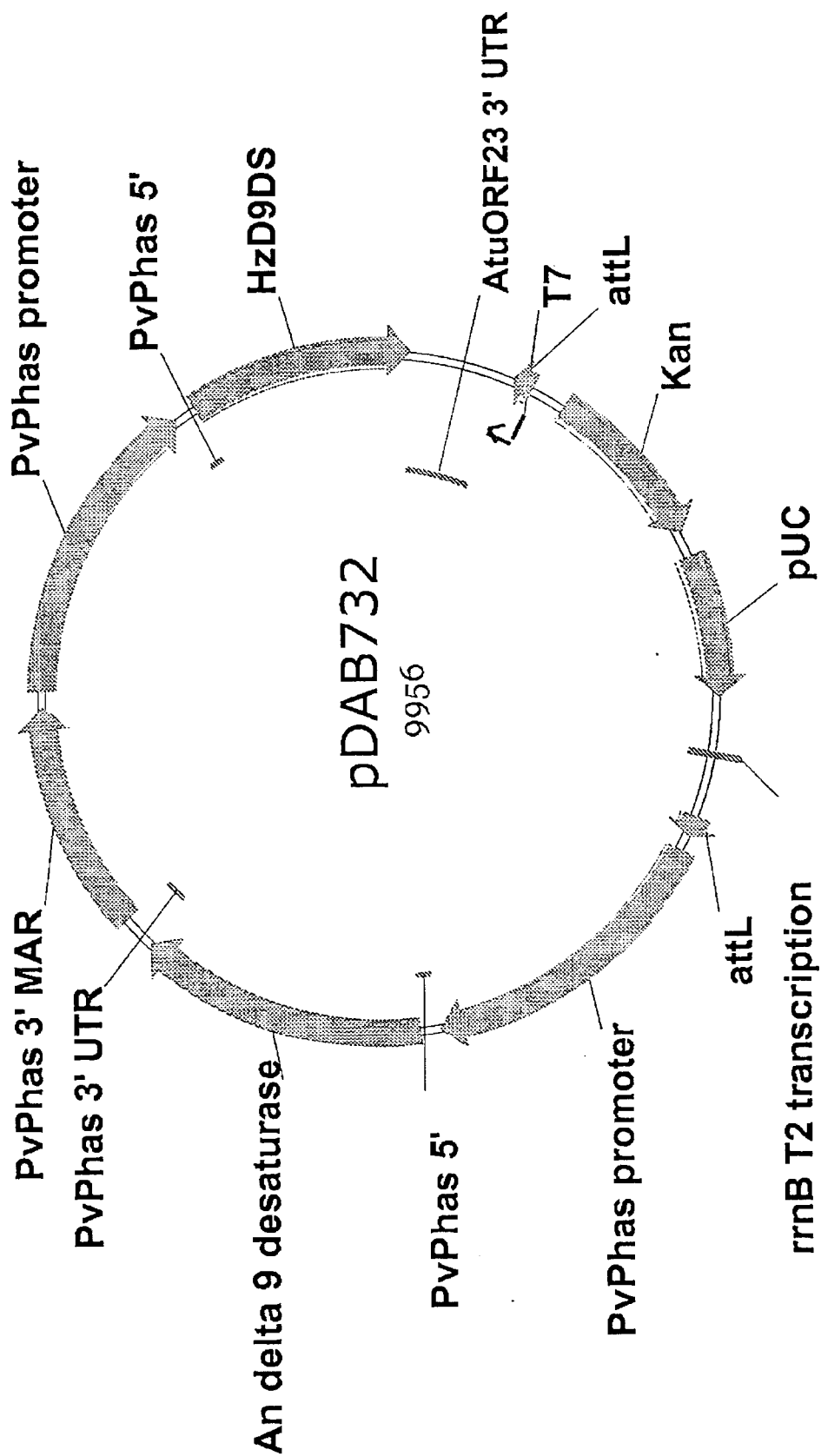


Fig. 10

SUBSTITUTE SHEET (RULE 26)

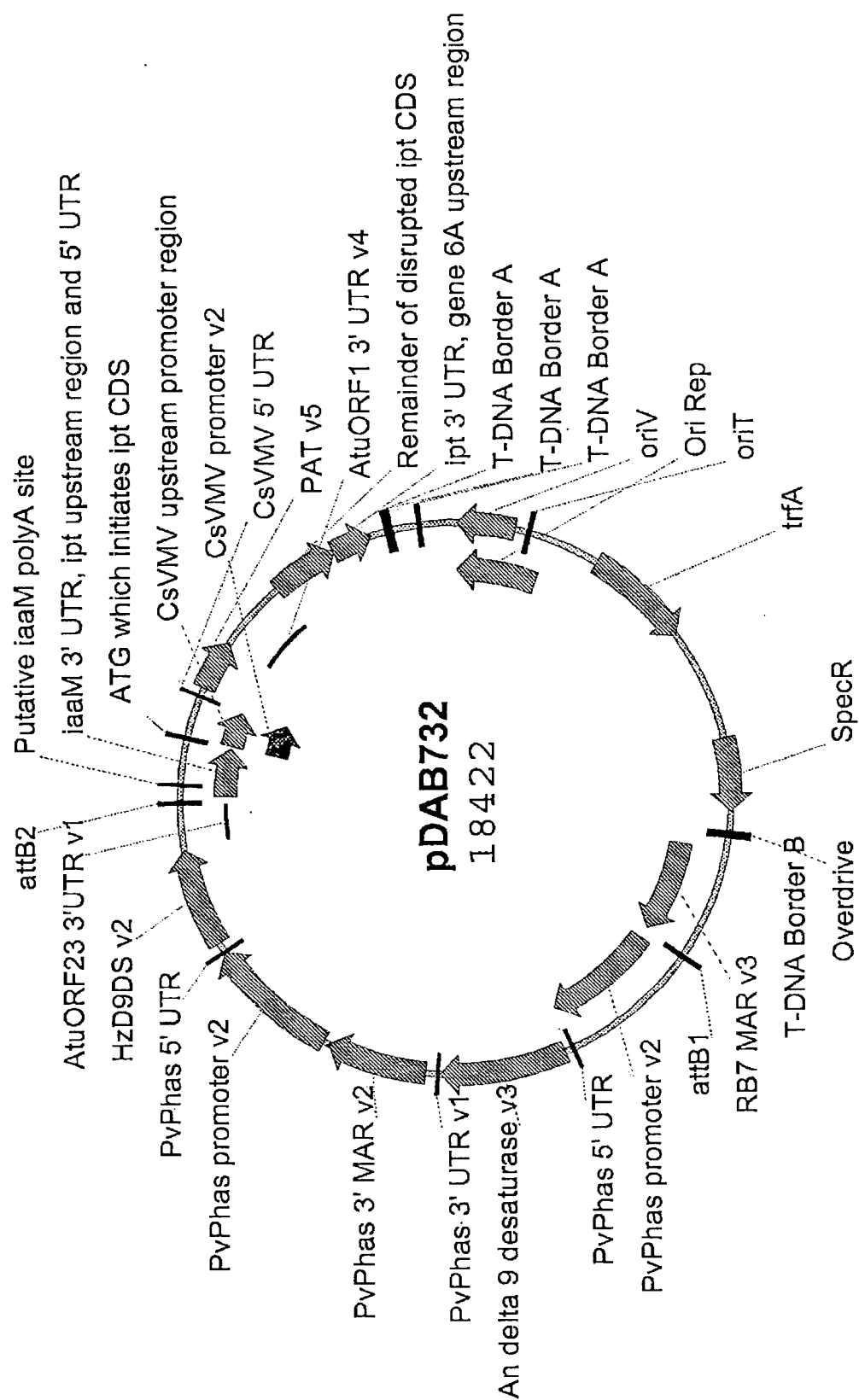


Fig. 11

SUBSTITUTE SHEET (RULE 26)

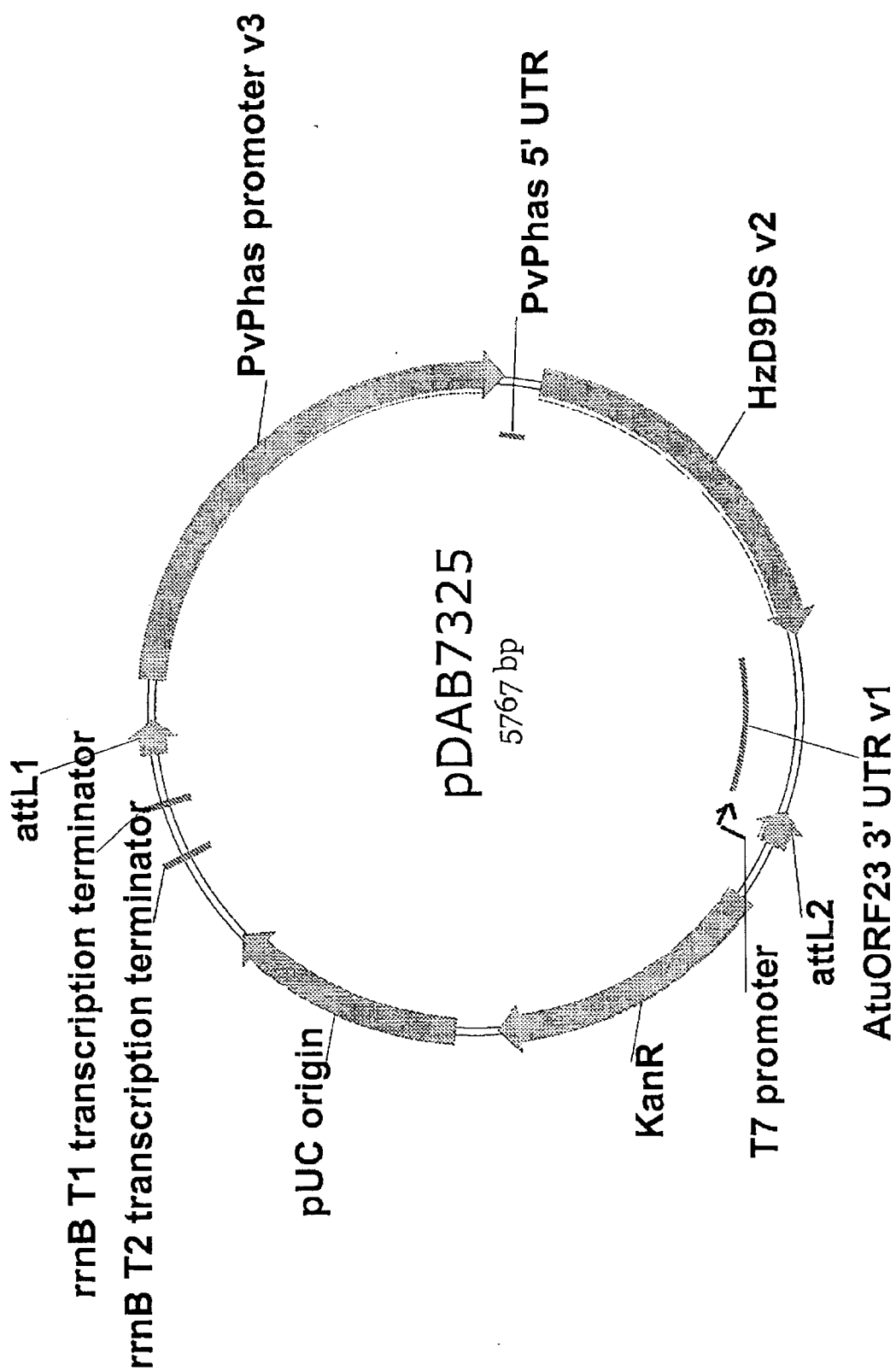


Fig. 12

SUBSTITUTE SHEET (RULE 26)

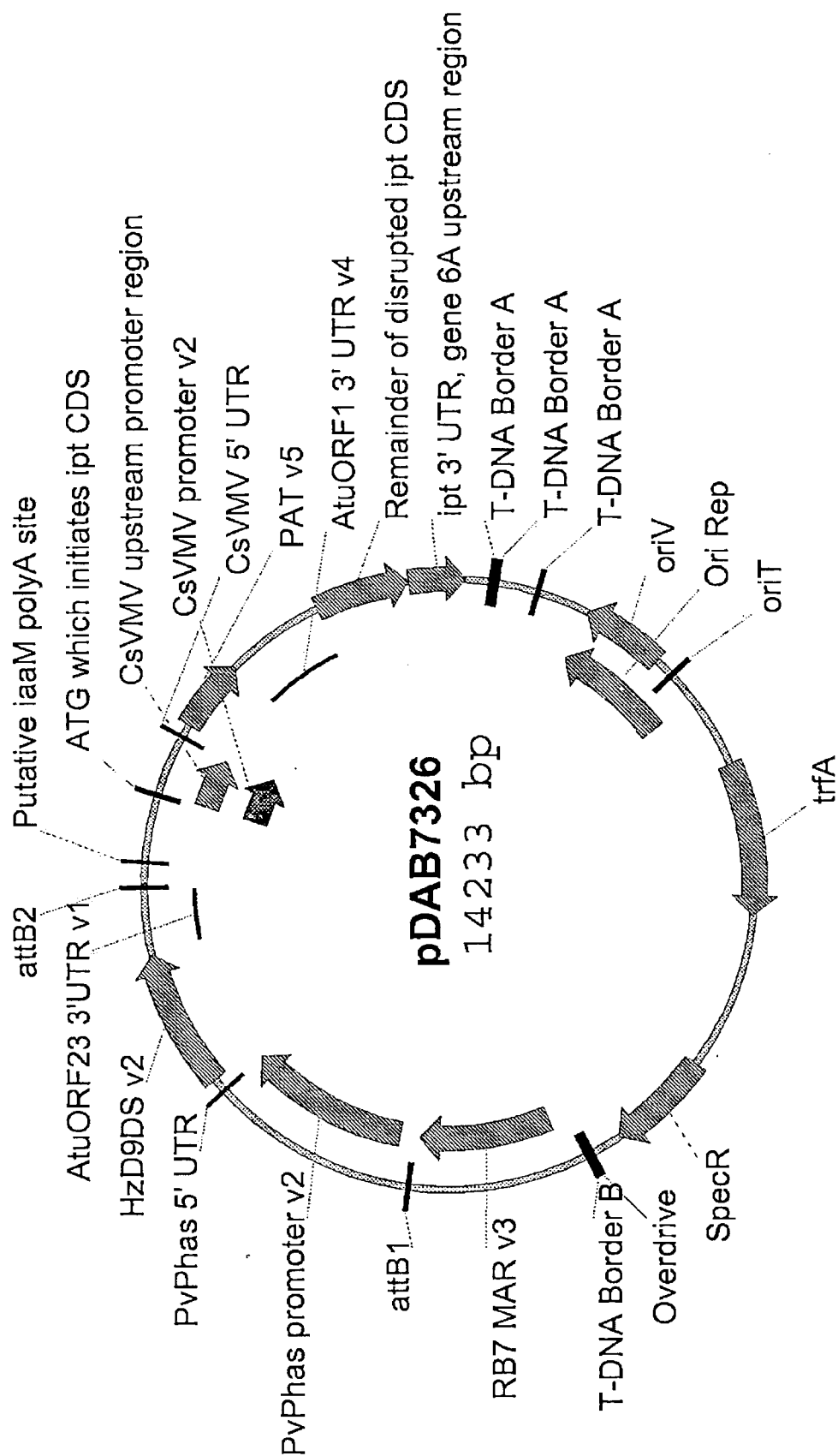


Fig. 13

SUBSTITUTE SHEET (RULE 26)

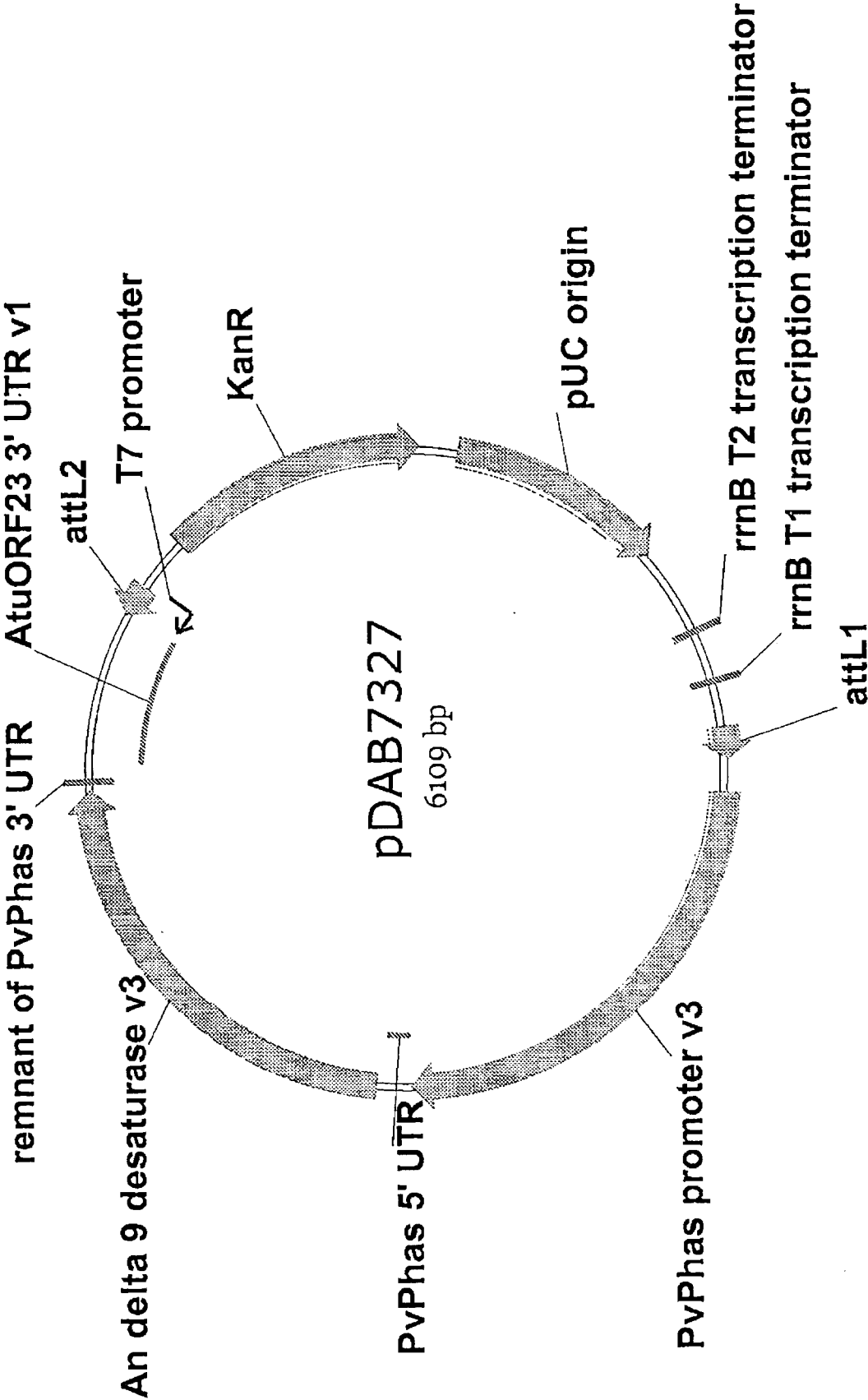


Fig. 14

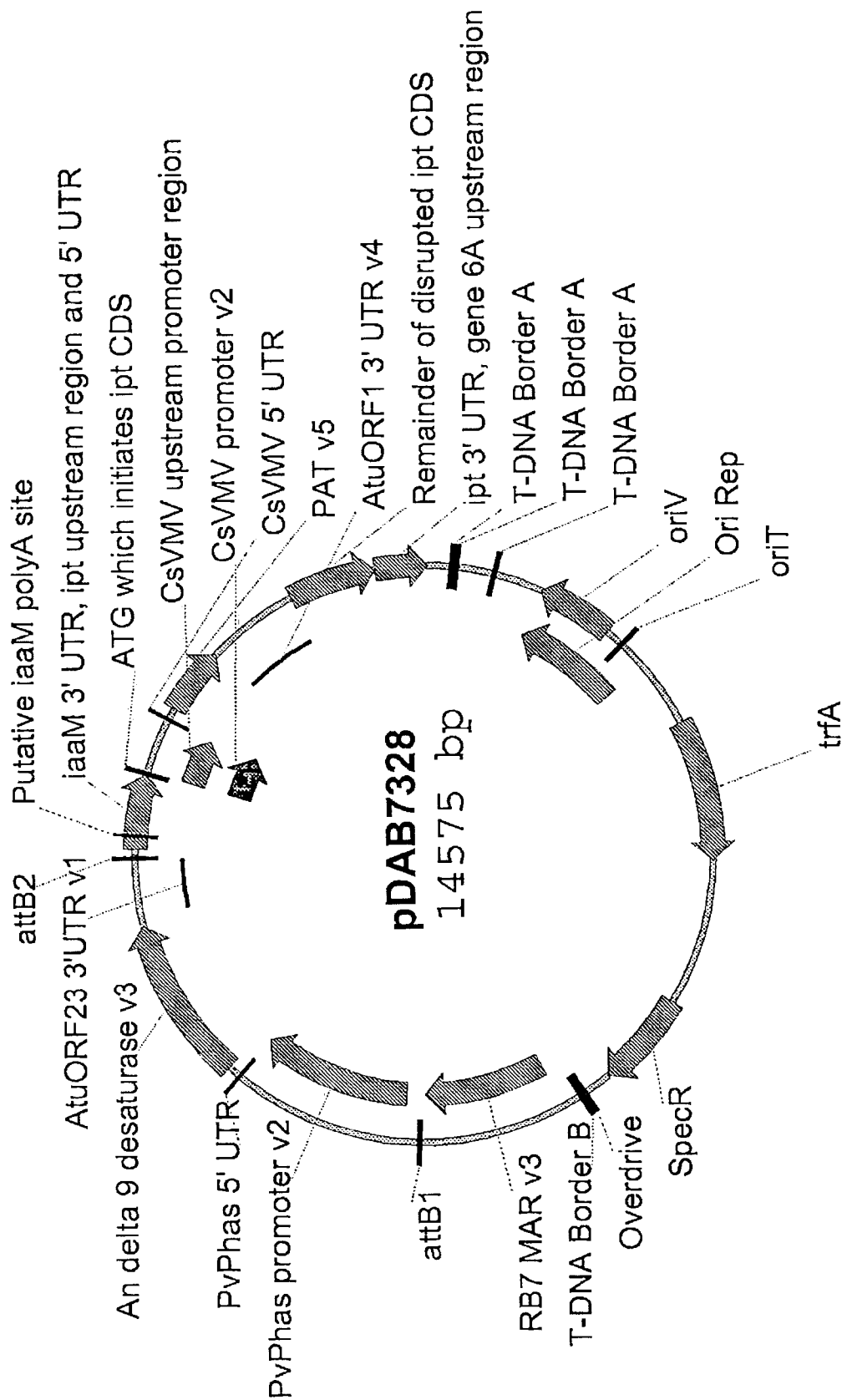


Fig. 15

SUBSTITUTE SHEET (RULE 26)

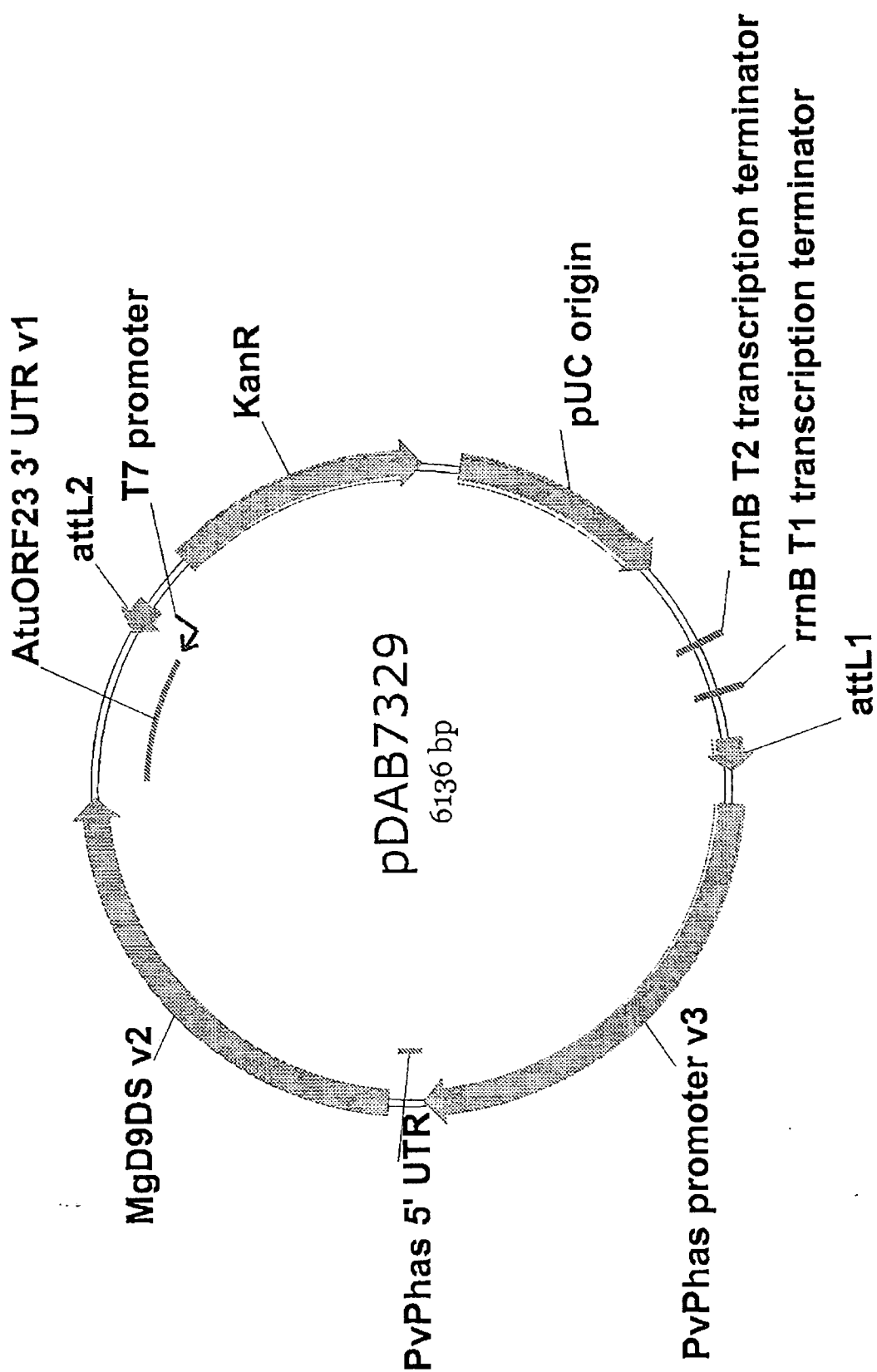


Fig. 16

SUBSTITUTE SHEET (RULE 26)

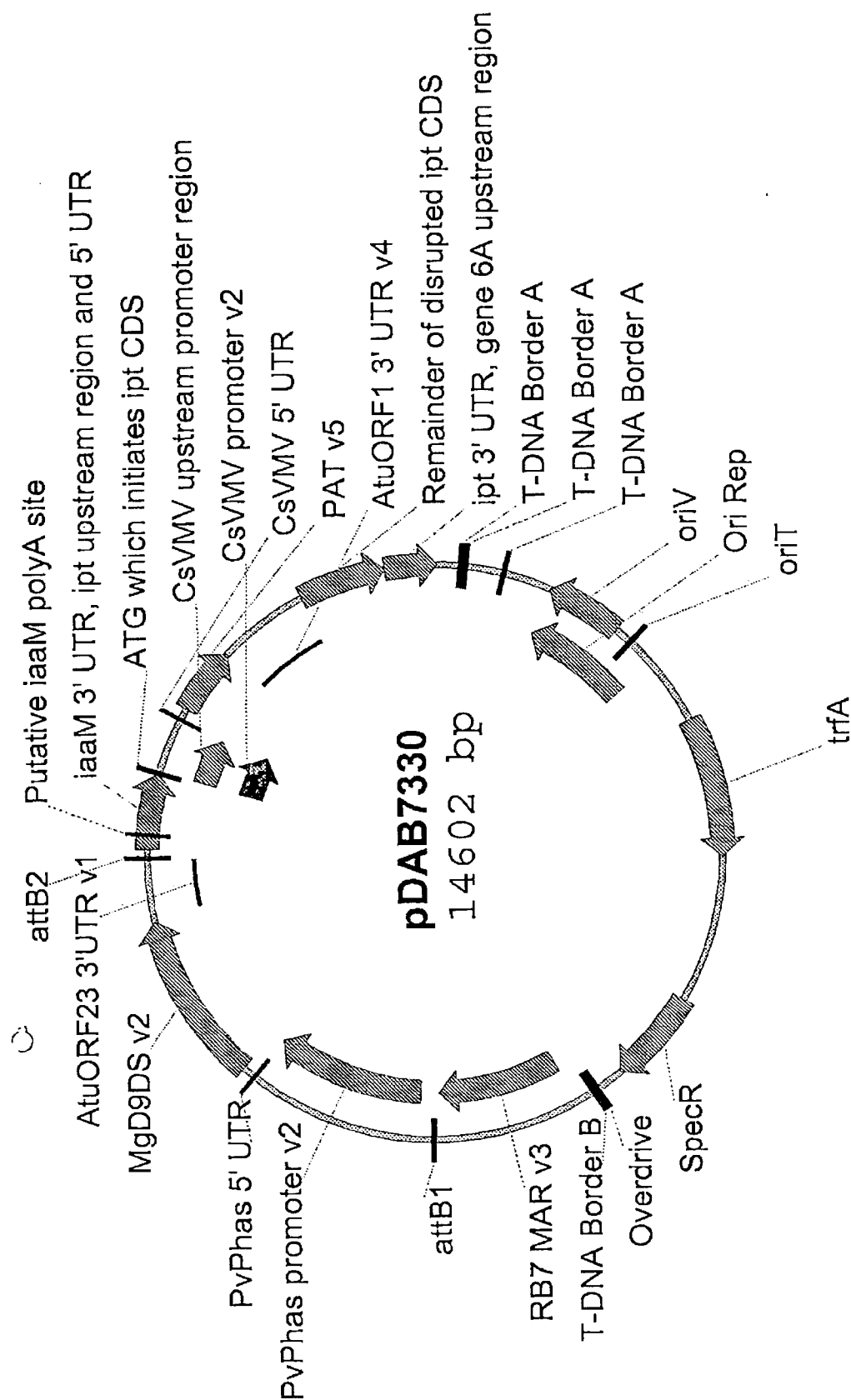


Fig. 17

SUBSTITUTE SHEET (RULE 26)

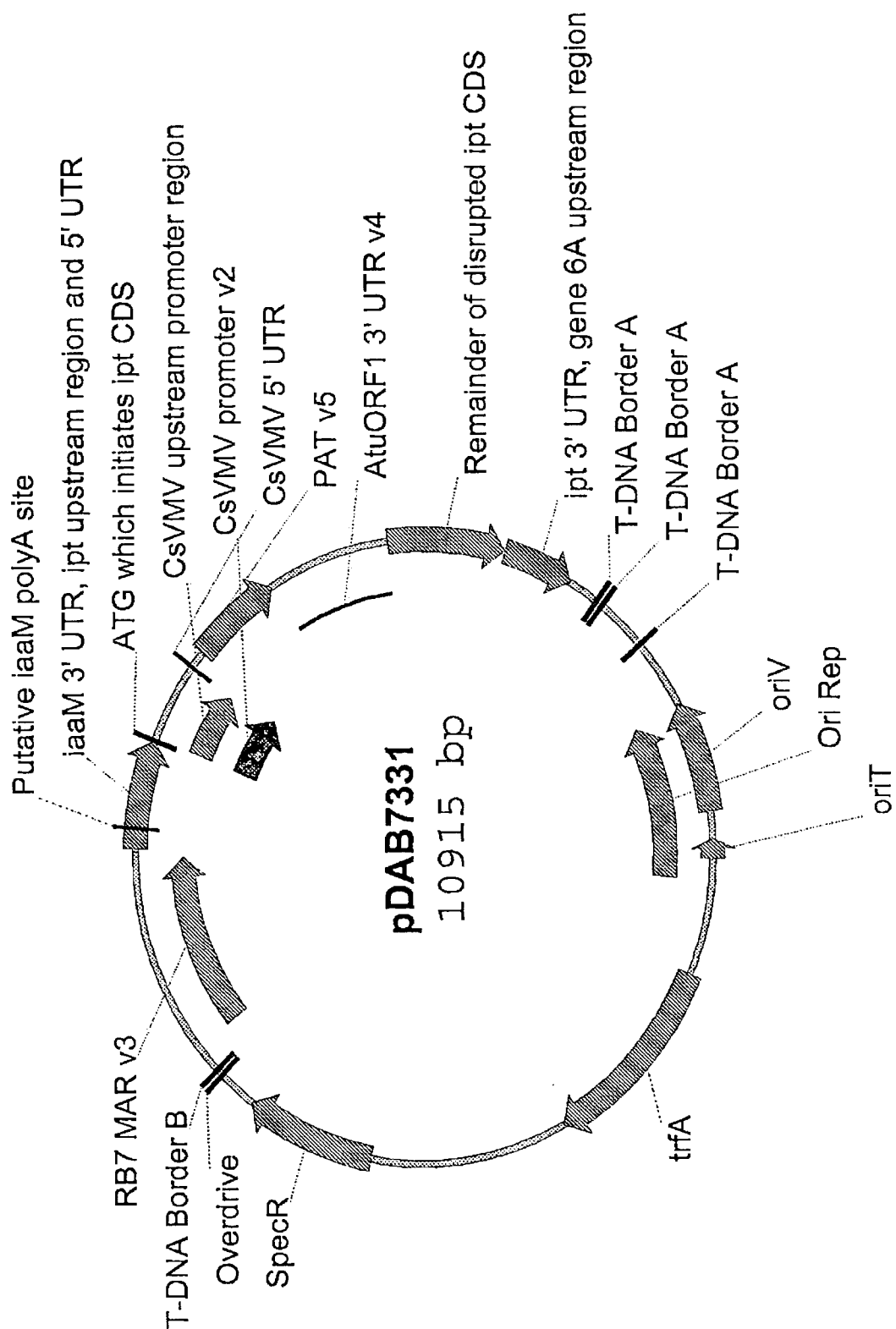


Fig. 18

SUBSTITUTE SHEET (RULE 26)

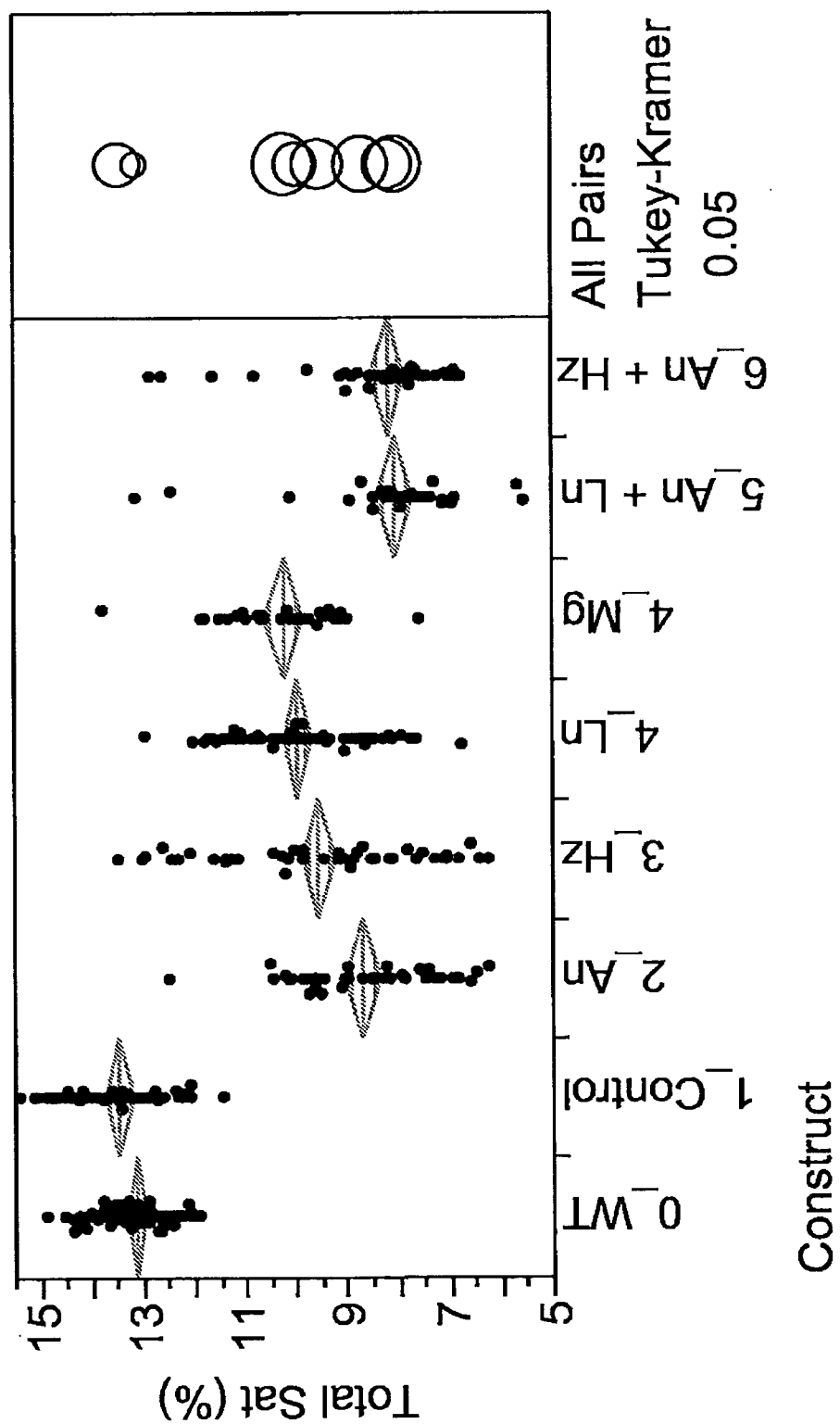


Fig. 19

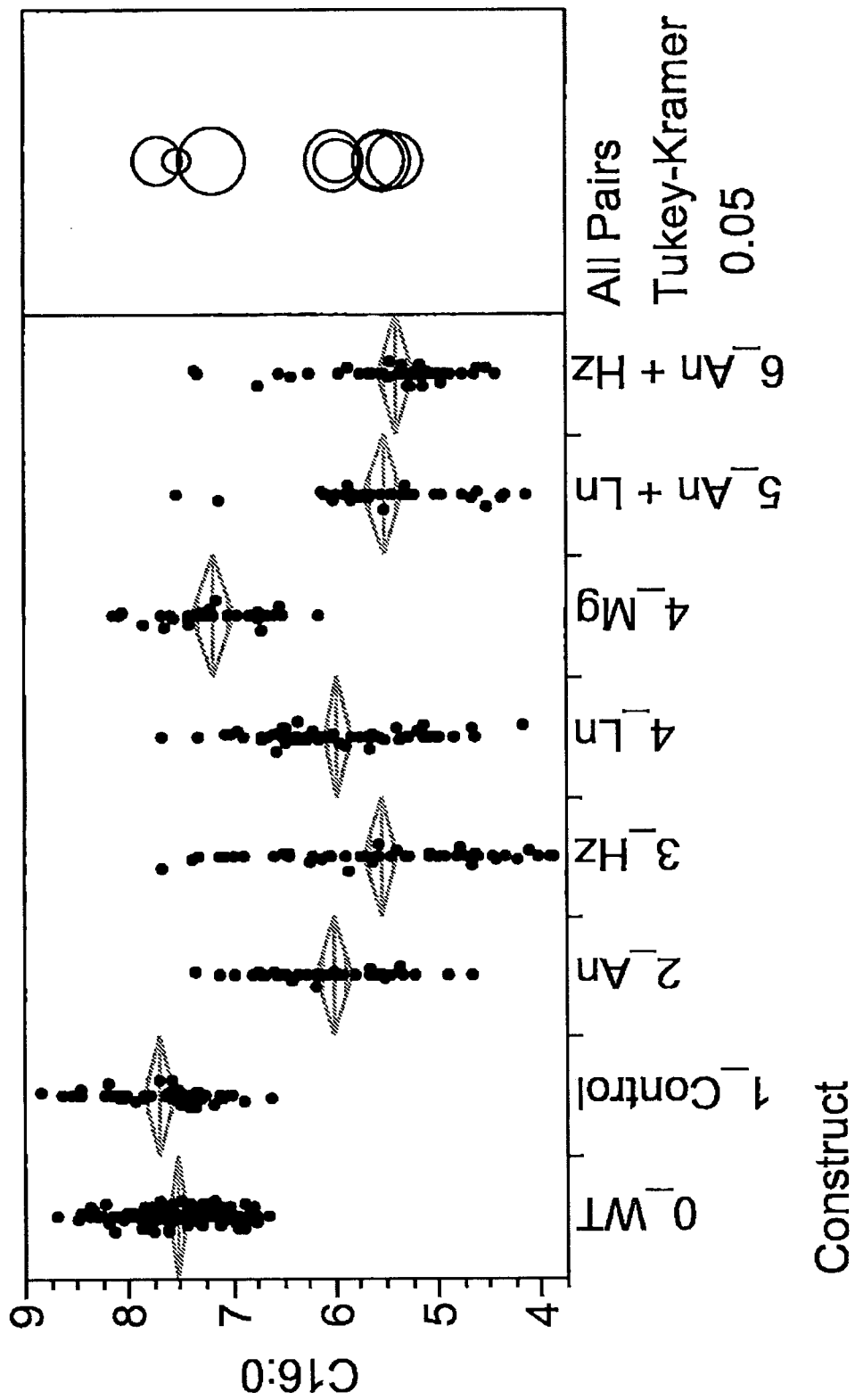


Fig. 20

SUBSTITUTE SHEET (RULE 26)

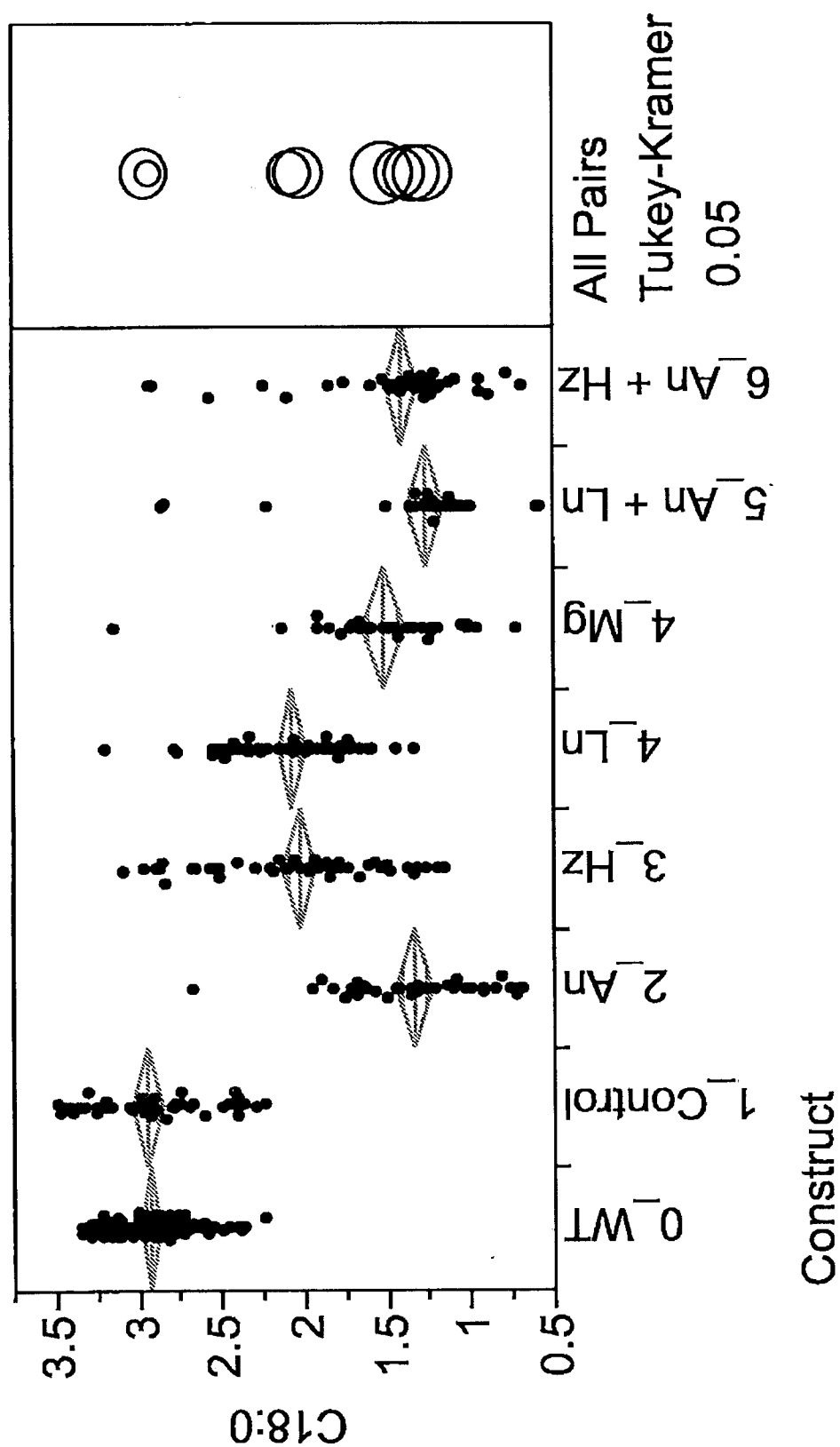


Fig. 21

SUBSTITUTE SHEET (RULE 26)

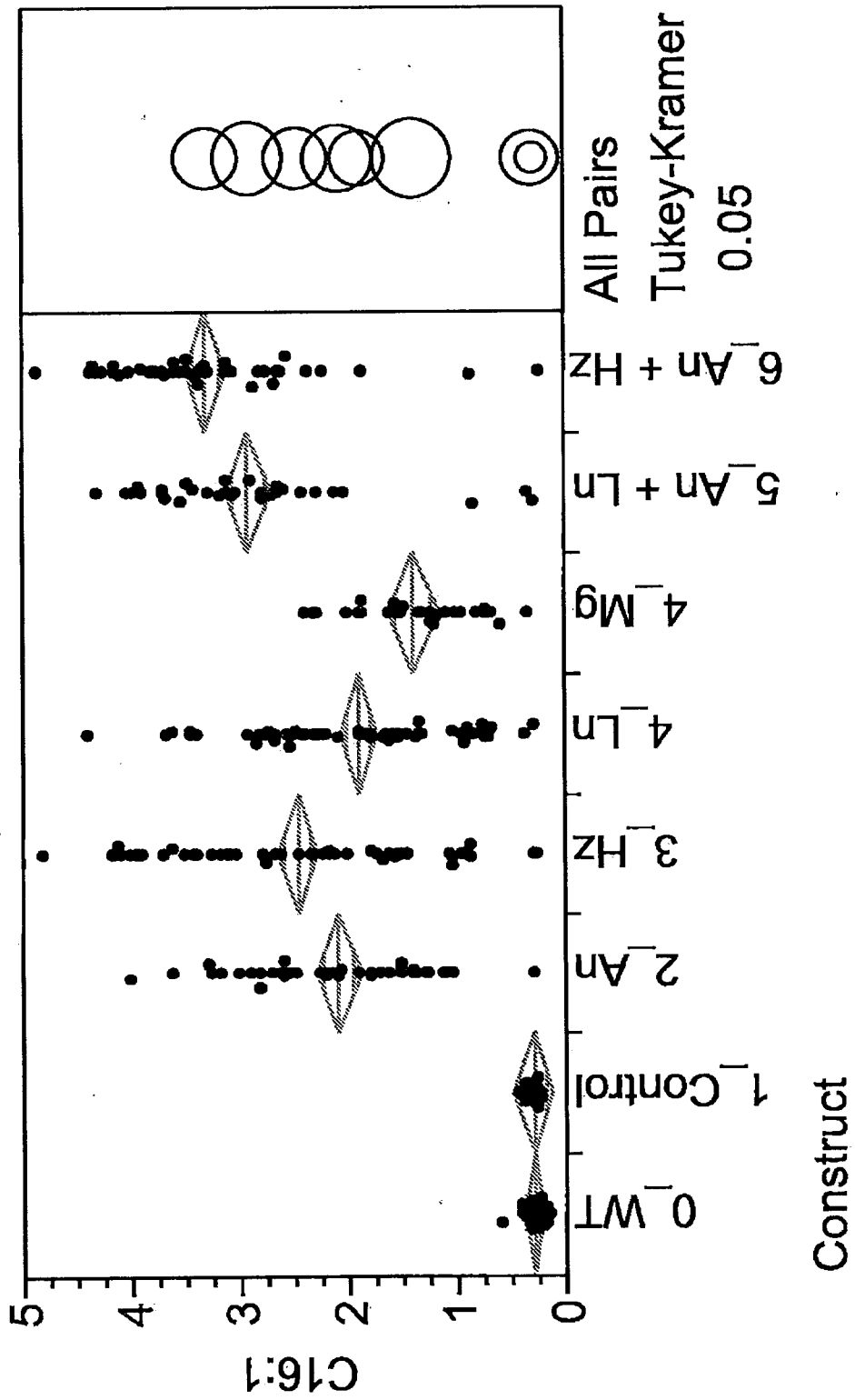


Fig. 22

SUBSTITUTE SHEET (RULE 26)

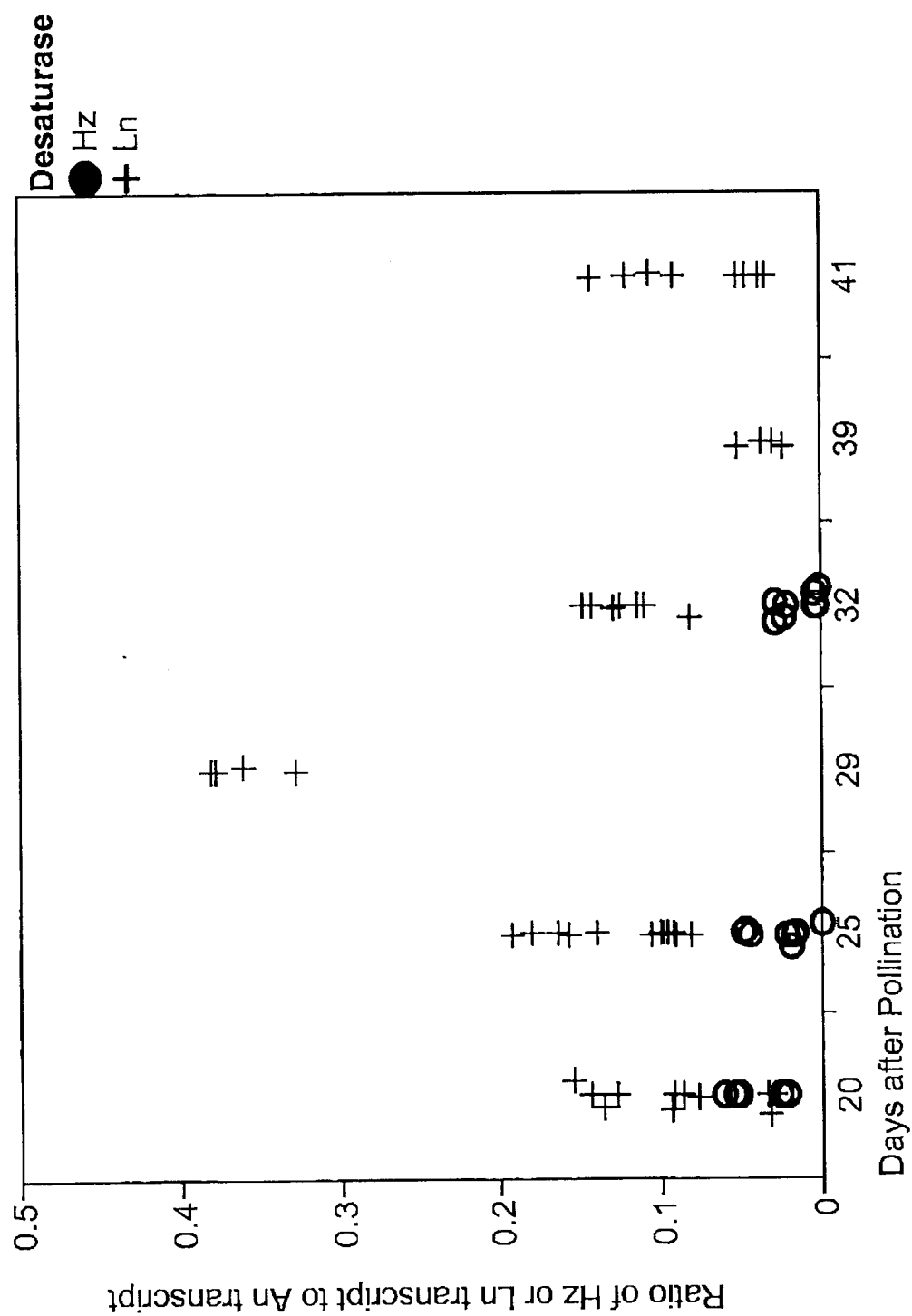


Fig. 23

SUBSTITUTE SHEET (RULE 26)