



(86) Date de dépôt PCT/PCT Filing Date: 2004/02/12

(87) Date publication PCT/PCT Publication Date: 2004/08/26

(45) Date de délivrance/Issue Date: 2013/04/30

(85) Entrée phase nationale/National Entry: 2005/07/05

(86) N° demande PCT/PCT Application No.: US 2004/005757

(87) N° publication PCT/PCT Publication No.: 2004/071178

(30) Priorité/Priority: 2003/02/12 (US60/446,833)

(51) Cl.Int./Int.Cl. *C12N 15/82* (2006.01),
A01H 5/00 (2006.01)

(72) Inventeurs/Inventors:
KINNEY, ANTHONY J., US;
LIU, ZHAN-BIN, US

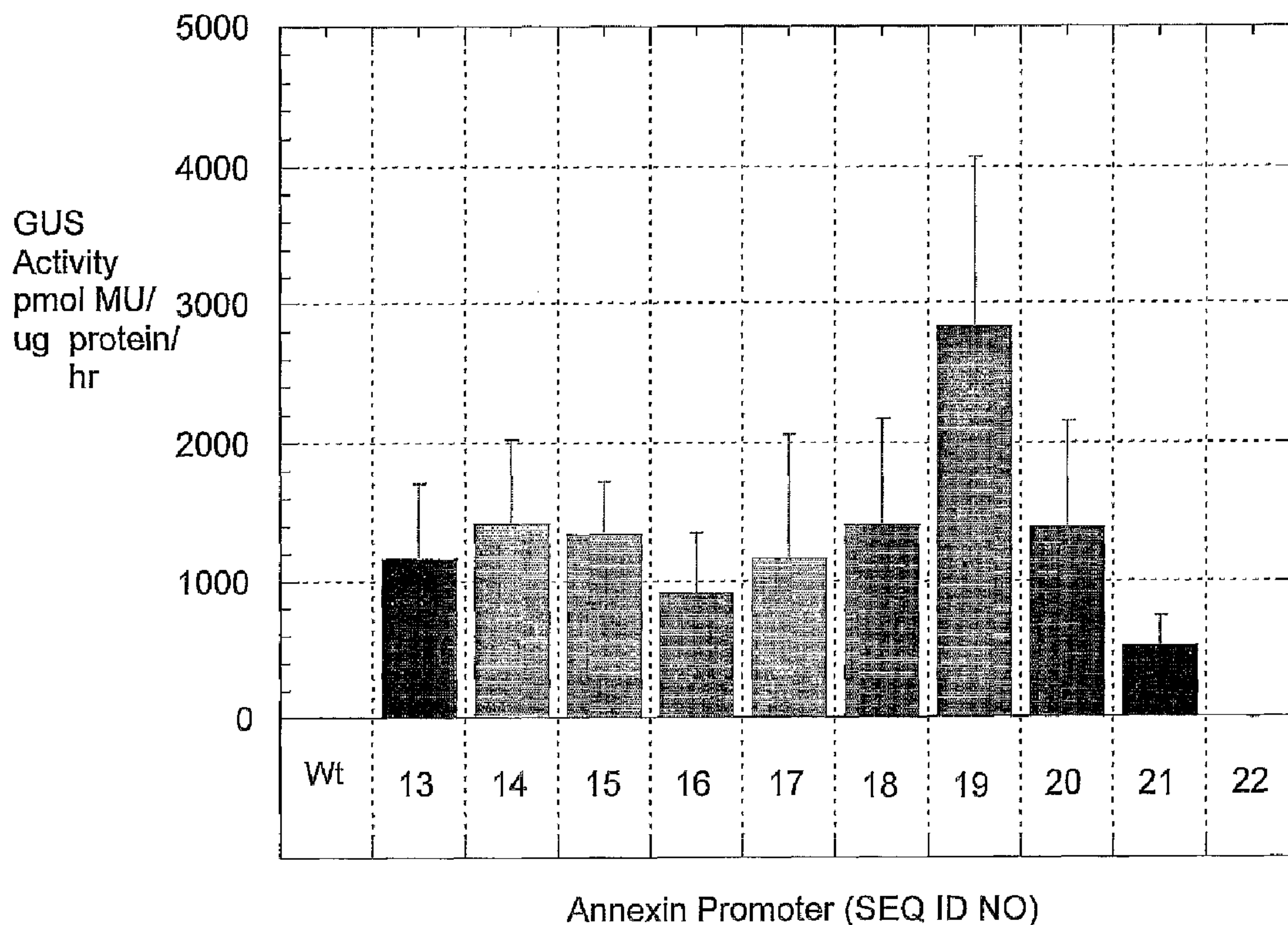
(73) Propriétaire/Owner:
E. I. DU PONT DE NEMOURS AND COMPANY, US

(74) Agent: TORYS LLP

(54) Titre : PROMOTEUR DE L'ANNEXINE ET SON UTILISATION DANS L'EXPRESSION DE GENES
TRANSGENIQUES DANS DES PLANTES

(54) Title: ANNEXIN PROMOTER AND ITS USE IN EXPRESSION OF TRANSGENIC GENES IN PLANTS

Soybean Annexin Promoter Deletion



(57) Abrégé/Abstract:

A seed specific plant annexin and P34 promoters and subfragments thereof and their use in promoting the expression of one or more heterologous nucleic acid fragments in plants are described.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
26 August 2004 (26.08.2004)

PCT

(10) International Publication Number
WO 2004/071178 A3

(51) International Patent Classification⁷: C12N 15/82,
A01H 5/00

(21) International Application Number:
PCT/US2004/005757

(22) International Filing Date: 12 February 2004 (12.02.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/446,833 12 February 2003 (12.02.2003) US

(71) Applicant (for all designated States except US): **E. I. DU PONT DE NEMOURS AND COMPANY** [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **KINNEY, Anthony, J.** [US/US]; 609 Lore Avenue, Wilmington, DE 19809 (US). **LIU, Zhan-Bin** [US/US]; 1357 Wooded Knoll, West Chester, PA 19382 (US).

(74) Agent: **CHRISTENBURY, Lynne, M.**; E.I. Du Pont De Nemours and Compagny, Legal Patent Records Center, 4417 Lancaster Pike, Wilmington, Delaware 19805 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

(88) Date of publication of the international search report:
24 March 2005

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ANNEXIN AND P34 PROMOTERS AND USE IN EXPRESSION OF TRANSGENIC GENES IN PLANTS

(57) Abstract: A seed specific plant annexin and P34 promoters and subfragments thereof and their use in promoting the expression of one or more heterologous nucleic acid fragments in plants are described.



WO 2004/071178 A3

ANNEXIN PROMOTER AND ITS USE IN EXPRESSION OF TRANSGENIC GENES IN PLANTS

This application claims the benefit of U.S. Provisional Application
5 No. 60/446,833, filed February 12, 2003, the disclosure of which is hereby
incorporated by reference in its entirety.

FIELD OF THE INVENTION

This invention relates to a plant promoter, in particular, to annexin and P34
promoters and subfragments thereof and their use in regulating expression of at
10 least one heterologous nucleic acid fragment in plants.

BACKGROUND OF THE INVENTION

Recent advances in plant genetic engineering have opened new doors to
engineer plants having improved characteristics or traits, such as, resistance to plant
diseases, insect resistance, herbicidal resistance, enhanced stability or shelf-life of
15 the ultimate consumer product obtained from the plants and improvement of the
nutritional quality of the edible portions of the plant. Thus, a desired gene (or genes)
from a source different than the plant, but engineered to impart different or improved
characteristics or qualities, can be incorporated into the plant's genome. This new
gene (or genes) can then be expressed in the plant cell to exhibit the desired
20 phenotype such as a new trait or characteristic.

The proper regulatory signals must be present and be in the proper location
with respect to the gene in order to obtain expression of the newly inserted gene in
the plant cell. These regulatory signals include a promoter region, a 5' non-
translated leader sequence and a 3' transcription termination/polyadenylation
25 sequence.

A promoter is a DNA sequence that directs cellular machinery of a plant to
produce RNA from the contiguous coding sequence downstream (3') of the
promoter. The promoter region influences the rate, developmental stage, and cell
type in which the RNA transcript of the gene is made. The RNA transcript is
30 processed to produce messenger RNA (mRNA) which serves as a template for
translation of the RNA sequence into the amino acid sequence of the encoded
polypeptide. The 5' non-translated leader sequence is a region of the mRNA
upstream of the protein coding region that may play a role in initiation and translation
of the mRNA. The 3' transcription termination/polyadenylation signal is a non-
35 translated region downstream of the protein coding region that functions in the plant
cells to cause termination of the RNA transcript and the addition of polyadenylate
nucleotides to the 3' end of the RNA.

It has been shown that certain promoters are able to direct RNA synthesis at a higher rate than others. These are called "strong promoters". Certain other promoters have been shown to direct RNA production at higher levels only in particular types of cells or tissues and are often referred to as "tissue specific promoters". In this group, many seed storage protein genes' promoters have been well characterized and widely used, such as the phaseolin gene promoter of *Phaseolus vulgaris*, the helianthinin gene of sunflower, the β -conglycinin gene of soybean (Chen et al., (1989) *Dev. Genet.* 10, 112-122), the napin gene promoter of *Brassica napus* (Ellerstrom et al, (1996) *Plant Mol. Biol.* 32, 1019-1027), the oleosin gene promoters of *Brassica* and *Arabidopsis* (Keddie et al, (1994) *Plant Mol. Biol.* 24, 327-340; Li, (1997) Texas A&M Ph.D. dissertation, pp. 107-128; Plant et al, (1994) *Plant Mol. Biol.* 25, 193-205). Another class of tissue specific promoters is described in, U.S. Patent No. 5,589,583, issued to Klee et al. on December 31, 1996; these plant promoters are capable of conferring high levels of transcription of chimeric genes in meristematic tissues and/or rapidly dividing cells. In contrast to tissue-specific promoters, "inducible promoters" direct RNA production in response to certain environmental factors, such as heat shock, light, hormones, ion concentrations etc. (Espartero et al, (1994) *Plant Mol. Biol.* 25, 217-227; Gomez-Gomez and Carrasco, (1998) *Plant Physiol.* 117, 397-405; Holtorf et al, (1995) *Plant Mol. Biol.* 29, 637-646; MacDowell et al, (1996) *Plant Physiol.* 111, 699-711; Mathur et al, (1992) *Biochem. Biophys. Acta* 1137, 338-348; Mett et al, (1996) *Transgenic Res.* 5, 105-113; Schoffl et al, (1989) *Mol. Gen. Genet.* 217, 246-253; Ulmasov et al, (1995) *Plant Physiol.* 108, 919-927).

Since the patterns of expression of a chimeric gene (or genes) introduced into a plant are controlled using promoters, there is an ongoing interest in the isolation and identification of novel promoters which are capable of controlling expression of a chimeric gene or (genes). Of particular interest are promoters that express only in the developing seeds. Another desirable feature of a promoter would be an expression pattern that occurs very soon after pollination in the developing seed.

SUMMARY OF THE INVENTION

This invention concerns an isolated nucleic acid fragment comprising a promoter wherein said promoter consists essentially of the nucleotide sequence set forth in SEQ ID NOs:1, 2, 13-22 or said promoter consists essentially of a fragment or subfragment that is substantially similar and functionally equivalent to the nucleotide sequence set forth in SEQ ID NOs:1, 2, 13-22.

In a second embodiment, this invention concerns a chimeric gene comprising at least one heterologous nucleic acid fragment operably linked to the promoter of the invention.

WO 2004/071178

PCT/US2004/005757

In a third embodiment, this invention concerns plants comprising this chimeric gene and seeds obtained from such plants.

In a fourth embodiment, this invention concerns a method of increasing or decreasing the expression of at least one heterologous nucleic acid fragment in a
5 plant cell which comprises:

- (a) transforming a plant cell with the chimeric gene described above;
- (b) growing fertile mature plants from the transformed plant cell of step (a);
- (c) selecting plants containing the transformed plant cell wherein the
10 expression of the heterologous nucleic acid fragment is increased or decreased.

In a fifth embodiment, this invention concerns an isolated nucleic acid fragment comprising a seed specific plant annexin, or P34, promoter.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCES

15 The invention can be more fully understood from the following detailed description, the drawings and the Sequence Descriptions that form a part of this application. The Sequence Descriptions contain the three letter codes for amino acids.

20 SEQ ID NO:1 is the DNA sequence comprising a 2012 nucleotide soybean annexin promoter.

SEQ ID NO:2 is the DNA sequence comprising a 1408 nucleotide soybean P34 promoter.

25 SEQ ID NO:3 is an oligonucleotide primer used in the first PCR amplification of the annexin promoter.

SEQ ID NO:4 is an oligonucleotide primer used in the second nested PCR amplification of the annexin promoter.

SEQ ID NO:5 is an oligonucleotide primer used in the first PCR amplification of the P34 promoter.

30 SEQ ID NO:6 is an oligonucleotide primer used in the second nested PCR amplification of the P34 promoter.

SEQ ID NO:7 is an oligonucleotide primer used in the PCR amplification of the annexin promoter when paired with SEQ ID NO:8 or 11.

35 SEQ ID NO:8 is an oligonucleotide primer used in the PCR amplification of the annexin promoter when paired with SEQ ID NO:7.

SEQ ID NO:9 is an oligonucleotide primer used in the PCR amplification of the P34 promoter when paired with SEQ ID NO:10 or 12.

SEQ ID NO:10 is an oligonucleotide primer used in the PCR amplification of the P34 promoter when paired with SEQ ID NO:9.

SEQ ID NO:11 is an oligonucleotide primer used in the PCR amplification of the annexin promoter when paired with SEQ ID NO:7.

5 SEQ ID NO:12 is an oligonucleotide primer used in the PCR amplification of the annexin promoter when paired with SEQ ID NO:9.

SEQ ID NO:13 is a 93.6% truncated form of the annexin promoter (SEQ ID NO:1).

10 SEQ ID NO:14 is a 85.4% truncated form of the annexin promoter (SEQ ID NO:1).

SEQ ID NO:15 is a 77.2% truncated form of the annexin promoter (SEQ ID NO:1).

SEQ ID NO:16 is a 67.9% truncated form of the annexin promoter (SEQ ID NO:1).

15 SEQ ID NO:17 is a 57.7% truncated form of the annexin promoter (SEQ ID NO:1).

SEQ ID NO:18 is a 48.1% truncated form of the annexin promoter (SEQ ID NO:1).

20 SEQ ID NO:19 is a 38.3% truncated form of the annexin promoter (SEQ ID NO:1).

SEQ ID NO:20 is a 29.0% truncated form of the annexin promoter (SEQ ID NO:1).

SEQ ID NO:21 is a 21.2% truncated form of the annexin promoter (SEQ ID NO:1).

25 SEQ ID NO:22 is a 8.6% truncated form of the annexin promoter (SEQ ID NO:1).

30 Figure 1. Soybean Annexin Promoter-GUS Expression in Arabidopsis. The dark developing seeds are staining blue due to GUS specific expression in the seeds. This demonstrates that the annexin promoter is capable of directing seed specific expression of a reporter construct. Untransformed seeds are not blue and show up as pale seeds.

Figure 2. Soybean P34 Promoter-GUS Expression in Arabidopsis. As in Figure 1 the blue staining seeds are transformed with the P34 promoter-GUS construct and shows that P34 is capable of directing seed-specific expression.

35 Figure 3. Soybean Seed Promoter Temporal Expression Patterns. Annexin, P34, beta-conglycinin beta subunit, beta-conglycinin alpha' subunit, glycinin, Kunitz trypsin inhibitor, and 2S albumin promoter expression patterns are shown on a

WO 2004/071178

PCT/US2004/005757

timeline of soybean seed development. The times are "days after fertilization" (DAF). The annexin promoter is the earliest known seed specific promoter.

Figure 4. GLA Accumulation in Soybean Somatic Embryos. The expression of delta-6 desaturase in soybean seeds allows for the accumulation of gamma-linolenic acid (GLA, not normally found in soybean seeds). Expression of delta-6 desaturase by the seed specific promoters from beta-conglycinin alpha' subunit, annexin, glycinin, and P34 are all capable of generating GLA in transgenic soybeans. The levels of GLA produced by annexin is comparable to levels obtained by the strong beta-conglycinin and glycinin promoters.

Figure 5. Deletion Analysis of the Soybean Annexin Promoter. The full-length soybean annexin promoter (SEQ ID NO:1) was truncated to form deletion fragments that are tested for promoter activity (SEQ ID NOs:13-22). The regulatory elements discussed in Example 3 are shown.

Figure 6. Promoter Strength Assays. The full-length and truncated promoters shown in Figure 5 were fused to a GUS reporter and transformed into Arabidopsis. Seeds from Arabidopsis transformants were assayed for GUS activity to assess the relative strengths of the various promoters. The results are shown with the standard deviations from the assays.

DETAILED DESCRIPTION OF THE INVENTION

In the context of this disclosure, a number of terms shall be utilized.

As used herein, an "isolated nucleic acid fragment" is a polymer of ribonucleotides (RNA) or deoxyribonucleotides (DNA) that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

The terms "polynucleotide", "polynucleotide sequence", "nucleic acid sequence", and "nucleic acid fragment"/"isolated nucleic acid fragment" are used interchangeably herein. These terms encompass nucleotide sequences and the like. A polynucleotide may be a polymer of RNA or DNA that is single- or double-stranded, that optionally contains synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, synthetic DNA, or mixtures thereof. Nucleotides (usually found in their 5'-monophosphate form) are referred to by a single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deoxycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridylate, "T" for deoxythymidylate, "R" for purines (A or G),

“Y” for pyrimidines (C or T), “K” for G or T, “H” for A or C or T, “I” for inosine, and “N” for any nucleotide.

The terms “subfragment that is functionally equivalent” and “functionally equivalent subfragment” are used interchangeably herein. These terms refer to a portion or subsequence of an isolated nucleic acid fragment in which the ability to alter gene expression or produce a certain phenotype is retained whether or not the fragment or subfragment encodes an active enzyme. For example, the fragment or subfragment can be used in the design of chimeric genes to produce the desired phenotype in a transformed plant. Chimeric genes can be designed for use in co-suppression or antisense by linking a nucleic acid fragment or subfragment thereof, whether or not it encodes an active enzyme, in the appropriate orientation relative to a plant promoter sequence.

The terms “substantially similar” and “corresponding substantially” as used herein refer to nucleic acid fragments wherein changes in one or more nucleotide bases does not affect the ability of the nucleic acid fragment to mediate gene expression or produce a certain phenotype. These terms also refer to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid fragment relative to the initial, unmodified fragment. It is therefore understood, as those skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences.

Moreover, the skilled artisan recognizes that substantially similar nucleic acid sequences encompassed by this invention are also defined by their ability to hybridize, under moderately stringent conditions (for example, 0.5 X SSC, 0.1% SDS, 60° C) with the sequences exemplified herein, or to any portion of the nucleotide sequences reported herein and which are functionally equivalent to the promoter of the invention. Preferred substantially similar nucleic acid sequences encompassed by this invention are those sequences that are 80% identical to the nucleic acid fragments reported herein or which are 80% identical to any portion of the nucleotide sequences reported herein. More preferred are nucleic acid fragments which are 90% identical to the nucleic acid sequences reported herein, or which are 90% identical to any portion of the nucleotide sequences reported herein. Most preferred are nucleic acid fragments which are 95% identical to the nucleic acid sequences reported herein, or which are 95% identical to any portion of the nucleotide sequences reported herein. It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying related polynucleotide sequences. Useful examples of percent identities are those listed above, or also preferred is any integer percentage from 80% to 100%.

WO 2004/071178

PCT/US2004/005757

Sequence alignments and percent similarity calculations may be determined using the Megalign™ program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences are performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS*.

5 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are GAP PENALTY=10, GAP LENGTH PENALTY=10, KTUPLE=2,
10 GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. A "substantial portion" of an amino acid or nucleotide sequence comprises enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to afford putative identification of that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison
15 and identification using algorithms such as BLAST (Altschul, S. F., et al., (1993) *J. Mol. Biol.* 215:403-410) and Gapped Blast (Altschul, S. F. et al., (1997) *Nucleic Acids Res.* 25:3389-3402).

"Desaturase" is a polypeptide which can desaturate one or more fatty acids to produce a mono- or poly-unsaturated fatty acid or precursor which is of interest.

20 "Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" or "recombinant DNA construct", which are used interchangeably, refers to any gene that is not a
25 native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers
30 to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

35 A "heterologous nucleic acid fragment" refers to a nucleic acid fragment comprising a nucleic acid sequence that is different from the nucleic acid sequence comprising the plant promoter of the invention.

“Coding sequence” refers to a DNA sequence that codes for a specific amino acid sequence. “Regulatory sequences” refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to, promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

“Promoter” refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an “enhancer” is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as “constitutive promoters”. New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg (1989, *Biochemistry of Plants* 15:1-82). It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of some variation may have identical promoter activity. An “intron” is an intervening sequence in a gene that is transcribed into RNA but is then excised in the process of generating the mature mRNA. The term is also used for the excised RNA sequences. An “exon” is a portion of the sequence of a gene that is transcribed and is found in the mature messenger RNA derived from the gene, but is not necessarily a part of the sequence that encodes the final gene product.

Among the most commonly used promoters are the nopaline synthase (NOS) promoter (Ebert et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:5745-5749), the octapine synthase (OCS) promoter, caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S promoter (Lawton et al. (1987) *Plant Mol. Biol.* 9:315-324), the CaMV 35S promoter (Odell et al. (1985) *Nature* 313:810-812), and the figwort mosaic virus 35S promoter, the light inducible promoter from the small subunit of rubisco, the Adh promoter (Walker et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:6624-66280), the sucrose synthase promoter (Yang et al. (1990) *Proc.*

WO 2004/071178

PCT/US2004/005757

Natl. Acad. Sci. U.S.A. 87:4144-4148), the R gene complex promoter (Chandler et al. (1989) *Plant Cell* 1:1175-1183), the chlorophyll a/b binding protein gene promoter, etc. Other commonly used promoters are, the promoters for the potato tuber ADPGPP genes, the sucrose synthase promoter, the granule bound starch synthase promoter, the glutelin gene promoter, the *maize waxy* promoter, Brittle gene promoter, and Shrunk 2 promoter, the acid chitinase gene promoter, and the zein gene promoters (15 kD, 16 kD, 19 kD, 22 kD, and 27 kD; Perdersen et al. (1982) *Cell* 29:1015-1026). A plethora of promoters is described in WO 00/18963, published on April 6, 2000.

10

Examples of a seed-specific promoter include, but are not limited to, the promoter for β -conglycinin (Chen et al. (1989) *Dev. Genet.* 10: 112-122), the napin promoter, and the phaseolin promoter. Other tissue-specific promoters that may be used to accomplish the invention include, but are not limited to, the chloroplast glutamine synthase (GS2) promoter (Edwards et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:3459-3463), the chloroplast fructose-1,6-biophosphatase promoter (Lloyd et al. (1991) *Mol. Gen. Genet.* 225:209-2216), the nuclear photosynthetic (ST-LS1) promoter (Stockhaus et al. (1989) *EMBO J.* 8:2445-2451), the serine/threonine kinase (PAL) promoter, the glucoamylase promoter, the promoters for the *Cab* genes (*cab6*, *cab-1*, and *cab-1R*, Yamamoto et al. (1994) *Plant Cell Physiol.* 35:773-778; Fejes et al. (1990) *Plant Mol. Biol.* 15:921-932; Lubberstedt et al. (1994) *Plant Physiol.* 104:997-1006; Luan et al. (1992) *Plant Cell* 4:971-981), the pyruvate orthophosphate dikinase promoter (Matsuoka et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:9586-9590), the LhcB promoter (Cerdan et al. (1997) *Plant Mol. Biol.* 33:245-255), the *PsbP* promoter (Kretsch et al. (1995) *Plant Mol. Biol.* 28:219-229), the SUC2 sucrose H⁺ symporter promoter (Truernit et al. (1995) *Planta* 196:564-570), and the promoters for the thylakoid membrane genes (*psaD*, *psaF*, *psaE*, *PC*, *FNR*, *atpC*, *atpD*), etc.

The "translation leader sequence" refers to a DNA sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner, R. and Foster, G. D. (1995) *Molecular Biotechnology* 3:225).

The "3' non-coding sequences" refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or

gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al., (1989) *Plant Cell* 1:671-680.

5 "RNA transcript" refers to a product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When an RNA transcript is a perfect complementary copy of a DNA sequence, it is referred to as a primary transcript or it may be a RNA sequence derived from posttranscriptional processing of a primary transcript and is referred to as a mature RNA. "Messenger RNA" ("mRNA") refers
10 to RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a DNA that is complementary to and synthesized from an mRNA template using the enzyme reverse transcriptase. The cDNA can be single-stranded or converted into the double-stranded by using the klenow fragment of DNA polymerase I. "Sense" RNA refers to RNA transcript that includes mRNA and
15 so can be translated into protein within a cell or *in vitro*. "Antisense RNA" refers to a RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks expression or transcripts accumulation of a target gene (U.S. Patent No. 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, *i.e.* at the 5' non-coding sequence, 3' non-coding
20 sequence, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

"Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell. "Antisense RNA" refers to a RNA transcript that is
25 complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene (U.S. Patent No. 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, *i.e.*, at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that may
30 not be translated but yet has an effect on cellular processes.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (*i.e.*, that the coding
35 sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the production of a functional end-product *e.g.*, a mRNA or a protein (precursor or mature).

The term "expression cassette" as used herein, refers to a discrete nucleic acid fragment into which a nucleic acid sequence or fragment can be moved.

Expression or overexpression of a gene involves transcription of the gene and translation of the mRNA into a precursor or mature protein. "Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. "Co-suppression" refers to the production of sense RNA transcripts capable of suppressing the expression or transcript accumulation of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020). The mechanism of co-suppression may be at the DNA level (such as DNA methylation), at the transcriptional level, or at post-transcriptional level.

"Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein. "Co-suppression" refers to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020). Co-suppression constructs in plants previously have been designed by focusing on overexpression of a nucleic acid sequence having homology to an endogenous mRNA, in the sense orientation, which results in the reduction of all RNA having homology to the overexpressed sequence (see Vaucheret et al. (1998) *Plant J.* 16:651-659; and Gura (2000) *Nature* 404:804-808). The overall efficiency of this phenomenon is low, and the extent of the RNA reduction is widely variable. Recent work has described the use of "hairpin" structures that incorporate all, or part, of an mRNA encoding sequence in a complementary orientation that results in a potential "stem-loop" structure for the expressed RNA (PCT Publication WO 99/53050 published on October 21, 1999 and PCT Publication WO 02/00904 published on January 3, 2002). This increases the frequency of co-suppression in the recovered transgenic plants. Another variation describes the use of plant viral sequences to direct the suppression, or "silencing", of proximal mRNA encoding sequences (PCT Publication WO 98/36083 published on August 20, 1998). Neither of these co-suppressing phenomena have been elucidated mechanistically at the molecular level, although genetic evidence has been obtained that may lead to the identification of potential components (Elmayan et al. (1998) *Plant Cell* 10:1747-1757).

"Altered expression" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ significantly from the amount of the gene product(s) produced by the corresponding wild-type organisms.

“Transformation” refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as “transgenic” organisms. The preferred method of corn cell transformation is use of particle-accelerated or “gene gun” transformation technology (Klein et al. (1987) *Nature (London)* 327:70-73; U.S. Patent No. 4,945,050).

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989 (hereinafter “Sambrook et al., 1989”) or Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (eds.), *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1990 (hereinafter “Ausubel et al., 1990”).

“PCR” or “Polymerase Chain Reaction” is a technique for the synthesis of large quantities of specific DNA segments, consists of a series of repetitive cycles (Perkin Elmer Cetus Instruments, Norwalk, CT). Typically, the double stranded DNA is heat denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps comprises a cycle.

An “expression construct” is a plasmid vector or a subfragment thereof comprising the instant chimeric gene. The choice of plasmid vector is dependent upon the method that will be used to transform host plants. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the chimeric gene. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., (1985) *EMBO J.* 4:2411-2418; De Almeida et al., (1989) *Mol. Gen. Genetics* 218:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis.

Although the annexin, or P34, polypeptides are known to be present in seeds, the promoters responsible for expression of these polypeptides, and the developmental timing of these promoters, have not been previously described. It was not possible to predict, before the studies reported herein, whether any annexin, or P34, gene was controlled by a seed-specific promoter. It is demonstrated herein

that seed-specific annexin, or P34, promoters do, in fact, exist in plants, and that such promoters can be readily isolated and used by one skilled in the art.

This invention concerns an isolated nucleic acid fragment comprising a seed-specific plant annexin, or P34, promoter. This invention also concerns an isolated nucleic acid fragment comprising a promoter wherein said promoter consists essentially of the nucleotide sequence set forth in SEQ ID NOs:1 or 2, or said promoter consists essentially of a fragment or subfragment that is substantially similar and functionally equivalent to the nucleotide sequence set forth in SEQ ID NOs:1 or 2. A nucleic acid fragment that is functionally equivalent to the instant annexin, or P34, promoter is any nucleic acid fragment that is capable of controlling the expression of a coding sequence or functional RNA in a similar manner to the annexin, or P34, promoter. The expression patterns of annexin, or P34, promoters are set forth in Examples 2 and 3.

The promoter activity of the soybean genomic DNA fragment upstream of the annexin, or P34, protein coding sequence was assessed by linking the fragment to a reporter gene, the *E. coli* β -glucuronidase gene (GUS) (Jefferson (1987) Plant Mol. Biol. Rep. 5:387-405), transforming the annexin, or P34, promoter::GUS expression cassette into *Arabidopsis*, and analyzing GUS expression in various cell types of the transgenic plants (see Example 2). GUS expression was restricted to the seeds although all parts of the transgenic plants were analyzed. These results indicated that the nucleic acid fragment contained seed specific promoters.

It is clear from the disclosure set forth herein that one of ordinary skill in the art could readily isolate a plant annexin, or P34, promoter from any plant by performing the following procedure:

1) obtaining an annexin, or P34, cDNA from a desired plant by any of a variety of methods well known to those skilled in the art including, but not limited to, (a) random sequencing of ESTs from a cDNA library and characterizing the ESTs via a BLAST search as described above; or (b) hybridizing a cDNA library to a known plant annexin, or P34, cDNA; or (c) PCR amplification using oligonucleotide primers designed from known annexin, or P34, cDNAs;

2) fragmenting genomic DNA with a restriction enzyme leaving blunt-ends and annealing adaptors onto the ends of the fragments. Using primers specific for the 5' end of the annexin or P34 transcript, and primers specific for the adaptors, to amplify the promoter region in a polymerase chain reaction.

3) operably linking the nucleic acid fragment containing the annexin, or P34, promoter sequence to a suitable reporter gene ; there are a variety of reporter genes that are well known to those skilled in the art, including the bacterial GUS gene, the

firefly luciferase gene, and the green fluorescent protein gene; any gene for which an easy and reliable assay is available can serve as the reporter gene

4) transforming a chimeric annexin, or P34, promoter::reporter gene expression cassette into an appropriate plant for expression of the promoter. There are a variety of appropriate plants which can be used as a host for transformation that are well known to those skilled in the art, including the dicots, *Arabidopsis*, tobacco, soybean, oilseed rape, peanut, sunflower, safflower, cotton, tomato, potato, cocoa and the monocots, corn, wheat, rice, barley and palm. The terms "oilseed rape" and "oilseed Brassica" are used interchangeably herein.

5) testing for expression of an annexin, or P34, promoter in various cell types of transgenic plant tissues, e.g., leaves, roots, flowers, seeds, transformed with the chimeric annexin, or P34, promoter::reporter gene expression cassette by assaying for expression of the reporter gene product. A strong seed-specific annexin, or P34, promoter will produce high level expression of the reporter in seeds without producing detectable expression in other plant tissues.

In another aspect, this invention concerns a recombinant DNA construct comprising at least one heterologous nucleic acid fragment operably linked to any promoter, or combination of promoter elements, of the present invention. Recombinant DNA constructs can be constructed by operably linking the nucleic acid fragment of the invention, i.e., any one annexin, or P34, promoter or a fragment or a subfragment that is substantially similar and functionally equivalent to any portion of the nucleotide sequence set forth in SEQ ID NOs:1, 2, or 13-22, to a heterologous nucleic acid fragment. Any heterologous nucleic acid fragment can be used to practice the invention. The selection will depend upon the desired application or phenotype to be achieved. The various nucleic acid sequences can be manipulated so as to provide for the nucleic acid sequences in the proper orientation. It is believed that various combinations of promoter elements as described herein may be useful in practicing the present invention.

Plasmid vectors comprising the instant recombinant DNA constructs can then be constructed. The choice of plasmid vector is dependent upon the method that will be used to transform host cells. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the chimeric gene.

Methods for transforming dicots, primarily by use of *Agrobacterium tumefaciens*, and obtaining transgenic plants have been published, among others, for cotton (U.S. Patent No. 5,004,863, U.S. Patent No. 5,159,135); soybean (U.S. Patent No. 5,569,834, U.S. Patent No. 5,416,011); *Brassica* (U.S. Patent No. 5,463,174); peanut (Cheng et al. (1996) *Plant Cell Rep.* 15:653-657, McKently

et al. (1995) *Plant Cell Rep.* 14:699-703); papaya (Ling, K. et al. (1991) *Bio/technology* 9:752-758); and pea (Grant et al. (1995) *Plant Cell Rep.* 15:254-258). For a review of other commonly used methods of plant transformation see Newell, C.A. (2000) *Mol. Biotechnol.* 16:53-65. One of these methods of transformation uses *Agrobacterium rhizogenes* (Tepfler, M. and Casse-Delbart, F. (1987) *Microbiol. Sci.* 4:24-28). Transformation of soybeans using direct delivery of DNA has been published using PEG fusion (PCT publication WO 92/17598), electroporation (Chowrira, G.M. et al. (1995) *Mol. Biotechnol.* 3:17-23; Christou, P. et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:3962-3966), microinjection, or particle bombardment (McCabe, D.E. et. al. (1988) *BiolTechnology* 6:923; Christou et al. (1988) *Plant Physiol.* 87:671-674).

There are a variety of methods for the regeneration of plants from plant tissue. The particular method of regeneration will depend on the starting plant tissue and the particular plant species to be regenerated. The regeneration, development and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art (Weissbach and Weissbach, (1988) *In.: Methods for Plant Molecular Biology*, (Eds.), Academic Press, Inc., San Diego, CA). This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil. Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

In addition to the above discussed procedures, practitioners are familiar with the standard resource materials which describe specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.), generation of recombinant DNA fragments and recombinant expression constructs and the screening and isolating of clones, (see for example, Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press; Maliga et al. (1995) *Methods in Plant Molecular Biology*, Cold Spring Harbor Press; Birren et al. (1998) *Genome Analysis: Detecting Genes*, 1, Cold Spring Harbor, New York; Birren et al. (1998) *Genome Analysis: Analyzing*

DNA, 2, Cold Spring Harbor, New York; Plant Molecular Biology: A Laboratory Manual, eds. Clark, Springer, New York (1997)).

The bacterial GUS gene can be successfully expressed in *Arabidopsis* embryos (see Figures 1 and 2). Furthermore, a gene encoding delta-6 desaturase from *M. alpina* also successfully expressed by this promoter in transgenic soybeans, as depicted in Figure 4. This further validates the application of the annexin, or P34, promoter of the invention in plant genetic engineering practice.

The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression of the chimeric genes (Jones *et al.*, (1985) *EMBO J.* 4:2411-2418; De Almeida *et al.*, (1989) *Mol. Gen. Genetics* 218:78-86). Thus, multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by northern analysis of mRNA expression, western analysis of protein expression, or phenotypic analysis. Also of interest are seeds obtained from transformed plants displaying the desired expression profile.

The level of activity of the annexin, or P34, promoter is comparable to that of many known strong promoters, such as the CaMV 35S promoter (Atanassova *et al.*, (1998) *Plant Mol. Biol.* 37:275-285; Battraw and Hall, (1990) *Plant Mol. Biol.* 15:527-538; Holtorf *et al.*, (1995) *Plant Mol. Biol.* 29:637-646; Jefferson *et al.*, (1987) *EMBO J.* 6:3901-3907; Wilmink *et al.*, (1995) *Plant Mol. Biol.* 28:949-955), the *Arabidopsis* oleosin promoters (Plant *et al.*, (1994) *Plant Mol. Biol.* 25:193-205; Li, (1997) Texas A&M University Ph.D. dissertation, pp. 107-128), the *Arabidopsis* ubiquitin extension protein promoters (Callis *et al.*, 1990), a tomato ubiquitin gene promoter (Rollfinke *et al.*, 1998), a soybean heat shock protein promoter (Schoffl *et al.*, 1989), and a maize H3 histone gene promoter (Atanassova *et al.*, 1998).

Expression of chimeric genes in most plant cell makes the annexin, or P34, promoter of the instant invention especially useful when seed specific expression of a target heterologous nucleic acid fragment is required. Another useful feature of the annexin promoter is its expression profile in developing seeds. The annexin promoter of the invention is most active in developing seeds at early stages (within 10 days after pollination) and is largely quiescent in later stages (see Figure 3). The expression profile of the claimed annexin promoter is different from that of many seed-specific promoters, e.g., seed storage protein promoters, which often provide highest activity in later stages of development (Chen *et al.*, (1989) *Dev. Genet.* 10:112-122; Ellerstrom *et al.*, (1996) *Plant Mol. Biol.* 32:1019-1027; Keddie *et al.*, (1994) *Plant Mol. Biol.* 24:327-340; Plant *et al.*, (1994) *Plant Mol. Biol.* 25:193-205; Li, (1997) Texas A&M University Ph.D. dissertation, pp. 107-128). The P34 promoter has a more conventional expression profile but remains distinct from other known

seed specific promoters (see Figure 3). Thus, the annexin, or P34, promoter will be a very attractive candidate when overexpression, or suppression, of a gene in embryos is desired at an early developing stage. For example, it may be desirable to overexpress a gene regulating early embryo development or a gene involved in the metabolism prior to seed maturation.

Methods of isolating seed oils are well known in the art (Young et al, Processing of Fats and Oils, in "The Lipid Handbook" (Gunstone et al eds.) Chapter 5, pp 253-257; London, Chapman & Hall, 1994).

Another general application of the annexin, or P34, promoter of the invention is to construct chimeric genes that can be used to reduce expression of at least one heterologous nucleic acid fragment in a plant cell. To accomplish this a chimeric gene designed for cosuppression of a heterologous nucleic acid fragment can be constructed by linking the fragment to the annexin, or P34, promoter of the present invention. (See U.S. Patent No. 5,231,020, and PCT Publication WO 99/53050 published on October 21, 1999, PCT Publication WO 02/00904 published on January 3, 2002, and PCT Publication WO 98/36083 published on August 20, 1998, for methodology to block plant gene expression via cosuppression.) Alternatively, a chimeric gene designed to express antisense RNA for a heterologous nucleic acid fragment can be constructed by linking the fragment in reverse orientation to the annexin, or P34, promoter of the present invention. (See U.S. Patent No. 5,107,065 for methodology to block plant gene expression via antisense RNA.) Either the cosuppression or antisense chimeric gene can be introduced into plants via transformation. Transformants wherein expression of the heterologous nucleic acid fragment is decreased or eliminated are then selected.

This invention also concerns a method of increasing or decreasing the expression of at least one heterologous nucleic acid fragment in a plant cell which comprises:

- (a) transforming a plant cell with the chimeric genes described herein;
- (b) growing fertile mature plants from the transformed plant cell of step (a);
- (c) selecting plants containing a transformed plant cell wherein the expression of the heterologous nucleic acid fragment is increased or decreased.

Transformation and selection can be accomplished using methods well-known to those skilled in the art including, but not limited to, the methods described herein.

EXAMPLES

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. Techniques in molecular biology were typically performed as described in

WO 2004/071178

PCT/US2004/005757

Ausubel, F. M., et al., (1990, Current Protocols in Molecular Biology, John Wiley and Sons, New York) or Sambrook, J. et al., (1989, Molecular cloning - A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). It should be understood that these Examples, while indicating preferred
 5 embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in
 10 addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

15

EXAMPLE 1Isolation of Soybean Annexin and P34 Promoters

The soybean annexin and P34 promoters were isolated using a polymerase chain reaction (PCR) based approach. Soybean genomic DNA was digested to completion with a DNA restriction enzyme that generates blunt ends (DraI, EcoRV,
 20 PvuII, or StuI, for example) according to standard protocols. The Universal GenomeWalker™ system from Clontech™ (user manual PT3042-1) was used to ligate adaptors to the ends of the genomic DNA fragments. Nested primers are also supplied that are specific for the adaptor sequence (AP1 and AP2, for the first and second adaptor primer respectively). Two gene specific primers (GSP1 and GSP2)
 25 were designed for the soybean annexin gene based on the 5' coding sequences in annexin cDNA in DuPont EST database. The oligonucleotide sequences of the GSP1 and GSP2 primers have the sequences shown below (SEQ ID NO:3 and 4).

SEQ.ID NO:3 5'-GCCCCCATCCTTTGAAAGCCTGT-3'

SEQ ID NO:4 5'-CGCGGATCCGAGAGCCTCAGCATCTTGAGCAGAA-3'

30 The underlined bases are the recognition site for the restriction enzyme BamH I. The AP2 primer from the GenomeWalker™ kit contains a Sal I restriction site.

The AP1 and the GSP1 primers were used in the first round PCR using each of the adaptor ligated genomic DNA populations (DraI, EcoRV, PvuII, or StuI) under conditions defined in the GenomeWalker™ protocol. Cycle conditions were 94°C
 35 for 4 minutes; 94°C for 2 second and 72°C for 3 min, 7 cycles; 94°C for 2 second and 67°C for 3 minutes, 32 cycles; 67°C for 4 minutes. The products from each of the first run PCRs were diluted 50-fold. One microliter from each of the diluted products was used as templates for the second PCR with the AP2 and GSP2 as

primers. Cycle conditions were 94°C for 4 minutes; 94°C for 2 second and 72°C for 3 min, 5 cycles; 94°C for 2 second and 67°C for 3 minutes, 20 cycles; 67°C for 3 minutes. Agarose gels were run to determine which PCR gave an optimal fragment length. A 2.1 kb genomic fragment was detected and isolated from the
 5 EcoRV-digested genomic DNA reaction. The genomic fragment was digested with BamH I and Sal I and cloned into Bluescript KS⁺ vector for sequencing. Finally, sequencing data indicated that this genomic fragment contained a 2012 bp soybean annexin promoter sequence as shown in SEQ ID NO:1.

Two gene specific primers (GSP3 and GSP4) were designed for the soybean
 10 P34 gene based on the 5' coding sequences in P34 cDNA in NCBI Genebank (J05560). The oligonucleotide sequences of the GSP3 and GSP4 primers have the sequences shown below (SEQ ID NOs:5 and 6).

SEQ ID NO:5 5'-GGTCCAATATGGAACGATGAGTTGATA-3'

SEQ ID NO:6 5'-CGCGGATCCGCTGGAACTAGAAAGAGAGACCTAAGA-3'

15 The AP1 and the GSP3 primers were used in the first round PCR using the same conditions defined in the GenomeWalker™ system protocol. The cycle conditions used for soybean annexin promoter did not work well for the soybean P34 promoter reactions. A modified PCR protocol was used. Cycle conditions were: 94°C for 4 minutes; 94°C for 2 second and 74°C for 3 min, 6 cycles in which annealing
 20 temperature drops 1°C every cycle; 94°C for 2 second and 69°C for 3 minutes, 32 cycles; 69°C for 4 minutes. The products from the 1st run PCR were diluted 50-fold. One microliter of the diluted products were used as templates for the 2nd PCR with the AP2 and GSP4 as primers. Cycle conditions were: 94°C for 4 minutes; 94°C for 2 second and 74°C for 3 min, 6 cycles in which annealing
 25 temperature drops 1°C every cycle; 94°C for 2 second and 69°C for 3 minutes, 20 cycles; 69°C for 3 minutes. A 1.5 kb genomic fragment was amplified and isolated from the Pvu II-digested GenomeWalker library. The genomic fragment was digested with BamH I and Sal I and cloned into Bluescript KS⁺ vector for sequencing. Sequencing data indicated that this genomic fragment contained a
 30 1408bp soybean P34 promoter sequence as shown in SEQ ID NO:2.

EXAMPLE 2

Construction of GUS Reporter Constructs linked to Soybean Annexin Promoter or P34 Promoter and Expression in Arabidopsis Seeds

Two oligonucleotides were designed to re-amplify the annexin promoter with
 35 either BamH I or Nco I sites (underlined below in SEQ ID NOs: 7 and 8, respectively). The oligonucleotide sequences of these two oligonucleotides are shown in SEQ ID NOs:7 and 8.

SEQ ID NO:7 5'-CGCGGATCCATCTTAGGCCCTTGATTATATGGTGTTT-3'

WO 2004/071178

PCT/US2004/005757

SEQ ID NO:8 5'-CCTTGACCATGGAAGTATTGCTTCTTAGTTAACCTTTCC-3'

The re-amplified annexin promoter fragment was digested with BamH I and Nco I, purified and cloned into the BamH I and Nco I sites of plasmid pG4G to make the fusion between the soybean annexin promoter-GUS fusion (pJS86). The plasmid pG4G has been described in US Patent No. 5,968,793 .

Two oligonucleotides with either BamH I or Nco I sites at the 5' ends were designed to re-amplify the P34 promoter. The oligonucleotide sequences of these two PCR primers are shown in SEQ ID NOs:9 and 10.

10 SEQ ID NO:9 5'-CGCGGATCCAACTAAAAAAGCTCTCAAATTACATTTTGAG-3'

SEQ ID NO:10 5'-CCTTGACCATGGCTTGGTGGGAAGAATTTTATGATTGAAATT-3'. The re-amplified p34 promoter fragment was digested with BamH I and Nco I, purified and cloned into the BamH I and Nco I sites of plasmid pG4G to make the fusion between the soybean p34 promoter-GUS fusion (pJS87).

15 The chimeric promoter-GUS recombinant constructs were cloned as a BamH I-Sal I fragment into the *Agrobacterium tumefaciens* binary vector pZBL120 to create pJS90 and pJS91. The binary vector pZBL120 is the same as the pZBL1 binary vector as described in US Patent No. 5,968,793 (ATCC# 209128) except the NOS promoter was replaced with a 963 bp 35S promoter (NCBI accession number V00141 from nucleotide 6494 to 7456) in the Nos/P-nptII-OCS 3' gene. The new 35S promoter-nptII-OCS 3' gene serves as a kanamycin resistance plant selection marker in pZBL120. The pJS90 and pJS91 binary vector constructions were transformed into *Agrobacterium tumefaciens* LBA4404, which was then used to inoculate *Arabidopsis* plants by Vacuum infiltration (Guang-Ning Ye et. al., *Plant Journal* 19, 249-257, 1999). The *Arabidopsis* seeds of primary transformants were selected by 100mg/l Kan on MS culture plates. The Kan resistant seedlings were transferred into soil and analyzed for GUS activity in seeds, leaves, stems, flowers and silique coats. The GUS activity was analyzed by histochemical staining by X-Gluc and quantitative fluorometric MUG GUS assay as described by Jefferson (25 *Plant Mol. Biol. Rep.* 5:387-405, 1987).

35 As shown in Figure 1 and Figure 2, both soybean annexin promoter and P34 promoter provide very specific GUS expression in seeds (dark seeds are stained blue in the figures). Other parts of transformed plants, such as leaves, stems, flowers and silique coats, did not exhibit GUS staining (data not shown). The annexin promoter is much stronger than the p34 promoter is for seed specific expression. As shown in Figure 3, the annexin gene is expressed in a very early stage of seed development, as compared to a mid-late stage gene P34 and other seed storage protein genes.

EXAMPLE 3Construction of Annexin Promoter-M. alpina Delta-6 Desaturase Constructs and Polyunsaturated Fatty Acid Production in Transgenic Soybean Somatic Embryos

Based on the sequences of cloned soybean annexin promoter, another oligo
 5 with Not I site at the 5' end was designed and used with BamH I primer (SEQ ID
 No.7) to re-amplify the annexin promoter. The oligonucleotide sequence of this Not
 I-containing oligo is shown in SEQ ID No.11.

SEQ ID No. 11: GAATTCCGCGGCCGCTGAAGTATTGCTTCTTAGTTAACCTTTCC

Based on the sequences of cloned soybean P34 promoter, another oligo with NotI
 10 site at the 5' end was designed and used with BamH I primer (SEQ ID No. 9) to re-
 amplify the P34 promoter. The oligonucleotide sequence of this NotI-containing
 oligo is shown in SEQ ID No.12.

SEQ ID No. 12:

GAATTCCGCGGCCGCAACTTGGTGGGAAGATTATGATTGAAA

15 The re-amplified annexin and P34 promoter fragment was digested with BamH I and
 Not I, purified and cloned into the BamH I and Not I sites of plasmid pZBL115 to
 make pJS88 and pJS89. The pZBL115 plasmid contains the origin of replication
 from pRB322, the bacterial HPT hygromycin resistance gene driven by T7 promoter
 and T7 terminator, and a 35S promoter-HPT-Nos3' gene to serve as a hygromycin
 20 resistant plant selection marker. M. alpina delta 6 desaturase gene was cloned into
 Not I site of pJS88 and pJS89 in the sense orientation to make plant expression
 cassettes pJS92 and pJS93. The pJS92 and pJS93 were transformed into a
 soybean somatic embryo system. The matured transgenic embryos were analyzed
 for novel GLA (γ -linolenic acid) production by HPLC/GC.

25 As shown in Figure 4, GLA accumulation in soybean somatic embryos was
 detected when the M. alpina delta 6 desaturase gene was under the control of a
 variety of soybean seed specific promoters. With very strong seed specific
 promoters such as soybean beta-conglycinin alpha' subunit promoter, soybean
 Glycinin Gy1 promoter, GLA level is about 35-40%. With soybean annexin
 30 promoter, the level of GLA reaches about 40% of total fatty acids. As for soybean
 P34 promoter, GLA level is about 8%. All these results demonstrated that the
 soybean annexin and P34 promoters are functional in soybean somatic embryos to
 produce a novel fatty acid GLA.

EXAMPLE 4Identification Seed-Specific Consensus Elements in Annexin and P34 Promoters

The soybean annexin promoter contains the consensus core promoter
 sequences known as CCAAT box, TATA box and transcription start site. The
 annexin promoter also contains several seed-specific/ABA responsive elements,

such as the RY-G-box seed-specific coupling elements (CATGCAA, CATGCCT, CATGCAG, CTACGTCA, TAACGTGC), ACAC elements (CCTACACTCT, CCAACACTGG, TATACACTCC, TGTACACATA, TTCACACCAT, ACAACACTTT, CTAACACGAT), GTGT elements (ATGGTGTTTA, GTAGTGTGAA, AATGTGTTAT, CATGTGTAAA) and AT-rich sequences. All these conserved elements, individually or in combination, can be very important for the temporal and tissue-specific gene expression of the soybean annexin promoter.

The soybean P34 promoter contains two putative TATA boxes (TATATA and TATATATA). The P34 promoter also contains several seed-specific/ABA responsive elements, such as the RY-G-box seed-specific coupling elements (CATGCAG, CATGCAA, CATGCTA, ACACGTTA, AGACGTGT, GGACGTATACACGTTT, TTACGTAT), ACAC elements (CAACACGT, AACACACAT, ATACACGT), GTGT elements (GACGTGTACG, GCAGTGTCTGA, CATGTGTGAA, ACTGTGTGCT, TTTGTGTTAG). It is interesting to notice that there are two overlapping ACAC element/ACGT elements and one overlapping ACGT element/GTGT element within the promoter, which may play a very important role for both seed-specific and ABA-regulated gene expressions. All these conserved elements, individually or in combination, may be very important for the temporal and tissue-specific gene expression of the soybean P34 promoter.

EXAMPLE 5

Deletion and Site-directed Mutagenesis of Annexin and P34 Promoters

In order to further define the transcriptional elements controlling temporal and tissue-specific gene expression of these new soybean seed specific promoters, a series of 5' unidirectional deletions of the promoters were made using PCRs. PCRs were also used to make internal deletion and site-directed mutagenesis in the promoters. All these deletion or mutated promoter-GUS constructs were transferred into binary vectors and transformed into transgenic Arabidopsis (as described in Example 2).

Figure 5 shows the ten different deletion fragments that were tested for the annexin promoter (SEQ ID NOs:13-22). The consensus elements identified in Example 4 are shown as boxes. The fragment lengths are 1883 bp, 1719 bp, 1553 bp, 1367 bp, 1160 bp, 967 bp, 770 bp, 584 bp, 425 bp, and 174 bp (SEQ ID NOs:13-22 respectively).

Analysis of the relative promoter strengths and their tissue-specificity of expression was performed by histochemical GUS staining with X-Gluc and quantitative fluorometric MUG GUS assay (as described in Example 2). The results shown in Table 1 and Figure 6 demonstrate that all of the annexin promoters tested, except the shortest (-174 bp, SEQ ID NO:22), retain high levels of promoter activity.

The -174 promoter may retain some very low level activity. The highest promoter activity is seen with the -770 promoter (SEQ ID NO:19).

<u>TABLE 1</u>		
	GUS (pmol MU/ug protein.hr)	Standard Deviation
WT seeds	-0.19173	0.55127
-1883	1164.2	543.89
-1719	1418.8	606.94
-1553	1340.4	379.76
-1367	913.87	434.69
-1160	1161.2	895.42
-967	1407.9	760.74
-770	2831.7	1233.1
-584	1388.2	760.81
-425	519.22	221.11
-174	0.99894	1.9657

BB1531 Canada Seq Lst.txt

SEQUENCE LISTING

<110> E.I. du Pont de Nemours and Company

<120> Annexin and P34 Promoters and Use in Expression of Transgenic Genes in Plants

<130> 53388-1765/DPC

<140> 2,512,588

<141> 12 February 2004

<160> 22

<170> Microsoft Office 97

<210> 1

<211> 2012

<212> DNA

<213> Glycine max

<400> 1

atccttaggcc	cttgattata	tggtgtttag	atggattcac	atgcaagttt	ttattttcaat	60
cccttttcct	ttgaataact	gaccaagaac	aacaagaaaa	aaaaaaaaag	aaaaggatca	120
ttttgaaagg	atatttttcg	ctcctattca	aatactgtat	ttttaccaaa	aaaactgtat	180
ttttcctaca	ctctcaagct	ttgtttttcg	cttcgactct	catgatttcc	ttcatatgcc	240
aatcactcta	tttataaatg	gcataaggta	gtgtgaacaa	ttgcaaagct	tgtcatcaaa	300
agcttgcaat	gtacaaatta	atgtttttca	tgcctttcaa	aattatctgc	accccctagc	360
tattaatcta	acatctaagt	aaggctagtg	aattttttcg	aatagtcatg	cagtgcatta	420
atttccccgt	gactattttg	gctttgactc	caacactggc	cccgtacatc	cgtccctcat	480
tacatgaaaa	gaaatattgt	ttatattcct	aattaaaaat	attgtccctt	ctaaattttc	540
atatagttaa	ttattatatt	acttttttct	ctatttctatt	agttctatct	tcaaattatt	600
atttatgcat	atgtaaagta	cattatatatt	ttgctatatata	cttaaataatt	tctaaattat	660
taaaaaaaga	ctgatatgaa	aaattttattc	tttttaaagc	tatatcattt	tatatatact	720
ttttcttttc	ttttctttca	ttttctattc	aattttaataa	gaaataaatt	ttgtaaattt	780
ttattttatca	attttataaaa	atatttttact	ttatatgttt	tttcacattt	ttgttaaaca	840
aatcatatca	ttatgattga	aagagaggaa	attgacagtg	agtaataagt	gatgagaaaa	900
aaatgtgtta	tttcctaaaa	aaaacctaaa	caaacatgta	tctactctct	atttcatcta	960
tctctcattt	catttttctc	tttatctctt	tctttatttt	tttatcatat	catttcacat	1020
taattatttt	tactctcttt	atttttttct	tctatccctc	tcttatttcc	actcatatat	1080
acactccaaa	attggggcat	gccttttatca	ctactctatc	tcctccacta	aatcatttaa	1140
atgaaactga	aaagcattgg	caagtctcct	cccctcctca	agtgatttcc	aactcagcat	1200
tggcatctga	ttgattcagt	atatctattg	catgtgtaaa	agtctttcca	caatacataa	1260
ctattaatta	atcttaaata	aataaaggat	aaaatatttt	tttttcttca	taaaatttaa	1320
atatgttatt	ttttgttttag	atgtatatcc	gaataaatct	aaatatatga	taatgatttt	1380
ttatatgtat	taaacatata	atcaatatata	aatatgatat	ttttttatat	aggttgtaca	1440
cataatttta	taaggataaa	aaatatgata	aaaataaatt	ttaaataatt	ttatatattac	1500
gagaaaaaaa	aatatatttag	ccataaataa	atgaccagca	tatttttaca	ccttagtaat	1560
tcataaattc	ctatatgtat	atttgaaatt	aaaaacagat	aatcgttaag	ggaaggaatc	1620
ctacgtcatc	tcttgccatt	tgttttttcat	gcaaacagaa	agggacgaaa	aaccacctca	1680
ccatgaatca	ctcttcacac	cattttttact	agcaaacaag	tctcaacaac	tgaagccagc	1740
tctcttttccg	tttctttttta	caacactttc	tttgaaatag	tagtattttt	ttttcacatg	1800
atttattaac	gtgccaaaag	atgcttattg	aatagagtgc	acatttgtaa	tgtactacta	1860
attagaacat	gaaaaagcat	tgttcttaaca	cgataatcct	gtgaaggcgt	taactccaaa	1920
gatccaattt	cactatataa	attgtgacga	aagcaaaatg	aattcacata	gctgagagag	1980
aaaggaaagg	ttaactaaga	agcaataactt	ca			2012

BB1531 Canada Seq Lst.txt

<210> 2
 <211> 1408
 <212> DNA
 <213> Glycine max

<400> 2
 aactaaaaaa agctctcaaa ttacattttg agttgtttca ggttccattg ctttattgct 60
 aaaactccaa ctaaaataac aaatagcaca tgcaggtgca aacaacacgt tactctgatg 120
 aaggtgatgt gcctctagca gtctagctta tgaggctcgc tgcttatcaa cgattcatca 180
 ttccccaaga cgtgtacgca gattaaacaa tggacaaaac ttcaatcgat tatagaataa 240
 taatttttaac agtgccgact tttttctgta aacaaaaggc cagaatcata tcgcacatca 300
 tcttgaatgc agtgtcgagt ttggaccatt tgagtacaaa gccaatattg aatgattttt 360
 cgatttttaca tgtgtgaatc agacaaaagt gcatgcaatc acttgcaagt aaattaagga 420
 tactaatcta ttcctttcat tttatatgct ccacttttat ataaaaaaat atacattatt 480
 atatatgcat tattaattat tgcagtatta tgctattggg tttatggccc tgctaaataa 540
 cctaaatgag tctaactatt gcatatgaat caaatgaagg aagaatcatg atctaaacct 600
 gagtacccaa tgcaataaaa tgcgtcctat tacctaaact tcaaacacac attgccatcg 660
 gacgtataaa ttaatgcata taggttattt tgagaaaaga aaacatcaaa agctctaaaa 720
 cttcttttta ctttgaaata agctgataaa aatacgcttt aaatcaactg tgtgctgtat 780
 ataagctgca atttcacatt ttaccaaacc gaaacaagaa tggtaacagt gaggcaaaaa 840
 tttgaaaaat gtcctacttc acattcacat caaatgaatt acaactaaat aaataaacat 900
 cgtgattcaa gcagtaatga aagtcgaaat cagatagaat atacacgttt aacatcaatt 960
 gaattttttt ttaaattgat atatacaagt ttactatttt atatataatg aaaatttcatt 1020
 ttgtgttagc acaaaactta cagaaagaga taaattttta ataaagagaa ttatatccaa 1080
 ttttataatc caaaataatc aaattaaaga atattggcta gatagaccgg cttttttcact 1140
 gcccctgctg gataatgaaa attcatatca aaacaataca gaagttctag tttaataata 1200
 aaaaagttgg caaactgtca ttccctgttg gtttttaagc caaatcacaa ttcaattacg 1260
 tatcagaaat taattttaa ccaatatata gctacgaggg aacttcttca gtcattacta 1320
 gctagctcac taatcactat atatacgaca tgctacaagt gaagtgacca tatcttaatt 1380
 tcaaatcata aaattcttcc accaagtt 1408

<210> 3
 <211> 24
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: PCR primer

<400> 3
 gcccccatc ctttgaaagc ctgt 24

<210> 4
 <211> 34
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: PCR primer

<400> 4
 cgcggatccg agagcctcag catcttgagc agaa 34

<210> 5
 <211> 27
 <212> DNA
 <213> Artificial Sequence

BB1531 Canada Seq Lst.txt

<220>
 <223> Description of Artificial Sequence: PCR primer
 <400> 5
 ggtccaatat ggaacgatga gttgata 27

<210> 6
 <211> 35
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Description of Artificial Sequence: PCR primer
 <400> 6
 cgcgatccg ctggaactag aagagagacc taaga 35

<210> 7
 <211> 37
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Description of Artificial Sequence: PCR primer
 <400> 7
 cgcgatcca tcttaggcc ttgattatat ggtgttt 37

<210> 8
 <211> 39
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Description of Artificial Sequence: PCR primer
 <400> 8
 ccttgaccat ggaagtattg cttcttagtt aacctttcc 39

<210> 9
 <211> 41
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Description of Artificial Sequence: PCR primer
 <400> 9
 cgcgatcca actaaaaaaaa gctctcaaatacattttga g 41

<210> 10
 <211> 42
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Description of Artificial Sequence: PCR primer

BB1531 Canada Seq Lst.txt

<400> 10
ccttgaccat ggcttggtgg aagaatttta tgatttgaaa tt 42

<210> 11
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 11
gaattcgcgg ccgctgaagt attgcttctt agttaacctt tcc 43

<210> 12
<211> 44
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer

<400> 12
gaattcgcgg ccgcaacttg gtggaagaat tttatgattt gaaa 44

<210> 13
<211> 1883
<212> DNA
<213> Glycine max

<400> 13
gatatattttc gctcctattc aaatactgta tttttaccaa aaaaactgta tttttcctac 60
actctcaagc tttgtttttc gcttcgactc tcatgatttc cttcatatgc caatcactct 120
atttataaat ggcataaggt agtgtgaaca attgcaaagc ttgtcatcaa aagcttgcaa 180
tgtacaaatt aatgtttttc atgcctttca aaattatctg cacccttag ctattaatct 240
aacatctaag taaggctagt gaattttttc gaatagtcatt gcagtgcatt aatttccccg 300
tgactatttt ggctttgact ccaacactgg ccccgtagat ccgtccctca ttacatgaaa 360
agaaatattg tttatattct taattaaaaa tattgtccct tctaaatttt catatagtta 420
attattatat tacttttttc tctattctat tagttctatt ttcaaattat tatttatgca 480
tatgtaaagt acattatatt tttgctatat acttaaatat ttctaaatta ttaaaaaaag 540
actgatatga aaaattttatt ctttttaaaag ctatatcatt ttatatatac tttttctttt 600
cttttctttc attttctatt caatttaata agaaataaat tttgtaaatt tttatttatc 660
aattttataaa aatatttttac tttatatggt ttttcacatt tttgttaaac aaatcatatc 720
attatgattg aaagagagga aattgacagt gagtaataag tgatgagaaa aaaatgtgtt 780
atttcctaaa aaaaacctaa acaaacatgt atctactctc tatttcattc atctctcatt 840
tcattttttc ctttatctct ttctttatatt ttttatcata tcatttcaca ttaattatatt 900
ttactctctt tatttttttct ctctatccct ctcttatttc cactcatata tacactccaa 960
aattggggca tgcctttatc actactctat ctctccact aaatcattta aatgaaactg 1020
aaaagcattg gcaagtctcc tcccctcctc aagtgatttc caactcagca ttggcatctg 1080
attgattcag tatactctatt gcatgtgtaa aagtctttcc acaatacata actattaatt 1140
aatcttaaat aaataaagga taaaatattt ttttttcttc ataaaattaa aatatgttat 1200
tttttgttta gatgtatatt cgaataaatc taaatatatg ataagtattt tttatattga 1260
ttaaacatat aatcaatatt aaatatgata tttttttata taggttgtag acataatttt 1320
ataaggataa aaaatatgat aaaaataaat tttaaatatt tttatatatta cgagaaaaaa 1380
aaatatttta gccataaata aatgaccagc atattttaca acctagtaga ttcataaatt 1440
cctatatgta tatttgaaat taaaaacaga taatcggtta gggaagggaat cctacgtcat 1500
ctcttgccat ttgtttttca tgcaaacaga aagggacgaa aaaccacctc accatgaatc 1560
actcttcaca ccattttttac tagcaaacaa gtctcaacaa ctgaagccag ctctctttcc 1620
gtttcttttt acaacacttt ctttgaaata gtagtatatt tttttcacat gatttattaa 1680

BB1531 Canada Seq Lst.txt

cgtgccaaaa	gatgcttatt	gaatagagtg	cacatttgta	atgtactact	aattagaaca	1740
tgaaaaagca	ttgtttctaac	acgataatcc	tgtgaaggcg	ttaactccaa	agatccaatt	1800
tcactatata	aattgtgacg	aaagcaaaat	gaattcacat	agctgagaga	gaaaggaaag	1860
gttaactaag	aagcaatact	tca				1883

<210> 14
 <211> 1719
 <212> DNA
 <213> Glycine max

<400> 14

catcaaaagc	ttgcaatgta	caaattaatg	tttttcatgc	ctttcaaaat	tatctgcacc	60
ccctagctat	taatctaaca	tctaagtaag	gctagtgaat	tttttcgaat	agtcatgcag	120
tgcatthaatt	tccccgtgac	tatttttggt	ttgactccaa	cactggcccc	gtacatccgt	180
ccctcattac	atgaaaagaa	atattgttta	tattcttaat	taaaaatatt	gtcccttcta	240
aattttcata	tagttaatta	ttataattact	tttttctcta	ttctattagt	tctattttca	300
aattattatt	tatgcatatg	taaagtacat	tatatattttg	ctatatactt	aaatattttct	360
aaattatttaa	aaaaagactg	atatgaaaaa	tttattcttt	ttaaagctat	atcatttttat	420
atatactttt	tcttttcttt	tctttcattt	tctattcaat	ttaataagaa	ataaatttttg	480
taaattttta	tttatcaatt	tataaaaaata	ttttacttta	tatgtttttt	cacattttttg	540
ttaaacaaat	catatcatta	tgattgaaag	agaggaaatt	gacagtgagt	aataagtgat	600
gagaaaaaaa	tgtgttattt	cctaaaaaaa	acctaaacaa	acatgtatct	actctctatt	660
tcacttatct	ctcatttcat	ttttctcttt	atctctttct	ttattttttt	atcatatcat	720
ttcacattaa	ttattttttac	tctcttttatt	ttttctctct	atccctctct	tatttccact	780
catatataca	ctccaaaatt	ggggcatgcc	tttatcacta	ctctatctcc	tccactaaat	840
cattttaaag	aaactgaaaa	gcattggcaa	gtctcctccc	ctcctcaagt	gatttccaac	900
tcagcattgg	catctgattg	attcagtata	tctattgcat	gtgtaaaagt	ctttccacaa	960
tacataacta	ttaattaatc	ttaaataaat	aaaggataaa	atattttttt	ttcttcataa	1020
aattaaaata	tgttattttt	tgtttagatg	tatattcgaa	taaatctaaa	tatatgataa	1080
tgatttttta	tattgattaa	acatatatac	aatattaaat	atgatatttt	tttatatagg	1140
ttgtacacat	aattttataa	ggataaaaaa	tatgataaaa	ataaatttta	aatattttta	1200
tattttacgag	aaaaaaaaat	atttttagcca	taaataaatg	accagcatat	tttacaacct	1260
tagtaattca	taaattccta	tatgtatatt	tgaaattaaa	aacagataat	cgtaaaggga	1320
aggaatccta	cgatcatctt	tgccatttgt	ttttcatgca	aacagaaaag	gacgaaaaac	1380
cacctcacca	tgaatcactc	ttcacaccat	ttttactagc	aaacaagtct	caacaactga	1440
agccagctct	ctttccggtt	cttttttaca	cactttcttt	gaaatagtag	tatttttttt	1500
tcacatgatt	tattaacgtg	ccaaaagatg	cttattgaat	agagtgcaca	tttgtaatgt	1560
actactaatt	agaacatgaa	aaagcattgt	tctaacacga	taatcctgtg	aaggcgtaa	1620
ctccaaagat	ccaatttcac	tatataaatt	gtgacgaaag	caaaatgaat	tcacatagct	1680
gagagagaaa	ggaaagggtta	actaagaagc	aatacttca			1719

<210> 15
 <211> 1553
 <212> DNA
 <213> Glycine max

<400> 15

ccccgtacat	ccgtccctca	ttacatgaaa	agaaatattg	tttatattct	taattaaaaa	60
tattgtccct	tctaaatttt	catatagtta	attattatat	tacttttttc	tctattctat	120
tagttctatt	ttcaaattat	tatttatgca	tatgtaaagt	acatttatatt	tttgctatat	180
acttaaatat	ttctaaatta	ttaaaaaaag	actgatatga	aaaattttatt	cttttttaaag	240
ctatatcatt	ttatatatac	tttttctttt	cttttctttc	attttctatt	caattttaata	300
agaaataaat	tttgtaaatt	tttattttatc	aatattataaa	aatatttttac	tttatatggt	360
ttttcacatt	tttgttaaac	aaatcatatc	attatgattg	aaagagagga	aattgacagt	420
gagtaataag	tgatgagaaa	aaaatgtgtt	atttcctaaa	aaaaacctaa	acaaacatgt	480
atctactctc	tatttcatct	atctctcatt	tcattttttct	ctttatctct	ttctttattt	540
ttttatcata	tcattttcaca	ttaattattt	ttactctctt	tattttttct	ctctatccct	600
ctcttatttt	cactcatata	tacactccaa	aattggggca	tgccctttatc	actactctat	660
ctcctccact	aatcatttta	aatgaaactg	aaaagcattg	gcaagtctcc	tcccctctc	720

BB1531 Canada Seq Lst.txt

aagtgatttc	caactcagca	ttggcatctg	attgattcag	tatatctatt	gcatgtgtaa	780
aagtctttcc	acaatacata	actattaatt	aatcttaaat	aaataaagga	taaaatattt	840
ttttttcttc	ataaaattaa	aatatgttat	tttttgttta	gatgtatatt	cgaataaatc	900
taaatatatg	ataatgattt	tttatattga	ttaaacatat	aatcaatatt	aaatatgata	960
ttttttttata	taggttgtac	acataatttt	ataaggataa	aaaatatgat	aaaaataaat	1020
tttaaatatt	tttatattta	cgagaaaaaa	aaatatttta	gccataaata	aatgaccagc	1080
atatttttaca	accttagtaa	ttcataaatt	cctatatgta	tatttgaaat	taaaaacaga	1140
taatcgttaa	gggaaggaat	cctacgtcat	ctcttgccat	ttgtttttca	tgcaaacaga	1200
aagggacgaa	aaaccacctc	accatgaatc	actcttcaca	ccattttttac	tagcaaacaa	1260
gtctcaacaa	ctgaagccag	ctctctttcc	gtttcttttt	acaacacttt	ctttgaaata	1320
gtagtatttt	tttttcacat	gatttattaa	cgtgccaaaa	gatgcttatt	gaatagagtg	1380
cacatttgta	atgtactact	aattagaaca	tgaaaaagca	ttgttctaac	acgataatcc	1440
tgtgaaggcg	ttaactccaa	agatccaatt	tcactatata	aattgtgacg	aaagcaaaat	1500
gaattcacat	agctgagaga	gaaaggaaag	gttaactaag	aagcaatact	tca	1553

<210> 16
 <211> 1367
 <212> DNA
 <213> Glycine max

<400> 16

atattttctaa	attattaaaa	aaagactgat	atgaaaaaatt	tattctttttt	aaagctatat	60
catttttata	atactttttc	ttttcttttc	tttcattttc	tattcaattt	aataagaaat	120
aaatttttga	aattttttatt	tatcaattta	taaaaaatatt	ttactttata	tgttttttca	180
catttttgtt	aaacaaatca	tatcattatg	attgaaagag	aggaaattga	cagtgagtaa	240
taagtgatga	gaaaaaaatg	tgttattttcc	taaaaaaaac	ctaaacaaac	atgtatctac	300
tctctatttc	atctatctct	catttcattt	ttctctttat	ctctttcttt	atttttttat	360
catatcattt	cacattaatt	attttttactc	tctttatttt	ttctctctat	ccctctctta	420
tttccactca	tatatacact	ccaaaattgg	ggcatgcctt	tatcactact	ctatctcctc	480
cactaaatca	tttaaatgaa	actgaaaagc	attggcaagt	ctcctcccct	cctcaagtga	540
tttccaactc	agcattggca	tctgattgat	tcagtatatc	tattgcatgt	gtaaaagtct	600
ttccacaata	cataactatt	aattaatctt	aaataaataa	aggataaaat	attttttttt	660
cttcataaaa	ttaaaatatg	ttattttttg	tttagatgta	tattcgaata	aatctaaata	720
tatgataatg	atttttttata	ttgattaaac	atataatcaa	tattaaatat	gatatttttt	780
tatataggtt	gtacacataa	ttttataagg	ataaaaaata	tgataaaaat	aaatttttaa	840
tattttttata	tttacgagaa	aaaaaaatat	tttagccata	aataaatgac	cagcatattt	900
tacaacctta	gtaattcata	aattcctata	tgtatatattg	aaattaaaaa	cagataatcg	960
ttaagggaag	gaatcctacg	tcattctcttg	ccatttggtt	ttcatgcaa	cagaaaggga	1020
cgaaaaacca	cctcaccatg	aatcactctt	cacaccattt	ttactagcaa	acaagtctca	1080
acaactgaag	ccagctctct	ttccgtttct	ttttacaaca	ctttctttga	aatagtagta	1140
tttttttttc	acatgattta	ttaacgtgcc	aaaagatgct	tattgaatag	agtgcacatt	1200
tgtaatgtac	tactaattag	aacatgaaaa	agcattgttc	taacacgata	atcctgtgaa	1260
ggcgttaact	ccaaagatcc	aatttcta	tataaattgt	gacgaaagca	aaatgaattc	1320
acatagctga	gagagaaagg	aaaggttaac	taagaagcaa	tacttca		1367

<210> 17
 <211> 1160
 <212> DNA
 <213> Glycine max

<400> 17

atgattgaaa	gagaggaaat	tgacagtgag	taataagtga	tgagaaaaaa	atgtgttatt	60
tcctaaaaaa	aacctaaaca	aacatgtatc	tactctctat	ttcatctatc	tctcatttca	120
tttttctctt	tatctctttc	tttatttttt	tatcatatca	tttcacatta	attattttta	180
ctctctttat	tttttctctc	tatccctctc	ttattttccac	tcataataac	actccaaaat	240
tggggcatgc	ctttatcact	actctatctc	ctccactaaa	tcattttaaat	gaaactgaaa	300
agcattggca	agtctcctcc	cctcctcaag	tgattttcaa	ctcagcattg	gcatctgatt	360
gattcagtat	atctattgca	tgtgtaaaaag	tctttccaca	atacataact	attaattaat	420
cttaataaaa	taaaggataa	aatatttttt	tttcttcata	aaattaaaaat	atgtttatttt	480

BB1531 Canada Seq Lst.txt

ttgttttagat	gtatatctga	ataaatctaa	atatatgata	atgatttttt	atattgatta	540
aacatataat	caatattaaa	tatgatattt	ttttatatag	gttgtagaca	taattttata	600
aggataaaaa	atatgataaa	aataaatttt	aaatattttt	atattttacga	gaaaaaaaaa	660
tatttttagcc	ataaataaat	gaccagcata	ttttacaacc	ttagtaattc	ataaattcct	720
atatgtatat	ttgaaattaa	aaacagataa	tcgttaaggg	aaggaatcct	acgtcatctc	780
ttgccatttg	tttttcatgc	aaacagaaaag	ggacgaaaaa	ccacctcacc	atgaatcact	840
cttcacacca	tttttactag	caaacaagtc	tcaacaactg	aagccagctc	tctttccggt	900
tctttttaca	acactttctt	tgaaatagta	gtattttttt	ttcacatgat	ttattaacgt	960
gccaaaagat	gcttattgaa	tagagtgcac	atttgtaatg	tactactaat	tagaacatga	1020
aaaagcattg	ttctaacacg	ataatcctgt	gaaggcggtta	actccaaaga	tccaatttca	1080
ctatataaat	tgtgacgaaa	gcaaaatgaa	ttcacatagc	tgagagagaa	aggaaagggt	1140
aactaagaag	caatacttca					1160

<210> 18
 <211> 967
 <212> DNA
 <213> Glycine max

<400> 18

ttctctctat	ccctctctta	tttccactca	tatatacact	ccaaaattgg	ggcatgcctt	60
tatcactact	ctatctcctc	cactaaatca	tttaaatgaa	actgaaaagc	attggcaagt	120
ctcctcccct	cctcaagtga	tttccaactc	agcattggca	tctgattgat	tcagtatatc	180
tattgcatgt	gtaaaagtct	ttccacaata	cataactatt	aattaatctt	aaataaataa	240
aggataaaat	attttttttt	cttcataaaa	ttaaaatatg	ttattttttg	tttagatgta	300
tattcgaata	aatctaaata	tatgataatg	atttttttata	ttgattaaac	atataatcaa	360
tattaaatat	gatatttttt	tatatagggt	gtacacataa	ttttataagg	ataaaaaata	420
tgataaaaaat	aaatttttaa	tattttttata	tttacgagaa	aaaaaaatat	tttagccata	480
aataaatgac	cagcatatct	tacaacctta	gtaattcata	aattcctata	tgtatatttg	540
aaattaaaaa	cagataatcg	ttaagggaag	gaatcctacg	tcattctctg	ccatttggtt	600
ttcatgcaaa	cagaaaggga	cgaaaaacca	cctcaccatg	aatcactctt	cacaccattt	660
ttactagcaa	acaagtctca	acaactgaag	ccagctctct	ttccgtttct	ttttacaaca	720
ctttctttga	aatagtagta	tttttttttc	acatgattta	ttaacgtgcc	aaaagatgct	780
tattgaatag	agtgcacatt	tgtaatgtac	tactaattag	aacatgaaaa	agcattgttc	840
taacacgata	atcctgtgaa	ggcggttaact	ccaagatcc	aatttcacta	tataaattgt	900
gacgaaagca	aatgaattc	acatagctga	gagagaaagg	aaagggttaac	taagaagcaa	960
tacttca						967

<210> 19
 <211> 770
 <212> DNA
 <213> Glycine max

<400> 19

tctttccaca	atacataact	attaattaat	cttaaataaa	taaaggataa	aatatttttt	60
tttcttcata	aaattaaaaat	atgttatattt	ttgttttagat	gtatatctga	ataaatctaa	120
atatatgata	atgatttttt	atattgatta	aacatataat	caatattaaa	tatgatattt	180
ttttatatag	gttgtagaca	taattttata	aggataaaaa	atatgataaa	aataaatttt	240
aaatattttt	atattttacga	gaaaaaaaaa	tatttttagcc	ataaataaat	gaccagcata	300
ttttacaacc	ttagtaattc	ataaattcct	atatgtatat	ttgaaattaa	aaacagataa	360
tcgttaaggg	aaggaatcct	acgtcatctc	ttgccatttg	tttttcatgc	aaacagaaaag	420
ggacgaaaaa	ccacctcacc	atgaatcact	cttcacacca	tttttactag	caaacaagtc	480
tcaacaactg	aagccagctc	tctttccggt	tctttttaca	acactttctt	tgaaatagta	540
gtattttttt	ttcacatgat	ttattaacgt	gccaaaagat	gcttattgaa	tagagtgcac	600
atttgtaatg	tactactaat	tagaacatga	aaaagcattg	ttctaacacg	ataatcctgt	660
gaaggcggtta	actccaaaga	tccaatttca	ctatataaat	tgtgacgaaa	gcaaaatgaa	720
ttcacatagc	tgagagagaa	aggaaagggt	aactaagaag	caatacttca		770

<210> 20

BB1531 Canada Seq Lst.txt

<211> 584
 <212> DNA
 <213> Glycine max

<400> 20
 ataggttgta cacataattt tataaggata aaaaatatga taaaaataaa ttttaaatat 60
 ttttatattt acgagaaaaa aaaatatatt agccataaat aaatgaccag catattttac 120
 aaccttagta attcataaat tcctatatgt atatttgaaa ttaaaaacag ataatcgta 180
 agggaaggaa tcctacgtca tctcttgcca tttgtttttc atgcaaacag aaagggacga 240
 aaaaccacct caccatgaat cactcttcac accattttta ctagcaaaca agtctcaaca 300
 actgaagcca gctctctttc cgtttctttt tacaacactt tctttgaaat agtagtattt 360
 ttttttcaca tgatttatta acgtgccaaa agatgcttat tgaatagagt gcacatttgt 420
 aatgtactac taattagaac atgaaaaagc attgttctaa cacgataatc ctgtgaaggc 480
 gttaactcca aagatccaat ttcactatat aaattgtgac gaaagcaaaa tgaattcaca 540
 tagctgagag agaaaggaaa ggtaactaa gaagcaatac ttca 584

<210> 21
 <211> 425
 <212> DNA
 <213> Glycine max

<400> 21
 attaaaaaca gataatcggt aagggaagga atcctacgtc atctcttgcc atttgttttt 60
 catgcaaaca gaaagggacg aaaaaccacc tcaccatgaa tcactcttca caccattttt 120
 actagcaaac aagtctcaac aactgaagcc agctctcttt ccgtttcttt ttacaacact 180
 ttctttgaaa tagtagtatt tttttttcac atgatttatt aacgtgccaa aagatgctta 240
 ttgaatagag tgcacatttg taatgtacta ctaattagaa catgaaaaag cattgttcta 300
 acacgataat cctgtgaagg cgtaactcc aaagatccaa tttcactata taaattgtga 360
 cgaaagcaaa atgaattcac atagctgaga gagaaaggaa aggttaacta agaagcaata 420
 cttca 425

<210> 22
 <211> 174
 <212> DNA
 <213> Glycine max

<400> 22
 gcacatttgt aatgtactac taattagaac atgaaaaagc attgttctaa cacgataatc 60
 ctgtgaaggc gttaactcca aagatccaat ttcactatat aaattgtgac gaaagcaaaa 120
 tgaattcaca tagctgagag agaaaggaaa ggtaactaa gaagcaatac ttca 174

What is claimed is:

1. An isolated nucleic acid fragment comprising a seed-specific promoter wherein said promoter:

- 5 (i) comprises of the nucleotide sequence set forth in SEQ ID NO: 1, or a nucleotide sequence with at least 95% identity to the sequence set forth in SEQ ID NO: 1 and which is capable of controlling expression of a coding sequence or functional RNA in a similar manner to the sequence set forth in SEQ ID NO: 1; or
- 10 (ii) is a fragment of the nucleotide sequence set forth in SEQ ID NO: 1 and comprises the nucleotide sequence set forth in any one of SEQ ID NOs: 13-22, or a nucleotide sequence with at least 95% identity to any of the sequences set forth in any one of SEQ ID NOs: 13-22 and which is capable of controlling expression of a coding sequence or functional RNA in a similar manner to the corresponding sequence set forth in
15 any one of SEQ ID NOs: 13-22.

2. The isolated nucleic acid fragment of claim 1 wherein the the promoter comprises the nucleotide sequence set forth in any one of SEQ ID Nos: 1, or 13-20.

20 3. An isolated nucleic acid fragment as claimed in any one of Claims 1 or 2 wherein said promoter is a soybean promoter.

4. A recombinant expression construct comprising at least one heterologous nucleic acid fragment operably linked to any one of the isolated nucleic acid fragments of any one of Claims 1, 2 or 3.

25 5. A plant cell comprising in its genome the recombinant expression construct of Claim 4.

6. The plant cell of Claim 5 wherein said plant cell is from a dicotyledonous plant.

7. The plant cell of Claim 6 wherein the plant cell is a soybean cell.

30 8. The recombinant expression construct of Claim 4 wherein the heterologous nucleic acid fragment encodes an enzyme related to production of at least one long chain polyunsaturated fatty acid.

9. A method for regulating expression of at least one heterologous nucleic acid fragment in plant which comprises:

- 35 (a) transforming a plant cell with the recombinant expression construct of Claim 4;
- (b) growing fertile mature plants from transformed plant cell of step (a); and

- (c) selecting a plant comprising a transformed plant cell expressing the heterologous nucleic acid fragment.

10. The method of Claim 9 wherein the plant is a soybean plant.

5 11. The method of Claim 9 wherein the heterologous nucleic acid fragment encodes an enzyme related to production of at least one long chain polyunsaturated fatty acid.

12. A method for regulating expression of at least one heterologous nucleic acid fragment in a plant which comprises:

- 10 (a) transforming a plant cell with a recombinant expression construct comprising at least one heterologous nucleic acid fragment operably linked to the isolated nucleic acid fragment of any of Claims 1, 2 or 3;
- (b) growing a fertile mature plant from transformed plant cell of step (a); and
- 15 (c) selecting a plant comprising a transformed plant cell expressing the heterologous nucleic acid fragment during early seed development.

Figure 1: Soybean Annexin Promoter-GUS in Arabidopsis

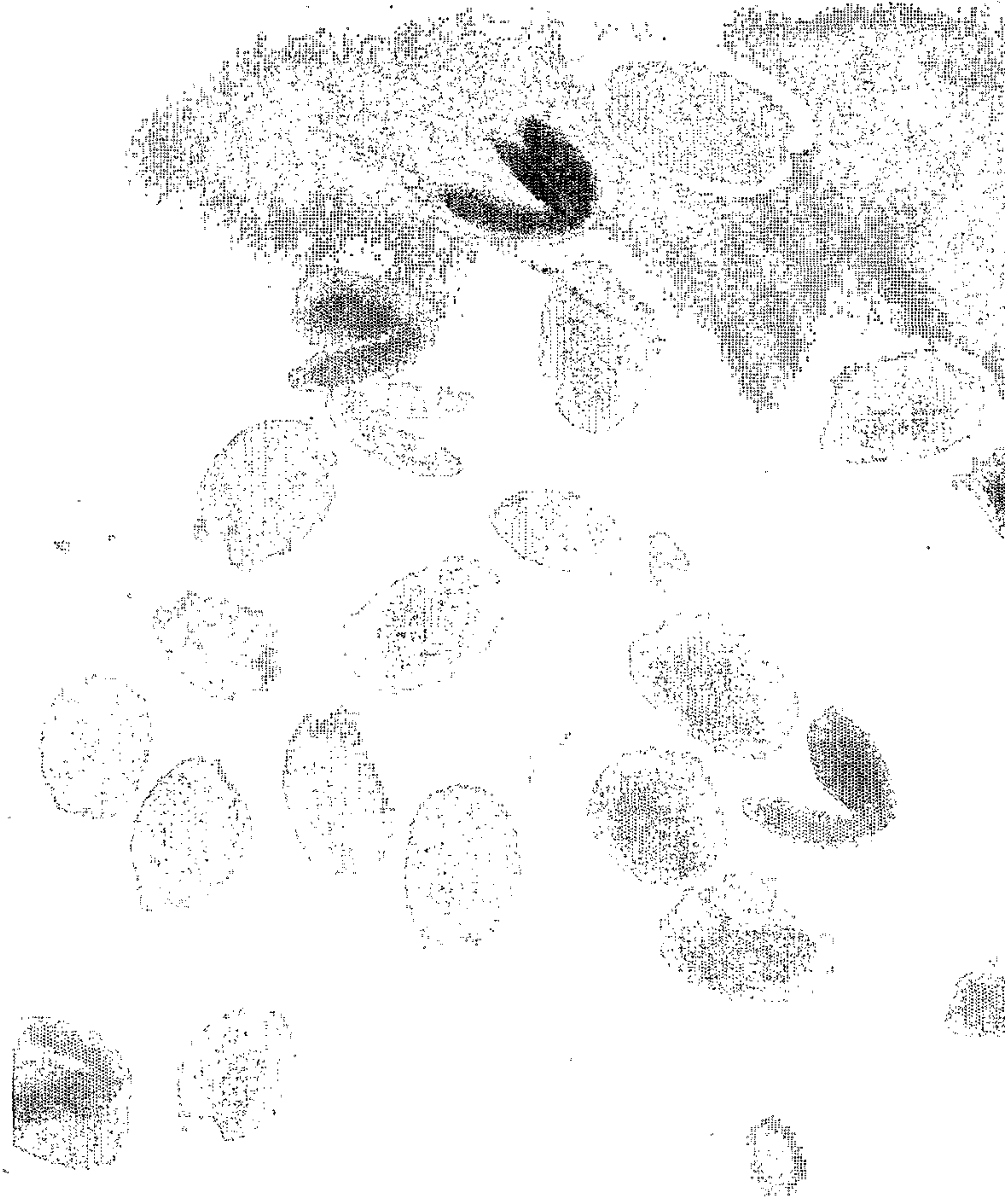


Figure 2: Soybean P34 Promoter-GUS in Arabidopsis

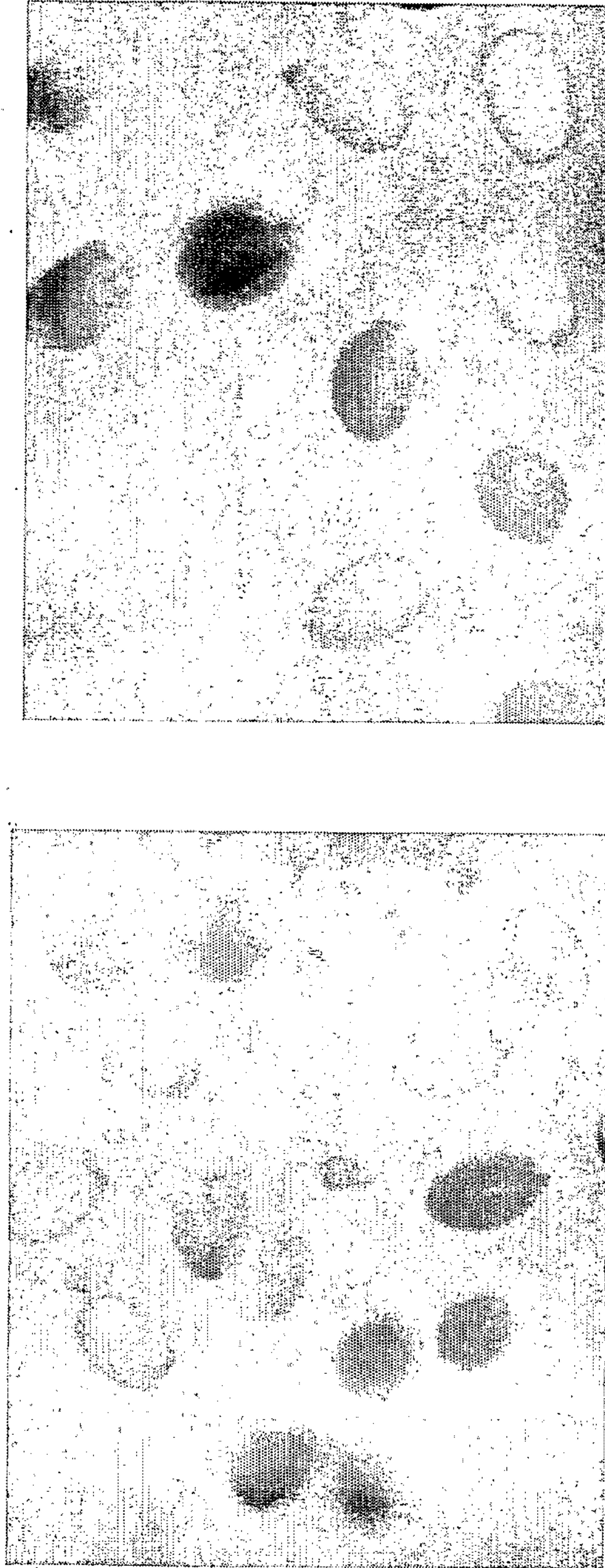


Figure 3: Soybean Seed Promoter Temporal Expression Patterns

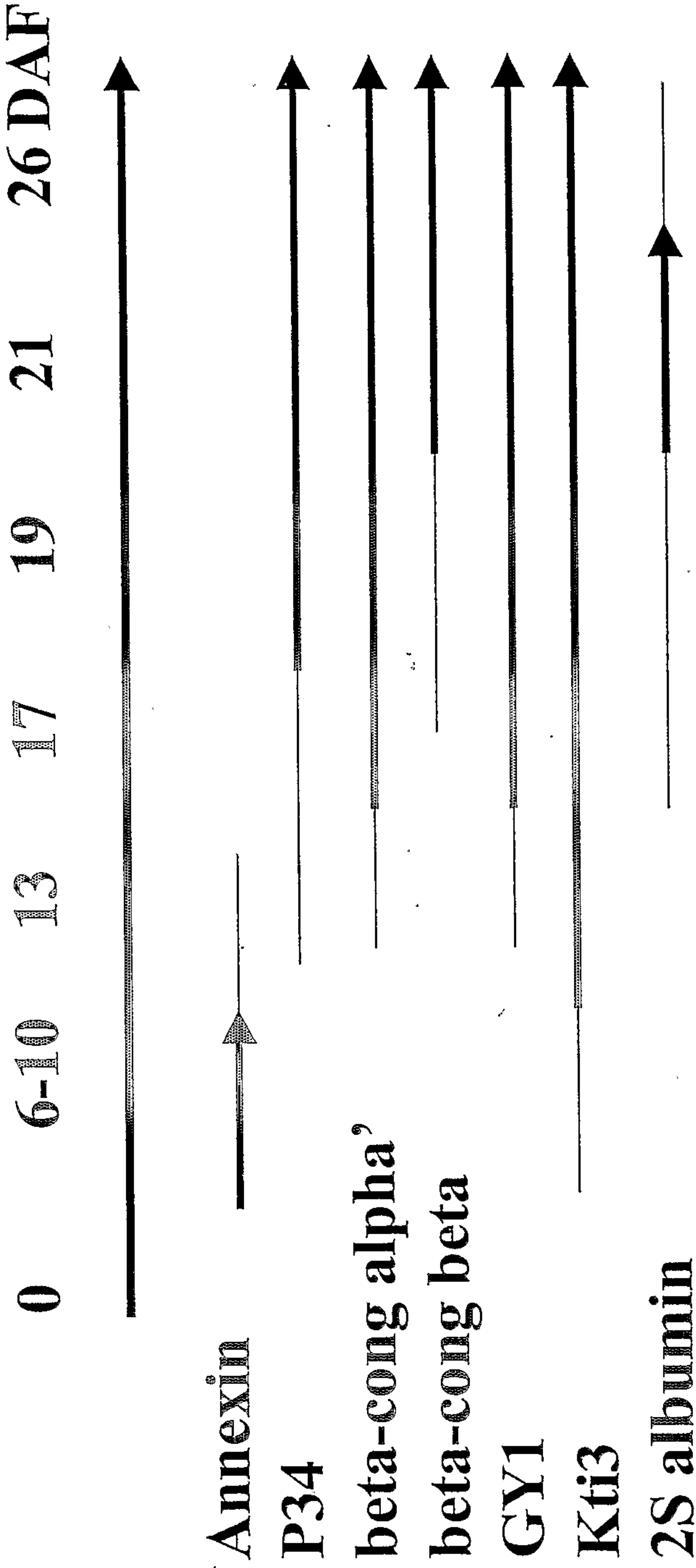


Figure 4: GLA Accumulation in Soybean Somatic Embryos

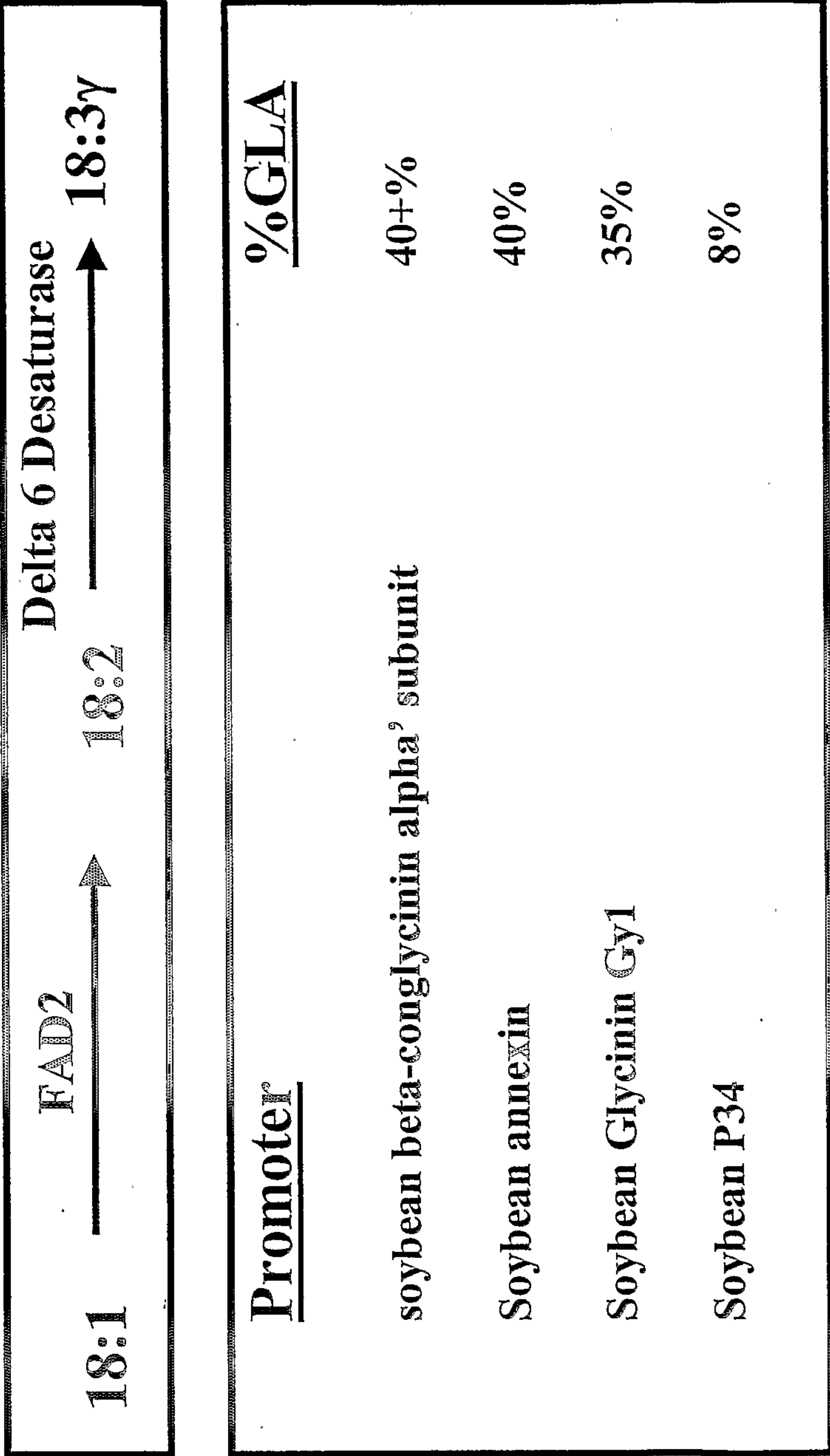


Figure 5: Deletion analyses of soybean Annexin promoter

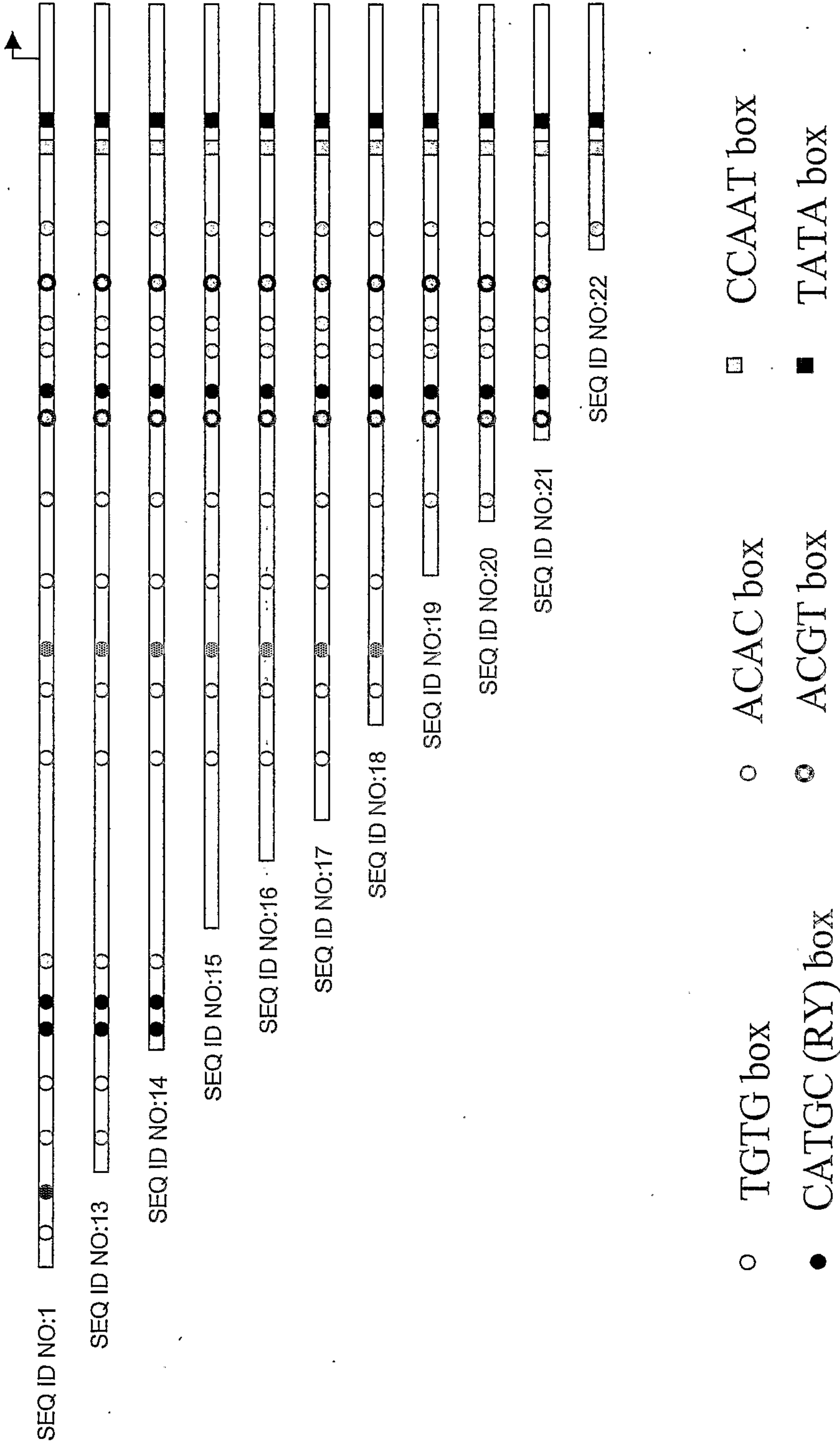
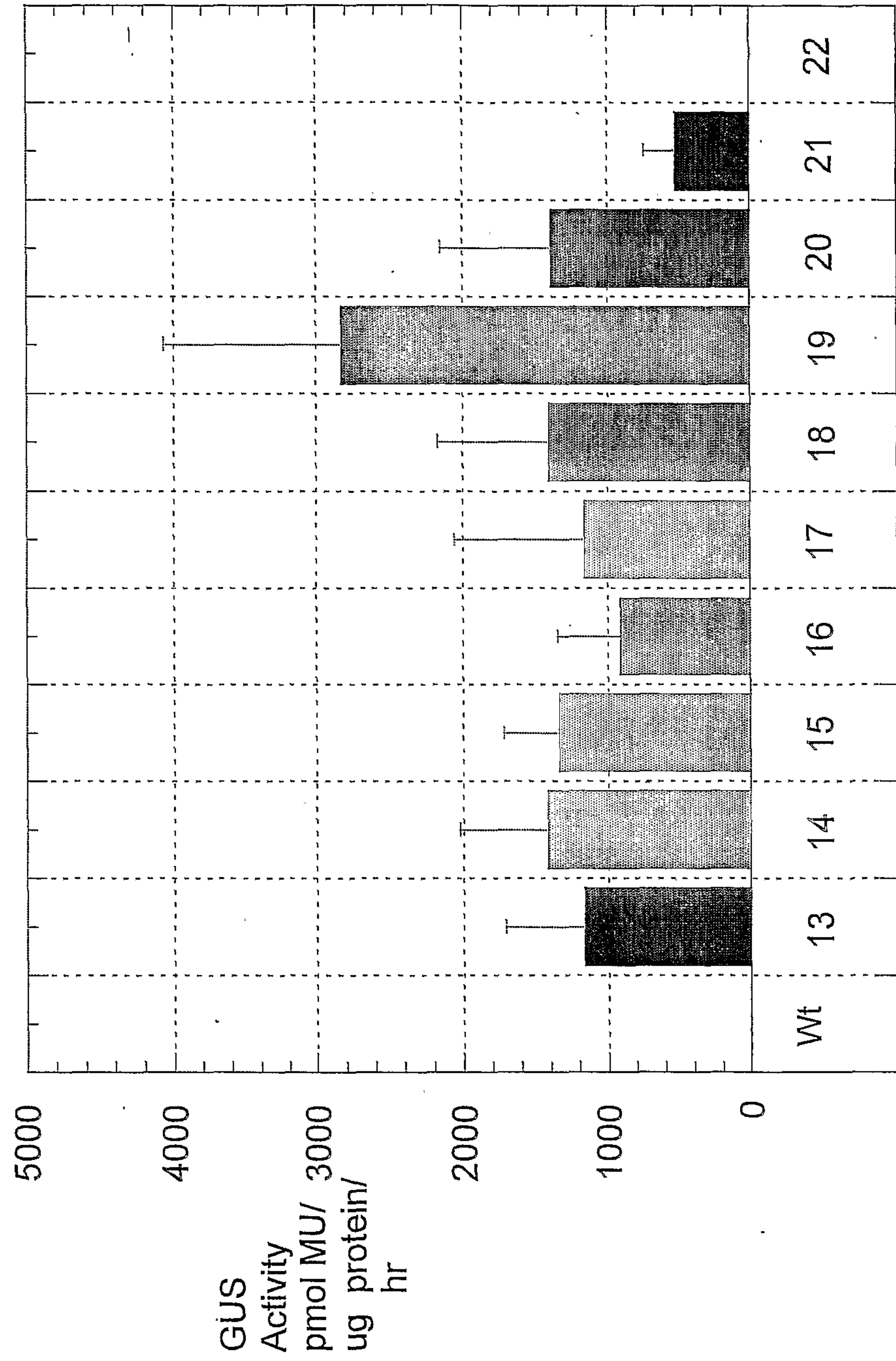


FIGURE 6

Soybean Annexin Promoter Deletion



Annexin Promoter (SEQ ID NO)

Soybean Annexin Promoter Deletion

