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(71) Demandeur/Applicant:
CALGENE LLC, US

(72) Inventeur/Inventor:
DEHESH, KATAYOON, US

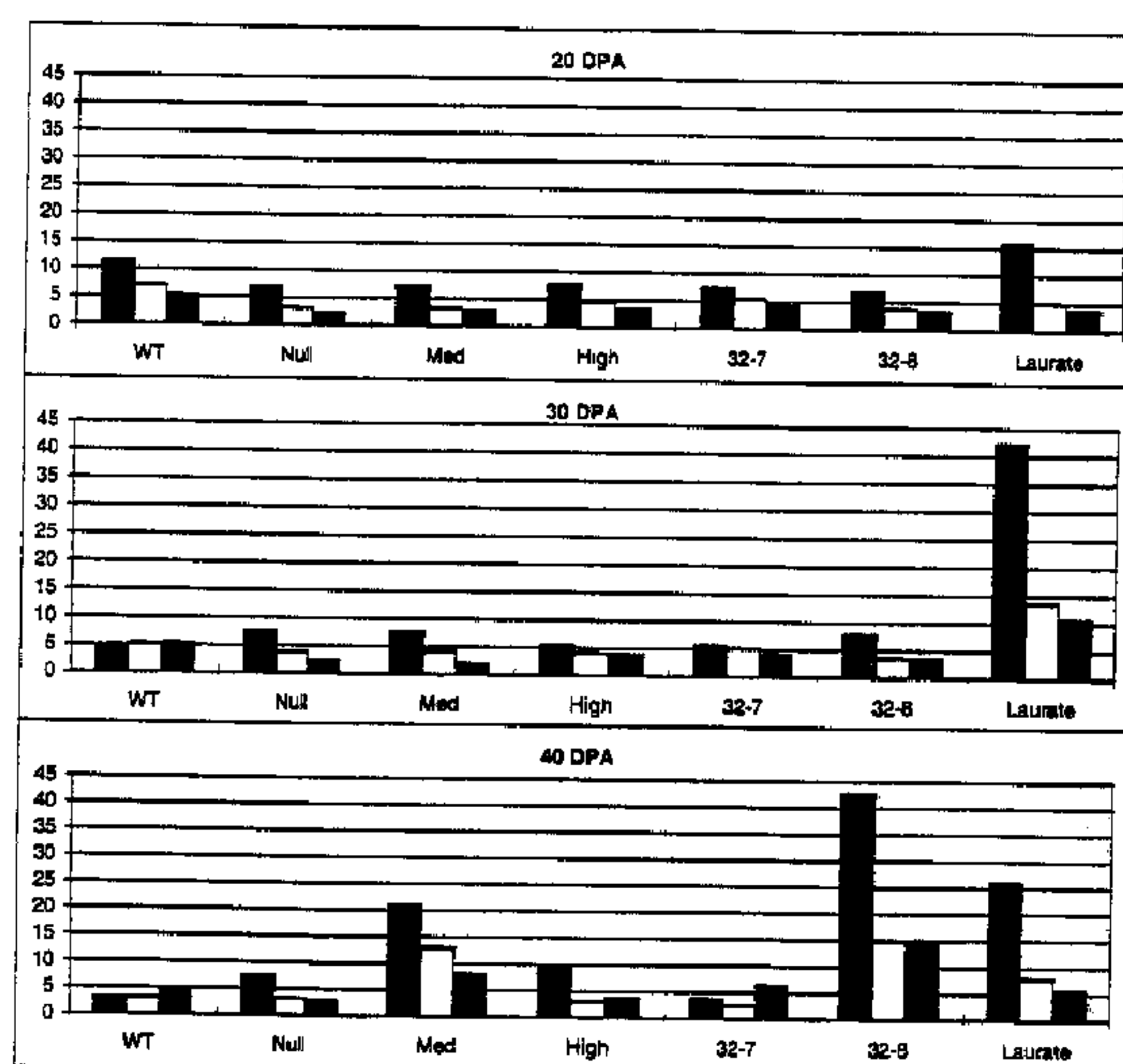
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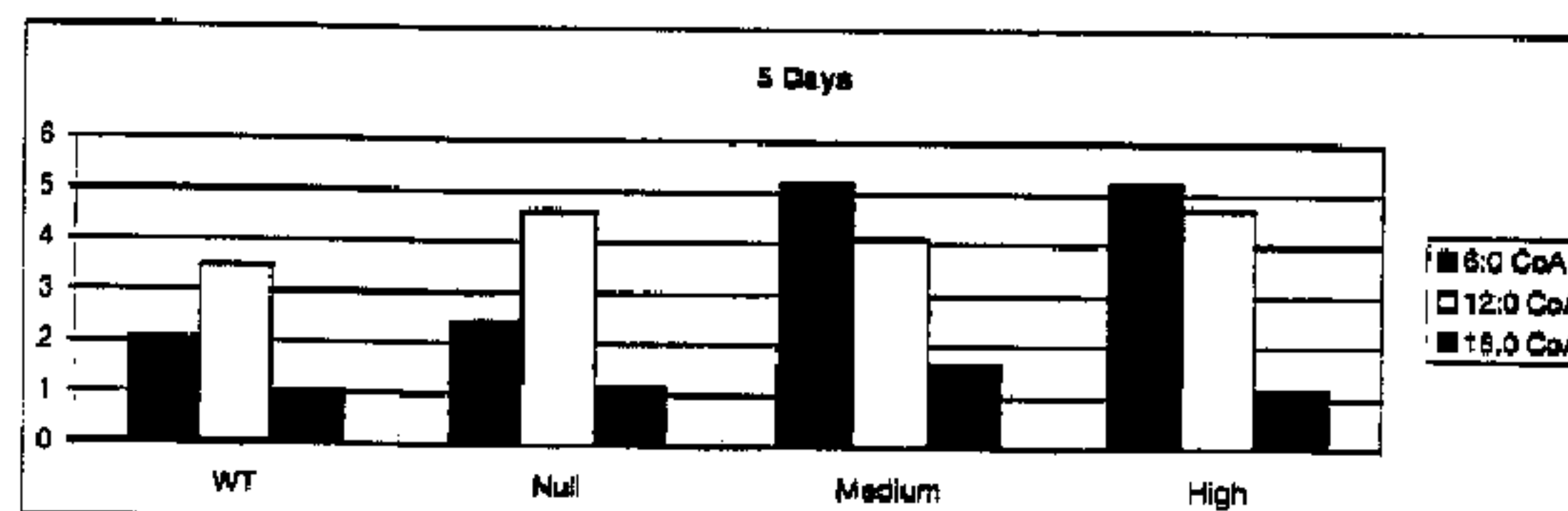
(54) Title: NUCLEIC ACID SEQUENCES ENCODING PROTEINS INVOLVED IN FATTY ACID BETA-OXIDATION AND
METHODS OF USE

ACOX Activity (mU/mg)

A. Developing Seeds



B. Germinating Seeds



(57) Abrégé/Abstract:

The invention provides novel polynucleotide and polypeptide sequences involved in fatty acid beta-oxidation and to methods of producing such polypeptides using recombinant techniques. In addition, methods are provided for using such sequences to alter lipid levels in plants by altering fatty acid beta-oxidation in host plant cells.

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C12N 15/11(74) Agent: EAGLE, Alissa, M.; Calgene LLC, 1920 Fifth
Street, Davis, CA 95616 (US).

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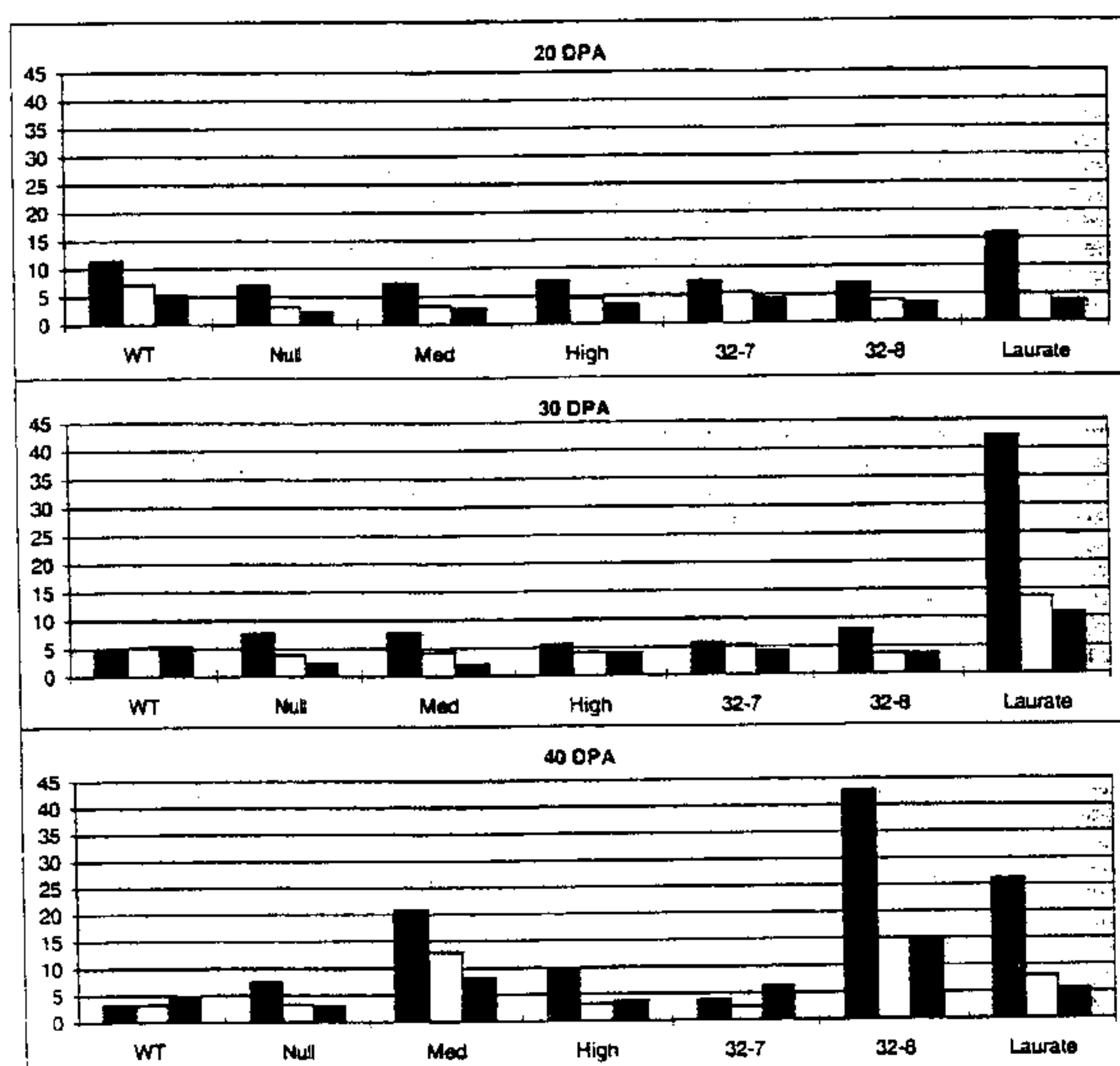
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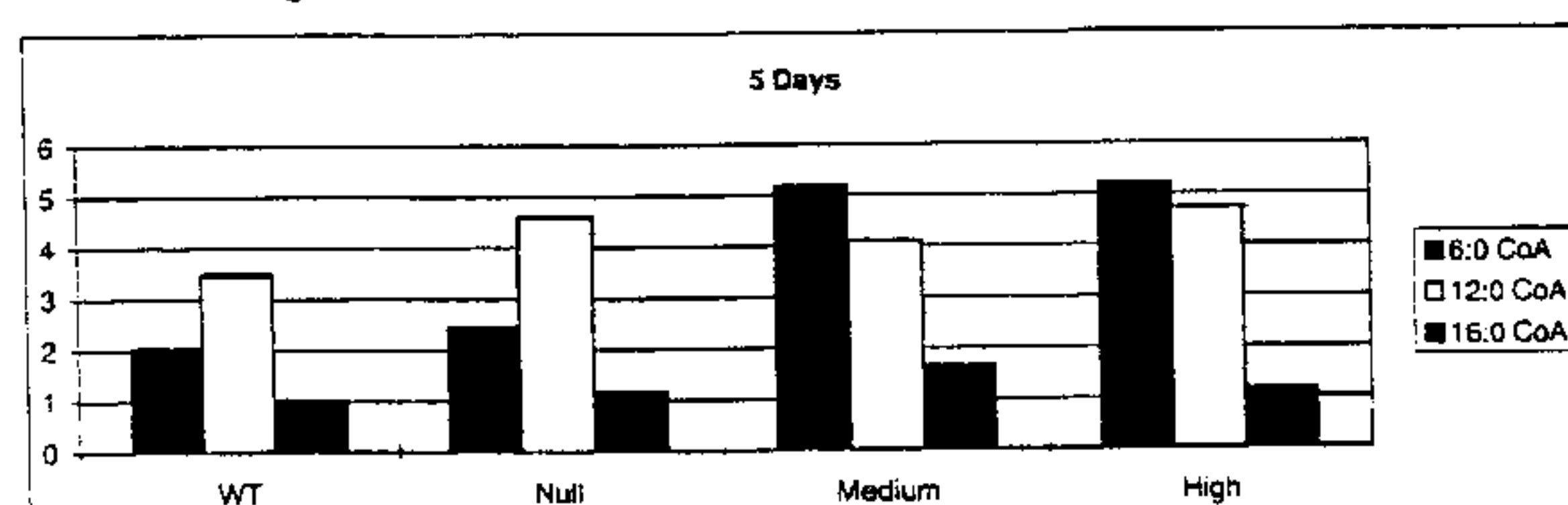
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29 November 2001(71) Applicant: CALGENE LLC [US/US]; 1920 Fifth Street,
Davis, CA 95616 (US).(72) Inventor: DEHESH, Katayoon; 521 Crownpointe Circle,
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ning of each regular issue of the PCT Gazette.(54) Title: NUCLEIC ACID SEQUENCES ENCODING PROTEINS INVOLVED IN FATTY ACID BETA-OXIDATION AND
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NUCLEIC ACID SEQUENCES ENCODING PROTEINS INVOLVED IN FATTY ACID BETA-OXIDATION AND METHODS OF USE

5

INTRODUCTION

This application claims the benefit of U.S. Provisional Application Number 60/138,162 filed June 8, 1999.

10

Technical Field

The present invention is directed to nucleic acid and amino acid sequences and constructs, and methods related thereto.

15 Background

Fatty acids are organic acids having a hydrocarbon chain of from about 4 to 24 carbons. Many different kinds of fatty acids are known which differ from each other in chain length, and in the presence, number and position of double bonds. In cells, fatty acids typically exist in covalently bound forms, the carboxyl portion being referred to as a fatty acyl group. The chain length and degree of saturation of these molecules is often depicted by the formula CX:Y, where "X" indicates number of carbons and "Y" indicates number of double bands. As the carbon chain of fatty acyl molecules always contains an even number of carbons, the formula "C₂X" may also be used to represent carbon chain length.

Fatty acyl groups are major components of many lipids, and their long, non-polar hydrocarbon chain is responsible for the water-insoluble nature of these lipid molecules. The type of covalent linkage of the fatty acyl group to other factors can vary. For example, in biosynthetic reactions they may be covalently bound via a thioester linkage to an acyl carrier protein (ACP) or to CoenzymeA (CoA), depending on the particular enzymatic reaction. In waxes, fatty acyl groups are linked to fatty alcohols via an ester linkage, and triacylglycerols have three fatty acyl groups linked to a glycerol molecule via an ester linkage.

30

The fatty acid composition of an oil determines its physical and chemical properties, and thus its uses. Plants, especially plant species which synthesize large amounts of oils in plant seeds, are an important source of oils both for edible and industrial uses.

5 A wide range of novel vegetable oils compositions and/or improved means to obtain or manipulate fatty acid compositions, from biosynthetic or natural plant sources, are needed for a variety of intended uses. Plant breeding, even with mutagenesis, cannot meet this need and provide for the introduction of any oil traits which are outside of the target plant's gene pool.

Various oils compositions are now in demand. For example, edible oil sources
10 containing the minimum possible amounts of saturates, palmitate (C16:0) and stearate (C18:0) saturated fatty acids, are desired for dietary reasons and alternatives to current sources of highly saturated oil products, such as tropical oils, are also needed. Generating a spread of C4, C6 and C8 short chain 3-keto fatty acids could become a key improvement in polyhydroxybutyrate (PHB)-based biodegradable plastics made in bacteria and plants.

15 Medium-chain fatty acids have special importance in the detergent and lubricant industries or in the formulation of edible oils with reduced caloric value or other health benefits. *See* for example, U.S. Pat. No. 4,863,753 and Barch, A.C. & Babayan, V.K., *Am. J. Clin. Nat.* (1982) 36:950-962. Longer chain fatty acids may have certain other utilities, i.e., C16 and C18 have particular uses in margarine and other solid oil-based products and very long chain fatty acids
20 also have specialized uses, i.e., C22 is used to make peanut butter smoother. As such, a ready source of a variety of fatty acid lengths, including storage lipids which have incorporated differing chain length fatty acids in desired ratios, are desired for a variety of industrial and food use fields. Improved yield of current oilseed crops and the development of novel plant fatty acid compositions and oils products are also needed. Examples of novel plant fatty acid
25 and oils products include fatty alcohols, epoxy fatty acids (e.g., biodegradable paint thinner), long chain liquid wax (e.g., jojoba oil substitute), hydroxylated fatty acids (motor lubricants) or cyclopropanated fatty acids (motor lubricants).

There is a need for improved means to obtain or manipulate compositions fatty acids from biosynthetic or natural plant sources. For example, novel oil products, improved sources

of synthetic triacylglycerols (triglycerides), alternative sources of commercial oils, such as tropical oils (*i.e.*, palm kernel and coconut oils), and plant oils found in trace amounts from natural sources are desired for a variety of industrial and food uses. Or, the ability to increase total oil production in plants may provide for novel applications of seed oils for use in human and animal nutrition.

SUMMARY OF THE INVENTION

The present invention is directed to fatty acid β -oxidation polynucleotides, and in particular to acyl-CoA oxidase (ACOX) polynucleotides. The present invention further provides 3-ketoacyl-CoA thiolase (thiolase) polynucleotides. The polynucleotides of the present invention include those derived from plant sources.

One aspect of the present invention relates to oligonucleotides which include partial or complete ACOX or thiolase encoding sequences.

It is also an aspect of the present invention to provide recombinant DNA constructs which can be used for transcription or transcription and translation (expression) of ACOX and/or thiolase. In particular, constructs are provided which are capable of transcription or transcription and translation in host cells. Particularly preferred constructs are those capable of suppression of endogenous host cell ACOX and/or thiolase.

In another aspect of the present invention, methods are provided for production of ACOX and or thiolase in a host cell or progeny thereof. In particular, host cells are transformed or transfected with a DNA construct which can be used for transcription or transcription and translation of ACOX and/or thiolase. The recombinant cells which contain ACOX and/or thiolase are also part of the present invention.

In a further aspect, the present invention relates to methods of using polynucleotide and polypeptide sequences to modify the fatty acid content as well as composition, particularly in seed tissue of oilseed crops. Plant cells having such a modified fatty acid content are also contemplated herein.

In yet a further aspect, the present invention relates to methods of using polynucleotide and polypeptide sequences to inhibit or delay the germination of seeds.

The modified plants, seeds and oils obtained by the expression of the plant ACOX proteins are also considered part of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Provides ACOX activity comparisons in 20, 30 and 40 day post anthesis (dpa) developing seeds of wild-type *Brassica*, null lines, medium and high level MCFA containing lines, and laurate producing lines. Each line was examined for short chain specific ACOX activity (6:0 CoA), medium chain specific ACOX activity (12:0 CoA), and long chain specific activity (16:0 CoA), represented by the left bar, middle bar and right bar on the graph respectively.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the subject invention, polynucleotides and polypeptides involved in plant fatty acid β -oxidation and methods for the use of such polynucleotides and polypeptides are provided. In particular, polynucleotides and polypeptides related to acyl-CoA oxidase polynucleotides. The present invention further provides 3-ketoacyl-CoA thiolase polynucleotides and polypeptides..

Beta-oxidation is responsible for the catabolism of fatty acids to produce succinate for the production of sucrose. Fatty acyl-CoA is the substrate for fatty acid β -oxidation in peroxisomes and mitochondria, which consists of four steps. The first step of β -oxidation is catalyzed by Acyl-CoA oxidase (hereinafter also referred to as ACOX). The second and third steps are catalyzed by a single enzyme which has both enoyl-CoA hydratase and β -hydroxyacyl-CoA dehydrogenase activities. The fourth step is catalyzed by 3-ketoacyl-CoA thiolase (herein after also referred to as thiolase). The acetyl-CoA produced as a result of β -oxidation is metabolized further to produce succinate by the glyoxylate pathway.

A first aspect of the present invention relates to isolated polynucleotides involved in plant fatty acid β -oxidation. In particular, isolated ACOX and thiolase polynucleotides are provided. The polynucleotide sequences of the present invention include isolated polynucleotides that encode the polypeptides of the invention having a deduced amino acid sequence selected from the group of sequences set forth in the Sequence Listing and to other polynucleotide sequences closely related to such sequences and variants thereof.

The invention provides a polynucleotide sequence identical over its entire length to each coding sequence as set forth in the Sequence Listing. The invention also provides the

coding sequence for the mature polypeptide or a fragment thereof, as well as the coding sequence for the mature polypeptide or a fragment thereof in a reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, pro-, or prepro- protein sequence. The polynucleotide can also include non-coding sequences, including for example, but not limited to, non-coding 5' and 3' sequences, such as the transcribed, untranslated sequences, termination signals, ribosome binding sites, sequences that stabilize mRNA, introns, polyadenylation signals, and additional coding sequence that encodes additional amino acids. For example, a marker sequence can be included to facilitate the purification of the fused polypeptide. Polynucleotides of the present invention also include polynucleotides comprising a structural gene and the naturally associated sequences that control gene expression.

The invention also includes polynucleotides of the formula:



wherein, at the 5' end, X is hydrogen, and at the 3' end, Y is hydrogen or a metal, R_1 and R_3 are any nucleic acid residue, n is an integer between 1 and 3000, preferably between 1 and 1000 and R_2 is a nucleic acid sequence of the invention, particularly a nucleic acid sequence selected from the group set forth in the Sequence Listing and preferably SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23. In the formula, R_2 is oriented so that its 5' end residue is at the left, bound to R_1 , and its 3' end residue is at the right, bound to R_3 . Any stretch of nucleic acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

The invention also relates to variants of the polynucleotides described herein that encode for variants of the polypeptides of the invention. Variants that are fragments of the polynucleotides of the invention can be used to synthesize full-length polynucleotides of the invention. Preferred embodiments are polynucleotides encoding polypeptide variants wherein 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues of a polypeptide sequence of the invention are substituted, added or deleted, in any combination. Particularly preferred are substitutions, additions, and deletions that are silent such that they do not alter the properties or activities of the polynucleotide or polypeptide.

Further preferred embodiments of the invention that are at least 50%, 60%, or 70% identical over their entire length to a polynucleotide encoding a polypeptide of the invention, and polynucleotides that are complementary to such polynucleotides. More preferable are polynucleotides that comprise a region that is at least 80% identical over its entire length to a

polynucleotide encoding a polypeptide of the invention and polynucleotides that are complementary thereto. In this regard, polynucleotides at least 90% identical over their entire length are particularly preferred, those at least 95% identical are especially preferred. Further, those with at least 97% identity are highly preferred and those with at least 98% and 99% identity are particularly highly preferred, with those at least 99% being the most highly preferred.

Preferred embodiments are polynucleotides that encode polypeptides that retain substantially the same biological function or activity as the mature polypeptides encoded by the polynucleotides set forth in the Sequence Listing.

The invention further relates to polynucleotides that hybridize to the above-described sequences. In particular, the invention relates to polynucleotides that hybridize under stringent conditions to the above-described polynucleotides. As used herein, the terms "stringent conditions" and "stringent hybridization conditions" mean that hybridization will generally occur if there is at least 95% and preferably at least 97% identity between the sequences. An example of stringent hybridization conditions is overnight incubation at 42°C in a solution comprising 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 micrograms/milliliter denatured, sheared salmon sperm DNA, followed by washing the hybridization support in 0.1x SSC at approximately 65°C. Other hybridization and wash conditions are well known and are exemplified in Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly Chapter 11.

One skilled in the art will readily recognize that antibody preparations, nucleic acid probes (DNA and RNA) and the like may be prepared and used to screen and recover "homologous" or "related" sequences from a variety of plant sources. Homologous sequences are found when there is an identity of sequence, which may be determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions between a known ACOX or thiolase and a candidate source. Conservative changes, such as Glu/Asp, Val/Ile, Ser/Thr, Arg/Lys and Gln/Asn may also be considered in determining sequence homology. Amino acid sequences are considered homologous by as little as 25% sequence identity between the two complete mature proteins. (*See generally*, Doolittle, R.F., *OF URFS and ORFS* (University Science Books, CA, 1986.).

Thus, other ACOXs and thiolases may be obtained from the specific sequences provided herein. Furthermore, it will be apparent that one can obtain natural and synthetic

sequences, including modified amino acid sequences and starting materials for synthetic-protein modeling from the exemplified ACOX and thiolase sequences and from sequences which are obtained through the use of such exemplified sequences. Modified amino acid sequences include sequences which have been mutated, truncated, increased and the like,
5 whether such sequences were partially or wholly synthesized. Sequences which are actually purified from plant preparations or are identical or encode identical proteins thereto, regardless of the method used to obtain the protein or sequence, are equally considered naturally derived.

For immunological screening, antibodies to the protein can be prepared by injecting
10 rabbits or mice with the purified protein or portion thereof, such methods of preparing antibodies being well known to those in the art. Either monoclonal or polyclonal antibodies can be produced, although typically polyclonal antibodies are more useful for gene isolation. Western analysis may be conducted to determine that a related protein is present in a crude extract of the desired plant species, as determined by cross-reaction with the antibodies to the
15 encoded proteins. When cross-reactivity is observed, genes encoding the related proteins are isolated by screening expression libraries representing the desired plant species. Expression libraries can be constructed in a variety of commercially available vectors, including lambda gt11, as described in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

20 The invention also provides a polynucleotide consisting essentially of a polynucleotide sequence obtainable by screening an appropriate library containing the complete gene for a polynucleotide sequence set forth in the Sequence Listing under stringent hybridization conditions with a probe having the sequence of said polynucleotide sequence or a fragment thereof; and isolating said polynucleotide sequence. Fragments useful for
25 obtaining such a polynucleotide include, for example, probes and primers as described herein.

As discussed herein regarding polynucleotide assays of the invention, for example, polynucleotides of the invention can be used as a hybridization probe for RNA, cDNA, or genomic DNA to isolate full length cDNAs or genomic clones encoding a polypeptide and to isolate cDNA or genomic clones of other genes that have a high sequence similarity to a
30 polynucleotide set forth in the Sequence Listing. Such probes will generally comprise at least 15 bases. Preferably such probes will have at least 30 bases and can have at least 50 bases. Particularly preferred probes will have between 30 bases and 50 bases, inclusive.

The coding region of each gene that comprises or is comprised by a polynucleotide sequence set forth in the Sequence Listing may be isolated by screening using a DNA sequence provided in the Sequence Listing to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the invention is then
5 used to screen a library of cDNA, genomic DNA or mRNA to identify members of the library which hybridize to the probe. For example, synthetic oligonucleotides are prepared which correspond to the ACOX EST sequences. The oligonucleotides are used as primers in polymerase chain reaction (PCR) techniques to obtain 5' and 3' terminal sequence of ACOX genes. Alternatively, where oligonucleotides of low degeneracy can be prepared from
10 particular ACOX peptides, such probes may be used directly to screen gene libraries for ACOX gene sequences. In particular, screening of cDNA libraries in phage vectors is useful in such methods due to lower levels of background hybridization.

Typically, a sequence obtainable from the use of nucleic acid probes will show 60-70% sequence identity between the target sequence and the encoding sequence used as a
15 probe. However, lengthy sequences with as little as 50-60% sequence identity may also be obtained. The nucleic acid probes may be a lengthy fragment of the nucleic acid sequence, or may also be a shorter, oligonucleotide probe. When longer nucleic acid fragments are employed as probes (greater than about 100 bp), one may screen at lower stringencies in order to obtain sequences from the target sample which have 20-50% deviation (i.e., 50-80%
20 sequence homology) from the sequences used as probe. Oligonucleotide probes can be considerably shorter than the entire nucleic acid sequence encoding an ACOX or thiolase enzyme, but should be at least about 10, preferably at least about 15, and more preferably at least about 20 nucleotides. A higher degree of sequence identity is desired when shorter regions are used as opposed to longer regions. It may thus be desirable to identify regions of
25 highly conserved amino acid sequence to design oligonucleotide probes for detecting and recovering other related ACOX and thiolase genes. Shorter probes are often particularly useful for polymerase chain reactions (PCR), especially when highly conserved sequences can be identified. (*See, Gould, et al., PNAS USA (1989) 86:1934-1938.*)

Another aspect of the present invention relates to plant fatty acid β -oxidation
30 polypeptides. In particular, ACOX and thiolase polypeptides are provided. Such polypeptides include isolated polypeptides set forth in the Sequence Listing, as well as polypeptides and fragments thereof, particularly those polypeptides which exhibit ACOX or thiolase activity and also those polypeptides which have at least 50%, 60% or 70% identity,

preferably at least 80% identity, more preferably at least 90% identity, and most preferably at least 95% identity to a polypeptide sequence selected from the group of sequences set forth in the Sequence Listing, and also include portions of such polypeptides, wherein such portion of the polypeptide preferably includes at least 30 amino acids and more preferably includes at least 50 amino acids.

“Identity”, as is well understood in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, “identity” also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as determined by the match between strings of such sequences. “Identity” can be readily calculated by known methods including, but not limited to, those described in *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York (1988); *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M. and Griffin, H.G., eds., Humana Press, New Jersey (1994); *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press (1987); *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., Stockton Press, New York (1991); and Carillo, H., and Lipman, D., *SIAM J Applied Math*, 48:1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available programs. Computer programs which can be used to determine identity between two sequences include, but are not limited to, GCG (Devereux, J., et al., *Nucleic Acids Research* 12(1):387 (1984); suite of five BLAST programs, three designed for nucleotide sequences queries (BLASTN, BLASTX, and TBLASTX) and two designed for protein sequence queries (BLASTP and TBLASTN) (Coulson, *Trends in Biotechnology*, 12: 76-80 (1994); Birren, et al., *Genome Analysis*, 1: 543-559 (1997)). The BLAST X program is publicly available from NCBI and other sources (*BLAST Manual*, Altschul, S., et al., NCBI NLM NIH, Bethesda, MD 20894; Altschul, S., et al., *J. Mol. Biol.*, 215:403-410 (1990)). The well known Smith Waterman algorithm can also be used to determine identity.

Parameters for polypeptide sequence comparison typically include the following:

Algorithm: Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970)

Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, *Proc. Natl. Acad. Sci USA* 89:10915-10919 (1992)

Gap Penalty: 12

Gap Length Penalty: 4

A program which can be used with these parameters is publicly available as the “gap” program from Genetics Computer Group, Madison Wisconsin. The above parameters along
5 with no penalty for end gap are the default parameters for peptide comparisons.

Parameters for polynucleotide sequence comparison include the following:

Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970)

Comparison matrix: matches = +10; mismatches = 0

Gap Penalty: 50

10 Gap Length Penalty: 3

A program which can be used with these parameters is publicly available as the “gap” program from Genetics Computer Group, Madison Wisconsin. The above parameters are the default parameters for nucleic acid comparisons.

The invention also includes polypeptides of the formula:

15
$$X-(R_1)_n-(R_2)-(R_3)_n-Y$$

wherein, at the amino terminus, X is hydrogen, and at the carboxyl terminus, Y is hydrogen or a metal, R_1 and R_3 are any amino acid residue, n is an integer between 1 and 1000, and R_2 is an amino acid sequence of the invention, particularly an amino acid sequence selected from the group set forth in the Sequence Listing and preferably SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14,
20 16, 18, 20, 22, and 24. In the formula, R_2 is oriented so that its amino terminal residue is at the left, bound to R_1 , and its carboxy terminal residue is at the right, bound to R_3 . Any stretch of amino acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

Polypeptides of the present invention include isolated polypeptides encoded by a
25 polynucleotide comprising a sequence selected from the group set forth in the Sequence Listing and preferably SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24..

The polypeptides of the present invention can be mature protein or can be part of a fusion protein.

Fragments and variants of the polypeptides are also considered to be a part of the
30 invention. A fragment is a variant polypeptide which has an amino acid sequence that is entirely the same as part but not all of the amino acid sequence of the previously described polypeptides. The fragments can be “free-standing” or comprised within a larger polypeptide of which the fragment forms a part or a region, most preferably as a single continuous region.

Preferred fragments are biologically active fragments which are those fragments that mediate activities of the polypeptides of the invention, including those with similar activity or improved activity or with a decreased activity. Also included are those fragments that antigenic or immunogenic in an animal, particularly a human.

5 Variants of the polypeptide also include polypeptides that vary from the sequences set forth in the Sequence Listing by conservative amino acid substitutions, substitution of a residue by another with like characteristics. In general, such substitutions are among Ala, Val, Leu and Ile; between Ser and Thr; between Asp and Glu; between Asn and Gln; between Lys and Arg; or between Phe and Tyr. Particularly preferred are variants in which 5 to 10; 1
10 to 5; 1 to 3 or one amino acid(s) are substituted, deleted, or added, in any combination.

Variants that are fragments of the polypeptides of the invention can be used to produce the corresponding full length polypeptide by peptide synthesis. Therefore, these variants can be used as intermediates for producing the full-length polypeptides of the invention.

15 The polynucleotides and polypeptides of the invention can be used, for example, in the transformation of host cells, such as plant host cells, as further discussed herein.

The invention also provides polynucleotides that encode a polypeptide that is a mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids within the mature polypeptide (for example, when the mature form of the protein has more than one
20 polypeptide chain). Such sequences can, for example, play a role in the processing of a protein from a precursor to a mature form, allow protein transport, shorten or lengthen protein half-life, or facilitate manipulation of the protein in assays or production. It is contemplated that cellular enzymes can be used to remove any additional amino acids from the mature protein.

25 A precursor protein, having the mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. The inactive precursors generally are activated when the prosequences are removed. Some or all of the prosequences may be removed prior to activation. Such precursor protein are generally called proproteins.

Once the desired nucleic acid sequence is obtained, it may be manipulated in a variety
30 of ways. Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally occurring sequence. In addition, all or part of the sequence may be synthesized. In the structural gene, one or more codons may be

modified to provide for a modified amino acid sequence, or one or more codon mutations may be introduced to provide for a convenient restriction site or other purpose involved with construction or expression. The structural gene may be further modified by employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like.

5 Of interest in the present invention is the use of the polynucleotide sequences in the preparation of DNA constructs. Furthermore, of particular interest is the use of the nucleic acid sequences of the present invention for the production of expression cassettes for use in transformation of host cells. Such transformed host cells can provide a ready source of the enzyme for a wide variety of uses, including, but not limited to, enzyme assays, fermentation,
10 biotransformation, and the like.

Suitable host cells include both prokaryotic and eukaryotic cells. In particular, host cells for use in the methods of the present invention include fungal cells, including yeast, mammalian cells, insect cells, bacterial cells, and plant cells.

Of particular interest is the use of the nucleotide sequences in recombinant DNA
15 constructs to direct the transcription or transcription and translation (expression) of the ACOX sequences of the present invention in a host plant cell. The expression constructs generally comprise a promoter functional in a host plant cell operably linked to a nucleic acid sequence encoding an ACOX of the present invention and a transcriptional termination region functional in a host plant cell.

20 Those skilled in the art will recognize that there are a number of promoters which are functional in plant cells, and have been described in the literature. Chloroplast and plastid specific promoters, chloroplast or plastid functional promoters, and chloroplast or plastid operable promoters are also envisioned.

One set of promoters are constitutive promoters such as the CaMV35S or FMV35S
25 promoters that yield high levels of expression in most plant organs. Enhanced or duplicated versions of the CaMV35S and FMV35S promoters are useful in the practice of this invention (Odell, *et al.* (1985) *Nature* 313:810-812; Rogers, U.S. Patent Number 5,378, 619). In addition, it may also be preferred to bring about expression of the ACOX gene in specific tissues of the plant, such as leaf, stem, root, tuber, seed, fruit, etc., and the promoter chosen
30 should have the desired tissue and developmental specificity.

Of particular interest is the expression of the nucleic acid sequences of the present invention from transcription initiation regions which are preferentially expressed in a plant seed tissue. Examples of such seed preferential transcription initiation sequences include

those sequences derived from sequences encoding plant storage protein genes or from genes involved in fatty acid biosynthesis in oilseeds. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl *et al.*, *Seed Sci. Res.* 1:209:219 (1991)), phaseolin, zein, soybean trypsin inhibitor, ACP, stearyl-ACP desaturase, soybean α' subunit of β -conglycinin (soy 7s, (Chen *et al.*, *Proc. Natl. Acad. Sci.*, 83:8560-8564 (1986))) and oleosin.

It may be advantageous to direct the localization of proteins conferring ACOX to a particular subcellular compartment, for example, to the mitochondrion, peroxisomes, endoplasmic reticulum, vacuoles, chloroplast or other plastidic compartment. For example, where the genes of interest of the present invention will be targeted to the peroxisome the constructs will also employ the use of peroxisome targeting sequences. Such sequences are referred to herein as peroxisome targeting sequences (PTS). Such peroxisome targeting sequences are known in the art, see for example Olsen, *et al.* (1993) *Plant Cell* 5:941-952. Alternatively, the targeting sequence can be obtained from the native ACOX or thiolase protein. Sequences for targeting preferred proteins to other specific subcellular compartments are described, for example, by; Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun.* 196:1414-1421; and, Shah *et al.* (1986) *Science* 233:478-481.

Depending upon the intended use, the expression constructs may contain the nucleic acid sequence which encodes the entire ACOX or thiolase protein, or a portion thereof. For example, where antisense inhibition of a given ACOX or thiolase protein is desired, the entire ACOX sequence is not required. Furthermore, where ACOX or thiolase sequences used in constructs are intended for use as probes, it may be advantageous to prepare constructs containing only a particular portion of a ACOX or thiolase encoding sequence, for example a sequence which is discovered to encode a highly conserved ACOX or thiolase region.

The skilled artisan will recognize that there are various methods for the inhibition of expression of endogenous sequences in a host cell. Such methods include, but are not limited to antisense suppression (Smith, *et al.* (1988) *Nature* 334:724-726), co-suppression (Napoli, *et al.* (1989) *Plant Cell* 2:279-289), ribozymes (PCT Publication WO 97/10328), and combinations of sense and antisense Waterhouse, *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:13959-13964. Methods for the suppression of endogenous sequences in a host cell

typically employ the use of at least a portion of the sequence to be suppressed in the expression construct. Such sequences may be homologous to coding as well as non-coding regions of the endogenous sequence. Depending on the method of suppression employed, sequences complementary to the host cells endogenous sequence can be used in the expression constructs.

Regulatory transcript termination regions may be provided in plant expression constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the ACOX or thiolase of the present invention or a convenient transcription termination region derived from a different gene source, for example, the transcript termination region which is naturally associated with the transcript initiation region. The skilled artisan will recognize that any convenient transcript termination region which is capable of terminating transcription in a plant cell may be employed in the constructs of the present invention.

Alternatively, constructs may be prepared to direct the expression of the ACOX or thiolase sequences directly from the host plant cell plastid. Such constructs and methods are known in the art and are generally described, for example, in Svab, *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-8530 and Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913-917 and in U.S. Patent Number 5,693,507.

A plant cell, tissue, organ, or plant into which the recombinant DNA constructs containing the expression constructs have been introduced is considered transformed, transfected, or transgenic. A transgenic or transformed cell or plant also includes progeny of the cell or plant and progeny produced from a breeding program employing such a transgenic plant as a parent in a cross and exhibiting an altered phenotype resulting from the presence of a ACOX nucleic acid sequence.

Plant expression or transcription constructs having a sequence of the present invention as the DNA sequence of interest for increased or decreased expression thereof may be employed with a wide variety of plant life, particularly, plant life involved in the production of vegetable oils for edible and industrial uses. Most especially preferred are temperate oilseed crops. Plants of interest include, but are not limited to, rapeseed (Canola and High Erucic Acid varieties), sunflower, safflower, cotton, soybean, peanut, coconut and oil palms, and corn. Depending on the method for introducing the recombinant constructs into the host cell, other DNA sequences may be required. Importantly, this invention is applicable to

dicotyledons and monocotyledons species alike and will be readily applicable to new and/or improved transformation and regulation techniques.

Of particular interest, is the use of the expression constructs of the present invention in plants to produce plants or plant parts, including, but not limited to leaves, stems, roots, reproductive, and seed, with a modified fatty acid composition and/or yield of the fatty acid content.

Thus, the present invention provides methods for enhancing the content or altering the composition of fatty acids and compounds containing such fatty acids, such as oils, waxes, fats, and storage proteins. Such methods employ the use of the expression constructs described herein for the modification of the host plant cell's β -oxidation pathway.

The present invention further provides methods for inhibiting or postponing germination in seeds having an introduced expression construct providing for the suppression of the host cells endogenous ACOX and/or thiolase in the seed of the host cell.

The present invention also provides methods for the production of particular fatty acids in host plant cells.

The expression constructs of the present invention can be used in conjunction with additional expression constructs employing nucleic acid sequences encoding fatty acid biosynthetic proteins. Such sequences encoding fatty acid biosynthetic proteins are known in the art and include, but are not limited to, thioesterases (see for example, U.S. Patent Number 5,667,997), β -ketoacyl-ACP synthases (KAS)(see for example U.S. Patent Number 5,475,099) , desaturases, and the like.

Furthermore, more than one additional sequence encoding fatty acid biosynthetic protein can be used in conjunction with the expression constructs of the present invention. For example, the expression constructs of the present invention can be used in conjunction with expression constructs providing the expression of a thioesterase and an expression construct providing for the expression of a β -ketoacyl-ACP synthase (see for example, PCT Publication WO 98/46776).

Thus, the present invention also provides methods for the production of particular fatty acids in a host plant cell. Such methods use the expression constructs of the present invention in conjunction with at least one additional expression construct having a nucleic acid sequence encoding a protein involved in fatty acid biosynthesis. Preferably the ACOX and/or thiolase expression construct used provides for the suppression of endogenous host

plant cells ACOX and/or thiolase. The additional expression constructs employed can provide for the expression or suppression of fatty acid biosynthesis proteins.

It is contemplated that the gene sequences may be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences. Thus, all or a
5 portion of the desired structural gene (that portion of the gene which encodes the protein) may be synthesized using codons preferred by a selected host. Host-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a desired host species.

To confirm the activity and specificity of the proteins encoded by the identified
10 nucleic acid sequences as ACOX or thiolase proteins, *in vitro* assays are performed in insect cell cultures using baculovirus expression systems. Such baculovirus expression systems are known in the art and are described by Lee, *et al.* U.S. Patent Number 5,348,886, the entirety of which is herein incorporated by reference.

In addition, other expression constructs may be prepared to assay for protein activity
15 utilizing different expression systems. Such expression constructs are transformed into yeast or prokaryotic host and assayed for ACOX or thiolase activity. Such expression systems are known in the art and are readily available through commercial sources.

The method of transformation in obtaining such transgenic plants is not critical to the instant invention, and various methods of plant transformation are currently available.
20 Furthermore, as newer methods become available to transform crops, they may also be directly applied hereunder. For example, many plant species naturally susceptible to *Agrobacterium* infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left
25 and right borders, more particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation. In addition, techniques of microinjection, DNA particle bombardment, and electroporation have been developed which allow for the transformation of various monocot and dicot plant species.

30 Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral

immunity or the like. Depending upon the number of different host species the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used
5 which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall formation), the latter being permissible, so long as the *vir* genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a
10 mixture of normal plant cells and gall.

In some instances where *Agrobacterium* is used as the vehicle for transforming host plant cells, the expression or transcription construct bordered by the T-DNA border region(s) will be inserted into a broad host range vector capable of replication in *E. coli* and *Agrobacterium*, there being broad host range vectors described in the literature. Commonly
15 used is pRK2 or derivatives thereof. See, for example, Ditta, *et al.*, (*Proc. Nat. Acad. Sci., U.S.A.* (1980) 77:7347-7351) and EPA 0 120 515, which are incorporated herein by reference. Alternatively, one may insert the sequences to be expressed in plant cells into a vector containing separate replication sequences, one of which stabilizes the vector in *E. coli*, and the other in *Agrobacterium*. See, for example, McBride and Summerfelt (*Plant Mol. Biol.*
20 (1990) 14:269-276), wherein the pRiHRI (Jouanin, *et al.*, *Mol. Gen. Genet.* (1985) 201:370-374) origin of replication is utilized and provides for added stability of the plant expression vectors in host *Agrobacterium* cells.

Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed *Agrobacterium* and transformed plant cells. A
25 number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, kanamycin, the aminoglycoside G418, hygromycin, or the like. The particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

For transformation of plant cells using *Agrobacterium*, explants may be combined and
30 incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for

regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of vegetable oils.

There are several possible ways to obtain the plant cells of this invention which contain multiple expression constructs. Any means for producing a plant comprising a construct having a DNA sequence encoding the expression construct of the present invention, and at least one other construct having another DNA sequence encoding an enzyme are encompassed by the present invention. For example, the expression construct can be used to transform a plant at the same time as the second construct either by inclusion of both expression constructs in a single transformation vector or by using separate vectors, each of which express desired genes. The second construct can be introduced into a plant which has already been transformed with the ACOX or thiolase expression construct, or alternatively, transformed plants, one expressing the ACOX or thiolase construct and one expressing the second construct, can be crossed to bring the constructs together in the same plant.

Thus, by expression of the nucleic acid sequences encoding the plant β -oxidation sequences of the present invention in a host cell, it is possible to modify the lipid content and/or composition of the host cell. Furthermore, it is also possible to inhibit or delay the germination of seeds of plant containing the expression constructs of the present invention.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

Example 1 Characterization of Developing and Germinating Seeds

Seeds of *Brassica* plants containing the constructs pCGN5401 for the expression of ChKAS A and pCGN4804 for the expression of the *Cuphea pulcheriama* FatB1 thioesterase (described in WO 98/46776, the entirety of which is incorporated herein by reference) were analyzed for activity of enzymes involved in β -oxidation. Acyl-CoA oxidase assays were performed on wild-type *Brassica napus* plants as well as on transgenic plants containing different levels of medium chain fatty acids. Plants were grouped into three groups, high level MCFA containing lines, medium level MCFA containing lines, and null lines.

The results demonstrate that short, medium, and long chain acyl-CoA oxidase enzymes are active at all time points in all seeds examined during seed development. The short chain ACOX is the most active enzyme at all time points, with the exception of 40 days

post anthesis (dpa) wild-type seeds. The highest activity for short chain ACOX as contrasted to the wild-type is measured at 30 dpa laurate producing seed (U.S. Patent Number 5,344,771, the entirety of which is incorporated herein by reference) and 40 dpa MCFA (lines containing pCGN5401 and pCGN4804) (Figure 1).

Furthermore, five day old germinating seeds from wild-type, null, medium, and high MCFA producing *Brassica* lines were assayed for ACOX activity. The results demonstrate that in MCFA producing seeds, short chain ACOX was most active whereas in the wild-type and the null, the medium chain ACOX showed the highest activity (Figure 1).

Example 2 Identification of Acyl CoA Oxidase and Thiolase Sequences

Nucleic acid sequences were identified in genomic DNA sequence databases using various ACOX sequences. The results of the searches in databases containing *Arabidopsis* sequences identified two un-annotated sequences related to ACOX and one un-annotated sequence related to peroxisomal thiolases. These sequences are referred to as At ACX1-1 (SEQ ID NO:3), At ACX3 (SEQ ID NO:7), and At PED1-1 (SEQ ID NO:13), respectively. In addition, related sequences are also identified in databases containing genomic DNA sequences from *Brassica*. These sequences are referred to as Br ACX1 (SEQ ID NO:17), Br ACX2 (SEQ ID NO:19), Br PED1 (SEQ ID NO:21), Br PKT2 (SEQ ID NO:23) and Br SACOX (SEQ ID NO:26).

Sequence alignments between the ACOX sequences identified herein and previously identified ACOX sequences are compared to identify the similarity between the sequences. Both nucleic acid as well as amino acid sequences are aligned.

Example 4 Expression constructs

4A. *E. coli* Expression Constructs

Constructs are prepared to direct the expression of the *Arabidopsis* and *Brassica* ACOX and thiolase sequences in *E. coli*. The entire coding region of the ACOX and thiolase sequence is amplified using polymerase chain reaction (PCR). The PCR products was subcloned into PQE30 (Qiagen). Double stranded DNA sequence was obtained to verify that no errors were introduced by PCR amplification. The plasmid pCGN10407 contains the coding sequence of AtACX2 for expression in *E. coli*. The plasmid pCGN10408 contains the coding sequence of BrACX2 for expression in *E. coli*. The plasmid pCGN10409 contains the coding sequence of AtACX3 for expression in *E. coli*. The plasmid pCGN10410 contains the

coding sequence of AtACX1 for expression in *E. coli*. The plasmid pCGN10411 contains the coding sequence of AtACX1-1 for expression in *E. coli*. The plasmid pCGN10412 contains the coding sequence of BrACX1 for expression in *E. coli*.

5 4B. Plant Expression Construct Preparation

A plasmid containing the napin cassette derived from pCGN3223 (described in USPN 5,639,790, the entirety of which is incorporated herein by reference) was modified to make it more useful for cloning large DNA fragments containing multiple restriction sites, and to allow the cloning of multiple napin fusion genes into plant binary transformation vectors. An
 10 adapter comprised of the self annealed oligonucleotide of sequence
 CGCGATTAAATGGCGCGCCCTGCAGGCGGCCGCCTGCAGGGCGCGCCATTAA
 AT (SEQ ID NO:25) was ligated into the cloning vector pBC SK+ (Stratagene) after digestion with the restriction endonuclease BssHII to construct vector pCGN7765. Plasmids
 pCGN3223 and pCGN7765 were digested with NotI and ligated together. The resultant
 15 vector, pCGN7770, contains the pCGN7765 backbone with the napin seed specific expression cassette from pCGN3223.

The construct pCGN9873 contains the BrACX1 coding sequence in the antisense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation
 20 region.

The construct pCGN9874 contains the BrACX2 coding sequence in the antisense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region.

25 The construct pCGN9875 contains the BrPKT2 coding sequence in the antisense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region.

30 The construct pCGN9876 contains the AtACX1 coding sequence in the antisense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region.

The construct pCGN9877 contains the BrACX2 coding sequence in the antisense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region.

5 The construct pCGN9878 contains the AtPED1 coding sequence in the antisense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region.

10 The construct pCGN9879 contains the AtPKT2 coding sequence in the antisense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region.

15 The construct pCGN10413 contains the AtACX1 coding sequence in the sense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region. The AtACX1 expression construct is oriented in the same direction of transcription as the Cpu Fat B1 expression construct.

20 The construct pCGN10414 contains the AtACX1 coding sequence in the sense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region. The AtACX1 expression construct is oriented in the opposite direction of transcription from the Cpu Fat B1 expression construct.

25 The construct pCGN10416 contains the BrACX2 coding sequence in the sense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region. The BrACX2 expression construct is oriented in the opposite direction of transcription from the Cpu Fat B1 expression construct.

30 The construct pCGN10417 contains the AtPED1 coding sequence in the sense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region. The AtPED1 expression construct is oriented in the same direction of transcription as the Cpu Fat B1 expression construct.

The construct pCGN10419 contains the AtPKT2 coding sequence in the sense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region. The AtPKT2 expression construct is oriented in the same direction of transcription as the Cpu Fat B1 expression construct.

The construct pCGN10421 contains the AtACX1-1 coding sequence in the sense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region. The AtACX1-1 expression construct is oriented in the same direction of transcription as the Cpu Fat B1 expression construct.

The construct pCGN10423 contains the AtACX1-1 coding sequence in the antisense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region. The AtACX1-1 expression construct is oriented in the opposite direction of transcription from the Cpu Fat B1 expression construct.

The construct pCGN10424 contains the AtPED1-1 coding sequence in the sense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region. The AtPED1-1 expression construct is oriented in the same direction of transcription as the Cpu Fat B1 expression construct.

The construct pCGN10425 contains the AtPED1-1 coding sequence in the sense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region. The AtPED1-1 expression construct is oriented in the opposite direction of transcription from the Cpu Fat B1 expression construct.

The construct pCGN10426 contains the AtPED1-1 coding sequence in the antisense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation region. The AtPED1-1 expression construct is oriented in the same direction of transcription as the Cpu Fat B1 expression construct.

The construct pCGN10417 contains the BrPED1 coding sequence in the antisense orientation under the control of the napin transcription initiation region and the *Cuphea pullcherrima* Fat B1 coding sequence under the control of the napin transcription initiation

region. The BrPED1 expression construct is oriented in the same direction of transcription as the Cpu Fat B1 expression construct.

Example 5 Plant Transformation

5 A variety of methods have been developed to insert a DNA sequence of interest into the genome of a plant host to obtain the transcription or transcription and translation of the sequence to effect phenotypic changes.

The plant binary constructs described above are used in plant transformation to direct the expression of the ACOX and thiolase sequences from plant tissues.

10 Transgenic *Brassica* plants are obtained by *Agrobacterium*-mediated transformation as described by Radke *et al.* (*Theor. Appl. Genet.* (1988) 75:685-694; *Plant Cell Reports* (1992) 11:499-505). Transgenic *Arabidopsis thaliana* plants may be obtained by *Agrobacterium*-mediated transformation as described by Valverkens *et al.*, (*Proc. Nat. Acad. Sci.* (1988) 85:5536-5540), or as described by Bent *et al.* ((1994), *Science* 265:1856-1860), or
15 Bechtold *et al.* ((1993), *C.R.Acad.Sci, Life Sciences* 316:1194-1199). Other plant species may be similarly transformed using related techniques.

Alternatively, microprojectile bombardment methods, such as described by Klein *et al.* (*Bio/Technology* 10:286-291) may also be used to obtain nuclear transformed plants.

20 Example 7 Analysis of Transgenic Plants

Transgenic plants containing the expression constructs described above ACOX and thiolase sequences are analyzed for oil composition and content using techniques known in the art. Furthermore, seeds of transgenic plants are screened for germination rates.

25 The above results demonstrate that the nucleic acid sequences identified encode proteins which are involved in fatty acid β -oxidation. Such sequences find use in preparing expression constructs for plant transformations. The expression constructs provide a means for modifying the lipid content and composition in host plant cells, as well as for the inhibition or postponing of germination of seeds of plants containing constructs having such
30 sequences.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as

if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that
5 certain changes and modifications may be practiced within the scope of the appended claim.

SEQUENCE LISTING

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<120> Nucleic Acid Sequences encoding Proteins
Involved in Fatty Acid Beta-Oxidation and Methods of Use.

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<140> New Application

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Leu Asn Glu Glu Glu Ala Gly Arg Leu Arg His Phe Ile Asp Gln Pro
85     90     95
Ala Tyr Val Asp Leu His Trp Gly Met Phe Val Pro Ala Ile Lys Gly
100    105    110
Gln Gly Thr Glu Glu Gln Gln Lys Lys Trp Leu Ser Leu Ala Asn Lys
115    120    125
Met Gln Ile Ile Gly Cys Tyr Ala Gln Thr Glu Leu Gly His Gly Ser
130    135    140
Asn Val Gln Gly Leu Glu Thr Thr Ala Thr Phe Asp Pro Lys Thr Asp
145    150    155    160
Glu Phe Val Ile His Thr Pro Thr Gln Thr Ala Ser Lys Trp Trp Pro
165    170    175
Gly Gly Leu Gly Lys Val Ser Thr His Ala Val Val Tyr Ala Arg Leu
180    185    190
Ile Thr Asn Gly Lys Asp Tyr Gly Ile His Gly Phe Ile Val Gln Leu
195    200    205
Arg Ser Leu Glu Asp His Ser Pro Leu Pro Asn Ile Thr Val Gly Asp
210    215    220
Ile Gly Thr Lys Met Gly Asn Gly Ala Tyr Asn Ser Met Asp Asn Gly
225    230    235    240
Phe Leu Met Phe Asp His Val Arg Ile Pro Arg Asp Gln Met Leu Met
245    250    255
Arg Leu Ser Lys Val Thr Arg Glu Gly Glu Tyr Val Pro Ser Asp Val
260    265    270
Pro Lys Gln Leu Val Tyr Gly Thr Met Val Tyr Val Arg Gln Thr Ile
275    280    285
Val Ala Asp Ala Ser Asn Ala Leu Ser Arg Ala Val Cys Ile Ala Thr

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	Leu	Leu	Ala	Ser	Ala	Tyr	Ala	Phe	Arg	Phe	Val	Gly	Glu	Trp	Leu	Lys
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	Leu	Gln	Cys	Arg	Ser	Gly	Val	Gln	Lys	Ala	Glu	Asp	Trp	Leu	Asn	Pro
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	Asp	Val	Val	Leu	Glu	Ala	Phe	Glu	Ala	Arg	Ala	Leu	Arg	Met	Ala	Val
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	Thr	Cys	Ala	Lys	Asn	Leu	Ser	Lys	Phe	Glu	Asn	Gln	Glu	Gln	Gly	Phe
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	Leu	Ile	Val	Val	Ser	Lys	Phe	Ile	Ala	Lys	Leu	Glu	Gln	Asp	Ile	Gly
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	Leu	Tyr	Leu	Leu	His	Lys	His	Leu	Gly	Asp	Phe	Leu	Ser	Thr	Asn	Cys
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	Tyr	Thr	Gln	Val	Arg	Pro	Asn	Ala	Val	Ala	Leu	Val	Asp	Ala	Phe	Asn
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 <213> Arabidopsis sp.

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<211> 664

<212> PRT

<213> Arabidopsis sp.

<400> 4

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 35           40           45
Glu Lys Ser Lys Arg Ala Val Met Ser Arg Lys Glu Leu Phe Lys Asn
 50           55           60
Thr Leu Arg Lys Ser Val His Ala Trp Lys Leu Ile Asn Glu Leu Arg
 65           70           75           80
Leu Ser Asp Glu Glu Gly Leu Lys Leu Arg Ser Phe Met Asp Gln Pro
 85           90           95
Gly Phe Leu Asp Leu His Trp Gly Met Phe Val Pro Ala Ile Lys Gly
100          105          110
Gln Gly Thr Glu Glu Gln Gln Gln Lys Trp Leu Ser Leu Ala Thr Lys

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- 5 -

- 6 -

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 <213> Arabidopsis sp.

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			20					25					30		
Pro	Leu	Val	Gln	Thr	Glu	Thr	Cys	Ser	Ala	Arg	Ser	Lys	Lys	Leu	Asp
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Val	Asn	Gly	Glu	Ala	Leu	Ser	Leu	Tyr	Met	Arg	Gly	Lys	His	Ile	Asp
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Ile	Gln	Glu	Lys	Ile	Phe	Asp	Phe	Phe	Asn	Ser	Arg	Pro	Asp	Leu	Gln
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Thr	Pro	Ile	Glu	Ile	Ser	Lys	Asp	Asp	His	Arg	Glu	Leu	Cys	Met	Asn
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Gln	Leu	Ile	Gly	Leu	Val	Arg	Glu	Ala	Gly	Val	Arg	Pro	Phe	Arg	Tyr
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Ser	Val	Asp	Met	Ser	Leu	Gly	Ile	Lys	Met	Gly	Val	Gln	Tyr	Ser	Leu
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Trp	Gly	Gly	Ser	Val	Ile	Asn	Leu	Gly	Thr	Lys	Lys	His	Arg	Asp	Lys
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Tyr	Phe	Asp	Gly	Ile	Asp	Asn	Leu	Asp	Tyr	Thr	Gly	Cys	Phe	Ala	Met
				165					170					175	
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			180					185					190		
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Arg	Asp	Gly	Thr	Tyr	Thr	Ser	Ser	Leu	Pro	Thr	Ile	Asn	Lys	Arg	Phe
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Gly	Ala	Thr	Leu	Gly	Glu	Leu	Val	Gly	Gly	Arg	Val	Gly	Leu	Ala	Tyr
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Ala	Ser	Val	Gly	Val	Leu	Lys	Ile	Ser	Ala	Thr	Ile	Ala	Ile	Arg	Tyr
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Ser Leu Leu Arg Gln Gln Phe Gly Pro Pro Lys Gln Pro Glu Val Ser
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 Ile Leu Asp Tyr Gln Ser Gln Gln His Lys Leu Met Pro Met Leu Ala
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 Ser Thr Tyr Ala Tyr His Phe Ala Thr Val Tyr Leu Val Glu Lys Tyr
 385 390 395 400
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 Ala Leu Ser Ala Gly Leu Lys Ser Tyr Val Thr Ser Tyr Thr Ala Lys
 420 425 430
 Ala Leu Ser Val Cys Arg Glu Ala Cys Gly Gly His Gly Tyr Ala Ala
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 Pro Val Thr Ala Arg Trp Glu Gly Glu Asp His Leu Arg Asp Pro Lys
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 Pro Asp Pro Ser Ala Lys Ala Ala Leu Lys Leu Ala Cys Asp Leu Tyr
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 Tyr Val Ala Pro Asn Lys Ala Lys Ala Ile His Lys Leu Thr Glu Tyr
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<211> 2025

<212> DNA

<213> Arabidopsis sp.

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<213> Arabidopsis sp.

<220>

<221> VARIANT

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Asp Val Lys Glu Met Arg Lys Leu Leu Asp Gly His Asn Val Val Asp
          50          55          60
Arg Asp Trp Ile Tyr Gly Leu Met Met Gln Ser Asn Leu Phe Asn Arg
65          70          75          80
Lys Glu Arg Gly Gly Lys Ile Phe Val Ser Pro Asp Tyr Asn Gln Thr
          85          90          95
Met Glu Gln Gln Arg Glu Ile Thr Met Lys Arg Ile Trp Tyr Leu Leu
100          105          110
Glu Asn Gly Val Phe Lys Gly Trp Leu Thr Glu Thr Gly Pro Glu Ala

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- 10 -

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Ser Val Lys Asp Val Leu Gly Leu Val Arg Ser Met Tyr Ala Leu Ile
      595      600      605
Ser Leu Glu Glu Asp Pro Ser Leu Leu Arg Tyr Gly Tyr Leu Ser Gln
      610      615      620
Asp Asn Val Gly Asp Val Arg Arg Glu Val Ser Lys Leu Cys Gly Glu
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Leu Arg Pro His Ala Leu Ala Leu Val Thr Ser Phe Gly Ile Pro Asp
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      675

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 <212> PRT
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 35          40          45
Tyr His Phe Asn Asp Leu Leu Thr Pro Glu Glu Gln Ala Ile Arg Lys
 50          55          60
Lys Val Arg Glu Cys Met Glu Lys Glu Val Ala Pro Ile Met Thr Glu
 65          70          75          80
Tyr Trp Glu Lys Ala Glu Phe Pro Phe His Ile Thr Pro Lys Leu Gly
 85          90          95
Ala Met Gly Val Ala Gly Gly Ser Ile Lys Gly Tyr Gly Cys Pro Gly
 100          105          110
Leu Ser Ile Thr Ala Asn Ala Ile Ala Thr Ala Glu Ile Ala Arg Val
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Pro Asp Asn Gly Ser Asp Ala Ser Gly Leu Gly Thr Thr Ala Thr Lys
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Val Glu Gly Gly Trp Lys Ile Asn Gly Gln Lys Arg Trp Ile Gly Asn
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Ser Thr Phe Ala Asp Leu Leu Ile Ile Phe Ala Arg Asn Thr Thr Thr
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 245          250          255
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 325          330          335
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 340          345          350
Thr Gly Gln Met Thr Pro Gly Gln Ala Ser Leu Gly Lys Ala Trp Ile
 355          360          365
Ser Ser Lys Ala Arg Glu Thr Ala Ser Leu Gly Arg Glu Leu Leu Gly
 370          375          380
Gly Asn Gly Ile Leu Ala Asp Phe Leu Val Ala Lys Ala Phe Cys Asp
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420

425

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<213> Arabidopsis sp.

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<211> 1677

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Lys	Asp	Ser	Ala	Ala	Tyr	Gln	Tyr	Gly	Asp	Asp	Val	Val	Ile	Val	Ala
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Ala	Gln	Arg	Thr	Ala	Leu	Cys	Lys	Ala	Lys	Arg	Gly	Ser	Phe	Lys	Asp
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Thr	Phe	Pro	Asp	Glu	Leu	Leu	Ala	Ser	Val	Leu	Arg	Ala	Leu	Ile	Glu
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Lys	Thr	Asn	Val	Asn	Pro	Ser	Glu	Val	Gly	Asp	Ile	Val	Val	Gly	Thr
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Phe	Tyr	Ala	Gly	Phe	Pro	Glu	Thr	Val	Pro	Ile	Arg	Thr	Val	Asn	Arg
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Gln	Cys	Ser	Ser	Gly	Leu	Gln	Ala	Val	Ala	Asp	Val	Ala	Ala	Ala	Ile
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 260 265 270
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Ser Pro Val Asn Cys Val Ser Glu Val Ser Pro Met Ala Ala Phe Gly
          35          40          45
Asp Asp Ile Val Ile Val Ala Ala Tyr Arg Thr Ala Ile Cys Lys Ala
          50          55          60
Arg Arg Gly Gly Phe Lys Asp Thr Leu Pro Asp Asp Leu Leu Ala Ser
65          70          75          80
Val Leu Lys Ala Val Val Glu Arg Thr Ser Leu Asp Pro Ser Glu Val
          85          90          95
Gly Asp Ile Val Val Gly Thr Val Ile Ala Pro Gly Ser Gln Arg Ala
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Met Glu Cys Arg Val Ala Ala Tyr Phe Ala Gly Phe Pro Asp Ser Val
          115          120          125
Pro Val Arg Thr Val Asn Arg Gln Cys Ser Ser Gly Leu Gln Ala Val
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Ala Asp Val Ala Ala Ser Ile Arg Ala Gly Tyr Tyr Asp Ile Gly Ile
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Phe His Gly Ser Asn Pro Arg Ala Gln Asp Phe Pro Lys Ala Arg Asp
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Gly Val Thr Arg Glu Glu Gln Asp Met Ala Ala Val Glu Ser His Lys
210          215          220
Arg Ala Ala Ala Ala Ile Ala Ser Gly Lys Leu Lys Asp Glu Ile Ile

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Met	Lys	Arg	Ser	Leu	Ala	Met	Lys	Lys	Gly	Leu	Pro	Ile	Leu	Gly	Val
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Phe	Arg	Ser	Phe	Ala	Val	Thr	Gly	Val	Glu	Pro	Ser	Val	Met	Gly	Ile
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Tyr	Val	Tyr	Ser	Cys	Lys	Lys	Leu	Glu	Leu	Asp	Met	Glu	Lys	Val	Asn
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 <212> DNA
 <213> Brassica sp.

<400> 17

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 <212> PRT
 <213> Brassica sp.

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Glu Lys Ser Asp Arg Ala Arg Leu Ser Arg Lys Glu Leu Phe Lys Ser
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Thr Leu Lys Lys Cys Ala His Ala Trp Lys Arg Ile Ile Glu Leu Arg
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Leu Thr Glu Glu Glu Ala Gly Arg Leu Arg Phe Phe Val Asp Gln Pro
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Ala Phe Val Asp Leu His Trp Gly Met Phe Val Pro Ala Ile Lys Gly
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Gln Gly Thr Glu Glu Gln Gln Glu Lys Trp Leu Ser Leu Ala Asn Lys
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Met Gln Ile Ile Gly Cys Tyr Ala Gln Thr Glu Leu Gly His Gly Ser
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Asn Val Gln Gly Leu Glu Thr Thr Ala Thr Phe Asp Pro Lys Thr Asp
145         150         155         160
Glu Phe Val Ile His Ser Pro Thr Gln Thr Ser Ser Lys Trp Trp Pro
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Gly Gly Leu Gly Lys Val Ser Thr His Ala Val Val Tyr Ala Arg Leu
          180         185         190
Ile Thr Asp Gly Lys Asp Tyr Gly Val His Gly Phe Ile Val Gln Leu
          195         200         205
Arg Ser Leu Glu Asp His Ser Pro Leu Pro Asn Ile Ile Val Gly Asp
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Ile Gly Thr Lys Met Gly Asn Gly Ala Tyr Asn Ser Met Asp Asn Gly

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WO 00/75350

PCT/US00/16149

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 <212> DNA
 <213> Brassica sp.

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 <212> PRT
 <213> Brassica sp.

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Lys	Val	His	Glu	Phe	Tyr	Asn	Ser	Arg	Pro	Asp	Leu	Gln	Thr	Pro	Leu
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Leu Arg Glu Ser Met	Ser Ser Tyr Leu Ala Gln	Pro Asn Pro Val Thr
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Ala Arg Trp Glu Gly	Glu Asp His Leu Arg Asp	Pro Lys Phe Gln Leu
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Asp Ala Phe Arg Tyr	Arg Thr Ser Arg Leu Leu	Gln Ser Val Ala Met
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Ser Ala Arg Ala Gly	Leu Lys Leu Val Cys	Asp Leu Tyr Ala Leu Asp
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Arg Ile Trp Asn Asp	Ile Gly Thr Tyr Arg	Asn Val Asp Tyr Val Ala
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<211> 490

<212> PRT

<213> Brassica sp.

<400> 22

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Ser Lys Arg Gly Asn Phe Lys Asp Thr Tyr Pro Asp Asp Leu Leu Ala
 65     70     75     80
Pro Val Leu Arg Ala Leu Ile Glu Lys Thr Asn Leu Asp Pro Ser Glu
 85     90     95
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115    120    125
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130    135    140
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Ile Gly Ala Gly Leu Glu Ser Met Thr Thr Asn Pro Met Ala Trp Glu
165    170    175
Gly Ser Val Asn Pro Ala Val Lys Lys Phe Glu Gln Ala Gln Asn Cys
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Ala	Ser	Arg	Gly	Arg	Ser	Arg	Leu								
		435					440								

Claims

What is Claimed is:

1. An isolated DNA sequence having a sequence selected from the group consisting
5 of SEQ ID NOs:1, 3, 5, 7, 9, 17, 19, and 26.
2. The isolated DNA sequence according to Claim 1, wherein said nucleic acid
sequence encodes acyl-CoA oxidase.
- 10 3. An isolated DNA sequence, wherein said nucleic acid sequence is complementary
to a polynucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9,
17, 19, and 26.
4. An isolated DNA sequence having the sequence selected from a group consisting
15 of SEQ ID NOs:11, 13, 15, 21, and 23.
5. The isolated DNA sequence according to Claim 4, wherein said nucleic acid
sequence encodes 3-keto thiolase.
- 20 6. An isolated DNA sequence wherein said nucleic acid sequence is complementary
to a polynucleotide sequence selected from the group consisting of SEQ ID NOs:11, 13, 15,
21, and 23.
7. A nucleic acid construct comprising as operably linked components in the 5' to 3'
25 direction of transcription:
a transcriptional initiation region; and
a polynucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5,
7, 9, 11, 13, 15, 17, 19, 21, 23, and 26.
- 30 8. A host cell comprising the DNA construct of Claim 7.
9. The host cell according to Claim 8, wherein said host cell is selected from the
group consisting of bacterial, insect, fungal, mammalian, and plant.

10. A plant comprising the host cell of Claim 9.

11. A method for producing a recombinant host cell, comprising:

5 transforming or transfecting a cell with a nucleic acid construct comprising a transcriptional initiation region and a polynucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, and 26, such that said host cell, under appropriate culture conditions, produces a protein encoded by said polynucleotide sequence.

10 12. The method according to claim 11 wherein said host cell is a plant cell.

13. A method of modifying the oil composition in a plant cell, said method comprising:

15 transforming a plant cell with a nucleic acid construct comprising a transcriptional initiation region and a polynucleotide sequence encoding an enzyme active in fatty acid beta-oxidation, or a fragment or variant thereof.

20 14. The method according to Claim 13, wherein said polynucleotide sequence encodes an enzyme selected from the group consisting of acyl-CoA oxidase and 3-ketoacyl-CoA thiolase.

25 15. The method according to Claim 14, wherein said polynucleotide sequence is obtained from a plant selected from the group consisting of *Arabidopsis* and *Brassica*.

16. The method according to Claim 13, wherein said polynucleotide sequence is in an antisense orientation.

30 17. The method according to Claim 13, wherein said polynucleotide sequence is in a sense orientation.

18. The method according to Claim 13, wherein the activity of the endogenous fatty acid beta-oxidation is suppressed.

19. The method according to Claim 13, wherein the activity of the endogenous fatty acid beta-oxidation is enhanced.

5 20. A method of modifying the lipid composition in a plant cell, said method comprising:

transforming a plant cell with a first nucleic acid construct comprising a transcriptional initiation region and a first polynucleotide sequence selected from the group consisting of a polynucleotide according to claim 1 and a polynucleotide according to claim 4,
10 such that said plant cell, under appropriate culture conditions, produces a protein involved in fatty acid beta-oxidation.

21. The method according to Claim 20 wherein said polynucleotide sequence is in an antisense orientation, whereby transcribed mRNA from said sequence is complementary to
15 the equivalent mRNA transcribed from the endogenous gene and the activity of said fatty acid beta-oxidation in said plant cell is suppressed.

22. The method according to Claim 21, wherein said polynucleotide sequence encodes an enzyme selected from the group consisting of acyl-CoA oxidase and 3-ketoacyl-CoA thiolase.
20

23. The method according to Claim 21, wherein said polynucleotide sequence is obtained from a plant selected from the group consisting of *Arabidopsis* and *Brassica*.

25 24. The method according to Claim 20, wherein said polynucleotide sequence is in a sense orientation.

25 25. The method according to Claim 20, wherein said method further comprises the step of, transforming a plant cell with a second nucleic acid construct having a transcriptional initiation region and a second polynucleotide sequence encoding a protein involved in fatty acid biosynthesis.
30

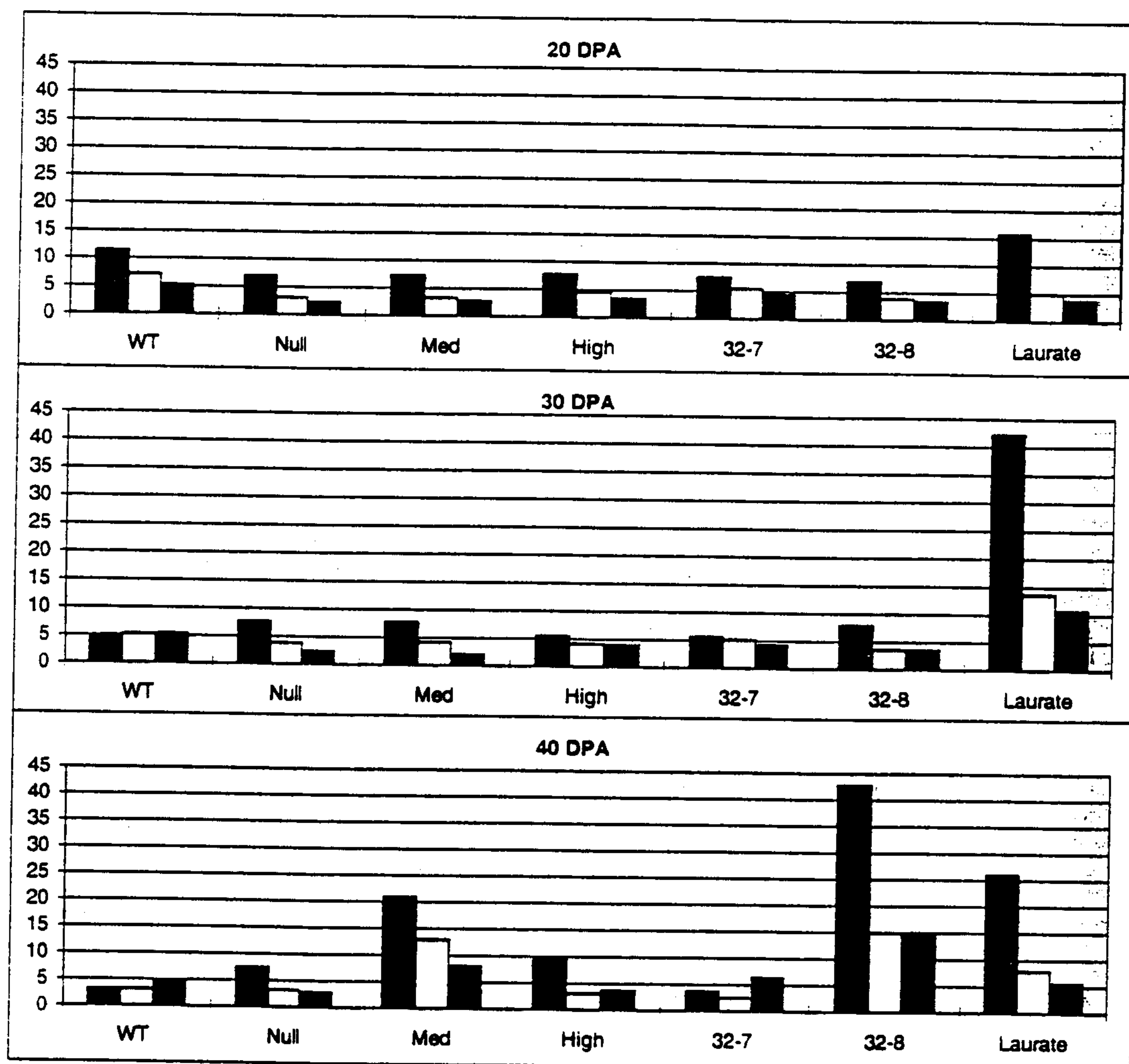
26. The method according to Claim 25, wherein said second polynucleotide sequence encodes a protein selected from the group consisting of beta-ketoacyl-ACP synthase, desaturase, and thioesterase.

5

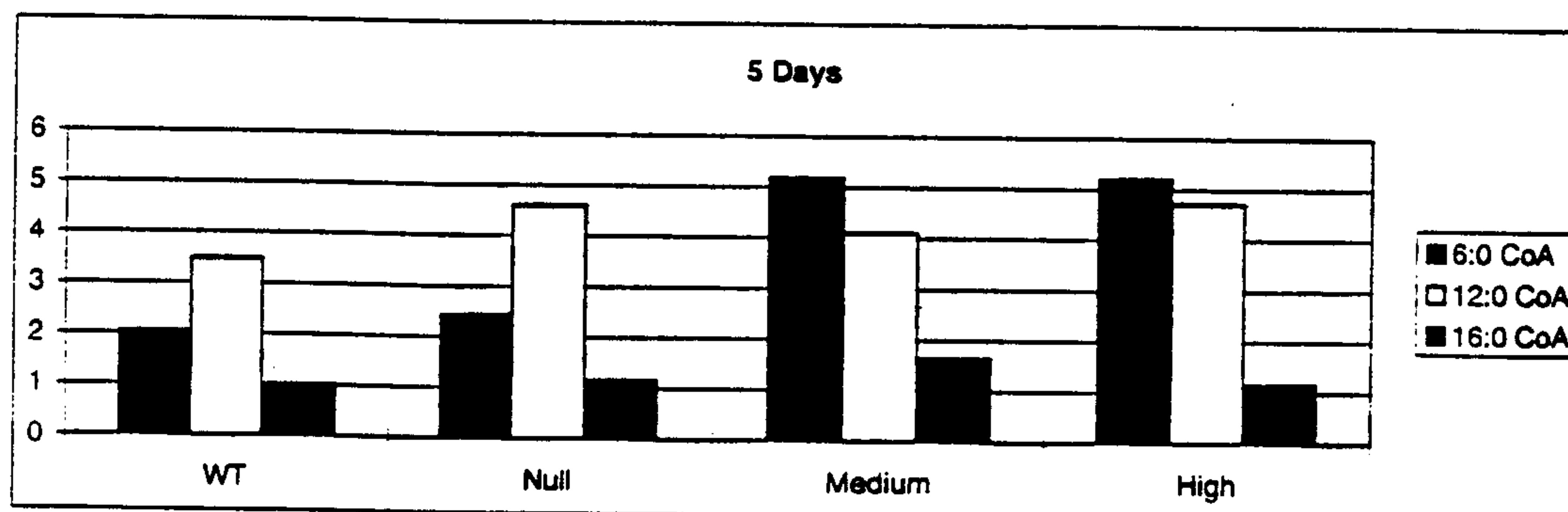
27. An isolated polypeptide having a sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 27.

ACOX Activity (mU/mg)

A. Developing Seeds

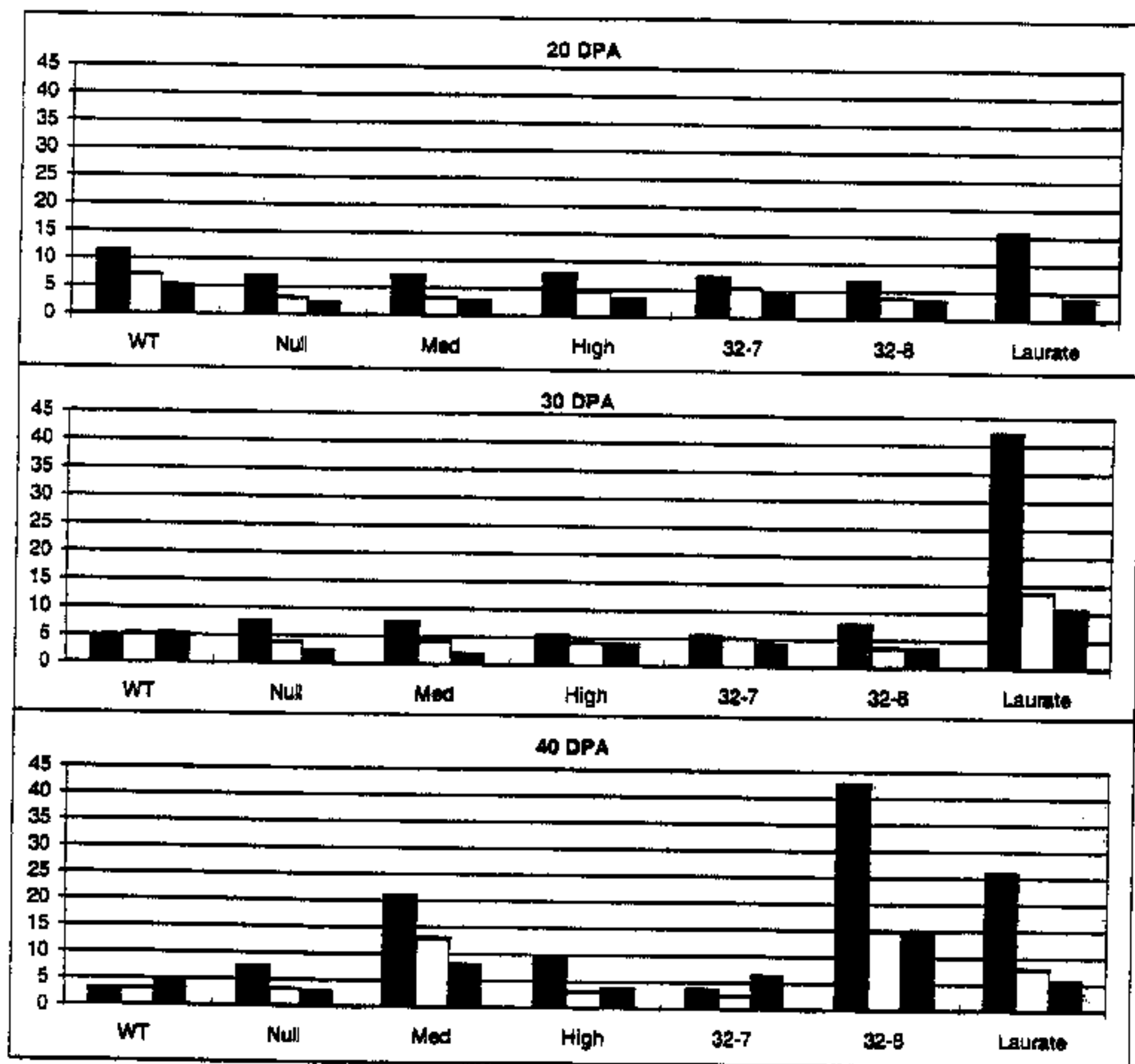


B. Germinating Seeds

FIGURE 1
1 / 1

ACOX Activity (mU/mg)

A. Developing Seeds



B. Germinating Seeds

