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# Full Length Transcript Promotor from Figwort Mosaic Caulimovirus (FMV) and Use to Express Chimeric Genes in Plant Cells

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United States Patent [19]
Maiti et al.

[11] Patent Number: 5,994,521
[45] Date of Patent: Nov. 30, 1999

- [54] FULL LENGTH TRANSCRIPT (FLT) PROMOTER FROM FIGWORT MOSAIC CAULIMOVIRUS (FMV) AND USE TO EXPRESS CHIMERIC GENES IN PLANT CELLS
[75] Inventors: Indu B. Maiti, Lexington, Ky.; Robert J. Shepherd, Portland, Oreg.
[73] Assignee: University of Kentucky Research Foundation, Lexington, Ky.
[21] Appl. No.: 08/675,090
[22] Filed: Jul. 3, 1996
[51] Int. Cl. C07H 19/00; C07H 21/04; C13N 15/00; A01G 1/00
[52] U.S. Cl. 536/22.1; 536/23.6; 536/24.1; 536/24.2; 435/440; 435/468; 435/240.1; 800/200; 800/205
[58] Field of Search 435/172.1, 240.1, 435/440, 468; 800/200, 205; 536/22.1, 23.6, 24.1, 24.2

13, pp. 6110-6114.
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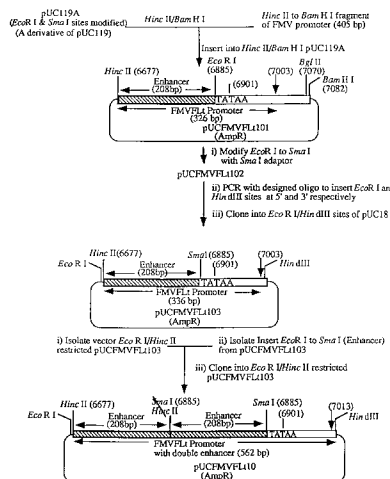
(List continued on next page.)

Primary Examiner—Ardin H. Marschel
Assistant Examiner—Jezia Riley
Attorney, Agent, or Firm—McDermott, Will & Emery

[57] ABSTRACT

Use of wild type and modified viral FLT promoters of FMV in the expression of chimeric genes in plant cells. The FLT promoter from FMV is modified with duplicated enhancer domains. The FLT promoter with its single or double enhancer domains is linked to heterologous coding sequences to form chimeric gene constructs. These genes have been shown to be expressed well in plant cells.

12 Claims, 17 Drawing Sheets



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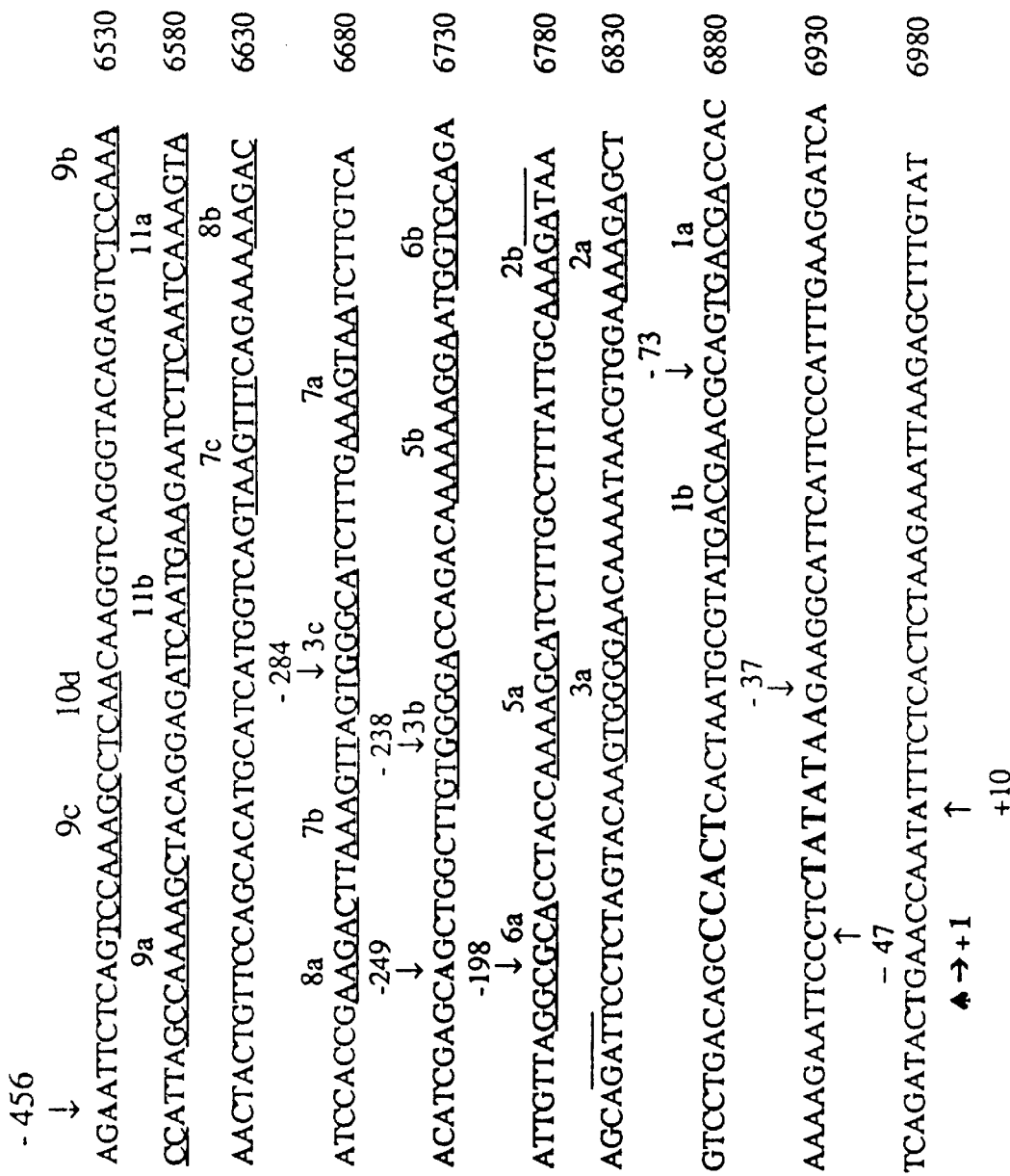


FIG. 1A

TO FIG. 1B

FROM FIG. 1A

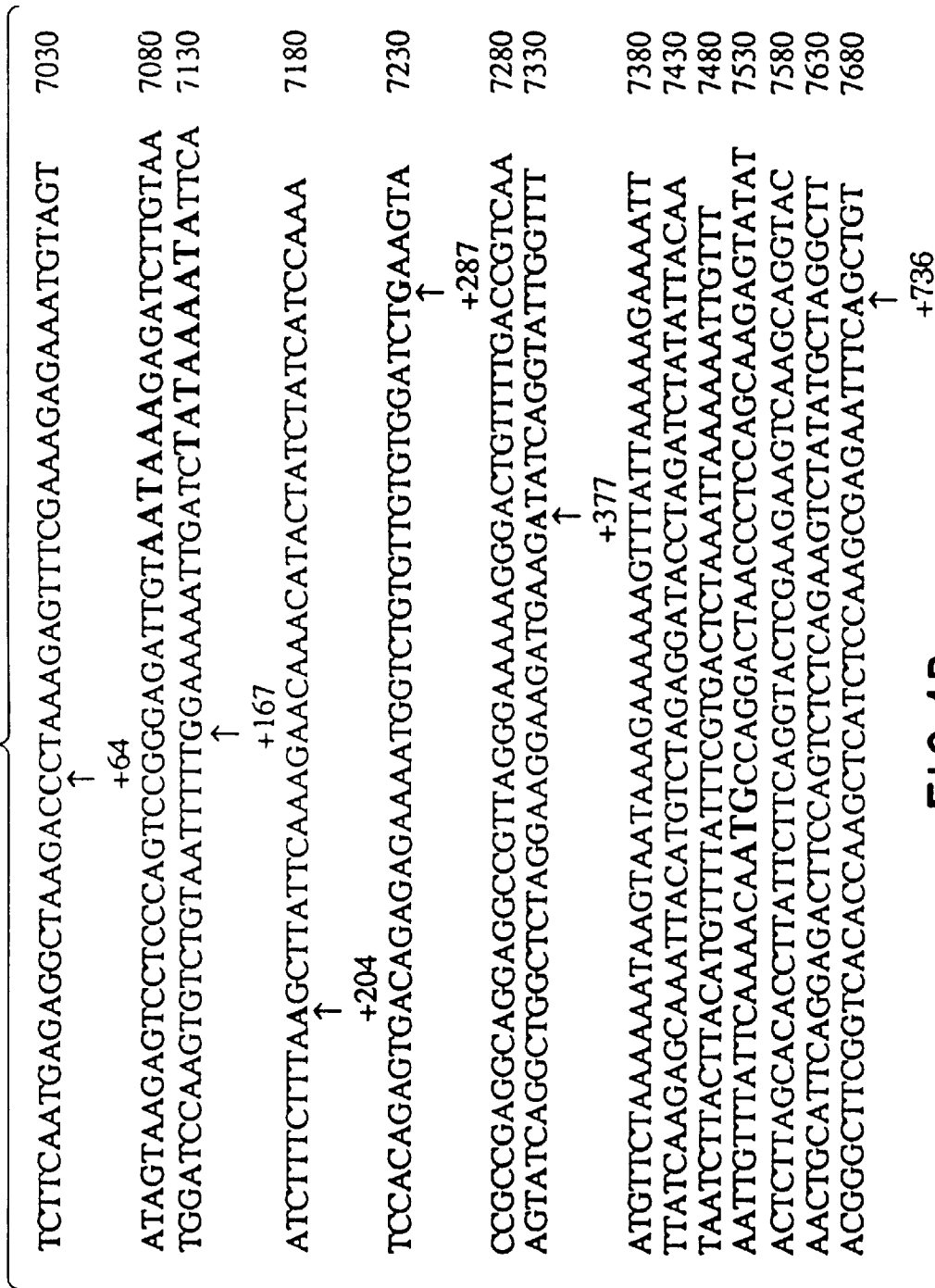
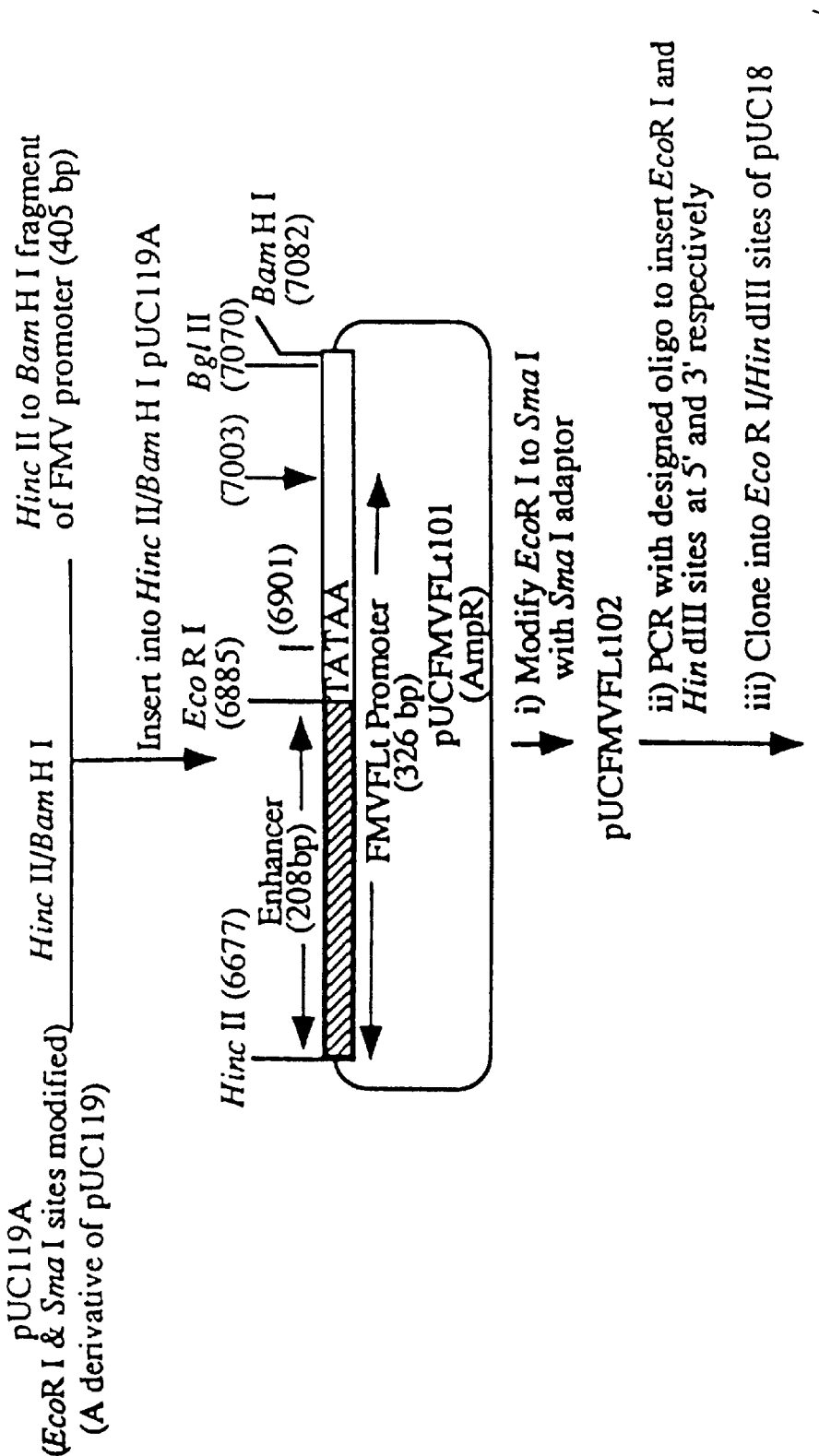


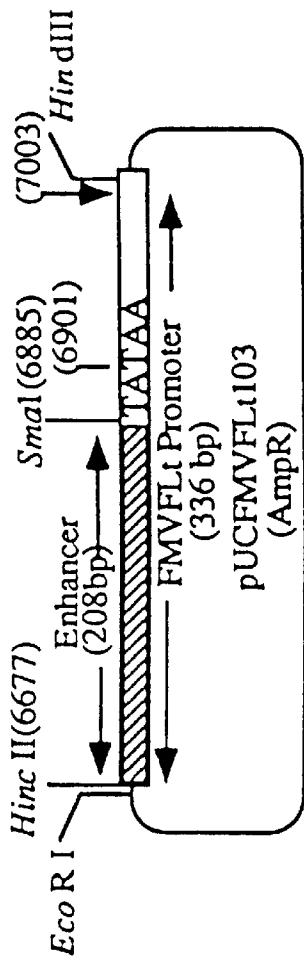
FIG. 1B



TO FIG. 2B

FIG. 2A

FROM FIG. 2A



i) Isolate vector *Eco* R I/*Hinc* II restricted pUCFMVFL103

ii) Isolate Insert *Eco* R I to *Sma* I (Enhancer) from pUCFMVFL103

iii) Clone into *Eco* R I/*Hinc* II restricted pUCFMVFL103

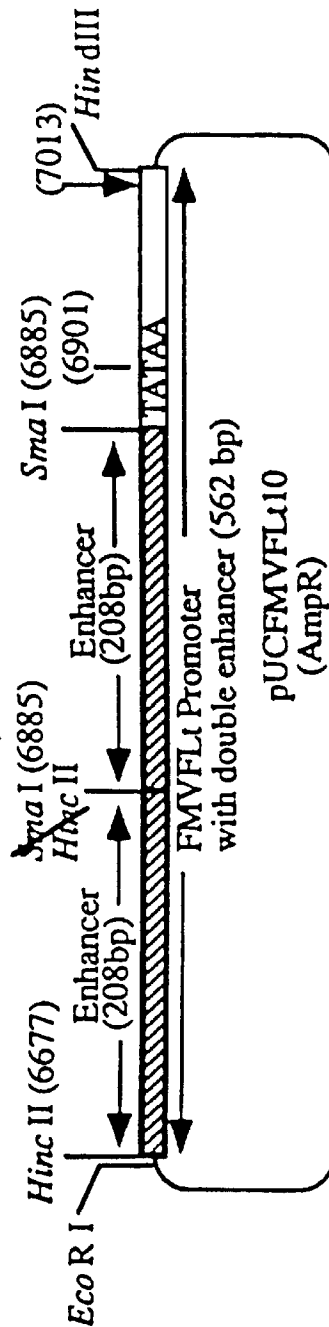


FIG. 2B

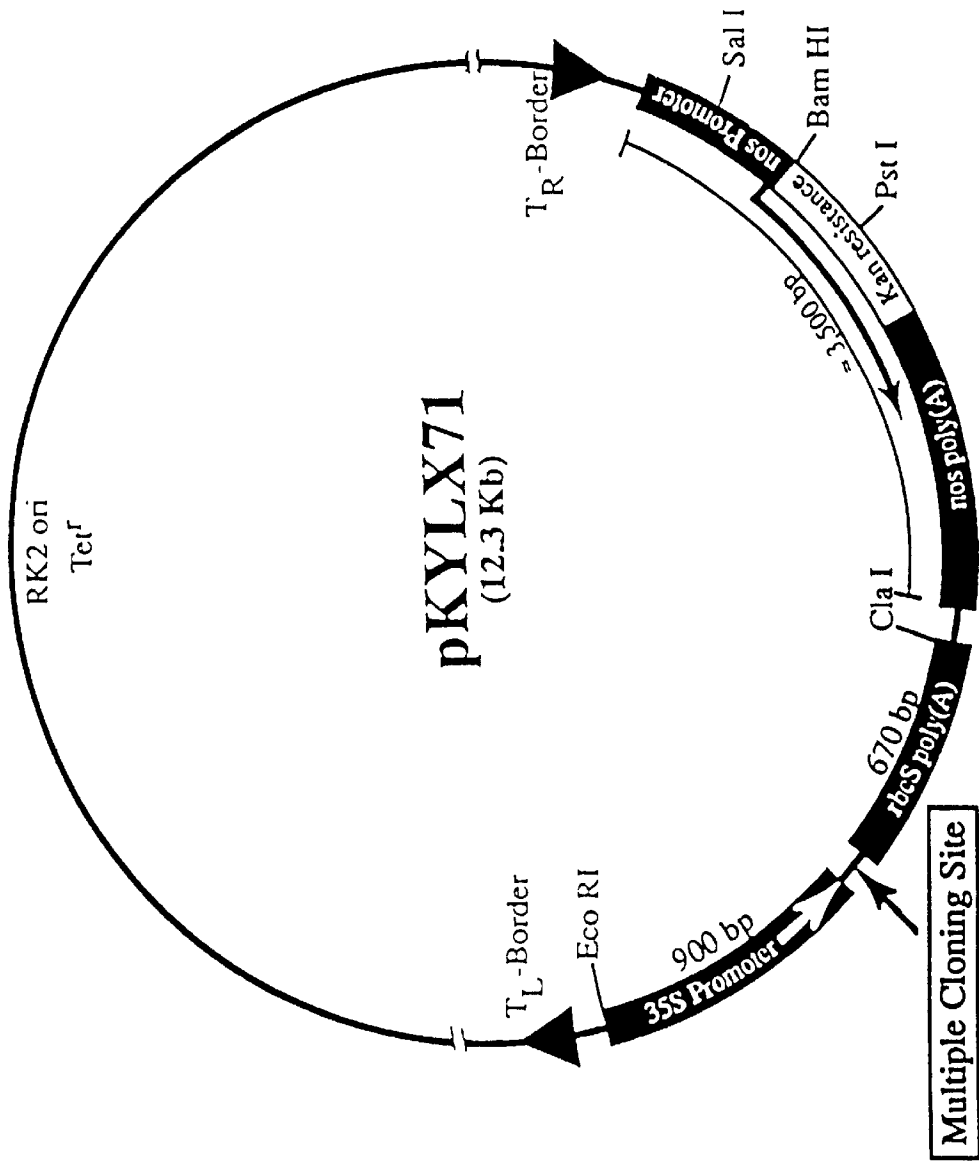
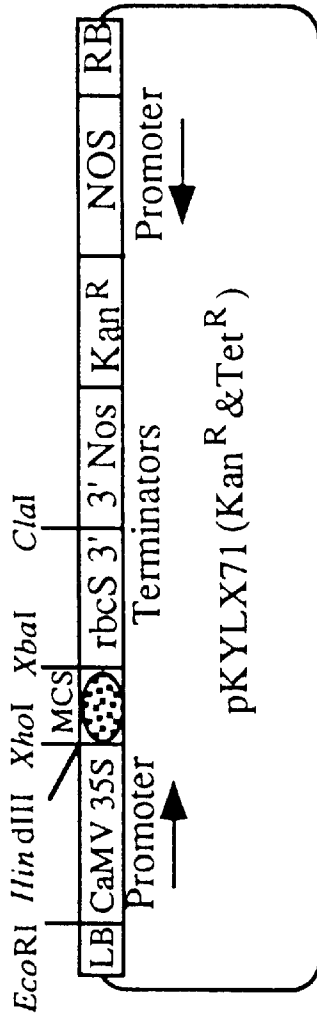


FIG. 3



Construction strategy of pKLF



1. Digest with *EcoRI* + *Hin dIII*

2. Isolate Vector

1. Digest with *EcoRI* + *Hin dIII*  
2. Isolate promoter fragment

3. Insert FMVFLt promoter fragment into *EcoRI/Hin dIII* digested pKYLX 71 vector

TO FIG. 4B

FIG. 4A

FROM FIG. 4A

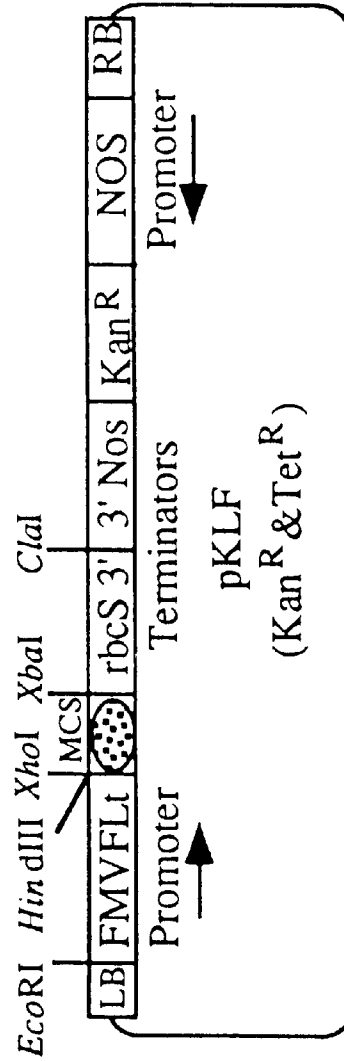
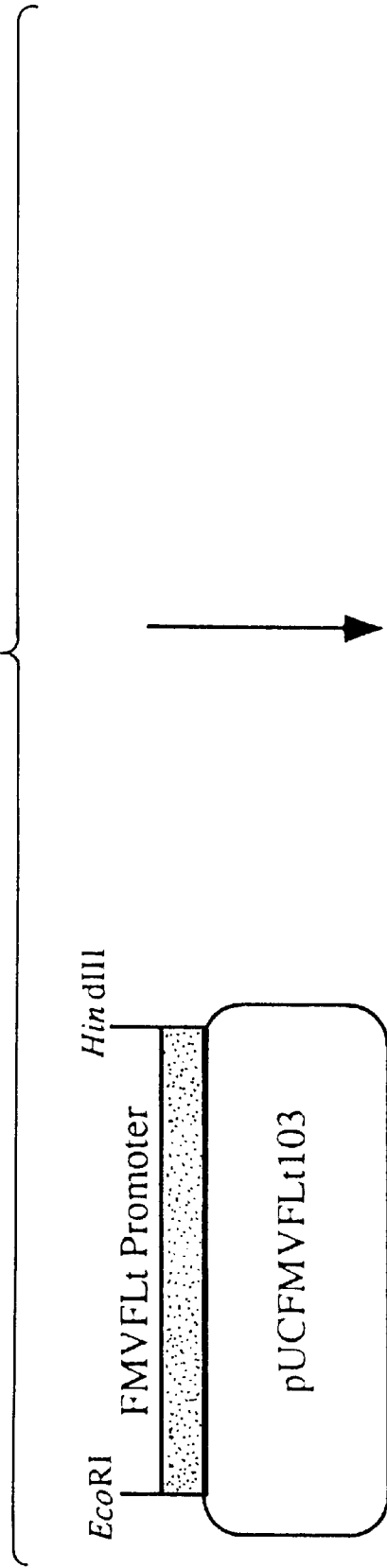
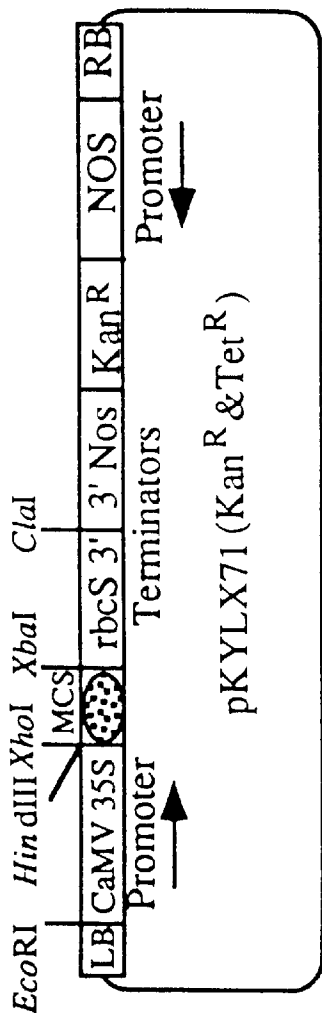


FIG. 4B

# Construction strategy of pKLF2



1. Digest with *EcoRI* + *Hin dIII*

2. Isolate Vector

1. Digest with *EcoRI* + *Hin dIII*

2. Isolate promoter fragment

3. Insert FMVFLt promoter fragment with duplicated enhancer into *EcoRI/Hin dIII* digested pKYLX 71 vector

TO FIG. 5B

FIG. 5A

FROM FIG. 5A

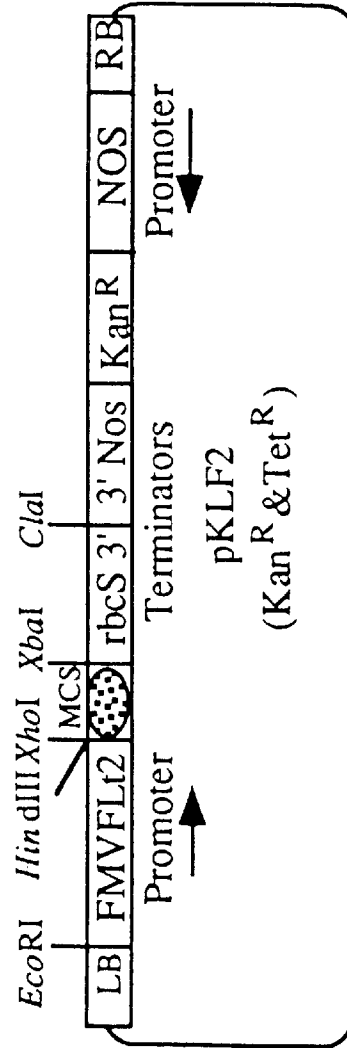
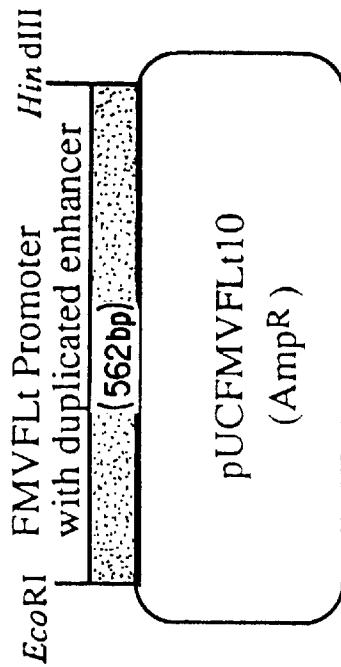


FIG. 5B

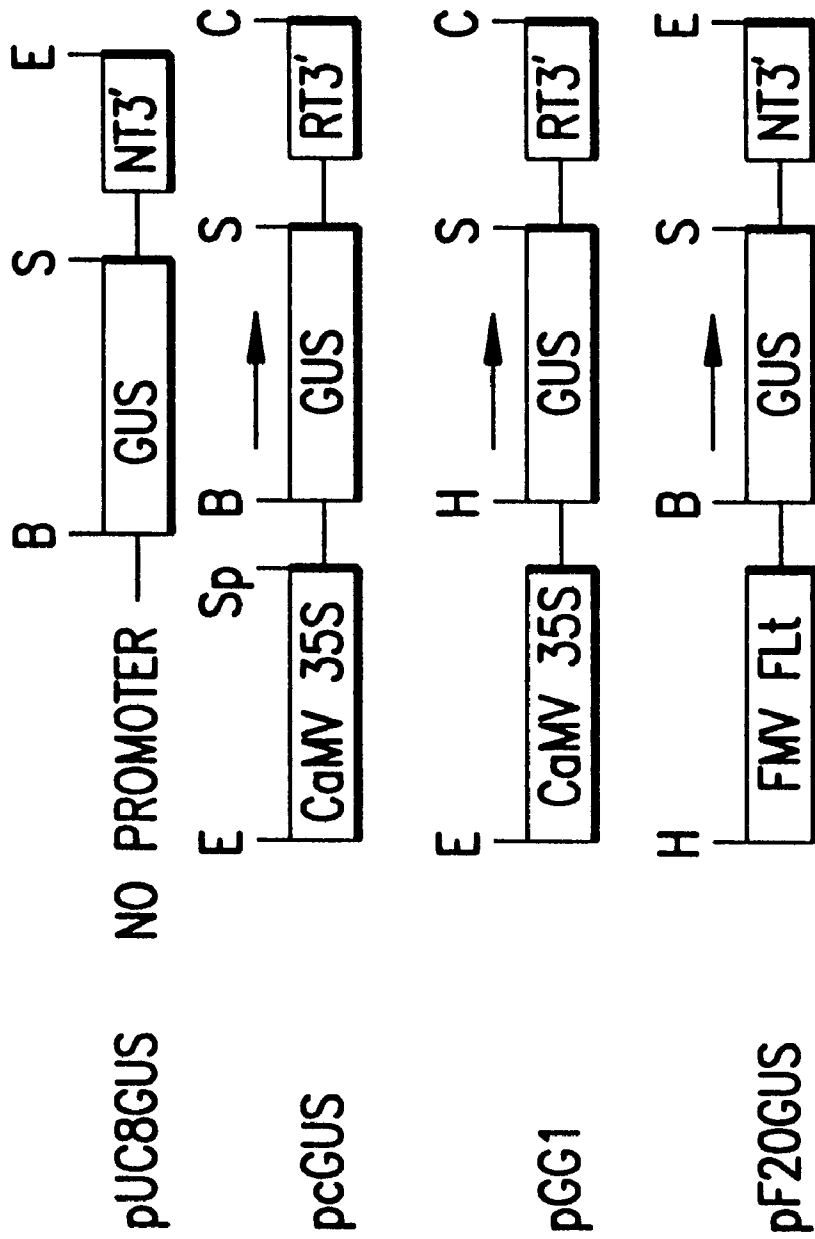


FIG. 6A

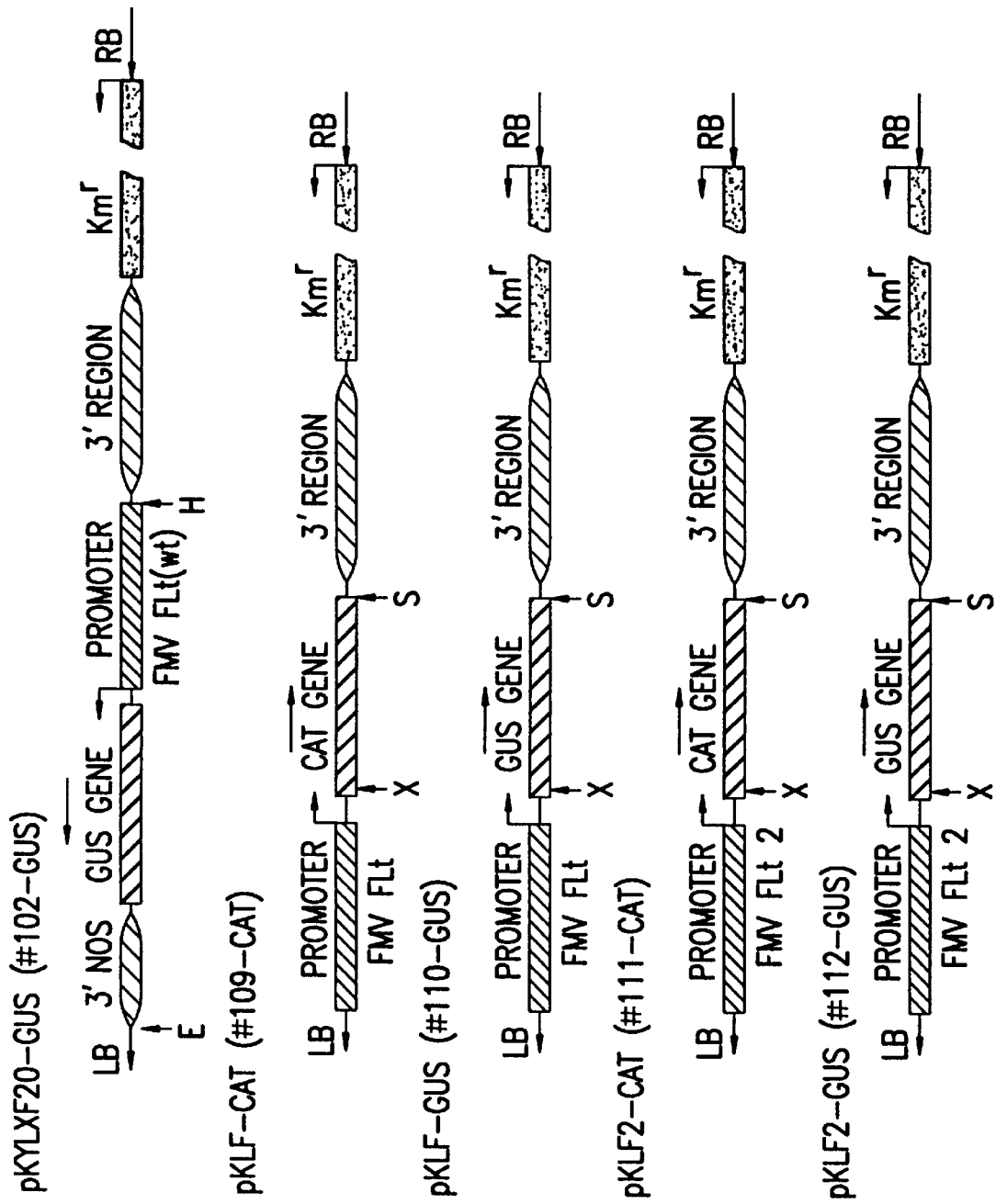


FIG. 6B

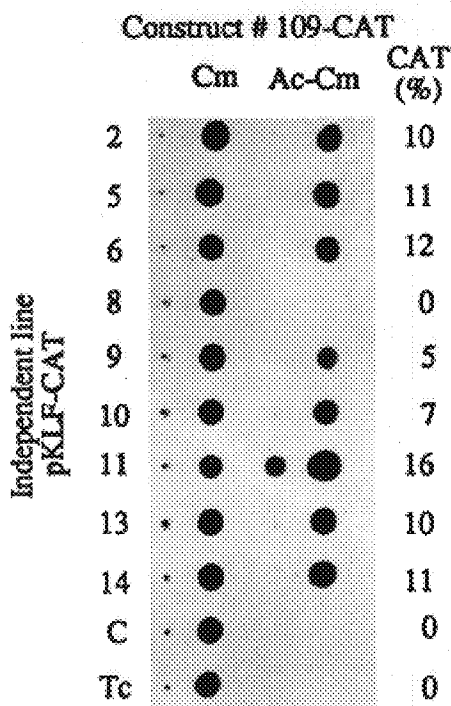


FIG. 7A

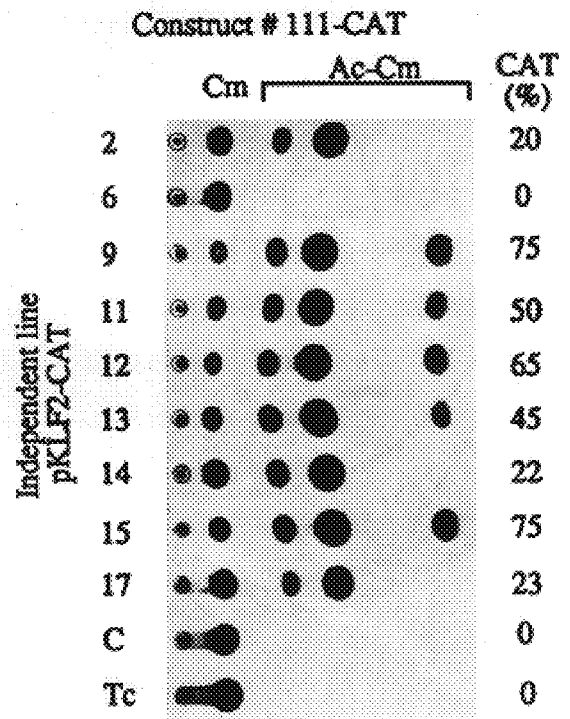


FIG. 7B

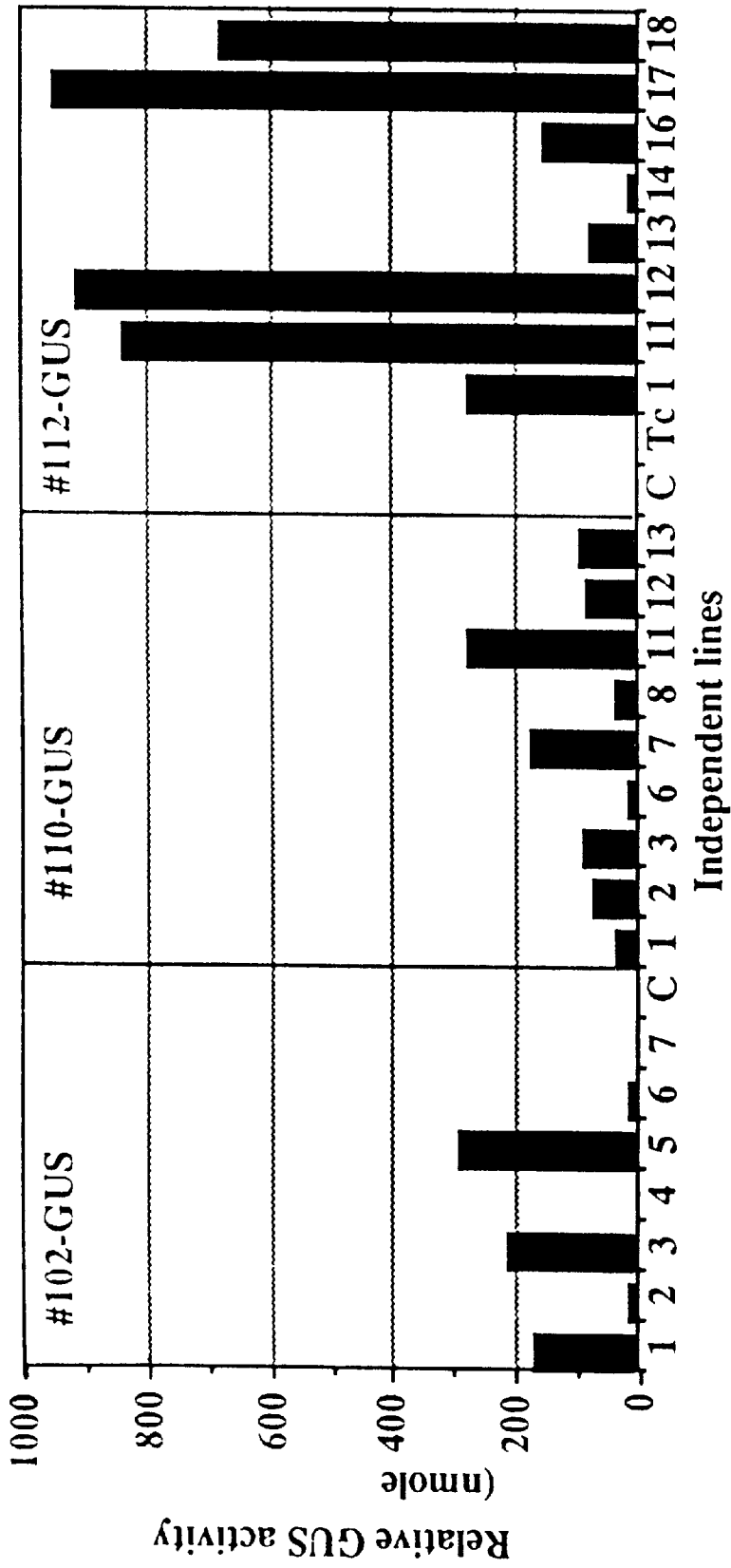


FIG. 8



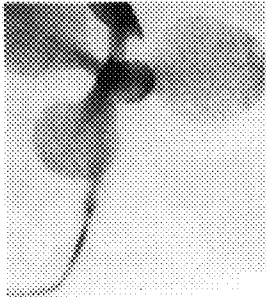


FIG. 9A

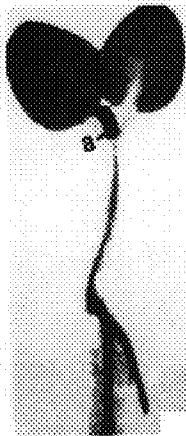


FIG. 9B

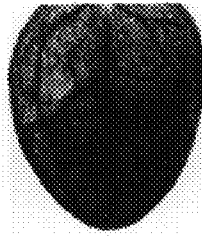


FIG. 9C



FIG. 9D



FIG. 9E

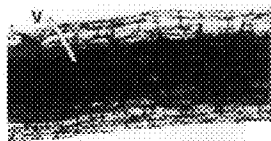


FIG. 9F

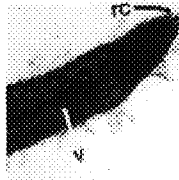


FIG. 9G

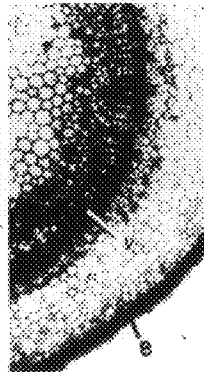


FIG. 9H

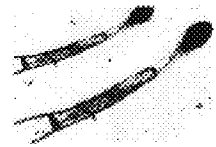


FIG. 9I

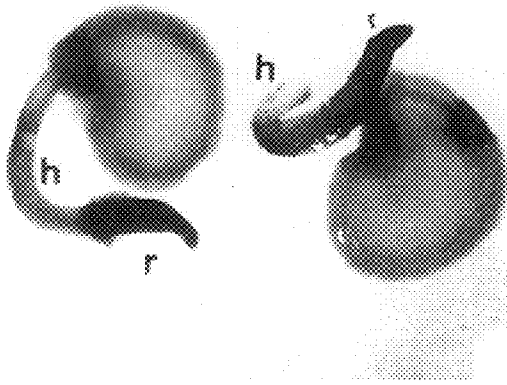


FIG. 9J

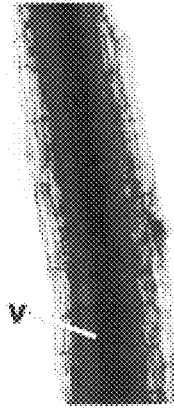


FIG. 9K

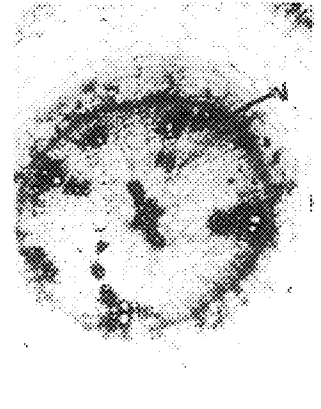


FIG. 9L

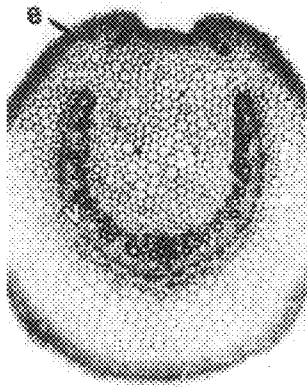


FIG. 9M

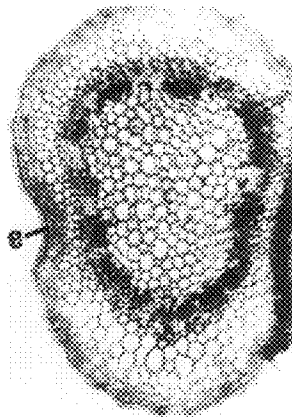


FIG. 9N

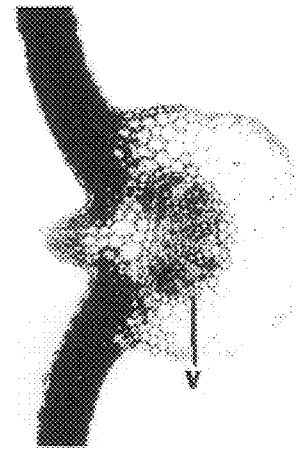
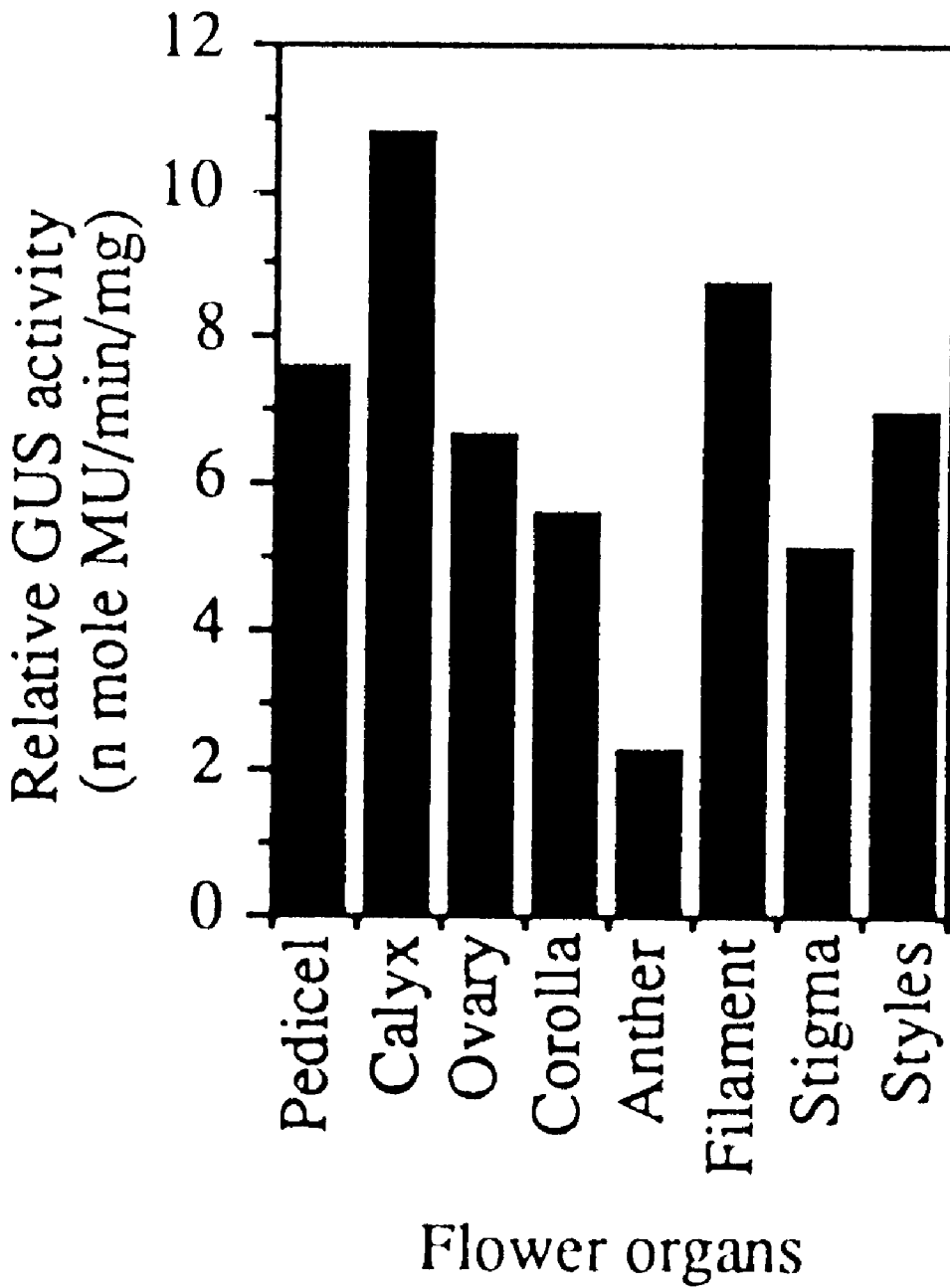


FIG. 9O



**FIG. 10**

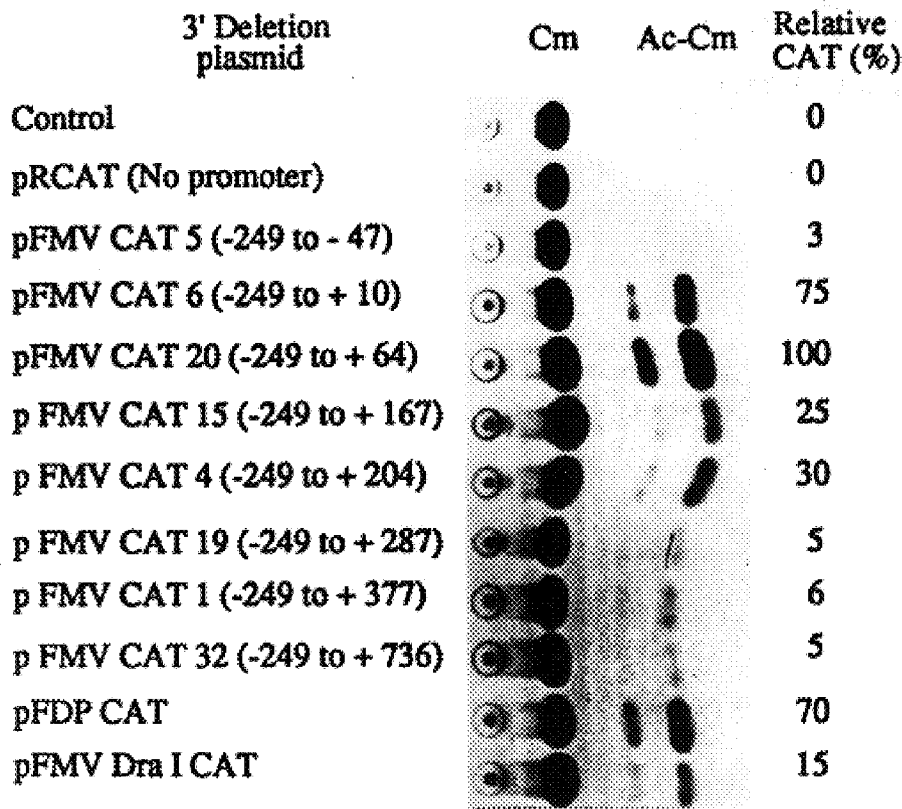


FIG. 11A

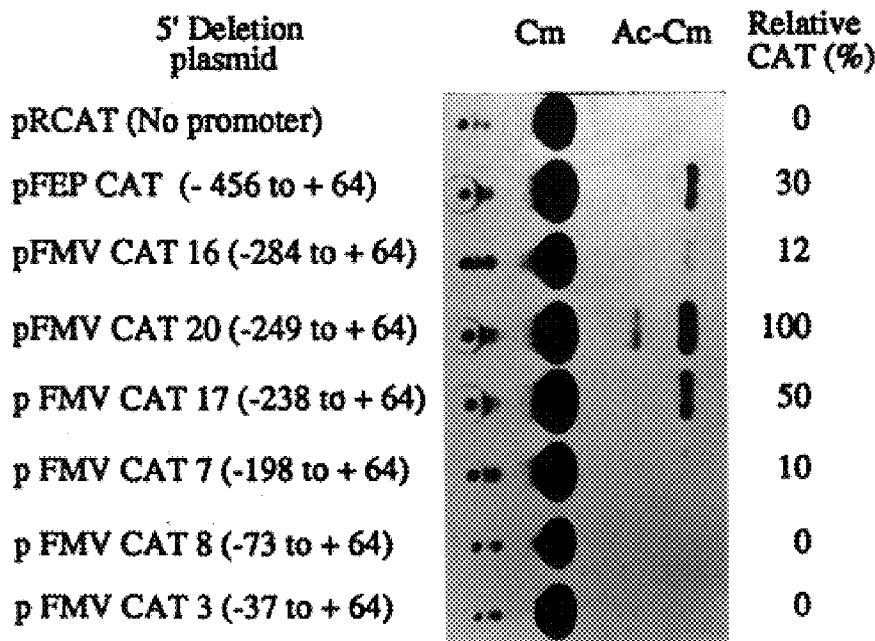


FIG. 11B

**FULL LENGTH TRANSCRIPT (FLT)  
PROMOTER FROM FIGWORT MOSAIC  
CAULIMOVIRUS (FMV) AND USE TO  
EXPRESS CHIMERIC GENES IN PLANT  
CELLS**

TECHNICAL FIELD

The present invention relates to the isolation, modification and use of wild type and modified viral FLT promoters of FMV in the expression of chimeric genes in plant cells. The FLT promoter from FMV is modified with duplicated enhancer domains. The FLT promoter with its single or double enhancer domains is linked to heterologous coding sequences to form chimeric gene constructs. These genes have been shown to be expressed well in plant cells. The FLT promoter with its double enhancer domain gives increased expression of genes compared to the FLT promoter with a single enhancer domain. This invention also includes plant cells, plant tissue, differentiated plants which express the chimeric genes of the invention.

BACKGROUND ART

A virus is a group of submicroscopic infective agents with double or single stranded DNA or RNA as core genetic material surrounded by a protein (and lipid in some case) shell called 'capsid' or 'coat'. It has no semipermeable membrane and it can multiply only in living cells using host cellular components. The short segment of the virus genetic material (FLT promoter) used in this invention can not infect plants or other organisms to cause disease. It is useful with selected foreign genes to obtain expression of these genes in other plants to confer useful properties to those transgenic plants.

Promoters from caulimoviruses

The following is a description of caulimoviruses also called plant pararetroviruses. Caulimoviruses derived their name from cauliflower mosaic virus (CaMV), the type member of the group (for reviews see Shepherd, 1989; Covey and Hull, 1992). More than a dozen types of caulimoviruses have been described to date. All have small circular DNA molecules as their genetic material. The genomes of CaMV (Gardner, et al., 1981) and four other members of this group, namely carnation etched ring virus (CERV), (Hull, et al., 1986), figwort mosaic virus (FMV), (Richins, et al., 1987) soybean chlorotic mottle virus (SoCMV), (Hasegawa, 1989), and peanut chlorotic streak virus (PCISV) (Richins, 1993; Richins, et al., 1995) have been fully sequenced. CaMV is a circular double stranded DNA virus with a genome size of approximately 8 kb. It is organized into seven open reading frames (genes) and two intergenic regions.

In the case of CaMV, the polypeptides corresponding to the six genes (I to VI) have been detected in infected cells and their functions have been identified. The cell-to-cell movement function (Thomas, et al., 1993; Ducasse et al., 1995), aphid-transmission factor (Daubert et al., 1983; Woolson, et al., 1983), minor capsid protein (Giband, et al., 1986), major capsid protein (Daubert, et al., 1982), reverse transcriptase (Takatsui, et al., 1992), and inclusion body protein (Odell and Howell, 1980) are associated with ORFs I to VI respectively. The gene VII protein was not detected in vivo (Wurch, et al., 1991). Its function is not clearly established. However a sequence located with this ORF of FMV is involved in translation of viral genes (Gowda, et al., 1991).

The viral genome is replicated through reverse transcription of the terminally redundant full length transcript

(Bonneville and Hohn, 1993) by a virus encoded reverse transcriptase. Two major viral transcripts, known as 35S RNA and 19S RNA are synthesized exclusively from the minus strand DNA by the host RNA polymerase II (Odell, et al., 1981; Howell and Hull, 1978). The large intergenic region (L-IR) which resides between gene VI and VII, contains the promoter (35S) for the full length transcript which spans the entire viral genome (Dixon and Hohn, 1984; Scholthof, et al., 1992). The 35S RNA serve as template for minus strand DNA synthesis by viral gene V encoded reverse transcriptase (Gordon, et al., 1988). The small intergenic region (S-IR) residing between gene V and gene VI contains a promoter (19S) which transcribes gene VI only (Odell and Howell, 1980). The PCISV is apparently lacking the S-IR sequence, however both FMV (Scholthof, et al., 1992) and PCISV (Richins, 1993) have also been shown to have similar transcripts to the 19S and 35S RNA found in CAMV infected plant cells.

The CaMV 35S promoter, which spans about 941 base pair (bp) upstream from the transcription start site, has been shown to be active in various monocot and dicot cells. The cis-regulatory elements that are involved in directing transcription initiation reside within this region. The CaMV 35S promoter has a modular construction that includes an enhancer (Lam, 1994, and references there in) similar to those of other promoters like that of SV40 in mammalian systems (Ondek, et al., 1987; Schirm, et al., 1987; Fromental, et al., 1988). The 5' deletion analysis of CaMV35S promoter, studied in transformed tobacco calli or a protoplasts transient assay system, indicates that a promoter fragment of 343 bp upstream from the transcription start site is sufficient for high promoter activity (Odell, et al., 1985, Ow, et al., 1987).

The high CaMV35S promoter activity is the result of synergistic and combinatorial effects of enhancer elements residing in the -343 to -46 region upstream of the TATA element promoter (-46 to +8) (Fang, et al., 1989, Benfey, et al., 1989, Benfey and Chua, 1990, Benfey, et al., 1990a and Benfey et al., 1990b).

U.S. Pat. No. 5,378,619 to Rogers discloses a full length transcript promoter from the figwort mosaic virus. U.S. Pat. No. 4,940,835 to Shaw discloses the cauliflower mosaic virus 35S promoter. The latter patent claims chimeric plant genes containing the cauliflower mosaic virus promoter sequence. The patent does not disclose a double full length transcript promoter from the figwort mosaic virus in a transgenic plant and its expression advantages.

Several protein binding sequence motifs have been identified in the enhancer region of the 35S promoter (Lam, et al., 1989; Lam and Chua, 1989; Prat, et al., 1989; Bouchez, et al., 1989, Yanagisawa and Izui, 1992). Identical or similar sequence motifs are also present in promoters of other caulimoviruses (Bouchez, et al., 1989; Sanger, et al., 1990; Cooke and Penon, 1990; Richins, et al., 1993). Two nuclear binding protein factors, known as Activating Sequence Factor-1 and -2 (ASF-1 and ASF-2) from tobacco have been well characterized. ASF-1 binds to the activating sequence as-1 (-82 to -62) region of 35S promoter. Two TGACG motifs within this site are essential for DNA-protein interaction (Lam, et al., 1989). The as-1 motif is also found in full length transcript promoters from other caulimovirus including FMV (Sanger, et al., 1990, and present studies), PCISV (Richins, 1993) and MMV (Shepherd group, unpublished observation).

Single or multiple copies of enhancer sequences from the CaMV 35S promoter can increase homo- and heterologous promoter activity in an orientation-independent manner

(Kay, et al., 1987; Ow, et al., 1987; Odell, et al., 1988; Fang, et al., 1989; Driesen, et al., 1993; Omirulleh, et al., 1993). The enhancement of promoter activity was proportional to the copy number of the enhancer sequence (Kay, et al., 1987; Ow, et al., 1987; Omirulleh, et al., 1993). Similar observation was made when single or multiple copies of the enhancer sequence was inserted upstream of the TATA element of the CaMV19S promoter (Ow, et al., 1987; Driesen, et al., 1993), rbcS-3A promoter (Fang, et al., 1989) and the nos promoter (Odel, et al., 1988).

U.S. Pat. No. 5,463,175 to Barry et al. discloses the figwort mosaic virus promoter. U.S. Patent No. 5,503,999 to Jilka et al. discloses the cauliflower mosaic virus 35S promoter and the figwort mosaic virus 35S promoter. U.S. Pat. No. 5,145,783 to Kishore et al. discloses the cauliflower mosaic virus 35S promoter. Figwort mosaic virus promoter is also disclosed.

U.S. Pat. No. 5,242,412 to Brown et al. discloses the figwort mosaic virus 35S promoter. U.S. Pat. No. 5,510,253 to Mitsky et al. discloses the figwort mosaic virus promoter. U.S. Pat. No. 5,512,466 to Klee et al. discloses the cauliflower mosaic virus 35S promoter and the figwort mosaic virus promoter.

U.S. Pat. No. 5,304,730 to Lawson et al. discloses the figwort mosaic virus 35S promoter. PCT Publication WO 94/24848 discloses a transgenic plant in which a chimeric gene comprising a wound inducible promoter which shows enhanced resistance to insect infection. Examples of vectors at least with a pKYLX4, pKYLX5 and pKYLX71 vectors.

U.S. Pat. No. 5,106,739 to Comai et al. discloses a caulimovirus 35S enhanced mannopine synthase promoter and method for using the promoter. The patent also discloses the use of a double CaMV 35S promoter in a construct used to create transgenic plants.

Proceedings of the National Academy of Sciences, Volume 90, page 6110-6114, July 1993, entitled "Plants that express a potyvirus proteinase gene are resistant to virus infection". This publication discloses pKYLX71:35S vector.

The Journal of Cellular Biochemistry, Supplement 16F, Apr. 3-16, 1992, discloses a binary vector PKYLX71-GUS. In Vitro Cellular & Development Biology, March 19, 1992, Volume 28, No. 3, Abstract P-1119 discloses PKYLX71-GUS vector. Molecular & General Genetics, Volume 220, page 389-392, Spring 1990, discloses expression of the caulimovirus 35S-GUS gene in transgenic rice plants.

Chemical Abstracts, Volume 119, Abstract No. 197251n discloses transgenic plants with increased solids content. The plants are made with a construct including a CaMV 35S promoter. Plant Physiology, June 1995, Volume 108, No. 2, discloses in Abstract 803, the expression of heterologous genes following electroporation of the marine diatom. Electroporation induced loading of plasmid CaMV35S.

The engineering of novel traits into plants and other crops promises to be an area of great agricultural importance (Maiti and Hunt, 1992; Wagner, 1992). Plant genetic engineering techniques allow researchers to introduce heterologous genes of interest into plants cells to obtain the desired qualities in the plants of choice. Plant genetic engineering has led to a rapid progress in production of economically valuable germplasm with improved characteristics or traits such as insect resistance, virus resistance, fungal resistance, herbicide resistance, bacterial or nematode pathogen resistance, cold or drought tolerance, improved nutritional value, seed oil modification, delayed ripening of fruits, and male sterility, to name a few.

These newly created germplasms provide a enhanced development in breeding programs for crops improvement

as well as a better understanding of gene regulation and organization in transgenic plants. The expression of useful foreign traits in plants is a major focus in plant biotechnology. Plant metabolic engineering is the application of genetic engineering methods to modify the nature of chemical metabolites in plants. For metabolic engineering where multiple genes need to be inserted into one cell, the use of different strong constitutive promoters is desirable in order to avoid genetic instability caused by recombination between identical or closely related promoter sequences taken from plants themselves. Through use of the present promoter sequence the introduced genes can be transcribed to messenger RNA and then translated to resultant proteins to exhibit new traits or characters.

Besides developing useful traits in crops, the present invention provides a further understanding of molecular pathways involved in disease development and secondary metabolism in plants. Moreover, by engineering plants with specific foreign genes, the responses of plants to abiotic and biotic stress and stress related metabolism can be analyzed. The invention described herein in developing gene vectors with newly defined promoters of the caulimoviruses advances this effort.

A wide variety of well-characterized genes of animal, human, bacterial and of plant origin, including those of several viruses, are available for engineering plants. For the most effective expression of this wide selection of genes either constitutive or regulated, versatile gene expression vectors are required. At the University of Kentucky, Dr. Arthur Hunt and his colleagues have developed a series of plant expression vectors (Scharidl, et al., 1987) with a constitutive 35S promoter from cauliflower mosaic virus (CaMV) which have been successfully used to produce transgenic plants (Maiti, et al., 1988, 1989, 1991, 1993, 1994, 1995; Graybosh, et al., 1989; Berger, et al., 1989; Yeargan, et al., 1992; Liod, et al., 1992).

The present invention, develops additional useful promoters from FMV for high level expression of foreign genes in transgenic tobacco. These vectors are useful for both direct DNA uptake by isolated protoplasts and Ti plasmid-mediated gene transfer.

Enhanced levels of transcription via highly active promoters are essential for high levels of gene expression. The most widely used promoter for plant transformation, as described earlier, has been the 35S promoter of CaMV. It is active in a wide variety of plants and tissues. It is also the most thoroughly characterized promoter with respect to the sequence elements active in its transcriptional activity (Benfey and Chua, 1990). Kay, et al., 1987 showed that the transcriptional activity of the CaMV 35S promoter could be increased approximately tenfold by making a tandem duplication of 250 base pairs of upstream sequence.

Similar observation have been made with other promoters (McNeall, et al., 1989). The present inventors have constructed and tested a construct with the FMV FLt promoter.

The Monsanto Co. has recently patented 35S and the 19S promoters of CaMV, and the full length transcript promoter from FMV. In both cases cloned DNA material was provided to Monsanto Co. by the present investigator, Dr. Shepherd, University of Kentucky, Lexington, Ky. The present inventors have overcome the deficiencies of prior transgenic plant promoters and have now developed new, unique promoters of equal or better expression strength.

#### SUMMARY OF THE INVENTION

The present inventions are applicable to plant genetic engineering. Specifically, the present inventions relate to the

promoters from figwort mosaic virus and these promoters direct the expression of genes in plant cells.

Thus an object of the present invention is to provide a plasmid comprising a chimeric gene comprising a full length transcript (FLt) promoter and at least one enhancer domain of the figwort mosaic virus (FMV), operably linked to a heterologous gene sequence which is heterologous to said promoter.

Another object of the invention provides a plasmid with a single, a double, or multiple enhancer domains. In a preferred embodiment the plasmid of claim 1, further comprises a 5' non-translated leader sequence from figwort mosaic virus. The plasmid may optionally include a region of homology to an *Agrobacterium tumefaciens* vector and a T-DNA border region from *Agrobacterium tumefaciens*, wherein said chimeric gene is located between the T-DNA border and the region of homology. Examples of plasmids of the invention include intermediate plasmids pUCFMV FLt 10, PUCFMV Flt 101, PUCFMV Flt 102, and PUCFMV Flt 103.

In a preferred embodiment the plasmid full length transcript (FLt) promoter consists of the 3' portion of gene VI and the intergenic region including nucleotides 6481 to 7030 of the FMV genome. The heterologous gene is preferably downstream from the promoter and is capable of being expressed in a transgenic plant.

In an additional embodiment the plasmid comprises a) a FMV FLt promoter with single enhancer domain; b) a 3' nontranslated polyadenylation sequence of *rbcS E9* gene; and (c) a structural sequence encoding neomycin phosphotransferase II.

Still another object of the invention is to provide for an expression vector comprising a chimeric gene including a full length transcript (FLt) promoter and at least one enhancer domain of the figwort mosaic virus (FMV), operably linked to a heterologous gene sequence which is heterologous to the promoter.

The expression vector may comprise a disarmed plant tumor inducing plasmid of *Agrobacterium tumefaciens*, and is preferably selected from pKLF, pKLF2, pKLF2-GUS, pKLF2-CAT, pKLF20-GUS.

The invention also provides for a plant cell transformed with the plasmid comprising a chimeric gene comprising a full length transcript (FLt) promoter and at least one enhancer domain of the figwort mosaic virus (FMV), operably linked to a heterologous gene sequence which is heterologous to said promoter.

Transgenic plants comprising the plasmid are also within the scope of the invention. Transgenic plants are preferably selected from crop plants including, but not limited to cotton, soy bean, alfalfa, oilseed rape, flax, tomato, sugar beet, sunflower, potato, tobacco, maize, wheat, rice, lettuce and banana plants. Any crop plant which is modifiable with the plasmid of the invention is included within the scope of this application.

The heterologous gene is expressed in plant tissues including but not limited to plant tissues selected from calyx, filament, pedicel, style, ovary, corolla, anther, stigma, leaf, stem, embryo, seed and root tissues.

The invention provides for a chimeric gene or DNA which is transcribed and translated in plant cells, said chimeric gene comprising a region comprising an FMV FLt promoter and at least one enhancer domain, wherein said region is free of a FMV protein-encoding DNA sequence and a DNA sequence which is heterologous the promoter.

The above and other objects of the invention will become readily apparent to those of skill in the relevant art from the following detailed description and figures, wherein only the preferred embodiments of the invention are shown and described, simply by way of illustration of the best mode of carrying out the invention. As is readily recognized the invention is capable of modifications within the skill of the relevant art without departing from the spirit and scope of the invention.

#### BRIEF DESCRIPTION OF DRAWINGS

FIGS. 1A and 1B. The DNA sequence of the full length transcript (FLt) promoter from the figwort mosaic virus (FMV) strain DxS (Richins et al., 1987). The nucleotide sequence (FMV coordinates 6481 to 7680, a 1200 bp fragment) includes the 3' end of gene VI, and part of the large intergenic region, presented in the 5' to 3' direction of the transcript from left to right.

FIGS. 2A and 2B. Construction strategy of FMV FLt promoter with its single and double enhancer domains. Number in parenthesis indicate nucleotide position in the FMV genome.

FIG. 3. Physical map of pKYLX71.

FIGS. 4A and 4B. Physical map of pKLF.

FIGS. 5A and 5B. Physical map of pKLF2.

FIGS. 6A and 6B. Schematic representation of chimeric GUS or CAT constructs used for assaying promoter activity in transient expression analysis (A) and transgenic plants (B).

FIGS. 7A and 7B. FMV FLt promoter activity in transgenic plants expressing a CAT reporter gene.

FIG. 8. A comparison of the wild type and modified FMV FLt promoter activity in transgenic *Nicotiana tabacum* cv Samsun NN (R0 progeny) expressing a GUS reporter gene.

FIGS. 9A-9O. Histochemical localization of GUS activity in developing transgenic tobacco.

FIG. 10. FMV FLt promoter activity in different flower organs.

FIGS. 11A and 11B. A deletion analysis of the FMV FLt promoter.

#### STATEMENT OF DEPOSIT

Plasmids pKLF and pKLF2 in *E. coli* TB1 have been deposited with the Agricultural Research Service (ARS) Patent Culture Collection (NRRL), 1815 North University Street, Peoria, Ill., USA, 61604, under the terms of the Budapest Treaty on Jun. 28, 1996. The deposit will be maintained for the life of the patent as required by Treaty. The recombinant bacteria may be cultured in LB medium in the presence of tetracycline (15 µg/ml).

#### DESCRIPTION OF THE INVENTION

The present invention includes: i) isolation of the promoter for the full length transcript (FLt) from figwort mosaic virus (FMV) strain DxS (Richins, et al. 1987) from the full length viral DNA clone as described below in Experimental Section. The modification includes duplication or multimerization of the enhancer domain of the FLt promoter from FMV. The FLt promoter sequence for FMV is shown in FIG. 1.; iii) Use of FMV promoter in a method for transforming plant cells, expression vectors including FMV promoter, a chimeric gene including FMV promoter sequence and transgenic plants, plant cells and seeds incorporating the FMV promoter in a chimeric gene.

The chimeric gene may preferably be composed of a promoter region, a 5' non-translated leader sequence the structural gene itself and a 3' polyadenylation sequence. The promoter is a DNA fragment composed of modular sequence which directs and regulates the expression of genes through transcription to messenger RNA. The proper regulatory signals/enhancer elements must be present in defined location in order to express the inserted gene into RNA and a resultant protein. The 3'-polyadenylation sequence is a non-translated region which signals the adenylation of the 3' end of the RNA in order to stabilize the RNA in the cytoplasm for subsequent translation into protein.

Certain promoters have a specific modular sequence which makes it either tissue specific, developmentally regulated or environmentally regulated for its selective expression of genes in cells. Promoters capable of directing RNA synthesis at higher rates compared to other promoters are desirable for many purposes. If these promoters are able to direct the expression of genes in most of tissues of plants, they are defined as constitutive promoters. The inventors have found that the CaMV 35S promoter is one of the strongest constitutive promoters. The transcriptional activity of the CaMV 35S promoter is the result of synergistic and combinatorial effect of enhancer elements residing upstream of the TATA element. Single or multiple copies of enhancer sequences from the CaMV 35S promoter can also increase homo- or heterologous promoter activity in an orientation-independent manner. The enhancement of promoter activity has been found to be related to the copy number of the enhancer sequence.

The inventors have developed expression vectors with the FMV promoter with its single and duplicated enhancer domains. The upstream enhancer elements of the strong constitutive promoter from the full length transcript of FMV has been doubled in a strategy to even further strengthen this promoter. Promoters from other caulimoviruses such as FMV, PCISV, and MMV as well as the better characterized CaMV 35S promoter will be useful for plant genetic engineering. The inventors have developed plant expression vectors with constitutive FLt promoters of FMV.

The primary objective of the present invention is to provide several strong and constitutive promoters to be used for expression of chimeric genes in transgenic plants. Another object of the present invention is to develop a strategy to further strengthen the promoters from the full length transcript of other member of the calimovirus (plant pararetrovirus) including FMV.

#### Experimental Procedures

Strains of FMV adapted to solanaceous plants have been described by Shepherd et al., 1987. Isolation of the promoter for the full-length RNA transcript (FLt) and characterization of its activity in protoplasts of tobacco has also been done in this laboratory (Gowda et al., 1889). The clone of the promoter (plasmid pFMV 20) was shown to give high levels of constitutive expression in tobacco cells (Gowda, et al., 1989). Later the FMV FLt promoter was shown to control transcription of an RNA transcript spanning the entire circular genome of FMV (Scholthof, et al., 1992); (Cooke, 1990). Another newly described caulimovirus PCISV (Reddy, et al., 1993; Richins et al., 1993) has been partially characterized in this laboratory. These investigations provide the materials (DNA clones) for the invention described herein.

Creation of plant expression vectors pKLF, pKLF2

Construction of a FMV FLt promoter with single and duplicated enhancer elements and creation of plasmids pKLF and pKLF2

The construction strategy for isolating the FMV FLt promoter and its enhancer domain is shown in FIG. 2. For the FLt promoter, a 406 bp Hinc II to Bam HI fragment (coordinates 6677 to 7082 of the FMV genome) was cloned into the corresponding sites of pUC119A (a modified pUC119 in which Eco RI and Sma I sites were destroyed by digesting with Eco RI and Sma I followed by ligation). The resulting plasmid was designated as pUCFMV FLt101. An Eco RI site located 6 bp upstream from the TATAA box was changed to a Sma I site using a Sma I adaptor. This change inserted 8 additional nucleotides (5'-ACCCGGGC-3') into the promoter sequence. The resulting plasmid was designated as pUCFMVFLt102. In the FLt promoter with its single enhancer domain, a 335 bp segment (position 6677 to 7003 of the FMV sequence) was amplified from pUCFMVFLt102 by PCR using appropriately designed oligonucleotides to insert an Eco RI at the 5' end and a Hin dIII site at the 3' end of the fragment. This promoter fragment was cloned into pUC18 at its Eco RI and Hin dIII sites. The resulting plasmid was designated pUCFMVFLt103.

The enhancer domain Eco RI to Sma I fragment of 213 bp was isolated from pUCFMVFLt103 and this fragment was cloned into the Eco RI and Hinc II sites of pUCFMVFLt103, the resulting plasmid named pUCFMVFLt10 contains the FLt promoter with its duplicated enhancer domain. The FLt promoter sequence with either single or double enhancer domains was inserted into a plant expression vector by substituting it for the CaMV35S promoter of pKYLX71 (Scharl et al., 1987). The physical map of pKYLX71 is shown (FIG. 3). The unique Eco RI and Hin dIII sites that flank the promoter were used for this purpose. The resulting expression vectors were designated as pKLF (FIG. 4) when a single enhancer domain was present or pKLF2 (FIG. 5) when a double enhancer domain was present. These plasmids have multiple cloning sites (MCS: 5'-Hin dIII, Bam HI, Xho I, Pst I, Sac I and Xba I-3') with the following unique sites: Hin dIII, Xho I, Sac I and Xba I.

Testing the Expression Vectors with a GUS or CAT reporter gene: Stable transformation and analysis of transgenic plants

The reporter genes CAT or GUS were tailored by PCR to include just the coding sequence with the initiation and termination codons of each gene, flanked by a Xho I site at the 5' end and a Sst I site at the 3' end. The PCR isolated fragment for the reporter gene (CAT or GUS) was digested with Xho I and Sst I, gel purified and cloned into the corresponding sites of plant expression vectors pKLF or pKLF2. The resulting constructs #109-CAT, 110-GUS, 111-CAT and 112-GUS (FIG. 6 B) were introduced into *Agrobacterium tumefaciens* strain C58C1:pGV3850 by triparental mating and tobacco (cv. Samsun NN) was transformed with the engineered *Agrobacterium* as described earlier (Maiti et al., 1993).

The construct #102 in pKYLXF20GUS contains an Eco RI to Hind III fragment from pF20GUS cloned into Eco RI/Hind III sites of pKYLX7 by replacing the CaMV 35S promoter flanked by Eco RI and Hind III sites.

To examine the integration of genes in transgenic plants, genomic DNA was isolated following the procedure (Thomson and Henry 1993) for PCR analysis. The integration of reporter CAT or GUS gene in the genome of transgenic plants (R0 and R1 progeny) was detected by PCR amplification using appropriately designed oligonucleotides specific for the CAT or GUS gene sequence. Specificity of each PCR product was tested by Southern hybridization with a GUS or CAT probe.

#### EXAMPLE 1

Comparative functional analysis of the CaMV35S and the FMV FLt promoters in transient expression experiments



using tobacco leaf protoplasts. To compare the relative strengths of the CaMV 35S and the FMV F<sub>L</sub>t promoters, we tested different vector constructs with the GUS gene in transient expression experiments in protoplasts of *Nicotiana edwardsonii*. The schematic maps of the plasmids used in these experiments are shown in FIG. 6A. Isolation of protoplasts from *Nicotiana edwardsonii* cell suspension cultures and electroporation of protoplasts with supercoiled plasmid DNA containing CAT or GUS has been described (Gowda et al., 1989; Kiernan et al., 1993). In brief, an aliquot containing 2×10<sup>6</sup> protoplasts was electroporated with 50 μg of plasmid DNA. After 20 hrs, 2×10<sup>5</sup> protoplasts were harvested for each CAT or GUS assay. CAT activity was determined according to the published method (Gorman et al., 1982).

Plant tissue extracts containing 5 μg of soluble protein were used for each CAT assay. The reaction was carried out at 37° C. for 30 min. The rates of reaction were in a linear range over the period of incubation. Fluorometric GUS assays to measure GUS activity of plant tissue extracts and histochemical GUS assays to determine the distribution of GUS activity in plants on embryos and seedlings, were performed according to published procedure (Jefferson et al., 1987). Protein in plant extracts was estimated (Bradford 1976) using BSA as a standard.

For the fluorometric assays, samples were homogenized in GUS extraction buffer (50 mM NaPO<sub>4</sub>, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na<sub>2</sub> EDTA, 0.1% Na Sarkosyl, 0.1% Triton X-100), and centrifuged for 10 min. at full speed in a microcentrifuge. Soluble protein (5 μg) from transgenic plant tissue extracts were incubated with 4-methyl umbelliferyl glucuronide (MUG) solution for 10 to 20 minutes after which fluorescence was measured. The activity remained linear with added enzymes. Fluorescence of a solution of 100 pmol 4-methyl umbelliferone (MU) in 0.2 M sodium carbonate was used for calibration. Fluorescence was measured on a minifluorometer (Model No TKO 100: Hoefer, San Francisco, Calif.), with an excitation wavelength of 365 nm and photodetector wavelength of 460 nm.

The results from the transient expression experiments are shown in Table 1. The gene constructs with the wild type F<sub>L</sub>t promoter (pFMV 20 GUS) with its single enhancer domain showed about 2.5 fold higher promoter activity than the CaMV 35S promoter-GUS construct (pGG1) in these assays. A control plasmid pc-GUS (CaMV 35S promoter-GUS-nos3' terminator) contains an extra out of frame ATG codon (as Sph I site GCATGC) in the multiple cloning site of pKYLX 7. The presence of this ATG codon causes about 7-8 fold less GUS activity compared to pGG1. The duplication of FMV F<sub>L</sub>t promoter enhancer domain in plasmid pKLF2-GUS increased the level of GUS activity about 4 fold as compared to pKLF-GUS with the single enhancer domain. This increase was also observed in a stably transformed system in intact tobacco plants. In pFMV 20 GUS, the F<sub>L</sub>t promoter with an Eco RI site located 6 bp upstream from the TATAA box was changed to a Sma I site using a Sma I adaptor. This change inserted 8 additional nucleotides (5'-ACCCGGGC-3') into the promoter sequence in pKLF-GUS.

#### EXAMPLE 2

Analysis of FMV F<sub>L</sub>t promoter activity in transgenic plants

The constructs shown in FIG. 6 B were transformed into tobacco plants via the *Agrobacterium* co-cultivation method as described in Maiti et al., 1988. Transformations were done using *Nicotiana tabacum* cv Samsun NN or *Datura*

*innoxia*. For screening of transformants tobacco plants arising from first generation seeds (R1 progeny), germination was done in presence of kanamycin (200 μg/ml). Primary transformants of tobacco were selected for resistance to kanamycin (300 mg/ml) and these were grown to maturity in the greenhouse. At least 8 to 10 independent lines were generated for each construct tested. The presence of the reporter genes, CAT or GUS, in genomic DNA from these transformants was detected by PCR amplification using primers based on sequences from the coding region of each gene. The specificities of PCR fragments were tested by Southern hybridization with CAT or GUS probes for the respective transformants (data not shown).

The expression levels of the CAT reporter gene in independent transformants developed for pKLF2CAT, pKLF2CAT are shown in FIG. 7 A & B. Individual plant lines generated from independent calli expressing the same gene showed variable CAT activity. Similar patterns of plant-to-plant variations in gene expression have been reported with many other plant promoters. It is believed that these variations are largely due to the difference in position of the integrated genes in the chromosome and the degree of co-suppression. Separate plant lines developed with pKLF2CAT showed more activity than any of the plants transformed with pKLF2CAT. On average, about 4.5 fold higher activity was exhibited by plants transformed with pKLF2CAT, which has a duplicated enhancer domain, not present in plants transformed with pKLF-CAT which has a single enhancer domain.

The wild type FMV F<sub>L</sub>t promoter in pKYLXF20GUS (construct #102) was compared with a modified FMV F<sub>L</sub>t promoter in pKLF2GUS (construct #110) and pKLF2GUS (construct #112). The expression of the GUS reporter gene in leaf extracts of primary transformants is shown in FIG. 8. Although variation in GUS activity in transgenic tobacco plants was considerable, the GUS expression in leaves in plants transformed with pKYLX GUS, (construct #102-GUS) are very similar to that of plants transformed with pKLF2GUS, which contains 8 additional base pairs just 6 bp upstream of TATA box (as a result of inserting a Sma I linker in its EcoRI site). Promoter activity was not affected by this insertion.

Although there is variability in GUS expression in several independent lines transformed with pKLF2GUS (construct #112), four lines had more activity (average of 3.5 fold greater activity) than any transformant of either pKLF2GUS or pKYLXF20GUS. Hence, the F<sub>L</sub>t promoter with a duplicated enhancer domain is more active than the F<sub>L</sub>t promoter with a single enhancer domain.

#### EXAMPLE 3

Expression levels in seedlings (R1 progeny) and young tobacco or *Datura* plants

In order to examine the promoter activity in various tissues during seedling development, the expression of the GUS reporter gene in seedlings (R1 progeny) transformed with pKYLXF20-GUS, or pKLF2-GUS was examined by fluorometric assay of tissue extracts and by histochemical staining of transverse sections of leaves, stems and roots. The FMV promoter activity was monitored in 14 day old seedlings grown aseptically on an MS-agar medium in the presence of kanamycin (300 μg/ml) and 3% sucrose. Several independent lines for each construct were studied. Comparison of activities of the F<sub>L</sub>t promoter indicated a gradient of expression in the following order; the highest level of activity was found in roots followed by leaves and stems. Stable transformants with a double enhancer gave about 5 fold more GUS activity in roots than those with a single enhancer domain.

The histochemical staining shown in FIG. 9 is representative of the staining patterns analyzed in plants expressing high levels of GUS activity. In seedlings and sections of young leaves stained for GUS, the intensity of staining was markedly greater in vascular tissues of young leaves, petioles, stems and roots. The intensity of GUS staining observed in vascular tissue was in the following order: roots>leaves>stems (FIG. 9). The histochemical GUS assay in leaves showed more activity in midribs, veins and other vascular tissue, and in trichomes than in leaf mesophyll and palisade cells. No GUS activity was detected in transgenic plants containing the construct #111-CAT gene (FIG. 9A).

#### EXAMPLE 4

Expression of the FMV FLt promoter in various flower organs

FLt promoter activity was examined in flowers from several independent primary lines of construct #110-GUS. Flower samples were collected one day before anthesis. A representative of this analysis with line #7 is shown in FIG. 10. In flowers the highest expression occurred in the calyx, followed by the filament, pedicel, styles, ovary, corolla and stigma. Expression was lowest in anthers. The activity in flowers is relatively low compared to leaves, stems or roots of plants. Similar observations have been made with the CaMV 35S promoter in transgenic tobacco (An et al., 1988).

The disarmed *Agrobacterium* strain transformed with plant expression vectors containing chimeric gene of interest can be used to engineer desired plants including but not limited to cotton, soybean, alfalfa, oilseed rape, flax, tomato, sugar beet, sunflower, potato, tobacco, maize, wheat, rice and lettuce, banana, etc. The use of DNA fragment or vectors including FMV promoter sequence tailored with heterologous DNA sequence in the transformation of plants by electroporation or particle gun transformation is within the scope of this invention. These embodiments and examples are provided in order to evaluate the practice of present invention. These examples serve mainly the illustrative purpose, and are not intended to limit the scope of the invention.

TABLE 1

Constructs	Relative GUS activity (%)
Control (TE buffer)	00
pUC8 GUS (No promoter)	00
pc-GUS (extra ATG)	7
pGG1 (CaMV35S)	35
pFMV 20-GUS (FMV FLt)	100
pKLF (FMV FLt modified)	100
pKLF 2 (2 X Enh FMV FLt)	410

Relative  $\beta$ -glucuronidase (GUS) activity of GUS fusion constructs containing different promoters electroporated into tobacco protoplasts. The GUS assay was carried out 20 hrs after electroporation. Assays and conditions were as described in the Methods. Promoter strength is presented as percentage of GUS activity normalized to pFMV 20 GUS for pUC based constructs or pKLF GUS for pKYLX7 based constructs, and represent the mean of three samples from at least two independent experiments, variation was within 12% of the presented value.

The inventors have found that the FLt promoter is a strong, constitutive promoter able to direct gene expression at a level comparable to or exceeding that of the 35S promoter of CaMV. There are extensive similarities in the

cis-elements of the 35S and FLt promoters. Both the 35S and FLt promoters contain a consensus TATA-box element (TATATAA) at positions -30 to -24 and -44 to -38 respectively, from the transcription start site. The weaker 19S promoter of CaMV (Guilley et al., 1982) and the FLt promoter of FMV strain M3 (Sanger et al., 1990) carry a non-consensus sequence (TATTAA) for the TATA-box element. There are three CCAAT-like boxes in the FMV FLt promoter: CCACT (-97 to -93), CACTAA (-92 to -87) and CCACA (-62 to -57); likewise in the 35S promoter these are: CCACT (-85 to -81), CACAAT (-64 to -59) and CCACT (-57 to -53) (Fang et al., 1989; Ow et al., 1987). In the FLt promoter the influence of the CCAAT-element was not evaluated. However this element has been analyzed for other promoters. The deletion of the 5'-most CCAAT-element drastically reduced the activity of the 35S promoter (Ow et al., 1987). In animal systems the importance of the CAAT box has been demonstrated (Bienz et al., 1986). However in the *rbcSE9* gene deletion of a putative CAAT-box that showed no negative effect on promoter activity (Morelli et al., 1985).

The region -80 to -63 of the FLt promoter contains the as-1 motif TGACGA repeat (Table 2), that is similar in sequence to that of the 35S promoter 'as-1' element (Lam 1994) at position -82 to -62. The nuclear protein factor ASF-1, mostly responsible for root specific expression, binds within this region, as demonstrated for the 35S promoter (Lam 1994). The as-1 element is important for high expression of promoter activity in young seedlings and leaves by interacting with other adjacent cis-acting units. The leaf-specific element 'as-2', GAT(A/C) repeat sequence of the 35S promoter, at position -105 to -85, interacts with the nuclear factor ASF-2 (Lam et al., 1989). In the 35S promoter, the 'as-2' motif is located 2 bp upstream of the 'as-1' motif. However, in the FLt promoter the 'as-2' like domain GAT(A/T), at position -163 to -151, is located 70 bp upstream of the 'as-1' domain. The as-2 motif has also been identified in similar positions compared to the CaMV 35S promoter in 12 different *Cab* (chlorophyll a/b binding protein) genes (Lam and Chua 1989).

Besides the CAAT and TATA boxes and the 'as-1' and 'as-2' domains, there are several repeat elements present in both the 35S and FLt promoter sequences which resemble the SV40 core enhancer sequence GTGG/C (Khoury and Gruss 1983). The sequences GTGGGGA (-138 to -132), GTGGGGA (-240 to -234) and GTGGGCA (-286 to -280) in the FLt promoter (Table 2 and FIG. 1) resemble the GTGGAAAAAG (SEQ ID NO:1), GTGGAAAAAG (-261 to -253) and GGTAATATC (-325 to -316) sequences of the 35S promoter (Fang et al., 1989). In the FLt promoter, the sequences AAAGA (-116 to -112), AAAGA (-165 to -162), AAAAGGA (-189 to -183), AAAAGCA (-225 to -119) have similarity with AAAGC (-111 to -107), AAAGA (-136 to -132), AAGATGG (-173 to -167) and AAGATGC (-200 to -194) sequences in the 35S promoter. The 5' deletion analysis of the FLt promoter showed that the heptamer sequence repeat, GTGGGGA at position -138 to -132, and at -240 to -234, seems to be important for FLt promoter activity. Further studies will be necessary to evaluate the impact of these upstream repeat sequences on promoter function.

The FMV FLt promoter with longer upstream sequence (-456 to +64) gives less expression compared to the fragment -249 to +64. (See FIG. 11 and section: FMV FLt promoter structure and deletion analysis, page 29 herein.) This result is in contrast to the 35S promoter where a fragment (-1600 to +1) with a longer upstream sequence

retained almost full activity compared to a promoter construct extending from -134 to +1 (Ow et al., 1987). It was reported that a promoter sequence isolated from FMV strain M3 exhibited more activity when it contained longer upstream regions (Sanger et al., 1990).

The 3'- and 5'-end deletion analysis of the FLt promoter/leader, studied in transient expression experiments in protoplasts, indicates that a promoter fragment of 313 bp extending from position -249 to +64 with respect to the transcription start site, gives maximum promoter activity. The FLt promoter gives better activity with the longer 3' leader sequence extending to +64 compared to +10 (FIG. 11). Untranslated plant viral leader sequences stimulate expression of a downstream reporter gene (Day Dowson et al., 1993). The 3' leader of the FLt promoter may have some sequence important for initiation of transcription or translation and transcript analysis will be needed to evaluate this putative effect.

Full activity may be due to the location and spacing of the enhancer motifs, specifically the 'as-2' motif in FMV which is apart from the 'as-1' motif, whereas in the 35S promoter both the 'as-1' and 'as-2' motifs are closely associated. In addition, the GTGGGGA repeat elements at position (-138 to -132) and (-240 to -234), separated by 94 nt, seem to be essential for full promoter expression. pFMV CAT 17 (-238 to +64) contains most of the repeat sequences, including one of the GTGGGGA sequences at (-138 to 132) and part of a second upstream GTGGGGA sequence at (-240 to -234). When part of these upstream elements is missing only 50% of full promoter expression is obtained. In pFMV CAT 20 (-249 to +64), inclusion of 11 nt upstream of -238 gives maximum promoter expression, suggesting the importance of the GTGGGGCA sequence. The sequence -249 to -47 upstream of the TATA box contains elements necessary for maximal promoter expression.

When tested with GUS reporter genes in protoplasts, the FLt promoter showed about 2.5-fold more GUS activity compared to the 35S promoter (see Table 1 in Example 1). The sequence from -256 to -55 was duplicated to develop an FLt promoter with a double enhancer domain. This was inserted into the plant expression vector pKLF2. Tandem duplication of enhancer elements of the FLt promoter in pKLF2-GUS or pKLF2-CAT increased gene expression approximately 4-6 fold in transgenic plants. These vectors may be useful for both direct uptake by protoplasts and Ti plasmid-mediated gene transfer.

The expression of useful foreign traits in plants is a major focus in plant biotechnology. For metabolic engineering, where multiple genes need to be inserted into a single cell during the course of transformation, the use of different strong constitutive promoters will be desirable in order to avoid genetic instability caused by recombination between identical promoter sequences.

#### EXAMPLE 5

Stable transformation and analysis of transgenic plants

The reporter genes CAT or GUS were tailored by PCR to include just the coding sequence with the initiation and termination codons of each gene, flanked by a Xho I site at the 5' end and a Sst I site at the 3' end.

Methods for this example are similar to those set forth in Example 2 above. These were introduced into plant expression vectors. The PCR isolated fragment for the reporter gene (CAT or GUS) was digested with Xho I and Sst I, gel purified and cloned into the corresponding sites of plant expression vectors pKLF or pKLF2. The resulting constructs #109-CAT, 110-GUS, 111-CAT and 112-GUS were introduced into *Agrobacterium tumefaciens* strain

C58C1:pGV3850 by triparental mating and tobacco (cv. Samsun NN) was transformed with the engineered *Agrobacterium* as described earlier (Maiti et al., 1993). The construct #102 in pKYLXF20GUS contains an Eco RI to Hin dIII fragment from pF20GUS cloned into the Eco RI/Hin dIII sites of pKYLX7 by replacing the CaMV 35S promoter flanked by Eco RI and Hind III sites.

To examine the integration of genes in transgenic plants, genomic DNA was isolated following the procedure of Thomson and Henry (1993) for PCR analysis. The integration of the reporter CAT or GUS genes in the genome of transgenic plants (R0 and R1 progeny) was detected by PCR amplification using appropriately designed oligonucleotides specific for the CAT or GUS gene sequence. Specificity of each PCR product was tested by Southern hybridization with a GUS or CAT probe.

#### EXAMPLE 6

Transient expression experiments, protoplast isolation, and electroporation

Isolation of protoplasts from *Nicotiana edwardsonii* cell suspension cultures and electroporation of protoplasts with supercoiled plasmid DNA containing CAT or GUS has been described (Gowda et al., 1989; Kiernan et al., 1993). (Methods are similar to those described in detail in Examples 1 and 2). In brief, an aliquot containing  $2 \times 10^6$  protoplasts was electroporated with  $50 \mu\text{g}$  of plasmid DNA. After 20 hr,  $2 \times 10^5$  protoplasts were harvested for each CAT or GUS assay.

#### EXAMPLE 7

Chloramphenicol acetyl transferase (CAT) and  $\beta$ -glucuronidase (GUS) assays

CAT activity was determined according to the published method (Gorman et al., 1982). Plant tissue extracts containing  $5 \mu\text{g}$  of soluble protein were used for each CAT assay. The reaction was carried out at  $37^\circ \text{C}$ . for 30 min. The rates of reaction were linear over the period of incubation. Fluorometric GUS assays to measure GUS activity of plant tissue extracts and histochemical GUS assays to determine the distribution of GUS activity in plants (embryos and seedlings), were performed according to published procedures (Jefferson et al., 1987). Protein in plant extracts was estimated (Bradford 1976) using BSA as a standard. For the fluorometric assays, samples were homogenized in GUS extraction buffer (50 mM  $\text{NaPO}_4$ , pH 7.0, 10 mM  $\beta$ -mercaptoethanol, 10 mM  $\text{Na}_2 \text{EDTA}$ , 0.1% Na Sarkosyl, 0.1% Triton X-100), and centrifuged for 10 min at full speed in a microcentrifuge. Soluble protein ( $5 \mu\text{g}$ ) from transgenic plant tissue extracts were incubated with 4-methyl umbelliferyl glucuronide (MUG) solution for 10 to 20 min, after which fluorescence was measured. Fluorescence of a solution of 100 p mol 4-methyl umbelliferone (MU) in 0.2 M sodium carbonate was used for calibration. Fluorescence was measured on a minifluorometer (Model No TKO 100: Hoefer, San Francisco, Calif.), with an excitation wavelength of 365 nm and photodetector wavelength of 460 nm.

For histochemical staining to detect GUS activity, seedlings or plant tissue sections were placed in GUS histochemical buffer (100 mM  $\text{NaPO}_4$ , 0.5 mM  $\text{K}_3[\text{Fe}(\text{CN})_6]$ , 0.5 mM  $\text{K}_4[\text{Fe}(\text{CN})_6]$ , 10 mM  $\text{EDTA}$ , 1 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (X-gluc) in vacuo for 10 min, followed by incubation at  $37^\circ \text{C}$ . for 1 to 18 hr. Samples were evaluated for the rate and intensity of color development in tissues.

## EXAMPLE 8

Structure of the FMV FLt promoter and analysis of deletion constructs

In order to define the regions of the FLt promoter needed for maximal expression, a 3'- and 5'-end deletion analysis was carried out on the appropriate DNA fragment from the FMV genome (Richins et al., 1987) (genome coordinates 6481 to 7680). The start site of transcription (ACTGAA, start site in bold letter, coordinate 6939 of the FMV genome) to produce the full length transcript (Scholthof et al., 1992) was determined by primer extension analysis (data not shown). The FLt promoter contains a consensus TATA box (TATAATA) at position -44 to -38, a CAAT box like sequence (CCACT) at position -97 to -93, an as-1 enhancer elements (TGACG), an as-2 motif and several repeat sequences resembling the SV40 core element GTGG/C sequence (Khoury and Gruss 1983) (Table 2).

TABLE 2

A list of DNA repeat sequences and other putative regulatory elements in the FMV FLt promoter, (FMV coordinates 6481 to 7680, of 1200 bp fragment)			
Designation of sequence	Repeat sequence or regulatory elements	Position	Spacing (nt) between successive domains
1a	TGACGA	-68 to -63,	6
1b	TGACGA	-80 to -75	
(as-1 motif)			
2a	AAAGA	-116 to -112	44
2b	AAAGA	-165 to -162	94
3a	GTGGGGGA	-138 to -132	
3b	GTGGGGGA	-240 to -234	40
3c	GTGGGCA	-286 to -280	4
4a	GATT	-163 to -160	
4b	GATA	-154 to -151	
(as-2 like)			
5a	AAAAGGA	-189 to -183	29
5b	AAAAGCA	-225 to -119	9
6a	GGCACA	-201 to -196	
6b	GGTGCA	-216 to -211	13
7a	AAAGTAA	-277 to -273	
7b	AAAGTTA	-293 to -287	26
7c	TAAGTTT	-326 to -320	8
8a	AAGAC	-300 to -296	
8b	AAGAC	-313 to -309	25
9a	TCCAAAGC	-447 to -440	
9b	TCCAAACC	-414 to -406	5
9c	GCCAAAAGC	-402 to -394	50
10a	TCAA	-438 to -435	
10b	TCAA	-384 to -380	10
10c	TCAA	-370 to -367	0
10d	TCAA	-366 to -363	9
11a	ATCAAAGTA	-385 to -377	
11b	ATCAATGAA	-367 to -359	
CAAT-box	CCACT	-91 to -93	50
TATA-box	TATATAA	-44 to -38	
polyA track	AATAAA	+125 to +130	
TATA like	TATAAAATA	+180 to +188	

The FMV FLt promoter was subcloned into pUC 119 from the FMV strain DxS genome (Richins et al., 1987). In order to define the 3' boundary of the promoter/leader for maximum expression of the reporter gene, a set of nested 3' deletion plasmids with the 5' end point at -249 were selected for analysis. These constructs were introduced into tobacco protoplasts by electroporation for transient expression assays. The construct pFMV CAT20 (-249 to +64), (Gowda et al., 1989) showed maximum expression, and for comparison with other constructs this value was considered to represent 100% of full activity. The constructs pFMV CAT 32, pFMV CAT 1 or pFMV CAT 19, with 3' ends at +736, +377 or +287 respectively, gave much lower CAT activity

than pFMV CAT20. Further deletion to +204 or +167 increased the promoter expression to 25-30% of full activity. However, this was still significantly lower than the activity of the plasmid pFMV CAT 20 (-249 to +64), which showed the maximum expression.

These results show that the leader sequence from +167 to +736 has a significant inhibitory effect on expression activity, probably through its effect on translation of the transcript. This depressing effect of the leader sequence on expression has been documented earlier for both FMV (Gowda et al., 1989) and CaMV (Baughman and Howell 1988). The 3' deletion to +10 in pFMV CAT 6 showed about 75% maximal promoter expression compared to pFMV CAT 20. This suggests that for better expression a longer 3' leader is necessary for the FLt promoter. FMV FLt promoter expression was significantly reduced by deleting the sequence to -47 in pFMV CAT 5 (TATA box region) from the 3' end, demonstrating the importance of a TATA box sequence in the FMV FLt promoter.

In the downstream sequence of the FMV FLt promoter, there is a TATA like sequence (TATAAAATA) at position +180 to +188 after the poly A signal (AATAAA) at position +125 to +130 (Table 2). An internal deletion mutant pFD-PCAT was generated from pFMV 19 (-249 to +287) by deleting a 199 bp segment (-53 to +144) containing the TATA box at position -44 to -38 and the poly A track at position +125 to +130. Interestingly, this promoter/leader mutant pFDP CAT showed expression of about 70% of full activity, suggesting that the TATAAAATA sequence may substitute for the upstream normal TATA -box in this context.

The mutation of the poly A signal AATAAA to the sequence TTTAAA in pFMV Dra I CAT derived from pFMV CAT 15 (-249 to +167) resulted in about 40% less activity compared to pFMVCAT15, indicating some importance of this sequence for proper expression in this context. However, from 3' deletion analysis, the 313 bp promoter fragment (-249 to +64) in pFMV CAT 20 was found to be sufficient for high expression of the reporter gene. Therefore, for 5' deletion analysis, the 3' end point was fixed at +64.

## EXAMPLE 9

The effect of 5' deletion on FMV FLt promoter function was evaluated by transient expression in protoplasts

A series of 5' deletion constructs with their 3' end at +64 with respect to the transcription start site were generated. The 5' deletion plasmid pFMV CAT 3, (-37 to +64) without a TATA box sequence, or pFMV CAT 8, (-73 to +64) with a TATA box sequence and part of an 'as-1' domain, showed no expression, suggesting dependency on additional upstream sequence elements. A similar effect has also been documented for the CaMV 35S promoter. The 5' deletion construct pFMV CAT 7, consisting of a fragment with sequence -198 to +64 from the start site containing the TATA box, the 'as-1' domain and the CCACT sequence, showed very little activity (10% of full activity), suggesting that further upstream elements are needed for promoter expression. In pFMV CAT 17 (-238 to +64), the level of expression is about 50% of the full activity shown by pFMV CAT 20 (-249 to +64). This suggests that sequence elements between -198 and -249 probably comprise a domain necessary in addition to downstream elements for maximal promoter expression. Interestingly, inclusion of the upstream sequence beyond -249 in the mutant pFMV CAT 16 (-284 to +64) reduced the promoter expression to 12% of full activity.

The construct pFEP CAT (-456 to +64) with a longer upstream segment dropped about 30% in its expression

compared to the pFMV CAT 20. The sequence between -249 and -284 may have a negative regulatory effect. On the basis of deletion analysis with the FLt promoter, the fragment -249 to +64 seems to be composed of cis-elements necessary for strong promoter activity. Consequently, this promoter fragment was tested for expression activity in transgenic plants.

Examples Heterologous Genes which may be used with FMV FLt Promoter

Plant genetic engineering techniques allow researchers to introduce heterologous genes of interest into plant cells to obtain the desired qualities. A strong constitutive promoter like FMV FLt promoter is useful to direct the any gene to be used for plant genetic engineering, a field of biotechnology which is leading a rapid progress in the production of economically valuable germplasm with improved characters or traits such as:

1. Insect resistance, (developed with Bt toxin gene,  $\alpha$ -amylase inhibitor gene).
2. Virus resistance, (developed with CP, protease or replicase gene).
3. Fungal resistance, (developed with chitinase gene, ribosome inhibiting protein gene, glucanase gene).
4. Herbicide resistance, (developed with acetolactate synthase, phosphinothricin acetyl transferase or bar gene, nitrilase gene, or 2,4-dichlorophenoxyacetate monooxygenase gene).
5. Bacterial or nematode pathogen resistance, (developed with  $\alpha$ -hordothionin gene, Bt toxin gene, beet cyst nematode resistant locus).
6. Cold or drought tolerance.
7. Improved nutritional value, (developed with seed storage protein genes).
8. Seed oil modification, (developed by controlling chain length and saturation with fatty acid synthesis genes including stearoyl-ACP desaturase, oleoyl-ACP thioesterase,  $\beta$ -ketoacyl-ACP synthase and acyl-ACP thioesterase).
9. Delayed ripening of fruits, (developed by controlling ethylene producing genes, ACC oxidase gene).
10. Male sterility.
11. Modification of carbohydrate (developed with anti-sense gene of granule bound starch synthase, branching enzyme encoding genes, glgB).
12. Protein/peptides controlling human disease (Therapeutic peptides, proteins such as RMP-7, AC137, antithrombin hirudin, growth hormone, interleukin could be produced in plant-based system) to name a few examples.

The above heterologous genes, and other heterologous genes may be inserted into plasmids pKLF and pKLF2 by genetic engineering methods known in the art. These newly created germplasms can enhance breeding programs for crop improvement, as well providing as a better understanding of gene regulation and organization in transgenic plants. Plant metabolic engineering is the application of genetic engineering methods to modify the nature of chemical metabolites in plants. For metabolic engineering where multiple genes need to be inserted into one cell, the use of different, strong, constitutive promoters is desirable in order to avoid genetic instability caused by recombination between identical or closely related promoter sequences taken from plants themselves. Through use of the promoter sequences of the invention the introduced genes can be transcribed to messenger RNA and then RNA translated to resultant proteins

that exhibit new traits or characters. The invention described herein, in developing gene vectors with newly defined promoters of the caulimoviruses, advances this effort.

A wide variety of well-characterized genes of animal, human, bacterial and of plant origin, including those of several viruses, are available for engineering plants. For the most effective expression of this wide selection of genes either constitutive or regulated, versatile gene expression vectors are required.

#### DETAILED DESCRIPTION OF THE FIGURES

FIG. 1 show the DNA sequence of the full length transcript promoter and the 3' leader sequence and the 5' portion of gene VII from the figwort mosaic virus (FMV) strain DxS (Richins et al., 1987). The nucleotide sequence (FMV coordinates 6481 to 7680, a 1200 bp fragment) includes the 3' end of gene VI, part of the large intergenic region, presented in the 5' to 3' direction of the transcript from left to right. The TATA box, CCACT box and poly A signal sequence (AATAA) are shown in bold. The transcription initiation site for the full length FMV transcript is indicated as +1, (position 6939 in the FMV genome). Repeat sequence domains (Ia, Ib to 11a, 11b as indicated, listed in Table 2) are under lined or overlined. These sequence motif may be important for the promoter activity.

FIG. 2 shows construction strategy of FMV FLt promoter with its single and double enhancer domains. Number in parenthesis indicate nucleotide position in the FMV genome.

FIG. 3 shows a physical map of pKYLX71.

FIG. 4 shows the construction strategy and physical map of pKLF.

FIG. 5 shows the construction strategy and physical map of pKLF2.

FIGS. 6 A and B. show a schematic representation of chimeric GUS or CAT constructs used for assaying promoter activity in transient expression analysis (A) and transgenic plants (B). The identity of the respective promoter is shown for each plasmid. GUS represents the gene for  $\beta$ -glucuronidase of *E. coli.*, CAT represents bacterial chloramphenicol acetyl transferase gene. The position XhoI (X), SacI (S), BamHI (B), EcoRI (E), HindIII (H), Cla I (C), SphI (Sp) used to assemble these plasmids are given. The position of the left and right T-DNA borders (LB and RB respectively) the rbcS polyadenylation signal (3' REGION) and the Kmr gene are illustrated. NT3' or RT3' represent the polyadenylation sequences from NOS or RbcS gene respectively.

FIG. 7 shows a FMV FLt promoter activity in transgenic plants expressing a CAT reporter gene: Comparison of the modified FMV FLt promoter activity in transgenic plants *Nicotiana tabacum* cv Samsun NN (R0 progeny) expressing a CAT reporter gene. Promoter activity was analyzed in independent lines developed with construct #109-CAT (A) with a FMV FLt-promoter single enhancer domain or construct #111-CAT (B) with FMV FLt2 promoter containing a duplicated enhancer domain. The plasmids carrying these constructs are pKLF-CAT and pKLF2-CAT respectively. CAT activity was determined in tissue extracts (5  $\mu$ g of total soluble protein) from fully expanded leaves. Enzyme activity (CAT %) is expressed as percent conversion of chloramphenicol (Cm) to acetylated chloramphenicol (Ac-Cm). Lanes with control untransformed tobacco leaf extract (C) from Samsun NN and transformed control (Tc) with the GUS reporter gene from transgenic tobacco leaf developed with construct #112-GUS.

FIG. 8. shows a comparison of the wild type and modified FMV FLt promoter activity in transgenic *Nicotiana tabacum* cv Samsun NN (R0 progeny) expressing a GUS reporter gene. Relative GUS activity of independent lines #1, 3, 4, 5, 6, 7 from transformation with construct #102-GUS (wild type FMV FLt promoter with a single enhancer domain-GUS); and independent lines #1, 2, 3, 6, 7, 8, 11, 12, 13 generated for construct #110-GUS (modified FMV FLt promoter and with a single enhancer domain-GUS) independent lines #1, 11, 12, 13, 14, 16, 17, and 18 from transformation with construct #112-GUS (FMV FLt promoter with a double enhancer-GUS). Soluble protein extract (5 µg) from fully expanded leaves of transformed lines were used for the GUS assays.

FIG. 9 shows a histochemical localization of GUS activity in developing transgenic tobacco (A to I) and *Datura innoxia* (J to O) plants containing the GUS reporter gene directed by the FLt promoter.

- A. Transgenic tobacco seedling (X10), (pKLF2 CAT #9, R1 progeny) with CAT gene; no GUS activity was detected.
- B. Seedling (X10), (pKLF2-GUS#12, R1 progeny) at 10 DAI; GUS activity was localized in the roots, root hairs, leaves, stems and apical meristematic region.
- C. Close up view of young leaf (X40) from 10 day old seedling (pKLF2 GUS, R1 progeny); more activity in veins.
- D. Mature leaf section (X5) from six week old plants (pKLF2GUS, R1 progeny); more GUS staining in midrib and veins.
- E to G. Roots from six week old seedlings (pKLF 2-GUS #12), Longitudinal section of a matured root (X40) stained for 2 hrs (E), prolonged staining for 18 hrs (F), and root tip (G); staining in the root was most intense at the tip (X20), vascular tissue and in root hairs.
- H. Transverse stem section (X20) from a seedling (pKLF2GUS #12, R1 progeny) at 14 DAI; GUS activity was localized at vascular (v) and epidermis (e) regions.
- I. Trichomes (X25), most intense GUS activity localized at head cells.
- J. Transgenic *Datura innoxia* seedlings (X25), (pKYLX20GUS, R1 progeny) at 12 DAI, grown axenically on filter paper. GUS activity is localized primarily in the root (root tip & hairs) and in the lower hypocotyl.
- K. and L. Roots (X25) from 4 week old *Datura innoxia* seedlings (pKYLX20GUS, R1 progeny) grown in greenhouse, longitudinal section (K) and cross-section (L); GUS activity was more intense in the vascular tissue.
- M to O. Transverse section of petiole (X25), (M); stem (X25), (N); and midrib (X25), (O) from 4 weeks old *Datura innoxia* seedlings (pKYLXF20GUS, R1 progeny) grown in greenhouse. GUS staining was more intense in the vascular system. Legend: a, apical meristem; e, epidermis; h, hypocotyl; v, vascular tissue; r, root; rc, root cap.

FIG. 10 shows FMV FLt promoter activity in different flower organs. Transgenic tobacco flowers were sampled one day before anthesis. GUS activity was measured in extracts from each type of tissue as indicated in figure.

FIG. 11 shows transient expression analysis of 3' and 5' deletion plasmids of the FMV FLt promoter. Downstream deletion end points of each plasmid are indicated in parenthesis. CAT activities are presented as percentage activity of

the 3' deletion construct pFMV20CAT. Each construct was assayed at least four times; variation was within 10% of presented value. Cm=chloramphenicol and Ac-Cm=acetylated chloramphenicol.

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- The purpose of the above description and examples is to illustrate some embodiments of the present invention without implying any limitation. It will be apparent to those of skill in the art that various modifications and variations may be made to the composition and method of the present invention without departing from the spirit or scope of the invention. All patents and publications cited herein are incorporated by reference in their entireties.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 2



-continued

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 10 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTGGAAAAG

10

## (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1199 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGAATTCCTCA GTCCAAAGCC TCAACAAGGT CAGGGTACAG AGTCTCCAAA CCATTAGCCA 60  
 AAAGCTACAG GAGATCAATG AAGAATCTTC AATCAAAGTA AACTACTGTT CCAGCACATG 120  
 CATCATGGTC AGTAAGTTTC AGAAAAGAC ATCCACCGAA GACTTAAAGT TAGTGGGCAT 180  
 CTTTGAAAGT AATCTTGTC ACATCGAGCA GCTGGCTTGT GGGGACCAGA CAAAAAGGA 240  
 ATGGTGACAGA ATTGTTAGGC GCACCTACCA AAAGCATCTT TGCCTTTATT GCAAAGATAA 300  
 AGCAGATTCC TCTAGTACAA GTGGGGAACA AAATAACGTG GAAAAGAGCT GTCCTGACAG 360  
 CCCACTCACT AATGCGTATG ACGAACGCAG TGACGACCAC AAAAGAATTC CCTCTATATA 420  
 AGAAGGCATT CATTCGCATT TGAAGGATCA TCAGATACTG AACCAATATT TCTCACTCTA 480  
 AGAAATTAAG AGCTTTGTAT TCTTCAATGA GAGGCTAAGA CCCTAAAGAG TTTCGAAAGA 540  
 GAAATGTAAG ATAGTAAGAG TCCTCCAGT CCGGGAGATT GTAATAAAGA GATCTTGTA 600  
 TGGATCCAAG TGCTGTAAT TTTTGGAAAA ATTGATCTAT AAAATATCA ATCTTTCTTT 660  
 AAGCTTATTC AAAGAACAAA CATACTATCT ATCATCCAAA TCCACAGAGT GACAGAGAGA 720  
 AAATGGTCTG TGTGTGTGG ATCTGAAGTA CCGCCGAGGC AGGAGGCCGT TAGGGAAAA 780  
 GGGACTGTTT TGACCGTCAA AGTATCAGGC TGGCTCTAGG AAGGAAGATG AAGATATCAG 840  
 GTATTGGTTT ATGTTCTAAA AAATAAGTAA TAAAGAAAA AGTTTATTAA AAAGAAAATT 900  
 TTATCAAGAG CAATTACAT GTCTAGAGGA TACCTAGATC TATATTACAA TAATCTTACT 960  
 TACATGTTTT ATTTCTGTAC TCTAAATTAA AAAATTGTTT AATTGTTTAT TCAAAACAAT 1020  
 GCCAGGACTA ACCCTCCAGC AAGAGTATAT ACTCTTAGCA CACCTTATTC TTCAGTACT 1080  
 CGAAGAAGTC AAGCAGGTAC AACTGCATTC AGGAGACTTC CAGTCTCTCA GAAGTCTATA 1140  
 TGCTAGGCTT ACGGGCTTCG GTCACACCAA GCTCATCTCC AAGCGAGAAT TTCAGCTGT 1199

We claim:

1. An isolated DNA molecule consisting essentially of restriction fragment Eco RI-Sma I of pUCFMV Flt103.
2. The isolated DNA molecule of claim 1 having the nucleotide sequence shown in SEQ ID NO: 2 from about residue 200 to about residue 405.
3. A plasmid comprising double enhancer regions from figwort mosaic virus, which enhancer regions have the nucleotide sequence shown in SEQ ID NO: 2 from about residue 200 to about residue 405.
4. The plasmid of claim 3, wherein the enhancer regions are contiguous.
5. A plasmid selected from the group consisting of pUCFMV Flt10, pUCFMV Ft101, pUCFMV Flt102, and pUCFMV Flt103.
6. An expression vector comprising a selectable marker gene, a promoter from figwort mosaic virus having a double enhancer region defined by Eco RI-Hind III restriction fragment from pUCFMV Flt10, and a multiple cloning site 3' to the promoter.

7. The expression vector of claim 6, wherein said enhancer region has the nucleotide sequence shown in SEQ ID NO: 2 from about residue 200 to about residue 405.
8. The expression vector of claim 6, further comprising a nucleotide sequence heterologous to the promoter, which is operably linked to said promoter.
9. The expression vector of claim 6, further comprising flanking left and right T-DNA border regions of *Agrobacterium tumefaciens*.
10. An expression vector selected from the group consisting of pKLF and pKLF2.
11. An expression vector comprising a selectable marker gene, a promoter region from figwort mosaic virus consisting essentially of the nucleotide sequence shown in SEQ ID NO: 2 from about residue 210 to about residue 522, and a multiple cloning site 3' to the promoter region.
12. The expression vector of claim 11, wherein the promoter region is that of pFMV CAT 20.

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