

**INCREASED SEED SIZE AND SEED NUMBER THROUGH
TRANSGENIC OVER EXPRESSION OF A GROWTH AND/OR
DEVELOPMENT RELATED GENE DURING EARLY EMBRYO
DEVELOPMENT**

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Patent Application No. 60/750,991, filed on December 15, 2005, incorporated by reference herein in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] The most important trait as a target for crop improvement is yield. Efforts to improve crop yields by developing new plant varieties can be divided into two approaches. One is to reduce crop yield losses by breeding or engineering crop varieties with increased resistance to abiotic stress conditions such as drought, cold, or salt or to biotic stress conditions resulting from pests or disease-causing pathogens. While this approach has value, it does not provide fundamentally improved crop yield in the absence of stress conditions.

[0003] The second approach is to breed or engineer new crop varieties in which the basic yield capacity is increased. Classical breeding programs have initially produced substantial gains in improved yield in a variety of crops. A commonly experienced pattern though has been substantial gains in yield initially followed by incremental further improvements that become smaller and more difficult to obtain.

[0004] More recently developed approaches based on molecular biology technologies have in principle offered the potential to achieve substantial improvement in crop yield by altering the timing, location, or level of expression of plant genes that play a role in plant growth and/or development. Substantial progress has been made over the past twenty years in identifying plant genes that have a role in plant growth and/or development. Because of the complexity of plant growth regulation and how it relates in the end to yield traits, it is still not obvious which, if any, of these genes would be a clear candidate to improve crop yield.

[0005] Much of the work that has been done to identify plant genes with a growth and/or development function has been done in the model plant system *Arabidopsis thaliana*. One such gene, called *REVOLUTA* (REV), was originally identified as a loss-of-function mutation in *Arabidopsis* called *rev1* (Talbert *et al.*, *Development* 121:2723-2735, 1995). This mutation had pleiotropic effects on plant growth and morphology. One phenotype of the *rev1* mutation that was of interest was significantly larger seeds. This phenotype was potentially desirable agriculturally but, unfortunately, it was accompanied by undesirable traits such as reduced numbers of flowers and seeds, infertility, and altered leaf morphology. The *rev1* mutant exhibits larger leaves, stems, and flowers in addition to larger seeds.

[0006] The REV gene was identified by a map based cloning approach (described in WO 01/33944, incorporated herein by reference in its entirety) and it proved to be a transcription factor that belonged to the home domain-leucine zipper (HD-ZIP) family of transcription factors. In plants, HD-Zip genes are involved in many developmental pathways, including vascular tissue development, trichome and root hair development, and light-regulated responses. Since *rev1* was likely to be a loss of function mutation, efforts were made to knock out REV function in transgenic *Arabidopsis* by expressing inverted repeat REV (REV-IR) constructs or via co-suppression triggered by strong over expression of the REV gene (WO 01/33944). REV-IR constructs gave a weak *rev1* phenotype. The *rev1* phenotype including larger, heavier seeds was also observed in REV over expression lines where strong constitutive expression of the REV transgene throughout the plant was driven by the constitutive 35S promoter. Interestingly, this effect correlated with increased REV mRNA levels indicating that it was not due to co-suppression of the endogenous REV gene. The fact that REV over expression and not suppression gave increased seed size was an important finding because it was unanticipated based on the prior work with the *rev1* mutant.

[0007] Strong constitutive expression of the REV transgene throughout the plant mimicked the large seed phenotype of the *rev1* mutant, but it also replicated undesirable phenotypes seen with the *rev1* mutant. While the ability to obtain the larger seed size phenotype by the technically straight forward approach of constitutive over expression of a REV transgene showed promise, the undesirable phenotypes meant that this approach would not be viable for commercial agricultural applications.

[0008] The agriculturally relevant part of the plant for many crop species is the seed. If the large seed trait could be obtained without the detrimental side effects of general constitutive

REV over expression, it would be of potentially great agricultural value. One conceivable approach to overcome this problem was to limit *REV* over expression specifically to the seed. While many seed-specific promoters are already known and characterized that could potentially be used to drive *REV* transgene expression, there was no evidence to suggest that, in principle, REV over expression limited to the seed would actually result in increased seed size. The natural function of a plant's endogenous *REV* gene in the seed is known to be in meristem initiation and adaxial cell fate determination (Otsuga *et al.*, *Plant J.* 25:223-236, 2001; McConnell and Barton, *Development* 125:2935-2942 (1998); McConnell *et al.*, *Nature* 411:709-713, 2001; Emery *et al.*, *Curr. Biol.* 13:1768-1774, 2003) and not in cell growth or division. Further, it is not known whether the large seed size phenotype in the 35S/REV over expression plants is due to over expression of REV in the seed or in the developing embryo or is instead due to effects on the overall growth and development of the plant caused by REV over expression throughout the plant's tissues.

[0009] In addition to the lack of any known biological function for REV in the seed that would have an effect on seed size determination, there is no information to indicate in what part of the seed or during what stage during seed development it might be best to attempt to over express a REV transgene to achieve an increase in seed size.

[0010] The present invention provides for the embryo-specific over expression of a growth and/or development related gene during an early stage of embryo development that results in an increase in seed size when compared to a wild-type plant that does not over express the gene. In a particular embodiment the *REV* gene was overexpressed using an early embryo-specific promoter. The increased seed size trait was achieved without the detrimental side effects seen with constitutive over expression of REV throughout the plant. In addition, embryo-specific over expression of REV gave the unpredicted result of an increase in total seed number per plant when compared to a wild-type plant. The increase in total seed number resulted from an increase in seed number per pod, an increase in the number of racemes per plant, an increase in the number of pods per raceme, a decrease in the rate of seed abortion, or a combination of these effects. Together, the increased seed size and increased seed number resulting from over expression of REV in the developing embryo lead to substantial increases in total yield when compared to a wild-type plant and demonstrates that a gene associated with plant growth and/or development can be overexpressed in association with an early embryo-specific promoter to increase plant yield.

BRIEF SUMMARY OF THE INVENTION

[0011] The present invention provides methods and compositions for increasing the seed size and/or seed number in transgenic plants. In particular, the present invention relates to the use of early phase-specific embryo promoters operatively associated with a gene associated with plant growth and/or development to provide for the over expression of the gene and/or a protein encoded by the gene in a developing seed of a transgenic plant. Over expression of the gene during this early stage of seed development in a transgenic plant provides for increased seed production and/or increased seed size in the transgenic plant when compared with the wild-type plant.

[0012] In a particular embodiment of the present invention the *REV* gene was operatively associated with an early phase-specific embryo promoter to provide for the over expression of REV protein in a developing seed of a transgenic plant. Over expression of REV during this early stage of seed development surprisingly resulted in increased seed size and increased seed production in the transgenic plant without the detrimental side effects that had been seen when REV was over expressed throughout the plant.

[0013] A method for increasing seed size in a plant comprising over expressing a gene associated with plant growth and/or development in a seed during early embryo development is also provided. In particular, the method comprises expressing the growth and/or development related gene in the seed under the control of an early phase-specific embryo promoter. The early phase-specific embryo promoter can be heterologous or homologous to the plant. In certain embodiments of the present invention the promoter is an early phase-specific promoter associated with an amino acid permease gene, such as AAP1, an oleate 12-hydroxylase:desaturase gene, a 2S2 albumin gene, a fatty acid elongase gene, such as FAE1, or a leafy cotyledon gene. Particular promoters useful in the present invention include the AAP1 promoter from *Arabidopsis thaliana*, an oleate 12-hydroxylase:desaturase gene promoter from *Lesquerella fendleri* (LFAH12), a 2S2 gene promoter or a leafy cotyledon gene LEC2 promoter from *Arabidopsis thaliana*.

[0014] In a particular embodiment of the present invention the *REV* gene was operatively associated with an early phase-specific embryo promoter. In this method, the *REV* gene was overexpressed in the early development of the seed and lead to an increase in seed size and seed number as compared with a wild-type plant.

[0015] The methods of the present invention can be used to increase the seed size and/or seed number in plants that are characterized as a monocot or a dicot. In particular, the methods can be used to increase the seed size and/or seed number in plants that are members of the *Brassicaceae*, (*Cruciferae*), *Gramineae*, *Malvaceae*, or *Leguminosae-Papilionoideae* families. Particular plants of interest for use of the methods of the present invention include canola, corn, camelina, cotton, alfalfa, soybean, wheat, rice, or barley.

[0016] The present invention also provides genetic constructs comprising a nucleic acid sequence for a gene associated with plant growth and/or development is operatively linked to one or more control sequences wherein the one or more control sequences are capable of promoting expression of the gene during embryo development. In particular, the genetic construct of the invention comprise a control sequence including an early phase-specific embryo promoter. The early phase specific embryo promoters can include the promoter associated with an amino acid permease gene (AAP1), an oleate 12-hydroxylase:desaturase gene, an 2S2 albumin gene (2S2), a fatty acid elongase gene (FAE1), or a leafy cotyledon gene (LEC2). Typical genetic constructs of the present invention comprise the AAP1 promoter from *Arabidopsis thaliana*, the oleate 12-hydroxylase:desaturase promoter from *Lesquerella fendleri* (LFAH12), the 2S2 gene promoter from *Arabidopsis thaliana*, the fatty acid elongase gene promoter from *Arabidopsis thaliana*, or the leafy cotyledon gene 2 promoter from *Arabidopsis thaliana*. Particular genetic constructs of the present invention comprise an early phase-specific embryo promoter operatively associated with a *REV* gene from *Arabidopsis*. The genetic construct can also include an operatively associated polyA sequence.

[0017] The present invention also provides methods for the production of a transgenic plant having increased seed size and/or seed number, the methods comprising: (a) introducing into a plant or into a plant cell, a genetic construct as set forth above and cultivating the plant or plant cell comprising the genetic construct under conditions promoting regeneration and mature plant growth. Typically, the methods produce a transgenic plant having increased seed size and/or seed number when compared to the corresponding wild-type plant. Transgenic plants comprising the genetic constructs can be monocotyledonous or a dicotyledonous plants, particularly where the monocotyledonous plant is a member of the *Gramineae* family. The plants from the *Gramineae* family of particular interest include rice, oat, corn, or wheat. Additionally, transgenic plants of the present invention are plants of the

Brassicaceae (Cruciferae), *Malvaceae*, or *Leguminosae-Papilionoideae* families. In particular wherein the transgenic plant is soybean, cotton, camelina, alfalfa, or canola.

[0018] The present invention also provides methods for selecting for a gene that increases plant yield when functionally associated with an early phase-specific embryo promoter; comprising construction an expression vector comprising a gene associated with plant growth and/or development functionally associated with a early phase-specific embryo promoter, transfecting a plant cell with the expression vector to form a transgenic plant; growing the transgenic plant and selecting those transgenic plants that have an increased yield. The genes that produce a transgenic plant with increased yield are selected for further development of transgenic plants. The genes that can be used in the present method include, for example, but are not limited to, the *CAP* (cyclase-associated protein) gene, the rice histone deacetylase 1 gene, *E2Fc* gene, *BKI* gene, the *BRI1* gene, *ARL* (Argos-Like) gene, the *bril* (brassinosteroid hormone perception) gene, *FATB* gene, Pepino gene, *RGS* (regulator of G protein signaling) gene, the *TIP1* gene, the *BB* (Big Brother) gene, the RHD2 gene, *INCW2* gene, the *MNI* gene, the *WAK2* gene, the *WAK4* gene, the *AP2* gene, and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figure 1 depicts the expression profiles of embryo-specific promoters during early embryo development. Approximate stages in *Arabidopsis* embryo development: early globular = 2 days after pollination (DAP), heart = 4 DAP, torpedo = 6 DAP, walking stick = 7 DAP, early maturation embryo = 8 DAP.

[0020] Figure 2 depicts an alignment of amino acid sequences of a region of REVOLUTA and non-REVOLUTA HD-ZIPIII class transcription factors. This region of the amino acid sequence contains a characteristic difference in amino acid sequence that defines HD-ZIPIII proteins as belonging to the REVOLUTA or non-REVOLUTA classes of proteins. The boxed portion of the sequences indicate the site of the amino acid sequence insertion that defines non-REVOLUTA HD-ZIPIII proteins. REVOLUTA proteins are defined by the lack of an amino acid sequence insertion. Athb-9 (*Arabidopsis*; SEQ ID NO:2); Athb-14 (*Arabidopsis*; SEQ ID NO:3); REV (*Arabidopsis*; SEQ ID NO:4); BnLfREV (*Bassica napis*; SEQ ID NO:5); OsREV1 (*Oryza sativa*; SEQ ID NO:6); ZmRLD1 (*Zea mays*; SEQ ID

NO:7); OsREV2 (*Oryza sativa*; SEQ ID NO:8); Athb-15 (*Arabidopsis*; SEQ ID NO:9); and Athb-8 (*Arabidopsis*; SEQ ID NO:10).

DETAILED DESCRIPTION OF THE INVENTION

[0021] The present invention provides methods and compositions useful for producing plants having an increased seed size and/or increased seed number when compared to a wild-type plant. In particular, the methods comprise over expression of a gene involved in plant growth and/or development in an embryo of a plant wherein the over expression of the transgene results in an increase in seed size and/or an increased number of seeds in the plant. In a particular embodiment the *REVOLUTA* (*REV*) gene was overexpressed. The *REV* transgene in the methods of the present invention is under the regulation of a promoter that initiates expression during embryo development and in particular initiates expression during early phase-specific embryo development. Unexpectedly, over expression of the *REV* transgene in early stage embryo development resulted in larger and/or more seeds.

[0022] As a first attempt to determine if seed size could be increased through over expression of a gene involved in plant growth and/or development the *REV* gene was targeted to the seed, a *REV* transgene construct comprising an embryo-specific promoter to drive *REV* expression was tested. The availability of a number of embryo-specific promoters with well characterized expression profiles during *Arabidopsis* embryo development made it possible to create transgenic plants in which *REV* over expression was targeted to several different but overlapping phases of embryo development. The *Arabidopsis REV* transgene constructs were introduced into canola for testing. The rationale for going directly to canola was that the *Arabidopsis* genome is very closely related to canola so the known expression characteristics of the promoters as determined in *Arabidopsis* would likely carry over to canola and that canola is a crop species. Demonstration of an effect on seed size in canola would have direct agricultural relevance. In a second example, the *Arabidopsis REV*

[0023] As used herein, a plant growth and/or development related gene is a gene that plays a role in determining growth rate, overall size, tissue size, or tissue number of a plant or plays a role in the plant developmental program leading to determination of tissue identity and morphology. Such growth and development related genes are identified when modification of their function by mutation, overexpression, or suppression of expression results in altered plant growth rate, overall plant size, tissue size or number, or altered development. Plant and growth related genes can exert their effects through a number of mechanisms some of which

include regulation of cell cycle, plant hormone synthesis/breakdown pathways, sensitivity to plant hormones, cell wall biosynthesis, cell identity determination, and the like.

[0024] A number of plant genes have been shown by overexpression or suppression analysis to play roles in growth and/or development. Examples of some, but not all, of the genes that are known to be involved in growth and/or development and that can be used or tested in the methods of the present invention are discussed herein below. The *Arabidopsis* *CAP* gene encodes a cyclase-associated protein that is involved in Ras-cAMP signaling and regulation of the actin cytoskeleton. Overexpression of *CAP* under a glucocorticoid-inducible promoter causes a loss of actin filaments and a reduction in the size of leaves due to reduced elongation of epidermal and mesophyll cells (Barrero *et al.*, *Annals of Botany* 91:599-603, 2003). Suppression of sucrose synthase gene expression in cotton leads to reduced cell fiber length and smaller and fewer fiber cells (Yong-Ling Ruan *et al.*, *Plant Cell* 15:952-964, 2003). Overexpression of the rice *histone deacetylase 1* gene with an ABA-inducible promoter in transgenic rice resulted in plants with an increase in growth rate and abnormal shoot and root tissue development compared to the wild-type (In-Cheol Jang *et al.*, *Plant J.* 33:531-541, 2003). Suppression of E2Fc by RNAi in *Arabidopsis* increases proliferative activity in leaves, meristems, and pericycle cells. Cells in organs were smaller but more numerous than wild type and there was a reduced ploidy level in the leaves (del Pozo *et al.*, *Plant Cell* 18:2224-2235, 2006). Suppression of the *BKI* gene by RNAi resulted in seedlings with increased hypocotyls lengths and overexpression of *BKI* gave dwarf plants (Xuelu and Chory, *Science* 313:1118-1122, 2006). Transgenic plants expressing a partially constitutive steroid receptor BRI1 have longer hypocotyls (Wang *et al.*, *Dev. Cell* 8:855-865, 2005). Suppression of Argos-Like (ARL) in *Arabidopsis* gave smaller cotyledons, leaves and other lateral organs, while overexpression gave the opposite effect. The change in organ size can be attributed to cell size rather than to cell number (Hu *et al.*, *Plant J.* 47:1-9, 2006).

[0025] Analysis of plants with mutations resulting in altered growth and/or developmental phenotypes has identified a number of genes that play roles in plant growth and development. A mutation affecting brassinosteroid hormone perception, *bri1-5*, results in a dwarf plant (Wang *et al.*, *Dev. Cell* 8:855-865, 2005). A T-DNA insertion (a knock-out) in the *Arabidopsis* *FATB* gene encoding an acyl-acyl carrier protein thioesterase leads to reduced growth rate, reduced fresh weight and low seed viability (Bonaventure *et al.*, *Plant Cell* 15:1020-1033, 2003). A loss-of-function mutation in Pepino, a putative anti-phosphatase, displayed tumor-like cell proliferation at the shoot apical meristem and produced

supernumerary abnormal leaves (Haberer *et al.*, *Dev. Genes Evol.* 212:542-550, 2002). The *Arabidopsis* *RGS* gene (regulator of G protein signaling) has the structure of a G-protein-coupled receptor (GPCR) and contains an RGS box. RGS proteins accelerate the deactivation of the G α subunit and thus reduce GPCR signaling. The null *rgs* mutant has increased cell elongation in hypocotyls grown in the dark and increased cell production in roots grown in light (Chen *et al.*, *Science* 301:1728-1731, 2003). The *Arabidopsis* *TIP1* gene plays a role in root hair development and also in cellular growth. The *tip1-2* mutant has smaller rosettes, reduced height and shorter internodes (Ryan *et al.*, *New Phytol.* 138:49-58, 1998 and Hemsley *et al.*, *Plant Cell* 17:2554-2563, 2005). Mutants (chromosomal rearrangement or T-DNA insertion) of the *Big Brother* (*BB*) gene that give very little or no Big Brother mRNA develop larger floral organs, more flower biomass and thicker stems. Conversely, overexpression of Big Brother leads to smaller floral organs, less flower biomass, thinner stems and reduced leaf size. BB may be altering cell number (Disch *et al.*, *Curr. Biol.* 16:272-279, 2006). The *RHD2* gene encodes an NADPH oxidase important for accumulation of reactive-oxygen species in root hairs and the subsequent activation of calcium channels. The *rhd2* mutant is defective in cell expansion of the tip growing cells of the root (Foreman *et al.*, *Nature* 422:442-446, 2003). The miniature mutation in maize causes a loss in the cell wall invertase, expressed from the *INCW2* gene. Cells of the *mn1* mutant are smaller than wild-type and *mn1* seed mutants only have 20% of the endosperm weight of wild type seeds. Expansion may be compromised in cells of the peripheral layers of the *mn1* endosperm and may lead to decreased mitotic activity of these cells (Vilhar *et al.*, *Plant Physiol.* 129: 23-30, 2002). A T-DNA insertion mutant of WAK2, *wak2-1*, has decreased cell elongation in roots. WAK2 may control cell expansion through regulation of vacuolar invertase activity. Expression of an inducible antisense of WAK2 or WAK4 in plants prevents cell elongation and produces dwarf plants (Wagner and Kohorn, *Plant Cell* 13:303-318, 2001, Lally *et al.*, *Plant Cell* 13:1317-1331, 2001, and Kohorn *et al.*, *Plant J.* 46:307-316, 2006). The *Arabidopsis* gene *AP2* plays a role in floral organ identity and establishment of floral meristem identity. Loss-of-function mutations in *AP2* gives increased seed mass compared to the wild type (Masa-Ohto *et al.*, *Proc. Nat'l. Acad. Sci. USA* 102:3123-3128, 2005). *teb* mutants have short roots, serrated leaves, and fasciation. They show defects in cell division that may be caused by a defect in G2/M cell cycle progression (Inagaki *et al.*, *Plant Cell* 18:879-892, 2006).

[0026] The terms "growth and/or development gene" or "growth and/or development transgene" are used herein to mean any polynucleotide sequence that encodes or facilitates the expression and/or production of a nucleotide or protein encoded by the gene. Thus the terms "growth and/or development gene" or "growth and/or development transgene" can include sequences that flank the nucleotide and/or protein encoding sequences. For example, the sequences can include those nucleotide sequences that are protein encoding sequences (exons), intervening sequences (introns), the flanking 5' and 3' DNA regions that contain sequences required for normal expression of the gene (i.e., the promoter and polyA addition regions, respectively, and any enhancer sequences).

[0027] The terms "growth and/or development protein," "growth and/or development homolog" or "growth and/or development associated ortholog" are used herein to mean protein having the ability to regulate growth rate, overall size, tissue size, or tissue number of a plant or regulate the plant developmental program leading to determination of tissue identity and morphology (when utilized in the practice of the methods of the present invention) and that have an amino acid sequence that is at least about 70 % identical, more typically at least about 75 % identical, and more typically at least about 80 % identical to the amino acid sequences for the gene.

[0028] As used herein an "embryo-specific gene" is a gene that is preferentially expressed during embryo development in a plant. For purposes of the present disclosure, embryo development begins with the first cell divisions in the zygote and continues through the late phase of embryo development (characterized by maturation, desiccation, dormancy), and ends with the production of a mature and desiccated seed. Embryo-specific genes can be further classified as "early phase-specific" and "late phase-specific". Early phase-specific genes are those expressed in embryos up to the end of embryo morphogenesis. Late phase-specific genes are those expressed from maturation through to production of a mature and desiccated seed. Examples of embryo-specific genes that initiate expression during early embryo development and are early phase-specific are presented in Figure 1.

[0029] A "heterologous sequence" is an oligonucleotide sequence that originates from a different species, or, if from the same species, is substantially modified from its original form. For example, a heterologous promoter operably linked to a structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, is substantially modified from its original form.

[0030] The term "vector" refers to a piece of DNA, typically double-stranded, which may have inserted into it a piece of foreign DNA. The vector or replicon may be for example, of plasmid or viral origin. Vectors contain "replicon" polynucleotide sequences that facilitate the autonomous replication of the vector in a host cell. The term "replicon" in the context of this disclosure also includes polynucleotide sequence regions that target or otherwise facilitate the recombination of vector sequences into a host chromosome. In addition, while the foreign DNA may be inserted initially into, for example, a DNA virus vector, transformation of the viral vector DNA into a host cell may result in conversion of the viral DNA into a viral RNA vector molecule. Foreign DNA is defined as heterologous DNA, which is DNA not naturally found in the host cell, which, for example, replicates the vector molecule, encodes a selectable or screenable marker or transgene. The vector is used to transport the foreign or heterologous DNA into a suitable host cell. Once in the host cell, the vector can replicate independently of or coincidental with the host chromosomal DNA, and several copies of the vector and its inserted DNA can be generated. Alternatively, the vector can target insertion of the foreign or heterologous DNA into a host chromosome. In addition, the vector can also contain the necessary elements that permit transcription of the inserted DNA into a mRNA molecule or otherwise cause replication of the inserted DNA into multiple copies of RNA. Some expression vectors additionally contain sequence elements adjacent to the inserted DNA that allow translation of the mRNA into a protein molecule. Many molecules of mRNA and polypeptide encoded by the inserted DNA can thus be rapidly synthesized.

[0031] The term "transgene vector" refers to a vector that contains an inserted segment of DNA, the "transgene," that is transcribed into mRNA or replicated as a RNA within a host cell. The term "transgene" refers not only to that portion of inserted DNA that is converted into RNA, but also those portions of the vector that are necessary for the transcription or replication of the RNA. In addition, a transgene need not necessarily comprise a polynucleotide sequence that contains an open reading frame capable of producing a protein.

[0032] The terms "transformed host cell," "transformed," and "transformation" refer to the introduction of DNA into a cell. The cell is termed a "host cell," and it may be a prokaryotic or a eukaryotic cell. Typical prokaryotic host cells include various strains of *E. coli*. Typical eukaryotic host cells are plant cells (*e.g.*, canola, cotton, camelina, alfalfa, soy, rice, oat, wheat, barley, or corn cells, and the like), yeast cells, insect cells, or animal cells. The introduced DNA is usually in the form of a vector containing an inserted piece of DNA. The

introduced DNA sequence may be from the same species as the host cell or from a different species from the host cell, or it may be a hybrid DNA sequence, containing some foreign DNA and some DNA derived from the host species.

[0033] The term "plant" includes whole plants, plant organs, (*e.g.*, leaves, stems, flowers, roots, and the like), seeds and plant cells (including tissue culture cells) and progeny of same. The class of plants which can be used in the methods of the present invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants, as well as certain lower plants such as algae. It includes plants of a variety of ploidy levels, including polyploidy, diploid and haploid.

[0034] A "heterologous sequence" is one that originates from a foreign species, or, if from the same species, is substantially modified from its original form. For example, a heterologous promoter operably linked to a structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, is substantially modified from its original form.

[0035] The terms "*REVOLUTA* gene" or "*REVOLUTA* transgene" are used herein to mean any polynucleotide sequence that encodes or facilitates the expression and/or production of a *REVOLUTA* protein. Thus the terms "*REVOLUTA* gene" or "*REVOLUTA* transgene" can include sequences that flank the *REVOLUTA* protein encoding sequences. For example, the sequences can include those nucleotide sequences that are protein encoding sequences (exons), intervening sequences (introns), the flanking 5' and 3' DNA regions that contain sequences required for normal expression of the *REVOLUTA* gene (*i.e.*, the promoter and polyA addition regions, respectively, and any enhancer sequences).

[0036] The terms "*REVOLUTA* protein," "*REVOLUTA* homolog" or "*REVOLUTA* ortholog" are used herein to mean protein having the ability to regulate plant cell division (when utilized in the practice of the methods of the present invention) and that have an amino acid sequence that is at least about 70 % identical, more typically at least about 75 % identical, and more typically at least about 80 % identical to the amino acid sequences for *REVOLUTA* described in WO 01/33944 (incorporated herein by reference in its entirety).

[0037] Alternatively, the terms "*REVOLUTA* protein" "*REVOLUTA* homolog", or "*REVOLUTA* ortholog" are used herein to mean *REVOLUTA* proteins that are identified as distinct from non-*REVOLUTA* members of the HD-ZIPIII class of plants transcription factors. The *REVOLUTA* members of the HD-ZIPIII class of proteins are characterized by

the lack or absence of a characteristic amino acid sequence insertion that is present in non-REVOLUTA HD-ZIPIII proteins between amino acid residues 146 and 147 of the REVOLUTA amino acid sequence described in WO 01/33944 and as shown in Figure 2. In Figure 2, Homeobox transcription factors from *Arabidopsis thaliana* designated athb-8, athb-9, athb-14 and athb-15 are non-REVOLUTA HD-ZIPIII proteins and all have a characteristic amino acid sequence insertion between amino acids 146 and 147 of the REVOLUTA amino acid sequence. The five REVOLUTA sequences in Figure 2, all lack an amino acid insertion at this location in the REVOLUTA amino acid sequence. The lack of this amino acid sequence insertion is a distinguishing and defining characteristic of REVOLUTA proteins.

[0038] The term "percent identity" means the percentage of amino acids or nucleotides that occupy the same relative position when two amino acid sequences, or two nucleic acid sequences are aligned side by side using a computer program such as one identified below. The term "percent similarity" is a statistical measure of the degree of relatedness of two compared protein sequences. The percent similarity is calculated by a computer program that assigns a numerical value to each compared pair of amino acids based on chemical similarity (*e.g.*, whether the compared amino acids are acidic, basic, hydrophilic, aromatic, and the like) and/or evolutionary distance as measured by the minimum number of base pair changes that would be required to convert a codon encoding one member of a pair of compared amino acids to a codon encoding the other member of the pair. Calculations are made after a best fit alignment of the two sequences has been made empirically by iterative comparison of all possible alignments. (*See for example, Henikoff et al., Proc. Natl. Acad. Sci. USA 89:10915-10919, 1992.*)

[0039] The term "substantial identity" of poly nucleotide sequences means that a polynucleotide comprises a sequence that has at least 60 % sequence identity, typically at least 70 %, more typically at least 80 % and most typically at least 90 %, compared to a reference sequence using the programs described below 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.

[0040] Amino acid sequence identity can be determined, for example, in the following manner. The portion of the amino acid sequence of the protein encoded by the growth and/or development associated gene, *e.g.*, REVOLUTA, can be used to search a nucleic acid

sequence database, such as the GenBank[®] database, using the program BLASTP version 2.0.9 (Atschul *et al.*, *Nucl. Acids Res.* 25:3389-3402, 1997). Sequence comparisons between two (or more) polynucleotides or polypeptides are typically performed by comparing sequences of the two sequences over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" as used herein refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence can be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

[0041] Optimal alignment of sequence for comparison can be conducted by local identity or similarity algorithms such as those described in Smith *et al.*, *Adv. Appl. Math.* 2:482, 1981, by the homology alignment algorithm of Needleman *et al.*, *J. Mol. Biol.* 48:443-453, 1970, by the search for similarity method of Pearson *et al.*, *Proc. Natl. Acad. Sci. USA* 85:2444-2448, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), or by visual inspection.

[0042] One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng *et al.*, *J. Mol. Evol.* 35:351-360, 1987. The method used is similar to the method described by Higgins *et al.*, *CABIOS* 5:151-153, 1989. The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most related sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their nucleotide or amino acid coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

[0043] Another example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al.*, *J. Mol. Biol.* 215:403-410, 1990. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information web site. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W , T , and X determine the sensitivity and the speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see, Henikoff *et al.*, *Proc. Natl. Acad. Sci. USA* 89:10915-10919, 1992) alignments (B) of 50, expectation (E) of 10, $M=5$, $N=-4$, and a comparison of both strands.

[0044] In addition to calculating percent sequence identity, the BLAST algorithm also performs statistical analysis of the similarity between two sequences (see *e.g.*, Karlin *et al.*, *Proc. Natl. Acad. Sci. USA* 90:5873-5877, 1993). One measure of similarity provided by the BLAST algorithm is the smallest sum probability ($P(N)$), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison test is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. Additional methods and algorithms for sequence alignment and analysis of sequence similarity are well known to the skilled artisan.

[0045] In the case where the inserted polynucleotide sequence is transcribed and translated to produce a functional polypeptide, one of skill will recognize that because of codon degeneracy a number of polynucleotide sequences will encode the same polypeptide. These variants are specifically covered by the terms "growth and/or development gene" and "growth

and/or development transgene", and specifically "*REVOLUTA* gene" and "*REVOLUTA* transgene". In addition, these terms specifically include those full length sequences substantially identical with a gene sequence and that encode a protein that retains the function of the gene product, *e.g.*, *REVOLUTA*. Two nucleic acid sequences or polynucleotides are said to be "identical" if the sequences of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described above. The term "complementary to" is used herein to mean that the complementary sequence is identical to all or a portion of a reference polynucleotide sequence.

[0046] Variations and alterations in the amino acid sequence of the growth and/or development associated gene and growth and/or development associated protein, *e.g.*, the *REVOLUTA* gene and *REVOLUTA* protein sequences are described in WO 01/33944, incorporated herein by reference. The gene of interest, such as the *REVOLUTA* gene, polynucleotide or polynucleotide sequence can be isolated from or obtained from any plant species. In a particular embodiment of the present invention the *REVOLUTA* gene sequence used is that from *Arabidopsis thaliana*, but the *REVOLUTA* gene from other species of interest can also be used. For example the nucleotide sequence and amino acid sequence for *REVOLUTA* from corn (*Zea mays*) is described in WO 2004/063379 (incorporated herein by reference in its entirety).

[0047] The terms "biological activity", "biologically active", "activity", "active", "biological function", "REV biological activity", and functionally active" refer to the ability of the protein of interest, such as *REVOLUTA* proteins to dimerize (or otherwise assemble into protein oligomers), or the ability to modulate or otherwise effect the dimerization of native wild-type (*e.g.*, endogenous) *REVOLUTA* protein. However, the terms are also intended to encompass the ability of a protein of interest, such as the *REVOLUTA* proteins, to bind and/or interact with other molecules, including for example, but not by limitation, DNA containing specific nucleotide sequences in promoter regions recognized by the protein, *e.g.*, the *REVOLUTA* protein, and which binding and/or interaction event(s) mediate plant cell division and ultimately confer a phenotype, or the ability to modulate or otherwise effect the binding and/or interaction of other molecules with native wild-type protein and which binding and/or interaction event(s) mediate plant cell division and ultimately confer a phenotype associated with the gene of interest.

[0048] REV phenotype as used herein is intended to refer to a phenotype conferred by a REV nucleic acid or protein and particularly encompasses the characteristic wherein the seed size or seed number is exhibited. Typically, a REV phenotype is determined by examination of a plant over expressing REV during early phase-specific embryo development, where the number and size of seeds from the plant can be compared to the number and size of seeds in the corresponding tissues of a parental or wild-type plant. Plants having the REV phenotype have a statistically significant change in the number and/or size of the seeds within a representative number of a plant population.

[0049] A promoter suitable for being operably linked to a plant growth and/or development associated gene and expressed using the described methods of the present invention typically has greater expression in embryo and lower or no expression in other plant tissues. Of particular interest are those promoter sequences that initiate expression in early phase-specific embryo development. An early phase-specific promoter is a promoter that initiates expression of a protein prior to day 7 after pollination (walking stick) in *Arabidopsis* or an equivalent stage in another plant species. Examples of promoter sequences of particular interest include a promoter for the amino acid permease gene (AAP1) (*e.g.*, the AAP1 promoter from *Arabidopsis thaliana*) (Hirner *et al.*, *Plant J.* 14:535-544, 1998), a promoter for the oleate 12-hydroxylase:desaturase gene (*e.g.*, the promoter designated LFAH12 from *Lesquerella fendleri*) (Broun *et al.*, *Plant J.* 13:201-210, 1998), a promoter for the 2S2 albumin gene (*e.g.*, the 2S2 promoter from *Arabidopsis thaliana*) (Guerche *et al.*, *Plant cell* 2:469-478, 1990), a fatty acid elongase gene promoter (FAE1) (*e.g.*, the FAE1 promoter from *Arabidopsis thaliana*) (Rossak *et al.*, *Plant Mol. Biol.* 46:717-715, 2001), and the leafy cotyledon gene promoter (LEC2) (*e.g.*, the LEC2 promoter from *Arabidopsis thaliana*) (Kroj *et al.* *Development* 130:6065-6073, 2003). Other early embryo-specific promoters of interest include, but are not limited to, Seedstick (Pinyopich *et al.*, *Nature* 424:85-88, 2003), Fbp7 and Fbp11 (Petunia Seedstick) (Colombo *et al.*, *Plant Cell.* 9:703-715, 1997), Banyuls (Devic *et al.*, *Plant J.* 19:387-398, 1999), agl-15 and agl-18 (Lehti-Shiu *et al.*, *Plant Mol. Biol.* 58:89-107, 2005), Phe1 (Kohler *et al.*, *Genes Develop.* 17:1540-1553, 2003), Per1 (Haslekas *et al.*, *Plant Mol Biol.* 36:833-845, 1998; Haslekas *et al.*, *Plant Mol. Biol.* 53:313-326, 2003), emb175 (Cushing *et al.*, *Planta* 221:424-436, 2005), L11 (Kwong *et al.*, *Plant Cell* 15:5-18, 2003), Lec1 (Lotan *et al.*, *Cell* 93:1195-1205, 1998), Fusca3 (Kroj *et al.*, *Development* 130:6065-6073, 2003), tt12 (Debeaujon *et al.*, *Plant Cell* 13:853-871, 2001), ttl6 (Nesi *et al.*, *Plant Cell* 14:2463-2479, 2002), A-RZf (Zou and Taylor, *Gene* 196:291-295, 1997), TtG1

(Walker *et al.*, *Plant Cell* 11:1337-1350, 1999; Tsuchiya *et al.*, *Plant J.* 37:73-81, 2004), Tt1 (Sagasser *et al.*, *Genes Dev.* 16:138-149, 2002), TT8 (Nesi *et al.*, *Plant Cell* 12:1863-1878, 2000), Gea-8 (carrot) (Lin and Zimmerman, *J. Exp. Botany* 50:1139-1147, 1999), Knox (rice) (Postma-Haarsma *et al.*, *Plant Mol. Biol.* 39:257-271, 1999), Oleosin (Plant *et al.*, *Plant Mol. Biol.* 25:193-205, 1994; Keddie *et al.*, *Plant Mol. Biol.* 24:327-340, 1994), ABI3 (Ng *et al.*, *Plant Mol. Biol.* 54:25-38, 2004; Parcy *et al.*, *Plant Cell* 6:1567-1582, 1994), and the like.

[0050] The promoters suitable for use in the present invention can be used either from the same species of plant to be transformed or can be from a heterologous species. Further, the promoter can be from the same species as for the *REV* transgene to be used or it can be from a heterologous species. Promoters for use in the methods of the present invention can also comprise a chimeric promoter which can include a combination of promoters that have an expression profile in common with one or more of those described above. In one embodiment of the present invention, the AAP1 gene promoter from *Arabidopsis thaliana* was combined with the *Arabidopsis thaliana REV* gene and used to construct transgenic canola (*Brassica napus*). Further, in an additional embodiment of the present invention the oleate 12-hydroxylase:desaturase gene promoter LFAH12 from *Lesquerella fendleri* was operatively linked to the *Arabidopsis thaliana REV* gene and used to construct transgenic canola (*Brassica napus*). Each of the above transgenic plants demonstrated the *REV* phenotype characteristic of the methods of the present invention wherein *REV* is over expressed in early embryo development resulting in increased seed size and/or seed number.

[0051] It should be noted that the early phase-specific promoters described above are only representative promoters that can be used in the methods of the present invention. Methods for identifying and characterizing promoter regions in plant genomic DNA are well known to the skilled artisan and include, for example, those described by Jordano *et al.*, *Plant Cell* 1:855-866, 1989; Bustos *et al.*, *Plant Cell* 1:839-854, 1989; Green *et al.*, *EMBO J.* 7:4035-4044, 1988; Meier *et al.*, *Plant Cell* 3:309-316, 1991; and Zhang *et al.*, *Plant Physiol.* 110:1069-1079, 1996.

[0052] Transgenic plants which over express *REV* in early phase embryos can be obtained, for example, by transferring transgenic vectors (*e.g.*, plasmids, virus, and the like) that encode an early phase-specific embryo promoter operatively linked to a gene that encodes *REVOLUTA* into a plant. Typically, when the vector is a plasmid the vector also includes a selectable marker gene, *e.g.*, the kanamycin gene encoding resistance to kanamycin. The

most common method of plant transformation is performed by cloning a target transgene into a plant transformation vector that is then transformed into *Agrobacterium tumifaciens* containing a helper Ti-plasmid as described in Hoeckeme *et al.*, (*Nature* 303:179-181, 1983). Additional methods are described in for example, Maloney *et al.*, *Plant Cell Reports* 8:238, 1989. The *Agrobacterium* cells containing the transgene vector can be incubated with leaf slices of the plant to be transformed as described by An *et al.* (*Plant Physiol.* 81:301-305, 1986; Hooykaas, *Plant Mol. Biol.* 13:327-336, 1989). Transformation of cultured plant host cells is typically accomplished through *Agrobacterium tumifaciens*, as described above. Cultures of host cells that do not have rigid cell membrane barriers are usually transformed using the calcium phosphate method as originally described by Graham *et al.* (*Virology* 52:546, 1978) and modified as described in Sambrook *et al.* (*Molecular Cloning : A Laboratory Manual* (2nd Ed., 1989 Cold Spring Harbor Laboratory Press, New York, NY). However, other methods for introducing DNA into cells such as Polybrene (Kawai *et al.*, *Mol. Cell. Biol.* 4:1172, 1984), protoplast fusion (Schaffner, *Proc. Natl. Acad. Sci. USA* 77:2163, 1980), electroporation (Neumann *et al.*, *EMBO J.* 1:841, 1982), and direct microinjection into nuclei (Capecchi, *Cell* 22:479, 1980) can also be used. Transformed plant calli can be selected through the selectable marker by growing the cells on a medium containing, *e.g.*, kanamycin, and appropriate amounts of phytohormone such as naphthalene acetic acid and benzyladenine for callus and shoot induction. The plant cells can then be regenerated and the resulting plants transferred to soil using techniques well known to those skilled in the art.

[0053] In addition to the methods described above, a large number of methods are well known in the art for transferring cloned DNA into a wide variety of plant species, including gymnosperms, angiosperms, monocots and dicots (see, *e.g.*, Glick and Thompson, eds., *Methods in Plant Molecular Biology and Biotechnology*, CRC Press, Boca Raton, Florida, 1993; Vasil, *Plant Mol. Biol.*, 25:925-937, 1994; and Komai *et al.*, *Current Opinions Plant Biol.* 1:161-165, 1998 (general review); Loopstra *et al.*, *Plant Mol. Biol.* 15:1-9, 1990; and Brasileiro *et al.*, *Plant Mol. Biol.* 17:441-452, 1990 (transformation of trees); Eimert *et al.*, *Plant Mol. Biol.* 19:485-490, 1992 (transformation of *Brassica*); Hiei *et al.*, *Plant J.* 6:271-282, 1994; Hiei *et al.*, *Plant Mol. Biol.* 35:205-218, 1997; Chan *et al.*, *Plant Mol. Biol.* 22:491-506, 1993; U.S. Patent Nos. 5,516,668 and 5,824,857 (rice transformation); and U.S. Patent Nos. 5,955,362 (wheat transformation); 5,969,213 (monocot transformation); 5,780,798 (corn transformation); 5,959,179 and 5,914,451 (soybean transformation).

Representative examples include electroporation-facilitated DNA uptake by protoplasts (Rhodes *et al.*, *Science* 240:204-207, 1988; Bates, *Meth. Mol. Biol.* 111:359-366, 1999; D'Halluin *et al.*, *Meth. Mol. Biol.* 111:367-373, 1999; U.S. Patent No. 5,914,451); treatment of protoplasts with polyethylene glycol (Lyznik *et al.*, *Plant Mol. Biol.* 13:151-161, 1989; Datta *et al.*, *Meth. Mol. Biol.*, 111:335-334, 1999); and bombardment of cells with DNA laden microprojectiles (Klein *et al.*, *Plant Physiol.* 91:440-444, 1989; Boynton *et al.*, *Science* 240:1534-1538, 1988; Register *et al.*, *Plant Mol. Biol.* 25:951-961, 1994; Barcelo *et al.*, *Plant J.* 5:583-592, 1994; Vasil *et al.*, *Meth. Mol. Biol.* 111:349-358, 1999; Christou, *Plant Mol. Biol.* 35:197-203, 1997; Finer *et al.*, *Curr. Top. Microbiol. Immunol.* 240:59-80, 1999). Additionally, plant transformation strategies and techniques are reviewed in Birch, *Ann. Rev. Plant Phys. Plant Mol. Biol.* 48:297, 1997; Forester *et al.*, *Exp. Agric.* 33:15-33, 1997. Minor variations make these technologies applicable to a broad range of plant species.

[0054] In the case of monocot transformation, particle bombardment is typically the method of choice. However, monocots such as maize can also be transformed by using *Agrobacterium* transformation methods as described in United States Patent 5,591,616. Another method to effect monocot transformation, *e.g.*, corn, mixes cells from embryogenic suspension cultures with a suspension of fibers (5 % w/v, Silar SC-9 whiskers) and plasmid DNA (1 µg/ul) and which is then placed either upright in a multiple sample head on a Vortex GENIE II vortex mixer (Scientific Industries, Inc., Bohemia, NY, USA) or horizontally in the holder of a MIXOMAT dental amalgam mixer (Degussa Canada Ltd., Burlington, Ontario, Canada). Transformation can then be carried out by mixing at full speed for about 60 seconds (for example with a Vortex GENIE II) or shaking at fixed speed for 1 second (MIXOMAT). This process results in the production of cell populations out of which stable transformants can be selected. Plants are regenerated from the stably transformed calluses and these plants and their progeny can be shown by Southern hybridization analysis to be transgenic. The principal advantages of the approach are its simplicity and low cost. Unlike particle bombardment, expensive equipment and supplies are not required. The use of whiskers for the transformation of plant cells, particularly maize, is described in, for example, United States Patent 5,464,765.

[0055] United States Patent 5,968,830 describes methods of transforming and regenerating soybean. United States Patent 5,969,215 describes transformation techniques for producing transformed *Beta vulgaris* plants, such as the sugar beet.

[0056] Each of the above transformation techniques has advantages and disadvantages. In each of the techniques, DNA from a plasmid is genetically engineered such that it contains not only the gene of interest, but also selectable and screenable marker genes. A selectable marker gene is used to select only those cells that have integrated copies of the plasmid (the construction is such that the gene of interest and the selectable and screenable genes are transferred as a unit). The screenable gene provides another check for the successful culturing of only those cells carrying the genes of interest.

[0057] Traditional *Agrobacterium* transformation with antibiotic resistance selectable markers can be problematical because of public opposition that such plants pose an undue risk-of spreading antibiotic tolerance to animals and humans. Such antibiotic markers can be eliminated from plants by transforming plants using the *Agrobacterium* techniques similar to those described in United States Patent 5,731,179. Antibiotic resistance issues can also be effectively avoided by the use of bar or pat coding sequences, such as is described in United States Patent Number 5,712,135. These preferred marker DNAs encode second proteins or polypeptides inhibiting or neutralizing the action of glutamine synthetase inhibitor herbicides phosphinothricin (glufosinate) and glufosinate anunonium salt (Basta, Ignite).

[0058] The plasmid containing one or more of these genes is introduced into either plant protoplasts or callus cells by any of the previously mentioned techniques. If the marker gene is a selectable gene, only those cells that have incorporated the DNA package survive under selection with the appropriate phytotoxic agent. Once the appropriate cells are identified and propagated, plants are regenerated. Progeny from the transformed plants must be tested to insure that the DNA package has been successfully integrated into the plant genome.

[0059] There are numerous factors that influence the success of transformation. The design and construction of the exogenous gene construct and its regulatory elements influence the integration of the exogenous sequence into the chromosomal DNA of the plant nucleus and the ability of the transgene to be expressed by the cell. A suitable method for introducing the exogenous gene construct into the plant cell nucleus in a non-lethal manner is essential. Importantly, the type of cell into which the construct is introduced must, if whole plants are to be recovered, be of a type which is amenable to regeneration, given an appropriate regeneration protocol.

[0060] Prokaryotes can also be used as host cells for the initial cloning steps of the present invention. Methods, vectors, plasmids and host cell systems are well known to the skilled

artisan that can be used for these initial cloning and expansion steps and will not be described herein.

[0061] In another embodiment of the present invention an early phase-specific embryo promoter can be inserted so as to be operatively linked to a gene encoding a plant growth and/or development associated gene, such *REV*, in the plant to be transformed using methods well known to the skilled artisan. Insertion of the promoter will allow for the early embryonic expression of the gene, *e.g.*, *REV*, in the developing seeds of the transgenic plant.

[0062] Transgenic plants of particular interest in the methods of the present invention include but are not limited to monocot and dicots particularly from the families *Brassicaceae* (*Cruciferae*), *Gramineae*, *Malvaceae*, or *Leguminosae-Papilionoideae*. Plants of particular interest within these families include, but are not limited to canola, corn, camelina cotton, wheat, rice, soybean, barley and other seed producing plants, as well as other plants including, but not limited to alfalfa, and the like, of agricultural interest which comprise in a particular embodiment of the present invention, a *REV* transgene under the control of an early phase-specific embryo promoter. The transgene can be from the same species as the transgenic plant, or the transgene can be from a heterologous plant. Of particular interest is a transgenic plant comprising the *REV* transgene from *Arabidopsis*. The early phase-specific embryo promoter can also be from the same species as the transgenic plant, or from a heterologous plant. For example, the early phase-specific embryo promoter can be from the same plant species as the *REV* transgene or even from another species of plant. Of particular interest are early phase-specific embryo promoters from *Arabidopsis* or *Lesquerella fendleri*, but the early phase-specific promoter can be obtained from another species of plant. Specific combinations of early phase-specific promoter and *REV* transgene that have been found to be suitable for the methods of the present invention include, but are not limited to (a) *Lesquerella fendleri* LFAH12 promoter/ *Arabidopsis* *REV*; (b) *Arabidopsis* AAP1 promoter/ *Arabidopsis* *REV*; (c) *Arabidopsis* LEC2 promoter/ *Arabidopsis* *REV*; and (d) *Arabidopsis* 2S2 promoter/ *Arabidopsis* *REV*. In a particular embodiment of the present invention, these transgene constructs have been used to transform canola, but can be used to transform other plant species. In particular, they can be used to produce transgenic plants having increased seed size and/or seed number in soybeans, corn, cotton, camelina, rice, wheat, barley, alfalfa, and other crops of agricultural interest.

[0063] The present invention also provides methods for selecting a growth and/or development associated gene that increases plant yield. In the method, a gene of interest is functionally associated with an early phase-specific embryo promoter in an expression plasmid or vector. The expression plasmid or vector comprising the gene of interest is transfected into a plant cell using a method known in the art to form a transgenic cell. The cell comprising the transgene is grown up and regenerated into a transgenic plant by known methods, including those disclosed above until transgenic plants are obtained. The transgenic plants are observed for increased yield as compared with a wild-type plant and those growth and/or development associated genes that were used to obtain the transgenic plants with increased yield are selected for further development. Transgenic plants comprising the selected growth and/or development associated gene can be further developed to provide plants of agricultural importance with a higher yield than the wild-type plants.

[0064] The following examples are provided merely as illustrative of various aspects of the invention and shall not be construed to limit the invention in any way.

Examples

[0065] The following example describes the construction of various expression vectors comprising a early phase-specific embryo promoter and a gene with a role in plant growth and/or development. In particular, the embryo specific promoters (a) *Lesquerella fendleri* LFAH12; (b) *Arabidopsis* AAP1; (c) *Arabidopsis* LEC2; (d) *Arabidopsis* 2S2, and (e) *Arabidopsis* FAE1 were operatively associated with the *Arabidopsis* *REVOLUTA* (*REV*) gene and used to produce transgenic canola plants.

Example 1: Transgenic Canola Plants Expressing Transgene Constructs Designed To Confer Embryo-Specific Expression Of *REVOLUTA*.

[0066] Constitutive over-expression of the *Arabidopsis* *REV* gene in transgenic *Arabidopsis* plants results in increased seed size relative to non-transgenic wild type *Arabidopsis* plants, but this trait is accompanied by negative pleiotropic effects. These effects include altered leaf morphology and reduced axillary shoot formation in *Arabidopsis*. Altered leaf morphology and overall stunted growth of plants are negative effects observed in transgenic canola over-expressing the *REV* gene at high constitutive levels throughout the plant. To obtain the increased seed size trait while avoiding undesirable effects in non-seed tissues, a strategy can be taken to target over-expression of the *REV* transgene specifically to the developing embryo in the seed. This can be accomplished using a transgene expression

construct in which an embryo-specific promoter drives the expression of the *REV* gene. Constitutive overexpression of other plant genes like *REV* involved in plant development and growth processes could also be reasonably expected to have negative pleiotropic effects on the transgenic plant. The use of embryo-specific promoters to evaluate plant genes that have roles in development and growth as possible yield enhancing genes offers a general approach to evaluate such plant genes for efficiency in increasing crop yield.

[0067] Five promoters that confer embryo-specific expression were selected for use in expression constructs designed to give transgenic expression of REV in canola embryos (*Brassica napus*) during early embryo development. These promoters include AAP1 (amino acid permease gene from *Arabidopsis thaliana*)(Hirner *et al.*, *Plant J.* 14:535-544, 1998),

GGTTGCATCT TTGAATACCT TTTTCTCATT TAGGCATAAC AATATAATAA
TTTGTTTTTT GTTTCATTT TCTTTTGGTG TCATCTTCAA AAATCTGTAA
ACCCAAAAGT TTGTATAACT TGTTTATTAA GATATTTTAA ATTAAATTTT
TTTTTTTGAC ATTTTAAAAA AATTATAAAG TGTTTATGA ATTTAAGGAG
TAAATAATAT TTATTTAGAA CACTATAAAT TAGTTTTACA AGTTCTTAGA
AATGTATCTG TAAATTTCAA AAAGGAAAAA TATAGCATTT AATTTTGAAG
ATTTTTTTCT ACATTATATA TATGATAAAA ATATTGTATT TTGTACTTTG
TAGTTACAAA AAGTCATTAT ATCAACAAAT CTAAATATAA AATATTTTTT
TATATATTAC TCCAAATTAA CTGTCAGAAT AAAAAAGAAG AATAATTATT
ACAGAATCTG AACATTAAAA TCGTCCCTCC ATATGTGGTC TCTGTCTAGT
CCAAAAGCAA TTTACACATC CCAAGCCGAA ACTATATTAA ATAAACATTT
TTTTTCTTT AACTAAAACA TTTATAACAT TTAACAATAA AAGTTAAAAA
TCGAACACGT ATAACGTATT TTTTACGTA TACGTCTTGT TGGCATATAT
GCTTAAAAAC TTCATTACAT ACATATACAA GTATGTCTAT ATATATGATA
TTATGCAAAC ACAAATCTGT TGACTATAAT TAGACTTCTT CATTACTCT
CTCTCTGACT TAAACATTT ATTTTATCTT CTTCTTGTTT TCTCTTCTC
TTTCTCTCA (SEQ ID NO:11);

LFAH12 (oleate 12-hydroxylase:desaturase gene from *Lesquerella fendleri*)(Broun *et al.*, *Plant J.* 13:201-210, 1998),

TCAGGAAGAT TAAGTCTTTG CTTGTTGTCT GATTTTCTTT AAATACATTA
AGAAATCGGT TATGAAGCTT CGTTTTTTGT GTTTTGGGAT TATGAAGCTG

TCTTTGGATA TTAGTTGCGG TTATTAGCAT GCTTCTCTTT TGTGTTTTGG
GGATGATGAA GCAGGGTCTC TCTATGTAAT GCATTTTGTG TGAAAACCTCA
GCTAATGCTA ATGCAATTC TTTTGAAACC TTTGTTATGT TTTCAAAAAT
ATTGAATAGG TTCTGTTATG GATTTATTTG CAAAAGCCAT TGATTAAATC
AAACCATTAC ATAAGAACAA CATTCAATTAT TAACTAATTA GAGATGCAAA
ACACAACATT ACATACAACA TCAGTGACTA ATTATTGAGA CAAAACAACA
TCACAGACAC AAACATTTCAT CTCATACATC ACTTAGAGAG ACACAAAAAG
CAACCAAACA CAACTATTCC GCCAACAACA ATTAGCTTCA TACGTTTTGC
TTCTCCTTTC AAGCCTTCAA TCATCTTCTC ACAGCCACGA ATCTGAGCCT
TCAATAATAA CATTTCTTCA TCGTGACACT TCTCACGGTT ATGAATGCAA
GCCTTTATGT CCTCTACTTC TTCTACTAAA GACACATCGG TCCACTTCCA
GGTGTGGAAT CCTCCTCTTT TGAAATTTTT CTCACAGGTA TGGAATAATC
TACCTGGGTT TTTTGGAGTT CTTGAGGTTT TGATCACAAC ACGGCATCCA
CATCGACAGG TCTTAGGAAA ACCACGAAGG TTATGATCTT CAAGCTCACT
GTCAAAAGAT AAAAACGAGT TTGAAGAAGA AGAAGGCATT ATCAATTTCA
GAGAATTTTG GAGAATTTTG AGAGATTGAG AATTGGGAAA TAAGAACCCT
AATCCCAAT TTATGAGATT GAAAATATAT CCGTTAGAGA AGAAACATAA
TGTTGTGCGT TTTAATTAGA AAAAATAGAG ATGGGCTTTA TCTTTTGTTA
AGAGTTTTGG GCTTGGGCTT GGGTTTTTGA TAAAAAATT AATTAAACCA
AAACGACGTC GTTTGGTTTA ATTGTTGTTA AAAAAAATT AAAACACCAA
AACGACGTCG TTTTGGTGTT ATTAACGGCC TAAAACGGA TTAATCCAT
AATCCGTCAG TCAACTAGGG TTACGGATGG TCAACGGCGT TTTTGCATAA
CGGAGGCACA GTTCAGGCTT AACGGAGTGG ACGGAATGGC TTTTAGGAA
GTTTGTAACC GGGGTCTTTT GTTTATGATG TATTTGTCCC CGTCGGCTAT
TGTTCAGGCC GTTTAGGCCT TTTTCTATA TACTGGAAAT AACTATTGTC
CAGACGAGTT ACTTCTCCAA CATATCAAGA AGTGTTACAA AGATGTGTTA
CGAAGCCATA AAACCTCAAAA CCCTAAGCCT AAACCCTAGA ACTTTCTAGC
ACGTTTATAC CTTCTCCTTT CTTTAGTTTC CTTTAAAGGC CTTCGTATCA
TAAGTTTTAT TTTTGCTTAA TACTAACACT AGAAAAAAC AATAATCAAC
ATAAACTAGG TTAAGTCGTG GATCTAATTT TATTGTGAAA ATGTAATTGC
TTCTCTTAAG AAAAGATTCA TAGCAAAATA TTCGCATCTT TCTTGTGAAT
CATCTTTTGT TTTTGGGGCT ATTAAGAGAAA AATTGAACTC ATGAAATGGT
GACAACTTTA TTCTAGAGGT AACAGAACAA AAATATAGGA ACAACACGTG
TTGTTCATAA ACTACACGTA TAATACTCAA GAAGATGAAT CTTTATAAGA

ATTTAGTTTT CTCATGAAAA CATAAAAAGT TTTGTCAATT GAAAGTGACA
 GTTGAAGCAA AGGAACAAAA GGATGGTTGG TGATGATGCT GAAATGAAAA
 TGTGTCATTC ATCAAATACT AAATACTACA TTA CTGTGCA CTGCCTACTT
 CTCCTCTTTC CTCCGCCACC CATTTTGGAC CCACGAGCCT TCCATTTAAA
 CCCTCTCTCG TGCTATTAC CAGAATAGAA GCCAAGAGAG AGAGAGAGAT
 TGTGCTGAGG ATCATTGTCT TCTTCATCGT TATTAACGTA AGTTTTTTTT
 TGACCACTTA TATCTAAAAT CTAGTACATG CAATAGATTA ATGACTGTTC
 CTTCTTTTGA TATTTTCAGC (SEQ ID NO:12);

2S2 (2S2 albumin gene from *Arabidopsis thaliana*)(Guerche *et al.*, *Plant Cell* 2:469-478, 1990), FAE1 (fatty acid elongase gene from *Arabidopsis thaliana*)(Rossak *et al.*, *Plant Mol. Biol.* 46:717-725, 2001),

and LEC2 (leafy cotyledon gene from *Arabidopsis thaliana*)(Kroj *et al.*, *Development* 130:6065-6073, 2003).

CTTTGTTTTG TAGAGTGTTT TATGGGTTAT GATTTCGAAA AGAAAAAAAAA
 TTGTGAGACA CTTAATAAAA TTATTTTCGAC AAAAAAAGTA GCTTGTATAA
 AAAAATCAGA TTTTAATT TAAGTAAGAACA AATTCCAATA TCCAATAGTT
 AAAAATAATT ATTTGTTCCG ATTAATCGAG TTTTGCAAAA TATGCACAAA
 ATCTATCAT GTACCATTTC TAAGACTATA TATTTGGTTA TATATTTTAT
 GCCGTGTGTT CTGATTCCAA TAAATTTTAT CGCATAGTAA ATTTTCTAAA
 AAGCAAAATT TTCTCAAAAG TGTACTAATG ACAATTAATT GAGTTTCTAC
 AAAATAAGAA TAACTATTGA CTCGATTTTC ACAAACACTAG TATGCTAAAT
 ATCACATTAC TTTTAAAATT AAATGGAATT ATCTTTTTC AATATTGGATA
 CGAATAATTT TTACACTAAA GTTATTTTAA TAAATAACC GTTTATTCAA
 AATATGTAAA GACGACAAAA ATATATATTA AATGGAAAAA CGACTAACTT
 AGTTTTTGCA AAATTAAATG GATTTGTCCT TTTCAATGTT TGAATACAAA
 AAAAAATCTA TAATAAGTTT ATTATATTAA AATAACCCGT TTTTTCAGAA
 TACGCAAAAA CGACAAAAAA ATATTAATTA CAAAGAAATT TAGTTTATA
 CAAAAATATG AATGGCTATT AATGGTGTTT ACTCTAAATT TAATTATTAT
 GCATTTATGC TAAATCTTTC TAAAGGTACA AAGATTCGTT TTTTCAATG
 TTTGAACTGC ATATTAAGGT ATAGATTTGG ACCTTAACAG AGTTAATATA
 TAAGGAAGAG AGCCAAGGAA CTCCAAAATA AAATAAAGAG CCTTCTCTCT

CTCTCTCTGA GAAAAAACAC ATATAGCCAA TGACCTTCTC GTGGTCTTCT
 GTGCCATAAA AGCCATTATA TACATTCAAA CACAATCTGG CGCCACATAT
 ACACATGTAC TAGTGTATGT ATATGTCCTA ACCTCTGTAT TCATATCTCT
 CTCCTTGTCT GAGTGGTGCG ATGGGTATCC CCATAAGCTG CAAACATTGA
 ACCATCTGCA ACATTTTGAC TCGTTTTCTT TTGTGTTTTT CCAACATCTG
 TCTCTTCTTC ACTCGCTCTC TCCTAATCAA TCTCCCCAAC GACCTCTCTT
 TTTTTTTGTT TCTTCACTCA GATCTCTCTC CCTCTCTCTC TCTCTCTCTC
 CGGGAAAA (SEQ ID NO:13)

[0068] All five promoters give early embryo expression in Arabidopsis. The expression profiles of the five promoters during early embryo development are represented schematically in Figure 1. The AAP1, LFAH12, 2S2, and FAE1 promoters are off in the earliest stage of embryo development. They become transcriptionally active at progressively later stages in development starting with AAP1 followed by LFAH12, 2S2, and then FAE1. All four promoters then remain active through later embryonic development stages. The LEC2 promoter has an inverse expression profile, is active in very early embryo development and then declines in activity gradually through later stages.

[0069] The AAP1, LFAH12, 2S2, FAE1, and LEC2 promoters were combined with either of two configurations of the REV coding region sequence in the *REV* transgene expression constructs. The AAP1, LFAH12, 2S2, FAE1, and LEC2 promoters were combined with the *REV* gene coding sequence including all introns as present in the *REV* gene. The AAP1 and LFAH12 promoters were also combined with a REV coding region sequence derived from a *REV* cDNA lacking introns.

Arabidopsis thaliana REV (AtRev) cDNA

ATGGAGATGG CGGTGGCTAA CCACCGTGAG AGAAGCAGTG ACAGTATGAA
 TAGACATTTA GATAGTAGCG GTAAGTACGT TAGGTACACA GCTGAGCAAG
 TCGAGGCTCT TGAGCGTGTC TACGCTGAGT GTCCTAAGCC TAGCTCTCTC
 CGTCGACAAC AATTGATCCG TGAATGTTCC ATTTTGGCCA ATATTGAGCC
 TAAGCAGATC AAAGTCTGGT TTCAGAACCG CAGGTGTCGA GATAAGCAGA
 GGAAAGAGGC GTCGAGGCTC CAGAGCGTAA ACCGGAAGCT CTCTGCGATG
 AATAAACTGT TGATGGAGGA GAATGATAGG TTGCAGAAGC AGGTTTCTCA
 GCTTGTCTGC GAAAATGGAT ATATGAAACA GCAGCTAACT ACTGTTGTTA
 ACGATCCAAG CTGTGAATCT GTGGTCACAA CTCCTCAGCA TTCGCTTAGA

GATGCGAATA GTCCTGCTGG ATTGCTCTCA ATCGCAGAGG AGACTTTGGC
AGAGTTCCTA TCCAAGGCTA CAGGAACTGC TGTTGATTG GGTTCAGATG
CCTGGGATGA AGCCTGGTCC GGATTCGGTT GGCATCTTTG CCATTTGCGA
AAGATGCAAT GGAGTGGCAG CTCGAGCCTG TGGTCTTGTT AGCTTAGAAC
CTATGAAGAT TGCAGAGATC CTCAAAGATC GGCCATCTTG GTTCCGTGAC
TGTAGGAGCC TTGAAGTTTT CACTATGTTC CCGGCTGGTA ATGGTGGCAC
AATCGAGCTT GTTTATATGC AGACGTATGC ACCAACGACT CTGGCTCCTG
CCCGCGATTT CTGGACCCTG AGATACACAA CGAGCCTCGA CAATGGGAGT
TTTGTGGTTT GTGAGAGGTC GCTATCTGGC TCTGGAGCTG GGCCTAATGC
TGCTTCAGCT TCTCAGTTTG TGAGAGCAGA AATGCTTTCT AGTGGGTATT
TAATAAGGCC TTGTGATGGT GGTGGTTCTA TTATTCACAT TGTCGATCAC
CTTAATCTTG AGGCTTGAG TGTTCCGGAT GTGCTTCGAC CCCTTTATGA
GTCATCCAAA GTCGTTGCAC AAAAAATGAC CATTTCCGCG TTGCGGTATA
TCAGGCAATT AGCCCAAGAG TCTAATGGTG AAGTAGTGTA TGGATTAGGA
AGGCAGCCTG CTGTTCTTAG AACCTTTAGC CAAAGATTAA GCAGGGGCTT
CAATGATGCG GTTAATGGGT TTGGTGACGA CGGGTGGTCT ACGATGCATT
GTGATGGAGC GGAAGATATT ATCGTTGCTA TTA ACTCTAC AAAGCATTG
AATAATATTT CTAATTCTCT TTCGTTCTT GGAGGCGTGC TCTGTGCCAA
GGCTTCAATG CTTCTCCAAA ATGTTCTCC TCGGGTTTTG ATCCGGTTCC
TTAGAGAGCA TCGATCTGAG TGGGCTGATT TCAATGTTGA TGCATATTCC
GCTGCTACAC TTAAAGCTGG TAGCTTTGCT TATCCGGGAA TGAGACCAAC
AAGATTCCT GGGAGTCAGA TCATAATGCC ACTAGGACAT ACAATTGAAC
ACGAAGAAAT GCTAGAAGTT GTTAGACTGG AAGGTCATTC TCTTGCTCAA
GAAGATGCAT TTATGTCACG GGATGTCCAT CTCCTTCAGA TTTGTACCGG
GATTGACGAG AATGCCGTTG GAGCTTGTT TGA ACTGATA TTTGCTCCGA
TTAATGAGAT GTTCCCGGAT GATGCTCCAC TTGTTCCCTC TGGATTCCGA
GTCATACCCG TTGATGCTAA AACGGGAGAT GTACAAGATC TGTTAACCGC
TAATCACCGT AACTAGACT TAACTTCTAG CCTTGAAGTCG GTCCATCACC
TGAGAATGCT TCTGGAACT CTTTTTCTAG CTCAAGCTCG AGATGTATTC
TCACTATCGC GTTTC AATTC CTTTTGAAA ACAACTTGCA AGAAAATGTT
GCTGGTATGG CTTGTCAGTA TGTGAGGAGC GTGATCTCAT CAGTTCAACG
TGTTGCAATG GCGATCTCAC CGTCTGGGAT AAGCCCGAGT CTGGGCTCCA
AATTGTCCCC AGGATCTCCT GAAGCTGTTA CTCTTGCTCA GTGGATCTCT
CAAAGTTAC AGTCATCACT TAGGCTCGGA GTTGCTGACG ATTGATTAC

TTGGAAGCGA CGACTCGGTA CTAAAACTTC TATGGGATCA CCAAGATGCC
 ATCCTGTGTT GCTCATTAAG GCCACAGCCA GTGTTCATGT TTGCGAACCA
 AGCTGGTCTA GACATGCTAG AGACAACACT TGTAGCCTTA CAAGATATAA
 CACTCGAAAA GATATTCGAT GAATCGGGTC GTAAGGCTAT CTGTTCGGAC
 TTCGCCAAGC TAATGCAACA GGGATTTGCT TGCTTGCCTT CAGGAATCTG
 TGTGTCAACG ATGGGAAGAC ATGTGAGTTA TGAACAAGCT GTTGCTTGGG
 AAGTGTTTGC TGCATCTGAA GAAAACAAC AACAATCTGC ATTGTCTTGC
 CTTCTCCTTT GTAAACTGGT CTTTTGTGTG A (SEQ ID NO:1).

REV Transgene Constructs

LFAH12-At REV gene-rev 3' UTR

[0070] A 2170 base pair (bp) region of the LFAH12 promoter was amplified from *Lesquerella fendleri* genomic DNA with *EcoRI*-LFAH12 (GAATTCTCAGGAAGATTAAGTCTTTGCTTG; SEQ ID NO: 14) and *SacI*-LFAH12 (GAGCTCGCTGAAAATATCAAAAGAAGGAACA; SEQ ID NO: 15) primers. These primers started 24 nucleotides and 15 nucleotides in from the 5' and 3' ends, respectively, of the published LFAH12 sequence ((GenBank® designations AF016103.1 or GI:3452128) (Broun *et al.*, *Plant J.* 13:201-210 (1998)). Several independent PCR reactions were cloned into pCR-Blunt (Invitrogen) and sequenced. The sequences of all were identical to one another (SEQ ID NO:12) and 97% identical to the published LFAH12 sequence. The differences could be due to the specific accession of *L. fendleri* used for the retrieval of the promoter. LFAH12 was moved to the pBluescript plasmid with *EcoRI* and the orientation of the promoter in pBluescript was determined to be *KpnI* on the 5' side of the promoter and *SpeI* on the 3' side of the promoter (pTG143). The At *REV* gene-rev 3' UTR cassette was taken as a *SpeI*-*KpnI* fragment from pTG95 (35S-At *REV* gene-rev 3'UTR in pCGN1547, patent WO 01/33944A1) and along with the LFAH12 promoter (*KpnI*-*SpeI* fragment from pTG143), was ligated into pCGN1547 binary vector (McBride *et al.*, *Plant Mol. Biol.* 14:269-276, 1990) that had been cut with *KpnI* in a three-way ligation to create LFAH12 promoter-At *REV* gene-rev 3' UTR in a tail-to-tail orientation with the plant NPTII expression cassette.

AAP1-At REV gene-rev 3' UTR

[0071] An 809 base pair (bp) region of the AAP1 promoter was amplified from *Arabidopsis thaliana* (ecotype Columbia) genomic DNA with *EcoRI*-AAP1

(GAATTCGGTTGCATCTTTGAATACCTTTTT; SEQ ID NO:16) and *SacI*-AAP1 (GAGCTCTGAGAGAAAGAGAAAGAGAGAACAA; SEQ ID NO:17) primers. The *SacI*-AAP1 primer started 6 nucleotides in from the 3' end of the published AAP1 sequence (GenBank[®] deposit designation X95622.1; GI:1566687) (Hirner *et al.*, *Plant J.* 14:535-544, 1998). The sequences of all PCR products were identical to one another, 98% identical to the published C24 ecotype AAP1 sequence, and 100 % identical to the annotated Col ecotype AAP1 sequence (GenBank[®] designation AC008051.3; GI:7462019) (SEQ ID NO:11). AAP1 was moved to the plasmid pBluescript with *EcoRI* and the orientation of the promoter in pBluescript was determined to be *KpnI* on the 5' side of the promoter and *SpeI* on the 3' side of the promoter (pTG145). The At *REV* gene-rev 3' UTR cassette was taken as a *SpeI*-*KpnI* fragment from pTG95 (35S-At *REV* gene-rev 3'UTR in pCGN1547, See WO 01/33944A1) and along with the AAP1 promoter (*KpnI*-*SpeI* fragment from pTG145), was ligated into pCGN1547 binary vector (McBride *et al.*, *Plant Mol. Biol.* 14:269-276, 1990) that had been cut with *KpnI* in a three-way ligation to create AAP1 promoter-At *REV* gene-rev 3' UTR in a head-to-tail orientation with the plant NPTII expression cassette.

LFAH12-At *REV* cDNA-rev 3' UTR

[0072] At *REV* cDNA was amplified from cDNA synthesized from total RNA isolated from *Arabidopsis* leaf of the Columbia ecotype. The primers used incorporated an *NcoI* site at the ATG and a *BamHI* site after the stop codon: *NcoI*-ATG rev CCATGGAGATGGCGGTGGC TAAC (SEQ ID NO:18) and *BamHI*-TGA rev GGATCCTCACACAAAAGACCAGTTTAC AAAGGA (SEQ ID NO:19). The resulting At *REV* cDNA PCR product was cloned into the plasmid pCR-Blunt and sequenced (pTG230). Using pTG95 as the template, *Rev* 3' UTR was amplified with primers that incorporated an *EcoRV* site at the 5' end and *NotI*/*KpnI* at the 3' end: *EcoRV* revUTR GATATCTTCGATTGACAGAAAAAG (SEQ ID NO:20) and *NotI*/*KpnI* revUTR GCGGCCGCGGTACCCTCAACCAACCACATGGAACCA (SEQ ID NO:21). The resulting At *REV* 3'UTR was cloned into the plasmid pCR-Blunt and sequenced. *Rev* 3' UTR was moved to the plasmid pBluescript with *EcoRV* and *NotI* sites (pTG234). *Rev* 3' UTR was then taken from pTG234 and ligated into pTG230 with *EcoRV* and *NotI* sites (pTG239). The At *REV* cDNA-rev 3'UTR cassette was taken as a *SpeI*-*KpnI* fragment from pTG239 and along with the LFAH12 promoter (*KpnI*-*SpeI* fragment from pTG143), was ligated into the pCGN1547 binary vector in a three-way ligation to create LFAH12-At *REV* cDNA-rev 3'UTR in a head-to-tail orientation with the plant NPTII expression cassette (pTG241).

AAP1-At *REV* cDNA-rev 3'UTR

[0073] The At *REV* cDNA-rev 3'UTR cassette was taken as a *SpeI*-*KpnI* fragment from pTG239 and along with the AAP1 promoter (*KpnI*-*SpeI* fragment from pTG145), was ligated into the pCGN1547 binary vector in a three-way ligation to create AAP1-At *REV* cDNA-rev 3'UTR in a head-to-tail orientation with the plant NPTII expression cassette (pTG242).

FAE1-At *REV* gene-rev 3'UTR

[0074] The 933 bp FAE1 promoter was amplified from a CD vector with *KpnI* and *SpeI* sites, cloned into the plasmid pCR-Blunt, and sequenced (pTG238). FAE1 promoter was then moved into the plasmid pBluescript with *EcoRI* (pTG243). The At *REV* gene-rev 3'UTR cassette was taken as a *SpeI*-*KpnI* fragment from pTG95 and along with the FAE1 promoter (*KpnI*-*SpeI* fragment from pTG243), was ligated into the pCGN1547 binary vector (McBride *et al.*, *Plant Mol. Biol.* 14:269-276, 1990) that had been cut with *KpnI* in a three-way ligation to create FAE1 promoter-At *REV* gene-rev 3'UTR in a head-to-tail orientation with the plant NPTII expression cassette (pTG248).

2S2-At *REV* gene-rev3'UTR

[0075] The 1379 bp 2S2 promoter was excised from CD plasmid p4163 (2S2- At *REV* *SwaI* fragment and ligated into pBluescript at the *EcoRV* site. The orientation of the promoter in pBluescript was determined to be *KpnI* on the 5' side of the promoter and *BamHI* on the 3' side of the promoter (pTG251). The At *REV* gene-rev3'UTR cassette was taken as a *BamHI*-*KpnI* fragment from pTG138(35S-At *REV* gene-rev3'UTR in TOPO) and along with the 2S2 promoter (*KpnI*-*BamHI* fragment from pTG251), was ligated into pCGN1547 binary vector (McBride *et al.*, *Plant Mol. Biol.* 14:269-276, 1990) that had been cut with *KpnI* in a three-way ligation to create 2S2 promoter-At *REV* gene-rev3'UTR in a head-to-tail orientation with the plant NPTII expression cassette.

LEC2-At *REV* gene-rev3'UTR

[0076] The 1256 bp LEC2 promoter was excised from CD plasmid p5217 with *ApaI* and *PstI* and cloned into the plasmid pBluescript at the same sites (pTG252). The promoter was then taken as a *KpnI*-*BamHI* fragment into pTG112 (At *REV* gene in the plasmid pCR-Blunt) linearized at the same sites to give pTG280, which is the LEC2-At *REV* gene in the pCR-Blunt plasmid. Using pTG234 as the template, Rev 3'UTR was amplified with primers that

incorporated a *NotI* site at the 5' end and *NotI/KpnI* at the 3' end: *Not* REV UTR start GCGGCCGCTTCGATTGACAGAAAAAG (SEQ ID NO:22) and *NotKpn* Rev UTR GCGGCCGCGGTACCCTCAACCAACCACATGGAACCA (SEQ ID NO:23). The resulting At REV 3'UTR was cloned into the plasmid pCR-Blunt and sequenced (pTG281). The Rev 3'UTR was then moved to pTG280 using the *NotI* sites and screened for proper orientation. The resulting plasmid was LEC2-At REV gene-rev 3'UTR in the plasmid pCR-Blunt (pTG283). LEC2-At REV gene-rev 3'UTR was moved as a *KpnI* fragment to a pCGN1547 plasmid linearized with *KpnI* and screened for head-to-tail orientation with the plant NPTII expression cassette (pTG288).

Canola (*Brassica napus*) Transformation

[0077] The double haploid canola variety DH12075 was transformed with the *REV* transgene expression constructs using an *Agrobacterium*-mediated transformation method based on that of Maloney *et al.* (Maloney *et al.*, *Plant Cell Reports* 8:238, 1989)

[0078] Sterilized seeds were germinated on ½ MS (Murashige & Skoog) media with 1% sucrose in 15 X 60 mm Petri dishes for 5 days with approximately 40 to about 60 seeds per plate. A total of approximately 1500 seeds were germinated for each transformation construct. Seeds were not fully submerged in the germination medium. Germinated seedlings were grown in a tissue culture room at 25°C, on a 16 hour light/8 hour dark cycle.

[0079] Cotyledons were cut just above the apical meristem without obtaining any of the meristem tissue. This was done by gently gripping the two petioles with forceps immediately above the apical meristem region. Care was taken not to crush the petioles with the forceps. Using the tips of the forceps as a guide, petioles were cut using a scalpel with a sharp No. 12 blade. Cotyledons were released onto a 15mm X 100mm plate of co-cultivation medium. Properly cut cotyledons separate easily. If they did not, there was a very good chance that meristematic tissue had been obtained and such cotyledons were not used. Each plate held approximately 20 cotyledons. Cotyledon explants were inoculated with *Agrobacterium* after every few plates that were prepared to avoid wilting which would have a negative impact on following stages of the protocol.

[0080] REV tranformation constructs were introduced into *Agrobacterium tumefaciens* by electroporation. *Agrobacterium* harboring the REV transformation construct was grown in AB medium with appropriate antibiotics for two days shaking at 28°C. To inoculate

cotyledon explants, a small volume of *Agrobacterium* culture was added to a 10 mm x 35 mm Petri dish. The petiole of each explant was dipped into the *Agrobacterium* culture and the cut end placed into co-cultivation medium in a Petri dish. The plates were sealed and placed in a tissue culture room at 25°C, 16 hour light/8 hour dark for 3 days.

[0081] After 3 days, explants were transferred in sets of ten to fresh 25 mm x 100 mm Petri dishes containing shoot induction medium. This medium contained a selection agent (20 mg/l Kanamycin) and hormone (4.5 mg/l a brassinosteroid (BA)). Only healthy-looking explants were transferred. Explants were kept on shoot induction medium for 14 to 21 days. At this time, green calli and possibly some shoot development and some non-transformed shoots may could be observed. Non-transformed shoots were easily recognized by their white and purple color. Kanamycin-sensitive shoots were removed by cutting them away and all healthy-looking calli were transferred to fresh plates of shoot induction medium. The explants were kept on these plates for another 14 to 21 days.

[0082] After 2 to 3 weeks, shoots that were dark green in color were transferred to plates containing shoot elongation medium. This medium contained a selection agent (20 mg/l Kanamycin) but did not contain any hormones. Five shoots were transferred to each plate. The plates were sealed and returned to the tissue culture room. Transformed shoots that appeared vitrious were transferred to shoot elongation medium containing phloroglucinol (150 mg/l). Shoots that became healthy and green were returned to shoot elongation medium plates. Repeated transfers of vitrious shoots to fresh plates of the same medium were required in some cases to obtain normal looking shoots.

[0083] Shoots with normal morphology were transferred to 4 oz. baby food jars with rooting medium containing 0.5 mg/l indole butyric acid. Any excess callus was cut away when transferring shoots to the jars. Shoots could be maintained in jars indefinitely by transferring them to fresh jars containing 0.2 mg/l indole butyric acid approximately every 6 weeks.

[0084] Once a good root system had formed, the T₀ generation shoots were removed from jars, agar removed from the roots, and the plantlet transferred to potting soil. Each independent T₀ plantlet represented an independent occurrence of insertion of the transgene into the canola genome and was referred to as an event. A transparent cup was placed over the plantlet for a few days, allowing the plant to acclimatize to the new environment. Once

the plant had hardened, the cup was removed. The T₀ transgenic events were then grown to maturity in the greenhouse and T₁ seeds collected.

T₀ Event Characterization

[0085] The number of transgene insertion site loci was determined in each event by Southern analysis. *REV* transgene expression in the T₀ events was verified by Northern analysis or end-point RT-PCR. *REV* expression data were obtained for a single time point in embryo development, 19 days after pollination (DAP). From these data it was concluded that, at this developmental time point, the LFAH12 and 2S2 albumin promoters were driving higher levels of *REV* RNA production than the AAP1 promoter. The 2S2/*REV* constructs gave moderate to high level expression in many events. The expression data demonstrated that all five promoters were functional in driving *REV* transgene expression in transgenic canola plants.

[0086] T₀ plants were successfully generated for all seven *REV* transgene constructs. The tested constructs included (a) AAP1/*REV* gene; (b) AAP1/*REV*cDNA; (c) LFAH12/*REV* gene; (d) LFAH12/*REV*cDNA; (e) 2S2/*REV* gene; (f) FAE1/*REV* gene; and (g) LEC2/*REV* gene.

Example 2. Evaluation of the Effect of *REV* Transgene Expression During Embryo Development on Canola Yield in Replicated Field Trials.

[0087] In this example the transgenic canola plants comprising the *Arabidopsis REV* transgene under the control of the various embryo specific promoters were tested in field trials.

Advancement of Transgenic *REV* Events to Field Trials.

[0088] T₀ events were selected for advancement to field trials based on a combination of transgene expression and transgene insertion locus number. Events with verified transgene expression and a single transgene insertion locus were assigned the highest priority to be carried forward to field testing. In some instances, events with multiple insertion loci were selected if the presence of multiple genes gave a high overall transgene expression level due to gene dosage.

[0089] T₁ seeds from selected events were grown as segregating T₁ populations in field plots. Each event was planted as a two row, twenty four plant plot. For events with a single transgene insertion locus, segregation of the transgene among the twenty four T₁ plants would produce a distribution of approximately six null plants lacking the transgene, twelve heterozygous plants, and six homozygous plants. Each T₁ plant was individually bagged before flowering to prevent out-crossing. T₂ seeds from each of the twenty four T₁ plants were harvested separately.

[0090] The T₂ seed stocks were used to identify which of the twenty four parent T₁ plants were null, heterozygous, or homozygous. Approximately thirty T₂ seeds from each T₁ plant were germinated on filter paper in petri dishes with a solution containing the antibiotic G418, an analog of kanamycin. Since the plants were co-transformed with the *nptII* resistance gene as a selectable marker, only those seeds carrying the transgene would germinate and continue to grow. If all the seeds on a plate proved to be sensitive to G418, then the T₁ parent was identified as a null line. If all the seeds on a plate were resistant to G418, then the T₁ parent was identified as a homozygous line. If approximately one quarter of the seeds on a plate were sensitive and the rest resistant, the T₁ parent was identified as a heterozygous line. T₂ seeds from homozygous T₁ parents from the same transformation event were bulked to generate homozygous seed stocks for field trial testing. T₂ seeds from null T₁ parents from the same transformation event were bulked to generate null sibling seed stocks for field trial testing.

Field Trial Design

[0091] The use of embryo-specific promoters to evaluate the potential of the *REV* transgene as a yield enhancing gene was tested in the transgenic canola lines by comparing each transgenic line directly with its null sibling in the field in large scale replicated trials. Since the null sibling arises from segregation of the transgene in the T₁ generation, the null and homozygous siblings are nearly identical genetically. The only significant difference is the presence or absence of the *REV* transgene. This near genetic identity makes the null sibling the optimal control for evaluation of the effect of the *REV* transgene. As the main objective of the trial was the comparison of the transgenic line from an event to its null segregant, a split plot design was chosen. This design gives a high level of evaluation to the interaction between the transgenic and non-transgenic subentries and the differences between transgenic

subplots between events (the interaction of subplot and main plot) and a lower level of evaluation to the differences between overall events or the main plot.

[0092] Field trials were conducted at multiple locations across prairie environments to assess yield phenotypes under the range of environmental conditions in which canola is typically grown. At all locations, each transgenic event was physically paired with its null sibling in adjacent plots. Each plot pair of homozygous and null siblings was replicated four times at each trial location. The locations of the four replicate plot pairs in each trial were randomly distributed at each trial location. Plots were 1.6 m by 6 m and planted at a density of approximately 142 seeds per square meter. Plants were grown to maturity using standard agronomic practices typical to commercial production of canola.

Example 3. Expression of a *REV* Transgene from an Embryo-specific Promoter to Increase Seed Yield In Transgenic Canola.

[0093] All plots at each yield field trial location were individually harvested with a combine. Total seed yield data were collected as total seed weight adjusted for moisture content from each plot. For every transgenic event in each trial, the mean of the total yield from the four replicate plots of each homozygous line was compared to the mean of the total yield from the four replicate plots of the associated null sibling line. This comparison was used to evaluate the effect of the *REV* transgene on total seed yield. Results from each of the multiple trial locations were combined to give an across trials analysis of the effect of the *REV* transgene on total seed yield. Statistical analysis of variance at each trial location permitted the assignment of a threshold for significance ($P = 0.05$) for differences in total seed yield between homozygous transgenic lines and their null siblings.

[0094] The transgenic *REV* canola lines that showed a statistically significant increase in total seed yield are summarized in Table 1. Transgenic lines showing statistically significant increases in total yield were identified for all seven promoter/*REV* transgene constructs. These results demonstrate that over expression of *REV* using an embryo-specific promoter results in increased seed yield. The greatest impact on yield was obtained with the LEC2 promoter suggesting that over expression of *REV* may be most effective in the earliest stage of embryo development since the LEC2 promoter is maximally active very early in development with decreasing transcriptional activity through the later stages of embryo development. The AAP1, LFAH12, and 2S2 promoters initiate transcriptional activity at progressively later stages in embryo development but were also effective in increasing yield.

The LFAH12 promoter was notable for the number of independent events that were recovered that showed significantly increased yield indicating a high degree of penetrance for the trait using this promoter. Only one event with a moderate increase in yield was recovered for the FAE1 promoter possibly suggesting that promoters that initiate transcriptional activity in earlier stages of embryo development provide the best means for yield increase and demonstrate that early embryonic overexpression of a gene can be used to evaluate whether a gene involved in plant growth and/or development has potential to increase crop yield in transgenic plants.

Table 1. Change in total seed yield in homozygous *REV* plants relative to their null siblings. All values are statistically significant ($P = 0.05$).

Event	Promoter	Transgene	% Yield Increase
TG6-21	AAP1	REV gene	7.8
TG12-6	AAP1	REV cDNA	7.9
TG5-32	LFAH12	REV gene	15.5
TG11-4	LFAH12	REV cDNA	18.9
TG11-13	LFAH12	REV cDNA	8.9
TG11-17	LFAH12	REV cDNA	16.7
TG11-18	LFAH12	REV cDNA	5.8
TG11-23	LFAH12	REV cDNA	9.4
TG20-5	2S2	REV gene	25.6
TG10-16	FAE1	REV gene	5.6
TG19-8	LEC2	REV gene	59.8

Example 4. Increased Seed Size In Transgenic Canola Expressing Rev During Embryo Development Using Embryo-Specific Promoters.

[0095] All seeds harvested from individual plots in the field trials were dried at low heat in an oven to uniform moisture content. For each seed sample for each plot in the field trials, one thousand seeds were counted out using an Agriculex (Guelph, Ontario, Canada) seed counter. The weight of each one thousand seed sample was then measured. The mean of the thousand seed weight (TSW) from the four replicate plots of each homozygous line was compared to the mean of TSW from the four replicate plots of the associated null sibling line. The differences in TSW values between homozygous transgenic *REV* lines and their null sibling lines were used as a measure of differences in seed size that could be attributed to the REV transgene. Statistical analysis of variance at each trial location permitted the assignment of a threshold for significance ($P = 0.05$) for differences in TSW between homozygous transgenic lines and their null siblings.

[0096] The transgenic *REV* canola lines that showed a statistically significant increase in seed size as indicated by TSW are summarized in Table 2. Transgenic lines showing statistically significant increases in TSW were identified for six of the seven promoter/*REV* transgene constructs. Effects of the LEC2 and LFAH12 promoters driving *REV* expression on seed size were consistent with effects observed on total yield using these promoters. The LEC2/*REV* construct gave a strong positive increase in seed size indicating that over expression of *REV* in very early embryo development is effective. The LFAH12 promoter again showed a strong penetrance of the trait with six independent events showing statistically significant increases in seed size. The AAP1 and 2S2 promoters driving *REV* over expression also gave statistically significant increases in seed size. No events carrying the FAE1/*REV* transgene construct showed a significant increase in TSW. Since the activity of the FAE1 promoter initiates the latest in embryo development of the five promoters tested, the lack of an effect suggests that promoters that initiate transcriptional activity in the earlier stages of embryo development provide a means to increase yield when associated with a gene that affects plant growth and development, such as *REV*. In addition, these promoters can be used to evaluate other plant genes associated with growth and/or development for their potential to increase yield, such as seed size and/or number, in transgenic plants..

Table 2. Change in seed size as indicated by 1000 seed weight. All values are statistically significant (P = 0.05). Value indicated by an * was significant at one trial location.

Event	Promoter	Transgene	% 1000 Seed Weight Increase
TG6-21-15	AAP1	REV gene	3.9
TG12-6-6	AAP1	REV cDNA	3.6*
TG5-32-23	LFAH12	REV gene	5.8
TG5-61	LFAH12	REV gene	4.8
TG11-4	LFAH12	REV cDNA	7.9
TG11-13-9	LFAH12	REV cDNA	4.8
TG11-17-19	LFAH12	REV cDNA	3.1
TG11-18-19	LFAH12	REV cDNA	4.8
TG20-15	2S2	REV gene	2.7
TG19-8	LEC2	REV gene	3.1
TG19-23	LEC2	REV gene	7.7

Example 5. Increased Seed Size And Yield In Transgenic Canola Expressing Rev During Embryo Development Using Embryo-Specific Promoters.

[0097] One conceivable outcome for an increased seed size phenotype can be a compensating response by the plant that results in decreased seed number to maintain a constant net allocation of resources to seed production. Table 3 summarizes six independent transgenic *REV* events that demonstrated both a statistically significant increased seed size and an increase in total yield in field trials. Simultaneous increases in seed size and total yield were observed in events carrying AAP1/REV, LFAH12/REV, 2S2/REV, and LEC2/REV transgene constructs indicating that all four promoters were effective in increasing seed size without eliciting a reduction in seed number as a compensating response by the plant.

[0098] Comparison of TSW values with total yield values in Table 3 revealed that the increased seed size did not account for the observed total increases in yield. Increased seed size was one component contributing to total yield. The remainder of the increased yield was due to increased seed number. In most cases, increased seed number was the major component of increased total yield. Increased seed number could result from an increase in seed number per pod, an increase in the number of racemes, an increase in the number of pods per raceme, a decrease in seed abortion rate, or a combination of these effects.

[0099] The absence of events carrying the FAE1/REV transgene construct from the list of events showing both increase seed size and total yield indicates that promoters that initiate transcriptional activity in the earlier stages of embryo development provide the best means for evaluating plant genes involved in plant development and growth for their potential to increase seed size and yield in transgenic plants. It also appears that the greatest efficacy for yield increase may be obtained with promoters that initiate transcriptional activity in the earlier stages of embryo development.

Table 3. Independent transgenic *REV* events showing both increased seed size and total yield. Values are statistically significant ($P = 0.05$) except ** which was not statistically significant but positive at all four trial locations. Values marked with * were significant at one trial location.

Event	Promoter	Transgene	% 1000 Seed Weight Increase	% Yield Increase
TG6-21	AAP1	REV gene	3.9	7.8
TG12-6-6	AAP1	REV cDNA	16.2*	103.9*
TG5-32-14	LFAH12	REV gene	5.3	5.7
TG11-4	LFAH12	REV cDNA	7.9	18.9
TG20-15	2S2	REV gene	2.7	8.6**
TG19-8	LEC2	REV gene	3.1	59.8

Example 6. Transgenic Soybean Plants Expressing Transgene Constructs Designed to Confer Embryo-Specific Expression of REVOLUTA.

[0100] Based on the successful use of the LFAH12 embryo-specific promoter to drive REVOLUTA transgene expression in transgenic canola plants (Tables 1, 2, and 3.) to obtain yield and seed size increase, an LFAH12-AtREVgene construct was built for transformation of soybean. Soybean, like canola, is a dicot and the high conservation of *REVOLUTA* genes across plant species made it likely that the *Arabidopsis REVOLUTA* gene would function when introduced into transgenic soybean plants.

REV Transgene Construct

LFAH12-AtREV gene-rev 3' UTR

[0101] A 2170 bp region of the LFAH12 promoter was amplified from *Lesquerella fendleri* genomic DNA with *EcoRI*-LFAH12 (GAATTCTCAGGAAGATTAAGTCTTTGCTTG; SEQ ID NO: 14) and *SacI*-LFAH12 (GAGCTCGCTGAAAATATCAAAAGAAGGAACA; SEQ ID NO: 15) primers. These primers started 24 nucleotides and 15 nucleotides in from the 5' and 3' ends, respectively, of the published LFAH12 sequence ((GenBank® designations AF016103.1 or GI:3452128) (Broun *et al.*, *Plant J.* 13:201-210 (1998)). Several independent PCR reactions were cloned into pCR-Blunt (Invitrogen) and sequenced. The sequences of all were identical to one another and 97 % identical to the published LFAH12 sequence. The differences could be due to the specific accession of *L. fendleri* used for the retrieval of the

promoter. LFAH12 was moved to the pBluescript II plasmid with *EcoRI* and the orientation of the promoter in pBluescript was determined to be *KpnI* on the 5' side of the promoter and *SpeI* on the 3' side of the promoter (pTG143). The At REV gene-rev 3' UTR cassette was taken as a *SpeI-KpnI* fragment from pTG95 (35S-At REV gene-rev 3'UTR in pCGN1547, patent WO 01/33944A1) and along with the LFAH12 promoter (*KpnI-SpeI* fragment from pTG143), was ligated into pCGN1547 binary vector (McBride *et al.*, *Plant Mol. Biol.* 14:269-276, 1990) that had been cut with *KpnI* in a three-way ligation to create LFAH12 promoter-At REV gene-rev 3' UTR in a head-to-tail orientation with the plant NPTII expression cassette (pTG171). The LFAH12pr-At REV gene-rev 3' UTR cassette was excised from pTG171 with *KpnI*, blunted with T4 polymerase and cloned into pBluescript II that had been cut with *SmaI* to give pTG464 (TG-GM6).

Soybean Transformation

[0102] The soybean variety X5 was transformed with the *REV* transgene expression construct using the biolistic method to introduce DNA on gold particles into soybean cells grown in suspension culture. Linear DNA containing the *REV* transgene expression construct was co-transformed with a hygromycin selectable marker gene on a separate linear DNA fragment.

[0103] To prepare DNA for insertion into soybean cells, 2.5 µl *REV* DNA and 2.5 µl pHYG^R DNA were added to gold particles. 50 µl CaCl₂ was added and vortexed 5 sec. Followed by addition of 20 µl spermidine and vortexed 5 sec. Precipitate was allowed to settle to the bottom of the tube. The supernatant was removed and the pellet washed twice with 200 µl 100 % ethanol. The supernatant was removed and the pellet resuspended in 120 µl 100 % ethanol. 8 µl was aliquoted per disk for insertion.

[0104] After biolistics insertion, cells were grown in suspension culture in flasks for 12 days. Cultures were then transferred to new flasks containing 30 ml of 2.4 % Sugar + Finer's Light (F.L.) + 170 µl of 10 µg/µl Hygromycin (hyg). For the following four weeks, media was changed weekly by transferring to a new flask with 2.4% Sugar + F.L. + hyg.

[0105] Transformed green calli developed in the flasks during the fifth through tenth weeks. The contents of flasks with green calli were poured into sterile 100 x 15 mm dishes or lids. Using sterile forceps, green calli were teased from brown cells and transferred to 26-

well plates filled with 1 - 2 ml 2.4 % Sugar + F.L.+Hyg/well. 1 green callus (clone)/well. The goal was to get 20 - 30 clones/flask.

[0106] Media was changed in the plates every 2 weeks using a sterile 10-mL pipette and automatic pipettor to suck all media from all wells. Medium was replaced with fresh 2.4 % Sugar +Hyg. Plates were monitored for suitable proembryogenic cultures. These were green with several proembryogenic projections and showed rapid division and growth. Once a suitable cell mass was identified by microscopic observation, it was transferred onto a sterile dish or lid where as much dead cell matter as possible was removed. The remaining piece of tissue was transferred to a regular 30 ml flask of 2.4 % Sugar + Hyg. Clones were allowed to grow about 3 to 5 weeks between media changes.

[0107] When clones were grown sufficiently, they were split. Only the 5 best pieces per clone were kept. These were green with several proembryogenic projections. Smooth, green colonies were not desired. The clones were subcultured every 3 - 5 weeks until they were sufficiently large. At this point, clones were transferred to a soybean regeneration medium with activated charcoal to remove 2,4-D (OMSM6AC, Simmonds and Donaldson, *Plant Cell Reports* 19:485-490, 2000, incorporated herein by reference in its entirety) as the first step of regeneration.

[0108] Clones were grown no more than six days on OMSM6AC containing activated charcoal (AC) to remove 2,4-D from tissue. Clones were then transferred to soybean embryo germination medium (OMSM6G, Simmonds and Donaldson, *supra*) for three weeks. This medium allows for the development of embryos. After three weeks, the best 20 embryos were chosen and transferred to soybean maturation medium (OMSM6PH, Simmonds and Donaldson, *supra*). The best embryos were single (as opposed to fused) embryos with 1 or 2 cotyledons and a visible meristem. Embryos were transferred so that the meristem was parallel to agar surface. Clones were maintained on OMSM6PH for four weeks during which time embryos turned a yellow color. Once yellow, they were ready to be desiccated and regenerated to plants.

[0109] Embryos were desiccated in Magenta boxes sterilized 30 min with 3 layers of H₂O-saturated Whatman filters at bottom. Embryos were desiccated ten days and then transferred to soybean rooting medium (B5/2 medium, Simmonds and Donaldson, *supra*) in plates for about 10 to 12 days. Plantlets were ready to go to Sunshine Mix (flats) when the shoot grew

to the lid or when leaves were open. Transgenic plantlets were grown in flats until the 1st trifoliate leaf appeared and then transferred to 6 inch pots to grow to seed.

Example 7. Expressing a *REV* Transgene from an Embryo-specific Promoter to Determine Increased Seed Size in Transgenic Soybean

[0110] T₀ events harboring the LFAH12-AtREV gene-rev 3' UTR (TG GM6) transgene construct were selected for advancement for phenotype evaluation based on a combination of transgene expression and transgene insertion locus number. Events with verified transgene expression and a single transgene insertion locus were assigned the highest priority to be carried forward for seed expansion and field testing. In some instances, events with multiple insertion loci were selected if the presence of multiple genes gave a high overall transgene expression level due to gene dosage. T₁ seeds from selected T₀ events were grown as segregating T₁ populations in a greenhouse. Seeds were harvested from all T₁ plants individually. T₂ generation plants for each event were grown and typed by PCR to determine which T₂ lines were homozygous, heterozygous, or null segregants for the *REV* transgene. Seeds from each T₂ line were harvested, counted, and weighed. Based on the total seed weight and seed number for each T₂ line, a thousand seed weight (TSW) value was calculated for each line. The average TSW was then calculated for the homozygous, heterozygous, and null segregant classes of seeds for each event.

[0111] Two independent transformation events with REV expression driven by the LFAH12 embryo-specific promoter showed statistically significant increases in seed size for both homozygous and heterozygous lines relative to null segregant lines. The results summarized in Table 4 demonstrate that overexpression of a *REV* transgene during early embryo development.

Table 4. Use of an embryo-specific promoter to evaluate the capacity of *REV* transgene to increase seed size in soybean as indicated by 1000 seed weight in homozygous and heterozygous transgenic plants relative to their null segregant sibling lines.

Event	Zygosity	Number of lines	Avg. 1000 Seed Weight (g)	Standard Error	Seed Size Increase (%)
2A4	Homozygous	32	69.8	1.03	5.8

Event	Zygoty	Number of lines	Avg. 1000 Seed Weight (g)	Standard Error	Seed Size Increase (%)
2A4	Heterozygous	79	70.6	0.58	7.0
2A4	Null	38	66.0	0.97	NA
2B2	Homozygous	3	83.6	5.90	14.1
2B2	Heterozygous	19	79.5	2.33	8.5
2B2	Null	14	73.2	2.29	NA

Example 8. Expressing a *REV* Transgene from an Embryo-specific Promoter to Determine Increased Total Seed Yield in Transgenic Soybean.

[0112] To test the effect of *REV* transgene expression driven by an embryo-specific promoter on total seed yield, T₃ seeds from homozygous and null segregant sibling lines were pooled and planted in the field during the summer growing season. All seeds harvested from individual plots in the field were dried at low heat in an oven to uniform moisture content before measuring seed weights. One transgenic event with REV expression driven by the LFAH12 embryo-specific promoter showed both increased total seed yield of 22.7 % and increased seed size of 8.0 % relative to its null sibling (Table 5).

Table 5. Use of an embryo-specific promoter to evaluate the capacity of a *REV* transgene to increase both seed size and yield in soybean comparing homozygous transgenic plants to null segregant sibling plants.

Event	Zygoty	Total Seed Weight/100 Plants (g)	Total Seed Increase (%)	Avg. 1000 Seed Weight (g)	Seed Size Increase (%)
2B2	Homozygous	1552	22.7	90.0	8.0
2B2	Null	1265	NA	83.3	NA

[0113] The previous examples are provided to illustrate, but not to limit, the scope of the claimed inventions. Other variants of the inventions will be readily apparent to those of ordinary skill in the art and encompassed by the appended claims. All publications, patents, patent applications and other references cited herein and are also incorporated by reference herein in their entirety.

WHAT IS CLAIMED IS:

1. A method for increasing seed size in a plant comprising over expressing a plant growth and development associated gene in a seed during early embryo development.
2. The method according to claim 1, wherein the growth and/or development related gene expression in the seed is under the control of an early phase-specific embryo promoter.
3. The method according to claim 2, wherein the early phase-specific embryo promoter is heterologous to the plant.
4. The method according to claim 2, wherein the early phase-specific embryo promoter is homologous to the plant.
5. The method according to claim 2, wherein the early phase-specific promoter is the promoter associated with an amino acid permease gene (AAP1), an oleate 12-hydroxylase:desaturase gene, an 2S2 albumin gene (2S2), a fatty acid elongase gene (FAE1), or a leafy cotyledon gene (LEC2).
6. The method according to claim 5, wherein the AAP1 promoter is the AAP1 promoter from *Arabidopsis thaliana*, the oleate 12-hydroxylase:desaturase promoter is the oleate 12-hydroxylase:desaturase gene promoter from *Lesquerella fendleri* (LFAH12), the 2S2 gene promoter is from *Arabidopsis thaliana*, the fatty acid elongase gene promoter is from *Arabidopsis thaliana*, or the leafy cotyledon gene promoter is from *Arabidopsis thaliana*.
7. The method according to any of claims 1 through 6, wherein the plant growth and/or development associated gene is a *REV* gene, a *CAP* (cyclase-associated protein) gene, a rice histone deacetylase 1 gene, a *E2Fc* gene, a *BKI* gene, a *BRI1* gene, a *ARL* (Argos-Like) gene, a *bril* (brassinosteroid hormone perception) gene, a *FATB* gene, a Pepino gene, a *RGS* (regulator of G protein signaling) gene, a *TIP1* gene, a *BB* (Big Brother) gene, a *RHD2* gene, a *INCW2* gene, a *MNI* gene, a *WAK2* gene, a *WAK4* gene, or a *AP2* gene.

8. The method according to claim 7, wherein the growth and/or development associated gene is *REV*.
9. The method according to claim 8, wherein the *REV* gene is derived from *Arabidopsis thaliana*.
10. The method according to any of claims 1 through 9, wherein the plant is a monocot or a dicot.
11. The method according to claim 10, wherein the plant is a member of the *Brassicaceae* (*Cruciferae*), *Gramineae*, *Malvaceae*, or *Leguminosae-Papilionoideae* families.
12. The method according to claim 11, wherein the plant is canola, corn, camelina, cotton, alfalfa, soybean, wheat, rice, or barley.
13. The method according to claim 12, wherein the plant is canola or soybean, the plant growth and/or development related gene is *REV*, and the early phase-specific promoter is the promoter associated with an amino acid permease gene (*AAP1*), an oleate 12-hydroxylase:desaturase gene, an 2S2 albumin gene (*2S2*), a fatty acid elongase gene (*FAE1*), or a leafy cotyledon gene (*LEC2*).
14. The method according to claim 13, wherein the *AAP1* promoter is the *AAP1* promoter from *Arabidopsis thaliana*, the oleate 12-hydroxylase:desaturase promoter is the oleate 12-hydroxylase:desaturase gene promoter from *Lesquerella fendleri* (*LFAH12*), the 2S2 gene promoter is from *Arabidopsis thaliana*, the fatty acid elongase gene promoter is from *Arabidopsis thaliana*, or the leafy cotyledon gene promoter is from *Arabidopsis thaliana*.
15. A method for increasing the number of seeds obtainable from a plant comprising over expressing a plant growth and development associated gene in a seed during early embryo development.
16. The method according to claim 15, wherein the growth and/or development related gene expression in the seed is under the control of an early phase-specific embryo promoter.

17. The method according to claim 16, wherein the early phase-specific embryo promoter is heterologous to the plant.

18. The method according to claim 16, wherein the early phase-specific embryo promoter is homologous to the plant.

19. The method according to any of claims 16 through 18, wherein the early phase-specific promoter is the promoter associated with an amino acid permease gene (AAP1), an oleate 12-hydroxylase:desaturase gene, an 2S2 albumin gene (2S2), a fatty acid elongase gene (FAE1), or a leafy cotyledon gene (LEC2).

20. The method according to claim 19, wherein the AAP1 promoter is the AAP1 promoter from *Arabidopsis thaliana*, the oleate 12-hydroxylase:desaturase promoter is the oleate 12-hydroxylase:desaturase gene promoter from *Lesquerella fendleri* (LFAH12), the 2S2 gene promoter is from *Arabidopsis thaliana*, the fatty acid elongase gene promoter is from *Arabidopsis thaliana*, or the leafy cotyledon gene promoter is from *Arabidopsis thaliana*.

21. The method according to any of claims 15 through 20, wherein the plant growth and/or development associated gene is a *REV* gene, a *CAP* (cyclase-associated protein) gene, a rice histone deacetylase 1 gene, a *E2Fc* gene, a *BKI* gene, a *BRI1* gene, a *ARL* (Argos-Like) gene, a *bril* (brassinosteroid hormone perception) gene, a *FATB* gene, a Pepino gene, a *RGS* (regulator of G protein signaling) gene, a *TIP1* gene, a *BB* (Big Brother) gene, a *RHD2* gene, a *INCW2* gene, a *MNI* gene, a *WAK2* gene, a *WAK4* gene, or a *AP2* gene.

22. The method according to claim 21, wherein the growth and/or development associated gene is *REV*.

23. The method according to claim 22, wherein the *REV* gene is derived from *Arabidopsis thaliana*.

24. The method according to any of claims 15 through 23, wherein the plant is a monocot or a dicot.

25. The method according to claim 24, wherein the plant is a member of the *Brassicaceae* (*Cruciferae*), *Gramineae*, *Malvaceae*, or *Leguminosae-Papilionoideae* families.

26. The method according to claim 25, wherein the plant is canola, corn, camelina, cotton, alfalfa, soybean, wheat, rice, or barley.

27. The method according to claim 26, wherein the plant is canola or soybean, the plant growth and/or development related gene is *REV*, and the early phase-specific promoter is the promoter associated with an amino acid permease gene (AAP1), an oleate 12-hydroxylase:desaturase gene, an 2S2 albumin gene (2S2), a fatty acid elongase gene (FAE1), or a leafy cotyledon gene (LEC2).

28. The method according to claim 27, wherein the AAP1 promoter is the AAP1 promoter from *Arabidopsis thaliana*, the oleate 12-hydroxylase:desaturase promoter is the oleate 12-hydroxylase:desaturase gene promoter from *Lesquerella fendleri* (LFAH12), the 2S2 gene promoter is from *Arabidopsis thaliana*, the fatty acid elongase gene promoter is from *Arabidopsis thaliana*, or the leafy cotyledon gene promoter is from *Arabidopsis thaliana*.

29. A genetic construct comprising a nucleic acid sequence encoding a plant growth and/or development associated gene operatively linked to one or more control sequences wherein the one or more control sequences are capable of promoting expression of the plant growth and/or development associated gene during embryo development.

30. The genetic construct according to claim 29, wherein the control sequence is an early phase-specific embryo promoter.

31. The genetic construct according to claim 30, wherein the early phase specific embryo promoter is the promoter associated with an amino acid permease gene (AAP1), an oleate 12-hydroxylase:desaturase gene, an 2S2 albumin gene (2S2), a fatty acid elongase gene (FAE1), or a leafy cotyledon gene (LEC2).

32. The genetic construct according to claim 31, wherein the AAP1 promoter is the AAP1 promoter from *Arabidopsis thaliana*, the oleate 12-hydroxylase:desaturase promoter is the oleate 12-hydroxylase:desaturase gene promoter from *Lesquerella*

fendleri (LFAH12), the 2S2 gene promoter is from *Arabidopsis thaliana*, the fatty acid elongase gene promoter is from *Arabidopsis thaliana*, or the leafy cotyledon gene promoter is from *Arabidopsis thaliana*.

33. The genetic constructs according to any of claims 29 through 32, wherein the plant growth and/or development associated gene is a *REV* gene, gene, a *CAP* (cyclase-associated protein) gene, a rice histone deacetylase 1 gene, a *E2Fc* gene, a *BKI* gene, a *BRI1* gene, a *ARL* (Argos-Like) gene, a *bril* (brassinosteroid hormone perception) gene, a *FATB* gene, a Pepino gene, a *RGS* (regulator of G protein signaling) gene, a *TIP1* gene, a *BB* (Big Brother) gene, a *RHD2* gene, a *INCW2* gene, a *MN1* gene, a *WAK2* gene, a *WAK4* gene, or a *AP2* gene.

34. The genetic construct according to claim 33, wherein the plant growth and/or development gene is a *REV* gene.

35. The genetic construct according to claim 34, wherein the *REV* gene is an *Arabidopsis thaliana REV* gene.

36. The genetic construct of any of claims 29 through 35, wherein the construct further comprises a polyA sequence.

37. A method for the production of a transgenic plant having increased seed size, the method comprising: (a) introducing into a plant or into a plant cell, a genetic construct according to any of claims 29 through 36; and (b) cultivating the plant or plant cell comprising the genetic construct under conditions promoting regeneration and mature plant growth.

38. The method according to claim 37, wherein the plant or plant cell is derived from a plant which is a monocot or a dicot.

39. The method according to claim 38, wherein the plant is a member of the *Brassicaceae* (*Cruciferae*), *Gramineae*, *Malvaceae*, or *Leguminosae-Papilionoideae* families.

40. The method according to claim 39, wherein the plant is canola, corn, camelina, cotton, alfalfa, soybean, wheat, rice, or barley.

41. The method according to claim 40, wherein the plant is canola or soybean.
42. A method for the production of a transgenic plant having increased seed number, the method comprising: (a) introducing into a plant or into a plant cell, a genetic construct according to any of claims 29 through 36; and (b) cultivating the plant or plant cell comprising the genetic construct under conditions promoting regeneration and mature plant growth.
43. The method according to claim 42, wherein the plant or plant cell is derived from a plant which is a monocot or a dicot.
44. The method according to claim 43, wherein the plant is a member of the *Brassicacea* (*Cruciferae*), *Gramineae*, *Malvaceae*, or *Leguminosae-Papilionoideae* families.
45. The method according to claim 44, wherein the plant is canola, corn, camelina, cotton, alfalfa, soybean, wheat, rice, or barley.
46. The method according to claim 45, wherein the plant is canola or soybean.
47. A method for the production of a transgenic plant having increased seed size and increased seed number, the method comprising: (a) introducing into a plant or into a plant cell, a genetic construct according to any of claims 29 through 36; and (b) cultivating the plant or plant cell comprising the genetic construct under conditions promoting regeneration and mature plant growth.
48. The method according to claim 47, wherein the plant or plant cell is derived from a plant which is a monocot or a dicot.
49. The method according to claim 48, wherein the plant is a member of the *Brassicacea* (*Cruciferae*), *Gramineae*, *Malvaceae*, or *Leguminosae-Papilionoideae* families.
50. The method according to claim 49, wherein the plant is canola, corn, camelina, cotton, alfalfa, soybean, wheat, rice, or barley.

51. The method according to claim 50, wherein the plant is canola or soybean.

52. A transgenic plant comprising the genetic construct according to any of claims 29 through 36 and having increased seed size when compared to the corresponding wild-type plant, having increased seed number when compared to the corresponding wild-type plant, or having increased seed size and seed number when compared to the corresponding wild-type plant.

53. The transgenic plant according to claim 52, wherein the plant or plant cell is derived from a plant which is a monocot or a dicot.

54. The transgenic plant according to claim 53, wherein the plant is a member of the *Brassicaceae* (*Cruciferae*), *Gramineae*, *Malvaceae*, or *Leguminosae-Papilionoideae* families.

55. The transgenic plant according to claim 54, wherein the plant is canola, corn, camelina, cotton, alfalfa, soybean, wheat, rice, or barley.

56. The transgenic plant according to claim 55, wherein the plant is canola or soybean.

57. The transgenic plant according to claim 53, wherein the monocotyledonous plant is rice, oat, corn, or wheat.

58. The transgenic plant according to claim 57, wherein the plant is a member of the *Brassicaceae* (*Cruciferae*), *Malvaceae*, or *Leguminosae-Papilionoideae* families.

59. The transgenic plant according to claim 53, wherein the dicotyledonous plant is soybean, cotton, camelina, alfalfa, or canola.

60. The transgenic plant according to any of claims 52 through 59, wherein the plant is a whole plant, a plant organ, a seed, a plant cells, or the progeny thereof.

61. The transgenic plant according to claim 60, wherein the plant organ is a leaf, a stem, a flower, or a root.

62. The transgenic plant according to claim 60, wherein the plant cell is a tissue culture cell.

63. A method for selecting for a gene that increases plant yield when functionally associated with an early phase-specific embryo promoter; comprising:

constructing an expression vector comprising a gene associated with plant growth and/or development functionally associated with a early phase-specific embryo promoter;

transfecting a plant cell with the expression vector to form a transgenic plant;

growing the transgenic plant; and

selecting the growth and/or development associated gene in the transgenic plants that have increased yield when compared to the wild-type plant.

64. The method according to claim 63, wherein the growth and/or development associated gene is a *CAP* (cyclase-associated protein) gene, a rice histone deacetylase 1 gene, a *E2Fc* gene, a *BKI* gene, a *BR11* gene, a *ARL* (Argos-Like) gene, a *bril* (brassinosteroid hormone perception) gene, a *FATB* gene, a Pepino gene, a *RGS* (regulator of G protein signaling) gene, a *TIP1* gene, a *BB* (Big Brother) gene, a *RHD2* gene, a *INCW2* gene, a *MNI* gene, a *WAK2* gene, a *WAK4* gene, or a *AP2* gene.

65. The method according to claim 63, wherein the early phase specific embryo promoter is the promoter associated with an amino acid permease gene (*AAP1*), an oleate 12-hydroxylase: desaturase gene, an 2S2 albumin gene (*2S2*), a fatty acid elongase gene (*FAE1*), or a leafy cotyledon gene (*LEC2*).

66. The method according to claim 63, wherein the *AAP1* promoter is the *AAP1* promoter from *Arabidopsis thaliana*, the oleate 12-hydroxylase: desaturase promoter is the oleate 12-hydroxylase:desaturase gene promoter from *Lesquerella fendleri* (*LFAH12*), the 2S2 gene promoter is from *Arabidopsis thaliana*, the fatty acid elongase gene promoter is from *Arabidopsis thaliana*, or the leafy cotyledon gene promoter is from *Arabidopsis thaliana*.