



US 20110041219A1

(19) **United States**(12) **Patent Application Publication**  
**COOK et al.**(10) **Pub. No.: US 2011/0041219 A1**(43) **Pub. Date: Feb. 17, 2011**(54) **PROMOTER, PROMOTER CONTROL  
ELEMENTS, AND COMBINATIONS, AND  
USES THEREOF**(75) Inventors: **Zhihong COOK**, Woodland Hills,  
CA (US); **Yiwen Fang**, Los  
Angeles, CA (US); **Ken Feldmann**,  
Newbury Park, CA (US); **Edward  
A. Kiegle**, Chester, VT (US); **Shing  
Kwok**, Woodland Hills, CA (US);  
**Yu-Ping Lu**, Oak Park, CA (US);  
**Leonard Medrano**, Azusa, CA  
(US); **Roger Pennell**, Malibu, CA  
(US); **Richard Schneeberger**,  
Carlsbad, CA (US); **Chuan-Yin  
Wu**, Newbury Park, CA (US)

Correspondence Address:

**BIRCH STEWART KOLASCH & BIRCH**  
**PO BOX 747**  
**FALLS CHURCH, VA 22040-0747 (US)**(73) Assignee: **CERES, INC.**, Thousand Oaks, CA  
(US)(21) Appl. No.: **12/891,723**(22) Filed: **Sep. 27, 2010****Related U.S. Application Data**(60) Division of application No. 11/600,953, filed on Nov.  
14, 2006, now Pat. No. 7,851,608, which is a divisionof application No. 10/957,569, filed on Sep. 30, 2004,  
now Pat. No. 7,402,667, which is a continuation-in-  
part of application No. 10/950,321, filed on Sep. 23,  
2004, now Pat. No. 7,173,121.(60) Provisional application No. 60/505,689, filed on Sep.  
23, 2003, provisional application No. 60/511,460,  
filed on Oct. 14, 2003, provisional application No.  
60/518,075, filed on Nov. 6, 2003, provisional appli-  
cation No. 60/527,611, filed on Dec. 4, 2003, provi-  
sional application No. 60/529,352, filed on Dec. 12,  
2003, provisional application No. 60/544,771, filed on  
Feb. 13, 2004.**Publication Classification**(51) **Int. Cl.**  
**A01H 5/00** (2006.01)  
**C07H 21/00** (2006.01)  
**C12N 15/63** (2006.01)  
**C12N 5/04** (2006.01)  
(52) **U.S. Cl.** ..... **800/298; 536/24.1; 435/320.1;**  
435/419(57) **ABSTRACT**

The present invention is directed to promoter sequences and promoter control elements, polynucleotide constructs comprising the promoters and control elements, and methods of identifying the promoters, control elements, or fragments thereof. The invention further relates to the use of the present promoters or promoter control elements to modulate transcript levels.

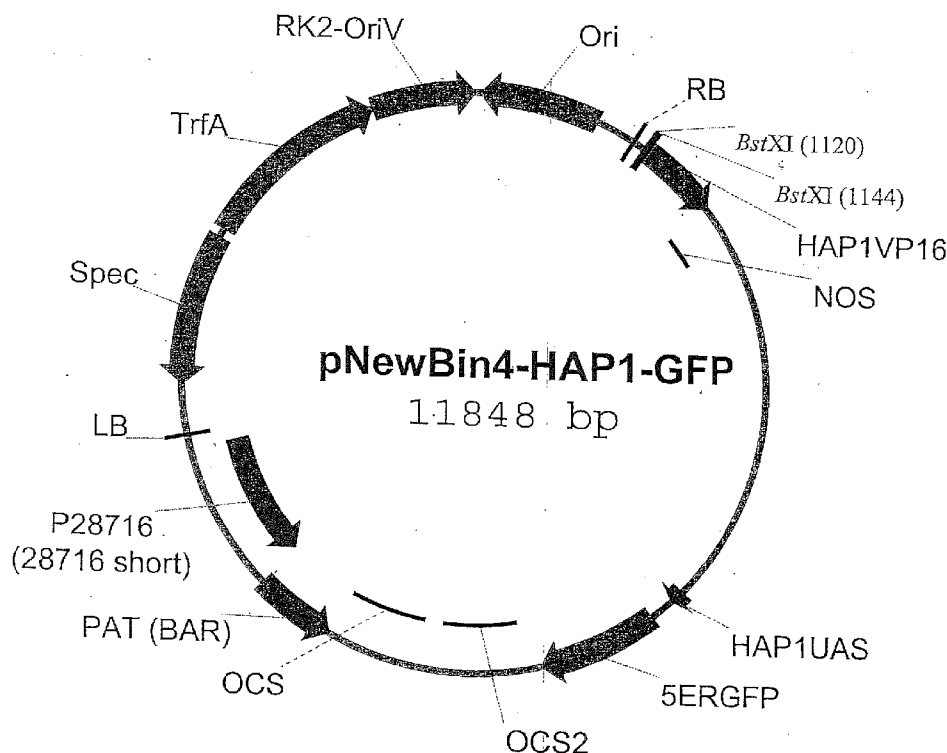
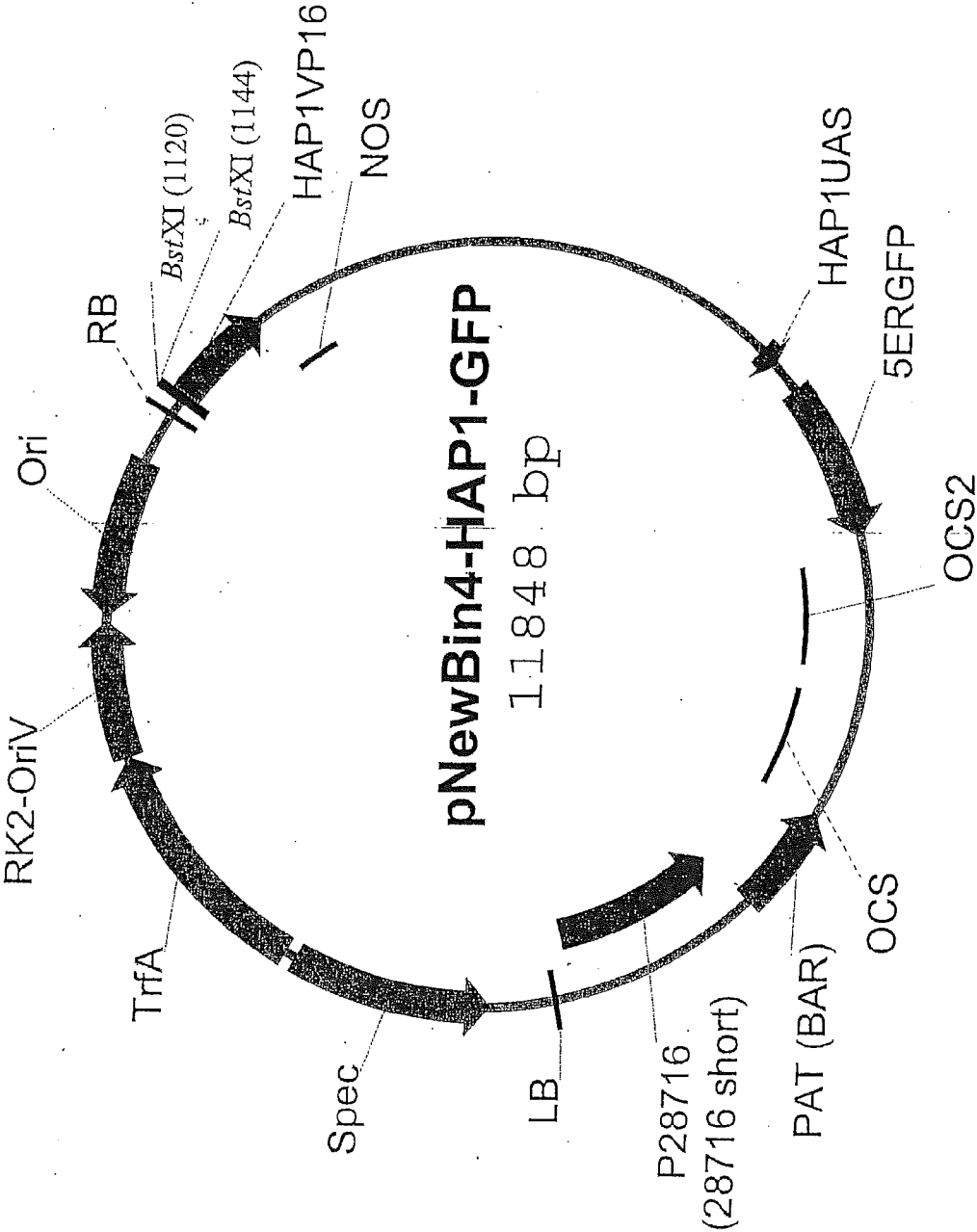


FIGURE 1



# PROMOTER, PROMOTER CONTROL ELEMENTS, AND COMBINATIONS, AND USES THEREOF

## CROSS REFERENCES TO RELATED APPLICATIONS

[0001] This application is a divisional of co-pending application Ser. No. 11/600,953 filed on Nov. 14, 2006, and for which priority is claimed under 35 U.S.C. §120; the entire contents of which are hereby incorporated by reference. The Non-Provisional application Ser. No. 11/600,953 claims priority of application Ser. No. 10/957,569 filed Sep. 30, 2004, which is a Continuation in part of Application No. 60/505,689 filed Sep. 23, 2004, entitled "Promoter, Promoter Control Elements, And Combinations", and Uses Thereof, which claims priority under 35 U.S.C. §119(e) on U.S. Provisional Application No. 60,505,689 filed on Sep. 23, 2003; Application No. 60,511,460 filed on Oct. 14, 2003; Application No. 60/518,075 filed on Nov. 6, 2003; Application No. 60/527,611 filed on Dec. 4, 2003; Application No. 60/529,352 filed on Dec. 12, 2003 and Application No. 60/544,771 filed on Feb. 13, 2005, each of which are incorporated by reference in its entirety.

## FIELD OF THE INVENTION

[0002] The present invention relates to promoters and promoter control elements that are useful for modulating transcription of a desired polynucleotide. Such promoters and promoter control elements can be included in polynucleotide constructs, expression cassettes, vectors, or inserted into the chromosome or as an exogenous element, to modulate in vivo and in vitro transcription of a polynucleotide. Host cells, including plant cells, and organisms, such as regenerated plants therefrom, with desired traits or characteristics using polynucleotides comprising the promoters and promoter control elements of the present invention are also a part of the invention.

## BACKGROUND OF THE INVENTION

[0003] This invention relates to the field of biotechnology and, in particular, to specific promoter sequences and promoter control element sequences which are useful for the transcription of polynucleotides in a host cell or transformed host organism.

[0004] One of the primary goals of biotechnology is to obtain organisms, such as plants, mammals, yeast, and prokaryotes having particular desired characteristics or traits. Examples of these characteristic or traits abound and may include, for example, in plants, virus resistance, insect resistance, herbicide resistance, enhanced stability or additional nutritional value. Recent advances in genetic engineering have enabled researchers in the field to incorporate polynucleotide sequences into host cells to obtain the desired qualities in the organism of choice. This technology permits one or more polynucleotides from a source different than the organism of choice to be transcribed by the organism of choice. If desired, the transcription and/or translation of these new polynucleotides can be modulated in the organism to exhibit a desired characteristic or trait. Alternatively, new patterns of transcription and/or translation of polynucleotides

endogenous to the organism can be to produced. Both approaches can be used at the same time.

## SUMMARY OF THE INVENTION

[0005] The present invention is directed to isolated polynucleotide sequences that comprise promoters and promoter control elements from plants, especially *Arabidopsis thaliana*, *Glycine max*, *Oryza sativa*, and *Zea mays*, and other promoters and promoter control elements functional in plants.

[0006] It is an object of the present invention to provide isolated polynucleotides that are promoter sequences. These promoter sequences comprise, for example,

[0007] (1) a polynucleotide having a nucleotide sequence as set forth in Table 1, in the section entitled "The predicted promoter sequence" or fragment thereof;

[0008] (2) a polynucleotide having a nucleotide sequence having at least 80% sequence identity to a sequence as set forth in Table 1, in the section entitled "The predicted promoter sequence" or fragment thereof; and

[0009] (3) a polynucleotide having a nucleotide sequence which hybridizes to a sequence as set forth in Table 1, in the section entitled "The predicted promoter sequence" under a condition establishing a  $T_m$ -20° C.

[0010] It is another object of the present invention to provide isolated polynucleotides that are promoter control element sequences. These promoter control element sequences comprise, for example,

[0011] (1) a polynucleotide having a nucleotide sequence as set forth in Table 1, in the section entitled "The predicted promoter sequence" or fragment thereof;

[0012] (2) a polynucleotide having a nucleotide sequence having at least 80% sequence identity to a sequence as set forth in Table 1, in the section entitled "The predicted promoter sequence" or fragment thereof; and

[0013] (3) a polynucleotide having a nucleotide sequence which hybridizes to a sequence as set forth in Table 1, in the section entitled "The predicted promoter sequence" under a condition establishing a  $T_m$ -20° C.

[0014] Promoter or promoter control element sequences of the present invention are capable of modulating preferential transcription.

[0015] In another embodiment, the present promoter control elements are capable of serving as or fulfilling the function, for example, as a core promoter, a TATA box, a polymerase binding site, an initiator site, a transcription binding site, an enhancer, an inverted repeat, a locus control region, or a scaffold/matrix attachment region.

[0016] It is yet another object of the present invention to provide a polynucleotide that includes at least a first and a second promoter control element. The first promoter control element is a promoter control element sequence as discussed above, and the second promoter control element is heterologous to the first control element. Moreover, the first and second control elements are operably linked. Such promoters may modulate transcript levels preferentially in a tissue or under particular conditions.

[0017] In another embodiment, the present isolated polynucleotide comprises a promoter or a promoter control element as described above, wherein the promoter or promoter control element is operably linked to a polynucleotide to be transcribed.

[0018] In another embodiment of the present vector, the promoter and promoter control elements of the instant invention are operably linked to a heterologous polynucleotide that is a regulatory sequence.

[0019] It is another object of the present invention to provide a host cell comprising an isolated polynucleotide or vector as described above or fragment thereof. Host cells include, for instance, bacterial, yeast, insect, mammalian, and plant. The host cell can comprise a promoter or promoter control element exogenous to the genome. Such a promoter can modulate transcription in cis- and in trans-.

[0020] In yet another embodiment, the present host cell is a plant cell capable of regenerating into a plant.

[0021] It is yet another embodiment of the present invention to provide a plant comprising an isolated polynucleotide or vector described above.

[0022] It is another object of the present invention to provide a method of modulating transcription in a sample that contains either a cell-free system of transcription or host cell. This method comprises providing a polynucleotide or vector according to the present invention as described above, and contacting the sample of the polynucleotide or vector with conditions that permit transcription.

[0023] In another embodiment of the present method, the polynucleotide or vector preferentially modulates

- [0024] (a) constitutive transcription,
- [0025] (b) stress induced transcription,
- [0026] (c) light induced transcription,
- [0027] (d) dark induced transcription,
- [0028] (e) leaf transcription,
- [0029] (f) root transcription,
- [0030] (g) stem or shoot transcription,
- [0031] (h) silique transcription,
- [0032] (i) callus transcription,
- [0033] (j) flower transcription,

[0034] (k) immature bud and inflorescence specific transcription, or

[0035] (l) senescing induced transcription

[0036] (m) germination transcription.

Other and further objects of the present invention will be made clear or become apparent from the following description.

## BRIEF DESCRIPTION OF THE TABLES AND FIGURES

Table 1

[0037] Table 1 consists of the Expression Reports for each promoter of the invention providing the nucleotide sequence for each promoter and details for expression driven by each of the nucleic acid promoter sequences as observed in transgenic plants. The results are presented as summaries of the spatial expression, which provides information as to gross and/or specific expression in various plant organs and tissues. The observed expression pattern is also presented, which gives details of expression during different generations or different developmental stages within a generation. Additional information is provided regarding the associated gene, the GenBank reference, the source organism of the promoter, and the vector and marker genes used for the construct. The following symbols are used consistently throughout the Table:

[0038] T1: First generation transformant

[0039] T2: Second generation transformant

[0040] T3: Third generation transformant

[0041] (L): low expression level

[0042] (M): medium expression level

[0043] (H): high expression level

[0044] Each row of the table begins with heading of the data to be found in the section. The following provides a description of the data to be found in each section:

Heading in Table 1	Description
Promoter	Identifies the particular promoter by its construct ID.
Modulates the gene:	This row states the name of the gene modulated by the promoter
The GenBank description of the gene:	This field gives the Locus Number of the gene as well as the accession number.
The predicted promoter sequence:	Identifies the nucleic acid promoter sequence in question.
The promoter was cloned from the organism:	Identifies the source of the DNA template used to clone the promoter.
The experimental promoter sequence:	Identifies the nucleic acid sequence in planta driving expression of the reporter gene.
The promoter was cloned in the vector:	Identifies the vector used into which a promoter was cloned.
When cloned into the vector the promoter was operably linked to a marker, which was the type:	Identifies the type of marker linked to the promoter. The marker is used to determine patterns of gene expression in plant tissue.
Promoter-marker vector was tested in:	Identifies the organism in which the promoter-marker vector was tested.
Generation screened: T1 Mature T2 Seedling T2 Mature T3 Seedling	Identifies the plant generation(s) used in the screening process. T1 plants are those plants subjected to the transformation event while the T2 generation plants are from the seeds collected from the T1 plants and T3 plants are from the seeds of T2 plants.
The spatial expression of the promoter-marker vector was found observed in and would be useful in expression in any or all of the following:	Identifies the specific parts of the plant where various levels of GFP expression are observed. Expression levels are noted as either low (L), medium (M), or high (H).

-continued

Heading in Table 1	Description
Observed expression pattern of the promoter-marker vector was in: T1 mature: T2 seedling: The promoter can be of use in the following trait and sub-trait areas: (search for the trait and sub-trait table) The promoter has utility in:	Identifies a general explanation of where GFP expression in different generations of plants was observed.  Identifies which traits and subtraits the promoter cDNA can modulate  Identifies a specific function or functions that can be modulated using the promoter cDNA.
Misc. promoter information:	
Bidirectionality:	“Bidirectionality” is determined by the number of base pairs between the promoter and the start codon of a neighboring gene. A promoter is considered bidirectional if it is closer than 200 bp to a start codon of a gene 5' or 3' to the promoter.
Exons:	“Exons” (or any coding sequence) identifies if the promoter has overlapped with either the modulating gene's or other neighboring gene's coding sequence. A “fail” for exons means that this overlap has occurred.
Repeats:	“Repeats” identifies the presence of normally occurring sequence repeats that randomly exist throughout the genome. A “pass” for repeats indicates a lack of repeats in the promoter.
An overlap in an exon with the endogenous coding sequence to the promoter occurs at base pairs:	Identifies the specific nucleotides overlapping the UTR region or exon of a neighboring gene. The orientation relative to the promoter is designated with a 5' or 3'.
The Ceres cDNA ID of the endogenous coding sequence to the promoter:	Identifies the number associated with the Ceres cDNA that corresponds to the endogenous cDNA sequence of the promoter.
cDNA nucleotide sequence:	The nucleic acid sequence of the Ceres cDNA matching the endogenous cDNA region of the promoter.
Coding sequence:	A translated protein sequence of the gene modulated by a protein encoded by a cDNA
Microarray Data shows that the coding sequence was expressed in the following experiments, which shows that the promoter would useful to modulate expression in situations similar to the following:	Microarray data is identified along with the corresponding experiments along with the corresponding gene expression. Gene expression is identified by a “+” or a “-” in the “SIGN(LOG_RATIO)” column. A “+” notation indicates the cDNA is upregulated while a “-” indicates that the cDNA is downregulated. The “SHORT_NAME” field describes the experimental conditions.
The parameters for the microarray experiments listed above by EXPT_REP_ID and Short_Name are as follow below:	Parameters for microarray experiments include age, organism, specific tissues, age, treatments and other distinguishing characteristics or features.

Table 2

**[0045]** Table 1 provides the results of differential expression experiments indicating if the expression levels were increased (“+”) or decreased (“-”). Such increase or decrease expression levels indicates the utility of the corresponding promoter. The following Table 2 correlates the various differential expression experiments with the utility for the promoter that would be understood from an increased or decreased expression. Table 2 includes three columns, the first column (“EXPT\_REP\_ID”) lists the microarray experiments by their experimental prep ID number and correspond to the same number listings in Table 1 in the “Microarray data” section. The second column lists the Short\_Name of the experiment that corresponds to the EXPT\_REP\_ID. When a cDNA is differentially expressed in an experiment, identified by its EXPT\_REP\_ID, the cDNA and its endogenous promoter can be used to modulate the traits and subtraits listed in the third column

FIG. 1

**[0046]** FIG. 1 is a schematic representation of the vector pNewBin4-HAP1-GFP. The definitions of the abbreviations used in the vector map are as follows:

**[0047]** Ori—the origin of replication used by an *E. coli* host

**[0048]** RB—sequence for the right border of the T-DNA from pMOG800

**[0049]** BstXI—restriction enzyme cleavage site used for cloning

**[0050]** HAP1VP16—coding sequence for a fusion protein of the HAP1 and VP16 activation domains

**[0051]** NOS—terminator region from the nopaline synthase gene

**[0052]** HAP1UAS—the upstream activating sequence for HAP1

**[0053]** 5ERGFP—the green fluorescent protein gene that has been optimized for localization to the endoplasmic reticulum

- [0054] OCS2—the terminator sequence from the octopine synthase 2 gene
- [0055] OCS—the terminator sequence from the octopine synthase gene
- [0056] p28716 (a.k.a 28716 short)—promoter used to drive expression of the PAT (BAR) gene
- [0057] PAT (BAR)—a marker gene conferring herbicide resistance
- [0058] LB—sequence for the left border of the T-DNA from pMOG800
- [0059] Spec—a marker gene conferring spectinomycin resistance
- [0060] TrfA—transcription repression factor gene
- [0061] RK2-OriV—origin of replication for *Agrobacterium*

## DETAILED DESCRIPTION OF THE INVENTION

### 1. Definitions

[0062] Chimeric: The term “chimeric” is used to describe polynucleotides or genes, as defined supra, or constructs wherein at least two of the elements of the polynucleotide or gene or construct, such as the promoter and the polynucleotide to be transcribed and/or other regulatory sequences and/or filler sequences and/or complements thereof, are heterologous to each other.

[0063] Constitutive Promoter: Promoters referred to herein as “constitutive promoters” actively promote transcription under most, but not necessarily all, environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcript initiation region and the 1' or 2' promoter derived from T-DNA of *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes, such as the maize ubiquitin-1 promoter, known to those of skill.

[0064] Core Promoter: This is the minimal stretch of contiguous DNA sequence that is sufficient to direct accurate initiation of transcription by the RNA polymerase II machinery (for review see: Struhl, 1987, *Cell* 49: 295-297; Smale, 1994, In *Transcription: Mechanisms and Regulation* (eds R. C. Conaway and J. W. Conaway), pp 63-81/Raven Press, Ltd., New York; Smale, 1997, *Biochim Biophys. Acta* 1351: 73-88; Smale et al., 1998, *Cold Spring Harb. Symp. Quant. Biol.* 58: 21-31; Smale, 2001, *Genes & Dev.* 15: 2503-2508; Weis and Reinberg, 1992, *FASEB J.* 6: 3300-3309; Burke et al., 1998, *Cold Spring Harb. Symp. Quant. Biol.* 63: 75-82). There are several sequence motifs, including the TATA box, initiator (Inr), TFIIB recognition element (BRE) and downstream core promoter element (DPE), that are commonly found in core promoters, however not all of these elements occur in all promoters and there are no universal core promoter elements (Butler and Kadonaga, 2002, *Genes & Dev.* 16: 2583-2592).

[0065] Domain: Domains are fingerprints or signatures that can be used to characterize protein families and/or parts of proteins. Such fingerprints or signatures can comprise conserved (1) primary sequence, (2) secondary structure, and/or (3) three-dimensional conformation. A similar analysis can be applied to polynucleotides. Generally, each domain has been associated with either a conserved primary sequence or a sequence motif. Generally these conserved primary sequence motifs have been correlated with specific in vitro and/or in vivo activities. A domain can be any length, including the entirety of the polynucleotide to be transcribed.

Examples of domains include, without limitation, AP2, helix, homeobox, zinc finger, etc.

[0066] Endogenous: The term “endogenous,” within the context of the current invention refers to any polynucleotide, polypeptide or protein sequence which is a natural part of a cell or organisms regenerated from said cell. In the context of promoter, the term “endogenous coding region” or “endogenous cDNA” refers to the coding region that is naturally operably linked to the promoter.

[0067] Enhancer/Suppressor: An “enhancer” is a DNA regulatory element that can increase the steady state level of a transcript, usually by increasing the rate of transcription initiation. Enhancers usually exert their effect regardless of the distance, upstream or downstream location, or orientation of the enhancer relative to the start site of transcription. In contrast, a “suppressor” is a corresponding DNA regulatory element that decreases the steady state level of a transcript, again usually by affecting the rate of transcription initiation. The essential activity of enhancer and suppressor elements is to bind a protein factor(s). Such binding can be assayed, for example, by methods described below. The binding is typically in a manner that influences the steady state level of a transcript in a cell or in an in vitro transcription extract.

[0068] Exogenous: As referred to within, “exogenous” is any polynucleotide, polypeptide or protein sequence, whether chimeric or not, that is introduced into the genome of a host cell or organism regenerated from said host cell by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include *Agrobacterium*-mediated transformation (of dicots—e.g. Salomon et al. *EMBO J.* 3:141 to (1984); Herrera-Estrella et al. *EMBO J.* 2:987 (1983); of monocots, representative papers are those by Escudero et al., *Plant J.* 10:355 (1996), Ishida et al., *Nature Biotechnology* 14:745 (1996), May et al., *Bio/Technology* 13:486 (1995)), biolistic methods (Armaleo et al., *Current Genetics* 17:97 1990)), electroporation, in planta techniques, and the like. Such a plant containing the exogenous nucleic acid is referred to here as a T<sub>0</sub> for the primary transgenic plant and T<sub>1</sub> for the first generation. The term “exogenous” as used herein is also intended to encompass inserting a naturally found element into a non-naturally found location.

[0069] Gene: The term “gene,” as used in the context of the current invention, encompasses all regulatory and coding sequence contiguously associated with a single hereditary unit with a genetic function. Genes can include non-coding sequences that modulate the genetic function that include, but are not limited to, those that specify polyadenylation, transcriptional regulation, DNA conformation, chromatin conformation, extent and position of base methylation and binding sites of proteins that control all of these. Genes encoding proteins are comprised of “exons” (coding sequences), which may be interrupted by “introns” (non-coding sequences). In some instances complexes of a plurality of protein or nucleic acids or other molecules, or of any two of the above, may be required for a gene's function. On the other hand a gene's genetic function may require only RNA expression or protein production, or may only require binding of proteins and/or nucleic acids without associated expression. In certain cases, genes adjacent to one another may share sequence in such a way that one gene will overlap the other. A gene can be found within the genome of an organism, in an artificial chromosome, in a plasmid, in any other sort of vector, or as a separate isolated entity.

**[0070]** Heterologous sequences: “Heterologous sequences” are those that are not operatively linked or are not contiguous to each other in nature. For example, a promoter from corn is considered heterologous to an *Arabidopsis* coding region sequence. Also, a promoter from a gene encoding a growth factor from corn is considered heterologous to a sequence encoding the corn receptor for the growth factor. Regulatory element sequences, such as UTRs or 3' end termination sequences that do not originate in nature from the same gene as the coding sequence originates from, are considered heterologous to said coding sequence. Elements operatively linked in nature and contiguous to each other are not heterologous to each other.

**[0071]** Homologous: In the current invention, a “homologous” gene or polynucleotide or to polypeptide refers to a gene or polynucleotide or polypeptide that shares sequence similarity with the gene or polynucleotide or polypeptide of interest. This similarity may be in only a fragment of the sequence and often represents a functional domain such as, examples including without limitation a DNA binding domain or a domain with tyrosine kinase activity. The functional activities of homologous polynucleotide are not necessarily the same.

**[0072]** Inducible Promoter: An “inducible promoter” in the context of the current invention refers to a promoter, the activity of which is influenced by certain conditions, such as light, temperature, chemical concentration, protein concentration, conditions in an organism, cell, or organelle, etc. A typical example of an inducible promoter, which can be utilized with the polynucleotides of the present invention, is PARSK1, the promoter from an *Arabidopsis* gene encoding a serine-threonine kinase enzyme, and which promoter is induced by dehydration, abscisic acid and sodium chloride (Wang and Goodman, Plant J. 8:37 (1995)). Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, the presence or absence of a nutrient or other chemical compound or the presence of light.

**[0073]** Modulate Transcription Level: As used herein, the phrase “modulate transcription” describes the biological activity of a promoter sequence or promoter control element. Such modulation includes, without limitation, includes up- and down-regulation of initiation of transcription, rate of transcription, and/or transcription levels.

**[0074]** Mutant: In the current invention, “mutant” refers to a heritable change in nucleotide sequence at a specific location. Mutant genes of the current invention may or may not have an associated identifiable phenotype.

**[0075]** Operable Linkage: An “operable linkage” is a linkage in which a promoter sequence or promoter control element is connected to a polynucleotide sequence (or sequences) in such a way as to place transcription of the polynucleotide sequence under the influence or control of the promoter or promoter control element. Two DNA sequences (such as a polynucleotide to be transcribed and a promoter sequence linked to the 5' end of the polynucleotide to be transcribed) are said to be operably linked if induction of promoter function results in the transcription of mRNA encoding the polynucleotide and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter sequence to direct the expression of the protein, antisense RNA or ribozyme, or (3) interfere with the ability of the DNA template to be transcribed. Thus, a

promoter sequence would be operably linked to a polynucleotide sequence if the promoter was capable of effecting transcription of that polynucleotide sequence.

**[0076]** Optional Promoter Fragments: The phrase “optional promoter fragments” is used to refer to any sub-sequence of the promoter that is not required for driving transcription of an operationally linked coding region. These fragments comprise the 5' UTR and any exon(s) of the endogenous coding region. The optional promoter fragments may also comprise any exon(s) and the 3' or 5' UTR of the gene residing upstream of the promoter (that is, 5' to the promoter). Optional promoter fragments also include any intervening sequences that are introns or sequence that occurs between exons or an exon and the UTR.

**[0077]** Orthologous: “Orthologous” is a term used herein to describe a relationship between two or more polynucleotides or proteins. Two polynucleotides or proteins are “orthologous” to one another if they serve a similar function in different organisms. In general, orthologous polynucleotides or proteins will have similar catalytic functions (when they encode enzymes) or will serve similar structural functions (when they encode proteins or RNA that form part of the ultrastructure of a cell).

**[0078]** Percentage of sequence identity: “Percentage of sequence identity,” as used herein, is determined by comparing two optimally aligned sequences over a comparison window, where the fragment of the polynucleotide or amino acid sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman Add. APL. Math. 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman Proc. Natl. Acad. Sci. (USA) 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis.), or by inspection. Given that two sequences have been identified for comparison, GAP and BESTFIT are preferably employed to determine their optimal alignment. Typically, the default values of 5.00 for gap weight and 0.30 for gap weight length are used.

**[0079]** Plant Promoter: A “plant promoter” is a promoter capable of initiating transcription in plant cells and can modulate transcription of a polynucleotide. Such promoters need not be of plant origin. For example, promoters derived from plant viruses, such as the CaMV35S promoter or from *Agrobacterium tumefaciens* such as the T-DNA promoters, can be plant promoters. A typical example of a plant promoter of plant origin is the maize ubiquitin-1 (ubi-1) promoter known to those of skill.

**[0080]** Plant Tissue: The term “plant tissue” includes differentiated and undifferentiated tissues or plants, including but not limited to roots, stems, shoots, cotyledons, epicotyl,

hypocotyl, leaves, pollen, seeds, tumor tissue and various forms of cells in culture such as single cells, protoplast, embryos, and callus tissue. The plant tissue may be in plants or in organ, tissue or cell culture.

**[0081]** Preferential Transcription: “Preferential transcription” is defined as transcription that occurs in a particular pattern of cell types or developmental times or in response to specific stimuli or combination thereof. Non-limitative examples of preferential transcription include: high transcript levels of a desired sequence in root tissues; detectable transcript levels of a desired sequence in certain cell types during embryogenesis; and low transcript levels of a desired sequence under drought conditions. Such preferential transcription can be determined by measuring initiation, rate, and/or levels of transcription.

**[0082]** Promoter: A “promoter” is a DNA sequence that directs the transcription of a to polynucleotide. Typically a promoter is located in the 5' region of a polynucleotide to be transcribed, proximal to the transcriptional start site of such polynucleotide. More typically, promoters are defined as the region upstream of the first exon; more typically, as a region upstream of the first of multiple transcription start sites; more typically, as the region downstream of the preceding gene and upstream of the first of multiple transcription start sites; more typically, the region downstream of the polyA signal and upstream of the first of multiple transcription start sites; even more typically, about 3,000 nucleotides upstream of the ATG of the first exon; even more typically, 2,000 nucleotides upstream of the first of multiple transcription start sites. The promoters of the invention comprise at least a core promoter as defined above. Frequently promoters are capable of directing transcription of genes located on each of the complementary DNA strands that are 3' to the promoter. Stated differently, many promoters exhibit bidirectionality and can direct transcription of a downstream gene when present in either orientation (i.e. 5' to 3' or 3' to 5' relative to the coding region of the gene). Additionally, the promoter may also include at least one control element such as an upstream element. Such elements include UARs and optionally, other DNA sequences that affect transcription of a polynucleotide such as a synthetic upstream element.

**[0083]** Promoter Control Element: The term “promoter control element” as used herein describes elements that influence the activity of the promoter. Promoter control elements include transcriptional regulatory sequence determinants such as, but not limited to, enhancers, scaffold/matrix attachment regions, TATA boxes, transcription start locus control regions, UARs, URRs, other transcription factor binding sites and inverted repeats.

**[0084]** Public sequence: The term “public sequence,” as used in the context of the instant application, refers to any sequence that has been deposited in a publicly accessible database prior to the filing date of the present application. This term encompasses both amino acid and nucleotide sequences. Such sequences are publicly accessible, for example, on the BLAST databases on the NCBI FTP web site (accessible at [ncbi.nlm.nih.gov/ftp](http://ncbi.nlm.nih.gov/ftp)). The database at the NCBI FTP site utilizes “gi” numbers assigned by NCBI as a unique identifier for each sequence in the databases, thereby providing a non-redundant database for sequence from various databases, including GenBank, EMBL, DDBJ, (DNA Database of Japan) and PDB (Brookhaven Protein Data Bank).

**[0085]** Regulatory Sequence: The term “regulatory sequence,” as used in the current invention, refers to any nucleotide sequence that influences transcription or translation initiation and rate, or stability and/or mobility of a transcript or polypeptide product. Regulatory sequences include, but are not limited to, promoters, promoter control elements, protein binding sequences, 5' and 3' UTRs, transcriptional start sites, termination sequences, polyadenylation sequences, introns, certain sequences within amino acid coding sequences such as secretory signals, protease cleavage sites, etc.

**[0086]** Related Sequences: “Related sequences” refer to either a polypeptide or a nucleotide sequence that exhibits some degree of sequence similarity with a reference sequence.

**[0087]** Specific Promoters: In the context of the current invention, “specific promoters” refers to a subset of promoters that have a high preference for modulating transcript levels in a specific tissue or organ or cell and/or at a specific time during development of an organism. By “high preference” is meant at least 3-fold, preferably 5-fold, more preferably at least 10-fold still more preferably at least 20-fold, 50-fold or 100-fold increase in transcript levels under the specific condition over the transcription under any other reference condition considered. Typical examples of temporal and/or tissue or organ specific promoters of plant origin that can be used with the polynucleotides of the present invention, are: PTA29, a promoter which is capable of driving gene transcription specifically in tapetum and only during another development (Koltonow et al., *Plant Cell* 2:1201 (1990); RCc2 and RCc3, promoters that direct root-specific gene transcription in rice (Xu et al., *Plant Mol. Biol.* 27:237 (1995); TobRB27, a root-specific promoter from tobacco (Yamamoto et al., *Plant Cell* 3:371 (1991)). Examples of tissue-specific promoters under developmental control include promoters that initiate transcription only in certain tissues or organs, such as root, ovule, fruit, seeds, or flowers. Other specific promoters include those from genes encoding seed storage proteins or the lipid body membrane protein, oleosin. A few root-specific promoters are noted above. See also “Preferential transcription”.

**[0088]** Stringency: “Stringency” as used herein is a function of probe length, probe composition (G+C content), and salt concentration, organic solvent concentration, and temperature of hybridization or wash conditions. Stringency is typically compared by the to parameter  $T_m$ , which is the temperature at which 50% of the complementary molecules in the hybridization are hybridized, in terms of a temperature differential from  $T_m$ . High stringency conditions are those providing a condition of  $T_m-5^\circ\text{C}$ . to  $T_m-10^\circ\text{C}$ . Medium or moderate stringency conditions are those providing  $T_m-20^\circ\text{C}$ . to  $T_m-29^\circ\text{C}$ . Low stringency conditions are those providing a condition of  $T_m-40^\circ\text{C}$ . to  $T_m-48^\circ\text{C}$ . The relationship of hybridization conditions to  $T_m$  (in  $^\circ\text{C}$ .) is expressed in the mathematical equation

$$T_m = 81.5 - 16.6(\log_{10} [\text{Na}^+]) + 0.41(\%G+C) - (600/N) \quad (1)$$

where N is the length of the probe. This equation works well for probes 14 to 70 nucleotides in length that are identical to the target sequence. The equation below for  $T_m$  of DNA-DNA hybrids is useful for probes in the range of 50 to greater than 500 nucleotides, and for conditions that include an organic solvent (formamide).

$$T_m = 81.5 + 16.6 \log \{ [\text{Na}^+] / (1 + 0.7[\text{Na}^+]) \} + 0.41(\%G+C) - 500/L \cdot 0.63(\%\text{formamide}) \quad (2)$$



where L is the length of the probe in the hybrid. (P. Tijessen, "Hybridization with Nucleic Acid Probes" in *Laboratory Techniques in Biochemistry and Molecular Biology*, P. C. van der Vliet, ed., c. 1993 by Elsevier, Amsterdam.) The  $T_m$  of equation (2) is affected by the nature of the hybrid; for DNA-RNA hybrids  $T_m$  is 10-15° C. higher than calculated, for RNA-RNA hybrids  $T_m$  is 20-25° C. higher. Because the  $T_m$  decreases about 1° C. for each 1% decrease in homology when a long probe is used (Bonner et al., J. Mol. Biol. 81:123 (1973)), stringency conditions can be adjusted to favor detection of identical genes or related family members.

**[0089]** Equation (2) is derived assuming equilibrium and therefore, hybridizations according to the present invention are most preferably performed under conditions of probe excess and for sufficient time to achieve equilibrium. The time required to reach equilibrium can be shortened by inclusion of a hybridization accelerator such as dextran sulfate or another high volume polymer in the hybridization buffer.

**[0090]** Stringency can be controlled during the hybridization reaction or after hybridization has occurred by altering the salt and temperature conditions of the wash solutions used. The formulas shown above are equally valid when used to compute the stringency of a wash solution. Preferred wash solution stringencies lie within the ranges stated above; high stringency is 5-8° C. below  $T_m$ , medium or moderate stringency is 26-29° C. below  $T_m$  and low stringency is 45-48° C. below  $T_m$ .

**[0091]** Substantially free of: A composition containing A is "substantially free of" B when at least 85% by weight of the total A+B in the composition is A. Preferably, A comprises at least about 90% by weight of the total of A+B in the composition, more preferably at least about 95% or even 99% by weight. For example, a plant gene can be substantially free of other plant genes. Other examples include, but are not limited to, ligands substantially free of receptors (and vice versa), a growth factor substantially free of other growth factors and a transcription binding factor substantially free of nucleic acids.

**[0092]** Suppressor: See "Enhancer/Suppressor"

**[0093]** TATA to start: "TATA to start" shall mean the distance, in number of nucleotides, between the primary TATA motif and the start of transcription.

**[0094]** Transgenic plant: A "transgenic plant" is a plant having one or more plant cells that contain at least one exogenous polynucleotide introduced by recombinant nucleic acid methods.

**[0095]** Translational start site: In the context of the present invention, a "translational start site" is usually an ATG or AUG in a transcript, often the first ATG or AUG. A single protein encoding transcript, however, may have multiple translational start sites.

**[0096]** Transcription start site: "Transcription start site" is used in the current invention to describe the point at which transcription is initiated. This point is typically located about 25 nucleotides downstream from a TFIID binding site, such as a TATA box. Transcription can initiate at one or more sites within the gene, and a single polynucleotide to be transcribed may have multiple transcriptional start sites, some of which may be specific for transcription in a particular cell-type or tissue or organ. "+1" is stated relative to the transcription start site and indicates the first nucleotide in a transcript.

**[0097]** Upstream Activating Region (UAR): An "Upstream Activating Region" or "UAR" is a position or orientation dependent nucleic acid element that primarily directs tissue,

organ, cell type, or environmental regulation of transcript level, usually by affecting the rate of transcription initiation. Corresponding DNA elements that have a transcription inhibitory effect are called herein "Upstream Repressor Regions" or "URR"s. The essential activity of these elements is to bind a protein factor. Such binding can be assayed by methods described below. The binding is typically in a manner that influences the steady state level of a transcript in a cell or in vitro transcription extract.

**[0098]** Untranslated region (UTR): A "UTR" is any contiguous series of nucleotide bases that is transcribed, but is not translated. A 5' UTR lies between the start site of the transcript and the translation initiation codon and includes the +1 nucleotide. A 3' UTR lies between the translation termination codon and the end of the transcript. UTRs can have particular functions such as increasing mRNA message stability or translation attenuation. Examples of 3' UTRs include, but are not limited to polyadenylation signals and transcription termination sequences.

**[0099]** Variant: The term "variant" is used herein to denote a polypeptide or protein or polynucleotide molecule that differs from others of its kind in some way. For example, polypeptide and protein variants can consist of changes in amino acid sequence and/or charge and/or post-translational modifications (such as glycosylation, etc). Likewise, polynucleotide variants can consist of changes that add or delete a specific UTR or exon sequence. It will be understood that there may be sequence variations within sequence or fragments used or disclosed in this application. Preferably, variants will be such that the sequences have at least 80%, preferably at least 90%, 95, 97, 98, or 99% sequence identity. Variants preferably measure the primary biological function of the native polypeptide or protein or polynucleotide.

## 2. Introduction

**[0100]** The polynucleotides of the invention comprise promoters and promoter control elements that are capable of modulating transcription.

**[0101]** Such promoters and promoter control elements can be used in combination with native or heterologous promoter fragments, control elements or other regulatory sequences to to modulate transcription and/or translation.

**[0102]** Specifically, promoters and control elements of the invention can be used to modulate transcription of a desired polynucleotide, which includes without limitation:

- [0103]** (a) antisense;
- [0104]** (b) ribozymes;
- [0105]** (c) coding sequences; or
- [0106]** (d) fragments thereof.

The promoter also can modulate transcription in a host genome in cis- or in trans-.

**[0107]** In an organism, such as a plant, the promoters and promoter control elements of the instant invention are useful to produce preferential transcription which results in a desired pattern of transcript levels in a particular cells, tissues, or organs, or under particular conditions.

## 3. Table of Contents

**[0108]** The following description of the present invention is outlined in the following table of contents.

**[0109] A. Identifying and Isolating Promoter Sequences of the Invention****[0110]** (1) Cloning Methods**[0111]** (2) Chemical Synthesis**[0112] B. Generating a “core” promoter sequence****[0113] C. Isolating Related Promoter Sequences****[0114]** (1) Relatives Based on Nucleotide Sequence Identity**[0115]** (2) Relatives Based on Coding Sequence Identity**[0116]** (3) Relatives based on Common Function**[0117] D. Identifying Control Elements****[0118]** (1) Types of Transcription Control Elements**[0119]** (2) Those Described by the Examples**[0120]** (3) Those Identifiable by Bioinformatics**[0121]** (4) Those Identifiable by In Vitro and In Vivo Assays**[0122]** (5) Non-Natural Control Elements**[0123] E. Constructing Promoters and Control Elements****[0124]** (1) Combining Promoters and Promoter Control Elements**[0125]** (2) Number of Promoter Control Elements**[0126]** (3) Spacing Between Control Elements**[0127] F. Vectors****[0128]** (1) Modification of Transcription by Promoters and Promoter Control Elements**[0129]** (2) Polynucleotide to be Transcribed**[0130]** (3) Other Regulatory Elements**[0131]** (4) Other Components of Vectors**[0132] G. Insertion of Polynucleotides and Vectors Into a Host Cell****[0133]** (1) Autonomous of the Host Genome**[0134]** (2) Integrated into the Host Genome**[0135] H. Utility****A. Identifying and Isolating Promoter Sequences of the Invention**

**[0136]** The promoters and promoter control elements of the present invention are presented in Table 1 in the section entitled “The predicted promoter” sequence and were identified from *Arabidopsis thaliana* or *Oryza sativa*. Additional promoter sequences encompassed by the invention can be identified as described below.

**[0137]** (1) Cloning Methods

**[0138]** Isolation from genomic libraries of polynucleotides comprising the sequences of the promoters and promoter control elements of the present invention is possible using known techniques.

**[0139]** For example, polymerase chain reaction (PCR) can amplify the desired polynucleotides utilizing primers designed from sequences in the row titled “The spatial expression of the promoter-marker-vector”. Polynucleotide libraries comprising genomic sequences can be constructed according to Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> Ed. (1989) Cold Spring Harbor Press, Cold Spring Harbor, N.Y.), for example.

**[0140]** Other procedures for isolating polynucleotides comprising the promoter sequences of the invention include, without limitation, tail-PCR, and 5' rapid amplification of cDNA ends (RACE). See, for tail-PCR, for example, Liu et al., *Plant J* 8(3): 457-463 (September 1995); Liu et al., *Genomics* 25: 674-681 (1995); Liu et al., *Nucl. Acids Res.* 21(14): 3333-3334 (1993); and Zoe et al., *BioTechniques*

27(2): 240-248 (1999); for RACE, see, for example, *PCR Protocols: A Guide to Methods and Applications*, (1990) Academic Press, Inc.

**[0141]** (2) Chemical Synthesis

**[0142]** In addition, the promoters and promoter control elements described in Table 1 in the section entitled “The predicted promoter” sequence can be chemically synthesized according to techniques in common use. See, for example, Beaucage et al., *Tet. Lett.* (1981) 22: 1859 and U.S. Pat. No. 4,668,777.

**[0143]** Such chemical oligonucleotide synthesis can be carried out using commercially available devices, such as, Biosearch 4600 or 8600 DNA synthesizer, by Applied Biosystems, a division of Perkin-Elmer Corp., Foster City, Calif., USA; and Expedite by Perceptive Biosystems, Framingham, Mass., USA.

**[0144]** Synthetic RNA, including natural and/or analog building blocks, can be synthesized on the Biosearch 8600 machines, see above.

**[0145]** Oligonucleotides can be synthesized and then ligated together to construct the desired polynucleotide.

**B. Generating Reduced and “Core” Promoter Sequences**

**[0146]** Included in the present invention are reduced and “core” promoter sequences. The reduced promoters can be isolated from the promoters of the invention by deleting at least one 5' UTR, exon or 3' UTR sequence present in the promoter sequence that is associated with a gene or coding region located 5' to the promoter sequence or in the promoter's endogenous coding region.

**[0147]** Similarly, the “core” promoter sequences can be generated by deleting all 5' UTRs, exons and 3' UTRs present in the promoter sequence and the associated intervening sequences that are related to the gene or coding region 5' to the promoter region and the promoter's endogenous coding region.

**[0148]** This data is presented in the row titled “Optional Promoter Fragments”.

**C. Isolating Related Promoter Sequences**

**[0149]** Included in the present invention are promoter and promoter control elements that are related to those described in Table 1 in the section entitled “The predicted promoter to sequence”. Such related sequence can be isolated utilizing

**[0150]** (a) nucleotide sequence identity;**[0151]** (b) coding sequence identity; or**[0152]** (c) common function or gene products.

Relatives can include both naturally occurring promoters and non-natural promoter sequences. Non-natural related promoters include nucleotide substitutions, insertions or deletions of naturally-occurring promoter sequences that do not substantially affect transcription modulation activity. For example, the binding of relevant DNA binding proteins can still occur with the non-natural promoter sequences and promoter control elements of the present invention.

**[0153]** According to current knowledge, promoter sequences and promoter control elements exist as functionally important regions, such as protein binding sites, and spacer regions. These spacer regions are apparently required for proper positioning of the protein binding sites. Thus, nucleotide substitutions, insertions and deletions can be tolerated in these spacer regions to a certain degree without loss of function.

**[0154]** In contrast, less variation is permissible in the functionally important regions, since changes in the sequence can interfere with protein binding. Nonetheless, some variation in the functionally important regions is permissible so long as function is conserved.

**[0155]** The effects of substitutions, insertions and deletions to the promoter sequences or promoter control elements may be to increase or decrease the binding of relevant DNA binding proteins to modulate transcript levels of a polynucleotide to be transcribed. Effects may include tissue-specific or condition-specific modulation of transcript levels of the polypeptide to be transcribed. Polynucleotides representing changes to the nucleotide sequence of the DNA-protein contact region by insertion of additional nucleotides, changes to identity of relevant nucleotides, including use of chemically-modified bases, or deletion of one or more nucleotides are considered encompassed by the present invention.

**[0156]** (1) Relatives Based on Nucleotide Sequence Identity

**[0157]** Included in the present invention are promoters exhibiting nucleotide sequence identity to those described in Table 1 in the section entitled "The predicted promoter sequence".

#### DEFINITION

**[0158]** Typically, such related promoters exhibit at least 80% sequence identity, preferably at least 85%, more preferably at least 90%, and most preferably at least 95%, even more preferably, at least 96%, 97%, 98% or 99% sequence identity compared to those shown in Table 1 in the section entitled "The predicted promoter" sequence. Such sequence identity can be calculated by the algorithms and computers programs described above.

**[0159]** Usually, such sequence identity is exhibited in an alignment region that is at least 75% of the length of a sequence shown in Table 1 in the section entitled "The predicted promoter" sequence or corresponding full-length sequence; more usually at least 80%; more usually, at least 85%, more usually at least 90%, and most usually at least 95%, even more usually, at least 96%, 97%, 98% or 99% of the length of a sequence shown in Table 1 in the section entitled "The predicted promoter sequence".

**[0160]** The percentage of the alignment length is calculated by counting the number of residues of the sequence in region of strongest alignment, e.g., a continuous region of the sequence that contains the greatest number of residues that are identical to the residues between two sequences that are being aligned. The number of residues in the region of strongest alignment is divided by the total residue length of a sequence in Table 1 in the section entitled "The predicted promoter sequence".

**[0161]** These related promoters may exhibit similar preferential transcription as those promoters described in Table 1 in the section entitled "The predicted promoter sequence".

**[0162]** Construction of Polynucleotides

**[0163]** Naturally occurring promoters that exhibit nucleotide sequence identity to those shown in Table 1 in the section entitled "The predicted promoter sequence" can be isolated using the techniques as described above. More specifically, such related promoters can be identified by varying stringencies, as defined above, in typical hybridization procedures such as Southern blots or probing of polynucleotide libraries, for example.

**[0164]** Non-natural promoter variants of those shown in Table 1 can be constructed using cloning methods that incorporate the desired nucleotide variation. See, for example, Ho, S. N., et al. *Gene* 77:51-59 1989, describing a procedure site directed mutagenesis using PCR.

**[0165]** Any related promoter showing sequence identity to those shown in Table can be chemically synthesized as described above.

**[0166]** Also, the present invention includes non-natural promoters that exhibit the above-sequence identity to those in Table 1.

**[0167]** The promoters and promoter control elements of the present invention may also be synthesized with 5' or 3' extensions, to facilitate additional manipulation, for instance.

**[0168]** The present invention also includes reduced promoter sequences. These sequences have at least one of the optional promoter fragments deleted.

**[0169]** Core promoter sequences are another embodiment of the present invention. The core promoter sequences have all of the optional promoter fragments deleted.

**[0170]** Testing of Polynucleotides

**[0171]** Polynucleotides of the invention were tested for activity by cloning the sequence into an appropriate vector, transforming plants with the construct and assaying for marker gene expression. Recombinant DNA constructs were prepared which comprise the polynucleotide sequences of the invention inserted into a vector suitable for transformation of plant cells. The construct can be made using standard recombinant DNA techniques (Sambrook et al. 1989) and can be introduced to the species of interest by *Agrobacterium*-mediated transformation or by other means of transformation as referenced below.

**[0172]** The vector backbone can be any of those typical in the art such as plasmids, viruses, artificial chromosomes, BACs, YACs and PACs and vectors of the sort described by

**[0173]** (a) BAC: Shizuya et al., *Proc. Natl. Acad. Sci. USA* 89: 8794-8797 (1992); Hamilton et al., *Proc. Natl. Acad. Sci. USA* 93: 9975-9979 (1996);

**[0174]** (b) YAC: Burke et al., *Science* 236:806-812 (1987);

**[0175]** (c) PAC: Sternberg N. et al., *Proc Natl Acad Sci USA*, January; 87(1):103-7 (1990);

**[0176]** (d) Bacteria-Yeast Shuttle Vectors: Bradshaw et al., *Nucl Acids Res* 23: 4850-4856 (1995);

**[0177]** (e) Lambda Phage Vectors: Replacement Vector, e.g., Frischauf et al., *J. Mol. Biol* 170: 827-842 (1983); or Insertion vector, e.g., Huynh et al., In: Glover N M (ed) *DNA Cloning: A practical Approach*, Vol. 1 Oxford: IRL Press (1985); T-DNA gene fusion vectors: Walden et al., *Mol Cell Biol* 1: 175-194 (1990); and

**[0178]** (g) Plasmid vectors: Sambrook et al., *infra*.

**[0179]** Typically, the construct comprises a vector containing a sequence of the present invention operationally linked to any marker gene. The polynucleotide was identified as a promoter by the expression of the marker gene. Although many marker genes can be used, Green Fluorescent Protein (GFP) is preferred. The vector may also comprise a marker gene to that confers a selectable phenotype on plant cells. The marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or phosphinotricin. Vectors can also include origins of replication, scaffold attachment regions (SARs), markers, homologous sequences, introns, etc.

**[0180]** Promoter Control Elements of the Invention

**[0181]** The promoter control elements of the present invention include those that comprise a sequence shown in Table 1 in the section entitled "The predicted promoter sequence" and fragments thereof. The size of the fragments of the row titled "The predicted promoter sequence" can range from 5 bases to 10 kilobases (kb). Typically, the fragment size is no smaller than 8 bases; more typically, no smaller than 12; more typically, no smaller than 15 bases; more typically, no smaller than 20 bases; more typically, no smaller than 25 bases; even more typically, no more than 30, 35, 40 or 50 bases.

**[0182]** Usually, the fragment size is no larger than 5 kb bases; more usually, no larger than 2 kb; more usually, no larger than 1 kb; more usually, no larger than 800 bases; more usually, no larger than 500 bases; even more usually, no more than 250, 200, 150 or 100 bases.

**[0183]** Relatives Based on Nucleotide Sequence Identity

**[0184]** Included in the present invention are promoter control elements exhibiting nucleotide sequence identity to those described in Table 1 in the section entitled "The predicted promoter sequence" of fragments thereof.

**[0185]** Typically, such related promoters exhibit at least 80% sequence identity, preferably at least 85%, more preferably at least 90%, and most preferably at least 95%, even more preferably, at least 96%, 97%, 98% or 99% sequence identity compared to those shown in Table 1 in the section entitled "The predicted promoter sequence". Such sequence identity can be calculated by the algorithms and computers programs described above.

**[0186]** Promoter Control Element Configuration

**[0187]** A common configuration of the promoter control elements in RNA polymerase II promoters is shown below: For more description, see, for example, "Models for prediction and recognition of eukaryotic promoters", T. Werner, *Mammalian Genome*, 10, 168-175 (1999).

**[0188]** Promoters are generally modular in nature. Promoters can consist of a basal promoter which functions as a site for assembly of a transcription complex comprising an RNA polymerase, for example RNA polymerase II. A typical transcription complex will include additional factors such as TF<sub>II</sub>B, TF<sub>II</sub>D, and TF<sub>II</sub>E. Of these, TF<sub>II</sub>D appears to be the only one to bind DNA directly. The promoter might also contain one or more promoter control elements such as the elements discussed above. These additional control elements may function as binding sites for additional transcription factors that have the function of modulating the level of transcription with respect to tissue specificity and of transcriptional responses to particular environmental or nutritional factors, and the like.

**[0189]** One type of promoter control element is a polynucleotide sequence representing a binding site for proteins. Typically, within a particular functional module, protein binding sites constitute regions of 5 to 60, preferably 10 to 30, more preferably 10 to 20 nucleotides. Within such binding sites, there are typically 2 to 6 nucleotides which specifically contact amino acids of the nucleic acid binding protein.

**[0190]** The protein binding sites are usually separated from each other by 10 to several hundred nucleotides, typically by 15 to 150 nucleotides, often by 20 to 50 nucleotides.

**[0191]** Further, protein binding sites in promoter control elements often display dyad symmetry in their sequence. Such elements can bind several different proteins, and/or a

plurality of sites can bind the same protein. Both types of elements may be combined in a region of 50 to 1,000 base pairs.

**[0192]** Binding sites for any specific factor have been known to occur almost anywhere in a promoter. For example, functional AP-1 binding sites can be located far upstream, as in the rat bone sialoprotein gene, where an AP-1 site located about 900 nucleotides upstream of the transcription start site suppresses expression. Yamauchi et al., *Matrix Biol.*, 15, 119-130 (1996). Alternatively, an AP-1 site located close to the transcription start site plays an important role in the expression of Moloney murine leukemia virus. Sap et al., *Nature*, 340, 242-244, (1989).

**[0193]** (2) Those Identifiable by Bioinformatics

**[0194]** Promoter control elements from the promoters of the instant invention can be identified utilizing bioinformatic or computer driven techniques.

**[0195]** One method uses a computer program AlignACE to identify regulatory motifs in genes that exhibit common preferential transcription across a number of time points. The program identifies common sequence motifs in such genes. See, Roth et al., *Nature Biotechnol.* 16: 949-945 (1998); Tavazoie et al., *Nat Genet.* 1999 July; 22(3):281-5;

**[0196]** Genomatix, also makes available a GEMS Launcher program and other programs to identify promoter control elements and configuration of such elements. Genomatix is located in Munich, Germany.

**[0197]** Other references also describe detection of promoter modules by models independent of overall nucleotide sequence similarity. See, for instance, Klingenhoff et al., *Bioinformatics* 15, 180-186 (1999).

**[0198]** Protein binding sites of promoters can be identified as reported in "Computer-assisted prediction, classification, and delimitation of protein binding sites in nucleic acids", Frech, et al., *Nucleic Acids Research*, Vol. 21, No. 7, 1655-1664, 1993.

**[0199]** Other programs used to identify protein binding sites include, for example, Signal Scan, Prestidge et al., *Comput. Appl. Biosci.* 12: 157-160 (1996); Matrix Search, Chen et al., *Comput. Appl. Biosci.* 11: 563-566 (1995), available as part of Signal Scan 4.0; MatInspector, Ghosh et al., *Nucl. Acid Res.* 21: 3117-3118 (1993) available <http://www.gsf.de/cgi-bin/matsearch.pl>; ConsInspector, Frech et al., *Nucl. Acids Res.* 21: 1655-1664 (1993), available at <ftp://ariane.gsf.de/pub/dos;TFSearch>; and TESS.

**[0200]** Frech et al., "Software for the analysis of DNA sequence elements of transcription", *Bioinformatics & Sequence Analysis*, Vol. 13, no. 1, 89-97 (1997) is a review of different software for analysis of promoter control elements. This paper also reports the usefulness of matrix-based approaches to yield more specific results.

**[0201]** For other procedures, see, Fickett et al., *Curr. Op. Biotechnol.* 11: 19-24 (2000); and Quandt et al., *Nucleic Acids Res.*, 23, 4878-4884 (1995).

**[0202]** (3) Those Identifiable by In-Vitro and In-Vivo Assays

**[0203]** Promoter control elements also can be identified with in-vitro assays, such as transcription detection methods; and with in-vivo assays, such as enhancer trapping protocols.

**[0204]** In-Vitro Assays

**[0205]** Examples of in-vitro assays include detection of binding of protein factors that bind promoter control elements. Fragments of the instant promoters can be used to identify the location of promoter control elements. Another

option for obtaining a promoter control to element with desired properties is to modify known promoter sequences. This is based on the fact that the function of a promoter is dependent on the interplay of regulatory proteins that bind to specific, discrete nucleotide sequences in the promoter, termed motifs. Such interplay subsequently affects the general transcription machinery and regulates transcription efficiency. These proteins are positive regulators or negative regulators (repressors), and one protein can have a dual role depending on the context (Johnson, P. F. and McKnight, S. L. *Annu. Rev. Biochem.* 58:799-839 (1989)).

**[0206]** One type of in-vitro assay utilizes a known DNA binding factor to isolate DNA fragments that bind. If a fragment or promoter variant does not bind, then a promoter control element has been removed or disrupted. For specific assays, see, for instance, B. Luo et al., *J. Mol. Biol.* 266:470 (1997), S. Chusacultanaichai et al., *J. Biol. Chem.* 274:23591 (1999), D. Fabbro et al., *Biochem. Biophys. Res. Comm.* 213:781 (1995)).

**[0207]** Alternatively, a fragment of DNA suspected of conferring a particular pattern of specificity can be examined for activity in binding transcription factors involved in that specificity by methods such as DNA footprinting (e.g. D. J. Cousins et al., *Immunology* 99:101 (2000); V. Kolla et al., *Biochem. Biophys. Res. Comm.* 266:5 (1999)) or “mobility-shift” assays (E. D. Fabiani et al., *J. Biochem.* 347:147 (2000); N. Sugiura et al., *J. Biochem.* 347:155 (2000)) or fluorescence polarization (e.g. Royer et al., U.S. Pat. No. 5,445,935). Both mobility shift and DNA footprinting assays can also be used to identify portions of large DNA fragments that are bound by proteins in unpurified transcription extracts prepared from tissues or organs of interest.

**[0208]** Cell-free transcription extracts can be prepared and used to directly assay in a reconstitutable system (Narayan et al., *Biochemistry* 39:818 (2000)).

**[0209]** In-Vivo Assays

**[0210]** Promoter control elements can be identified with reporter genes in in-vivo assays with the use of fragments of the instant promoters or variants of the instant promoter polynucleotides.

**[0211]** For example, various fragments can be inserted into a vector, comprising a basal or “core” promoter, for example, operably linked to a reporter sequence, which, when transcribed, can produce a detectable label. Examples of reporter genes include those encoding luciferase, green fluorescent protein, GUS, neo, cat and bar. Alternatively, reporter sequence can be detected utilizing AFLP and microarray techniques.

**[0212]** In promoter probe vector systems, genomic DNA fragments are inserted upstream of the coding sequence of a reporter gene that is expressed only when the cloned fragment contains DNA having transcription modulation activity (Neve, R. L. et al., *Nature* 277:324-325 (1979)). Control elements are disrupted when fragments or variants lacking any transcription modulation activity. Probe vectors have been designed for assaying transcription modulation in *E. coli* (An, G. et al., *J. B. act.* 140:400-407 (1979)) and other bacterial hosts (Band, L. et al., *Gene* 26:313-315 (1983); Achen, M. G., *Gene* 45:45-49 (1986)), yeast (Goodey, A. R. et al., *Mol. Gen. Genet.* 204:505-511 (1986)) and mammalian cells (Pater, M. M. et al., *J. Mol. App. Gen.* 2:363-371 (1984)).

**[0213]** A different design of a promoter/control element trap includes packaging into retroviruses for more efficient delivery into cells. One type of retroviral enhancer trap was

described by von Melchner et al. (*Genes Dev.* 1992; U.S. Pat. No. 5,364,783). The basic design of this vector includes a reporter protein coding sequence engineered into the U3 portion of the 3' LTR. No splice acceptor consensus sequences are included, limiting its utility to work as an enhancer trap only. A different approach to a gene trap using retroviral vectors was pursued by Friedrich and Soriano (*Genes Dev.* 1991), who engineered a lacZ-neo fusion protein linked to a splicing acceptor. LacZ-neo fusion protein expression from trapped loci allows not only for drug selection, but also for visualization of  $\beta$ -galactosidase expression using the chromogenic substrate, X-gal.

**[0214]** A general review of tools for identifying transcriptional regulatory regions of genomic DNA is provided by J. W. Fickett et al. (*Curr. Opin. Biotechnol.* 11:19 (2000)).

**[0215]** (4) Non-Natural Control Elements

**[0216]** Non-natural control elements can be constructed by inserting, deleting or substituting nucleotides into the promoter control elements described above. Such control elements are capable of transcription modulation that can be determined using any of the assays described above.

D. Constructing Promoters with Control Elements

**[0217]** (1) Combining Promoters and Promoter Control Elements

**[0218]** The promoter polynucleotides and promoter control elements of the present invention, both naturally occurring and synthetic, can be combined with each other to produce the to desired preferential transcription. Also, the polynucleotides of the invention can be combined with other known sequences to obtain other useful promoters to modulate, for example, tissue transcription specific or transcription specific to certain conditions. Such preferential transcription can be determined using the techniques or assays described above.

**[0219]** Fragments, variants, as well as full-length sequences those shown in Table 1 in the section entitled “The predicted promoter sequence” and relatives are useful alone or in combination.

**[0220]** The location and relation of promoter control elements within a promoter can affect the ability of the promoter to modulate transcription. The order and spacing of control elements is a factor when constructing promoters.

**[0221]** (2) Number of Promoter Control Elements

**[0222]** Promoters can contain any number of control elements. For example, a promoter can contain multiple transcription binding sites or other control elements. One element may confer tissue or organ specificity; another element may limit transcription to specific time periods, etc. Typically, promoters will contain at least a basal or core promoter as described above. Any additional element can be included as desired. For example, a fragment comprising a basal or “core” promoter can be fused with another fragment with any number of additional control elements.

**[0223]** (3) Spacing Between Control Elements

**[0224]** Spacing between control elements or the configuration or control elements can be determined or optimized to permit the desired protein-polynucleotide or polynucleotide interactions to occur.

**[0225]** For example, if two transcription factors bind to a promoter simultaneously or relatively close in time, the binding sites are spaced to allow each factor to bind without steric hinderance. The spacing between two such hybridizing control elements can be as small as a profile of a protein bound to a control element. In some cases, two protein binding sites

can be adjacent to each other when the proteins bind at different times during the transcription process.

**[0226]** Further, when two control elements hybridize the spacing between such elements will be sufficient to allow the promoter polynucleotide to hairpin or loop to permit the two elements to bind. The spacing between two such hybridizing control elements can be as small as a t-RNA loop, to as large as 10 kb.

**[0227]** Typically, the spacing is no smaller than 5 bases; more typically, no smaller than 8; more typically, no smaller than 15 bases; more typically, no smaller than 20 bases; more typically, no smaller than 25 bases; even more typically, no more than 30, 35, 40 or 50 bases.

**[0228]** Usually, the fragment size is no larger than 5 kb bases; more usually, no larger than 2 kb; more usually, no larger than 1 kb; more usually, no larger than 800 bases; more usually, no larger than 500 bases; even more usually, no more than 250, 200, 150 or 100 bases.

**[0229]** Such spacing between promoter control elements can be determined using the techniques and assays described above.

#### **[0230]** (4) Other Promoters

**[0231]** The following are promoters that are induced under stress conditions and can be combined with those of the present invention: *ldh1* (oxygen stress; tomato; see Germain and Ricard. 1997. *Plant Mol Biol* 35:949-54), *GPx* and *CAT* (oxygen stress; mouse; see Franco et al. 1999. *Free Radic Biol Med* 27:1122-32), *ci7* (cold stress; potato; see Kirch et al. 1997. *Plant Mol. Biol.* 33:897-909), *Bz2* (heavy metals; maize; see Marrs and Walbot. 1997. *Plant Physiol* 113:93-102), *HSP32* (hyperthermia; rat; see Raju and Maines. 1994. *Biochim Biophys Acta* 1217:273-80); *MAPKAPK-2* (heat shock; *Drosophila*; see Larochelle and Suter. 1995. *Gene* 163:209-14).

**[0232]** In addition, the following examples of promoters are induced by the presence or absence of light can be used in combination with those of the present invention: *Topoisomerase II* (pea; see Reddy et al. 1999. *Plant Mol Biol* 41:125-37), *chalcone synthase* (soybean; see Wingender et al. 1989. *Mol Gen Genet.* 218:315-22) *mdm2* gene (human tumor; see Saucedo et al. 1998. *Cell Growth Differ* 9:119-30), *Clock* and *BMAL1* (rat; see Namihira et al. 1999. *Neurosci Lett* 271:1-4, *PHYA* (*Arabidopsis*; see Canton and Quail 1999. *Plant Physiol* 121:1207-16), *PRB-1b* (tobacco; see Sessa et al. 1995. *Plant Mol Biol* 28:537-47) and *Ypr10* (common bean; see Walter et al. 1996. *Eur J Biochem* 239: 281-93).

**[0233]** The promoters and control elements of the following genes can be used in combination with the present invention to confer tissue specificity: *MipB* (iceplant; Yamada et al. 1995. *Plant Cell* 7:1129-42) and *SUCS* (root nodules; broad-bean; Kuster et al. 1993. *Mol Plant Microbe Interact* 6:507-14) for roots, *OsSUT1* (rice; Hirose et al. 1997. *Plant Cell Physiol* 38:1389-96) for leaves, *Msg* (soybean; Stomvik et al. 1999. *Plant Mol Biol* 41:217-31) for siliques, *cell* (*Arabidopsis*; Shani et al. 1997. *Plant Mol Biol* 34(6):837-42) to and *ACT11* (*Arabidopsis*; Huang et al. 1997. *Plant Mol Biol* 33:125-39) for inflorescence.

**[0234]** Still other promoters are affected by hormones or participate in specific physiological processes, which can be used in combination with those of present invention. Some examples are the *ACC synthase* gene that is induced differently by ethylene and brassinosteroids (mung bean; Yi et al. 1999. *Plant Mol Biol* 41:443-54), the *TAPG1* gene that is

active during abscission (tomato; Kalaitzis et al. 1995. *Plant Mol Biol* 28:647-56), and the *1-aminocyclopropane-1-carboxylate synthase* gene (carnation; Jones et al. 1995. *Plant Mol Biol* 28:505-12) and the *CP-2/cathepsin L* gene (rat; Kim and Wright. 1997. *Biol Reprod* 57:1467-77), both active during senescence.

#### E. Vectors

**[0235]** Vectors are a useful component of the present invention. In particular, the present promoters and/or promoter control elements may be delivered to a system such as a cell by way of a vector. For the purposes of this invention, such delivery may range from simply introducing the promoter or promoter control element by itself randomly into a cell to integration of a cloning vector containing the present promoter or promoter control element. Thus, a vector need not be limited to a DNA molecule such as a plasmid, cosmid or bacterial phage that has the capability of replicating autonomously in a host cell. All other manner of delivery of the promoters and promoter control elements of the invention are envisioned. The various T-DNA vector types are a preferred vector for use with the present invention. Many useful vectors are commercially available.

**[0236]** It may also be useful to attach a marker sequence to the present promoter and promoter control element in order to determine activity of such sequences. Marker sequences typically include genes that provide antibiotic resistance, such as tetracycline resistance, hygromycin resistance or ampicillin resistance, or provide herbicide resistance. Specific selectable marker genes may be used to confer resistance to herbicides such as glyphosate, glufosinate or broxynil (Comai et al., *Nature* 317: 741-744 (1985); Gordon-Kamm et al., *Plant Cell* 2: 603-618 (1990); and Stalker et al., *Science* 242: 419-423 (1988)). Other marker genes exist which provide hormone responsiveness.

#### **[0237]** (1) Modification of Transcription by Promoters and Promoter Control Elements

**[0238]** The promoter or promoter control element of the present invention may be operably linked to a polynucleotide to be transcribed. In this manner, the promoter or promoter control element may modify transcription by modulate transcript levels of that polynucleotide when inserted into a genome.

**[0239]** However, prior to insertion into a genome, the promoter or promoter control element need not be linked, operably or otherwise, to a polynucleotide to be transcribed. For example, the promoter or promoter control element may be inserted alone into the genome in front of a polynucleotide already present in the genome. In this manner, the promoter or promoter control element may modulate the transcription of a polynucleotide that was already present in the genome. This polynucleotide may be native to the genome or inserted at an earlier time.

**[0240]** Alternatively, the promoter or promoter control element may be inserted into a genome alone to modulate transcription. See, for example, Vaucheret, H et al. (1998) *Plant J* 16: 651-659. Rather, the promoter or promoter control element may be simply inserted into a genome or maintained extrachromosomally as a way to divert transcription resources of the system to itself. This approach may be used to downregulate the transcript levels of a group of polynucleotide(s).

**[0241]** (2) Polynucleotide to be Transcribed

**[0242]** The nature of the polynucleotide to be transcribed is not limited. Specifically, the polynucleotide may include sequences that will have activity as RNA as well as sequences that result in a polypeptide product. These sequences may include, but are not limited to antisense sequences, ribozyme sequences, spliceosomes, amino acid coding sequences, and fragments thereof.

**[0243]** Specific coding sequences may include, but are not limited to endogenous proteins or fragments thereof, or heterologous proteins including marker genes or fragments thereof.

**[0244]** Promoters and control elements of the present invention are useful for modulating metabolic or catabolic processes. Such processes include, but are not limited to, secondary product metabolism, amino acid synthesis, seed protein storage, oil development, pest defense and nitrogen usage. Some examples of genes, transcripts and peptides or polypeptides participating in these processes, which can be modulated by the present invention: are tryptophan decarboxylase (tdc) and strictosidine synthase (str1), dihydrodipicolinate synthase (DHDPS) and aspartate kinase (AK), 2S albumin and alpha-, beta-, and gamma-zeins, to ricinoleate and 3-ketoacyl-ACP synthase (KAS), *Bacillus thuringiensis* (Bt) insecticidal protein, cowpea trypsin inhibitor (CpTI), asparagine synthetase and nitrite reductase. Alternatively, expression constructs can be used to inhibit expression of these peptides and polypeptides by incorporating the promoters in constructs for antisense use, co-suppression use or for the production of dominant negative mutations.

**[0245]** (3) Other Regulatory Elements

**[0246]** As explained above, several types of regulatory elements exist concerning transcription regulation. Each of these regulatory elements may be combined with the present vector if desired.

**[0247]** (4) Other Components of Vectors

**[0248]** Translation of eukaryotic mRNA is often initiated at the codon that encodes the first methionine. Thus, when constructing a recombinant polynucleotide according to the present invention for expressing a protein product, it is preferable to ensure that the linkage between the 3' portion, preferably including the TATA box, of the promoter and the polynucleotide to be transcribed, or a functional derivative thereof, does not contain any intervening codons which are capable of encoding a methionine.

**[0249]** The vector of the present invention may contain additional components. For example, an origin of replication allows for replication of the vector in a host cell. Additionally, homologous sequences flanking a specific sequence allows for specific recombination of the specific sequence at a desired location in the target genome. T-DNA sequences also allow for insertion of a specific sequence randomly into a target genome.

**[0250]** The vector may also be provided with a plurality of restriction sites for insertion of a polynucleotide to be transcribed as well as the promoter and/or promoter control elements of the present invention. The vector may additionally contain selectable marker genes. The vector may also contain a transcriptional and translational initiation region, and a transcriptional and translational termination region functional in the host cell. The termination region may be native with the transcriptional initiation region, may be native with the polynucleotide to be transcribed, or may be derived from another source. Convenient termination regions are available

from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau et al., (1991) Mol. Gen. Genet. 262:141-144; Proudfoot (1991) Cell 64:671-674; Sanfacon et al. (1991) Genes Dev. 5:141-149; Mogen et al. (1990) Plant Cell 2:1261-1272; Munroe et al. (1990) Gene 91:151-158; Ballas et al. 1989) Nucleic Acids Res. 17:7891-7903; Joshi et al. (1987) Nucleic Acid Res. 15:9627-9639.

**[0251]** Where appropriate, the polynucleotide to be transcribed may be optimized for increased expression in a certain host cell. For example, the polynucleotide can be synthesized using preferred codons for improved transcription and translation. See U.S. Pat. Nos. 5,380,831, 5,436,391; see also and Murray et al., (1989) Nucleic Acids Res. 17:477-498.

**[0252]** Additional sequence modifications include elimination of sequences encoding spurious polyadenylation signals, exon intron splice site signals, transposon-like repeats, and other such sequences well characterized as deleterious to expression. The G-C content of the polynucleotide may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. The polynucleotide sequence may be modified to avoid hairpin secondary mRNA structures.

**[0253]** A general description of expression vectors and reporter genes can be found in Gruber, et al., "Vectors for Plant Transformation, in Methods in Plant Molecular Biology & Biotechnology" in Glich et al., (Eds. pp. 89-119, CRC Press, 1993). Moreover GUS expression vectors and GUS gene cassettes are available from Clontech Laboratories, Inc., Palo Alto, Calif. while luciferase expression vectors and luciferase gene cassettes are available from Promega Corp. (Madison, Wis.). GFP vectors are available from Aurora Biosciences.

**F. Polynucleotide Insertion into a Host Cell**

**[0254]** The polynucleotides according to the present invention can be inserted into a host cell. A host cell includes but is not limited to a plant, mammalian, insect, yeast, and prokaryotic cell, preferably a plant cell.

**[0255]** The method of insertion into the host cell genome is chosen based on convenience. For example, the insertion into the host cell genome may either be accomplished by vectors that integrate into the host cell genome or by vectors which exist independent of the host cell genome.

**[0256]** (1) Polynucleotides Autonomous of the Host Genome

**[0257]** The polynucleotides of the present invention can exist autonomously or independent of the host cell genome. Vectors of these types are known in the art and include, for example, certain type of non-integrating viral vectors, autonomously replicating plasmids, artificial chromosomes, and the like.

**[0258]** Additionally, in some cases transient expression of a polynucleotide may be desired.

**[0259]** (2) Polynucleotides Integrated into the Host Genome

**[0260]** The promoter sequences, promoter control elements or vectors of the present invention may be transformed into host cells. These transformations may be into protoplasts or intact tissues or isolated cells. Preferably expression vectors are introduced into intact tissue. General methods of culturing plant tissues are provided for example by Maki et al. "Procedures for Introducing Foreign DNA into Plants" in Methods in Plant Molecular Biology & Biotechnology, Glich et al. (Eds. pp. 67-88 CRC Press, 1993); and by Phillips et al.

"Cell-Tissue Culture and In-Vitro Manipulation" in Corn & Corn Improvement, 3rd Edition 10 Sprague et al. (Eds. pp. 345-387) American Society of Agronomy Inc. et al. 1988.

[0261] Methods of introducing polynucleotides into plant tissue include the direct infection or co-cultivation of plant cell with *Agrobacterium tumefaciens*, Horsch et al., Science, 227:1229 (1985). Descriptions of *Agrobacterium* vector systems and methods for *Agrobacterium*-mediated gene transfer provided by Gruber et al. supra.

[0262] Alternatively, polynucleotides are introduced into plant cells or other plant tissues using a direct gene transfer method such as microprojectile-mediated delivery, DNA injection, electroporation and the like. More preferably polynucleotides are introduced into plant tissues using the microprojectile media delivery with the biolistic device. See, for example, Tomes et al., "Direct DNA transfer into intact plant cells via microprojectile bombardment" In: Gamborg and Phillips (Eds.) Plant Cell, Tissue and Organ Culture: Fundamental Methods, Springer Verlag, Berlin (1995).

[0263] In another embodiment of the current invention, expression constructs can be used for gene expression in callus culture for the purpose of expressing marker genes encoding peptides or polypeptides that allow identification of transformed plants. Here, a promoter that is operatively linked to a polynucleotide to be transcribed is transformed into plant cells and the transformed tissue is then placed on callus-inducing media. If the transformation is conducted with leaf discs, for example, callus will initiate along the cut edges. Once callus growth has initiated, callus cells can be transferred to callus shoot-inducing or callus root-inducing media. Gene expression will occur in the callus cells developing on the appropriate media: callus root-inducing promoters will be activated on callus root-inducing media, etc. Examples of such peptides or polypeptides useful as transformation markers include, but are not limited to barstar, glyphosate, chloramphenicol acetyltransferase (CAT), kanamycin, spectinomycin, streptomycin or other antibiotic resistance enzymes, green fluorescent protein (GFP), and  $\beta$ -glucuronidase (GUS), etc. Some of the exemplary promoters of the row titled "The predicted promoter sequence" will also be capable of sustaining expression in some tissues or organs after the initiation or completion of regeneration. Examples of these tissues or organs are somatic embryos, cotyledon, hypocotyl, epicotyl, leaf, stems, roots, flowers and seed.

[0264] Integration into the host cell genome also can be accomplished by methods known in the art, for example, by the homologous sequences or T-DNA discussed above or using the cre-lox system (A. C. Vergunst et al., *Plant Mol. Biol.* 38:393 (1998)).

#### G. Utility

[0265] Common Uses

[0266] In yet another embodiment, the promoters of the present invention can be used to further understand developmental mechanisms. For example, promoters that are specifically induced during callus formation, somatic embryo formation, shoot formation or root formation can be used to explore the effects of overexpression, repression or ectopic expression of target genes, or for isolation of trans-acting factors.

[0267] The vectors of the invention can be used not only for expression of coding regions but may also be used in exon-trap cloning, or promoter trap procedures to detect differential gene expression in various tissues, K. Lindsey et al., 1993

"Tagging Genomic Sequences That Direct Transgene Expression by Activation of a Promoter Trap in Plants", *Transgenic Research* 2:3347. D. Auch & Reth, et al., "Exon Trap Cloning: Using PCR to Rapidly Detect and Clone Exons from Genomic DNA Fragments", *Nucleic Acids Research*, Vol. 18, No. 22, p. 674.

[0268] Entrapment vectors, first described for use in bacteria (Casadaban and Cohen, 1979, *Proc. Nat. Aca. Sci. U.S.A.*, 76: 4530; Casadaban et al., 1980, *J. Bacteriol.*, 143: 971) permit selection of insertional events that lie within coding sequences. Entrapment vectors can be introduced into pluripotent ES cells in culture and then passed into the germline via chimeras (Gossler et al., 1989, *Science*, 244: 463; Skarnes, 1990, *Biotechnology*, 8: 827). Promoter or to gene trap vectors often contain a reporter gene, e.g., lacZ, lacking its own promoter and/or splice acceptor sequence upstream. That is, promoter gene traps contain a reporter gene with a splice site but no promoter. If the vector lands in a gene and is spliced into the gene product, then the reporter gene is expressed.

[0269] Recently, the isolation of preferentially-induced genes has been made possible with the use of sophisticated promoter traps (e.g. WET) that are based on conditional auxotrophy complementation or drug resistance. In one IVET approach, various bacterial genome fragments are placed in front of a necessary metabolic gene coupled to a reporter gene. The DNA constructs are inserted into a bacterial strain otherwise lacking the metabolic gene, and the resulting bacteria are used to infect the host organism. Only bacteria expressing the metabolic gene survive in the host organism; consequently, inactive constructs can be eliminated by harvesting only bacteria that survive for some minimum period in the host. At the same time, constitutively active constructs can be eliminated by screening only bacteria that do not express the reporter gene under laboratory conditions. The bacteria selected by such a method contain constructs that are selectively induced only during infection of the host. The IVET approach can be modified for use in plants to identify genes induced in either the bacteria or the plant cells upon pathogen infection or root colonization. For information on IVET see the articles by Mahan et al. in *Science* 259:686-688 (1993), Mahan et al. in *PNAS USA* 92:669-673 (1995), Heithoff et al. in *PNAS USA* 94:934-939 (1997), and Wanget al. in *PNAS USA*. 93:10434 (1996).

[0270] Constitutive Transcription

[0271] Use of promoters and control elements providing constitutive transcription is desired for modulation of transcription in most cells of an organism under most environmental conditions. In a plant, for example, constitutive transcription is useful for modulating genes involved in defense, pest resistance, herbicide resistance, etc.

[0272] Constitutive up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase defense, pest and herbicide resistance may require constitutive up-regulation of transcription. In contrast, constitutive transcriptional down-regulation may be desired to inhibit those genes, transcripts, and/or polypeptides that lower defense, pest and herbicide resistance.

[0273] Typically, promoter or control elements that provide constitutive transcription produce transcription levels that are statistically similar in many tissues and environmental conditions observed.



[0274] Calculation of P-value from the different observed transcript levels is one means of determining whether a promoter or control element is providing constitutive up-regulation. P-value is the probability that the difference of transcript levels is not statistically significant. The higher the P-value, the more likely the difference of transcript levels is not significant. One formula used to calculate P-value is as follows:

$$\int \varphi(x) dx, \text{ integrated from } a \text{ to } \infty,$$

where  $\varphi(x)$  is a normal distribution

$$\text{where } a = \frac{|Sx - \mu|}{\sigma(\text{all Samples except } Sx)}$$

where  $Sx$  = the intensity of the sample of interest

where  $\mu$  =

is the average of the intensities of all samples except  $Sx$ ,

$$= \frac{(\sum S1 \dots Sn) - Sx}{n - 1}$$

where  $\sigma(S1 \dots S11, \text{ not including } Sx) =$

the standard deviation of all sample intensities except  $Sx$ .

The P-value from the formula ranges from 1.0 to 0.0.

[0275] Usually, each P-value of the transcript levels observed in a majority of cells, tissues, or organs under various environmental conditions produced by the promoter or control element is greater than  $10^{-8}$ ; more usually, greater than  $10^{-7}$ ; even more usually, greater than  $10^{-6}$ ; even more usually, greater than  $10^{-5}$  or  $10^{-4}$ .

[0276] For up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.

[0277] Stress Induced Preferential Transcription

[0278] Promoters and control elements providing modulation of transcription under oxidative, drought, oxygen, wound, and methyl jasmonate stress are particularly useful for producing host cells or organisms that are more resistant to biotic and abiotic stresses. In a plant, for example, modulation of genes, transcripts, and/or polypeptides in response to oxidative stress can protect cells against damage caused by oxidative agents, such as hydrogen peroxide and other free radicals.

[0279] Drought induction of genes, transcripts, and/or polypeptides are useful to increase the viability of a plant, for example, when water is a limiting factor. In contrast, genes, transcripts, and/or polypeptides induced during oxygen stress can help the flood tolerance of a plant.

[0280] The promoters and control elements of the present invention can modulate stresses similar to those described in, for example, stress conditions are VuPLD1 (drought stress; Cowpea; see Pham-Thi et al. 1999. Plant molecular Biology. 1257-65), pyruvate decarboxylase (oxygen stress; rice; see Rivosal et al. 1997. Plant Physiol. 114(3): 1021-29), chromoplast specific carotenoid gene (oxidative stress; *capsicum*; see Bouvier et al. 1998. Journal of Biological Chemistry 273: 30651-59).

[0281] Promoters and control elements providing preferential transcription during wounding or induced by methyl jasmonate can produce a defense response in host cells or organisms. In a plant, for example, preferential modulation of

genes, transcripts, and/or polypeptides under such conditions is useful to induce a defense response to mechanical wounding, pest or pathogen attack or treatment with certain chemicals.

[0282] Promoters and control elements of the present invention also can trigger a response similar to those described for cf9 (viral pathogen; tomato; see O'Donnell et al. 1998. The Plant journal: for cell and molecular biology 14(1): 137-42), hepatocyte growth factor activator inhibitor type 1 (HAI-1), which enhances tissue regeneration (tissue injury; human; Koono et al. 1999. Journal of Histochemistry and Cytochemistry 47: 673-82), copper amine oxidase (CuAO), induced during ontogenesis and wound healing (wounding; chick-pea; Rea et al. 1998. FEBS Letters 437: 177-82), proteinase inhibitor II (wounding; potato; see Pena-Cortes et al. 1988. Planta 174: 84-89), protease inhibitor II (methyl jasmonate; tomato; see Farmer and Ryan. 1990. Proc Natl Acad Sci USA 87: 7713-7716), two vegetative storage protein genes VspA and VspB (wounding, jasmonic acid, and water deficit; soybean; see Mason and Mullet. 1990. Plant Cell 2: 569-579).

[0283] Up-regulation and transcription down-regulation are useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase oxidative, flood, or drought tolerance may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to inhibit those genes, transcripts, and/or polypeptides that lower such tolerance.

[0284] Typically, promoter or control elements, which provide preferential transcription in wounding or under methyl jasmonate induction, produce transcript levels that are statistically significant as compared to cell types, organs or tissues under other conditions.

[0285] For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.

[0286] Light Induced Preferential Transcription

[0287] Promoters and control elements providing preferential transcription when induced by light exposure can be utilized to modulate growth, metabolism, and development; to increase drought tolerance; and decrease damage from light stress for host cells or organisms. In a plant, for example, modulation of genes, transcripts, and/or polypeptides in response to light is useful

[0288] (1) to increase the photosynthetic rate;

[0289] (2) to increase storage of certain molecules in leaves or green parts only, e.g., silage with high protein or starch content;

[0290] (3) to modulate production of exogenous compositions in green tissue, e.g., certain feed enzymes;

[0291] (4) to induce growth or development, such as fruit development and maturity, during extended exposure to light;

[0292] (5) to modulate guard cells to control the size of stomata in leaves to prevent water loss, or

[0293] (6) to induce accumulation of beta-carotene to help plants cope with light induced stress.

The promoters and control elements of the present invention also can trigger responses similar to those described in: abscisic acid insensitive3 (ABI3) (dark-grown *Arabidopsis* seedlings, see Rohde et al. 2000. The Plant Cell 12: 35-52), asparagine synthetase (pea root nodules, see Tsai, F. Y.; Coruzzi, G. M. 1990. EMBO J. 9: 323-32), *mdm2* gene (human tumor; see Saucedo et al. 1998. Cell Growth Differ 9: 119-30).

[0294] Up-regulation and transcription down-regulation are useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase drought or light tolerance may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to inhibit those genes, transcripts, and/or polypeptides that lower such tolerance.

[0295] Typically, promoter or control elements, which provide preferential transcription in cells, tissues or organs exposed to light, produce transcript levels that are statistically significant as compared to cells, tissues, or organs under decreased light exposure (intensity or length of time).

[0296] For preferential up-regulation of transcription, promoter and control elements produce to transcript levels that are above background of the assay.

[0297] Dark Induced Preferential Transcription

[0298] Promoters and control elements providing preferential transcription when induced by dark or decreased light intensity or decreased light exposure time can be utilized to time growth, metabolism, and development, to modulate photosynthesis capabilities for host cells or organisms. In a plant, for example, modulation of genes, transcripts, and/or polypeptides in response to dark is useful, for example,

[0299] (1) to induce growth or development, such as fruit development and maturity, despite lack of light;

[0300] (2) to modulate genes, transcripts, and/or polypeptide active at night or on cloudy days; or

[0301] (3) to preserve the plastid ultra structure present at the onset of darkness.

The present promoters and control elements can also trigger response similar to those described in the section above.

[0302] Up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase growth and development may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to inhibit those genes, transcripts, and/or polypeptides that modulate photosynthesis capabilities.

[0303] Typically, promoter or control elements, which provide preferential transcription under exposure to dark or decrease light intensity or decrease exposure time, produce transcript levels that are statistically significant.

[0304] For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.

[0305] Leaf Preferential Transcription

[0306] Promoters and control elements providing preferential transcription in a leaf can modulate growth, metabolism, and development or modulate energy and nutrient utilization in host cells or organisms. In a plant, for example, preferential modulation of genes, transcripts, and/or polypeptide in a leaf, is useful, for example,

[0307] (1) to modulate leaf size, shape, and development;

[0308] (2) to modulate the number of leaves; or

[0309] (3) to modulate energy or nutrient usage in relation to other organs and tissues

[0310] Up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase growth, for example, may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to inhibit energy usage in a leaf to be directed to the fruit instead, for instance.

[0311] Typically, promoter or control elements, which provide preferential transcription in the cells, tissues, or organs

of a leaf, produce transcript levels that are statistically significant as compared to other cells, organs or tissues.

[0312] For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.

[0313] Root Preferential Transcription

[0314] Promoters and control elements providing preferential transcription in a root can modulate growth, metabolism, development, nutrient uptake, nitrogen fixation, or modulate energy and nutrient utilization in host cells or organisms. In a plant, for example, preferential modulation of genes, transcripts, and/or in a leaf, is useful

[0315] (1) to modulate root size, shape, and development;

[0316] (2) to modulate the number of roots, or root hairs;

[0317] (3) to modulate mineral, fertilizer, or water uptake;

[0318] (4) to modulate transport of nutrients; or

[0319] (4) to modulate energy or nutrient usage in relation to other organs and tissues.

[0320] Up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase growth, for example, may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to inhibit nutrient usage in a root to be directed to the leaf instead, for instance.

[0321] Typically, promoter or control elements, which provide preferential transcription in cells, tissues, or organs of a root, produce transcript levels that are statistically significant as compared to other cells, organs or tissues.

[0322] For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.

[0323] Stem/Shoot Preferential Transcription

[0324] Promoters and control elements providing preferential transcription in a stem or shoot can modulate growth, metabolism, and development or modulate energy and nutrient utilization in host cells or organisms. In a plant, for example, preferential modulation of genes, transcripts, and/or polypeptide in a stem or shoot, is useful, for example,

[0325] (1) to modulate stem/shoot size, shape, and development; or

[0326] (2) to modulate energy or nutrient usage in relation to other organs and tissues

[0327] Up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase growth, for example, may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to inhibit energy usage in a stem/shoot to be directed to the fruit instead, for instance.

[0328] Typically, promoter or control elements, which provide preferential transcription in the cells, tissues, or organs of a stem or shoot, produce transcript levels that are statistically significant as compared to other cells, organs or tissues.

[0329] For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.

[0330] Fruit and Seed Preferential Transcription

[0331] Promoters and control elements providing preferential transcription in a silique or fruit can time growth, development, or maturity; or modulate fertility; or modulate energy and nutrient utilization in host cells or organisms. In a plant, for example, preferential modulation of genes, transcripts, and/or polypeptides in a fruit, is useful

[0332] (1) to modulate fruit size, shape, development, and maturity;

- [0333] (2) to modulate the number of fruit or seeds;
- [0334] (3) to modulate seed shattering;
- [0335] (4) to modulate components of seeds, such as, storage molecules, starch, protein, oil, vitamins, anti-nutritional components, such as phytic acid;
- [0336] (5) to modulate seed and/or seedling vigor or viability;
- [0337] (6) to incorporate exogenous compositions into a seed, such as lysine rich proteins;
- [0338] (7) to permit similar fruit maturity timing for early and late blooming flowers; or
- [0339] (8) to modulate energy or nutrient usage in relation to other organs and tissues.
- [0340] Up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase growth, for example, may to require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to inhibit late fruit maturity, for instance.
- [0341] Typically, promoter or control elements, which provide preferential transcription in the cells, tissues, or organs of siliques or fruits, produce transcript levels that are statistically significant as compared to other cells, organs or tissues.
- [0342] For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.
- [0343] Callus Preferential Transcription
- [0344] Promoters and control elements providing preferential transcription in a callus can be useful to modulating transcription in dedifferentiated host cells. In a plant transformation, for example, preferential modulation of genes, transcripts, in callus is useful to modulate transcription of a marker gene, which can facilitate selection of cells that are transformed with exogenous polynucleotides.
- [0345] Up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase marker gene detectability, for example, may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to increase the ability of the calluses to later differentiate, for instance.
- [0346] Typically, promoter or control elements, which provide preferential transcription in callus, produce transcript levels that are statistically significant as compared to other cell types, tissues, or organs. Calculation of P-value from the different observed transcript levels is one means of determining whether a promoter or control element is providing such preferential transcription.
- [0347] Usually, each P-value of the transcript levels observed in callus as compared to, at least one other cell type, tissue or organ, is less than  $10^{-4}$ ; more usually, less than  $10^{-5}$ ; even more usually, less than  $10^{-6}$ ; even more usually, less than  $10^{-7}$  or  $10^{-8}$ .
- [0348] For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.
- [0349] Flower Specific Transcription
- [0350] Promoters and control elements providing preferential transcription in flowers can modulate pigmentation; or modulate fertility in host cells or organisms. In a plant, for example, preferential modulation of genes, transcripts, and/or polypeptides in a flower, is useful,
- [0351] (1) to modulate petal color; or
- [0352] (2) to modulate the fertility of pistil and/or stamen.
- [0353] Up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase pigmentation, for example, may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to inhibit fertility, for instance.
- [0354] Typically, promoter or control elements, which provide preferential transcription in flowers, produce transcript levels that are statistically significant as compared to other cells, organs or tissues.
- [0355] For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.
- [0356] Immature Bud and Inflorescence Preferential Transcription
- [0357] Promoters and control elements providing preferential transcription in a immature bud or inflorescence can time growth, development, or maturity; or modulate fertility or viability in host cells or organisms. In a plant, for example, preferential modulation of genes, transcripts, and/or polypeptide in a fruit, is useful,
- [0358] (1) to modulate embryo development, size, and maturity;
- [0359] (2) to modulate endosperm development, size, and composition;
- [0360] (3) to modulate the number of seeds and fruits; or
- [0361] (4) to modulate seed development and viability.
- [0362] Up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase growth, for example, may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to decrease endosperm size, for instance.
- [0363] Typically, promoter or control elements, which provide preferential transcription in immature buds and inflorescences, produce transcript levels that are statistically significant as compared to other cell types, organs or tissues.
- [0364] For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.
- [0365] Senescence Preferential Transcription
- [0366] Promoters and control elements providing preferential transcription during senescence can be used to modulate cell degeneration, nutrient mobilization, and scavenging of free radicals in host cells or organisms. Other types of responses that can be modulated include, for example, senescence associated genes (SAG) that encode enzymes thought to be involved in cell degeneration and nutrient mobilization (Arabidopsis; see Hensel et al. 1993. Plant Cell 5: 553-64), and the CP-2/cathepsin L gene (rat; Kim and Wright. 1997. Biol Reprod 57: 1467-77), both induced during senescence.
- [0367] In a plant, for example, preferential modulation of genes, transcripts, and/or polypeptides during senescencing is useful to modulate fruit ripening.
- [0368] Up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase scavenging of free radicals, for example, may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to inhibit cell degeneration, for instance.
- [0369] Typically, promoter or control elements, which provide preferential transcription in cells, tissues, or organs dur-

ing senescence, produce transcript levels that are statistically significant as compared to other conditions.

**[0370]** For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.

**[0371]** Germination Preferential Transcription

**[0372]** Promoters and control elements providing preferential transcription in a germinating seed can time growth, development, or maturity; or modulate viability in host cells or organisms. In a plant, for example, preferential modulation of genes, transcripts, and/or polypeptide in a germinating seed, is useful,

**[0373]** (1) to modulate the emergence of they hypocotyls, cotyledons and radical; or

**[0374]** (2) to modulate shoot and primary root growth and development;

**[0375]** Up-regulation and transcription down-regulation is useful for these applications. For instance, genes, transcripts, and/or polypeptides that increase growth, for example, may require up-regulation of transcription. In contrast, transcriptional down-regulation may be desired to decrease endosperm size, for instance.

**[0376]** Typically, promoter or control elements, which provide preferential transcription in a germinating seed, produce transcript levels that are statistically significant as compared to other cell types, organs or tissues.

**[0377]** For preferential up-regulation of transcription, promoter and control elements produce transcript levels that are above background of the assay.

#### Microarray Analysis

**[0378]** A major way that a cell controls its response to internal or external stimuli is by regulating the rate of transcription of specific genes. For example, the differentiation of cells during organogenesis into forms characteristic of the organ is associated with the selective activation and repression of large numbers of genes. Thus, specific organs, tissues and cells are functionally distinct due to the different populations of mRNAs and protein products they possess. Internal signals program the selective activation and repression programs. For example, internally synthesized hormones produce such signals. The level of hormone can be raised by increasing the level of transcription of genes encoding proteins concerned with hormone synthesis.

**[0379]** To measure how a cell reacts to internal and/or external stimuli, individual mRNA levels can be measured and used as an indicator for the extent of transcription of the gene. Cells can be exposed to a stimulus, and mRNA can be isolated and assayed at different time points after stimulation. The mRNA from the stimulated cells can be compared to control cells that were not stimulated. The mRNA levels that are higher in the stimulated cell versus the control indicate a stimulus-specific response of the cell. The same is true of mRNA levels that are lower in stimulated cells versus the control condition.

**[0380]** Similar studies can be performed with cells taken from an organism with a defined mutation in their genome as compared with cells without the mutation. Altered mRNA levels in the mutated cells indicate how the mutation causes transcriptional changes. These transcriptional changes are associated with the phenotype that the mutated cells exhibit that is different from the phenotype exhibited by the control cells.

**[0381]** Applicants have utilized microarray techniques to measure the levels of mRNAs in cells from plants transformed with a construct containing the promoter or control elements of the present invention together with their endogenous cDNA sequences. In general, transformants with the constructs were grown to an appropriate stage, and tissue samples were prepared for the microarray differential expression analysis. In this manner it is possible to determine the differential expression for the cDNAs under the control of the endogenous promoter under various conditions.

#### Microarray Experimental Procedures and Results

##### Procedures

##### 1. Sample Tissue Preparation

**[0382]** Tissue samples for each of the expression analysis experiments were prepared as follows:

**[0383]** (a) Roots

**[0384]** Seeds of *Arabidopsis thaliana* (Ws) were sterilized in full strength bleach for less than 5 min, washed more than 3 times in sterile distilled deionized water and plated on MS agar plates. The plates were placed at 4° C. for 3 nights and then placed vertically into a growth chamber having 16 hr light/8 hr dark cycles, 23° C., 70% relative humidity and ~11,000 LUX. After 2 weeks, the roots were cut from the agar, flash frozen in liquid nitrogen and stored at -80° C.

**[0385]** (b) Rosette Leaves, Stems, and Siliques

**[0386]** *Arabidopsis thaliana* (Ws) seed was vernalized at 4° C. for 3 days before sowing in Metro-mix soil type 350. Flats were placed in a growth chamber having 16 hr light/8 hr dark, 80% relative humidity, 23° C. and 13,000 LUX for germination and growth. After 3 weeks, rosette leaves, stems, and siliques were harvested, flash frozen in liquid nitrogen and stored at -80° C. until use. After 4 weeks, siliques (<5 mm, 5-10 mm and >10 mm) were harvested, flash frozen in liquid nitrogen and stored at -80° C. until use. 5 week old whole plants (used as controls) were harvested, flash frozen in liquid nitrogen and kept at -80° C. until RNA was isolated.

**[0387]** (c) Germination

**[0388]** *Arabidopsis thaliana* seeds (ecotype Ws) were sterilized in bleach and rinsed with sterile water. The seeds were placed in 100 mm petri plates containing soaked autoclaved to filter paper. Plates were foil-wrapped and left at 4° C. for 3 nights to vernalize. After cold treatment, the foil was removed and plates were placed into a growth chamber having 16 hr light/8 hr dark cycles, 23° C., 70% relative humidity and ~11,000 lux. Seeds were collected 1 d, 2 d, 3 d and 4 d later, flash frozen in liquid nitrogen and stored at -80° C. until RNA was isolated.

**[0389]** (d) Abscissic Acid (ABA)

**[0390]** Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4° C. for 4 days to vernalize. They were then transferred to a growth chamber having grown 16 hr light/8 hr dark, 13,000 LUX, 70% humidity, and 20° C. and watered twice a week with 1 L of 1× Hoagland's solution. Approximately 1,000 14 day old plants were sprayed with 200-250 mls of 100 µM ABA in a 0.02% solution of the detergent Silwet L-77. Whole seedlings, including roots, were harvested within a 15 to 20 minute time period at 1 hr and 6 hr after treatment, flash-frozen in liquid nitrogen and stored at -80° C.

**[0391]** Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a

growth chamber having 16 hr light (25° C.)/8 hr dark (20° C.), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 100  $\mu$ M ABA for treatment. Control plants were treated with water. After 6 hr and 24 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80° C.

**[0392]** (e) Brassinosteroid Responsive

**[0393]** Two separate experiments were performed, one with epi-brassinolide and one with the brassinosteroid biosynthetic inhibitor brassinazole. In the epi-brassinolide experiments, seeds of wild-type *Arabidopsis thaliana* (ecotype Wassilewskija) and the brassinosteroid biosynthetic mutant dwf4-1 were sown in trays and left at 4° C. for 4 days to vernalize. They were then transferred to a growth chamber having 16 hr light/8 hr dark, 11,000 LUX, 70% humidity and 22° C. temperature. Four week old plants were sprayed with a 1  $\mu$ M solution of epi-brassinolide and shoot parts (unopened floral primordia and shoot apical meristems) harvested three hours later. Tissue was flash-frozen in liquid nitrogen and stored at -80° C. In the brassinazole experiments, seeds of wild-type *Arabidopsis thaliana* (ecotype Wassilewskija) were grown as described above. Four week old plants were sprayed with a 1  $\mu$ M solution of brassinazole and shoot parts (unopened floral primordia and shoot apical meristems) harvested three hours later. Tissue was flash-frozen in liquid nitrogen and stored at -80° C.

**[0394]** In addition to the spray experiments, tissue was prepared from two different mutants; (1) a dwf4-1 knock out mutant and (2) a mutant overexpressing the dwf4-1 gene.

**[0395]** Seeds of wild-type *Arabidopsis thaliana* (ecotype Wassilewskija) and of the dwf4-1 knock out and overexpressor mutants were sown in trays and left at 4° C. for 4 days to vernalize. They were then transferred to a growth chamber having 16 hr light/8 hr dark, 11,000 LUX, 70% humidity and 22° C. temperature. Tissue from shoot parts (unopened floral primordia and shoot apical meristems) was flash-frozen in liquid nitrogen and stored at -80° C.

**[0396]** Another experiment was completed with seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4° C. for 4 days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr. dark) conditions, 13,000 LUX light intensity, 70% humidity, 20° C. temperature and watered twice a week with 1 L 1 $\times$  Hoagland's solution (recipe recited in Feldmann et al., (1987) Mol. Gen. Genet. 208: 1-9 and described as complete nutrient solution). Approximately 1,000 14 day old plants were sprayed with 200-250 mls of 0.1  $\mu$ M Epi-Brassinolite in 0.02% solution of the detergent Silwet L-77. At 1 hr. and 6 hrs. after treatment aerial tissues were harvested within a 15 to 20 minute time period and flash-frozen in liquid nitrogen.

**[0397]** Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25° C.)/8 hr dark (20° C.), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 0.1  $\mu$ M epi-brassinolide for treatment. Control plants were treated with distilled deionized water. After 24 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80° C.

**[0398]** (f) Nitrogen: High to Low

**[0399]** Wild type *Arabidopsis thaliana* seeds (ecotype Ws) were surface sterilized with 30% Clorox, 0.1% Triton X-100 for 5 minutes. Seeds were then rinsed with 4-5 exchanges of sterile double distilled deionized water. Seeds were vernalized at 4° C. for 2-4 days in darkness. After cold treatment, seeds were plated on modified 1 $\times$ MS media (without to  $\text{NH}_4\text{NO}_3$  or  $\text{KNO}_3$ ), 0.5% sucrose, 0.5 g/L MES pH5.7, 1% phytagar and supplemented with  $\text{KNO}_3$  to a final concentration of 60 mM (high nitrate modified 1 $\times$ MS media). Plates were then grown for 7 days in a Percival growth chamber at 22° C. with 16 hr. light/8 hr dark.

**[0400]** Germinated seedlings were then transferred to a sterile flask containing 50 mL of high nitrate modified 1 $\times$ MS liquid media. Seedlings were grown with mild shaking for 3 additional days at 22° C. in 16 hr. light/8 hr dark (in a Percival growth chamber) on the high nitrate modified 1 $\times$ MS liquid media.

**[0401]** After three days of growth on high nitrate modified 1 $\times$ MS liquid media, seedlings were transferred either to a new sterile flask containing 50 mL of high nitrate modified 1 $\times$ MS liquid media or to low nitrate modified 1 $\times$ MS liquid media (containing 20 mM  $\text{KNO}_3$ ). Seedlings were grown in these media conditions with mild shaking at 22° C. in 16 hr light/8 hr dark for the appropriate time points and whole seedlings harvested for total RNA isolation via the Trizol method (LifeTech.). The time points used for the microarray experiments were 10 min. and 1 hour time points for both the high and low nitrate modified 1 $\times$ MS media.

**[0402]** Alternatively, seeds that were surface sterilized in 30% bleach containing 0.1% Triton X-100 and further rinsed in sterile water, were planted on MS agar, (0.5% sucrose) plates containing 50 mM  $\text{KNO}_3$  (potassium nitrate). The seedlings were grown under constant light (3500 LUX) at 22° C. After 12 days, seedlings were transferred to MS agar plates containing either 1 mM  $\text{KNO}_3$  or 50 mM  $\text{KNO}_3$ . Seedlings transferred to agar plates containing 50 mM  $\text{KNO}_3$  were treated as controls in the experiment. Seedlings transferred to plates with 1 mM  $\text{KNO}_3$  were rinsed thoroughly with sterile MS solution containing 1 mM  $\text{KNO}_3$ . There were ten plates per transfer. Root tissue was collected and frozen in 15 mL Falcon tubes at various time points which included 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 9 hours, 12 hours, 16 hours, and 24 hours.

**[0403]** Maize 35A19 Pioneer hybrid seeds were sown on flats containing sand and grown in a Conviron growth chamber at 25° C., 16 hr light/8 hr dark, ~13,000 LUX and 80% relative humidity. Plants were watered every three days with double distilled deionized water. Germinated seedlings are allowed to grow for 10 days and were watered with high nitrate modified 1 $\times$ MS liquid media (see above). On day 11, young corn seedlings were removed from the sand (with their roots intact) and rinsed briefly in high nitrate modified 1 $\times$ MS liquid media. The equivalent of half a flat of seedlings were then submerged (up to their roots) in a beaker containing either 500 mL of high or low nitrate modified 1 $\times$ MS liquid media (see above for details).

**[0404]** At appropriate time points, seedlings were removed from their respective liquid media, the roots separated from the shoots and each tissue type flash frozen in liquid nitrogen and stored at -80° C. This was repeated for each time point. Total RNA was isolated using the Trizol method (see above) with root tissues only.

[0405] Corn root tissues isolated at the 4 hr and 16 hr time points were used for the microarray experiments. Both the high and low nitrate modified 1×MS media were used.

[0406] (g) Nitrogen: Low to High

[0407] *Arabidopsis thaliana* ecotype Ws seeds were sown on flats containing 4 L of a 1:2 mixture of Grace Zonolite vermiculite and soil. Flats were watered with 3 L of water and vernalized at 4° C. for five days. Flats were placed in a Conviron growth chamber having 16 hr light/8 hr dark at 20° C., 80% humidity and 17,450 LUX. Flats were watered with approximately 1.5 L of water every four days. Mature, bolting plants (24 days after germination) were bottom treated with 2 L of either a control (100 mM mannitol pH 5.5) or an experimental (50 mM ammonium nitrate, pH 5.5) solution. Roots, leaves and siliques were harvested separately 30, 120 and 240 minutes after treatment, flash frozen in liquid nitrogen and stored at -80° C.

[0408] Hybrid maize seed (Pioneer hybrid 35A19) were aerated overnight in deionized water. Thirty seeds were plated in each flat, which contained 4 liters of Grace zonolite vermiculite. Two liters of water were bottom fed and flats were kept in a Conviron growth chamber with 16 hr light/8 hr dark at 20° C. and 80% humidity. Flats were watered with 1 L of tap water every three days. Five day old seedlings were treated as described above with 2 L of either a control (100 mM mannitol pH 6.5) solution or 1 L of an experimental (50 mM ammonium nitrate, pH 6.8) solution. Fifteen shoots per time point per treatment were harvested 10, 90 and 180 minutes after treatment, flash frozen in liquid nitrogen and stored at -80° C.

[0409] Alternatively, seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were left at 4° C. for 3 days to vernalize. They were then sown on vermiculite in a growth chamber having 16 hours light/8 hours dark, 12,000-14,000 LUX, 70% humidity, and 20° C. They were bottom-watered with tap water, twice weekly. Twenty-four days old plants were sprayed with either water (control) or 0.6% ammonium nitrate at 4 µL/cm<sup>2</sup> of tray surface. Total shoots and some primary roots were cleaned of vermiculite, flash-frozen in liquid nitrogen and stored at -80° C.

[0410] (h) Methyl Jasmonate

[0411] Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4° C. for 4 days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr. dark, 13,000 LUX, 70% humidity, 20° C. temperature and watered twice a week with 1 L of a 1× Hoagland's solution. Approximately 1,000 14 day old plants were sprayed with 200-250 mls of 0.001% methyl jasmonate in a 0.02% solution of the detergent Silwet L-77. At 1 hr and 6 hrs after treatment, whole seedlings, including roots, were harvested within a 15 to 20 minute time period, flash-frozen in liquid nitrogen and stored at -80° C.

[0412] Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25° C.)/8 hr dark (20° C.), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 0.001% methyl jasmonate for treatment. Control plants were treated with water. After 24 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80° C.

[0413] (i) Salicylic Acid

[0414] Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4° C. for 4 days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr. dark, 13,000 LUX, 70% humidity, 20° C. temperature and watered twice a week with 1 L of a 1× Hoagland's solution. Approximately 1,000 14 day old plants were sprayed with 200-250 mls of 5 mM salicylic acid (solubilized in 70% ethanol) in a 0.02% solution of the detergent Silwet L-77. At 1 hr and 6 hrs after treatment, whole seedlings, including roots, were harvested within a 15 to 20 minute time period flash-frozen in liquid nitrogen and stored at -80° C.

[0415] Alternatively, seeds of wild-type *Arabidopsis thaliana* (ecotype Columbia) and mutant CS3726 were sown in soil type 200 mixed with osmocote fertilizer and Marathon insecticide and left at 4° C. for 3 days to vernalize. Flats were incubated at room temperature with continuous light. Sixteen days post germination plants were sprayed with 2 mM SA, 0.02% SilwetL-77 or control solution (0.02% SilwetL-77). Aerial parts or flowers were harvested 1 hr, 4 hr, 6 hr, 24 hr and 3 weeks post-treatment flash frozen and stored at -80° C.

[0416] Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 to rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25° C.)/8 hr dark (20° C.), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 2 mM SA for treatment. Control plants were treated with water. After 12 hr and 24 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80° C.

[0417] (j) Drought Stress

[0418] Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in pots and left at 4° C. for three days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr dark, 150,000-160,000 LUX, 20° C. and 70% humidity. After 14 days, aerial tissues were cut and left to dry on 3 MM Whatman paper in a Petri-plate for 1 hour and 6 hours. Aerial tissues exposed for 1 hour and 6 hours to 3 MM Whatman paper wetted with 1× Hoagland's solution served as controls. Tissues were harvested, flash-frozen in liquid nitrogen and stored at -80° C.

[0419] Alternatively, *Arabidopsis thaliana* (Ws) seed was vernalized at 4° C. for 3 days before sowing in Metromix soil type 350. Flats were placed in a growth chamber with 23° C., 16 hr light/8 hr. dark, 80% relative humidity, ~13,000 LUX for germination and growth. Plants were watered with 1-1.5 L of water every four days. Watering was stopped 16 days after germination for the treated samples, but continued for the control samples. Rosette leaves and stems, flowers and siliques were harvested 2 d, 3 d, 4 d, 5 d, 6 d and 7 d after watering was stopped. Tissue was flash frozen in liquid nitrogen and kept at -80° C. until RNA was isolated. Flowers and siliques were also harvested on day 8 from plants that had undergone a 7 d drought treatment followed by 1 day of watering. Control plants (whole plants) were harvested after 5 weeks, flash frozen in liquid nitrogen and stored as above.

[0420] Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25° C.)/8 hr dark (20° C.), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were

carefully removed from the sand and placed in empty 1-liter beakers at room temperature for treatment. Control plants were placed in water. After 1 hr, 6 hr, 12 hr and 24 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ .

**[0421]** (k) Osmotic Stress

**[0422]** Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at  $4^{\circ}\text{C}$ . for three days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr dark, 12,000-14,000 LUX,  $20^{\circ}\text{C}$ ., and 70% humidity. After 14 days, the aerial tissues were cut and placed on 3 MM Whatman paper in a petri-plate wetted with 20% PEG (polyethylene glycol-M<sub>r</sub> 8,000) in 1× Hoagland's solution. Aerial tissues on 3 MM Whatman paper containing 1× Hoagland's solution alone served as the control. Aerial tissues were harvested at 1 hour and 6 hours after treatment, flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**[0423]** Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light ( $25^{\circ}\text{C}$ .)/8 hr dark ( $20^{\circ}\text{C}$ .), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 10% PEG (polyethylene glycol-M<sub>r</sub> 8,000) for treatment. Control plants were treated with water. After 1 hr and 6 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ .

**[0424]** Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light ( $25^{\circ}\text{C}$ .)/8 hr dark ( $20^{\circ}\text{C}$ .), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 150 mM NaCl for treatment. Control plants were treated with water. After 1 hr, 6 hr, and 24 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ .

**[0425]** (l) Heat Shock Treatment

**[0426]** Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at  $4^{\circ}\text{C}$ . for three days to vernalize before being transferred to a growth chamber with 16 hr light/8 hr dark, 12,000-14,000 Lux, 70% humidity and  $20^{\circ}\text{C}$ ., fourteen day old plants were transferred to a  $42^{\circ}\text{C}$ . growth chamber and aerial tissues were harvested 1 hr and 6 hr after transfer. Control plants were left at  $20^{\circ}\text{C}$ . and aerial tissues were harvested. Tissues were flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**[0427]** Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light ( $25^{\circ}\text{C}$ .)/8 hr dark ( $20^{\circ}\text{C}$ .), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers containing  $42^{\circ}\text{C}$ . water for treatment. Control plants were treated with water at  $25^{\circ}\text{C}$ . After 1 hr and 6 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ .

**[0428]** (m) Cold Shock Treatment

**[0429]** Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at  $4^{\circ}\text{C}$ . for three days to vernalize before being transferred to a growth chamber having 16 hr

light/8 hr dark, 12,000-14,000 LUX,  $20^{\circ}\text{C}$ . and 70% humidity. Fourteen day old plants were transferred to a  $4^{\circ}\text{C}$ . dark growth chamber and aerial tissues were harvested 1 hour and 6 hours later. Control plants were maintained at  $20^{\circ}\text{C}$ . and covered with foil to avoid exposure to light. Tissues were flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**[0430]** Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light ( $25^{\circ}\text{C}$ .)/8 hr dark ( $20^{\circ}\text{C}$ .), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers containing  $4^{\circ}\text{C}$ . water for treatment. Control plants were treated with water at  $25^{\circ}\text{C}$ . After 1 hr and 6 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ .

**[0431]** (n) *Arabidopsis* Seeds

**[0432]** Fruits (Pod+Seed) 0-5 mm

**[0433]** Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at  $4^{\circ}\text{C}$ . for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and  $22^{\circ}\text{C}$ . temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Silique lengths were then determined and used as an approximate determinant for to embryonic stage. Siliques 0-5 mm in length containing post fertilization through pre-heart stage [0-72 hours after fertilization (HAF)] embryos were harvested and flash frozen in liquid nitrogen.

**[0434]** Fruits (Pod+Seed) 5-10 mm

**[0435]** Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at  $4^{\circ}\text{C}$ . for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and  $22^{\circ}\text{C}$ . temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Silique lengths were then determined and used as an approximate determinant for embryonic stage. Siliques 5-10 mm in length containing heart-through early upturned-U-stage [72-120 hours after fertilization (HAF)] embryos were harvested and flash frozen in liquid nitrogen.

**[0436]** Fruits (Pod+Seed)>10 mm

**[0437]** Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at  $4^{\circ}\text{C}$ . for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and  $22^{\circ}\text{C}$ . temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Silique lengths were

then determined and used as an approximate determinant for embryonic stage. Siliques >10 mm in length containing green, late upturned-U-stage [>120 hours after fertilization (HAF)-9 days after flowering (DAF)] embryos were harvested and flash frozen in liquid nitrogen.

**[0438]** Green Pods 5-10 mm (Control Tissue for Samples 72-74)

**[0439]** Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4° C. for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22° C. temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Silique lengths were then determined and used as an approximate determinant for embryonic stage. Green siliques 5-10 mm in length containing developing seeds 72-120 hours after fertilization (HAF)] were opened and the seeds removed. The remaining tissues (green pods minus seed) were harvested and flash frozen in liquid nitrogen.

**[0440]** Green Seeds from Fruits >10 mm

**[0441]** Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4° C. for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22° C. temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Silique lengths were then determined and used as an approximate determinant for embryonic stage. Green siliques >10 mm in length containing developing seeds up to 9 days after flowering (DAF)] were opened and the seeds removed and harvested and flash frozen in liquid nitrogen.

**[0442]** Brown Seeds from Fruits >10 mm

**[0443]** Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4° C. for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22° C. temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Silique lengths were then determined and used as an approximate determinant for embryonic stage. Yellowing siliques >10 mm in length containing brown, dessicating seeds >11 days after flowering (DAF)] were opened and the seeds removed and harvested and flash frozen in liquid nitrogen.

**[0444]** Green/Brown Seeds from Fruits >10 mm

**[0445]** Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4° C. for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity,

and 22° C. temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Silique lengths were then determined and used as an approximate determinant for embryonic stage. Green siliques >10 mm in length containing both green and brown seeds >9 days after flowering (DAF)] were opened and the seeds removed and harvested and flash frozen in liquid nitrogen.

**[0446]** Mature Seeds (24 Hours after Imbibition)

**[0447]** Mature dry seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown onto moistened filter paper and left at 4° C. for two to three days to vernalize. Imbibed seeds were then transferred to a growth chamber [16 hr light: 8 hr dark conditions, 7000-8000 LUX light intensity, 70% humidity, and 22° C. temperature], the emerging seedlings harvested after 48 hours and flash frozen in liquid nitrogen.

**[0448]** Mature Seeds (Dry)

**[0449]** Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4° C. for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22° C. temperature and taken to maturity. Mature dry seeds are collected, dried for one week at 28° C., and vernalized for one week at 4° C. before used as a source of RNA.

**[0450]** (o) Herbicide Treatment

**[0451]** *Arabidopsis thaliana* (Ws) seeds were sterilized for 5 min with 30% bleach, 50 µl to Triton in a total volume of 50 ml. Seeds were vernalized at 4° C. for 3 days before being plated onto GM agar plates at a density of about 144 seeds per plate. Plates were incubated in a Percival growth chamber having 16 hr light/8 hr dark, 80% relative humidity, 22° C. and 11,000 LUX for 14 days.

**[0452]** Plates were sprayed (~0.5 mls/plate) with water, Finale (1.128 g/L), Glean (1.88 g/L), RoundUp (0.01 g/L) or Trimec (0.08 g/L). Tissue was collected and flash frozen in liquid nitrogen at the following time points: 0, 1, 2, 4, 8, 12 and 24 hours. Frozen tissue was stored at -80° C. prior to RNA isolation.

**[0453]** (p) Root Tips

**[0454]** Seeds of *Arabidopsis thaliana* (ecotype Ws) were placed on MS plates and vernalized at 4° C. for 3 days before being placed in a 25° C. growth chamber having 16 hr light/8 hr dark, 70% relative humidity and about 3 W/m<sup>2</sup>. After 6 days, young seedlings were transferred to flasks containing B5 liquid medium, 1% sucrose and 0.05 mg/l indole-3-butyric acid. Flasks were incubated at room temperature with 100 rpm agitation. Media was replaced weekly. After three weeks, roots were harvested and incubated for 1 hr with 2% pectinase, 0.2% cellulase, pH 7 before straining through a #80 (Sigma) sieve. The root body material remaining on the sieve (used as the control) was flash frozen and stored at -80° C. until use. The material that passed through the #80 sieve was strained through a #200 (Sigma) sieve and the material remaining on the sieve (root tips) was flash frozen and stored at -80° C. until use. Approximately 10 mg of root tips were collected from one flask of root culture.

**[0455]** Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25° C.)/8 hr dark (20° C.),



75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 8 days. Seedlings were carefully removed from the sand and the root tips (~2 mm long) were removed and flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ . The tissues above the root tips (~1 cm long) were cut, treated as above and used as control tissue.

**[0456]** (q) Imbibed Seed

**[0457]** Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in covered flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light ( $25^{\circ}\text{C}$ .)/8 hr dark ( $20^{\circ}\text{C}$ .), 75% relative humidity and to 13,000-14,000 LUX. One day after sowing, whole seeds were flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ . Two days after sowing, embryos and endosperm were isolated and flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ . On days 3-6, aerial tissues, roots and endosperm were isolated and flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ .

**[0458]** (r) Flowers (Green, White or Buds)

**[0459]** Approximately 10  $\mu\text{l}$  of *Arabidopsis thaliana* seeds (ecotype Ws) were sown on 350 soil (containing 0.03% marathon) and vernalized at  $4^{\circ}\text{C}$  for 3 days. Plants were then grown at room temperature under fluorescent lighting until flowering. Flowers were harvested after 28 days in three different categories. Buds that had not opened at all and were completely green were categorized as "flower buds" (also referred to as green buds by the investigator). Buds that had started to open, with white petals emerging slightly were categorized as "green flowers" (also referred to as white buds by the investigator). Flowers that had opened mostly (with no silique elongation) with white petals completely visible were categorized as "white flowers" (also referred to as open flowers by the investigator). Buds and flowers were harvested with forceps, flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA was isolated.

**[0460]** (s) Ovules

**[0461]** Seeds of *Arabidopsis thaliana* heterozygous for *pistillata* (pi) [ecotype Landsberg *erecta* (Ler)] were sown in pots and left at  $4^{\circ}\text{C}$ . for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 76% humidity, and  $24^{\circ}\text{C}$ . temperature. Inflorescences were harvested from seedlings about 40 days old. The inflorescences were cut into small pieces and incubated in the following enzyme solution (pH 5) at room temperature for 0.5-1 hr.: 0.2% pectolyase Y-23, 0.04% pectinase, 5 mM MES, 3% Sucrose and MS salts (1900 mg/l  $\text{KNO}_3$ , 1650 mg/l  $\text{NH}_4\text{NO}_3$ , 370 mg/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 170 mg/l  $\text{KH}_2\text{PO}_4$ , 440 mg/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 6.2 mg/l  $\text{H}_3\text{BO}_3$ , 15.6 mg/l  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 8.6 mg/l  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 mg/l  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.025 mg/l  $\text{CuCO}_3 \cdot 5\text{H}_2\text{O}$ , 0.025 mg/l  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.83 mg/l KI, 27.8 mg/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 37.3 mg/l Disodium EDTA, pH 5.8). At the end of the incubation the mixture of inflorescence material and enzyme solution was passed through a size 60 sieve and then through a sieve with a pore size of 125  $\mu\text{m}$ . Ovules greater than 125  $\mu\text{m}$  in diameter were collected, rinsed twice in B5 liquid medium (2500 mg/l  $\text{KNO}_3$ , 250 mg/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 150 mg/l  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 150 mg/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 134 mg/l  $(\text{NH}_4)_2\text{CaCl}_2 \cdot \text{SO}_4$ , 3 mg/l  $\text{H}_3\text{BO}_3$ , 10 mg/l  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 2  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 mg/l  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.025 mg/l  $\text{CuCO}_3 \cdot 5\text{H}_2\text{O}$ , 0.025 mg/l  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.75 mg/l KI, 40 mg/l EDTA sodium ferric salt, 20 g/l sucrose, 10 mg/l Thiamine hydrochloride, 1 mg/l Pyridoxine hydrochloride, 1 mg/l Nicotinic acid, 100 mg/l myo-

inositol, pH 5.5)), rinsed once in deionized water and flash frozen in liquid nitrogen. The supernatant from the 125  $\mu\text{m}$  sieving was passed through subsequent sieves of 50  $\mu\text{m}$  and 32  $\mu\text{m}$ . The tissue retained in the 32  $\mu\text{m}$  sieve was collected and mRNA prepared for use as a control.

**[0462]** (t) Wounding

**[0463]** Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at  $4^{\circ}\text{C}$ . for three days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr dark, 12,000-14,000 LUX, 70% humidity and  $20^{\circ}\text{C}$ . After 14 days, the leaves were wounded with forceps. Aerial tissues were harvested 1 hour and 6 hours after wounding. Aerial tissues from unwounded plants served as controls. Tissues were flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**[0464]** Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light ( $25^{\circ}\text{C}$ .)/8 hr dark ( $20^{\circ}\text{C}$ .), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were wounded (one leaf nicked by scissors) and placed in 1-liter beakers of water for treatment. Control plants were treated not wounded. After 1 hr and 6 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ .

**[0465]** (u) Nitric Oxide Treatment

**[0466]** Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at  $4^{\circ}\text{C}$ . for three days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr dark, 12,000-14,000 LUX,  $20^{\circ}\text{C}$ . and 70% humidity. Fourteen day old plants were sprayed with 5 mM sodium nitroprusside in a 0.02% Silwett L-77 solution. Control plants were sprayed with a 0.02% Silwett L-77 solution. Aerial tissues were harvested 1 hour and 6 hours after spraying, flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**[0467]** Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light ( $25^{\circ}\text{C}$ .)/8 hr dark ( $20^{\circ}\text{C}$ .), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 5 mM nitroprusside for treatment. Control plants were treated with water. After 1 hr, 6 hr and 12 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$ .

**[0468]** (v) Root Hairless Mutants

**[0469]** Plants mutant at the *rhl* gene locus lack root hairs. This mutation is maintained as a heterozygote.

**[0470]** Seeds of *Arabidopsis thaliana* (Landsberg *erecta*) mutated at the *rhl* gene locus were sterilized using 30% bleach with 1 ul/ml 20% Triton-X 100 and then vernalized at  $4^{\circ}\text{C}$ . for 3 days before being plated onto GM agar plates. Plates were placed in growth chamber with 16 hr light/8 hr. dark,  $23^{\circ}\text{C}$ ., 14,500-15,900 LUX, and 70% relative humidity for germination and growth.

**[0471]** After 7 days, seedlings were inspected for root hairs using a dissecting microscope. Mutants were harvested and the cotyledons removed so that only root tissue remained. Tissue was then flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**[0472]** *Arabidopsis thaliana* (Landsberg *erecta*) seedlings grown and prepared as above were used as controls.

[0473] Alternatively, seeds of *Arabidopsis thaliana* (Landsberg erecta), heterozygous for the rh11 (root hairless) mutation, were surface-sterilized in 30% bleach containing 0.1% Triton X-100 and further rinsed in sterile water. They were then vernalized at 4° C. for 4 days before being plated onto MS agar plates. The plates were maintained in a growth chamber at 24° C. with 16 hr light/8 hr dark for germination and growth. After 10 days, seedling roots that expressed the phenotype (i.e. lacking root hairs) were cut below the hypocotyl junction, frozen in liquid nitrogen and stored at -80° C. Those seedlings with the normal root phenotype (heterozygous or wt) were collected as described for the mutant and used as controls.

[0474] w) Ap2

[0475] Seeds of *Arabidopsis thaliana* (ecotype Landsberg erecta) and floral mutant *apetala2* (Jofuku et al., 1994, Plant Cell 6:1211-1225) were sown in pots and left at 4° C. for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light, 8 hr dark) conditions 7000-8000 LUX light intensity, 70% humidity and 22° C. temperature. Inflorescences containing immature floral buds (stages 1-7; Bowman, 1994) as well as the inflorescence meristem were harvested and flash-frozen. Polysomal polyA+ RNA was isolated from tissue according to Cox and Goldberg, 1988).

[0476] x) Salt

[0477] *Arabidopsis thaliana* ecotype Ws seeds were vernalized at 4° C. for 3 days before sowing in flats containing vermiculite soil. Flats were placed at 20° C. in a Conviron growth chamber having 16 hr light/8 hr dark. Whole plants (used as controls) received water. Other plants were treated with 100 mM NaCl. After 6 hr and 72 hr, aerial and root tissues were harvested and flash frozen in liquid nitrogen prior to storage at -80° C.

[0478] y) Petals

[0479] *Arabidopsis thaliana* ecotype Ws seeds were vernalized at 4° C. for 3 days before sowing in flats containing vermiculite soil. Flats were watered placed at 20° C. in a Conviron growth chamber having 16 hr light/8 hr dark. Whole plants (used as the control) and petals from inflorescences 23-25 days after germination were harvested, flash frozen in liquid nitrogen and stored at -80° C.

[0480] z) Pollen

[0481] *Arabidopsis thaliana* ecotype Ws seeds were vernalized at 4° C. for 3 days before sowing in flats containing vermiculite soil. Flats were watered and placed at 20° C. in a Conviron growth chamber having 16 hr light/8 hr dark. Whole plants (used as controls) and pollen from plants 38 dap was harvested, flash frozen in liquid nitrogen and stored at -80° C.

[0482] aa) Interploidy Crosses

[0483] Interploidy crosses involving a 6x parent are lethal. Crosses involving a 4x parent are complete and analyzed. The imbalance in the maternal/paternal ratio produced from the cross can lead to big seeds. *Arabidopsis thaliana* ecotype Ws seeds were vernalized at 4° C. for 3 days before sowing. Small siliques were harvested at 5 days after pollination, flash frozen in liquid nitrogen and stored at -80° C.

[0484] bb) Line Comparisons

[0485] Alkaloid 35S over-expressing lines were used to monitor the expression levels of terpenoid/alkaloid biosynthetic and P450 genes to identify the transcriptional regulatory points in the biosynthesis pathway and the related P450 genes. *Arabidopsis thaliana* ecotype Ws seeds were vernalized at 4° C. for 3 days before sowing in vermiculite soil

(Zonolite) supplemented by Hoagland solution. Flats were placed in Conviron growth chambers under long day conditions (16 hr light, 23° C./8 hr dark, 20° C.) Basta spray and selection of the overexpressing lines was conducted about 2 weeks after germination. Approximately 2-3 weeks after bolting (approximately 5-6 weeks after germination), stem and siliques from the over-expressing lines and from wild-type plants were harvested, flash frozen in liquid nitrogen and stored at -80° C.

[0486] cc) DMT-II

[0487] Demeter (*dmt*) is a mutant of a methyl transferase gene and is similar to *fie*. *Arabidopsis thaliana* ecotype Ws seeds were vernalized at 4° C. for 3 days before sowing. Cauline leaves and closed flowers were isolated from 35S::DMT and *dmt* -/- plant lines, flash frozen in liquid nitrogen and stored at -80° C.

[0488] dd) CS6630 Roots and Shoots

[0489] *Arabidopsis thaliana* ecotype Ws seeds were vernalized at 4° C. for 3 days before sowing on MS media (1%) sucrose on bacto-agar. Roots and shoots were separated 14 days after germination, flash frozen in liquid nitrogen and stored at -80° C.

[0490] ee) CS237

[0491] CS237 is an ethylene triple response mutant that is insensitive to ethylene and which has an *etr1-1* phenotype. *Arabidopsis thaliana* CS237 seeds were vernalized at 4° C. for 3 days before sowing. Aerial tissue was collected from mutants and wild-type Columbia ecotype plants, flash frozen in liquid nitrogen and stored at -80° C.

[0492] ff) Guard Cells

[0493] *Arabidopsis thaliana* ecotype Ws seeds were vernalized at 4° C. for 3 days before sowing. Leaves were harvested, homogenized and centrifuged to isolate the guard cell containing fraction. Homogenate from leaves served as the control. Samples were flash frozen in liquid nitrogen and stored at -80° C. Identical experiments using leaf tissue from canola were performed.

[0494] gg) 3642-1

[0495] 3642-1 is a T-DNA mutant that affects leaf development. This mutant segregates 3:1, wild-type:mutant. *Arabidopsis thaliana* 3642-1 mutant seeds were vernalized at 4° C. for 3 days before sowing in flats of MetroMix 200. Flats were placed in the greenhouse, watered and grown to the 8 leaf, pre-flower stage. Stems and rosette leaves were harvested from the mutants and the wild-type segregants, flash frozen and stored at -80° C.

[0496] hh) Caf

[0497] Carple factory (Caf) is a double-stranded RNase protein that is hypothesized to process small RNAs in *Arabidopsis*. The protein is closely related to a *Drosophila* protein named DICER that functions in the RNA degradation steps of RNA interference. *Arabidopsis thaliana* Caf mutant seeds were vernalized at 4° C. for 3 days before sowing in flats of MetroMix 200. Flats were placed in the greenhouse, watered and grown to the 8 leaf, pre-flower stage. Stems and rosette leaves were harvested from the mutants and the wild-type segregants, flash frozen and stored at -80° C.

## 2. Microarray Hybridization Procedures

[0498] Microarray technology provides the ability to monitor mRNA transcript levels of thousands of genes in a single experiment. These experiments simultaneously hybridize two differentially labeled fluorescent cDNA pools to glass slides that have been previously spotted with cDNA clones of

the same species. Each arrayed cDNA spot will have a corresponding ratio of fluorescence that represents the level of disparity between the respective mRNA species in the two sample pools. Thousands of polynucleotides can be spotted on one slide, and each experiment generates a global expression pattern.

#### Coating Slides

**[0499]** The microarray consists of a chemically coated microscope slide, referred herein as a “chip” with numerous polynucleotide samples arrayed at a high density. The poly-L-lysine coating allows for this spotting at high density by providing a hydrophobic surface, reducing the spreading of spots of DNA solution arrayed on the slides. Glass microscope slides (Gold Seal #3010 manufactured by Gold Seal Products, Portsmouth, N.H., USA) were coated with a 0.1% W/V solution of Poly-L-lysine (Sigma, St. Louis, Mo.) using the following protocol:

**[0500]** 1. Slides were placed in slide racks (Shandon Lipshaw #121). The racks were then put in chambers (Shandon Lipshaw #121).

**[0501]** 2. Cleaning solution was prepared:

70 g NaOH was dissolved in 280 mL ddH<sub>2</sub>O.

420 mL 95% ethanol was added. The total volume was 700 mL (=2×350 mL); it was stirred until completely mixed. If the solution remained cloudy, ddH<sub>2</sub>O was added until clear.

**[0502]** 3. The solution was poured into chambers with slides; the chambers were covered with glass lids. The solution was mixed on an orbital shaker for 2 hr.

**[0503]** 4. The racks were quickly transferred to fresh chambers filled with ddH<sub>2</sub>O. They were rinsed vigorously by plunging racks up and down. Rinses were repeated 4× with fresh ddH<sub>2</sub>O each time, to remove all traces of NaOH-ethanol.

**[0504]** 5. Polylysine solution was prepared:

0 mL poly-L-lysine+70 mL tissue culture PBS in 560 mL water, using plastic graduated cylinder and beaker.

**[0505]** 6. Slides were transferred to polylysine solution and shaken for 1 hr.

**[0506]** 7. The rack was transferred to a fresh chambers filled with ddH<sub>2</sub>O. It was plunged up and down 5× to rinse.

**[0507]** 8. The slides were centrifuged on microtiter plate carriers (paper towels were placed below the rack to absorb liquid) for 5 min @ 500 rpm. The slide racks were transferred to empty chambers with covers.

**[0508]** 9. Slide racks were dried in a 45 C oven for 10 min.

**[0509]** 10. The slides were stored in a closed plastic slide box.

**[0510]** 11. Normally, the surface of lysine coated slides was not very hydrophobic immediately after this process, but became increasingly hydrophobic with storage. A hydrophobic surface helped ensure that spots didn't run together while printing at high densities. After they aged for 10 days to a month the slides were ready to use. However, coated slides that have been sitting around for long periods of time were usually too old to be used. This was because they developed opaque patches, visible when held to the light, and these resulted in high background hybridization from the fluorescent probe. Alternatively, pre-coated glass slides were purchased from TeleChem International, Inc. (Sunnyvale, Calif., 94089; catalog number SMM-25, Supramine substrates).

#### PCR Amplification of cDNA Clone Inserts

**[0511]** Polynucleotides were amplified from *Arabidopsis* cDNA clones using insert specific probes. The resulting 100 uL PCR reactions were purified with Qiaquick 96 PCR purification columns (Qiagen, Valencia, Calif., USA) and eluted in 30 uL of 5 mM Tris. 8.5 uL of the elution were mixed with 1.5 uL of 20×SSC to give a final spotting solution of DNA in 3×SSC. The concentrations of DNA generated from each clone varied between 10-100 ng/uL, but were usually about 50 ng/uL.

#### Arraying of PCR Products on Glass Slides

**[0512]** PCR products from cDNA clones were spotted onto the poly-L-Lysine coated glass slides using an arrangement of quill-tip pins (ChipMaker 3 spotting pins; Telechem, International, Inc., Sunnyvale, Calif., USA) and a robotic arrayer (PixSys 3500, Cartesian Technologies, Irvine, Calif., USA). Around 0.5 nl of a prepared PCR product was spotted at each location to produce spots with approximately 100 um diameters. Spot center-to-center spacing was from 180 um to 210 um depending on the array. Printing was conducted in a chamber with relative humidity set at 50%.

**[0513]** Slides containing maize sequences were purchased from Agilent Technology (Palo Alto, Calif. 94304).

#### Post-Processing of Slides

**[0514]** After arraying, slides were processed through a series of steps—rehydration, UV cross-linking, blocking and denaturation—required prior to hybridization. Slides were rehydrated by placing them over a beaker of warm water (DNA face down), for 2-3 sec, to distribute the DNA more evenly within the spots, and then snap dried on a hot plate (DNA side, face up). The DNA was then cross-linked to the slides by UV irradiation (60-65 mJ; 2400 Stratalinker, Stratagene, La Jolla, Calif., USA).

**[0515]** Following this a blocking step was performed to modify remaining free lysine groups, and hence minimize their ability to bind labeled probe DNA. To achieve this the arrays were placed in a slide rack. An empty slide chamber was left ready on an orbital shaker. The rack was bent slightly inwards in the middle, to ensure the slides would not run into each other while shaking. The blocking solution was prepared as follows:

3×350-ml glass chambers (with metal tops) were set to one side, and a large round Pyrex dish with dH<sub>2</sub>O was placed ready in the microwave. At this time, 15 ml sodium borate was prepared in a 50 ml conical tube.

**[0516]** 6-g succinic anhydride was dissolved in approx. 325-350 mL 1-methyl-2-pyrrolidinone. Rapid addition of reagent was crucial.

**[0517]** a Immediately after the last flake of the succinic anhydride dissolved, the 15-mL sodium borate was added.

**[0518]** b Immediately after the sodium borate solution mixed in, the solution was poured into an empty slide chamber.

**[0519]** c. The slide rack was plunged rapidly and evenly in the solution. It was vigorously shaken up and down for a few seconds, making sure slides never left the solution.

**[0520]** d. It was mixed on an orbital shaker for 15-20 min. Meanwhile, the water in the Pyrex dish (enough to cover slide rack) was heated to boiling.

**[0521]** Following this, the slide rack was gently plunge in the 95 C water (just stopped boiling) for 2 min. Then the slide

rack was plunged 5× in 95% ethanol. The slides and rack were centrifuged for 5 min @ 500 rpm. The slides were loaded quickly and evenly onto the carriers to avoid streaking. The arrays were used immediately or store in slide box.

**[0522]** The Hybridization process began with the isolation of mRNA from the two tissues (see “Isolation of total RNA” and “Isolation of mRNA”, below) in question followed by their conversion to single stranded cDNA (see “Generation of probes for hybridization”, below). The cDNA from each tissue was independently labeled with a different fluorescent dye and then both samples were pooled together. This final differentially labeled cDNA pool was then placed on a processed microarray and allowed to hybridize (see “Hybridization and wash conditions”, below).

#### Isolation of Total RNA

**[0523]** Approximately 1 g of plant tissue was ground in liquid nitrogen to a fine powder and transferred into a 50-ml centrifuge tube containing 10 ml of Trizol reagent. The tube was vigorously vortexed for 1 min and then incubated at room temperature for 10-20 min on an orbital shaker at 220 rpm. Two ml of chloroform was added to the tube and the solution vortexed vigorously for at least 30-sec before again incubating at room temperature with shaking. The sample was then centrifuged at 12,000×g (10,000 rpm) for 15-20 min at 4° C. The aqueous layer was removed and mixed by inversion with 2.5 ml of 1.2 M NaCl/0.8 M to Sodium Citrate and 2.5 ml of isopropyl alcohol added. After a 10 min incubation at room temperature, the sample was centrifuged at 12,000×g (10,000 rpm) for 15 min at 4° C. The pellet was washed with 70% ethanol, re-centrifuged at 8,000 rpm for 5 min and then air dried at room temperature for 10 min. The resulting total RNA was dissolved in either TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or DEPC (diethylpyrocarbonate) treated deionized water (RNase-free water). For subsequent isolation of mRNA using the Qiagen kit, the total RNA pellet was dissolved in RNase-free water.

#### Isolation of mRNA

**[0524]** mRNA was isolated using the Qiagen Oligotex mRNA Spin-Column protocol (Qiagen, Valencia, Calif.). Briefly, 500 µl OBB buffer (20 mM Tris-Cl, pH 7.5, 1 M NaCl, 2 mM EDTA, 0.2% SDS) was added to 500 µl of total RNA (0.5-0.75 mg) and mixed thoroughly. The sample was first incubated at 70° C. for 3 min, then at room temperature for 10 minutes and finally centrifuged for 2 min at 14,000-18,000×g. The pellet was resuspended in 400 µl OW2 buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA) by vortexing, the resulting solution placed on a small spin column in a 1.5 ml RNase-free microcentrifuge tube and centrifuged for 1 min at 14,000-18,000×g. The spin column was transferred to a new 1.5 ml RNase-free microcentrifuge tube and washed with 400 µl of OW2 buffer. To release the isolated mRNA from the resin, the spin column was again transferred to a new RNase-free 1.5 ml microcentrifuge tube, 20-100 µl 70° C. OEB buffer (5 mM Tris-Cl, pH 7.5) added and the resin resuspended in the resulting solution via pipeting. The mRNA solution was collected after centrifuging for 1 min at 14,000-18,000×g.

**[0525]** Alternatively, mRNA was isolated using the Stratagene Poly(A) Quik mRNA Isolation Kit (Stratagene, La Jolla, Calif.). Here, up to 0.5 mg of total RNA (maximum volume of 1 ml) was incubated at 65° C. for 5 minutes, snap cooled on ice and 0.1× volumes of 10× sample buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0) 5 M NaCl) added.

The RNA sample was applied to a prepared push column and passed through the column at a rate of ~1 drop every 2 sec. The solution collected was reapplied to the column and collected as above. 200 µl of high salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 NaCl) was applied to the column and passed through the column at a rate of ~1 drop every 2 sec. This step was repeated and followed by three low salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM to EDTA, 0.1 M NaCl) washes performed in a similar manner mRNA was eluted by applying to the column four separate 200 µl aliquots of elution buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) preheated to 65° C. Here, the elution buffer was passed through the column at a rate of 1 drop/sec. The resulting mRNA solution was precipitated by adding 0.1× volumes of 10× sample buffer, 2.5 volumes of ice-cold 100% ethanol, incubating overnight at -20° C. and centrifuging at 14,000-18,000×g for 20-30 min at 4° C. The pellet was washed with 70% ethanol and air dried for 10 min. at room temperature before resuspension in RNase-free deionized water.

#### Preparation of Yeast Controls

**[0526]** Plasmid DNA was isolated from the following yeast clones using Qiagen filtered maxiprep kits (Qiagen, Valencia, Calif.): YAL022c(Fun26), YAL031c(Fun21), YBR032w, YDL131w, YDL182w, YDL194w, YDL196w, YDR050c and YDR116c. Plasmid DNA was linearized with either BsrBI (YAL022c(Fun26), YAL031c(Fun21), YDL131w, YDL182w, YDL194w, YDL196w, YDR050c) or AflIII (YBR032w, YDR116c) and isolated.

#### In Vitro Transcription of Yeast Clones

**[0527]** The following solution was incubated at 37° C. for 2 hours: 17 µl of isolated yeast insert DNA (1 µg), 20 µl 5× buffer, 10 µl 100 mM DTT, 2.5 µl (100 U) RNasin, 20 µl 2.5 mM (ea.) rNTPs, 2.7 µl (40 U) SP6 polymerase and 27.8 µl RNase-free deionized water. 2 µl (2 U) Ampli DNase I was added and the incubation continued for another 15 min. 10 µl 5M NH<sub>4</sub>OAC and 100 µl phenol:chloroform:isoamyl alcohol (25:24:1) were added, the solution vortexed and then centrifuged to separate the phases. To precipitate the RNA, 250 µl ethanol was added and the solution incubated at -20° C. for at least one hour. The sample was then centrifuged for 20 min at 4° C. at 14,000-18,000×g, the pellet washed with 500 µl of 70% ethanol, air dried at room temperature for 10 min and resuspended in 100 µl of RNase-free deionized water. The precipitation procedure was then repeated.

**[0528]** Alternatively, after the two-hour incubation, the solution was extracted with phenol:chloroform once before adding 0.1 volume 3M sodium acetate and 2.5 volumes of 100% ethanol. The solution was centrifuged at 15,000 rpm, 4° C. for 20 minutes and the pellet resuspended in RNase-free deionized water. The DNase I treatment was carried out at 37° C. to for 30 minutes using 2 U of Ampli DNase I in the following reaction condition: 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>. The DNase I reaction was then stopped with the addition of NH<sub>4</sub>OAC and phenol:chloroform:isoamyl alcohol (25:24:1), and RNA isolated as described above.

**[0529]** 0.15-2.5 ng of the in vitro transcript RNA from each yeast clone were added to each plant mRNA sample prior to labeling to serve as positive (internal) probe controls.

## Generation of Probes for Hybridization

**[0530]** Generation of Labeled Probes for Hybridization from First-Strand cDNA

**[0531]** Hybridization probes were generated from isolated mRNA using an Atlas™ Glass Fluorescent Labeling Kit (Clontech Laboratories, Inc., Palo Alto, Calif., USA). This entails a two step labeling procedure that first incorporates primary aliphatic amino groups during cDNA synthesis and then couples fluorescent dye to the cDNA by reaction with the amino functional groups. Briefly, 5 µg of oligo(dT)<sub>18</sub> primer d(TTTTTTTTTTTTTTTTTT) was mixed with Poly A+ mRNA (1.5-2 µg mRNA isolated using the Qiagen Oligotex mRNA Spin-Column protocol or the Stratagene Poly(A) Quik mRNA Isolation protocol (Stratagene, La Jolla, Calif., USA)) in a total volume of 25 µl. The sample was incubated in a thermocycler at 70° C. for 5 min, cooled to 48° C. and 10 µl of 5× cDNA Synthesis Buffer (kit supplied), 5 µl 10× dNTP mix (dATP, dCTP, dGTP, dTTP and aminoallyl-dUTP; kit supplied), 7.5 µl deionized water and 2.5 µl MMLV Reverse Transcriptase (500 U) added. The reaction was then incubated at 48° C. for 30 minutes, followed by 1 hr incubation at 42° C. At the end of the incubation the reaction was heated to 70° C. for 10 min, cooled to 37° C. and 0.5 µl (5 U) RNase H added, before incubating for 15 min at 37° C. The solution was vortexed for 1 min after the addition of 0.5 µl 0.5 MEDTA and 5 µl of QuickClean Resin (kit supplied) then centrifuged at 14,000-18,000×g for 1 min. After removing the supernatant to a 0.45 µm spin filter (kit supplied), the sample was again centrifuged at 14,000-18,000×g for 1 min, and 5.5 µl 3 M sodium acetate and 137.5 µl of 100% ethanol added to the sample before incubating at -20° C. for at least 1 hr. The sample was then centrifuged at 14,000-18,000×g at 4° C. for 20 min, the resulting pellet washed with 500 µl 70% ethanol, air-dried at room temperature for 10 min and resuspended in 10 µl of 2× fluorescent labeling buffer (kit provided). 10 µl each of the fluorescent dyes Cy3 and Cy5 (Amersham Pharmacia (Piscataway, N.J., USA); prepared according to Atlas™ kit directions of Clontech) were added and the sample incubated in the dark at room temperature for 30 min

**[0532]** The fluorescently labeled first strand cDNA was precipitated by adding 2 µl 3M sodium acetate and 50 µl 100% ethanol, incubated at -20° C. for at least 2 hrs, centrifuged at 14,000-18,000×g for 20 min, washed with 70% ethanol, air-dried for 10 min and dissolved in 100 µl of water.

**[0533]** Alternatively, 3-4 µg mRNA, 2.5 (~8.9 ng of in vitro translated mRNA) µl yeast control and 3 µg oligo dTV (TTTTTTTTTTTTTTTTT(A/C/G)) were mixed in a total volume of 24.7 µl. The sample was incubated in a thermocycler at 70° C. for 10 min before chilling on ice. To this, 8 µl of 5× first strand buffer (SuperScript II RNase H—Reverse Transcriptase kit from Invitrogen (Carlsbad, Calif. 92008); cat no. 18064022), 0.8° C. of aa-dUTP/dNTP mix (50×; 25 mM dATP, 25 mM dGTP, 25 mM dCTP, 15 mM dTTP, 10 mM aminoallyl-dUTP), 4 µl of 0.1 M DTT and 2.5 µl (500 units) of Superscript R.T.II enzyme (Stratagene) were added. The sample was incubated at 42° C. for 2 hours before a mixture of 10° C. of 1M NaOH and 10° C. of 0.5 MEDTA were added. After a 15 minute incubation at 65° C., 25 µl of 1 M Tris pH 7.4 was added. This was mixed with 450 µl of water in a Microcon 30 column before centrifugation at 11,000×g for 12 min. The column was washed twice with 450 µl (centrifugation at 11,000 g, 12 min) before eluting the sample by invert-

ing the Microcon column and centrifuging at 11,000×g for 20 seconds. Sample was dehydrated by centrifugation under vacuum and stored at -20° C.

**[0534]** Each reaction pellet was dissolved in 9 µl of 0.1 M carbonate buffer (0.1 M sodium carbonate and sodium bicarbonate, pH=8.5-9) and 4.5 µl of this placed in two microfuge tubes. 4.5 µl of each dye (in DMSO) were added and the mixture incubated in the dark for 1 hour. 4.5 µl of 4 M hydroxylamine was added and again incubated in the dark for 15 minutes.

**[0535]** Regardless of the method used for probe generation, the probe was purified using a Qiagen PCR cleanup kit (Qiagen, Valencia, Calif., USA), and eluted with 100 µl EB (kit provided). The sample was loaded on a Microcon YM-30 (Millipore, Bedford, Mass., USA) spin column and concentrated to 4-5 µl in volume. Probes for the maize microarrays were generated using the Fluorescent Linear Amplification Kit (cat. No. G2556A) from Agilent Technologies (Palo Alto, Calif.).

## Hybridization and Wash Conditions

**[0536]** The following Hybridization and Washing Condition were developed:

## Hybridization Conditions:

**[0537]** Labeled probe was heated at 95° C. for 3 min and chilled on ice. Then 25 mL of the hybridization buffer which was warmed at 42° C was added to the probe, mixing by pipeting, to give a final concentration of:

50% formamide

**[0538]** 4×SSC

**[0539]** 0.03% SDS

5×Denhardt's solution

0.1 µg/ml single-stranded salmon sperm DNA

**[0540]** The probe was kept at 42° C. Prior to the hybridization, the probe was heated for 1 more min, added to the array, and then covered with a glass cover slip. Slides were placed in hybridization chambers (Telechem, Sunnyvale, Calif.) and incubated at 42° C. overnight.

## Washing Conditions:

**[0541]** A. Slides were washed in 1×SSC+0.03% SDS solution at room temperature for 5 minutes,

**[0542]** B. Slides were washed in 0.2×SSC at room temperature for 5 minutes,

**[0543]** C. Slides were washed in 0.05×SSC at room temperature for 5 minutes.

**[0544]** After A, B, and C, slides were spun at 800×g for 2 min to dry. They were then scanned.

**[0545]** Maize microarrays were hybridized according to the instructions included Fluorescent Linear Amplification Kit (cat. No. G2556A) from Agilent Technologies (Palo Alto, Calif.).

## Scanning of Slides

**[0546]** The chips were scanned using a ScanArray 3000 or 5000 (General Scanning, Watertown, Mass., USA). The chips were scanned at 543 and 633 nm, at 10 µm resolution to

measure the intensity of the two fluorescent dyes incorporated into the samples hybridized to the chips.

#### Data Extraction and Analysis

**[0547]** The images generated by scanning slides consisted of two 16-bit TIFF images representing the fluorescent emissions of the two samples at each arrayed spot. These images were then quantified and processed for expression analysis using the data extraction software Imagene™ (Biodiscovery, Los Angeles, Calif., USA). Imagene output was subsequently analyzed using the analysis program Genespring™ (Silicon Genetics, San Carlos, Calif., USA). In Genespring, the data was imported using median pixel intensity measurements derived from Imagene output. Background subtraction, ratio calculation and normalization were all conducted in Genespring. Normalization was achieved by breaking the data in to 32 groups, each of which represented one of the 32 pin printing regions on the microarray. Groups consist of 360 to 550 spots. Each group was independently normalized by setting the median of ratios to one and multiplying ratios by the appropriate factor.

#### **[0548]** Results

**[0549]** The results of the microarray experiments are set forth in Table 1 in the section entitled "Microarray Data" which shows the results of the differential expression experiments for the mRNAs, as reported by their corresponding cDNA ID number, that were differentially transcribed under a particular set of conditions as compared to a control sample. The cDNA ID numbers correspond to those utilized. Increases in mRNA abundance levels in experimental plants versus the controls are denoted with the plus sign (+). Likewise, reductions in mRNA abundance levels in the experimental plants are denoted with the minus (−) sign.

**[0550]** The Table 1 section entitled "Microarray Data" is organized according to the clone number with each set of experimental conditions being denoted by the term "Expt Rep ID:" followed by a "short name". The row titled "Microarray Experiment Parameters" links each "short name" with a short description of the experiment and the parameters.

**[0551]** The sequences showing differential expression in a particular experiment (denoted by either a "+" or "−" in the column in Table 1 entitled "SIGNCLOG\_RATIO") thereby show utility for a function in a plant, and these functions/utilities are described in detail below, where the title of each section (i.e. a "utility section") is correlated with the particular differential expression experiment in the section of Table 1 entitled "Microarray Experiment Parameters".

#### Organ-Affecting Genes, Gene Components, Products (Including Differentiation and Function)

##### Root Genes

**[0552]** The economic values of roots arise not only from harvested adventitious roots or tubers, but also from the ability of roots to funnel nutrients to support growth of all plants and increase their vegetative material, seeds, fruits, etc. Roots have four main functions. First, they anchor the plant in the soil. Second, they facilitate and regulate the molecular signals and molecular traffic between the plant, soil, and soil fauna. Third, the root provides a plant with nutrients gained from the soil or growth medium. Fourth, they condition local soil chemical and physical properties.

**[0553]** Root genes are active or potentially active to a greater extent in roots than in most other organs of the plant.

These genes and gene products can regulate many plant traits from yield to stress tolerance. Root genes can be used to modulate root growth and development.

#### **[0554]** Differential Expression of the Sequences in Roots

**[0555]** The relative levels of mRNA product in the root versus the aerial portion of the plant was measured. Specifically, mRNA was isolated from roots and root tips of *Arabidopsis* plants and compared to mRNA isolated from the aerial portion of the plants utilizing microarray procedures.

#### Root Hair Genes, Gene Components and Products

**[0556]** Root hairs are specialized outgrowths of single epidermal cells termed trichoblasts. In many and perhaps all species of plants, the trichoblasts are regularly arranged around the perimeter of the root. In *Arabidopsis*, for example, trichoblasts tend to alternate with non-hair cells or atrichoblasts. This spatial patterning of the root epidermis is under genetic control, and a variety of mutants have been isolated in which this spacing is altered or in which root hairs are completely absent.

**[0557]** The root hair development genes of the instant invention are useful to modulate one or more processes of root hair structure and/or function including (1) development; (2) interaction with the soil and soil contents; (3) uptake and transport in the plant; and (4) interaction with microorganisms.

#### **[0558]** 1.) Development

The surface cells of roots can develop into single epidermal cells termed trichoblasts or root hairs. Some of the root hairs will persist for the life of the plant; others will gradually die back; some may cease to function due to external influences. These genes and gene products can be used to modulate root hair density or root hair growth; including rate, timing, direction, and size, for example. These genes and gene products can also be used to modulate cell properties such as cell size, cell division, rate and direction and number, cell elongation, cell differentiation, lignified cell walls, epidermal cells (including trichoblasts) and root apical meristem cells (growth and initiation); and root hair architecture such as leaf cells under the trichome, cells forming the base of the trichome, trichome cells, and root hair responses. In addition these genes and gene products can be used to modulate one or more of the growth and development processes in response to internal plant programs or environmental stimuli in, for example, the seminal system, nodal system, hormone responses, Auxin, root cap abscission, root senescence, gravitropism, coordination of root growth and development with that of other organs (including leaves, flowers, seeds, fruits, and stems), and changes in soil environment (including water, minerals, Ph, and microfauna and flora).

#### **[0559]** 2.) Interaction with Soil and Soil Contents

Root hairs are sites of intense chemical and biological activity and as a result can strongly modify the soil they contact. Roots hairs can be coated with surfactants and mucilage to facilitate these activities. Specifically, roots hairs are responsible for nutrient uptake by mobilizing and assimilating water, reluctant ions, organic and inorganic compounds and chemicals. In addition, they attract and interact with beneficial microfauna and flora. Root hairs also help to mitigate the effects of toxic ions, pathogens and stress. Thus, root hair genes and gene products can be used to modulate traits such as root hair surfactant and mucilage (including composition and secretion rate and time); nutrient uptake (including water, nitrate and other sources of nitrogen, phosphate, potassium, and

micronutrients (e.g. iron, copper, etc.); microbe and nematode associations (such as bacteria including nitrogen-fixing bacteria, mycorrhizae, nodule-forming and other nematodes, and nitrogen fixation); oxygen transpiration; detoxification effects of iron, aluminum, cadmium, mercury, salt, and other soil constituents; pathogens (including chemical repellents) glucosinolates (GSL1), which release pathogen-controlling isothiocyanates; and changes in soil (such as Ph, mineral excess and depletion), and rhizosphere.

#### **[0560]** 3.) Transport of Materials in Plants

Uptake of the nutrients by the root and root hairs contributes a source-sink effect in a plant. The greater source of nutrients, the more sinks, such as stems, leaves, flowers, seeds, fruits, etc. can draw sustenance to grow. Thus, root hair development genes and gene products can be used to modulate the vigor and yield of the overall plant as well as distinct cells, organs, or tissues of a plant. The genes and gene products, therefore, can modulate plant nutrition, growth rate (such as whole plant, including height, flowering time, etc., seedling, coleoptile elongation, young leaves, stems, flowers, seeds and fruit) and yield, including biomass (fresh and dry weight during any time in plant life, including maturation and senescence), number of flowers, number of seeds, seed yield, number, size, weight and harvest index (content and composition, e.g. amino acid, jasmonate, oil, protein and starch) and fruit yield (number, size, weight, harvest index, and post harvest quality).

#### Reproduction Genes, Gene Components and Products

**[0561]** Reproduction genes are defined as genes or components of genes capable of modulating any aspect of sexual reproduction from flowering time and inflorescence development to fertilization and finally seed and fruit development. These genes are of great economic interest as well as biological importance. The fruit and vegetable industry grosses over \$1 billion USD a year. The seed market, valued at approximately \$15 billion USD annually, is even more lucrative.

#### Inflorescence and Floral Development Genes, Gene Components and Products

**[0562]** During reproductive growth the plant enters a program of floral development that culminates in fertilization, followed by the production of seeds. Senescence may or may not follow. The flower formation is a precondition for the sexual propagation of plants and is therefore essential for the propagation of plants that cannot be propagated vegetatively as well as for the formation of seeds and fruits. The point of time at which the merely vegetative growth of plants changes into flower formation is of vital importance for example in agriculture, horticulture and plant breeding. Also the number of flowers is often of economic importance, for example in the case of various useful plants (tomato, cucumber, zucchini, cotton etc.) with which an increased number of flowers may lead to an increased yield, or in the case of growing ornamental plants and cut flowers.

**[0563]** Flowering plants exhibit one of two types of inflorescence architecture: indeterminate, to in which the inflorescence grows indefinitely, or determinate, in which a terminal flower is produced. Adult organs of flowering plants develop from groups of stem cells called meristems. The identity of a meristem is inferred from structures it produces: vegetative meristems give rise to roots and leaves, inflorescence meristems give rise to flower meristems, and flower meristems

give rise to floral organs such as sepals and petals. Not only are meristems capable of generating new meristems of different identity, but their own identity can change during development. For example, a vegetative shoot meristem can be transformed into an inflorescence meristem upon floral induction, and in some species, the inflorescence meristem itself will eventually become a flower meristem. Despite the importance of meristem transitions in plant development, little is known about the underlying mechanisms.

**[0564]** Following germination, the shoot meristem produces a series of leaf meristems on its flanks. However, once floral induction has occurred, the shoot meristem switches to the production of flower meristems. Flower meristems produce floral organ primordia, which develop individually into sepals, petals, stamens or carpels. Thus, flower formation can be thought of as a series of distinct developmental steps, i.e. floral induction, the formation of flower primordia and the production of flower organs. Mutations disrupting each of the steps have been isolated in a variety of species, suggesting that a genetic hierarchy directs the flowering process (see for review, Weigel and Meyerowitz, In Molecular Basis of Morphogenesis (ed. M. Bernfield). 51st Annual Symposium of the Society for Developmental Biology, pp. 93-107, New York, 1993).

**[0565]** Expression of many reproduction genes and gene products is orchestrated by internal programs or the surrounding environment of a plant. These genes can be used to modulate traits such as fruit and seed yield

#### Seed and Fruit Development Genes, Gene Components and Products

**[0566]** The ovule is the primary female sexual reproductive organ of flowering plants. At maturity it contains the egg cell and one large central cell containing two polar nuclei encased by two integuments that, after fertilization, develops into the embryo, endosperm, and seed coat of the mature seed, respectively. As the ovule develops into the seed, the ovary matures into the fruit or silique. As such, seed and fruit development requires the orchestrated transcription of numerous polynucleotides, some of which are ubiquitous, others that are embryo-specific and still others that are expressed only in the endosperm, seed coat, or fruit. Such genes are termed fruit development responsive genes and can be used to modulate seed and fruit growth and development such as seed size, seed yield, seed composition and seed dormancy.

#### **[0567]** Differential Expression of the Sequences in Siliques, Inflorescences and Flowers

The relative levels of mRNA product in the siliques relative to the plant as a whole was measured.

#### **[0568]** Differential Expression of the Sequences in Hybrid Seed Development

**[0569]** The levels of mRNA product in the seeds relative to those in a leaf and floral stems was measured.

#### Development Genes, Gene Components and Products

#### Imbibition and Germination Responsive Genes, Gene Components and Products

**[0570]** Seeds are a vital component of the world's diet. Cereal grains alone, which comprise ~90% of all cultivated seeds, contribute up to half of the global per capita energy intake. The primary organ system for seed production in flowering plants is the ovule. At maturity, the ovule consists of a haploid female gametophyte or embryo sac surrounded by

several layers of maternal tissue including the nucleus and the integuments. The embryo sac typically contains seven cells including the egg cell, two synergids, a large central cell containing two polar nuclei, and three antipodal cells. That pollination results in the fertilization of both egg and central cell. The fertilized egg develops into the embryo. The fertilized central cell develops into the endosperm. And the integuments mature into the seed coat. As the ovule develops into the seed, the ovary matures into the fruit or silique. Late in development, the developing seed ends a period of extensive biosynthetic and cellular activity and begins to desiccate to complete its development and enter a dormant, metabolically quiescent state. Seed dormancy is generally an undesirable characteristic in agricultural crops, where rapid germination and growth are required. However, some degree of dormancy is advantageous, at least during seed development. This is particularly true for cereal crops because it prevents germination of grains while still on the ear of the parent plant (preharvest sprouting), a phenomenon that results in major losses to the agricultural industry. Extensive domestication and breeding of crop species have ostensibly reduced the level of dormancy mechanisms present in the seeds of their wild ancestors, although under some adverse environmental conditions, dormancy may reappear. By contrast, weed seeds frequently mature with inherent dormancy mechanisms that allow some seeds to persist in the soil for many years before completing germination.

**[0571]** Germination commences with imbibition, the uptake of water by the dry seed, and the activation of the quiescent embryo and endosperm. The result is a burst of intense metabolic activity. At the cellular level, the genome is transformed from an inactive state to one of intense transcriptional activity. Stored lipids, carbohydrates and proteins are catabolized fueling seedling growth and development. DNA and organelles are repaired, replicated and begin functioning. Cell expansion and cell division are triggered. The shoot and root apical meristem are activated and begin growth and organogenesis. Schematic 4 summarizes some of the metabolic and cellular processes that occur during imbibition. Germination is complete when a part of the embryo, the radicle, extends to penetrate the structures that surround it. In *Arabidopsis*, seed germination takes place within twenty-four (24) hours after imbibition. As such, germination requires the rapid and orchestrated transcription of numerous polynucleotides. Germination is followed by expansion of the hypocotyl and opening of the cotyledons. Meristem development continues to promote root growth and shoot growth, which is followed by early leaf formation.

#### Imbibition and Germination Genes

**[0572]** Imbibition and germination includes those events that commence with the uptake of water by the quiescent dry seed and terminate with the expansion and elongation of the shoots and roots. The germination period exists from imbibition to when part of the embryo, usually the radicle, extends to penetrate the seed coat that surrounds it. Imbibition and germination genes are defined as genes, gene components and products capable of modulating one or more processes of imbibition and germination described above. They are useful to modulate many plant traits from early vigor to yield to stress tolerance.

**[0573]** Differential Expression of the Sequences in Germinating Seeds and Imbibed Embryos

**[0574]** The levels of mRNA product in the seeds versus the plant as a whole was measured.

#### Hormone Responsive Genes, Gene Components and Products

##### Abscissic Acid Responsive Genes, Gene Components and Products

**[0575]** Plant hormones are naturally occurring substances, effective in very small amounts, which act as signals to stimulate or inhibit growth or regulate developmental processes in plants. Abscissic acid (ABA) is a ubiquitous hormone in vascular plants that has been detected in every major organ or living tissue from the root to the apical bud. The major physiological responses affected by ABA are dormancy, stress stomatal closure, water uptake, abscission and senescence. In contrast to Auxins, cytokinins and gibberellins, which are principally growth promoters, ABA primarily acts as an inhibitor of growth and metabolic processes.

**[0576]** Changes in ABA concentration internally or in the surrounding environment in contact with a plant results in modulation of many genes and gene products. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield. While ABA responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different ABA responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants. In addition, the combination of an ABA responsive polynucleotide and/or gene product with another environmentally responsive polynucleotide is also useful because of the interactions that exist between hormone-regulated pathways, stress and defense induced pathways, nutritional pathways and development.

**[0577]** Differential Expression of the Sequences in ABA Treated Plants

**[0578]** The relative levels of mRNA product in plants treated with ABA versus controls treated with water were measured.

##### Brassinosteroid Responsive Genes, Gene Components and Products

**[0579]** Plant hormones are naturally occurring substances, effective in very small amounts, which act as signals to stimulate or inhibit growth or regulate developmental processes in plants. Brassinosteroids (BRs) are the most recently discovered, and least studied, class of plant hormones. The major physiological response affected by BRs is the longitudinal growth of young tissue via cell elongation and possibly cell division. Consequently, disruptions in to BR metabolism, perception and activity frequently result in a dwarf phenotype. In addition, because BRs are derived from the sterol metabolic pathway, any perturbations to the sterol pathway can affect the BR pathway. In the same way, perturbations in the BR pathway can have effects on the later part of the sterol pathway and thus the sterol composition of membranes.



**[0580]** Changes in BR concentration in the surrounding environment or in contact with a plant result in modulation of many genes and gene products. These genes and/or products are responsible for effects on traits such as plant biomass and seed yield. These genes were discovered and characterized from a much larger set of genes by experiments designed to find genes whose mRNA abundance changed in response to application of BRs to plants.

**[0581]** While BR responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different BR responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factors and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants. In addition, the combination of a BR responsive polynucleotide and/or gene product with another environmentally responsive polynucleotide is useful because of the interactions that exist between hormone-regulated pathways, stress pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common or overlapping pathways.

**[0582]** Differential Expression of the Sequences in Epi-Brassinolide or Brassinazole Plants

**[0583]** The relative levels of mRNA product in plants treated with either epi-brassinolide or brassinazole were measured.

Metabolism Affecting Genes, Gene Components and Products

Nitrogen Responsive Genes, Gene Components and Products

**[0584]** Nitrogen is often the rate-limiting element in plant growth, and all field crops have a fundamental dependence on exogenous nitrogen sources. Nitrogenous fertilizer, which is usually supplied as ammonium nitrate, potassium nitrate, or urea, typically accounts for 40% of the costs associated with crops, such as corn and wheat in intensive agriculture. Increased efficiency of nitrogen use by plants should enable the production of higher yields with existing fertilizer inputs and/or enable existing yields of crops to be obtained with lower fertilizer input, or better yields on soils of poorer quality. Also, higher amounts of proteins in the crops could also be produced more cost-effectively. "Nitrogen responsive" genes and gene products can be used to alter or modulate plant growth and development.

**[0585]** Differential Expression of the Sequences in Whole Seedlings, Shoots and Roots

**[0586]** The relative levels of mRNA product in whole seedlings, shoots and roots treated with either high or low nitrogen media were compared to controls.

Viability Genes, Gene Components and Products

**[0587]** Plants contain many proteins and pathways that when blocked or induced lead to cell, organ or whole plant death. Gene variants that influence these pathways can have profound effects on plant survival, vigor and performance.

The critical pathways include those concerned with metabolism and development or protection against stresses, diseases and pests. They also include those involved in apoptosis and necrosis. Viability genes can be modulated to affect cell or plant death.

Herbicides are, by definition, chemicals that cause death of tissues, organs and whole plants. The genes and pathways that are activated or inactivated by herbicides include those that cause cell death as well as those that function to provide protection.

**[0588]** Differential Expression of the Sequences in Herbicide Treated Plants and Herbicide Resistant Mutants

**[0589]** The relative levels of mRNA product in plants treated with herbicide and mutants resistant to herbicides were compared to control plants.

Stress Responsive Genes, Gene Components and Products

Wounding Responsive Genes, Gene Components and Products

**[0590]** Plants are continuously subjected to various forms of wounding from physical attacks to including the damage created by pathogens and pests, wind, and contact with other objects. Therefore, survival and agricultural yields depend on constraining the damage created by the wounding process and inducing defense mechanisms against future damage.

**[0591]** Plants have evolved complex systems to minimize and/or repair local damage and to minimize subsequent attacks by pathogens or pests or their effects. These involve stimulation of cell division and cell elongation to repair tissues, induction of programmed cell death to isolate the damage caused mechanically and by invading pests and pathogens, and induction of long-range signaling systems to induce protecting molecules, in case of future attack. The genetic and biochemical systems associated with responses to wounding are connected with those associated with other stresses such as pathogen attack and drought.

**[0592]** Wounding responsive genes and gene products can be used to alter or modulate traits such as growth rate; whole plant height, width, or flowering time; organ development (such as coleoptile elongation, young leaves, roots, lateral roots, tuber formation, flowers, fruit, and seeds); biomass; fresh and dry weight during any time in plant life, such as at maturation; number of flowers; number of seeds; seed yield, number, size, weight, harvest index (such as content and composition, e.g., amino acid, nitrogen, oil, protein, and carbohydrate); fruit yield, number, size, weight, harvest index, post harvest quality, content and composition (e.g., amino acid, carotenoid, jasmonate, protein, and starch); seed and fruit development; germination of dormant and non-dormant seeds; seed viability, seed reserve mobilization, fruit ripening, initiation of the reproductive cycle from a vegetative state, flower development time, insect attraction for fertilization, time to fruit maturity, senescence; fruits, fruit drop; leaves; stress and disease responses; drought; heat and cold; wounding by any source, including wind, objects, pests and pathogens; uv and high light damage (insect, fungus, virus, worm, nematode damage).

Cold Responsive Genes, Gene Components and Products

**[0593]** The ability to endure low temperatures and freezing is a major determinant of the geographical distribution and productivity of agricultural crops. Even in areas considered suitable for the cultivation of a given species or cultivar, can

give rise to yield decreases and crop failures as a result of aberrant, freezing temperatures. Even modest increases (1-2° C.) in the freezing tolerance of certain crop species would have a dramatic impact on agricultural productivity in some areas. The development of genotypes with increased freezing tolerance would provide a more reliable means to minimize crop losses and diminish the use of energy-costly practices to modify the microclimate.

**[0594]** Sudden cold temperatures result in modulation of many genes and gene products, including promoters. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield.

**[0595]** Manipulation of one or more cold responsive gene activities is useful to modulate growth and development.

**[0596]** Differential Expression of the Sequences in Cold Treated Plants

**[0597]** The relative levels of mRNA product in cold treated plants were compared to control plants.

#### Heat Responsive Genes, Gene Components and Products

**[0598]** The ability to endure high temperatures is a major determinant of the geographical distribution and productivity of agricultural crops. Decreases in yield and crop failure frequently occur as a result of aberrant, hot conditions even in areas considered suitable for the cultivation of a given species or cultivar. Only modest increases in the heat tolerance of crop species would have a dramatic impact on agricultural productivity. The development of genotypes with increased heat tolerance would provide a more reliable means to minimize crop losses and diminish the use of energy-costly practices to modify the microclimate.

**[0599]** Changes in temperature in the surrounding environment or in a plant microclimate results in modulation of many genes and gene products.

**[0600]** Differential Expression of the Sequences in Heat Treated Plants

**[0601]** The relative levels of mRNA product in heat treated plants were compared to control plants.

#### Drought Responsive Genes, Gene Components and Products

**[0602]** The ability to endure drought conditions is a major determinant of the geographical distribution and productivity of agricultural crops. Decreases in yield and crop failure frequently occur as a result of aberrant, drought conditions even in areas considered suitable for the cultivation of a given species or cultivar. Only modest increases in the drought to tolerance of crop species would have a dramatic impact on agricultural productivity. The development of genotypes with increased drought tolerance would provide a more reliable means to minimize crop losses and diminish the use of energy-costly practices to modify the microclimate.

**[0603]** Drought conditions in the surrounding environment or within a plant, results in modulation of many genes and gene products.

**[0604]** Differential Expression of the Sequences in Drought Treated Plants and Drought Mutants

**[0605]** The relative levels of mRNA product in drought treated plants and drought mutants were compared to control plants.

#### Methyl Jasmonate (Jasmonate) Responsive Genes, Gene Components and Products

**[0606]** Jasmonic acid and its derivatives, collectively referred to as jasmonates, are naturally occurring derivatives

of plant lipids. These substances are synthesized from linolenic acid in a lipoxygenase-dependent biosynthetic pathway. Jasmonates are signalling molecules which have been shown to be growth regulators as well as regulators of defense and stress responses. As such, jasmonates represent a separate class of plant hormones. Jasmonate responsive genes can be used to modulate plant growth and development.

#### Differential Expression of the Sequences in Methyl Jasmonate Treated Plants

**[0607]** The relative levels of mRNA product in methyl jasmonate treated plants were compared to control plants.

#### Salicylic Acid Responsive Genes, Gene Components and Products

**[0608]** Plant defense responses can be divided into two groups: constitutive and induced. Salicylic acid (SA) is a signaling molecule necessary for activation of the plant induced defense system known as systemic acquired resistance or SAR. This response, which is triggered by prior exposure to avirulent pathogens, is long lasting and provides protection against a broad spectrum of pathogens. Another induced defense system is the hypersensitive response (HR). HR is far more rapid, occurs at the sites of pathogen (avirulent pathogens) entry and precedes SAR. SA is also the key signaling molecule for this defense pathway.

**[0609]** Differential Expression of the Sequences in Salicylic Acid Treated Plants

**[0610]** The relative levels of mRNA product in salicylic acid treated plants were compared to control plants.

#### Osmotic Stress Responsive Genes, Gene Components and Products

**[0611]** The ability to endure and recover from osmotic and salt related stress is a major determinant of the geographical distribution and productivity of agricultural crops. Osmotic stress is a major component of stress imposed by saline soil and water deficit. Decreases in yield and crop failure frequently occur as a result of aberrant or transient environmental stress conditions even in areas considered suitable for the cultivation of a given species or cultivar. Only modest increases in the osmotic and salt tolerance of a crop species would have a dramatic impact on agricultural productivity. The development of genotypes with increased osmotic tolerance would provide a more reliable means to minimize crop losses and diminish the use of energy-costly practices to modify the soil environment. Thus, osmotic stress responsive genes can be used to modulate plant growth and development.

**[0612]** Differential Expression of the Sequences in PEG Treated Plants

**[0613]** The relative levels of mRNA product in PEG treated plants were compared to control plants.

#### Shade Responsive Genes, Gene Components and Products

**[0614]** Plants sense the ratio of Red (R): Far Red (FR) light in their environment and respond differently to particular ratios. A low R:FR ratio, for example, enhances cell elongation and favors flowering over leaf production. The changes in R:FR ratios mimic and cause the shading response effects in plants. The response of a plant to shade in the canopy structures of agricultural crop fields influences crop yields significantly. Therefore manipulation of genes regulating the shade avoidance responses can improve crop yields. While phyto-

chromes mediate the shade avoidance response, the downstream factors participating in this pathway are largely unknown. One potential downstream participant, ATHB-2, is a member of the HD-Zip class of transcription factors and shows a strong and rapid response to changes in the R:FR ratio. ATHB-2 overexpressors have a thinner root mass, smaller and fewer leaves and longer hypocotyls and petioles. This elongation arises from longer to epidermal and cortical cells, and a decrease in secondary vascular tissues, paralleling the changes observed in wild-type seedlings grown under conditions simulating canopy shade. On the other hand, plants with reduced ATHB-2 expression have a thick root mass and many larger leaves and shorter hypocotyls and petioles. Here, the changes in the hypocotyl result from shorter epidermal and cortical cells and increased proliferation of vascular tissue. Interestingly, application of Auxin is able to reverse the root phenotypic consequences of high ATHB-2 levels, restoring the wild-type phenotype. Consequently, given that ATHB-2 is tightly regulated by phytochrome, these data suggest that ATHB-2 may link the Auxin and phytochrome pathways in the shade avoidance response pathway.

**[0615]** Shade responsive genes can be used to modulate plant growth and development.

**[0616]** Differential Expression of the Sequences in Far-Red Light Treated Plants

**[0617]** The relative levels of mRNA product in far-red light treated plants were compared to control plants.

#### Viability Genes, Gene Components and Products

**[0618]** Plants contain many proteins and pathways that when blocked or induced lead to cell, organ or whole plant death. Gene variants that influence these pathways can have profound effects on plant survival, vigor and performance. The critical pathways include those concerned with metabolism and development or protection against stresses, diseases and pests. They also include those involved in apoptosis and necrosis. The applicants have elucidated many such genes and pathways by discovering genes that when inactivated lead to cell or plant death.

**[0619]** Herbicides are, by definition, chemicals that cause death of tissues, organs and whole plants. The genes and pathways that are activated or inactivated by herbicides include those that cause cell death as well as those that function to provide protection. The applicants have elucidated these genes.

**[0620]** The genes defined in this section have many uses including manipulating which cells, tissues and organs are selectively killed, which are protected, making plants resistant to herbicides, discovering new herbicides and making plants resistant to various stresses.

**[0621]** Viability genes were also identified from a much larger set of genes by experiments designed to find genes whose mRNA products changed in concentration in response to applications of different herbicides to plants. Viability genes are characteristically differentially transcribed in response to fluctuating herbicide levels or concentrations, whether internal or external to an organism or cell. The MA\_diff Table reports the changes in transcript levels of various viability genes.

#### Early Seedling-Phase Specific Responsive Genes, Gene Components and Products

**[0622]** One of the more active stages of the plant life cycle is a few days after germination is complete, also referred to as

the early seedling phase. During this period the plant begins development and growth of the first leaves, roots, and other organs not found in the embryo. Generally this stage begins when germination ends. The first sign that germination has been completed is usually that there is an increase in length and fresh weight of the radicle. Such genes and gene products can regulate a number of plant traits to modulate yield. For example, these genes are active or potentially active to a greater extent in developing and rapidly growing cells, tissues and organs, as exemplified by development and growth of a seedling 3 or 4 days after planting a seed.

**[0623]** Rapid, efficient establishment of a seedling is very important in commercial agriculture and horticulture. It is also vital that resources are approximately partitioned between shoot and root to facilitate adaptive growth. Phototropism and geotropism need to be established. All these require post-germination process to be sustained to ensure that vigorous seedlings are produced. Early seedling phase genes, gene components and products are useful to manipulate these and other processes.

#### Guard Cell Genes, Gene Components and Products

**[0624]** Scattered throughout the epidermis of the shoot are minute pores called stomata. Each stomatal pore is surrounded by two guard cells. The guard cells control the size of the stomatal pore, which is critical since the stomata control the exchange of carbon dioxide, oxygen, and water vapor between the interior of the plant and the outside atmosphere. Stomata open and close through turgor changes driven by ion fluxes, which occur mainly through the guard cell plasma membrane and tonoplast. Guard cells are known to respond to a number of external stimuli such as changes in light intensity, carbon dioxide and water vapor, for example. Guard cells can also sense and rapidly respond to internal stimuli including changes in ABA, to auxin and calcium ion flux.

**[0625]** Thus, genes, gene products, and fragments thereof differentially transcribed and/or translated in guard cells can be useful to modulate ABA responses, drought tolerance, respiration, water potential, and water management as examples. All of which can in turn affect plant yield including seed yield, harvest index, fruit yield, etc.

To identify such guard cell genes, gene products, and fragments thereof, Applicants have performed a microarray experiment comparing the transcript levels of genes in guard cells versus leaves. Experimental data is shown below.

#### Nitric Oxide Responsive Genes, Gene Components and Products

**[0626]** The rate-limiting element in plant growth and yield is often its ability to tolerate suboptimal or stress conditions, including pathogen attack conditions, wounding and the presence of various other factors. To combat such conditions, plant cells deploy a battery of inducible defense responses, including synergistic interactions between nitric oxide (NO), reactive oxygen intermediates (ROS), and salicylic acid (SA). NO has been shown to play a critical role in the activation of innate immune and inflammatory responses in animals. At least part of this mammalian signaling pathway is present in plants, where NO is known to potentiate the hypersensitive response (HR). In addition, NO is a stimulator molecule in plant photomorphogenesis.

[0627] Changes in nitric oxide concentration in the internal or surrounding environment, or in contact with a plant, results in modulation of many genes and gene products.

[0628] In addition, the combination of a nitric oxide responsive polynucleotide and/or gene product with other environmentally responsive polynucleotides is also useful because of the interactions that exist between hormone regulated pathways, stress pathways, pathogen stimulated pathways, nutritional pathways and development.

[0629] Nitric oxide responsive genes and gene products can function either to increase or dampen the above phenotypes or activities either in response to changes in nitric oxide concentration or in the absence of nitric oxide fluctuations. More specifically, these genes and gene products can modulate stress responses in an organism. In plants, these genes and gene products are useful for modulating yield under stress conditions. Measurements of yield include seed yield, seed size, fruit yield, fruit size, etc.

#### Shoot-Apical Meristem Genes, Gene Components and Products

[0630] New organs, stems, leaves, branches and inflorescences develop from the stem apical meristem (SAM). The growth structure and architecture of the plant therefore depends on the behavior of SAMs. Shoot apical meristems (SAMs) are comprised of a number of morphologically undifferentiated, dividing cells located at the tips of shoots. SAM genes elucidated here are capable of modifying the activity of SAMs and thereby many traits of economic interest from ornamental leaf shape to organ number to responses to plant density.

[0631] In addition, a key attribute of the SAM is its capacity for self-renewal. Thus, SAM genes of the instant invention are useful for modulating one or more processes of SAM structure and/or function including (I) cell size and division; (II) cell differentiation and organ primordia. The genes and gene components of this invention are useful for modulating any one or all of these cell division processes generally, as in timing and rate, for example. In addition, the polynucleotides and polypeptides of the invention can control the response of these processes to the internal plant programs associated with embryogenesis, and hormone responses, for example.

[0632] Because SAMs determine the architecture of the plant, modified plants will be useful in many agricultural, horticultural, forestry and other industrial sectors. Plants with a different shape, numbers of flowers and seed and fruits will have altered yields of plant parts. For example, plants with more branches can produce more flowers, seed or fruits. Trees without lateral branches will produce long lengths of clean timber. Plants with greater yields of specific plant parts will be useful sources of constituent chemicals.

#### GFP Experimental Procedures and Results

##### Procedures

[0633] The polynucleotide sequences of the present invention were tested for promoter activity using Green Fluorescent Protein (GFP) assays in the following manner

[0634] Approximately 1-2 kb of genomic sequence occurring immediately upstream of the ATG translational start site of the gene of interest was isolated using appropriate primers tailed with BstXI restriction sites. Standard PCR reactions using these primers and genomic DNA were conducted. The resulting product was isolated, cleaved with BstXI and cloned

into the BstXI site of an appropriate vector, such as pNew-Bin4-HAP1-GFP (see FIG. 1).

#### [0635] Transformation

[0636] The following procedure was used for transformation of plants

##### 1. Stratification of WS-2 Seed.

[0637] Add 0.5 ml WS-2 (CS2360) seed to 50 ml of 0.2% Phytagar in a 50 ml Corning tube and vortex until seeds and Phytagar form a homogenous mixture.

[0638] Cover tube with foil and stratify at 4° C. for 3 days.

##### 2. Preparation of Seed Mixture.

[0639] Obtain stratified seed from cooler.

[0640] Add seed mixture to a 1000 ml beaker.

[0641] Add an additional 950 ml of 0.2% Phytagar and mix to homogenize.

##### 3. Preparation of Soil Mixture.

[0642] Mix 24 L SunshineMix #5 soil with 16 L Thermo-Rock vermiculite in cement mixer to make a 60:40 soil mixture.

[0643] Amend soil mixture by adding 2 Tbsp Marathon and 3 Tbsp Osmocote and mix contents thoroughly.

[0644] Add 1 Tbsp Peters fertilizer to 3 gallons of water and add to soil mixture and mix thoroughly.

[0645] Fill 4-inch pots with soil mixture and round the surface to create a slight dome.

[0646] Cover pots with 8-inch squares of nylon netting and fasten using rubber bands.

[0647] Place 14 4-inch pots into each no-hole utility flat.

##### 4. Planting.

[0648] Using a 60 ml syringe, aspirate 35 ml of the seed mixture.

[0649] Exude 25 drops of the seed mixture onto each pot.

[0650] Repeat until all pots have been seeded.

[0651] Place flats on greenhouse bench, cover flat with clear propagation domes, place 55% shade cloth on top of flats and subirrigate by adding 1 inch of water to bottom of each flat.

##### 5. Plant Maintenance.

[0652] 3 to 4 days after planting, remove clear lids and shade cloth.

[0653] Subirrigate flats with water as needed.

[0654] After 7-10 days, thin pots to 20 plants per pot using forceps.

[0655] After 2 weeks, subirrigate all plants with Peters fertilizer at a rate of 1 Tsp per gallon water.

[0656] When bolts are about 5-10 cm long, clip them between the first node and the base of stem to induce secondary bolts.

[0657] 6 to 7 days after clipping, perform dipping infiltration.

##### 6. Preparation of *Agrobacterium*.

[0658] Add 150 ml fresh YEB to 250 ml centrifuge bottles and cap each with a foam plug (Identi-Plug).

- [0659] Autoclave for 40 min at 121° C.
- [0660] After cooling to room temperature, uncap and add 0.1 ml each of carbenicillin, spectinomycin and rifampicin stock solutions to each culture vessel.
- [0661] Obtain *Agrobacterium* starter block (96-well block with *Agrobacterium* cultures grown to an OD<sub>600</sub> of approximately 1.0) and inoculate one culture vessel per construct by transferring 1 ml from appropriate well in the starter block.
- [0662] Cap culture vessels and place on Lab-Line incubator shaker set at 27° C. and 250 RPM.
- [0663] Remove after *Agrobacterium* cultures reach an OD<sub>600</sub> of approximately 1.0 (about 24 hours), cap culture vessels with plastic caps, place in Sorvall SLA 1500 rotor and centrifuge at 8000 RPM for 8 min at 4° C.
- [0664] Pour out supernatant and put bottles on ice until ready to use.
- [0665] Add 200 ml Infiltration Media (IM) to each bottle, resuspend *Agrobacterium* pellets and store on ice.

#### 7. Dipping Infiltration.

- [0666] Pour resuspended *Agrobacterium* into 16 oz polypropylene containers.
- [0667] Invert 4-inch pots and submerge the aerial portion of the plants into the *Agrobacterium* suspension and let stand for 5 min.
- [0668] Pour out *Agrobacterium* suspension into waste bucket while keeping polypropylene container in place and return the plants to the upright position.
- [0669] Place 10 covered pots per flat.
- [0670] Fill each flat with 1-inch of water and cover with shade cloth.
- [0671] Keep covered for 24 hr and then remove shade cloth and polypropylene containers.
- [0672] Resume normal plant maintenance.
- [0673] When plants have finished flowering cover each pot with a ciber plant sleeve.
- [0674] After plants are completely dry, collect seed and place into 2.0 ml micro tubes and store in 100-place cryogenic boxes.

#### Recipes:

##### 0.2% Phytagar

- [0675] 2 g Phytagar
- [0676] 1 L nanopure water
- [0677] Shake until Phytagar suspended
- [0678] Autoclave 20 min

##### YEB (for 1 L)

- [0679] 5 g extract of meat
- [0680] 5 g Bacto peptone
- [0681] 1 g yeast extract
- [0682] 5 g sucrose
- [0683] 0.24 g magnesium sulfate
- [0684] While stirring, add ingredients, in order, to 900 ml nanopure water
- [0685] When dissolved, adjust pH to 7.2
- [0686] Fill to 1 L with nanopure water
- [0687] Autoclave 35 min

##### Infiltration Medium (IM) (for 1 L)

- [0688] 2.2 g MS salts
- [0689] 50 g sucrose

- [0690] 5 ul BAP solution (stock is 2 mg/ml)
- [0691] While stirring, add ingredients in order listed to 900 ml nanopure water
- [0692] When dissolved, adjust pH to 5.8.
- [0693] Volume up to 1 L with nanopure water.
- [0694] Add 0.02% Silwet L-77 just prior to resuspending *Agrobacterium*
- [0695] High Throughput Screening-T1 Generation
  1. Soil Preparation. Wear Gloves at all Times.
    - [0696] In a large container, mix 60% autoclaved SunshineMix #5 with 40% vermiculite.
    - [0697] Add 2.5 Tbsp of Osmocote, and 2.5 Tbsp of 1% granular Marathon per 25 L of soil.
    - [0698] Mix thoroughly.
  2. Fill Com-Packs with Soil.
    - [0699] Loosely fill D601 Com-Packs level to the rim with the prepared soil.
    - [0700] Place filled pot into utility flat with holes, within a no-hole utility flat.
    - [0701] Repeat as necessary for planting. One flat set should contain 6 pots.

#### 3. Saturate Soil.

- [0702] Evenly water all pots until the soil is saturated and water is collecting in the bottom of the flats.
- [0703] After the soil is completely saturated, dump out the excess water.

#### 4. Plant the Seed.

#### 5. Stratify the Seeds.

- [0704] After sowing the seed for all the flats, place them into a dark 4° C. cooler.
- [0705] Keep the flats in the cooler for 2 nights for WS seed. Other ecotypes may take longer. This cold treatment will help promote uniform germination of the seed.
- 6. Remove Flats from Cooler and Cover with Shade Cloth. (Shade Cloth is Only Needed in the Greenhouse)
  - [0706] After the appropriate time, remove the flats from the cooler and place onto growth racks or benches.
  - [0707] Cover the entire set of flats with 55% shade cloth. The cloth is necessary to cut down the light intensity during the delicate germination period.
  - [0708] The cloth and domes should remain on the flats until the cotyledons have fully expanded. This usually takes about 4-5 days under standard greenhouse conditions.

#### 7. Remove 55% Shade Cloth and Propagation Domes.

- [0709] After the cotyledons have fully expanded, remove both the 55% shade cloth and propagation domes.

#### 8. Spray Plants with Finale Mixture. Wear Gloves and Protective Clothing at all Times.

- [0710] Prepare working Finale mixture by mixing 3 ml concentrated Finale in 48 oz of water in to the Poly-TEK sprayer.
- [0711] Completely and evenly spray plants with a fine mist of the Finale mixture.
- [0712] Repeat Finale spraying every 3-4 days until only transformants remain. (Approximately 3 applications are necessary.)
- [0713] When satisfied that only transformants remain, discontinue Finale spraying.

## 9. Weed Out Excess Transformants.

**[0714]** Weed out excess transformants such that a maximum number of five plants per pot exist evenly spaced throughout the pot.

**[0715]** GFP Assay

**[0716]** Tissues are dissected by eye or under magnification using INOX 5 grade forceps and placed on a slide with water and coverslipped. An attempt is made to record images of observed expression patterns at earliest and latest stages of development of tissues listed below. Specific tissues will be preceded with High (H), Medium (M), Low (L) designations.

Flower	pedicel, receptacle, nectary, sepal, petal, filament, anther, pollen, carpel, style, papillae, vascular, epidermis, stomata, trichome
Silique	stigma, style, carpel, septum, placenta, transmitting tissue, vascular, epidermis, stomata, abscission zone, ovule
Ovule	Pre-fertilization: inner integument, outer integument, embryo sac, funiculus, chalaza, micropyle, gametophyte Post-fertilization: zygote, inner integument, outer integument, seed coat, primordia, chalaza, micropyle, early endosperm, mature endosperm, embryo
Embryo	suspensor, preglobular, globular, heart, torpedo, late, mature, provascular, hypophysis, radicle, cotyledons, hypocotyl
Stem	epidermis, cortex, vascular, xylem, phloem, pith, stomata, trichome
Leaf	petiole, mesophyll, vascular, epidermis, trichome, primordia, stomata, stipule, margin

**[0717]** T1 Mature: These are the T1 plants resulting from independent transformation events. These are screened between stage 6.50-6.90 (means the plant is flowering and that 50-90% of the flowers that the plant will make have developed) which is 4-6 weeks of age. At this stage the mature plant possesses flowers, siliques at all stages of development, and fully expanded leaves. We do not generally differentiate between 6.50 and 6.90 in the report but rather just indicate 6.50. The plants are initially imaged under UV with a Leica Confocal microscope. This allows examination of the plants on a global level. If expression is present, they are imaged using scanning laser confocal microscopy.

**[0718]** T2 Seedling: Progeny are collected from the T1 plants giving the same expression to pattern and the progeny (T2) are sterilized and plated on agar-solidified medium containing M&S salts. In the event that there was no expression in the T1 plants, T2 seeds are planted from all lines. The seedlings are grown in Percival incubators under continuous light at 22° C. for 10-12 days. Cotyledons, roots, hypocotyls, petioles, leaves, and the shoot meristem region of individual seedlings were screened until two seedlings were observed to have the same pattern. Generally found the same expression pattern was found in the first two seedlings. However, up to 6 seedlings were screened before “no expression pattern” was recorded. All constructs are screened as T2 seedlings even if they did not have an expression pattern in the T1 generation.

**[0719]** T2 Mature: The T2 mature plants were screened in a similar manner to the T1 plants. The T2 seeds were planted in the greenhouse, exposed to selection and at least one plant screened to confirm the T1 expression pattern. In instances

where there were any subtle changes in expression, multiple plants were examined and the changes noted in the tables.

**[0720]** T3 Seedling: This was done similar to the T2 seedlings except that only the plants for which we are trying to confirm the pattern are planted.

## Image Data:

**[0721]** Images are collected by scanning laser confocal microscopy. Scanned images are taken as 2-D optical sections or 3-D images generated by stacking the 2-D optical sections collected in series. All scanned images are saved as TIFF files by imaging software, edited in Adobe Photoshop, and labeled in Powerpoint specifying organ and specific expressing tissues.

## Instrumentation:

## Microscope

## Inverted Leica DM IRB

**[0722]** Fluorescence filter blocks:

Blue excitation BP 450-490; long pass emission LP 515.

Green excitation BP 515-560; long pass emission LP 590

## Objectives

HC PL FLUOTAR 5×/0.5

**[0723]** HCPL APO 10×/0.4 IMM water/glycerol/oil

HCPL APO 20×/0.7 IMM water/glycerol/oil

HCXL APO 63×/1.2 IMM water/glycerol/oil

## Leica TCS SP2 Confocal Scanner

**[0724]** Spectral range of detector optics 400-850 nm.

Variable computer controlled pinhole diameter.

Optical zoom 1-32×.

## Four Simultaneous Detectors:

**[0725]** Three channels for collection of fluorescence or reflected light.

One channel for transmitted light detector.

## Laser Sources:

**[0726]** Blue Ar 458/5 mW, 476 nm/5 mW, 488 nm/20 mW, 514 nm/20 mW.

Green HeNe 543 nm/1.2 mW

Red HeNe 633 nm/10 mW

## Results

**[0727]** The section in Table 1 entitled “The spatial expression of the promoter-marker-vector” presents the results of the GFP assays as reported by their corresponding cDNA ID number, construct number and line number. Unlike the microarray results, which measure the difference in expression of the endogenous cDNA under various conditions, the GFP data gives the location of expression that is visible under the imaging parameters. Table 3 summarizes the results of the spatial expression results for each promoter.

## Explanation of Table 1

**[0728]** Table 1 includes various information about each promoter or promoter control element of the invention including the nucleotide sequence, the spatial expression promoted by each promoter, and the corresponding results from different expression experiments.

<hr/> <p style="text-align: center;">Lengthy table referenced here</p> <p style="text-align: center;">US20110041219A1-20110217-T00001</p> <p>Please refer to the end of the specification for access instructions.</p> <hr/>	<hr/> <p style="text-align: center;">Lengthy table referenced here</p> <p style="text-align: center;">US20110041219A1-20110217-T00003</p> <p>Please refer to the end of the specification for access instructions.</p> <hr/>
<hr/> <p style="text-align: center;">Lengthy table referenced here</p> <p style="text-align: center;">US20110041219A1-20110217-T00002</p> <p>Please refer to the end of the specification for access instructions.</p> <hr/>	<hr/> <p><b>[0729]</b> The invention being thus described, it will be apparent to one of ordinary skill in the art that various modifications of the materials and methods for practicing the invention can be made. Such modifications are to be considered within the scope of the invention as defined by the following claims.</p> <p><b>[0730]</b> Each of the references from the patent and periodical literature cited herein is hereby expressly incorporated in its entirety by such citation.</p> <hr/>

#### LENGTHY TABLES

The patent application contains a lengthy table section. A copy of the table is available in electronic form from the USPTO web site (<http://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US20110041219A1>). An electronic copy of the table will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

#### SEQUENCE LISTING

The patent application contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (<http://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US20110041219A1>). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

What is claimed is:

1. An isolated nucleic acid molecule capable of modulating transcription wherein the nucleic acid molecule shows at least 80% sequence identity to one of the promoter sequences in Table 1, or a complement thereof.

2. The isolated nucleic acid molecule of claim 1, wherein said nucleic acid is capable of functioning as a promoter.

3. The isolated nucleic acid molecule of claim 2, wherein said nucleic acid comprises a reduced promoter nucleotide sequence having a sequence consisting of one of the promoter sequences in Table 1 having at least one of the corresponding optional promoter fragments identified in Table 1.

4. The isolated nucleic acid molecule of claim 2, wherein said nucleic acid comprises a reduced promoter nucleotide sequence having a sequence consisting of one of the promoter sequences in Table 1 having all of the corresponding optional promoter fragments identified in Table 1.

5. The isolated nucleic acid molecule of claim 1, wherein said nucleic acid molecule is capable of modulating transcription during the developmental times, or in response to a stimuli, or in a cell, tissue, or organ as set forth in Table 1 in the section "The spatial expression of the promoter-marker-vector".

6. A vector construct comprising:

a) a first nucleic acid capable of modulating transcription wherein the nucleic acid molecule shows at least 80% sequence identity to one of the promoter sequences in Table 1; and

b) a second nucleic acid having to be transcribed, wherein said first and second nucleic acid molecules are heterologous to each other and are operably linked together.

7. The vector construct according to claim 6, wherein said nucleic acid comprises a reduced promoter nucleotide sequence having a sequence consisting of one of the promoter sequences in Table 1 having at least one of the corresponding optional promoter fragments identified in Table 1 deleted therefrom.

8. The vector construct according to claim 6, wherein said nucleic acid comprises a reduced promoter nucleotide sequence having a sequence consisting of one of the promoter sequences in Table 1 having all of the corresponding optional promoter fragments identified in Table 1 deleted therefrom.

9. A host cell comprising an isolated nucleic acid molecule according to claim 1, wherein said nucleic acid molecule is flanked by exogenous sequence.

10. The host cell according to claim 9, wherein said nucleic acid comprises a reduced promoter nucleotide sequence having a sequence consisting of one of the promoter sequences in

Table 1 having at least one of the corresponding optional promoter fragments identified in Table 1 deleted therefrom.

**11.** The host cell according to claim **9**, wherein said nucleic acid comprises a reduced promoter nucleotide sequence having a sequence consisting of one of the promoter sequences in Table 1 having all of the corresponding optional promoter fragments identified in Table 1 deleted therefrom.

**12.** A host cell comprising a vector construct of claim **6**.

**13.** A method of modulating transcription by combining, in an environment suitable for transcription:

a) a first nucleic acid molecule capable of modulating transcription wherein the nucleic acid molecule shows at least 80% sequence identity to one of the promoter sequences in Table 1; and

b) a second molecule to be transcribed;

wherein the first and second nucleic acid molecules are heterologous to each other and operably linked together.

**14.** The method of claim **13**, wherein said nucleic acid comprises a reduced promoter nucleotide sequence having a

sequence consisting of one of the promoter sequences in Table 1 having at least one of the corresponding optional promoter fragments identified in Table 1 deleted therefrom.

**15.** The method of claim **13**, wherein said nucleic acid comprises a reduced promoter nucleotide sequence having a sequence consisting of one of the promoter sequences in Table 1 having all of the corresponding optional promoter fragments identified in Table 1 deleted therefrom.

**16.** The method according to any one of claims **13-15**, wherein said first nucleic acid molecule is capable of modulating transcription during the developmental times, or in response to a stimuli, or in a cell tissue, or organ as set forth in Table 1 in the section entitled "The spatial expression of the promoter-marker-vector" wherein said first nucleic acid molecule is inserted into a plant cell and said plant cell is regenerated into a plant.

**17.** A plant comprising a vector construct according to claim **6**.

\* \* \* \* \*