UNITED STATES DISTRICT COURT
SOUTHERN DISTRICT OF IOWA
CENTRAL DIVISION

MONSANTO COMPANY **Plaintiff**	)	
v.	)	**CASE NO.: 05-597**
TERRY LEE, BARRY LEE, HAROLD LEE, and LEE & SONS FARMS, **Defendants**	)	

COMPLAINT AND JURY DEMAND

COMES NOW the Plaintiff, Monsanto Company, through undersigned counsel, and for its Complaint at law against Terry Lee, Barry Lee, Harold Lee, and Lee & Sons Farms (hereinafter "Defendants") makes the following allegations:

THE PLAINTIFF

1. Monsanto is a company organized and existing under the laws of the State of Delaware with its principal place of business in St. Louis, Missouri. It is authorized to do and is doing business in Arkansas and this judicial district.

THE DEFENDANTS

2. Defendant Terry Lee, is an individual who has attained the age of majority and is a resident and domiciliary of Keokuk County, Iowa.

3. Defendant Barry Lee, is an individual who has attained the age of majority and is a resident and domiciliary of Keokuk County, Iowa.

4. Defendant Harold Lee, is an individual who has attained the age of majority and is a resident and domiciliary of Keokuk County, Iowa.

5. Upon information and belief, Lee & Sons Farms is a business entity organized and existing under the laws of the state of Iowa with its principle place of business in Keokuk County, Iowa.

JURISDICTION AND VENUE

6. Subject matter jurisdiction is conferred upon this court pursuant to 28 U.S.C. §1331, in that one or more of Monsanto's claims arise under the laws of the United States, as well as 28 U.S.C. §1338, granting district courts original jurisdiction over any civil action regarding patents.

7. Venue is proper in this district pursuant to 28 U.S.C. §1400 as the Defendants reside in this judicial district and a substantial number of the events giving rise to Monsanto's claims of patent infringement occurred within this judicial district.

GENERAL ALLEGATIONS

8. Monsanto is in the business of developing, manufacturing, licensing, and selling agricultural biotechnology, agricultural chemicals, and agricultural products. After the investment of substantial time, expense, and expertise, Monsanto developed a plant biotechnology that involves the transfer of a gene into crop seed that causes the plant to be resistant to glyphosate-based herbicides such as Roundup Ultra®[1], Roundup UltraMAX®[2], Roundup WeatherMAX®[3], and Touchdown®[4].

9. This new biotechnology has been utilized by Monsanto in soybeans. The genetically

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[1] Roundup Ultra® is a registered trademark of Monsanto Company.

[2] Roundup UltraMAX® is a registered trademark of Monsanto Company.

[3] Roundup WeatherMAX® is a registered trademark of Monsanto Company.

[4] Touchdown® is a registered trademark of Syngenta.

improved soybeans are marketed by Monsanto as Roundup Ready®[5] soybeans.

10. The Roundup® family of herbicides are non-selective, glyphosate-based herbicides manufactured by Monsanto, which will cause severe injury or death to soybean varieties that do not contain the Roundup Ready® technology.

11. Monsanto's Roundup Ready® seed technology is protected under United States Patent Number 5,352,605, which is attached hereto as Exhibit "A". The 5,352,605 patent was issued and assigned to Monsanto prior to the events giving rise to this action.

12. Since the Roundup Ready® technology was commercially introduced, Monsanto has labeled all bags of Roundup Ready® soybeans sold in the United States with the required statutory notice that its Roundup Ready® technology was patented. In particular, each bag of Roundup Ready® soybean seed sold in the United States was, and continues to be, marked with notice of United States Patent Number 5,352,605.

13. Monsanto licenses the use of Roundup Ready® seed technology to soybean producers at the retail marketing level through a limited use license commonly referred to as a Technology Agreement, or a limited use invoice license.

14. Among other things, the express terms of the limited use license prohibits licenses from saving harvested Roundup Ready® soybeans for planting purposes, or from selling, transferring or supplying saved Roundup Ready® soybeans to others for planting. The use of the seed is limited to the production of a single commercial crop.

15. Monsanto does not authorize the planting of saved (commonly referred to as bin run and/or brown bag) Roundup Ready® soybeans.

16. Defendants farm land in Keokuk County, Iowa, upon which they produce soybeans.

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[5] Roundup Ready® is a registered trademark of Monsanto Company.

17. Defendants planted Roundup Ready® soybeans in the 2005 growing season.

18. In late summer and early fall of 2005, Monsanto requested information about the Defendants' 2005 soybean farming operations, and particularly concerning their use of Roundup Ready® soybeans. The information sought included the number of acres planted, the source of the soybean seed planted, and the Defendants' permission to inspect their 2005 records and soybean fields.

19. The purpose of asking the Defendants for this information was to determine the extent of their use of Roundup Ready® soybeans and to ascertain whether they had planted saved Roundup Ready® soybeans. This information can only be obtained from the growers and by an inspection and sampling of the soybean fields.

20. Defendants refused to speak with investigators concerning their soybean production operation. Defendant Terry Lee did agree with outside counsel for Monsanto to provide authorization for the inspection of the Defendants' 2005 soybean records, but he has never responded with written authorization.

21. Upon information and belief, the Defendants have planted and used saved Roundup Ready® soybean seed during 2005 in contravention of Monsanto's patent rights.

22. Upon information and belief, the Defendants have knowingly, willfully and intentionally planted and used saved Roundup Ready® soybeans without authorization from Monsanto in violation of Monsanto's patent rights.

COUNT ONE-PATENT INFRINGEMENT-Patent No. 5,352,605

23. Each and every allegation set forth in the above-numbered paragraphs is hereby incorporated by reference just as if it was explicitly set forth hereunder.

24. On October 4, 1994, United States Patent Number 5,352,605 was duly and legally issued to Monsanto for an invention in Chimeric Genes for Transforming Plant Cells Using Viral Promoters, and since that date, Monsanto has been the owner of this patent. This invention is in the fields of genetic engineering and plant biology.

25. Monsanto placed the required statutory notice that its Roundup Ready® technology was protected by United States Patent Number 5,352,605 on the labeling of all bags containing Roundup Ready® soybean seed in compliance with 35 U.S.C. §287.

26. Defendants' conduct, as set forth above, constitutes the unauthorized use of a patented invention within the United States during the term of Patent Number 5,352,605, all in violation of 35 U.S.C. § 271. Accordingly, Monsanto has a right of civil action against the Defendants pursuant to 35 U.S.C. §281.

27. Upon information and belief, the Defendants have and may be continuing to infringe Monsanto's patent by making, using, offering for sale, selling, or otherwise transferring Roundup Ready® soybean seed embodying the patented invention without authorization from Monsanto, and will continue to do so unless enjoined by this court.

28. Pursuant to 35 U.S.C. §283, Monsanto is entitled to injunctive relief in accordance with the principles of equity to prevent the infringement of rights secured by its patents.

29. Pursuant to 35 U.S.C. §284, Monsanto is entitled to damages adequate to compensate for the infringement, although in no event less than a reasonable royalty, together with interest and costs to be taxed to the infringer. Further, damages should be trebled pursuant to 35 U.S.C. §284 in light of the Defendants' knowing, willful, conscious, and deliberate infringement of the patent rights at issue.

30. The infringing activity of the Defendants brings this cause within the ambit of the exceptional case contemplated by 35 U.S.C. §285, thus Monsanto requests the award of reasonable attorneys fees and costs.

31. Monsanto demands trial by jury.

WHEREFORE, Monsanto Company prays that process and due form of law issue to the Defendants, Terry Lee, Barry Lee, Harold Lee and Lee & Sons Farm, requiring them to appear and answer, all and singular, the allegations of this complaint, and that after due proceedings are had, there be judgment in favor of Monsanto Company and against the Defendants, providing the following remedies to Monsanto:

1. Entry of judgment for damages, together with interest and costs, to compensate Monsanto for the Defendants' patent infringement;

2. Trebling of damages awarded for the infringement of patents together with reasonable attorney's fees;

3. Entry of an order prohibiting the Defendants from planting, transferring, or selling the infringing articles to a third party;

4. Entry of a permanent injunction against the Defendants to prevent them from using, saving, cleaning, or planting any of Monsanto's proprietary seed technologies, without express written permission from Monsanto;

5. Entry of judgment for costs, expenses, and reasonable attorney's fees incurred by Monsanto; and

6. Such other relief as the Court may deem appropriate.

Respectfully submitted,

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US005352605A

United States Patent [19]

Fraley et al.

[11] Patent Number: 5,352,605

[45] Date of Patent: Oct. 4, 1994

[54] CHIMERIC GENES FOR TRANSFORMING PLANT CELLS USING VIRAL PROMOTERS

[75] Inventors: Robert T. Fraley, Ballwin; Robert B. Horsch; Stephen G. Rogers, both of St. Louis, all of Mo.

[73] Assignee: Monsanto Company, St. Louis, Mo.

[21] Appl. No.: 146,621

[22] Filed: Oct. 28, 1993

Related U.S. Application Data 03

[63] Continuation of Ser. No. 625,637, Dec. 7, 1990, abandoned, which is a continuation of Ser. No. 931,492, Nov. 17, 1986, abandoned, which is a continuation-in-part of Ser. No. 485,568, Apr. 15, 1983, abandoned, which is a continuation-in-part of Ser. No. 458,414, Jan. 17, 1983, abandoned.

[51] Int. Cl.^{5} C12N 5/00; C12N 15/00; C07H 21/04

[52] U.S. Cl. 435/240.4; 435/172.3; 435/320.1; 536/23.2; 536/24.1

[58] Field of Search 536/23.2, 24.1; 435/172.3, 240.4, 320.1; 800/205

[56] References Cited

U.S. PATENT DOCUMENTS

4,536,475	8/1985	Anderson	435/172.3
5,034,322	7/1991	Rogers et al.	435/172.3

FOREIGN PATENT DOCUMENTS

0116718B1	5/1990	European Pat. Off.	C12N 15/00
0116718	5/1990	European Pat. Off.	C12N 15/00
0131623	3/1991	European Pat. Off.	C12N 15/11
0142924	4/1992	European Pat. Off.	C12N 15/05
WO82/03087	9/1982	PCT Int'l Appl.	C12N 15/00

OTHER PUBLICATIONS

Guilley et al. 1982. Cell 30(3): 763-773.
Zambryski et al. 1983. EMBO J 2(12): 2143-2150.
Goodman et al. 1987. Science 236: 48-54.
Ursic et al., *Biochemical and Biophysical Research Communications*, 101, 3, pp. 1031-1037 (1981).
Beck et al., *Gene*, 19, pp. 327-336 (1982).
Herrera-Estrela et al., *EMBO*, 6 pp. 987-995 (1983).
Maliga et al., *Molec. Gen. Genet.*, 157, pp. 291-296 (1977).
De Greve et al., *Nature*, 30, pp. 752-755 (1982).
Portetelle et al., *Annales De Gembloux*, 87, 3, pp. 101-123 (1981).
Larkins et al. 1985. J. Cell. Biochem. Suppl. 9C:264.
Barton et al. 1987. Plant Physiol. 85:1103-1109.
Berry-Lowe et al., J. Mol. & Appl. Gent., 1(6): 483-498 (1982).
Bevan et al., Nature, 304: 184-187 (1983).
Cairns et al., Febs Letters, 96(2): 295-297 (1978).
Cairns et al., PNAS, 75(11): 5557-5559 (1978).
Chilton et al., PNAS, 77: 4060-4064 (1977).
Chilton et al., Stadler Symp., 13:39-51 (1981).
Chilton et al., Nature, 295: 432-434 (1982).
Chilton et al., The Fifteenth Miami Winter Symposium, 17-21 Jan. 1983, 14-15, Ahmad et al., (1983).
Colbere-Gerapin et al, J. Mol. Biol., 150: 1-14 (1981).
Condit et al., Miami Winter Symposium, Jan. 17-21, p. 564 (1983).
Davey et al., Transformation in plants: potential and reality—Conference paper from University of Nottingham (1982).

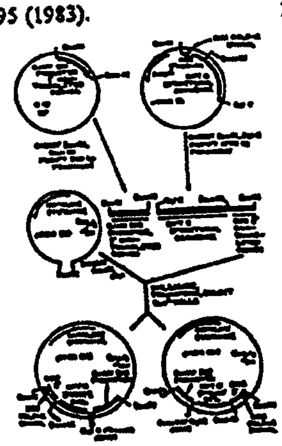
(List continued on next page.)

Primary Examiner—David T. Fox
Attorney, Agent, or Firm—Lawrence M. Lavin, Jr.; Dennis R. Hoerner, Jr.; Howard C. Stanley

[57] ABSTRACT

In one aspect the present invention relates to the use of viral promoters in the expression of chimeric genes in plant cells. In another aspect this invention relates to chimeric genes which are capable of being expressed in plant cells, which utilize promoter regions derived from viruses which are capable of infecting plant cells. One such virus comprises the cauliflower mosaic virus (CaMV). Two different promoter regions have been derived from the CaMV genome and ligated to heterologous coding sequences to form chimeric genes. These chimeric genes have been shown to be expressed in plant cells. This invention also relates to plant cells, plant tissue, and differentiated plants which contain and express the chimeric genes of this invention.

19 Claims, 10 Drawing Sheets



OTHER PUBLICATIONS

DeGreve et al., J. Mol. Appl. Genet., 1(6): 499-511 (1983).
Depicker et al., J. Mol. & Appl. Genet. 1(6): 561-573 (1982).
Depicker et al., Conference Paper, Davis, pp. 143-176 (1982).
Dix et al., Molec. Gen. Genet., 157: 285-290 (1977).
Fraley et al., PNAS, 80: 4803-4807 (1983).
Fraley et al., Miami Winter Symposia, Advances in Gene Technology: Molecular Genetics of Plants and Animals, 20: 211-221 (1983).
Franck et al., Cell, 21: 285-294 (1980).
Gardner, "Genetic Engineering of Plants-An Agricultural Perspective", Kosuge et al., (eds) pp. 121-142 (1982).
Garfinkel et al., Cell, 27:143-153 (1981).
Gronebom et al., Nature, 294: 773-776 (1981).
Hernalsteens et al., Nature, 287: 654-656 (1980).
Herrera-Estrella et al., Nature, 303: 209-213 (1983).
Hohn et al., "Current Topics in Microbiology and Immunology" Henle et al. (eds) vol. 96, pp. 193-236 (1982).
Holsters et al., Mol Gen Genet, 185: 283-290 (1982).
Howell et al., Science, 208: 1265-1267 (1980).
Jimenez et al., Nature, 287: 869-871 (1980).
Kemp et al., Genetic Engineering-Application to Agriculture, pp. 215-228, (1983).
Lebeurier et al., Gene, 12: 139-146 (1980).
Leemans, Universite Libre de Bruxelles, Thesis, 1-25; 114-125 (1982).
Leemans et al., J. Mol. & Appl. Genet. 1(2): 149-164 (1981).
Leemans et al., EMBO, 1(1): 147-152 (1982).
Leemans et al., "Molecular Biology of Plant Tumors" Chap. 21, pp. 537-545 (1982).
Liu et al., PNAS, 79: 2812-2816 (1982).
McKnight et al., J. of Virology, 37(2): 673-682 (1981).
Matzke et al., J. Mol. & Appl. Genet., 1: 39-49 (1981).
Meagher et al., "Genome Organization and Expression in Plants" Leaver, C. J. (ed), NATO Advance Study Institute Series, 29: 63-75 (1980).
Mulligan et al., Nature, 277: 108-114 (1979).
Mulligan et al., Science, 209: 1422-1427 (1980).
Mulligan et al., PNAS, 75(4): 2072-2076 (1981).
O'Hare et al., PNAS 78(3): 1527-1531 (1981).
Old et al., "Principles of Gene Manipulation", U. of Calif. Press, 1st ed. vol. 2 pp. 9-23 (1980).
Old et al., "Principles of Gene Manipulation", U. of Calif. Press, 2nd Ed., vol. 2 pp. 121-210 (1980).
Olszewski et al., Cell, 29: 395-402 (1982).
Otten et al., Mol Gen Genet, 183: 209-213 (1981).
Schell et al., abstract from "Broadening the Genetic Base of Crops", Harten et al. (eds) (1978).
Schell et al., abstract from "Plant Improvement and Somatic Cell Genetics" Vasil et al., (eds) (1982).
Schell et al., Biotechnology, 175-180 (1983).
Schell et al., The Fifteenth Miami Winter Symposium, 17-21 Jan. 1983, pp. 191-209. (1983).
Schroeder at al., "Plant Cell Culture in Crop Improvement", Sen et al., (eds) pp. 287-297 (1983).
Watson, "Molecular Biology of the Gene" 3rd ed., W. A. Benjamin, Inc. (publisher), pp. 482-483 (1977).
Willmitzer et al., Nature, 287: 359-361 (1980).
Wilmitzer et al., EMBO, 1(1): 139-146 (1982).
Colbere-Garapin et al., "A New Dominant Hybrid Selective Marker for Higher Eukaryotic Cells," *J. Mol. Biol.* (1981) vol. 150, pp. 1-14.
Guilley et al., "Transcription of Cauliflower Mosaic Virus DNA: Detection of Promoter Sequences, and Characterization of Transcripts," *Cell* (1982) vol. 30, pp. 763-773.
Condit et al., Miami Winter Symposiumm Jan. 17-21, 1983, Abstract: "Multiple Viral Specific Transcripts from the Genome of Cauliflower Mosaic Virus".
Howell et al., "Cloned Cauliflower Mosaic Virus DNA Infects Turnips (*Brassica rapa*)" (1980) *Science*, vol. 208, pp. 1265-1267.
McKnight et al., "Isolation and Mapping of Small Cauliflower Mosaic Virus DNA Fragments Active as Promoters in *Escherichia coli*" (1981) *Journal of Virology* vol. 37, No. 2, pp. 673-682 (Abstract Only).
Gardner, R. C., "Plant Viral Vectors: CaMV as an Experimental Tool," Genetic Engineering of Plants, an Agricultural Perspective, Proceedings of a Symposium held Aug. 15-19, 1982 at the University of California, Davis, Calif., Kusuge et al., Ed., pp. 124-125, 128 and 138.
Leemans et al., "Ti Plasmids and Directed Genetic Engineering" (1982) *Molecular Biology of Plant Tumors*, pp. 537-545.
Hohn et al, "Cauliflower Mosaic Virus on Its Way to Becoming a Useful Plant Vector" (1982) *Current Topics in Microbiology and Immunology* vol. 96, pp. 193-236.
Lebeurier et al, "Infectivities of Native and Cloned DNA of Cauliflower Mosaic Virus" (1980) *Gene*, vol. 12, pp. 139-146.
Davey et al., Conference paper from University of Nottingham (1982) Derwent Abstract 028990, DBA Accession No: 84-12265.

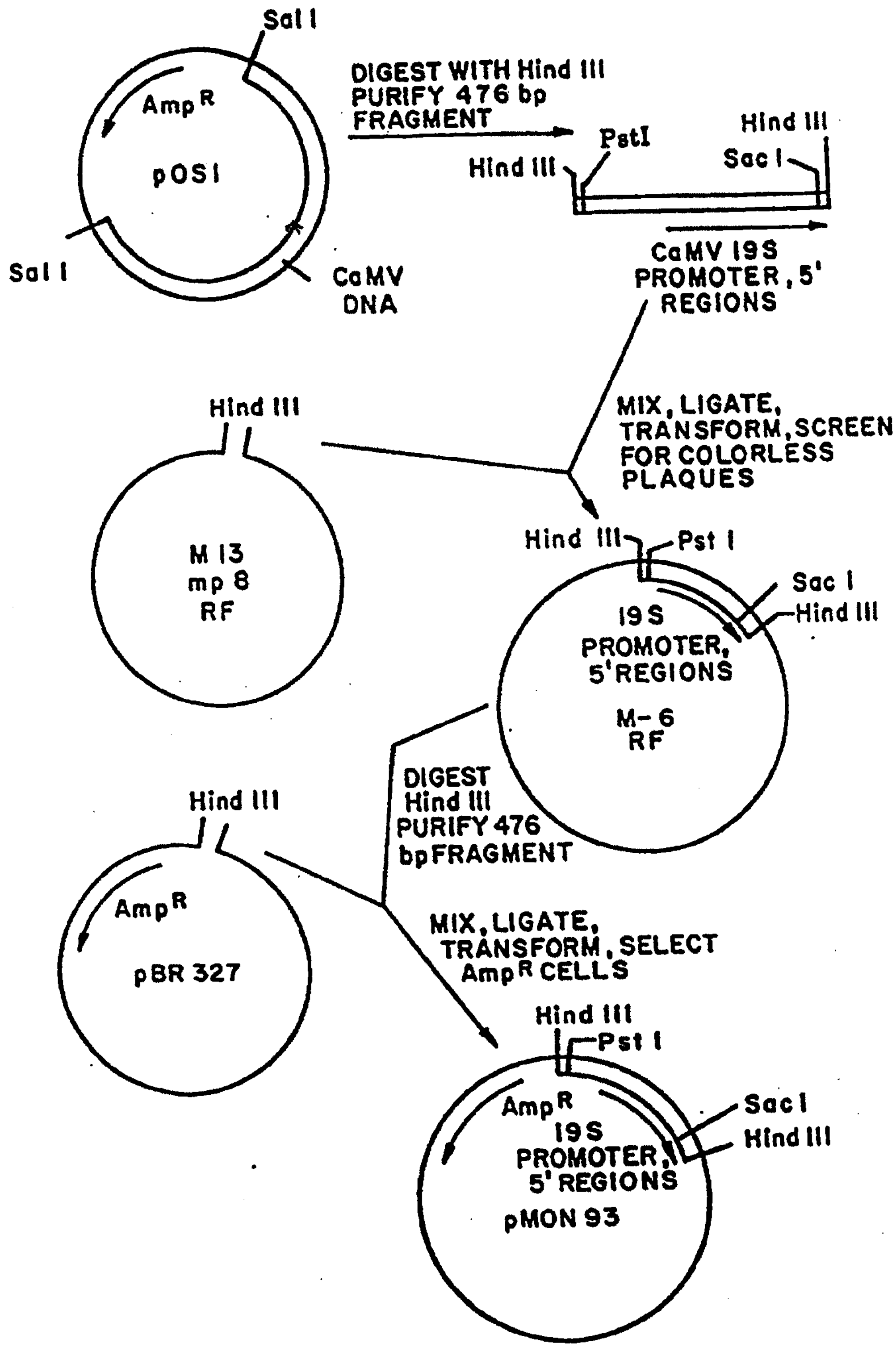

Sal I
Amp R
pOS1
Sal I
CaMV DNA
DIGEST WITH Hind III PURIFY 476 bp FRAGMENT
PstI
Hind III
Hind III
Sac I
CaMV 19S PROMOTER, 5' REGIONS
MIX, LIGATE, TRANSFORM, SCREEN FOR COLORLESS PLAQUES
Hind III
M 13 mp 8 RF
Hind III
Pst I
Sac I
Hind III
19 S PROMOTER, 5'REGIONS
M-6 RF
DIGEST Hind III PURIFY 476 bp FRAGMENT
Hind III
Amp R
pBR 327
MIX, LIGATE, TRANSFORM, SELECT Amp R CELLS
Hind III
Pst I
Amp R
19 S PROMOTER, 5' REGIONS
Sac I
Hind III
pMON 93

Figure 1

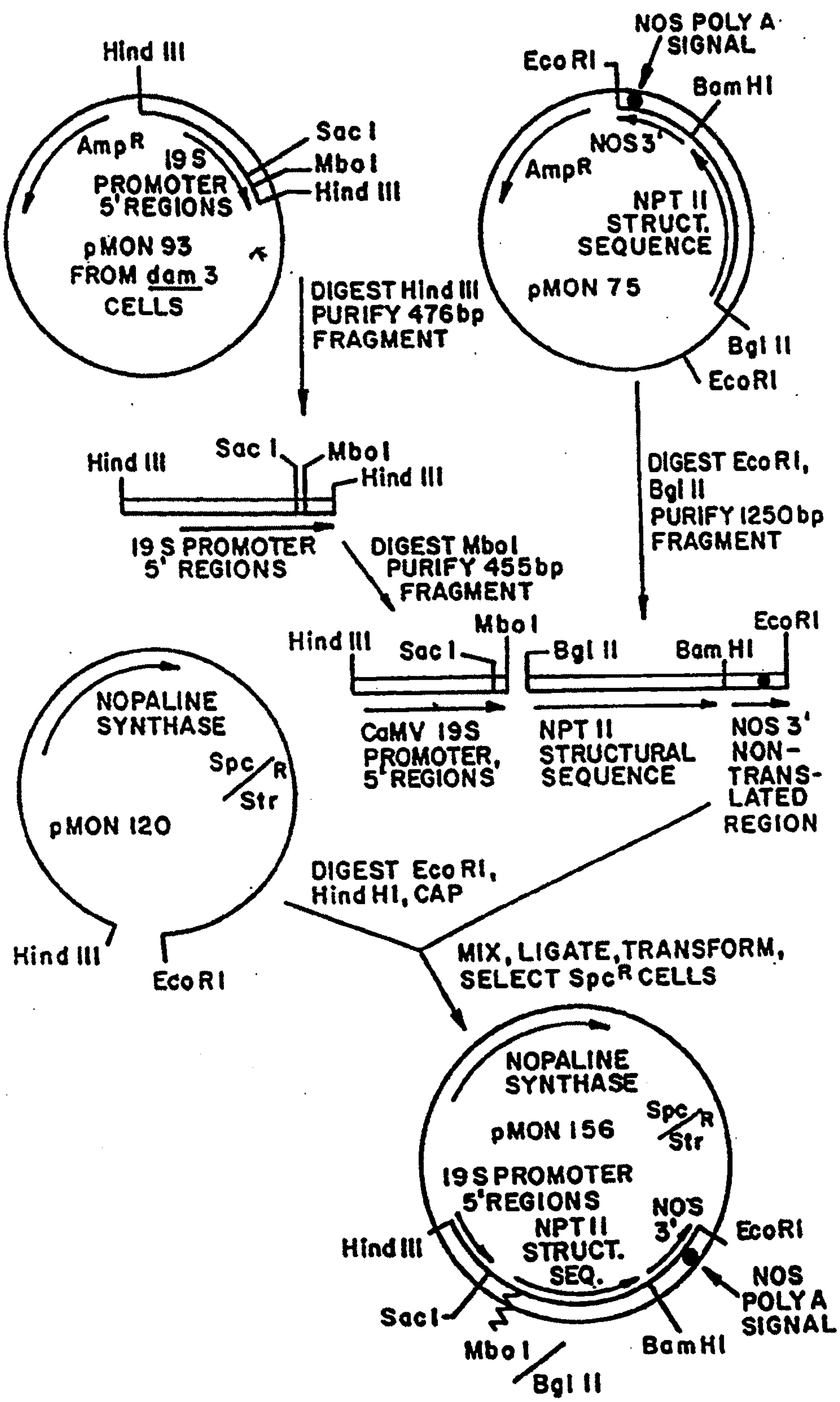

Hind III
AmpR
19S PROMOTER 5'REGIONS
Sac I
Mbo I
Hind III
pMON 93 FROM dam 3 CELLS
DIGEST Hind III PURIFY 476bp FRAGMENT
NOS POLY A SIGNAL
EcoRI
BamHI
NOS 3'
AmpR
NPT II STRUCT. SEQUENCE
pMON 75
Bgl II
EcoRI
DIGEST EcoRI, Bgl II PURIFY 1250bp FRAGMENT
Hind III
Sac I
MboI
Hind III
19 S PROMOTER 5' REGIONS
DIGEST MboI PURIFY 455bp FRAGMENT
Hind III
Sac I
Mbo I
Bgl II
Bam HI
EcoRI
CaMV 19S PROMOTER, 5' REGIONS
NPT II STRUCTURAL SEQUENCE
NOS 3' NON-TRANSLATED REGION
NOPALINE SYNTHASE
Spc/Str R
pMON 120
Hind III
EcoRI
DIGEST EcoRI, Hind HI, CAP
MIX, LIGATE, TRANSFORM, SELECT SpcR CELLS
NOPALINE SYNTHASE
pMON 156
Spc/Str R
19S PROMOTER 5'REGIONS
NPT II STRUCT. SEQ.
NOS 3'
EcoRI
NOS POLY A SIGNAL
Hind III
Sac I
Mbo I/Bgl II
BamHI

Figure 2

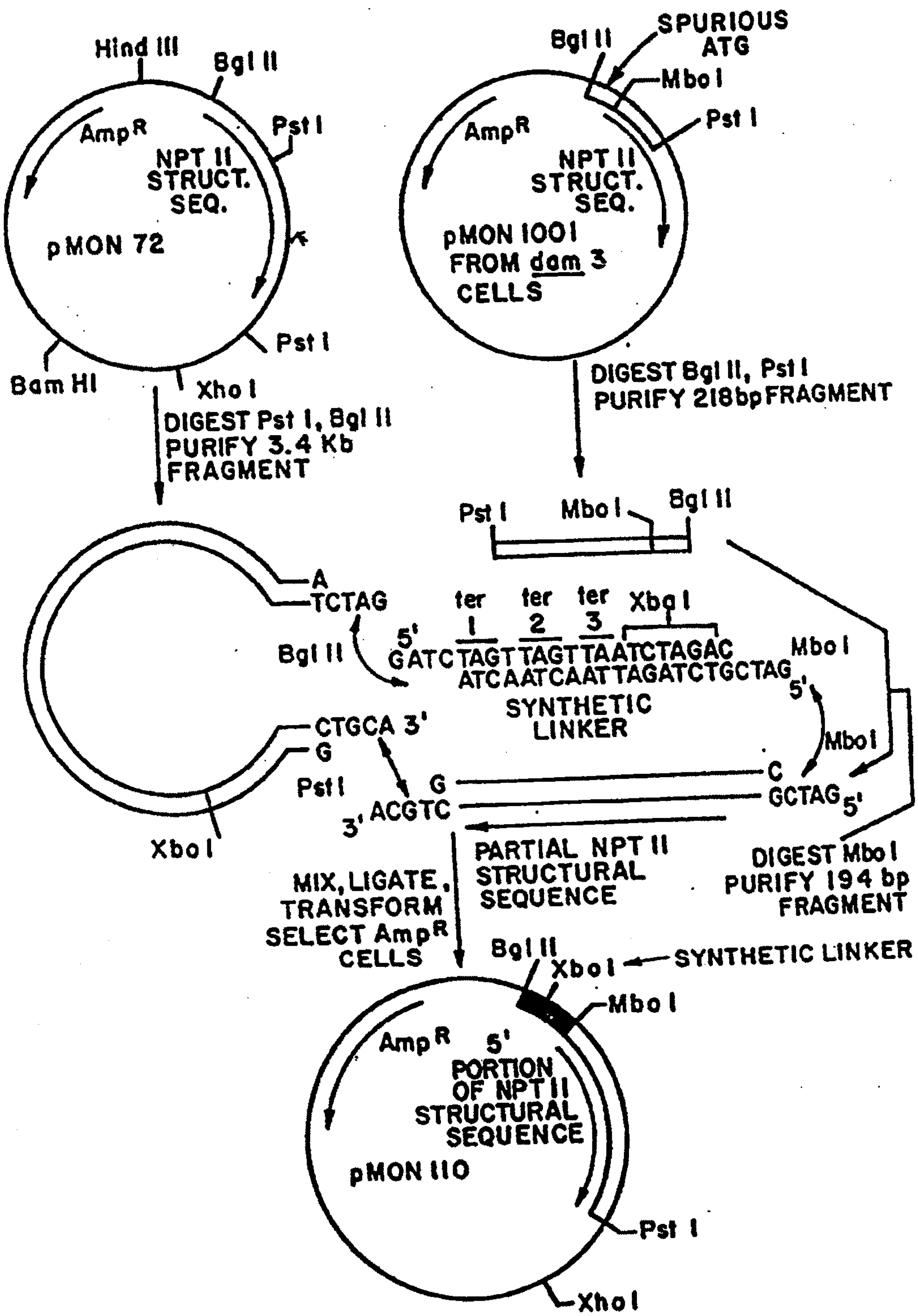

Hind III
Bgl II
Pst I
AmpR
NPT II STRUCT. SEQ.
pMON 72
Pst I
Bam HI
Xho I
SPURIOUS ATG
Bgl II
Mbo I
Pst I
AmpR
NPT II STRUCT. SEQ.
pMON 1001 FROM dam 3 CELLS
DIGEST Pst I, Bgl II
PURIFY 3.4 Kb FRAGMENT
DIGEST Bgl II, Pst I
PURIFY 218bp FRAGMENT
Pst I
Mbo I
Bgl II
A
TCTAG
Bgl II
ter 1
ter 2
ter 3
Xba I
5'
GATCTAGTTAGTTAATCTAGAC
ATCAATCAATTAGATCTGCTAG
5'
Mbo I
SYNTHETIC LINKER
CTGCA 3'
G
Pst I
G
3' ACGTC
C
GCTAG 5'
Mbo I
Xbo I
PARTIAL NPT II STRUCTURAL SEQUENCE
DIGEST Mbo I
PURIFY 194 bp FRAGMENT
MIX, LIGATE, TRANSFORM SELECT AmpR CELLS
Bgl II
Xba I
SYNTHETIC LINKER
Mbo I
AmpR
5' PORTION OF NPT II STRUCTURAL SEQUENCE
pMON 110
Pst I
Xho I

Figure 3

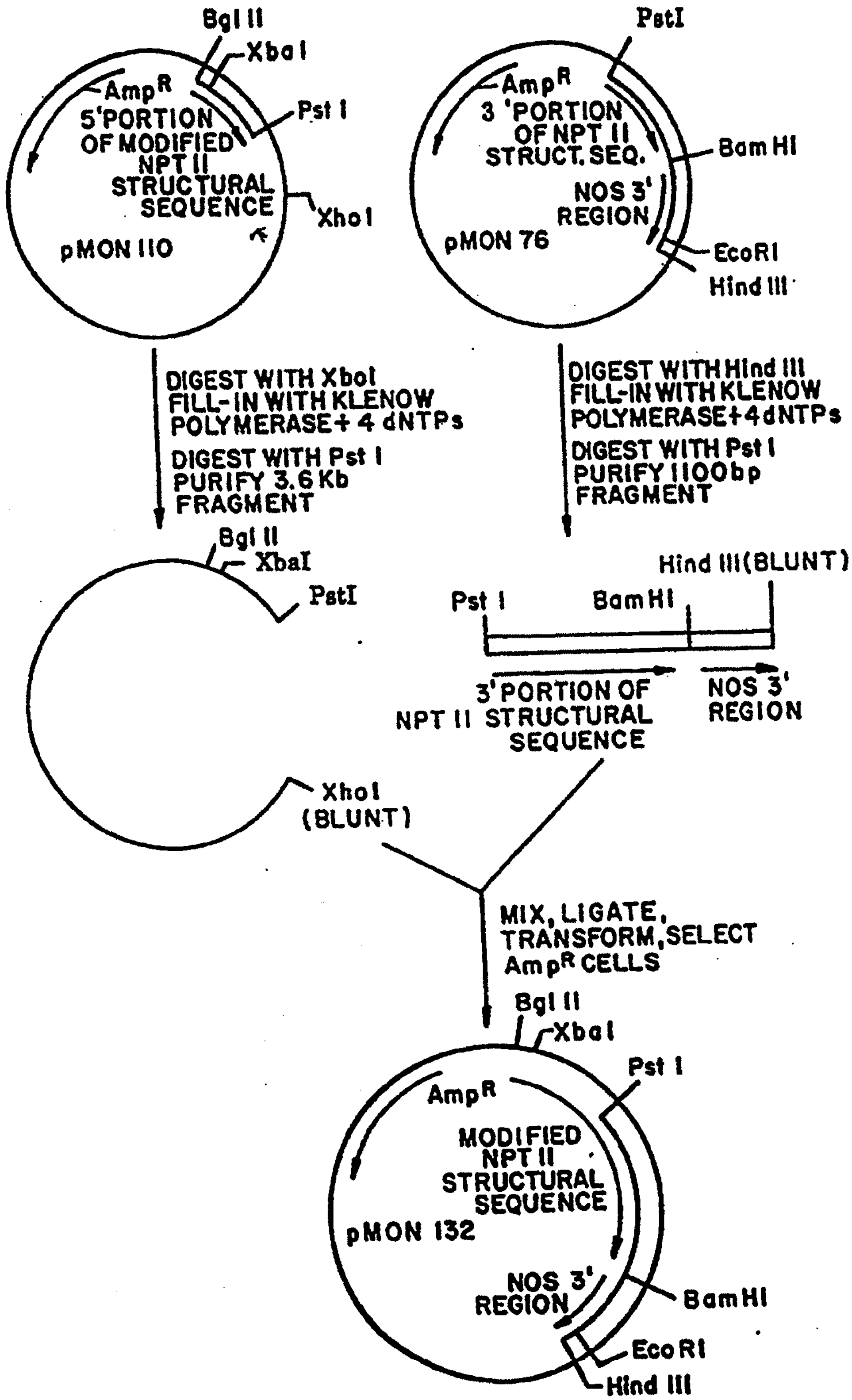

Bgl II
Xba I
Pst I
AmpR
5'PORTION OF MODIFIED NPT II STRUCTURAL SEQUENCE
Xho I
pMON 110
PstI
AmpR
3 'PORTION OF NPT II STRUCT. SEQ.
Bam HI
NOS 3' REGION
EcoRI
Hind III
pMON 76
DIGEST WITH XboI FILL-IN WITH KLENOW POLYMERASE+ 4 dNTPs
DIGEST WITH Pst I PURIFY 3.6 Kb FRAGMENT
DIGEST WITH Hind III FILL-IN WITH KLENOW POLYMERASE+4dNTPs
DIGEST WITH Pst I PURIFY 1100bp FRAGMENT
Bgl II
XbaI
PstI
XhoI (BLUNT)
Hind III(BLUNT)
Pst I
Bam HI
3'PORTION OF NPT II STRUCTURAL SEQUENCE
NOS 3' REGION
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
Bgl II
Xba I
Pst I
AmpR
MODIFIED NPT II STRUCTURAL SEQUENCE
pMON 132
NOS 3' REGION
BamHI
Eco RI
Hind III

Figure 4

US005352605A

United States Patent [19]

Fraley et al.

[11] Patent Number: 5,352,605

[45] Date of Patent: Oct. 4, 1994

[54] CHIMERIC GENES FOR TRANSFORMING PLANT CELLS USING VIRAL PROMOTERS

[75] Inventors: Robert T. Fraley, Ballwin; Robert B. Horsch; Stephen G. Rogers, both of St. Louis, all of Mo.

[73] Assignee: Monsanto Company, St. Louis, Mo.

[21] Appl. No.: 146,621

[22] Filed: Oct. 28, 1993

Related U.S. Application Data 03

[63] Continuation of Ser. No. 625,637, Dec. 7, 1990, abandoned, which is a continuation of Ser. No. 931,492, Nov. 17, 1986, abandoned, which is a continuation-in-part of Ser. No. 485,568, Apr. 15, 1983, abandoned, which is a continuation-in-part of Ser. No. 458,414, Jan. 17, 1983, abandoned.

[51] Int. Cl.[5] C12N 5/00; C12N 15/00; C07H 21/04

[52] U.S. Cl. 435/240.4; 435/172.3; 435/320.1; 536/23.2; 536/24.1

[58] Field of Search 536/23.2, 24.1; 435/172.3, 240.4, 320.1; 800/205

[56] References Cited

U.S. PATENT DOCUMENTS

4,536,475 8/1985 Anderson 435/172.3
5,034,322 7/1991 Rogers et al. 435/172.3

FOREIGN PATENT DOCUMENTS

0116718B1 5/1990 European Pat. Off. C12N 15/00
0116718 5/1990 European Pat. Off. C12N 15/00
0131623 3/1991 European Pat. Off. C12N 15/11
0142924 4/1992 European Pat. Off. C12N 15/05
WO82/03087 9/1982 PCT Int'l Appl. C12N 15/00

OTHER PUBLICATIONS

Guilley et al. 1982. Cell 30(3): 763–773.
Zambryski et al. 1983. EMBO J 2(12): 2143–2150.
Goodman et al. 1987. Science 236: 48–54.
Ursic et al., *Biochemical and Biophysical Research Communications*, 101, 3, pp. 1031–1037 (1981).
Beck et al., *Gene*, 19, pp. 327–336 (1982).
Herrera-Estrela et al., *EMBO*, 6 pp. 987–995 (1983).
Maliga et al., *Molec. Gen. Genet.*, 157, pp. 291–296 (1977).
De Greve et al., *Nature*, 30, pp. 752–755 (1982).
Portetelle et al., *Annales De Gembloux*, 87, 3, pp. 101–123 (1981).
Larkins et al. 1985. J. Cell. Biochem. Suppl. 9C:264.
Barton et al. 1987. Plant Physiol. 85:1103–1109.
Berry-Lowe et al., J. Mol. & Appl. Gent., 1(6): 483–498 (1982).
Bevan et al., Nature, 304: 184–187 (1983).
Cairns et al., Febs Letters, 96(2): 295–297 (1978).
Cairns et al., PNAS, 75(11): 5557–5559 (1978).
Chilton et al., PNAS, 77: 4060–4064 (1977).
Chilton et al., Stadler Symp., 13:39–51 (1981).
Chilton et al., Nature, 295: 432–434 (1982).
Chilton et al., The Fifteenth Miami Winter Symposium, 17–21 Jan. 1983, 14–15, Ahmad et al., (1983).
Colbere-Garapin et al, J. Mol. Biol., 150: 1–14 (1981).
Condit et al., Miami Winter Symposium, Jan. 17–21, p. 564 (1983).
Davey et al., Transformation in plants: potential and reality—Conference paper from University of Nottingham (1982).

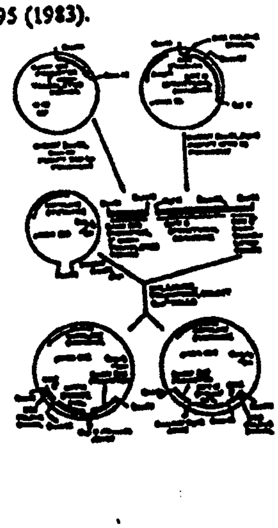
(List continued on next page.)

Primary Examiner—David T. Fox
Attorney, Agent, or Firm—Lawrence M. Lavin, Jr.; Dennis R. Hoerner, Jr.; Howard C. Stanley

[57] ABSTRACT

In one aspect the present invention relates to the use of viral promoters in the expression of chimeric genes in plant cells. In another aspect this invention relates to chimeric genes which are capable of being expressed in plant cells, which utilize promoter regions derived from viruses which are capable of infecting plant cells. One such virus comprises the cauliflower mosaic virus (CaMV). Two different promoter regions have been derived from the CaMV genome and ligated to heterologous coding sequences to form chimeric genes. These chimeric genes have been shown to be expressed in plant cells. This invention also relates to plant cells, plant tissue, and differentiated plants which contain and express the chimeric genes of this invention.

19 Claims, 10 Drawing Sheets



OTHER PUBLICATIONS

DeGreve et al., J. Mol. Appl. Genet., 1(6): 499-511 (1983).
Depicker et al., J. Mol. & Appl. Genet. 1(6): 561-573 (1982).
Depicker et al., Conference Paper, Davis, pp. 143-176 (1982).
Dix et al., Molec. Gen. Genet., 157: 285-290 (1977).
Fraley et al., PNAS, 80: 4803-4807 (1983).
Fraley et al., Miami Winter Symposia, Advances in Gene Technology: Molecular Genetics of Plants and Animals, 20: 211-221 (1983).
Franck et al., Cell, 21: 285-294 (1980).
Gardner, "Genetic Engineering of Plants-An Agricultural Perspective", Kosuge et al., (eds) pp. 121-142 (1982).
Garfinkel et al., Cell, 27:143-153 (1981).
Groneborn et al., Nature, 294: 773-776 (1981).
Hernalsteens et al., Nature, 287: 654-656 (1980).
Herrera-Estrella et al., Nature, 303: 209-213 (1983).
Hohn et al., "Current Topics in Microbiology and Immunology" Henle et al. (eds) vol. 96, pp. 193-236 (1982).
Holsters et al., Mol Gen Genet, 185: 283-290 (1982).
Howell et al., Science, 208: 1265-1267 (1980).
Jimenez et al., Nature, 287: 869-871 (1980).
Kemp et al., Genetic Engineering-Application to Agriculture, pp. 215-228, (1983).
Lebeurier et al., Gene, 12: 139-146 (1980).
Leemans, Universite Libre de Bruxelles, Thesis, 1-25; 114-125 (1982).
Leemans et al., J. Mol. & Appl. Genet. 1(2): 149-164 (1981).
Leemans et al., EMBO, 1(1): 147-152 (1982).
Leemans et al., "Molecular Biology of Plant Tumors" Chap. 21, pp. 537-545 (1982).
Liu et al., PNAS, 79: 2812-2816 (1982).
McKnight et al., J. of Virology, 37(2): 673-682 (1981).
Matzke et al., J. Mol. & Appl. Genet., 1: 39-49 (1981).
Meagher et al., "Genome Organization and Expression in Plants" Leaver, C. J. (ed), NATO Advance Study Institute Series, 29: 63-75 (1980).
Mulligan et al., Nature, 277: 108-114 (1979).
Mulligan et al., Science, 209: 1422-1427 (1980).
Mulligan et al., PNAS, 75(4): 2072-2076 (1981).
O'Hare et al., PNAS 78(3): 1527-1531 (1981).
Old et al., "Principles of Gene Manipulation", U. of Calif. Press, 1st ed. vol. 2 pp. 9-23 (1980).
Old et al., "Principles of Gene Manipulation", U. of Calif. Press, 2nd Ed., vol. 2 pp. 121-210 (1980).
Olszewski et al., Cell, 29: 395-402 (1982).
Otten et al., Mol Gen Genet, 183: 209-213 (1981).
Schell et al., abstract from "Broadening the Genetic Base of Crops", Harten et al. (eds) (1978).
Schell et al., abstract from "Plant Improvement and Somatic Cell Genetics" Vasil et al., (eds) (1982).
Schell et al., Biotechnology, 175-180 (1983).
Schell et al., The Fifteenth Miami Winter Symposium, 17-21 Jan. 1983, pp. 191-209. (1983).
Schroeder at al., "Plant Cell Culture in Crop Improvement", Sen et al., (eds) pp. 287-297 (1983).
Watson, "Molecular Biology of the Gene" 3rd ed., W. A. Benjamin, Inc. (publisher), pp. 482-483 (1977).
Willmitzer et al., Nature, 287: 359-361 (1980).
Wilmitzer et al., EMBO, 1(1): 139-146 (1982).
Colbere-Garapin et al., "A New Dominant Hybrid Selective Marker for Higher Eukaryotic Cells," *J. Mol. Biol.* (1981) vol. 150, pp. 1-14.
Guilley et al., "Transcription of Cauliflower Mosaic Virus DNA: Detection of Promoter Sequences, and Characterization of Transcripts," *Cell* (1982) vol. 30, pp. 763-773.
Condit et al., Miami Winter Symposiumm Jan. 17-21, 1983, Abstract: "Multiple Viral Specific Transcripts from the Genome of Cauliflower Mosaic Virus".
Howell et al., "Cloned Cauliflower Mosaic Virus DNA Infects Turnips (*Brassica rapa*)" (1980) *Science*, vol. 208, pp. 1265-1267.
McKnight et al., "Isolation and Mapping of Small Cauliflower Mosaic Virus DNA Fragments Active as Promoters in *Escherichia coli*" (1981) *Journal of Virology* vol. 37, No. 2, pp. 673-682 (Abstract Only).
Gardner, R. C., "Plant Viral Vectors: CaMV as an Experimental Tool," Genetic Engineering of Plants, an Agricultural Perspective, Proceedings of a Symposium held Aug. 15-19, 1982 at the University of California, Davis, Calif., Kusuge et al., Ed., pp. 124-125, 128 and 138.
Leemans et al., "Ti Plasmids and Directed Genetic Engineering" (1982) *Molecular Biology of Plant Tumors*, pp. 537-545.
Hohn et al, "Cauliflower Mosaic Virus on Its Way to Becoming a Useful Plant Vector" (1982) *Current Topics in Microbiology and Immunology* vol. 96, pp. 193-236.
Lebeurier et al., "Infectivities of Native and Cloned DNA of Cauliflower Mosaic Virus" (1980) *Gene*, vol. 12, pp. 139-146.
Davey et al., Conference paper from University of Nottingham (1982) Derwent Abstract 028990, DBA Accession No: 84-12265.

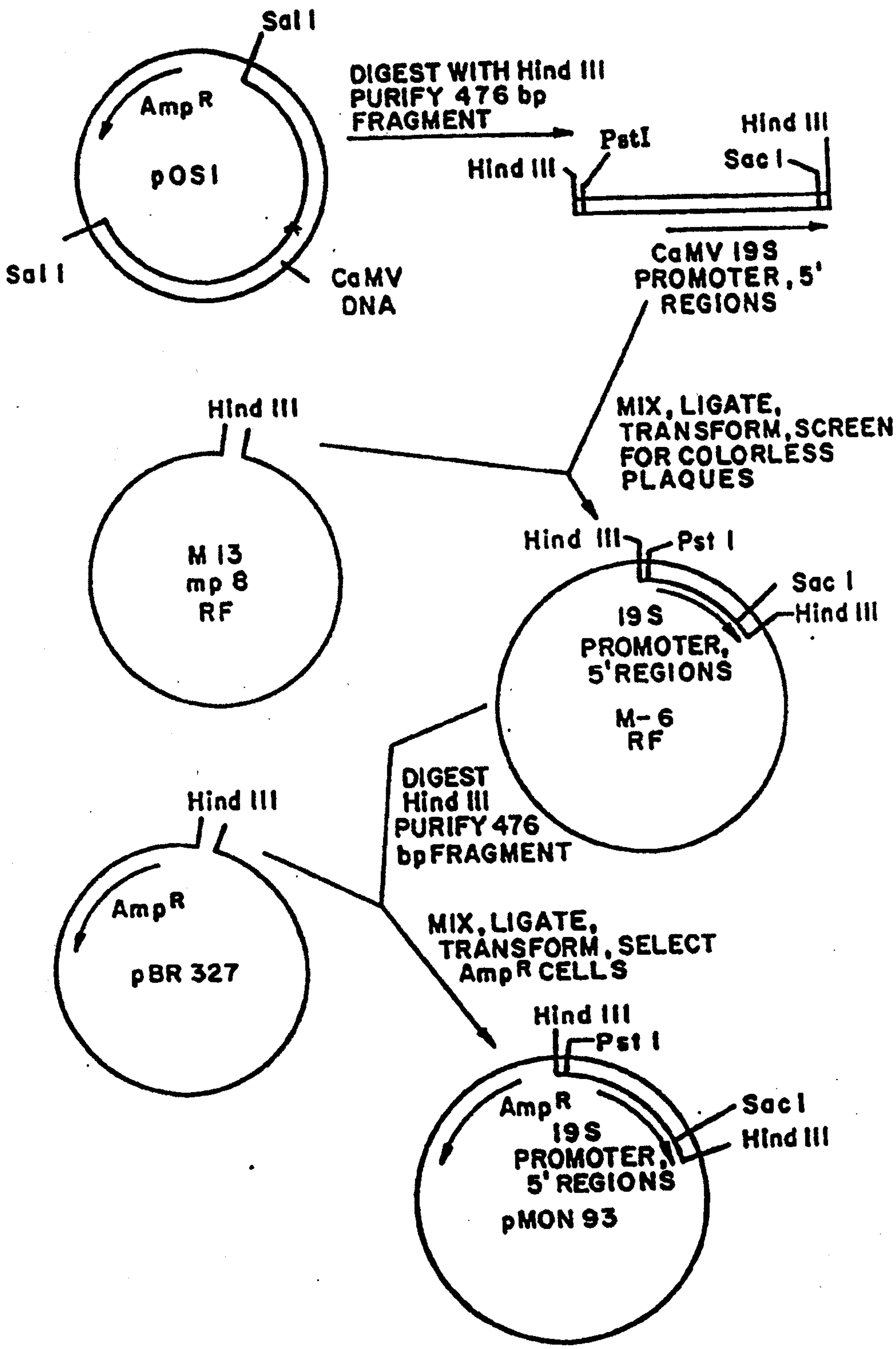

Sal I
Amp R
pOS1
Sal I
CaMV DNA
DIGEST WITH Hind III
PURIFY 476 bp
FRAGMENT
PstI
Hind III
Sac I
Hind III
CaMV 19S
PROMOTER, 5'
REGIONS
Hind III
M 13
mp 8
RF
MIX, LIGATE,
TRANSFORM, SCREEN
FOR COLORLESS
PLAQUES
Hind III
Pst I
Sac I
Hind III
19 S
PROMOTER,
5' REGIONS
M-6
RF
DIGEST
Hind III
PURIFY 476
bp FRAGMENT
Hind III
Amp R
pBR 327
MIX, LIGATE,
TRANSFORM, SELECT
Amp R CELLS
Hind III
Pst I
Amp R
19 S
PROMOTER,
5' REGIONS
Sac I
Hind III
pMON 93

Figure 1

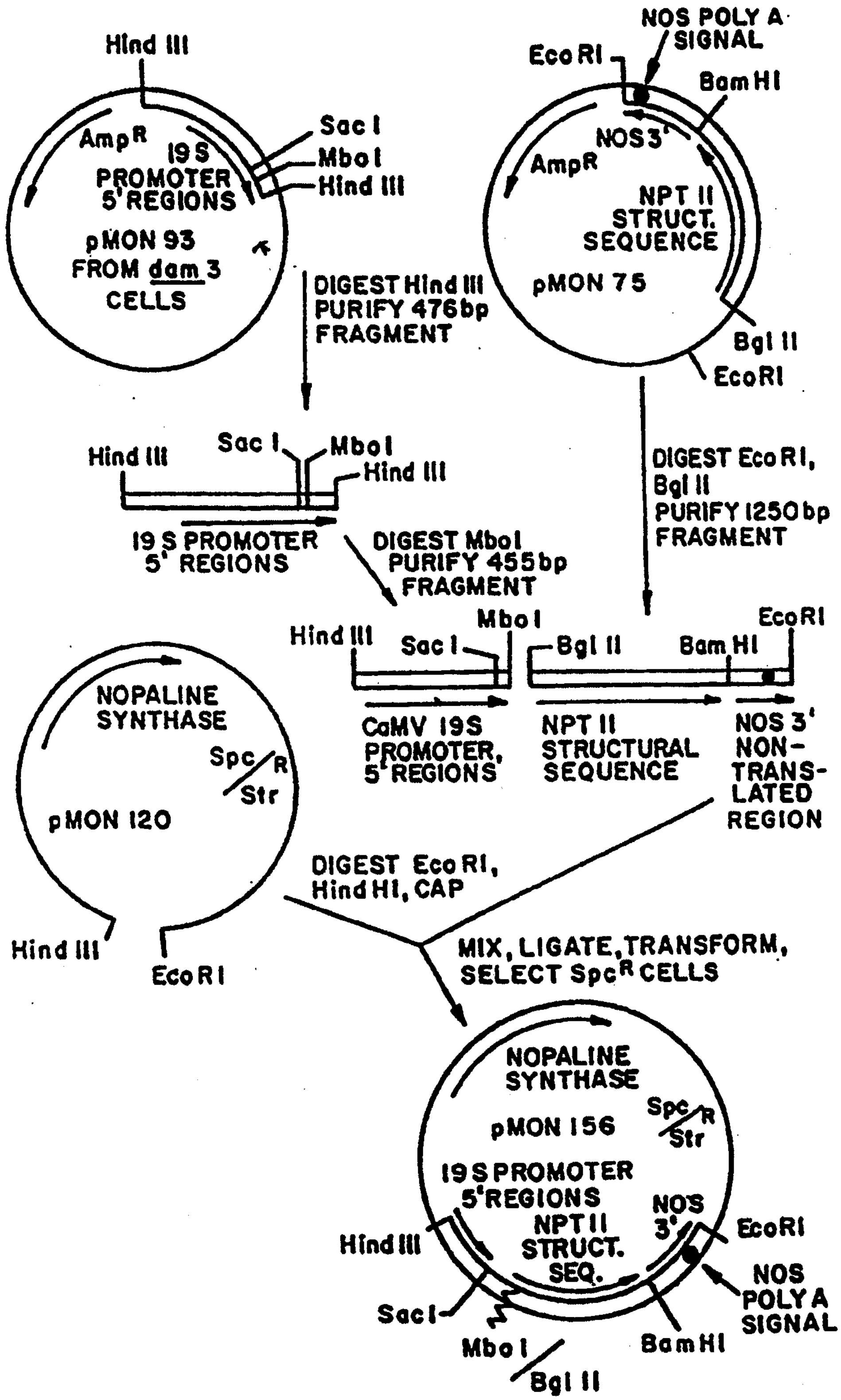

Hind III
AmpR
19 S PROMOTER 5' REGIONS
Sac I
Mbo I
Hind III
pMON 93 FROM dam 3 CELLS
NOS POLY A SIGNAL
Eco RI
Bam HI
NOS 3'
AmpR
NPT II STRUCT. SEQUENCE
pMON 75
Bgl II
EcoRI
DIGEST Hind III PURIFY 476bp FRAGMENT
Hind III
Sac I
Mbo I
Hind III
19 S PROMOTER 5' REGIONS
DIGEST Mbo I PURIFY 455bp FRAGMENT
DIGEST EcoRI, Bgl II PURIFY 1250bp FRAGMENT
Hind III
Sac I
Mbo I
Bgl II
Bam HI
EcoRI
CaMV 19S PROMOTER, 5' REGIONS
NPT II STRUCTURAL SEQUENCE
NOS 3' NON-TRANSLATED REGION
NOPALINE SYNTHASE
SpcR/Str
pMON 120
Hind III
EcoRI
DIGEST EcoRI, Hind HI, CAP
MIX, LIGATE, TRANSFORM, SELECT SpcR CELLS
NOPALINE SYNTHASE
pMON 156
SpcR/Str
19 S PROMOTER 5' REGIONS
NPT II STRUCT. SEQ.
NOS 3'
EcoRI
NOS POLY A SIGNAL
Hind III
Sac I
Mbo I/Bgl II
BamHI

Figure 2

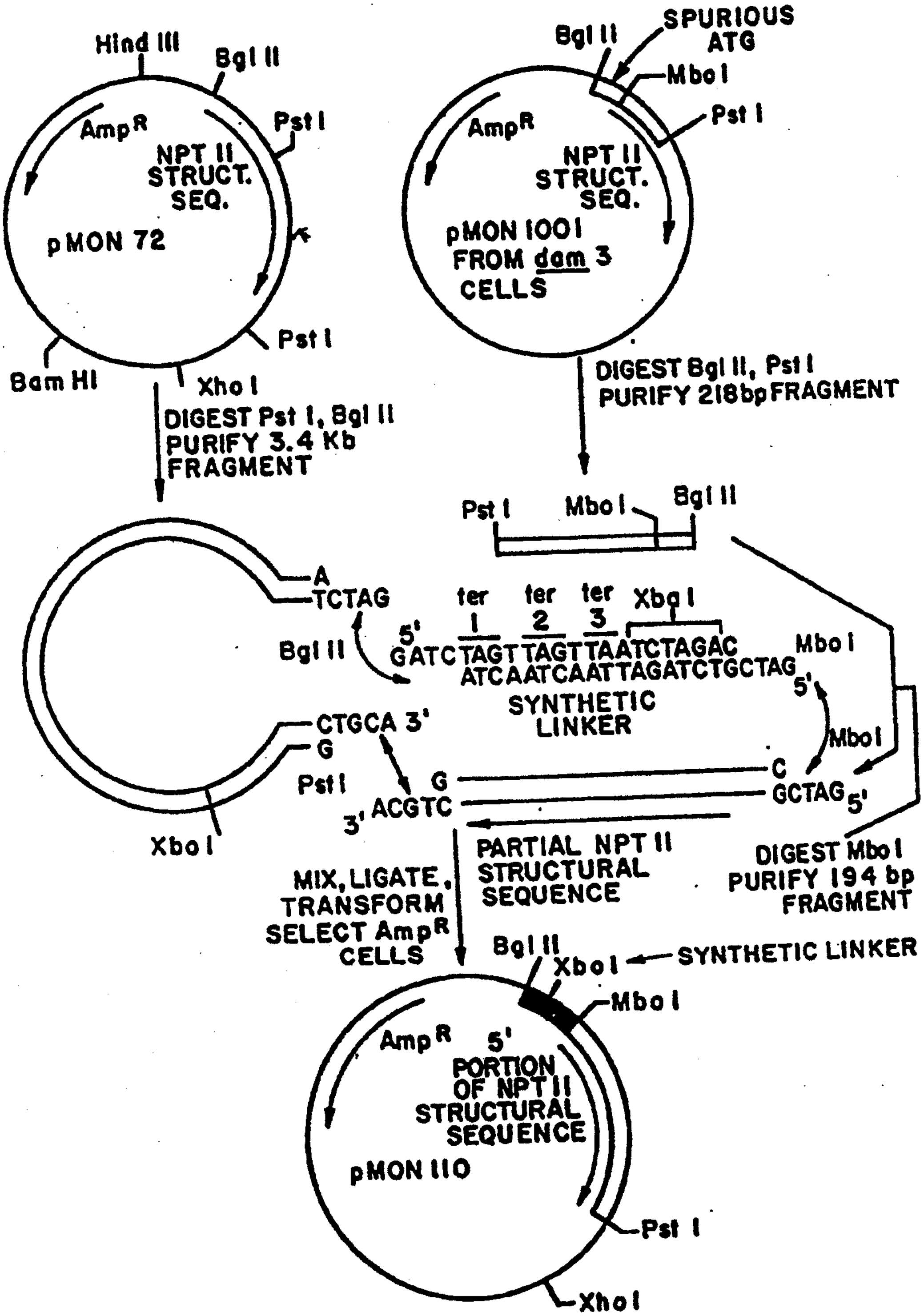
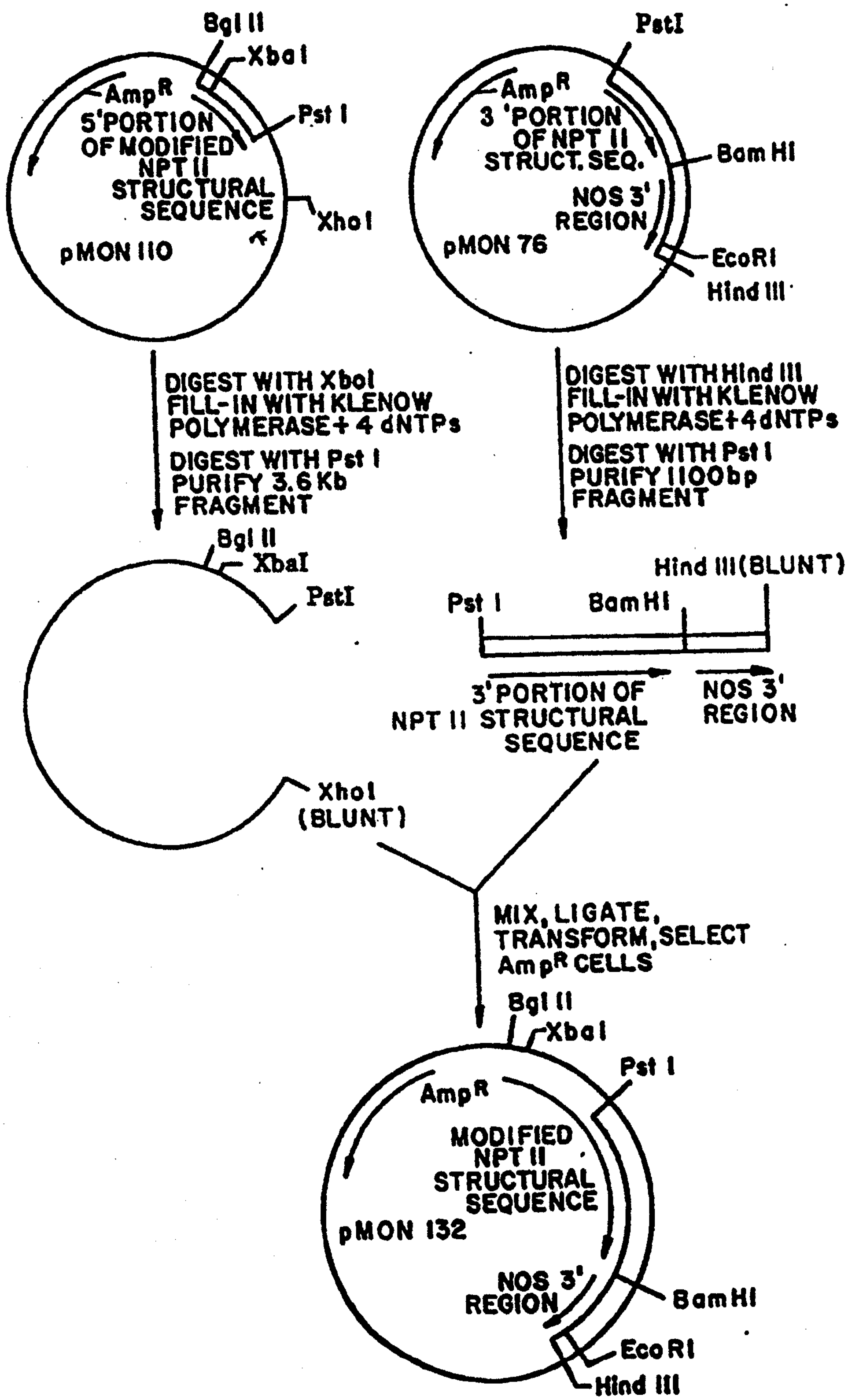
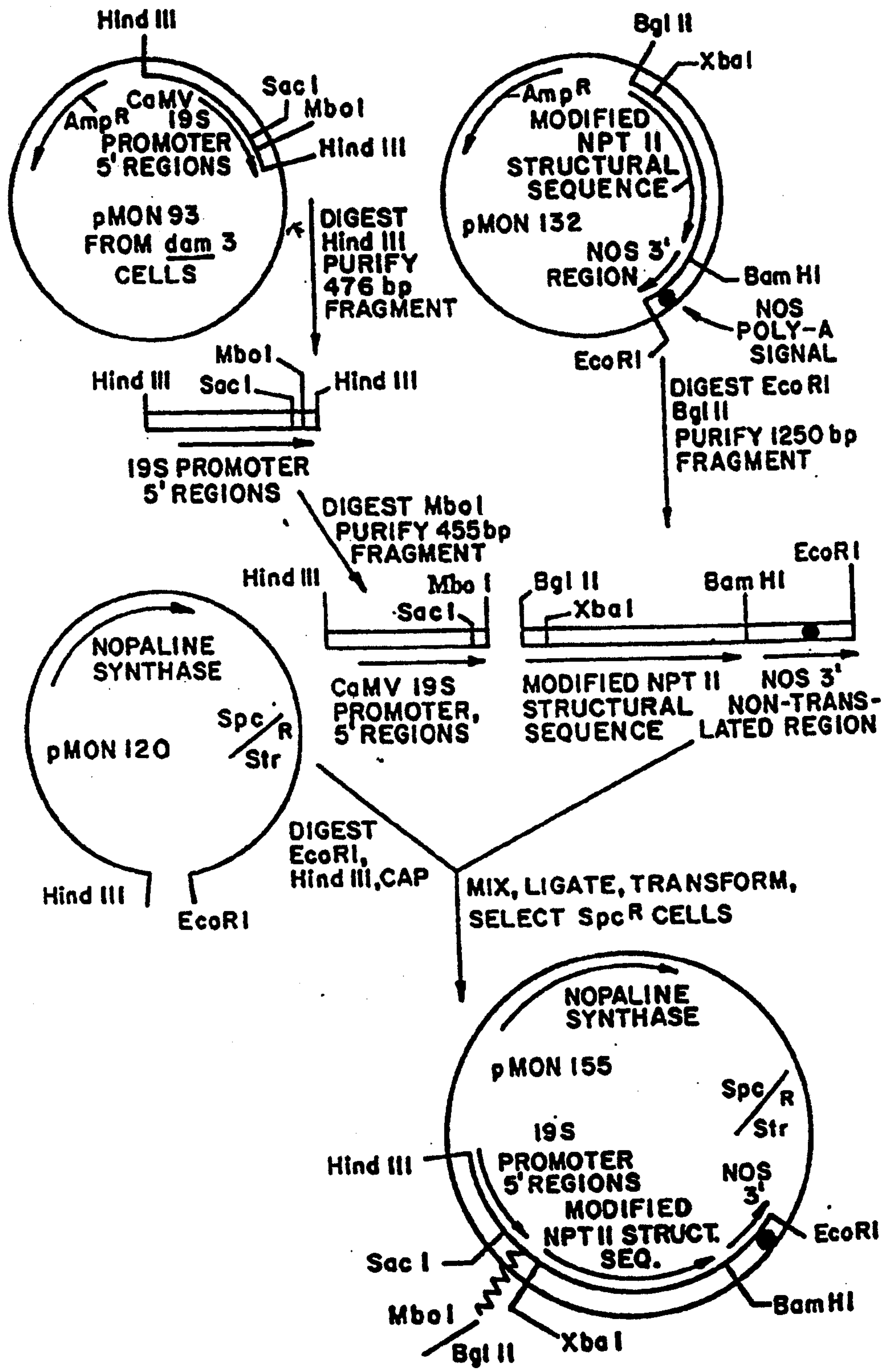

Hind III
Bgl II
Pst I
AmpR
NPT II STRUCT. SEQ.
pMON 72
Pst I
Bam HI
Xho I
DIGEST Pst I, Bgl II
PURIFY 3.4 Kb FRAGMENT
SPURIOUS ATG
Bgl II
Mbo I
Pst I
AmpR
NPT II STRUCT. SEQ.
pMON 1001 FROM dam 3 CELLS
DIGEST Bgl II, Pst I
PURIFY 218bp FRAGMENT
Pst I
Mbo I
Bgl II
A
TCTAG
ter 1
ter 2
ter 3
Xba I
5'
GATCTAGTTAGTTAATCTAGAC
ATCAATCAATTAGATCTGCTAG
5'
Mbo I
Bgl II
SYNTHETIC LINKER
CTGCA 3'
G
Mbo I
Pst I
G
ACGTC
3'
C
GCTAG
5'
Xbo I
PARTIAL NPT II STRUCTURAL SEQUENCE
DIGEST Mbo I
PURIFY 194 bp FRAGMENT
MIX, LIGATE, TRANSFORM SELECT AmpR CELLS
Bgl II
Xbo I
SYNTHETIC LINKER
Mbo I
AmpR
5' PORTION OF NPT II STRUCTURAL SEQUENCE
pMON 110
Pst I
Xho I

Figure 3



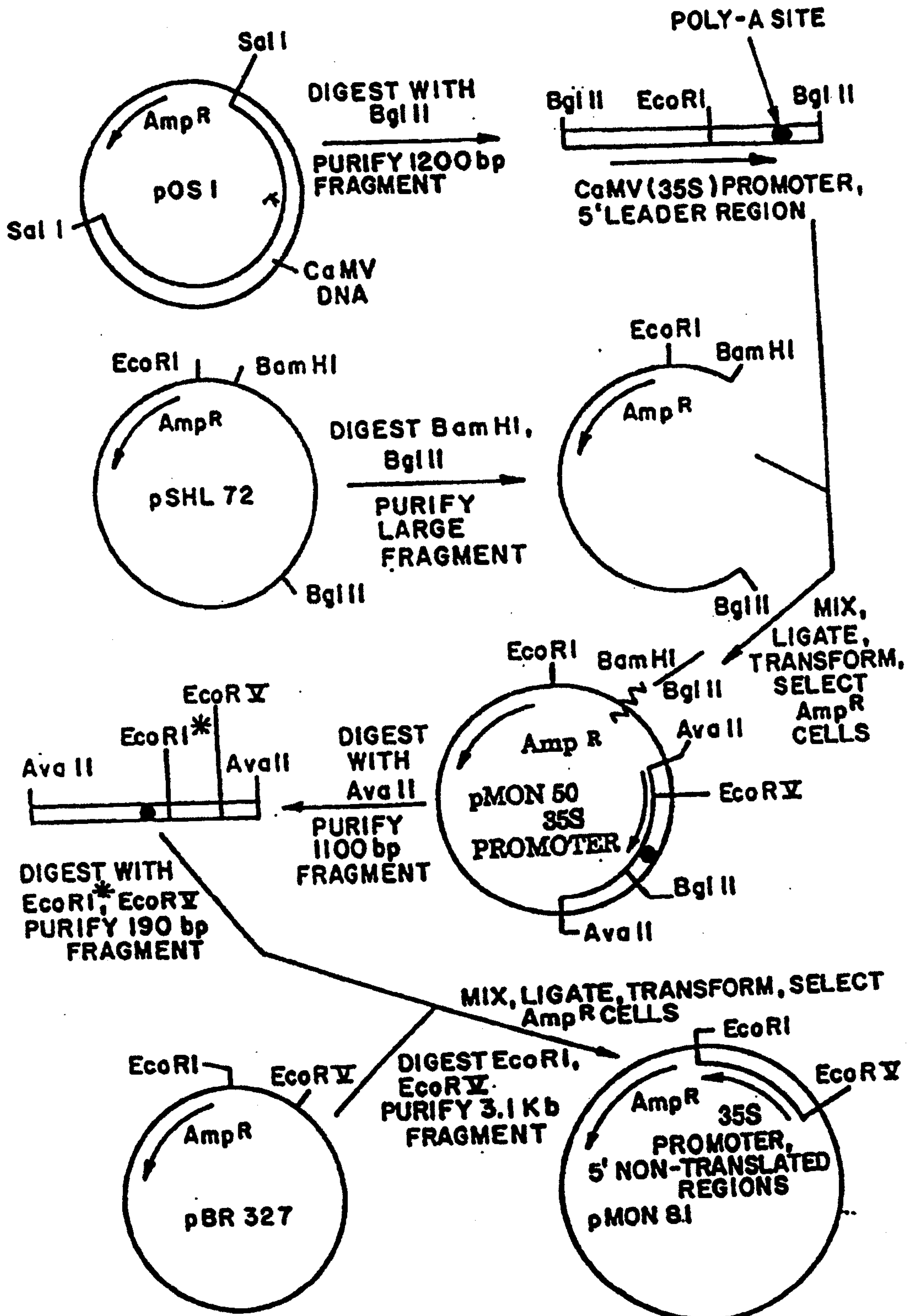
Bgl II
Xba I
Amp^R
5'PORTION OF MODIFIED NPT II STRUCTURAL SEQUENCE
Pst I
Xho I
pMON 110
PstI
Amp^R
3 'PORTION OF NPT II STRUCT. SEQ.
Bam HI
NOS 3' REGION
pMON 76
EcoRI
Hind III
DIGEST WITH XboI
FILL-IN WITH KLENOW POLYMERASE+ 4 dNTPs
DIGEST WITH Pst I
PURIFY 3.6 Kb FRAGMENT
DIGEST WITH Hind III
FILL-IN WITH KLENOW POLYMERASE+4dNTPs
DIGEST WITH Pst I
PURIFY 1100bp FRAGMENT
Bgl II
XbaI
PstI
XhoI (BLUNT)
Hind III(BLUNT)
Pst I
BamHI
3'PORTION OF NPT II STRUCTURAL SEQUENCE
NOS 3' REGION
MIX, LIGATE, TRANSFORM, SELECT Amp^R CELLS
Bgl II
Xba I
Pst I
Amp^R
MODIFIED NPT II STRUCTURAL SEQUENCE
pMON 132
NOS 3' REGION
BamHI
Eco RI
Hind III

Figure 4



Hind III
AmpR
CaMV 19S PROMOTER 5' REGIONS
Sac I
Mbo I
Hind III
pMON 93 FROM dam 3 CELLS
DIGEST Hind III PURIFY 476 bp FRAGMENT
Bgl II
Xba I
AmpR
MODIFIED NPT II STRUCTURAL SEQUENCE
pMON 132
NOS 3' REGION
Bam HI
NOS POLY-A SIGNAL
EcoRI
DIGEST EcoRI Bgl II PURIFY 1250 bp FRAGMENT
Mbo I
Hind III
Sac I
Hind III
19S PROMOTER 5' REGIONS
DIGEST Mbo I PURIFY 455bp FRAGMENT
Hind III
Mbo I
Sac I
Bgl II
Xba I
Bam HI
EcoRI
CaMV 19S PROMOTER, 5' REGIONS
MODIFIED NPT II STRUCTURAL SEQUENCE
NOS 3' NON-TRANS-LATED REGION
NOPALINE SYNTHASE
pMON 120
SpcR/Str
Hind III
EcoRI
DIGEST EcoRI, Hind III, CAP
MIX, LIGATE, TRANSFORM, SELECT SpcR CELLS
NOPALINE SYNTHASE
pMON 155
SpcR/Str
19S PROMOTER 5' REGIONS
MODIFIED NPT II STRUCT. SEQ.
NOS 3'
Hind III
Sac I
Mbo I
Bgl II
Xba I
Bam HI
EcoRI

Figure 5



Sal I
AmpR
pOS I
Sal I
CaMV DNA
DIGEST WITH Bgl II
PURIFY 1200 bp FRAGMENT
POLY-A SITE
Bgl II
EcoRI
Bgl II
CaMV (35S) PROMOTER, 5' LEADER REGION
EcoRI
BamHI
AmpR
pSHL 72
Bgl II
DIGEST BamHI, Bgl II
PURIFY LARGE FRAGMENT
EcoRI
BamHI
AmpR
Bgl II
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
EcoRI
BamHI
Bgl II
Ava II
Amp R
pMON 50
35S PROMOTER
EcoRV
Bgl II
Ava II
DIGEST WITH Ava II
PURIFY 1100 bp FRAGMENT
EcoRV
EcoRI*
Ava II
Ava II
DIGEST WITH EcoRI*, EcoRV
PURIFY 190 bp FRAGMENT
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
EcoRI
EcoRV
AmpR
pBR 327
DIGEST EcoRI, EcoRV
PURIFY 3.1 Kb FRAGMENT
EcoRI
EcoRV
AmpR
35S PROMOTER, 5' NON-TRANSLATED REGIONS
pMON 81

Figure 6

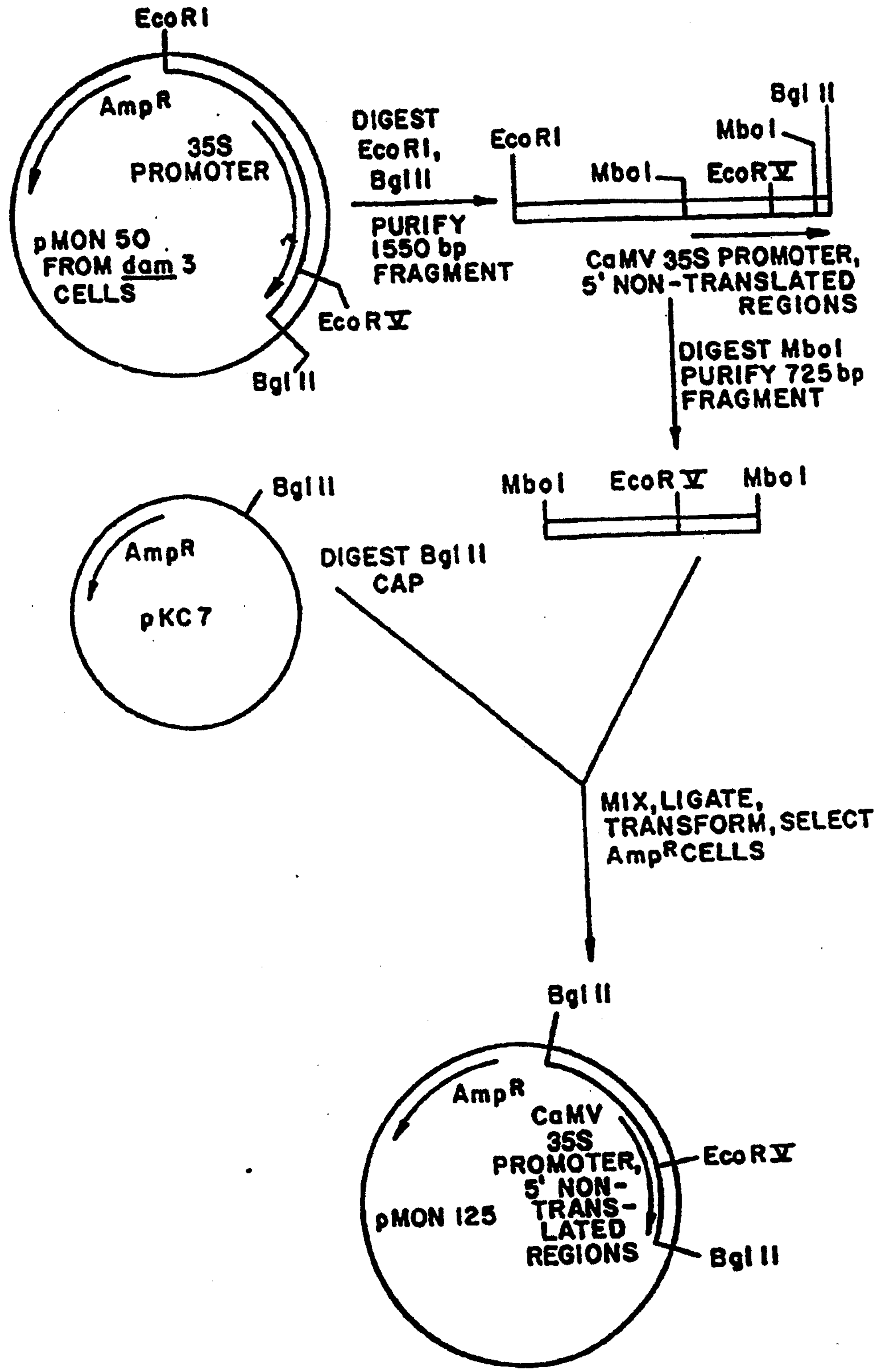

EcoRI
AmpR
35S PROMOTER
pMON 50 FROM dam 3 CELLS
EcoRV
Bgl II
DIGEST EcoRI, BglII
PURIFY 1550 bp FRAGMENT
EcoRI
MboI
EcoRV
MboI
Bgl II
CaMV 35S PROMOTER, 5' NON-TRANSLATED REGIONS
DIGEST MboI PURIFY 725 bp FRAGMENT
MboI
EcoRV
MboI
BglII
AmpR
pKC7
DIGEST Bgl II CAP
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
Bgl II
AmpR
CaMV 35S PROMOTER, 5' NON-TRANS-LATED REGIONS
pMON 125
EcoRV
Bgl II

Figure 7

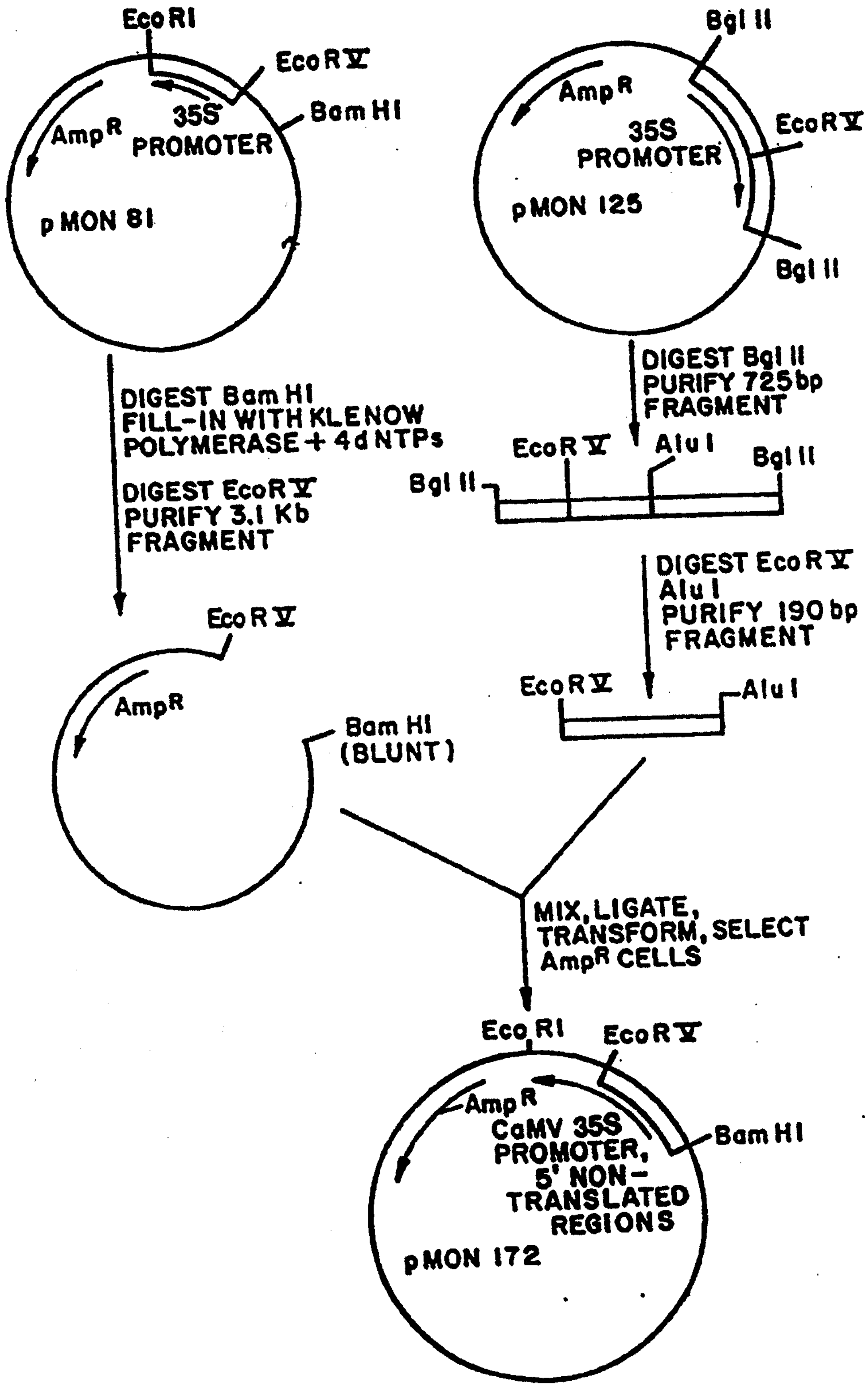

EcoRI
EcoRV
BamHI
AmpR
35S PROMOTER
pMON 81
BglII
AmpR
35S PROMOTER
EcoRV
pMON 125
BglII
DIGEST BamHI
FILL-IN WITH KLENOW POLYMERASE + 4dNTPs
DIGEST EcoRV
PURIFY 3.1 Kb FRAGMENT
DIGEST BglII
PURIFY 725bp FRAGMENT
EcoRV
AluI
BglII
BglII
DIGEST EcoRV
AluI
PURIFY 190bp FRAGMENT
EcoRV
AmpR
BamHI (BLUNT)
EcoRV
AluI
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
EcoRI
EcoRV
AmpR
CaMV 35S PROMOTER, 5' NON-TRANSLATED REGIONS
BamHI
pMON 172

Figure 8

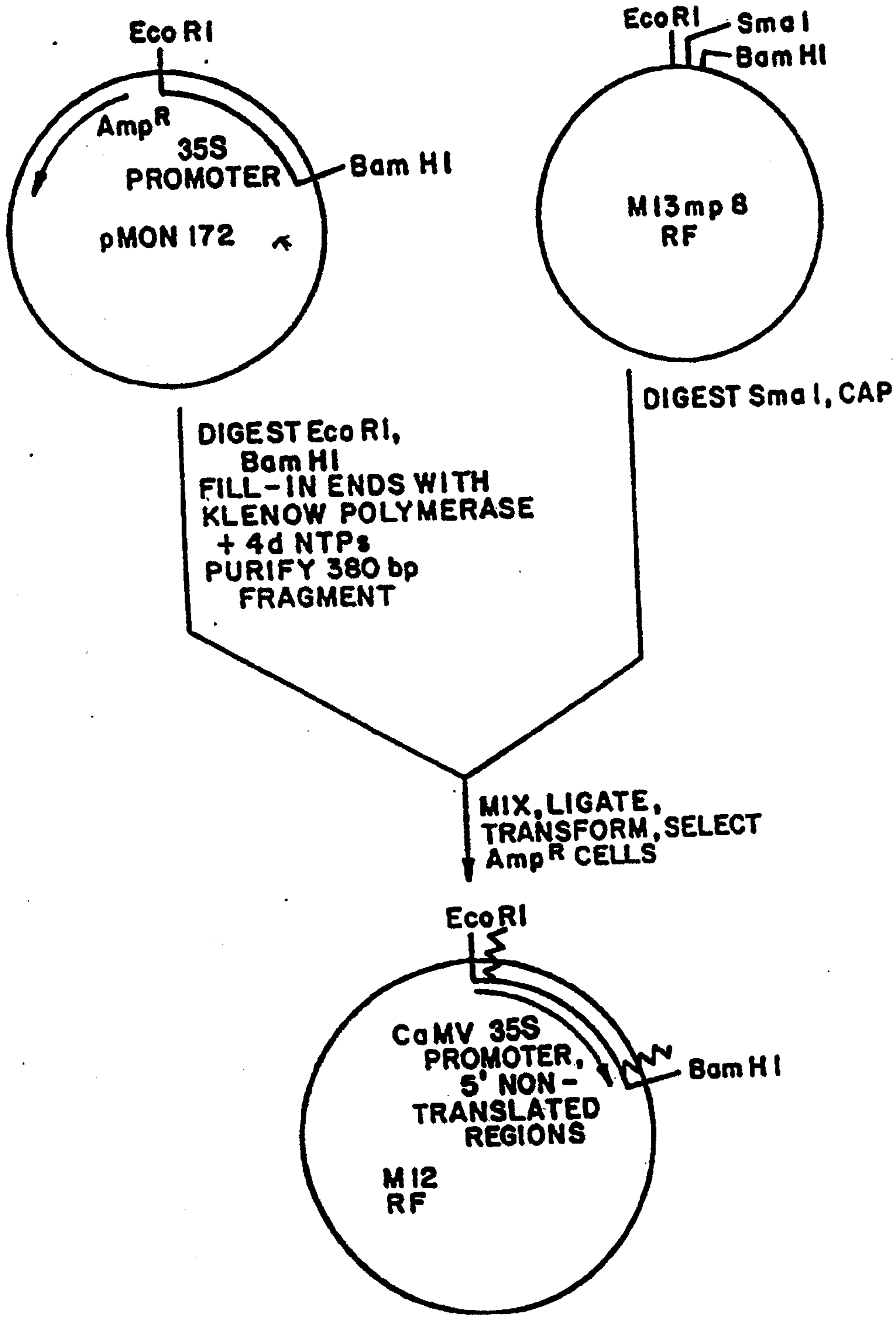
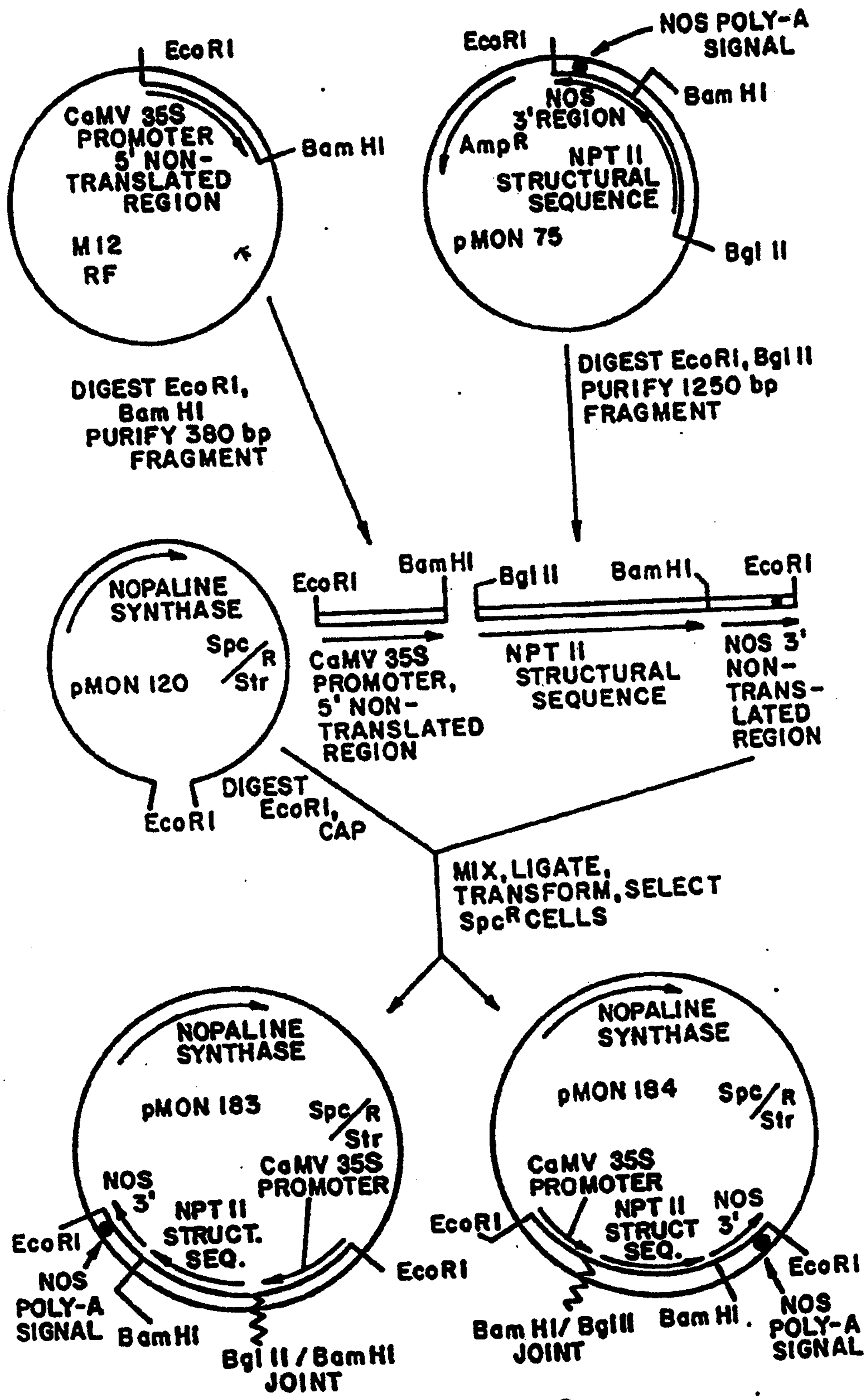

Eco RI
AmpR
35S PROMOTER
Bam HI
pMON 172
EcoRI
Sma I
Bam HI
M13mp8 RF
DIGEST Eco RI, Bam HI FILL-IN ENDS WITH KLENOW POLYMERASE + 4d NTPs PURIFY 380 bp FRAGMENT
DIGEST Sma I, CAP
MIX, LIGATE, TRANSFORM, SELECT AmpR CELLS
EcoRI
CaMV 35S PROMOTER, 5' NON-TRANSLATED REGIONS
Bam HI
M12 RF

Figure 9



EcoRI
CaMV 35S PROMOTER 5' NON-TRANSLATED REGION
Bam HI
M12 RF
EcoRI
NOS POLY-A SIGNAL
NOS 3' REGION
Bam HI
AmpR
NPT II STRUCTURAL SEQUENCE
pMON 75
Bgl II
DIGEST EcoRI, Bam HI PURIFY 380 bp FRAGMENT
DIGEST EcoRI, Bgl II PURIFY 1250 bp FRAGMENT
NOPALINE SYNTHASE
Spc/Str R
pMON 120
EcoRI
DIGEST EcoRI, CAP
EcoRI
BamHI
CaMV 35S PROMOTER, 5' NON-TRANSLATED REGION
Bgl II
BamHI
EcoRI
NPT II STRUCTURAL SEQUENCE
NOS 3' NON-TRANSLATED REGION
MIX, LIGATE, TRANSFORM, SELECT SpcR CELLS
NOPALINE SYNTHASE
pMON 183
Spc/Str R
CaMV 35S PROMOTER
NOS 3'
NPT II STRUCT. SEQ.
EcoRI
NOS POLY-A SIGNAL
BamHI
Bgl II / BamHI JOINT
EcoRI
NOPALINE SYNTHASE
pMON 184
Spc/Str R
CaMV 35S PROMOTER
NPT II STRUCT SEQ.
NOS 3'
EcoRI
Bam HI/ BglII JOINT
Bam HI
EcoRI
NOS POLY-A SIGNAL

Figure 10

CHIMERIC GENES FOR TRANSFORMING PLANT CELLS USING VIRAL PROMOTERS

RELATED APPLICATIONS

This is a File Wrapper continuation of application Ser. No. 07/625,637, filed Dec. 7, 1990, now abandoned, which is a continuation of U.S. Ser. No. 06/931,492, filed Nov. 17, 1986, now abandoned, which is a continuation-in-part of U.S. Ser. No. 06/485,568, filed Apr. 15, 1983, now abandoned, which is a continuation-in-part of U.S. Ser. No. 06/458,414, filed Jan. 17, 1983, now abandoned.

TECHNICAL FIELDS

This invention is in the fields of genetic engineering and plant biology.

BACKGROUND ART

A virus is a microorganism comprising single or double stranded nucleic acid (DNA or RMA) contained within a protein (and possibly lipid) shell called a "capsid" or "coat". A virus is smaller than a cell, and it does not contain most of the components and substances necessary to conduct most biochemical processes. Instead, a virus infects a cell and uses the cellular processes to reproduce itself.

The following is a simplified description of how a DNA-containing virus infects a cell; RNA viruses will be disregarded in this introduction for the sake of clarity. First, a virus attaches to or enters a cell, normally called a "host" cell. The DNA from the virus (and possibly the entire viral particle) enters the host cell where it usually operates as a plasmid (a loop of extrachromosomal DNA). The viral DNA is transcribed into messenger RNA, which is translated into one or more polypeptides. Some of these polypeptides are assembled into new capsids, while others act as enzymes to catalyze various biochemical reactions. The viral DNA is also replicated and assembled with the capsid polypeptides to form new viral particles. These viral particles may be released gradually by the host cell, or they may cause the host cell to lyse and release them. The released viral particles subsequently infect new host cells. For more background information on viruses see, e.g., Stryer, 1981 and Matthews, 1970 (note: all references cited herein, other than patents, are listed with citations after the examples).

As used herein, the term "virus" includes phages and viroids, as well as replicative intermediates. As used herein, the phrases "viral nucleic acid" and DNA or RNA derived from a virus" are construed broadly to include any DNA or RNA that is obtained or derived from the nucleic acid of a virus. For example, a DNA strand created by using a viral RNA strand as a template, or by chemical synthesis to create a known sequence of bases determined by analyzing viral DNA, would be regarded as viral nucleic acid.

The host range of any virus (i.e., the variety of cells that a type of virus is capable of infecting) is limited. Some viruses are capable of efficient infection of only certain types of bacteria; other viruses can infect only plants, and may be limited to certain genera; some viruses can infect only mammalian cells. Viral infection of a cell requires more than mere entry of the viral DNA or RNA into the host cell; viral particles must be reproduced within the cell. Through various assays, those skilled in the art can readily determine whether any

particular type of virus is capable of infecting any particular genus, species, or strain of cells. As used herein, the term "plant virus" is used to designate a virus which is capable of infecting one or more types of plant cells, regardless of whether it can infect other types of cells.

With the possible exception of viroids (which are poorly understood at present), every viral particle must contain at least one gene which can be "expressed" in infected host cells. The expression of a gene requires that a segment of DNA or RNA must be transcribed into or function as a strand of messenger RNA (mRNA), and the mRNA must be translated into a polypeptide. Most viruses have about 5 to 10 different genes, all of which are expressed in a suitable host cell.

In order to be expressed in a cell, a gene must have a promoter which is recognized by certain enzymes in the cell. Gene promoters are discussed in some detail in the parent application Ser. No. 458,414 cited above, the contents of which are incorporated herein by reference. Those skilled in the art recognize that the expression of a particular gene to yield a polypeptide is dependent upon two distinct cellular processes. A region of the 5' end of the gene called the promoter, initiates transcription of the gene to produce a mRNA transcript. The mRNA is then translated at the ribosomes of the cell to yield an encoded polypeptide. Therefore, it is evident that although the promoter may function properly, ultimate expression of the polypeptide depends at least in part on post-transcriptional processing of the mRNA transcript.

Promoters from viral genes have been utilized in a variety of genetic engineering applications. For example, chimeric genes have been constructed using various structural sequences (also called coding sequences) taken from bacterial genes, coupled to promoters taken from viruses which can infect mammalian cell(the most commonly used mammalian viruses are designated as Simian Virus 40 (SV40) and Herpes Simplex Virus (HSV)). These chimeric genes have been used to transform mammalian cells. See, e.g., Mulligan et al 1979; Southern and Berg 1982. In addition, chimeric genes using promoters taken from viruses which can infect bacterial cells have been used to transform bacterial cells; see, e.g., the phage lambda P_L promoter discussed in Maniatis et al, 1982.

Several researchers have theorized that it might be possible to utilize plant viruses as vectors for transforming plant cells. See, e.g., Hohn et al, 1982. In general, a "vector" is a DNA molecule useful for transferring one or more genes into a cell. Usually, a desired gene is inserted into a vector, and the vector is then used to infect the host cell.

Several researchers have theorized that it might be possible to create chimeric genes which are capable of being expressed in plant cells, by using promoters derived from plant virus genes. See, e.g., Hohn et al, 1982, at page 216.

However, despite the efforts of numerous research teams, prior to this invention no one had succeeded in (1) creating a chimeric gene comprising a plant virus promoter coupled to a heterologous structural sequence and (2) demonstrating the expression of such a gene in any type of plant cell.

CAULIFLOWER MOSAIC VIRUS (CaMV)

The entire DNA sequence of CaMV has been published. Gardner et al, 1981; Hohn et al, 1982. In its most common form, the CaMV genome is about 8000 bp long. However, various naturally occurring infective mutants which have deleted about 500 bp have been discovered; see Howarth et al 1981. The entire CaMV genome is transcribed into a single mRNA, termed the "full-length transcript" having a sedimentation coefficient of about 35S. The promoter for the full-length mRNA (hereinafter referred to as "CaMV(35S)") is located in the large intergenic region about 1 kb counterclockwise from Gap 1 (see Guilley et al, 1982).

CaMV is believed to generate at least eight proteins; the corresponding genes are designated as Genes I through VIII. Gene VI is transcribed into mRNA with a sedimentation coefficient of 19S. The 19S mRNA is translated into a protein designated as P66, which is an inclusion body protein. The 19S mRNA is promoted by the 19S promoter, located about 2.5 kb counterclockwise from Gap 1.

SUMMARY OF THE INVENTION

In one aspect, the present invention relates to the use of viral promoters in the expression of chimeric genes in plant cells. In another aspect this invention relates to chimeric genes which are capable of being expressed in plant cells, which utilize promoter regions derived from viruses which are capable of infecting plant cells. One such virus comprises the cauliflower mosaic virus (CaMV). Two different promoter regions have been derived from the CaMV genome and ligated to heterologous coding sequences to form chimeric genes. These chimeric genes have been proven to be expressed in plant cells. This invention also relates to plant cells, plant tissue (including seeds and propagules), and differentiated plants which have been transformed to contain viral promoters and express the chimeric genes of this invention, and to polypeptides that are generated in plant cells by the chimeric genes of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The figures herein are schematic representations; they have not been drawn to scale.

FIG. 1 represents the creation and structure of plasmid pMON93.

FIG. 2 represents the creation and structure of plasmid pMON156.

FIG. 3 represents the creation and structure of plasmid pMON110.

FIG. 4 represents the creation and structure of plasmid pMON132.

FIG. 5 represents the creation and structure of plasmid pMON155.

FIG. 6 represents the creation and structure of plasmid pMON81.

FIG. 7 represents the creation and structure of plasmid pMON125.

FIG. 8 represents the creation and structure of plasmid pMON172.

FIG. 9 represents the creation and structure of phage M12.

FIG. 10 represents the creation and structure of plasmids pMON183 and pMON184.

DETAILED DESCRIPTION OF THE INVENTION

In one preferred embodiment of this invention, a chimeric gene was created which contained the following elements:

1. a promoter region and a 5' non-translated region derived from the CaMV (19S) gene, which codes for the P66 protein;
2. a partial coding sequence from the CaMV (19S) gene, including an ATG start codon and several internal ATG sequences, all of which were in the same frame as a TGA termination sequence immediately inside the desired ATG start codon of the NPTII gene;
3. a structural sequence derived from a neomycin phosphotransferase II (NPTII) gene; this sequence was preceded by a spurious ATG sequence, which was in the same reading frame as a TGA sequence within the NPTII structural sequence; and,
4. a 3' non-translated region, including a poly-adenylation signal, derived from a nopaline synthase (NOS) gene.

This chimeric gene, referred to herein as the CaMV(19S)-NPTII-NOS gene, was inserted into plasmid pMON120 (described in the parent application, Ser. No. 458,414; ATCC accession number 39263) to create a plasmid designated as pMON156. Plasmid pMON156 was inserted into an *Agrobacterium tumefaciens* cell, where it formed a co-integrate Ti plasmid by means of a single crossover event with a Ti plasmid in the *A. tumefaciens* cell, using a method described in the parent application. The chimeric gene in the co-integrate plasmid was within a modified T-DNA region in the Ti plasmid, surrounded by left and right T-DNA borders.

A. tumefaciens cells containing the co-integrate Ti plasmids with the CaMV(19S)-NPTII-NOS genes were used to infect plant cells, using a method described in the parent application. Some of the plant cells were genetically transformed, causing them to become resistant to an antibiotic (kanamycin) at concentrations which are toxic to untransformed plant cells.

A similar chimeric gene was created and assembled in a plasmid designated as pMON155. This chimeric gene resembled the gene in pMON156, with two exceptions:

1. an oligonucleotide linker having stop codons in all three reading frames was inserted between the CaMV(19S) partial structural sequence and the NPTII structural sequence; and,
2. the spurious ATG sequence on the 5' side of the NPTII structural sequence was deleted.

The construction of this chimeric gene is described in Example 2. This gene was inserted into *A. tumefaciens* cells and subsequently into plant cells. Its level of expression was apparently higher than the expression of the similar gene in pMON156, as assayed by growth on higher concentrations of kanamycin.

CREATION OF PLASMIDS pMON183 and 184; CaMV(35S)

In an alternate preferred embodiment of this invention, a chimeric gene was created comprising

(1) a promoter region which causes transcription of the 35S mRNA of cauliflower mosaic virus, CaMV(35S);
(2) a structural sequence which codes for NPTII; and
(3) a nopaline synthase (NOS) 3' non-translated region.

The assembly of this chimeric gene is described in Example 3. This gene was inserted into plant cells and it caused them to become resistant to kanamycin.

Petunia plants cannot normally be infected by CaMV. Those skilled in the art may determine through routine experimentation whether any particular plant viral promoter (such as the CaMV promoter) will function at satisfactory levels in any particular type of plant cell, including plant cells that are outside of the normal host range of the virus from which the promoter was derived.

It is possible to regenerate genetically transformed plant cells into differentiated plants. One method for such regeneration was described in U.S. patent application entitled "Genetically Transformed Plants", Ser. No. 458,402, now abandoned. That application was filed simultaneously with, and incorporated by reference into, the parent application of this invention. The methods of application Ser. No. 458,402, now abandoned, may be used to create differentiated plants (and their progeny) which contain and express chimeric genes having plant virus promoters.

It is possible to extract polypeptides generated in plant cells by chimeric genes of this invention from the plant cells, and to purify such extracted polypeptides to a useful degree of purity, using methods and substances known to those skilled in the art.

Those skilled in the art will recognize, or may ascertain using no more than routine experimentation, numerous equivalents to the specific embodiments described herein. Such equivalents are within the scope of this invention, and are covered by the claims below.

EXAMPLES

Example 1: Creation and Use of pMON156

Plasmids which contained CaMV DNA were a gift to Monsanto Company from Dr. R. J. Shepherd, University of California, Davis. To the best of Applicants' knowledge and belief, these plasmids (designated as pOS1) were obtained by inserting the entire genome of a CaMV strain designated as CM4-184 (Howarth et al, 1981) into the Sal I restriction site of a pBR322 plasmid (Bolivar et al, 1978). *E. coli* cells transformed with pOS1 were resistant to ampicillin (Amp^R) and sensitive to tetracycline (Tet^S).

Various strains of CaMV suitable for isolation of CaMV DNA which can be used in this invention are publicly available; see, e.g., ATCC Catalogue of Strains II, p. 387 (3rd edition, 1981).

pOS1 DNA was cleaved with HindIII. Three small fragments were purified after electrophoresis on an 0.8% agarose gel using NA-45 membrane (Schleicher and Schuell, Keene NH). The smallest fragment, about 500 bp in size, contains the 19S promoter. This fragment was further purified on a 6% acrylamide gel. After various manipulations which did not change the sequence of this fragment (shown in FIG. 1), it was digested with MboI to created 455 bp HindIII-MboI fragment. This fragment was mixed with a 1250 bp fragment obtained by digesting pMON75 (described and shown in FIG. 9 of the parent application Ser. No. 458,414, now abandoned,) with BglII and EcoRI. This fragment contains the NPTII structural sequence and the NOS 3' non-translated region. The two fragments were ligated by their compatible MboI and BglII overhangs to create a fragment containing the CaMV(19S)-NPTII-NOS chimeric gene. This fragment was inserted into pMON120 (described and shown in FIG. 10 of the parent application, Ser. No. 458,414, now abandoned; ATCC accession number 39263) which had been cleaved with HindIII and EcoRI. The resulting plasmid was designated as pMON156, as shown in FIG. 2.

Plasmid pMON156 was inserted into *E. coli* cells and subsequently into *A. tumefaciens* cells where it formed a co-integrate Ti plasmid having the CaMV(19S)-NPTII-NOS chimeric gene surrounded by T-DNA borders. *A. tumefaciens* cells containing the co-integrate plasmids were co-cultivated with petunia cells. The foregoing methods are described in detail in a separate application, entitled "Plasmids for Transforming Plant Cells" Ser. No. 458,411, now abandoned, which was filed simultaneously with and incorporated by reference into parent application, Ser. No. 458,414, now abandoned.

The co-cultivated petunia cells were cultured on media containing kanamycin, an antibiotic which is toxic to petunia cells. Kanamycin is inactivated by the enzyme NPTII, which does not normally exist in plant cells. Some of the co-cultivated petunia cells survived and produced colonies on media containing up to 50 ug/ml kanamycin. This indicated that the CaMV(19S)-NPTII-NOS genes were expressed in petunia cells. These results were confirmed by Southern blot analysis of transformed plant cell DNA.

Example 2: Creation of pMON155

Plasmid pMON72 was obtained by inserting a 1.8 kb HindIII-BamHI fragment from bacterial transposon Tn5 (which contains an NPTII structural sequence) into a $PstI^-$ pBR327 plasmid digested with HindIII and BamHI. This plasmid was digested with BglII and PstI to remove the NPTII structural sequence.

Plasmid pMON1001 (described and shown in FIG. 6 of the parent application) from dam^- cells was digested with BglII and PstI to obtain a 218 bp fragment with a partial NPTII structural sequence. This fragment was digested with MboI to obtain a 194 bp fragment.

A triple ligation was performed using (a) the large PstI-BglII fragment of pMON72; (b) PstI-MboI fragment from pMON1001; and (c) a synthetic linker with BglII and MboI ends having stop codons in all three reading frames. After transformation of *E. coli* cells and selection for ampicillin resistant colonies, plasmid DNA from Amp^R colonies was analyzed. A colony containing a plasmid with the desired structure was identified. This plasmid was designated pMON110, as shown on FIG. 3.

In order to add the 3' end of the NPTII structural sequence to the 5' portion in pMON110, pMON110 was treated with XhoI. The resulting overhanging end was filled in to create a blunt end by treatment with Klenow polymerase and the four deoxy-nucleotide triphosphates (dNTP's), A, T, C, and G. The Klenow polymerase was inactivated by heat, the fragment was digested with PstI, and a 3.6 kb fragment was purified. Plasmid pMON76 (described and shown in FIG. 9 of the parent application) was digested with HindIII, filled in to create a blunt end with Klenow polymerase and the four dNTP's, and digested with PstI. An 1100 bp fragment was purified, which contained part of the NPTII structural sequence, and a nopaline synthase (NOS) 3' non-translated region. This fragment was ligated with the 3.6 kb fragment from pMON110. The mixture was used to transform *E. coli* cells; Amp R cells were selected, and a colony having a plasmid with the desired structure was identified. This plasmid was designated pMON132, as shown on FIG. 4. Plasmid pMON93 (shown on FIG. 1) was digested with HindIII, and a 476 bp fragment was isolated. This fragment was digested with MboI, and a 455 bp HindIII-MboI fragment was purified which contained the CaMV (19S) promoter region, and 5' non-translated region.

Plasmid pMON132 was digested with EcoRI and BglII to obtain a 1250 bp fragment with (1) the synthetic linker equipped with stop codons in all three reading frames; (2) the NPTII structural sequence; and (3) the NOS 3' non-translated region. These two fragments were joined together through the compatible MboI nd BglII ends to create a CaMV (19S)-NPTII-NOS chimeric gene.

This gene was inserted into pMON120, which was digested with HindIII and EcoRI, to create plasmid pMON155, as shown in FIG. 5.

Plasmid pMON155 was inserted into *A. tumefaciens* GV3111 cells containing a Ti plasmid, pTiB6S3. The pMON155 plasmid formed a cointegrate plasmid with the Ti plasmid by means of a single crossover event. Cells which contain this co-integrate plasmid have been deposited with the American Type Culture Center, and have been assigned ATCC accession number 39336. A fragment which contains the chimeric gene of this invention can be obtained by digesting the co-integrate plasmid with HindIII and EcoRI, and purifying the 1.7 kb fragment. These cells have been used to transform petunia cells, allowing the petunia cells to grow on media containing at least 100 ug/ml kanamycin.

Example 3: Creation of pMON183 and 184

Plasmid pOS1 (described in Example 1) was digested with BglII, and a 1200 bp fragment was purified. This fragment contained the 35S promoter region and part of the 5' non-translated region. It was inserted into plasmid pSHL72 which had been digested with BamHI and BglII (pSHL72 is functionally equivalent to pAGO60, described in Colbere-Garapin et al, 1981). The resulting plasmid was designated as pMON50, as shown on FIG. 6.

The cloned BglII fragment contains a region of DNA that acts as a polyadenylation site for the 35S RNA transcript. This polyadenylation region was removed as follows: pMON50 was digested with AvaII and an 1100 bp fragment was purified. This fragment was digested with EcoRI* and EcoRV. The resulting 190 bp EcoRV-EcoRI* fragment was purified and inserted into plasmid pBR327, which had been digested with EcoRI* and EcoRV. The resulting plasmid, pMON81, contains the CaMV 35S promoter on a 190 bp EcoRV-EcoRI* fragment, as shown in FIG. 6.

To make certain the entire promoter region of CaMV(35S) was present in pMON81, a region adjacent to the 5' (EcoRV) end of the fragment was inserted into pMON81 in the following way. Plasmid pMON50 prepared from dam⁻ cells was digested with EcoRI and BglII and the resultant 1550 bp fragment was purified and digested with MboI. The resulting 725 bp MboI fragment was purified and inserted into the unique BglII site of plasmid pKC7 (Rao and Rogers, 1979) to give plasmid pMON125, as shown in FIG. 7. The sequence of bases adjacent to the two MboI ends regenerates BglII sites and allows the 725 bp fragment to be excised with BglII.

To generate a fragment carrying the 35S promoter, the 725 bp BglII fragment was purified from pMON125 and was subsequently digested with EcoRV and AluI to yield a 190 bp fragment. Plasmid pMON81 was digested with BamHI, treated with Klenow polymerase and digested with EcoRV. The 3.1 kb EcoRV-BamHI(-blunt) fragment was purified, mixed with the 190 bp EcoRV-AluI fragment and treated with DNA ligase. Following transformation and selection of ampicillin-resistant cells, plasmid pMON172 was obtained which carries the CaMV(35S) promoter sequence on a 380 bp BamHI-EcoRI fragment, as shown on FIG. 8. This fragment does not carry the polyadenylation region for the 35S RNA. Ligation of the AluI end to the filled-in BamHI site regenerates the BamHI site.

To rearrange the restriction endonuclease sites adjacent to the CaMV(35S) promoter, the 380 bp BamHI-EcoRI fragment was purified from pMON172, treated with Klenow polymerase, and inserted into the unique smaI site of phage M13 mp8. One recombinant phage, M12, carried the 380 bp fragment in the orientation shown on FIG. 9. The replicative form DNA from this phage carries the 35S promoter fragment on an EcoRI(-5')-BamHI(3') fragment, illustrated below.

```
    EcoRI
1   |                                                                  70
GAATTCCCGATCcTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAAGGTGGCACTACAAATGCCAT
71                                                                    140
CATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCAC
141                                                                   210
CCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAT
                                                            TATA
211                                                           |       280
CTCCACTGACGTAAGGGATGACGCACAATCCCACTATACCTTCGCAAGACCCTTCCTCTATATAAGGAAGT
                     5'mRNA
281                    |                                              350
TCATTTCATTTGGAGAGGACACGCTGAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTATTTTCT
        Extra Translational Initiator              BamHI
351     |                                          |
CCATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTGGGGATCC
```

Plasmids carrying a chimeric gene CaMV(35S) promoter region-NPTII structural sequence-NOS 3' non-translated region) were assembled as follows. The 380 bp EcoRI-BamHI CaMV(35S) promoter fragment was purified from phage M12 RF DNA and mixed with the 1250 bp BglII-EcoRI NPTII-NOS fragment from pMON75. Joining of these two fragments through their compatible BamHI and BglII ends results in a 1.6 kb CaMV(35S)-NPTII-NOS chimeric gene. This gene was inserted into pMON120 at the EcoRI site in both orientations. The resultant plasmids, pMON183 and 184, appear in FIG. 10. These plasmids differ only in the direction of the chimeric gene orientation.

These plasmids were used to transform petunia cells, as described in Example 1. The transformed cells are capable of growth on media containing 100 ug/ml kanamycin.

COMPARISON OF CaMV(35S) AND NOS PROMOTERS

Chimeric genes carrying the nopaline synthase (NOS) promoter or the cauliflower mosaic virus full-length transcript promoter (CaMV(35S)) were constructed. In both cases, the promoters, which contain their respective 5' non-translated regions were joined to

al., 1982). The CaMV(35S) promoter sequence described above is listed below.

pMON273 CaMV 35S Promoter and 5' Leader

```
 EcoRI                                                                       70
1 |
GAATTCCCGATCc TATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAAGGTGGCACTACAAATGCCAT
71                                                                          140
CATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCAC
141                                                                         210
CCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAT
                                                                   TATA
211                                                                 |       280
CTCCACTGACGTAAGGGATGACGCACAATCCACTATACCTTCGCAAGACCCTTCCTCTATATAAGGAAGT
                  5'mRNA                                     BglII
281                 |                                          |  334
TCATTTCATTTGGAGAGGACACGCTGAAATCACCAGTCTCTCTCTACAAGATCT
```

a NPTII coding sequence in which the bacterial 5' leader had been modified so that a spurious ATG translational initiation signal (Southern and Berg, 1982) has been removed.

Plasmid pMON200 is a derivative of previously described intermediate vector pMON120 (ATCC accession number 39263). pMON200 contains a modified chimeric nopaline synthase-neomycin phosphotransferasenopaline synthase gene (NOS/NPTII/NOS) which confers kanamycin (Km^R) resistance to the transformed plant. The modified chimeric Km^R gene lacks an upstream ATG codon present in the bacterial leader sequence and a synthetic multilinker with unique HindIII, XhoI, BglII, XbaI, ClaI and EcoRI restriction sites.

Plasmid pMON273 is a derivative of pMON200 in which the nopaline synthase promoter of the chimeric NOS-NPTII-NOS gene has been replaced with the CaMV(35S) promoter.

The CaMV(35S) promoter fragment was isolated from plasmid pOS-1, a derivative of pBR322 carrying the entire genome of CM4-184 as a SalI insert (Howarth et al., 1981). The CM4-184 strain is a naturally occurring deletion mutant of strain CM1841. The nucleotide sequence of the CM1841 (Gardner et al., 1981) and Cabb-S (Franck et al., 1980) strains of CaMV have been published as well as some partial sequence for a different CM4-184 clone (Dudley et al., 1982). The nucleotide sequences of the 35S promoter regions of these three isolates are essentially identical. In the following the nucleotide numbers reflects the sequence of Gardner et al. (1981). The 35S promoter was isolated as an AluI (n 7143)-EcoRI* (n 7517) fragment which was inserted first into pBR322 cleaved with BamHI, treated with the Klenow fragment of DNA polymerase I and then cleaved with EcoRI. The promoter fragment was then excised from pBR322 with BamHI and EcoRI, treated with Klenow polymerase and inserted into the SmaI site of M13 mp8 so that the EcoRI site of the mp8 multilinker was at the 5' end of the promoter fragment. Site directed mutagenesis (Zoller and Smith, 1982) was then used to introduce a G at nucleotide 7464 to create a BglII site. The 35S promoter fragment was then excised from the M13 as a 330 bp EcoRI-BglII site. The 35S promoter fragment was then excised from the M13 as a 330 bp EcoRI-BglII fragment which contains the 35S promoter, 30 nucleotides of the 5' non-translated leader but does not contain any of the CaMV translational initiators nor the 35S transcript polyadenylation signal that is located 180 nucleotides downstream from the start of transcription (Covey et al., 1981; Guilley et

The 35S promoter fragment was joined to a 1.3 kb BglII-EcoRI fragment containing the Tn5 neomycin phosphotransferase II coding sequence modified so that the translational initiator signal in the bacterial leader sequence had been removed and the NOS 3' non-translated region and inserted into pMON120 to give pMON273.

These plasmids were transferred in *E. coli* strain JM101 and then mated into *Agrobacterium tumefaciens* strain GV3111 carrying the disarmed pTiB6S3-SE plasmid as described by Fraley et al. (1983).

Plant Transformation

Cocultivation of Petunia protoplasts with *A. tumefaciens*, selection of kanamycin resistant transformed callus and regeneration of transgenic plants was carried out as described in Fraley et al. (1984).

Preparation of DNAs

Plant DNA was extracted by grinding the frozen tissue in extraction buffer (50 mM TRIS-HCl pH 8.0, 50 mM EDTA, 50 mM NaCl, 400 ul/ml EtBr, 2% sarcosyl). Following low speed centrifugation, cesium chloride was added to the supernatant (0.85 gm/ml). The CsCl gradients were centrifuged at 150,000×g for 48 hours. The ethidium bromide was extracted with isopropanol, the DNA was dialyzed, and ethanol precipitated.

Southern Hybridization Analysis

10 ug of each plant DNA was digested, with BamHI for pMON200 plant DNAs and EcoRI for pMON273 plant DNAs. The fragments were separated by electrophoresis on a 0.8% agarose gel and transferred to nitrocellulose (Southern, 1975). The blots were hybridized (50% formamide, 3xSSC, 5X denhardt's, 0.1% SDS and 20 ug/ml tRNA) with nick-translated pMON273 plasmid DNA for 48-60 hours at 42° C.

Preparation of RNA from Plant Tissue

Plant leaves were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The ground frozen tissue was added to a 1:1 mixture of grinding buffer and PCE (1% Tri-iso-propylnaphtalenesulfonic acid, 6% p-Aminosalicylic acid, 100 mM NaCl, 1% SDS and 50 mM 2-mercaptoethanol; PCI [phenol: chloroform: isoamyl alcohol (24:24:1)] and homogenized immediately with a polytron. The crude homogenate was mixed for 10 min and the phases separated by centrifugation. The aqueous phase then was re-extracted with an equal volume of PCI. The aqueous phase was ethanol precipitated with one tenth volume of 3M NaAcetate and 2.5 volumes of ethanol. The nucleic acid pellet was resuspended in water. An equal volume of 4M lithium chloride LiCl was added and the mix was placed on ice for 1 hour or overnight. Following centrifugation, the pellet was resuspended in water the LiCl precipitation repeated 3 times. The final LiCl pellet was resuspended in water and ethanol precipitated.

Poly (A) containing RNA was isolated by passing total RNA over an Oligo d(T) cellulose Type III (Collaborative Research) column. Quantitation of the poly (A) containing RNA involved annealing an aliquot of the RNA to radio-labeled poly U [(uridylate 5,6-3H)-polyuridylic acid] (New England Nuclear), followed by RNase A treatment (10 ug per ml for 30 minutes at 37° C.). The reaction mix was spotted on DE-81 filter paper, washed 4× with 0.5M NaPhosphate (pH 7.5) and counted. Globin poly (A) containing RNA (BRL) was used as a standard.

Northern Hybridization Analysis

5 ug of poly (A) RNA from each plant source was treated with glyoxal and dimethysulfoxide (Maniatis, 1982). The RNAs were electrophoresed in 1.5% agarose gels (0.01M NaH_2HPO_4, pH 6.5) for 7 hours at 60 volts. The glyoxylated RNAs were electro-blotted (25 mM $NaH_2PO_4NaHPO_4$, pH 6.5) for 16 hours at 125 amps from the gel to GeneScreen® (New England Nuclear). The filters were hybridized as per manufacturer's instructions (50% formamide, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% ficoll, 5XSSC, 1.0% SDS, 100 u/ml tRNA and probe) for 48–60 hours at 42° C. with constant shaking. The nick-translated DNAs used as probes were the 1.3 kb BglII/EcoRI NPTII fragment purified from the pMON273 plasmid for detecting the NPTII transcript, and the petunia small subunit gene as an internal standard for comparing the amount of RNA per lane. The membranes were washed 2×100 ml of 2XSSC at room temperature for 5 minutes, 2×100 ml of 2XSSC/1.0% SDS at 65° C. for 30 minutes. The membranes were exposed to XAR-5 film with a DuPont intensifying screen at −80° C.

Neomycin Phosphotransferase Assay

The gel overlay assay was used to determine the steady state level of NPTII enzyme activity in each plant. Several parameters were investigated for optimizing the sensitivity of the assay in plant tissue. Early observations showed that the level of NPTII activity varied between leaves from different positions on the same plant. This variability was minimized when the plant extract was made from pooled tissue. A paper hole punch was used to collect 15 disks from both young and old leaves. Grinding the plant tissue in the presence of micro-beads (Ferro Corp) rather than glass beads increased the plant protein yield 4-fold.

To optimize detection of low levels of NPTII activity a saturation curve was prepared with 10–85 ug/lane of plant protein. For the pMON200 (NOS) plants, NPTII activity was not detectable at less than 50 ug/lane of total protein (2 hour exposure) while activity was detectable at 20 ug/lane for the pMON273 plants. There was a non-linear increase in NPTII activity for pMON200 NOS plants between 40 and 50 ug of protein per lane. This suggested that the total amount of protein may affect the stability of the NPTII enzyme. Supplementing plant cell extracts with 30–45 ug per lane of bovine serum albumin (BSA), resulted in a linear response; NPTII activity increased proportionately as plant protein levels increased. The addition of BSA appears to stabilize the enzyme, resulting in a 20-fold increase in the sensitivity of the assay. Experiments indicate that 25 ug/lane of pMON273 plant protein and 70 ug/lane of pMON200 plant protein was within the linear range of the assay in the presence of BSA. Elimination of SDS from the extraction buffer resulted in a 2-fold increase in assay sensitivity. Leaf disks were pooled from each plant for the assay. The tissue was homogenized with a glass rod in a microfuge tube with 150–200 ul of extraction buffer (20% glycerol, 10% β-mercaptoethanol, 125 mM Tris-HCl pH 6.8, 100 ug/ml bromophenol blue and 0.2% SDS). Following centrifugation in a microfuge for 20 minutes, total protein was determined using the Bradford assay. 25 ug of pMON273/3111SE plant protein or 70 ug of pMON200/3111SE plant protein, supplemented with BSA, was loaded on a native polyacrylamide gel as previously described. The polyacrylamide gel was equilibrated for 30 minutes in water and then 30 minutes in reaction buffer (67 mM TRIS-maleate pH 7.1, 43 mM $MgCl_2$, 400 mM NH_4Cl), transferred onto a glass plate, and overlaid with a 1.5% agarose gel. The overlay gel contained the neomycin phosphotransferase substrates: 450 uCi [γ-^{32}] ATP and 27 ug/ml neomycin sulfate (Sigma). After 1 hour at room temperature a sheet of Whatman P81 paper, two sheets of Whatman 3MM paper, a stack of paper towels and a weight were put on top of the agarose gel. The phosphorylated neomycin is positively charged and binds to the P81 phosphocellulose ion exchange paper. After blotting overnight, the P81 paper was washed 3× in 80° C. water, followed by 7 room temperature washes. The paper was air dried and exposed to XAR-5 film. Activity was quantitated by counting the ^{32}P-radioactivity in the NPTII spot. The NPTII transcript levels and enzyme activities in two sets of transgenic petunia plants were compared. In one set of plants (pMON273) the NPTII coding sequence is preceded by the CaMV(35S) promoter and leader sequences, in the other set of plants (PMON200) the NPTII coding region is preceded by the nopaline synthase promoter and leader sequences. The data indicates the pMON273 plants contain about a 30 fold greater level of NPTII transcript than the pMON200 plants, see Table I below.

TABLE I

QUANTITATION OF NPTII TRANSCRIPT LEVELS AND NPTII ACTIVITY IN pMON273 AND pMON200 PLANTS

Plant Number	Relative NPTII Transcript[a]	Relative NPTII Activity[b]
pMON 273		
3272	682	113
3271	519	1148
3349	547	447
3350	383	650
3343	627	1539
Average	551	779
pMON 200		
2782	0	0.22
2505	0	5.8
2822	0	0
2813	34	19
2818	0	1.0
3612	45	0.33
2823	97	23
Average	19	7
	~30-fold	~110-fold

TABLE I-continued

QUANTITATION OF NPTII TRANSCRIPT LEVELS AND NPTII ACTIVITY IN pMON273 AND pMON200 PLANTS

Plant Number	Relative NPTII Transcript[a]	Relative NPTII Activity[b]
	difference	difference

[a] Numbers derived from silver grain quantitation of autoradiogram. The RNA per lane was determined by filter hybridization to a petunia small subunit gene. The NPTII transcript values obtained with the NPTII probe were normalized for the amount of RNA in each lane.

[b] Numbers represent quantitation of NPT assay. Values were obtained by scintillation counting of 32-P-NPTII spots on the PE-81 paper used in the NPT assay as previously described. Values have been adjusted for the different amounts of protein loaded on the gels (25 ug) for pMON273 and 70 ug for pMON200 plants).

Consistent with this observation is the finding that the pMON273 leaf extracts have higher NPTII enzyme activity than the pMON200 leaf extracts. In several of the transgenic plants, there is a substantial variation in both RNA and enzyme levels which cannot be accounted for by the slight difference in gene copy num-

al., 1981). The CM4-184 strain is a naturally occurring deletion mutant of strain CM1841. The references to nucleotide numbers in the following discussion are those for the sequence of CM1841 (Gardner et al., 1981). A 476 bp fragment extending from the HindIII site at bp 5372 to the HindIII site at bp 5848 was cloned into M13 mp8 for site directed mutagenesis (Zoller and Smith, 1982) to insert an XbaI (5'-TCTAGA) site immediately 5' of the first ATG translational initiation signal in the 19S transcript (Dudley et al., 1982). The resulting 400 bp HindIII-XbaI fragment was isolated and joined to the 1.3 kb XbaI-EcoRI fragment of pMON273 which carries the neomycin phosphotransferase II (NPTII) coding sequence modified so that the extra ATG translational initiation signal in the bacterial leader had been removed and the nopaline synthase 3' nontranslated region (NOS). The resulting 1.7 kb HindIII-EcoRI fragment was inserted into pMON120 between the EcoRI and HindIII sites to give pMON203. The complete sequence of the 19S promoter-NPTII leader is given below.

```
   HindIII                                                                          70
 11
 AAGCTTTAAAGCTGCAGAAAGGAATTACCACAGCAATGACAAAGAGACATTGGCGGTAATAAATACTATA
 71                                                                                140
 AAGAAATTCAGTATTTATCTAACTCCTGTTCATTTTCTGATTAGGACAGATAATACTCATTTCAAGAGTT
 141                                                                               210
 TTGTTAACCTTAATTACAAAGGAGATTCAAAACTTGGAAGAAACATCAGATGGCAAGCATGGCTTAGCCA
 211                                                                               280
 CTATTCGTTTGATGTTGAACATATTAAAGGAACCGACAACCACTTTGCGGACTTCCTTTCAAGAGAATTC
 281                                                                               350
 AATAAGGTTAATTCCTAATTGAAATCCGAAGATAAGATTCCCACACACTTGTGGCTGATATCAAAAAGGC
           TATA                                      5'  mRNA
 351        |                                         |||          402
 TACTACCTATATAAACACATCTCTGGAGACTGAGAAAATCAGACCTCCAAGC
      XbaI                 NPTII Initiator Signal
       |                    |
 TCTAGACGATCGTTTCGC ATG
```

ber. Such "position effects" have been reported in transgenic mice and fruit flies and have not yet been adequately explained at the molecular level. Although, there is not a clear correlation between insert copy number and level of chimeric gene expression, the fact that 4 of the 7 pMON200 transgenic plants contain 2 copies of the NOS-NPTII-NOS gene would suggest that the differential expression of the CaMV(35S) promoter is actually slightly underestimated in these studies.

The constructs described in this comparative example have identical coding regions and 3' non-translated regions, indicating that the differences in the steady state transcript levels of these chimeric genes is a result of the 5' sequences.

COMPARISON OF CaMV19S AND CaMV(35S) PROMOTERS

Chimeric genes were prepared comprising either the CaMV19S or CaMV(35S) promoters. As in the above example, the promoters contained their respective 5' non-translated regions and were joined to a NPTII coding sequence in which the bacterial 5' leader had been modified to remove a spurious ATG translational initiation signal. The constructs tested were pMON203 and pMON204 containing the CaMV19S/NPTII/NOS gene and pMON273 containing the CaMV(35S)/NPTII/NOS gene.

Construction of pMON203

The CaMV 19S promoter fragment was isolated from plasmid pOS-1, a derivative of pBR322 carrying the entire genome of CM4-184 as a SalI insert (Howarth et

Construction of pMON204

The 400 bp HindIII-XbaI fragment containing the CaMV19S promoter was joined to a synthetic linker with the sequence:

```
      XbaI          BglII
       |              |
5'-TCTAGACTCCTTACAACAGATCT
```

to add a BglII site to the 3' end of the promoter fragment. The HindIII-BglII fragment was joined to the 1.3 kb BglII-EcoRI fragment of pMON128 that contains the natural, unmodified NPTII coding sequence joined to the NOS 3' nontranslated signals and inserted into the EcoRI and HindIII sites of pMON120. The resulting plasmid is pMON204. The CaMV 19S promoter signals in this plasmid are identical to those in pMON203. The only difference is the sequence of the 5' nontranslated leader sequence which in pMON204 contains the extra ATG signal found in the bacterial leader of NPTII and contains extra bases from the synthetic linker and bacterial leader sequence.

Petunia leaf discs were transformed and plants regenerated as described above. The gel overlay assay was used to determine NPTII levels in transformants.

Quantitation was done by scintillation counting of 32P-neomycin, the end product of neomycin phosphotransferase activity. The average NPTII enzyme level determined for CaMV(35S) (pMON273) plants was 3.6 times higher than that determined for CaMV(19S) (pMON203 & 204) plants.

QUANTITATION OF NPTII ACTIVITY LEVELS IN pMON203, pMON204, AND pMON273 PLANTS

Construct	Plant Number	Relative NPTII Activity[a]	Average	
pMON203	4283	499,064	398,134	
pMON203	4248	297,204		
				356,203
pMON204	4275	367,580	314,273	
pMON204	4280	260,966		
pMON273	3350	1,000,674	1,302,731	
pMON273	3271	1,604,788		

$$\frac{35s}{19s} \quad \frac{1,302,731}{356,203} = 3.6$$

[a]Numbers represent quantitation of NPT assay. Values were obtained by scintillation counting of ^{32}P-NPTII spots on the PE-81 paper used in the NPT assay as previously described.

REFERENCE

F. Bolivar, *Gene* 4: 121 (1978)

F. Colbere-Garapin et al, *J. Mol. Biol.* 150: 1 (1981)

S. N. Covey, G. P. Lomonosoff and R. Hull (1981) *Nucleic Acids Res.* 9, 6735–6747.

R. Dudley et al (1982) *Virology* 117: 19.

R. T. Fraley, et al. (1983) *Proc. Natl. Acad. Sci.* USA 80:4803–4807.

R. T. Fraley, R. B. Horsch, A. Matzke, M. D. Chilton, W. S. Chilton and P. R. Sanders (1984) *Plant Molecular Biology* 3, 371–378.

A. Frank., H. Guilley, G. Joward, K. Richards and L. Hirth (1980) *Cell* 21, 285–294.

R. C. Gardner et al, *Nucleic Acids Research* Vol. 9 No. 12: 287 (1981)

G. Guilley et al, *Cell* 30: 763 (1982)

T. Hohn et al, in Gene Cloning in Organisms Other than *E. coli* , p. 193, Hofschneider and Goebel, eds. (Springer Verlag, N.Y., 1982)

A. S. Howarth et al, *Virology* 112:678 (1981)

T. Maniatis et al, Molecular Cloning—A Laboratory Manual (Cold Spring Harbor, Lab, 1982)

R. E. F. Matthews (ed.) *Plant Virology* (Academic Press, N.Y., 1970).

R. C. Mulligan et al, *Nature* 277: 108 (1979).

R. N. Rao and S. Rogers, *Gene* 7: 79 (1979).

S. Rogers et al., (1985) *Plant Mol. Rep.* 3:111.

P. J. Southern & P. Berg, *J. Mol. Appl. Gen.* 1 327 (1982).

L. Stryer, *Biochemistry,* 2nd. ed. (Freeman and Co. San Francisco, 1981).

M. Zoller et al., (1982) *Nucleic Acids Res.* 10:6487.

We claim:

1. A chimeric gene which is expressed in plant cells comprising a promoter from a cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV (35S) promoter isolated from CaMV protein-encoding DNA sequences and a CaMV (19S) promoter isolated from CaMV protein-encoding DNA sequences, and a structural sequence which is heterologous with respect to the promoter.

2. A chimeric gene of claim 1 in which the promoter is the CaMV(35S) promoter.

3. A chimeric gene of claim 1 in which the promoter is the CaMV(19S) promoter.

4. A plant cell which comprises a chimeric gene that contains a promoter from cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV (35S) promoter and a CaMV (19S) promoter, wherein said promoter is isolated from CaMV protein-encoding

DNA sequences, and a structural sequence which is heterologous with respect to the promoter.

5. A plant cell of claim 4 in which the promoter is the CaMV(35S) promoter.

6. A plant cell of claim 4 in which the promoter is the CaMV(19S) promoter.

7. An intermediate plant transformation plasmid which comprises a region of homology to an *Agrobacterium tumefaciens* vector, a T-DNA border region from *Agrobacterium tumefaciens* and a chimeric gene, wherein the chimeric gene is located between the T-DNA border and the region of homology, said chimeric gene comprising a promoter from cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV(35S) promoter and a CaMV(19S) promoter, and a structural sequence which is heterologous with respect to the promoter.

8. A plant transformation vector which comprises a disarmed plant tumor inducing plasmid of *Agrobacterium tumefaciens* and a chimeric gene, wherein the chimeric gene contains a promoter from cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV(35S) promoter and a CaMV(19S) promoter, and a structural sequence which is heterologous with respect to the promoter.

9. A plant transformation vector of claim 8 in which the promoter is the CaMV(35S) promoter.

10. A plant transformation vector of claim 8 in which the promoter is the CaMV(19S) promoter.

11. The chimeric gene of claim 1 comprising in the 5' to 3' direction:

(1) the CaMV(35S) promoter,

(2) a structural sequence encoding neomycin phosphotransferase II, and

(3) a 3' non-translated polyadenylation sequence of nopaline synthase.

12. The chimeric gene of claim 1 comprising in the 5' to 3' direction:

(1) the CaMV(19S) promoter,

(2) a structural sequence encoding neomycin phosphotransferase II, and

(3) a 3' non-translated polyadenylation sequence of nopaline synthase.

13. A DNA construct comprising:

(A) a CaMV promoter selected from the group consisting of (1) a CaMV 35S promoter isolated from CaMV protein-encoding DNA sequences and (2) a CaMV 19S promoter isolated from CaMV protein-encoding DNA sequences, and

(B) a DNA sequence of interest heterologous to (A), wherein (B) is under the regulatory control of (A) when said construct is transcribed in a plant cell.

14. A chimeric gene which is transcribed and translated in plant cells, said chimeric gene comprising a promoter from cauliflower mosaic virus, said promoter selected from the group consisting of:

a) a CaMV 35S promoter region free of CaMV protein-encoding DNA sequences and

b) a CaMV 19S promoter region free of CaMV protein-encoding DNA sequences,

and a DNA sequence which is heterologous with respect to the promoter.

15. A chimeric gene which is expressed in plants cells comprising a promoter from a cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV(35S) promoter region free of CaMV protein-encoding DNA sequences and a CaMV(19S) promoter

region free of CaMV protein-encoding DNA sequences, and a DNA sequence which is heterologous with respect to the promoter.

16. A chimeric gene which is transcribed in plants cells comprising a promoter from a cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV(35S) promoter free of CaMV protein-encoding DNA sequences and a CaMV(19S) promoter free of CaMV protein-encoding DNA sequences, a DNA sequence which is heterologous with respect to the promoter and a 3' non-translated polyadenylation signal sequence.

17. A plant cell which comprises a chimeric gene where said chimeric gene comprises a promoter from cauliflower mosaic virus, said promoter selected from the group consisting of a CaMV(35S) promoter and a CaMV(19S) promoter, wherein said promoter is free of CaMV protein-encoding DNA sequences, and a DNA sequence which is heterologous with respect to the promoter and a 3' non-translated polyadenylation signal sequence.

18. An intermediate plasmid of claim 7 in which the promoter is the CaMV(19S) promoter.

19. An intermediate plasmid of claim 7 in which the promoter is the CaMV(35S) promoter.

* * * * *