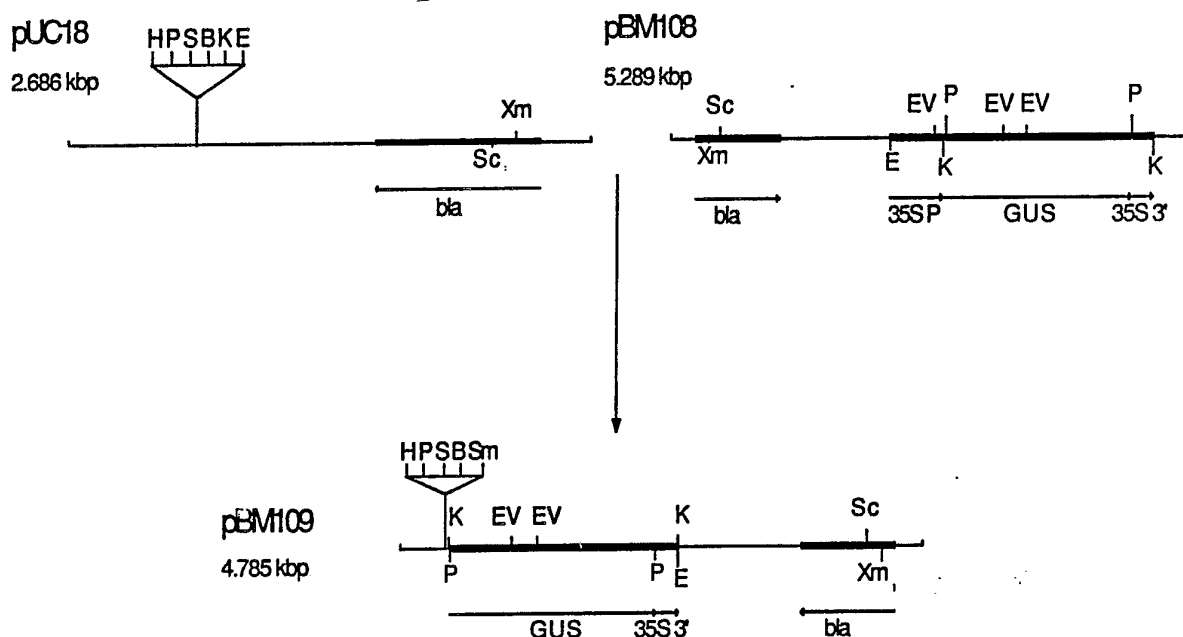




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(21) International Application Number: PCT/US89/00973 (22) International Filing Date: 15 March 1989 (15.03.89) (31) Priority Application Numbers: 174,744 247,185 (32) Priority Dates: 29 March 1988 (29.03.88) 21 September 1988 (21.09.88) (33) Priority Country: US (71) Applicants: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US). STATE OF OREGON acting by and through THE OREGON STATE BOARD OF HIGHER EDUCATION ON BEHALF OF OREGON STATE UNIVERSITY [US/US]; Corvallis, OR 97331 (US). (72) Inventors: LITTS, James, Cooper ; 1302 L Street, Davis, CA 95616 (US). MARCOTTE, William, Richard, Jr. ; 2007 Ferguson Drive, Wilmington, DE 19808 (US). QUATRANO, Ralph, Stephen ;			1900 Greenhill Avenue, Wilmington, DE 19806 (US). (74) Agent: MORRISSEY, Bruce, W.; E.I. du Pont de Nemours and Company, 1007 Market Street, Wilmington, DE 19898 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>Without international search report and to be republished upon receipt of that report.</i>

(54) Title: DNA PROMOTER FRAGMENTS FROM WHEAT**(57) Abstract**

The preparation and use of nucleic acid promoter fragments homologous to the Em gene of wheat to bring the expression of selected genes in plants under external control are described. The Em promoter fragment is responsive to abscisic acid (ABA) and other compounds possessing ABA-like activity. Through transformation of protoplasts and plant cells with recombinant DNA constructs incorporating such promoter fragments, operably linked selected genes are expressed in response to ABA and compounds possessing ABA-like activity. The application of such promoter fragments and constructs to transient assay systems to predict the likelihood of stable transformation in plants is disclosed.

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TITLE

5 DNA PROMOTER FRAGMENTS FROM WHEAT

RELATED APPLICATION

 This application is a continuation-in-part of
 copending application U.S.S.N. 174,744 filed
10 March 29, 1988.

FIELD OF THE INVENTION

 This invention relates to the preparation of
 nucleic acid promoter fragments homologous to the Em
15 gene of wheat which are responsive to compounds
 possessing abscisic acid-like activity, and their
 incorporation into recombinant DNA constructs to
 bring the expression of nucleic acid sequences for
 selected gene products operably linked to the
20 promoter fragments under external control in
 protoplasts and plants.

BACKGROUND OF THE INVENTION

 The external control of the expression of
25 selected gene products in plants through the
 application of chemical substances can provide
 important agronomic and foodstuff benefits. The
 ability to externally control the expression of
 introduced genes in transgenic plants could be used
30 to (1) prolong or extend the accumulation of
 desirable nutritional food reserve in seeds, roots,
 or tubers, (2) produce and accumulate products in
 plant tissues that are convenient for harvest and
 isolation, and (3) initiate expression of a toxin at
35 the site of pathogen attack, possibly avoiding
 contamination of the ultimate food product with the

toxin. These and other benefits have been
5 unattainable in the field since means to bring known
plant genes under external control in the field have
not been available.

While technology exists to transform plants
with the genes for selected products, their
10 expression is either continuous throughout the life
cycle (controlled by a constitutive promoter), or is
regulated by the developmentally timed program of
maturation inherent in each organ/tissue/cell (stage
or tissue specific promoters). Continuous expression
15 precludes production at particular stages, in
specific tissues or in response to environmentally
unpredictable events. Such expression could place a
major penalty on yield, due to greatly increased
energy demands accompanying prolonged synthesis of
20 the product. Tissue or stage specific expression,
although valuable for the temporal and spatial
accumulation of products, is under the variable
timing of the developmental program of the plant.
Precise developmental control of gene expression
25 would necessitate the isolation of a multitude of
stage and tissue specific promoters in all crop
plants of interest. It would be desirable to
externally control the expression of an introduced
gene product by the application of a specific
30 chemical which can induce a specific promoter to
activate the accumulation of a desired gene product.
Hence, one chemical/promoter combination can be
interactive at any stage or in any tissue available
to the chemical, throughout the life cycle of any one
35 of a large number of transformable plants, to control
any desired gene product.

Certain genes can be induced by environmental
5 factors such as light, heat shock and anaerobiosis.
The promoters of these inducible genes from plants
have been extensively analyzed (c.f., Kuhlemeier
et al., Ann. Rev. Pl. Physiol., 38:221-257 (1987)).
However, the inducers of these genes (i.e., light,
10 temperature and O₂ levels) are not easily or
practically controllable under normal agronomic
techniques.

Numerous chemicals, both natural and synthetic,
have been shown to affect the growth and development
15 of plants. A number of these plant growth regulators
occur naturally, such as the hormones auxin,
gibberellic acid, cytokinins, ethylene and abscisic
acid (c.f., Davies, P. (Ed.) Plant Hormones and Their
Roles In Plant Growth and Development, Martinus
20 Nijhoff Publ. (1987)), while others have equally
dramatic effects such as salicylic acid (Hooft
Vanhuijsduijnen et al., J. Gen. Virol., 67:235-2143
(1986)). When these growth regulators are applied to
various plant cells/tissues/organs, a change in the
25 metabolism of the plant is observed which has been
shown with each of the above regulators to result
from new gene expression. Some products of these
genes, as well as some genes themselves, have been
isolated and characterized. The instant invention
30 focuses on the phytohormone abscisic acid (ABA),
3-methyl-5-(1'-hydroxy-4'-oxo-2',6',6'-trimethyl-2'-
cyclohexen-1'-yl)-cis, trans-2,4-pentadienoic acid
(c.f. Addicott, Absciscic Acid, Praeger Publ., N.Y.
pp. 607 (1983)). ABA is involved in numerous
35 processes such as stomatal closure, bud/seed
dormancy, inhibition of seed germination, as well as
in responses of plants to physical perturbations or

stresses such as temperature and water (c.f. Zeevaart
5 and Creelman, Ann. Rev. Plant Physiol., 39:439-473
(1988)). Numerous compounds have been described that
mimic ABA in these physiological process, i.e., they
are ABA-like (c.f. Walton, In Abscisic Acid (Ed.
Addicott), Chapt. 4, pp. 113-146 (1983)). Our
10 immediate interest focuses on the involvement of ABA
in two of these key plant processes; accumulation of
storage reserves in seeds and the response of plants
to water stress (drought).

During seed formation in a wide variety of
15 seeds, the endogenous ABA level increases
dramatically, as it does in water-stressed tissue.
Genes from the seeds of six plants (cotton, soybean,
rice, maize, wheat and rapeseed) have been isolated
that respond to ABA (when tissue is treated in vitro
20 with the phytohormone) by accumulating the products
of these same genes. The response is an increase in
the mRNA levels of these specific genes (c.f.,
Quatrano, Oxford Surveys Pl. Mol. Cell. Biol.,
3:467-477 (1986)). Although the characterization of
25 some of these genes has been extensive, only in the
case of the β -conglycinin gene from soybean has an
ABA-regulated gene been expressed in a transgenic
plant; in petunia and tobacco by Bray et al., Planta
172:364-370 (1987) and in tobacco by Barker et al.,
30 Proc. Natl. Acad. Sci. (USA) 85:458-462 (1988).

The goal in each case is to identify the DNA
sequences that control the expression of these genes
in a tissue-specific, stage-specific and
environmentally regulated manner. Bray et al.
35 demonstrated this specific regulation in transgenic
plants when a 4.2 kilobase (kb) fragment of the
natural gene was transferred. In addition to the

coding region, it contained a 1.1 kb 5' fragment and
5 a 1.3 kb 3' fragment. Barker et al. transferred to
tobacco a much larger fragment (12.3 kb) that
contained more genes than the β -conglycinin gene.
Neither group characterized further, which part(s) of
these large fragments might be responsible for the
10 specific ABA induction effect, nor did they report
treating transformed tissue with ABA to determine if
the foreign gene would respond to hormone addition.

Gomez et al., Nature 334:262-264 (1988),
reported that when immature maize embryos, like
15 wheat, are cultured in the presence of ABA they
express a new set of genes in response to the
hormone. One ABA-regulated gene (pMAH9), isolated in
this report, codes for a 15.4 Kd protein. The mRNA
for this protein is also expressed when mature leaf
20 tissue is wounded or water stressed. The complete
sequence of the coding region and parts of the 3' and
5' flanking regions are given, but no information is
reported as to the sequence within the promoter
region which is essential for ABA regulation.

25 Mundy and Chua, EMBO Journal, 7:2279-2286
(1988), characterized the full genomic sequence of a
gene from rice (RAB 21) which is induced by ABA, as
well as by salt (NaCl), and water stress in numerous
tissues, e.g., roots, leaves, embryos, suspension
30 cultures. Although the complete sequence of the
coding region (approximately 1 kb) and the 5'
regulatory or promoter region (approximately 1.5 kb)
is given, no information is reported as to the
sequences within the promoter region which are
35 essential for ABA regulation of the coding
sequences. There is no teaching regarding the
isolation of a promoter fragment, or its potential

utility to achieve external control of plant gene
5 expression. The authors themselves (p. 2285) state
that "the regulatory roles of these different GC-rich
repeats remains to be established...." Applicant's
invention lies in the isolation of promoter
fragments, their use in constructs to transform
10 protoplasts and plants, and in achieving the external
control of plant gene expression.

Other seed-specific genes that have not been
shown to be regulated by ABA, or by any other
hormone, have been analyzed in the same manner. For
15 example, Chen et al., Proc. Natl. Acad. Sci. (USA)
83:8560-8564 (1986) showed that the region between
-159 and -257 base pairs (bp) 5' to the transcription
start site of the α -conglycinin gene is the
required region for seed specificity. More recently,
20 Chen et al., EMBO Journal, 7:297-302 (1988),
constructed a chimeric gene comprising a 170 bp
fragment from the α -conglycinin promoter region
(-78 to -257) in different positions and orientations
to the constitutive viral 35S promoter linked to the
25 reporter gene chloramphenicol acetyl transferase
(CAT). They showed that the 170 bp 5' fragment
enhances expression of the CAT gene in a
tissue-specific and temporally regulated manner.

However, delineation of the specific regulatory
30 regions required these investigators to assay for
expression in transgenic plants at the stage of seed
formation. This required long periods of time until
the plants flowered and set seed (several months).
Transient assays for promoter analysis have been
35 reported and represent a faster way to delineate
those promoter fragments that are regulatory.

However, none of these reports which follow involve
5 the use of a chemical inducer.

Ebert et al., Proc. Natl. Acad. Sci. (USA)
84:5745-5749 (1987), disclose studies of the active
fragment of DNA constituting the nopaline synthase
promoter. This promoter is constitutive rather than
10 inducible, and while of bacterial origin, operates in
a wide range of plant tissues. A construction was
made such that the promoter controlled the expression
of the reporter gene CAT. The authors reported that
a fragment of 33 bp (-97 to -130) of DNA was
15 necessary to promote expression of the CAT gene.
They reported further that the presence of two copies
of the fragment tripled the expression of the CAT
gene. These results from stably transformed tobacco
tissue were repeatable in a transient assay using
20 tobacco protoplasts. Comparison of the level of CAT
gene expression controlled by the fragments in both
the transient expression of tobacco protoplasts and
stably transformed tissues resulted in some
differences. The authors nevertheless indicated
25 their belief that such transient assays are valuable
for "the study of upstream elements and trans-acting
factors".

Howard, et al., Planta, 170:535-540 (1987),
studied the anaerobic induction of the maize alcohol
30 dehydrogenase (Adhl) gene by electroporating gene
fragments of Adhl into maize protoplasts from
suspension culture cells, subjecting these
protoplasts to reduced oxygen levels and assaying for
Adhl expression 20 hours later. To facilitate
35 measurement of Adhl gene expression regulated by
anaerobiosis, they fused the 5' promoter or
regulatory fragment of the native Adhl gene (1096

base pairs) to CAT. Their results demonstrated the
5 normal regulation of an inducible promoter from a
monocot maize gene (i.e., Adh1) when electroporated
into protoplasts derived from a homologous cell
culture system. They showed that just the Adh1
promoter fragment, without the coding and 3' regions
10 of the Adh1 gene, is sufficient for anaerobic
induction of the CAT gene.

Lee et al., Plant Physiology 85:327-330 (1987),
have reported further definition of the size of the
DNA fragment responsible for anaerobic induction of
15 the maize Adh1 gene. Utilizing a plasmid vector that
facilitated placement of any desired gene adjacent
and 3' to the promoter, they inserted the gene for
CAT next to the Adh1 gene. Using this plasmid, they
transformed maize protoplasts and measured the
20 production of CAT 24 hours later. By altering the
size of the promoter DNA sequences in the
construction, Lee et al. determined that 146 bp 5' to
the transcription start site were sufficient to place
the production of CAT under anaerobic induction.
25 However, the expression of CAT was increased 5x or 8x
by the addition of 266 or 955 bp, respectively, of
contiguous 5' promoter sequences.

Walker et al., Proc. Natl. Acad. Sci (USA)
84:6624-6628 (1987), continued the studies of the DNA
30 sequences of the 5' promoter region of the maize Adh1
gene required for gene expression induced by
anaerobic conditions in a transient assay. They
determined that control of anaerobic induction of
gene expression resided in two sequences from the
35 promoter: those being the sequence between -133 and
-124 bp and the sequence between -113 and -99 bp (5'

to the transcription start site). Both sequences are
5 necessary. Attachment of this 40 bp element to an
unrelated viral promoter confers anaerobic regulation.

Ellis et al., EMBO Journal 6: 11-16 (1987),
indicate that when the fragment of DNA between base
pairs -1094 and +106 bp of the maize Adh1 gene was
10 placed next to the sequence of DNA encoding CAT and
the construction was stably transformed into tobacco
cells, only extremely low levels of CAT gene
expression could be observed under appropriate
anaerobic conditions. In fact, only CAT messenger
15 RNA was detected. However, promoter elements from
the octopine synthase gene of bacteria, or those from
the Cauliflower Mosaic Virus (CaMV), linked 5' to the
Adh1 promoter, stimulated the expression of the CAT
gene and permitted detection of CAT after anaerobic
20 induction. The fragment of DNA, consisting of 247 bp
adjacent and 5' to the transcription start site of
the structural gene for Adh1, was sufficient to put
the expression of the CAT gene under anaerobic
control. Therefore, anaerobic control by the 247 bp
25 fragment of DNA was maintained even when the octopine
synthase and CaMV 35S promoters, which are
constitutive promoters, were present. The region of
the Adh1 promoter responsible for anaerobic induction
demonstrated in transient assays by Howard et al.,
30 Lee et al., and Walker et al. were similar to the
region showing anaerobic induction in stably
transformed plants by Ellis et al.

Patents have been issued to animal and
microbial systems in which selected gene sequences
35 have been induced by chemicals in the environment
that interact with certain regulatory sequences.
U.S. Patent 4,579,821, issued to Palmiter and

Brinster, discloses the isolation of
5 promoter/regulator sequences of the mouse
metallothionein-I gene and its use to control the
expression of selected DNA sequences operably linked
to the promoter by exposure to heavy metal ions or
steroid hormones. The expression of thymidine kinase
10 fused to the metallothionein-I promoter was obtained
in differentiated cells of adult mice upon admin-
istration of cadmium or dexamethasone. U.S. Patent
4,703,005, issued to Nakata and Shinagawa, discloses
the isolation of a gene for phosphate-binding protein
15 (phoS) to which was fused a foreign gene 3' to phoS.
The foreign gene is controlled by phosphate in the
culture medium.

Despite considerable effort to characterize the
molecular basis of the response of plant tissue to
20 hormones, the signal/transduction pathway from
chemical to gene is not well understood. While
reports of plant promoter sequences stimulated by
light and anaerobic stress have appeared, no
disclosures of inducible plant promoters responsive
25 to chemical substances which can be applied in the
field to control gene expression have appeared. At
this time, a clear need exists for promoter sequences
and recombinant constructs for the transformation of
plants which would enable external control of
30 selected genes which can confer agronomic
advantages. Further, this specificity of expression
should be amenable to external control through
exposure to chemical substances which can be readily
manipulated.

SUMMARY OF THE INVENTION

5 A means to externally control the expression of selected genes in plant cells, plants and protoplasts through exposure to a chemical substance has been discovered. The Em gene of wheat is an inducible gene, responsive to ABA, which is accumulated and
10 implicated in the physiological events of embryo maturation and drought stress protection. Applicants have discovered novel promoter fragments derived from the Em gene which can be used to achieve external control of selected genes, and it is believed that
15 promoter regions from other plant genes homologous to the Em structural gene of wheat will function similarly. Specifically, one preferred aspect of the present invention is a nucleic acid promoter fragment comprising a nucleotide sequence from the 5' flanking
20 promoter region of a plant gene homologous to the Em structural gene of wheat, said promoter fragment being responsive in transformed plant cells to compounds possessing ABA-like activity such that the expression of DNA sequences for selected gene
25 products operably linked on the 3' side of said promoter fragment can be controlled with the proviso that such promoter fragments are not responsive to phaseic acid. Preferred is a nucleic acid promoter fragment comprising a nucleotide sequence from the 5'
30 flanking promoter region of the Em structural gene of wheat, said promoter fragment being responsive in plant cells to compounds possessing ABA-like activity such that the expression of nucleic acid sequences for selected gene products operably linked on the 3'
35 side of said promoter fragment can be controlled. The invention is thus broadly applicable for securing control of many genetic processes, ranging from the

alteration of existing regulation of endogenous genes
5 in plant cells, to securing selective regulation of
expression of selected foreign genes stably
incorporated in plant cells. Preferred compounds
possessing ABA-like activity include ABA, Compound C,
Compound D, Compound E, and Compound F (as defined
10 herein).

Presently, preferred promoter fragments for use
in practice of the invention are derived from the Em
gene of wheat, specifically, an 1850 bp nucleic acid
promoter fragment. More preferred is a 640 bp
15 nucleic acid promoter fragment. Most preferred, by
virtue of ease of use in constructs and effective use
in plants, are a 254 bp and a 50 bp nucleic acid
promoter fragment derived from the regulatory DNA
sequence controlling the expression of the Em
20 structural gene of wheat. Such promoter fragments
are also defined by their nucleic acid sequence.

Another aspect of this invention involves a
recombinant DNA construct capable of modifying the
properties of a protoplast comprising a nucleic acid
25 promoter fragment of the Em gene of wheat, and a DNA
sequence for a selected gene product operably linked
to said promoter fragment such that upon exposure to
a compound possessing ABA-like activity said DNA
sequence for a selected gene product is expressed.
30 Another embodiment involves a recombinant DNA
construct capable of transforming a plant cell
comprising a nucleic acid promoter fragment of the Em
gene of wheat, a DNA sequence for a selected gene
product operably linked to said promoter fragment,
35 and a suitable regulatory sequence such that upon
exposure to a compound possessing ABA-like activity
said DNA sequence for a selected gene product is

expressed. These constructs may be incorporated into
5 DNA plasmid vectors to provide useful tools in the
transformation of a wide variety of plant cells,
plant tissues and plants.

Yet another aspect of this invention involves a
transient assay method to evaluate the likelihood of
10 expression of a selected gene product in a stably
transformed plant comprising the steps of (a)
preparing protoplasts from rice, (b) transferring to
said protoplasts a recombinant DNA construct
incorporating a nucleic acid promoter fragment of the
15 Em gene of wheat and an operably linked DNA sequence
for a selected gene product, (c) incubating said
protoplasts containing said construct with a compound
possessing ABA-like activity, and (d) measuring the
amount of said selected gene product expressed in
20 response to said compounds.

Another process embodiment of the invention
involves a method for controlling the expression of a
selected gene product in a plant comprising the steps
of (a) transforming said plant with a recombinant DNA
25 construct incorporating a nucleic acid promoter
fragment of the Em gene of wheat and an operably
linked DNA sequence for a selected gene product, and
a suitable regulatory sequence, (b) exposing said
plant to a compound possessing ABA-like activity, and
30 (c) causing said plant to express said selected gene
product at a desired time.

A final aspect of the invention involves a
nucleic acid fragment for controlling over-production
of a selected gene product in plants in response to
35 drought conditions comprising a nucleic acid promoter
fragment responsive to ABA inserted into the CaMV 35S
promoter sequence. In this embodiment, it is

believed that environmental factors leading to
5 increased levels of ABA within the plant will trigger
over-production of selected gene products which will
protect the plant.

Characterization of the regulation of the
promoter fragment of the Em gene of wheat has been
10 carried out utilizing rice protoplasts. This system
is preferred based upon the rapid response of
transformed rice protoplasts to compounds possessing
ABA-like activity, the ability to regenerate
transgenic rice plants from protoplasts and to
15 predict the response in a stably transformed plant.

Further, to easily and rapidly measure the
response of a compound possessing ABA-like activity,
a reporter gene is operably linked to the promoter
fragment. The reporter gene chosen is that for the
20 enzyme β -glucuronidase (GUS) for several reasons; no
background enzyme activity is detected in plant
cells, an extremely sensitive and quantitative
fluorometric assay is available, and the enzyme
activity is very stable. A recent review of the
25 merits of using GUS is provided by Jefferson, Pl.
Mol. Biol. Rep., 5:387-405 (1988).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a physical map of plasmid pBM108 as
30 derived from plasmids pRAJ260 and pDH51.

Figure 2 is a physical map of plasmid pBM109 as
derived from plasmids pBM108 and pUC18.

Figure 3 is the nucleotide sequence of the Em
gene 5' flanking region from -1328 to +6 from the
35 translational start codon. The translational start
codon, ATG, is underlined.

Figure 4 is a physical map of plasmid pBM113 as
5 derived from plasmids pBM109 and pEAS41.8.

Figure 5 is a physical map of plasmids pBM113Kp
and pBM311 as derived from plasmids pBM109 and pBM113.

Figure 6 is a physical map of plasmids
pBM113Δ220.10, pBM113Δ220.9 and pBM113Δ240.13
10 as derived from plasmid pBM113.

Figure 7 is a physical plasmid map of plasmid
pBM117 as derived from plasmids pDH51 and pBM109.

Figure 8 is a physical map of plasmid pBM119 as
derived from plasmids pUC18 and pRAJ275.

15 Figure 9 is a physical map of plasmids pCS102
and pBM120 as derived from plasmids pDH51 and pBM119.

Figure 10 is a physical map of plasmid series
pBM165 and plasmid series pBM167 as derived from
plasmids pDH51, a 56 bp oligonucleotide, and plasmid
20 pBM120.

Figure 11 is a physical map of plasmid pBM152
as derived from plasmids pDH51 and pBM120.

Figure 12 is a physical map of plasmid pBM1170
as derived from plasmids pBM117 and pBI101.1.

25 Figure 13 is a physical map of plasmid pBM1130
as derived from plasmids pBM113Kp and pBI101.1.

Figure 14 is a physical map of plasmid pBM3110
as derived from plasmids pBM311 and pBI101.1.

30

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides nucleic acid
promoter fragments of the Em gene of wheat which are
responsive to compounds possessing ABA-like activity
35 to bring the expression of DNA sequences for selected
gene products under external control. A major
storage protein found in mature wheat embryos is

synthesized early during the process of seed
5 maturation. Its synthesis can be monitored by the
accumulation of a radioactive amino acid
³⁵S-methionine. Based on these characteristics,
Grzelczak et al., Canad. J. Biochem. 60:389-397
(1982), named this protein "early-methionine-
10 labeled", or Em. Em begins to accumulate in embryos
by 21 days post anthesis, i.e., Stage III. When
embryos are removed from the grain at Stage II (15
days) or III and cultured in vitro, they germinate
precociously into normal seedlings and Em is not
15 accumulated. Williamson and Quatrano, Europ. J.
Biochem., 152:501-507 (1985) showed that if 10^{-6} to
 10^{-4} M ABA is present in the culture medium, both
the Em mRNA and protein are rapidly synthesized and
accumulated. They further studied the regulation of
20 the native Em gene in wheat by application of ABA to
intact mature embryos (Williamson and Quatrano, Plant
Physiol., 86:208-215 (1988)). Em is one of a family
of about 50 proteins that appear to be regulated in
this manner by ABA. The Em gene of wheat, encoding
25 the Em protein, includes the coding region for the
protein, and all necessary regulatory sequences
upstream (5') and downstream (3') and/or within the
coding region, such as the upstream (5') promoter
region. Another storage protein from wheat embryos,
30 a 7S globulin, is included in this family (Williamson
and Quatrano, Plant Physiol., 86:208-215 (1988)).
This globulin class of storage proteins has members
in the monocot maize (Khavkin et al., Planta,
143:11-20 (1978)) and has been extensively studied
35 and characterized in dicot seeds, especially the
legumes (Casey et al., Oxford Surveys Pl. Mol. Cell
Biol., 3:1-95 (1986)). Based on the distribution of

this class of storage proteins in seeds of many
5 plants, we would expect that proteins and genes
homologous to Em from wheat would be represented in
all seed plants.

In the context of this disclosure, a number of
terms shall be utilized. As used herein, the term
10 "promoter region" refers to a sequence of DNA,
usually upstream (5') to the coding sequence, which
controls the expression of the coding region by
providing the recognition for RNA polymerase and/or
other factors required for transcription to start at
15 the correct site. A "promoter fragment" constitutes
a fraction of the DNA sequence of the promoter
region. "Nucleic acid" refers to a large molecule
which can be single-stranded or double-stranded,
composed of monomers (nucleotides) containing a
20 sugar, phosphate and either a purine or pyrimidine.
In higher plants, deoxyribonucleic acid (DNA) is the
genetic material while ribonucleic acid (RNA) is
involved in the translation of the information in DNA
into proteins. The term "nucleotide sequence" refers
25 to a polymer of DNA or RNA which can be
single-stranded or double-stranded, optionally
containing synthetic, non-natural or altered
nucleotide bases capable of incorporation into DNA or
RNA polymers. As used herein, "DNA sequences for
30 selected gene products" refers to a gene of DNA
sequences that codes for a specific protein.
"Regulatory sequence", as used herein, refers to a
nucleotide sequence located upstream (5'), within,
and/or downstream (3') to a DNA sequence for a
35 selected gene product whose transcription and
expression is controlled by the regulatory sequence
in conjunction with the protein synthetic apparatus

of the cell. The term "recombinant DNA construct" refers to a plasmid, virus, autonomously replicating sequence, phage or nucleotide sequence, linear or circular, of a single-stranded or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product into a plant cell. As used herein, "monocotyledonous plant" or monocot refers to a plant whose seeds have only one cotyledon, or organ of the embryo which stores and absorbs food. Similarly, "dicotyledonous plant" or dicot, refers to a plant whose seeds have two cotyledons. A "protoplast" refers to a plant cell without a cell wall or extracellular matrix.

As used herein, "transformation" means processes by which cell/tissue/plant acquire properties encoded on a nucleic acid molecule that has been transferred to the cell/tissue/plant. "Transferring" refers to methods to transfer DNA into cells including microinjection, permeabilizing the cell membrane with various physical (e.g., electroporation) or chemical (e.g., polyethylene glycol, PEG) treatments, or by integration into the host nuclear DNA by the bacterial vector Agrobacterium tumefaciens. As used herein, "exposing" a protoplast or a plant to a chemical substance refers to treating, incubating, contacting said protoplast or plant with the substance. The term, "operably linked" refers to the chemical fusion of two fragments of DNA in a proper orientation and reading frame to be transcribed into functional RNA. As used herein, the term "homologous to" refers to

proteins or nucleic acids having similar sequences of amino acids or nucleotides respectively, i.e., proteins or nucleic acids with the same structural or functional properties. The term "expression", as used herein, is intended to mean the transcription and translation to gene product from a gene coding for the sequence of the gene product. In the expression, a DNA chain coding for the sequence of the gene product is first transcribed to a complementary RNA which is called a messenger RNA and, then, the thus transcribed messenger RNA is translated into the above-mentioned gene product. Expression which is constitutive and further enhanced by an externally controlled promoter fragment thereby producing excess copies of messenger RNA resulting in the accumulation of more than normal quantities of the selected gene product, is referred to as "over-production". The "translation start codon" refers to a unit of three nucleotides (codon) in a nucleic acid that specifies the initiation of the structural gene or protein sequence. "Chemical inducers" are compounds which interact with promoters to trigger the expression of operably-linked structural genes, selected gene products or reporter genes such as GUS.

"ABA-like" compounds are compounds that mimic the effect of ABA in one or more physiological processes or bioassays, such as stomatal closure, bud/seed dormancy, inhibition of seed germination and responses of plants to physical stress (c.f. Addicott, Abscisic Acid, Praeger Publ., N.Y. 607 pp. (1983); Addicott and Lyon, Ann. Rev. Plant Physiol. 20:139-164 (1969); Zeevaart and Creelman, Ann. Rev. Plant Physiol. 39:439-473 (1988)).

"Transformed plant cell(s)" can include a
5 protoplast(s) (plant cell without a cell wall) an
isolated cell(s) or a cell(s) within a transformed
plant.

The techniques of DNA recombination used
throughout this invention are known to those skilled
10 in the art and are generally described in Maniatis et
al., Molecular Cloning: A Laboratory Manual, Cold
Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
(1982).

15 Enzymatic Treatments of DNA

1. Restriction Enzyme Digestions

The restriction enzyme digestion buffers and
digestion conditions used are those supplied by the
manufacturer of each particular enzyme. When
20 recommended by the manufacturer, dithiothreitol (DTT)
is added from a separate sterile stock solution to
the recommended concentration. Enzyme is added to
give 5-10 units per microgram of DNA and the reaction
mixture is adjusted to the appropriate final volume
25 with H₂O (usually 10-20 µl). The restriction
enzyme reaction mixtures used routinely contained
0.7-2.0 µg plasmid DNA. The reaction mixtures are
mixed by vortexing and then incubated at the
appropriate temperature for 1.5 hours. Digestion of
30 DNA with multiple enzymes is done concomitantly when
the optimal salt and temperature conditions of the
separate enzymes are similar. When these conditions
are sufficiently different, digestions are done
sequentially beginning with the enzyme requiring the
35 lowest salt concentration. Subsequent reactions are
supplemented to the appropriate buffer conditions for
the enzyme used.

2. Ligation

5 For ligation of DNA fragments, T4 DNA ligase from Bethesda Research Laboratories, Gaithersburg, MD 20877 and the buffer supplied with the enzyme (50 mM Tris/HCl pH7.6, 10 mM MgCl₂, 1mM ATP, 1mM DTT, and 5% (w/v) polyethylene glycol (PEG) is used. One to
10 two units of enzyme are added per microgram of DNA. The reaction mixtures are incubated at either 16°C overnight or at 37°C for 2-4 hours.

3. Fill-in/Chew-back Reactions

It is necessary, on occassion, to fill-in or
15 chew-back the overhang of a particular restriction site prior to ligation. Fill-in is used for those enzymes that leave a 5' overhang at the site of restriction and chew-back is used for those enzymes that leave a 3' overhang. The fill-in reaction
20 utilizes the Klenow fragment of E. coli DNA polymerase I. The reaction mixture is 1 unit of Klenow fragment, 16 µM of deoxynucleotide triphosphates (dNTPs) (all four) in 10-50 mM Tris/HCl (pH 7.5-8.0), 0-50 mM NaCl and is performed at 37°C
25 for 15 minutes. When convenient, the reaction is performed in the same tube in which the reaction with the restriction enzyme(s) took place. In those cases, the salt concentration is adjusted to a final concentration of less than 50 mM. When secondary
30 restriction digestion(s) are to be performed, the Klenow fragment is denatured by heating it to 65°C for 10 minutes to avoid unwanted filling-in of secondary restriction site ends.

T4 DNA polymerase may be used for filling-in
35 restriction sites which leave 5' overhangs and for chewing-back restriction sites which leave 3' overhangs. The reaction mixture for both reactions

is 10 units of T4 polymerase, 300 μ M dNTPs (all
5 four), 40 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM
DTT, 50 mM NaCl, 50 μ g/ml bovine serum albumin
(BSA) and is performed at 11°C for 20 minutes.
Similarly, when secondary restriction digestions are
to be performed, the T4 polymerase is denatured by
10 heating to 75°C for 10 minutes to avoid unwanted
filling-in or chewing-back of secondary restriction
site ends.

4. Exonuclease III-Mediated Deletion

This procedure is designed for the construction
15 of fragments of DNA reduced by the progressive,
unidirectional deletion of DNA. This is accomplished
by digesting appropriately prepared DNA with
exonuclease III (exoIII) and removing aliquots at
increasing intervals of time during the digestion.
20 The resulting successively smaller fragments of DNA
may be sequenced to determine the exact endpoint of
the deletions. There are several commercially
available systems which use exonuclease III (exoIII)
to create such a deletion series (e.g.
25 Promega-Biotec, Madison, WI 53711, Erase-A-Base
System, Cat# E5750).

The procedure is performed as follows: The DNA
molecule of interest (usually a plasmid) must contain
at least two unique restriction sites. One of these
30 sites (the first) will be the one from which the
deletion reaction will proceed and may be adjacent to
or within the DNA to be deleted. The second site
must be in a position such that the first site lies
between the second site and the DNA to be deleted.
35 The molecule is cleaved with the restriction enzymes
(as described above) to prepare for the deletion
reaction. The restriction site from which the

deletion is to proceed must produce a 5' protruding
5 end or a blunt end (sensitive to *exoIII* digestion).
The other restriction site must either produce a 4
base 3' protruding end, or must be filled-in with
 α -phosphorothioate nucleotides (insensitive to
exoIII digestion). When one end of the molecule must
10 be protected from *exoIII* digestion with the
 α -phosphorothioate nucleotides, the plasmid is
digested with this enzyme first. The ends will then
be filled-in in the presence of 40 μ M each of the
four α -phosphorothioate nucleotides, 1 mM DTT, 20
15 mM Tris/HCl (pH 8.0), 100 mM $MgCl_2$ and 50 units/ml
Klenow fragment at 37°C for 10 minutes.

The sample is then heated to 70°C to inactivate
the Klenow fragment and the second restriction
digestion is performed. If the second restriction
20 enzyme is incompatible with the salt concentrations
in the Klenow reaction, the sample must first be
extracted with an equal volume of
phenol: $CHCl_3$:isoamyl alcohol (25:24:1), and the DNA
isolated by precipitation from the aqueous phase by
25 the addition of 2 volumes of ethanol. After the
second restriction digestion is finished, the sample
is extracted and the DNA again precipitated by
addition of ethanol.

The *exoIII* digestion may now be performed on
30 the isolated DNA. The procedure outlined below is
adapted for digesting approximately 5 μ g of double-
cut plasmid DNA and taking samples at 25 intervals of
time: The DNA sample is dissolved in 60 μ l of 1 x
exoIII buffer (66 mM Tris/HCl (pH8.0), 6.6 mM
35 $MgCl_2$) and 250-500 units of *exoIII* nuclease is
added, mixed rapidly and the sample is incubated at
37°C. Aliquots (2.5 μ l) are removed at various

times from the reaction tube and added to 7.5 μ l
5 aliquots of S1 nuclease mix and kept on ice. S1
nuclease mix consists of 40 mM potassium acetate
(pH4.6), 0.33 mM NaCl, 1.35 mM $ZnCl_2$, 6.67 mM
glycerol containing 0.3 units of S1 nuclease/ μ l.
After all the samples are taken, the S1 mix tubes
10 containing the aliquots of the different time
intervals are removed from the ice and incubated at
room temperature for 30 minutes. To stop the S1
reaction, 1 μ l of S1 stop buffer (0.3 M Tris base,
50 mM ethylenediaminetetraacetic acid (EDTA) is added
15 and the samples are heated to 70°C for 10 minutes.

Aliquots (2 μ l) are removed from the sample
tubes and are analyzed on a 1% agarose gel to
determine the extent of digestion. The remainder of
each of the samples are transferred to 37°C and
20 1 μ l of Klenow mix (1 x Klenow buffer-see above
containing 0.10-0.15 unit Klenow fragment/ μ l) is
added. After a 3 minute incubation period, 1 μ l of
dNTP mix (0.125 mM each of each of the four dNTPs) is
added and the incubation is continued for 5
25 additional minutes.

The samples are then moved to room temperature
and 40 μ l of ligase mix (50 mM Tris/HCl (pH 7.6),
10 mM $MgCl_2$, 1 mM ATP, 5% PEG, 1 mM DTT containing
0.005 units T4 ligase/ μ l) is added. The tubes are
30 mixed well and allowed to incubate at room
temperature for 1 hour.

After ligation, the samples are transformed
into E. coli as described in the section entitled
"Transformation of Bacterial Cells". Individual
35 transformants are analysed by isolation of plasmid
DNA (small-scale) followed by restriction digestion
analysis and/or by sequencing (see below).

5 Gel Electrophoresis of DNA

For polyacrylamide gel electrophoresis of DNA, the Tris-Borate-EDTA (TBE) buffer described by Bethesda Research Laboratories, Gaithersburg, MD 20877, which consists of 89 mM Tris-89 mM borate (pH 8.3), 2.5 mM Na₂EDTA is used. The gels used consisted of 5% acrylamide and 0.2% bis-acrylamide dissolved in 100 ml 1 x TBE. To this solution, 0.225 ml of an aqueous 25% ammonium persulfate solution is added.

15 After adding 55 µl of N,N,N',N'-Tetra-methylethylenediamine (TEMED), the solution is pipetted into a gel mold. A 0.75 mm comb and 0.75 mm spacers are used and approximately 0.5-1.0 µg of DNA is loaded per well. Electrophoresis is carried out at 150-250 volts in 1 x TBE. After electrophoresis, the gel is stained in an aqueous solution of ethidium bromide (0.5 µg/ml) and the DNA is visualized on an ultraviolet transilluminator. The gel is photographed using a Polaroid camera and Polaroid 57 film (Polaroid Tech. Photo, Cambridge, MA 02139).

DNA is recovered from polyacrylamide gels as follows: the desired band, visualized by ethidium bromide staining, is cut from the gel, placed in an Eppendorf tube and minced with a teflon pestle. An equal volume of a 0.5 M ammonium acetate, 1 mM EDTA solution is added and the tube is incubated at 37°C overnight. The following day, the tube is centrifuged at 14,000 x g in a microfuge for 5 minutes at room temperature, the supernatant is

removed, 1/2 volume of fresh elution buffer is added
5 to the minced polyacrylamide and the contents are
mixed by vortexing.

The tube is again centrifuged, as above, for 5
minutes and the supernatant removed and pooled with
the supernatant from the first centrifugation. The
10 pooled supernatants are passed over a small glass
wool column to remove any residual polyacrylamide gel
pieces and the DNA in the sample is precipitated two
times consecutively, each time by addition of 2
volumes of ethanol and incubation in dry ice-ethanol.
15 The DNA is collected by centrifugation of the sample
in a microfuge, as above, for 15 minutes at room
temperature. The pellet is then resuspended in the
buffer of choice depending on the nature of the next
manipulation.

20 Agarose gel electrophoresis of DNA is performed
in 1% agarose gels using the buffer described above
for polyacrylamide gels. Ethidium bromide is added
to the agarose to a final concentration of 0.5
 $\mu\text{g/ml}$ prior to pouring the gel. No ethidium
25 bromide is included in the running buffer. After
electrophoresis, the DNA is visualized on an
ultraviolet transilluminator and photographed as
described above. DNA is recovered from agarose gels
by electroelution onto DEAE-cellulose paper
30 (Schleicher & Schuell, Inc. Keene, NH 03431, Cat#
NA45). The DNA is eluted from the paper by
incubation at 65°C in 1 x Tris-EDTA (TE) (10mM
Tris/HCl, 1 mM EDTA, pH 8.0) containing 150 mM NaCl
for 15-20 minutes. To aid the elution of the DNA,
35 the contents of the tubes are mixed by vortexing them
for 30 seconds every 5 minutes of incubation. The
eluted DNA is precipitated by addition of 2 volumes

of ethanol and incubation of the resultant mixture in
5 a dry ice-ethanol bath until the contents in the
sample becomes a slurry. The DNA is pelleted by
centrifugation of the tubes in a microfuge at 14,000
x g for 15 minutes at room temperature and
resuspended in 150 μ l TE. The resuspended DNA is
10 extracted once with phenol:ChCl₃:isoamyl alcohol
(25:24:1) and the aqueous phase is transferred to a
fresh tube. The aqueous solution is made 0.3 M with
respect to sodium acetate by addition of 3.0 M sodium
acetate and the DNA is precipitated by addition of 2
15 volumes of ethanol as before. After centrifugation,
as already described, the resulting DNA pellet is
resuspended in the appropriate buffer depending on
the next manipulation.

20 Transformation of Bacterial Cells

Transformation of E. coli with plasmid DNA is
performed as described by Davis et al., A Manual for
Genetic Engineering. Advanced Bacterial Genetics,
Cold Spring Harbor Laboratory, Cold Spring Harbor,
25 N. Y., (1982). Cultures are grown from single
colonies of E. coli in a rich medium (i.e.,
Luria-Bertani (LB) medium) to early log phase (OD @
550 = about 0.3). The bacterial cells are pelleted
by centrifugation in a clinical centrifuge at 1,400 x
30 g for 10 minutes at 4°C. The cells are resuspended
in 1/2 volume of ice-cold CaCl₂ (50 mM) solution by
gentle vortexing, and the resulting suspension is
incubated on ice for 20 minutes. The cells are
collected again by centrifugation as above and
35 resuspended in 1/20 volume ice-cold CaCl₂. These
are now competent E. coli cells.

Ligation mixtures are diluted at least 50% with
5 TE prior to adding competent E. coli cells.
Typically, a 1, 2, and 5 µl aliquot of each
ligation mixture is placed in pre-chilled, sterile
Eppendorf tubes and TE is added to a final volume of
10 µl. E. coli cells (200 µl of competent cells)
10 are added to each tube and the tubes are incubated on
ice for 40 minutes. The transformation tubes are
heat shocked by incubation for 2 minutes at 42°C
after which they are returned to ice for 2-3
minutes. One ml of rich medium is added to each tube
15 and the tubes are incubated at 37°C for 30-60 minutes.

The cells are pelleted by gentle centrifugation
(in a microfuge at 7,000 x g), most of the medium is
decanted and the pelleted cells are resuspended in
the 100-200 µl of medium left in tube.

20 The contents of each transformation tube are
spread on an LB-agar plate containing 100 µg/ml of
ampicillin or the required amount of other
antibiotics as appropriate (antibiotic plates). The
antibiotic-resistant cells which grow are analyzed
25 for the presence of the appropriate DNA construct by
isolation of plasmid DNA (small scale), restriction
enzyme analysis (below) and by sequence analysis
(below).

30 Preparations of Plasmid DNA

.1. Large Scale Isolation of Plasmid DNA

Plasmid-harboring strains of gram negative
bacteria are grown to saturation overnight at 37°C in
LB medium (described by Maniatis et al., Molecular
35 Cloning: a Laboratory Manual, p 440, Cold Spring
Harbor Laboratory, Cold Spring Harbor, N. Y. (1982)),
containing 125 µg/ml ampicillin or the appropriate

concentration of whatever antibiotic is used. The
5 bacterial cells are harvested by centrifugation, as
above, washed once with 10 mM MgSO_4 , collected
again by centrifugation, then lysed by an alkaline
lysis procedure essentially as described by Maniatis
et al., supra. The procedure is as follows:

10 The cells from a 500 ml culture are resuspended
in 10 ml of ice-cold lysis solution and incubated at
room temperature for 5 minutes. Lysis solution
consists of 50 mM glucose, 25 mM Tris/HCl (pH 8.0),
10 mM EDTA and 5 mg/ml of lysozyme, the lysozyme
15 being added just prior to use. After the addition of
20 ml of a solution of freshly-made, ice-cold 0.2 N
NaOH, 1% sodium dodecyl sulfate (SDS) the contents
are thoroughly mixed and incubated on ice for 10
minutes. Then, 15 ml of a 5 M potassium acetate
20 solution is added, and the contents are mixed by
vortexing for 30 sec. The mixture is then incubated
on ice for an additional 10 minutes. The 5 M
potassium acetate solution is prepared by addition of
60 ml of 5 M potassium acetate and 11.5 ml of glacial
25 acetic acid to 28.5 ml of H_2O . The resulting
solution is 3 M with respect to potassium and 5 M
with respect to acetate.

The tubes are then centrifuged at 47,500 x g
for 20 minutes at 4°C and the supernatant is
30 transferred to a fresh tube. Nucleic acid is
precipitated from the supernatant by addition of 0.6
volumes of isopropanol followed by incubation for 15
minutes at room temperature. The nucleic acid is
pelleted by centrifugation at 12,000 x g at room
35 temperature for 30 minutes. The supernatant is
discarded and the remaining nucleic acid pellet is

washed once with 70% ethanol. The pellet is
5 resuspended in 5 ml TE, and further purified as
described below.

The resulting plasmid DNA is purified by
banding it two times successively in cesium
chloride-ethidium bromide (0.97 g/ml and 0.43 g/ml
10 respectively) equilibrium density gradients by
centrifugation at 220,000 x g at 20°C for 14-18
hours. The bands containing the plasmid DNA are
removed from the tubes by inserting a 23 gauge needle
(connected to a 5 ml syringe) through the side of the
15 tube. Ethidium bromide is removed from the sample by
extraction with H₂O-saturated n-butanol. The
extraction is repeated until there is no longer a
visible pink color in the aqueous phase. The
extracted plasmid DNA is mixed with 2.0 volumes H₂O
20 to dilute the CsCl. The sample is then mixed with
2.5 volumes of 95% ethanol and placed at -70°C for 30
minutes. The DNA is pelleted by centrifugation at
12,000 x g for 30 minutes at 4°C. The DNA pellet is
washed once with 70 percent ethanol then resuspended
25 in 0.36 ml of 1 x TE and transferred to an Eppendorf
tube (1.5 ml). Sodium acetate (3M) is added to a
final concentration of 0.3 M (1/10 volume) and the
plasmid DNA is re-precipitated by the addition of 2
volumes of ethanol. The nucleic acid is pelleted by
30 centrifugation and dissolved in a final volume of
0.5-2.0 ml of TE and stored at -20°C.

2. Small Scale Isolation of plasmid DNA

Each of a number of single, well-isolated
antibiotic-resistant bacterial colonies from
35 antibiotic plates is used to inoculate separate
tubes containing 2 ml of LB medium with ampicillin at

125 µg/ml or other antibiotic and the resulting
5 cultures are grown for at least 4 hours at 37°C with shaking.

Bacteria from the same culture are streaked on an antibiotic plate and incubated at 37°C overnight. This is called a master plate and will be the source
10 of bacteria for subsequent manipulations. To obtain plasmids from them, the cultures are processed as follows: A portion of each culture (typically 1.5 ml) is transferred to a 1.5 ml Eppendorf tube and the cells are harvested by centrifugation at 14,000 x g
15 for 1 minute. The pelleted cells are resuspended in 100 µl of ice-cold, lysis solution (previously described) to which 4 mg/ml lysozyme is added just prior to use. The tubes are incubated at room temperature for 5 minutes, then 200 µl of a
20 solution of freshly-made, ice-cold 0.2 N NaOH, 1% SDS are added. The tubes are mixed by inversion several times followed by incubation on ice for 5 minutes. Then, 150 µl of a 5 M potassium acetate solution (previously described) is added, and the contents of
25 the tubes are mixed by vortexing for 10 seconds while the tubes are in an inverted position.

After they are incubated on ice for an additional 5 minutes, the tubes are then centrifuged at 14,000 x g for 5 minutes at 4°C. The supernatant
30 is transferred to a fresh tube and extracted with an equal volume of phenol:CHCl₃:isoamyl alcohol (25:24:1). The tubes are again centrifuged, as above, at room temperature for 5 minutes in a microfuge and the aqueous phase is transferred to a
35 fresh tube. The nucleic acid is precipitated from the solution by addition of 2 volumes of absolute ethanol, mixing by vortexing for 10 sec, and

incubation for 2 minutes at room temperature. The
5 tubes are again centrifuged, as above, at room
temperature for 5 minutes. The ethanol is removed
and the remaining nucleic acid pellet is washed once
with 70% ethanol. The pellet is resuspended in 50
µl of TE containing 20 µg/ml RNase A.

10 The presence of the desired plasmid is
determined by digestion of a 10 µl aliquot of the
recovered DNA with the appropriate restriction
endonucleases. The fragments produced in the
digestion are analyzed by electrophoresis on agarose
15 or polyacrylamide gels as described above. The
master plate is used to initiate cultures originating
from single colonies for large scale isolation of the
plasmid and also for the preparation of bacterial
stocks which are stored at -70°C in LB medium
20 containing 20% glycerol.

DNA Sequence Determination and Analysis

Plasmid DNA was prepared as described above for
large scale isolation. The protocol used is a
25 combination of the method of Zagursky, et al. (Gene
Anal. Tech., 2:89-94 (1985)) and the method supplied
with the Sequenase® Sequencing Kit (Cat. #70700)
available commercially from U.S. Biochemical Corp.,
Cleveland, Ohio 44122. The plasmid DNA is sequenced
30 as follows: Three µg of an aqueous plasmid sample,
in a total volume of 8 µl, is placed into a tube, 2
µl of 2 N NaOH is added and mixed well. The tubes
are incubated at room temperature for 5 minutes after
which 1 µl of a primer oligonucleotide, 6 µl of
35 H₂O, and 3 µl of 3 M NaOAc (pH 5.2) are added and
the samples are mixed. The plasmid and primer are
co-precipitated by addition of 75 µl ethanol and

incubation in a dry ice/ethanol bath for 20 minutes.

- 5 The nucleic acids are pelleted at 14,000 x g for 5 minutes at room temperature. The pellet is resuspended in 8 μ l H₂O and 2 μ l of 5 x Sequenase® buffer is added. Sequenase® buffer (5x) is 200 mM Tris/HCl pH7.5, 100 mM MgCl₂, 250 mM NaCl. These are called the annealing tubes.

The samples are incubated at 65°C for 2 minutes and allowed to slowly cool to room temperature by placing the samples in a beaker of 65°C H₂O and allowing the H₂O to cool at room temperature.

- 15 While the samples are cooling, 4 tubes are labelled for each reaction (one each G, A, T, and C) and 2.5 μ l of the 4 termination mixes (supplied with the kit) is added to the appropriately labelled tubes and the tubes are capped. These are called the termination tubes. Termination mixes all contain 160 μ M dITP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP and 50 mM NaCl. In addition, the G termination mix contains 1.6 μ M ddGTP, the A termination mix contains 8 μ M ddATP, the T termination mix contains 8 μ M ddTTP, and the C termination mix contains 8 μ M ddCTP. The termination tubes are then prewarmed to 37°C.

- After the annealing tubes cool to below 35°C, 1 μ l of 100 mM DTT, 2 μ l labeling mix, and 30 0.5 μ l of ³⁵S-dATP (500 Ci/mmol) are added and the tubes are mixed. Labeling mix is 3 μ M dITP, 1.5 μ M dCTP, and 1.5 μ M dTTP. The Sequenase® enzyme is then added (6.5 units/annealing), the samples are mixed and incubated at 37°C for 5 35 minutes. These are now called the labeling tubes. The contents of the labeling tubes are then aliquoted into the termination tubes, 3.5 μ l into each (G, A,

T, C), and the incubation is continued at 37°C for an
5 additional 5 minutes. After the termination
reaction, 4 µl of stop solution is added. Stop
solution is 95% formamide, 20 mM EDTA, 0.05%
bromphenol blue, and 0.05% xylene cyanol.

The samples are heated to 75°C for 2 minutes
10 and electrophoresed on a 6% polyacrylamide sequencing
gel. The gel is 6% polyacrylamide (acrylamide:bis-
acrylamide = 19:1), 8 M urea, and 1 x TBE buffer (see
above). The running buffer is also 1 x TBE. The gel
is electrophoresed at 22 mA (constant current), dried
15 down on Whatman 3MM chromatography paper and exposed
to Kodak XAR X-ray film for 1-2 days at room
temperature.

Plasmid Constructions

20 Plasmids may be constructed using restriction
enzymes, polymerases and other procedures as
described above and which are well known to those
skilled in the art and described in Maniatis et al.,
supra. The promoter fragments of DNA of this
25 invention may be obtained by digesting plasmid
pEAS41.8 which has been deposited in the American
Type Culture Collection, Rockville, MD 20852-1776,
accession number 67647, with the restriction
endonucleases AhaII and SalI and isolating resulting
30 DNA fragments with about 1500 and 354 bp. These
fragments are joined together, in the proper
orientation and order (hereafter referred to as the
1850 bp promoter fragment) and placed 5' to a
reporter gene or any gene whose expression is to be
35 controlled by abscisic acid. The construct resulting
from the linkage of the promoter fragment of DNA and
a desired gene may be placed in a plasmid vector

capable of growing in bacteria, for convenience in
5 preparing quantities of the construct. The 1850 bp
promoter fragment of DNA may be further reduced in
length to contain 640 bp, 254 bp or 50 bp by suitable
enzymatic digestions, isolations and ligations. When
operably linked to a gene or DNA sequence, said
10 640 bp, 254 bp and 50 bp promoter fragments retain
their ability to respond to the presence of suitable
concentrations of ABA. The molecular manipulations
of DNA to construct the plasmids disclosed below were
done as described in the section above and include
15 digesting DNA with specific endonucleases, isolating
fragments from gels, filling-in certain restriction
site overhangs, ligating desired DNA fragments, and
transforming bacterial cells. Several such plasmids
are produced in the EXAMPLES but these are not meant
20 to be all inclusive.

Isolation of Protoplasts

Rice suspension cultures, initiated from
immature embryos or anther-derived callus, are
25 maintained by weekly subculture at a 1:4 dilution
ratio with fresh liquid N6 medium as described by Chu
et al., Sci Sinica 18: 659-668 (1975) containing 2
mg/ml 2,4-dichlorophenoxyacetic acid and 3% (w/v)
sucrose, pH 6.0. Protoplasts are isolated from
30 suspensions of rice cells 4-6 days after subculture
by overnight incubation (16-18 h) in 4 ml of enzyme
solution per gram of cells and agitation of the
mixture on a rotary shaker at 30 rpm at 25°C. The
enzyme solution contained 2% (w/v) cellulase
35 "Onozuka" RS and 0.5% (w/v) Macerozyme (both from
Yakult Honsha, Nishinomiya, Japan) and 13% (w/v)
mannitol, pH 5.6. Released protoplasts are filtered

through a 60 μ m mesh size nylon screen, transferred
5 to 50 ml Pyrex test tubes and washed twice by
centrifugation at 80 x g for 10 minutes in Krens' F
solution (140 mM NaCl, 3.6 mM KCl, 0.75 mM
Na₂HPO₄·7H₂O, 5 mM glucose, 125 mM CaCl₂,
pH7.0). Protoplasts are purified by resuspending the
10 pellet in N6 medium with 17% (w/v) sucrose,
centrifuging at 80 x g for 20 minutes and collecting
the floating layer. Cell counts are made with a
Fuchs-Rosenthal hemocytometer.

15 Transformation of Protoplasts

Protoplasts are transformed as follows:
Protoplasts ($1-5 \times 10^6$) are centrifuged gently
(80 x g) for 2 minutes in sterile tubes. The
supernatant is removed and discarded and the tubes
20 are shaken gently to loosen the protoplasts from the
bottom of the tube. The DNA sample, with which the
protoplasts are to be transformed, in 5 μ l or less
of TE, is added to the protoplasts. The tubes are
shaken gently to disperse the cells in the DNA
25 solution then 100 μ l of a solution containing 40%
PEG (Polysciences Inc., Warrington PA 18976, CAT#
1102) and 3 mM CaCl₂ is added. The resulting
mixture is mixed gently for 10 seconds, then 1.0 ml
of Krens' F solution is added to dilute out the PEG.
30 The tubes are incubated on ice for 15-20
minutes and then centrifuged at 80 x g for 4
minutes. The supernatant is removed and the
protoplasts are washed once with 1.0 ml of a solution
of Krens' F solution. The protoplasts are
35 resuspended in protoplast medium (0.2 mM KH₂PO₄,
1 mM KNO₃, 1 mM MgSO₄, 1 μ M KI, 0.1 μ M
CuSO₄, 10 mM CaCl₂, 10% mannitol, pH 6.5) at a

density of 1×10^6 protoplasts per ml and incubated
5 at 25°C, in the dark, for the desired periods of
time, with or without ABA, phaseic acid, Compound C
or Compound D.

GUS Assay

10 Protoplasts are harvested by centrifugation at
80 x g, and are resuspended in 0.5 ml 1x GUS lysis
buffer (50 mM NaPO_4 (pH 7.0), 10 mM 2-mercap-
toethanol, 10 mM EDTA, 0.1% Triton X-100, 0.1%
N-lauroylsarcosine). The suspension containing the
15 lysed protoplasts is transferred to Eppendorf tubes
and centrifuged in a microfuge at 14,000 x g for 10
minutes. It is possible to freeze the suspension of
lysed cells in the extraction buffer if the assay is
to be performed at a later date. Forty-five μl of
20 the supernatant is transferred to a tube containing
755 μl H_2O for analysis of protein content.
Protein content is determined using the Bio-Rad
Laboratories, Richmond, CA 94804, microassay. For
the assay, 0.2 ml of the dye concentrate is added to
25 the 800 μl sample and the absorbance is measured on
a spectrophotometer at a wavelength of 595 nm. A
standard curve must be calculated using a protein
such as bovine serum albumin and the amount of
protein in the experimental samples is read off the
30 standard curve. The protein content, so determined,
is multiplied by a factor of 2 to give the protein
content in 90 μl of extract (which is the amount of
extract in a single time point of the assay-see
below). Of the remaining supernatant fluid, 405 μl
35 is transferred to a fresh tube for analysis of GUS
activity.

The substrate for the assay is

5 4-methyl-umbelliferyl- β -D-glucuronide (4-MUG) and was obtained from Sigma Chemical Co., St. Louis MO 63178, Cat# 9130) in 1 x GUS buffer. It is at a concentration of 1 mM in the assay. One/tenth volume (45 μ l) of a pre-warmed (37°C) 10 mM 4-MUG stock is

10 added to the pre-warmed 405 μ l protoplast extract, and a 100 μ l aliquot is immediately transferred to a well of a 24-well microtiter dish containing 0.9 ml 0.2 M Na_2CO_3 . Similar aliquots are removed at 0.5 hour, 1 hour, and 2 hours or at 1 hour, 2 hours

15 and 4 hours, and the microtiter dishes are stored in the dark between time points. The dishes may be examined for the fluorescence of 4-methyl umbelliferone (4-MU), the product of enzyme digested 4-MUG, by means of an ultraviolet transilluminator.

20 Individual samples, from each time point, may be examined on a fluorimeter for quantitative analysis. In the case where quantitative analysis is desired, the excitation wavelength should be set at 365 nm and the emission wavelength should be set at 455 nm.

25 Standards of 4-MU (Sigma Chemical Co., Cat# 1508), are also measured on the fluorimeter (typically at concentrations of 100 nM and 1 μ M) and the specific activity is calculated as the picomoles of 4-MU produced per μ g protein per hour of assay.

30

Transformation of Plants

There are two general forms in which foreign genes can be expressed in plant cells; as fixed integrates into the nuclear DNA (genome) of the host

35 cell (stable expression), or not integrated but freely active in the cell (transient expression). To test for stable expression of genes requires weeks or

months to regenerate transformed tissue and in many
5 cases considerable space in growth chambers and
greenhouses to grow the required amount of
tissue/plants. If one is testing the effect of
numerous recombinant DNA constructs (e.g., 50-100)
for regulation of the foreign genes in separately
10 transformed plants, the time and space required
becomes considerable. If on the other hand, one has
fast (hours), reliable transient expression systems
to quickly test in cells, in a preliminary screen,
which of the 50-100 constructs would be the most
15 likely ones to show the natural regulation in stable
transformants, significant time and money would be
saved. Such a system would be even more important
for most monocots (including the cereals), since
these plants cannot be regenerated from single
20 cells. With the exception of rice (rice plants have
been regenerated from protoplasts by Kyoizuka et al.,
Mol. Gen. Genet., 206:408-413 (1987)), genes
introduced into single cells from monocots cannot be
monitored in stable, transgenic monocot plants for
25 regulatory sequences. Using the methods of this
invention, one can determine if inducible cereal
promoters can be recognized in rice protoplasts using
a transient assay, and if so, to quickly determine
those sequences that are required for the induction.
30 Furthermore, such a transient assay would allow the
rapid screening of numerous analogs of a chemical
inducer of gene expression such as ABA, to optimize
structure/expression activity. Knowing that the
induction works in rice cells, and that the key
35 regulatory regions of the gene and specific moieties
of the chemical inducer have been initially
identified, one could introduce these sequences into

a relatively few stable transformants of rice.

- 5 Confirmation of the correlation between results obtained in transient expression systems with those from stable integration is provided by the Ebert et al. and Ellis et al. references discussed supra. These two studies, one using a constitutive bacterial
10 promoter, the other an inducible promoter from a plant, both operably linked to an easily measured reporter gene, CAT, demonstrate that transient assays in monocot (maize) cells can predict sequences that are regulatory in stable transformants of the dicot
15 tobacco.

The main approach used to identify operative recombinant DNA constructs of general utility is to place regulatory sequences derived from monocots in cells that are easy to transform, i.e., dicots such
20 as tobacco, to determine if their sequences are recognized in transgenic plants. In fact, the Ebert et al. and Ellis et al. references demonstrate that when a viral enhancer element was operably linked to a maize inducible promoter (Adhl), and this construct
25 stably introduced into tobacco plants, stable transformants were successfully induced by low oxygen levels.

Similarly, we have introduced the Em promoter from wheat (monocot), operably linked to the reporter
30 gene GUS, into tobacco plants via the bacterial vector Agrobacterium tumefaciens to test for ABA induction of GUS in stable transformants (transgenic plants). Since Em gene expression was initially detected only in embryo tissue of wheat seeds in
35 response to ABA, the regulatory sequences within the promoter may specify ABA-regulated expression only in embryo tissue. To determine if both these regulatory

components of the Em promoter (i.e., tissue
5 specificity and ABA inducibility) are represented in
the same or different segments requires analyses of
GUS expression throughout the life cycle of
transgenic plants. Although the regulatory sequence
responsible for ABA induction can be determined from
10 transient expression in single-celled protoplasts,
those sequences responsible for tissue specificity
must be determined in multi-cellular, transgenic
plants. Initially, we introduced ABA-regulatory
regions of the Em promoter (as defined in the
15 transient assay) operably linked to GUS into the
tobacco plant genome via Agrobacterium. We then
determined that the regulation throughout the life
cycle of tobacco was identical to the normal
regulation of the Em gene in wheat. We precociously
20 induced GUS expression in very young seeds by
exposure to exogenous ABA, and have shown the
expression of GUS in mature seed. We demonstrated
that the region of the promoter responding to ABA in
protoplasts (defined in the transient system)
25 includes that region regulating tissue specificity in
transgenic plants. In addition, this demonstrates
that these regulatory sequences from the monocot
wheat are recognized and operable in the dicot
tobacco.

30

Chemical Inducers

To bring plant gene expression under external
chemical control in the field requires that the
chemical not only be able to induce specific gene
35 expression in transgenic plants, but that the
chemical have unique traits that will allow it to be
effective under field conditions, e.g., light stable,

ability to be translocated within the plant, etc.

- 5 Equally important will be a lack of toxicity or additional physiological effects on the plant. For example, ABA is a natural growth regulator found in all seed plants (c.f., Davies, P. (Ed.) Plant Hormones and Their Roles In Plant Growth and
- 10 Development., Martinus Nijhoff Publ. (1987)). It is light sensitive and will have pronounced physiological effects when applied to certain plant parts (c.f., Zeevaart and Creelman, Ann. Rev. Plant Physiol., 39:439-473 (1988)). For example, when
- 15 sprayed on leaves, ABA will cause stomates to close and thereby prevent gaseous exchange between the plant and the atmosphere. ABA has also been shown to inhibit seed germination, and to play a role in bud/seed dormancy, leaf senescence and in responses
- 20 of plants to various physical stresses such as temperature and water. Numerous compounds have been described that mimic the effect of ABA on one or more of these processes (or any other ABA-mediated process) and are referred to as "ABA-like". If
- 25 ABA-like compounds are to be used as chemical inducers of selected genes at all stages, their effects on these key physiological processes must be minimized or eliminated.

- The transient assay and the experiments with
- 30 transgenic plants outlined above will provide excellent tests which will allow us to optimize for field applicability traits as well as gene inducibility effectiveness of chemical inducers that are ABA-like. For example, Compound C
- 35 [2-fluoro-5-l-hydroxy-2,6,6-trimethyl-4-oxo-cyclohex-2-en-1-yl/-3-methyl-2,4-pentadienoic acid] differs from ABA by addition of a fluorine molecule in the

structure, possibly making it more light stable.

5 Compound D [1,4-dihydroxy- β ,2,6,6-tetramethyl-2-cyclohexane-1-penta-cis-2,trans-4-dienoic acid] differs in the introduction of a hydroxyl in place of an oxygen. Both of these compounds are structurally very similar to ABA and are functionally "ABA-like",

10 i.e., they both inhibit seed germination. Jung and Grossman reported (J. Plant. Physiol. 121:361-367 (1985) and J. Agron. Crop Sci. 153:14-22 (1984)) that various terpenoid derivatives are very "ABA-like" in their ability to produce certain physiological

15 effects, e.g., stomatal closure, increase leaf senescence and increased chilling/freezing resistance (Flores et al., J. Plant Physiol. 132:362-369 (1988)). These terpenoid derivatives are described in two German patents by Bliesener et al. (1981 - DOS

20 P 31 43 721) and Sauter et al. (1981 - DOS P 31 43 720). The effect of two such derivatives, Compound E [1,4-Dioxaspiro[4,5]dec-6-en-8-ol,2,7,9,9-tetramethyl-8-[3 methyl-4-(4-methyl-1,3-dioxolan-2-yl)-3-buten-1-ynyl]-] and Compound F [1,4 Dioxaspiro[4,5]dec-6-en-8-ol,8-(5,5-dimethoxy-3-methyl-

25 3-penten-1-ynyl)-2,7,9,9-tetramethyl-] are clearly ABA-like when assayed for their ability to close stomates and promote leaf senescence (Jung and Grossmann, J. Plant Physiol., 121:361-367 (1985)) as

30 well as increased chilling and freezing resistance (Flores et al., J. Plant Physiol., 132:362-369 (1988)). We would predict that all such ABA-like compounds might serve as chemical inducers of our Em promoter/GUS construct. Using our specific and rapid

35 transient assay in protoplasts, a series of ABA-like chemical inducers, that may have reduced or no effects on the physiological processes mentioned

above (separately determined in bioassays for these
5 processes) can be tested for their ability to induce
gene expression. Screening of numerous compounds at
different concentrations through our transient assay
and these bioassays could result in an ideal chemical
inducer in the field, i.e., one which induces gene
10 expression at a concentration that is without other
effects on the plant or plant processes.

In addition to screening various ABA-like
compounds for the most effective inducer, one can
also identify in the transient assay, the smallest
15 and most responsive promoter sequence to these
compounds. One could delete sections of the promoter
until the smallest fragment is identified,
facilitating subsequent manipulations of this
ABA-regulatory sequence into other promoters/genes.
20 In addition, one can make base substitutions in this
small fragment to further delineate the specificity
of control for each inducer. The combined use of
this rapid preliminary screen in protoplasts, with
testing of the inducers on transgenic plants under
25 field conditions will result in the optimal
structure/function combination of chemical inducer
and controlling sequence for ideal control of gene
expression in the field.

30 Stable Transformation of Tobacco

In order to introduce DNA constructions into
plants, the constructions are mobilized from E. coli
strain HB101 into Agrobacterium tumefaciens, strain
LBA4404. E. coli strain HB101, harboring the plasmid
35 pRK2013, is used as a helper for the plasmid
mobilization in a triparental mating. LBA4404 is
grown overnight at 28°C in 5 ml of minimal A-sucrose

medium (37.5 mM $(\text{NH}_4)_2\text{SO}_4$, 165 mM KH_2PO_4 ,
5 300 mM K_2HPO_4 , 8.5 mM sodium citrate, 1 mM
 MgSO_4 , 0.2% sucrose, 50 $\mu\text{g/ml}$ thiamine). The
HB101 derivatives are grown at 37°C in Luria-Bertani
(LB) broth containing the appropriate antibiotic for
3 to 6 hours (to approximately $6-8 \times 10^8$
10 cells/ml). The cells from all cultures are pelleted
by centrifugation and resuspended in 5 ml LB broth
(to remove antibiotics from the medium).

The bacterial strains are mixed as follows:
100 μl of each of the HB101 derivatives and
15 200 μl of LBA4404 are placed in a 1.5 ml
microcentrifuge tube, mixed well and pelleted
gently. The bacterial pellet is resuspended in
50 μl LB broth and the mixture is pipetted onto a
sterile nitrocellulose disk (Millipore HA type
20 filters). Each nitrocellulose disk is transferred to
a stack of 6-8 sterile Whatman #1 filter paper disks
to remove the excess liquid from the culture and
thereby concentrate the bacteria in the mixture. The
nitrocellulose disks are then placed onto LB agar in
25 a 150 mm petri dish and incubated for approximately
16 hours at 28°C.

Following the incubation, the disk is sterily
transferred to a 15 ml tube containing 5 ml 10 mM
 MgSO_4 . The tube is vortexed to release the
30 bacteria into the liquid. The bacteria (100 μl and
50 μl aliquots) are plated on 2 minimal A-sucrose
plates containing the appropriate antibiotic(s) and
incubated at 28°C for 3 days. After the incubation,
resistant colonies are selected and the presence of
35 the desired construct is confirmed by preparation of
plasmid DNA (small-scale procedure from above)
followed by restriction enzyme analysis.

Recombinant DNA constructs are stably
5 integrated into the nuclear DNA of tobacco via
Agrobacterium tumifaciens infection of tobacco leaf
disks. Standard aseptic techniques for the
manipulation of sterile media and axenic
plant/bacterial cultures are followed, including the
10 use of a laminar flow hood for all transfers. Potted
tobacco plants for leaf disk infections are grown in
a growth chamber maintained for a 12 hour, 24°C day,
12 hour, 20°C night cycle, with approximately 80%
relative humidity, under mixed cool white fluorescent
15 and incandescent lights. Tobacco leaf disk
infections are carried out essentially by the method
of Horsch et al., Science, 227:1229-1231 (1985).

Young leaves, not fully expanded and
approximately 6-10 cm in length are harvested with a
20 scalpel from approximately 4-6 week old tobacco
plants. The leaves are surface sterilized for 20
minutes by submerging them in approximately 500 ml of
a 10% Clorox, 0.1% SDS solution and then rinsing them
3 times with sterile deionized water. Leaf disks, 8
25 mm in diameter, are prepared from whole leaves using
a sterile paper punch.

Leaf disks are inoculated by submerging them
for several minutes in 20 ml of a 1:20 dilution of an
overnight YEB broth culture of Agrobacteria carrying
30 the plasmid of interest. After inoculation, the leaf
disks are placed in petri dishes containing .1N1B
agar medium (Murashige Minimal Organics Medium
(Gibco, Grand Island, N.Y. 14072) with 0.1 mg/l
thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic
35 acid, 8 gm/l agar, 0.1 mg/l naphthalene acetic acid
(NAA), and 1 mg/l benzylaminopurine (BAP), pH 5.8).
The plates are sealed with parafilm and incubated

under mixed fluorescent and "Gro and Sho" plant
5 lights (General Electric) for 3 days in a culture
room maintained at approximately 25°C.

To eliminate Agrobacteria from the leaf disks
and to select for the growth of transformed tobacco
cells, the leaf disks are transferred to fresh .1N1B
10 medium containing 500 mg/L cefotaxime and 100 mg/L
kanamycin. Cefotaxime is kept as a frozen 100 mg/ml
stock and added aseptically (filter sterilized
through a 0.45 µm filter) to the media after
autoclaving. A fresh kanamycin stock (50 mg/ml) is
15 made for each use and is filter sterilized into the
autoclaved media.

Leaf disks are incubated under the growth
conditions described above for 3 weeks and then
transferred to fresh media of the same composition.
20 Approximately 1-2 weeks later shoots which develop on
kanamycin-selected explants are excised with a
sterile scalpel and planted in MX⁻ medium (.1N1B
agar medium from above without hormones), containing
200 mg/l cefotaxime and 100 mg/L kanamycin. Shoots
25 which root in kanamycin are transferred to soil and
grown in a growth chamber as described above. As
plants develop flower buds, each inflorescence is
bagged to ensure self pollination.

Seeds of transformed plants are harvested at
30 various developmental stages to assay for the
expression of GUS. After removing the capsule from
the plant, half the capsule wall is cut and removed
using a sterile scalpel. Immature seeds are
harvested by scraping them off the placenta into
35 Ependorf tubes. This procedure is repeated for the
other half of the capsule. Mature seed is freely
released after forming a hole in the capsule wall.

Seeds are ground in Ependorf tubes using disposable
5 pestles (Kontes, Vineland, N.J. 08360) and 1 ml GUS
lysis buffer. The samples are vortexed and then
centrifuged at 14K rpm for 15 minutes. The
supernatant is used for GUS enzyme assay and protein
determination (see above).

10 To analyze the effect of ABA on expression of
GUS in immature seed, young seeds are harvested as
described above and placed on MX⁻ medium with and
without 10⁻⁴M ABA. Seeds are incubated in darkness
at 25°C for 20-48 hours, then transferred into
15 Ependorf tubes using disposable cell scrapers
(Costar, Cambridge, MA 02139). Each sample is split
into two Ependorf tubes, then ground and assayed as
described above.

The present invention is further defined in the
20 following EXAMPLES, in which all parts and
percentages are by weight and degrees are Celsius,
unless otherwise stated. It should be understood
that these EXAMPLES, while indicating preferred
embodiments of the invention, are given by way of
25 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
30 invention to adapt it to various usages and
conditions.

EXAMPLE 1

35 Construction of plasmids pBM107 and pBM108

Plasmid pDH51 was disclosed in Pietrzak, et
al., Nucl. Acids Res., 14: 5857-5868 (1986) and
served as the source of Cauliflower Mosaic Virus

(CaMV) 35S promoter sequence, a polylinker sequence
5 and the CaMV 35S 3' region. Plasmid pRAJ260 served
as the source of the *E. coli uidA* gene, which encodes
a β -glucuronidase enzyme (GUS). This plasmid is
described by Jefferson et al., Proc. Natl. Acad. Sci.
(USA) 83: 8447-8451 (1986). The coding sequence for
10 the GUS gene was cleaved from the vector sequences by
digesting pRAJ260 with the restriction enzyme PstI as
described above. This digestion results in the GUS
gene being present on a 1.8 kb fragment. One such
fragment was then isolated from a 1% agarose gel,
15 (described above) and ligated into PstI-digested
pDH51. Individual plasmids were analyzed until one
was identified in which the PstI insert was present
in the same 5' to 3' orientation as the 35S
promoter. The resulting plasmid, pBM107, contains
20 the *E. coli* gene for GUS located between the CaMV 35S
promoter sequence and the CaMV 35S 3' sequence.
Plasmid pBM107 was then digested with the restriction
enzymes SmaI and SalI. The 5' overhang of the SalI
site was filled-in by incubation of the cleaved
25 plasmid for 15 minutes at 37°C with the Klenow
fragment of DNA polymerase I and 16 μ M
deoxynucleotide triphosphates (dNTPs). The resultant
blunt-ended fragment was ligated as described above,
transformed into *E. coli* and a clone was identified
30 in which the plasmid no longer contained a BamHI
site. Such a clone would have lost the smaller
fragment. This resulted in the removal of the
restriction endonuclease sites SmaI, BamHI, XbaI, and
SalI from the polylinker region of pBM107. The
35 resulting plasmid is pBM108. This plasmid contains

the GUS gene and the CaMV 35S 3' region on a 2099 bp
5 KpnI fragment. The construction of this plasmid is
diagrammed in Figure 1.

Construction of plasmid pBM109

The 2099 bp KpnI fragment was cleaved from
10 pBM108 with the restriction endonuclease KpnI,
isolated on a 1 % agarose gel as described above, and
inserted into plasmid pUC18 at its KpnI restriction
endonuclease site. The fragment was inserted in the
opposite 5' to 3' orientation with respect to the
15 lacZ gene which already resided in pUC18. The
resulting plasmid, pBM109, is extremely useful
because of the presence of multiple unique
restriction sites just 5' relative to the GUS gene.
Putative promoter sequences may be cloned into these
20 sites thereby allowing the use of the GUS assay as a
means of delineating promoter activity. Plasmid
pBM109 has been deposited in the American Type Culture
Collection (ATCC) Rockville, MD 20852-1776 on
March 2, 1988 and bears the ATCC accession number
25 67648. The construction of this plasmid is
diagrammed in Figure 2.

Construction of plasmids pBM112 and pBM113

The source of the inducible promoter DNA
30 sequence of the wheat Em gene is plasmid pEAS41.8,
which has been deposited in the American Type Culture
Collection (ATCC), Rockville, MD 20852-1776 on
March 2, 1988 and bears the ATCC accession number
67647.

35 Plasmid pEAS41.8 was digested with the
restriction endonuclease AhaII and the 5' overhang
was filled-in as described. The plasmid was then

digested with the restriction endonuclease SalI and
5 electrophoresed on a 1% agarose gel. Two fragments
were isolated: a SalI fragment of about 1500 bp and a
SalI-AhaII fragment of 354 bp. This 354 bp fragment
corresponds to positions 981-1334 of Figure 3 and
consists of 348 bp of Em promoter sequence plus 6 bp
10 of Em coding region. The 1500 bp SalI fragment
represents sequences from position 981 in Figure 3 to
a site about 1500 bp 5' of 981. The plasmid pBM112
was constructed by ligating the 354 bp
SalI/blunt-ended-AhaII fragment into SalI/SmaI
15 digested pBM109.

The 1500 bp SalI fragment was then ligated into
SalI-digested pBM112. A clone was identified in
which the 1500 bp SalI fragment was in the proper
orientation to the SalI/AhaII fragment and this
20 plasmid is called pBM113. The construction of this
plasmid, which contains the approximately 1850 bp
promoter fragment, is diagrammed in Figure 4. This
SalI-SalI-AhaII fragment insertion resulted in the
translational initiation codon (ATG) of the Em gene
25 coding region being 5' relative to and in frame with
the translational initiation codon for the GUS gene.
Therefore, this fusion created a gene whose protein
product is a chimera containing 12 amino acids added
to the natural amino terminus of GUS (assuming the
30 plant translation initiation signals are
preferentially recognized in plant systems).

EXAMPLE 2

35 Construction of plasmids pBM113Kp and pBM311

Plasmid pBM109 was digested with 0.5 units
KpnI/μg DNA (total reaction volume 50 μl) and 10

5 μ l aliquots of the reaction were removed at time
points of 2, 5, 10, 20 and 30 minutes of digestion.
The aliquots were placed in tubes pre-warmed to 70°C
which contained 1 μ l of 25 mM EDTA in agarose gel
tracking dye. The aliquots remained at 70°C for 10
minutes to stop the reaction. The aliquots, which
10 contained partial digestion products, were then
electrophoresed on a 1% agarose gel. The band which
corresponded to linearized pBM109 (4785 bp) was
eluted as already described.

Plasmid pBM113 was digested with the
15 restriction endonuclease KpnI. This generated a 652
bp fragment of DNA containing a portion of the
approximately 1850 bp of DNA from the wheat genome
present. This 652 bp fragment represents 640 bp of
Em promoter sequence, 6 bp of Em coding region and 6
20 bp of polylinker sequence. This 640 bp promoter
fragment corresponds to position 689 to 1328 in
Figure 3. The 652 bp fragment was isolated and
ligated into the linearized pBM109. This ligation
should, theoretically, contain four different
25 constructs due to the presence of two KpnI sites in
pBM109 and the possibility that the 652 bp fragment,
having two identical ends, could have inserted in
either orientation in either site. Two of these
constructs were to be isolated, namely, those into
30 which the KpnI fragment had been inserted 5' relative
to the GUS gene coding region, one in each
orientation. After transforming E. coli with the
ligation mixture, the transformants were screened for
the appropriate constructs by small scale plasmid
35 isolation, etc. as described above. The plasmid
pBM113Kp contains the KpnI fragment in the forward
orientation (with respect to the GUS gene) and has

exactly reconstructed the junction found in the
5 originating plasmid pBM113. Thus, plasmid pBM113Kp
is a derivative of the plasmid pBM109 into which the
652 bp KpnI fragment of pBM113 has been inserted into
the KpnI site 5' relative to the initiation codon of
the GUS gene. The plasmid pBM311 contains the same
10 652 bp fragment in the same KpnI site but in the
opposite orientation. The construction of these
plasmids is diagrammed in Figure 5.

EXAMPLE 3

15

Construction of Plasmids pBM113A220.10, pBM113A220.9, pBM113A240.13, and pBM117

Plasmid pBM113 was subjected to exoIII-mediated
digestion as described in the section entitled
20 "Exonuclease III-Mediated Deletion". Plasmid DNA
(10 μ g) was cleaved with HindIII to completion and
the overhang was filled-in with α -phosphorothioate
nucleotides as described. The reaction was then
extracted and the DNA ethanol precipitated, as
25 described. The linearized, filled-in DNA molecule
was then cleaved with BglII, which cleaves 683 bp 5'
relative to the KpnI site. The sample was extracted
and precipitated as described above. This produced a
molecule which was resistant to exoIII digestion at
30 the HindIII site but sensitive to exoIII digestion at
the BglII site.

ExoIII digestion was performed on the prepared
plasmid as described in the section entitled
"Exonuclease III-Mediated Deletion" and aliquots were
35 removed at 10 second intervals beginning at 40
seconds and continuing to 250 seconds. E. coli cells
were transformed with the ligated samples from each

time interval, and the transformed cells were plated
5 on LB plates containing 100 µg/ml ampicillin. The
plates were incubated at 37°C overnight and placed at
4°C the following day.

Bacteria originating from individual
transformants from the samples of the various time
10 intervals were analyzed by isolation of plasmid DNA
(small scale) and restriction enzyme digestion. The
isolated plasmids were digested with the restriction
enzymes KpnI and PvuII concomitantly. These enzymes
were chosen for the following reasons:

15 1. Deletions were not of interest until the
KpnI site which defines the 5' border of the Em
promoter fragment known to be present in pBM113Kp was
lost. The loss of the KpnI site is indicative of the
removal of sufficient DNA so that the fragment
20 remaining has less of the promoter region than that
in pBM113Kp.

2. The presence of two PvuII sites between the
KpnI site mentioned above and the translational
initiation codon (ATG) of the construct would allow
25 estimation of the deletion endpoint using the sizes
of the fragments produced upon digestion.

Several transformants harboring plasmids of
interest were selected. The plasmids are named
pBM113Δ220.10, pBM113Δ220.9, and pBM113Δ240.13.
30 The construction of these plasmids is diagrammed in
Figure 6. [The Δ in the plasmid name refers to
Deletion, and the number refers to the time interval
(seconds) of digestion]. The endpoints of the
deletions were determined by sequence analysis (see
35 above) and found to be at -291 bp, -254 bp, and -192
bp from the translational initiation codon (ATG)

of the Em gene, respectively. These positions
5 correspond to positions 1038, 1075 and 1137 in
Figure 3, respectively.

Construction of Plasmid pBM117

Plasmid pBM117 is a derivative of the plasmid
10 pBM109 into which the promoter region of the 35S gene
of CaMV has been cloned. This was done by cleavage
of plasmid pDH51 with the restriction endonucleases
NcoI and SmaI.

The overhang produced by NcoI cleavage was
15 filled-in as described in the section entitled
"Enzymatic Treatments of DNA". The resulting
fragments of the cleavage reaction were separated by
electrophoresis on a 1% agarose gel and a fragment
535 bp in length, containing the 35S promoter region,
20 was isolated from the gel as described in the section
entitled "Gel Electrophoresis of DNA".

The plasmid pBM109 was cleaved with the
restriction endonuclease SmaI which results in the
linearization of the molecule. The 535 bp fragment
25 isolated as described in the previous paragraph, was
ligated (as described in the section entitled
"Enzymatic Treatments of DNA") into the SmaI-cleaved
pBM109. After transforming E. coli with the ligation
mixture, as described in the section entitled
30 "Transformation of Bacterial Cells", a clone was
identified which harbored a plasmid containing the
35S promoter fragment of DNA, 5' relative to the GUS
encoding region of DNA, and in the proper
orientation. The construction of this plasmid is
35 diagrammed in Figure 7.

EXAMPLE 45 Construction of Plasmids pBM119, pCS102 and pBM120

Plasmid pRAJ275 served as the source of the β -glucuronidase gene (GUS) and has been described by Jefferson in Plant Molecular Biology Reporter 5:387-405 (1987). The GUS gene in plasmid pRAJ275
10 differs from that in pRAJ260 in that the sequence surrounding the initiator codon ATG has been changed to introduce two new restriction sites, SalI, and NcoI and in the introduction of a single restriction site for the restriction enzyme EcoRI at the 3' end
15 of the gene. The NcoI site contains the translational start codon (ATG) and is flanked immediately 5' by the SalI site. The GUS gene was cleaved from plasmid pRAJ275 by sequential cleavage of the plasmid, as described above, with SalI, after
20 which the SalI site was filled-in as described above, followed by cleavage with EcoRI. This digestion results in the GUS gene being on a 1857 bp fragment. One such fragment was isolated from a 1% agarose gel (described above) and ligated into pUC18 which had
25 been cleaved identically as was pRAJ275 (i.e., SalI digestion, after which the end was filled-in, followed by EcoRI digestion). Individual plasmids were analyzed until one was identified which carried the GUS gene and the resulting plasmid is pBM119.
30 The construction of this plasmid is diagrammed in Figure 8.

Plasmid pDH51, which served as the source of the 35S 3' sequences, was cleaved with the restriction enzymes NcoI and SphI after which the
35 ends of the fragment were treated with T4 polymerase, as described, to create blunt ends. This treatment results in the liberation of a 568 bp fragment

containing the 35S promoter region plus the
5 polylinker region of pDH51 and a 2857 bp fragment
consisting of the remainder of pDH51, which carries
the 35S 3' end. The 2857 bp fragment was isolated
from a 1% agarose gel, ligated, and individual
plasmids were screened for the presence of a 214 bp
10 EcoRI fragment which would carry the 35S 3' region.
The resulting plasmid is pCS102. The construction of
this plasmid is diagrammed in Figure 9.

Plasmid pCS102 was cleaved with EcoRI and the
15 214 bp fragment carrying the 35S 3' end was isolated
on a 1% agarose gel and ligated into EcoRI-cut
pBM119. Individual plasmids were analyzed until one
was isolated which carried the 35S 3' region 3' of the
GUS gene and in the proper 5' to 3' orientation. The
20 resulting plasmid is pBM120 and the construction of
this plasmid is diagrammed in Figure 9.

EXAMPLE 5

Construction of Plasmids pBM165 and pBM167

25 The series of plasmids designated pBM165 are
derivatives of plasmid pDH51 into which an
oligonucleotide (oligo) has been inserted into the
35S promoter region. The oligonucleotide was
inserted into the EcoRV site at -90 bp from the start
30 of transcription from the 35S promoter. The
oligonucleotide was synthesized using an ABS DNA
Synthesizer and includes a 25 bp region of the Em
promoter bearing sequence homology to a region of a
conglycinin promoter plus 19 bp of contiguous Em 5'
35 flanking sequences and 7 bp of contiguous Em 3'
flanking sequences. This 50 bp promoter sequence
corresponds to coordinates 1091 to 1140 in Figure 3.

In addition, the ends of the oligo contain 1/2 an
5 EcoRV site such that the oligo may be inserted into
the EcoRV site and excised with the same enzyme.
Therefore, the oligo is 56 bp in length, and is
hereafter referred to as "the 56 bp oligo". To
construct the pBM165 series of plasmids, the plasmid
10 pDH51 was digested with the restriction enzyme EcoRV
and ligated in the presence of the 56 bp oligo.
Individual plasmids were analyzed for the presence of
the oligo and plasmids were isolated in which the
orientation and copy number of the oligo are as
15 follows:

pBM165-2: This plasmid contains a single copy
of the oligo in the same 5' to 3' orientation as the
sequence occurs in the Em promoter.

20 pBM165-4: This plasmid contains two copies of
the oligo, both of which are in the same 5' to 3'
orientation as the sequence occurs in the Em promoter
(i.e., head-to-tail).

Plasmid pBM165-2 has been deposited in the
American Type Culture Collection (ATCC), Rockville,
25 MD 10852-1776 on September 15, 1988 and bears the
ATCC accession number 67797.

The series of plasmids designated pBM167 are
derivatives of pBM120 into which the 35S promoter
regions from the plasmids pBM165-2 and pBM165-4 have
30 been inserted. Plasmid pBM120 was cleaved with the
restriction enzyme SalI and the ends of the plasmid
were filled-in as described. The plasmids pBM165-2
and pBM165-4 were cleaved with the restriction
enzymes SmaI and EcoRI after which the EcoRI ends of
35 the plasmid were filled-in as described. This
digestion results in the 35S promoter fragment
containing the 56 bp oligo being on a 599 bp fragment

for plasmids pBM165-2 and on a 655 bp fragment for
5 plasmid pBM165-4. These fragments were isolated from
a 1% agarose gel and were ligated to the SalI-cut,
filled-in pBM120. Individual plasmids were analyzed
and isolates were identified which had incorporated
the promoter regions in the proper orientation. The
10 number and orientation of oligonucleotide insertions
were confirmed by DNA sequence analysis, as described
above. These plasmids are the pBM167 series and they
are as follows:

pBM167-11: This plasmid contains the promoter
15 region from the plasmid pBM165-2.

pBM167-4: This plasmid contains the promoter
region from the plasmid pBM165-4.

The construction of plasmid series pBM165 and
plasmid series pBM167 are diagrammed in Figure 10.
20 The 35S promoter, not containing the 56 bp oligo, was
isolated from pDH51 and inserted into plasmid pBM120
exactly as described for the construction of the
plasmid series pBM167 above. The resulting plasmid
is pBM152 and the construction of this plasmid is
25 diagrammed in Figure 11.

EXAMPLE 6

Construction of Binary Plasmids pBM1170, pBM1130 and pBM3110

30 Binary plasmids are plasmids which may be
propagated in several hosts. Many plasmids of this
type are useful for the Agrobacterium-mediated
transformation of certain species of plants,
especially tobacco. Binary constructs were made for
the stable transformation of tobacco with several of
35 the promoter-GUS constructs used in the transient
system described above. The binary vector used for

the construction of these binary plasmids was
5 pBI101.1 and has been described by Jefferson et al.
EMBO 6:3901-3907 (1987). Plasmid pBI101.1 is a
derivative of plasmid pBIN19 into which a
"promoter-less" GUS cassette has been inserted.
Therefore, this plasmid has a plant selectable
10 marker, in the form of a neomycin phosphotransferase
gene (nptII), driven by the nopaline synthase
promoter, which encodes resistance to the antibiotic
kanamycin. All constructs were made by the ligation
of gel-purified HindIII/EcoRI fragments.
15 Digestion of pBI101.1 with these two enzymes
results in the liberation of an approximately 2 kbp
fragment containing the "promoter-less" GUS cassette
from the vector. This fragment is not used in the
constructs. The remaining vector fragment
20 (approximately 10 kbp) was gel purified as described
above. Digestion of plasmid pBM117 with the same two
enzymes results in the liberation of a 2687 bp
fragment from the vector sequences containing a 35S
promoter-GUS-35S 3' cassette. This 2687 bp fragment
25 will be called fragment A. Similarly, digestion of
the plasmids pBM113Kp and pBM311 with the same
enzymes results in the liberation of 2802 bp
fragments from the vector sequences. These fragments
(B and C, respectively) contain the Em
30 promoter-GUS-35S 3' cassettes with the Em promoter in
the forward orientation in fragment B and in the
reverse orientation in fragment C. All three of
these fragments (A, B and C) were individually
ligated into the binary vector sequences purified
35 above (the approximately 10 kbp fragment from
pBI101.1).

Individual binary plasmids were identified
5 which now contained the relevant promoter-GUS
cassette. As such, these plasmids now contain a
selectable plant marker (kanamycin resistance) and an
appropriate cassette containing an experimental
promoter-GUS-35S 3' construct. Plasmids pBM1170,
10 pBM1130 and pBM3110 are binary derivatives of
plasmids pBM117, pBM113Kp and pBM311, respectively.
The construction of these plasmids are diagrammed in
Figures 12, 13 and 14, respectively. These binary
constructs were introduced into the Gram-negative
15 bacterium Agrobacterium tumefaciens by the method of
tri-parental mating and subsequently introduced into
tobacco tissue by the method of leaf disk
transformation (see section on "Transformation of
Plants" and "Stable Transformation of Tobacco").

20

EXAMPLE 7

Response of an 1850 bp Promoter Fragment (pBM113) to ABA

25 Protoplasts were isolated, as described in the
section entitled "Isolation of Protoplasts" from a
suspension culture of rice. The protoplasts were
either not exposed to DNA or were transformed with
plasmid pBM113 DNA as described above in the section
30 entitled "Transformation of Protoplasts". The
culture of protoplasts which did not receive DNA
contained 1×10^6 protoplasts. The culture to be
transformed with plasmid DNA contained 4×10^6
protoplasts and these were exposed to 4 μ g of
35 pBM113 plasmid DNA. The culture with 1×10^6
protoplasts, which served as a control, was
resuspended in 1.0 ml of protoplasts medium after the

transformation protocol and was incubated for 2
5 days. The culture containing transformed protoplasts
was split into 4 equal aliquots each containing 1×10^6
protoplasts and the 4 aliquots were incubated
in protoplast medium either lacking ABA, or
containing 10^{-5} M ABA, 10^{-6} M ABA, or 10^{-7} M ABA,
10 respectively. These cultures were also incubated for
2 days.

After the 2 days incubation, the protoplasts
were collected by centrifugation at 80 x g. The
protoplast medium was removed from the tubes and the
15 cells were lysed by addition of 0.5 ml of GUS lysis
buffer as described in the section entitled "GUS
Assay". Each culture of lysed protoplasts was then
assayed for GUS activity as described above in the
section entitled "GUS Assay". The specific plasmid
20 DNA with which the protoplasts were transformed, the
presence or absence of ABA, the concentrations of ABA
in which the cultures were incubated and the results
from the assay are given below in Table 1.

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30

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5 Table 1

<u>Sample</u>	<u>Treatment</u>		<u>Specific Activity*</u> (Units)
	<u>Source of Input DNA</u>	<u>[ABA]</u>	
10 (1)	NO DNA	0	0.25
(2)	pBM113	0	3.87
(3)	pBM113	10 ⁻⁵ M	1167.65
(4)	pBM113	10 ⁻⁶ M	251.42
(5)	pBM113	10 ⁻⁷ M	4.74

15 *Ten units are equivalent to 5.57 pmoles of 4-MU/ μ g protein/hour in this experiment.

The results shown in Table 1 demonstrate the following points:

20 1. There is no significant endogenous GUS activity in the protoplasts in the absence of DNA (Treatment Sample 1).

2. There is a 300-fold increase in the level of expression generated by the fragment of the Em promoter in the presence of the highest concentration of ABA relative to the level of expression in the
25 absence of ABA (Compare Treatment Samples 2 and 3).

3. The level of induction of the Em promoter-GUS construct is dependent on the concentration of ABA in the medium (Treatment Samples 3-5).

30 Therefore, there is an induction of GUS expression in protoplasts containing the Em promoter-GUS construct (plasmid pBM113) only when ABA is present in the medium. In addition, the level of induction is dependent on the concentration of ABA.

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EXAMPLE 8Time course of ABA Effect

The source of protoplasts used for transformation is identical to that used in EXAMPLE 7. Protoplasts either did not receive added DNA or they were transformed with plasmid DNA as described in the section entitled "Transformation of Protoplasts".

For those protoplasts which did not receive exogenous DNA (i.e., no DNA in Table 2) a culture containing 2×10^6 protoplasts was subjected to the transformation protocol except that it did not receive any DNA. The culture was then divided into 4 equal aliquots (each containing 0.5×10^6 protoplasts). Two of these four aliquots were incubated in protoplast medium without ABA while the other two contained 10^{-5} M ABA. The protoplasts from two samples, one without ABA and one with ABA, were collected immediately after protoplast medium was added. The protoplasts in the other two samples, one without ABA and one with ABA, were collected after 6 hours of incubation in the dark at 25°C.

For those protoplasts which received pBM117 plasmid DNA (to serve as the positive control), a culture containing 2×10^6 protoplasts was transformed with 2 μ g of plasmid DNA then divided and incubated as the cultures above which did not receive DNA (i.e., the four samples were harvested either immediately after addition of protoplast medium (one each, without ABA and with ABA) or at 6 hours (again, one each, without ABA and with ABA)).

The plasmid pBM113 contains the 1850 bp
5 promoter sequence of this invention operably linked
to the GUS gene. A protoplast suspension containing
5 x 10⁶ protoplasts was transformed by the addition
of 5 µg of plasmid DNA during the transformation
protocol.

10 The protoplast suspension was split into 2
equal aliquots (2.5 x 10⁶ protoplasts each), one
without ABA and one with ABA (10⁻⁵M) and incubated
as described.

15 Samples from the protoplast cultures (0.5 x
10⁶ protoplasts), one each, without ABA and with
ABA, were removed immediately after the addition of
protoplast medium, and at 1, 2, 4, and 6 hours of
incubation. All the protoplast samples were stored
20 at -70°C after collection in GUS lysis buffer and
assayed the following day for the presence of GUS
activity as described in the section entitled "GUS
Assay". The results are shown in Table 2.

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Table 2

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<u>Treatment</u>		<u>Specific Activity*</u> (Units)				
<u>Source of</u> <u>Input DNA</u>	<u>[ABA]</u>	<u>0**</u>	<u>1**</u>	<u>2**</u>	<u>4**</u>	<u>6**</u>
10 NO DNA	0	0.30	ND***	ND	ND	0.11
pBM117	0	0.30	ND	ND	ND	10.00
pBM113	0	0.17	0.55	1.91	5.99	36.31
NO DNA	10 ⁻⁵ M	0.17	ND	ND	ND	0.16
pBM117	10 ⁻⁵ M	0.17	ND	ND	ND	9.21
pBM113	10 ⁻⁵ M	0.11	4.38	42.93	290.09	712.80

15

*Ten units are equivalent to a specific activity of 9.2 pmoles 4-MU/μg protein/hour in this experiment.

**Incubation period in hours.

***ND = not done

20

The results in Table 2 demonstrate the following:

1. There is no significant GUS activity intrinsic to the system either in the presence or absence of ABA (No DNA treatment).

25

2. There is no significant effect mediated by ABA on the 35S promoter-GUS construct (pBM117) implying that the effect is specific for promoter sequences normally modulated by ABA in vivo.

3. There is a time-dependent effect of ABA on the Em promoter-GUS construct (pBM113).

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EXAMPLE 9

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Effect of Phaseic Acid on Em Promoter

The protoplasts for this example are from the same source as that for EXAMPLE 7. Protoplasts were transformed with either calf thymus (CT) DNA, with
10 pBM113 plasmid DNA or pBM117 plasmid DNA by the procedure described in the section entitled "Transformation of Protoplasts". CT DNA is non-specific DNA derived from calf thymus and is used as a negative control in protoplast transformation.

15 Protoplast suspensions containing 1×10^6 protoplasts were used. Protoplast suspensions receiving CT DNA were transformed by the addition of 25 μ g of CT DNA and those being transformed with DNA from pBM117 received 5 μ g plasmid DNA and 20
20 μ g of CT DNA.

Protoplast suspensions to be transformed with DNA from plasmid pBM113 contained 2.0×10^6 protoplasts, 10.0 μ g plasmid DNA and 15 μ g CT DNA. After the transformation procedure, the
25 suspensions transformed with CT DNA and DNA from pBM117 were each divided into two equal aliquots (0.5×10^6 protoplasts each) and incubated in protoplast medium. One aliquot was without ABA while the other contained 10^{-4} M ABA. The suspension transformed
30 with DNA from pBM113 was divided into 3 aliquots (0.5×10^6 protoplasts each) and similarly incubated in protoplast medium either without ABA, with 10^{-4} M ABA or with 10^{-4} M phaseic acid (PA).

35

After the incubation period (4 hours), the
5 protoplasts in each culture were collected by
centrifugation and stored at -70°C in GUS lysis
buffer. All aliquots were later assayed for enzyme
activity. The results are shown in Table 3.

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TABLE 3

5	Treatment			Specific Activity* (Units)
	Source of Input DNA	[ABA]	[PA]**	
10	CT	0	0	2.02
	CT	10 ⁻⁴ M	0	1.81
	pBM117	0	0	10.00
	pBM117	10 ⁻⁴ M	0	9.20
	pBM113	0	0	8.24
	pBM113	10 ⁻⁴ M	0	31.51
	pBM113	0	10 ⁻⁴ M	8.36

15

*Ten units are equivalent to 2.18 pmoles
4-MU/ μ g protein/hour in this experiment.

**Phaseic Acid

The results from Table 3 demonstrate that
20 phaseic acid, a structural analog and the first
breakdown product of ABA in vivo, does not induce
expression of the Em promoter-GUS construct. This is
consistent with previous results in which phaseic
acid does not mediate the in vivo physiological
25 effects attributed to ABA in wheat embryos, i.e.,
phaseic acid does not inhibit seed germination.
Non-specific, non-plant DNA (CT) did not respond to
ABA.

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EXAMPLE 10Equivalence of pBM113 and pBM113Kp

The source of protoplasts for this example is
10 the same as that used in EXAMPLE 7. For this
example, several cultures, each containing 2×10^6
protoplasts either received no DNA or were
transformed, as described in EXAMPLE 7, with plasmid
DNA (2 μ g) of the plasmids pBM117, pBM113,
15 pBM113Kp or pBM311. Those suspensions which did not
receive DNA, or received either pBM117 DNA or pBM311
DNA were divided into 2 equal aliquots after the
transformation protocol, placed in protoplast medium,
and incubated for 18 hours either in the presence of
20 10^{-4} M ABA or without added ABA. Transformations
with pBM113 and pBM113Kp were each done in duplicate
and the duplicates were pooled.

The pooled duplicates were then split into
four (4) equal aliquots. The transformed protoplasts
25 were incubated for 18 hours in protoplast medium at
25°C in the dark. The protoplast medium contained
the concentration of ABA shown in the table below.
The results of the GUS assay of these treatments are
given in Table 4.

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TABLE 4

5	Treatment			
	Sample	Source of Input DNA	[ABA]	Specific Activity* (Units)
10	(1)	NO DNA	0	0.17
	(2)	NO DNA	10 ⁻⁴ M	0.15
	(3)	pBM117	0	10.00
	(4)	pBM117	10 ⁻⁴ M	8.24
	(5)	pBM113	0	17.15
	(6)	pBM113	10 ⁻⁴ M	563.05
	(7)	pBM113	10 ⁻⁵ M	233.20
	(8)	pBM113	10 ⁻⁶ M	109.77
	(9)	pBM113Kp	0	18.50
	(10)	pBM113Kp	10 ⁻⁴ M	1170.09
15	(11)	pBM113Kp	10 ⁻⁵ M	403.70
	(12)	pBM113Kp	10 ⁻⁶ M	130.61
	(13)	pBM311	0	0.21
	(14)	pBM311	10 ⁻⁴ M	0.45

20 *Ten units are equivalent to 6.59 pmoles 4-MU produced/ μ g protein/hour in this experiment.

The results represented in Table 4 demonstrate:

- 25 1. Expression of GUS activity from plasmids pBM113 and pBM113Kp is regulated by the presence of ABA (Treatment Samples 5 and 6; 9 and 10).
2. The induction of GUS expression from both plasmids, pBM113 and pBM113Kp, is not only regulated by ABA, but the level of expression is dependent on the concentration of ABA in the medium (Treatment Samples 6-8; 10-12).
- 30 3. The plasmid pBM113Kp, which contains only 652 bp of the Em promoter region, behaves similarly to the plasmid pBM113, which contains approximately 1850 bp of the Em promoter region (Treatment Samples 5-8; 9-12).
- 35 4. Plasmid pBM311, which contains only 652 bp of the Em promoter region linked to GUS in the wrong orientation (unoperably linked), did not significantly express GUS activity when treated with ABA (Treatment Samples 13-14). Furthermore, the low

levels of activity seen with the same promoter
fragment operably linked to GUS (pBM113Kp) when
5 incubated without ABA, is totally abolished when
pBM311 is used (compare Treatment Samples 9 and 13).

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EXAMPLE 11Response of a 254 bp Promoter Fragment to ABA

The source of protoplasts for this EXAMPLE is the same as that used in EXAMPLE 7. Transformations were performed using 2×10^6 protoplasts and 2 μ g of plasmid DNA (except for the control samples which did not receive DNA). Each suspension of transformed protoplasts was split equally (1×10^6 protoplasts) into protoplast medium either without ABA or with 10^{-4} M ABA and incubated at 25°C in the dark for 18 hours. The protoplasts were then collected and assayed for GUS activity. The results are given in Table 5.

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TABLE 5

5	Treatment			
Sample	Source of Input DNA	[ABA]	Specific Activity* (Units)	
(1)	NO DNA	0	0.86	
(2)	pBM117	0	10.00	
10	(3) pBM113Δ220.9	0	21.83	
	(4) pBM113Δ220.9	10 ⁻⁴ M	282.90	
	(5) pBM113Δ220.10	0	21.29	
	(6) pBM113Δ220.10	10 ⁻⁴ M	802.31	
	(7) pBM113Δ240.13	0	1.83	
	(8) pBM113Δ240.13	10 ⁻⁴ M	4.3	

15 *Ten units are equivalent to 1.86 pmoles 4-MU
produced/μg protein/hour in this experiment.

The results of the above experiment demonstrate:

20 1. Plasmids containing fragments of the Em
promoter to -291 bp (pBM113Δ220.10) and -254 bp
(pBM113Δ220.9) 5' from the translation start codon
(ATG) operably linked to GUS still retain the ability
to be induced by ABA (Treatment Samples 3 and 4; 5
and 6).

25 2. A plasmid containing a fragment of the Em
promoter to -192 bp (pBM113Δ240.13) 5' from the
translation start codon (ATG) operably linked to GUS
cannot be induced by ABA (Treatment Samples 7 and 8).

30 3. Deletion to -192 bp from the ATG not only
prevents ABA enhancement of expression from the Em
promoter region but also abolishes the low level of
expression seen in the absence of ABA using longer Em
promoter fragments (Compare Treatment Sample 7 with 3
and 5).

35 4. Plasmids pBM113Δ220.9 and pBM113Δ240.13
define a region of 62 bp in the Em promoter (-192 to
-254) at least part of which is necessary for the ABA
induction (ABA regulatory region).

EXAMPLES 12/135 Effect of two ABA analogs (Compounds C and D) on
Em Promoter

6 The source of protoplasts for this EXAMPLE is
7 the same as in EXAMPLE 7. The transformation was
8 performed in duplicate using 50×10^6 protoplasts
9 and 100 μ g of plasmid DNA. The transformed
10 protoplasts were split four ways and incubated in
11 culture medium containing either ABA, Compound C,
12 Compound D, or with no additions, at 25°C in the dark
13 for 18 hours. The protoplasts were then collected
14 and assayed for GUS activity as described. The
15 results are given in Table 6.

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TABLE 6

5	<u>Sample</u>	<u>Source of Input DNA</u>	<u>Additions to Culture Medium</u>	<u>Specific Activity (pmoles 4-MU/μg protein/hour)</u>
10	1	No DNA	- - - - -	2.05
	2	pBM113Kp	- - - - -	3.24
	3	pBM113Kp	ABA	50.25
	4	pBM113Kp	Compound D	38.89
15	5	pBM113Kp	Compound C	8.40

The results in Table 6 demonstrate the following:

20 1. Compounds C and D, both structural and functional analogs of ABA, induce expression of GUS activity from plasmid pBM113Kp at a concentration of 10^{-4} M.

2. Compounds D and ABA induce GUS activity approximately 20-fold over no additions, while Compound C had an approximate 4-fold stimulation.

25 3. These results and those of EXAMPLE 8 confirm that this transient expression system can rapidly and specifically determine if close structural and/or functional analogs of ABA possess gene-inducing activity, and can be used as a screen to identify analogs as potential inducers of gene expression.

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EXAMPLE 14

5 The results from EXAMPLE 11 clearly demonstrate
that at least part of the DNA sequences necessary for
ABA-mediated regulation of gene expression reside in
a 62 bp region between -192 and -254 bp from the ATG
codon of the Em coding region (as defined by plasmids
10 pBM113Δ240.13 and pBM113Δ220.9, respectively).
Within this region is a sequence, 25 bp in length,
which bears close homology (76%) to a sequence from
the β-conglycinin gene of the dicot soybean.
β-conglycinin expression is also developmentally
15 regulated by ABA (see page 4). This region of
homology is at the promoter proximal end of, and is
completely contained within, the 62 bp region. The
presence of this region of homology prompted the
synthesis of the 56 bp oligo (see above). The
20 introduction of the 56 bp oligo into a foreign
promoter will cause expression from the foreign
promoter to be influenced by ABA.

 The protoplasts for this example are from the
same source as that for EXAMPLE 7. Protoplasts were
25 transformed with either no DNA, with pBM117 plasmid
DNA, or with pBM167 plasmid DNA by the procedure
described in the section entitled "Transformation of
Protoplasts". The pBM167 series of plasmids is as
described in EXAMPLE 5.

30 Protoplast suspensions containing 4×10^6
protoplasts were used and were transformed by the
addition of either no DNA or by the addition of
4 μg plasmid DNA. After the transformation

procedure, the suspensions were divided into two
5 equal aliquots (2×10^6 protoplasts each) and
incubated in protoplast medium. One aliquot of each
sample was incubated without ABA while the other was
incubated with 10^{-4} M ABA. After the incubation
period, the protoplasts in each culture were
10 collected by centrifugation and lysed by addition of
0.5 ml GUS assay/lysis buffer. All aliquots were
assayed for GUS enzyme activity and the results are
shown in Table 7.

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TABLE 7

Treatment				
	Sample	Source of Input DNA	[ABA]	Specific Activity (pmoles 4MU/ μ g protein/hour)
5				
10	1	No DNA	0	1.02
	2	No DNA	10^{-4} M	1.02
	3	pBM152	0	1777.13
	4	pBM152	10^{-4} M	1730.42
15	5	pBM167-11	0	1108.50
	6	pBM167-11	10^{-4} M	2081.18
	7	pBM167-4	0	104.39
20	8	pBM167-4	10^{-4} M	560.51

The results in Table 7 demonstrate the following points:

25 1. There is no significant GUS activity intrinsic to the rice protoplast system, in the presence or absence of ABA (Treatment Samples 1 and 2).

30 2. There is no significant effect mediated by ABA on the 35S promoter-GUS construct, pBM152 (Treatment Samples 3 and 4). The 35S promoter is unresponsive to ABA.

35 3. There is a decrease of over 30% in GUS activity directed by the 35S promoter upon insertion of one copy of the 56 bp oligo into this promoter (Compare Treatment Samples 3 and 5). When two tandem copies of the 56 bp oligo are inserted into the 35S promoter, a decrease of over 90% in GUS activity is observed (Compare Treatment Samples 3 and 7). The reason for this decrease is not clear.

4. However, there is a significant increase in GUS activity over these levels when protoplasts containing plasmids with the 56 bp oligo inserted in the 35S promoter are treated with ABA: about two-fold with one copy of the 56 bp oligo (plasmid pBM167-11, and, over five-fold with two copies (plasmid pBM167-4). (Compare Treatment Samples 5 with 6, and 7 with 8.)

These results clearly demonstrate that the 56 bp oligo contains ABA-regulatory sequences that confer ABA regulation to the normally ABA-unresponsive 35S promoter.

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EXAMPLE 155 Expression of GUS activity in maturing seeds of
transgenic tobacco

Binary plasmids containing the plasmids pBM1170
(³⁵S-GUS) and pBM1130 (Em-GUS) were introduced into
the Gram-negative bacterium Agrobacterium tumefaciens
10 by the method of triparental mating and subsequently
introduced into tobacco tissue by the leaf disk
transformation protocol. Transgenic tobacco plants
were selected and grown as described earlier. At
various times after flowering, seeds at different
15 stages of maturation were collected and assayed for
GUS activity. Detailed procedures are described in
the section "Stable Transformation of Tobacco".

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TABLE 8

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	<u>Sample</u>	<u>Promoter Construction</u>	<u>Stages of Seed Maturity</u>	<u>Specific Activity*</u> (pmole 4MU/min/ µg protein)
10	1	pBM1170	Very Immature (white)	1,037
	2	pBM1170	Immature (tan)	307
	3	pBM1170	Mature (brown)	269
15	4	pBM1130	Very Immature (white)	2
	5	pBM1130	Immature (tan)	8
	6	pBM1130	Mature (brown)	29,000

20 *Each value is the average of 2 samples taken from the same capsule of a single transformed tobacco plant. Seeds from untransformed tobacco plants showed no GUS activity. Values represent activity over time zero in the enzyme assay.

The results of the above experiment demonstrate:

25 1. Tobacco plants transformed with plasmid pBM1170 (Samples 1-3) show similar levels of accumulated GUS activity in seeds at various stages of maturity; from the very immature (white seeds), the immature (tan seeds) and the dry mature seed (dark brown). 35S is a constitutive promoter in higher plants and clearly shows its predicted activity throughout seed development.

30 2. Tobacco plants transformed with plasmid pBM1130 (Samples 4-6) show increasing amounts of accumulated GUS activity in seeds at later stages of maturation. This pattern of GUS accumulation in later stages of transgenic tobacco seeds is similar to increasing Em expression in normal wheat seeds indicating that the fragment of the Em promoter
35 (650 bp) linked to GUS is being regulated in a manner predictable from its normal expression in wheat.

3. The Em promoter fragment from wheat
(monocot) is recognized and regulated in a normal
5 pattern in tobacco (dicot), presumably by the
increasing endogenous levels of ABA occurring in late
seed development.

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EXAMPLE 165 Expression of GUS activity in transgenic tobacco
 seeds in response to ABA

Binary plasmids pBM1170 (35S-GUS), pBM1130
(Em-GUS) and pBM3110 (mE-GUS) were introduced into
Agrobacterium and used to transform tobacco leaf
10 disks by the methods outlined in EXAMPLE 15 and in
the section Stable Transformation of Tobacco.
Immature seeds (tan in color) were removed from the
capsule as described in Stable Transformation of
Tobacco and incubated for 24 hours on MX-medium with
15 or without ABA.

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TABLE 9

5	<u>Sample</u>	<u>Promoter Construction</u>	<u>[ABA]</u>	<u>Specific Activity*</u> (pmole 4MU/min/ μg protein)
	1	pBM1170	0	29
	2	pBM1170	10 ⁻⁴ M	33
10	3	pBM1130	0	50
	4	pBM1130	10 ⁻⁴ M	160
	5	pBM3110	0	0
15	6	pBM3110	10 ⁻⁴ M	0

*Samples 1 and 2 each represent an average of 4 seed samples (immature seed-tan) taken from two capsules of a single transformant. Samples 3 and 4 each represent an average of 4 seed samples (immature seed-tan) taken from two capsules of another single transformant. Samples 5 and 6 represent an average of 2 seed samples (immature seed-tan) taken from one capsule of one transformant. Values represent values over time zero in the enzyme assay. Seeds from untransformed tobacco plants showed no GUS activity.

1. Tobacco plants transformed with plasmid pBM1170 (Samples 1 and 2) showed no stimulation of GUS activity when immature seeds were incubated with 10⁻⁴M ABA. These results in stable transformants are identical to the results obtained with the same constructs in the transient system (see EXAMPLE 10, Table 4).

2. Tobacco plants transformed with plasmid pBM1130 (Samples 3 and 4) showed approximately a 3-fold increase of GUS activity when immature seeds were incubated in 10⁻⁴M ABA. When the promoter is reversed (Samples 5 and 6) no GUS activity is expressed. These results are identical to the results obtained with the same constructs in the transient system (see EXAMPLE 10, Table 4).

3. Results 1) and 2) demonstrate the ability of the transient assay to accurately predict expression of GUS in transgenic plants.

4. The EM promoter fragment from wheat
(monocot) is recognized and regulated by ABA in
transgenic tobacco (dicot) in the same manner as in
wheat, i.e., isolated immature wheat embryos when
treated with ABA express the Em gene.

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EXAMPLE 17

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Development of Drought Tolerance in Plants

The Em protein of wheat has been implicated in protection of the embryo during seed desiccation (McCubbin et al., Can. J. Biochem. Cell Biol.,
10 63:803-811 (1985) and Litts et al., Nucl. Acids Res. 15:3607-3618 (1987)). Normally during the life cycle of the wheat plant, Em is accumulated only in the embryo of seeds. It will be valuable to express this protein, with its natural ability to bind water, not
15 just in seeds but in whole plants or specific tissue(s) that are experiencing unpredictable drought or water stress. Such expression and accumulation of the Em protein will allow the plant or its affected parts to retain water.

20 We will use the information gained in the construction of this invention to protect plants under drought conditions. A general response of plants to water stress or drought is to accumulate large amounts of ABA in affected tissue (e.g.,
25 Guerrero and Mullet, Plant Physiol. 80:588-591 (1986)). The ABA regulatory region of the Em promoter will be used to over-produce the Em protein under conditions of water stress, resulting in the plant becoming tolerant to these adverse conditions.

30 In Example 14, we described a "56 bp oligo", containing a 50 bp region of the wheat Em promoter, that was sufficient for ABA regulation of GUS. We have operably linked this inducible ABA regulatory region of the Em promoter with the constitutive 35S
35 promoter of CaMV by synthesizing the 56 bp oligo such that it can be inserted into the EcoRV site present in the 35S promoter. The 35S promoter will provide

the polymerase binding sequences necessary for
5 transcription that have been removed from the ABA
regulatory fragment. This chimeric promoter will
then be operably linked to the coding region of Em.
Such a construct, when stably transformed into
plants, will direct the low level expression of the
10 Em protein constitutively throughout the plant.
However, when endogenous levels of ABA increase under
drought conditions, an over-production of the Em
protein will result due to the presence of the ABA
regulatory sequence, thereby protecting the tissue
15 experiencing the water stress.

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CLAIMS

5 What is claimed is:

1. A nucleic acid promoter fragment
comprising a nucleotide sequence from the 5' flanking
promoter region of a plant gene homologous to the Em
10 structural gene of wheat, said promoter fragment
being responsive in transformed plant cells to
compounds possessing ABA-like activity such that the
expression of DNA sequences for selected gene
products operably linked on the 3' side of said
15 promoter fragment can be controlled, with the proviso
that said promoter fragment is not responsive to
phaseic acid.

2. A nucleic acid promoter fragment of Claim
1 wherein said compound possessing ABA-like activity
20 is selected from the group consisting of ABA,
Compound C, Compound D, Compound E, and Compound F.

3. A nucleic acid promoter fragment of Claim
2 wherein said compound possessing ABA-like activity
is ABA.

25 4. A nucleic acid promoter fragment of
Claim 1 comprising a nucleotide sequence from the 5'
flanking promoter region of a plant gene homologous
to the Em structural gene of wheat, said promoter
fragment being responsive in transformed
30 monocotyledonous plant cells to compounds possessing
ABA-like activity such that the expression of DNA
sequences for selected gene products operably linked
on the 3' side of said promoter fragment can be
controlled.

35 5. A nucleic acid promoter fragment of
Claim 1 comprising the 5' flanking promoter region of
the Em structural gene of wheat.

6. A nucleic acid promoter fragment of
5 Claim 5 comprising the nucleotide sequence of about
1850 base pairs running in the 5' direction from base
pair position 0 to about base pair position -1850
with reference to the translation start codon ATG of
the Em structural gene.

10 7. A nucleic acid promoter fragment of
Claim 5 comprising the nucleotide sequence of 640
base pairs running in the 5' direction from base pair
position 0 to base pair position -640 with reference
to the translation start codon ATG of the Em
15 structural gene.

8. A nucleic acid promoter fragment of
Claim 5 comprising the nucleotide sequence of 254
base pairs running in the 5' direction from base pair
position 0 to base pair position -254 with reference
20 to the translation start codon ATG of the Em
structural gene.

9. A nucleic acid promoter fragment of
Claim 5 comprising the nucleotide sequence of 50 base
pairs running in the 5' direction from base pair
25 position -189 to base pair position -238 with
reference to the translation start codon ATG of the
Em structural gene.

10. A substantially pure preparation of the
5' flanking promoter region of a gene from a plant
30 homologous to the Em structural gene of wheat capable
of being operably linked on the 3' side to a
nucleotide sequence for a selected gene product and
incorporated into a recombinant DNA construct for the
transformation of a plant cell such that said
35 promoter region is responsive to a compound
possessing ABA-like activity to control the
expression of said nucleotide sequence for a selected
gene product in said plant.

11. A promoter region of Claim 10 wherein
5 said gene is the Em gene of wheat and said compound
possessing ABA-like activity is ABA.

12. A nucleic acid promoter fragment of Claim
5 comprising the nucleotide sequence of 1328 base
pairs corresponding to base pairs 1 to 1328 in
10 Figure 3, and any mutation or derivative of said
nucleotide sequence.

13. A nucleic acid promoter fragment of Claim
5 comprising the nucleotide sequence of 640 base
pairs corresponding to base pairs 689 to 1328 in
15 Figure 3, and any mutation or derivative of said
nucleotide sequence.

14. A nucleic acid promoter fragment of Claim
5 comprising the nucleotide sequence of 254 base
pairs corresponding to base pairs 1075 to 1328 in
20 Figure 3, and any mutation or derivative of said
nucleotide sequence.

15. A nucleic acid promoter fragment of Claim
5 comprising the nucleotide sequence of 50 base pairs
corresponding to base pairs 1091-1140 in Figure 3,
25 and any mutation or derivative of said nucleotide
sequence.

16. A recombinant DNA construct capable of
transforming a protoplast comprising a nucleic acid
promoter fragment of Claims 1, 2, 3, 4, 5, 6, 7, 8,
30 9, 12, 13, 14 or 15 and a DNA sequence for a selected
gene product operably linked to said promoter
fragment such that upon exposure of a transformed
protoplast to a compound possessing ABA-like activity
said DNA sequence for a selected gene product is
35 expressed.

17. A recombinant DNA construct capable of
5 transforming a protoplast comprising a nucleic acid
promoter fragment of Claim 1 and a DNA sequence for a
selected gene product operably linked to said
promoter fragment wherein said DNA sequence for a
selected gene product is the sequence for
10 β -glucuronidase.

18. A recombinant DNA construct capable of
transforming a protoplast comprising a nucleic acid
promoter fragment of Claim 1 and a DNA sequence for a
selected gene product operably linked to said
15 promoter fragment wherein said protoplast is a rice
protoplast.

19. A recombinant DNA construct capable of
transforming a plant cell comprising a nucleic acid
promoter fragment of Claims 1, 2, 3, 4, 5, 6, 7, 8,
20 9, 12, 13, 14 or 15, a DNA sequence for a selected
gene product operably linked to said promoter
fragment, and a suitable regulatory sequence such
that upon exposure of said transformed plant cell to
a compound possessing ABA-like activity said DNA
25 sequence for a selected gene product is expressed.

20. A plant containing a nucleic acid
promoter fragment of Claims 1, 2, 3, 4, 5, 6, 7, 8,
9, 12, 13, 14 or 15.

21. A rice plant containing a nucleic acid
30 promoter fragment of Claim 1.

22. A tobacco plant containing a nucleic acid
promoter fragment of Claim 1.

23. Seed obtained by growing a plant of Claim
20.

35 24. A plant protoplast containing the nucleic
acid promoter fragment of Claims 1, 2, 3, 4, 5, 6, 7,
8, 9, 12, 13, 14 or 15.

25. A rice protoplast containing the nucleic
5 acid promoter fragment of Claim 1.

26. A transient assay method to evaluate the
likelihood of expression of a selected gene product
in a stably transformed plant comprising the steps of
(a) preparing protoplasts of rice plants, (b)
10 transferring to said protoplasts a recombinant DNA
construct of Claim 16, (c) incubating said
protoplasts containing said construct with a compound
possessing ABA-like activity, and (d) measuring the
amount of said selected gene product expressed in
15 response to said compound possessing ABA-like
activity.

27. The method of Claim 26 wherein said DNA
sequence for a selected gene product is the sequence
for β -glucuronidase.

20 28. The method of Claim 26 wherein said
compound possessing ABA-like activity is ABA.

29. A method for controlling the expression
of a selected gene product in a plant comprising the
steps of (a) transforming said plant with a
25 recombinant DNA construct of Claim 16, (b) exposing
said plant to a compound possessing ABA-like
activity, and (c) causing said plant to express said
selected gene product at a desired time.

30. A method of Claim 29 wherein said
30 compound possessing ABA-like activity is selected
from the group consisting of ABA, Compound C,
Compound D, Compound E, and Compound F.

31. A nucleic acid fragment for controlling
over-production of a selected gene product in plants
35 under drought conditions comprising a nucleic acid
promoter fragment of Claim 15 inserted into the CaMV
35S promoter sequence.

32. A recombinant DNA construct capable of
5 transforming a plant comprising a nucleic acid
fragment of Claim 31, the Em structural gene of wheat
operably linked to said fragment, such that upon
encountering low-water conditions resulting in the
endogenous production of ABA, said plant produces the
10 Em structural protein.

33. A plant containing a recombinant DNA
construct of Claim 32.

34. Seed obtained by growing the plant of
Claim 33.

15 35. A plant of Claim 34 wherein said plant is
rice.

36. The Em promoter fragment inducer compound
2-fluoro-5-l-hydroxy-2,6,6-trimethyl-4-oxo-cyclohex-
2-en-1-yl/-3-methyl-2,4-pentadienoic acid.

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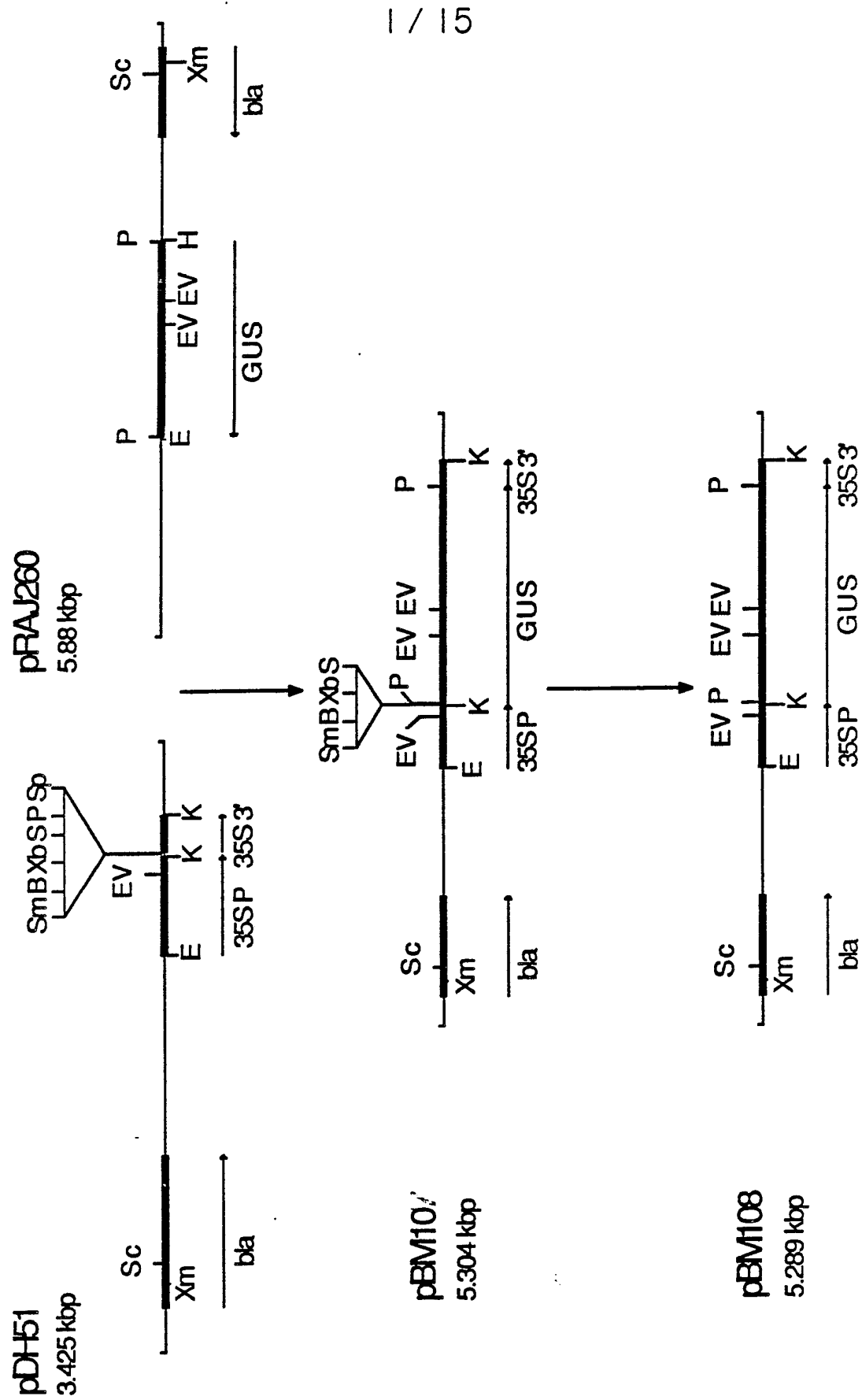


FIG. 1

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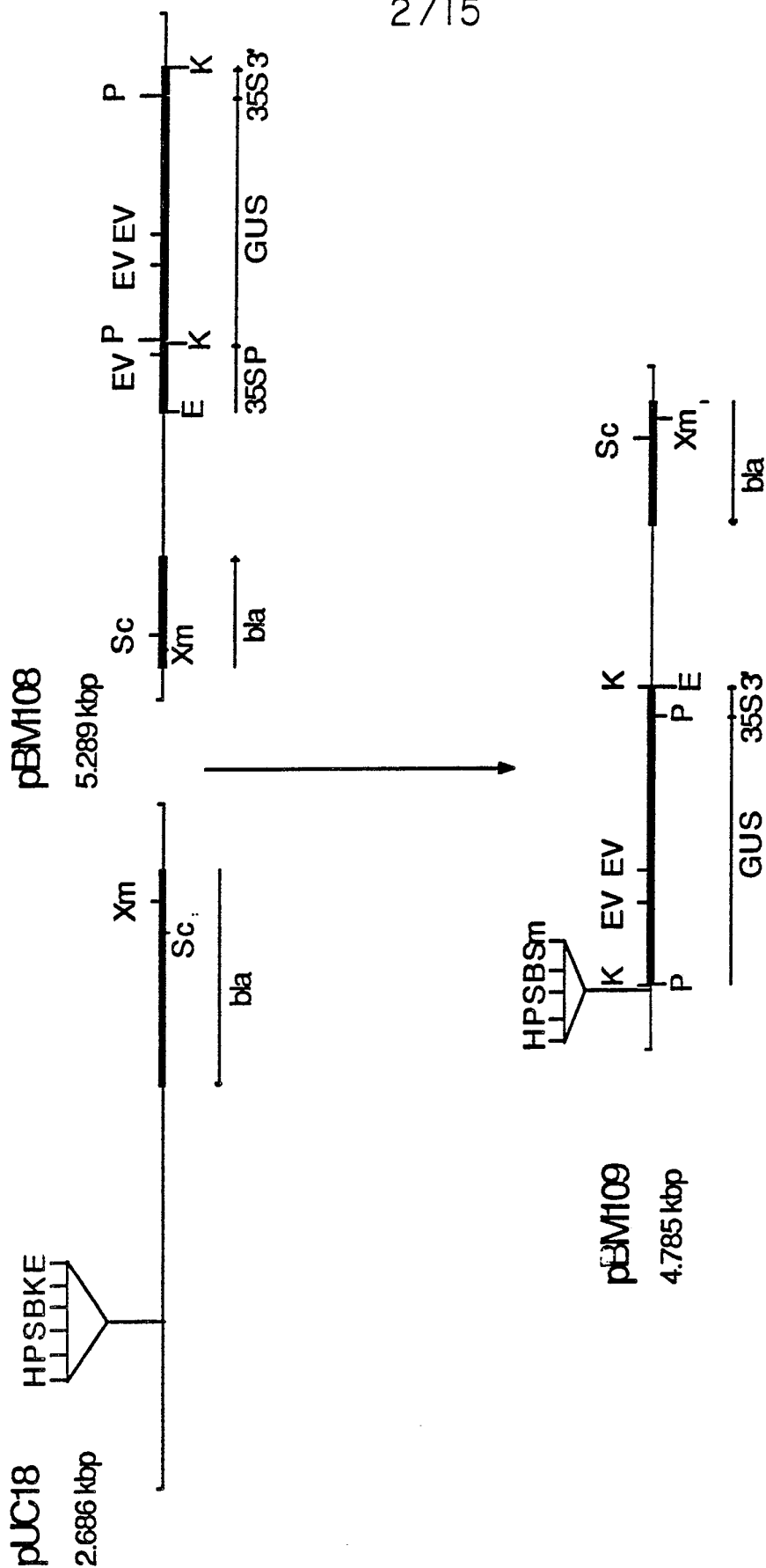


FIG. 2

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1 AGATCTAAAC TAACACGTGT CCTTACAAA AATAAGTGA AATAGAAAAC
51 ACATAGAATA TAGATATAAA CTAGCAAAAG AGCCCGTGCG TTGCAACGGG
101 AGAATAAATA ATACATGTTC TTAACCCCAAT AACCATGACT CAAGACCCCTA
151 ATGTGTTTCA GTCCTTTATT TTCACATGGC ATCACATTTG TGTGTTGAT
201 GGTGGTCTCA GTGCTCACAT AAAGAAAGCG TGTTTGAATG CGGTCAGTTC
251 TAAGGCATCT CTCCTCGGCA CACTCGCTCG AAATAAAATA AGAAATATGT
301 TCTTTTTC CCGGAGGTTT TTCACAGGTG TGCATGCCGTG GTTATTGATG
351 TTTTCTTTCC ACTCTATCAG TAAAAAGAA AACACATCA TACATTATAG
401 ATTAAGTTCA CACAAAATAA TATTTAGAA ATATTTAAAC GCTAAAAATA
451 GCATCATATT TAGATTCTAC ACATTTTCT AATCAAGTTT CATATATAAC
501 ATGTTAAAT TGGAAATTACG GTTAAAGA TATTGATAAT TTTGTTTAGA
551 TAAACTGTCA GTGGGTTTG TGTAAACG AAAAAACGA TTCGGTTGAC
601 TTAAGTATGG ACAGTGGGTT GATTAAACA TCAGAGGGTT TTGCGAGAAA

FIG. 3

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651 ATGCAAAATAA AAGGTTCCGG AGTGAGTTAA ACGGGTACCA CCGCAGTTTA
701 TTTACGAAAA ATAGAGGGTT TTTTAAAAA AAATATATGA CGGGCGACCA
751 GAAACCCAAT TTGCTTTATT ATTATTAAAA AAATAAATAA AATAGTAAAA
801 ATAAAGATAA AAATAAAAT ATAATAAAAT GGCTGCATGC ATCTCCGACG
851 CAGAGGCCGG GGTCACTCTT TAAAAAGAA GAAATATAAA TATATTAGAG
901 CAAACCATAC GAAGAAATGG CATGACGATC GGTTCACGGC CAGTCTCCGA
951 TCGAGGCCGG CCGCTACAAA CGTACACGGG TCGACAAATGC ATGCATGCAA
1001 GCAGAGTCTT GAGCTTCTCG TCCCCTTCCT CGGATAACTC CATGCCCTGC
1051 GAGGGCACGC CCATTACGTG TTGTCTTCCA GGCCCTTGCC GGACACGTGG
1101 CGCGACAGCA GGGACAACGA GCAGGCCGAC GCACGTCCGC GTCGCTGCAC
1151 ACGTGCCGCC TCCGTGCTTC ACGACGCACC GCGCCCTCC AGCTATAAAA
1201 ACACGGCGTA TGGCTCGTCT TCTCCACCAT CGATCATTTG CTCGAGCTCG
1251 AGCGCCAGCA GTTGCAATACA CCACACACGC ATCCACACGT CCGTTTCAGG
1301 AACCTTAGCG GTCGAGCACC TGTTAGCAAT GGCG

F I G. 3 (Cont.)

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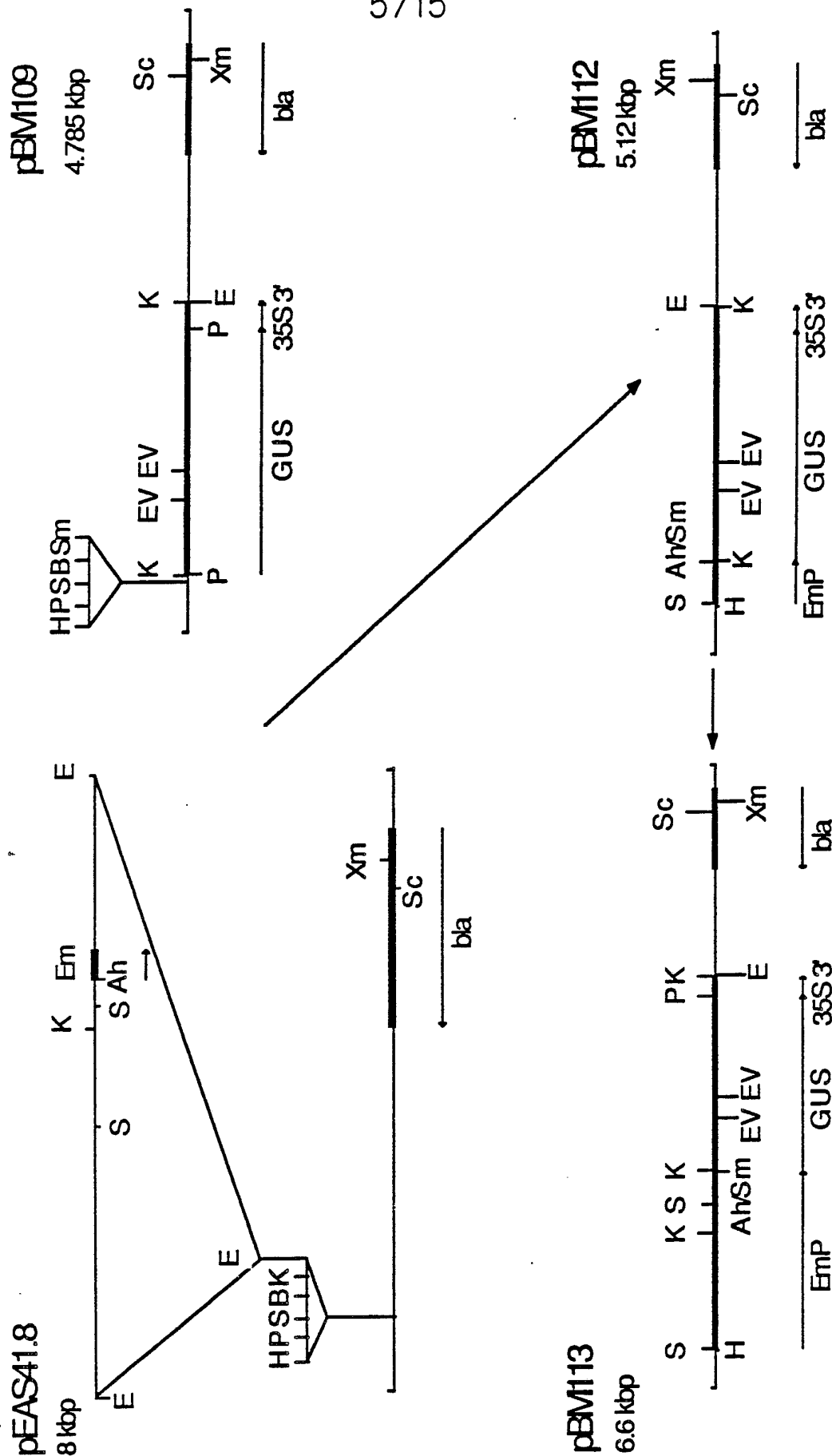


FIG. 4

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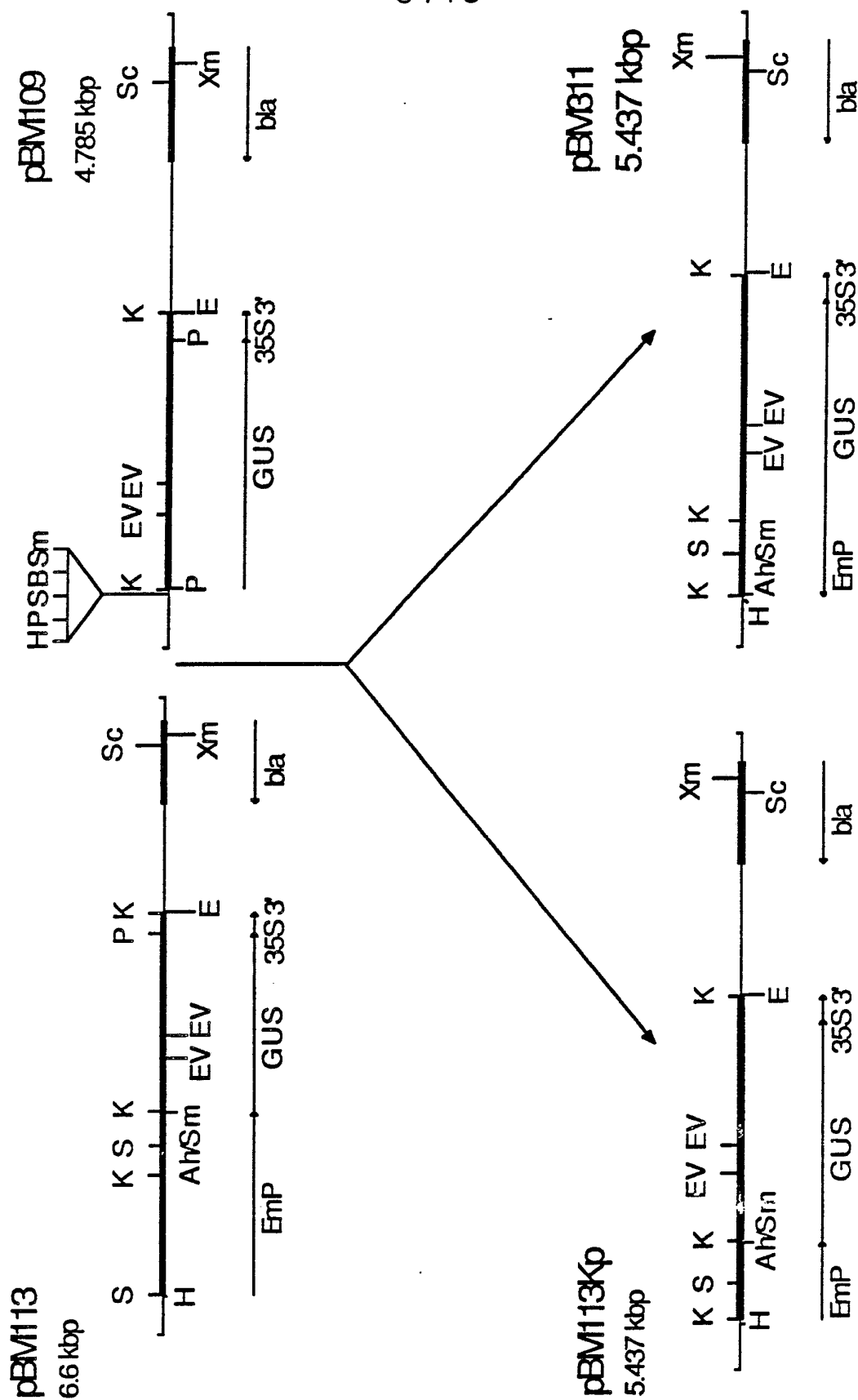


FIG. 5

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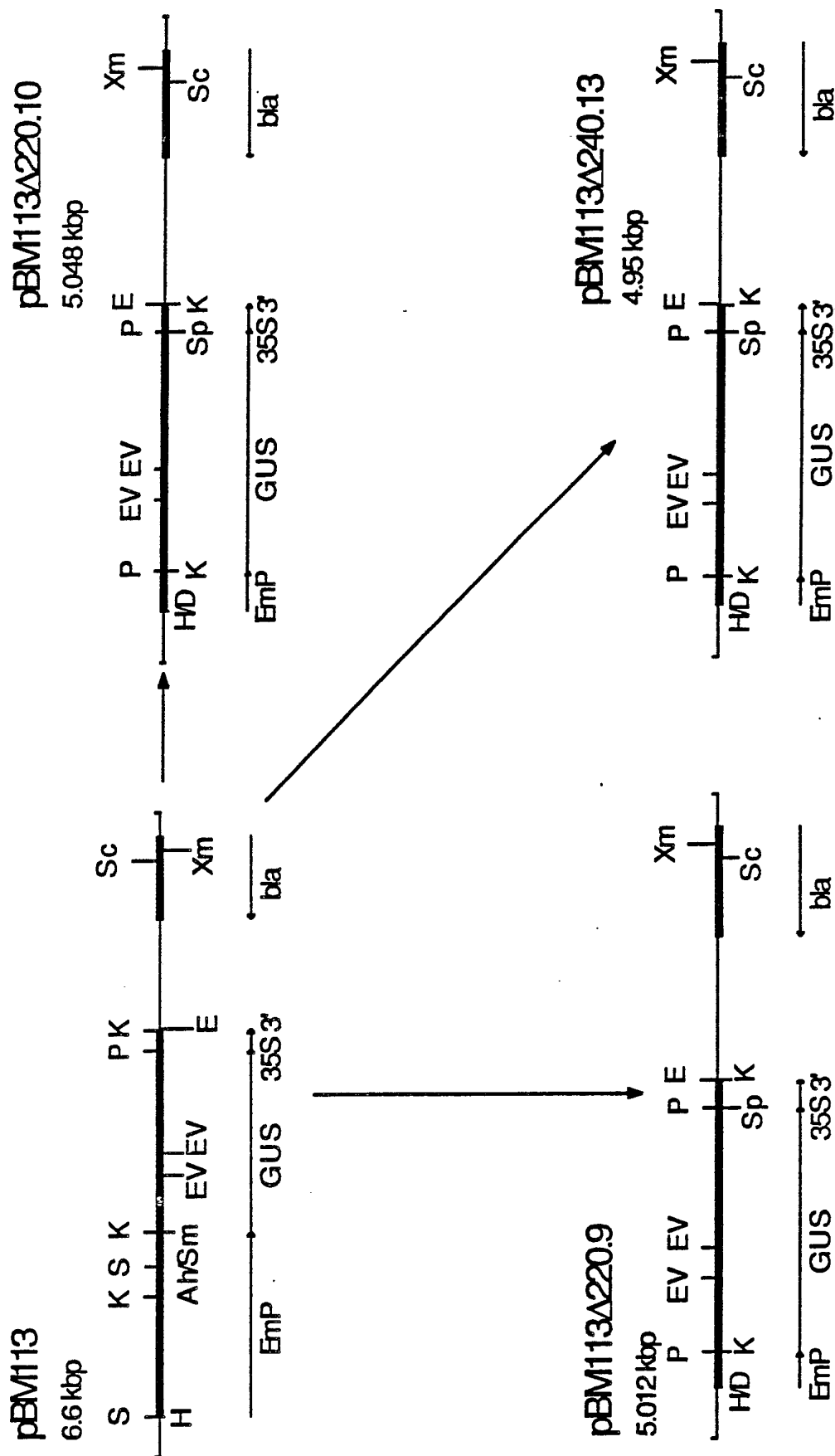
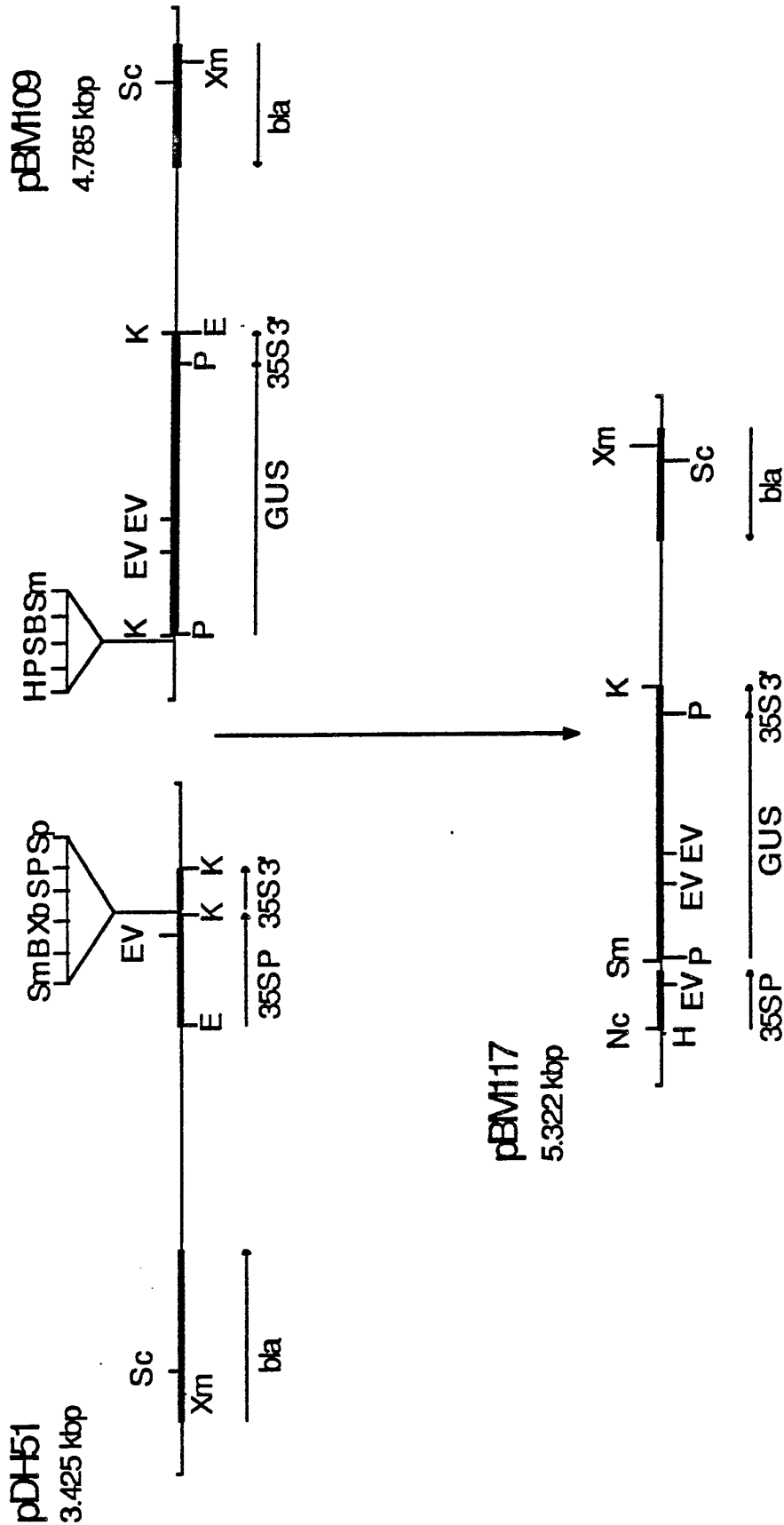


FIG. 6



F I G. 7

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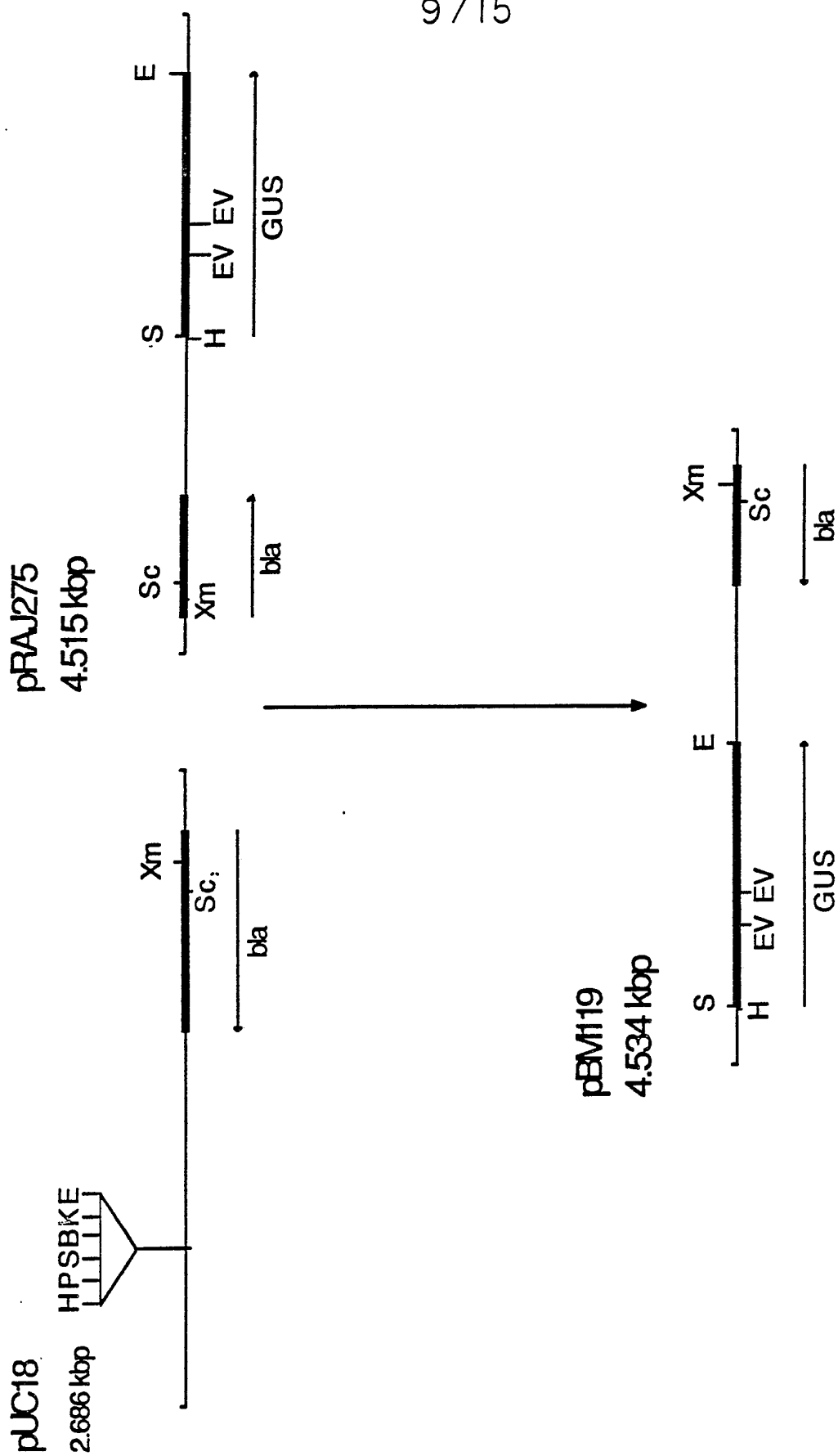
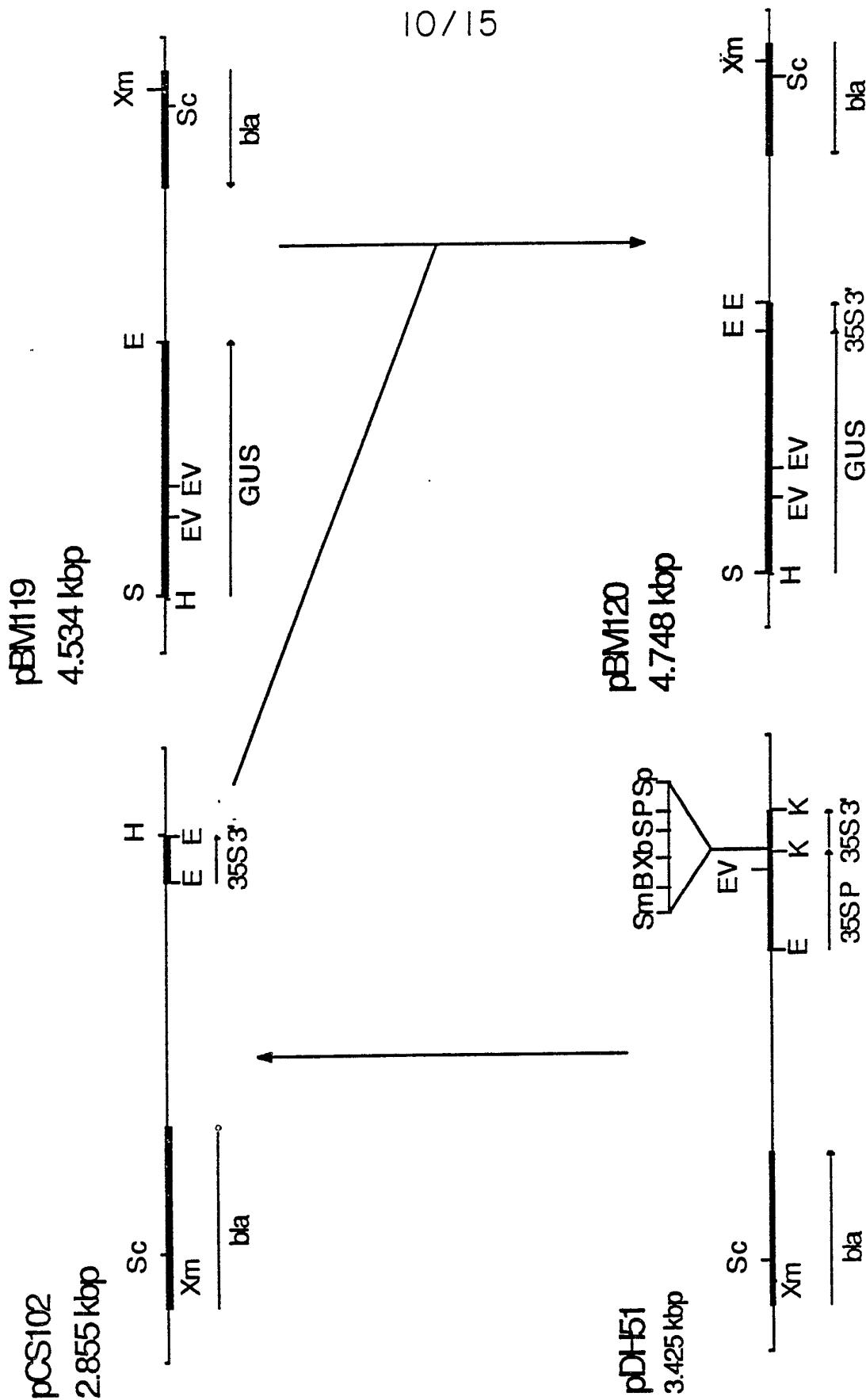


FIG. 8

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F I G. 9

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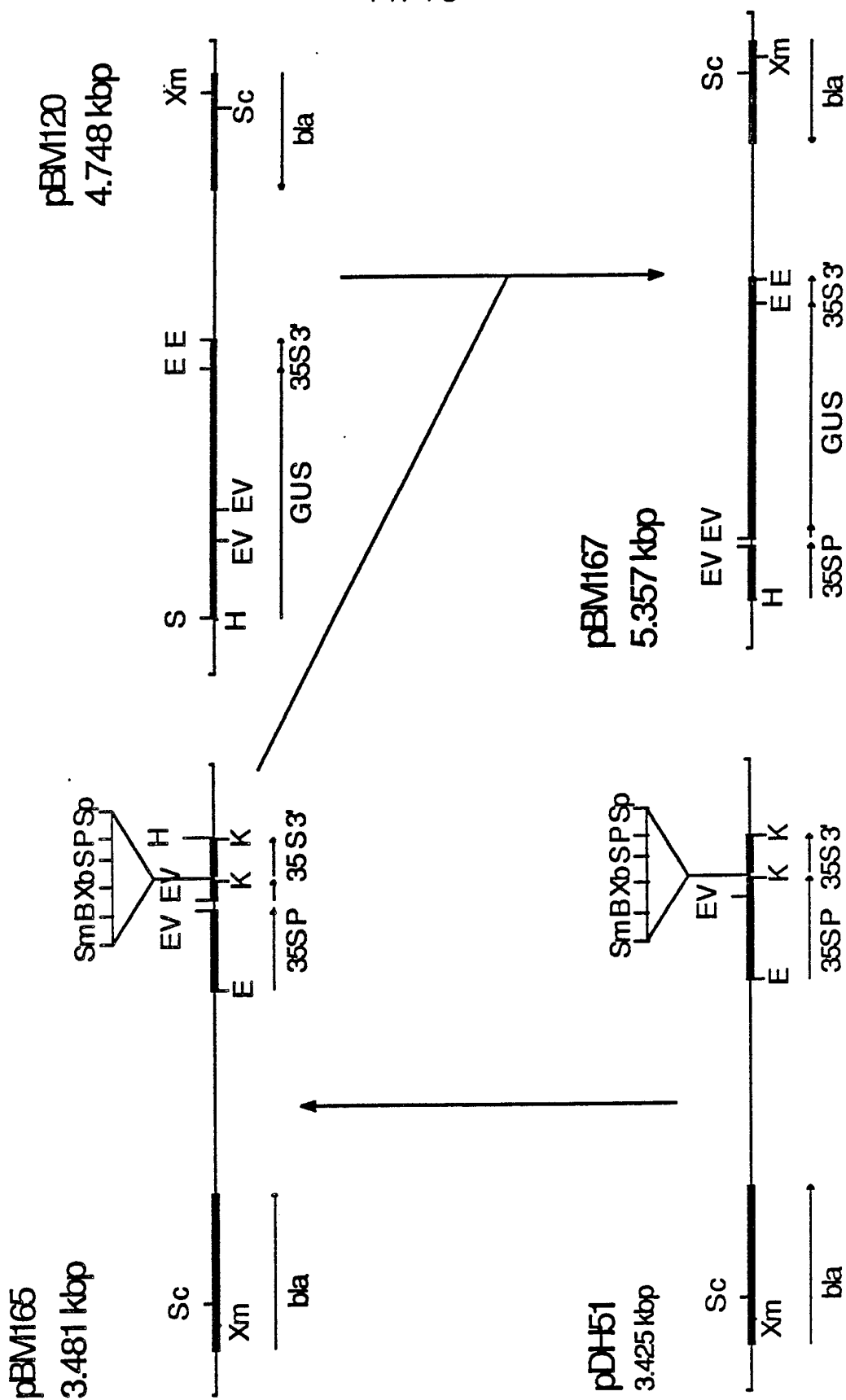


FIG. 10

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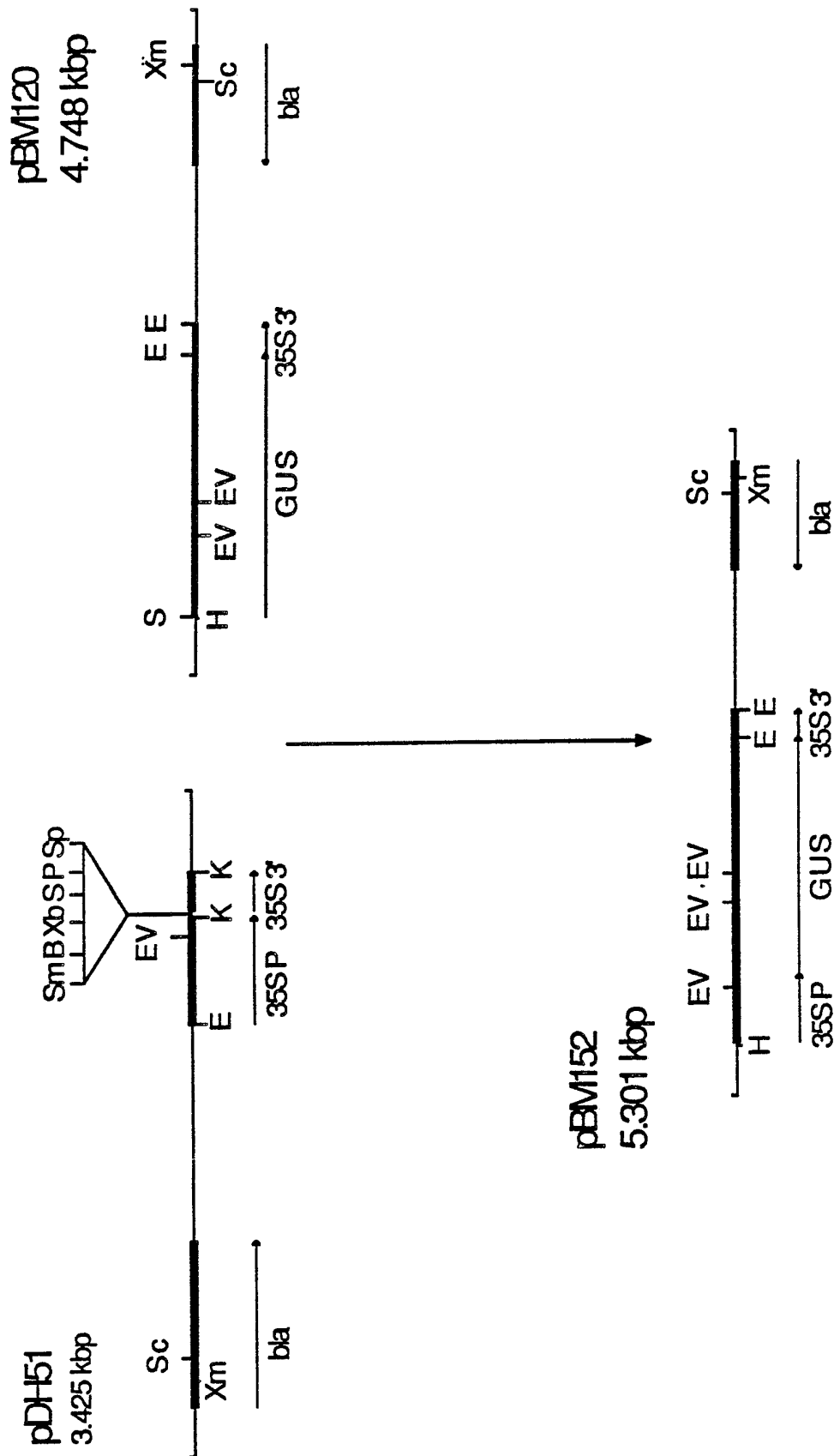


FIG. 11

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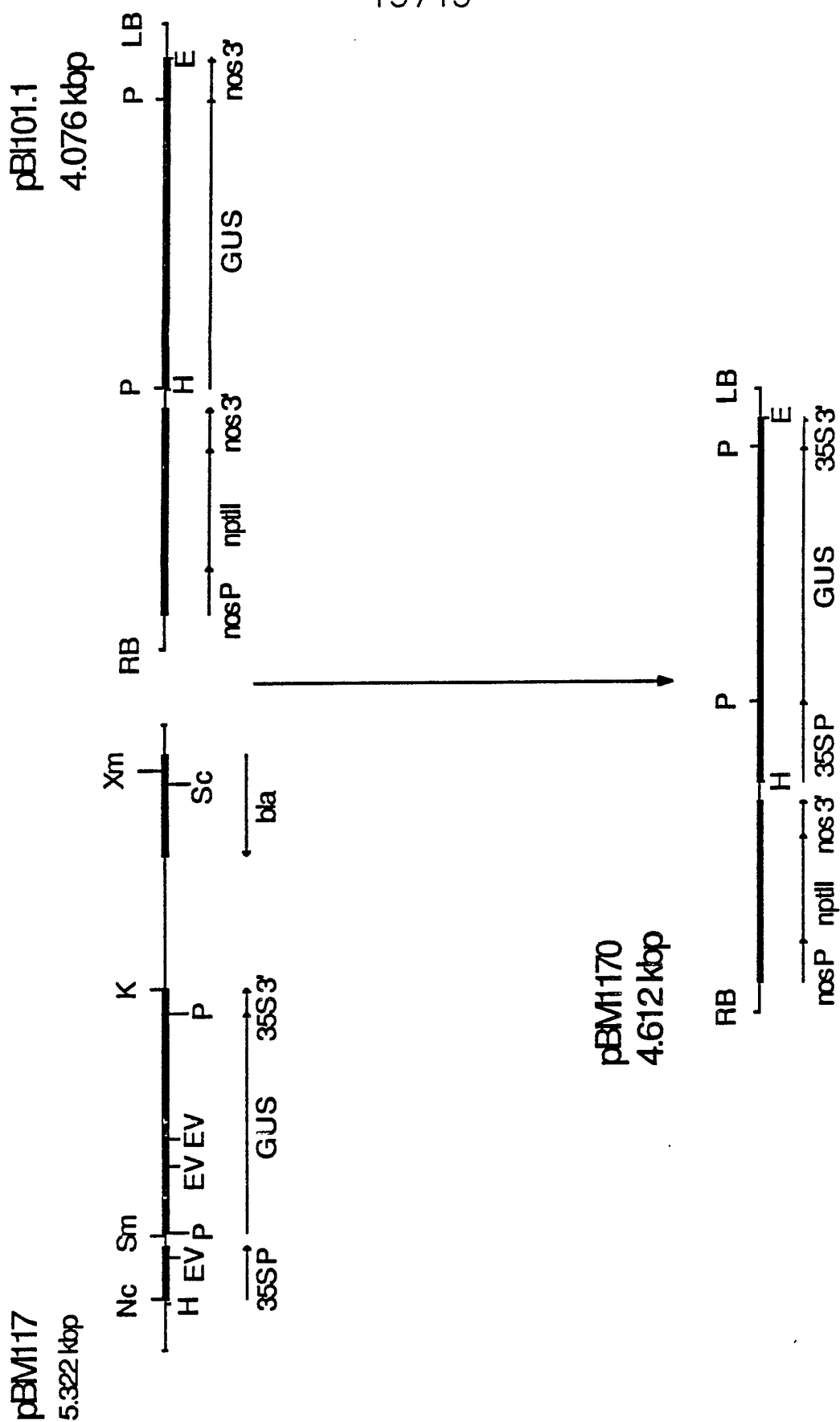


FIG. 12

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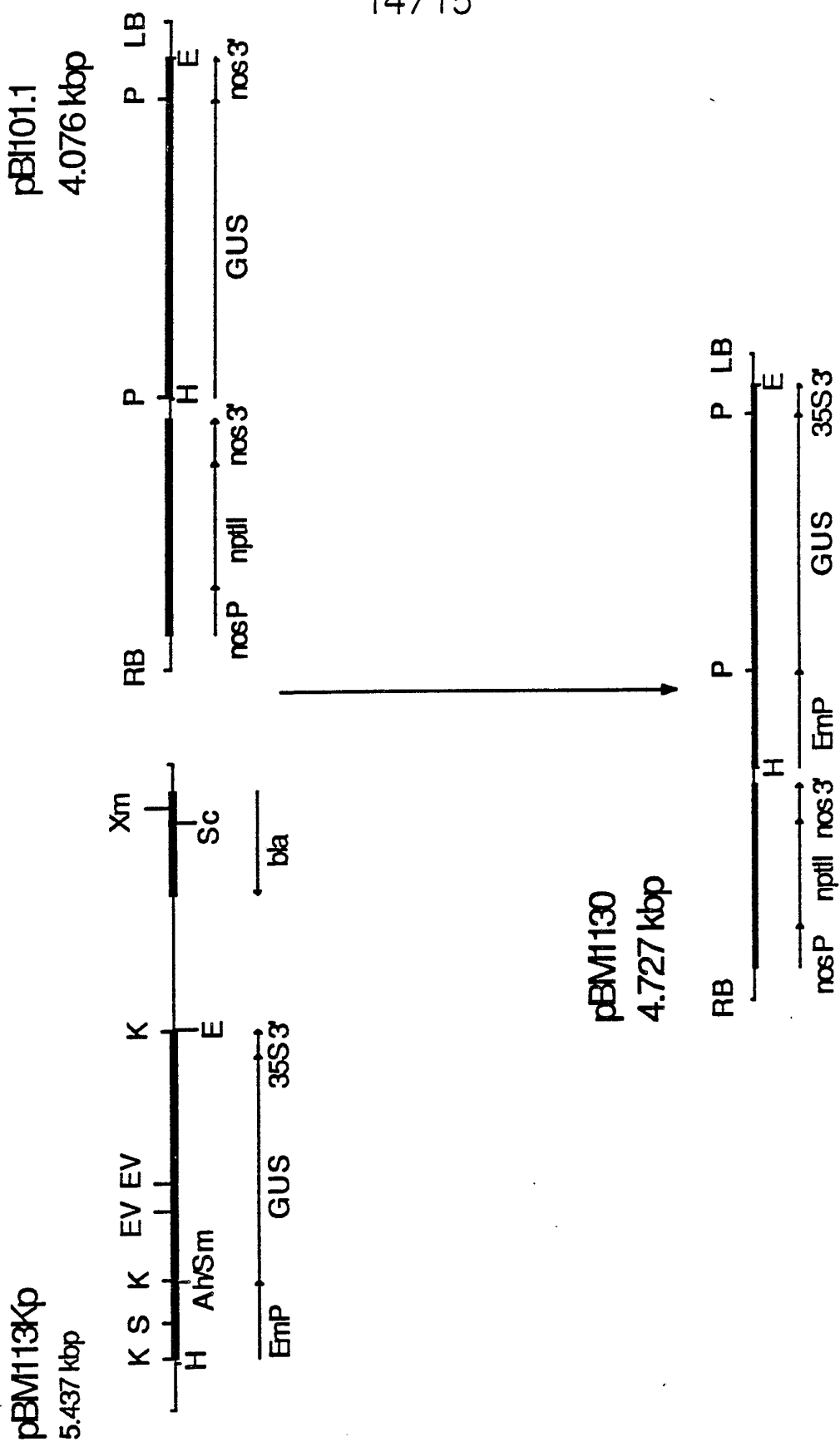


FIG. 13

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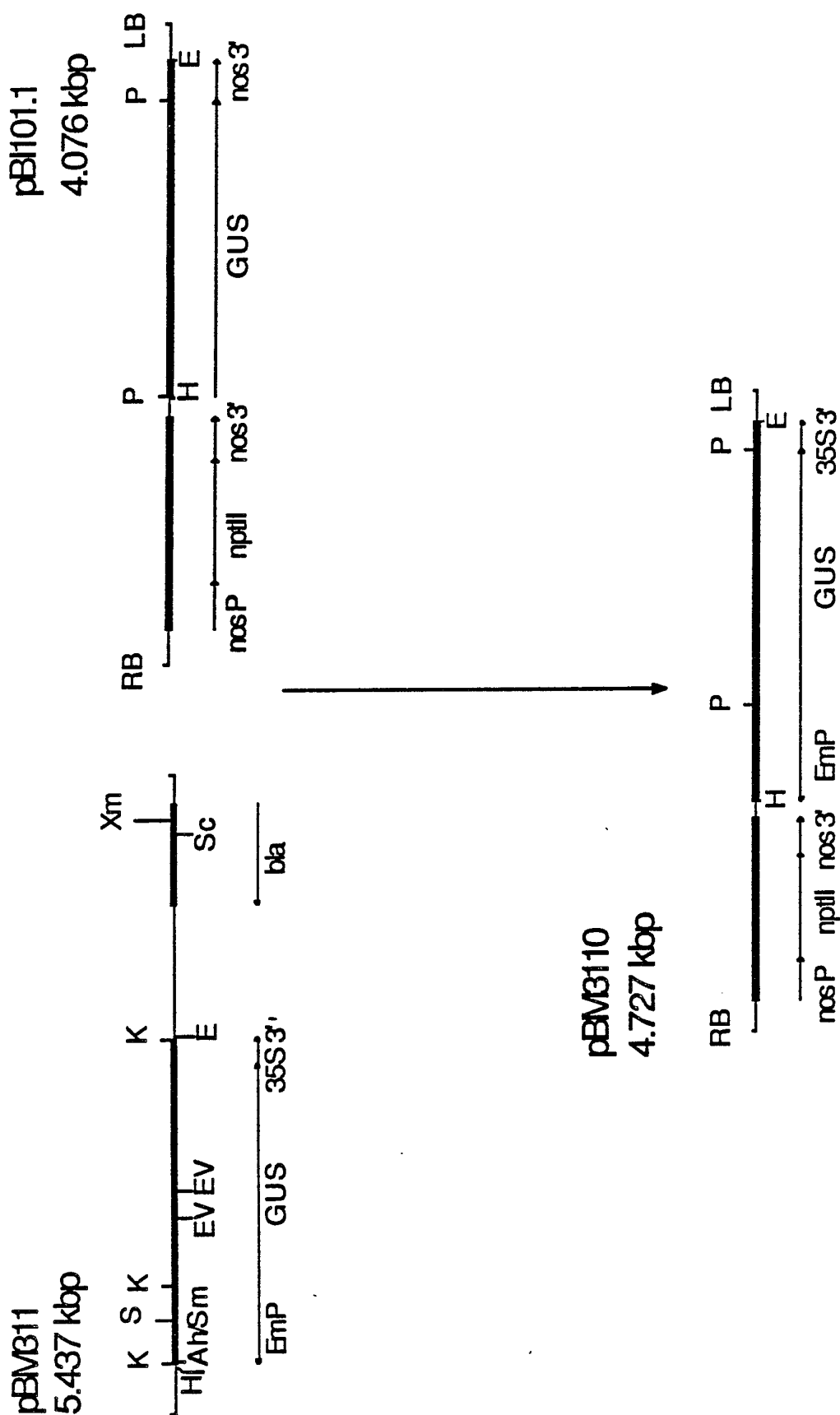


FIG. 14