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(72) Inventeurs/Inventors:
OHLROGGE, JOHN B., US;
RUUSKA, SARI A., AU;
LI, YONGHUA, US

(73) Propriétaire/Owner:
BOARD OF TRUSTEES OF MICHIGAN STATE UNIVERSITY, US

(74) Agent: SMART & BIGGAR

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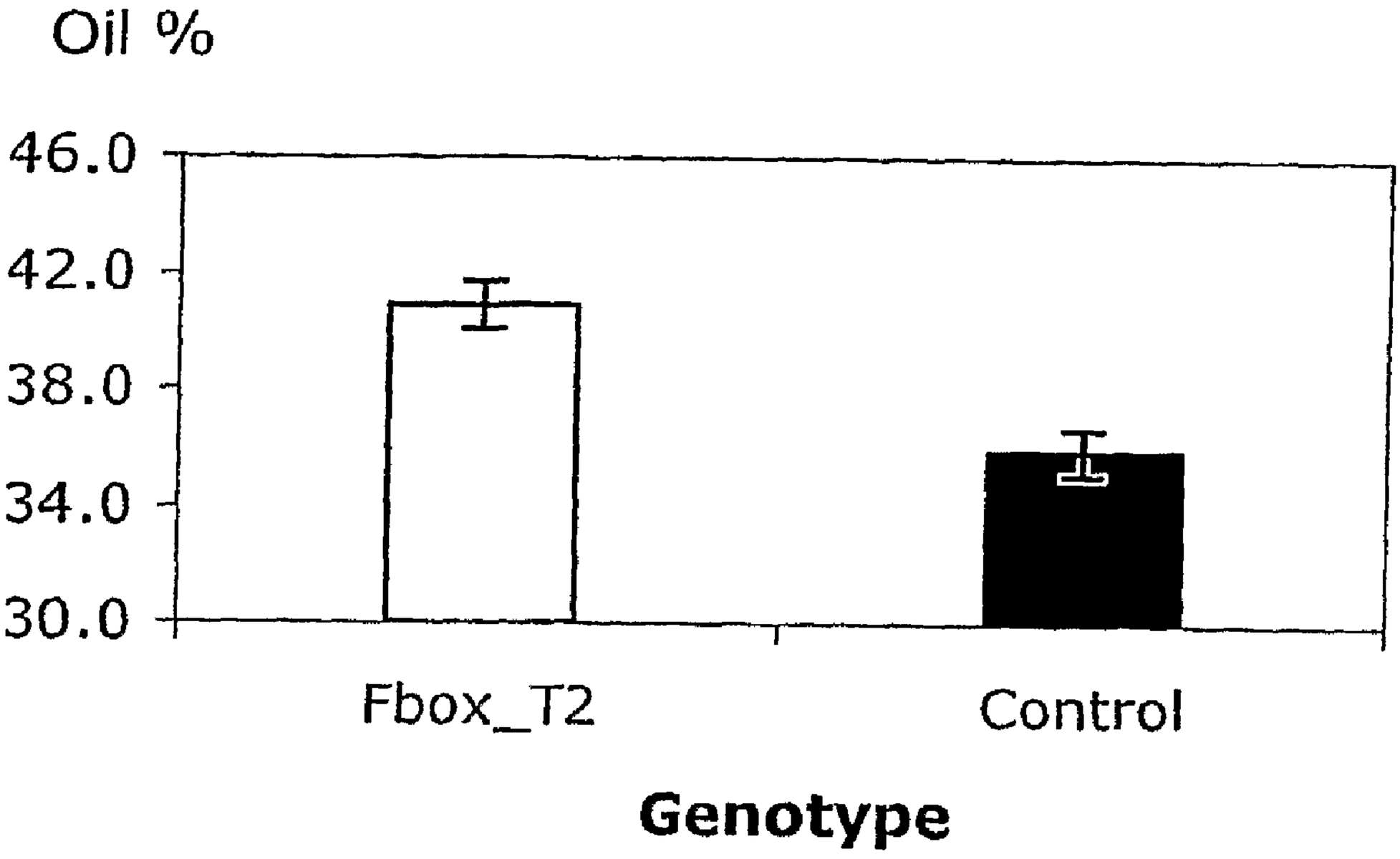

(57) Abrégé/Abstract:
Disclosed are genetic constructs, transgenic plant cells and transgenic plants, as well as associated methods, for increasing oil production in a plant using F-box gene sequences. The present provides a genetic construct for the overexpression of an F-box protein in plants comprising: (a) a gene promoter; and (b) a nucleic acid molecule encoding an F-box protein functionally linked to the gene promoter.

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Abstract

Disclosed are genetic constructs, transgenic plant cells and transgenic plants, as well as associated methods, for increasing oil production in a plant using F-box gene sequences. The present provides a genetic construct for the overexpression of an F-box protein in plants comprising: (a) a gene promoter; and (b) a nucleic acid molecule encoding an F-box protein functionally linked to the gene promoter.

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F-BOX PROTEIN TARGETED PLANT OIL PRODUCTION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This Application claims the benefit of priority to U.S. Provisional Application No. 60/720,424, filed September 26, 2005.

FIELD OF THE INVENTION

[0002] This invention relates to the field of agriculture and plant engineering. In particular, this invention relates to methods and compositions for increasing oil content in transgenic plants, including the oil content of *Arabidopsis thaliana* seeds.

1. BACKGROUND OF THE INVENTION

[0003] Plant oils represent a renewable resource of highly reduced carbon. Current world vegetable oil production is estimated at 87 million metric tons with an approximate market value of some 40 billion U.S. dollars. The majority of vegetable oil currently goes directly to human consumption and as much as 25% of human caloric intake in developed countries is derived from plant fatty acids (Broun *et al.* (1999) Ann. Rev. Nutr. 19: 197-216). In addition to their importance in human nutrition, plant fatty acids are also major ingredients of nonfood products such as soaps, detergents, lubricants, biofuels, cosmetics, and paints (see Ohlrogge (1994) Plant Physiol. 104: 821-6). While the demand for vegetable oils has increased steadily, production capacity to meet this demand is more than adequate and prices of vegetable oils have remained below or near 0.6 U.S. dollars per kilogram. This low cost of production has stimulated interest in use of vegetable oils as renewable alternatives to petroleum-derived chemical feedstocks.

[0004] Fatty acids are the most abundant form of reduced carbon chains available from nature and have diverse uses ranging from food to industrial feedstocks. Plants represent a significant renewable source of fatty acids because many species accumulate them in the form of triacylglycerol as major storage components in seeds. With the advent of plant transformation technology, metabolic engineering of oilseed fatty acids has become possible and transgenic plant oils represent some of the first successes in design

of modified plant products. For example, the transfer of a California bay plant thioesterase gene into the seeds on non-laurate (12:0)-accumulating plants, *Arabidopsis* and *Brassica napus* (rapeseed) resulted in the alteration of the fatty acid acyl chain elongation process to produce laurate up to 24% and 58% of total seed fatty acids, respectively (see Voelker *et al.* (1992) Sci. 257: 72-4; and Voelker *et al.* (1996) Plant J. 9: 229-41 respectively). Thus, the transfer of a single gene into a plant can dramatically alter the type of fatty acids produced.

[0005] However, such success with a single gene is the exception rather than the rule (for review, see Thelen and Ohlrogge (2002) Metabol. Engineer. 4:12-21). Moreover, to be economically useful for both human consumption and industrial uses, an actual increase in seed oil fatty acid content, rather than just a change in the type of fatty acid produced, would be highly desirable. While the production of malonlyl-CoA by acetyl-CoA carboxylase is a key regulatory step in the *de novo* synthesis of fatty acids, attempts to increase the rate of this apparently rate-limiting step by genetic engineering have met with, at best, modest success. Furthermore, the overexpression of several individual fatty acid synthase enzymes has not resulted in an increased flux of fatty acid biosynthesis (reviewed in Thelan and Ohlrogge (2002) Metabol. Engineer. 4:12-21).

[0006] Accordingly, there remains a need for genetic engineering strategies that will increase the total amount of fatty acid produced by plants.

2. SUMMARY OF THE INVENTION

[0007] The invention is based, in part, upon the unexpected finding that overexpression of F-box genes in plants results in the increased production of plant oil. In particular, the overexpression of the *Arabidopsis thaliana* F-box protein (GenBank Accession Nos. NM_111499 (cDNA) and NP_566277 (protein) using the seed specific promoter phaseolin, produced seeds with a higher oil content phenotype (see Figures 1-5).

[0008] Accordingly, in one aspect, the invention provides a genetic construct for the overexpression of an F-box protein in plants. In general, such genetic constructs of the invention include a gene promoter sequence; and a sequence encoding a plant F-box protein that is functionally linked to the gene promoter sequence. In some embodiments, the F-box protein is a plant F-box protein or encoded by a plant F-box gene. In other

embodiments, the F-box protein is a non-plant F-box protein or encoded by a non-plant F-box gene, such as an animal (e.g., a mammal) F-box protein or gene. In some embodiments, the gene promoter sequence is a seed-specific promoter. In particularly useful embodiments, the gene promoter sequence is phaseolin. In certain particularly useful embodiments, the genetic construct comprises a pBBV-PHAS expression vector.

[0009] In further embodiment, the genetic constructs of the invention comprise a sequence that encodes the plant F-box protein has a polypeptide sequence that is at least 75% identical to the polypeptide sequence of SEQ ID NO: 1. In other embodiments, the plant F-box protein has a polypeptide sequence that is at least 90% identical to the polypeptide sequence of SEQ ID NO: 1. In still further useful embodiments, the genetic constructs of the invention comprise an plant F-box protein having the polypeptide sequence of SEQ ID NO: 1. In particular embodiments, the plant F-box protein is encoded by the nucleic acid sequence of SEQ ID NO: 2. In further embodiments, the F-box protein is encoded by a nucleic acid that hybridizes to the nucleic acid sequence of SEQ ID NO: 2. In particularly useful embodiments, the F-box protein is encoded by a nucleic acid that hybridizes under stringent conditions to the nucleic acid sequence of SEQ ID NO: 2.

[0010] In another aspect, the invention provides transgenic plant cells comprising any of the above-described genetic constructs of the invention. In certain useful embodiments, the transgenic plant cell is an *Arabidopsis thaliana*. In other embodiments, the transgenic plant cell is an agricultural crop plant, such as corn or wheat. In other embodiments, the transgenic plant cell is an oil-producing agricultural crop such as soybean, palm, rapeseed or sunflower.

[0011] In a further aspect, the invention provides a recombinant *Arabiposis thaliana* plant cell having an heterologous genetic construct that includes a gene promoter sequence functionally linked to a plant F-box encoding sequence. In certain particularly useful embodiments, the F-box sequence encoded corresponds to the plant F-box polypeptide of SEQ ID NO: 1 and the promoter is phaseolin.

[0012] In yet another aspect, the invention provides a plant having a transgenic plant cell of the invention, as described above, or a part, propagule or progeny thereof comprising one of the genetic constructs of the invention.

[0013] In still another aspect, the invention provides a method of modifying oil production in a plant by, first, stably incorporating into the genome of the plant a genetic construct of the invention, as described above, to provide a transformed plant, and, then, regenerating the transformed plant so that expression of the incorporated genetic construct modifies oil production in the plant. In particular embodiments, the plant modified is *Arabidopsis thaliana*. In other embodiments, the plant is an agricultural crop plant, such as corn or wheat. In further embodiments, the plant is an oil-producing agricultural crop such as soybean, palm, rapeseed or sunflower.

[0013a] In an embodiment, the invention relates to a method of modifying oil production in a plant comprising: stably incorporating into the genome of the plant a genetic construct as described herein to provide a transformed plant; and regenerating the transformed plant, wherein expression of the incorporated genetic construct increases oil production in the plant as compared to a wild type plant.

[0013b] In an embodiment, the invention relates to a method of increasing oil production in *Arabidopsis thaliana* comprising: stably incorporating into the genome of the *Arabidopsis thaliana* plant a genetic construct having a phaseolin gene promoter functionally linked to a nucleic acid molecule encoding an *Arabidopsis* F-box polypeptide of SEQ ID NO: 1; and regenerating the transformed plant, wherein expression of the incorporated genetic construct increases oil production in the *Arabidopsis thaliana* plant as compared to a wild type plant.

[0013c] In another aspect, the invention relates to the use of a plant comprising the plant cell as described herein for producing progeny.

[0013d] In another aspect, the invention relates to the use of a plant comprising the plant cell as described herein for producing plant oil.

3. BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1 is a graphical representation of experiments demonstrating the increased oil content of transgenic phaseolin-F-box lines compared with vector control lines.

Data shown are average of 18 lines (T2) and error bars are SE.

[0015] Figure 2 is a graphical representation of experiments demonstrating total seed FAMEs of F-box transgenic lines compared to WT, and vector control lines. Error bars are SE based on 18 lines: 3 duplicate for each line.

[0016] Figure 3 is a graphical representation of experiments demonstrating total seed FAMEs of transgenic lines versus WT and vector control lines. Large dots are the two lines with highest oil content and were named Fbox2 and Fbox3 later chosen for next generation analysis.

[0017] Figure 4 is a graphical representation of experiments showing the fatty acid profile and fatty acid molar ratio composition of F-box transgenic lines versus control plants.

[0018] Figure 5 is a graphical representation of seed weight determination. Around 400 seeds were counted for both wild type seeds and transformants with F-box genes. Counted seeds were stored in a desiccator overnight then determined using an analytical balance. Error bars are SE based on 6 replicates.

[0019] Figure 6 is a graphical representation of experiments demonstrating total FAMEs per seed in F-box T3 seeds versus WT and vector control lines. The error bars are SE based on data obtained from 12 plants for F-box2 and F-box3 lines; 6 plants for WT

and vector control lines. The two lines here named F-box2 and F-box3 are the two data points indicated by large dots in Figure 3.

[0020] Figure 7 is a graphical representation of experiments demonstrating total seed FAMEs of transgenic lines (F-box2 and F-box3) versus WT and vector control lines.

[0021] Figure 8 (A) is a graphical representation of experiments demonstrating a C: N ratio analysis for two independent lines F-box2 and F-box3. Error bars are SE based on six replicates. Figure 8 (B) is a graphical representation of experiments demonstrating the positive correlation between oil content and C:N ratio. Open columns are overexpressors F-box2 and F-box3; grey columns are Wild type Arabidopsis seeds.

[0022] Figure 9 (A) is a graphical representation of experiments demonstrating a FAME analysis of F-box-KO lines.

[0023] Figure 9 (B) is a graphical representation of experiments demonstrating a fatty acid profile of KO lines and WT. Error bars are SE based on nine independent replicates.

[0024] Figure 10A is a schematic representation of the polypeptide sequence of an *Arabidopsis thaliana* F-box protein corresponding to GenBank Accession No. NP_566277 (SEQ ID NO: 1).

[0025] Figure 10B is a schematic representation of the nucleotide sequence of an *Arabidopsis thaliana* F-box protein corresponding to GenBank Accession No. NM_111499 (SEQ ID NO: 2). The predicted initiation and termination codons of the F-box protein open reading frame are underlined.

[0026] Figure 11 is a schematic representation of the nucleic acid sequence (SEQ ID NO: 6) of pGATE-PHASE: F-box, with the inserted F-box encoding sequences shown in capital letters.

[0027] Figure 12 is a diagrammatic representation of the seed-specific pGATE-PHASE: F-box vector showing the site of cloning behing the seed-specific phaseolin promoter.

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4. DETAILED DESCRIPTION OF THE INVENTION

[0028] The patent and scientific literature referred to herein establishes knowledge that is available to those of skill in the art.

4.1. General

[0029] The invention is based in part upon the unexpected discovery that overexpression of plant F-box proteins increases the oil content of plant seeds. In particular, the overexpression of the *Arabidopsis thaliana* F-box protein (GenBank Accession Nos. NM_111499 (cDNA) and NP_566277 (protein) using the seed specific promoter phaseolin, produced seeds with a higher fatty acid content phenotype (see Figures 1-5). Accordingly, in general, the invention provides transgenic plants incorporating heterologous F-box genes that increase the oil content of one or more developmental stages of a vascular plant.

[0030] Fatty acids stored in plant seeds are usually unbranched compounds with an even number of carbons ranging from 12 to 22 and with 0 to 3 *cis* double bonds. However, numerous variations on this theme exist in nature particularly with regard to additional functional groups such as hydroxyl, epoxy, cyclopropene, or acetylenic. Plants represent a large reservoir of fatty acid diversity, synthesizing at least 200 different types of fatty acids (see van de Loo *et al.*, (1993) "Unusual fatty acids"in Plant Lipids (T. Moore, Ed.), pp. 91-126, CRC, Boca Raton, FL). Human use, however, has been predominantly restricted to a select few fatty acids that accumulate in domesticated plants. The four most important oilseed crops are, in descending order, soybean, oil palm, rapeseed, and sunflower, which together account for 65% of current worldwide vegetable oil production. The abundant fatty acids produced in these major commercial oilseeds comprise just four of the more than 200 possibilities, namely linoleate, palmitate, laurate, and oleate.

[0031] With regard to lipid nomenclature, a simple shorthand notation based on molecule length and the number and position of double bonds has been developed to

designate fatty acids. For example, the common monounsaturated fatty acid oleic acid (octadecenoic acid) is designated 18:1. The first value, 18, represents the number of carbons. The second value, 1, indicates the number of double bonds. In addition, the position of the double bonds, counting from the carboxyl group is designated by delta (Δ) and oleic acid can be more fully designated as 18:1 Δ9. The double bonds in naturally occurring fatty acids are almost exclusively *cis* isomers, and usually no designation for the type of double bond is used unless it is a *trans* isomer, as in 16:1 Δ3t. Some authors also designate the positions of the double bonds relative to the terminal methyl carbon. Thus, an omega-3 fatty acid contains a double bond 3 carbons from the methyl end of the fatty acid (e.g., 18:3 Δ9, 12, 15 is an omega-3 fatty acid). The position at which a fatty acid is esterified to the glycerol backbone of glycerolipids is designated *sn*-3 (the terminal hydroxyl that is phosphorylated in glycerol 3-phosphate), *sn*-2 (the central hydroxyl), and *sn*-1 (the terminal hydroxyl that is not phosphorylated).

[0032] For both edible and industrial uses, an increase in seed oil content is desirable and has been a major goal of oilseed engineering. However, to be economically useful, such a change must not come at the expense of overall seed yield or at the loss of other high-value components. For example, soybean is the largest source of vegetable oil, comprising 30% of the world market, and now constitutes over 80% of all dietary vegetable oils in the United States. Although termed an oilseed, soybean contains only 18-22% oil on a seed dry-weight basis and is grown principally as a high-protein meal for animal feeds. Thus, increasing oil in soybean will in most cases not be useful if it comes at the expense of high-value soy protein that drives the crop's economics. By comparison, other oilseed crops (except cotton) are grown primarily for their oil and produce seeds with 40-60% oil. The wide range of seed oil percentage observed in nature suggests that this pathway might be amenable to metabolic engineering, particularly in "low-oil" oilseeds, provided the key mechanisms which control oil content are identified.

4.2 Definitions

[0033] For convenience, the meaning of certain terms and phrases employed in the specification, examples, and appended claims are provided below.

[0034] The term "agonist", as used herein, is meant to refer to an agent that mimics or upregulates (*e.g.* potentiates or supplements) a bioactivity. For example, an F-box agonist can be a wild-type F-box protein or derivative thereof having at least one bioactivity of the wild-type F-box protein. An agonist can also be a compound which increases the interaction of a bioactive polypeptide with another molecule, for example, a receptor. Agonists can be any class of molecule, such as a small molecule, including a nucleic acid, protein, carbohydrate, lipid or combination thereof.

[0035] The term "allele", which is used interchangeably herein with "allelic variant" refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for the gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene or allele. Alleles of a specific gene can differ from each other in a single nucleotide, or several nucleotides, and can include substitutions, deletions, and insertions of nucleotides. Frequently occurring sequence variations include transition mutations (i.e. purine to purine substitutions and pyrimidine to pyrimidine substitutions, *e.g.* A to G or C to T), transversion mutations (*i.e.* purine to pyrimidine and pyrimidine to purine substitutions, *e.g.* A to T or C to G), and alteration in repetitive DNA sequences (*e.g.* expansions and contractions of trinucleotide repeat and other tandem repeat sequences). An allele of a gene can also be a form of a gene containing a mutation. The term "allelic variant of a polymorphic region of a gene" refers to a region of a locus gene having one or several nucleotide sequence differences found in that region of the gene in other individuals.

[0036] As used herein, the term "F-box" or "F-box protein" refers to the amino acid sequences of proteins involved in proteolysis, including but not limited to proteins involved in the ubiquitin-ligase complex obtained from any species, including plant species, from any source whether natural, synthetic, semi-synthetic, or recombinant. The F-box is a sequence of 35-45 amino acids and allows the F-box proteins to enter into complexes with other ubiquitin-dependent protein degradation components (*e.g.*, Skp1). Thus, the F-box proteins may bind Skp1, and may contain a motif that displays a sequence similarity to Grr1 or Cdc4 or to the Arabidopsis thaliana F-box protein shown in Figure 10A. This conserved structural motif is included in the sequence alignments

shown in Figure 7 of U.S. Patent No. 6,573,094
(*i.e.*, the amino acid residues that are shared by the F-box proteins shown). However, it is not intended that the term be limited to the exact sequences set forth herein. In some embodiments, the F-box proteins may further comprise additional motifs, in particular motifs involved in protein-protein interaction. These additional motifs included, but are not limited to leucine-rich repeats, and WD-40. In certain embodiments, the F-box protein is from a vascular plant, while in particularly useful embodiments, the F-box protein is from *Arabidopsis thaliana*.

[0037] The term "antagonist" as used herein is meant to refer to an agent that downregulates (*e.g.* suppresses or inhibit;) at least bioactivity. An antagonist can be a compound that inhibits or decreases the interaction between one protein and another molecule, *e.g.*, a substrate. Accordingly, a useful antagonist is a compound that inhibits or decreases binding to a substrate and thereby blocks enzyme function. An antagonist can also be a compound that downregulates expression of a gene or genes or which reduces the amount of a gene product translated. The target bioactivity antagonist can be a dominant negative form of a polypeptide possessing that bioactivity, for example F-box antagonists would include a form of an F-box polypeptide which is capable of interacting with other components of the ubiquitin-dependent pathway, but which interferes with the function of the resulting complex (*i.e.* a dominant negative form of the target bioactivity). An antagonist can also be an antisense nucleic acid, or a ribozyme capable of interacting specifically with a traget bioactivity-encoding mRNA. Yet other antagonists are molecules that bind to a target bioactivity and inhibit its action. Such molecules include peptides such as those which will bind the active site of an enzyme and prevent it from interacting with substrate. Yet other target bioactivity antagonists include antibodies which interact specifically with an epitope of the target polypeptide, such that binding interferes with the biological function of the polypeptide. In yet another useful embodiment, the antagonist is a small molecule, such as a molecule capable of inhibiting the interaction between a target enzyme and its substrate.

[0038] "Asexual propagation" refers to producing progeny by regenerating an entire plant from leaf cuttings, stem cuttings, root cuttings, single plant cells (protoplasts) and callus.

[0039] The term "catalytic site" refers to the portion of a molecule that is capable of binding a reactant and improving the rate of a reaction. Catalytic sites may be present on polypeptides or proteins, enzymes, organics, organo-metal compounds, metals and the like. A catalytic site may be made up of separate portions present on one or more polypeptide chains or compounds. These separate catalytic portions associate together to form a larger portion of a catalytic site. A catalytic site may be formed by a polypeptide or protein that is bonded to a metal.

[0040] "Cells", "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[0041] A "chimeric polypeptide" or "fusion polypeptide" is a fusion of a first amino acid sequence encoding one of the subject polypeptides with a second amino acid sequence defining a domain (*e.g.* polypeptide portion) foreign to and not substantially homologous with any domain of the subject polypeptide. A chimeric polypeptide may present a foreign domain which is found (albeit in a different polypeptide) in an organism which also expresses the first polypeptide, or it may be an "interspecies", "intergenic", etc. fusion of polypeptide structures expressed by different kinds of organisms. In general, a fusion polypeptide can be represented by the general formula X-polypeptide-Y, wherein polypetide represents a first or subject protein or polypeptide, and X and Y are independently absent or represent amino acid sequences which are not related to the first sequence in an organism, including naturally occurring mutants.

[0042] As used herein, "conservatively modified variations" of a particular nucleic acid sequence refer to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, COG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can

be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of "conservatively modified variations." Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence. Furthermore, one of skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are "conservatively modified variations" where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[0043] As described herein, sequences may be optimized for expression in a particular host cell used to produce the protein (*e.g*, a plant cell such as a tomato, or a cloning and expression system such as a yeast cell). Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence are substituted with different amino acids with highly similar properties (see, the definitions section, supra), are also readily identified as being highly similar to a particular amino acid sequence, or to a particular nucleic acid sequence which encodes an amino acid. Such conservatively substituted variations of any particular sequence are a feature of the present invention.

[0044] A "delivery complex" shall mean a targeting means (*e.g.* a molecule that results in higher affinity binding of a gene, protein, polypeptide or peptide to a target cell surface and/or increased cellular or nuclear uptake by a target cell). Examples of targeting means include: sterols (*e.g.* cholesterol), lipids (*e.g.* a cationic lipid, virosome or liposome), viruses (*e.g.* tobacco mosaic virus) or target cell specific binding agents (*e.g.*

ligands recognized by target cell specific receptors). Useful complexes are sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the gene, protein, polypeptide or peptide is released in a functional form.

[0045] As is well known, genes may exist in single or multiple copies within the genome of an individual. Such duplicate genes may be identical or may have certain modifications, including nucleotide substitutions, additions or deletions, which all still code for polypeptides having substantially the same activity. The term "DNA sequence encoding a target polypeptide" may thus refer to one or more genes within a particular individual. Moreover, certain differences in nucleotide sequences may exist between individual organisms, which are called alleles. Such allelic differences may or may not result in differences in amino acid sequence of the encoded polypeptide yet still encode a polypeptide with the same biological activity.

[0046] The phrases "disruption of the gene" and "targeted disruption" or any similar phrase refers to the site specific interruption of a native DNA sequence so as to prevent expression of that gene in the cell as compared to the wild-type copy of the gene. The interruption may be caused by deletions, insertions or modifications to the gene, or any combination thereof.

[0047] The term "enzymatic site" refers to the portion of a protein molecule that contains a catalytic site. Most enzymatic sites exhibit a very high selective substrate specificity. An enzymatic site may be comprised of two or more enzymatic site portions present on different segments of the same polypeptide chain. These enzymatic site portions are associated together to form a greater portion of an enzymatic site. A portion of an enzymatic site may also be a metal.

[0048] The term "enzyme" refers to a protein, polypeptide, peptide RNA molecule, or multimeric protein capable of accelerating or producing by catalytic action some change in a substrate for which it is often specific.

[0049] The term "epitope" refers to portion of a molecule that is specifically recognized by an immunoglobulin product. It is also referred to as the determinant or antigenic determinant.

[0050] As used herein, an "immunoglobulin" is a multimeric protein containing the immunologically active portions of an immunoglobulin heavy chain and immunoglobulin light chain covalently coupled together and capable of specifically combining with antigen.

[0051] As used herein, a Fab fragment is a multimeric protein consisting of the portion of an immunoglobulin molecule containing the immunologically active portions of an immunoglobulin heavy chain and an immunoglobulin light chain covalently coupled together and capable of specifically combining with antigen. Fab fragments are typically prepared by proteolytic digestion of substantially intact immunoglobulin molecules with papain using methods that are well known in the art. However, a Fab fragment may also be prepared by expressing in a suitable host cell the desired portions of immunoglobulin heavy chain and immunoglobulin light chain using methods well known in the art.

[0052] As used herein, an F[v]fragment: A multimeric protein consisting of the immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region covalently coupled together and capable of specifically combining with antigen. F[v]fragments are typically prepared by expressing in suitable host cell the desired portions of immunoglobulin heavy chain variable region and immunoglobulin light chain variable region using methods well known in the art.

[0053] As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide of the present invention, including both exon and (optionally) intron sequences. A "recombinant gene" refers to nucleic acid encoding such regulatory polypeptides, which may optionally include intron sequences which are either derived from a chromosomal DNA. Exemplary recombinant genes include those which encode an F-box polypeptide activity.

[0054] As used herein, "heterologous DNA" or "heterologous nucleic acid" include DNA that does not occur naturally as part of the genome in which it is present or which is found in a location or locations in the genome that differs from that in which it occurs in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Generally, although not necessarily, such DNA encodes RNA and proteins that are not normally produced by the cell in which it is

expressed. Heterologous DNA may also be referred to as foreign DNA, Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, isolated DNA that encodes an F-box protein.

[0055] "Homology" or "identity" or "similarity" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are identical at that position. A degree of homology or similarity or identity between nucleic acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleic acid sequences. A degree of identity of amino acid sequences is a function of the number of identical amino acids at positions shared by the amino acid sequences. A degree of homology or similarity of amino acid sequences is a function of the number of amino acids, i.e. structurally related, at positions shared by the amino acid sequences. An "unrelated" or "non-homologous" sequence shares less than 40% identity, or less than 25% identity, with one of the sequences of the present invention.

[0056] "Inactivation", with respect to genes of the host cell, means that production of a functional gene product is prevented or inhibited. Inactivation may be achieved by deletion of the gene, mutation of the promoter so that expression does not occur, or mutation of the coding sequence so that the gene product is inactive (constitutively or inducibly). Inactivation may be partial or total.

[0057] The term "interact" as used herein is meant to include detectable relationships or association (*e.g.* biochemical interactions) between molecules, such as interaction between protein-protein, protein-nucleic acid, nucleic acid-nucleic acid, and protein-small molecule or nucleic acid-small molecule in nature.

[0058] The term "isolated" as also used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs, or RNAs, respectively, that are present in the natural source of the macromolecule. For example, isolated nucleic acids encoding the subject polypeptides typically include no more than 10 kilobases (kb)

of nucleic acid sequence which naturally immediately flanks that gene in genomic DNA, or no more than 5 kb of such naturally occurring flanking sequences, and most usefully less than 1.5 kb of such naturally occurring flanking sequence. The term isolated as used herein also refers to a nucleic acid or polypeptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. The term "isolated" is also used herein to refer to polypeptides which are isolated from other cellular proteins and is meant to encompass both purified and recombinant polypeptides.

[0059] The term "knock-out" refers to partial or complete suppression of the expression of an endogenous gene. This is generally accomplished by deleting a portion of the gene or by replacing a portion with a second sequence, but may also be caused by other modifications to the gene such as the introduction of stop codons, the mutation of critical amino acids, the removal of an intron junction, *etc*.

[0060] The term "marker" or "marker sequence" or similar phrase means any gene that produces a selectable genotype or a selectable phenotype. It includes such examples as the spectinomycin gene (spcR), neo gene (neoR), green fluorescent protein (GFP) gene, TK gene, β-galactosidase gene, *etc*. The marker sequence may be any sequence known to those skilled in the art that serves these purposes, although typically the marker sequence will be a sequence encoding a protein that confers a selectable trait, such as an antibiotic resistance gene, or an enzyme that can be detected and that is not typically found in the cell. The marker sequence may also include regulatory regions such as a promoter or enhancer that regulates the expression of that protein. However, it is also possible to transcribe the marker using endogenous regulatory sequences. In one embodiment of the present invention, the marker facilitates separation of transfected from untransfected cells by fluorescence activated cell sorting, for example by the use of a fluorescently labeled antibody or the expression of a fluorescent protein such as GFP. Other DNA sequences that facilitate expression of marker genes may also be incorporated into the DNA constructs of the present invention. These sequences include, but are not limited to transcription initiation and termination signals, translation signals, post-

translational modification signals, intron splicing junctions, ribosome binding sites, and polyadenylation signals, to name a few. The marker sequence may also be used to append sequence to the target gene. For example, it may be used to add a stop codon to truncate F-box translation. The use of selectable markers is well known in the art and need not be detailed herein. The term "modulation" as used herein refers to both upregulation (i.e., activation or stimulation (*e.g.*, by agonizing or potentiating)) and downregulation (i.e. inhibition or suppression (*e.g.*, by antagonizing, decreasing or inhibiting)).

[0061] A "mutated gene" or "mutation" refers to an allelic form of a gene, which is capable of altering the phenotype of a subject having the mutated gene relative to a subject which does not have the mutated gene. If a subject must be homozygous for this mutation to have an altered phenotype, the mutation is said to be recessive. If one copy of the mutated gene is sufficient to alter the genotype of the subject, the mutation is said to be dominant. If a subject has one copy of the mutated gene and has a phenotype that is intermediate between that of a homozygous and that of a heterozygous subject (for that gene), the mutation is said to be co-dominant.

[0062] As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should be understood to include either single- or double-stranded forms of nucleic acid, and, as equivalents, analogs of either RNA or DNA. Such nucleic acid analogs may be composed of nucleotide analogs, and, as applicable to the embodiment being described, may be single-stranded (such as sense or antisense) or double-stranded polynucleotides.

[0063] The phrase "nucleotide sequence complementary to the nucleotide sequence set forth in SEQ ID NO: x" refers to the nucleotide sequence of the complementary strand of a nucleic acid strand having SEQ ID NO: x. The term "complementary strand" is used herein interchangeably with the term "complement". The complement of a nucleic acid strand can be the complement of a coding strand or the complement of a non-coding strand. When referring to double stranded nucleic acids, the complement of a nucleic acid having SEQ ID NO: x refers to the complementary strand of the strand having SEQ ID NO: x or to any nucleic acid having the nucleotide sequence of the complementary strand of SEQ ID NO: x. When referring to a single stranded nucleic acid having the nucleotide sequence SEQ ID NO: x, the complement of this nucleic acid is a nucleic acid having a

nucleotide sequence which is complementary to that of SEQ ID NO: x. The nucleotide sequences and complementary sequences thereof are always given in the 5' to 3' direction.

[0064] The phrase "operably linked" refers to functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates transcription of RNA corresponding to the second sequence.

[0065] The term "percent identical" refers to sequence identity between two amino acid sequences or between two nucleotide sequences. Identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (*e.g.*, similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used, including FASTA, BLAST or ENTREZ. FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, *e.g.*, default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md. In one embodiment, the percent identity of two sequences can be determined by the GCG program with a, gap weight of 1, *e.g.*, each amino acid gap is weighted as if it were a single amino acid or nucleotide mismatch between the two sequences.

[0066] The term "plant" includes whole plants, plant organs (*e.g.*, leaves, stems, flowers, roots, etc.), seeds and plant cells and progeny of same. The class of plants which can be used in the method of the invention is as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants, as well as certain lower plants such as algae. It includes plants of a variety of ploidy levels, including polyploid, diploid and haploid. Useful crop plants of the invention include, but are not limited to, corn (*Zea mays*), *Brassica* spp. (*e.g.*, canola (*B. napus*), *B. rapa*, *B. juncea*), particularly those *Brassica* species useful as sources of

seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (*e.g.*, pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (Olea europaea), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

[0067] The term "plant" farther includes the following classes of plant species:

[0068] . Dicotyledon (dicot): A flowering plant whose embryos have two seed halves or cotyledons. Examples of dicots are: tobacco; tomato; the legumes including alfalfa; oaks; maples; roses; mints; squashes; daisies; walnuts; cacti; violets; and buttercups.

[0069] Monocotyledon (monocot): A flowering plant whose embryos have one cotyledon or seed leaf. Non-limiting examples of monocots are: lilies; grasses; corn; grains, including oats, wheat and barley; orchids; irises; onions and palms.

[0070] "Lower plant", refers to a non-flowering plant including ferns, gymnosperms, conifers, horsetails, club mosses, liver warts, hornworts, mosses, red algae, brown algae, gametophytes, sporophytes of pteridophytes, and green algae.

[0071] In general, plants of interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, millet, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, Brassica, maize, alfalfa, palm, coconut, flax, castor, olive etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, *etc.*

[0072] The term "promoter" refers to a region of nucleic acid subsequences located upstream and/or downstream from the start of transcription which aid in the recognition, binding and/or initiation of RNA polymerase or other transcription proteins which initiate transcription of an associated gene. A "plant promoter" is a promoter capable of initiating transcription in plant cells. A "plant leucine aminopeptidase promoter" is a promoter derived from a leucine aminopeptidase gene, *e.g.*, by cloning, isolating or recombinantly modifying a native promoter from a leucine aminopeptidase gene.

[0073] A "recombinant nucleic acid" comprises or is encoded by one or more nucleic acid which is derived from a nucleic acid which wag artificially constructed. For example, the nucleic acid can comprise or be encoded by a cloned nucleic acid formed by joining heterologous nucleic acids as taught, *e.g.*, in Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods In Enzymology Vol. 152 Academic Press, Inc., San Diego, Calif. (Berger) and in Sambrook et al. Molecular Cloning-A Laboratory Manual (2nd ed.) Vol. 1-3 (1989) (Sambrook) and in Current Protocols In Molecular Biology, Ausubel, F. M., et al., eds., Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1996 Supplement) (Ausubel). Alternatively, the nucleic acid can be synthesized chemically.

[0074] As used herein, a "reporter gene construct" is a nucleic acid that includes a "reporter gene" operatively linked to a transcriptional regulatory sequences. Transcription of the reporter gene is controlled by these sequences. The transcriptional regulatory sequences include the promoter and other regulatory regions, such as enhancer sequences, that modulate the activity of the promoter, or regulatory sequences that modulate the activity or efficiency of the RNA polymerase that recognizes the promoter, or regulatory sequences are recognized by effector molecules.

[0075] As used herein, the term "nucleic acid" refers to polynucleotides or oligonucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also he understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs and as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

[0076] As used herein, the term "promoter" means a DNA sequence that regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in cells. The term encompasses "tissue specific" promoters, i.e. promoters, which effect expression of the selected DNA sequence only in specific cells (*e.g.* cells of a specific tissue). The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well, The term also encompasses non-tissue specific promoters and promoters that constitutively express or that are inducible (i.e. expression levels can be controlled).

[0077] The terms "protein", "polypeptide" and "peptide" are used interchangeably herein when referring to a gene product.

[0078] The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA, techniques, wherein generally, DNA encoding a specific polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant target gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native target polypeptide, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the polypeptide.

[0079] As used herein, "recombinant cells" include any cells that have been modified by the introduction of heterologous DNA. Control cells include cells that are substantially identical to the recombinant cells, but do not express one or more of the proteins encoded by the heterologous DNA, *e.g.*, do not include or express a recombinant F-box gene.

[0080] "Small molecule" as used herein, is meant to refer to a composition, which has a molecular weight of less than about 5 kD and most usefully less than about 4 kD. Small molecules can be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon containing) or inorganic molecules. Many pharmaceutical companies have extensive libraries of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts, which can be screened with any of the assays of the invention to identify compounds that modulate a target bioactivity.

[0081] As used herein, the term "specifically hybridizes" or "specifically detects" refers to the ability of a nucleic acid molecule of the invention to hybridize to at least approximately 6, 12, 20, 30, 50, 100, 150, 200, 300, 350, 400 or 425 consecutive nucleotides of a gene, most usefully a plant F-box gene.

[0082] The term "substantially homologous", when used in connection with amino acid sequences, refers to sequences which are substantially identical to or similar in sequence, giving rise to a homology in conformation and thus to similar biological activity. The term is not intended to imply a common evolution of the sequences.

[0083] As used herein, the term "transfection" means the introduction of a nucleic acid, *e.g.*, via an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. Methods for transformation which are known in the art include any electrical, magnetic, physical, biological or chemical means. As used herein, "transfection" includes such specific techniques as electroporation, magnetoporation, Ca^{++} treatment, injection, bombardment, retroviral infection and lipofection, among others. "Transformation" as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of a target polypeptide or, in the case of anti-sense expression from the transferred gene, the expression of a naturally-occurring form of the target polypeptide is disrupted.

[0084] As used herein, the term "transgene" means a nucleic acid sequence (encoding, *e.g.*, one of the target polypeptides, or an antisense transcript thereto) which has been introduced into a cell. A transgene could be partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (*e.g.*, it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can also be present in a cell in the form of an episome. A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of a selected nucleic acid.

[0085] A "transgenic plant" refers to any plant, in which one or more of the cells of the plant contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic plants described herein, the transgene causes cells to express a recombinant form of one of the target polypeptides, *e.g.* either agonistic or antagonistic forms. However, transgenic plants in which the recombinant target gene is silent are also contemplated, as for example, FLP or CRE recombinant dependent constructs. Moreover, "transgenic plant" also includes those recombinant animals in which gene disruption of one or more plant genes is caused by human intervention, including both recombination and antisense techniques. A "transgenic plant" is, further, one which has been genetically modified to contain and express heterologous DNA sequences, either as regulatory RNA molecules or as proteins. As specifically exemplified herein, a transgenic plant is genetically modified to contain and express at least one heterologous DNA sequence operably linked to and under the regulatory control of transcriptional control sequences which function in plant cells or tissue or in whole plants. As used herein, a transgenic plant also refers to progeny of the initial transgenic plant where those progeny contain and are capable of expressing the heterologous coding sequence under the regulatory control of the plant-expressible transcription control sequences described herein. Seeds containing transgenic embryos are encompassed within this definition, as are cuttings and other plant materials for vegetative propagation of a transgenic plant.

[0086] When plant expression of a heterologous gene or coding sequence of interest is desired, that coding sequence is operably linked in the sense orientation to a suitable promoter and advantageously under the regulatory control of DNA sequences which quantitatively regulate transcription of a downstream sequence in plant cells or tissue or in plants, in the same orientation as the promoter, so that a sense (i.e., functional for

translational expression) mRNA is produced. A transcription termination signal, for example, as polyadenylation signal, functional in a plant cell is advantageously placed downstream of the metal or organometal resistance coding sequence, and a selectable marker which can be expressed in a plant, can be covalently linked to the inducible expression unit so that after this DNA molecule is introduced into a plant cell or tissue, its presence can be selected and plant cells or tissue not so transformed will be killed or prevented from growing. In the present invention, the mercury resistance coding sequence can serve as a selectable marker for transformation of plant cells or tissue. Where constitutive gene expression is desired, suitable plant-expressible promoters include the 35S or 19S promoters of Cauliflower Mosaic Virus, the nos, ocs or mas promoters of Agrobacterium tumefaciens Ti plasmids, and others known to the art. Where tissue specific expression of the plant-expressible metal resistance coding sequence is desired, the skilled artisan will choose from a number of well-known sequences to mediate that form of gene expression. Environmentally regulated promoters are also well known in the art, and the skilled artisan can choose from well known transcription regulatory sequences to achieve the desired result.

[0087] "Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In some embodiments, transcription of a recombinant gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of the protein.

[0088] The term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of useful vector is an episome, *i.e.*, a nucleic acid capable of extra-chromosomal replication. Particularly useful vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression

vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

[0089] The term "wild-type allelle" refers to an allele of a gene which, when present in two copies in a subject results in a wild-type phenotype. There can be several different wild-type alleles of a specific gene, since certain nucleotide changes in a gene may not affect the phenotype of a subject having two copies of the gene with the nucleotide changes.

4.3 F-box Nucleic Acid and Polypeptide Sequences

[0090] The F-box nucleic acid and protein sequences of the invention include the *Arabidopsis thaliana* F-box protein (GenBank Accession Nos. NM_111499 (cDNA) and NP_566277 (protein), see Figures 10B and 10A, respectively).

[0091] Other equivalent nucleic acids and polypeptides include those discernable from the following summary of this F-box protein family (At3g06240).

SELECTED PROTEIN SIMILARITIES

[0092] Comparison of sequences in UniGene with proteins supported by a complete genome. The alignments can suggest function of a gene.

[0093] *A. thaliana* ref:NP_566277.1 - expressed protein [Arabidopsis thaliana] 100 % / 416 aa (see ProtEST)

GENE EXPRESSION

[0094] Tissues and development stages from this gene's sequences survey gene expression. Links to other NCBI expression resources.

[0095] cDNA sources: mixture of silique and flower ; green siliques ; roots ; seed ; adult vegetative tissue; flower buds; flowers and buds; inflorescence lacking open or older flowers.

[0096] GEO profiles: Gene expression profiles in the NCBI Gene Expression Omnibus database .

MAPPING POSITION

[0097] Genomic location specified by transcript mapping, radiation hybrid mapping, genetic mapping or cytogenetic mapping.

[0098] Arabidopsis Chromosome: III

SEQUENCES

[0099] Sequences representing this gene; mRNAs, ESTs, and gene predictions supported by transcribed sequences.

[0100] NM_111499.3 Arabidopsis thaliana F-box family protein (At3g06240) mRNA, complete cds P

[0101] AY084423.1 Arabidopsis thaliana clone 108003 mRNA, complete sequence P

[0102] AK118303.1 Arabidopsis thaliana At3g06240 mRNA for unknown protein, complete cds, clone: RAFL19-58-P14 P

[0103] BT006048.1 Arabidopsis thaliana clone U50970 putative F-box protein family (At3g06240) mRNA, complete cds P

[0104] BX824678.1 Arabidopsis thaliana Full-length cDNA Complete sequence from clone GSLTPGH64ZA06 of Hormone Treated Callus of strain col-0 of Arabidopsis thaliana (thale cress) P

[0105] BX829355.1 Arabidopsis thaliana Full-length cDNA Complete sequence from clone GSLTSIL94ZF11 of Silique of strain col-0 of Arabidopsis thaliana (thale cress) P

[0106] EST Sequences (19)

BP842389.1 Clone RAFL21-54-K05 5' read

BP825839.1 Clone RAFL22-69-O13 mixture of silique and flower 5' read

BP824403.1 Clone RAFL22-09-H01 mixture of silique and flower 5' read

BP817574.1 Clone RAFL22-45-F24 mixture of silique and flower 5' read

BP652696.1 Clone RAFL19-94-A01 mixture of silique and flower 3' read

BP644487.1 Clone RAFL19-65-J12 mixture of silique and flower 3' read

BP643288.1 Clone RAFL19-61-H05 mixture of silique and flower 3' read

CF773451.1 Clone 17B03 inflorescence lacking open or older flowers

BX838532.1 Clone GSLTFB50ZF02 flowers and buds 5' read P

BX836858.1 Clone GSLTLS79ZF01 adult vegetative tissue 5' read

BX837508.1 Clone GSLTLS79ZE01 adult vegetative tissue 5' read P

AU239163.1 Clone RAFL19-58-P14 mixture of silique and flower 5' read P

AU230440.1 Clone RAFL19-58-P14 mixture of silique and flower 3' read

AV562416.1 Clone SQ169f12F green siliques 3' read A

AV551881.1 Clone RZ15g09R roots 5' read

AV540406.1 Clone RZ15g09F roots 3' read P

AV534311.1 Clone FB078a02F flower buds 3' read P

BE525819.1 Clone 600034345R1 seed 5' read

BE523193.1 Clone M35B4 seed 5' read P.

Homologous F-box Proteins

[0107] The invention further provides F-box protein encoding genes that are homologous to those specifically described herein (*e.g.*, the *Arabidopsis thaliana* F-box Protein shown in Figure 10A, and the corresponding encoding sequence demarcated in Figure 10B). For example, equivalent nucleotide sequences include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as intragenus variants; and also include sequences that differ from the nucleotide sequence encoding the portion of the a protein represented herein due to the degeneracy of the genetic code. Equivalent nucleic acids also include nucleotide sequences that hybridize under stringent conditions (i.e., equivalent to about 20-27° C. below the melting temperature (Tm) of the DNA duplex formed in about 1 M salt) to a nucleotide sequence of an heterologous gene of the invention.

[0108] Particularly useful nucleic acids encode polypeptides comprising an amino acid sequence which is at least 70% identical, 80% identical or 85% identical with an amino acid sequence of the invention. Nucleic acids encoding polypeptides, particularly polypeptides retaining an activity of one of the subject heterologous genes which confer an F-box activity, and comprising an amino acid sequence which is at least about 90%, or at least about 95%, and most usefully at least about 98-99% identical with an amino acid sequence of the invention are also within the scope of the invention.

[0109] Another aspect of the invention provides nucleic acid that hybridizes under high or low stringency conditions to nucleic acid which encodes a polypeptide identical or homologous with an amino acid sequence of the invention. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45° C., followed by a wash of 2.0 x SSC at 50° C, are known to those skilled in the art or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989) 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0.x.SSC at 50° C to a high stringency of about 0.2 x SSC at 50° C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22° C, to high stringency conditions at about 65° C.

[0110] Isolated nucleic acids encoding an heterologous protein of the present invention, yet which differ from the nucleotide sequences referenced herein due to degeneracy in the genetic code, are also within the scope of the invention. Such nucleic acids are understood to be capable of encoding functionally equivalent polypeptides (i.e., a polypeptide having at least a portion of the biological activity of a protein encoded by the enumerated sequences). For instance, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the protein will exist even within the same species. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-4% of the nucleotides) of a gene encoding a protein may exist among individual cells of a given species, *e.g.,* amongst a population of *Arabidopsis thaliana* cells, due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

[0111] Fragments of the nucleic acid encoding portions of the subject heterologous F-box such as a fragments which retain the ability to interact with the ubiquitin proteolytic pathway, are also within the scope of the invention. As used herein, such fragments refer to nucleotide sequences having fewer nucleotides than the coding sequence of the gene,

yet still include enough of the coding sequence so as to encode a polypeptide with at least some of the activity of the full-length protein activity.

[0112] In yet a further embodiment, the recombinant regulatory genes may further include, additional nucleotide sequences. For instance, the recombinant gene can include nucleotide sequences of a PCR fragment generated by amplifying the gene from a genomic DNA library, e.g., 5' and 3' non-coding sequences of either of the subject genes.

[0113] Nucleic acids within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of the recombinant polypeptides.

[0114] As indicated by the examples set out below, a nucleic acid encoding one of the subject proteins may be obtained from mRNA present in a sample of eukaryotic cells, such as those of a vascular plant. It will also be possible to obtain nucleic acids encoding the subject proteins from genomic DNA obtained from such cells. For example, a gene encoding one of the subject F-box proteins can be cloned from either a cDNA or a genomic library from other plant species in accordance with protocols described herein, as well as those generally known in the art. For instance, a cDNA encoding an heterologous protein can be obtained by isolating total mRNA from a plant, generating double stranded cDNAs from the total mRNA, cloning the cDNA into a suitable plasmid or bacteriophage vector, and isolating clones expressing the subject protein using any one of a number of known techniques, *e.g.*, oligonucleotide probes, western blot analysis, or complementation. Genes encoding related proteins can also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention. The nucleic acid of the invention can be DNA or RNA.

[0115] Moreover, the nucleotide sequence determined from the cloning of the subject heterologous genes will permit the generation of probes designed for use in identifying the heterologous transgenic DNA as well as for detecting the presence of the corresponding heterologous mRNA. For example, the subject nucleic acids may be used following transgenic targeting to confirm the presence and integrity of the introduced sequence as well as the amount and specificity of expression in transgenic progeny. For instance, the present invention provides a probe/primer comprising a substantially

purified oligonucleotide, wherein the oligonucleotide comprises a region of nucleotide sequence which hybridizes under stringent conditions to at least 10, 25, 50, or 100 consecutive nucleotides of sense or anti-sense sequence of one of the subject nucleic acids, or naturally occurring mutants thereof. In some embodiments, the probe/primer further comprises a label group attached thereto and able to be detected, *e.g.* the label group is selected from the group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors.

Other F-box Proteins

[0116] Still other F-box proteins for use in the invention are known in the art and specifically described elsewhere (see, *e.g.*, U.S. Patent Nos. 6,573,094 and 6,232,081). Other known plant F-box proteins include the F-box protein TIR1, an auxin receptor (see Dharmasiri *et al.* (2005) Nature 435: 441-5; GenBank Accession No. Q570C0; GI: 68053009)), as well as three additional F-box proteins, termed AFB1, 2, and 3, which also regulate auxin response (see Dharmasiri *et al.* (2005) Dev. Cell 9:109-119). Indeed, the *Arabidopsis* genome alone encodes nearly 700 F-box proteins (see Gagne *et al.* (2002) Proc. Natl. Acad. Sci. USA 99: 11519-24). Still other plant F-box proteins are known in the art and available to the skilled artisan using only routine methods in gene cloning.

Vectors

[0117] This invention also provides expression vectors which include a nucleotide sequence encoding one of the subject polypeptides and operably linked to at least one regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Plant egulatory sequences are art-recognized. Accordingly, the term regulatory sequence includes promoters, enhancers and other expression control elements. Exemplary plant regulatory sequences are described in Yusibo *et al.* (1999) Curr. Top. Micro. Immun. 240: 81-94 and Hood *et al.* (1999) Adv. Exp. Med. Biol. 464: 127-47. For instance, any of a wide variety of expression control sequences-sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors

to express DNA sequences encoding the Zostera genetic trait-conferring proteins and nucleic acids of this invention. Such useful expression control sequences, include, for example, the constitutive maize ubiquitin promoter (ubi promoter) (Christensen *et al.* (1992) Plant Mol. Biol. 18: 675-89; Cornejo *et al.* (1993) Plant Mol. Biol. 23: 567-81) and the potato PinII terminator sequence (An *et al.* (1989) Plant Cell 1: 115-22). Other useful expression control sequences are those derived from plant viruses such as: the 35S promoter, which is derived from Cauliflower Mosaic Virus sequences; and the TMV coat protein promoter, such as that contained in the cloning vector designated "30B" which is derived from the Tobacco Mosaic Virus. Also included in certain aspects of the invention are non-plant transcriptional regulatory sequences such as early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, *e.g.*, Pho5, the promoters of the yeast a-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

[0118] The recombinant construct of the present invention may include a selectable marker for propagation of the construct. For example, a construct to be propagated in bacteria preferably contains an antibiotic resistance gene, such as one that confers resistance to kanamycin, tetracycline, streptomycin, or chloramphenicol. Suitable vectors for propagating the construct include plasmids, cosmids, bacteriophages or viruses, to name but a few.

[0119] In addition, the recombinant constructs may include plant-expressible selectable or screenable marker genes for isolating, identifying or tracking of plant cells transformed by these constructs. Selectable markers include, but are not limited to, genes

that confer antibiotic resistances (e.g., resistance to kanamycin or hygromycin) or herbicide resistance (e.g., resistance to sulfonylurea, phosphinothricin, or glyphosate). Screenable markers include, but are not limited to, the genes encoding beta-glucuronidase (Jefferson (1987) Plant Mol. Biol. Rep. 5:387-405), luciferase (Ow *et al.* (1986) Sci. 234:856-859), B and C1 gene products that regulate anthocyanin pigment production (Goffet *et al.* (1990) EMBO J 9:2517-2522).

[0120] The present invention may also utilize the *Agrobacterium* system for transforming plants, the recombinant DNA constructs additionally comprise at least the right T-DNA border sequence flanking the DNA sequences to be transformed into plant cell. In some embodiments, the sequences to be transferred in flanked by the right and left T-DNA border sequences. The proper design and construction of such T-DNA based transformation vectors are well known to those skilled in the art.

[0121] This invention also pertains to a host cell transfected with a recombinant gene in order that it may express a recombinant protein of the present invention. The host cell may be any prokaryotic or eukaryotic cell. For example, a plant F-box protein of the present invention may be expressed in bacterial cells, such as *E. coli*, insect cells, yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

[0122] Another aspect of the present invention concerns recombinant forms of the subject plant proteins. The term "recombinant protein" refers to a protein of the present invention which is produced by recombinant DNA techniques, wherein generally DNA encoding the protein is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant gene encoding one of the subject proteins, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of the native (or "authentic") form of the plant protein, or an amino acid sequence similar thereto, which is generated by mutation so as to include substitutions and/or deletions relative to a naturally occurring form of the protein. To illustrate, recombinant proteins particularly useful in the present invention, in addition to those having an amino acid sequence of the native proteins, are those recombinant proteins having amino acid sequences which are at least 70% homologous, or 80% homologous and most usefully 90% homologous with an ammo acid sequence of the present

invention. A polypeptide which having an amino acid sequence that is at least about 95%, or at least about 98%, and most usefully identical to one of the polypeptide sequences of the invention are also within the scope of the invention. Thus, the present invention pertains to recombinant proteins which are derived, for example from *Arabidopsis thaliana* F-box genes and which have amino acid sequences evolutionarily related to a sequence encoded by an orthologous gene from another plant protein, wherein "evolutionarily related to" refers to polypeptides having amino acid sequences which have arisen naturally (e.g. by allelic variance), as well as mutational variants of the regulatory proteins which are derived, for example, by combinatorial mutagenesis.

4.4 Transgenic Plants and Plant Cells

[0123] Techniques for stably incorporating genetic constructs into the genome of target plants are well known in the art and include *Agrobacterium tumefaciens*-mediated introduction, electroporation, protoplast fusion, injection into reproductive organs, injection into immature embryos, high velocity projectile introduction and the like. The choice of technique will depend upon the target plant to be transformed. For example, dicotyledonous plants and certain monocots and gymnosperms may be transformed by Agrobacterium Ti plasmid technology (as described, for example by Bevan, (1984) Nucl. Acids Res. 12:8711-8721). Targets for the introduction of the genetic constructs of the present invention include tissues, such as leaf tissue, dissociated cells, protoplasts, seeds, embryos, meristematic regions; cotyledons, hypocotyls, and the like.

[0124] Once the cells are transformed, cells having the inventive genetic construct incorporated in their genome may be selected by means of a marker, such as the kanamycin resistance marker discussed above. Transgenic cells may then be cultured in an appropriate medium to regenerate whole plants, using techniques well known in the art. In the case of protoplasts, the cell wall is allowed to reform under appropriate osmotic conditions. In the case of seeds or embryos, an appropriate germination or callus initiation medium is employed. For explants, an appropriate regeneration medium is used. Regeneration of plants is well established for many species. For a review of regeneration of forest trees see Dunstan *et al.* Somatic embryogenesis in woody plants as described in Thorpe, T. A. ed., In Vitro Embryogenesis of Plants. Vol. 20 in Current Plant Science and

Biotechnology in Agriculture, Chapter 12, pp. 471-540, 1995. Specific protocols for the regeneration of spruce are discussed by Roberts *et al.*, Somatic Embryogenesis of Spruce is described in: Synseed Applications of synthetic seed to crop improvement. Redenbaugh, K, ed., CRC Press, Chapter 23, pp. 427-449, 1993. The resulting transformed plants may be reproduced sexually or asexually, using methods well known in the art, to give successive generations of transgenic plants.

[0125] As discussed above, the production of RNA in target plant cells can be controlled by choice of the promoter sequence. A target plant may be transformed with more than one genetic construct of the present invention, thereby modulating the activity of more than one polypeptide, affecting polypeptide activity in more than one tissue, or affecting polypeptide activity at more than one expression time. Similarly, a genetic construct may be assembled containing more than one open reading frame coding for an inventive polypeptide or more than one non-coding region of a gene coding for such a polypeptide. The polynucleotides of the present invention may also be employed in combination with other known sequences encoding polypeptides involved in plant cell signaling.

[0126] The isolated polynucleotides of the present invention may be employed as probes to isolate other related F-box DNA sequences, including those from other plant species, using techniques well known to those of skill in the art, such as routinely used DNA hybridization and PCR techniques.

[0127] The inventive polynucleotides, polypeptides and antibodies to such polypeptides may be used to screen for molecules that interact with such polynucleotides and/or polypeptides and that thereby modulate cell signaling. Techniques for performing such assays are well known in the art. Similarly, the polynucleotides and polypeptides of the present invention may be employed in studies designed to elucidate the mechanism of cell signaling pathways.

[0128] The invention provides numerous methods for the transformation of plants with an heterologous F-box gene or genes which contribute to the production of oils in the recipient plant, the details of which methods are further described below. In some embodiments, the heterologous gene is introduced by transformation, and the introduced gene is expressed stably over the life of the plant and is further capable of being

transmitted to the plant's offspring. In general, it is desirable for the transgene to be integrated into the nuclear DNA, although the plastid genome may be an appropriate target for some constructs.

[0129] The transformation of crop and other plants can be effected by a number of methods known in the field of plant biotechnology. The method for transformation will vary with the plant species to be transformed and the desired pattern and stability of transgene expression. For example, particle bombardment methods have been shown to be effective in transforming many plant species, including those previously considered recalcitrant to transformation. This method is commonly used in the transformation of monocotyledonous plants such as corn. Another plant transformation method available is *Agrobacterium*-mediated gene transfer, which is commonly used to transform dicotyledonous crops.

[0130] Still other methods available for plant transformation do not rely upon tissue culture for the recovery of transgenic plants, thereby allowing the production of transgenics from plant species for which no reliable method of tissue culture exists. For example, microtargeting of particle-bound DNA into shoot meristematic tissue produces transgenic flowering parts from which transgenic seeds arise (Sautter *et al.* (1991) Biotech. 9: 1080-85). Transgenic seeds can also be created by electrophoresing DNA into meristematic tissue (Griesbach (1994) Plant Sci. 102: 81-89; Burchi *et al.* (1995) J Genetl Breeding 49: 163-8). This method has proven successful in the transformation of several plant species including orchids, chrysantehemums, carnations, lisianthus, peppers, and even woody plant species such as plum (*Plumus domestica*).

[0131] In general, the invention provides methods and reagents for the genetic engineering of a target host plant, such as a crop plant, with an heterologous nucleic acid which encode an F-box protein function that contributes to the production of oils in the recipient plant. One method for transformation makes use of the aforementioned common soil bacterium *Agrobacterium* (see Birch (1997) Ann. Rev. Plant Physiol. Plant Mol. Biol. 48: 297-326). This method involves a modified transfer-DNA (T-DNA) vector which carries the desired nucleic acid fragment between the T-DNA border regions (specific 25 base pair direct repeat regions). The resulting vector is transferred into an *Agrobacterium* host and the target host plant is inoculated with the transformed

recombinant bacterium. Virulence genes products of *Agrobacterium* then actively recognize, excise, transport, and integrate the T-DNA region into the host plant genome.

[0132] *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature (see, *e.g.* Horsch, *et al.* (1984) Science 233:496-8, and Fraley, *et al.* (1983) Proc. Nat. Acad. Sci. USA 80:4803. Agrobacterium-mediated transformation is a particularly useful method of transformation of dicots.

[0133] The natural host range of *Agrobacterium* is limited and so this approach to transformation is not practicable in some target host plants, particularly cereal crops and other monocotyledonous species. For such crops, the invention provides alternative approaches to transformation such as direct uptake of naked DNA into protoplasts or tissues using electroporation or particle gun bombardment. In this method, the co-transformation of a selectable marker gene along with the gene of interest allows the preferential growth of the transformed cells in cell culture. Successive manipulations of the chemical composition of the culture medium, especially the plant hormones, allows the regeneration of complete plants. This method has allowed the recovery of genetically engineered plants in virtually all crop plants.

[0134] One representative method for direct transformation of the transgene construct is by particle bombardment of target plant tissues with high-velocity microprojectiles (see, *e.g.,* Finer *et al.* (1999) Curr. Top. Micro. and Immun. 240: 60-80 for review). This method utilizes a particle accelerator or "gene blaster" to penetrate the outer surface layers of the plant tissue or protoplast (Sanford (1988) Trends Biotech.l 6: 299-302). Biolistics, a combination of "biological" and "ballistics", describes a technique which utilizes instrumentation to accelerate DNA coated microprojectiles into cells, past the cell wall and cell membrane. The microprojectile is generally small enough (0.5-5.0 mm) to enter the plant cell without too much damage, yet large enough to have the mass to penetrate the cell wall and carry an appropriate amount of DNA on its surface into the interior of the plant cell.

[0135] A number of different particle gun designs may be used. The basis of all of these designs is to coat the DNA onto small dense particles and accelerate the particles towards a target tissue. The particles usually consist of either gold or tungsten spherical

particles which are between 0.5 and 5.0 mm in diameter. Gold particles are chemically inert, generally more uniform in size than tungsten particles and produce no cytootoxic effects. Accordingly, gold particles are generally more effective than tungsten particles. Ideally the particles used for bombardment should have good initial affinity for DNA, yet freely release the DNA once inside the target cell cytoplasm or nucleus.

[0136] To prepare DNA-coated microprojectiles, washed gold or tungsten particles are mixed with plasmid DNA. The DNA is bound on the particles using either ethanol or calcium chloride precipitation methods, which are known in the art. Spermidine may be added to the mixture, possibly protecting the DNA from degradation and/or altering its conformation. After precipitation, the particles may be washed, resuspended and either dried or stored on ice as an aqueous suspension until needed.

[0137] The particle gun may utilize a macrocarrier, which supports or carries the particles and is accelerated along with the particles towards the target. The macrocarrier is usually retained by a stopping plate or screen before it collides with the target, whereas the particles continue along their course. In most cases, the particles are accelerated under partial vacuum in a vacuum chamber to reduce air drag. Particle penetration is controlled by modifying the intensity of the explosive burst, by changing the distance that the particles must travel to reach the target tissue or by using different sized particles. A commercial hand-held device (the Helios Gene Gun) is available from BioRad Laboratories (Hercules, Calif.). A helium-modified bombardment device, which utilizes continual build-up of helium back-pressure delivered to a calibrated rupture disc which transmits a shock wave to a second disc or macrocarrier that holds the DNA-coated particles, is also available from BioRad (i.e. the PDS-1000/He unit). A high voltage electrical discharge gun which causes rapid vaporization of a water droplet which in turn transmits a shock wave to a mylar sheet coated with DNA-bound particles has also been developed (see McCabe and Christou (1993) Plant Cell Tiss. Organ. Cult. 33: 227-236). Yet another device for particle bombardment is a microtargeting device, which does not utilize a macrocarrier (Sautter *et al.* (1991) Bio/Technology 9: 1080-5). This device accelerates small amounts of a DNA/particle mixture in a focused stream of high-pressure nitrogen. The DNA is not precipitate on the gold particles, but is delivered as a mixture.

[0138] A variety of different plant tissues have been used as targets for particle bombardment-mediated transformation. Selection of the appropriate target tissue is dependent on multiple factors. For rapid gene expression analysis, various plasmid constructs can be introduced into different tissues and transient expression can be quickly analyzed to assess promoter activity without the production of stably transformed plants (see *e.g.* Iida et al. (1995) Plant Cell Rep. 14: 539-44). Almost any tissue can be used for transient expression studies as long as the cell wall is penetrable by the DNA-coated particles. For example, embryogenic plant cell cultures have been used successfully for the production of transformed plants (see *e.g.* Fromm *et al.* (1990) Bio/Technology 8: 833-9). Shoot apical meristem transformation results in chimeric plants, where the transformed cells directly give rise to germ-line tissue and the introduced DNA is then passed onto progeny plants. Bombardment of shoot meristematic tissues followed by tissue culture expansion of the transformed cells has been used to produce genetically-transmissible transgenic plant lines (McCable *et al.* (1988) Bio/Technology 6: 923-6). In addition to embryogenic cultures and shoot tips, other tissues that have been subjected to particle bombardment include leaves (Klein *et al.* (1988) Proc. Nat. Acad. Sci. USA 85: 8502-5), root sections (Seki *et al.* (1991) Appl. Microbiol. Biotech. 36: 228-30), stem sections (Loopstra *et al.* (1992) Can. J. Res. 22: 993-6), pollen (Twell *et al.* (1989) Plant Physiol. 91-1270-4), styles (Clark and Sims (1994) Plant Physiol. 106: 25-36), cereal aleurone cells (Kim *et al.* (1992) Mol. Gen. Genet. 232: 383-93) and tassel primordia (Dupeuis and Pace (1993) Plant Cell Rep. 12: 607-11). In certain instances, the plant tissue that is selected for particle bombardment-mediated transformation be relatively new, as long-term cell cultures can result in abnormalities that may compromise the usefulness of the transgenic plant--such as infertility of the subsequent transgenic progeny (see Rhodes *et al.* (1988) Biotech. 6: 56-60).

[0139] In certain instances, the magnitude of transgene expression varies markedly with the site of insertion and the nature of the inserted sequence(s). For example, while T-DNA mediated transfer typically results in the insertion of a single complete intact DNA fragment at a single locus, direct DNA transfer approaches frequently result in long concatamers of the transferred DNA (see *e.g.* Czernilofsky *et al.* (1986) DNA 5: 473-82). Such multiple tandem insertions are associated with transcriptional "silencing"

phenomena in certain instances. Furthermore, the site of insertion within the plant genome frequently affects the strength of expression of the transgene--a phenomenon know as "position effect." Accordingly, the invention provides methods for mitigating interference with the expression of the transgene. For example, position effects can be mitigated by flanking transgenes with specific matrix-associated regions which insulate transcriptional regulation from the effects of surrounding chromatin (see, *e.g.*, Mlynarova *et al.* (1994) Plant Cell 7: 599-609). For example, scaffold attachment regions (SARs, also known as matrix attachment regions or MARs) may be included in the transgene vector construct. Usefully, the SARs are ligated to the flanking regions of the gene of interest. These sequences are known in the art (e.g. a tobacco SAR is described in Breyne *et al.* (1992) Plant Cell 4: 463-71; and Allen *et al.* (1996) Plant Cell 8: 899-913). Furthermore, transgene silencing mediated by homology-dependent processes can be avoided by utilizing transgenic plant lines which avoid multiple tandem or inverted repeat insertion patterns, and by limiting homology of the inserted transgene with any corresponding endogenous host gene(s) by engineering conserved codon replacements within the transgene construct where appropriate. When the transgene is inserted as one intact DNA fragment at a single locus, its expression generally behaves in a highly consistent manner. Such transgenic loci exhibit the expected additive gene action both within loci (hemizygous versus homozygous) and between loci (dihybrids between homozygous transgenic individuals). Loss of transgene unction is rare in such transgenic lines (approximately one in ten thousand), which is consistent with the performance of many endogenous plant genes. Optimized transgenic plants of the invention may be obtained by screening candidate plants for persistent expression of the transgene through multiple generations of breeding or rounds of vegetative propogation.

5. EXAMPLES

[0140] The following examples are offered by way of illustration and not by way of limitation.

5.1 Materials and Methods

[0141] AT3G06240: F -box family protein, contains F-box domain Pfam:PF00646

[0142] PRIMERS for the original PCR

[0143] date 02/28/2002

[0144] Forward primer (Fbox-For)

[0145] 5'-CAC CAA ACA ATG GAG GCG ACG AAG AGA G-3'

[0146] Reverse primer (Fbox-Rev_1)

[0147] 5'-ATC TTA GCA AAA AGC AGA CTT C-3'

[0148] These primers were used to successfully RT-PCR the following sequence.

CACC AAACA ATGGAGGCGACGAAGAGAGAAAGACGGA
GAGAAGATGACGACGGCGAAAAAGCTTCACCGGAATCACTCGTTCTTCCACCAGAGATCA
TTACAGAAATTCTTCTCCGATTACCAGCCAAATCGATCGGGCGATTCAGGTGCGTATCAA
AGCTCTTTTGCACTTTATCGTCGGATCCAGGGTTCGCGAAGATTCACCTCGATCTGATCC
TTCGAAACGAATCCGTAAGATCGCTCCACCGTAAGCTCATTGTGTCTTCACATAATCTGT
ACTCGTTAGATTTCAATTCGATCGGTGACGGAATTAGGGATTTAGCGGCTGTGGAACACA
ATTATCCTCTTAAAGACGATCCAAGCATTTTCTCTGAGATGATTAGGAATTACGTGGGGG
ACCATCTGTACGATGATCGTCGCGTGATGCTTAAGCTGAATGCGAAATCGTATCGAAGAA
ACTGGGTTGAGATCGTTGGATCTTCCAATGGTTTAGTGTGTATCTCTCCTGGTGAAGGAG
CTGTTTTCTTGTATAATCCAACTACCGGAGATTCCAAGAGATTACCTGAAAATTTTCGTC
CCAAATCTGTAGAATACGAAAGAGATAATTTCCAAACTTATGGATTTGGTTTCGATGGTC
TCACTGATGATTACAAATTGGTGAAGCTTGTTGCTACCAGTGAAGATATTCTCGATGCTA
GTGTCTATTCCTTGAAGGCTGACTCATGGAGACGGATCTGCAATTTGAATTATGAGCACA
ACGATGGCTCCTACACGTCCGGTGTGCATTTCAACGGTGCGATTCACTGGGTGTTCACAG
AGAGTAGGCACAACCAAAGAGTGGTTGTAGCATTTGATATTCAAACTGAGGAGTTTCGAG
AGATGCCAGTGCCTGATGAAGCTGAAGATTGTTCCCATAGGTTTAGCAACTTTGTGGTCG
GAAGTCTCAATGGACGTCTCTGTGTGGTCAATAGTTGCTACGATGTGCATGATGATATAT
GGGTGATGAGTGAGTACGGTGAAGCTAAATCCTGGAGCAGAATTCGAATCAACTTGTTGT
ATAGGTCGATGAAACCGCTCTGTTCGACTAAGAACGATGAAGAGGTTCTTCTGGAGCTTG
ATGGAGACCTGGTGTTGTACAACTTTGAAACCAATGCATCGAGTAATCTAGGAATTTGTG
GGGTTAAGCTCAGTGACGGGTTCGAGGCAAATACATACGTAGAGAGCCTCATATCACCCA
ACTCTTATG GTATAGAGAGCTGA GGAAGTCTGCTTTTTGCTAAGAT

[0149] One of the purified PCR products, Fbox_like1, was then cloned into GATEWAY pENTR/D-TOPO vector. This was designated as "Entry Clone" and was

used to sub-clone the desired sequence in to GATEWAY expression vectors (Invitrogen, Carlsbad, California cat.number pENTR/D-TOPO, K240020). Further sequence information is available from Invitrogen.

[0150] One of the Fbox entry clone plasmids was chosen for full length sequencing (coded **Fbox-like –LIG1).** The clone was sequenced completely using internal primers as well and was found to be full-length and without PCR errors.

[0151] Sub-cloning of the Fbox-like1 gene fragment to the GATEWAY expression vector pGATE-Phas is shown in Figure 12. The vector was constructed by inserting Gateway Reading Frame Cassette b into PmeI site of pBBV-Phas parent vector.

[0152] The complete pGATE-Phas sequence is shown in Figure 11 (SEQ ID NO: 6). The short underlined sequences on either side of the insert were removed during the ligation reaction, *ie* this is where the insert goes. Subcloning and *Agrobacterium* transformation of the Fbox construct, was then completed. Before *Agrobacterium* transformation, the orientation of the inserts were checked by sequencing, and the correct ones were utilized.

[0153] After Agrobacterium transformation the presence of the construct was confirmed by PCR. Finally, a mixture of 3 independent *Agrobacterium* transformation events were used for transformation into *Arabidopsis*.

5.2 Results of F-box Overexpression

[0154] Lines that have positive increase in oil content have been taken to T3 and/or T4 generation and reanalyzed.

[0155] Screening has shown that over expression of F-box (At3g06240), when driven under a seed specific promoter phaseolin, produced seeds with higher oil phenotype is shown in Figures 1-5. Also the data obtained for the next (T3) generation is shown in Figures 6-7, and further analysis of other aspects of the transgenic seeds is shown in Figure 8-9. In particular, Figure 1 shows the oil content of transgenic phaseolin-F-box lines compared with vector control lines. Data shown are average of 18 lines (T2) and error bars are SE. Figure 2 shows the total seed FAMEs of F-box transgenic lines compared to WT, and vector control lines (error bars are SE based on 18 lines: 3 duplicate for each line). Figure 3 shows the total seed FAMEs of transgenic lines versus

WT and vector control lines (large dots are the two lines with highest oil content and were named F-box2 and F-box3 later chosen for next generation analysis). Figure 4 shows the fatty acid profile and fatty acid molar ratio composition of F-box transgenic lines versus control plants. Figure 5 shows the seed weight determination (around 400 seeds were counted for both wild type seeds and transformants with F-box genes and counted seeds were stored in desiccators overnight, and then determined using an analytical balance and error bars are SE based on 6 replicates).

[0156] Data in above section show that there is an >10% increase in oil content in F-box overexpressor lines comparing to wild type plants. This increase is confirmed both at a per mg basis and at a per seed basis. To further confirm this result, it is essential to determine changes in oil content for next generation. Furthermore, there is a small yet significant decrease in 18:1 and 18:2 in F-box overexpressors comparing to wild type, and there is a significant increase in 18:3 observed. The reason for this is not yet clear.

[0157] Evaluation of F-box gene at T3 generation: T2 plants from F-box2 and F-box3 lines (see Figure 3) were grown under standard conditions. To eliminate the potential effect of BASTA on seed metabolism, transformants were screened by PCR rather than by BASTA spray. F-box2 and F-box3 lines were grown together with WT and vector control lines. FAME analysis of T3 seeds was shown in Figure 6.

[0158] Figure 6 shows the total FAMEs per seed in F-box T3 seeds versus WT and vector control lines (the error bars are SE based on data obtained from 12 plants for F-box2 and F-box3 lines; 6 plants for WT and vector control lines; and the two lines here named F-box2 and F-box3 are the two data points indicated by big dots in Figure 3). Figure 7 shows the total seed FAMEs of transgenic lines (F-box2 and F-box3) versus WT and vector control lines.

[0159] C: N ratio analysis of T2 seeds: The two lines F-box2 and F-box3 were chosen for C: N ratio analysis. This analysis will confirm the changes the seed storage compounds (oil versus protein) (see Figure 8). Figure 8(a) shows the C: N ratio analysis for two independent lines F-box2 and F-box3. Error bars are SE based on six replicates. Figure 8(b) shows the positive correlation between oil content and C:N ratio further that confirms the result (open columns are overexpressors F-box2 and F-box3; grey columns are Wild type *Arabidopsis* seeds).

5.3 Results of F-box knock-out

[0160] KO (knock-out) analysis of F-box gene: Salk T-DNA insertion lines (SALK_048969 and SALK_048962) were ordered, and homozygous plants were picked up by PCR from line SALK_048969, which has a T-DNA insertion at the last exon of the gene. Homozygous plants together with WT plants were grown to maturity; seeds were analyzed for total FAMEs (see Figure 9).

[0161] Figure 9 (a) shows the FAME analysis of F-box-KO lines, and Figure 9(b) shows the fatty acid profile of KO lines and WT (error bars are SE based on nine independent replicates). As shown above, there were no observable differences in oil content between WT and F-box_KO line; however there was a significant increase in 18:1 and 18:2 in F-box_KO comparing to wild type, and there was a significant decrease in 18:3 observed. This mirrors the profile observed with the overexpression lines (See Figure 4).

[0162] Therefore, through this detailed analysis of F-box transgenic lines, it has been shown that overexpression of an F-box gene in *Arabidopsis* can increase total oil content by more than 10%. Indeed, this increase was confirmed in two generations and the analysis was validated with two different approaches (total FAMEs and C: N ratio analysis).

Equivalents

[0163] Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described specifically herein.

DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D'UN TOME.

CECI EST LE TOME __1___ DE __2___

NOTE: Pour les tomes additionels, veillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1___ OF __2___

NOTE: For additional volumes please contact the Canadian Patent Office.

75712-66

CLAIMS:

1. A genetic construct for the overexpression of an F-box protein in plants comprising:

(a) a gene promoter; and

(b) a nucleic acid molecule encoding an F-box protein functionally linked to the gene promoter, wherein the F-box protein comprises a polypeptide comprising a sequence that over its entirety is at least 90% identical to the sequence of SEQ ID NO: 1.

2. The genetic construct of claim 1, wherein the F-box protein is a plant F-box protein.

3. The genetic construct of claim 1 or 2, wherein the gene promoter is a seed-specific promoter.

4. The genetic construct of claim 3, wherein the gene promoter is phaseolin.

5. The genetic construct of claim 1, comprising a pBBV-PHAS expression vector.

6. The genetic construct of any one of claims 1 to 4, wherein the F-box protein comprises a polypeptide comprising the sequence of SEQ ID NO: 1.

7. The genetic construct of any one of claims 1 to 4, wherein the F-box protein is encoded by a nucleic acid molecule that hybridizes under stringent conditions to the nucleic acid complement of SEQ ID NO: 2, wherein the stringent conditions comprise hybridization in 6x SSC at about 45°C, followed by a wash in 2x SSC at about 50°C.

8. The genetic construct of any one of claims 1 to 4, wherein the F-box protein is encoded by the nucleic acid molecule of SEQ ID NO: 2.

9. A transgenic plant cell comprising a genetic construct of any one of claims 1 to 8.

10. The transgenic plant cell of claim 9, wherein the plant is *Arabidopsis thaliana*.

11. The transgenic plant cell of claim 9, wherein the plant is selected from the group consisting of soybean, palm, rapeseed and sunflower.

12. A recombinant *Arabidopsis thaliana* plant cell comprising a heterologous genetic construct having a gene promoter functionally linked to a nucleic acid molecule encoding a plant F-box protein, wherein the plant F-box protein comprises a polypeptide comprising a sequence that over its entirety is at least 90% identical to the sequence of SEQ ID NO: 1.

13. The recombinant *Arabidopsis thaliana* plant cell of claim 12, wherein the nucleic acid molecule encoding the plant F-box protein encodes the polypeptide of SEQ ID NO: 1 and the promoter is phaseolin.

14. Use of a plant comprising the plant cell of any one of claims 9 to 13 for producing progeny.

15. Use of a plant comprising the plant cell of any one of claims 9 to 13 for producing plant oil.

16. A method of modifying oil production in a plant comprising:

stably incorporating into the genome of the plant a genetic construct according to any one of claims 1 to 8 to provide a transformed plant; and

regenerating the transformed plant,

wherein expression of the incorporated genetic construct increases oil production in the plant as compared to a wild type plant.

17. The method of claim 16, wherein the plant is *Arabidopsis thaliana*.

18. The method of claim 16, wherein the plant is selected from the group consisting of soybean, palm, rapeseed and sunflower.

19. A method of increasing oil production in *Arabidopsis thaliana* comprising:

stably incorporating into the genome of the *Arabidopsis thaliana* plant a genetic construct having a phaseolin gene promoter functionally linked to a nucleic acid molecule encoding an *Arabidopsis* F-box polypeptide of SEQ ID NO: 1; and

regenerating the transformed plant,

wherein expression of the incorporated genetic construct increases oil production in the *Arabidopsis thaliana* plant as compared to a wild type plant.

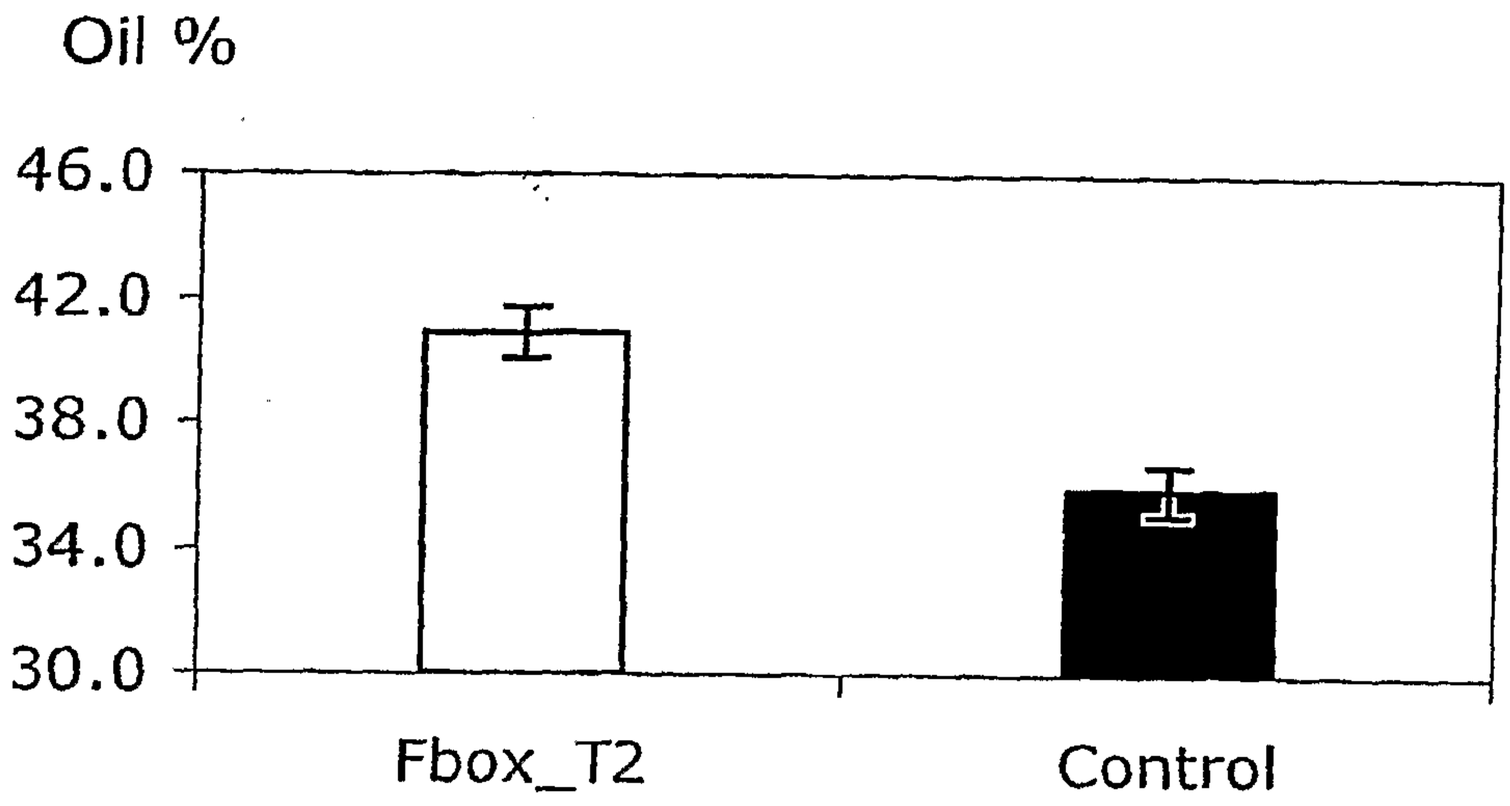
Oil %
46.0
42.0
38.0
34.0
30.0
Fbox_T2
Control
Genotype

FIG. 1

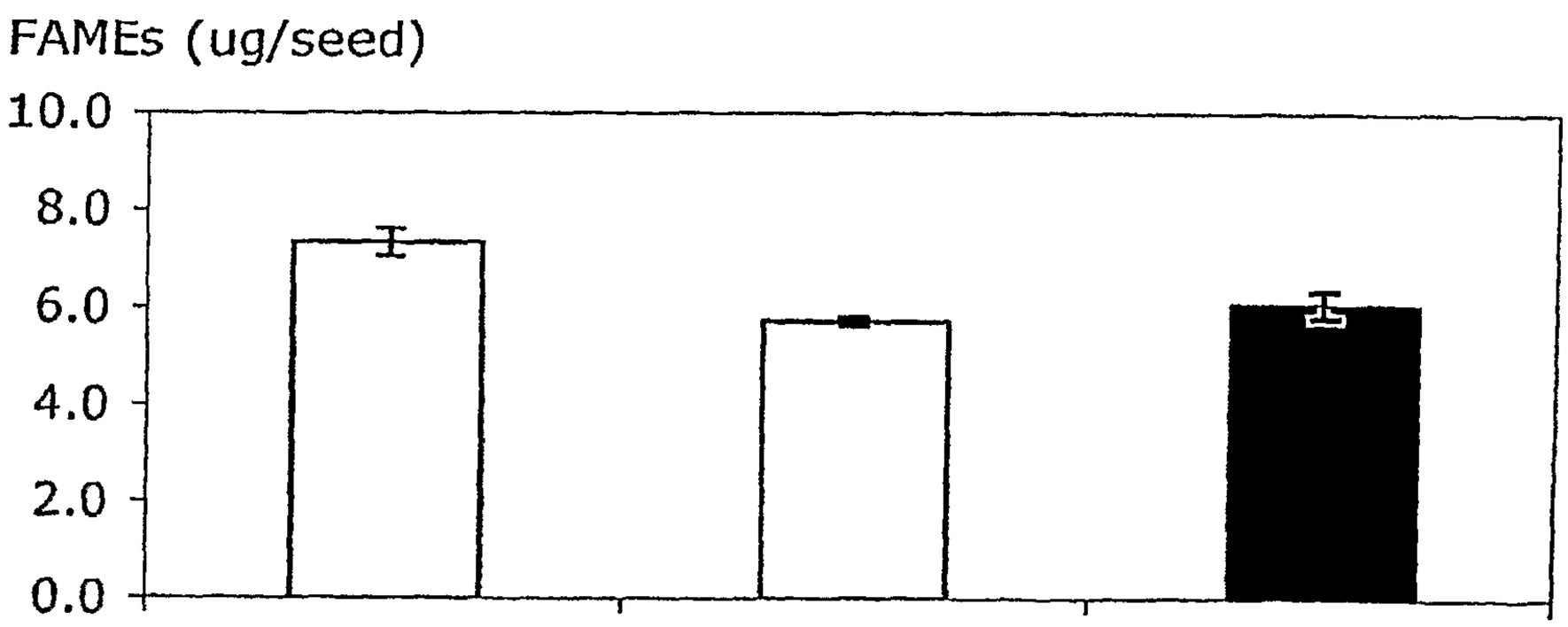
FAMEs (ug/seed)
10.0
8.0
6.0
4.0
2.0
0.0
Fbox_T2
Control
WT
Genotype

FIG. 2

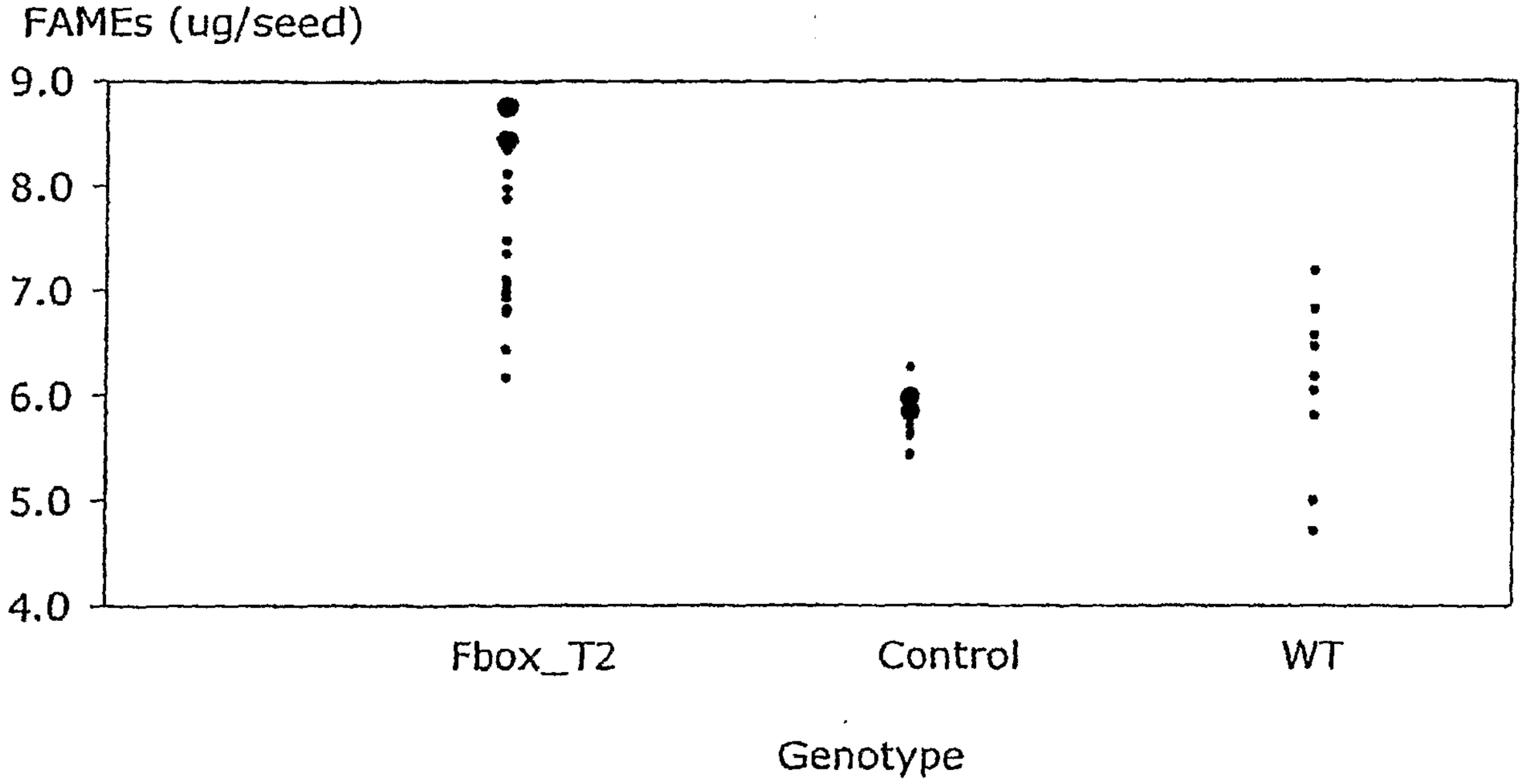
FAMEs (ug/seed)
9.0
8.0
7.0
6.0
5.0
4.0
Fbox_T2
Control
WT
Genotype

FIG. 3

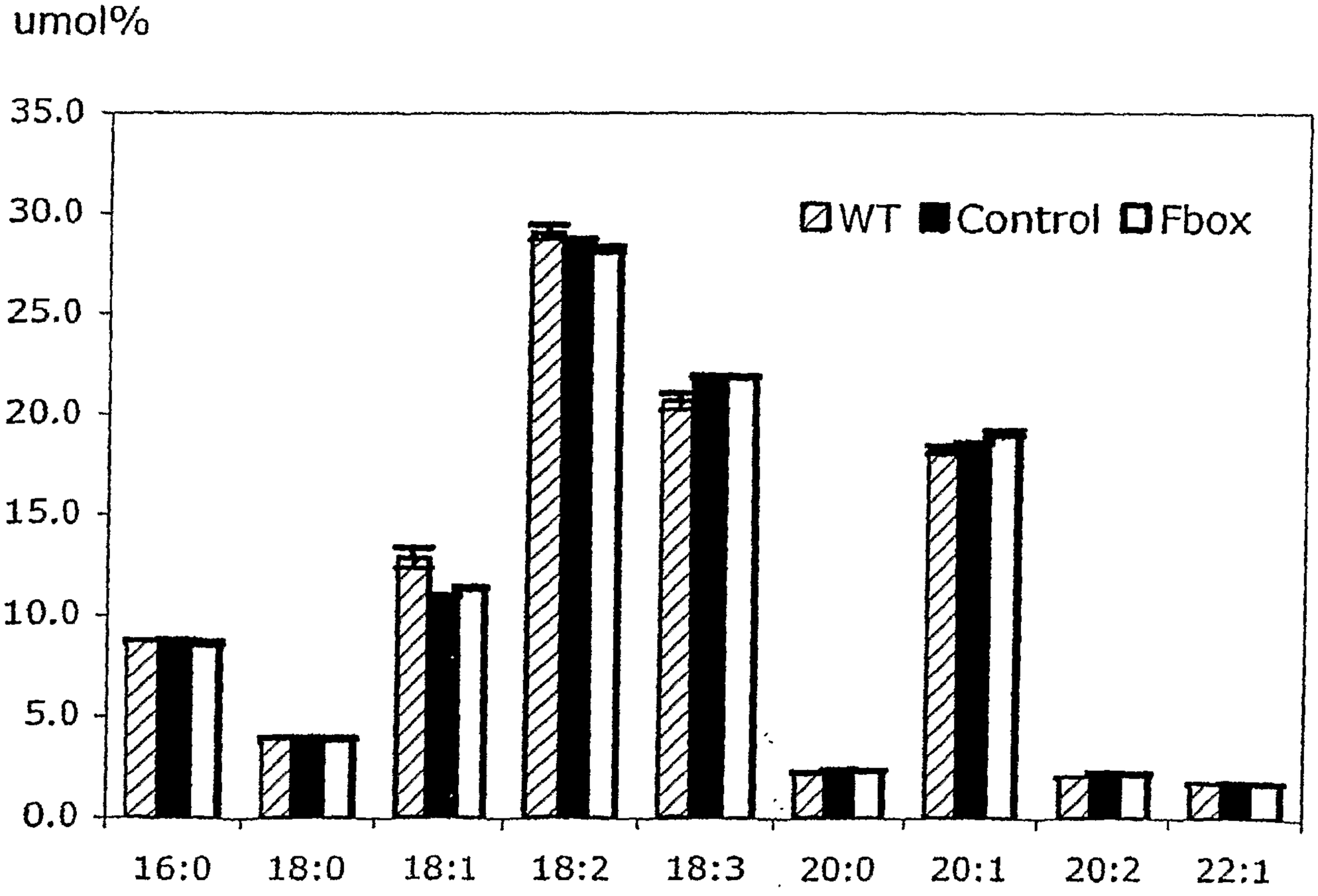
umol%
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
WT
Control
Fbox
16:0
18:0
18:1
18:2
18:3
20:0
20:1
20:2
22:1

FIG. 4

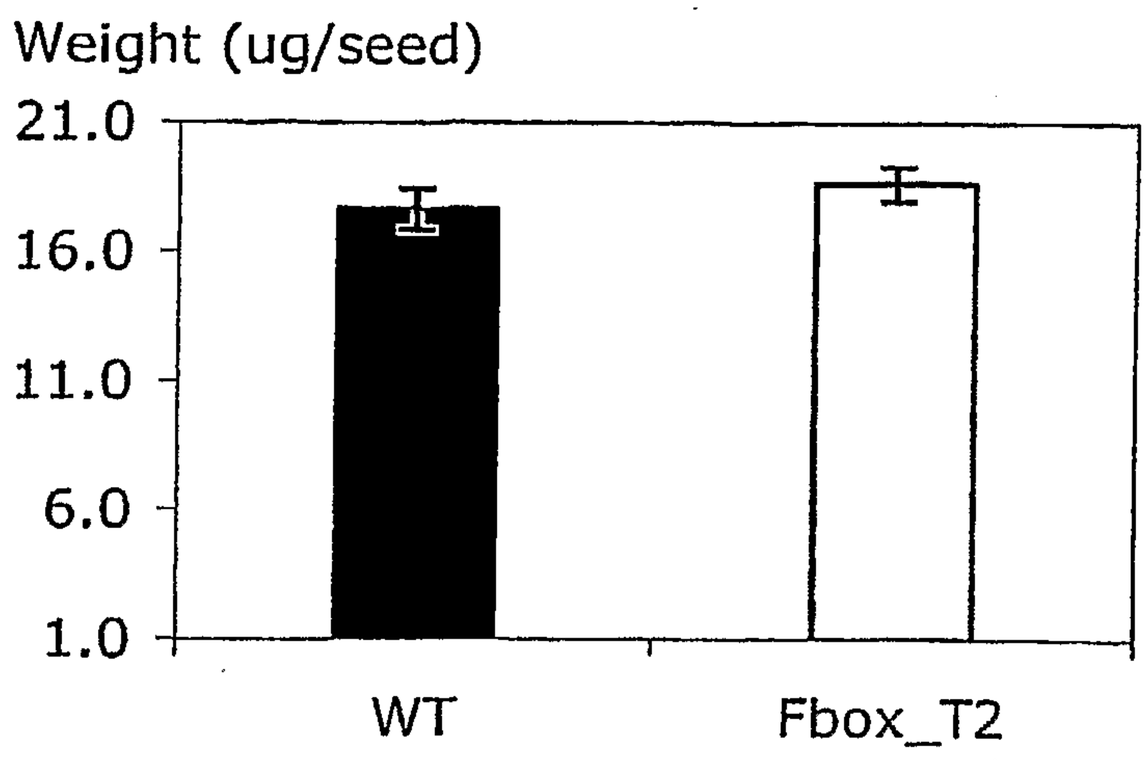
Weight (ug/seed)
21.0
16.0
11.0
6.0
1.0
WT
Fbox_T2

FIG. 5

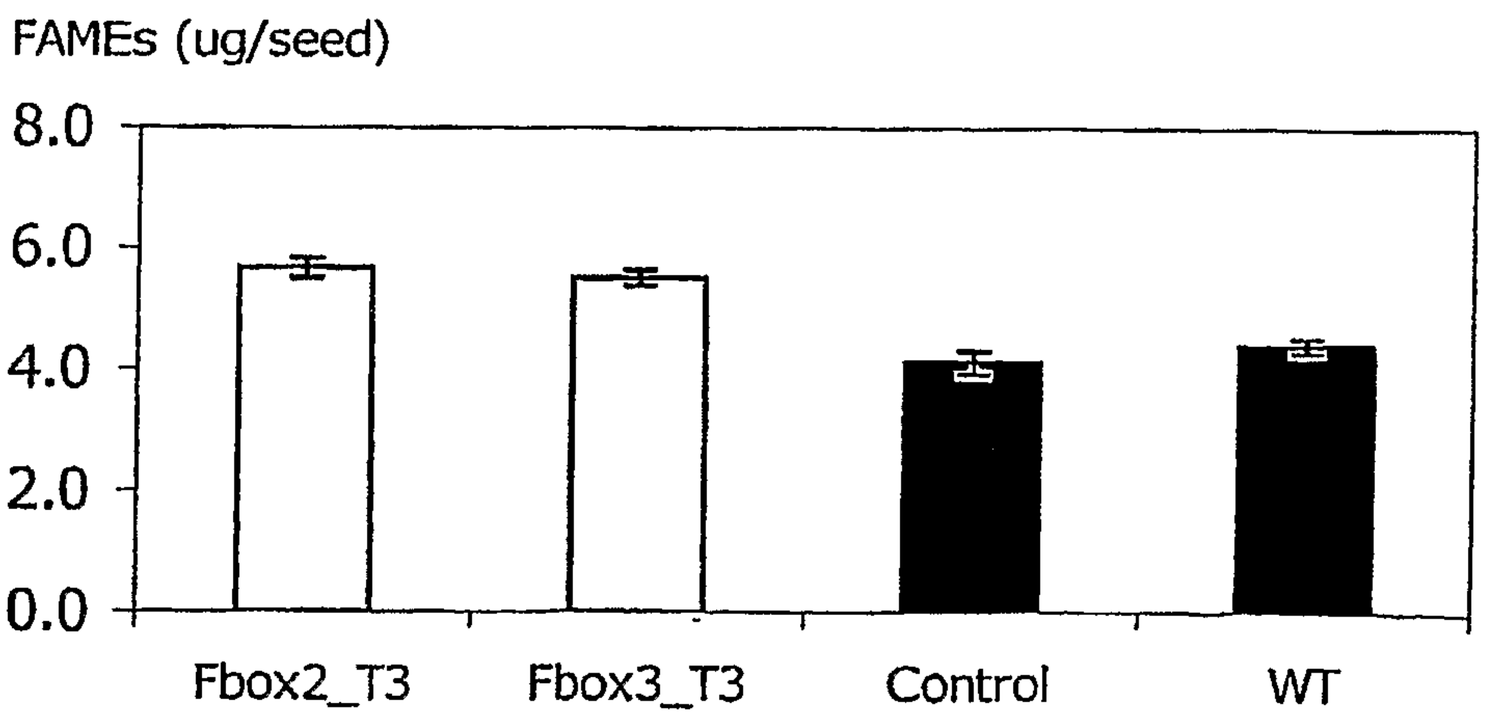
FAMEs (ug/seed)
8.0
6.0
4.0
2.0
0.0
Fbox2_T3
Fbox3_T3
Control
WT

FIG. 6

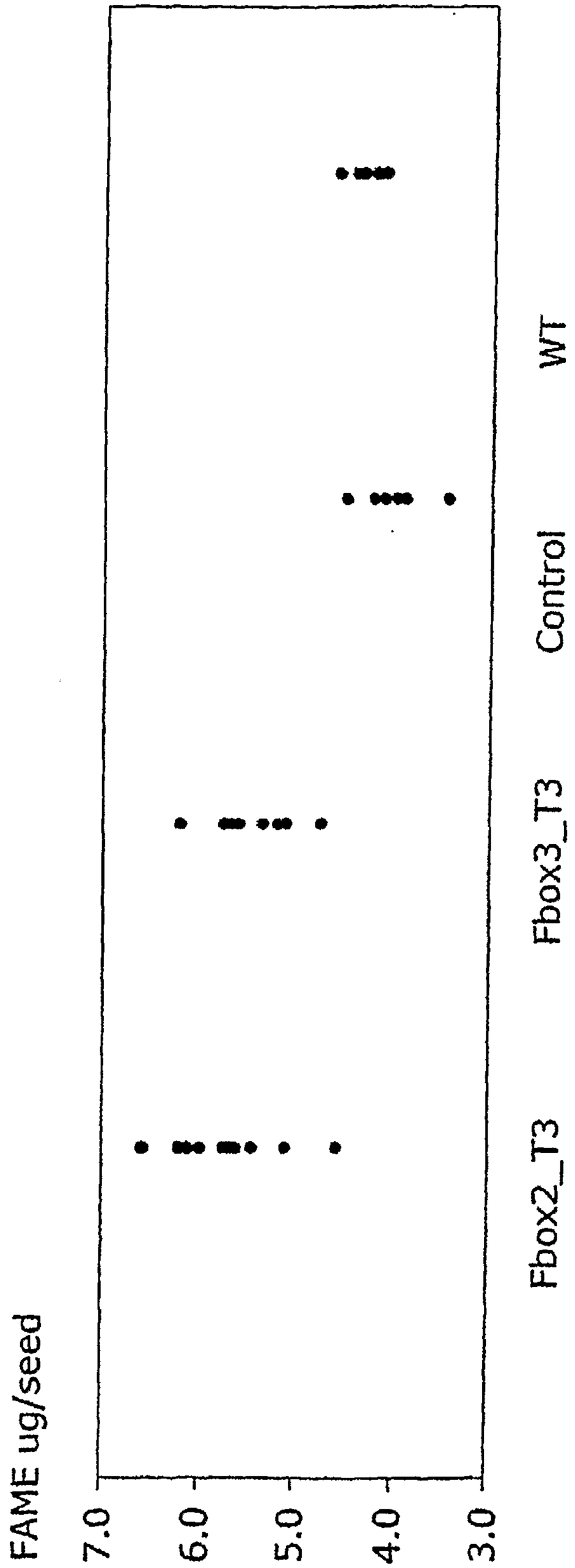

FIG. 7

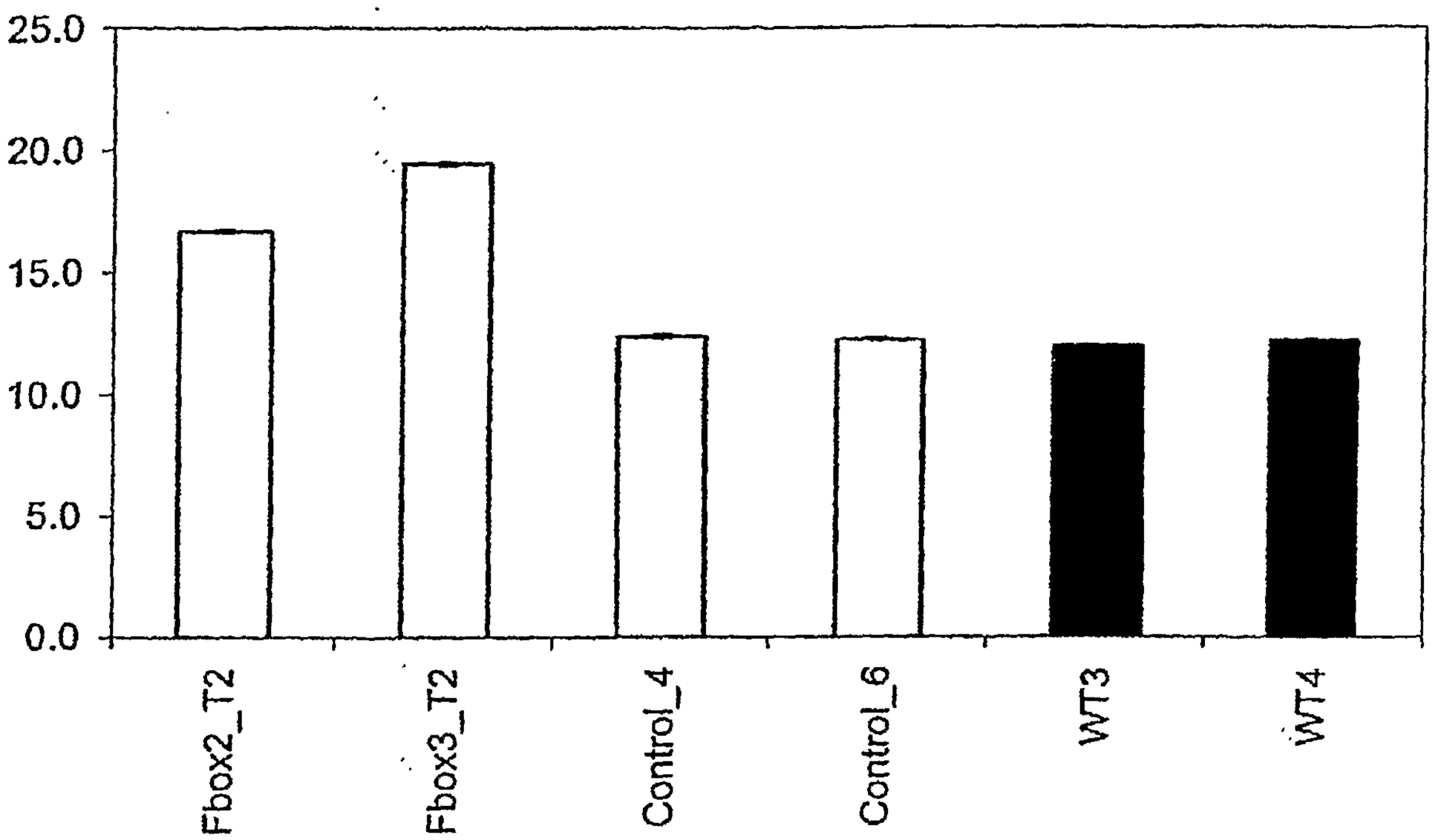
C:N ratio
25.0
20.0
15.0
10.0
5.0
0.0
Fbox2_T2
Fbox3_T2
Control_4
Control_6
WT3
WT4

FIG. 8A

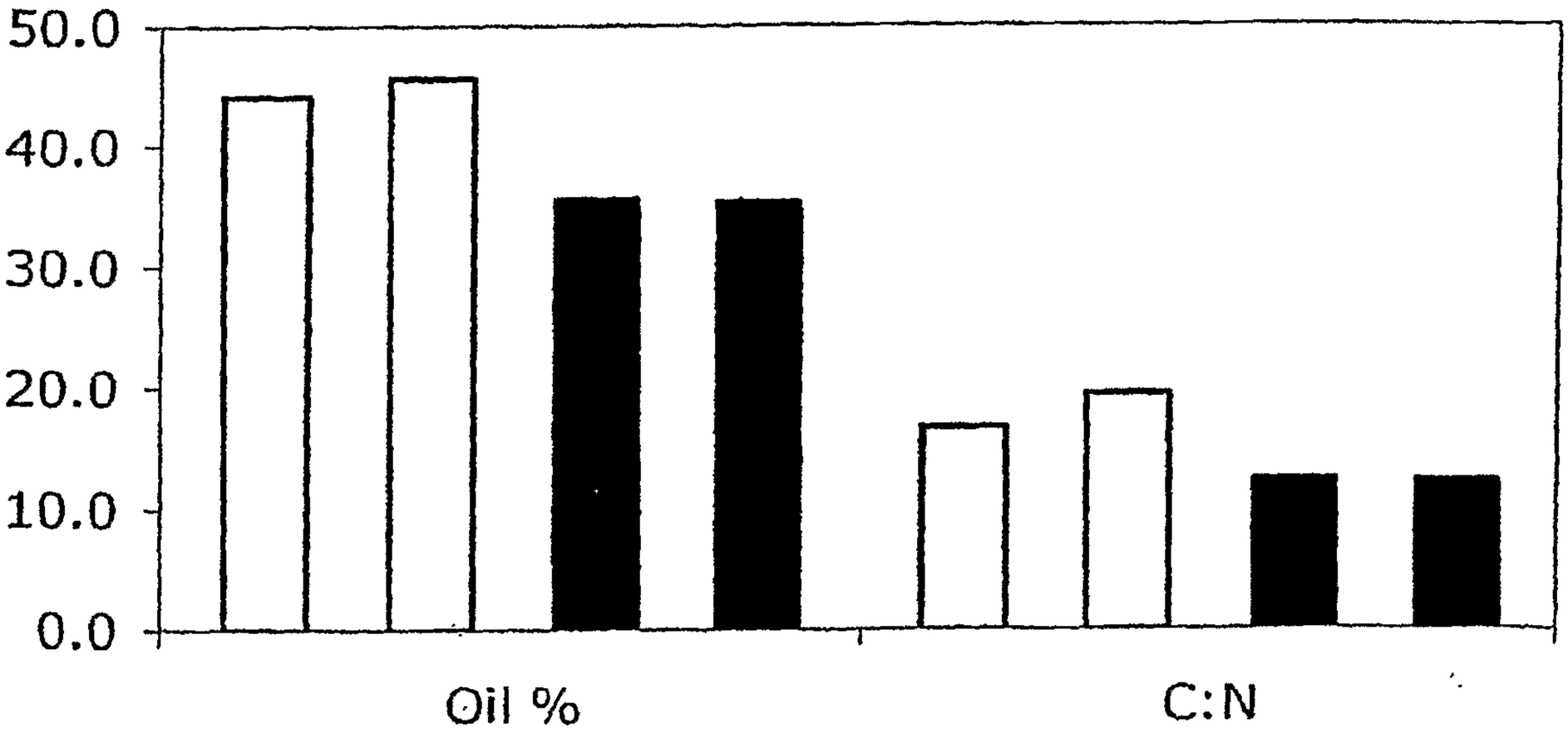
50.0
40.0
30.0
20.0
10.0
0.0
Oil %
C:N

FIG. 8B

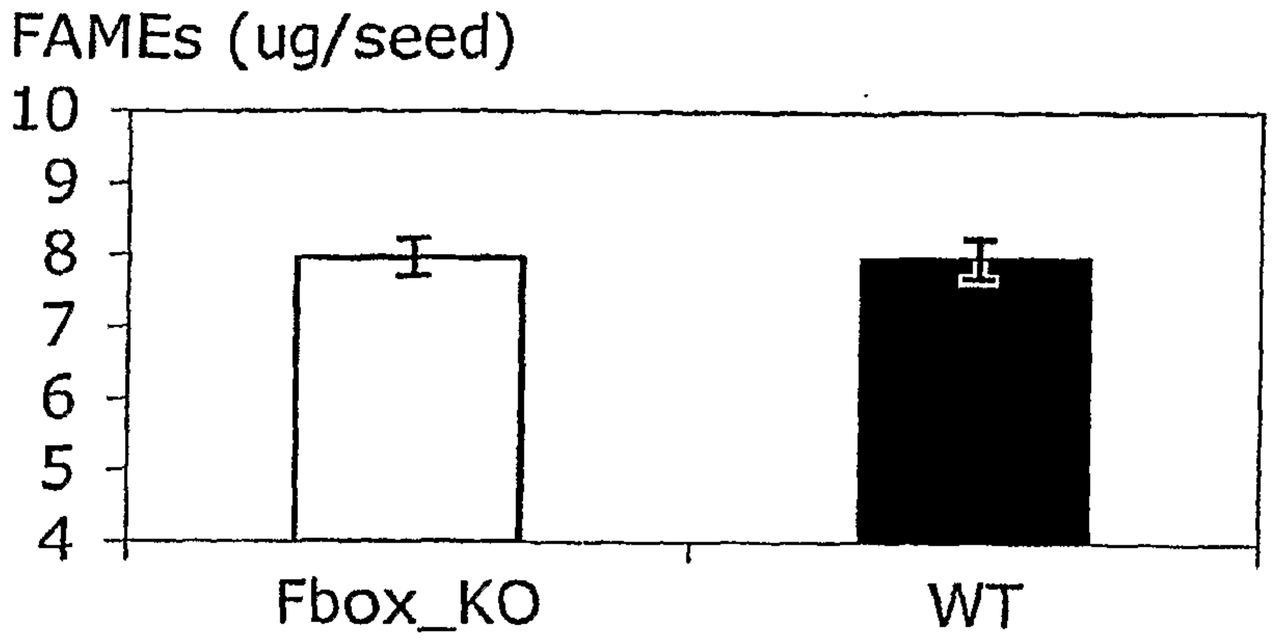
FAMEs (ug/seed)
10
9
8
7
6
5
4
Fbox_KO
WT

FIG. 9A

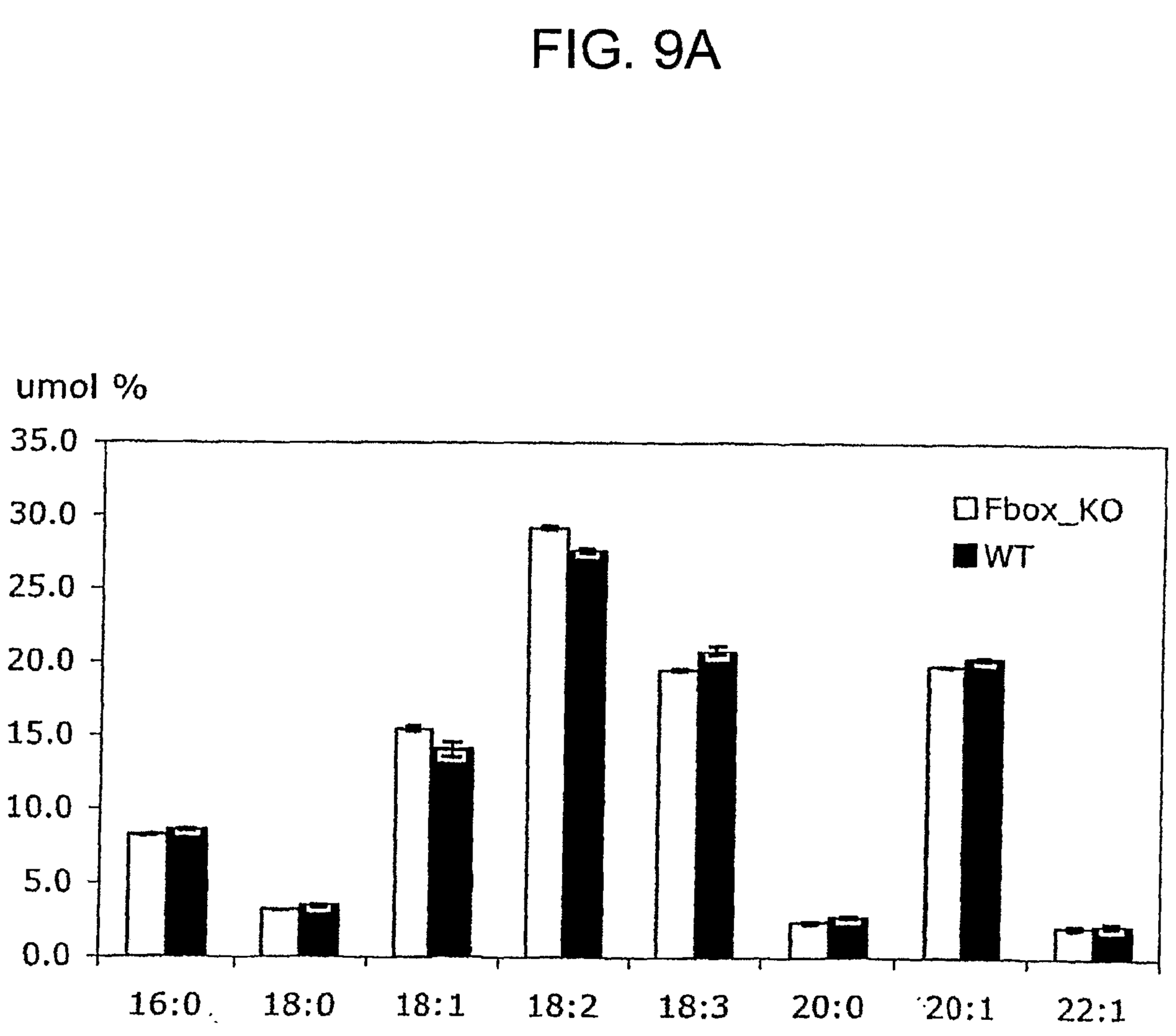
umol %
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Fbox_KO
WT
16:0
18:0
18:1
18:2
18:3
20:0
20:1
22:1

FIG. 9B

POLYPEPTIDE SEQUENCE OF ARABIDOPSIS THALIANA F-BOX PROTEIN (GENBANK ACCESSION NO. NP_566277 (SEQ ID NO. 1))

```
  1 MKAIQLLWEA IMEATKRERR REDDDGEKAS PESLVLPPEI ITEILLRLPA KSIGRFRCVS
 61 KLFCTLSSDP GFAKIHLDLI LRNESVRSLH RKLIVSSHNL YSLDFNSIGD GIRDLAAVEH
121 NYPLKDDPSI FSEMIRNYVG DHLYDDRRVM LKLNAKSYRR NWVEIVGSSN GLVCISPGEG
181 AVFLYNPTTG DSKRLPENFR PKSVEYERDN FQTYGFGFDG LTDDYKLVKL VATSEDILDA
241 SVYSLKADSW RRICNLNYEH NDGSYTSGVH FNGAIHWVFT ESRHNQRVVV AFDIQTEEFR
301 EMPVPDEAED CSHRFSNFVV GSLNGRLCVV NSCYDVHDDI WVMSEYGEAK SWSRIRINLL
361 YRSMKPLCST KNDEEVLLEL DGDLVLYNFE TNASSNLGIC GVKLSDGFEA NTYVESLISP
421 NSYGIES
```

FIG. 10A

FIG. 10B-1

FIG. 10B-2

FIG. 10B-3

FIG. 10B

DNA SEQUENCE OF ARABIDOPSIS THALIANA F-BOX PROTEIN (GENBANK ACCESSION NO. NM_111499 (SEQ ID NO. 2))

```
  1 TTTTTCAAAT CAAATCAGAA TACATTGATT CTGTATATCT TATTGAAAAA TCCATCAATT
 61 TACATCAACA ATTTTATATC TAATAATTAA TTTAAAGAGA AAATTTATAA AAGTTTATTA
121 GAGCAAATAA CTCAAACTCG GATTTTATAG TCGTTATGAC CCGGTTTGAC TATTGAACCG
181 TTTAACCGAG AAATTGGGAC TCAATTAAGA CAACCGAAAC TAGACCCGGA TCCAGTGTTA
241 GCGGGCTAGA TTAAGGTGTC GGGTCATAGC GGAGAAGCAA CCAGACGCCA ACAATGAAAG
301 CGATCCAGTT GCTGTGGGAA GCGATAATGG AGGCGACGAA GAGAGAAAGA CGGAGAGAAG
361 ATGACGACGG CGAAAAAGCT TCACCGGAAT CACTCGTTCT TCCACCAGAG ATCATTACAG
421 AAATTCTTCT CCGATTACCA GCCAAATCGA TCGGGCGATT CAGGTGCGTA TCAAAGCTCT
```

FIG. 10B-1

481 TTTGCACTTT ATCGTCAGAT CCAGGGTTCG CGAAGATTCA CCTCGATCTG ATCCTTCGAA

541 ACGAATCCGT AAGATCGCTC CACCGTAAGC TCATTGTGTC TTCACATAAT CTGTACTCGT

601 TAGATTTCAA TTCGATCGGT GACGGAATTA GGGATTTAGC GGCTGTGGAA CACAATTATC

661 CTCTTAAAGA CGATCCAAGC ATTTTCTCTG AGATGATTAG GAATTACGTG GGGGACCATC

721 TGTACGATGA TCGTCGCGTG ATGCTTAAGC TGAATGCGAA ATCGTATCGA AGAAACTGGG

781 TTGAGATCGT TGGATCTTCC AATGGTTTAG TGTGTATCTC TCCTGGTGAA GGAGCTGTTT

841 TCTTGTATAA TCCAACTACC GGAGATTCCA AGAGATTACC TGAAAATTTT CGTCCCAAAT

901 CTGTAGAATA CGAAAGAGAT AATTTCCAAA CTTATGGATT TGGTTTCGAT GGTCTCACTG

961 ATGATTACAA ATTGGTGAAG CTTGTTGCTA CCAGTGAAGA TATTCTCGAT GCTAGTGTCT

1021 ATTCCTTGAA GGCTGACTCA TGGAGACGGA TCTGCAATTT GAATTATGAG CACAACGATG

1081 GCTCCTACAC GTCCGGTGTG CATTTCAACG GTGCGATTCA CTGGGTGTTC ACAGAGAGTA

FIG. 10B-2

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```
1141 GGCACAACCA AAGAGTGGTT GTAGCATTTG ATATTCAAAC CGAGGAGTTT CGAGAGATGC
1201 CAGTGCCTGA TGAAGCTGAA GATTGTTCCC ATAGGTTTAG CAACTTTGTG GTCGGAAGTC
1261 TCAATGGACG TCTCTGTGTG GTCAATAGTT GCTACGATGT GCATGATGAT ATATGGGTGA
1321 TGAGTGAGTA CGGTGAAGCT AAATCCTGGA GCAGAATTCG AATCAACTTG TTGTATAGGT
1381 CGATGAAACC GCTCTGTTCG ACTAAGAACG ATGAAGAGGT.TCTTCTGGAG CTTGATGGAG
1441 ACCTGGTGTT GTACAACTTT GAAACCAATG CATCGAGTAA TCTAGGAATT TGTGGGGTTA
1501 AGCTCAGTGA CGGGTTCGAG GCAAATACAT ACGTAGAGAG CCTCATATCA CCCAACTCTT
1561 ATGGTATAGA GAGCTGAGGA AGTCTGCTTT TTGCTAAGAT ATAATAAACC AACATTCGGA
1621 TTAGAAATGT TTTAGAAACA TAATCATGTA ATATGTATCA TGTAATTAAC AACGAATGGT
1681 CAATGGGTAT TTTAAGTTTC TTTCTCCT
```

FIG. 10B-3

SUBSTITUTE SHEET (RULE 26)

FIG. 11A
FIG. 11B
FIG. 11C
•
•
•
FIG. 11M

FIG. 11

(SEQ ID NO:6)

```
  1  ggccgcaaca gaggtggatg gacagacccg ttcttacacc ggactgggcg
 51  cgggatagga tattcagatt gggatgggat tgagcttaaa gccggcgctg
101  agaccatgct caaggtaggc aatgtcctca gcgtcgagcc cggcatctat
151  gtcgagggca ttggtggagc gcgcttcggg gataccgtgc ttgtaactga
201  gaccggatat gaggccctca ctccgcttga tcttggcaaa gatatttgac
251  gcatttatta gtatgtgtta attttcattt gcagtgcagt attttctatt
301  cgatctttat gtaattcgtt acaattaata aatattcaaa tcagattatt
351  gactgtcatt tgtatcaaat cgtgtttaat ggatattttt attataatat
401  tgatgatatc tcaatcaaaa cgtagataat aataatattt atttaatatt
451  tttgcgtcgc acagtgaaaa tctatatgag attacaaaat accgacaaca
501  ttatttaaga tacatagaca ttaaccctga gactgttgga cagagctcat
551  tggtacctca gatctgggta actggcctaa ctggccttgg aggagctggc
601  aactcaaaat ccctttgcca aaaaccaaca tcatgccatc caccatgctt
651  gtatccagct gcgcgcaatg taccccgggc tgtgtatccc aaagcctcat
701  gcaacctaac agatggatcg tttggaaggc ctataacagc aaccacagac
751  ttaaaacctt gcgcctccat agacttaagc aaatgtgtgt acaatgtgga
801  tcctaggccc aacctttgat gcctatgtga cacgtaaaca gtactctcaa
851  ctgtccaatc gtaagcgttc ctagccttcc agggcccagc gtaagcaata
901  ccagccacaa caccctcaac ctcagcaacc aaccaagggt atctatcttg
```

```
 951  caacctctct agatcatcaa tccactcttg tggtgtttgt ggctctgtcc
1001  taaagttcac tgtagacgtc tcaatgtaat ggttaacgat atcacaaacc
1051  gcggccatat cagctgctgt agctggccta atctcaactg gtctcctctc
1101  cggagaagcc atggtttgga tccacaaact tacaaatttc tctgaagttg
1151  tatcctcagt acttcaaaga aaatagctta caccaatttt ttcttgtttt
1201  cacaaatgcc gaacttggtt ccttatatag gaaaactcaa gggcaaaaat
1251  gacacggaaa aatataaaag gataagtagt gggggataag attcctttgt
1301  gataaggtta ctttccgccc ttacattttc caccttacat gtgtcctcta
1351  tgtctctttc acaatcaccg accttatctt cttcttttca ttgttgtcgt
1401  cagtgcttac gtcttcaaga ttcttttctt cgcctggttc ttctttttca
1451  atttctacgt attcttcttc gtattctggc agtataggat cttgtatctg
1501  tacattcttc atttttgaac ataggttgca tatgtgccgc atattgatct
1551  gcttcttgct gagctcacat aatacttcca tagtttttcc cgtaaacatt
1601  ggattcttga tgctacatct tggataatta ccttctggcc ggccgcgaat
1651  tcgttggtag ggtgctagga aacttgtttt tggggttttg tataagggtt
1701  gaaacatccc tgaagtgtct cattttattt tatttattct ttgctgataa
1751  aaaaataaaa taaaagaagc taagcacacg gtcaaccatt gctctactgc
1801  taaaagggtt atgtgtagtg ttttactgca taaattatgc agcaaacaag
```

FIG. 11B

SUBSTITUTE SHEET (RULE 26)

```
1851  acaactcaaa ttaaaaaatt tcctttgctt gtttttttgt tgtctctgac
1901  ttgactttct tgtggaagtt ggttgtataa ggattgggac accattgtcc
1951  ttcttaattt aattttattc tttgctgata aaaaaaaaaa atttcatata
2001  gtgttaaata ataatttgtt aaataaccaa aaagtcaaat atgtttactc
2051  tcgtttaaat aattgagatt cgttccagca aggctaaacg attgtataga
2101  tttatgacaa tatttacttt tttatagata aatgttatat tataataaat
2151  ttatatacat atattatatg ttatttatta tttattatta ttttaaatcc
2201  ttcaatattt tatcaaacca actcataatt tttttttttat ctgtaagaag
2251  caataaaatt aaatagaccc actttaagga tgatccaacc tttatacaga
2301  gtaagagagt tcaaatagta ccttttcata tacatatcaa ctaaaatatt
2351  agaaatatca tggatcaaac cttataaaga cattaaataa gtggataagt
2401  ataatatata aatgggtagt atataatata taaatggata caaacttctc
2451  tctttataat tgttatgtct ccttaacatc ctaatataat acataagtgg
2501  gtaatatata atatataaat ggagacaaac ttcttccatt ataattgtta
2551  tgtcttctta acacttatgt ctcgttcaca atgctaaagt tagaattgtt
```

FIG. 11C

SUBSTITUTE SHEET (RULE 26)

```
2601  tagaaagtct tatagtacac atttgttttt gtactatttg aagcattcca
2651  taagccgtca cgattcagat gatttataat aataagagga aatttatcat
2701  agaacaataa ggtgcataga tagagtgtta atatatcata acatcctttg
2751  tttattcata gaagaagtga gatggagctc agttattata ctgttacatg
2801  gtcggataca atattccatg ctctccatga gctcttacac ctacatgcat
2851  tttagttcat actggtgacc ctcgaggcga tcgctttggc cggccattta
2901  aatggcgcgc ctttgcccgg gctttcctgc aggGTTTTATC AACCACTTTG
2951  TACAAGAAAG CTGAACGAGA AACGTAAAAT GATATAAATA TCAATATATT
3001  AAATTAGATT TTGCATAAAA AACAGACTAC ATAATACTGT AAAACACAAC
3051  ATATCCAGTC ACTATGGTCG ACCTGCAGAC TGGCTGTGTA TAAGGGAGCC
3101  TGACATTTAT ATTCCCCAGA ACATCAGGTT AATGGCGTTT TTGATGTCAT
3151  TTTCGCGGTG GCTGAGATCA GCCACTTCTT CCCCGATAAC GGAGACCGGC
3201  ACACTGGCCA TATCGGTGGT CATCATGCGC CAGCTTTCAT CCCCGATATG
3251  CACCACCGGG TAAAGTTCAC GGGAGACTTT ATCTGACAGC AGACGTGCAC
3301  TGGCCAGGGG GATCACCATC CGTCGCCCGG GCGTGTCAAT AATATCACTC
3351  TGTACATCCA CAAACAGACG ATAACGGCTC TCTCTTTTAT AGGTGTAAAC
3401  CTTAAACTGC ATTTCACCAG TCCCTGTTCT CGTCAGCAAA AGAGCCGTTC
3451  ATTTCAATAA ACCGGGCGAC CTCAGCCATC CCTTCCTGAT TTTCCGCTTT
3501  CCAGCGTTCG GCACGCAGAC GACGGGCTTC ATTCTGCATG GTTGTGCTTA
```

FIG. 11D

SUBSTITUTE SHEET (RULE 26)

```
3551  CCAGACCGGA GATATTGACA TCATATATGC CTTGAGCAAC TGATAGCTGT
3601  CGCTGTCAAC TGTCACTGTA ATACGCTGCT TCATAGCACA CCTCTTTTTG
3651  ACATACTTCG GGTATACATA TCAGTATATA TTCTTATACC GCAAAAATCA
3701  GCGCGCAAAT ACGCATACTG TTATCTGGCT TTTAGTAAGC CGGATCCAGA
3751  TCTTTACGCC CCGCCCTGCC ACTCATCGCA GTACTGTTGT AATTCATTAA
3801  GCATTCTGCC GACATGGAAG CCATCACAGA CGGCATGATG AACCTGAATC
3851  GCCAGCGGCA TCAGCACCTT GTCGCCTTGC GTATAATATT TGCCCATGGT
3901  GAAAACGGGG GCGAAGAAGT TGTCCATATT GGCCACGTTT AAATCAAAAC
3951  TGGTGAAACT CACCCAGGGA TTGGCTGAGA CGAAAAACAT ATTCTCAATA
4001  AACCCTTTAG GGAAATAGGC CAGGTTTTCA CCGTAACACG CCACATCTTG
4051  CGAATATATG TGTAGAAACT GCCGGAAATC GTCGTGGTAT TCACTCCAGA
4101  GCGATGAAAA CGTTTCAGTT TGCTCATGGA AAACGGTGTA ACAAGGGTGA
4151  ACACTATCCC ATATCACCAG CTCACCGTCT TTCATTGCCA TACGGAATTC
4201  CGGATGAGCA TTCATCAGGC GGGCAAGAAT GTGAATAAAG GCCGGATAAA
4251  ACTTGTGCTT ATTTTTCTTT ACGGTCTTTA AAAAGGCCGT AATATCCAGC
4301  TGAACGGTCT GGTTATAGGT ACATTGAGCA ACTGACTGAA ATGCCTCAAA
4351  ATGTTCTTTA CGATGCCATT GGGATATATC AACGGTGGTA TATCCAGTGA
4401  TTTTTTTCTC CATTTTAGCT TCCTTAGCTC CTGAAAATCT CGCCGGATCC
4451  TAACTCAAAA TCCACACATT ATACGAGCCG GAAGCATAAA GTGTAAAGCC
4501  TGGGGTGCCT AATGCGGCCG CCATAGTGAC TGGATATGTT GTGTTTTACA
4551  GTATTATGTA GTCTGTTTTT TATGCAAAAT CTAATTTAAT ATATTGATAT
4601  TTATATCATT TTACGTTTCT CGTTCAGCTT TTTTGTACAA ACTTGTTGAT
```

FIG. 11E

4651 **aaac**ttaatt aaggatccta gagtagtatt gaatatgagt tgggttgggg
4701 tattatagta gtagagtagt agtactctgg atggatggat gatgaaagaa
4751 gtgagtgata ttagaggtat ttataggtat tatataagag agaaggtggt
4801 tggaacatgc atggagattt gggcatggga tgacacgcat atgcaggttg
4851 acgtgtgttg aagtgaagaa attgaggtgg cggaagagaa tgaatatata
4901 caggtggttg tggtgatgat gaagaaaaag gcaatgtgtt tgtgtgtggg
4951 ttgagatggg tgagccattt aaagtgcatg ttaagcacgt gttgctttgc
5001 atggcattta gacatacatg gacgcggcga tcttgatcag ccagtgacta
5051 atttgagttg gttgtgtgat tgcgttttgt ctctctgttt tgtcttttct
5101 ctttgttctt tgtctttttc ttgcgcaagc atccatgcat gaaccaaaag
5151 accacagagt gtcatggcaa cccacagtaa ttccagttac ggacttacat
5201 accaagaaaa ggtaaaagca ataagaaata tatgaaaatt agtccaccat
5251 aaatcttata gtttatggtt tagggtaaca ctctaacact ctactacatt
5301 acttatattt actttaaact atctataaaa caatttaaac atagtagaat
5351 aataaatcaa tagtcacaaa ttcaacaatt aaacttaaaa ttaaaaaagt
5401 aatattttaa ttatatctaa ttaatttttt agaagtaata ttgagtattt

FIG. 11F

SUBSTITUTE SHEET (RULE 26)

```
5451  gatatatgaa atcttgaata tagtaactat tattaaaatt actttagaag
5501  atgtgtctcg catgtaaaag cagatcttca gttacttccg tagtgtcaaa
5551  tgggaattat agaattttgc ataacatgac ttgcttcaga aataccacaa
5601  atcattgttt ggtgaaattt tcattgtata aaaaaaatac aatgataatt
5651  ggatttttt attcaaagaa aaaaatggct agttgtgtca ctgggtgttg
5701  ctttaagatc agtcgaataa aaaaattatg gagttaaatt ttattacttt
5751  tgaaacaact tattattatg agatttacgt ggttgaaaaa tatttgataa
5801  atatatttt aaaatataaa atgggaaatc cttcttaagg taaagaattt
5851  gtttatattg tatattaaac atttatatga agaagaataa gaataaatca
5901  ttatgctttc taccaacgct aaaattaagt aaattatata tttcaatatg
5951  aaaatgttag actacattaa agatagacgg gacttcataa aattttatgc
6001  ggtttgaaaa tgtttaacaa ataataaatt tgtagggata tcgtgtgcgg
6051  aagcgtgata atttcaacca aagattatga gaaattaaag taacaagtaa
6101  agtgagaatg ataccagaat ttttaggtgg aaaaccccctt taaatagagg
6151  taaaaaacca ccggcgagag agccaaaact ttcactataa tgatactggg
6201  agtacaatgg cggccggggc tgcaattgat ccggtgagta atattgtacg
6251  gctaagagcg aatttggcct gtagacctca attgcgagct ttctaatttc
6301  aaactattcg ggcctaactt ttggtgtgat gatgctgact ggcaggatat
6351  ataccgttgt aatttgagct cgtgtgaata agtcgctgtg tatgtttgtt
6401  tgattgtttc tgttggagtg cagcccattt caccggacaa gtcggctaga
```

FIG. 11G

SUBSTITUTE SHEET (RULE 26)

```
6451  ttgatttagc cctgatgaac tgccgagggg aagccatctt gagcgcggaa
6501  tgggaatgga tcgaaccggg agcacaggat gacgcctaac aattcattca
6551  agccgacacc gcttcgcggc gcggcttaat tcaggagtta aacatcatga
6601  gggaagcggt gatcgccgaa gtatcgactc aactatcaga ggtagttggc
6651  gtcatcgagc gccatctcga accgacgttg ctggccgtac atttgtacgg
6701  ctccgcagtg gatggcggcc tgaagccaca cagtgatatt gatttgctgg
6751  ttacggtgac cgtaaggctt gatgaaacaa cgcggcgagc tttgatcaac
6801  gacctttttgg aaacttcggc ttcccctgga gagagcgaga ttctccgcgc
6851  tgtagaagtc accattgttg tgcacgacga catcattccg tggcgttatc
6901  cagctaagcg cgaactgcaa tttggagaat ggcagcgcaa tgacattctt
6951  gcaggtatct tcgagccagc cacgatcgac attgatctgg ctatcttgct
7001  gacaaaagca agagaacata gcgttgcctt ggtaggtcca gcggcggagg
7051  aactctttga tccggttcct gaacaggatc tatttgaggc gctaaatgaa
7101  accttaacgc tatggaactc gccgcccgac tgggctggcg atgagcgaaa
7151  tgtagtgctt acgttgtccc gcatttggta cagcgcagta accggcaaaa
7201  tcgcgccgaa ggatgtcgct gccgactggg caatggagcg cctgccggcc
7251  cagtatcagc ccgtcatact tgaagctagg caggcttatc ttggacaaga
7301  agatcgcttg gcctcgcgcg cagatcagtt ggaagaattt gttcactacg
```

FIG. 11H

SUBSTITUTE SHEET (RULE 26)

```
7351  tgaaaggcga gatcaccaag gtagtcggca aataatgtct aacaattcgt
7401  tcaagccgac gccgcttcgc ggcgcggctt aactcaagcg ttagagagct
7451  ggggaagact atgcgcgatc tgttgaaggt ggttctaagc ctcgtacttg
7501  cgatggcatt tcgatcgaaa ggggtacaaa ttcccactaa gcgctcgggg
7551  gctgagaaag cccagtaagg aaacaactgt aggttcgagt cgcgagatcc
7601  cccggaacca aaggaagtag gttaaacccg ctccgatcag gccgagccac
7651  gccaggccga gaacattggt tcctgtaggc atcgggattg gcggatcaaa
7701  cactaaagct actggaacga gcagaagtcc tccggccgcc agttgccagg
7751  ccgtaaaggt gagcagaggc acgggaggtt gccacttgcg ggtcagcacg
7801  gttccgaacg ccatggaaac cgcccccgcc aggcccgctg cgacgccgac
7851  aggatctagc gctgcgtttg gtgtcaacac caacagcgcc acgcccgcag
7901  ttccgcaaat agcccccagg accgccatca atcgtatcgg gctacctagc
7951  agagcggcag agatgaacac gaccatcagc ggctgcacag cgcctaccgt
8001  cgccgcgacc cgcccggcag gcggtagacc gaaataaaca acaagctcca
8051  gaatagcgaa atattaagtg cgccgaggat gaagatgcgc atccaccaga
8101  ttcccgttgg aatctgtcgg acgatcatca cgagcaataa acccgccggc
8151  aacgcccgca gcagcatacc ggcgacccct cggcctcgct gttcgggctc
8201  cacgaaaacg ccggacagat gcgccttgtg agcgtccttg gggccgtcct
8251  cctgtttgaa gaccgacagc ccaatgatct cgccgtcgat gtaggcgccg
```

FIG. 11I

SUBSTITUTE SHEET (RULE 26)

```
8301  aatgccacgg catctcgcaa ccgttcagcg aacgcctcca tgggcttttt
8351  ctcctcgtgc tcgtaaacgg acccgaacat ctctggagct ttcttcaggg
8401  ccgacaatcg gatctcgcgg aaatcctgca cgtcggccgc tccaagccgt
8451  cgaatctgag ccttaatcac aattgtcaat tttaatcctc tgtttatcgg
8501  cagttcgtag agcgcgccgt gcgcccgagc gatactgagc gaagcaagtg
8551  cgtcgagcag tgcccgcttg ttcctgaaat gccagtaaag cgctggctgc
8601  tgaaccccca gccggaactg accccacaag gccctagcgt ttgcaatgca
8651  ccaggtcatc attgacccag gcgtgttcca ccaggccgct gcctcgcaac
8701  tcttcgcagg cttcgccgac ctgctcgcgc cacttcttca cgcgggtgga
8751  atccgatccg cacatgaggc ggaaggtttc cagcttgagc gggtacggct
8801  cccggtgcga gctgaaatag tcgaacatcc gtcgggccgt cggcgacagc
8851  ttgcggtact tctcccatat gaatttcgtg tagtggtcgc cagcaaacag
8901  cacgacgatt tcctcgtcga tcaggacctg gcaacgggac gttttcttgc
8951  cacggtccag gacgcggaag cggtgcagca gcgacaccga ttccaggtgc
9001  ccaacgcggt cggacgtgaa gcccatcgcc gtcgcctgta ggcgcgacag
9051  gcattcctcg gccttcgtgt aataccggcc attgatcgac cagcccaggt
9101  cctggcaaag ctcgtagaac gtgaaggtga tcggctcgcc gatagggggtg
9151  cgcttcgcgt actccaacac ctgctgccac accagttcgt catcgtcggc
9201  ccgcagctcg acgccggtgt aggtgatctt cacgtccttg ttgacgtgga
```

FIG. 11J

SUBSTITUTE SHEET (RULE 26)

```
9251   aaatgacctt gttttgcagc gcctcgcgcg ggattttctt gttgcgcgtg
9301   gtgaacaggg cagagcgggc cgtgtcgttt ggcatcgctc gcatcgtgtc
9351   cggccacggc gcaatatcga acaaggaaag ctgcatttcc ttgatctgct
9401   gcttcgtgtg tttcagcaac gcggcctgct tggcctcgct gacctgtttt
9451   gccaggtcct cgccggcggt ttttcgcttc ttggtcgtca tagttcctcg
9501   cgtgtcgatg gtcatcgact tcgccaaacc tgccgcctcc tgttcgagac
9551   gacgcgaacg ctccacggcg gccgatggcg cgggcagggc aggggagcc
9601   agttgcacgc tgtcgcgctc gatcttggcc gtagcttgct ggaccatcga
9651   gccgacggac tggaaggttt cgcggggcgc acgcatgacg gtgcggcttg
9701   cgatggtttc ggcatcctcg gcggaaaacc ccgcgtcgat cagttcttgc
9751   ctgtatgcct tccggtcaaa cgtccgattc attcaccctc cttgcgggat
9801   tgccccgact cacgccgggg caatgtgccc ttattcctga tttgacccgc
9851   ctggtgcctt ggtgtccaga taatccacct tatcggcaat gaagtcggtc
9901   ccgtagaccg tctggccgtc cttctcgtac ttggtattcc gaatcttgcc
9951   ctgcacgaat accagcgacc ccttgcccaa atacttgccg tgggcctcgg
10001  cctgagagcc aaaacacttg atgcggaaga agtcggtgcg ctcctgcttg
10051  tcgccggcat cgttgcgcca ctcttcatta accgctatat cgaaaattgc
10101  ttgcggcttg ttagaattgc catgacgtac ctcggtgtca cgggtaagat
```

FIG. 11K

SUBSTITUTE SHEET (RULE 26)

```
10151  taccgataaa ctggaactga ttatggcnnc tcgaaattcc ctcggtcttg
10201  ccttgctcgt cggtgatgta cttcaccagc tccgcgaagt cgctcttctt
10251  gatggagcgc atggggacgt gcttggcaat cacgcgcacc ccccggccgt
10301  tttagcggct aaaaaaagtca tggctctgcc ctcgggcgga ccacgcccat
10351  catgaccttg ccaagctcgt cctgcttctc ttcgatcttc gccagcaggg
10401  cgaggatcgt ggcatcaccg aaccgcgccg tgcgcgggtc gtcggtgagc
10451  cagagtttca gcaggccgcc caggcggccc aggtcgccat tgatgcgggc
10501  cagctcgcgg acgtgctcat agtccacgac gcccgtgatt ttgtagccct
10551  ggccgacggc cagcaggtag gccgacaggc tcatgccggc cgccgccgcc
10601  ttttcctcaa tcgctcttcg ttcgtctgga aggcagtaca ccttgatagg
10651  tgggctgccc ttcctggttg gcttggtttc atcagccatc cgcttgccct
10701  catctgttac gccggcggta gccggccagc ctcgcagagc aggattcccg
10751  ttgagcaccg ccaggtgcga ataagggaca gtgaagaagg aacacccgct
10801  cgcgggtggg cctacttcac ctatcctgcc cggctgacgc cgttggatac
10851  accaaggaaa gtctacacga acccttggc aaaatcctgt atatcgtgcg
10901  aaaaaggatg gatataccga aaaaatcgct ataatgaccc cgaagcaggg
10951  ttatgcagcg gaaaagatcc gtcgaccctt tccgacgctc accgggctgg
11001  ttgccctcgc cgctgggctg gcggccgtct atggccctgc aaacgcgcca
11051  gaaacgccgt cgaagccgtg tgcgagacac cgcggccgcc ggcgttgtgg
11101  ataccacgcg gaaaacttgg ccctcactga cagatgaggg gcggacgttg
```

FIG. 11L

SUBSTITUTE SHEET (RULE 26)

```
11151  acacttgagg ggccgactca cccggcgcgg cgttgacaga tgaggggcag
11201  gctcgatttc ggccggcgac gtggagctgg ccagcctcgc aaatcggcga
11251  aaacgcctga ttttacgcga gtttcccaca gatgatgtgg acaagcctgg
11301  ggataagtgc cctgcggtat tgacacttga ggggcgcgac tactgacaga
11351  tgaggggcgc gatccttgac acttgagggg cagagtgatg acagatgagg
11401  ggcgcaccta ttgacatttg aggggctgtc cacaggcaga aaatccagca
11451  tttgcaaggg tttccgcccg tttttcggcc accgctaacc tgtcttttaa
11501  cctgctttta aaccaatatt tataaacctt gtttttaacc agggctgcgc
11551  cctggcgcgt gaccgcgcac gccgaagggg ggtgcccccc cttctcgaac
11601  cctcccggcc cgctaacgcg ggcctcccat ccccccaggg gctgcgcccc
11651  tcggccgcga acggcctcac cccaaaaatg gcaggccaag cttgcttggt
11701  cgttccggta cgtaccgtga acgtcggctc gattgtacct gcgttcaaat
11751  actttgcgat cgtgttgcgc gcctgcccgg tgcgtcggct gatctcacgg
11801  atcgactgct tctctcgcaa cgccatccga cggatgatgt ttaaaagtcc
11851  catgtggatc actccgttgc cccgtcgctc accgtgttgg ggggaaggtg
11901  cacatggctc agttctcaat ggaaattatc tgcctaaccg gctcagttct
11951  gcgtagaaac caacatgcaa gctccaccgg gtgcaaagcg gcagcggcgg
12001  caggatatat tcaattgtaa atggcttcat gtccgggaaa tctacatgga
12051  tcagcaatga gtatgatggt caatatggag aaaaagaaag agtaattacc
12101  aatttttttt caattcaaaa atgtagatgt ccgcagcgtt attataaaat
12151  gaaagtacat tttgataaaa cgacaaatta cgatccgtcg tatttatagg
12201  cgaaagcaat aaacaaatta ttctaattcg gaaatcttta tttcgacgtg
12251  tctacattca cgtccaaatg ggggcggcga att
```

FIG. 11M

SUBSTITUTE SHEET (RULE 26)

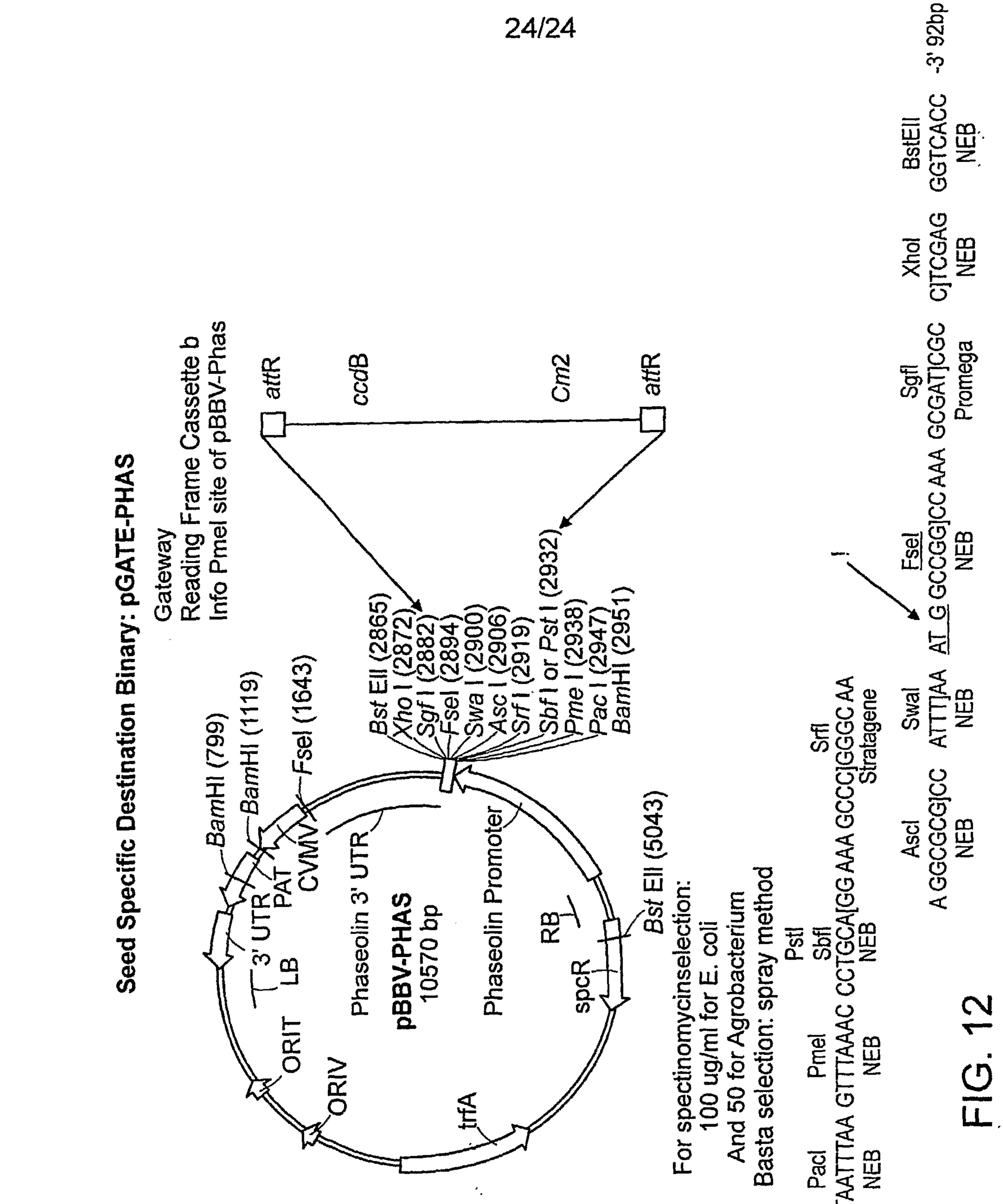
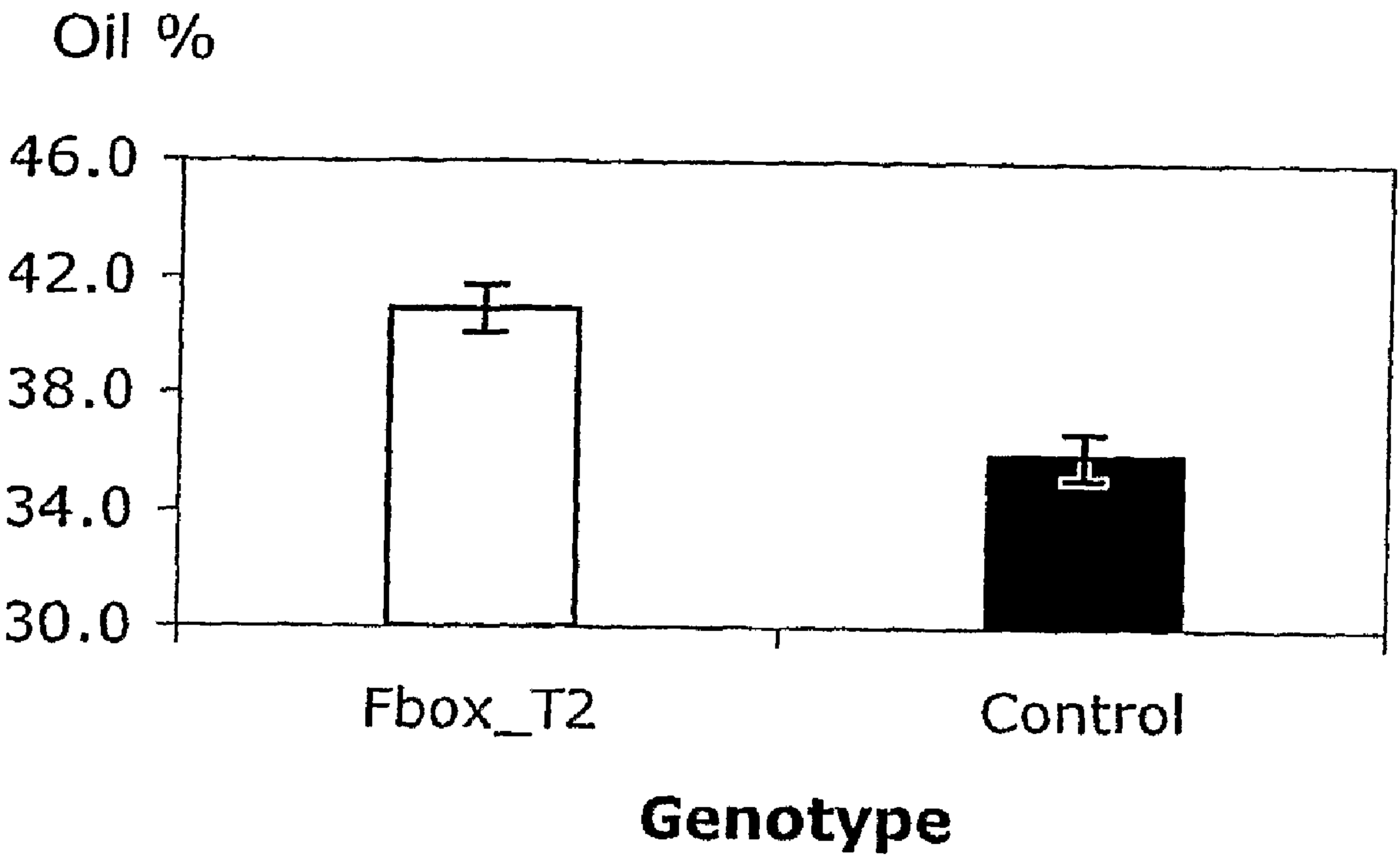

Seed Specific Destination Binary: pGATE-PHAS
Gateway
Reading Frame Cassette b
Info PmeI site of pBBV-Phas
attR
ccdB
Cm2
attR
Bst EII (2865)
Xho I (2872)
Sgf I (2882)
Fse I (2894)
Swa I (2900)
Asc I (2906)
Srf I (2919)
Sbf I or Pst I (2932)
Pme I (2938)
Pac I (2947)
BamHI (2951)
BamHI (799)
BamHI (1119)
FseI (1643)
3' UTR
PAT
CVMV
Phaseolin 3' UTR
pBBV-PHAS
10570 bp
Phaseolin Promoter
RB
spcR
Bst EII (5043)
ORIT
ORIV
trfA
LB
For spectinomycinselection:
100 ug/ml for E. coli
And 50 for Agrobacterium
Basta selection: spray method
BamHI NEB
PstI NEB
SbfI NEB
PmeI NEB
PacI NEB
SrfI Stratagene
AscI NEB
SwaI NEB
FseI NEB
SgfI Promega
XhoI NEB
BstEII NEB
-3' 92bp
5'-GGATCC TTAATTTAA GTTTAAAC CCTGCA]GG AAA GCCC]GGGC AA
ATTT]AA AT G GCCGG]CC AAA GCGAT]CGC
A GGCGCGC]CC
GGTCACC

FIG. 12


Oil %
46.0
42.0
38.0
34.0
30.0
Fbox_T2
Control
Genotype