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(54) Title: MODULATION OF ACC SYNTHASE IMPROVES PLANT YIELD UNDER LOW NITROGEN CONDITIONS

(57) Abstract: The invention provides methods for improving plant yield, particularly under nitrogen limiting conditions. According to the invention, applicants have discovered that modulating ACC synthase activity in plants improves yield of plants, even when grown under low nitrogen conditions. The same plants, while demonstrating improved yield over non-modified plants, exhibited no deleterious effects under normal nitrogen conditions. The invention further provides methods using recombinant expression cassettes, host cells and transgenic plants.

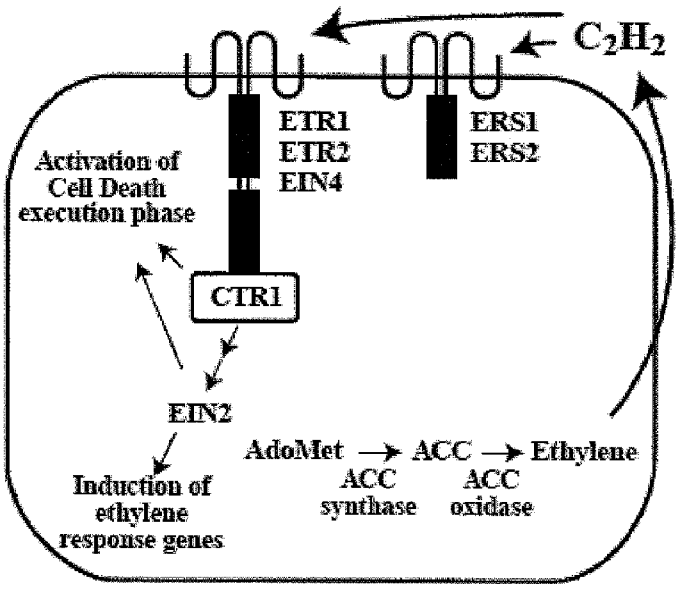

FIG. 1

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MODULATION OF ACC SYNTHASE IMPROVES PLANT YIELD UNDER LOW NITROGEN CONDITIONS

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS A TEXT FILE VIA EFS-WEB

The official copy of the sequence listing is submitted concurrently with the specification as a text file via EFS-Web, in compliance with the American Standard Code for Information Interchange (ASCII), with a file name of 388171SEQLIST.TXT, a creation date of April 13, 2010, and a size of 200 KB. The sequence listing filed via EFS-Web is part of the specification and is hereby incorporated in its entirety by reference herein.

FIELD OF THE INVENTION

The invention relates generally to the field of molecular biology, specifically the modulation of ACC synthase activity to improve plant yield and nitrogen stress tolerance.

BACKGROUND OF THE INVENTION

The domestication of many plants has correlated with dramatic increases in yield. Most phenotypic variation occurring in natural populations is continuous and is effected by multiple gene influences. The identification of specific genes responsible for the dramatic differences in yield in domesticated plants has become an important focus of agricultural research.

Nitrogen utilization efficiency (NUE) genes affect yield and have utility for improving the use of nitrogen in crop plants, especially maize. Increased nitrogen use efficiency can result from enhanced uptake and assimilation of nitrogen fertilizer and/or the subsequent remobilization and reutilization of accumulated nitrogen reserves, as well as increased tolerance of plants to stress situations such as low nitrogen environments. The genes can be used to alter the genetic composition of the plants, rendering them more productive with current fertilizer application standards or maintaining their productive rates with significantly reduced fertilizer or reduced nitrogen availability. Improving NUE in corn would increase corn harvestable yield

per unit of input nitrogen fertilizer, both in developing nations where access to nitrogen fertilizer is limited and in developed nations where the level of nitrogen use remains high. Nitrogen utilization improvement also allows decreases in on-farm input costs, decreased use and dependence on the non-renewable energy sources required for nitrogen fertilizer production and reduces the environmental impact of nitrogen fertilizer manufacturing and agricultural use.

SUMMARY OF THE INVENTION

Methods and compositions for improving plant yield are provided. In some embodiments, plant yield is improved under stress, particularly abiotic stress, such as nitrogen limiting conditions. Methods of improving plant yield include inhibiting the ethylene synthesis pathway, such as, for example, inhibiting the activity of at least one 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. The activity of an ACC synthase can be inhibited using any method known in the art, including but not limited to the disruption of an ACC synthase gene, or a decrease in the expression of the gene through the use of co-suppression, antisense, or RNA silencing or interference.

Inhibiting the activity of at least one ACC synthase in a plant can improve the nitrogen stress tolerance of the plant and such plants can maintain their productive rates with significantly less nitrogen fertilizer input and/or exhibit enhanced uptake and assimilation of nitrogen fertilizer and/or remobilization and reutilization of accumulated nitrogen reserves. In addition to an overall increase in yield, the improvement of nitrogen stress tolerance through the inhibition of ACC synthase can also result in increased root mass and/or length, increased ear, leaf, seed, and/or endosperm size, and/or improved standability. Accordingly, in some embodiments, the methods further comprise growing said plants under nitrogen limiting conditions and optionally selecting those plants exhibiting greater tolerance to the low nitrogen levels.

Further, methods and compositions are provided for improving yield under abiotic stress, which include evaluating the environmental conditions of an area of cultivation for abiotic stressors (e.g., low nitrogen levels in the soil) and planting seeds or plants having reduced ethylene synthesis, which in some embodiments, is due to reduced activity of at least one ACC synthase, in stressful environments.

Constructs and expression cassettes comprising nucleotide sequences that can efficiently reduce the expression of an ACC synthase are also provided herein.

DESCRIPTION OF THE FIGURES

Figure 1 is a schematic illustration of the ethylene biosynthetic and signaling genes in plants, e.g., Arabidopsis. Ethylene is generated from methionine by a well-defined pathway involving the conversion of S-adenosyl-L-methionine (SAM or Ado Met) to the cyclic amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) which is facilitated by ACC synthase. ACC synthase is an aminotransferase which catalyzes the rate limiting step in the formation of ethylene by converting S-adenosylmethionine to ACC.

Ethylene is then produced from the oxidation of ACC through the action of ACC oxidase (also known as the ethylene forming enzyme) with hydrogen cyanide as a secondary product that is detoxified by β-cyanoalanine synthase. Finally, ethylene is metabolized by oxidation to CO_2 or to ethylene oxide and ethylene glycol.

Figure 2, panels A-C, illustrate the ACS2 hairpin construct. Panel A is a schematic diagram of a PHP plasmid containing an ubiquitin promoter (UBI1ZM PRO) driving expression of the ACS2 hairpin (a terminal repeat consisting of TR1 and TR2). RB represents the Agrobacterium right border sequence. A 4126 bp fragment of the 49682 bp cassette is illustrated. Panel B presents the sequence of ZM-ACS2 TR1 (SEQ ID NO: 12) and Panel C presents the sequence of ZM-ACS2 TR2 (SEQ ID NO: 13).

Figure 3, panels A-C, illustrate the ACS6 hairpin construct. Panel A is a schematic diagram of a PHP plasmid containing an ubiquitin promoter (UBI1ZM PRO) driving expression of the ACS6 hairpin (a terminal repeat consisting of TR1 and TR2). RB represents the Agrobacterium right border sequence. A 3564 bp fragment of the 49108 bp cassette is illustrated. Panel B presents the sequence of ZM-ACS6 TR1 (SEQ ID NO: 14) and Panel C presents the sequence of ZM-ACS6 TR2 (SEQ ID NO: 15).

Figure 4 is a schematic of an improved ACS6 inhibition expression cassette, which is set forth in SEQ ID NO:57.

Figure 5 shows the yield of transformed plants of the invention under flowering stress in Environment 1. Each bar represents a separate transformation event. Average yield of transgene-negative segregants is shown (139 bu/a) as control (CN). A total of 74% of the events yielded nominally more than the control plants. Plants representing 18 transgenic events outyielded the control at $P<0.10$.

Figure 6 shows the yield of transformed plants of the invention under grain-fill stress in Environment 2. Each bar represents a separate transformation event. Average yield of transgene-negative segregants is shown (176 bu/a) as control (CN). Thirteen

events out-yielded the CN at P<0.10. Of these, eight had also shown significant improvement under flowering stress.

Figure 7 shows the yield, as a percent of control, of transformed plants of the invention (indicated by a circle), as well as plants transformed using an alternative ACS6 inhibition vector (indicated by a square) under grain fill stress in Environment 3. Each data point represents a separate transformation event. NS = not significant. The control plants are bulked transgene-negative segregants. As can be seen, 64% of the events of the invention had significantly superior yield; only 17% of the alternative ACS6 inhibition events had significantly superior yield, relative to the control.

Figure 8 shows the yield, as a percent of control, of transformed plants of the invention (indicated by a circle), as well as plants transformed using an alternative ACS6 inhibition vector (indicated by a square) under rain-fed conditions in Environment 4. Each data point represents a separate transformation event. NS = not significant. The control plants are bulked transgene-negative segregants. As can be seen, all points exhibiting statistically significant increases in yield represent events of the invention disclosed herein. In addition, all points exhibiting statistically significant decreases in yield are events containing the alternative ACS6 inhibition vector.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the surprising finding that modulation of ACC synthase (ACS) improves plant responses in low nitrogen conditions, with no deleterious effect on plant performance under normal nitrogen conditions. In fact, plants with ACS inhibition constructs actually had superior yield not only in low nitrogen conditions, but also under normal nitrogen conditions. Accordingly, methods for improving plant yield, particularly under abiotic stress, by modulating the ethylene synthesis pathway are provided.

Ethylene is generated from methionine by a well-defined pathway involving the conversion of S-adenosyl-L-methionine (SAM or Ado Met) to the cyclic amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) which is facilitated by ACC synthase. ACC synthase is an aminotransferase which catalyzes the rate limiting step in the formation of ethylene by converting S-adenosylmethionine to ACC. Ethylene is then produced from the oxidation of ACC through the action of ACC oxidase (also known as the ethylene forming enzyme) with hydrogen cyanide as a secondary product that is detoxified by β-cyanoalanine synthase. ACC oxidase is encoded by multigene families

in which individual member's exhibit tissue-specific regulation and/or are induced in response to environmental and chemical stimuli. Activity of ACC oxidase can be inhibited by anoxia and cobalt ions. The ACC oxidase enzyme is stereospecific and uses cofactors, e.g., Fe^{+2}, O_2, ascorbate, etc. Finally, ethylene is metabolized by oxidation to carbon dioxide (CO_2) or to ethylene oxide and ethylene glycol. See, Figure 1.

In some embodiments of the presently disclosed methods, the activity of at least one ACC synthase is modulated or inhibited to enhance plant yield and improve nitrogen stress tolerance. An "ACC synthase" is an enzyme having amino transferase activity that catalyzes the conversion of S-adenosylmethionine to ACC. Non-limiting examples of ACC synthases include ACS 1 through ACS11. In maize, this includes ACS2, ACS6 and/or ACS 7. In dicots, ACC synthase is part of a larger superfamily of amino transferases with nine members being ACS genes. The genes fall into three different classes which are distinguished by their C-terminal structure and their post-translational regulation. In maize and other monocots, there are only 3 members and one member falls into each class. See, Table 4 in Example 16 for a non-limiting list of some publically available ACS sequences which may be used for the invention.

The term "ACC synthase polypeptide" refers to one or more amino acid sequences and is inclusive of fragments, variants, homologs, alleles or precursors (e.g., preproproteins or proproteins) thereof that retain the function of catalyzing the conversion of S-adenosylmethionine to ACC. An "ACC synthase protein" comprises an ACC synthase polypeptide. Unless otherwise stated, the term "ACC synthase nucleic acid" means a nucleic acid comprising a polynucleotide ("ACC synthase polynucleotide") encoding an ACC synthase polypeptide.

As used herein the term "modulation of ACC synthase activity" shall be interpreted to mean any change in an ACC synthase biological activity, which can include an altered level of ACC synthase present in a plant cell, altered efficacy of the enzyme or any other means which affects one or more of the biological properties of ACC synthase in relation to its role in converting S-adenosylmethionine to ACC in the formation of ethylene. Accordingly, "inhibition of ACC synthase activity" encompasses a reduction in the efficacy of the enzyme, or a reduction in the level of ACC synthase present in a plant cell, for example, due to a reduction in the expression of an ACC synthase gene.

In other embodiments, other steps along the ethylene synthesis pathway could be modulated to improve plant yield or nitrogen stress tolerance of a plant. For example, the rate of conversion of SAM to polyamines could be increased, or the level or activity of ACC oxidase could be decreased, or the level or activity of ACC could be increased, or the level or activity of SAM could be increased, or some combination of these and/or other modifications in the ethylene synthesis pathway could occur as a result of the genetic modulation described herein. While not wishing to be bound by any theory, it is postulated that modification of one or more steps towards ethylene synthesis results in decreased ethylene activity. In any event, the invention is directed to increasing plant yield under abiotic stress conditions, and in some embodiments, improving nitrogen stress tolerance, resulting from modulated expression of an ACC synthase gene, regardless of the precise effect of that modulation on the ethylene synthesis pathway, ethylene production or ethylene activity.

The methods of the invention provide for an improved yield of plants. As used herein, "yield" may include reference to bushels per acre of a grain crop at harvest, as adjusted for grain moisture (15% typically for maize, for example) and/or the volume of biomass generated (for forage crops such as alfalfa and plant root size for multiple crops). Grain moisture is measured in the grain at harvest. The adjusted test weight of grain is determined to be the weight in pounds per bushel, adjusted for grain moisture level at harvest. Biomass is measured as the weight of harvestable plant material generated.

In some embodiments of the presently disclosed methods, the modulation of the ethylene synthesis pathway results in improved nitrogen stress tolerance of a plant. As used herein, a plant having "improved nitrogen stress tolerance" shall include but is not limited to, plants that have improved tolerance to low nitrogen conditions, plants that maintain their productive rates with significantly less nitrogen fertilizer input, enhanced uptake and assimilation of nitrogen fertilizer and/or remobilization and reutilization of accumulated nitrogen reserves, or any combination thereof, compared to a corresponding control plant (e.g., non-modified plant).

The term "low nitrogen conditions" or "nitrogen limiting conditions" as used herein shall be interpreted to mean any environmental condition in which plant-available nitrogen is less than would be optimal for expression of maximum yield potential.

The methods of the invention provide for improved plant performance in nitrogen limiting conditions. This performance may be demonstrated in a number of ways including a modulation of root development, shoot and leaf development, and/or reproductive tissue development.

Accordingly, methods for modulating root development in a plant are provided. By "modulating root development" is intended any alteration in the development of the plant root under nitrogen limiting conditions when compared to a control plant. Such alterations in root development include, but are not limited to, alterations in the growth rate of the primary root, the fresh root weight, the extent of lateral and adventitious root formation, the vasculature system, meristem development or radial expansion.

Methods for modulating root development of a plant in nitrogen limiting conditions are provided. The methods comprise modulating the level and/or activity of an ACC synthase polypeptide in the plant. In one method, an ACC synthase sequence inhibition construct is provided to the plant. In another method, the nucleotide sequence is provided by introducing into the plant a polynucleotide comprising an ACC synthase inhibiting nucleotide sequence, expressing the same and thereby modifying root development under conditions of low nitrogen. In still other methods, the ACC synthase inhibition nucleotide construct introduced into the plant is stably incorporated into the genome of the plant. A change in ACC synthase activity can result in at least one or more of the following alterations to root development, including, but not limited to, alterations in root biomass and length when the plant is grown under nitrogen limiting conditions.

As used herein, "root growth" encompasses all aspects of growth of the different parts that make up the root system at different stages of its development in both monocotyledonous and dicotyledonous plants. It is to be understood that enhanced root growth can result from enhanced growth of one or more of its parts including the primary root, lateral roots, adventitious roots, etc.

Methods of measuring such developmental alterations in the root system are known in the art. See, for example, US Patent Application Publication Number 2003/0074698 and Werner, *et al.*, (2001) *PNAS* 18:10487-10492, both of which are herein incorporated by reference.

As discussed elsewhere herein, one of skill will recognize the appropriate promoter to use to modulate root development in the plant. Exemplary promoters for

this embodiment include constitutive promoters and root-preferred promoters. Exemplary root-preferred promoters have been disclosed elsewhere herein.

Stimulating root growth and increasing root mass in the presence of low nitrogen or nitrogen associated stress by decreasing the activity and/or level of an ACC synthase polypeptide also finds use in improving the standability of a plant. The term "resistance to lodging" or "standability" refers to the ability of a plant to fix itself to the soil. For plants with an erect or semi-erect growth habit, this term also refers to the ability to maintain an upright position under adverse (environmental) conditions. This trait relates to the size, depth and morphology of the root system. In addition, stimulating root growth and increasing root mass in nitrogen limiting conditions by altering the level and/or activity of the ACC synthase polypeptide also finds use in promoting *in vitro* propagation of explants.

Furthermore, higher root biomass production has a direct effect on the yield and an indirect effect of production of compounds produced by root cells or transgenic root cells or cell cultures of said transgenic root cells.

Accordingly, the present invention further provides plants having modulated root development in nitrogen limiting conditions when compared to the root development of a control plant. In normal conditions no such modulation is observed.

Methods are also provided for modulating shoot and leaf development in a plant, particularly under nitrogen limiting conditions. By "modulating shoot and/or leaf development" is intended any alteration in the development of the plant shoot and/or leaf in nitrogen limiting conditions. Such alterations in shoot and/or leaf development include, but are not limited to, alterations in shoot meristem development, in leaf number, leaf size, leaf and stem vasculature, internode length and leaf senescence. As used herein, "leaf development" and "shoot development" encompasses all aspects of growth of the different parts that make up the leaf system and the shoot system, respectively, at different stages of their development, both in monocotyledonous and dicotyledonous plants. Methods for measuring such developmental alterations in the shoot and leaf system are known in the art. See, for example, Werner, *et al.*, (2001) *PNAS* 98:10487-10492 and US Patent Application Publication Number 2003/0074698, each of which is herein incorporated by reference.

The method for modulating shoot and/or leaf development in a plant in low nitrogen conditions comprises modulating the activity and/or level of an ACC synthase polypeptide. In one embodiment, an ACC synthase nucleotide sequence can be

provided by introducing into the plant a polynucleotide comprising an ACC synthase nucleotide sequence inhibition construct, expressing the same and thereby modifying shoot and/or leaf development in nitrogen limiting conditions. In other embodiments, the ACC synthase inhibition nucleotide construct introduced into the plant is stably incorporated into the genome of the plant.

A change in ACC synthase activity can result in at least one or more of the following alterations in shoot and/or leaf development under low nitrogen conditions, including, but not limited to, changes in leaf number, altered leaf surface, altered vasculature, internodes and plant growth and alterations in leaf senescence, when compared to a control plant in the same conditions.

As discussed elsewhere herein, one of skill will recognize the appropriate promoter to use to modulate shoot and leaf development of the plant. Exemplary promoters for this embodiment include constitutive promoters, shoot-preferred promoters, shoot meristem-preferred promoters and leaf-preferred promoters. Exemplary promoters have been disclosed elsewhere herein.

Methods for modulating reproductive tissue development, particularly under nitrogen limiting conditions are provided. In one embodiment, methods are provided to modulate floral development in a plant. By "modulating floral development" is intended any alteration in a structure of a plant's reproductive tissue as compared to a control plant in which the activity or level of the ACC synthase polypeptide has not been modulated. "Modulating floral development" further includes any alteration in the timing of the development of a plant's reproductive tissue (i.e., a delayed or an accelerated timing of floral development) when compared to a control plant in which the activity or level of the ACC synthase polypeptide has not been modulated. Macroscopic alterations may include changes in size, shape, number or location of reproductive organs, the developmental time period that these structures form or the ability to maintain or proceed through the flowering process in times of environmental stress. Microscopic alterations may include changes to the types or shapes of cells that make up the reproductive organs.

The method for modulating floral development in a plant comprises modulating ACC synthase activity in a plant. Such methods can comprise introducing an ACC synthase nucleotide sequence into the plant and changing the activity of the ACC synthase polypeptide. In some embodiments, the ACC synthase nucleotide construct introduced into the plant is stably incorporated into the genome of the plant. Altering

expression of the ACC synthase sequence of the invention can modulate floral development during periods of stress. Such methods are described elsewhere herein. Accordingly, the present invention further provides plants having modulated floral development when compared to the floral development of a control plant. Compositions include plants having an altered level/activity of ACC synthase polypeptide and having an altered floral development. Compositions also include plants having a modified level/activity of the ACC synthase polypeptide wherein the plant maintains or proceeds through the flowering process in times of stress.

As discussed elsewhere herein, one of skill will recognize the appropriate promoter to use to modulate floral development of the plant or to increase seed size and/or seed weight. Exemplary promoters of this embodiment include constitutive promoters, inducible promoters, seed-preferred promoters, embryo-preferred promoters and endosperm-preferred promoters.

Thus, a plant having reduced ACC synthase activity can have at least one of the following phenotypes in nitrogen limiting conditions, including but not limited to: increased overall plant yield, increased root mass, increased root length, increased leaf size, increased ear size, increased seed size, increased endosperm size, improved standability, alterations in the relative size of embryos and endosperms leading to changes in the relative levels of protein, oil and/or starch in the seeds, altered floral development, changes in leaf number, altered leaf surface, altered vasculature, altered internodes, alterations in leaf senescence, absence of tassels, absence of functional pollen bearing tassels, or increased plant size when compared to a non-modified plant under conditions of low nitrogen.

Any method known in the art to reduce or eliminate the activity of an ACC synthase polypeptide can be used to improve nitrogen stress tolerance of a plant. In some embodiments, a polynucleotide is introduced into a plant that may inhibit the expression of the ACC synthase polypeptide directly, by preventing transcription or translation of the ACC synthase messenger RNA, or indirectly, by encoding a polypeptide that inhibits the transcription or translation of an ACC synthase gene encoding an ACC synthase polypeptide. Methods for inhibiting or eliminating the expression of a gene in a plant are well known in the art, and any such method may be used in the present invention to inhibit the expression of the ACC synthase polypeptide. In other embodiments, a polynucleotide that encodes a polypeptide that inhibits the activity of an ACC synthase polypeptide is introduced into a plant. In yet other

embodiments, the activity of an ACC synthase is inhibited through disruption of an ACC synthase gene. Many methods may be used to reduce or eliminate the activity of an ACC synthase polypeptide. In addition, more than one method may be used to reduce the activity of a single ACC synthase polypeptide.

In some embodiments, the ACC synthase activity is reduced through the disruption of at least one ACC synthase gene or a reduction in the expression of at least one ACC synthase gene. As used herein, an "ACC synthase gene" refers to a gene that encodes an ACC synthase polypeptide. An ACC synthase gene can encode one or more ACC synthases and in some embodiments can comprise, e.g., at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5% or more sequence identity to SEQ ID NO: 1 (gACS2), SEQ ID NO: 2 (gACS6) or SEQ ID NO: 3 (gACS7). Many ACS genes are known to those of skill in the art and are readily available through sources such as GENBANK and the like and Table 4 in Example 16 lists several. The expression of any ACS gene may be reduced according to the invention.

In accordance with the present invention, the expression of an ACC synthase is inhibited if the transcript or protein level of the ACC synthase is statistically lower than the transcript or protein level of the same ACC synthase in a plant that has not been genetically modified or mutagenized to inhibit the expression of that ACC synthase. In particular embodiments of the invention, the transcript or protein level of the ACC synthase in a modified plant according to the invention is less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, or less than 5% of the protein level of the same ACC synthase in a plant that is not a mutant or that has not been genetically modified to inhibit the expression of that ACC synthase. The expression level of the ACC synthase may be measured directly, for example, by assaying for the level of ACC synthase expressed in the cell or plant, or indirectly, for example, by measuring the ACC synthase activity in the cell or plant. The activity of an ACC synthase protein is "eliminated" according to the invention when it is not detectable by at least one assay method. Methods for assessing ACC synthase activity are known in the art and include measuring levels of ACC or ethylene, which can be recovered and assayed from cell extracts. For example, internal concentrations of ACC

can be assayed by gas chromatography-mass spectroscopy, in acidic plant extracts as ethylene after decomposition in alkaline hypochlorite solution, etc. The concentration of ethylene can be determined by, e.g., gas chromatography-mass spectroscopy, etc. See, e.g., Nagahama, *et al.*, (1991) *J. Gen. Microbiol.* 137:2281 2286. For example, ethylene can be measured with a gas chromatograph equipped with, e.g., an alumina based column (such as an HP-PLOT A1203 capillary column) and a flame ionization detector. methods.

In other embodiments of the invention, the activity of one or more ACC synthases is reduced or eliminated by transforming a plant cell with an expression cassette comprising a polynucleotide encoding a polypeptide that inhibits the activity of one or more ACC synthases. The activity of an ACC synthase is inhibited according to the present invention if the activity of that ACC synthase in the transformed plant or cell is statistically lower than the activity of that ACC synthase in a plant that has not been genetically modified to inhibit the activity of at least one ACC synthase. In particular embodiments of the invention, an ACC synthase activity of a modified plant according to the invention is less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, or less than 5% of that ACC synthase activity in an appropriate control plant that has not been genetically modified to inhibit the expression or activity of that ACC synthase.

In other embodiments, the activity of an ACC synthase may be reduced or eliminated by disrupting at least one gene encoding the ACC synthase. The disruption inhibits expression or activity of at least one ACC synthase protein compared to a corresponding control plant cell lacking the disruption. In one embodiment, the at least one endogenous ACC synthase gene comprises two or more endogenous ACC synthase genes or subsequences thereof (e.g., any two or more of ACS2, ACS6 and ACS7, e.g., ACS2 and ACS6). Similarly, in another embodiment, the at least one endogenous ACC synthase gene comprises three or more endogenous ACC synthase genes. In certain embodiments, the disruption results in reduced or decreased ethylene production by the knockout plant cell as compared to the control plant cell. The disruption results in the plant's improved performance in low nitrogen conditions as compared to a control plant in similar conditions.

In another embodiment, the disruption step comprises insertion of one or more transposons, where the one or more transposons are inserted into the at least one

endogenous ACC synthase gene. In yet another embodiment, the disruption comprises one or more point mutations in the at least one endogenous ACC synthase gene. The disruption can be a homozygous disruption in the at least one ACC synthase gene. Alternatively, the disruption is a heterozygous disruption in the at least one ACC synthase gene. In certain embodiments, when more than one ACC synthase gene is involved, there is more than one disruption, which can include homozygous disruptions, heterozygous disruptions or a combination of homozygous disruptions and heterozygous disruptions.

Detection of expression products is performed either qualitatively (by detecting presence or absence of one or more product of interest) or quantitatively (by monitoring the level of expression of one or more product of interest). In one embodiment, the expression product is an RNA expression product. Aspects of the invention optionally include monitoring an expression level of a nucleic acid, polypeptide or chemical (e.g., ACC, ethylene, etc.) as noted herein for detection of ACC synthase, ethylene production, nitrogen utilization or tolerance to low nitrogen conditions, etc. in a plant or in a population of plants.

Thus, many methods may be used to reduce or eliminate the activity of an ACC synthase. More than one method may be used to reduce the activity of a single plant ACC synthase. In addition, combinations of methods may be employed to reduce or eliminate the activity of two or more different ACC synthases. Non-limiting examples of methods of reducing or eliminating the expression of a plant ACC synthase are given below.

In some embodiments of the present invention, a polynucleotide is introduced into a plant that upon introduction or expression, inhibits the expression of an ACC synthase polypeptide of the invention. The term "expression" as used herein refers to the biosynthesis of a gene product, including the transcription and/or translation of said gene product. For example, for the purposes of the present invention, an expression cassette capable of expressing a polynucleotide that inhibits the expression of at least one ACC synthase polypeptide is an expression cassette capable of producing an RNA molecule that inhibits the transcription and/or translation of at least one ACC synthase polypeptide of the invention. The "expression" or "production" of a protein or polypeptide from a DNA molecule refers to the transcription and translation of the coding sequence to produce the protein or polypeptide, while the "expression" or "production" of a protein or polypeptide from an RNA molecule refers to the translation

of the RNA coding sequence to produce the protein or polypeptide. Further, "expression" of a gene can refer to the transcription of the gene into a non-protein coding transcript.

As used herein, "polynucleotide" includes reference to a deoxyribopolynucleotide, ribopolynucleotide or analogs thereof that have the essential nature of a natural ribonucleotide in that they hybridize, under stringent hybridization conditions, to substantially the same nucleotide sequence as naturally occurring nucleotides and/or allow translation into the same amino acid(s) as the naturally occurring nucleotide(s). A polynucleotide can be full-length or a subsequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the term includes reference to the specified sequence as well as the complementary sequence thereof. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including *inter alia,* simple and complex cells.

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide nucleic acids).

By "encoding" or "encoded," with respect to a specified nucleic acid, is meant comprising the information for transcription into a RNA and in some embodiments, translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g., as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal" genetic code. However, variants of the universal code, such as is present in some plant, animal, and fungal mitochondria, the bacterium *Mycoplasma capricolum* (Yamao, *et al.*, (1985)

Proc. Natl. Acad. Sci. USA 82:2306-9) or the ciliate *Macronucleus*, may be used when the nucleic acid is expressed using these organisms.

Examples of polynucleotides that inhibit the expression of an ACC synthase polypeptide are given below.

In some embodiments of the invention, inhibition of the expression of an ACC synthase polypeptide may be obtained by sense suppression or cosuppression. For cosuppression, an expression cassette is designed to express an RNA molecule corresponding to all or part of a messenger RNA encoding an ACC synthase polypeptide in the "sense" orientation. Over expression of the RNA molecule can result in reduced expression of the native gene. Accordingly, multiple plant lines transformed with the cosuppression expression cassette are screened to identify those that show the greatest inhibition of ACC synthase polypeptide expression.

The polynucleotide used for cosuppression may correspond to all or part of the sequence encoding the ACC synthase polypeptide, all or part of the 5' and/or 3' untranslated region of an ACC synthase polypeptide transcript or all or part of both the coding sequence and the untranslated regions of a transcript encoding an ACC synthase polypeptide. A polynucleotide used for cosuppression or other gene silencing methods may share 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 85%, 80%, or less sequence identity with the target sequence, which in some embodiments is SEQ ID NO:4, 5, or 6. When portions of the polynucleotides (e.g., SEQ ID NO:4, 5, or 6) are used to disrupt the expression of the target gene, generally, sequences of at least 15, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 200, 300, 400, 450, 500, 550, 600, 650, 700, 750, 800, 900, or 1000 contiguous nucleotides or greater may be used. In some embodiments where the polynucleotide comprises all or part of the coding region for the ACC synthase polypeptide, the expression cassette is designed to eliminate the start codon of the polynucleotide so that no protein product will be translated.

Cosuppression may be used to inhibit the expression of plant genes to produce plants having undetectable protein levels for the proteins encoded by these genes. See, for example, Broin, *et al.*, (2002) *Plant Cell* 14:1417-1432. Cosuppression may also be used to inhibit the expression of multiple proteins in the same plant. See, for example, US Patent Number 5,942,657. Methods for using cosuppression to inhibit the expression of endogenous genes in plants are described in Flavell, *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:3490-3496; Jorgensen, *et al.*, (1996) *Plant Mol. Biol.* 31:957-

973; Johansen and Carrington, (2001) *Plant Physiol.* 126:930-938; Broin, *et al.*, (2002) *Plant Cell* 14:1417-1432; Stoutjesdijk, *et al.*, (2002) *Plant Physiol.* 129:1723-1731; Yu, *et al.*, (2003) *Phytochemistry* 63:753-763 and US Patent Numbers 5,034,323, 5,283,184 and 5,942,657, each of which is herein incorporated by reference. The efficiency of cosuppression may be increased by including a poly-dT region in the expression cassette at a position 3' to the sense sequence and 5' of the polyadenylation signal. See, US Patent Publication Number 2002/0048814, herein incorporated by reference. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, optimally greater than about 65% sequence identity, more optimally greater than about 85% sequence identity, most optimally greater than about 95% sequence identity. See, US Patent Numbers 5,283,184 and 5,034,323, herein incorporated by reference.

In some embodiments of the invention, inhibition of the expression of the ACC synthase polypeptide may be obtained by antisense suppression. For antisense suppression, the expression cassette is designed to express an RNA molecule complementary to all or part of a messenger RNA encoding the ACC synthase polypeptide. Over expression of the antisense RNA molecule can result in reduced expression of the native gene. Accordingly, multiple plant lines transformed with the antisense suppression expression cassette are screened to identify those that show the greatest inhibition ACC synthase polypeptide expression.

The polynucleotide for use in antisense suppression may correspond to all or part of the complement of the sequence encoding the ACC synthase polypeptide, all or part of the complement of the 5' and/or 3' untranslated region of the ACC synthase transcript or all or part of the complement of both the coding sequence and the untranslated regions of a transcript encoding the ACC synthase polypeptide.

In addition, the antisense polynucleotide may be fully complementary (i.e., 100% identical to the complement of the target sequence) or partially complementary (i.e., less than 100%, including but not limited to, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 85%, 80%, identical to the complement of the target sequence, which in some embodiments is SEQ ID NO:4, 5, or 6) to the target sequence. Antisense suppression may be used to inhibit the expression of multiple proteins in the same plant. See, for example, US Patent Number 5,942,657. Furthermore, portions of the antisense nucleotides may be used to disrupt the

expression of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, 300, 400, 450, 500, 550 or greater may be used.

Methods for using antisense suppression to inhibit the expression of endogenous genes in plants are described, for example, in Liu, *et al.*, (2002) *Plant Physiol.* 129:1732-1743 and US Patent Number 5,759,829 and 5,942,657, each of which is herein incorporated by reference. Efficiency of antisense suppression may be increased by including a poly-dT region in the expression cassette at a position 3' to the antisense sequence and 5' of the polyadenylation signal. See, US Patent Application Publication Number 2002/0048814, herein incorporated by reference.

In some embodiments of the invention, inhibition of the expression of an ACC synthase polypeptide may be obtained by double-stranded RNA (dsRNA) interference. For dsRNA interference, a sense RNA molecule like that described above for cosuppression and an antisense RNA molecule that is fully or partially complementary to the sense RNA molecule are expressed in the same cell, resulting in inhibition of the expression of the corresponding endogenous messenger RNA.

Expression of the sense and antisense molecules can be accomplished by designing the expression cassette to comprise both a sense sequence and an antisense sequence. Alternatively, separate expression cassettes may be used for the sense and antisense sequences. Multiple plant lines transformed with the dsRNA interference expression cassette or expression cassettes are then screened to identify plant lines that show the greatest inhibition of ACC synthase polypeptide expression. Methods for using dsRNA interference to inhibit the expression of endogenous plant genes are described in Waterhouse, *et al.*, (1998) *Proc. Natl. Acad. Sci. USA* 95:13959-13964, Liu, *et al.*, (2002) *Plant Physiol.* 129:1732-1743 and WO 99/49029, WO 99/53050, WO 99/61631 and WO 00/49035, each of which is herein incorporated by reference.

In some embodiments of the invention, inhibition of the expression of an ACC synthase polypeptide may be obtained by hairpin RNA (hpRNA) interference or intron-containing hairpin RNA (ihpRNA) interference. These methods are highly efficient at inhibiting the expression of endogenous genes. See, Waterhouse and Helliwell, (2003) *Nat. Rev. Genet.* 4:29-38 and the references cited therein.

For hpRNA interference, the expression cassette is designed to express an RNA molecule that hybridizes with itself to form a hairpin structure that comprises a single-stranded loop region and a base-paired stem. The base-paired stem region comprises a sense sequence corresponding to all or part of the endogenous messenger RNA

encoding the gene whose expression is to be inhibited, and an antisense sequence that is fully or partially complementary to the sense sequence. The antisense sequence may be located "upstream" of the sense sequence (i.e., the antisense sequence may be closer to the promoter driving expression of the hairpin RNA than the sense sequence). The base-paired stem region may correspond to a portion of a promoter sequence controlling expression of the gene to be inhibited. A polynucleotide designed to express an RNA molecule having a hairpin structure comprises a first nucleotide sequence and a second nucleotide sequence that is the complement of the first nucleotide sequence, and wherein the second nucleotide sequence is in an inverted orientation relative to the first nucleotide sequence.

Thus, the base-paired stem region of the molecule generally determines the specificity of the RNA interference. The sense sequence and the antisense sequence are generally of similar lengths but may differ in length. Thus, these sequences may be portions or fragments of at least 10, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 50, 70, 90, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400, 500, 600, 700, 800, 900 nucleotides in length, or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 kb in length. The loop region of the expression cassette may vary in length. Thus, the loop region may be at least 100, 200, 300, 400, 500, 600, 700, 800, 900 nucleotides in length, or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 kb in length.

hpRNA molecules are highly efficient at inhibiting the expression of endogenous genes and the RNA interference they induce is inherited by subsequent generations of plants. See, for example, Chuang and Meyerowitz, (2000) *Proc. Natl. Acad. Sci. USA* 97:4985-4990; Stoutjesdijk, *et al.*, (2002) *Plant Physiol.* 129:1723-1731 and Waterhouse and Helliwell, (2003) *Nat. Rev. Genet.* 4:29-38. Methods for using hpRNA interference to inhibit or silence the expression of genes are described, for example, in Chuang and Meyerowitz, (2000) *Proc. Natl. Acad. Sci. USA* 97:4985-4990; Stoutjesdijk, *et al.*, (2002) *Plant Physiol.* 129:1723-1731; Waterhouse and Helliwell, (2003) *Nat. Rev. Genet.* 4:29-38; Pandolfini *et al.*, *BMC Biotechnology* 3:7 and US Patent Application Publication Number 2003/0175965, each of which is herein incorporated by reference. A transient assay for the efficiency of hpRNA constructs to silence gene expression *in vivo* has been described by Panstruga, *et al.*, (2003) *Mol. Biol. Rep.* 30:135-140, herein incorporated by reference.

For ihpRNA, the interfering molecules have the same general structure as for hpRNA, but the RNA molecule additionally comprises an intron in the loop of the

hairpin that is capable of being spliced in the cell in which the ihpRNA is expressed. The use of an intron minimizes the size of the loop in the hairpin RNA molecule following splicing, and this increases the efficiency of interference. See, for example, Smith, *et al.*, (2000) *Nature* 407:319-320. In fact, Smith, *et al.*, show 100% suppression of endogenous gene expression using ihpRNA-mediated interference. In some embodiments, the intron is the ADH1 intron 1. Methods for using ihpRNA interference to inhibit the expression of endogenous plant genes are described, for example, in Smith, *et al.*, (2000) *Nature* 407:319-320; Wesley, *et al.*, (2001) *Plant J.* 27:581-590; Wang and Waterhouse, (2001) *Curr. Opin. Plant Biol.* 5:146-150; Waterhouse and Helliwell, (2003) *Nat. Rev. Genet.* 4:29-38; Helliwell and Waterhouse, (2003) *Methods* 30:289-295 and US Patent Application Publication Number 2003/0180945, each of which is herein incorporated by reference.

The expression cassette for hpRNA interference may also be designed such that the sense sequence and the antisense sequence do not correspond to an endogenous RNA. In this embodiment, the sense and antisense sequence flank a loop sequence that comprises a nucleotide sequence corresponding to all or part of the endogenous messenger RNA of the target gene. Thus, it is the loop region that determines the specificity of the RNA interference. See, for example, WO 02/00904; Mette, *et al.*, (2000) *EMBO J* 19:5194-5201; Matzke, *et al.*, (2001) *Curr. Opin. Genet. Devel.* 11:221-227; Scheid, *et al.*, (2002) *Proc. Natl. Acad. Sci., USA* 99:13659-13662; Aufsaftz, *et al.*, (2002) *Proc. Nat'l. Acad. Sci.* 99(4):16499-16506; Sijen, *et al.*, *Curr. Biol.* (2001) 11:436-440), herein incorporated by reference.

Amplicon expression cassettes comprise a plant virus-derived sequence that contains all or part of the target gene but generally not all of the genes of the native virus. The viral sequences present in the transcription product of the expression cassette allow the transcription product to direct its own replication. The transcripts produced by the amplicon may be either sense or antisense relative to the target sequence (i.e., the messenger RNA for the ACC synthase polypeptide). Methods of using amplicons to inhibit the expression of endogenous plant genes are described, for example, in Angell and Baulcombe, (1997) *EMBO J.* 16:3675-3684, Angell and Baulcombe, (1999) *Plant J.* 20:357-362 and US Patent Number 6,635,805, each of which is herein incorporated by reference.

In some embodiments, the polynucleotide expressed by the expression cassette of the invention is catalytic RNA or has ribozyme activity specific for the messenger

RNA of the ACC synthase polypeptide. Thus, the polynucleotide causes the degradation of the endogenous messenger RNA, resulting in reduced expression of the ACC synthase polypeptide. This method is described, for example, in US Patent Number 4,987,071, herein incorporated by reference.

In some embodiments of the invention, inhibition of the expression of an ACC synthase polypeptide may be obtained by RNA interference by expression of a polynucleotide encoding a micro RNA (miRNA). miRNAs are regulatory agents consisting of about 22 ribonucleotides. miRNA are highly efficient at inhibiting the expression of endogenous genes. See, for example Javier, *et al.*, (2003) *Nature* 425:257-263, herein incorporated by reference.

For miRNA interference, the expression cassette is designed to express an RNA molecule that is modeled on an endogenous miRNA gene. The miRNA gene encodes an RNA that forms a hairpin structure containing a 22-nucleotide sequence that is complementary to another endogenous gene (target sequence). For suppression of ACC synthase expression, the 22-nucleotide sequence is selected from an ACC synthase transcript sequence and contains 22 nucleotides of said ACC synthase sequence in sense orientation and 21 nucleotides of a corresponding antisense sequence that is complementary to the sense sequence. miRNA molecules are highly efficient at inhibiting the expression of endogenous genes, and the RNA interference they induce is inherited by subsequent generations of plants.

In some embodiments, polypeptides or polynucleotide encoding polypeptides can be introduced into a plant, wherein the polypeptide is capable of inhibiting the activity of an ACC synthase polypeptide. The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The terms "residue" or "amino acid residue" or "amino acid" are used interchangeably herein to refer to an amino acid that is incorporated into a protein, polypeptide, or peptide (collectively "protein"). The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids.

In one embodiment, the polynucleotide encodes a zinc finger protein that binds to a gene encoding an ACC synthase polypeptide, resulting in reduced expression of the gene. In particular embodiments, the zinc finger protein binds to a regulatory region of an ACC synthase gene. In other embodiments, the zinc finger protein binds to a messenger RNA encoding an ACC synthase polypeptide and prevents its translation. Methods of selecting sites for targeting by zinc finger proteins have been described, for example, in US Patent Number 6,453,242, and methods for using zinc finger proteins to inhibit the expression of genes in plants are described, for example, in US Patent Application Publication Number 2003/0037355, each of which is herein incorporated by reference.

In some embodiments of the invention, the polynucleotide encodes an antibody that binds to at least one ACC synthase polypeptide and reduces the activity of the ACC synthase polypeptide. In another embodiment, the binding of the antibody results in increased turnover of the antibody-ACC synthase complex by cellular quality control mechanisms. The expression of antibodies in plant cells and the inhibition of molecular pathways by expression and binding of antibodies to proteins in plant cells are well known in the art. See, for example, Conrad and Sonnewald, (2003) *Nature Biotech.* 21:35-36, incorporated herein by reference.

In some embodiments of the present invention, the activity of an ACC synthase polypeptide is reduced or eliminated by disrupting the gene encoding the ACC synthase polypeptide. The gene encoding the ACC synthase polypeptide may be disrupted by any method known in the art. For example, in one embodiment, the gene is disrupted by transposon tagging. In another embodiment, the gene is disrupted by mutagenizing plants using random or targeted mutagenesis and selecting for plants that have reduced nitrogen utilization activity.

In one embodiment of the invention, transposon tagging is used to reduce or eliminate the ACC synthase activity of one or more ACC synthase polypeptides. Transposon tagging comprises inserting a transposon within an endogenous ACC synthase gene to reduce or eliminate expression of the ACC synthase polypeptide.

In this embodiment, the expression of one or more ACC synthase polypeptides is reduced or eliminated by inserting a transposon within a regulatory region or coding region of the gene encoding the ACC synthase polypeptide. A transposon that is within an exon, intron, 5' or 3' untranslated sequence, a promoter or any other regulatory

sequence of an ACC synthase gene may be used to reduce or eliminate the expression and/or activity of the encoded ACC synthase polypeptide.

Methods for the transposon tagging of specific genes in plants are well known in the art. See, for example, Maes, *et al.*, (1999) *Trends Plant Sci.* 4:90-96; Dharmapuri and Sonti, (1999) *FEMS Microbiol. Lett.* 179:53-59; Meissner, *et al.*, (2000) *Plant J.* 22:265-274; Phogat, *et al.*, (2000) *J. Biosci.* 25:57-63; Walbot, (2000) *Curr. Opin. Plant Biol.* 2:103-107; Gai, *et al.*, (2000) *Nucleic Acids Res.* 28:94-96; Fitzmaurice, *et al.*, (1999) *Genetics* 153:1919-1928). In addition, the TUSC process for selecting Mu insertions in selected genes has been described in Bensen, *et al.*, (1995) *Plant Cell* 7:75-84; Mena, *et al.*, (1996) *Science* 274:1537-1540 and US Patent Number 5,962,764, each of which is herein incorporated by reference.

Additional methods for decreasing or eliminating the expression of endogenous genes in plants are also known in the art and can be similarly applied to the instant invention. These methods include other forms of mutagenesis, such as ethyl methanesulfonate-induced mutagenesis, deletion mutagenesis and fast neutron deletion mutagenesis used in a reverse genetics sense (with PCR) to identify plant lines in which the endogenous gene has been deleted. For examples of these methods see, Ohshima, *et al.*, (1998) *Virology* 243:472-481; Okubara, *et al.*, (1994) *Genetics* 137:867-874 and Quesada, *et al.*, (2000) *Genetics* 154:421-436, each of which is herein incorporated by reference. In addition, a fast and automatable method for screening for chemically induced mutations, TILLING (Targeting Induced Local Lesions In Genomes), using denaturing HPLC or selective endonuclease digestion of selected PCR products is also applicable to the instant invention. See, McCallum, *et al.*, (2000) *Nat. Biotechnol.* 18:455-457, herein incorporated by reference.

Mutations that impact gene expression or that interfere with the function of the encoded protein are well known in the art. Insertional mutations in gene exons usually result in null-mutants. Mutations in conserved residues are particularly effective in inhibiting the activity of the encoded protein. Conserved residues of plant ACC synthase polypeptides suitable for mutagenesis with the goal to eliminate ACC synthase activity have been described. Such mutants can be isolated according to well-known procedures, and mutations in different ACC synthase loci can be stacked by genetic crossing. See, for example, Gruis, *et al.*, (2002) *Plant Cell* 14:2863-2882.

In another embodiment of this invention, dominant mutants can be used to trigger RNA silencing due to gene inversion and recombination of a duplicated gene locus. See, for example, Kusaba, *et al.*, (2003) *Plant Cell* 15:1455-1467.

The invention encompasses additional methods for reducing or eliminating the activity of one or more ACC synthase polypeptides. Examples of other methods for altering or mutating a genomic nucleotide sequence in a plant are known in the art and include, but are not limited to, the use of RNA:DNA vectors, RNA:DNA mutational vectors, RNA:DNA repair vectors, mixed-duplex oligonucleotides, self-complementary RNA:DNA oligonucleotides and recombinogenic oligonucleobases. Such vectors and methods of use are known in the art. See, for example, US Patent Numbers 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972 and 5,871,984, each of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO 99/25821 and Beetham, *et al.*, (1999) *Proc. Natl. Acad. Sci. USA* 96:8774-8778, each of which is herein incorporated by reference.

Where polynucleotides are used to decrease or inhibit ACC synthase activity, it is recognized that modifications of the exemplary sequences disclosed herein may be made as long as the sequences act to decrease or inhibit expression of the corresponding mRNA. Thus, for example, polynucleotides having at least 70%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the exemplary sequences disclosed herein (e.g., SEQ ID NO:4, 5, or 6) may be used. Furthermore, portions or fragments of the exemplary sequences or portions or fragments of polynucleotides sharing a particular percent sequence identity to the exemplary sequences may be used to disrupt the expression of the target gene. Generally, fragments or sequences of at least 10, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 220, 240, 250, 260, 280, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, or more contiguous nucleotides, or greater of, for example, SEQ ID NO:4, 5, or 6 may be used. It is recognized that in particular embodiments, the complementary sequence of such sequences may be used. For example, hairpin constructs comprise both a sense sequence fragment and a complementary, or antisense, sequence fragment corresponding to the gene of interest. Antisense constructs may share less than 100% sequence identity with the gene of interest, and may comprise portions or fragments of the gene of interest, so long as the object of the embodiment is achieved, *i.e.*, so long as expression of the gene of interest is decreased.

The ACC synthase nucleic acids that may be used for the present invention comprise at least one ACC synthase polynucleotide selected from the group consisting of:

(a) a polynucleotide encoding an ACC synthase polypeptide and conservatively modified and polymorphic variants thereof;

(b) a polynucleotide having at least 70% sequence identity with polynucleotides of (a);

(c) a fragment of a polynucleotide encoding an ACC synthase polypeptide; and

(d) complementary sequences of polynucleotides of (a), (b), or (c).

Thus, in some embodiments, the method comprises introducing at least one polynucleotide sequence comprising an ACC synthase nucleic acid sequence, or subsequence thereof, into a plant cell, such that the at least one polynucleotide sequence is linked to a promoter in a sense or antisense orientation, and where the at least one polynucleotide sequence comprises, e.g., at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, about 99.5% or more sequence identity to SEQ ID NO: 1 (gACS2), SEQ ID NO: 2 (gACS6) or SEQ ID NO: 3 (gACS7) or a subsequence thereof or a complement thereof. In another embodiment, the disruption is effected by introducing into the plant cell at least one polynucleotide sequence comprising one or more subsequences of an ACC synthase nucleic acid sequence configured for RNA silencing or interference.

In other embodiments, the methods of the invention are practiced with a polynucleotide comprising a member selected from the group consisting of: (a) a polynucleotide or a complement thereof, comprising, e.g., at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, about 99.5% or more sequence identity to SEQ ID NO: 1 (gACS2), SEQ ID NO: 2 (gACS6), SEQ ID NO: 3 (gACS7), SEQ ID NO: 4 (ACS2 cDNA), SEQ ID NO: 5 (ACS6 cDNA), or SEQ ID NO: 6 (ACS7 cDNA) or a subsequence thereof, or a conservative variation thereof; (b) a polynucleotide, or a complement thereof, encoding a polypeptide sequence of SEQ ID NO: 7 (ACS 2), SEQ ID NO: 8 (ACS6) or SEQ ID

NO: 9 (ACS7) or a subsequence thereof, or a conservative variation thereof; (c) a polynucleotide, or a complement thereof, that hybridizes under stringent conditions over substantially the entire length of a polynucleotide subsequence comprising at least 100 contiguous nucleotides of SEQ ID NO: 1 (gACS2), SEQ ID NO: 2 (gACS6), SEQ ID NO: 3 (gACS7), SEQ ID NO: 4 (ACS2 cDNA), SEQ ID NO: 5 (ACS6 cDNA), or SEQ ID NO: 6 (ACS7 cDNA) or that hybridizes to a polynucleotide sequence of (a) or (b); and (d) a polynucleotide that is at least about 85% identical to a polynucleotide sequence of (a), (b) or (c). In certain embodiments, the polynucleotide inhibits ethylene production when expressed in a plant.

In particular embodiments, a heterologous polynucleotide is introduced into a plant, wherein the heterologous polynucleotide is selected from the group consisting of: a) a nucleic acid comprising an ACC synthase nucleic acid; b) a nucleic acid comprising at least 15 contiguous nucleotides of the complement of an ACC synthase nucleic acid; and c) a nucleic acid encoding a transcript that is capable of forming a double-stranded RNA (e.g., a hairpin) and mediated RNA interference of an ACC synthase nucleic acid, wherein said nucleic acid comprises a first nucleotide sequence comprising at least 21 contiguous nucleotides of an ACC synthase nucleic acid, and a second nucleotide sequence comprising the complement of said first nucleotide sequence.

In other particular embodiments, the methods comprise introducing into a plant a heterologous polynucleotide selected from the group consisting of: a) the nucleotide sequence set forth in SEQ ID NO:1, 2, 3, 4, 5, or 6, or a complete complement thereof; b) a nucleotide sequence having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater sequence identity to SEQ ID NO: 1, 2, 3, 4, 5, or 6, or a complete complement thereof; c) a nucleotide sequence encoding the polypeptide sequence of SEQ ID NO: 7, 8, or 9; d) a nucleotide sequence encoding a polypeptide sequence having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater sequence identity to SEQ ID NO: 7, 8, or 9; e) a nucleotide sequence comprising at least 15 contiguous nucleotides of SEQ ID NO:1, 2, 3, 4, 5, or 6; f) a nucleotide sequence comprising at least 15 contiguous nucleotides of the complement of SEQ ID NO:1, 2, 3, 4, 5, or 6; and g) a nucleotide sequence encoding a transcript that is capable of forming a double-

stranded RNA (e.g., hairpin) and mediating RNA interference of an ACC synthase nucleic acid, wherein said nucleotide sequence comprises at least 21 contiguous nucleotides of SEQ ID NO:1, 2, 3, 4, 5, or 6, and the complement thereof. In other embodiments, the heterologous polynucleotide comprises at least 500 contiguous nucleotides of SEQ ID NO: 1, 2, 3, 4, 5, or 6 and the complement thereof. In some of these embodiments, the heterologous polynucleotide encodes a transcript that is capable of forming a double-stranded RNA (e.g., hairpin) and mediating RNA interference of an ACC synthase nucleic acid. In some of these embodiments, the plant comprises an mRNA encoded by a polynucleotide having the target sequence set forth in SEQ ID NO:1, 2, 3, 4, 5, or 6.

In yet other particular embodiments, the methods comprise introducing into a plant a heterologous polynucleotide comprising a sequence that encodes a transcript having a hairpin structure, wherein the sequence comprises a first nucleotide sequence having the sequence set forth in SEQ ID NO:14 and a second nucleotide sequence having the sequence set forth in SEQ ID NO:15. In other embodiments, the heterologous polynucleotide that comprises a sequence that encodes a transcript having a hairpin structure comprises a first nucleotide sequence having the sequence set forth in SEQ ID NO: 51 and a second nucleotide sequence having the sequence set forth in SEQ ID NO:52. In other embodiments, the methods comprise introducing into a plant a construct comprising SEQ ID NO:53, 54, 55, 56, or 57.

Methods are provided for improving yield under low nitrogen conditions comprising planting seeds or plants having a reduced activity of at least one ACC synthase in an area of cultivation having nitrogen limiting conditions.

Prior to the planting of the seeds or plants in the area of cultivation having nitrogen limiting conditions, the environment can be evaluated to determine if nitrogen limiting conditions are present, including measuring the amount of nitrogen or nitrogen fertilizer in the soil. As used herein, an "area of cultivation" comprises any region in which one desires to grow a plant. Such areas of cultivations include, but are not limited to, a field in which a plant is cultivated (such as a crop field, a sod field, a tree field, a managed forest, a field for culturing fruits and vegetables, etc), a greenhouse, a growth chamber, etc.

The present invention provides methods utilizing, *inter alia*, isolated nucleic acids of RNA, DNA, homologs, paralogs and orthologs and/or chimeras thereof,

comprising an ACC synthase polynucleotide. This includes naturally occurring as well as synthetic variants and homologs of the sequences.

The terms "isolated" or "isolated nucleic acid" or "isolated protein" refer to material, such as a nucleic acid or a protein, which is substantially or essentially free from components which normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (*i.e.*, sequences located at the 5′ and 3′ ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived.

Sequences homologous, i.e., that share significant sequence identity or similarity, to those provided herein derived from maize, *Arabidopsis thaliana* or from other plants of choice, can also be used in the methods of the invention. Homologous sequences can be derived from any plant including monocots and dicots and in particular agriculturally important plant species, including but not limited to, crops such as soybean, wheat, corn (maize), potato, cotton, rice, rape, oilseed rape (including canola), sunflower, alfalfa, clover, sugarcane and turf, or fruits and vegetables, such as banana, blackberry, blueberry, strawberry and raspberry, cantaloupe, carrot, cauliflower, coffee, cucumber, eggplant, grapes, honeydew, lettuce, mango, melon, onion, papaya, peas, peppers, pineapple, pumpkin, spinach, squash, sweet corn, tobacco, tomato, tomatillo, watermelon, rosaceous fruits (such as apple, peach, pear, cherry and plum) and vegetable brassicas (such as broccoli, cabbage, cauliflower, Brussels sprouts and kohlrabi). Other crops, including fruits and vegetables, whose phenotype can be changed and which comprise homologous sequences include barley; rye; millet; sorghum; currant; avocado; citrus fruits such as oranges, lemons, grapefruit and tangerines, artichoke, cherries; nuts such as the walnut and peanut; endive; leek; roots such as arrowroot, beet, cassava, turnip, radish, yam and sweet potato and beans. The homologous sequences may also be derived from woody species, such pine, poplar and eucalyptus or mint or other labiates. In addition, homologous sequences may be derived from plants that are evolutionarily-related to crop plants, but which may not

have yet been used as crop plants. Examples include deadly nightshade (*Atropa belladona*), related to tomato; jimson weed (*Datura strommium*), related to peyote, and teosinte (*Zea* species), related to corn (maize).

Homologous sequences as described above can comprise orthologous or paralogous sequences. Several different methods are known by those of skill in the art for identifying and defining these functionally homologous sequences. Three general methods for defining orthologs and paralogs are described; an ortholog, paralog or homolog may be identified by one or more of the methods described below.

Orthologs and paralogs are evolutionarily related genes that have similar sequence and similar functions. Orthologs are structurally related genes in different species that are derived by a speciation event. Paralogs are structurally related genes within a single species that are derived by a duplication event.

Within a single plant species, gene duplication may result in two copies of a particular gene, giving rise to two or more genes with similar sequence and often similar function known as paralogs. A paralog is therefore a similar gene formed by duplication within the same species. Paralogs typically cluster together or in the same clade (a group of similar genes) when a gene family phylogeny is analyzed using programs such as CLUSTAL (Thompson, *et al.*, (1994) *Nucleic Acids Res.* 22:4673-4680; Higgins, *et al.*, (1996) *Methods Enzymol.* 266:383-402). Groups of similar genes can also be identified with pair-wise BLAST analysis (Feng and Doolittle, (1987) *J. Mol. Evol.* 25:351-360).

For example, a clade of very similar MADS domain transcription factors from Arabidopsis all share a common function in flowering time (Ratcliffe, *et al.*, (2001) *Plant Physiol.* 126:122-132) and a group of very similar AP2 domain transcription factors from Arabidopsis are involved in tolerance of plants to freezing (Gilmour, *et al.*, (1998) *Plant J.* 16:433-442). Analysis of groups of similar genes with similar function that fall within one clade can yield sub-sequences that are particular to the clade. These sub-sequences, known as consensus sequences, can not only be used to define the sequences within each clade, but define the functions of these genes; genes within a clade may contain paralogous sequences, or orthologous sequences that share the same function (see also, for example, Mount, (2001), in *Bioinformatics: Sequence and Genome Analysis* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., page 543.)

Speciation, the production of new species from a parental species, can also give rise to two or more genes with similar sequence and similar function. These genes, termed orthologs, often have an identical function within their host plants and are often interchangeable between species without losing function. Because plants have common ancestors, many genes in any plant species will have a corresponding orthologous gene in another plant species. Once a phylogenic tree for a gene family of one species has been constructed using a program such as CLUSTAL (Thompson, *et al.*, (1994) *Nucleic Acids Res.* 22:4673-4680; Higgins, *et al.*, (1996) *supra*) potential orthologous sequences can be placed into the phylogenetic tree and their relationship to genes from the species of interest can be determined. Orthologous sequences can also be identified by a reciprocal BLAST strategy. Once an orthologous sequence has been identified, the function of the ortholog can be deduced from the identified function of the reference sequence.

Orthologous genes from different organisms have highly conserved functions, and very often essentially identical functions (Lee, *et al.*, (2002) *Genome Res.* 12:493-502; Remm, *et al.*, (2001) *J. Mol. Biol.* 314:1041-1052). Paralogous genes, which have diverged through gene duplication, may retain similar functions of the encoded proteins. In such cases, paralogs can be used interchangeably with respect to certain embodiments of the instant invention (for example, transgenic expression of a coding sequence).

ACC synthase polynucleotides, such as those disclosed herein, can be used to isolate homologs, paralogs and orthologs. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the ACC synthase polynucleotide.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single

specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like. By "amplified" is meant the construction of multiple copies of a nucleic acid sequence or multiple copies complementary to the nucleic acid sequence using at least one of the nucleic acid sequences as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario), Q-*Beta* Replicase systems, transcription-based amplification system (TAS) and strand displacement amplification (SDA). See, e.g., *Diagnostic Molecular Microbiology*: *Principles and Applications*, Persing, *et al.*, eds., American Society for Microbiology, Washington, DC (1993). The product of amplification is termed an amplicon.

In hybridization techniques, all or part of a known polynucleotide is used as a probe that selectively hybridizes to other nucleic acids comprising corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (*i.e.,* genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the ACC synthase sequences disclosed herein. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, the entire ACC synthase sequences disclosed herein, or one or more portions thereof, may be used as probes capable of specifically hybridizing to corresponding ACC synthase sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among ACC synthase sequences and are at least about 10, 12, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 50, 60, 70, 80, 90, or more nucleotides in length. Such probes may be used to amplify corresponding ACC synthase sequences from a chosen plant by PCR. This technique may be used to isolate additional coding sequences from a desired plant or as a diagnostic assay to determine the presence of coding sequences in a plant. Hybridization techniques include hybridization screening of plated nucleic acid (e.g., DNA) libraries (either plaques or colonies; see, for

example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). By "nucleic acid library" is meant a collection of isolated DNA or RNA molecules, which comprise and substantially represent the entire transcribed fraction of a genome of a specified organism. Construction of exemplary nucleic acid libraries, such as genomic and cDNA libraries, is taught in standard molecular biology references such as Berger and Kimmel, (1987) *Guide To Molecular Cloning Techniques*, from the series *Methods in Enzymology,* vol. 152, Academic Press, Inc., San Diego, CA; Sambrook, *et al.*, (1989) *Molecular Cloning*: *A Laboratory Manual,* 2nd ed., vols. 1-3; and *Current Protocols in Molecular Biology*, Ausubel, *et al.*, eds, Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (1994 Supplement).

Hybridization of such sequences may be carried out under stringent conditions. The terms "stringent conditions" or "stringent hybridization conditions" include reference to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which can be up to 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Optimally, the probe is approximately 500 nucleotides in length, but can vary greatly in length from less than 500 nucleotides to equal to the entire length of the target sequence.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide or Denhardt's. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency

conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C and a wash in 0.1X SSC at 60 to 65°C. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, (1984) *Anal. Biochem.*, 138:267-84: $T_m = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\%\ form) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3 or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9 or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15 or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology - Hybridization with Nucleic Acid Probes,* part I, chapter 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," Elsevier, New York (1993); and *Current Protocols in Molecular Biology*, chapter 2, Ausubel, *et al.*, eds, Greene Publishing and Wiley-Interscience, New York (1995). Unless otherwise stated, in the present application high stringency is defined as hybridization in 4X SSC, 5X Denhardt's (5 g Ficoll, 5 g polyvinypyrrolidone, 5 g bovine serum albumin in 500ml of

water), 0.1 mg/ml boiled salmon sperm DNA and 25 mM Na phosphate at 65°C and a wash in 0.1X SSC, 0.1% SDS at 65°C.

The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 40% sequence identity, preferably 60-90% sequence identity and most preferably 100% sequence identity (i.e., complementary) with each other.

The term "hybridization complex" includes reference to a duplex nucleic acid structure formed by two single-stranded nucleic acid sequences selectively hybridized with each other.

As used herein, "consisting essentially of" means the inclusion of additional sequences to an object polynucleotide where the additional sequences do not selectively hybridize, under stringent hybridization conditions, to the same cDNA as the polynucleotide and where the hybridization conditions include a wash step in 0.1X SSC and 0.1% sodium dodecyl sulfate at 65°C.

The ACC synthase nucleotide sequences can be used to generate variant nucleotide sequences having the nucleotide sequence of the 5'-untranslated region, 3'-untranslated region, or promoter region that is approximately 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99% identical to the original nucleotide sequence. These variants are then associated with natural variation in the germplasm for component traits related to NUE. The associated variants are used as marker haplotypes to select for the desirable traits.

Variant amino acid sequences of the ACC synthase polypeptides are generated. In this example, one or more amino acid is altered. Specifically, the open reading frames are reviewed to determine the appropriate amino acid alteration. The selection of the amino acid to change is made by consulting the protein alignment (with the other orthologs and other gene family members from various species). An amino acid is selected that is deemed not to be under high selection pressure (not highly conserved) and which is rather easily substituted by an amino acid with similar chemical characteristics (i.e., similar functional side-chain). Using a protein alignment, an appropriate amino acid can be changed. Once the targeted amino acid is identified, the procedure outlined herein is followed. Variants having about 70%, 75%, 80%, 85%,

90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher nucleic acid sequence identity are generated using this method. These variants are then associated with natural variation in the germplasm for component traits related to NUE. The associated variants are used as marker haplotypes to select for the desirable traits.

The present invention also includes polynucleotides optimized for expression in different organisms. For example, for expression of the polynucleotide in a maize plant, the sequence can be altered to account for specific codon preferences and to alter GC content as according to Murray, *et al*, *supra*. Maize codon usage for 28 genes from maize plants is listed in Table 4 of Murray, *et al.*, *supra.*

The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refer to those nucleic acids that encode identical or conservatively modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine 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" and represent one species of conservatively modified variation. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine; one exception is *Micrococcus rubens*, for which GTG is the methionine codon (Ishizuka, *et al.*, (1993) *J. Gen. Microbiol.* 139:425-32) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid, which encodes a polypeptide of the present invention, is implicit in each described polypeptide sequence and incorporated herein by reference.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" when the alteration results in the substitution of an amino acid with a chemically similar amino acid. Thus, any number of amino acid residues selected from the group of integers consisting of from 1 to 15 can be so altered. Thus, for example, 1, 2, 3, 4, 5, 7 or 10

alterations can be made. Conservatively modified variants typically provide similar biological activity as the unmodified polypeptide sequence from which they are derived. For example, substrate specificity, enzyme activity or ligand/receptor binding is generally at least 30%, 40%, 50%, 60%, 70%, 80% or 90%, preferably 60-90% of the native protein for its native substrate. 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).

See also, Creighton, *Proteins*, W.H. Freeman and Co. (1984).

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides or polypeptides: (a) "reference sequence," (b) "comparison window," (c) "sequence identity," (d) "percentage of sequence identity" and (e) "substantial identity."

As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence or the complete cDNA or gene sequence.

As used herein, "comparison window" means includes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence may be compared to a reference sequence and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100 or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of nucleotide and amino acid sequences for comparison are well known in the art. The local homology algorithm (BESTFIT) of Smith and Waterman, (1981) *Adv. Appl. Math* 2:482, may conduct optimal alignment of sequences for comparison; by the homology alignment algorithm (GAP) of Needleman and Wunsch, (1970) *J. Mol. Biol.* 48:443-53; by the search for similarity method (Tfasta and Fasta) of Pearson and Lipman, (1988) *Proc. Natl. Acad. Sci. USA* 85:2444; by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California, GAP, BESTFIT, BLAST, FASTA and TFASTA in the Wisconsin Genetics Software Package®, Version 8 (available from Genetics Computer Group (GCG® programs (Accelrys, Inc., San Diego, CA).). The CLUSTAL program is well described by Higgins and Sharp, (1988) *Gene* 73:237-44; Higgins and Sharp, (1989) *CABIOS* 5:151-3; Corpet, *et al.*, (1988) *Nucleic Acids Res.* 16:10881-90; Huang, *et al.*, (1992) *Computer Applications in the Biosciences* 8:155-65, and Pearson, *et al.*, (1994) *Meth. Mol. Biol.* 24:307-31. The preferred program to use for optimal global alignment of multiple sequences is PileUp (Feng and Doolittle, (1987) *J. Mol. Evol.*, 25:351-60 which is similar to the method described by Higgins and Sharp, (1989) *CABIOS* 5:151-53 and hereby incorporated by reference). The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, *Current Protocols in Molecular Biology*, Chapter 19, Ausubel *et al.*, eds., Greene Publishing and Wiley-Interscience, New York (1995).

GAP uses the algorithm of Needleman and Wunsch, *supra*, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap

extension penalty values in Version 10 of the Wisconsin Genetics Software Package® are 8 and 2, respectively. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 100. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package® is BLOSUM62 (see, Henikoff and Henikoff, (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using the BLAST 2.0 suite of programs using default parameters (Altschul, *et al.*, (1997) *Nucleic Acids Res.* 25:3389-402).

As those of ordinary skill in the art will understand, BLAST searches assume that proteins can be modeled as random sequences. However, many real proteins comprise regions of nonrandom sequences, which may be homopolymeric tracts, short-period repeats, or regions enriched in one or more amino acids. Such low-complexity regions may be aligned between unrelated proteins even though other regions of the protein are entirely dissimilar. A number of low-complexity filter programs can be employed to reduce such low-complexity alignments. For example, the SEG (Wooten and Federhen, (1993) *Comput. Chem.* 17:149-63) and XNU (Claverie and States, (1993) *Comput. Chem.* 17:191-201) low-complexity filters can be employed alone or in combination.

As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences, which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by

conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences, which differ by such conservative substitutions, are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, (1988) *Computer Applic. Biol. Sci.* 4:11-17, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has between 50-100% sequence identity, preferably at least 50% sequence identity, preferably at least 60% sequence identity, preferably at least 70%, more preferably at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial

identity of amino acid sequences for these purposes normally means sequence identity of between 55-100%, preferably at least 55%, preferably at least 60%, more preferably at least 70%, 80%, 90% and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two nucleic acid molecules hybridize to each other under stringent conditions as described elsewhere herein. However, the degeneracy of the genetic code allows for many nucleic acid substitutions that lead to variety in the nucleotide sequence that code for the same amino acid, hence it is possible that the DNA sequence could code for the same polypeptide but not hybridize to each other under stringent conditions. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross-reactive with the polypeptide encoded by the second nucleic acid.

The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with between 55-100% sequence identity to a reference sequence; in some embodiments, at least 55% sequence identity, 60%, 70%, 80%, at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the reference sequence over a specified comparison window. In some embodiments, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch, *supra*. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. In addition, a peptide can be substantially identical to a second peptide when they differ by a non-conservative change if the epitope that the antibody recognizes is substantially identical. Peptides which are "substantially similar" share sequences as noted above, except that residue positions which are not identical may differ by conservative amino acid changes.

The nucleic acids used in the presently disclosed methods can be made using (a) standard recombinant methods, (b) synthetic techniques, or combinations thereof. In some embodiments, the polynucleotides of the present invention will be cloned, amplified or otherwise constructed from a fungus or bacteria.

The nucleic acids may conveniently comprise sequences in addition to a polynucleotide useful in the methods of the present invention. For example, a multi-cloning site comprising one or more endonuclease restriction sites may be inserted into the nucleic acid to aid in isolation of the polynucleotide. Also, translatable sequences may be inserted to aid in the isolation of the translated polynucleotide useful in the methods of the present invention. For example, a hexa-histidine marker sequence provides a convenient means to purify proteins useful in the methods of the present invention. The nucleic acid useful in the methods of the present invention - excluding the polynucleotide sequence - is optionally a vector, adapter or linker for cloning and/or expression of a polynucleotide of the present invention. Additional sequences may be added to such cloning and/or expression sequences to optimize their function in cloning and/or expression, to aid in isolation of the polynucleotide, or to improve the introduction of the polynucleotide into a cell. Typically, the length of a nucleic acid for use in the methods of the present invention less the length of its polynucleotide of the present invention is less than 20 kilobase pairs, often less than 15 kb and frequently less than 10 kb. Use of cloning vectors, expression vectors, adapters and linkers is well known in the art. Exemplary nucleic acids include such vectors as: M13, lambda ZAP Express, lambda ZAP II, lambda gt10, lambda gt11, pBK-CMV, pBK-RSV, pBluescript II, lambda DASH II, lambda EMBL 3, lambda EMBL 4, pWE15, SuperCos 1, SurfZap, Uni-ZAP, pBC, pBS+/-, pSG5, pBK, pCR-Script, pET, pSPUTK, p3'SS, pGEM, pSK+/-, pGEX, pSPORTI and II, pOPRSVI CAT, pOPI3 CAT, pXT1, pSG5, pPbac, pMbac, pMC1neo, pOG44, pOG45, pFRTβGAL, pNEOβGAL, pRS403, pRS404, pRS405, pRS406, pRS413, pRS414, pRS415, pRS416, lambda MOSSlox and lambda MOSElox. Optional vectors for the present invention, include but are not limited to, lambda ZAP II and pGEX. For a description of various nucleic acids see, e.g., Stratagene Cloning Systems, Catalogs 1995, 1996, 1997 (La Jolla, CA) and Amersham Life Sciences, Inc, Catalog '97 (Arlington Heights, IL).

The nucleic acids used in the methods of the present invention can also be prepared by direct chemical synthesis by methods such as the phosphotriester method of Narang, *et al.*, (1979) *Meth. Enzymol.* 68:90-9; the phosphodiester method of Brown, *et al.*, (1979) *Meth. Enzymol.* 68:109-51; the diethylphosphoramidite method of Beaucage, *et al.*, (1981) *Tetra. Letts.* 22(20):1859-62; the solid phase phosphoramidite triester method described by Beaucage, *et al.*, *supra*, e.g., using an automated

synthesizer, e.g., as described in Needham-VanDevanter, *et al.*, (1984) *Nucleic Acids Res.* 12:6159-68 and the solid support method of US Patent Number 4,458,066. Chemical synthesis generally produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence or by polymerization with a DNA polymerase using the single strand as a template. One of skill will recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

In general, translational efficiency has been found to be regulated by specific sequence elements in the 5' non-coding or untranslated region (5' UTR) of the RNA. Positive sequence motifs include translational initiation consensus sequences (Kozak, (1987) *Nucleic Acids Res.*15:8125) and the 5<G> 7 methyl GpppG RNA cap structure (Drummond, *et al.*, (1985) *Nucleic Acids Res.* 13:7375). Negative elements include stable intramolecular 5' UTR stem-loop structures (Muesing, *et al.*, (1987) *Cell* 48:691) and AUG sequences or short open reading frames preceded by an appropriate AUG in the 5' UTR (Kozak, *supra,* Rao, *et al.*, (1988) *Mol. and Cell. Biol.* 8:284). Accordingly, the present invention provides 5' and/or 3' UTR regions for modulation of translation of heterologous coding sequences.

Further, the polypeptide-encoding segments of the polynucleotides used in the present invention can be modified to alter codon usage. Altered codon usage can be employed to alter translational efficiency and/or to optimize the coding sequence for expression in a desired host or to optimize the codon usage in a heterologous sequence for expression in maize. Codon usage in the coding regions of the polynucleotides useful in the methods of the present invention can be analyzed statistically using commercially available software packages such as "Codon Preference" available from the University of Wisconsin Genetics Computer Group. See, Devereaux, *et al.*, (1984) *Nucleic Acids Res.* 12:387-395); or MacVector 4.1 (Eastman Kodak Co., New Haven, Conn.). Thus, the present invention provides a codon usage frequency characteristic of the coding region of at least one of the polynucleotides useful in the methods of the present invention. The number of polynucleotides (3 nucleotides per amino acid) that can be used to determine a codon usage frequency can be any integer from 3 to the number of polynucleotides of the present invention as provided herein. Optionally, the polynucleotides will be full-length sequences. An exemplary number of sequences for statistical analysis can be at least 1, 5, 10, 20, 50 or 100.

When the nucleic acid is prepared or altered synthetically, advantage can be taken of known codon preferences of the intended host where the nucleic acid is to be expressed. For example, although nucleic acid sequences of the present invention may be expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledonous plants or dicotyledonous plants as these preferences have been shown to differ (Murray, *et al.*, (1989) *Nucleic Acids Res.* 17:477-98, herein incorporated by reference). Thus, the maize preferred codon for a particular amino acid might be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants is listed in Table 4 of Murray, *et al.*, *supra*.

Polynucleotides used in the methods of the present invention can be obtained through sequence shuffling using ACC synthase-encoding polynucleotides. Sequence shuffling is described in PCT Publication Number 96/19256. See also, Zhang, *et al.*, (1997) *Proc. Natl. Acad. Sci. USA* 94:4504-9 and Zhao, *et al.*, (1998) *Nature Biotech* 16:258-61. Generally, sequence shuffling provides a means for generating libraries of polynucleotides having a desired characteristic, which can be selected or screened for. Libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides, which comprise sequence regions, which have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. The population of sequence-recombined polynucleotides comprises a subpopulation of polynucleotides which possess desired or advantageous characteristics and which can be selected by a suitable selection or screening method. The characteristics can be any property or attribute capable of being selected for or detected in a screening system, and may include properties of: an encoded protein, a transcriptional element, a sequence controlling transcription, RNA processing, RNA stability, chromatin conformation, translation, or other expression property of a gene or transgene, a replicative element, a protein-binding element, or the like, such as any feature which confers a selectable or detectable property. In some embodiments, the selected characteristic will be an altered K_m and/or K_{cat} over the wild-type protein as provided herein. In other embodiments, a protein or polynucleotide generated from sequence shuffling will have a ligand binding affinity greater than the non-shuffled wild-type polynucleotide. In yet other embodiments, a protein or polynucleotide generated from sequence shuffling will have an altered pH optimum as compared to the non-shuffled

wild-type polynucleotide. The increase in such properties can be at least 110%, 120%, 130%, 140% or greater than 150% of the wild-type value.

The components for practicing the methods of the invention may be included in a kit, comprising polynucleotides encoding ACC synthase or their complements or nucleic acids configured for RNA interference of ACC synthase, with instructional materials for improving plant yield under low nitrogen conditions. In some of these embodiments, the kit comprises a nucleic acid comprising the sequence of SEQ ID NO:1, 2, 3, 4, 5, or 6, or a complete complement thereof; a nucleic acid comprising at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater sequence identity to SEQ ID NO:1, 2, 3, 4, 5, or 6, or a complete complement thereof; a nucleic acid encoding the polypeptide sequence of SEQ ID NO:8 or a polypeptide sequence having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater sequence identity to SEQ ID NO:8; or a nucleic acid configured for RNA silencing or interference, wherein said nucleic acid comprises a polynucleotide with at least 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, or more contiguous nucleotides of SEQ ID NO:1, 2, 3, 4, 5, or 6, and the complement of said polynucleotide.

The present invention further provides the use of recombinant expression cassettes comprising a nucleic acid useful in the methods of the present invention. As used herein, a "recombinant expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements, which permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid to be transcribed and a promoter.

A nucleic acid sequence coding for the desired polynucleotide or polypeptide useful in the methods of the present invention, for example a polynucleotide encoding a nucleic acid that can reduce the expression of an ACC synthase gene, can be used to construct a recombinant expression cassette which can be introduced into the desired host cell. A recombinant expression cassette will typically comprise a polynucleotide

useful in the methods of the present invention operably linked to transcriptional initiation regulatory sequences which will direct the transcription of the polynucleotide in the intended host cell, such as tissues of a transformed plant.

For example, plant expression vectors may include (1) a cloned plant gene under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (e.g., one conferring inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific/selective expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site and/or a polyadenylation signal.

As used herein "operably linked" includes reference to a functional linkage between a first sequence, such as a promoter, and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

As used herein "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells. Exemplary plant promoters include, but are not limited to, those that are obtained from plants, plant viruses, and bacteria which comprise genes expressed in plant cells such *Agrobacterium* or *Rhizobium*. Examples are promoters that preferentially initiate transcription in certain tissues, such as leaves, roots, seeds, fibres, xylem vessels, tracheids or sclerenchyma. Such promoters are referred to as "tissue- preferred." A "cell type" specific promoter primarily drives expression in certain cell types in one or more organs, for example, vascular cells in roots or leaves. An "inducible" or "regulatable" promoter is a promoter which is under environmental control. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions or the presence of light. Another type of promoter is a developmentally regulated promoter, for example, a promoter that drives expression during pollen development. Tissue preferred, cell type specific, developmentally regulated and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is a promoter, which is active under most environmental conditions. Constitutive

promoters are classified as providing for a range of constitutive expression. Thus, some are weak constitutive promoters and others are strong constitutive promoters. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By "low level" is intended at levels of about 1/10,000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Conversely, a "strong promoter" drives expression of a coding sequence at a "high level," or about 1/10 transcripts to about 1/100 transcripts to about 1/1,000 transcripts.

A plant promoter fragment can be employed which will direct expression of a polynucleotide useful in the methods of the present invention in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (US Patent Number 5,633,439), the *Nos* promoter, the rubisco promoter, the GRP1-8 promoter, the 35S promoter from cauliflower mosaic virus (CaMV), as described in Odell, *et al.*, (1985) *Nature* 313:810-2; rice actin (McElroy, *et al.*, (1990) *Plant Cell* 163-171); ubiquitin (Christensen, *et al.*, (1992) *Plant Mol. Biol.* 12:619-632 and Christensen, *et al.*, (1992) *Plant Mol. Biol.* 18:675-89); pEMU (Last, *et al.*, (1991) *Theor. Appl. Genet.* 81:581-8); MAS (Velten, *et al.*, (1984) *EMBO J.* 3:2723-30) and maize H3 histone (Lepetit, *et al.*, (1992) *Mol. Gen. Genet.* 231:276-85 and Atanassvoa, *et al.*, (1992) *Plant Journal* 2(3):291-300); ALS promoter, as described in PCT Application Number WO 96/30530 and other transcription initiation regions from various plant genes known to those of skill. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611. In some embodiments, the ubiquitin promoter is used for expression in monocot plants.

Alternatively, the plant promoter can direct expression of a polynucleotide of the present invention in a specific tissue or may be otherwise under more precise environmental or developmental control. Such promoters are referred to here as "inducible" promoters. Environmental conditions that may effect transcription by inducible promoters include pathogen attack, anaerobic conditions or the presence of light. Examples of inducible promoters are the Adh1 promoter, which is inducible by

hypoxia or cold stress, the Hsp70 promoter, which is inducible by heat stress and the PPDK promoter, which is inducible by light.

Generally, it will be beneficial to express the gene from an inducible promoter, particularly from a pathogen-inducible promoter. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol.* 89:245-254; Uknes *et al.* (1992) *Plant Cell* 4:645-656; and Van Loon (1985) *Plant Mol. Virol.* 4:111-116. See also WO 99/43819, herein incorporated by reference.

Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol.* 9:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions* 2:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet.* 2:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA* 93:14972-14977. See also, Chen *et al.* (1996) *Plant J.* 10:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:2507-2511; Warner *et al.* (1993) *Plant J.* 3:191-201; Siebertz *et al.* (1989) *Plant Cell* 1:961-968; U.S. Patent No. 5,750,386 (nematode-inducible); and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero *et al.* (1992) *Physiol. Mol. Plant Path.* 41:189-200).

Additionally, as pathogens find entry into plants through wounds or insect damage, a wound-inducible promoter may be used in the constructions of the invention. Such wound-inducible promoters include potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath.* 28:425-449; Duan *et al.* (1996) *Nature Biotechnology* 14:494-498); wun1 and wun2, U.S. Patent No. 5,428,148; win1 and win2 (Stanford *et al.* (1989) *Mol. Gen. Genet.* 215:200-208); systemin (McGurl *et al.* (1992) *Science* 225:1570-1573); WIP1 (Rohmeier *et al.* (1993) *Plant Mol. Biol.* 22:783-792; Eckelkamp *et al.* (1993) *FEBS Letters* 323:73-76); MPI gene (Corderok *et al.* (1994) *Plant J.* 6(2):141-150); and the like, herein incorporated by reference.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters

are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Examples of promoters under developmental control include promoters that initiate transcription only, or preferentially, in certain tissues, such as leaves, roots, fruit, seeds or flowers. The operation of a promoter may also vary depending on its location in the genome. Thus, an inducible promoter may become fully or partially constitutive in certain locations.

Tissue-preferred promoters can be utilized to target expression of a polynucleotide useful in methods of the present invention within a particular plant tissue. Tissue-preferred promoters include Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

Leaf-preferred promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kwon *et al.* (1994) *Plant Physiol.* 105:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor *et al.* (1993) *Plant J.* 3:509-18; Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6):1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590.

Root-preferred promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See,

for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.* 14(3):433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell* 2(7):633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1):69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see *EMBO J.* 8(2):343-350). The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4):759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

"Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination). See Thompson *et al.* (1989) *BioEssays* 10:108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); milps (myo-inositol-1-phosphate synthase) (see WO 00/11177 and U.S. Patent No. 6,225,529; herein incorporated by reference). Gamma-zein is an endosperm-specific promoter. Globulin

1 (Glb-1) is a representative embryo-specific promoter. For dicots, seed-specific promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, gamma-zein, waxy, shrunken 1, shrunken 2, Globulin 1, etc. See also WO 00/12733, where seed-preferred promoters from *end1* and *end2* genes are disclosed; herein incorporated by reference.

The regulatory regions (*i.e.*, promoters, transcriptional regulatory regions, and translational termination regions) and/or the ACC synthase polynucleotide may be native/analogous to the host cell or to each other. Alternatively, the regulatory regions and/or the ACC synthase polynucleotide may be heterologous to the host cell or to each other. As used herein, "heterologous" in reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from that from which the polynucleotide was derived, or, if from the same/analogous species, one or both are substantially modified from their original form, or the promoter is not the native promoter for the operably linked polynucleotide. Likewise, a heterologous protein may originate from a foreign species or, if from the same species, is substantially modified from its original form by deliberate human intervention.

If polypeptide expression is desired, it is generally desirable to include a polyadenylation region at the 3'-end of a polynucleotide coding region. The polyadenylation region can be derived from a variety of plant genes, or from T-DNA. The 3' end sequence to be added can be derived from, for example, the nopaline synthase or octopine synthase genes, or alternatively from another plant gene, or less preferably from any other eukaryotic gene. Examples of such regulatory elements include, but are not limited to, 3' termination and/or polyadenylation regions such as those of the *Agrobacterium tumefaciens* nopaline synthase (nos) gene (Bevan, *et al.*, (1983) *Nucleic Acids Res.* 12:369-85); the potato proteinase inhibitor II (PINII) gene (Keil, *et al.*, (1986) *Nucleic Acids Res.* 14:5641-50 and An, *et al.*, (1989) *Plant Cell* 1:115-22) and the CaMV 19S gene (Mogen, *et al.*, (1990) *Plant Cell* 2:1261-72).

An intron sequence can be added to the 5' untranslated region or the coding sequence of the partial coding sequence to increase the amount of the mature message

that accumulates in the cytosol. Inclusion of a spliceable intron in the transcription unit in both plant and animal expression constructs has been shown to increase gene expression at both the mRNA and protein levels up to 1000-fold (Buchman and Berg, (1988) *Mol. Cell Biol.* 8:4395-4405; Callis, *et al.*, (1987) *Genes Dev.* 1:1183-200). Such intron enhancement of gene expression is typically greatest when placed near the 5' end of the transcription unit. Use of maize introns Adh1-S intron 1, 2 and 6, the Bronze-1 intron are known in the art. See generally, *The Maize Handbook*, Chapter 116, Freeling and Walbot, eds., Springer, New York (1994).

Plant signal sequences, including, but not limited to, signal-peptide encoding DNA/RNA sequences which target proteins to the extracellular matrix of the plant cell (Dratewka-Kos, *et al.*, (1989) *J. Biol. Chem.* 264:4896-900), such as the *Nicotiana plumbaginifolia* extension gene (DeLoose, *et al.*, (1991) *Gene* 99:95-100); signal peptides which target proteins to the vacuole, such as the sweet potato sporamin gene (Matsuka, *et al.*, (1991) *Proc. Natl. Acad. Sci. USA* 88:834) and the barley lectin gene (Wilkins, *et al.*, (1990) *Plant Cell*, 2:301-13); signal peptides which cause proteins to be secreted, such as that of PRIb (Lind, *et al.*, (1992) *Plant Mol. Biol.* 18:47-53) or the barley alpha amylase (BAA) (Rahmatullah, *et al.*, (1989) *Plant Mol. Biol.* 12:119, and hereby incorporated by reference) or signal peptides which target proteins to the plastids such as that of rapeseed enoyl-Acp reductase (Verwaert, *et al.*, (1994) *Plant Mol. Biol.* 26:189-202) are useful in the invention.

The vector comprising the sequences from a polynucleotide useful in the methods of the present invention will typically comprise a marker gene, which confers a selectable phenotype on plant cells. Usually, the selectable marker gene will encode antibiotic resistance, with suitable genes including genes coding for resistance to the antibiotic spectinomycin (e.g., the aada gene), the streptomycin phosphotransferase (SPT) gene coding for streptomycin resistance, the neomycin phosphotransferase (NPTII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (HPT) gene coding for hygromycin resistance, genes coding for resistance to herbicides which act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) gene containing mutations leading to such resistance in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides which act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the *bar* gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, and the

ALS gene encodes resistance to the herbicide chlorsulfuron. Other genes that confer resistance to herbicidal compounds, such as such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D) can be used. Additional selectable markers include phenotypic markers such as β-galactosidase and fluorescent proteins such as green fluorescent protein (GFP) (Su et *al.* (2004) *Biotechnol Bioeng 85:*610-9 and Fetter *et al.* (2004) *Plant Cell 16:*215-28), cyan florescent protein (CYP) (Bolte *et al.* (2004) *J. Cell Science 117*:943-54 and Kato *et al.* (2002) *Plant Physiol 129*:913-42), and yellow florescent protein (PhiYFP™ from Evrogen, see, Bolte *et al.* (2004) *J. Cell Science 117*:943-54). For additional selectable markers, see generally, Yarranton (1992) *Curr. Opin. Biotech.* 3:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao *et al.* (1992) *Cell* 71:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell* 48:555-566; Brown *et al.* (1987) *Cell* 49:603-612; Figge *et al.* (1988) *Cell* 52:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA* 86:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-2553; Deuschle *et al.* (1990) *Science* 248:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res.* 19:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry* 27:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature* 334:721-724. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers, *et al.* (1987), *Meth. Enzymol.* 153:253-77. These vectors are plant integrating vectors in that on transformation, the vectors integrate a portion of vector DNA into the genome of the host plant. Exemplary

A. tumefaciens vectors useful herein are plasmids pKYLX6 and pKYLX7 of Schardl, *et al.*, (1987) *Gene* 61:1-11 and Berger, *et al.*, (1989) *Proc. Natl. Acad. Sci. USA*, 86:8402-6. Another useful vector herein is plasmid pBI101.2 that is available from CLONTECH Laboratories, Inc. (Palo Alto, CA). As used herein, "vector" includes reference to a nucleic acid used in transfection of a host cell and into which can be inserted a polynucleotide. Vectors are often replicons. Expression vectors permit transcription of a nucleic acid inserted therein as described elsewhere herein.

One may express a protein in a recombinantly engineered cell such as bacteria, yeast, insect, mammalian or preferably plant cells. The cells produce the protein in a non-natural condition (e.g., in quantity, composition, location and/or time), because they have been genetically altered through human intervention to do so.

By "host cell" is meant a cell, which comprises a heterologous nucleic acid sequence of the invention, which contains a vector and supports the replication and/or expression of the expression vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, plant, amphibian or mammalian cells. Preferably, host cells are monocotyledonous or dicotyledonous plant cells, including but not limited to maize, sorghum, sunflower, soybean, wheat, alfalfa, rice, cotton, sugarcane, canola, lawn grass, barley, millet and tomato. In some embodiments, the monocotyledonous host cell is a maize host cell.

As used herein "recombinant" includes reference to a cell or vector that has been modified by the introduction of a heterologous nucleic acid, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention or may have reduced or eliminated expression of a native gene. The term "recombinant" as used herein does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein useful in the methods of the present invention. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

In brief summary, the expression of isolated nucleic acids encoding a protein of the present invention will typically be achieved by operably linking, for example, the DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. As described above, typical expression vectors contain transcription and translation terminators, initiation sequences and promoters useful for regulation of the expression of the DNA encoding a protein useful in the methods of the present invention. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter, such as ubiquitin, to direct transcription, a ribosome binding site for translational initiation and a transcription/translation terminator.

One of skill would recognize that modifications could be made to a protein useful in the methods of the present invention without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of *E. coli;* however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang, *et al.*, (1977) *Nature* 198:1056), the tryptophan (trp) promoter system (Goeddel, *et al.*, (1980) *Nucleic Acids Res.* 8:4057) and the lambda derived P L promoter and N-gene ribosome binding site (Shimatake, *et al.*, (1981) *Nature* 292:128). The inclusion of selection markers in DNA vectors transfected in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline or chloramphenicol.

The vector is selected to allow introduction of the gene of interest into the appropriate host cell. Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected

with the plasmid vector DNA. Expression systems for expressing a protein useful in the methods of the present invention are available using *Bacillus sp.* and *Salmonella* (Palva, *et al.*, (1983) *Gene* 22:229-35; Mosbach, *et al.*, (1983) *Nature* 302:543-5). The pGEX-4T-1 plasmid vector from Pharmacia is the preferred *E. coli* expression vector for the present invention.

A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, the present invention can be expressed in these eukaryotic systems. In some embodiments, transformed/transfected plant cells, as discussed *infra*, are employed as expression systems for production of the proteins of the instant invention.

Synthesis of heterologous proteins in yeast is well known. Sherman, *et al.*, (1982) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory is a well recognized work describing the various methods available to produce the protein in yeast. Two widely utilized yeasts for production of eukaryotic proteins are *Saccharomyces cerevisiae* and *Pichia pastoris*. Vectors, strains and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase and an origin of replication, termination sequences and the like as desired.

A protein useful in the methods of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates or the pellets. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay of other standard immunoassay techniques.

The sequences encoding proteins useful in the methods of the present invention can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect or plant origin. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21 and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (e.g., the CMV promoter, a HSV *tk* promoter or *pgk* (phosphoglycerate kinase) promoter), an enhancer (Queen, *et al.*, (1986) *Immunol. Rev.* 89:49) and necessary processing information sites, such as ribosome binding sites,

RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site) and transcriptional terminator sequences. Other animal cells useful for production of proteins of the present invention are available, for instance, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (7^{th} ed., 1992).

Appropriate vectors for expressing proteins of the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (see, e.g., Schneider, (1987) *J. Embryol. Exp. Morphol.* 27:353-65).

As with yeast, when higher animal or plant host cells are employed, polyadenlyation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenlyation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, *et al.*, (1983) *J. Virol.* 45:773-81). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors (Saveria-Campo, "Bovine Papilloma Virus DNA a Eukaryotic Cloning Vector," in *DNA Cloning*: *A Practical Approach,* vol. II, Glover, ed., IRL Press, Arlington, VA, pp. 213-38 (1985)).

In addition, the ACC synthase polynucleotide placed in the appropriate plant expression vector can be used to transform plant cells. The polypeptide can then be isolated from plant callus or the transformed cells can be used to regenerate transgenic plants. Such transgenic plants can be harvested, and the appropriate tissues (seed or leaves, for example) can be subjected to large scale protein extraction and purification techniques.

Numerous methods for introducing foreign polynucleotides into plants are known and can be used to insert an ACC synthase polynucleotide into a plant host, including biological and physical plant transformation protocols. See, e.g., Miki, *et al.*, "Procedure for Introducing Foreign DNA into Plants," in *Methods in Plant Molecular Biology and Biotechnology*, Glick and Thompson, eds., CRC Press, Inc., Boca Raton, pp. 67-88 (1993). The methods chosen vary with the host plant and include chemical transfection methods such as calcium phosphate, microorganism-mediated gene transfer such as *Agrobacterium* (Horsch, *et al.*, *Science* 227:1229-31 (1985)), electroporation, micro-injection and biolistic bombardment.

The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon or transiently expressed (e.g., transfected mRNA). When a polynucleotide or polypeptide is introduced into a plant, "introducing" is intended to mean presenting to the plant the polynucleotide or polypeptide in such a manner that the sequence gains access to the interior of a cell of the plant. The methods of the invention do not depend on a particular method for introducing a sequence into a plant, only that the polynucleotide or polypeptides gains access to the interior of at least one cell of the plant. Methods for introducing polynucleotide or polypeptides into plants are known in the art, including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods.

Expression cassettes and vectors and *in vitro* culture methods for plant cell or tissue transformation and regeneration of plants are known and available. See, e.g., Gruber, *et al.*, "Vectors for Plant Transformation," in *Methods in Plant Molecular Biology and Biotechnology*, *supra*, pp. 89-119.

The polynucleotides or polypeptides may be introduced into the plant by one or more techniques typically used for direct delivery into cells. Such protocols may vary depending on the type of organism, cell, plant or plant cell, i.e. monocot or dicot, targeted for gene modification. Suitable methods of transforming plant cells include microinjection (Crossway, *et al.*, (1986) *Biotechniques* 4:320-334 and US Patent Number 6,300,543), electroporation (Riggs, *et al.*, (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, direct gene transfer (Paszkowski *et al.*, (1984) *EMBO J.* 3:2717-2722) and ballistic particle acceleration (see, for example, Sanford, *et al.*, US Patent Number 4,945,050; WO 91/10725 and McCabe, *et al.*, (1988) *Biotechnology* 6:923-926). Also see, Tomes, *et al.*, "Direct DNA Transfer into Intact Plant Cells Via Microprojectile Bombardment". pp. 197-213 in *Plant Cell, Tissue and Organ Culture, Fundamental Methods*. eds. Gamborg and Phillips, Springer-Verlag Berlin Heidelberg New York, 1995; US Patent Number 5,736,369 (meristem); Weissinger, *et al.*, (1988) *Ann. Rev. Genet.* 22:421-477; Sanford, *et al.*, (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou, *et al.*, (1988) *Plant Physiol.* 87:671-674 (soybean); Datta, *et al.*, (1990) *Biotechnology* 8:736-740 (rice); Klein, *et al.*, (1988) *Proc. Natl. Acad. Sci. USA*

85:4305-4309 (maize); Klein, *et al.*, (1988) *Biotechnology* 6:559-563 (maize); WO 91/10725 (maize); Klein, *et al.*, (1988) *Plant Physiol.* 91:440-444 (maize); Fromm, *et al.*, (1990) *Biotechnology* 8:833-839 and Gordon-Kamm, *et al.*, (1990) *Plant Cell* 2:603-618 (maize); Hooydaas-Van Slogteren and Hooykaas, (1984) *Nature* (London) 311:763-764; Bytebierm, *et al.*, (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet, *et al.*, (1985) *In The Experimental Manipulation of Ovule Tissues*, ed. Chapman, *et al.*, pp. 197-209, Longman, NY (pollen); Kaeppler, *et al.*, (1990) *Plant Cell Reports* 9:415-418; and Kaeppler, *et al.*, (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); US Patent Number 5,693,512 (sonication); D'Halluin, *et al.*, (1992) *Plant Cell* 4:1495-1505 (electroporation); Li, *et al.*, (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford, (1995) *Annals of Botany* 75:407-413 (rice); Osjoda, *et al.*, (1996) *Nature Biotech.* 14:745-750; Agrobacterium mediated maize transformation (US Patent Number 5,981,840); silicon carbide whisker methods (Frame, *et al.*, (1994) *Plant J.* 6:941-948); laser methods (Guo, *et al.*, (1995) *Physiologia Plantarum* 93:19-24); sonication methods (Bao, *et al.*, (1997) *Ultrasound in Medicine & Biology* 23:953-959; Finer and Finer, (2000) *Lett Appl Microbiol.* 30:406-10; Amoah, *et al.*, (2001) *J Exp Bot* 52:1135-42); polyethylene glycol methods (Krens, *et al.*, (1982) *Nature* 296:72-77); protoplasts of monocot and dicot cells can be transformed using electroporation (Fromm, *et al.*, (1985) *Proc. Natl. Acad. Sci. USA* 82:5824-5828) and microinjection (Crossway, *et al.*, (1986) *Mol. Gen. Genet.* 202:179-185), all of which are herein incorporated by reference.

The most widely utilized method for introducing an expression vector into plants is based on the natural transformation system of *Agrobacterium*. *A. tumefaciens* and *A. rhizogenes* are plant pathogenic soil bacteria, which genetically transform plant cells. The Ti and Ri plasmids of *A. tumefaciens* and *A. rhizogenes*, respectively, carry genes responsible for genetic transformation of plants. See, e.g., Kado, (1991) *Crit. Rev. Plant Sci.* 10:1. Descriptions of the *Agrobacterium* vector systems and methods for *Agrobacterium*-mediated gene transfer are provided in Gruber, *et al.*, *supra*; Miki, *et al.*, *supra* and Moloney, *et al.*, (1989) *Plant Cell Reports* 8:238.

Similarly, the gene can be inserted into the T-DNA region of a Ti or Ri plasmid derived from *A. tumefaciens* or *A. rhizogenes*, respectively. Thus, expression cassettes can be constructed as above, using these plasmids. Many control sequences are known which when coupled to a heterologous coding sequence and transformed into a host organism show fidelity in gene expression with respect to tissue/organ specificity of the

original coding sequence. See, e.g., Benfey and Chua, (1989) *Science* 244:174-81. Particularly suitable control sequences for use in these plasmids are promoters for constitutive leaf-specific expression of the gene in the various target plants. Other useful control sequences include a promoter and terminator from the nopaline synthase gene (NOS). The NOS promoter and terminator are present in the plasmid pARC2, available from the American Type Culture Collection and designated ATCC Deposit Number 67238. If such a system is used, the virulence (*vir*) gene from either the Ti or Ri plasmid must also be present, either along with the T-DNA portion or via a binary system where the *vir* gene is present on a separate vector. Such systems, vectors for use therein and methods of transforming plant cells are described in US Patent Number 4,658,082; US Patent Application Serial Number 913,914, filed October 1, 1986, as referenced in US Patent Number 5,262,306, issued November 16, 1993 and Simpson, *et al.*, (1986) *Plant Mol. Biol.* 6:403-15 (also referenced in the '306 patent), all incorporated by reference in their entirety.

Once constructed, these plasmids can be placed into *A. rhizogenes* or *A. tumefaciens* and these vectors used to transform cells of plant species, which are ordinarily susceptible to *Fusarium* or *Alternaria* infection. Several other transgenic plants are also contemplated by the present invention including but not limited to soybean, corn, sorghum, alfalfa, rice, clover, cabbage, banana, coffee, celery, tobacco, cowpea, cotton, melon and pepper. The selection of either *A. tumefaciens* or *A. rhizogenes* will depend on the plant being transformed thereby. In general *A. tumefaciens* is the preferred organism for transformation. Most dicotyledonous plants, some gymnosperms, and a few monocotyledonous plants (e.g., certain members of the *Liliales* and *Arales*) are susceptible to infection with *A. tumefaciens*. *A. rhizogenes* also has a wide host range, embracing most dicots and some gymnosperms, which includes members of the *Leguminosae*, *Compositae* and *Chenopodiaceae*. Monocot plants can now be transformed with some success. EP Patent Application Number 604 662 A1 discloses a method for transforming monocots using *Agrobacterium*. EP Patent Application Number 672 752 A1 discloses a method for transforming monocots with *Agrobacterium* using the scutellum of immature embryos. Ishida, *et al.*, discuss a method for transforming maize by exposing immature embryos to *A. tumefaciens* (*Nature Biotechnology* 14:745-50 (1996)).

Once transformed, these cells can be used to regenerate transgenic plants. As used herein, "transgenic plant" includes reference to a plant, which comprises within its

genome a heterologous polynucleotide. Generally, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant expression cassette. "Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition or spontaneous mutation.

For example, whole plants can be infected with these vectors by wounding the plant and then introducing the vector into the wound site. Any part of the plant can be wounded, including leaves, stems and roots. Alternatively, plant tissue, in the form of an explant, such as cotyledonary tissue or leaf disks, can be inoculated with these vectors, and cultured under conditions, which promote plant regeneration. Roots or shoots transformed by inoculation of plant tissue with *A. rhizogenes* or *A. tumefaciens*, containing the gene coding for the fumonisin degradation enzyme, can be used as a source of plant tissue to regenerate fumonisin-resistant transgenic plants, either via somatic embryogenesis or organogenesis. Examples of such methods for regenerating plant tissue are disclosed in Shahin, (1985) *Theor. Appl. Genet.* 69:235-40; US Patent Number 4,658,082; Simpson, *et al.*, *supra* and US Patent Application Serial Numbers 913,913 and 913,914, both filed October 1, 1986, as referenced in US Patent Number 5,262,306, issued November 16, 1993, the entire disclosures therein incorporated herein by reference.

Despite the fact that the host range for *Agrobacterium*-mediated transformation is broad, some major cereal crop species and gymnosperms have generally been recalcitrant to this mode of gene transfer, even though some success has recently been achieved in rice (Hiei, *et al.*, (1994) *The Plant Journal* 6:271-82). Several methods of plant transformation, collectively referred to as direct gene transfer, have been developed as an alternative to *Agrobacterium*-mediated transformation.

A generally applicable method of plant transformation is microprojectile-mediated transformation, where DNA is carried on the surface of microprojectiles

measuring about 1 to 4 μm. The expression vector is introduced into plant tissues with a biolistic device that accelerates the microprojectiles to speeds of 300 to 600 m/s which is sufficient to penetrate the plant cell walls and membranes (Sanford, *et al.*, (1987) *Part. Sci. Technol.* 5:27; Sanford, (1988) *Trends Biotech* 6:299; Sanford, (1990) *Physiol. Plant* 79:206 and Klein, *et al.*, (1992) *Biotechnology* 10:268).

Another method for physical delivery of DNA to plants is sonication of target cells as described in Zang, *et al.*, (1991) *BioTechnology* 9:996. Alternatively, liposome or spheroplast fusions have been used to introduce expression vectors into plants. See, e.g., Deshayes, *et al.*, (1985) *EMBO J.* 4:2731 and Christou, *et al.*, (1987) *Proc. Natl. Acad. Sci. USA* 84:3962. Direct uptake of DNA into protoplasts using $CaCl_2$ precipitation, polyvinyl alcohol, or poly-L-ornithine has also been reported. See, e.g., Hain, *et al.*, (1985) *Mol. Gen. Genet.* 199:161 and Draper, *et al.*, (1982) *Plant Cell Physiol.* 23:451.

Electroporation of protoplasts and whole cells and tissues has also been described. See, e.g., Donn, *et al.*, (1990) *Abstracts of the VIIth Int'l. Congress on Plant Cell and Tissue Culture IAPTC*, A2-38, p. 53; D'Halluin, *et al.*, (1992) *Plant Cell* 4:1495-505 and Spencer, *et al.*, (1994) *Plant Mol. Biol.* 24:51-61.

In addition to modulating ethylene synthesis, the methods of the invention can be used along with sequences or methods that alter additional phenotypes in the plant. Various changes in phenotype are of interest including modifying the fatty acid composition in a plant, altering the amino acid content of a plant, altering a plant's pathogen defense mechanism, and the like. These results can be achieved by providing expression of heterologous products or increased expression of endogenous products in plants. Alternatively, the results can be achieved by providing for a reduction of expression of one or more endogenous products, particularly enzymes or cofactors in the plant. These changes result in a change in phenotype of the transformed plant.

Genes of interest are reflective of the commercial markets and interests of those involved in the development of the crop. Crops and markets of interest change, and as developing nations open up world markets, new crops and technologies will emerge also. In addition, as our understanding of agronomic traits and characteristics such as yield and heterosis increase, the choice of genes for transformation will change accordingly. General categories of genes of interest include, for example, those genes involved in information, such as zinc fingers, those involved in communication, such as kinases and those involved in housekeeping, such as heat shock proteins. More specific

categories of transgenes, for example, include genes encoding important traits for agronomics, insect resistance, disease resistance, herbicide resistance, sterility, grain characteristics and commercial products. Genes of interest include, generally, those involved in oil, starch, carbohydrate or nutrient metabolism as well as those affecting kernel size, sucrose loading and the like.

In certain embodiments the nucleic acid sequences of the present invention can be used in combination ("stacked") with other polynucleotide sequences of interest in order to create plants with a desired phenotype. The combinations generated can include multiple copies of any one or more of the polynucleotides of interest. The polynucleotides of the present invention may be stacked with any gene or combination of genes to produce plants with a variety of desired trait combinations, including but not limited to traits desirable for animal feed such as high oil genes (e.g., US Patent Number 6,232,529); balanced amino acids (e.g., hordothionins (US Patent Numbers 5,990,389; 5,885,801; 5,885,802 and 5,703,049); barley high lysine (Williamson, *et al.*, (1987) *Eur. J. Biochem.* 165:99-106 and WO 98/20122) and high methionine proteins (Pedersen, *et al.*, (1986) *J. Biol. Chem.* 261:6279; Kirihara, *et al.*, (1988) *Gene* 71:359 and Musumura, *et al.*, (1989) *Plant Mol. Biol.* 12:123)); increased digestibility (e.g., modified storage proteins (US Patent Application Serial Number 10/053,410, filed November 7, 2001) and thioredoxins (US Patent Application Serial Number 10/005,429, filed December 3, 2001)), the disclosures of which are herein incorporated by reference. The polynucleotides of the present invention can also be stacked with traits desirable for insect, disease or herbicide resistance (e.g., *Bacillus thuringiensis* toxic proteins (US Patent Numbers 5,366,892; 5,747,450; 5,736,514; 5,723,756; 5,593,881; Geiser, *et al.*, (1986) *Gene* 48:109); lectins (Van Damme, *et al.*, (1994) *Plant Mol. Biol.* 24:825); fumonisin detoxification genes (US Patent Number 5,792,931); avirulence and disease resistance genes (Jones, *et al.*, (1994) *Science* 266:789; Martin, *et al.*, (1993) *Science* 262:1432; Mindrinos, *et al.*, (1994) *Cell* 78:1089); acetolactate synthase (ALS) mutants that lead to herbicide resistance such as the S4 and/or Hra mutations; inhibitors of glutamine synthase such as phosphinothricin or basta (e.g., bar gene); and glyphosate resistance (EPSPS gene)) and traits desirable for processing or process products such as high oil (e.g., US Patent Number 6,232,529); modified oils (e.g., fatty acid desaturase genes (US Patent Number 5,952,544; WO 94/11516)); modified starches (e.g., ADPG pyrophosphorylases (AGPase), starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes

(SDBE)) and polymers or bioplastics (e.g., US Patent Number 5.602,321; beta-ketothiolase, polyhydroxybutyrate synthase and acetoacetyl-CoA reductase (Schubert, *et al.*, (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhydroxyalkanoates (PHAs)), the disclosures of which are herein incorporated by reference. One could also combine the polynucleotides of the present invention with polynucleotides affecting agronomic traits such as male sterility (e.g., see, US Patent Number 5,583,210), stalk strength, flowering time or transformation technology traits such as cell cycle regulation or gene targeting (e.g., WO 99/61619; WO 00/17364; WO 99/25821), the disclosures of which are herein incorporated by reference.

In one embodiment, sequences of interest improve plant growth and/or crop yields. For example, sequences of interest include agronomically important genes that result in improved primary or lateral root systems. Such genes include, but are not limited to, nutrient/water transporters and growth induces. Examples of such genes, include but are not limited to, maize plasma membrane H^+-ATPase (MHA2) (Frias, *et al.*, (1996) *Plant Cell* 8:1533-44); AKT1, a component of the potassium uptake apparatus in *Arabidopsis*, (Spalding, *et al.*, (1999) *J Gen Physiol* 113:909-18); RML genes which activate cell division cycle in the root apical cells (Cheng, *et al.*, (1995) *Plant Physiol* 108:881); maize glutamine synthetase genes (Sukanya, *et al.*, (1994) *Plant Mol Biol* 26:1935-46) and hemoglobin (Duff, *et al.*, (1997) *J. Biol. Chem* 27:16749-16752, Arredondo-Peter, *et al.*, (1997) *Plant Physiol.* 115:1259-1266; Arredondo-Peter, *et al.*, (1997) *Plant Physiol* 114:493-500 and references sited therein). The sequence of interest may also be useful in expressing antisense nucleotide sequences of genes that that negatively affects root development.

Additional, agronomically important traits such as oil, starch and protein content can be genetically altered in addition to using traditional breeding methods. Modifications include increasing content of oleic acid, saturated and unsaturated oils, increasing levels of lysine and sulfur, providing essential amino acids and also modification of starch. Hordothionin protein modifications are described in US Patent Numbers 5,703,049, 5,885,801, 5,885,802 and 5,990,389, herein incorporated by reference. Another example is lysine and/or sulfur rich seed protein encoded by the soybean 2S albumin described in US Patent Number 5,850,016, and the chymotrypsin inhibitor from barley, described in Williamson, *et al.*, (1987) *Eur. J. Biochem.* 165:99-106, the disclosures of which are herein incorporated by reference.

Derivatives of the coding sequences can be made by site-directed mutagenesis to increase the level of preselected amino acids in the encoded polypeptide. For example, the gene encoding the barley high lysine polypeptide (BHL) is derived from barley chymotrypsin inhibitor, US Patent Application Serial Number 08/740,682, filed November 1, 1996 and WO 98/20133, the disclosures of which are herein incorporated by reference. Other proteins include methionine-rich plant proteins such as from sunflower seed (Lilley, *et al.*, (1989) *Proceedings of the World Congress on Vegetable Protein Utilization in Human Foods and Animal Feedstuffs*, ed. Applewhite (American Oil Chemists Society, Champaign, Illinois), pp. 497-502, herein incorporated by reference); corn (Pedersen, *et al.*, (1986) *J. Biol. Chem.* 261:6279; Kirihara, *et al.*, (1988) *Gene* 71:359, both of which are herein incorporated by reference) and rice (Musumura, *et al.*, (1989) *Plant Mol. Biol.* 12:123, herein incorporated by reference). Other agronomically important genes encode latex, Floury 2, growth factors, seed storage factors and transcription factors.

Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer and the like. Such genes include, for example, *Bacillus thuringiensis* toxic protein genes (US Patent Numbers 5,366,892; 5,747,450; 5,736,514; 5,723,756; 5,593,881 and Geiser, *et al.*, (1986) *Gene* 48:109) and the like.

Genes encoding disease resistance traits include detoxification genes, such as against fumonosin (US Patent Number 5,792,931); avirulence (avr) and disease resistance (R) genes (Jones, *et al.*, (1994) *Science* 266:789; Martin, *et al.*, (1993) *Science* 262:1432 and Mindrinos, *et al.*, (1994) *Cell* 78:1089) and the like.

Herbicide resistance traits may include genes coding for resistance to herbicides that act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) gene containing mutations leading to such resistance, in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides that act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the bar gene) or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, the *nptII* gene encodes resistance to the antibiotics kanamycin and geneticin and the ALS-gene mutants encode resistance to the herbicide chlorsulfuron.

Sterility genes can also be encoded in an expression cassette and provide an alternative to physical detasseling. Examples of genes used in such ways include male

tissue-preferred genes and genes with male sterility phenotypes such as QM, described in US Patent Number 5,583,210. Other genes include kinases and those encoding compounds toxic to either male or female gametophytic development.

The quality of grain is reflected in traits such as levels and types of oils, saturated and unsaturated, quality and quantity of essential amino acids and levels of cellulose. In corn, modified hordothionin proteins are described in US Patent Numbers 5,703,049, 5,885,801, 5,885,802 and 5,990,389.

Commercial traits can also be encoded on a gene or genes that could increase for example, starch for ethanol production, or provide expression of proteins. Another important commercial use of transformed plants is the production of polymers and bioplastics such as described in US Patent Number 5,602,321. Genes such as β-Ketothiolase, PHBase (polyhydroxyburyrate synthase) and acetoacetyl-CoA reductase (see, Schubert, *et al*., (1988) *J. Bacteriol.* 170:5837-5847) facilitate expression of polyhyroxyalkanoates (PHAs).

Exogenous products include plant enzymes and products as well as those from other sources including procaryotes and other eukaryotes. Such products include enzymes, cofactors, hormones, and the like. The level of proteins, particularly modified proteins having improved amino acid distribution to improve the nutrient value of the plant, can be increased. This is achieved by the expression of such proteins having enhanced amino acid content.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5: 81-84. These plants may then be grown and either pollinated with the same transformed strain or different strains; the resulting progeny having the desired phenotypic characteristic can then be identified. Two or more generations may be grown to ensure that the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure that stable transformants exhibiting the desired phenotypic characteristic have been achieved. In this manner, the present invention provides transformed seed (also referred to as “transgenic seed”) having a nucleotide construct of the invention, for example, a cassette of the invention, stably incorporated into their genome.

As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. The term plant also includes plant protoplasts, plant calli, and plant

clumps. Plant cell, as used herein includes, without limitation, cells in or from seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen and microspores. Plant cells can be part of an intact plant or part of a plant, such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, kernels, ears, cobs, husks, stalks, roots, root tips, anthers, and the like. Grain is intended to mean the mature seed produced by commercial growers for purposes other than growing or reproducing the species. Progeny, variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced polynucleotides.

The present invention may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plant species of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *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. In particular embodiments of the presently disclosed methods, the plant is *Zea mays*.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa*

spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum.

Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). In specific embodiments, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.). In other embodiments, corn and soybean and sugarcane plants are optimal, and in yet other embodiments corn plants are optimal.

Other 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, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, 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.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of botany, microbiology, tissue culture, molecular biology, chemistry, biochemistry and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Langenheim and Thimann, (1982) *Botany: Plant Biology and Its Relation to Human Affairs*, John Wiley; *Cell Culture and Somatic Cell Genetics of Plants*, vol. 1, Vasil, ed. (1984); Stanier, *et al.*, (1986) *The Microbial World*, 5th ed., Prentice-Hall; Dhringra and Sinclair, (1985) *Basic Plant Pathology Methods*, CRC Press; Maniatis, *et al.*, (1982) *Molecular Cloning: A Laboratory Manual*; *DNA Cloning*, vols. I and II, Glover, ed. (1985); *Oligonucleotide Synthesis*, Gait, ed. (1984); *Nucleic Acid Hybridization*, Hames and Higgins, eds. (1984) and the series *Methods in Enzymology*, Colowick and Kaplan, eds, Academic Press, Inc., San Diego, CA.

Units, prefixes and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

Numeric ranges are inclusive of the numbers defining the range. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "a polypeptide" is understood to represent one or more polypeptides. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

Throughout this specification and the claims, the words "comprise," "comprises," and "comprising" are used in a non-exclusive sense, except where the context requires otherwise.

As used herein, the term "about," when referring to a value is meant to encompass variations of, in some embodiments ± 50%, in some embodiments ± 20%, in some embodiments ± 10%, in some embodiments ± 5%, in some embodiments ± 1%, in some embodiments ± 0.5%, and in some embodiments ± 0.1% from the specified amount, as such variations are appropriate to perform the disclosed methods or employ the disclosed compositions.

Further, when an amount, concentration, or other value or parameter is given as either a range, preferred range, or a list of upper preferable values and lower preferable values, this is to be understood as specifically disclosing all ranges formed from any pair of any upper range limit or preferred value and any lower range limit or preferred value, regardless of whether ranges are separately disclosed. Where a range of numerical values is recited herein, unless otherwise stated, the range is intended to include the endpoints thereof, and all integers and fractions within the range. It is not intended that the scope of the presently disclosed subject matter be limited to the specific values recited when defining a range.

This invention can be better understood by reference to the following non-limiting examples. It will be appreciated by those skilled in the art that other embodiments of the invention may be practiced without departing from the spirit and the scope of the invention as herein disclosed and claimed.

EXAMPLES

EXAMPLE 1. ACC Synthase Knockouts by Hairpin RNA Expression

As noted previously, knockout plant cells and plants can be produced, for example, by introduction of an ACC synthase polynucleotide sequence configured for RNA silencing or interference. This example describes hairpin RNA expression cassettes for improving plant nitrogen utilization phenotype, e.g., in maize. As noted previously, knockout of ACC synthase(s), e.g., by hpRNA expression, can result in plants or plant cells having reduced expression (up to and including no detectable expression) of one or more ACC synthases.

Expression of hairpin RNA (hpRNA) molecules specific for regions of ACC synthase genes (e.g., promoters, other untranslated regions or coding regions) in plants can alter nitrogen utilization potential of the plants, e.g., through RNA interference and/or silencing.

hpRNA constructs of ACS2 and ACS6 were generated by linking a ubiquitin promoter to an inverted repeat of a portion of the coding sequence of either the ACS2 or ACS6 gene (see, Figures 2 and 3). Each construct was transformed into maize using Agrobacterium-mediated transformation techniques. Nucleic acid molecules and methods for preparing the constructs and transforming maize were as previously described and known in the art and as described herein.

Expression of hpRNA specific for either ACS2 or ACS6 coding sequences resulted in maize plants that displayed no abnormalities in vegetative and reproductive growth. A total of 36 and 40 individual maize transgenic events were generated for ACS2- and ACS6-hairpin constructs, respectively.

Approximately 10 low-copy-number events per hpRNA construct were selected for additional backcrossing and transgene evaluation. Nitrogen utilization potential phenotype is evaluated for the backcrossed lines comprising the hpRNA transgene(s), e.g., as described herein (for example, by visual inspection, measurements of photosynthetic activity, determination of chlorophyll or protein content, grain yield, or the like, under normal conditions or under nitrogen-depleted, drought or other stress conditions).

Corn hybrids containing the inhibition constructs and nulls were planted in the field under nitrogen stress and normal nitrogen conditions. The planting density was 36,000 plants per acre and plants were fully irrigated. Under normal nitrogen conditions, 100 lbs nitrogen per acre was applied in the form of urea ammonium nitrate

(UAN) pre-plant, then another 150 lbs per acre UAN was applied as a sidedress at the V6 stage of development. Nitrogen stress was achieved through depletion of soil nitrogen reserves by planting corn with no added nitrogen for two years. After each season of depletion, corn grain and stover were removed to deplete organic matter sources of nitrogen through mineralization. Soil nitrate reserves were monitored to assess the level of depletion. To achieve the target level of stress, UAN was applied by fertigation between V2 and VT, for a total of 150 lbs nitrogen.

Transgenic events from the construct were nested together with the null to minimize the spatial effects of field variation. The grain yield of events containing the transgene was compared to the yield of a transgenic null. Statistical analysis was conducted to assess whether there is a significant improvement in yield compared with the transgenic null, taking into account row and column spatial effects.

- Treatments: Low nitrogen (LN) and Normal N (NN)
- Nested design, 6 reps in LN, 4 reps in NN

Table 1 below shows yield data (bushels per acre) of 7 events with the RNAi inhibition construct Ubi:ACS6 and wild type controls.

Table 1.

EVENT	Low Nitrogen	Normal Nitrogen
E5678.29.1.20	128	266*
E5678.29.1.22	162*	256*
E5678.29.1.26	148*	251*
E5678.29.1.3	150*	244
E5678.29.1.30	151*	236
E5678.29.1.32	160*	276*
E5678.29.1.33	125	265*
wild type	117	231
Lsd	14.9	20.0

* significantly different from wild type (P<0.1)

As can be seen, 5 out of 7 events showed superior yield to wild type in low nitrogen. Also interestingly, 5 out of 7 events also showed superior yield in normal nitrogen.

EXAMPLE 2: Improved ACC Synthase Inhibition by Hairpin RNA Expression

An improved hpRNA construct, the sequence of which is set forth in SEQ ID NO:53 (the expression cassette is set forth in SEQ ID NO:57 and depicted in Fig. 4), was generated by linking a ubiquitin promoter to a portion of the coding sequence of the ACS6 gene and its inverted repeat (SEQ ID NOs: 51 and 52), separated by an ADH1 intron.

Provided below is a general description of the improved hpRNA plasmid (SEQ ID NO:53):

DNA SEQ ID NO:53
UBI:ZM-ACS6 RNAi + UBI:MOPAT:PINII Co-integrate.
length: 51280 bp
storage type: Basic
form: Circular
Functional Map
CDS (10 signals)
MO-PAT
Start: 7109 End: 7660
SPC
Start: 9525 End: 10313 (Complementary)
SPECTINOMYCIN RESISTANCE
TET
Start: 14622 End: 15272 (Complementary)
tetracycline resistance
TET
Start: 15378 End: 16028 (Complementary)
tetracycline resistance
TRF A
Start: 17208 End: 19397 (Complementary)
CTL
Start: 24763 End: 31033 (Complementary)
VIR C1
Start: 34264 End: 34958

VIR C2

Start: 34961 End: 35569

VIR G

Start: 35680 End: 36483 (Complementary)

Agrobacterium virG (region approximated)

VIR B

Start: 36615 End: 46051 (Complementary)

Agrobacterium virB (region approximated)

Intron (3 signals)

UBI1ZM INTRON1 (PHI)

Start: 2196 End: 3208

ADH1 INTRON1 (PHI)

Start: 3791 End: 4327

Isolated from B73 at Pioneer (Notebook 4136.51)

UBI1ZM INTRON1 (PHI)

Start: 6060 End: 7072

Misc_feature (11 signals)

RB

Start: 1 End: 25

ALL STOPS

Start: 306 End: 339

A synthetic sequence of stop codons designed to stop all 6 open reading frames.

FRT5

Start: 452 End: 499

ALL STOPS

Start: 910 End: 943

A synthetic sequence of stop codons designed to stop all 6 open reading frames.

ATTB1

Start: 1155 End: 1175

ATTB2

Start: 4931 End: 4951 (Complementary)

FRT12

Start: 4998 End: 5045

FLP recombination target 12

FRT1

Start: 8004 End: 8051

PSB1

Start: 8052 End: 8146

A synthetic sequence designed to facilitate PCR analysis of recombined FRT sites.

ALL STOPS

Start: 8147 End: 8180

A synthetic sequence of stop codons designed to stop all 6 open reading frames.

LB

Start: 8325 End: 8350

tDNA Left border sequence from Japan Tobacco

Promoter_prokaryotic (2 signals)

UBI1ZM PRO

Start: 1218 End: 2113

Maize ubiquitin promoter

UBI1ZM PRO

Start: 5082 End: 5977

Maize ubiquitin promoter

Rep_origin (2 signals)

COLE1 ORI

Start: 11588 End: 11857 (Complementary)

ORI V

Start: 32041 End: 32751 (Complementary)

Terminator (2 signals)

IN2-1 (B) TERM

Start: 505 End: 902 (Complementary)

98bp deletion from 3'-end of the terminator. IN stands for INducible and 2-1 relates to an internal code used to designate the same

PINII TERM

Start: 7671 End: 7989

Potato PINII terminator

5'UTR (2 signals)

UBI1ZM 5UTR (PHI)

Start: 2114 End: 2195

UBI1ZM 5UTR (PHI)

Start: 5978 End: 6059

Misc_RNA (2 signals)

ZM-ACS6 (TR3)

Start: 3272 End: 3776 (Complementary)

Maize ACC synthase 6 (Aminocyclopropane carboxylate synthase)

Truncated fragment for gene silencing. PCR'd from genomic

ZM-ACS6 (TR4)

Start: 4332 End: 4874

Maize ACC synthase 6 (Aminocyclopropane carboxylate synthase)

Truncated fragment for gene silencing. PCR'd from genomic

Restriction Map

ApaLI: 14 sites G|TGCAC
CACGT|G

N1: 287

N2: 2517

N3: 3704

N4: 4391

N5: 6381

N6: 10036

N7: 10810

N8: 11376

N9: 11874

N10: 13564

N11: 45696

N12: 48291

N13: 48789

N14: 50471

AvaI: 37 sites C|YCGRG
GRGCY|C

N1: 1144

N2: 1909

N3: 3318

N4: 3666

N5: 4429

N6: 4777

N7: 4922

N8: 5072

N9: 5773

N10: 7116

N11: 10391

N12: 15995

N13: 16761

N14: 17741

N15: 20628

N16: 21650

N17: 22422

N18: 24908

N19: 28599

N20: 30815

N21: 31984

N22: 33215

N23: 33223

N24: 33231

N25: 34047

N26: 34545

N27: 35022

N28: 35445

N29: 35491

N30: 36128

N31: 36869

N32: 37358

N33: 37895

N34: 38948

N35: 41282

N36: 43949

N37: 51174

BamHI: 11 sites G|GATCC
CCTAG|G

N1: 3230

N2: 4329

N3: 4865

N4: 5066

N5: 7094

N6: 34434

N7: 35775

N8: 37634

N9: 38570

N10: 38770

N11: 43916

ClaI: 18 sites AT|CGAT
TAGC|TA

N1: 2445

N2: 2710

N3: 2935

N4: 6309

N5: 6574

N6: 6799

N7: 33943

N8: 34189

N9: 34622

N10: 36384

N11: 36728

N12: 36855

N13: 41483

N14: 42530

N15: 44527

N16: 46781

N17: 47943

N18: 48057

EcoRI: 11 sites G|AATTC
CTTAA|G

N1: 2609

N2: 3261

N3: 4834

N4: 4877

N5: 6473

N6: 13890

N7: 37680

N8: 40564

N9: 40976

N10: 42287

N11: 43685

HindIII: 8 sites A|AGCTT
TTCGA|A

N1: 649

N2: 1202

N3: 3864

N4: 33254

N5: 44317

N6: 45226

N7: 46347

N8: 47862

NcoI: 2 sites C|CATGG
GGTAC|C

N1: 17026

N2: 17554

PstI: 15 sites CTGCA|G
G|ACGTC

N1: 1218

N2: 3208

N3: 3777

N4: 5082

N5: 7072

N6: 12698

N7: 13142

N8: 33407

N9: 38321

N10: 41155

N11: 42206

N12: 42791

N13: 48045

N14: 49613

N15: 50049

SmaI: 10 sites CCC|GGG
GGG|CCC

N1: 1146

N2: 3320

N3: 3668

N4: 4431

N5: 4779

N6: 4924

N7: 5074

N8: 15997

N9: 16763

N10: 34049

Each construct was transformed into maize using Agrobacterium-mediated transformation techniques. Nucleic acid molecules and methods for preparing the constructs and transforming maize are as previously described and known in the art.

Transformed plants of the invention were evaluated for yield under four environments. Eight reps were grown under flowering stress in Environment 1, 6 reps were grown under grain fill stress in Environment 2, 6 reps were grown under grain fill stress in Environment 3, and 4 reps were grown under rain-fed conditions in Environment 4. Yields were compared with a highly repeated construct null (CN). The data are shown in Figures 5-8.

Figure 5 shows the yield of transformed plants of the invention under flowering stress in Environment 1. Each bar represents a separate transformation event. Average yield of transgene-negative segregants is shown (139 bu/a) as control (CN). A total of 74% of the events yielded nominally more than the control plants. Plants representing 18 transgenic events outyielded the control at $P<0.10$.

Figure 6 shows the yield of transformed plants of the invention under grain-fill stress in Environment 2. Each bar represents a separate transformation event. Average yield of transgene-negative segregants is shown (176 bu/a) as control (CN). Thirteen events out-yielded the CN at $P<0.10$. Of these, eight had also shown significant improvement under flowering stress.

Figure 7 shows the yield, as a percent of control, of transformed plants of the invention (indicated by a circle), as well as plants transformed using an alternative ACS6 inhibition vector (indicated by a square) under grain fill stress in Environment 3. Each data point represents a separate transformation event. NS = not significant. The control plants are bulked transgene-negative segregants. As can be seen, 64% of the events of the invention had significantly superior yield; only 17% of the alternative ACS6 inhibition events had significantly superior yield, relative to the control.

Figure 8 shows the yield, as a percent of control, of transformed plants of the invention (indicated by a circle), as well as plants transformed using an alternative ACS6 inhibition vector (indicated by a square) under rain-fed conditions in Environment 4. Each data point represents a separate transformation event. NS = not significant. The control plants are bulked transgene-negative segregants. As can be seen, all points exhibiting statistically significant increases in yield represent events of the invention disclosed herein. In addition, all points exhibiting statistically significant decreases in yield are events containing the alternative ACS6 inhibition vector.

Without being limited to any particular theory, it is speculated that the construct of the invention provides the documented improvement in yield by refining the modulation of ACS expression. For example, inclusion of the Adh1 intron within the ACS6 hairpin may result in ACS6 being downregulated to a lesser extent in plants of the invention than in plants transformed with the previous (alternative) ACS6 inhibition vector. Alternatively or additionally, the construct of the invention may impact expression of genes other than ACS6, for example ACS2.

Methods:

Protein Extraction

For total protein isolation, leaves of B73 or mutant plants are collected at the indicated times, quick-frozen in liquid nitrogen and ground to a fine powder. One ml of extraction buffer (20 mM HEPES (pH 7.6), 100 mM KCl, 10% Glycerol) is added to approximately 0.1 g frozen powder and mixed thoroughly. Samples are centrifuged 10 minutes at 10,000 rpm, the supernatant removed to a new tube and the concentration determined spectrophotometrically according to the methods of Bradford, (1976). See, Bradford, (1976) *Anal. Biochem.* 72:248-254.

Chlorophyll Extraction

Leaves are frozen in liquid nitrogen and ground to a fine powder. Samples of approximately 0.1 g are removed to a 1.5 ml tube and weighed. Chlorophyll is extracted 5x with 1 ml (or 0.8 ml) of 80% acetone. Individual extractions are combined and the final volume adjusted to 10 ml (or 15 ml) with additional 80% acetone. Chlorophyll content (a+b) is determined spectrophotometrically according to the methods of Wellburn, (1994). See, Wellburn, (1994) *J. Plant Physiol.* 144:307-313.

Measurement of Photosynthesis

Plants are grown in the field under normal and drought-stress conditions. Under normal conditions, plants are watered for eight hours twice a week. For drought-stressed plants, water is limited to approximately four hours per week for a period starting approximately one week before pollination and continuing through three weeks after pollination. During the period of limited water availability, drought-stressed plants may show visible signs of wilting and leaf rolling. Transpiration, stomatal conductance and CO_2 assimilation are determined with a portable TPS-1 Photosynthesis System (PP Systems). Each leaf on a plant is measured at forty days after pollination. Values typically represent a mean of six determinations.

DNA and RNA Purification

For total nucleic acid isolation, leaves of B73 are collected at desired times, quick-frozen in liquid nitrogen and ground to a fine powder. Ten ml of extraction buffer (100 mM Tris (pH 8.0), 50 mM EDTA, 200 mM NaCl, 1% SDS, 10 μ/ml β – mercaptoethanol) is added and mixed thoroughly until thawed. Ten ml of

Phenol/Chloroform (1:1, vol:vol) is added and mixed thoroughly. Samples are centrifuged 10 min at 8,000 rpm, the supernatant is removed to a new tube and the nucleic acid is precipitated at -20°C following addition of 1/10 vol 3M sodium acetate and 1 vol isopropanol. Total nucleic acid is pelleted by centrifugation at 8,000 rpm and resuspended in 1 ml TE. One half of the prep is used for DNA purification and the remaining half is used for RNA purification. (Alternatively, DNA or total nucleic acids can be extracted from 1 cm^2 of seedling leaf, quick-frozen in liquid nitrogen, and ground to a fine powder. 600 µl of extraction buffer [100 mM Tris (pH 8.0), 50 mM EDTA, 200 mM NaCl, 1% SDS, 10 µl/ml β –mercaptoethanol] is added and the sample mixed. The sample is extracted with 700 µl phenol/chloroform (1:1) and centrifuged for 10 minutes at 12,000 rpm. DNA is precipitated and resuspended in 600 µl H2O.)

For DNA purification, 500 µg Dnase-free Rnase is added to the tube and incubated at 37°C for 1 hr. Following Rnase digestion, an equal volume of Phenol/Chloroform (1:1, vol:vol) is added and mixed thoroughly. Samples are centrifuged 10 min at 10,000 rpm, the supernatant is removed to a new tube and the DNA precipitated at -20°C following addition of 1/10 vol 3M sodium acetate and 1 vol isopropanol. DNA is resuspended in sterile water and the concentration is determined spectrophotometrically. To determine DNA integrity, 20 mg of DNA is separated on a 1.8% agarose gel and visualized following staining with ethidium bromide. RNA is purified by 2 rounds of $LiCl_2$ precipitation according to methods described by Sambrook, *et al.*, *supra*.

Real-Time RT-PCR Analysis

Fifty µg total RNA is treated with RQ1.TM. Dnase (Promega) to ensure that no contaminating DNA is present. Two µg total RNA is used directly for cDNA synthesis using the Omniscript™ reverse transcription kit (Qiagen) with oligo-dT(20) as the primer.

Analysis of transcript abundance is accomplished using the QuantiTect™ SYBR Green PCR kit (Qiagen). Reactions contain 1.times. buffer, 0.5 µl of the reverse transcription reaction (equivalent to 50 ng total RNA) and 0.25 µM (final concentration) forward and reverse primers in a total reaction volume of 25 µl.

Reactions are carried out using an ABI PRISM 7700 sequence detection system under the following conditions: 95°C/15 minutes (1 cycle); 95C/30 sec, 62°C/30 sec,

72°C/2 minute (50 cycles); 72°C/5 minutes (1 cycle). Each gene is analyzed a minimum of four times.

All the primer combinations are initially run and visualized on an agarose gel to confirm the presence of a single product of the correct size. All amplification products are subcloned into the pGEM-T Easy vector system (Promega) to use for generation of standard curves to facilitate conversion of expression data to a copy/μg RNA basis.

Ethylene Determination

Ethylene is measured from the second fully-expanded leaf of seedlings at the 4-leaf stage or from the terminal 15 cm of leaves of plants 20, 30 or 40 days after pollination (DAP). Leaves are harvested at the indicated times and allowed to recover for 2 hr prior to collecting ethylene, between moist paper towels. Leaves are placed into glass vials and capped with a rubber septum. Following a 3- to 4-hour incubation, 0.9 mL of headspace is sampled from each vial and the ethylene content measured using a 6850 series gas chromatography system (Hewlett-Packard, Palo Alto, CA) equipped with a HP Plot alumina-based capillary column (Agilent Technologies, Palo Alto, CA). Tissue fresh weight is measured for each sample. Three replicates are typically measured and the average and standard deviation reported.

Western Blot Analysis

B73 leaves are collected at the indicated times and ground in liquid nitrogen to a fine powder. One ml of extraction buffer [20 mM HEPES (pH 7.6), 100 mM KCl, 10% glycerol, 1 mM PMSF] is added to approximately 0.1 g frozen powder and mixed thoroughly. Cell debris is pelleted by centrifugation at 10,000 rpm for 10 min and the protein concentration determined as described (Bradford, 1976). Antiserum raised against the large subunit of rice Rubisco is obtained from Dr. Tadahiko Mae (Tohoku University, Sendai, Japan). Protein extracts are resolved using standard SDS-PAGE and the protein transferred to 0.22 μm nitrocellulose membrane by electroblotting. Following transfer, the membranes are blocked in 5% milk, 0.01% thimerosal in TPBS (0.1% TWEEN® 20, 13.7 mM NaCl, 0.27 mM KCl, 1 mM Na2HPO4, 0.14 mM KH2PO4) followed by incubation with primary antibodies diluted typically 1:1000 to 1:2000 in TPBS with 1% milk for 1.5 hrs. The blots are then washed twice with TPBS and incubated with goat anti-rabbit horseradish peroxidase-conjugated antibodies (Southern Biotechnology Associates, Inc.) diluted to 1:5000 to 1:10,000 for 1 hr. The

blots are washed twice with TPBS and the signal detected typically between 1 to 15 min using chemiluminescence (Amersham Corp).

EXAMPLE 3. Yield of plants comprising improved ACS6 inhibition construct under reduced nitrogen

Plants comprising the improved ACS6 inhibition construct described in Example 2 were planted in the field under nitrogen-stress and normal-nitrogen conditions.

Nitrogen stress was achieved through targeted depletion of soil nitrogen reserves by previous corn production and/or limited application of nitrogen fertilitzer. In addition to cropping history, soil type and other environmental factors were taken into consideration in creating appropriate nitrogen-stress conditions.

The grain yield of plants containing the transgene was compared to the yield of a wild-type or transgenic null. The test used a randomized complete block design with six replications. Statistical analysis was conducted using ASReml to assess differences in yield, taking into account row and column spatial effects and autoregressive (AR1) adjustments.

Table 2 provides yield data in bushels/acre for plants representing 19 transformation events under nitrogen-stress conditions in two geographic locations. Yields marked with an asterisk are significantly greater than the control at $P<0.1$.

Table 2.

Event	**Location 1**	**Location 2**
2.12	121	202*
2.29	124*	199
2.32	123	211*
113.2.7	124*	206*
4.3	124*	204*
4.8	125*	203*
1.23	127*	208*
1.44	126*	207*
2.15	124*	205*
2.2	124	201
2.24	124*	198

2.38	125*	204*
2.49	123	202*
1.14	125*	210*
2.18	126*	206*
2.22	124*	208*
2.8	125*	205*
2.1	125*	206*
66.2.7	124*	202*
Control	120	197

Additional measurements were taken at Location 2, as follows. Average yield of the transgenic plants under normal-nitrogen conditions was 232 bushels per acre; under nitrogen-stress conditions, the average yield was 203 bushels per acre. Under nitrogen stress, growing-degree-units to pollen shed was 1273, compared to 1330 under normal-nitrogen conditions. In addition, plants grown in the nitrogen-stress environment showed a reduction in anthesis-silking interval (ASI) of 18. Barren count in the low-nitrogen environment was 1 on a 1 to 10 scale, where 10 is least favorable.

EXAMPLE 4. Low Nitrogen Seedling Assay Protocol

Seeds produced by transgenic plants are separated into transgene (heterozygous) and null seed using a seed color marker. Two different random assignments of treatments are made to each block of 54 pots, arranged as 6 rows of 9 columns and using 9 replicates of all treatments. In one case, null seed of 5 events of the same construct are mixed and used as control for comparison of the 5 positive events in this block, making up 6 treatment combinations in each block. In the second case, 3 transgenic positive treatments and their corresponding nulls are randomly assigned to the 54 pots of the block, making 6 treatment combinations for each block, containing 9 replicates of all treatment combinations. In the first case transgenic parameters are compared to a bulked construct null; in the second case, transgenic parameters are compared to the corresponding event null. In cases where there are 10, 15 or 20 events in a construct, the events are assigned in groups of 5 events, the variances calculated for

each block of 54 pots, but the block null means are pooled across blocks before mean comparisons are made.

Two seeds of each treatment are planted in 4-inch-square pots containing TURFACE® -MVP on 8-inch, staggered centers and watered four times each day with a solution containing the following nutrients:

1mM CaCl2	2mM MgSO4	0.5mM KH2PO4	
83ppm Sprint330			
3mM KCl	1mM KNO3	1uM ZnSO4	1uM MnCl2
3uM H3BO4	1uM MnCl2	0.1uM CuSO4	0.1uM NaMoO4

After emergence the plants are thinned to one seed per pot. Treatments routinely are planted on a Monday, emerge the following Friday and are harvested 18 days after planting. At harvest, plants are removed from the pots and the Turface® washed from the roots. The roots are separated from the shoot, placed in a paper bag and dried at 70°C for 70 hr. The dried plant parts (roots and shoots) are weighed and placed in a 50 ml conical tube with approximately 20 5/32 inch steel balls and ground by shaking in a paint shaker. Approximately, 30 mg of the ground tissue (weight recorded for later adjustment) is hydrolyzed in 2ml of 20% H_2O_2 and 6M H_2SO_4 for 30 min at 170°C. After cooling, water is added to 20 ml, mixed thoroughly, and a 50 µl aliquot removed and added to 950 µl 1M Na_2CO_3. The ammonia in this solution is used to estimate total reduced plant nitrogen by placing 100 µl of this solution in individual wells of a 96 well plate followed by adding 50 µl of OPA solution. Fluorescence, excitation = 360nM / emission = 530nM, is determined and compared to NH_4Cl standards dissolved in a similar solution and treated with OPA solution.

OPA solution - 5ul Mercaptoethanol + 1ml OPA stock solution (make fresh, daily) OPA stock - 50mg o-phthadialdehyde (OPA - Sigma #P0657) dissolved in 1.5ml methanol + 4.4ml 1M Borate buffer pH9.5 (3.09g H_3BO_4 + 1g NaOH in 50ml water) + 0.55ml 20% SDS (make fresh weekly)

Using these data the following parameters are measured and means are compared to null mean parameters using a Student's t test:

Total Plant Biomass
Root Biomass
Shoot Biomass
Root/Shoot Ratio
Plant N concentration
Total Plant N

Variance is calculated within each block using a nearest neighbor calculation as well as by Analysis of Variance (ANOV) using a completely random design (CRD) model. An overall treatment effect for each block was calculated using an F statistic by dividing overall block treatment mean square by the overall block error mean square.

EXAMPLE 5. Screening of Gaspe Bay Flint Derived Maize Lines Under Nitrogen Limiting Conditions

Transgenic plants will contain two or three doses of Gaspe Flint-3 with one dose of GS3 (GS3/(Gaspe-3)2X or GS3/(Gaspe-3)3X) and will segregate 1:1 for a dominant transgene. Plants will be planted in TURFACE®, a commercial potting medium and watered four times each day with 1 mM KNO_3 growth medium and with 2 mM KNO_3 or higher, growth medium. Control plants grown in 1 mM KNO_3 medium will be less green, produce less biomass and have a smaller ear at anthesis. Results are analyzed for statistical significance.

Expression of a transgene will result in plants with improved plant growth in 1 mM KNO_3 when compared to a transgenic null. Thus biomass and greenness will be monitored during growth and compared to a transgenic null. Improvements in growth, greenness and ear size at anthesis will be indications of increased nitrogen utilization efficiency.

EXAMPLE 6. Assays to Determine Alterations of Root Architecture in Maize

Transgenic maize plants are assayed for changes in root architecture at seedling stage, flowering time or maturity. Assays to measure alterations of root architecture of maize plants include, but are not limited to the methods outlined below. To facilitate manual or automated assays of root architecture alterations, corn plants can be grown in clear pots.

1) Root mass (dry weights). Plants are grown in Turface®, a growth medium that allows easy separation of roots. Oven-dried shoot and root tissues are weighed and a root/shoot ratio calculated.

2) Levels of lateral root branching. The extent of lateral root branching (e.g., lateral root number, lateral root length) is determined by sub-sampling a complete root system, imaging with a flat-bed scanner or a digital camera and analyzing with WinRHIZO™ software (Regent Instruments Inc.).

3) Root band width measurements. The root band is the band or mass of roots that forms at the bottom of greenhouse pots as the plants mature. The thickness of the root band is measured in mm at maturity as a rough estimate of root mass.

4) Nodal root count. The number of crown roots coming off the upper nodes can be determined after separating the root from the support medium (e.g., potting mix). In addition the angle of crown roots and/or brace roots can be measured. Digital analysis of the nodal roots and amount of branching of nodal roots form another extension to the aforementioned manual method.

All data taken on root phenotype are subjected to statistical analysis, normally a t-test to compare the transgenic roots with those of non-transgenic sibling plants. One-way ANOVA may also be used in cases where multiple events and/or constructs are involved in the analysis.

EXAMPLE 7: NUE assay of plant growth

Seeds of Arabidopsis thaliana (control and transgenic line), ecotype Columbia, are surface sterilized (Sánchez, *et al.*, 2002) and then plated on to Murashige and Skoog (MS) medium containing 0.8% (w/v) Bacto™-Agar (Difco). Plates are incubated for 3 days in darkness at 4°C to break dormancy (stratification) and transferred thereafter to growth chambers (Conviron, Manitoba, Canada) at a temperature of 20°C under a 16-h light/8-h dark cycle. The average light intensity is 120 μE/m2/s. Seedling are grown for 12 days and then transferred to soil based pots. Potted plants are grown on a nutrient-free soil LB2 Metro-Mix® 200 (Scott's Sierra Horticultural Products, Marysville, OH, USA) in individual 1.5-in pots (Arabidopsis system; Lehle Seeds,

Round Rock, TX, USA) in growth chambers, as described above. Plants are watered with 0.6 or 6.5 mM potassium nitrate in the nutrient solution based on Murashige and Skoog (MS free Nitrogen) medium. The relative humidity is maintained around 70%. 16-18 days later plant shoots are collected for evaluation of biomass and SPAD readings.

EXAMPLE 8. *Agrobacterium* mediated transformation into maize

Maize plants can be transformed to overexpress a nucleic acid sequence of interest in order to examine the resulting phenotype.

Agrobacterium-mediated transformation of maize is performed essentially as described by Zhao, *et al.*, (2006) *Meth. Mol. Biol.* 318:315-323 (see also, Zhao, *et al.*, (2001) *Mol. Breed.* 8:323-333 and US Patent Number 5,981,840 issued November 9, 1999, incorporated herein by reference). The transformation process involves bacterium inoculation, co-cultivation, resting, selection and plant regeneration.

1. Immature Embryo Preparation

Immature embryos are dissected from caryopses and placed in a 2 mL microtube containing 2 mL PHI-A medium.

2. Agrobacterium Infection and Co-Cultivation of Embryos

2.1 Infection Step

PHI-A medium is removed with 1 mL micropipettor and 1 mL *Agrobacterium* suspension is added. Tube is gently inverted to mix. The mixture is incubated for 5 min at room temperature.

2.2 Co-Culture Step

The *Agrobacterium* suspension is removed from the infection step with a 1 mL micropipettor. Using a sterile spatula the embryos are scraped from the tube and transferred to a plate of PHI-B medium in a 100x15 mm Petri dish. The embryos are oriented with the embryonic axis down on the surface of the medium. Plates with the embryos are cultured at 20°C, in darkness, for 3 days. L-Cysteine can be used in the co-cultivation phase. With the standard binary vector, the co-cultivation medium supplied with 100-400 mg/L L-cysteine is critical for recovering stable transgenic events.

3. Selection of Putative Transgenic Events

To each plate of PHI-D medium in a 100x15 mm Petri dish, 10 embryos are transferred, maintaining orientation, and the dishes are sealed with Parafilm®. The plates are incubated in darkness at 28°C. Actively growing putative events, as pale yellow embryonic tissue are expected to be visible in 6-8 weeks. Embryos that produce no events may be brown and necrotic, and little friable tissue growth is evident. Putative transgenic embryonic tissue is subcultured to fresh PHI-D plates at 2-3 week intervals, depending on growth rate. The events are recorded.

4. Regeneration of T0 plants

Embryonic tissue propagated on PHI-D medium is subcultured to PHI-E medium (somatic embryo maturation medium); in 100x25 mm Petri dishes and incubated at 28°C, in darkness, until somatic embryos mature, for about 10-18 days. Individual, matured somatic embryos with well-defined scutellum and coleoptile are transferred to PHI-F embryo germination medium and incubated at 28°C in the light (about 80 µE from cool white or equivalent fluorescent lamps). In 7-10 days, regenerated plants, about 10 cm tall, are potted in horticultural mix and hardened-off using standard horticultural methods.

<u>Media for Plant Transformation</u>

1. PHI-A: 4g/L CHU basal salts, 1.0 mL/L 1000X Eriksson's vitamin mix, 0.5mg/L thiamin HCL, 1.5 mg/L 2,4-D, 0.69 g/L L-proline, 68.5 g/L sucrose, 36g/L glucose, pH 5.2. Add 100µM acetosyringone, filter-sterilized before using.
2. PHI-B: PHI-A without glucose, increased 2,4-D to 2mg/L, reduced sucrose to 30 g/L and supplemented with 0.85 mg/L silver nitrate (filter-sterilized), 3.0 g/L Gelrite®, 100µM acetosyringone (filter-sterilized), pH 5.8.
3. PHI-C: PHI-B without Gelrite® and acetosyringone, reduced 2,4-D to 1.5 mg/L and supplemented with 8.0 g/L agar, 0.5 g/L Ms-morpholino ethane sulfonic acid (MES) buffer, 100mg/L carbenicillin (filter-sterilized).
4. PHI-D: PHI-C supplemented with 3mg/L bialaphos (filter-sterilized).

5. PHI-E: 4.3 g/L of Murashige and Skoog (MS) salts, (Gibco, BRL 11117-074), 0.5 mg/L nicotinic acid, 0.1 mg/L thiamine HCl, 0.5mg/L pyridoxine HCl, 2.0 mg/L glycine, 0.1 g/L myo-inositol, 0.5 mg/L zeatin (Sigma, cat.no. Z-0164), 1 mg/L indole acetic acid (IAA), 26.4 μg/L abscisic acid (ABA), 60 g/L sucrose, 3 mg/L bialaphos (filter-sterilized), 100 mg/L carbenicillin (fileter-sterilized), 8g/L agar, pH 5.6.
6. PHI-F: PHI-E without zeatin, IAA, ABA; sucrose reduced to 40 g/L; replacing agar with 1.5 g/L Gelrite®; pH 5.6.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm, *et al.*, (1990) *Bio/Technology* 8:833-839).

Phenotypic analysis of transgenic T0 plants and T1 plants can be performed. T1 plants can be analyzed for phenotypic changes. Using image analysis T1 plants can be analyzed for phenotypical changes in plant area, volume, growth rate and color analysis at multiple times during growth of the plants. Alteration in root architecture can be assayed as described herein.

Subsequent analysis of alterations in agronomic characteristics can be done to determine whether plants containing the nucleic acid sequence of interest have an improvement of at least one agronomic characteristic, when compared to the control (or reference) plants that have not been so transformed. The alterations may also be studied under various environmental conditions.

Expression constructs containing the nucleic acid sequence of interest that result in a significant alteration in root and/or shoot biomass, improved green color, larger ear at anthesis or yield will be considered evidence that the nucleic acid sequence of interest functions in maize to alter nitrogen use efficiency.

EXAMPLE 9. Electroporation of *Agrobacterium tumefaciens* LBA4404

Electroporation competent cells (40 μl), such as *Agrobacterium tumefaciens* LBA4404 (containing PHP10523), are thawed on ice (20-30 min). PHP10523 contains VIR genes for T-DNA transfer, an *Agrobacterium* low copy number plasmid origin of replication, a tetracycline resistance gene and a cos site for in vivo DNA biomolecular

recombination. Meanwhile the electroporation cuvette is chilled on ice. The electroporator settings are adjusted to 2.1 kV.

A DNA aliquot (0.5 µL JT (US 7,087,812) parental DNA at a concentration of 0.2 µg -1.0 µg in low salt buffer or twice distilled H_2O) is mixed with the thawed *Agrobacterium* cells while still on ice. The mix is transferred to the bottom of electroporation cuvette and kept at rest on ice for 1-2 min. The cells are electroporated (Eppendorf electroporator 2510) by pushing "Pulse" button twice (ideally achieving a 4.0 msec pulse). Subsequently 0.5 ml 2xYT medium (or SOCmedium) are added to cuvette and transferred to a 15 ml Falcon tube. The cells are incubated at 28-30°C, 200-250 rpm for 3 h.

Aliquots of 250 µl are spread onto #30B (YM + 50µg/mL Spectinomycin) plates and incubated 3 days at 28-30°C. To increase the number of transformants one of two optional steps can be performed:

Option 1: overlay plates with 30 µl of 15 mg/ml Rifampicin. LBA4404 has a chromosomal resistance gene for Rifampicin. This additional selection eliminates some contaminating colonies observed when using poorer preparations of LBA4404 competent cells.

Option 2: Perform two replicates of the electroporation to compensate for poorer electrocompetent cells.

Identification of transformants:

Four independent colonies are picked and streaked on AB minimal medium plus 50mg/mL Spectinomycin plates (#12S medium) for isolation of single colonies. The plates are incubated at 28°C for 2-3 days.

A single colony for each putative co-integrate is picked and inoculated with 4 ml #60A with 50 mg/l Spectinomycin. The mix is incubated for 24 h at 28°C with shaking. Plasmid DNA from 4 ml of culture is isolated using Qiagen Miniprep + optional PB wash. The DNA is eluted in 30 µl. Aliquots of 2 µl are used to electroporate 20 µl of DH10b + 20 µl of dd H_2O as per above.

Optionally a 15 µl aliquot can be used to transform 75-100 µl of Invitrogen™ Library Efficiency DH5α. The cells are spread on LB medium plus 50mg/mL Spectinomycin plates (#34T medium) and incubated at 37°C overnight.

Three to four independent colonies are picked for each putative co-integrate and inoculated 4 ml of 2xYT (#60A) with 50 μg/ml Spectinomycin. The cells are incubated at 37°C overnight with shaking.

The plasmid DNA is isolated from 4 ml of culture using QIAprep® Miniprep with optional PB wash (elute in 50 μl) and 8 μl are used for digestion with SalI (using JT parent and PHP10523 as controls).

Three more digestions using restriction enzymes BamHI, EcoRI and HindIII are performed for 4 plasmids that represent 2 putative co-integrates with correct SalI digestion pattern (using parental DNA and PHP10523 as controls). Electronic gels are recommended for comparison.

EXAMPLE 10. Particle-mediated bombardment for Transformation of Maize

A vector can be transformed into embryogenic maize callus by particle bombardment, generally as described by Tomes, *et al.*, Plant Cell, Tissue and Organ Culture: Fundamental Methods, Eds. Gamborg and Phillips, Chapter 8, pgs. 197-213 (1995) and as briefly outlined below. Transgenic maize plants can be produced by bombardment of embryogenically responsive immature embryos with tungsten particles associated with DNA plasmids. The plasmids typically comprise or consist of a selectable marker and an unselected structural gene, or a selectable marker and an ACC synthase polynucleotide sequence or subsequence, or the like.

Preparation of Particles

Fifteen mg of tungsten particles (General Electric), 0.5 to 1.8μ, preferably 1 to 1.8μ, and most preferably 1μ, are added to 2 ml of concentrated nitric acid. This suspension is sonicated at 0°C. for 20 minutes (Branson Sonifier Model 450, 40% output, constant duty cycle). Tungsten particles are pelleted by centrifugation at 10000 rpm (Biofuge) for one minute and the supernatant is removed. Two milliliters of sterile distilled water are added to the pellet, and brief sonication is used to resuspend the particles. The suspension is pelleted, one milliliter of absolute ethanol is added to the pellet and brief sonication is used to resuspend the particles. Rinsing, pelleting and resuspending of the particles are performed two more times with sterile distilled water and finally the particles are resuspended in two milliliters of sterile distilled water. The particles are subdivided into 250-μl aliquots and stored frozen.

Preparation of Particle-Plasmid DNA Association

The stock of tungsten particles are sonicated briefly in a water bath sonicator (Branson Sonifier Model 450, 20% output, constant duty cycle) and 50 µl is transferred to a microfuge tube. The vectors are typically cis: that is, the selectable marker and the gene (or other polynucleotide sequence) of interest are on the same plasmid.

Plasmid DNA is added to the particles for a final DNA amount of 0.1 to 10 µg in 10 µL total volume and briefly sonicated. Preferably, 10 µg (1 µg/µL in TE buffer) total DNA is used to mix DNA and particles for bombardment. Fifty microliters (50 µL) of sterile aqueous 2.5 M $CaCl_2$ are added and the mixture is briefly sonicated and vortexed. Twenty microliters (20 µL) of sterile aqueous 0.1 M spermidine are added and the mixture is briefly sonicated and vortexed. The mixture is incubated at room temperature for 20 minutes with intermittent brief sonication. The particle suspension is centrifuged and the supernatant is removed. Two hundred fifty microliters (250 µL) of absolute ethanol are added to the pellet, followed by brief sonication. The suspension is pelleted, the supernatant is removed and 60 µl of absolute ethanol are added. The suspension is sonicated briefly before loading the particle-DNA agglomeration onto macrocarriers.

Preparation of Tissue

Immature embryos of maize are the target for particle bombardment-mediated transformation. Ears from F1 plants are selfed or sibbed and embryos are aseptically dissected from developing caryopses when the scutellum first becomes opaque. This stage occurs about 9 13 days post-pollination and most generally about 10 days post-pollination, depending on growth conditions. The embryos are about 0.75 to 1.5 millimeters long. Ears are surface sterilized with 20 50% Clorox® for 30 minutes, followed by three rinses with sterile distilled water.

Immature embryos are cultured with the scutellum oriented upward, on embryogenic induction medium comprised of N6 basal salts, Eriksson vitamins, 0.5 mg/l thiamine HCl, 30 gm/l sucrose, 2.88 gm/l L-proline, 1 mg/l 2,4-dichlorophenoxyacetic acid, 2 gm/l Gelrite® and 8.5 mg/l $AgNO_3$. Chu, *et al.*, (1975) *Sci. Sin.* 18:659; Eriksson, (1965) *Physiol. Plant* 18:976. The medium is sterilized by autoclaving at 121°C. for 15 minutes and dispensed into 100x25 mm Petri dishes. $AgNO_3$ is filter-sterilized and added to the medium after autoclaving. The tissues are cultured in complete darkness at 28°C. After about 3 to 7 days, most usually about 4

days, the scutellum of the embryo swells to about double its original size and the protuberances at the coleorhizal surface of the scutellum indicate the inception of embryogenic tissue. Up to 100% of the embryos display this response, but most commonly, the embryogenic response frequency is about 80%.

When the embryogenic response is observed, the embryos are transferred to a medium comprised of induction medium modified to contain 120 gm/l sucrose. The embryos are oriented with the coleorhizal pole, the embryogenically responsive tissue, upwards from the culture medium. Ten embryos per Petri dish are located in the center of a Petri dish in an area about 2 cm in diameter. The embryos are maintained on this medium for 3 to 16 hours, preferably 4 hours, in complete darkness at 28°C just prior to bombardment with particles associated with plasmid DNAs containing the selectable and unselectable marker genes.

To effect particle bombardment of embryos, the particle-DNA agglomerates are accelerated using a DuPont PDS-1000 particle acceleration device. The particle-DNA agglomeration is briefly sonicated and 10 μl are deposited on macrocarriers and the ethanol is allowed to evaporate. The macrocarrier is accelerated onto a stainless-steel stopping screen by the rupture of a polymer diaphragm (rupture disk). Rupture is effected by pressurized helium. The velocity of particle-DNA acceleration is determined based on the rupture disk breaking pressure. Rupture disk pressures of 200 to 1800 psi are used, with 650 to 1100 psi being preferred and about 900 psi being most highly preferred. Multiple disks are used to effect a range of rupture pressures.

The shelf containing the plate with embryos is placed 5.1 cm below the bottom of the macrocarrier platform (shelf #3). To effect particle bombardment of cultured immature embryos, a rupture disk and a macrocarrier with dried particle-DNA agglomerates are installed in the device. The He pressure delivered to the device is adjusted to 200 psi above the rupture disk breaking pressure. A Petri dish with the target embryos is placed into the vacuum chamber and located in the projected path of accelerated particles. A vacuum is created in the chamber, preferably about 28 in Hg. After operation of the device, the vacuum is released and the Petri dish is removed.

Bombarded embryos remain on the osmotically-adjusted medium during bombardment, and 1 to 4 days subsequently. The embryos are transferred to selection medium comprised of N6 basal salts, Eriksson vitamins, 0.5 mg/l thiamine HCl, 30 gm/l sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid, 2 gm/l Gelrite®, 0.85 mg/l Ag NO_3 and 3 mg/l bialaphos (Herbiace, Meiji). Bialaphos is added filter-sterilized. The

embryos are subcultured to fresh selection medium at 10 to 14 day intervals. After about 7 weeks, embryogenic tissue, putatively transformed for both selectable and unselected marker genes, proliferates from a fraction of the bombarded embryos. Putative transgenic tissue is rescued and that tissue derived from individual embryos is considered to be an event and is propagated independently on selection medium. Two cycles of clonal propagation are achieved by visual selection for the smallest contiguous fragments of organized embryogenic tissue.

A sample of tissue from each event is processed to recover DNA. The DNA is restricted with a restriction endonuclease and probed with primer sequences designed to amplify DNA sequences overlapping the ACC synthase and non-ACC synthase portion of the plasmid. Embryogenic tissue with amplifiable sequence is advanced to plant regeneration.

For regeneration of transgenic plants, embryogenic tissue is subcultured to a medium comprising MS salts and vitamins (Murashige and Skoog, (1962) *Physiol. Plant* 15:473), 100 mg/l myo-inositol, 60 gm/l sucrose, 3 gm/l Gelrite®, 0.5 mg/l zeatin, 1 mg/l indole-3-acetic acid, 26.4 ng/l cis-trans-abscissic acid and 3 mg/l bialaphos in 100X25 mm Petri dishes and is incubated in darkness at 28°C until the development of well-formed, matured somatic embryos is seen. This requires about 14 days. Well-formed somatic embryos are opaque and cream-colored and are comprised of an identifiable scutellum and coleoptile. The embryos are individually subcultured to a germination medium comprising MS salts and vitamins, 100 mg/l myo-inositol, 40 gm/l sucrose and 1.5 gm/l Gelrite® in 100x25 mm Petri dishes and incubated under a 16 hour light:8 hour dark photoperiod and 40 meinsteinsm^{-2}sec^{-1} from cool-white fluorescent tubes. After about 7 days, the somatic embryos germinate and produce a well-defined shoot and root. The individual plants are subcultured to germination medium in 125x25 mm glass tubes to allow further plant development. The plants are maintained under a 16 hour light:8 hour dark photoperiod and 40 meinsteinsm^{-2}sec-1 from cool-white fluorescent tubes. After about 7 days, the plants are well-established and are transplanted to horticultural soil, hardened off and potted into commercial greenhouse soil mixture and grown to sexual maturity in a greenhouse. An elite inbred line is used as a male to pollinate regenerated transgenic plants.

EXAMPLE 11: Soybean embryo transformation

Soybean embryos are bombarded with a plasmid comprising a preferred promoter operably linked to a heterologous nucleotide sequence comprising an ACC synthase polynucleotide sequence or subsequence (e.g., SEQ ID NOS: 1 and 2), as follows. To induce somatic embryos, cotyledons of 3 5 mm in length are dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, then cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiply as early, globular-staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can be maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C with fluorescent lights on a 16:8 hour day/night schedule. Cultures are sub-cultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein, *et al.*, (1987) *Nature (London)* 327:70-73, US Patent Number 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell, *et al.*, (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from E. coli; Gritz, *et al.*, (1983) *Gene* 25:179-188) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of Agrobacterium tumefaciens. The expression cassette of interest, comprising the preferred promoter and a heterologous ACC synthase polynucleotide, can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µl of a 60 mg/ml 1 µm gold particle suspension is added (in order): 5 µl DNA (1 µg/µl), 20 µl spermidine (0.1 M) and 50 µl $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µl 70% ethanol and resuspended in 40 µl of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300 400 mg of a two-week-old suspension culture is placed in an empty 60X5 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media and eleven to twelve days post-bombardment with fresh media containing 50 mg/ml hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

EXAMPLE 12. Composition of cDNA Libraries; Isolation and Sequencing of cDNA Clones

cDNA libraries representing mRNAs from various tissues of *Canna edulis* (Canna), *Momordica charantia* (balsam pear), *Brassica* (mustard), *Cyamopsis tetragonoloba (*guar), *Zea mays* (maize), *Oryza sativa* (rice), *Glycine max* (soybean), *Helianthus annuus* (sunflower) and *Triticum aestivum* (wheat) are prepared. cDNA libraries may be prepared by any one of many methods available. For example, the cDNAs may be introduced into plasmid vectors by first preparing the cDNA libraries in Uni-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA).

Full-insert sequence (FIS) data is generated utilizing a modified transposition protocol. Clones identified for FIS are recovered from archived glycerol stocks as single colonies, and plasmid DNAs are isolated via alkaline lysis. Isolated DNA templates are reacted with vector primed M13 forward and reverse oligonucleotides in

a PCR-based sequencing reaction and loaded onto automated sequencers. Confirmation of clone identification is performed by sequence alignment to the original EST sequence from which the FIS request is made.

Confirmed templates are transposed via the Primer Island transposition kit (PE Applied Biosystems, Foster City, CA) which is based upon the *Saccharomyces cerevisiae* Ty1 transposable element (Devine and Boeke, (1994) *Nucleic Acids Res.* 22:3765-3772). The *in vitro* transposition system places unique binding sites randomly throughout a population of large DNA molecules. Multiple subclones are randomly selected from each transposition reaction, plasmid DNAs are prepared via alkaline lysis and templates are sequenced (ABI Prism dye-terminator ReadyReaction mix) outward from the transposition event site, utilizing unique primers specific to the binding sites within the transposon.

Sequence data is collected (ABI Prism Collections) and assembled using Phred and Phrap (Ewing, *et al.*, (1998) *Genome Res.* 8:175-185; Ewing and Green, (1998) *Genome Res.* 8:186-194). The resulting DNA fragment is ligated into a pBluescript vector using a commercial kit and following the manufacturer's protocol. This kit is selected from many available from several vendors including Invitrogen™ (Carlsbad, CA), Promega Biotech (Madison, WI) and Gibco-BRL (Gaithersburg, MD). The plasmid DNA is isolated by alkaline lysis method and submitted for sequencing and assembly using Phred/Phrap, as above.

EXAMPLE 13. Identification of cDNA Clones

cDNA clones encoding ACC synthase-like polypeptides were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul, *et al.*, (1993) *J. Mol. Biol.* 215:403-410; see also, the explanation of the BLAST algorithm on the world wide web site for the National Center for Biotechnology Information at the National Library of Medicine of the National Institutes of Health) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL and DDBJ databases). The cDNA sequences obtained as described in Example 11 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were

translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish and States, (1993) *Nat. Genet. 3*:266-272) provided by the NCBI. For convenience, the P-values (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

ESTs submitted for analysis are compared to the Genbank database as described above. ESTs that contain sequences more 5- or 3-prime can be found by using the BLASTn algorithm (Altschul, *et al.*, (1997) *Nucleic Acids Res.* 25:3389-3402.) against the Du Pont proprietary database comparing nucleotide sequences that share common or overlapping regions of sequence homology. Where common or overlapping sequences exist between two or more nucleic acid fragments, the sequences can be assembled into a single contiguous nucleotide sequence, thus extending the original fragment in either the 5- or 3-prime direction. Once the most 5-prime EST is identified, its complete sequence can be determined by Full Insert Sequencing. Homologous genes belonging to different species can be found by comparing the amino acid sequence of a known gene (from either a proprietary source or a public database) against an EST database using the tBLASTn algorithm. The tBLASTn algorithm searches an amino acid query against a nucleotide database that is translated in all 6 reading frames. This search allows for differences in nucleotide codon usage between different species and for codon degeneracy.

EXAMPLE 14. Preparation of a Plant Expression Vector

A PCR product obtained using methods that are known by one skilled in the art can be combined with the Gateway® donor vector, such as pDONR™/Zeo (Invitrogen™). Using the Invitrogen™ Gateway® Clonase™ technology, the homologous gene can then be transferred to a suitable destination vector to obtain a plant expression vector for use with *Arabidopsis* and corn.

EXAMPLE 15. Variants of ACC synthase Sequences

A. Variant Nucleotide Sequences of ACC synthase Proteins That Do Not Alter the Encoded Amino Acid Sequence

The ACC synthase nucleotide sequences are used to generate variant nucleotide sequences having the nucleotide sequence of the open reading frame with about 70%, 75%, 80%, 85%, 90% and 95% nucleotide sequence identity when compared to the starting unaltered ORF nucleotide sequence of the corresponding SEQ ID NO. These functional variants are generated using a standard codon table. While the nucleotide sequences of the variants are altered, the amino acid sequence encoded by the open reading frames does not change.

B. Variant Amino Acid Sequences of ACC synthase Polypeptides

Variant amino acid sequences of the ACC synthase polypeptides are generated. In this example, one amino acid is altered. Specifically, the open reading frames are reviewed to determine the appropriate amino acid alteration. The selection of the amino acid to change is made by consulting the protein alignment (with the other orthologs and other gene family members from various species). An amino acid is selected that is deemed not to be under high selection pressure (not highly conserved) and which is rather easily substituted by an amino acid with similar chemical characteristics (i.e., similar functional side-chain). Using the protein alignment, an appropriate amino acid can be changed. Once the targeted amino acid is identified, the procedure outlined in the following section C is followed. Variants having about 70%, 75%, 80%, 85%, 90% and 95% nucleic acid sequence identity are generated using this method.

C. Additional Variant Amino Acid Sequences of ACC synthase Polypeptides

In this example, artificial protein sequences are created having 80%, 85%, 90% and 95% identity relative to the reference protein sequence. This latter effort requires identifying conserved and variable regions from the alignment and then the judicious application of an amino acid substitutions table. These parts will be discussed in more detail below.

Largely, the determination of which amino acid sequences are altered is made based on the conserved regions among ACC synthase protein or among the other ACC synthase polypeptides. It is recognized that conservative substitutions can be made in

the conserved regions below without altering function. In addition, one of skill will understand that functional variants of the ACC synthase sequence of the invention can have minor non-conserved amino acid alterations in the conserved domain.

Artificial protein sequences are then created that are different from the original in the intervals of 80-85%, 85-90%, 90-95% and 95-100% identity. Midpoints of these intervals are targeted, with liberal latitude of plus or minus 1%, for example. The amino acids substitutions will be effected by a custom Perl script. The substitution table is provided below in Table 3.

Table 3. Substitution Table

Amino Acid	**Strongly Similar and Optimal Substitution**	**Rank of Order to Change**	**Comment**
I	L,V	1	50:50 substitution
L	I,V	2	50:50 substitution
V	I,L	3	50:50 substitution
A	G	4	
G	A	5	
D	E	6	
E	D	7	
W	Y	8	
Y	W	9	
S	T	10	
T	S	11	
K	R	12	
R	K	13	
N	Q	14	
Q	N	15	
F	Y	16	
M	L	17	First methionine cannot change
H		Na	No good substitutes

C		Na	No good substitutes
P		Na	No good substitutes

First, any conserved amino acids in the protein that should not be changed is identified and "marked off" for insulation from the substitution. The start methionine will of course be added to this list automatically. Next, the changes are made.

H, C and P are not changed in any circumstance. The changes will occur with isoleucine first, sweeping N-terminal to C-terminal. Then leucine, and so on down the list until the desired target it reached. Interim number substitutions can be made so as not to cause reversal of changes. The list is ordered 1-17, so start with as many isoleucine changes as needed before leucine, and so on down to methionine. Clearly many amino acids will in this manner not need to be changed. L, I and V will involve a 50:50 substitution of the two alternate optimal substitutions.

The variant amino acid sequences are written as output. Perl script is used to calculate the percent identities. Using this procedure, variants of the ACC synthase polypeptides are generating having about 80%, 85%, 90% and 95% amino acid identity to the starting unaltered ORF nucleotide sequence of SEQ ID NO: 1, 2 or 3.

EXAMPLE 16. ACS sequences from Genbank

The following are examples of publicly available ACS genes from Genbank which may be used for various crop species according to the invention.

Table 4.

Crop	**Genbank Accession Number**	**SEQ ID NO**
Arabidopsis	NM_116016-ACS1	16
Arabidopsis	NM_100030-ACS2	17
Arabidopsis	NM_179241-ACS2	18
Arabidopsis	AF334719-ACS2	19
Arabidopsis	NM_122719-ACS-3	20
Arabidopsis	NM_127846-ACS4	21
Arabidopsis	AF332404-ACS4	22
Arabidopsis	AK229087-ACS5	23
Arabidopsis	AF334720-ACS5	24
Arabidopsis	NM_125977-ACS5	25

Arabidopsis	NM_117199-ACS6	26
Arabidopsis	NM_118753-ACS7	27
Arabidopsis	NM_119939-ACS8	28
Arabidopsis	AF334712-ACS8	29
Arabidopsis	AF332391-ACS9	30
Arabidopsis	NM_104974-ACS10	31
Arabidopsis	NM_116873-ACS11	32
Oryza sativa	Z27244-ACC synthase	33
Oryza sativa	Z27243-ACC synthase	34
Oryza sativa	Z27242-ACC synthase	35
Oryza sativa	Z27241-ACC synthase	36
Oryza sativa	U65704-ACS5	37
Oryza sativa	U65703-ACS4	38
Oryza sativa	U65702-ACS3	39
Oryza sativa	U65701-ACS2	40
Oryza sativa	M96673-(ACC1 synthase)	41
Oryza sativa	M96672-(ACC1 synthase)	42
Glycine max	EU604829-ACS	43
Glycine max	X67100-ACC synthase	44
Glycine max	DQ273841-ACS	45
Glycine max	DQ273840-ACS	46
Potato	Z27235-ACS2	47
Potato	Z27234-ACS	48
Potato	L20634-ACS	49
Potato	U70842-ACS	50

Table 5. Sequence Listing Summary

SEQ ID NO	NT or PP	DESCRIPTION
1	nucleotide	maize ACS 2 (genomic)
2	nucleotide	maize ACS 6 (genomic)
3	nucleotide	maize ACS 7 (genomic)
4	nucleotide	maize ACS 2(cDNA)
5	nucleotide	maize ACS 6(cDNA)
6	nucleotide	maize ACS 7(cDNA)
7	polypeptide	maize ACS 2
8	polypeptide	maize ACS 6
9	polypeptide	maize ACS 7
10	nucleotide	maize ACC
11	polypeptide	maize ACC
12	nucleotide	ACS 2 RNAi hairpin TR1
13	nucleotide	ACS 2 RNAi hairpin TR2
14	nucleotide	ACS 6 RNAi hairpin TR1
15	nucleotide	ACS 6 RNAi hairpin TR2
16	nucleotide	NM_116016-ACS1
17	nucleotide	NM_100030ACS2
18	nucleotide	NM179241-ACS2
19	nucleotide	AF334719ACS2
20	nucleotide	NM_122719ACS3
21	nucleotide	NM_127846-ACS4
22	nucleotide	AF332404-ACS4
23	nucleotide	AK229087ACS5
24	nucleotide	AF334720ACS5
25	nucleotide	NM_125977-ACS5
26	nucleotide	NM_117199-ACS6
27	nucleotide	NM_118753ACS7
28	nucleotide	NM_119939-ACS8

29	nucleotide	AF334712-ACS8
30	nucleotide	AF332391-ACS9
31	nucleotide	NM_104974-ACS10
32	nucleotide	NM_116873ACS11
33	nucleotide	Z27244 ACC synthase
34	nucleotide	Z27243 ACC synthase
35	nucleotide	Z27242 ACC synthase
36	nucleotide	Z27241-ACC synthase
37	nucleotide	U65704-ACS5
38	nucleotide	U65703ACS4
39	nucleotide	U65702ACS3
40	nucleotide	U65701ACS2
41	nucleotide	M96673 (ACC1synthase)
42	nucleotide	M96672 (ACC1synthase)
43	nucleotide	EU604829-ACS
44	nucleotide	X67100-ACC synthase
45	nucleotide	DQ273841-ACS
46	nucleotide	DQ273840-ACS
47	nucleotide	Z27235-ACS2
48	nucleotide	Z27234-ACS
49	nucleotide	L20634-ACS
50	nucleotide	U70842-ACS
51	nucleotide	improved ACS 6 RNAi hairpin TR3 (3272-3776 of SEQ ID NO:54)
52	nucleotide	improved ACS 6 RNAi hairpin TR4 (4332-4874 of SEQ ID NO:54)
53	nucleotide	Entire improved ACS6 inhibition plasmid construct
54	nucleotide	Fragment of improved ACS6 inhibition construct comprising TR3, ADH1

		intron 1, and TR4 (3272-4874 of SEQ ID NO:53)
55	nucleotide	Fragment of improved ACS6 inhibition construct comprising UBIZm promoter, UBIZm 5' UTR, UBI1Zm Intron 1, TR3, ADH1 intron 1, and TR4 (1218-4874 of SEQ ID NO:53)
56	nucleotide	Fragment of improved ACS6 inhibition construct comprising UBIZm promoter, UBIZm 5'UTR, UBIZm Intron 1, TR3, ADH1 intron 1, TR4, ATTB2, FRT12, UBIZm promoter, UBIZm 5'UTR, UBIZm Intron 1, MO-PAT, and PinII terminator (1218-7989 of SEQ ID NO:53)
57	nucleotide	Complete improved ACS6 inhibition expression cassette (1-8350 of SEQ ID NO:53)

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated

drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

THAT WHICH IS CLAIMED:

1. A method of improving nitrogen stress tolerance in a plant comprising:

a) inhibiting ethylene synthesis in a plant by introducing into a plant a heterologous polynucleotide with means for reducing the activity of an ACC synthase or an ACC oxidase upon expression of said heterologous polynucleotide; and

b) growing said plant under nitrogen limiting conditions, whereby said heterologous polynucleotide is expressed and said plant demonstrates improved nitrogen stress tolerance compared to a control plant.

2. The method of claim 1, wherein said heterologous polynucleotide reduces the expression of an ACC synthase upon expression of said heterologous polynucleotide.

3. The method of claim 2, wherein said heterologous polynucleotide comprises a nucleic acid selected from the group consisting of:

a) a nucleic acid comprising an ACC synthase nucleic acid;

b) a nucleic acid comprising at least 15 contiguous nucleotides of the complement of an ACC synthase nucleic acid; and

c) a nucleic acid encoding a transcript that is capable of forming a double-stranded RNA and mediating RNA interference of an ACC synthase nucleic acid, wherein said nucleic acid comprises:

i) a first nucleotide sequence comprising at least 21 contiguous nucleotides of an ACC synthase nucleic acid; and

ii) a second nucleotide sequence comprising the complement of said first nucleotide sequence.

4. The method of claim 2, wherein said heterologous polynucleotide comprises a nucleotide sequence selected from the group consisting of:

a) the nucleotide sequence set forth in SEQ ID NO:1, 2, 3, 4, 5, or 6, or a complete complement thereof;

b) a nucleotide sequence having at least 90% sequence identity to SEQ ID NO:1, 2, 3, 4, 5, or 6, or a complete complement thereof;

c) a nucleotide sequence encoding the polypeptide sequence of SEQ ID NO:7, 8, or 9 or a complete complement thereof;

d) a nucleotide sequence encoding a polypeptide sequence having at least 95% identity to SEQ ID NO:7, 8, or 9 or a complete complement thereof;

e) a nucleotide sequence comprising at least 15 contiguous nucleotides of the complement of SEQ ID NO:1, 2, 3, 4, 5, or 6; and

f) a nucleotide sequence encoding a transcript that is capable of forming a double-stranded RNA and mediating RNA interference of an ACC synthase nucleic acid, wherein said nucleotide sequence comprises at least 21 contiguous nucleotides of SEQ ID NO:1, 2, 3, 4, 5, or 6 and the complement thereof.

5. The method of claim 4, wherein said plant is a maize plant.

6. The method of claim 4, wherein said plant comprises an mRNA encoded by a polynucleotide having the target sequence set forth in SEQ ID NO:1, 2, 3, 4, 5, or 6, wherein expression of said heterologous polynucleotide inhibits the expression of the mRNA.

7. The method of claim 2, wherein said heterologous polynucleotide comprises a nucleotide sequence selected from the group consisting of:

a) the nucleotide sequence set forth in SEQ ID NO:2 or 5, or a complete complement thereof;

b) a nucleotide sequence having at least 90% sequence identity to SEQ ID NO:2 or 5, or a complete complement thereof;

c) a nucleotide sequence encoding the polypeptide sequence of SEQ ID NO:8 or a complete complement thereof;

d) a nucleotide sequence encoding a polypeptide sequence having at least 95% identity to SEQ ID NO:8 or a complete complement thereof;

e) a nucleotide sequence comprising at least 15 contiguous nucleotides of the complement of SEQ ID NO:2 or 5; and

f) a nucleotide sequence encoding a transcript that is capable of forming a double-stranded RNA and mediating RNA interference of an ACC synthase nucleic acid, wherein said nucleotide sequence comprises at least 21 contiguous nucleotides of SEQ ID NO:2 or 5 and the complement thereof.

8. The method of claim 7, wherein said plant is a maize plant.

9. The method of claim 7, wherein said plant comprises an mRNA encoded by a polynucleotide having the target sequence set forth in SEQ ID NO:2 or 5, wherein expression of said heterologous polynucleotide inhibits the expression of the mRNA.

10. The method of claim 1, wherein said ACC synthase is selected from the group consisting of: ACC synthase 2, ACC synthase 6 and ACC synthase 7.

11. The method of claim 1, wherein said improved nitrogen stress tolerance compared to a control plant comprises at least one phenotype selected from the group consisting of:

a) increased yield;
b) increased root mass;
c) increased root length;
d) increased leaf size;
e) increased ear size;
f) increased seed size;
g) increased endosperm size; and
h) improved standability.

12. The method of claim 1, wherein said heterologous polynucleotide is operably linked to a promoter that functions in plants.

13. The method of claim 12, wherein the promoter that functions in plants is a tissue-preferred promoter, tissue-specific promoter, or an inducible promoter.

14. The method of claim 1, wherein the heterologous polynucleotide is introduced by a method selected from one of the following: electroporation, micro-projectile bombardment and Agrobacterium-mediated transfer.

15. The method of claim 1, wherein said plant is a monocot.

16. The method of claim 15, wherein said monocot is maize, wheat, rice, barley, sorghum, sugarcane, or rye.

17. The method of claim 1, wherein said plant is a dicot.

18. The method of claim 17, wherein said dicot is soybean, canola, Brassica or sunflower.

19. A method for improving nitrogen stress tolerance under low nitrogen conditions, said method comprising:

a) evaluating environmental conditions of an area of cultivation for nitrogen limiting conditions; and

b) planting a transgenic seed or a transgenic plant having a reduced activity of at least one ACC synthase or ACC oxidase in an area of cultivation having nitrogen limiting conditions.

20. The method of claim 19, wherein said plant or seed is stably transformed with a heterologous polynucleotide with means for decreasing the activity of an ACC synthase or ACC oxidase upon expression of said heterologous polynucleotide.

21. The method of claim 20, wherein said heterologous polynucleotide reduces the expression of an ACC synthase upon expression of said heterologous polynucleotide.

22. The method of claim 21, wherein said heterologous polynucleotide comprises a nucleic acid selected from the group consisting of:

a) a nucleic acid comprising an ACC synthase nucleic acid;

b) a nucleic acid comprising at least 15 contiguous nucleotides of the complement of an ACC synthase nucleic acid; and

c) a nucleic acid encoding a transcript that is capable of forming a double-stranded RNA and mediating RNA interference of an ACC synthase nucleic acid, wherein said nucleic acid comprises:

i) a first nucleotide sequence comprising at least 21 contiguous nucleotides of an ACC synthase nucleic acid; and

ii) a second nucleotide sequence comprising the complement of said first nucleotide sequence.

23. The method of claim 21, wherein said heterologous polynucleotide comprises a nucleotide sequence selected from the group consisting of:

a) the nucleotide sequence set forth in SEQ ID NO:1, 2, 3, 4, 5, or 6, or a complete complement thereof;

b) a nucleotide sequence having at least 90% sequence identity to SEQ ID NO:1, 2, 3, 4, 5, or 6, or a complete complement thereof;

c) a nucleotide sequence encoding the polypeptide sequence of SEQ ID NO:7, 8, or 9 or a complete complement thereof;

d) a nucleotide sequence encoding a polypeptide sequence having at least 95% identity to SEQ ID NO:7, 8, or 9;

e) a nucleotide sequence comprising at least 15 contiguous nucleotides of the complement of SEQ ID NO:1, 2, 3, 4, 5, or 6; and

f) a nucleotide sequence encoding a transcript that is capable of forming a double-stranded RNA and mediating RNA interference of an ACC synthase nucleic acid, wherein said nucleotide sequence comprises at least 21 contiguous nucleotides of SEQ ID NO:1, 2, 3, 4, 5, or 6 and the complement thereof.

24. The method of claim 23, wherein said plant is a maize plant.

25. The method of claim 23, wherein said plant comprises an mRNA encoded by a polynucleotide having the target sequence set forth in SEQ ID NO:1, 2, 3, 4, 5, or 6, wherein expression of said heterologous polynucleotide inhibits the expression of the mRNA.

26. The method of claim 21, wherein said heterologous polynucleotide comprises a nucleotide sequence selected from the group consisting of:

a) the nucleotide sequence set forth in SEQ ID NO:2 or 5, or a complete complement thereof;

b) a nucleotide sequence having at least 90% sequence identity to SEQ ID NO:2 or 5, or a complete complement thereof;

c) a nucleotide sequence encoding the polypeptide sequence of SEQ ID NO:8 or a complete complement thereof;

d) a nucleotide sequence encoding a polypeptide sequence having at least 95% identity to SEQ ID NO:8;

e) a nucleotide sequence comprising at least 15 contiguous nucleotides of the complement of SEQ ID NO:2 or 5; and

f) a nucleotide sequence encoding a transcript that is capable of forming a double-stranded RNA and mediating RNA interference of an ACC synthase nucleic acid, wherein said nucleotide sequence comprises at least 21 contiguous nucleotides of SEQ ID NO:2 or 5 and the complement thereof.

27. The method of claim 26, wherein said plant is a maize plant.

28. The method of claim 26, wherein said plant comprises an mRNA encoded by a polynucleotide having the target sequence set forth in SEQ ID NO:2 or 5, wherein expression of said heterologous polynucleotide inhibits the expression of the mRNA.

29. The method of claim 19, wherein said ACC synthase is selected from the group consisting of: ACC synthase 2, ACC synthase 6 and ACC synthase 7.

30. The method of claim 19, wherein said improved nitrogen stress tolerance compared to a control plant comprises at least one phenotype selected from the group consisting of:

a) increased yield;

b) increased root mass;

c) increased root length;

d) increased leaf size;

e) increased ear size;

f) increased seed size;

g) increased endosperm size; and

h) improved standability.

31. The method of claim 20, wherein said heterologous polynucleotide is operably linked to a promoter that functions in plants.

32. The method of claim 31, wherein the promoter that functions in plants is a tissue-preferred promoter, tissue-specific promoter, or an inducible promoter.

33. The method of claim 19, wherein said plant is a monocot.

34. The method of claim 33, wherein said monocot is maize, wheat, rice, barley, sorghum, sugarcane, or rye.

35. The method of claim 19, wherein said plant is a dicot.

36. The method of claim 35, wherein said dicot is soybean, canola, Brassica or sunflower.

37. An expression cassette comprising a promoter that functions in plants operably linked to a polynucleotide that comprises at least one of SEQ ID NO: 51 and SEQ ID NO: 52.

38. The expression cassette of claim 37, wherein said promoter is a constitutive promoter.

39. A construct comprising a nucleotide sequence selected from the group consisting of:

a) the nucleotide sequence set forth in SEQ ID NO: 53;

b) the nucleotide sequence set forth in SEQ ID NO: 54;

c) the nucleotide sequence set forth in SEQ ID NO:55;

d) the nucleotide sequence set forth in SEQ ID NO:56; and

e) the nucleotide sequence set forth in SEQ ID NO:57.

40. A plant cell comprising a heterologous polynucleotide configured for RNA silencing or interference, wherein said heterologous polynucleotide comprises at least one of SEQ ID NO: 51 and SEQ ID NO: 52.

41. The plant cell of claim 40, wherein said heterologous polynucleotide is operably linked to a promoter that functions in plants.

42. The plant cell of claim 40, wherein said heterologous polynucleotide has a nucleotide sequence selected from the group consisting of:

a) the nucleotide sequence set forth in SEQ ID NO: 53;

b) the nucleotide sequence set forth in SEQ ID NO: 54;

c) the nucleotide sequence set forth in SEQ ID NO:55;

d) the nucleotide sequence set forth in SEQ ID NO:56; and

e) the nucleotide sequence set forth in SEQ ID NO:57.

43. The plant cell of claim 40, wherein the plant cell is from a dicot or monocot.

44. The plant cell of claim 43, wherein the dicot or monocot is Zea mays, wheat, rice, sorghum, barley, oat, lawn grass, rye, soybean, sugarcane, Brassica or sunflower.

45. A plant regenerated from the plant cell of claim 40.

46. A method of inhibiting ethylene production in a plant, the method comprising inhibiting expression of one or more ACC synthase genes in the plant by introducing into the plant a heterologous polynucleotide having a nucleotide sequence selected from the group consisting of:

a) the nucleotide sequence set forth in SEQ ID NO: 51;

b) the nucleotide sequence set forth in SEQ ID NO: 52;

c) the nucleotide sequence set forth in SEQ ID NO: 53;

d) the nucleotide sequence set forth in SEQ ID NO: 54;

e) the nucleotide sequence set forth in SEQ ID NO: 55;

f) the nucleotide sequence set forth in SEQ ID NO: 56;

g) the nucleotide sequence set forth in SEQ ID NO: 57; and

h) the nucleotide sequences set forth in SEQ ID NO:51 and 52.

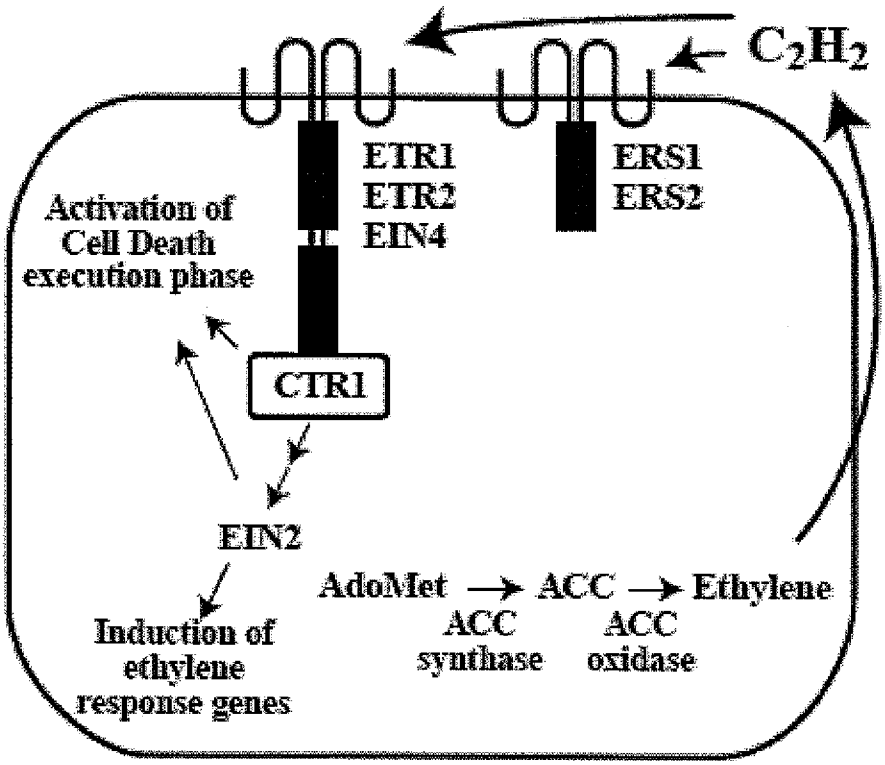
C_2H_2
ETR1
ETR2
EIN4
ERS1
ERS2
Activation of
Cell Death
execution phase
CTR1
EIN2
Induction of
ethylene
response genes
AdoMet → ACC → Ethylene
ACC
synthase
ACC
oxidase

FIG. 1

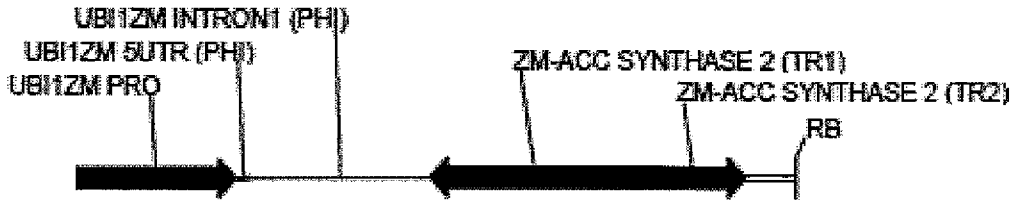

FIG. 2A

GGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTGATATGTAAACTGGTCTTTTATTCACCCTTGAG
CTACGCCTTCTGTGTCCTGAAGTTACGTGACAGGTTTTCAACAACAATTGATCGGGAGGTCTAAAT
TACCATACCCGATCGTTATTCTACCGTGTACTAACGTTAAACCAAACCATACATGCGATTTGGCAA
AACAGAAAAAAAGAACATATATACACGAAAAAATCGAGGACAAATGCGTCCTGCTTGACTAGCTGC
TTACCGCCGACGCCTGCTGACGCCTGTGCAACGTCTCTGTGTAATGTCAAGCTAGCTACTAATGCC
CTTTATTAGTCTTGTCTAATATTTTTGTCCACTTGTTTGCAGTTATGTGAGGGCACACCCTACAGC
CAGTCTTACCGAACGCTCGGTGACTAGCTAGCTGGCGTGGACCATCGGGGACTGCGGCGACAGCA
ACGCCAAGGGGCTGGGGATGGCGAGGTGCGAAGCGGTGGTTGCTCCCCGGCGCGGCAAGCTGAG
GCGGAGGGGCCGCGTGGCCGCCCAGCGCTCGGCCTTGGCCTTGCTGTGCTGGTGCTGGCGCACGA
AGCGGCGGATCCGGTCGAGCGCGACCTCCATGGTGTCGTCGTCCATGTTGGCGTAGCAGACGCGG
AACCAGCGGGGCTCGTTGCAGTGGAACGACGTGCCGGGCGACACGTTGAGCTTCACCCTGTGTAC
GATGACCCGCCACAGCTCCAGCTCCGCGTCGTGCGTCCTCTCCCGGAGCATGCCCCGCAGGTCCAT
CCACGAGAAGAGGCCCGCGTTGCCCGGCAGGCACGCGATGCCGACCTCGCGGAGGCCCGCGACGA
AGCGGTCGTGGCGCGCCCCAGCCGCCGCGCGCTCTCCGCGAGGAAGCGTGCCATGAACTCCGCG
TCGGCGAGCATCATCGCCAGGAAGTGCTGCGTCTGCGACGAGACGAGGCCGAAGCTGGACATCTT
GCGCGCGCAGGCCACCACGTCGTCGTTGTAGGAGTAGACGATGCCGACGCGGAAGCCCGGGAGGC
CGAAGTCCTTGGAGAGGCTGTACGCGATGTGGACGAGGTCCCTGTTGCAGCCCGGCGCGTCGCCG
CGCTCGATGACCTCGGCGATGCTCACGAAGCCCGGCTTGGCGAAGACGGAGCCCCGCGTA

FIG. 2B

GATCCGCCGCTTCGTGCGCCAGCACCAGCACAGCAAGGCCAAGGCCGAGCGCTGGCGGGCCACGC
GGCCCCTCCGCCTCAGCTTGCCGCGCCGGGGAGCAACCACCGCTTCGCACCTCGCCATCCCCAGCC
CCTTGGCGTTGCTGTCGCCGCAGTCCCCGATGGTCCACGCCAGCTAGCTAGTCACCGAGCGTTCGG
TAAGACTGGCTGTAGGGTGTGCCCTCACATAACTGCAAACAAGTGGACAAAAAAATATTAGACAAG
ACTAATAAAGGGCATTAGTAGCTAGCTTGACATTACACAGAGACGTTGCACAGGCGTCAGCAGGC
GTCGGCGGTAAGCAGCTAGTCAAGCAGGACGCATTTGTCCTCGATTTTTTCGTGTATATATGTTCTT
TTTTCTGTTTTGCCAAATCGCATGTATGGTTTGGTTTAACGTTAGTACACGGTAGAATAACGATCGG
GTATGGTAATTTAGACCTCCCGATCAATTGTTGTTGAAAACCTGTCACGTAACTTCAGGACACAGA
AGGCGTAGCTCAAGGGTTGAATAAAAGACCAGTTTACATATCAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAGGC

FIG. 2C

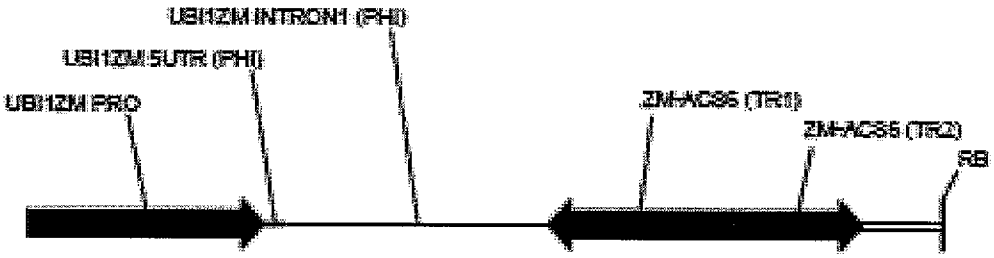

FIG. 3A

TAGCAGACGCGGAACCAGCCGGGCTCCCGGCAGTGGCAGGAGGAGCCCGGGGAGATGTTGAGCC
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GCCGCATGTTGACCCAGCAGAAGAGCCCCGCGTTGCTCTCCAGGCACTCGATGCCCACGGCCGCC
AGGCCCTCCGCCAGCTGCTCGCGCCGCTCCCTGATCCGCCGCGTGTTCTCCGCGATGTACCTCCGC
GTGAAGTCCCTGTCGCCCAGGAGCGACGCCAGGAGGTGCTGCGTCTGGGACGACACCAGGCCGAA
GCTCGACATCTTGGTGGCCGCGGAGACCACGCCGGCGTTGGACGAGTAGATGGCGCCCACGCGGA
ACCCCGGGAGGCCCAGGTCCTTGGACAGGCTGTACACCACGTGCACGCGGTCCGACAGCGGCCCA
ACGCCGACGACGCCGTCGTCCGTGGCGGCGCGCGCGGCCACCACCTCGAGGACGCTCACGAAGCC
CGGGTCCGCGAAGACCGTGCCCGAGTATATCTCGTCGCTCACCAGGTGGATGCCCTTGGCGGCCAC
GAAGTCCACCAGCATCTCCAGGTCGGCGGCGCGACGTGGTGCCCAGCGGGTTGGAAGGGTTGG
TGATGAGCACGCCCTTGACGCGCAGCCGCAGCTTCTGCGCGCGCCGGTA

FIG. 3B

CGCGCCGCCACGGACGACGGCGTCGTCGGCGTTGGGCCGCTGTCGGACCGCGTGCACGTGGTGTA
CAGCCTGTCCAAGGACCTGGGCCTCCCGGGGTTCCGCGTGGGCGCCATCTACTCGTCCAACGCCGG
CGTGGTCTCCGCGGCCACCAAGATGTCGAGCTTCGGCCTGGTGTCGTCCCAGACGGCAGCACCTCCT
GGCGTCGCTCCTGGGCGACAGGGACTTCACGCGGAAGGTACATCGCGGAGAACACGCGGCGGATCA
GGGAGCGGCGCGAGCAGCTGGCGGAGGGCCTGGCGGCCGTGGGCATCGAGTGCCTGGAGAGCAA
CGCGGGGCTCTTCTGCTGGGTCAACATGCGGCGCCTGATGCGGAGCCGGTCGTTCGAGGGCGAGA
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GCCGGGAGCCCGGCTGGTTCCGCGTCTGCTAA

FIG. 3C

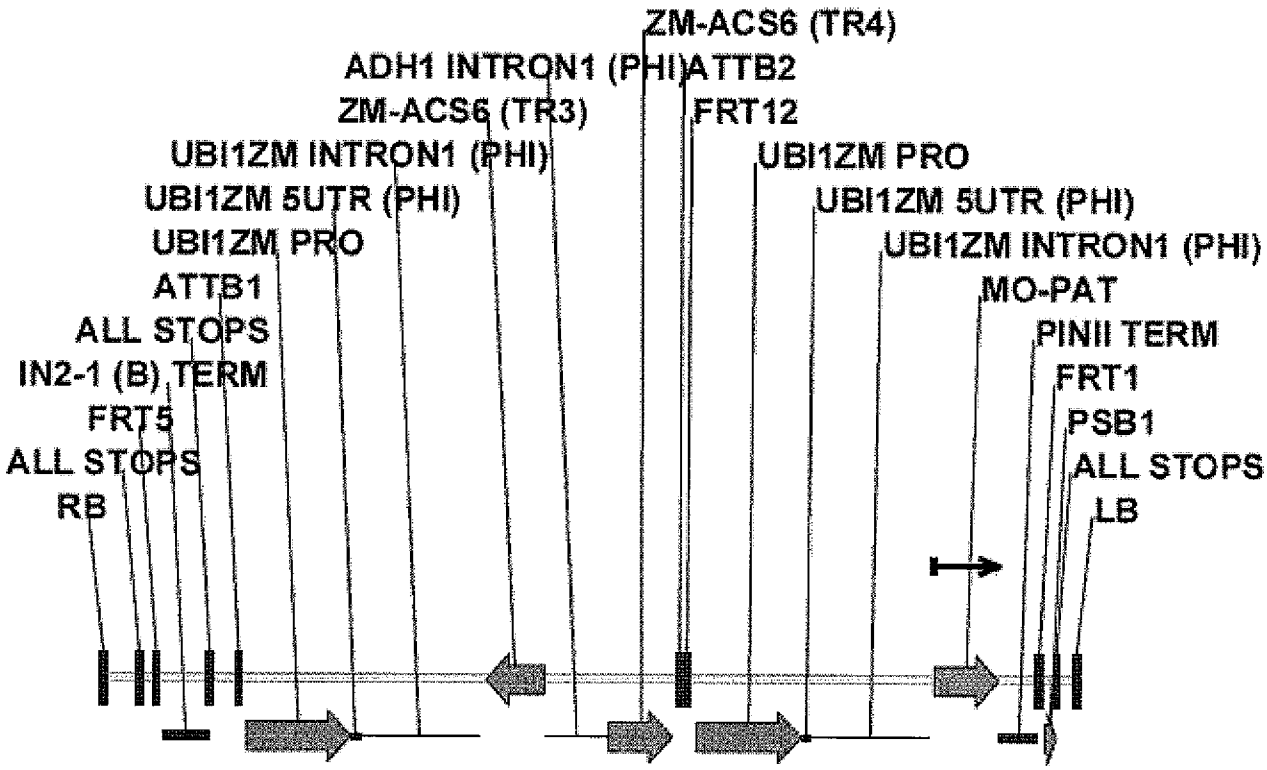

ZM-ACS6 (TR4)
ADH1 INTRON1 (PHI)
ATTB2
ZM-ACS6 (TR3)
FRT12
UBI1ZM INTRON1 (PHI)
UBI1ZM PRO
UBI1ZM 5UTR (PHI)
UBI1ZM 5UTR (PHI)
UBI1ZM PRO
UBI1ZM INTRON1 (PHI)
ATTB1
MO-PAT
ALL STOPS
PINII TERM
IN2-1 (B) TERM
FRT1
FRT5
PSB1
ALL STOPS
ALL STOPS
RB
LB

FIG. 4

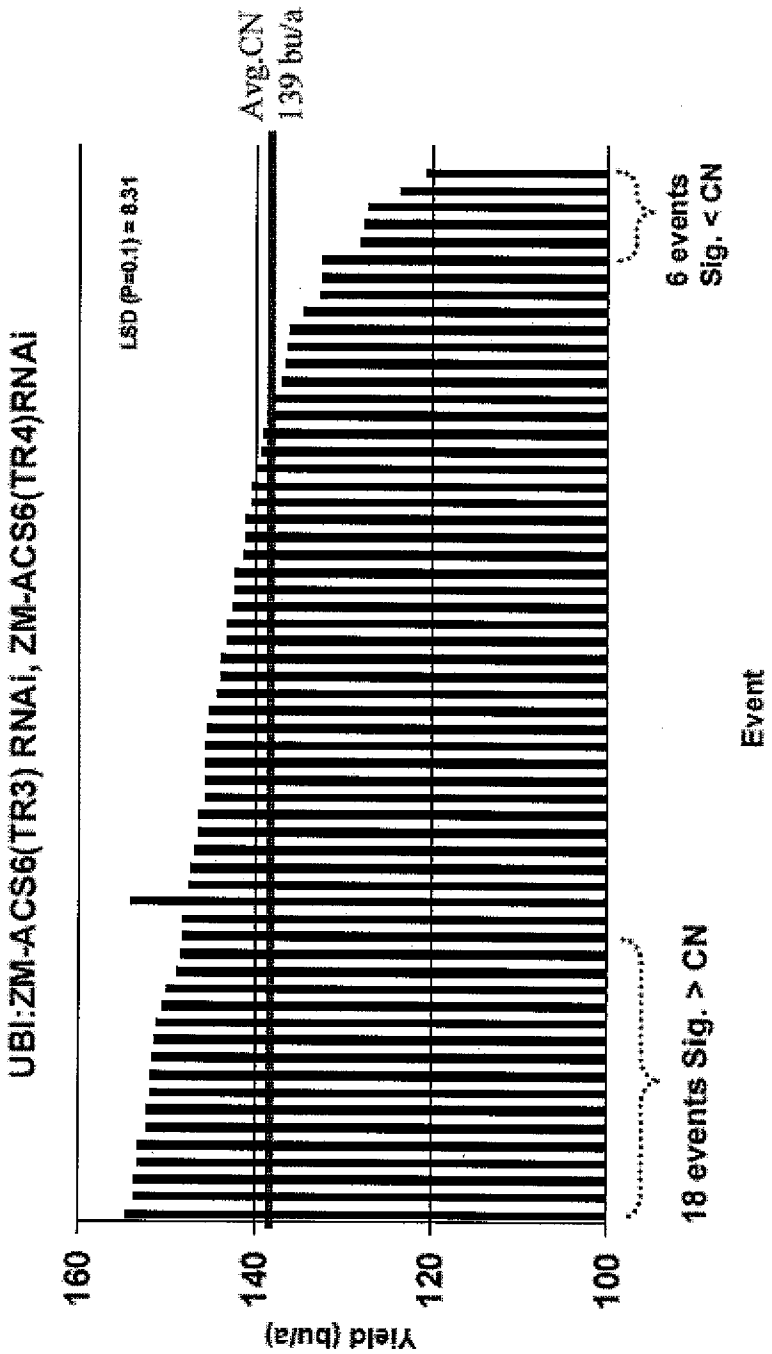

UBI:ZM-ACS6(TR3) RNAi, ZM-ACS6(TR4)RNAi
LSD (P=0.1) = 8.31
Avg.CN
139 bu/a
6 events
Sig. < CN
18 events Sig. > CN
Event
160
140
120
100
Yield (bu/a)

FIG. 5

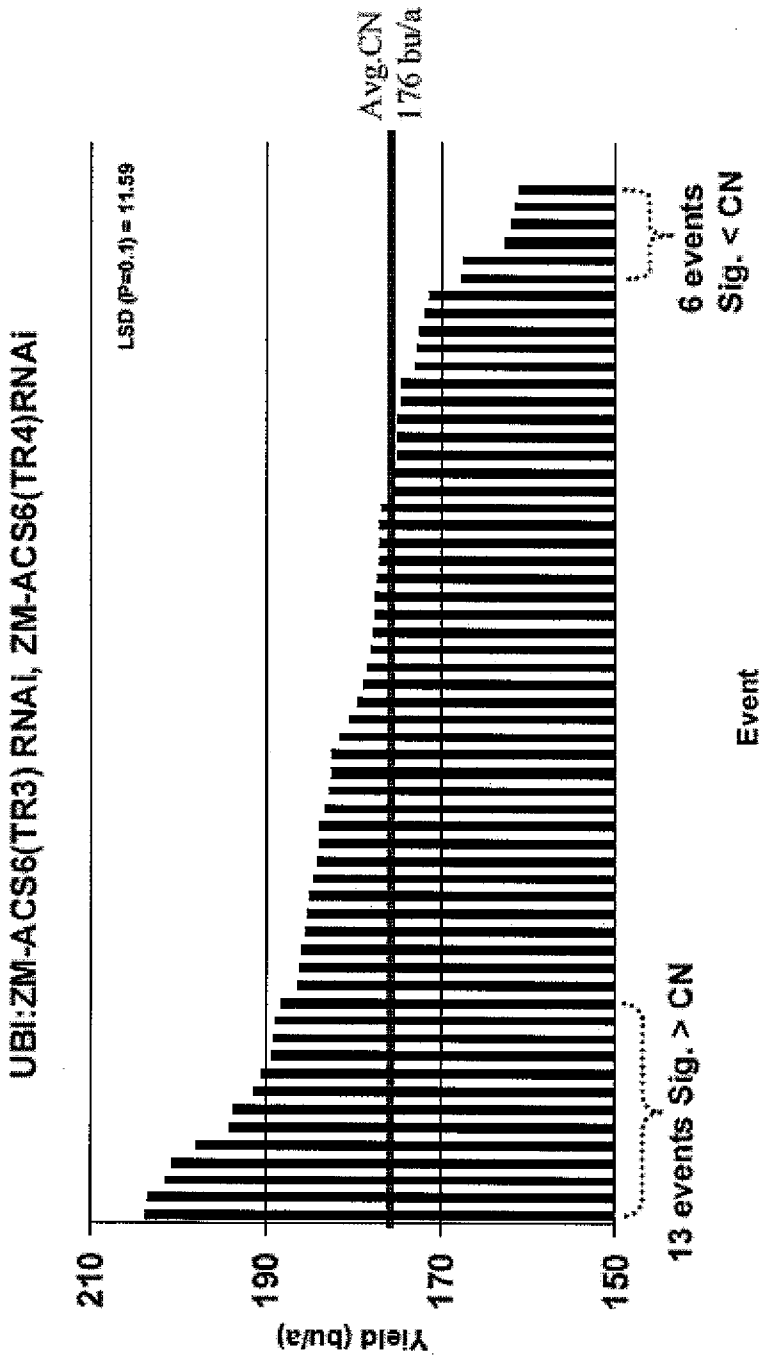
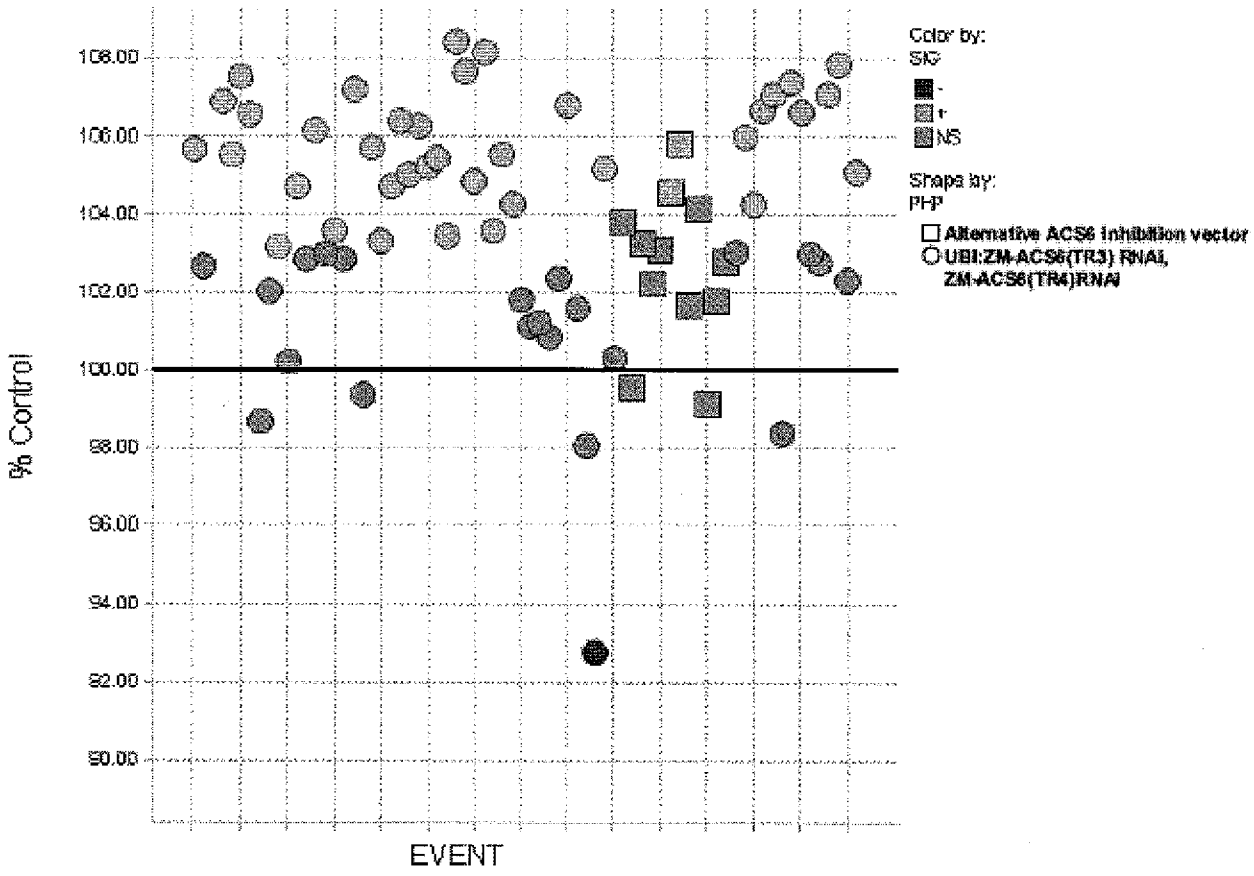

UBI:ZM-ACS6(TR3) RNAi, ZM-ACS6(TR4)RNAi
LSD (P=0.1) = 11.59
Avg.CN
176 bu/a
6 events
Sig. < CN
13 events Sig. > CN
Event
Yield (bu/a)
210
190
170
150

FIG. 6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106.00
104.00
102.00
100.00
98.00
96.00
94.00
92.00
90.00
EVENT
Color by:
SIG
-
+
NS
Shape by:
PHP
Alternative ACS6 inhibition vector
UBI:ZM-ACS6(TR3) RNAi,
ZM-ACS6(TR4)RNAi

FIG. 7

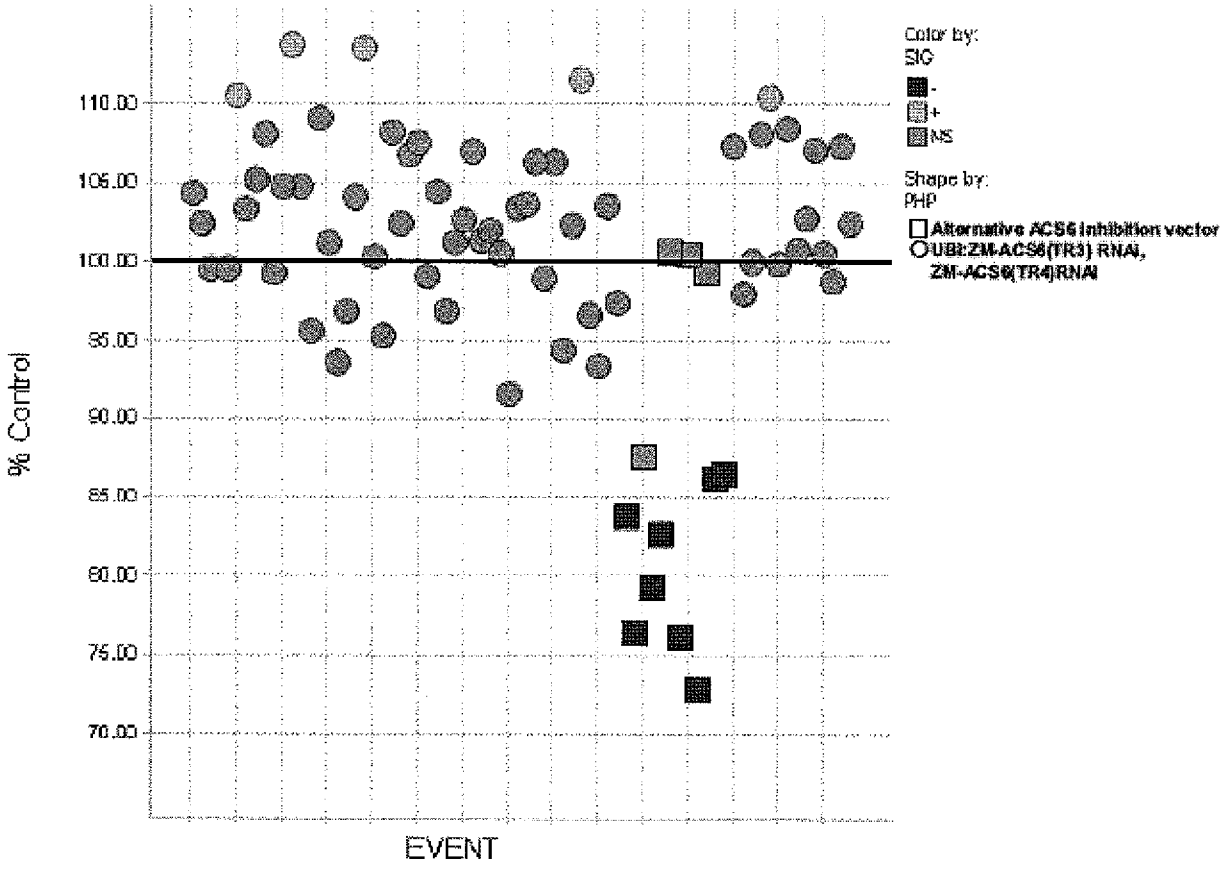
110.00
105.00
100.00
95.00
90.00
85.00
80.00
75.00
70.00
% Control
EVENT
Color by:
SIG
-
+
NS
Shape by:
PHP
Alternative ACS6 inhibition vector
UBI:ZM-ACS6(TR3) RNAi,
ZM-ACS6(TR4)RNAi

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/031008

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/88 C12N15/82
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2007/076115 A2 (ARCADIA BIOSCIENCES INC [US]; KRIDL JEAN [US]; DEPAUW MARY [CA]; SHRAW) 5 July 2007 (2007-07-05)	1-46
A	WO 2007/092704 A2 (PIONEER HI BRED INT [US]; HERSHEY HOWARD P [US]; SIMMONS CARL R [US];) 16 August 2007 (2007-08-16)	1-46
A	WO 2008/157375 A1 (PIONEER HI BRED INT [US]; LOUSSAERT DANIEL F [US]; O'NEILL DENNIS [US]) 24 December 2008 (2008-12-24)	1-46

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 July 2010	03/08/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Kania, Thomas

3

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WI SOO JIN ET AL: "Antisense expression of carnation cDNA encoding ACC synthase or ACC oxidase enhances polyamine content and abiotic stress tolerance in transgenic tobacco plants" MOLECULES AND CELLS, SEOUL, KR, vol. 13, no. 2, 30 April 2002 (2002-04-30) , pages 209-220, XP002338203 ISSN: 1016-8478 -----	1-46

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2010/031008

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CN 101378651 A	04-03-2009
		EP 1983819 A2	29-10-2008
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		EP 1991568 A2	19-11-2008
		US 2009320156 A1	24-12-2009
		US 2007300323 A1	27-12-2007
WO 2008157375 A1	24-12-2008	AR 067014 A1	30-09-2009
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		EP 2167652 A1	31-03-2010
		US 2008313775 A1	18-12-2008