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Section 29(1)
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AUSTRALIA

*Patents Act 1990*

648036

# NOTICE OF ENTITLEMENT

(To be filed before acceptance)

I, E.I. DU PONT DE NEMOURS AND COMPANY

of 1007 MARKET STREET
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U.S.A.

being the applicant in respect of Application No. 62729/90, state the following:-

*Part 1 - Must be completed for all applications*

The person(s) nominated for the grant of the patent has entitlement from the actual inventors by virtue of an assignment.

*Part 2 - not applicable*

*Part 3 - not applicable*

*Part 4 - Must be completed for PCT applications*

The person nominated for the grant of the patent has entitlement from the applicants of the applications listed in the declaration under Article 8 of the PCT by virtue of an assignment.

The basic applications listed in the declaration under Article 8 of the PCT are the first application(s) made in a Convention country in respect of the invention.

*Part 5 - not applicable*

*Part 6 - Must be completed if the application relates to a microorganism and relies on Section 6 of the Act*

The person nominated for the grant of the patent is the depositor of the deposits as listed hereafter

Deposit List:

| | |
|---|---|
| Deposit No.: | ATCC-67780 |
| Deposit Institution: | American Type Culture Collection |
| Deposit Date: | 30 August 1988 |

| | |
|---|---|
| Deposit No.: | ATCC-67781 |
| Deposit Institution: | American Type Culture Collection |
| Deposit Date: | 30 August 1988 |

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| Deposit No.: | ATCC-67992 |
| Deposit Institution: | American Type Culture Collection |
| Deposit Date: | 25 May 1989 |
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| Deposit Date: | 25 May 1989 |
| Deposit No.: | ATCC-67994 |
| Deposit Institution: | American Type Culture Collection |
| Deposit Date: | 25 May 1989 |
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| Deposit No.: | ATCC-68085 |
| Deposit Institution: | American Type Culture Collection |
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| Deposit Institution: | American Type Culture Collection |
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| Deposit No.: | ATCC-68214 |
| Deposit Institution: | American Type Culture Collection |
| Deposit Date: | 31 January 1990 |

*Part 7 - not applicable*

GRIFFITH HACK & CO.

Vivien Lantz

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Patent Attorneys for and
on behalf of the applicants

-6 JAN 1994

.....

Date

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(12) PATENT ABRIDGMENT (11) Document No. AU-B-62729/90
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 648036

(54) Title
EXPRESSION OF HERBICIDE METABOLIZING CYTOCHROMES P450

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(57) Claim

1. A fragment of DNA comprising a 2.4 kb BamHI fragment of DNA sequence encoding cytochrome P450 enzyme P450SU1 which causes metabolism of herbicide compounds when transformed into bacteria or plants.

3. A fragment of DNA of Claim 1 comprising a 2.4 kb BamHI fragment of DNA encoding enzyme P450SU1 and iron sulfur protein FeS-B isolated from S. griseolus.

14. A sequence of DNA encoding cytochrome P450 enzyme P450SU1 of Claim 1 comprising

```
130                 150                 170
ATGACCGATACCGCCACGACGCCCCAGACCACGGACGCACCCGCCTTCCCGAG
--+---------+---------+---------+---------+---------+
TACTGGCTATGGCGGTGCTGCGGGGTCTGGTGCCTGCGTGGGCGGAAGGGCTC
MetThrAspThrAlaThrThrProGlnThrThrAspAlaProAlaPheProSe
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.../2

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      190                 210                 230
CAACCGGAGCTGTCCCTACCAGTTACCGGACGGCTACGCCCAGCTCCGGGACACCCCCGG
---------+---------+---------+---------+---------+---------+
GTTGGCCTCGACAGGGATGGTCAATGGCCTGCCGATGCGGGTCGAGGCCCTGTGGGGGCC
rAsnArgSerCysProTyrGlnLeuProAspGlyTyrAlaGlnLeuArgAspThrProGl

      250                 270                 290
CCCCCTGCACCGGGTGACGCTCTACGACGGCCGTCAGGCGTGGGTGGTGACCAAGCACGA
---------+---------+---------+---------+---------+---------+
GGGGGACGTGGCCCACTGCGAGATGCTGCCGGCAGTCCGCACCCACCACTGGTTCGTGCT
yProLeuHisArgValThrLeuTyrAspGlyArgGlnAlaTrpValValThrLysHisGl

      310                 330                 350
GGCCGCGCGCAAACTGCTCGGCGACCCCCGGCTGTCCTCCAACCGGACGGACGACAACTT
---------+---------+---------+---------+---------+---------+
CCGGCGCGCGTTTGACGAGCCGCTGGGGGCCGACAGGAGGTTGGCCTGCCTGCTGTTGAA
uAlaAlaArgLysLeuLeuGlyAspProArgLeuSerSerAsnArgThrAspAspAsnPh

      370                 390                 410
CCCCGCCACGTCACCGCGCTTCGAGGCCGTCCGGGAGAGCCCGCAGGCGTTCATCGGCCT
---------+---------+---------+---------+---------+---------+
GGGGCGGTGCAGTGGCGCGAAGCTCCGGCAGGCCCTCTCGGGCGTCCGCAAGTAGCCGGA
eProAlaThrSerProArgPheGluAlaValArgGluSerProGlnAlaPheIleGlyLe

      430                 450                 470
GGACCCGCCCGAGCACGGCACCCGGCGGCGGATGACGATCAGCGAGTTCACCGTCAAGCG
---------+---------+---------+---------+---------+---------+
CCTGGGCGGGCTCGTGCCGTGGGCCGCCGCCTACTGCTAGTCGCTCAAGTGGCAGTTCGC
uAspProProGluHisGlyThrArgArgArgMetThrIleSerGluPheThrValLysAr

      490                 510                 530
GATCAAGGGCATGCGCCCCGAGGTCGAGGAGGTGGTGCACGGCTTCCTCGACGAGATGCT
---------+---------+---------+---------+---------+---------+
CTAGTTCCCGTACGCGGGGCTCCAGCTCCTCCACCACGTGCCGAAGGAGCTGCTCTACGA
gIleLysGlyMetArgProGluValGluGluValValHisGlyPheLeuAspGluMetLe

      550                 570                 590
GGCCGCCGGCCCGACCGCCGACCTGGTCAGTCAGTTCGCGCTGCCGGTGCCCTCCATGGT
---------+---------+---------+---------+---------+---------+
CCGGCGGCCGGGCTGGCGGCTGGACCAGTCAGTCAAGCGCGACGGCCACGGGAGGTACCA
uAlaAlaGlyProThrAlaAspLeuValSerGlnPheAlaLeuProValProSerMetVa

      610                 630                 650
GATCTGCCGACTCCTCGGCGTGCCCTACGCCGACCACGAGTTCTTCCAGGACGCGAGCAA
---------+---------+---------+---------+---------+---------+
CTAGACGGCTGAGGAGCCGCACGGGATGCGGCTGGTGCTCAAGAAGGTCCTGCGCTCGTT
lIleCysArgLeuLeuGlyValProTyrAlaAspHisGluPhePheGlnAspAlaSerLy
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      670                 690                 710
GCGGCTGGTGCAGTCCACGGACGCGCAGAGCGCGCTCACCGCGCGGAACGACCTCGCGGG
---------+---------+---------+---------+---------+---------+
CGCCGACCACGTCAGGTGCCTGCGCGTCTCGCGCGAGTGGCGCGCCTTGCTGGAGCGCCC
sArgLeuValGlnSerThrAspAlaGlnSerAlaLeuThrAlaArgAsnAspLeuAlaGl

      730                 750                 770
TTACCTGGACGGCCTCATCACCCAGTTCCAGACCGAACCGGGCGCGGGCCTGGTGGGCGC
---------+---------+---------+---------+---------+---------+
AATGGACCTGCCGGAGTAGTGGGTCAAGGTCTGGCTTGGCCCGCGCCCGGACCACCCGCG
yTyrLeuAspGlyLeuIleThrGlnPheGlnThrGluProGlyAlaGlyLeuValGlyAl

      790                 810                 830
TCTGGTCGCCGACCAGCTGGCCAACGGCGAGATCGACCGTGAGGAACTGATCTCCACCGC
---------+---------+---------+---------+---------+---------+
AGACCAGCGGCTGGTCGACCGGTTGCCGCTCTAGCTGGCACTCCTTGACTAGAGGTGGCG
aLeuValAlaAspGlnLeuAlaAsnGlyGluIleAspArgGluGluLeuIleSerThrAl

      850                 870                 890
GATGCTGCTCCTCATCGCCGGCCACGAGACCACGGCCTCGATGACCTCCCTCAGCGTGAT
---------+---------+---------+---------+---------+---------+
CTACGACGAGGAGTAGCGGCCGGTGCTCTGGTGCCGGAGCTACTGGAGGGAGTCGCACTA
aMetLeuLeuLeuIleAlaGlyHisGluThrThrAlaSerMetThrSerLeuSerValIl

      910                 930                 950
CACCCTGCTGGACCACCCCGAGCAGTACGCCGCCCTGCGCGCCGACCGCAGCCTCGTGCC
---------+---------+---------+---------+---------+---------+
GTGGGACGACCTGGTGGGGCTCGTCATGCGGCGGGACGCGCGGCTGGCGTCGGAGCACGG
eThrLeuLeuAspHisProGluGlnTyrAlaAlaLeuArgAlaAspArgSerLeuValPr

      970                 990                1010
CGGCGCGGTGGAGGAACTGCTCCGCTACCTCGCCATCGCCGACATCGCGGGCGGCCGCGT
---------+---------+---------+---------+---------+---------+
GCCGCGCCACCTCCTTGACGAGGCGATGGAGCGGTAGCGGCTGTAGCGCCCGCCGGCGCA
oGlyAlaValGluGluLeuLeuArgTyrLeuAlaIleAlaAspIleAlaGlyGlyArgVa

     1030                1050                1070
CGCCACGGCGGACATCGAGGTCGAGGGGCACCTCATCCGGGCCGGCGAGGGCGTGATCGT
---------+---------+---------+---------+---------+---------+
GCGGTGCCGCCTGTAGCTCCAGCTCCCCGTGGAGTAGGCCCGGCCGCTCCCGCACTAGCA
lAlaThrAlaAspIleGluValGluGlyHisLeuIleArgAlaGlyGluGlyValIleVa

     1090                1110                1130
CGTCAACTCGATAGCCAACCGGGACGGCACGGTGTACGAGGACCCGGACGCCCTCGACAT
---------+---------+---------+---------+---------+---------+
GCAGTTGAGCTATCGGTTGGCCCTGCCGTGCCACATGCTCCTGGGCCTGCGGGAGCTGTA
lValAsnSerIleAlaAsnArgAspGlyThrValTyrGluAspProAspAlaLeuAspI
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      1150                1170                1190
CCACCGCTCCGCGCGCCACCACCTCGCCTTCGGCTTCGGCGTGCACCAGTGCCTGGGCCA
---------+---------+---------+---------+---------+---------+
GGTGGCGAGGCGCGCGGTGGTGGAGCGGAAGCCGAAGCCGCACGTGGTCACGGACCCGGT
eHisArgSerAlaArgHisHisLeuAlaPheGlyPheGlyValHisGlnCysLeuGlyGl

      1210                1230                1250
GAACCTCGCCCGGCTGGAGCTGGAGGTCATCCTCAACGCCCTCATGGACCGCGTCCCGAC
---------+---------+---------+---------+---------+---------+
CTTGGAGCGGGCCGACCTCGACCTCCAGTAGGAGTTGCGGGAGTACCTGGCGCAGGGCTG
nAsnLeuAlaArgLeuGluLeuGluValIleLeuAsnAlaLeuMetAspArgValProTh

      1270                1290                1310
GCTGCGACTGGCCGTCCCCGTCGAGCAGTTGGTGCTGCGGCCGGGTACGACGATCCAGGG
---------+---------+---------+---------+---------+---------+
CGACGCTGACCGGCAGGGGCAGCTCGTCAACCACGACGCCGGCCCATGCTGCTAGGTCCC
rLeuArgLeuAlaValProValGluGlnLeuValLeuArgProGlyThrThrIleGlnGl

      1330                1350
CGTCAACGAACTCCCGGTCACCTGGTGA
---------+---------+--------
GCAGTTGCTTGAGGGCCAGTGGACCACT
yValAsnGluLeuProValThrTrpEnd
```

and any mutant or derivative of said sequence which causes metabolism of herbicide compounds when transformed into bacteria or plants.

OPI DATE 08/04/91 APPLN. ID 62729 / 90

PCT AOJP DATE 16/05/91 PCT NUMBER PCT/US90/04785

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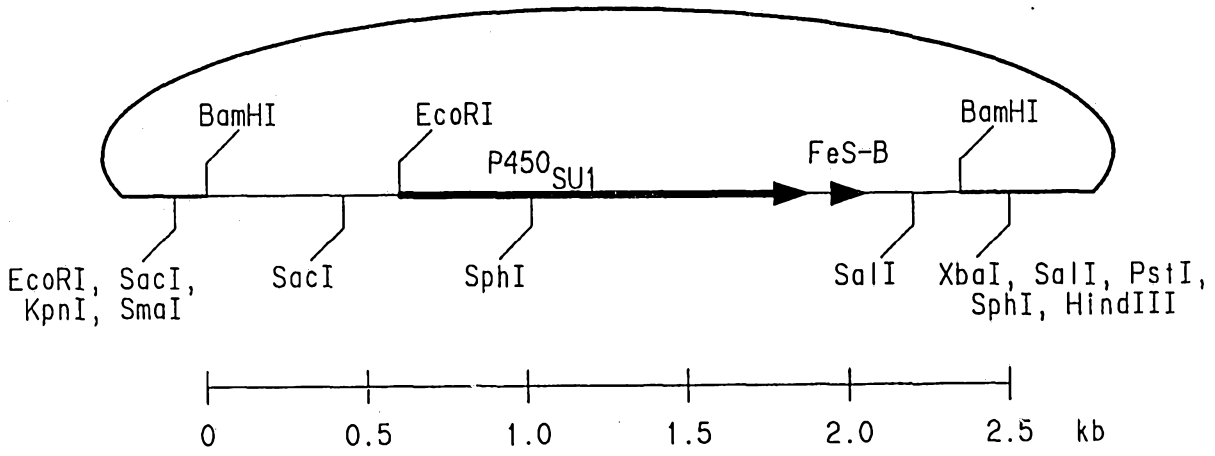
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(54) Title: EXPRESSION OF HERBICIDE METABOLIZING CYTOCHROMES P450




(57) Abstract

DNA sequences encoding herbicide metabolizing cytochrome P450 enzymes and iron-sulfur proteins that donate electrons to these enzymes, were introduced into plants and microorganisms rendering them able to produce the encoded gene products and to metabolize herbicides.

* See back of page

TITLE

EXPRESSION OF HERBICIDE METABOLIZING CYTOCHROMES P450

CROSS-REFERENCE TO RELATED APPLICATION

This is a continuation-in-part of U.S. Serial No. 07/464,499 filed January 12, 1990, which is a continuation-in-part of U.S. Serial No. 07/405,605 filed September 11, 1989.

FIELD OF THE INVENTION

The invention relates to the introduction of DNA sequences from Streptomyces griseolus into plants and microorganisms so that the recipient organisms produce the protein products of those genes and are thereby rendered capable of metabolizing the herbicide. These DNA sequences comprise those encoding herbicide metabolizing cytochromes P450 and iron-sulfur proteins that donate electrons to these cytochromes P450

BACKGROUND OF THE INVENTION

The use of herbicides in weed control is a widely accepted agricultural practice. Our understanding of herbicide metabolism and degradation is still in its infancy and is being actively investigated. Soil microorganisms were implicated in the degradation of herbicides by Joshi et al., Weed Sci. 33: 888-893, 1985. Sulfonylurea herbicides were shown to be co-metabolized by the soil bacterium Streptomyces griseolus by Romesser et al., Abstr. Ann. Mtg. Am. Soc. Microbiol. p. 248, 1985. Further study, as disclosed by Leto et al., Plant Physiol. 805: 5347 (1986) and Romesser et al., Biochem. Biophys. Res. Comm. 140: 650-659 (1986) showed that two cytochrome P450 enzymes designated P450SU1 and P450SU2, heme containing proteins of about 45,000 molecular weight, are synthesized in cells of the

bacterium Streptomyces griseolus when they are grown in a medium containing any of several herbicides. The synthesis of these proteins by S. griseolus is detectable by UV/vis difference spectroscopy as described by Romesser, et al., Biochem. Biophys. Res. Comm. 140: 650-659 (1986), analytical anion exchange and gel filtration chromatography as described by O'Keefe et al., Plant Physiol. 805: 5347 (1986) and LDS gel electrophoresis as described by Leto et al., Plant Physiol. 805: 5348 (1986). Romesser et al., Biochem. Biophys. Res. Comm. 140: 650-659 (1986) and O'Keefe et al., Recent Advances in Phytochemistry 21: 151-173 (1987), correlated the presence of P450 enzymes with the ability of this organism to carry out a variety of metabolic reactions on a number of sulfonylurea herbicides. Further, as discussed by Romesser et al., Biochem. Biophys. Res. Comm. 140: 650-659, 1986, crude cell-free extracts from S. griseolus exhibit sulfometuron methyl (10010) hydroxylase activity only when they are from cells grown in the presence of certain sulfonylureas, and difference spectra of the extracts resulting when chlorsulfuron (10013) or sulfometuron methyl (10010) is added suggest that the newly appearing cytochromes P450 bind to these compounds in a manner similar to substrate binding to cytochrome P450.

Additionally, genes that cause the breakdown of the active moieties of herbicidal compounds may be incorporated in plants and cause said plants to become resistant to the affected herbicide. Stalker et al., Science 242: 419-422 (1988) describe the transfer of the gene from Klebsiella ozaenae encoding a specific nitrilase that converts the herbicide bromoxynil to metabolite 3,5-dibromo-4-hydroxybenzoic acid into tobacco plants with the result that the tobacco plants became resistant to bromoxynil.

The major objects of the invention described here are the DNA sequences encoding the two cytochromes P450. Other objects are the sequences encoding their iron-sulfur protein electron donors. These sequences of this invention are from the bacterium Streptomyces griseolus ATCC11796. These two cytochromes P450 are capable of metabolizing sulfonylurea compounds and other herbicides. The two cytochromes P450 have been designated P450SU1 and P450SU2, and the two iron-sulfur proteins have been designated FeS-A and FeS-B.

In wild type Streptomyces griseolus, expression of cytochromes P450SU1, P450SU2, and iron-sulfur proteins FeS-A and FeS-B is induced by the addition of sulfonylurea compounds. Although many sulfonylurea compounds may be metabolized by these cytochromes P450, not all are good inducers of these proteins. Thus optimal metabolism of many sulfonylurea compounds by wild type organisms can only be achieved by first inducing the cytochromes P450 and iron-sulfur proteins with a sulfonylurea known to be a good inducer. Organisms producing the P450 enzymes constitutively or as a result of exposure to light would obviate the need for inducing organisms with sulfonylureas to make them capable of metabolizing said sulfonylureas.

Thus, another object of this invention is to obviate the need to induce the herbicide metabolizing cytochromes P450 and their iron-sulfur protein electron donors in organisms (bacteria and plants) by transforming said organisms with the genes of the herbicide metabolizing cytochromes P450 and where necessary, their iron-sulfur protein electron donors contained in plasmids which permit the constitutive

or light induced expression of the P450 enzymes and, where necessary, the iron sulfur proteins in the transformed organisms. Said transformed organisms are able to metabolize herbicides, both good and poor inducers, whenever they encounter them.

Typical cytochrome P-450 monooygenase systems from bacteria are similar to the P-450 CAM system from Pseudomonas putida (Sligar et al. in: Cytochrome P-450 Structure, Mechanism and Biochemistry, Ortiz de Montellano, ed. Plenum Press, NY (1986) pp. 429-504). This system is comprised of a flavoprotein reductase (putidaredoxin reductase), a low molecular weight iron-sulfur protein (putidaredoxin) and the cytochrome P-450 (P-450 CAM). This system of proteins functions to transfer reducing equivalents from a reduced pyridine nucleotide sequentially from putidaredoxin reductase, to putidaredoxin and then to P-450 CAM. It is important to note, however, that the specificity of the enzyme system for substrate resides solely on the P-450 protein, and that the reductase and iron sulfur proteins are only important insofar as they provide the reducing equivalents to the P-450 necessary for catalysis. Thus, another object of this invention is to place the genes for sulfonylurea or herbicide metabolism in other organisms in such a way as to utilize existing sources of reducing equivalents in these organisms to facilitate the function of the cytochrome P-450.

## SUMMARY OF THE INVENTION

The bacterium, Streptomyces griseolus, contains two inducible genes which produce certain P450 enzymes which metabolize herbicidal compounds. The two enzymes are called P450SU1 and P450SU2. It is

known that these enzymes operate effectively only when certain iron sulfur proteins are available and when reductase proteins capable of donating electrons to the iron-sulfur proteins are available. Genes for iron sulfur proteins in Streptomyces griseolus are located adjacent to and downstream of those for the P450 enzymes. The applicants have isolated the DNA sequences from S. griseolus which encode the P450 enzymes P450SU1 and P450SU2 and adjacent iron sulfur proteins, FeS-B and FeS-A. It has been found that either iron-sulfur protein FeS-A or FeS-B can transfer reducing equivalents to either enzyme. The DNA sequence comprising that for P450SU1 plus adjacent iron sulfur protein FeS-B is as detailed on pages 27 to 31 hereinafter starting at base pair number 128 and ending at base pair number 1578. The DNA sequence comprising that for P450SU2 plus its adjacent iron sulfur protein FeS-A is as detailed on pages 32 to 36 hereinafter starting at base pair number 195 and ending at base pair number 1646. The applicants have constructed novel plasmids comprising the DNA sequences for P450SU1 plus FeS-B (i.e., pCAO400, pCAO401, pCAO200SU1#12, pCAO200SU1-FeS-B#9 or pPAT108) and P450SU2 plus FeS-A (i.e., pCAO200-SU2-FeS-A#11 or pCS325) which can transform bacteria. Bacteria, preferably bacteria of the genus Streptomyces and most preferably Streptomyces lividans transformed with a plasmid selected from pCAO400, pCAO401, pCAO200SU1-FeS-B#9, or pPAT108 all comprising the DNA sequence encoding P450SU1 plus FeS-B produce the P450SU1 constitutively and metabolize herbicidal sulfonylurea compounds even though no iron-sulfur protein reductase gene has been introduced into these cells. Bacteria transformed

with the plasmid pCAO200SU2-FeS-A#11 or pCS325, comprising the DNA sequence encoding P450SU2 plus the iron sulfur protein FeS-A produce P450SU2 constitutively and can also metabolize herbicidal sulfonylurea compounds even though no iron-sulfur protein reductase gene has been added.

Another embodiment of this invention is a method for the preparation of metabolites of herbicide compounds comprising incubating sulfonylurea or other herbicide compounds with cultures of bacteria, preferably bacteria of the genus Streptomyces transformed with a plasmid selected from pCAO400, pCAO401, pCAO200SU1-FeS-B#9, pCAO200SU2-FeS-A#11, pPAT108 or pCS325.

Still another embodiment of this invention is a method for protecting plants in soil containing inhibitory amounts of herbicidal compounds comprising soaking seedlings of plants in cultures of bacteria, preferably bacteria of the genus Streptomyces and most preferably Streptomyces lividans transformed with a plasmid selected from pCAO400, pCAO401, pCAO200SU1-FeS-B#9 or pCAO200SU2-FeS-A#11 prior to transplanting the seedlings in the soil. A further embodiment of this invention is the bacteria coated seeds.

And another embodiment of this invention is the transformation of plants, in particular those of horticultural or agronomic utility, with these genes to make them capable of metabolizing sulfonylurea herbicides. For this purpose plasmids (i.e., pSU18, pSSU-SU111, pSSU-SU121, pCab-SU111, pCab-SU121, and pCab-SU131, pSuFe11, pSuFe21, pSuFe31 and pSuFe41) utilizing a fragment comprising sequences encoding P450SU1 and/or FeS-B with certain other DNA sequences

preceding and following the P450SU1 and/or FeS-B sequence have been engineered to transform plants with these genes. This may result in making said transformed plants susceptible to chemicals which lack, or contain only weakly, herbicidal activity by means of metabolizing the chemicals to compounds exhibiting greater plant toxicity.

Metabolism of herbicides by transformed plants can make them resistant to said herbicides and reduce the buildup of herbicide residues in the plant. Cytochrome P450-mediated metabolism of sulfonylureas from a less toxic to a more toxic form results in a conditionally lethal phenotype and could possibly be used for applications of tissue specific killing or for selection of events which disrupt gene expression.

Such transformed plants can include plants containing other mutant genes prior to their transformation with the P450SU1 or P450SU2 genes. Of particular interest are plants containing a mutant acetolactate synthase enzyme which prevents or decreases inhibition. This enzyme catalyses the first reaction in the synthesis of the amino acids valine, leucine, and isoleucine in plants and microorganisms. It is known that this enzyme in a variety of plants and microorganisms is quite sensitive to inhibition by sulfonylureas. It is theorized that transformed plants containing both mutant acetolactate synthase enzymes which decrease or prevent inhibition of the enzyme by sulfonylureas or other herbicides and P450 cytochrome enzymes enabling plants to metabolize herbicides would possibly result in plants showing even greater resistance to a wider variety of sulfonylurea

compounds than that seen in plants containing mutant acetolactate synthase alone.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a physical map, showing restriction endonuclease sites, of plasmid pUC18-SU1-BamHI.

Figures 2A and 2B are each a physical map, showing restriction endonuclease sites, of plasmids pCAO400 and pCAO401 respectively.

Figures 3A and 3B are each a physical map, showing restriction endonuclease sites, of plasmids pCAO200SU1-FeS-B#9 and pCAO200SU1#12 respectively.

Figure 4 is a physical map, showing restriction endonuclease sites, of plasmid pUC19-SU2-8.

Figure 5 shows Western blots as follows:

lane 1, 50 ng of purified cytochrome P450SU1;

lane 2, blank;

lane 3, protein from S. lividans C37 grown with no sulfonylurea;

lane 4, protein from S. lividans C37 induced for six hours with 120 ppm of 10001;

lane 5, S. lividans transformed with pCAO400 grown with no sulfonylurea;

lane 6, S. lividans transformed with pCAO400 induced for six hours with 120 ppm of 10001;

lane 7, 500 ng of purified cytochrome P450SU1.

Figure 6 shows Western blots as follows:

lanes 1, protein extract of S. lividans transformed with pCAO401 and induced with 120 ppm of 10001 for 24 hours;

lane 2, protein extract of S. lividans transformed with pCAO401 and induced with 120 ppm of 10001 for 6 hours;

lane 3, protein extract of S. lividans transformed with pCAO401 and induced with 120 ppm of 10001 for 3 hours;

lane 4, protein extract of S. lividans transformed with pCAO401 and grown for 24 hours;

lane 5, protein extract from S. lividans transformed with pCAO400 and induced with 120 ppm of 10001 for 24 hours;

lane 6, protein extract of S. lividans transformed with pCAO400 and induced with 120 ppm of 10001 for 6 hours;

lane 7, protein extract of S. lividans transformed with pCAO400 and induced with 120 ppm of 10001 for 3 hours;

lane 8, protein extract of S. lividans transformed with pCAO400 and grown for 24 hours;

lane 9, 100 ng of purified cytochrome P450SU1.

Figure 7 shows Western blots as follows:

lane 1, 100 ng of purified cytochrome P450SU1;

lane 2, 200 ng of purified cytochrome P450SU1;

lane 3, extracts of S. lividans transformed with pCAO200SU1#12;

lane 4, extracts of S. lividans transformed with pCAO200SU1-FeS-B#9.

Figure 8 is a physical map, showing restriction endonuclease sites, of pCAO200SU2-FeS-A#11.

Figure 9 shows Western blots as follows:

lane 1, 100 ng of purified cytochrome P450SU2;

lane 2, extract (30 µg of protein) of S. lividans transformed with pCAO200SU2-FeS-A#11 grown without sulfonylurea;

lane 3 extract (30 µg of protein) of S. lividans transformed with pCAO200SU1#12 grown without sulfonylurea;

lane 4 extract (30 µg of protein) of S. lividans transformed with pCAO200SU1-FeS-B#9 grown without sulfonylurea.

Figure 10A is a physical map showing restriction endonuclease sites of pSU17.

Figure 10B is a physical map showing restriction endonuclease sites of pSSU-SU11.

Figure 10C is a physical map showing restriction endonuclease sites of pSSU-SU12.

Figure 10D is a physical map showing restriction endonuclease sites of pCab-SU11.

Figure 10E is a physical map showing restriction endonuclease sites of pCab-SU12.

Figure 10F is a physical map showing restriction endonuclease sites of pCab-SU13.

Figure 11 depicts the N-dealkylation of 10015 to 10014.

Figure 12A is the UV Absorbance Spectra for 10015 and 10014 standards.

Figure 12B is the UV Absorbance Spectra for 10015 extracted from leaf and metabolite extracted from leaf.

Figure 13A depicts the metabolism of 10001 by tissues of transformed tobacco leaves to 10003 and 10002.

Figure 13B depicts the disappearance over time of 10001.

Figure 13C depicts the appearance over time of 10003.

Figure 13D depicts the appearance over time of 10002.

Figure 14 depicts the appearance of transformed and nontransformed tobacco plants 22 days after spraying with 10015.

Figure 15A is a physical map showing restriction endonuclease sites of plasmid pSuFe1.

Figure 15B is a physical map showing restriction endonuclease sites of plasmid pSuFe2.

Figure 15C is a physical map showing restriction endonuclease sites of plasmid pSuFe3.

Figure 15D is a physical map showing restriction endonuclease sites of plasmid pSuFe4.

In Figures 15A through 15D, H3 represents HindIII, BMI represents BamHI, NC1 represents NcoI, RI represents EcoRI, and BG2 represents BglII.

Figure 16A is a physical map showing restriction endonuclease sites of plasmid pPAT108.

Figure 16B is a physical map showing restriction endonuclease sites of plasmid pCS325.

Figures 17A to 17D are diagrams showing the construction of plasmid pSU17.

Figures 18A to 18D are diagrams showing the construction of plasmid pSUFe1.

Figures 19A and 19B are diagrams showing the construction of plasmids pSSU-SU11 and pSSU-SU12.

Figures 20A to 20C are diagrams showing the construction of plasmids pCab-SU11, pCab-SU12 and pCab-SU13.

Figures 21A to 21D are diagrams showing the construction of plasmids pSUFe3 and pSUFe4.

Figures 22A and 22B are diagrams showing the construction of plasmid pSUFe2.

Figures 23A and 23B are diagrams showing the construction of plasmids pAGS501 and pAGS502.

Figures 24A through 24D are diagrams showing the construction of plasmid pZ596.

Figure 25A is a physical map showing restriction endonuclease sites of plasmid pZ6A-SU1.

Figure 25B is a physical map showing restriction endonuclease sites of plasmid pZ6AT-SU1.

In Figures 17 through 22, P450SU1 represents the coding sequence for the cytochrome P450SU1 enzyme; CaMV35Sp is the 35S promoter of CaMV; Cab2215' is the 5' untranslated sequence of petunia gene for Cab22L; SSU301 is the coding sequence for the petunia gene for SSU; SSU3' is the 3' untranslated sequence of the petunia gene for SSU301; SSUp is the promoter of the petunia SSU301 gene; SSU-T is the sequence coding for the chloroplast transit peptide of the petunia SSU301 protein; SSU-M is the sequence coding for the mature petunia SSU301 protein; Cabp is the promoter of the petunia Cab22L gene; CabT is the sequence coding chloroplast transit; CabM is the sequence coding mature protein of petunia Cab22L protein; FeS-B is the sequence coding for FeS-B; and nos 3' is the 3' untranslated region from nopaline synthase gene. In Figures 23A and 23B, AMP means ampicillin resistance in bacteria, LB means left border of T-DNA, RB denotes right border of T-DNA, and NPT denotes kanamycin resistance in plants.

## DETAILS OF THE INVENTION

Definitions:

PIPES:piperazine-N, N'-bis(2-ethanesulfonic acid)

MOPS: 3-(N-morpholino)propanesulfonic acid

ATCC: American Type Culture Collection

depository located at 12301 Parklawn Drive, Rockville, Maryland 20852.

HPLC: high performance liquid chromatography

UV: ultraviolet light

Cytochromes P450SU1 and P450SU2: the names assigned to the two cytochrome P450 enzymes of this invention.

FeS-A and FeS-B: the names assigned to the two iron-sulfur proteins of this invention.

10001: N-[(4-chloro-6-methoxy-pyrimidin-2-yl)-aminocarbonyl]-2-ethoxycarbonylbenzenesulfonamide

10002: N-[(4-chloro-6-hydroxy-pyrimidin-2-yl)-aminocarbonyl]-2-ethoxycarbonylbenzenesulfonamide

10003: N-[(4-chloro-6-methoxy-pyrimidin-2-yl)-aminocarbonyl]-2-carboxybenzenesulfonamide

10004: 2-butyl-2,3-dihydro-N-[(4,6-dimethoxy-pyrimidin-2-yl)aminocarbonyl-1,2-benzisothiazole-7-sulfonamide-1,1-dioxide

10005: 2-(3-hydroxybutyl)-2,3-dihydro-N-[(4,6-dimethoxypyrimidin-2-yl)aminocarbonyl]-1,2-benziso-thiazole-7-sulfonamide-1,1-dioxide

10006: N-[(4-methoxy-6-methyl-1,3,5-triazin-yl)aminocarbonyl]-2-methoxycarbonylbenzenesulfonamide

10007: N-[(4-hydroxy-6-methyl-1,3,5-triazin-yl)aminocarbonyl]-2-methoxycarbonylbenzenesulfonamide

10008: N-[(4-methoxy-6-methyl-1,3,5-triazin-yl)aminocarbonyl]-2-carboxybenzenesulfonamide

10009: N-[(4-methoxy-6-hydroxymethyl-1,3,5-triazin-yl)aminocarbonyl]-2-methoxycarbonylbenzene-sulfonamide

10010: N-[(4,6-dimethylpyrimidin-2-yl)amino-carbonyl]-2-methoxycarbonylbenzenesulfonamide

10011: N-[(4-hydroxymethyl-6-methyl-pyrimidin-2-yl)aminocarbonyl]-2-methoxycarbonylbenzenesulfon-amide

10012: N-[(4-carboxy-6-methylpyrimidin-2-yl)aminocarbonyl]-2-methoxycarbonylbenzenesulfonamide

10013: N-[(4-methoxy-6-methyl-1,3,5 triazin-yl)aminocarbonyl]-2-chlorobenzenesulfonamide

10014: 2,3-dihydro-N-[(4,6-dimethoxypyrimidin-2-yl)aminocarbonyl]-1,2-benzisothiazol-7-sulfonamide-1,1-dioxide

10015: 2-methylethyl-2,3-dihydro-N-[(4,6-dimethoxypyrimidin-2-yl)aminocarbonyl]-1,2-benziso-thiazole-7-sulfonamide-1,1-dioxide

10016: N-[(4-methoxy-6-methylpyrimidin-2-yl)-aminocarbonyl]-5-dimethylamino-1-napthalenesulfonamide

10017: 3-cyclohexyl-1-methyl-6-dimethylamino-S-triazine-2,4(1H,3H)dione

10018: 4-amino-6-tert-butyl-3-(methylthio)-AS-triazin-5(4H)-one

10019: 3-(3-chloro-p-tolyl)-1,1-dimethylurea

10020: 7-chloro-5-fluoro-4-(2,3,4,5,6,7-hexa-hydro-1,3-dioxo-1H-isoindol-2-yl)-2,3-dihydro-2-benzo-furancarboxylic acid, methyl ester

10021: 2-[4-chloro-6-(ethylamino-1,3,5-triazin-2-yl)amino]-2-methylpropanenitrile

10022: 1-methyl-2(1H)-pyrimidinone

10023: 3,5-dibromo-4-hydroxybenzonitrile

10024: N-[(4,6-dimethoxypyrimidin-2-yl)amino-carbonyl]-2-ethyl-2,3-dihydro-1,2-benzisothiazole-7-sulfonamide-1,1-dioxide

10025: N-[(4,6-dimethoxypyrimidin-2-yl)amino-carbonyl]2,3-dihydro-2-(phenylmethyl)-1,2-benziso-thiazole-7-sulfonamide-1,1-dioxide

10026: N-[(4,6-dimethoxypyrimidin-2-yl)amino-carbonyl]-2-(2-fluoroethyl)-2,3-dihydro-1,2-benziso-thiazole-7-sulfonamide-1,1-dioxide

10027: N-[(4,6-dimethoxypyrimidin-2-yl)amino-carbonyl]-2,3-dihydro-2-propyl-1,2-benzisothiazole-7-sulfonamide-1,1-dioxide

10028: N-[(4,6-dimethoxypyrimidin-2-yl)amino-carbonyl]-2,3-dihydro-2-(2-propenyl)-1,2-benziso-thiazole-7-sulfonamide-1,1-dioxide

10029: N-[(4,6-dimethoxypyrimidin-2-yl)amino-carbonyl]-2-methyl-1,2-benzisothiazole-7-sulfonamide-1,1-dioxide

10030: N-[(4,6-dimethoxypyrimidin-2-yl)amino-carbonyl]-2,3-dihydro-2-(2-methylpropyl)-1,2-benz-isothiazole-7-sulfonamide-1,1-dioxide

10031: 2-acetyl-N-[(4,6-dimethoxypyrimidin-2-yl)aminocarbonyl]-2,3-dihydro-1,2-benzisothiazole-7-sulfonamide-1,1-dioxide

10032: N-[(4,6-dimethoxypyrimidin-2-yl)amino-carbonyl]-2,3-dihydro-2-(trimethylsilylmethyl)-1,2-benzisothiazole-7-sulfonamide-1,1-dioxide

10033: N-(2-chloro-6-methylphenyl)-5,7-dimethyl-1,2,4-triazolo-1,5A-pyrimidine-2-sulfonamide

10034: 2-[(4,5-dihydro-4-methyl-4-(1-methyl-ethyl)-1H-imidazol-2-yl)1-5-ethyl-3-pyridine-carboxylic acid

10035: 2-[4,5-dihydro-4-methyl-4-(1-methyl-ethyl)-5-oxo-1H-imidazol-2-yl]-3-quinolinecarboxylic acid

10036: N-(2,6-dichlorophenyl)-4,6-dimethyl-2-pyrimidinesulfonamide

In the context of this disclosure, a number of terms shall be utilized. As used herein, the terms "promoter" and "promoter region" refer to a sequence of DNA, usually upstream (5') to the protein coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at the correct

site. Promoter sequences are necessary but not always sufficient to drive the expression of the gene.

A "fragment" constitutes a fraction of the DNA sequence of the particular region.

"Nucleic acid" refers to a molecule which can be single stranded or double stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. In bacteria in higher plants, "deoxyribonucleic acid" (DNA) refers to the genetic material while "ribonucleic acid" (RNA) is involved in the translation of the information from DNA into proteins.

"Regulation" and "regulate" refer to the modulation of gene expression controlled by DNA sequence elements located primarily, but not exclusively upstream of (5' to) the transcription start of a gene. Regulation may result in an all or none response to a stimulation, or it may result in variations in the level of gene expression.

The term "coding sequence" refers to that portion of a gene encoding a protein, polypeptide, or a portion thereof, and excluding the regulatory sequences which drive the initiation of transcription. A coding sequence may be one normally found in the cell or it may be one not normally found in a cellular location wherein it is introduced, in which case it is termed a heterologous gene. A heterologous gene may be derived in whole or in part from any source known to the art, including a bacterial genome or episome, eukaryotic nuclear or plasmid DNA, cDNA, or chemically synthesized DNA. The coding sequence may constitute an uninterrupted coding region or it may include one or more introns bounded by appropriate splice junctions. The coding

sequence may be a composite of segments derived from different sources, naturally occurring or synthetic.

A "3' downstream region" (or "3' end") refers to that portion of a gene comprising a DNA segment, excluding the 5' sequence which drives the initiation of transcription and the coding sequence of the gene, that contain a polyadenylation signal in eukaryotes and any other regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal in eukaryotes is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5'-AATAAA-3', although variations are not uncommon.

The term "construction" or "construct" refers to a plasmid, virus, autonomously replicating sequence, phage or nucleotide sequence, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a plant cell.

As used herein, "plant" refers to whole plants and plant-derived tissues.

As used herein, "transformation" is the acquisition of new genes in a cell after the incorporation of nucleic acid (usually double stranded DNA).

The term, "operably linked" refers to the chemical fusion of two fragments of DNA in a proper

orientation and reading frame to be transcribed into functional RNA.

The term "expression" as used herein is intended to mean the transcription and translation to gene product from a gene coding for the sequence of the gene product. In the expression, a DNA chain coding for the sequence of gene product is first transcribed to a complementary RNA which is often a messenger RNA and, then, the thus transcribed messenger RNA is translated into the above-mentioned gene product if the gene product is a protein.

The "translation initiation signal" refers to a unit of three nucleotides (codon) in a nucleic acid that specifies the initiation of protein synthesis.

The term "plasmid" as used herein refers to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules.

The term "restriction endonuclease" refers to an enzyme which binds and cuts within a specific nucleotide sequence within double-stranded DNA.

The term "T-DNA" is the segment of DNA from a plasmid transferred from soil bacterium Agrobacterium to the genome of its plant host.

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

Materials and General Methods:

Restriction endonucleases, DNA polymerases, DNA ligase and other DNA modification enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, MD 20877; New England Biolabs, Beverly, MA 01915; and Boehringer-Mannheim Biochemicals, Indianapolis, IN 46250.

Media

Media for growth of Streptomyces cultures are YEME broth, sporulation broth, trypticase soy broth and minimal medium.

YEME broth consists of 340 g sucrose, 3.0 g yeast extract (Difco), 5.0 g peptone (Difco), 3.0 g malt extract broth (Oxoid) and 10 g glucose dissolved in water to 1 liter.

Sporulation broth consists of 1.0 g yeast extract (Difco), 1.0 g beef extract (Difco), 2.0 g tryptose (Difco), 10 g glucose and approximately 1 mg $FeSO_4$ dissolved in water to 1 liter.

Trypticase soy broth consists of 17.0 g pancreatic digest of casein, 3.0 g papaic digest of soybean meal, 5.0 g NaCl, 5.0 g $K_2HPO_4$, and 2.5 g glucose per liter of water.

Minimal medium consists of 0.5 g $K_2HPO_4$, 0.6 g L-asparagine, 0.3 g KOH, 0.4 g $MgSO_4.7H_2O$, .01 g $FeSO_4.7H_2O$, 3.07 g glycerol per liter of $H_2O$. To make solid medium, 15 g of agar is added per liter of medium.

Culturing of Streptomyces

Streptomyces cultures are grown in sporulation, YEME or trypticase soy broth at 30°C with shaking at 150-300 rpm in an orbital shaker.

Harvesting bacterial cells

Bacterial cells are harvested by centrifuging them at 6,000-12,000 x g for 10-20 minutes at 4°C. Cells are washed in 0.1 M PIPES buffer pH 7.0 or 0.1 M MOPS pH 7.2 and collected by again centrifuging them at 6,000-12,000 x g for 10-20 minutes at 4°C.

Cell extracts

Cell extracts from Streptomyces are obtained by resuspending harvested cells in 1 to 3 cell volumes of 0.1 M PIPES buffer pH 6.8-7.0 and disrupting them by means of a French pressure cell (20,000 psi). The cell debris is removed by centrifugation at 10,000-12,000 x g for 10-20 minutes in a micro-centrifuge at 4°C. The protein concentrations of each extract is quantitated using the method of Bradford (Anal. Biochem. 72: 247-254 (1976)).

Western blot analyses

Western blot analysis of proteins is performed by separating the proteins by SDS polyacrylamide gel electrophoresis (Laemmli, Nature 227: 680, 1970, herein incorporated by reference) and then transferring the proteins to nitrocellulose and detecting the protein of interest with antibody specific for the protein as described by Towbin et al., Proc. Natl. Acad. Sci. U.S.A. 76: 4350-4354 (1979) and Bio-Rad bulletin 885 85-0335. (Bio-Rad Laboratories, Richmond, CA 94804), each herein incorporated by reference. Antiserum to cytochrome P450SU1 was that described by O'Keefe et al., Recent Advances in Phytochemistry 21: 151-173 (1987), herein incorporated by reference. Antiserum to cytochrome P450SU2 was prepared as was that for P450SU1 except

that cytochrome P450SU2 was isolated from S. griseolus PH2042, a mutant that does not make cytochrome P450SU1.

HPLC analysis of herbicide compounds

Herbicides and their metabolites are measured by HPLC as described by Romesser et al., BBRC 140: 650-659 (1986), herein incorporated by reference, except that 0.1% $H_3PO_4$ is used in both solvents. Chromatographic identity and quantitation of the herbicides and their resulting metabolites is determined by comparing them chromatographically with authentic standard compounds. Identity of the resulting metabolites is also confirmed by UV spectroscopy.

Isolation of S. griseolus mutants without P450SU1

Mutants of S. griseolus that do not make cytochrome P450SU1, but do make cytochrome P450SU2 were isolated by treating spores of S. griseolus ATCC 11796 with 2 mg/ml nitrosoguanidine for 30 minutes at room temperature. The mutagenized spores were diluted and plated on a rich medium and incubated at 30°C until mature colonies had formed. Single colonies from these plates were then patched onto minimal medium. The colonies were incubated for several days, then, a soft agar overlay containing 20 mg/ml of the fluorescent sulfonylurea 10016 was poured over the plate followed by further incubation at 30°C. The plates were then viewed under short wave UV light. Large non-fluorescent zones were observed around a majority of the colonies that had metabolized the sulfonylurea. Those colonies which showed a reduced ability to metabolize 10016 (i.e.,

smaller non-fluorescent zones were observed) were considered potential mutants. A number of such colonies were isolated and found to make cytochrome P450SU2 but not cytochrome P450SU1. Three of these mutants, S. griseolus PH2001, PH2003 and PH2042, were used in the examples described below. These mutants have similar properties.

Amino acid sequencing

Cytochromes P450SU1 and P450SU2 were purified using the methods described by O'Keefe et al., Arch. Microbiol. 149: 406-412 (1988), herein incorporated by reference. Purified, native cytochromes P450SU1 and P450SU2 were reacted with iodoacetic acid to make the carboxymethyl-derivatives of each protein which were subsequently subjected to amino acid analysis and automated Edman degradation amino acid sequencing using methods well known to those skilled in the art (Methods of Protein Microcharacterization (1986), Humana Press, Inc., Clifton, NJ, J. E. Shively, ed., herein incorporated by reference). Two iron-sulfur proteins, FeS-A and FeS-B, which can be used in the reconstitution of cytochrome P450 enzymatic activity in the presence of cytochrome P450SU1 or P450SU2 and spinach ferredoxin:NADP oxidoreductase (commercially available) were purified from the same extracts of sulfonylurea induced S. griseolus cells used to purify cytochromes P450SU1 and P450SU2. The iron-sulfur proteins were collected as a single peak from the anion exchange column used in the P450 purification (O'Keefe et al., Arch Microbiol. 149: 406-412 (1988), herein incorporated by reference, and were detected by their spectral property of having nearly equal absorbance at both 460 nm and 420 nm.

The iron-sulfur proteins isolated in this way were subsequently concentrated by ultrafiltration. Determination of the acid labile iron and sulfide content confirmed the proteins to be iron-sulfur proteins. Carboxymethylation of the iron sulfur proteins and reverse phase chromatography separated the iron-sulfur protein preparation into two separate apoproteins designated FeS-A and FeS-B which were subjected to amino acid analysis and automated Edman degradation amino acid sequencing using methods well known to those skilled in the art (Methods of Protein Microcharacterization (1986), Humana Press, Inc., Clifton, NJ, J. E. Shively, ed.). The amino terminal amino acid sequences and amino acid compositions of P450SU1, P450SU2, FeS-A, and FeS-B are shown below.

Amino terminal amino acid sequence of P450SU1

5 10

$NH_2$-Thr-Asp-Thr-Ala-Thr-Thr-Pro-Gln-Thr-Thr-Asp-Ala-Pro-

15 20 25

Ala-Phe-Pro-Val-Asn-Arg-Ser-Cys-Pro-Tyr-Gln-Leu-X-Asp-

30

Gly-Tyr-Ala-Gln (amino acid 17 may be serine)

Amino terminal amino acid sequence of P450SU2

5 10

$NH_2$-Thr-Thr-Ala-Glu-X-Thr-Ala-Pro-Pro-Asp-Ala-Leu-Thr-

15 20 25

Val-Pro-Ala-Ser-Arg-Ala-Pro-Gly-Cys-Pro-Phe-Asp-Pro-Ala-

30

Pro-Asp-Val-Thr-Glu

Amino terminal amino acid sequence of FeS-A

5 10

$NH_2$-Met-Arg-Ile-His-Val-Asp-Gln-Asp-Lys-Cys-Cys-Gly-Ala-

15 20 25

Gly-Ser-Cys-Val-Leu-Ala-Ala-Pro-Asp-Val-Phe-Asp-Gln-Arg-

30 35 40

Glu-Glu-Asp-Gly-Ile-Val-Val-Leu-Leu-Asp-Thr-Ala-Pro-Pro-

43

Ala-Ala-

Amino terminal amino acid sequence of FeS-B

5 10

$NH_2$-Thr-Met-Arg-Val-Ser-Ala-Asp-Arg-Thr-Val-Cys-Val-Gly-

15 20 25

Ala-Gly-Leu-Cys-Ala-Leu-Thr-Ala-Pro-Gly-Val-Phe-Asp-Gln-

30 35 40

Asp-Asp-Asp-Gly-Ile-Val-Thr-Val-Leu-Thr-Ala-Glu-Pro-Ala-

43

Ala-Asp-

Amino acid compositions of cytochromes P450SU1, P450SU2, FeS-A, and FeS-B

| | Mole % of each Amino Acid | | | |
|---|---|---|---|---|
| Amino Acid | P450SU1 | P450SU2 | FeS-A | FeS-B |
| Cys | 0.7 | 1.3 | 4.7 | 3.4 |
| Asx | 9.5 | 9.0 | 12.5 | 13.2 |
| Thr | 6.7 | 7.3 | 6.0 | 9.8 |
| Ser | 4.3 | 3.3 | 1.6 | 2.9 |
| Glx | 11.4 | 9.2 | 8.2 | 7.9 |
| Pro | 6.7 | 6.9 | 6.5 | 4.3 |
| Gly | 7.8 | 7.1 | 4.9 | 9.0 |
| Ala | 11.4 | 12.3 | 18.9 | 14.7 |
| Val | 7.8 | 6.9 | 10.8 | 12.3 |

| | | | | |
|---|---|---|---|---|
| Met | 1.7 | 1.9 | 1.2 | 1.2 |
| Ile | 3.1 | 3.3 | 5.7 | 1.1 |
| Leu | 11.4 | 11.8 | 6.7 | 6.2 |
| Tyr | 1.9 | 0.9 | 0 | 0 |
| Phe | 3.1 | 3.1 | 1.6 | 1.5 |
| His | 2.6 | 2.4 | 3.0 | 1.4 |
| Lys | 1.4 | 1.2 | 1.7 | 0.3 |
| Trp | 0.5 | 0.5 | 0 | 0 |
| Arg | 8.1 | 11.6 | 6.1 | 10.9 |

Cloning the genes for cytochrome P450SU1, cytochrome P450SU2, FeS-A and FeS-B from Streptomyces griseolus ATCC11796

DNA encoding the gene for cytochrome P450SU1 was cloned from S. griseolus DNA. Bacteriophages containing the proper sequences of DNA were obtained by first identifying clones of transformed E. coli that expressed the SU1 protein. This was done by using antibody specific for cytochrome P450SU1 as described by O'Keefe et al., Recent Advances in Phytochemistry 21: 151-173 (1987), using methods well known to those skilled in the art (Young et al., Proc. Natl. Acad. Sci. U. S. A. 80: 1194-1198 (1983) and Young et al., Science 222: 778-782 (1983)), each of which is herein incorporated by reference. Restriction endonuclease maps (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY) were made of the isolated S. griseolus DNA and they indicated that a 2.4 kb BamHI restriction endonuclease fragment should contain the complete cytochrome P450SU1 coding sequence. This 2.4 kb restriction endonuclease fragment was cloned from S. griseolus DNA into the plasmid pUC18 using methods well known to those

skilled in the art (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY; Frischauf et al.,J. Mol. Biol. 170: 827-842 (1983), each herein incorporated by reference), to make the plasmid pUC18-SU1-BamHI. Subsequent DNA sequence analysis, shown below, indicated that the coding sequence for the FeS-B protein is also encoded on this 2.4 kb BamHI fragment, being just downstream from the sequence for SU1. The plasmid pUC18-SU1-BamHI has been deposited in the American Type Culture collection on 30 August 1988 and has ATCC accession number 67780. A restriction endonuclease map of pUC18-SU1-BamHI is shown in Fig. 1.

A 2.0 kb BamHI restriction endonuclease DNA fragment that cross-hybridized to the 2.4 kb BamHI fragment encoding cytochrome P450SU1 and FeS-B and which encodes cytochrome P450SU2 and FeS-A was obtained from S. griseolus mutant PH2001 and cloned using methods well known to those skilled in the art (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY). The 2.0 kb BamHI fragment was shown to encode both cytochrome P450SU2 and FeS-A as determined by DNA sequence analysis of the DNA. The 2.0 kb BamHI DNA fragment was subcloned into the plasmid pUC19 in E. coli and is called pUC19-SU2-8 and has been deposited in the American Type Culture collection on 30 August 1988 and has ATCC accession number 67781. A restriction endonuclease map of pUC19-SU2-8 is shown in Fig. 4.

The DNA sequence of cytochrome P450SU1 and FeS-B protein genes

By further restriction endonuclease mapping, it was determined that a 2.0 kb SacI-BamHI fragment of DNA derived from the 2.4 kb BamHI fragment in pUC18-SU1-BamHI contains the complete DNA coding sequence for the cytochrome P450SU1 and FeS-B proteins. That the 2.0 kb fragment contains the complete DNA coding sequence for the cytochrome P450SU1 and FeS-B proteins was determined by comparing all possible proteins encoded by the DNA sequence of the fragment with the molecular weight, amino acid composition, and N-terminal amino acid sequences of P-450SUI and the amino acid composition of N-terminal amino acid sequence of FeS-B, as shown above. The DNA sequence of the 2.0 kb SacI-BamHI fragment was determined from about 100 bp downstream of the SacI site through the BamHI site using methods well known to those skilled in the art (Messing, Methods in Enzymology 101: 20-78 (1983), herein incorporated by reference), and is shown as follows with the coding sequences of cytochrome P450SU1 and FeS-B, which start at base no. 128 and end at base no. 1578, indicated.

DNA Sequence of the DNA Containing the Coding Sequences for Cytochrome P450SU1 and FeS-B

```
        10                  30                  50
GCGGACAGGGGGACTCCTGAAGATGTCTGATAGAGGCCGTTGCGTTCTCTACGGGGGCAA
---------+---------+---------+---------+---------+---------+
CGCCTGTCCCCCTGAGGACTTCTACAGACTATCTCCGGCAACGCAAGAGATGCCCCCGTT
```

```
        70                  90                  110
GTCTATGCTCCGAAATAGAGAACATGGCGTTCTTTAAAGGTGAGAATTCTTGAATCGGAG
---------+---------+---------+---------+---------+---------+
CAGATACGAGGCTTTATCTCTTGTACCGCAAGAAATTTCCACTCTTAAGAACTTAGCCTC
                                          EcoRI

       130                 150                 170
TGGACCGATGACCGATACCGCCACGACGCCCCAGACCACGGACGCACCCGCCTTCCCGAG
---------+---------+---------+---------+---------+---------+
ACCTGGCTACTGGCTATGGCGGTGCTGCGGGGTCTGGTGCCTGCGTGGGCGGAAGGGCTC
P450SU1MetThrAspThrAlaThrThrProGlnThrThrAspAlaProAlaPheProSe
Start

       190                 210                 230
CAACCGGAGCTGTCCCTACCAGTTACCGGACGGCTACGCCCAGCTCCGGGACACCCCCGG
---------+---------+---------+---------+---------+---------+
GTTGGCCTCGACAGGGATGGTCAATGGCCTGCCGATGCGGGTCGAGGCCCTGTGGGGGCC
rAsnArgSerCysProTyrGlnLeuProAspGlyTyrAlaGlnLeuArgAspThrProGl

       250                 270                 290
CCCCCTGCACCGGGTGACGCTCTACGACGGCCGTCAGGCGTGGGTGGTGACCAAGCACGA
---------+---------+---------+---------+---------+---------+
GGGGGACGTGGCCCACTGCGAGATGCTGCCGGCAGTCCGCACCCACCACTGGTTCGTGCT
yProLeuHisArgValThrLeuTyrAspGlyArgGlnAlaTrpValValThrLysHisGl

       310                 330                 350
GGCCGCGCGCAAACTGCTCGGCGACCCCCGGCTGTCCTCCAACCGGACGGACGACAACTT
---------+---------+---------+---------+---------+---------+
CCGGCGCGCGTTTGACGAGCCGCTGGGGGCCGACAGGAGGTTGGCCTGCCTGCTGTTGAA
uAlaAlaArgLysLeuLeuGlyAspProArgLeuSerSerAsnArgThrAspAspAsnPh

       370                 390                 410
CCCCGCCACGTCACCGCGCTTCGAGGCCGTCCGGGAGAGCCCGCAGGCGTTCATCGGCCT
---------+---------+---------+---------+---------+---------+
GGGGCGGTGCAGTGGCGCGAAGCTCCGGCAGGCCCTCTCGGGCGTCCGCAAGTAGCCGGA
eProAlaThrSerProArgPheGluAlaValArgGluSerProGlnAlaPheIleGlyLe

       430                 450                 470
GGACCCGCCCGAGCACGGCACCCGGCGGCGGATGACGATCAGCGAGTTCACCGTCAAGCG
---------+---------+---------+---------+---------+---------+
CCTGGGCGGGCTCGTGCCGTGGGCCGCCGCCTACTGCTAGTCGCTCAAGTGGCAGTTCGC
uAspProProGluHisGlyThrArgArgArgMetThrIleSerGluPheThrValLysAr

       490                 510                 530
GATCAAGGGCATGCGCCCCGAGGTCGAGGAGGTGGTGCACGGCTTCCTCGACGAGATGCT
---------+---------+---------+---------+---------+---------+
CTAGTTCCCGTACGCGGGGCTCCAGCTCCTCCACCACGTGCCGAAGGAGCTGCTCTACGA
gIleLysGlyMetArgProGluValGluGluValValHisGlyPheLeuAspGluMetLe
```

```
       550                   570                   590
GGCCGCCGGCCCGACCGCCGACCTGGTCAGTCAGTTCGCGCTGCCGGTGCCCTCCATGGT
---------+---------+---------+---------+---------+---------+
CCGGCGGCCGGGCTGGCGGCTGGACCAGTCAGTCAAGCGCGACGGCCACGGGAGGTACCA
uAlaAlaGlyProThrAlaAspLeuValSerGlnPheAlaLeuProValProSerMetVa

       610                   630                   650
GATCTGCCGACTCCTCGGCGTGCCCTACGCCGACCACGAGTTCTTCCAGGACGCGAGCAA
---------+---------+---------+---------+---------+---------+
CTAGACGGCTGAGGAGCCGCACGGGATGCGGCTGGTGCTCAAGAAGGTCCTGCGCTCGTT
lIleCysArgLeuLeuGlyValProTyrAlaAspHisGluPhePheGlnAspAlaSerLy

       670                   690                   710
GCGGCTGGTGCAGTCCACGGACGCGCAGAGCGCGCTCACCGCGCGGAACGACCTCGCGGG
---------+---------+---------+---------+---------+---------+
CGCCGACCACGTCAGGTGCCTGCGCGTCTCGCGCGAGTGGCGCGCCTTGCTGGAGCGCCC
sArgLeuValGlnSerThrAspAlaGlnSerAlaLeuThrAlaArgAsnAspLeuAlaGl

       730                   750                   770
TTACCTGGACGGCCTCATCACCCAGTTCCAGACCGAACCGGGCGCGGGCCTGGTGGGCGC
---------+---------+---------+---------+---------+---------+
AATGGACCTGCCGGAGTAGTGGGTCAAGGTCTGGCTTGGCCCGCGCCCGGACCACCCGCG
yTyrLeuAspGlyLeuIleThrGlnPheGlnThrGluProGlyAlaGlyLeuValGlyAl

       790                   810                   830
TCTGGTCGCCGACCAGCTGGCCAACGGCGAGATCGACCGTGAGGAACTGATCTCCACCGC
---------+---------+---------+---------+---------+---------+
AGACCAGCGGCTGGTCGACCGGTTGCCGCTCTAGCTGGCACTCCTTGACTAGAGGTGGCG
aLeuValAlaAspGlnLeuAlaAsnGlyGluIleAspArgGluGluLeuIleSerThrAl

       850                   870                   890
GATGCTGCTCCTCATCGCCGGCCACGAGACCACGGCCTCGATGACCTCCCTCAGCGTGAT
---------+---------+---------+---------+---------+---------+
CTACGACGAGGAGTAGCGGCCGGTGCTCTGGTGCCGGAGCTACTGGAGGGAGTCGCACTA
aMetLeuLeuLeuIleAlaGlyHisGluThrThrAlaSerMetThrSerLeuSerValIl

       910                   930                   950
CACCCTGCTGGACCACCCCGAGCAGTACGCCGCCCTGCGCGCCGACCGCAGCCTCGTGCC
---------+---------+---------+---------+---------+---------+
GTGGGACGACCTGGTGGGGCTCGTCATGCGGCGGGACGCGCGGCTGGCGTCGGAGCACGG
eThrLeuLeuAspHisProGluGlnTyrAlaAlaLeuArgAlaAspArgSerLeuValPr

       970                   990                   1010
CGGCGCGGTGGAGGAACTGCTCCGCTACCTCGCCATCGCCGACATCGCGGGCGGCCGCGT
---------+---------+---------+---------+---------+---------+
GCCGCGCCACCTCCTTGACGAGGCGATGGAGCGGTAGCGGCTGTAGCGCCCGCCGGCGCA
oGlyAlaValGluGluLeuLeuArgTyrLeuAlaIleAlaAspIleAlaGlyGlyArgVa
```

```
      1030                1050                1070
CGCCACGGCGGACATCGAGGTCGAGGGGCACCTCATCCGGGCCGGCGAGGGCGTGATCGT
---------+---------+---------+---------+---------+---------+
GCGGTGCCGCCTGTAGCTCCAGCTCCCCGTGGAGTAGGCCCGGCCGCTCCCGCACTAGCA
lAlaThrAlaAspIleGluValGluGlyHisLeuIleArgAlaGlyGluGlyValIleVa

      1090                1110                1130
CGTCAACTCGATAGCCAACCGGGACGGCACGGTGTACGAGGACCCGGACGCCCTCGACAT
---------+---------+---------+---------+---------+---------+
GCAGTTGAGCTATCGGTTGGCCCTGCCGTGCCACATGCTCCTGGGCCTGCGGGAGCTGTA
lValAsnSerIleAlaAsnArgAspGlyThrValTyrGluAspProAspAlaLeuAspIl

      1150                1170                1190
CCACCGCTCCGCGCGCCACCACCTCGCCTTCGGCTTCGGCGTGCACCAGTGCCTGGGCCA
---------+---------+---------+---------+---------+---------+
GGTGGCGAGGCGCGCGGTGGTGGAGCGGAAGCCGAAGCCGCACGTGGTCACGGACCCGGT
eHisArgSerAlaArgHisHisLeuAlaPheGlyPheGlyValHisGlnCysLeuGlyGl

      1210                1230                1250
GAACCTCGCCCGGCTGGAGCTGGAGGTCATCCTCAACGCCCTCATGGACCGCGTCCCGAC
---------+---------+---------+---------+---------+---------+
CTTGGAGCGGGCCGACCTCGACCTCCAGTAGGAGTTGCGGGAGTACCTGGCGCAGGGCTG
nAsnLeuAlaArgLeuGluLeuGluValIleLeuAsnAlaLeuMetAspArgValProTh

      1270                1290                1310
GCTGCGACTGGCCGTCCCCGTCGAGCAGTTGGTGCTGCGCCCGGGTACGACGATCCAGGG
---------+---------+---------+---------+---------+---------+
CGACGCTGACCGGCAGGGGCAGCTCGTCAACCACGACGCCGGCCCATGCTGCTAGGTCCC
rLeuArgLeuAlaValProValGluGlnLeuValLeuArgProGlyThrThrIleGlnGl

      1330                1350                1370
CGTCAACGAACTCCCGGTCACCTGGTGACGGGGGAGAGGGGCAAGGACATGACCATGCGG
---------+---------+---------+---------+---------+---------+
GCAGTTGCTTGAGGGCCAGTGGACCACTGCCCCCTCTCCCCGTTCCTGTACTGGTACGCC
yValAsnGluLeuProValThrTrpEnd        FeS-B Start  MetThrMetArg

      1390                1410                1430
GTGAGTGCGGATCGGACGGTCTGCGTCGGTGCCGGGCTGTGTGCGCTGACGGCGCCGGGC
---------+---------+---------+---------+---------+---------+
CACTCACGCCTAGCCTGCCAGACGCAGCCACGGCCCGACACACGCGACTGCCGCGGCCCG
ValSerAlaAspArgThrValCysValGlyAlaGlyLeuCysAlaLeuThrAlaProGly

      1450                1470                1490
GTCTTCGACCAGGACGACGACGGGATCGTCACGGTGCTGACGGCCGAACCCGCCGCCGAC
---------+---------+---------+---------+---------+---------+
CAGAAGCTGGTCCTGCTGCTGCCCTAGCAGTGCCACGACTGCCGGCTTGGGCGGCGGCTG
ValPheAspGlnAspAspAspGlyIleValThrValLeuThrAlaGluProAlaAlaAsp
```

```
      1510                1530                1550
GACGACCGGCGCACCGCGCGCGAGGCCGGCCATCTCTGTCCGTCCGGTGCGGTCCGCGTC
---------+---------+---------+---------+---------+---------+
CTGCTGGCCGCGTGGCGCGCGCTCCGGCCGGTAGAGACAGGCAGGCCACGCCAGGCGCAG
AspAspArgArgThrAlaArgGluAlaGlyHisLeuCysProSerGlyAlaValArgVal

     1570                1590                1610
GTCGAGGACACGGAATAGGGTCAAGGACACGGAACAGGCGAGCGGGGATTCCGGCCGTCG
---------+---------+---------+---------+---------+---------+
CAGCTCCTGTGCCTTATCCCAGTTCCTGTGCCTTGTCCGCTCGCCCCTAAGGCCGGCAGC
ValGluAspThrGluEnd

      1630                1650                1670
GCCGGGGCGGTCTCCGGCCGACGGGCTGGGGCCGCCCGCGGTGCCGCCGCGCAGGCGAGG
---------+---------+---------+---------+---------+---------+
CGGCCCCGCCAGAGGCCGGCTGCCCGACCCCGGCGGGCGCCACGGCGGCGCGTCCGCTCC

      1690                1710                1730
CCGCCGGTGGCGCCCGGCACCCGCGGCGGCCGTCAGATCCACCCCTTCCGCGCCGCGTAC
---------+---------+---------+---------+---------+---------+
GGCGGCCACCGCGGGCCGTGGGCGCCGCCGGCAGTCTAGGTGGGGAAGGCGCGGCGCATG

      1750                1770                1790
AGAGCGACTTGGAAACGGGTGGTGGCGTCGGCGGCGCGGTTGAGCTGCTCCAACTGGCGG
---------+---------+---------+---------+---------+---------+
TCTCGCTCAACCTTTGCCCACCACCGCAGCCGCCGCGCCAACTCGACGAGGTTGACCGCC

      1810                1830                1850
GAGAGGGTGCGTCGACTGATGCCGAGCAGTTCGGCGATGGTCTCGTCCGTGACGCCGCTC
---------+---------+---------+---------+---------+---------+
CTCTCCCACGCAGCTGACTACGGCTCGTCAAGCCGCTACCAGAGCAGGCACTGCGGCGAG

      1870
CCCAGCAGCTCCAGGATCC
---------+---------
GGGTCGTCGAGGTCCTAGG
          BamHI
```

DNA Sequence of the cytochrome P450SU2 and FeS-A protein genes

The DNA sequence of the 2.0 kb BamHI fragment isolated from S. griseolus transformed with the plasmid pUC19-SU2-8 that contains the genes for cytochrome P450SU2 and the iron sulfur protein FeS-A was determined by methods well known to those skilled in the art and described by Messing, Methods in

Enzymology 101: 20-78 (1983). That the 2.0 kb BamHI DNA fragment encodes cytochrome P450SU2 and FeS-A was determined by comparing all possible proteins encoded by the DNA sequence to the known size, amino acid composition, and amino terminal amino acid sequence of P450SU2 and the known amino acid composition and amino terminal amino acid sequence of FeS-A. The DNA sequence of the fragment is shown as follows and the locations of the coding sequence for cytochrome P450SU2 and FeS-A, which start at base no. 195 and end at base no. 1646 are indicated.

DNA Sequence of the 2.0 kilobase BamHI DNA Fragment Containing the Coding Sequences for Cytochrome P450SU2 and FeS-A

```
        10                  30                  50
GGATCCGGCCACCGCCCGACCCGTCCGCACTCCGCCCCGCCGACCGTCGTCCATCCGCCC
---------+---------+---------+---------+---------+---------+
CCTAGGCCGGTGGCGGGCTGGGCAGGCGTGAGGCGGGGCGGCTGGCAGCAGGTAGGCGGG
BamHI

        70                  90                  110
CTGCGGCCATGCGGTTTGAGCCAACCTCGGTGCTGCCGCGATCTGCCCTTCCCTCCCCCG
---------+---------+---------+---------+---------+---------+
GACGCCGGTACGCCAAACTCGGTTGGAGCCACGACGGCGCTAGACGGGAAGGGAGGGGGC

       130                 150                 170
CCGGGCCTGCGTTAGCGTGACGACATCTTAATTACCTAAGTTAGGTAATTAGCTCACTCG
---------+---------+---------+---------+---------+---------+
GGCCCGGACGCAATCGCACTGCTGTAGAATTAATGGATTCAATCCATTAATCGAGTGCGC

       190                 210                 230
GAAGGACCGGCCGCATGACGACCGCAGAACGCACCGCTCCCCCCGACGCCCTCACCGTCC
---------+---------+---------+---------+---------+---------+
CTTCCTGGCCGGCGTACTGCTGGCGTCTTGCGTGGCGAGGGGGGCTGCGGGAGTGGCAGG
P450SU2       MetThrThrAlaGluArgThrAlaProProAspAlaLeuThrValP
Start

       250                 270                 290
CGGCCAGCCGCGCCCCCGGCTGCCCCTTCGACCCCGCGCCCGACGTCACCGAGGCGGCCC
---------+---------+---------+---------+---------+---------+
GCCGGTCGGCGCGGGGGCCGACGGGGAAGCTGGGGCGCGGGCTGCAGTGGCTCCGCCGGG
roAlaSerArgAlaProGlyCysProPheAspProAlaProAspValThrGluAlaAlaA
```

```
       310                 330                 350
GCACCGAACCGGTCACCCGGGCCACCCTCTGGGACGGCTCCTCCTGCTGGCTGGTGACGC
---------+---------+---------+------ --+---------+---------+
CGTGGCTTGGCCAGTGGGCCCGGTGGGAGACCCTGCCGAGGAGGACGACCGACCACTGCG
rgThrGluProValThrArgAlaThrLeuTrpAspGlySerSerCysTrpLeuValThrA

       370                 390                 410
GCCATCAGGACGTCCGCGCGGTCCTCGGCGACCCGCGCTTCAGCGCCGACGCCCACCGCA
---------+---------+---------+---------+---------+---------+
CGGTAGTCCTGCAGGCGCGCCAGGAGCCGCTGGGCGCGAAGTCGCGGCTGCGGGTGGCGT
rgHisGlnAspValArgAlaValLeuGlyAspProArgPheSerAlaAspAlaHisArgT

       430                 450                 470
CCGGCTTCCCCTTCCTGACCGCCGGCGGCCGCGAGATCATCGGCACCAACCCGACCTTCC
---------+---------+---------+---------+---------+---------+
GGCCGAAGGGGAAGGACTGGCGGCCGCCGGCGCTCTAGTAGCCGTGGTTGGGCTGGAAGG
hrGlyPheProPheLeuThrAlaGlyGlyArgGluIleIleGlyThrAsnProThrPheL

       490                 510                 530
TGCGCATGGACGACCCGGAGCACGCCCGACTGCGCCGGATGCTCACCGCCGACTTCATCG
---------+---------+---------+---------+---------+---------+
ACGCGTACCTGCTGGGCCTCGTGCGGGCTGACGCGGCCTACGAGTGGCGGCTGAAGTAGC
euArgMetAspAspProGluHisAlaArgLeuArgArgMetLeuThrAlaAspPheIleV

       550                 570                 590
TCAAGAAGGTCGAGGCGATGCGCCCCGAGGTGCAGCGCCTCGCCGACGACCTGGTCGACC
---------+---------+---------+---------+---------+---------+
AGTTCTTCCAGCTCCGCTACGCGGGGCTCCACGTCGCGGAGCGGCTGCTGGACCAGCTGG
alLysLysValGluAlaMetArgProGluValGlnArgLeuAlaAspAspLeuValAspA

       610                 630                 650
GGATGACCACCGGACGCACCTCCGCCGACCTGGTCACCGAGTTCGCGCTGCCGCTGCCGT
---------+---------+---------+---------+---------+---------+
CCTACTGGTGGCCTGCGTGGAGGCGGCTGGACCAGTGGCTCAAGCGCGACGGCGACGGCA
rgMetThrThrGlyArgThrSerAlaAspLeuValThrGluPheAlaLeuProLeuProS

       670                 690                 710
CCCTGGTGATCTGCCTGCTGCTCGGCGTCCCCTACGAGGACCACGCGTTCTTCCAGGAGC
---------+---------+---------+---------+---------+---------+
GGGACCACTAGACGGACGACGAGCCGCAGGGGATGCTCCTGGTGCGCAAGAAGGTCCTCG
erLeuValIleCysLeuLeuLeuGlyValProTyrGluAspHisAlaPhePheGlnGluA

       730                 750                 770
GCAGCCGGGTCCTGCTCACCCTGCGGTCCACTCCCGAGGAAGTCCGGGCCGCCCAGGACG
---------+---------+---------+---------+---------+---------+
CGTCGGCCCAGGACGAGTGGGACGCCAGGTGAGGGCTCCTTCAGGCCCGGCGGGTCCTGC
rgSerArgValLeuLeuThrLeuArgSerThrProGluGluValArgAlaAlaGlnAspG
```

```
      790                  810                  830
AGTTGCTGGAGTACCTCGCCCGGCTCGCCCGGACCAAGCGGGAGCGGCCGGACGACGCCA
---------+---------+---------+---------+---------+---------+
TCAACGACCTCATGGAGCGGGCCGAGCGGGCCTGGTTCGCCCTCGCCGGCCTGCTGCGGT
luLeuLeuGluTyrLeuAlaArgLeuAlaArgThrLysArgGluArgProAspAspAlaI

      850                  870                  890
TCATCAGCCGCCTGGTCGCCCGCGGCGAGCTCGACGACACCCAGATCGCCACCATGGGAC
---------+---------+---------+---------+---------+---------+
AGTAGTCGGCGGACCAGCGGGCGCCGCTCGAGCTGCTGTGGGTCTAGCGGTGGTACCCTG
leIleSerArgLeuValAlaArgGlyGluLeuAspAspThrGlnIleAlaThrMetGlyA

      910                  930                  950
GCCTGTTGCTGGTCGCCGGCCACGAGACGACCGCCAACATGACCGCGCTCTCCACCCTCG
---------+---------+---------+---------+---------+---------+
CGGACAACGACCAGCGGCCGGTGCTCTGCTGGCGGTTGTACTGGCGCGAGAGGTGGGAGC
rgLeuLeuLeuValAlaGlyHisGluThrThrAlaAsnMetThrAlaLeuSerThrLeuV

      970                  990                 1010
TGCTGCTGCGCAACCCCGACCAACTCGCCCGGCTGCGCGCCGAACCCGCGCTCGTCAAGG
---------+---------+---------+---------+---------+---------+
ACGACGACGCGTTGGGGCTGGTTGAGCGGGCCGACGCGCGGCTTGGGCGCGAGCAGTTCC
alLeuLeuArgAsnProAspGlnLeuAlaArgLeuArgAlaGluProAlaLeuValLysG

     1030                 1050                 1070
GCGCCGTCGAGGAGCTGCTGCGCTACCTGACGATCGTGCACAACGGCGTTCCCCGGATCG
---------+---------+---------+---------+---------+---------+
CGCGGCAGCTCCTCGACGACGCGATGGACTGCTAGCACGTGTTGCCGCAAGGGGCCTAGC
lyAlaValGluGluLeuLeuArgTyrLeuThrIleValHisAsnGlyValProArgIleA

     1090                 1110                 1130
CCACCGAGGACGTGCTCATCGGCGGCCGCACCATCGCCGCCGGCGAGGGCGTCCTGTGCA
---------+---------+---------+---------+---------+---------+
GGTGGCTCCTGCACGAGTAGCCGCCGGCGTGGTAGCGGCGGCCGCTCCCGCAGGACACGT
laThrGluAspValLeuIleGlyGlyArgThrIleAlaAlaGlyGluGlyValLeuCysM

     1150                 1170                 1190
TGATCAGCTCCGCCAACCGGGACGCCGAGGTGTTCCCCGGCGGCGACGACCTCGACGTGG
---------+---------+---------+---------+---------+---------+
ACTAGTCGAGGCGGTTGGCCCTGCGGCTCCACAAGGGGCCGCCGCTGCTGGAGCTGCACC
etIleSerSerAlaAsnArgAspAlaGluValPheProGlyGlyAspAspLeuAspValA

     1210                 1230                 1250
CCCGCGACGCCCGCCGCCACGTGGCCTTCGGCTTCGGCGTCCACCAGTGCCTGGGACAGC
---------+---------+---------+---------+---------+---------+
GGGCGCTGCGGGCGGCGGTGCACCGGAAGCCGAAGCCGCAGGTGGTCACGGACCCTGTCG
laArgAspAlaArgArgHisValAlaPheGlyPheGlyValHisGlnCysLeuGlyGlnP
```

```
      1270                1290                1310
CGTTGGCCAGGGTGGAGCTCCAGATCGCCATCGAAACGCTGCTGCGCCGCCTGCCGGACC
---------+---------+---------+---------+---------+---------+
GCAACCGGTCCCACCTCGAGGTCTAGCGGTAGCTTTGCGACGACGCGGCGGACGGCCTGG
roLeuAlaArgValGluLeuGlnIleAlaIleGluThrLeuLeuArgArgLeuProAspL

      1330                1350                1370
TGCGGCTGGCCGTGCCCCACGAGGAGATCCCGTTCCGCGGCGACATGGCGATCTACGGGG
---------+---------+---------+---------+---------+---------+
ACGCCGACCGGCACGGGGTGCTCCTCTAGGGCAAGGCGCCGCTGTACCGCTAGATGCCCC
euArgLeuAlaValProHisGluGluIleProPheArgGlyAspMetAlaIleTyrGlyV

      1390                1410                1430
TCCACTCGCTGCCGATCGCCTGGTAGCCCGGGCGCCCCCACCACCGACCACCACGCACCC
---------+---------+---------+---------+---------+---------+
AGGTGAGCGACGGCTAGCGGACCATCGGGCCCGCGGGGGTGGTGGCTGGTGGTGCGTGGG
alHisSerLeuProIleAlaTrpEnd

      1450                1470                1490
TTGGGAGCACCATGCGCATCCACGTCGACCAGGACAAGTGCTGCGGCGCCGGCAGTTGCG
---------+---------+---------+---------+---------+---------+
AACCCTCGTGGTACGCGTAGGTGCAGCTGGTCCTGTTCACGACGCCGCCGGCCGTCAACGC
FeS-A      MetArgIleHisValAspGlnAspLysCysCysGlyAlaGlySerCysV
Start

      1510                1530                1550
TCCTCGCCGCGCCCGACGTCTTCGACCAGCGGGAGGAGGACGGCATCGTGGTCCTCCTCG
---------+---------+---------+---------+---------+---------+
AGGAGCGGCGCGGGCTGCAGAAGCTGGTCGCCCTCCTCCTGCCGTAGCACCAGGAGGAGC
alLeuAlaAlaProAspValPheAspGlnArgGluGluAspGlyIleValValLeuLeuA

      1570                1590                1610
ACACCGCGCCGCCCGCCGCGCTGCACGACGCGGTCCGTGAGGCGGCGACCATCTGCCCCG
---------+---------+---------+---------+---------+---------+
TGTGGCGCGGCGGGCGGCGCGACGTGCTGCGCCAGGCACTCCGCCGCTGGTAGACGGGGC
spThrAlaProProAlaAlaLeuHisAspAlaValArgGluAlaAlaThrIleCysProA

      1630                1650                1670
CCGCCGCGATCACGGTGACCGACTGAGCCACCGGCCGCCCCGCCCGCCCGCGCCCCGGTC
---------+---------+---------+---------+---------+---------+
GGCGGCGCTAGTGCCACTGGCTGACTCGGTGGCCGGCGGGGCGGGCGGGCGCGGGGCCAG
laAlaAlaIleThrValThrAspEnd

      1690                1710                1730
CCCGCATCCCCCCGCGGCCCGGGGCGCGCCCCTAACCCGCCGCCCCGCACGCCGTCGCGC
---------+---------+---------+---------+---------+---------+
GGGCGTAGGGGGGCGCCGGGCCCCGCGCGGGGATTGGGCGGCGGGGCGTGCGGCAGCGCG
```

```
      1750                1770                1790
GCGCCGCCAGTGCCCGCAGCGCCGCCTCGGACGACGAACCCGGCACGGCGTGGTGGGTCA
---------+---------+---------+---------+---------+---------+
CGCGGCGGTCACGGGCGTCGCGGCGGAGCCTGCTGCTTGGGCCGTGCCGCACCACCCAGT

      1810                1830                1850
CCAGCGTCTGCCCCGGTTCGGCCGCCACGCGCAGCGTCCCGTAGGTCAGGGTCAGCGGGC
---------+---------+---------+---------+---------+---------+
GGTCGCAGACGGGGCCAAGCCGGCGGTGCGCGTCGCAGGGCATCCAGTCCCAGTCGCCCG

      1870                1890                1910
CCACCACCGGGTGGTCCAGCTGCTTCGTCCCGAAACCCTTGTCCTTGATGTCGTGCCGGG
---------+---------+---------+---------+---------+---------+
GGTGGTGGCCCACCAGGTCGACGAAGCAGGGCTTTGGGAACAGGAACTACAGCACGGCCC

      1930                1950                1970
CCCAGAAGCGCCGGAACTCCTCGCTCTGCACGGTCAGTTCGGTGATCCGCGCGGTCAGCG
---------+---------+---------+---------+---------+---------+
GGGTCTTCGCGGCCTTGAGGAGCGAGACGTGCCAGTCAAGCCACTAGGCGCGCCAGTCGC

      1990
CGGCGTCGTCCGGGATCC
---------+--------
GCCGCAGCAGGCCCTAGG
          BamHI
```

Plasmids for the constitutive expression of cytochrome P450SU1 in other organisms

Plasmids with which to transform other organisms to constitutively express cytochrome P450SU1 alone and P450SU1 and FeS-B together may be made as follows. Expression of the two genes (i.e., the DNA sequences) may be driven by the promoter and transcription signal of the genes from S. griseolus, or by any plasmid promoter(s) and translation signals that allow constitutive expression of exogenous coding sequences in the organism to be transformed. The non-regulated expression of the genes from these promoters in other organisms as exemplified by the example herein, in S. lividans, and as opposed to their regulated expression in S. griseolus is presumably due to the absence of the regulatory

factors (genes) in the other organisms that normally regulate the expression of cytochrome P450SU1 and FeS-B in S. griseolus.

pCAO400

This plasmid was made in E. coli by inserting the 2.4 kb BamHI fragment from pUC18-SU1-BamHI that contains the genes for both cytochrome P450SU1 and FeS-B into the unique BamHI site of pCAO170 (Omer et al., J. Bacteriol. 170:2174-2184, (1988)), using methods described in Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 390-400. The plasmid pCAO170 in E. coli CE170 has been deposited in the American Type Culture Collection on 29 August 1989 and has the ATCC accession number 68085. The resulting plasmid is called pCAO400.

pCAO401

This plasmid was made in E. coli by inserting the 2.0 kb BamHI-SacI fragment from pUC18-SU1-BamHI that contains the genes for both cytochrome P450SU1 and FeS-B into pCAO170 that had been digested with BamHI and SacI restriction endonucleases using methods as described by Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY. The resulting plasmid is called pCAO401.

pCAO200SU1-FeS-B #9

This plasmid was made similarly to the way pCAO400 above was made except that pCAO200 which can be made from pCAO170 (Omer et al., J. Bacteriol.

170:2174-2184, (1988)), was used instead of pCAO170 as the recipient of the 2.4 kb BamHI fragment.

pCAO200SU1#12

This plasmid was made by deleting the complete FeS-B protein coding sequence from the 2.4 kb BamHI DNA fragment. The deletion was made as described (Henikoff, Gene, 28:351-359 (1984); Messing, Methods in Enzymology 101: 20-78 (1983)). The resulting 1.8 kb DNA fragment still contains the sequences upstream of SU1, the complete cytochrome P450SU1 coding sequence, and 6 bp downstream of SU1. It is designated pUC118-SU-1.8. A BamHI site containing linker was inserted at the HindIII site downstream of the P450SU1 coding region and the resulting fragment was inserted in pUC118 creating pUC118-SU1-1.8(B). The 1.8 kb BamHI DNA fragment was isolated from pUC118-SU1-1.8(B) and inserted into the BamHI site of pCAO200 using methods well known to those skilled in the art (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY, pp 390-400) creating the plasmid pCAO200SU1#12.

Plasmid for the constitutive expression of cytochrome P450SU2 in other organisms

A plasmid for introducing the genes for cytochrome P450SU2 and FeS-A into S. lividans may be constructed as follows. The 2.0 kb BamHI fragment from pUC19-SU2-8 containing the genes for cytochrome P450SU2 and FeS-A can be cloned into the BamHI site of pCAO200 using methods well known to those skilled in the art creating pCAO200SU2-FeS-A#11 (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold

Spring Harbor Press, Cold Spring Harbor, NY, pp. 390-400). This fragment may also be cloned in other vectors.

The five plasmids, pCAO400, pCAO401 (Figs. 3A and 3B), pCAO200SU1-FeS-B.#9, pCAO200SU1#12 (Figs. 4A and 4B), and pCAOSU2-FeS-A (Fig. 8) were introduced into S. lividans JI1326 as described by Hopwood et al., Genetic Manipulation of Streptomyces. A Laboratory Manual, John Innes Foundation, Norwich, U. K., pp 108-109. S. lividans JI1326 has been deposited in the American Type Culture Collection on 29 August 1989 and has the ATCC accession number 53939. Transformants were selected for thiostrepton resistance which is encoded on the plasmids. These plasmids, which are based upon the SLP1 plasmid, site-specifically integrate into a unique locus in the S. lividans chromosome and are present in 1-2 copies per chromosome (Omer et al., J. Bacteriol. 170:2174-2184, (1988)).

While S. lividans can be transformed by the plasmids described above (including pCAO200SU1-FeS-B#9, pCAOSU2-FeS-A#11, pCAO400 and pCAO401) the host range of these SLP1-derived plasmids is limited (Kieser et al., 1982, Mol. Gen. Genet. 185:223-238). Broad host range plasmids such as those derived from the plasmids SCP2 or pIJ101 can be used to introduce into and allow expression of these genes in other Streptomyces species (Lydiate et al., 1985, Gene 35:223-235, Kieser et al., 1982, Mol. Gen. Genet. 185:223-238, Ward et al., 1986, Mol. Gen. Genet. 203:468-478). The 2.4 kb BamHI DNA fragment from pUC18-SU1 BamHI that contains the genes for P450SU1 and FeS-B can be cloned into the BamHI site of pIJ922 using methods well known to those

skilled in the art (Maniatis et al., 1982, A Guide to Molecular Cloning:A Laboratory Manual) creating pPAT108 (Figure 16A). The 2.0 kb BamHI DNA fragment from pUC19-SU2-8 can be cloned into the BglII site of pIJ425 using methods well-known to those skilled in the art (Maniatis et al., 1982, A Guide to Molecular Cloning:A Laboratory Manual) creating pCS325 (Figure 16B). The plasmids pPAT108 and pCS325 can be transformed into various Streptomyces species and will enable the transformed strains to constitutively metabolize herbicide chemicals.

Engineering plasmids with P450SU1 for the transformation of plants

For transcription and translation in plants, additional sequences must be added to the 5'-end and 3'-end of the DNA fragment comprising the cytochrome P450SU1 coding sequence. This yields a recombinant plasmid comprising A) a DNA fragment of the 2.4 kb BamHI fragment encoding enzyme P450SU1, a DNA fragment of the 2.0 kb SacI-BamHI fragment encoding enzyme P450SU1, a DNA fragment of the 1.8 kb BamHI fragment encoding enzyme P450SU1, or the 2.0 kb BamHI fragment encoding P450SU2 isolated from S. griseolus mutant PH2001; B) a DNA sequence of a plant promoter upstream and operably linked to said fragment; C) a 5'-untranslated sequence including a ribosomal binding site upstream and operably linked to said fragment; D) a DNA sequence downstream and operably linked to said fragment of a 3'-untranslated sequence which enables the mRNA transcribed from the plasmids to be polyadenylated on its 3'end.

This can be done using standard genetic engineering techniques as described in Maniatis et

al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY and Kunkel, T. A. et al. Proc. Natl. Acad. Sci. USA, 82:488-492 (1985).

Sources for a DNA fragment encoding the P450SU1 gene product include a 2.4 kb BamHI DNA fragment or a 2.0 kb BamHI-SacI DNA fragment from pUC18-SU1-BamHI. A source for a DNA fragment encoding the P450SU2 gene product is a 2.0 kb BamHI fragment in pUC19-SU2-8 isolated from S. griseolus mutant PH2001. A preferred source of a DNA fragment comprising the sequences encoding the P450SU1 gene product is the 2.4 kb BamHI DNA fragment from pUC18-SU1-BamHI. An alternative source for a similar cytochrome P450 gene would be the P450SU2 gene contained on a 2.0 kb BamHI fragment in pUC19-SU2-8.

Addition of promoter and 5'-untranslated sequences to the P450SU1 coding sequence.

A plant promoter and 5'-untranslated sequence including a ribosomal binding site must be added upstream of the DNA encoding cytochrome P450SU1 to permit the transcription and translation of the P450SU1 gene in plants. Among the promoters and 5'-untranslated sequences that will suffice for this purpose are those of the 35S promoter from the Cauliflower Mosaic Virus (CaMV) (Harpster et al. Mol. Gen. Genet. 212:182-190 (1988)), the light induced and tissue specific SSU301 gene from petunia (Dean et al. Mol. Gen. Genet. 206:465-474 (1987)), the light induced and tissue specific Cab22L gene from petunia (Dunsmuir, Nucleic Acids Res. 13:2503-2518 (1985) and Harpster et al. Mol. Gen. Genet. 212:182-190 (1988)), and the chemically inducible absicic acid-regulated

Em promoter from wheat (Marcotte et al., The Plant Cell 1:969-976 (1989)). Other plant promoters and 5'-untranslated regions from plant genes could also be used.

Addition of 3'-untranslated sequences to the P450SU1 coding sequence

Sequences must be added downstream of the DNA encoding cytochrome P450SU1 to enable the mRNA transcribed from the vectors to be polyadenylated on its 3'-end. A preferred source of such DNA is from the SSU301 gene from petunia (SSU301 encodes the small subunit of ribulose bisphosphate carboxylase) (Dean et al. Mol. Gen. Genet. 206:465-474 (1987)). This provides a poly A addition site on the P450SU1 encoding mRNA transcript when the 3'-tail region from the SSU301 gene is cotranscribed along with the p450SU1 coding sequence. Other plant genes could be used as sources of these 3'-untranslated sequences.

Addition to the cytochrome P450SU1 coding sequence of sequences encoding peptides that can facilitate the transport of cytochrome P450SU1 into the chloroplast of the plant cell

Plasmids consisting of the plant promoter, plant 5'-untranslated sequences, P450SU1 coding sequence, and plant 3'-untranslated sequences will express cytochrome P450SU1 in the cytoplasm of the plant cell. Localization of P450SU1 in chloroplasts instead of the cytoplasm may give better metabolism of herbicides because in chloroplasts there are electrons and iron-sulfur proteins that could deliver electrons to P450SU1 that might not be present in the cytoplasm. In order to express the cytochrome

P450SU1 protein in the chloroplasts of plants, constructions require in addition to the above mentioned components a transit peptide encoding sequence fused to the cytochrome P450SU1 sequence to direct the protein into the chloroplasts. Such sequences may be engineered using coding regions of the transit peptide sequences, or coding regions of the transit peptide sequence plus a portion of the coding region of the mature polypeptide, of nuclear genes encoding proteins that are normally imported into the chloroplasts of plants. Good sources of sequences for these fusions are the ribulose bisphosphate carboxylase (SSU) (Dean et al. Mol. Gen. Genet. 206:465-474 (1987)) and the chlorophyll a/b binding protein (Dunsmuir, Nucleic Acid Res. 13:2503-2518 (1985)) both from petunia. Other sources would be similar, nuclear encoded genes from other plants that encode proteins that are transported into chloroplasts. Fusions that only add the amino terminal amino acid sequence that is normally removed upon transport into chloroplasts and fusions that contain the normally removed peptide sequence and up to at least 27 amino acids of the mature transported protein can be added onto the amino terminus of cytochrome P450SU1 and downstream from the promoter and ribosomal binding site without preventing the expression of functional P450SU1 protein.

This technique will work for introducing into plants any soluble cytochrome P450 enzyme for which chloroplast ferredoxin acts as a reductant.

Engineering plasmids with P450SU1 and FeS-B for the transformation of plants

Although chloroplast ferredoxins are a source of reductant for cytochrome P450SU1, an alternative source is the FeS-B protein from S. griseolus which is the natural reductant for P450SU1. Thus plasmids that upon introduction into plant cells can direct the expression of both cytochrome P450SU1 and FeS-B are useful. To express both proteins in plant cells, modifications similar to those used to express cytochrome P450SU1 can also be done, on the same plasmid, to express FeS-B protein. Such proteins can be directed either to the cytoplasm or to chloroplasts of plant cells.

Such a recombinant plasmid resulting in expression in the cytoplasm comprises A) the DNA encoding for the cytochrome P450 or the DNA encoding for the cytochrome P450 and the DNA encoding for the FeS protein, B) one or more segments of the DNA sequence of a plant promoter upstream and operably linked to said encoding, C) one or more of a 5'-untranslated sequence including a ribosomal binding site upstream and operably linked to said encoding, and D) one or more of a DNA sequence downstream and operably linked to said encodings of a 3'-untranslated sequence which enables the mRNA transcribed from the plasmids to be polyadenylated on its 3' end.

Alternatively, a plasmid for targetting the proteins to the chloroplasts comprises A) the DNA encoding for the cytochrome P450 or the DNA encoding for the cytochrome P450 and the DNA encoding for the FeS protein, B) one or more segments of the DNA sequence of a plant promoter operably linked to said

encoding in the upstream position; C) one or more of a 5'-untranslated sequence including a ribosomal binding site operably linked to said encoding in the upstream position; D) one or more of a DNA sequence operably linked to said encoding in the downstream position of a 3'-untranslated sequence which enables the mRNA transcribed from the plasmids to be polyadenylated on its 3' end; and E) one or more of a transit peptide coding sequence or a transit peptide encoding sequence and additionally mature coding sequence of nuclear genes that encode proteins that are normally imported into the chloroplasts of plants operably linked to the DNA encoding for the amino terminus of the cytochrome P450, or to the DNA encodings for the amino terminae of the cytochrome P450 and FeS protein, and downstream from the promoter and ribosomal binding site.

The preferred plasmids for expression of cytochrome P450SU1 along with FeS-B in the cytoplasm and chloroplasts of plant cells are described in Example 19.

Introduction of plasmids into plants

DNAs containing the cytochrome P450SU1, P450SU2, FeS-A or FeS-B coding sequences with promoters from plant genes can be subcloned into T-DNA plasmids that mediate the transfer of these DNAs from Agrobacterium to plants (R. T. Fraley et al. Proc. Natl. Acad. Sci. U.S.A. 80:4803-4807 (1983), H. Klee et al. Annual Rev. Plant Physiol. 38:476-486 (1987) and references therein). This subcloning can be performed by methods well known to those skilled in the art (T. Maniatis et al., Molecular Cloning:a Laboratory Manual, Cold Spring

Harbor Press, Cold Spring Harbor, NY (1982) and T. A. Kunkel Proc. Natl. Acad. Sci. U.S.A. 82:488-492 (1985)). Although several different T-DNA plasmids were used in the examples presented here, pAGS502 could have been used for all of them since it contains unique HindIII, BamHI and EcoRI sites in the T-DNA region. Other T-DNA plasmids could also be used too as long as suitable restriction sites (i.e., HindIII, BamHI, EcoRI) are present in the region of the plasmid that is mobilized into plant cells. Alternatively, the restriction sites on the DNA fragments to be inserted into the T-DNA plasmids could be changed (T. A. Kunkel Proc. Natl. Acad. Sci. U.S.A. 82:488-492 (1985)) to allow insertion into most any restriction endonuclease site in a T-DNA plasmid.

Plasmids are mobilized into Agrobacterium such as A. tumifaciens strain LBA4404 (Hoekema et al. Nature 303:179-180, 1983) using tri-parental matings (Ruvkin and Ausubel, Nature 289:85-88, 1981), or the freeze-thaw method (Plant Molec. Biol. Manual, S. B. Gelvin and R. A. Schilperoot, Eds., A3:1-19, 1988). The resulting Agrobacterium strains are then cocultivated with protoplasts as described by van der Elzen et al. Plant Mol. Biol. 5:149-154(1985) or leaf disks as described by Horsch et al. Science 227:1229-1231,(1985) of Nicotiana tabacum cv. Wisconsin 38 and kanamycin resistant transformants selected. Kanamycin resistant transformed tobacco plants are regenerated from the transformed protoplasts or leaf disks and the plants are allowed to flower. Seed is obtained from each plant following self pollination.

Plants other than Nicotiana tabacum, including plants of horticultural or agronomic utility, such as such as vegetable or other crops, can be transformed in ways known to those skilled in the art (Gasser and Fraley, Science 244:1293-1299 (1989)). Using the Agrobacterium mediated T-DNA transfer of DNA the plasmids pSU18, pSSU-SU111, pSSU-SU121, pCab-SU111, pCabSU121, pCab-SU131, pSuFe11, pSuFe21, pSuFe31 and pSuFe41 can be mobilized into plant species that include, but are not limited to Lycopersicon esculentum, (tomato), (McCormick et al., Plan el Rep., 5:81-84 (1986)), Brassica napus, (oilse rape), (Pua et al., Bio/Technology. 5:815-( (1987)); Gossypium hirsutum, (cotton), (Umb al., Bio/Technology 5:263-266 (1987)) Glycine max, (soybean) (Hinchee et al., Bio/Technology, 6:915-921 (1988)), and Arabidopsis thaliana (Valvekens et al., Proc. Natl. Acad. Sci. USA 85:5536-5540 (1988)). The plasmids pSU18, pSSU-SU111, pSSU-SU121, pCab-SU111, pCab-SU121, pCab-SU131, pSUFe11, pSUFe21, pSUFe31 and pSUFe41 can be transformed into plant protoplasts as has been demonstrated for rice (Oryza sativa) (Toriyama et al., Bio/Technology, 6:1072-1074 (1988)) and maize (Zea mays L.) (Rhodes et al., Science, 140:204-207 (1988)). An additional alternative method to introduce the plasmids pSU18, pSSU-SU111, pSSU-SU121, pCab-SU111, pCab-SU121, pCab-SU131, pSUFe11, pSUFe21, pSUFe31 and pSUFe41 into plants is through the use of a "particle gun" (Klein et al., Nature, 327:70-73 (1987)). This method has been shown to work for Nicotiana tabacum, tobacco, (Klein et al., Proc. Natl. Acad. Sci., U.S.A., 85:8502-8505 (1988)) and Glycine max, soybeans, (McCabe et al., Bio/Technology, 6:923-926 (1988)) but is not necessarily limited to these species.

Following introduction of plasmids into plant cells by any of the above procedures, the plasmids or portions of these plasmids may be stably incorporated into the chromosomal DNA of the cell. In the case where plants are regenerated from single cells, all cells of the regenerated plant are expected to carry the integrated plasmid or plasmid parts. In the case where single cells within a regenerating multicellular structure are transformed, cells arising from the transformed cell(s) will give rise to sectors which carry the integrated plasmid or plasmid parts. In either case, the regenerated plants carrying the plasmid or plasmid parts are termed primary transformants. Depending on the species, the primary transformants can flower and give rise to gametes which fuse to form zygotes either by self pollination or by outcrossing with other plants of the same species.

Seed arising from either self pollination or outcrossing of a primary transformant contain embryos which are progeny of the primary transformant. A portion of the progeny plants may recieve chromosomes which carry copies of the plasmid or plasmid parts, depending on the number of copies of the plasmid or plasmid parts stably incorporated in the primary transformant, patterns of mendelian segregation, linkage relationships between the plasmids or plasmid parts where multiple copies exist, and whether or not the gametes arose from sectors carrying the integrated plasmid or plasmid parts. In like fashion these progeny plants may flower and give rise to subsequent generations of seed and plants carrying the plasmid or plasmid parts incorporated into the original primary transformant.

A similar situation pertains to the endosperm tissue of seed in cases where the endosperm is formed by sexual means.

A male sterility system for hybrid seed production

A means of inducing male sterility in plants generating the female parent to be used in a cross to produce hybrid seed would be very useful. Hybrid seed production is an important means of introducing desirable traits into agronomically valuable crop plants. For instance, quality traits such as oil content, herbicide resistance, disease resistance, adaptability to environmental conditions, and the like, can be hybridized in offspring so that the latter are invested with the most desirable traits of its parents. In addition, progeny from a hybrid cross may possess new qualities resulting from the combination of the two parental types, such as yield enhancement resulting from the phenomenon known as heterosis. Controlled cross-fertilization to produce hybrid seeds has been difficult to achieve commercially due to competing self-fertilization, which occurs in most crop plants.

Currently, hybrid seed production is performed by one of the following means: (a) mechanically removing or covering the male organs to prevent self-fertilization followed by exposing the male-disabled plants to plants with male organs that contain the trait(s) desired for crossing; (b) growing genetically male-sterile plants in the presence of plants with fertile male organs that contain the trait that is desired for crossing; or (c) treating plants with chemical hybridizing agents (CHA) that selectively sterilize male organs followed

by exposing the male-disabled plants to plants with fertile male organs that contain the trait that is desired for crossing. Some disadvantages to each of these methods include: (a) this is only possible for a few crops, such as corn; where the male and female organs are separate; and it is labor intensive and costly; (b) genetically male sterile lines are cumbersome to maintain, requiring crosses with restorer lines; (c) CHAs are not highly effective. The following method is applicable to a wide range of crops and allows selfing to maintain lines.

A plant is made to be receptive to male sterility induction by the introduction of the cytochrome p450SU1 or SU2 coding region under control of a suitable male organ-specific promoter. The resulting transgenic plant produces the cytochrome p450 enzyme only in its male organ. Such transgenic plants are male-fertile when grown normally. The p450-containing untreated fertile plant can be genetically crossed and propagated normally through seed production. Unlike normal plants, however, these plants can be rendered male-sterile by exposure to nontoxic chemical that is converted by the p450 enzyme into an active toxin. The toxin present in the male organ disrupts normal pollen development making the plant male sterile. The male sterility trait is only expressed when wanted, by contacting the plant with a selected protoxin; otherwise the transgenic plant behaves normally. Suitable protoxins include 10015 and other compounds that are sufficiently converted into 10014 by the cytochrome p450 enzyme.

EXAMPLES 1-3

Demonstration of constitutive expression of cytochrome P450SU1 in S. lividans strains that are transformed with pCAO400, pCAO401, pCAO200SU1-FeS-B#9 and pCAO200SU1#12

Cultures of S. lividans strains transformed with any one of the four plasmids pCAO400, pCAO401, pCAO200SU1-FeS-B#9 or pCAO200SU1#12 were grown in either sporulation broth or YEME broth media. The cultures were grown for approximately 24-36 hours at 30°C. An aliquot of cells was removed from the cultures at this time. If sulfonylurea induction of cytochrome P450SU1 was to be tested, a solution of the sulfonylurea 10001 that gave a final concentration in the culture of approximately 0.1-0.15 mg/ml was added to the culture remaining after the removal of the aliquots. Aliquots of cells were removed from the induced culture at various intervals up to 24 hours later and harvested and washed as described.

Western blot analyses for cytochrome P450SU1 was performed on cell extracts as described.

EXAMPLE 1

S. lividans transformed with pCAO400 and S. lividans C37 (Omer et. al., J. Bacteriol. 170:2174-2184, (1988), herein incorporated by reference), which contains the plasmid pCAO106 (from which pCAO170 the plasmid from which pCAO400 was derived), were grown as separate cultures in sporulation broth as described above. An aliquot of cells was removed from each culture before adding 10001 to a concentration of 0.12 mg/ml to the cells and another aliquot of cells was taken 24 hours after

adding 10001. Western blots were run on approximately 25 µg of protein from each aliquot of cells and analyzed for the presence of cytochrome P450SU1 by means of antiserum to cytochrome P450SU1 as described. The data in Fig. 5 show that S. lividans C37 (which does not contain the gene for cytochrome P450SU1) made no cytochrome P450SU1 whether or not it was induced with 10001. It also shows that cytochrome P450SU1 was made by S. lividans transformed with pCAO400 whether or not it had been induced with 10001.

EXAMPLE 2

S. lividans containing pCAO400 and S. lividans containing pCAO401 were used separately to inoculate 200 ml of sporulation broth and grown for approximately 36 hours. Fresh sporulation broth (100 ml) was added to each culture and a 30 ml aliquot was removed from each. At this time 36 mg of 10001 was added to each culture followed by the removal of 30 ml aliquots at 3, 6 and 24 hours. The cells in each aliquot were pelleted by means of centrifugation, washed and broken in a French pressure cell, as described. Approximately 25 µg of protein from each aliquot was used in a Western blot analysis for cytochrome P450SU1 by means of anti-P450SU1 antibody. The results shown in Fig. 6 indicate that cytochrome P450SU1 was produced by both S. lividans containing pCAO400 and S. lividans containing pCAO401, whether or not 10001 had been added.

EXAMPLE 3

S. lividans containing pCAO200, S. lividans containing pCAO200SU1-FeS-B#9 and S. lividans containing pCAO200SU1#12 were grown separately in 400 ml of YEME broth with shaking at 30°C for approximately 36 hours. S. griseolus ATCC 11796 was grown in 400 ml of sporulation broth for approximately 30 hours. Six hours before harvesting the cells from the cultures, 200 ml of YEME was added to the S. lividans cells. Six hours before harvesting, the S. griseolus culture was split into two 200 ml aliquots and 100 ml of fresh sporulation broth was added to each. At this time, 36 mg of 10001 was also added to one of the two S. griseolus cultures. The cells from each of the five cultures were harvested by means of centrifugation and washed twice with 50 mM MOPS pH 7.2 and an aliquot of the harvested cells was broken in a French pressure cell, as described. Approximately 30 µg of protein was used in a Western blot analysis for cytochrome P450SU1 using antiserum against cytochrome P450SU1. The results are shown in Fig. 7. No cytochrome P450SU1 was found in the S. lividans cells containing pCAO200 or in the S. griseolus culture not induced with 10001. Cytochrome P450SU1 was detected in the cultures of S. lividans transformed with either pCAO200SU1-FeS-B#9 or pCAO200SU1#12 and in the S. griseolus ATCC 11796 culture induced with 10001.

## EXAMPLES 4-9

## Metabolism of sulfonylurea compounds by S. lividans cells containing the genes for cytochrome P450SU1 and FeS-B

### EXAMPLE 4

Separate cultures (50 ml) inoculated with S. lividans C37, S. lividans transformed with pCAO400 or S. lividans transformed with pCAO401 in sporulation broth containing 0.12 mg/ml of 10001 were grown with shaking at 30°C. Aliquots (1.5 ml) of each culture were removed at 24, 32, 48 and 56 hours after inoculation and the supernatant of each aliquot was analyzed by HPLC for its concentration of 10001 and its metabolites 10002 or 10003.

The concentration (μM) of each compound is presented in Table 1.

TABLE 1

| Strain | Time (hours) | 10001 | 10002 | 10003 |
|---|---|---|---|---|
| S. lividans pCAO401 | 0 | 284 | 0 | 0 |
| | 24 | 230 | 32 | 26 |
| | 32 | 203 | 44 | 36 |
| | 48 | 123 | 63 | 46 |
| | 56 | 105 | 71 | 48 |
| S. lividans pCAO400 | 0 | 300 | 0 | 0 |
| | 24 | 269 | 17 | 16 |
| | 32 | 240 | 25 | 22 |
| | 48 | 184 | 39 | 28 |
| | 56 | 137 | 47 | 29 |
| S. lividans C37 | 0 | 325 | 1 | 0 |
| | 24 | 320 | 2 | 0 |
| | 32 | 295 | 3 | 0 |
| | 48 | 281 | 6 | 3 |
| | 56 | 280 | 7 | 3 |

EXAMPLE 5

A 100 ml culture of S. griseolus ATCC 11796 in sporulation broth and a 50 ml culture of S. lividans transformed with pCAO400 in YEME broth were prepared and incubated for 24 hours as described. At that time, the S. griseolus culture was split into two 50 ml aliquots into two separate flasks and 25 ml of fresh sporulation broth was added to each and 9 mg of 10001 was added to one of these cultures to induce cytochrome P450SU1 expression. Fresh YEME broth (25 ml) was added to the S. lividans culture. After an additional 6 hours growth at 30°C, the cells in each culture were harvested as described above, washed twice with MOPS (50 mM, pH 7.2) and recentrifuged. The cell pellets were resuspended in four cell volumes of MOPS (50 mM, pH 7.2) containing 0.2% glucose and about 100 µg/ml of 10004. These cell suspensions were incubated at 30°C with shaking and aliqouts were taken at 2 and 5.5 hours. The supernatant of each aliquot was analyzed by HPLC and the concentrations of the sulfonylurea compound 10004 and its metabolite 10005 were determined.

The concentration (µM) of 10004 and 10005 in the supernatant of each aliquot is shown in Table 2.

TABLE 2

| Cells | Time (hours) | 10004 | 10005 |
|---|---|---|---|
| none | 0 | 144 | 0 |
| S. griseolus | 2 | 55 | 72 |
| uninduced | 5.5 | 12 | 114 |
| S. griseolus | 2 | 0 | 122 |
| induced | 5.5 | 0 | 122 |
| S. lividans | 2 | 72 | 67 |
| pCAO400 | 5.5 | 0 | 125 |

EXAMPLE 6

S. lividans transformed respectively with pCAO200SU1-FeS-B#9, pCAO200SU1#12 and pCAO200 were cultured for 36 hours in 400 ml of YEME broth at 30°C. S. griseolus ATCC 11796 was similarly cultured in 400 ml of sporulation broth. Six hours before harvesting the cells, 150 ml of fresh YEME broth was added to the S. lividans cells, and the S. griseolus cells were split into two 200 ml cultures with 100 ml of fresh sporulation broth added to each. 10001 (36 mg) was added to one of the two S. griseolus cultures to induce cytochrome P450SU1. The cells were prepared as described above in Example 5 and the concentrations of the sulfonylurea compound 10006 and its metabolites 10007, 10008 or 10009 were determined.

The concentration (μM) of each compound is presented in Table 3.

TABLE 3

| Cells | Time (hours) | 10006 | 10007 | 10008 | 10009 |
|---|---|---|---|---|---|
| none | 0 | 362 | 0 | 0 | 0 |
| S. griseolus | 2 | 404 | 1 | 1 | 1 |
| ATCC 11796 | 4 | 394 | 2 | 1 | 1 |
| uninduced | 6 | 405 | 2 | 1 | 2 |
| S. griseolus | 2 | 369 | 4 | 3 | 4 |
| ATCC 11796 | 4 | 357 | 6 | 6 | 7 |
| induced | 6 | 355 | 8 | 5 | 9 |
| S. lividans | 2 | 326 | 12 | 18 | 15 |
| pCAO200 | 4 | 267 | 21 | 38 | 33 |
| SU1-FeS-B#9 | 6 | 232 | 30 | 55 | 49 |

| | | | | | |
|---|---|---|---|---|---|
| S. lividans | 2 | 376 | 2 | 1 | 0 |
| PCAO200SU1#12 | 4 | 384 | 2 | 1 | 1 |
| | 6 | 386 | 2 | 1 | 1 |
| S. lividans | 2 | 381 | 4 | 0 | 0 |
| pCAO200 | 4 | 377 | 1 | 0 | 0 |
| | 6 | 375 | 1 | 0 | 0 |

EXAMPLE 7

The cells were prepared as described above for Example 6 and it was determined to what extent the sulfonylurea compound 10010 was metabolized to compounds 10011 and 10012. The concentration (μM) of each compound is presented in Table 4.

TABLE 4

| Cells | Time (hours) | 10010 | 10011 | 10012 |
|---|---|---|---|---|
| none | 0 | 87 | 0 | 0 |
| S. griseolus | 2 | 70 | 12 | 0 |
| ATCC11796 | 4 | 49 | 20 | 2 |
| uninduced | 6 | 37 | 24 | 8 |
| S. griseolus | 2 | 40 | 32 | 2 |
| ATCC11796 | 4 | 8 | 46 | 11 |
| induced | 6 | 4 | 42 | 19 |
| S. lividans | 2 | 35 | 34 | 2 |
| pCAO200 | 4 | 11 | 37 | 12 |
| SU1-FeS-B#9 | 6 | 4 | 27 | 20 |
| S. lividans | 2 | 90 | 4 | 0 |
| pCAO200 | 4 | 85 | 7 | 0 |
| SU1#12 | 6 | 80 | 11 | 0 |
| S. lividans | 2 | 90 | 1 | 0 |
| pCAO200 | 4 | 89 | 2 | 0 |
| | 6 | 89 | 3 | 0 |

EXAMPLE 8

The cells were prepared as described above for Example 6 and it was determined to what extent the sulfonylurea compound 10001 was metabolized to compounds 10002 and 10003. The concentration (μM) of each compound is presented in Table 5.

TABLE 5

| Cells | Time (hours) | 10001 | 10002 | 10003 |
|---|---|---|---|---|
| none | 0 | 291 | 0 | 0 |
| S. griseolus | 2 | 244 | 20 | 14 |
| ATCC 11796 | 4 | 167 | 54 | 37 |
| uninduced | 6 | 86 | 86 | 62 |
| S. griseolus | 2 | 220 | 127 | 76 |
| ATCC 11796 | 4 | 36 | 203 | 128 |
| induced | 6 | 3 | 218 | 142 |
| S. lividans | 2 | 28 | 105 | 86 |
| pCAO200 | 4 | 0 | 116 | 95 |
| SU1-FeS-B#9 | 6 | 0 | 112 | 94 |
| S. lividans | 2 | 281 | 2 | 2 |
| pCAO200 | 4 | 276 | 4 | 4 |
| SU1#12 | 6 | 276 | 6 | 5 |
| S. lividans | 2 | 301 | 2 | 1 |
| pCAO200 | 4 | 287 | 1 | 1 |
| | 6 | 313 | 2 | 2 |

EXAMPLE 9

The cells were prepared as described above for Example 6 and it was determined to what extent the sulfonylurea compound 10004 was metabolized to compound 10005. The concentration (μM) of each compound is presented in Table 6.

TABLE 6

| Cells | Time (hours) | 10004 | 10005 |
|---|---|---|---|
| none | 0 | 114 | 0 |
| S. griseolus | 2 | 52 | 68 |
| ATCC 11796 | 4 | 0 | 114 |
| uninduced | 6 | 0 | 121 |
| S. griseolus | 2 | 0 | 122 |
| ATCC 11796 | 4 | 0 | 120 |
| induced | 6 | 2 | 118 |
| S. lividans | 2 | 2 | 35 |
| pCAO200 | 4 | 1 | 100 |
| SU1-FeS-B#9 | 6 | 2 | 100 |
| S. lividans | 2 | 81 | 43 |
| pCAO200 | 4 | 29 | 84 |
| SU1#12 | 6 | 0 | 109 |
| S. lividans | 2 | 97 | 22 |
| pCAO200 | 4 | 92 | 26 |
| | 6 | 85 | 30 |

Examples 4-9 demonstrate that the genes for cytochrome P450SU1 and FeS-B when expressed in S. lividans can metabolize sulfonylurea compounds to the same products that are produced by S. griseolus ATCC 11796. Expression in S. lividans strains that have the gene(s) for cytochrome P450SU1 with or without FeS-B, however, is constitutive, not requiring induction by compounds like 10001. For optimal metabolic activity of S. lividans strains expressing cytochrome P450SU1, the gene for its electron donor FeS-B must be present as well. Examples 6-9 demonstrate that expressing the genes for both P450SU1 and FeS-B in S. lividans enables S. lividans to metabolize several sulfonylurea compounds more readily than S. griseolus cells that have not

been previously induced with 10001. Such strains would be valuable for metabolizing sulfonylurea compounds that are poor inducers of cytochrome P450SU1 in S. griseolus ATCC 11796 since they can be metabolized by the S. lividans strains described without having to first induce with 10001 or some other sulfonylurea and later remove the inducing compound and its metabolites from the culture.

## EXAMPLE 10

### Constitutive expression of cytochrome P450SU2 and FeS-A in S. lividans

The plasmid made for the examples below was made by ligating the 2.0 kb BamHI fragment from pUC19-SU2-8 containing the genes for cytochrome P450SU2 and FeS-A into the BamHI site of pCAO200 which resulted in the plasmid pCAO200SU2-FeS-A#11. The plasmid pCAO200SU2-FeS-A#11 was transformed into Streptomyces lividans using methods selecting for resistance to the antibiotic thiostrepton encoded by the plasmid (Hopwood et al., Genetic Manipulation of Streptomyces. A Laboratory Manual, pp. 12-14 and 104-109, John Innes Foundation, Norwich, U. K., herein incorporated by reference). A restriction endonuclease map of pCAO200SU2-FeS-A#11 is shown in Fig. 8.

S. lividans containing the plasmid pCAO200SU2-FeS-A#11 was grown in YEME broth at 30°C as described in Example 1 and the level of cytochrome P450SU2 was analyzed by Western blot as in Examples 1-3. The results are shown in Fig. 9. As can be seen, cytochrome P450SU2 is expressed in S. lividans transformed with pCAO200-SU2-FeS-A in the absence of

sulfonylrurea induction. S. lividans cells transformed with pCAO200 do not produce cytochrome P450SU2.

## EXAMPLES 11-12

## Metabolism of sulfonylurea compounds by S. lividans cells containing the genes for cytochrome P450SU2 and FeS-A

### EXAMPLE 11

S. lividans transformed with pCAO200SU2-FeS-A#11 and S. lividans transformed with pCAO200 were cultured for 36 hours in 400 ml of YEME broth at 30°C. S. griseolus PH2001 (mutant without SU1) was cultured in 400 ml of sporulation broth at 30°C. Nine hours before harvesting the cells, the S. griseolus PH2001 culture was divided into two 200 ml cultures. Both received 100 ml of fresh sporulation broth and one received 36 mg of 10001 to induce cytochrome P450SU2. The cells were prepared as described above in Example 6 and the concentrations of the sulfonylurea compound 10001 and its metabolites 10002 and 10003 were determined.

The concentration (μM) of each compound is presented in Table 7.

TABLE 7

| Strain | Time (hours) | 10001 | 10002 | 10003 |
|---|---|---|---|---|
| none | 0 | 279 | 0 | 0 |
| S. griseolus | 2 | 271 | 7 | 1 |
| PH2001 | 4 | 264 | 12 | 2 |
| uninduced | 6 | 244 | 23 | 3 |

| | | | | |
|---|---|---|---|---|
| S. griseolus | 2 | 286 | 41 | 7 |
| PH2001 | 4 | 256 | 60 | 10 |
| induced | 6 | 234 | 71 | 13 |
| S. lividans | 2 | 279 | 7 | 1 |
| pCAO200SU2 | 4 | 273 | 12 | 2 |
| -FeS-A#11 | 6 | 251 | 15 | 3 |

EXAMPLE 12

The cells were prepared as described above for Example 11 and it was determined to what extent the sulfonylurea compound 10010 was metabolized to compound 10011.

The concentration (μM) of each compound is presented in Table 8.

TABLE 8

| Strain | Time (hours) | 10010 | 10011 |
|---|---|---|---|
| none | 0 | 88 | 0 |
| S. griseolus | 2 | 89 | 0 |
| PH2001 | 4 | 88 | 0 |
| uninduced | 6 | 89 | 1 |
| S. griseolus | 2 | 64 | 21 |
| PH2001 | 4 | 56 | 28 |
| induced | 6 | 50 | 32 |
| S. lividans | 2 | 90 | 1 |
| + pCAO200SU2 | 4 | 84 | 5 |
| -FeS-A#11 | 6 | 72 | 14 |

The results of Example 10 (the Western blot analysis) showed that bacteria containing the cytochrome P450SU2 gene produced cytochrome P450SU2 constitutively. This is in contrast to the case in S. griseolus strains in which P450SU2 is made in

detectable amounts only with the addition of inducing sulfonylurea compounds such as 10001 (O'Keefe et al. Recent Adv. in Phytochemistry 21: 151-137, (1987)). Results from Examples 11 and 12 (the metabolism experiments) show that constitutive expression of the cytochrome P450SU2 and FeS-A genes in S. lividans enables S. lividans to metabolize sulfonylurea compounds nearly to the same extent as S. griseolus PH2001. Also, S. lividans transformed with pCAO200SU2-FeS-A#11 metabolizes the sulfonylurea 10010, which is a poor inducer of cytochrome P450SU2 in S. griseolus, more readily than uninduced S. griseolus PH2001.

EXAMPLE 13

Prevention of sulfonylurea inhibition of plant growth

A 2 liter culture of S. lividans pCAO200SU1-FeS-B#9 was grown in YEME medium at 30°C until the culture was in late log phase of growth and the absorbance in a spectrophotometer at a wavelength of 600 nM was between about 1.0 and 1.3. Tomato seedlings (Lysopersicon esculentum cv. "Pixie") were seeded directly into soiless media, Oasis Wedges® (Smithers-Oasis, Kent, OH), fertilized with 500 ppm Peters'® fertilizer (20:19:18); and 300 ppm of iron was added weekly. As the tomato plants develop, roots ramify through the Oasis Wedges®. The tomato plants were transplanted to pots when they were 4 inches tall as follows.

Five inch standard round pots (without holes) were filled with Sassafras sandy loam (pH 6.7, 0.8% OM) and an oasis cube. The contents of the each pot was sprayed, prior to transplanting the tomato plants, with either 10001, in 25% dry flowable composition (25 DF) or 10010, 75 DF at rates of 16,

32, 64, 125 or 250 grams of active ingredient per hectare (g ai/ha). The Oasis cube was then removed and replaced by another containing a transplant tomato plant dipped in either water (treatment A), YEME medium (treatment B) or the culture of S. lividans pCAO200SU1-FeS-B#9 described above (treatment C). Six transplant tomato plants received each of the three treatments, and five transplant tomato plants (to serve as controls) received no treatment. The resulting tomato plants were grown in a greenhouse for 19 days and watered twice daily, after which they were evaluated for visual injury (100 = complete kill, other numbers = percentage of injury relative to controls [subjectively determined], 0 = no injury) with respect to the water dipped, no-herbicide control treatments. The plants were left in the greenhouse for one more week after which the fresh weights of the shoots of the plants were determined. The roots of the plants which received these treatments and controls were examined too.

Table 9 shows the visual injury ratings for the transplants which were determined by visual inspection 19 days after transplanting (DAT). Tomato plants which were planted into 10001 and 10010 showed different degrees of visual damage depending on which treatment they received. Tomato plants treated with treament C were significantly less injured by 10001 when the latter was applied at rates of 64, 125, and 250 g ai/ha than were the tomato plants which received treatments A or B. Tomato plants which had been transplanted into 10010 were injured to similar extents regardless of the treatment they received.

Weights of the fresh shoots of those plants which received 16 and 32 g ai/ha of 10010 and of all

those plants which received 10001 were determined (Table 10). The weights of the fresh shoots of tomatoes planted into 10010 receiving water, YEME or S. lividans treatments were not significantly different ($p = 0.05$) from each other and were all considerably less than those which did not receive any herbicide. While the shoots of tomatoes planted into 10001 (at application rates of 64, 125, or 250 g ai/ha) weighed considerably less than those which did not receive herbicide, they weighed significantly more when they had been dipped in S. lividans than when they were treated with water or YEME. Shoot weights of the tomatoes dipped in the cultures of pCAO200SU1-FeS-B#9 were approximately 3-4 times greater at the concentration of 250 g ai/ha of 10001 and 2-3 times at the concentration of 125 g ai/ha of 10001. The differences between the weights of the shoots from plants which received 10001 at application rates of 16 or 32 g ai/ha were not significant regardless of which additional treatment they received.

Visual examination of the root systems of selected treatments showed no signs of injury when the plants were dipped in water, YEME or pCAO200SU1-FeS-B#9 and did not receive herbicide. Apparently S. lividans produced no gross signs of damage or aberant morphology to the roots. When the plants received 10001, they all had roots with damage typical of that resulting from contact with sulfonylureas (stunted primary roots with poorly developed secondary roots). Presumably, dipping the plants in S. lividans depleted the level of 10001 in the soil solution in the vicinity of the roots to a low enough level to alleviate some shoot symptoms, but not enough to mitigate damage to the roots directly in contact with the soil.

TABLE 9

Visual Injury Ratings of Transplanted Tomatoes*

| Sulfonyl-urea | Dosage rate (g ai/ha) | Treatment A: (water) | Treatment B (YEME) | Treatment C (pCAO200SU1--FeS-B#9) |
|---|---|---|---|---|
| 10010 | 250 | 100,80,100, 90,100,100 | 100,80,80, 100,80,80 | 70,80,80, 100,90,90 |
| | 125 | 80,100,80, 90,100,100 | 100,70,80, 100,80,70 | 80,80,80 60,80,80 |
| | 64 | 80,100,80, 80,100,100 | 50,60,80, 70,100,80 | 60,100,100 80,80,100 |
| | 32 | 80,100,80, 100,100,100 | 70,70,60, 80,60,60 | 60,50,60 50,60,60 |
| | 16 | 60,80,90, 60,50,80, | 60,80,70, 80,60,50 | 60,60,60 60,60,60 |
| 10001 | 250 | 100,80,100, 100,90,80 | 80,70,80, 80,100,80 | 50,50,50 50,50,50 |
| | 125 | 100,100,90, 90,80,80 | 70,70,70, 70,80,60 | 50,50,50 50,50,50 |
| | 64 | 70,50,80, 70,70,60 | 60,60,80, 60,60,60 | 40,50,50 40,40,40 |
| | 32 | 40,100,70, 60,80,60 | 50,60,50, 50,80,60 | 40,40,40 100,40,40 |
| | 16 | 40,40,40, 60,40,40 | 60,50,50, 50,50,50 | 40,40,40 40,40,40 |
| none | 0 | 0,0,0 0,0, | 0,0,0, 30,30 | 0,0,0 20,20 |

* Scale of 0 to 100 with 100 = complete kill, 0 = no injury.

Mean value of visual ratings

| Sulfonyl-urea | Dosage rate (g ai/ha) | Treatment A (water) | Treatment B (YEME) | Treatment C (pCAO200SU1--FeS-B#9) |
|---|---|---|---|---|
| 10010 | 250 | 95.0 | 86.7 | 85 |
| | 125 | 91.7 | 83.3 | 76.7 |
| | 64 | 90.0 | 83.3 | 86.7 |
| | 32 | 93.3 | 66.7 | 56.7 |
| | 16 | 70.0 | 63.3 | 60 |
| 10001 | 250 | 91.7 | 81.7 | 50.0 |
| | 125 | 90 | 70 | 50 |
| | 64 | 66.7 | 63.3 | 43.3 |
| | 32 | 68.3 | 58.3 | 50 |
| | 16 | 43.3 | 51.7 | 40 |
| none | | 0 | 12 | 8 |

TABLE 10

Shoot Fresh Weights of Selected Treatments (grams)

| Sulfonyl-urea | Dosage rate (g ai/ha) | Treatment A (water) | Treatment B (YEME) | Treatment C (pCAO200SU1--FeS-B#9) |
|---|---|---|---|---|
| 10010 | 32 | 0.85,0.38,0.93 | 3.0,1.03,1.05 | 2.16,1.09,2.30 |
| | | 0.74,0.34,0.66 | 1.74,1.82,0.98 | 2.21,0.93,2.07 |
| | 16 | 0.56,1.45,2.54 | 1.42,3.10,1.65 | 2.86,1.19,3.27 |
| | | 2.14,0.78,0.18 | 1.56,1.49,0.28 | 2.70,1.62,0.95 |
| 10001 | 250 | 0.53,1.03,0.46 | 0.64,0.76,0.51 | 2.64,2.61,2.50 |
| | | 0.10,1.55,0.10 | 0.19,0.11,0.20 | 2.17,2.71,1.81 |
| | 125 | 1.86,0.73,1.88 | 1.13,1.24,1.68 | 3.06,3.69,4.00 |
| | | 0.1,0.10,0.10 | 0.63,1.11,0.58 | 1.41,1.55,2.60 |
| | 64 | 2.06,0.32,1.45 | 1.32,1.48,2.23 | 4.04,3.88,2.74 |
| | | 0.78,2.59,1.04 | 0.62,1.40,1.36 | 2.91,2.62,1.45 |
| | 32 | 2.43,3.30,1.16 | 0.53,2.82,1.86 | 4.02,2.19,3.22 |
| | | 5.36,0.80,1.90 | 1.26,1.84,2.26 | 4.10,3.10,3.59 |
| | 16 | 3.85,1.46,4.80 | 3.61,1.59,2.62 | 2.60,2.42,2,36 |
| | | 3.79,2.36,3.23 | 1.77,2.89,1.59 | 4.32,3.10,3.80 |
| none | | 14.8,17.1,12.6 | 16.9,15.0,5.0 | 11.2,12.09, |
| | | 16.28,14.77 | 11.4,16.5 | 15.75,7.8,7.54 |

Mean Shoot Fresh Weights (grams)

| Sulfonyl-urea | Dosage rate (g ai/ha) | Treatment A (water) | Treatment B (YEME) | Treatment C (pCAO200SU1--FeS-B#9) |
|---|---|---|---|---|
| 10010 | 32 | 0.65 | 1.60 | 1.79 |
| | 16 | 1.28 | 1.58 | 2.10 |
| 10001 | 250 | 0.63 | 0.40 | 2.41 |
| | 125 | 0.80 | 1.06 | 2.72 |
| | 64 | 1.37 | 1.40 | 2.94 |
| | 32 | 2.49 | 1.76 | 3.37 |
| | 16 | 3.25 | 2.35 | 3.1 |
| None | | 15.11 | 12.96 | 10.88 |

## EXAMPLE 14

Demonstration of metabolism of sulfonylurea compounds by S. griseolus, S. griseus, S. ambofaciens, and S. lividans strains that are transformed with pPAT108

S. griseolus PH2003, S. griseus PH4001, S. ambofaciens PH4002, and S. lividans JI1326 were transformed with the plasmid pPAT108. The transformed S. griseolus strain, PH3826, was cultured for 24 hours in 150 mls of sporulation broth with 5 ug/ml thiostrepton. The transformed S. griseus strain, PH3832, and the transformed S. ambofaciens strain, PH3834, were cultured for 24 hours in 150 mls of trypticase soy broth with 5 ug/ml thiostrepton. The transformed S. lividans strain, PH3822, was similarly cultured in 150 mls of YEME broth with 5 ug/ml thiostrepton. Three hours before harvesting the cells, 50 mls of fresh medium of the same type

with 5 ug/ml thiostrepton was added to each culture. The cells from each of the cultures were harvested by centrifugation, washed twice with 50 mM MOPS, pH7.2 and resuspended in five cell volumes of MOPS containing 0.2% glucose and 120 ug/ml of the sulfonylurea 10001. The cell suspensions were incubated at 30°C with shaking and aliquots were removed at 0.5, 1, 2 and 4 hours. The supernatant of each aliquot was analyzed by HPLC and the concentration of the sulfonylurea compound 10001 and its metabolites 10002 and 10003 were determined.

The concentration (uM) of each compound is presented in Table 11.

Table 11

| Strain | Time (hours) | 10001 | 10002 | 10003 |
|---|---|---|---|---|
| S. griseolus | 0.5 | 222 | 41 | 22 |
| PH3826 | 1.0 | 137 | 101 | 58 |
| | 2.0 | 19 | 171 | 97 |
| | 4.0 | 0 | 189 | 107 |
| S. griseus | 0.5 | 281 | 18 | 9 |
| PH3832 | 1.0 | 238 | 45 | 26 |
| | 2.0 | 177 | 82 | 46 |
| | 4.0 | 77 | 138 | 80 |
| S. ambofaciens | 0.5 | 299 | 6 | 1 |
| PH3834 | 1.0 | 278 | 11 | 6 |
| | 2.0 | 250 | 27 | 14 |
| | 4.0 | 191 | 64 | 33 |
| S. lividans | 0.5 | 294 | 2 | 1 |
| PH3822 | 1.0 | 284 | 5 | 3 |
| | 2.0 | 262 | 10 | 5 |
| | 4.0 | 232 | 25 | 15 |

| | | | | |
|---|---|---|---|---|
| S. griseus | 1.0 | 370 | 1 | 1 |
| PH4001 | 4.0 | 377 | 2 | 4 |
| S. ambofaciens | 1.0 | 406 | 0 | 0 |
| PH4002 | 4.0 | 412 | 0 | 0 |

EXAMPLE 15

Demonstration of metabolism of sulfonylurea compounds of S. griseolus, S. griseus, S. ambofaciens, and S. lividans strains that are transformed with pCS325.

S. griseolus PH2003, S. griseus PH4001, S. ambofaciens PH4002, and S. lividans JI1326 were transformed with the plasmid pCS325. Cells of the transformed strains of S. griseolus PH3809, S. griseus PH3817, S. ambofaciens PH3818, and S. lividans PH3816, were grown and treated as described in Example 14, and the concentrations of the sulfonylurea compound 10001 and its metabolites 10002 and 10003 were determined.

The concentration (uM) of each compound is presented in Table 12.

Table 12

| Strain | Time (hours) | 10001 | 10002 | 10003 |
|---|---|---|---|---|
| S. griseolus | 0.5 | 175 | 64 | 6 |
| PH3809 | 1.0 | 108 | 155 | 16 |
| | 2.0 | 0 | 236 | 24 |
| | 4.0 | 0 | 238 | 25 |
| S. griseus | 0.5 | 226 | 37 | 3 |
| PH3817 | 1.0 | 181 | 73 | 7 |
| | 2.0 | 69 | 167 | 16 |
| | 4.0 | 0 | 220 | 23 |

| | | | | |
|---|---|---|---|---|
| S. ambofaciens | 0.5 | 286 | 16 | 1 |
| PH3818 | 1.0 | 265 | 33 | 3 |
| | 2.0 | 210 | 67 | 7 |
| | 4.0 | 129 | 134 | 13 |
| S. lividans | 0.5 | 331 | 19 | 1 |
| PH3816 | 1.0 | 339 | 36 | 3 |
| | 2.0 | 299 | 68 | 7 |
| | 4.0 | 248 | 103 | 11 |

The results in Examples 14 and 15 showed that transformation of Streptomyces strains with broad host range plasmids containing the genes for P450SU1 and FeS-B or P450SU2 and FeS-A enabled these transformed strains to constitutively metabolize sulfonylurea compounds. The rate at which these transformed strains were able to carry out the metabolism of a sulfonylurea compound varied depending on the ability of endogenous reductases to provide reducing equivalents to the P450 as required for catalysis and on the copy number of the plasmid in the transformed strain.

## EXAMPLE 16

Metabolism of Non-sulfonylurea Herbicides by Bacteria Containing the Genes for P450SU1 or P450SU2.

Separate cultures (50 ml) inoculated with S. lividans C37, S. lividans transformed with pCAO200SU1-FeS-B#9, S. griseolus ATCC11796, S. griseolus PH2003 transformed with pIJ425, or S. griseolus PH2003 transformed with pCS325 were cultured in sporulation broth for 18 hours at 30°C with shaking. Each culture was then resuspended in 25 ml fresh sporulation broth and 3.0 mg herbicide added. In the case of cultures of S. griseolus

ATCC11796, a second culture containing herbicide and 3.0 mg 10001 was also prepared. Each culture was reincubated for 24 hours, then an aliquot of the medium was withdrawn and analyzed by HPLC. The percent conversion of herbicide was determined.

The percent conversion of herbicide is presented in Table 13. The results in Table 13 show that bacteria containing constitutively expressed P450SU1 metabolized the nonsulfonylurea herbicides 10017, 10018, and 10019. In addition bacteria containing constitutively expressed P450SU2 metabolized the nonsulfonylurea herbicides 10020, 10021, 10017, 10022 and 10018.

Table 13

Percent Conversion

| Strain | 10020 | 10021 | 10017 | 10022 | 10018 | 10019 | 10023 | 10001 |
|---|---|---|---|---|---|---|---|---|
| S. griseolus ATCC 11796 | 12 | NT | 1 | 1 | NT* | 0 | 0 | 100 |
| S.griseolus ATCC 11796, 10001 added | 33 | NT | 35 | 30 | NT* | 80 | 0 | 100 |
| S. lividans C37 | 17 | 0 | 19 | 50 | 0 | 0 | 0 | 0 |
| S. lividans pCAO200 SU1-FeS-B#9 | 19 | 23 | 47 | 42 | 56 | 35 | 0 | 56 |
| S. griseolus PH2003 pIJ425 | 7 | 7 | 6 | 0 | 10 | 0 | NT* | 100 |
| S. griseolus PH2003 pCS325 | 89 | 93 | 98 | 96 | 72 | 9 | NT* | 100 |

*NT = Not tested

EXAMPLE 17

Metabolism of 10015 Analogs to 10014 - Formation of Phytotoxic Metabolites by P450SU1

Cultures of S. griseolus ATCC11796 were cultivated in sporulation broth (50ml) at 30°C with shaking for 17 hours. Each culture was then resuspended in 25 ml fresh sporulation broth, 3.0 mg sulfonylurea was added, and the culture reincubated for 4 days. Aliquots of the medium were then analyzed by HPLC. Formation of 10014 was measured based on similarity of retention time and UV spectrum of the test metabolite to that formed by metabolism of 10015.

The percent conversion of sulfonylurea to 10014 is presented in Table 14. The results in Table 14 show that P450SU1 metabolized the nonphytotoxic sulfonylureas 10015, 10024, 10025, 10026, 10027 and 10028 to phytotoxic 10014.

Table 14

| Sulfonylurea | N-substitution | % Conversion to 10014 |
|---|---|---|
| 10029 | $-CH_3$ | 0 |
| 10024 | $-CH_2CH_3$ | 69 |
| 10027 | $-CH_2CH_2CH_3$ | <10 |
| 10004 | $-CH_2CH_2CH_2CH_3$ | 0 |
| 10015 | $-CH(CH_3)_2$ | 100 |
| 10030 | $-CH_2CH(CH_3)_2$ | 0 |
| 10025 | -benzyl | 50 |
| 10028 | $-CH_2CH=CH_2$ | 27 |
| 10031 | $-COCH_3$ | 0 |
| 10026 | $-CH_2CH_2F$ | 18 |
| 10032 | $-CH_2Si(CH_3)_3$ | 0 |

EXAMPLE 18

Prevention of sulfonylurea inhibition of plant growth

One liter cultures of S. griseolus ATCC 11796, S. lividans pCAO200, S. lividans pCAO200-#9-SU1-FeS-B or S. lividans pCAO200-#12-SU1 were grown in YEME medium (sporulation broth for the S. griseolus culture) at 30°C until the cultures were in late log phase of growth and the absorbance of each culture in a spectrophotometer at a wavelength of 600 nM was between 1.0 and 1.3. Tomato seedlings (Lycopersicon esculentum cv. "Pixie") were seeded directly into soiless media, Oasis Wedges® (Smithers-Oasis, Kent, OH), fertilized with 500 ppm Peter's® fertilizer (20:19:18); and 300 ppm of iron was added weekly. As the tomato plants develop, roots ramify through the Oasis Wedges®. The tomato plants were transplanted to pots when they were 4 inches tall as follows.

Five inch standard round pots (without holes) were filled with Sassafras sandy loam (ph 6.7, 0.8% OM) and a single Oasis cube and then treated preemergence with either Classic® (10001) 25 DF (16, 32, 64, 125 and 250 grams active ingredient/hectare [g ai/ha]) or Oust® (10010) 75 DF at rates of 4, 8, 16, 32 and 64 g ai/ha), both herbicides available from E. I. du Pont de Nemours and Company, Wilmington, Delaware. The oasis cube was then removed and replaced by a transplant tomato dipped in either the cultures described above of S. griseolus ATCC 11796 (treatment A), S. lividans pCAO200 (treatment B), S. lividans pCAO200-#9-SU1-FeS-B (treatment C), S. lividans pCAO200-#12-SU1 (treatment D) or into water (treatment E). Five transplants were tested for each treatment at each dosage rate. The pots were placed on a greenhouse bench for 22

days and watered twice daily, after which shoot fresh weights of treatments were determined. Roots of these treatments and controls were examined too. Plants, soil and pots treated with bacterial cultures were double bagged and disposed by incineration.

Table 15 shows the weights of the fresh shoots for the transplants which were determined 22 days after transplanting. When fresh weights were compared, the safening by treatment C (S. lividans-#9-SU1-FeS-B) was clear (P=0.05). Treatment C permitted significantly greater fresh weights than the water controls (treatment E) at 10001 rates of 32, 64 and 125 g ai/ha and at 10010 rates of 16 and 32 g ai/ha. At these herbicide application rates, treatment C gave greater safening than treatments A, B and D as well, which demonstrated the need for the inclusion of the DNA encoding FeS-B for the best safening. Shoot fresh weight of the tomatoes in treatment C (S. lividans pCAO200-#9-SU1-Fes-B) were approximately 2-3 times greater than those from the other treatments when planted into soil with 32, 64 and 125 g ai/ha of 10001 or 16 and 32 g ai/ha of 10010. The differences between the weights of the shoots from plants receiving the other herbicide treatments were not significantly different from those treated with water.

Visual examination of the root systems of the plants treated with any of the five treatments showed no signs of injury when they did not receive herbicide. When the plants were treated with herbicide, all had roots with damage typical of that resulting from contact with sulfonylureas (stunted primary roots with poorly developed secondary roots). This pointed to S. lividans expressing the

genes for cytochrome P450SU1 and FeS-B being able to deplete the level of herbicide within the transplanted cube, but since S. lividans probably did not colonize the roots of the transplant, damage still occurred when the roots came directly in contact with the treated soil.

TABLE 15

Shoot Fresh Weights of Tomatoes Transplanted into 10001 or 10010 after Various Treatments*

| Herbicide | Rate (g ai/ha) | Pretreatment** A | B | C | D | E |
|---|---|---|---|---|---|---|
| 10001 | 16 | 0.88 | 0.67 | 1.63 | 0.62 | 1.67 |
| | | (0.16) | (0.09) | (0.18) | (0.13) | (0.48) |
| | 32 | 0.74 | 0.47 | 1.62 | 0.82 | 1.07 |
| | | (0.20) | (0.05) | (0.15) | (0.19) | (0.25) |
| | 64 | 0.21 | 0.53 | 1.24 | 0.47 | 0.46 |
| | | (0.04 | (0.07) | (0.08) | (0.04) | (0.16) |
| | 125 | 0.49 | 0.38 | 1.01 | 0.35 | 0.23 |
| | | (0.13) | (0.11) | (0.29) | (0.05) | (0.02) |
| | 250 | 0.44 | 0.25 | 0.55 | 0.26 | 0.21) |
| | | (0.12) | (0.07) | (0.24) | (0.03) | (0.04) |
| 10010 | 4 | 1.16 | 0.73 | 0.88 | 0.88 | 2.00 |
| | | (0.11) | (0.14) | (0.12) | (0.27) | (0.24) |
| | 8 | 0.58 | 0.69 | 1.02 | 0.75 | 0.65 |
| | | (0.17) | (0.13) | (0.32) | (0.15) | (0.32) |
| | 16 | 0.32 | 0.65 | 0.98 | 0.56 | 0.39 |
| | | (0.06 | (0.15) | (0.13) | (0.11) | (0.25) |
| | 32 | 0.33 | 0.42 | 1.01 | 0.62 | 0.28 |
| | | (0.03 | (0.09) | (0.15) | (0.10) | (0.04) |
| | 64 | 0.30 | 0.39 | 0.46 | 0.29 | 0.34 |
| | | (0.06) | (0.12) | (0.14) | (0.05) | (0.09) |

| | | | | | | |
|---|---|---|---|---|---|---|
| None | --- | 14.14 (0.44) | 9.73 (1.90) | 14.25 (2.56) | 16.40 (2.91) | 23.73 (4.70) |

*The values in grams are the mean of five plants for each pretreatment and herbicide rate. The standard deviation in parentheses () is indicated below the mean weight value

**Key to Pretreatments:

A: Streptomyces griseolus ATCC11796

B: Streptomyces lividans pCAO200

C: S. lividans pCAO200-#9-SU1-FeS-B

D: S. lividans pCAO200-#12-SU1

E: water

EXAMPLE 19

Engineering plasmids with the P450SU1 and or FeS-B coding sequences for the transformation of plants

Sequences must be added to 5'-end and 3'-end of the cytochrome P450SU1 and FeS-B coding sequence in order to get transcription and translation of the cytochrome P450SU1 and FeS-B genes in plants. We have done so in ten plasmids that are described below. General descriptions of these ten plasmids are given first followed by detailed descriptions of how these plasmids were made.

A. Plasmid for cytoplasmic expression of cytochrome P450SU1 with or without FeS-B

The plasmid, pSU17, was prepared containing the P450SU1 coding sequence with the Cauliflower Mosaic Virus 35S promoter and the 5' untranslated region from the petunia chlorophyll a/b binding protein gene "Cab22L" (described in Harpster et al. Mol. Gen. Genet. 212:182-190 (1988) herein incorporated by reference) upstream of the P450SU1 coding sequence. The 3' untranslated region from the small subunit of ribulose bisphosphate carboxylase (SSU) gene "SSU301" from petunia (Dean et al. Mol. Gen. Genet.206:465-474 (1987)) was placed downstream of the P450SU1 coding sequence. For propogation in E. coli the pSU17 contained the sequences of the plasmid pUC118. A diagram of pSU17 is shown in Figure 10A. The construct pSU17 when introduced into plant cells expressed cytochrome P450SU1 in the cytoplasm.

The plasmid pSuFe1 contains two adjacent Cauliflower Mosaic Virus (CaMV) 35S promoters promoting transcription in opposite directions along

with the 60 bp region from the 5'-untranslated region of the small subunit of ribulose bis-phosphate carboxylase (SSU) from petunia to constitutively express cytochrome P450SU1 and FeS-B in the cytoplasm of plants. The 3'-untranslated region used for expression of both genes is from the gene for nopaline synthetase(nos) derived from T-DNA of Agrobacterium tumefaciens (Depicker et al., J. Mol. Appl. Genet. 1:561-573 (1982)). A diagram of pSuFel is shown in Fig. 15A.

B. Plasmids that encode cytochrome P450SU1 and or FeS-B proteins that additionally contain peptides that can facilitate the transport of cytochrome P450SU1 or FeS-B into chloroplasts of plant cells

In order to express the cytochrome P450SU1 or FeS-B proteins in the chloroplasts of plants, eight constructions were engineered using 5' promoter regions, coding regions of the transit peptide sequences and in some cases part of the mature coding sequences of genes encoding proteins normally imported into the chloroplasts of plants. The genes for normally imported proteins were those for ribulose bisphosphate carboxylase (SSU) and chlorophyll a/b binding protein (Cab) both from petunia. Plasmids that only added to the P450SU1 coding sequence the amino terminal amino acid sequence that is normally removed upon transport into chloroplasts and plasmids that added to the P450SU1 coding sequence the normally removed peptide and up to 27 amino acids of the mature transported protein were constructed. Plasmids that additionally contain the FeS-B coding sequence only added the DNA sequence that encodes the peptide normally removed upon transport into the chloroplast.

1. pSSU-SU11. This plasmid was prepared and contained the DNA encoding the first 69 amino acids of the SSU301 gene from petunia (Dean et al., Mol. Gen. Genet. 206:465-474 (1987) herein incorporated by reference) (57 amino acid chloroplast transit peptide and 12 amino acids of mature SSU301) added onto the $NH_2$-terminus of the P450SU1 coding sequence. The SSU301 promoter and 5' and 3' untranslated sequences (Dean et al., Mol. Gen. Genet. 206:465-474 (1987)) of the SSU301 gene provided transcription and translation signals for expression of this protein in plants. For propogation in E. coli pSSU-SU11 included the sequences of the plasmid pUC118. A diagram of pSSU-SU11 is shown in Figure 10B.

2. pSSU-SU12. This plasmid was prepared like pSSU-SU11 except that it contained only the DNA encoding the 57 amino acid chloroplast transit peptide of the petunia SSU301 gene added onto the amino terminus of P450SU1. A diagram of pSSU-SU12 is shown in Figure 10C.

3. pCab-SU13. This plasmid was prepared containing the DNA encoding the first 61 amino acids of the petunia Cab22L gene (Dunsmuir, Nucleic Acids Res. 13:2503-2518 (1985) herein incorporated by reference) (34 amino acids of the chloroplast transit peptide and 27 amino acids of the mature Cab22L protein) added onto the $NH_2$-terminus of cytochrome P450SU1. The promoter and 5'-untranslated region of the petunia Cab22L gene(Gidoni et al. Mol. Gen. Genet. 211:507-514 (1988) herein incorporated by

reference) and the 3'-untranslated region of the petunia SSU301 gene provided transcription and translation signals for expression in plant cells. For propogation in E. coli pCab-SU13 included the sequences of the plasmid pUC118. A diagram of pCab-SU13 is shown in Figure 10F.

4. pCab-SU11. This plasmid is similar to pCab-SU13 except that it contains the DNA encoding first 48 amino acids of the petunia Cab22L gene (Dunsmuir, Nucleic Acids Res. 13:2503-2518(1985)) (34 amino acids of the chloroplast transit peptide and 14 amino acids of the mature Cab22L protein) added onto the $NH_2$-terminus of cytochrome P450SU1. This plasmid can be prepared from pCab-SU13 by site-directed mutagenesis in which the 39 nucleotides encoding for the 13 additional amino acids of the Cab22L protein found in pCab-SU13 are removed from the plasmid using methods well known to those skilled in the art (Kunkel, T.A., et al. Proc. Natl. Acad. Sci. U.S.A., 82:488-492 (1985)) knowing that the DNA sequence spanning this area in pCab-SU13 is:

ATG AGG AAG ACT GCT ACC AAG GCC AAG CCT
$Met_1$- $Arg_2$- $Lys_3$- $Thr_4$- $Ala_5$- $Thr_6$- $Lys_7$- $Ala_8$- $Lys_9$- $Pro_{10}$-
Cab22L mature protein

GTC TCT TCT GGC AGC CCA TGG TAT
$Val_{11}$- $Ser_{12}$- $Ser_{13}$- $Gly_{14}$- $Ser_{15}$- $Pro_{16}$- $Trp_{17}$- $Tyr_{18}$-

GGT CCT GAT CGT GTC AAG TAC TTG
$Gly_{19}$- $Pro_{20}$- $Asp_{21}$- $Arg_{22}$- $Val_{23}$- $Lys_{24}$- $Tyr_{25}$- $Phe_{26}$-

GGC AGT ACT GAT ACC GCC
$Gly_{17}$- $Ser_1$- $Thr_2$- $Asp_3$- $Thr_4$- $Ala_5$
Cytochrome P450SU1

A diagram of pCab-SU11 is shown in Figure 10D.

5. pCab-SU12. This plasmid is similar to pCab-SU13 except that it contains the DNA encoding first 53 amino acids of the petunia Cab22L gene (Dunsmuir, Nucleic Acids Res. 13:2503-2518 (1985)) (34 amino acids of the chloroplast transit peptide and 19 amino acids of the mature Cab22L protein) added onto the $NH_2$-terminus of cytochrome P450SU1. This plasmid can be prepared from pCab-SU13 by site-directed mutagenesis in which the 24 nucleotides encoding for the 8 additional amino acids of the Cab22L protein found in pCab-SU13 are removed from the plasmid using methods well known to those skilled in the art (Kunkel, T.A. et al., Proc. Natl. Acad. Sci. U.S.A., 82:488-492 (1985)) knowing that the DNA sequence spanning this area in pCab-SU13 is as shown above. A diagram of pCab-SU13 is shown in Figure 10E.

6. The plasmid pSuFe2 contains two adjacent CaMV 35S promoters directing transcription in opposite directions along with the 60 bp region from the 5'-untranslated region of SSU from petunia. The cytochrome P450SU1 and FeS-B coding sequences, however, have sequences encoding the 57 amino acid chloroplast transit peptide from SSU added at the start of each coding sequence. The FeS-B gene contains the nos 3'-untranslated sequence while the P450SU1 gene contains the petunia SSU 3'-untranslated sequence. This construction constitutively expresses cytochrome P450SU1 and FeS-B and targets their resulting proteins to the chloroplasts or plastids of plants and upon entry into the chloroplast and processing of the transit peptide the mature P450SU1

or FeS-B protein will be present without any additional sequences. A diagram of pSuFe2 is shown in Figure 15B.

7. The plasmid pSuFe3 contains two adjacent SSU promoters from petunia directing transcription in opposite directions. These two promoters express cytochrome P450SU1 and FeS-B coding sequences that have had sequences encoding the 57 amino acid chloroplast transit peptide from SSU added at the start of each coding sequence. The FeS-B gene has the nos 3'-untranslated sequence while cytochrome P450SU1 has the petunia SSU 3'-untranslated sequence. This construction expresses both cytochrome P450SU1 and FeS-B in a light dependent fashion. The two proteins are also targeted to the chloroplast where, after proteolytic cleavage of the transit peptide, they exist without any additional sequences being added. A diagram of pSuFe3 is shown in Figure 15C.

8. The plasmid pSuFe4 is similar to pSuFe3 except that instead of the two SSU promoters being adjacent to one another, the nos 3'-untranslated sequence and petunia SSU 3'-untranslated sequence are adjacent to one another. All of the components of the two plasmids are otherwise the same. A diagram of pSuFe4 is shown in Figure 15D.

E. coli bacteria transformed by seven of the plasmids described above, i.e., two for cytoplasmic expression and five for chloroplast expression, were deposited in the American Type Culture Collection under the following access numbers. pCab-SU11 and pCab-SU12 can be made from pCab-SU13 as described above by those skilled in the art. pSuFe4 can be made from pSuFe3 as described below by those skilled in the art.



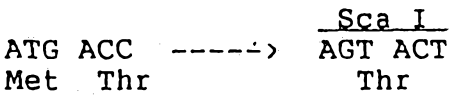
| P450SU1 construction | ATCC accession number | Date of deposit |
|---|---|---|
| pSU17 | 67995 | 25 May 1989 |
| pSSU-SU11 | 67994 | 25 May 1989 |
| pSSU-SU12 | 67993 | 25 May 1989 |
| pCab-SU13 | 67992 | 25 May 1989 |
| pSuFe1 | 68212 | 31 January 1990 |
| pSuFe2 | 68213 | 31 January 1990 |
| pSuFe3 | 68214 | 31 January 1990 |

Plasmids for expression of cytochrome P450SU1 with or without FeS-B in the cytoplasm of plant cells.

1. Construction of pSU17. Flow diagram is shown in Figures 17A to 17D.

Plasmids used in sequencing the genes for cytochrome P450SU1 and FeS-B were derived by exonuclease III deletion (Henikoff, Gene 28:351-359, 1984) from either end of the 2.4 kb BamHI DNA fragment that contains these genes. One of these plasmids, pSU12-1.8, has an endpoint 6bp downstream from the translation termination codon for P450SU1 while still containing the entire coding sequence for cytochrome P450SU1. This plasmid, pSU12-1.8, was used as a starting place to develop DNA constructions that would express the P450SU1 protein in plant cells. Addition of sequences to the 3'-end of the P450SU1 coding sequence are required for translation in plants. pSU12-1.8 was digested with Hind III and the site was filed in using the Klenow fragment of DNA polymerase I (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1982)). This

plasmid was cut with EcoRI and the approximately 1.3 kb EcoRI-blunt end DNA fragment containing the P450SU1 coding sequence was subcloned into EcoRI-HincII cut pUC118 creating pSU14. The 3' nontranslated sequence from the SSU301 gene (encoding the small subunit of ribulose bisphosphate carboxylase[SSU] from petunia) was fused to the 3'-end of the P450SU1 coding sequence as follows. pSSU3033, a plasmid containing the SSU301 gene with a BglII site at the TGA stop codon of the translational stop for SSU (C. Dean et al. The Plant Cell 1:201-208 (1989)) was cut with BglII and the ends blunted with the Klenow fragment of DNA polymerase I (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY(1982)) and then cut with BamHI. The resulting 1.45kb blunt end-BamHI DNA fragment containing the 3'-end of the SSU301 gene was subcloned into BamHI-HincII cut pUC118 and the resulting plasmid called pSSU3040. A three component ligation consisting of 1. the P450SU1 coding region from pSU14 (a 1.3kb EcoRI-PstI DNA fragment), 2. the 3'-untranslated region from the SSU301 gene (a 1.45 kb PstI-BamHI fragment from pSSU3040) and 3. BamHI-EcoRI cleaved pUC118 was performed (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY(1982)) to create pSU15. A ScaI site was introduced at the ATG start codon:




for P450SU1 in pSU15 by in vitro mutagenesis (Kunkel, T. A. PNAS 82: 488-492, (1985)) creating pSU16. This creates a P450SU1 "cassette" which was used in

further constructions to express the P450SU1 gene in plants. A plasmid, p35S(J):Cab22L-CH, that contains the Cauliflower Mosaic Virus(CaMV) 35S promoter and the 5' untranslated region from the petunia chlorophyll a/b binding protein gene "Cab 22L" (Harpster et al. Molecular and General Genetics, 212: 182-190, 1988) was used to provide a promoter for expression of P450SU1 in plants. A 1.2 kb EcoRI-NcoI(blunted with the Klenow fragment of DNA polymerase I, (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY(1982))) from p35S(J):Cab22L-CH was ligated to ScaI(blunt)-EcoRI cleaved pSU16 to create pSU17. When the filled in NcoI site from p35S(J):Cab22L-CH and the ScaI site from pSU17 were fused together in this construction the ATG start codon for cytochrome P450SU1 was regenerated. This construction, pSU17, when introduced into plants expressed cytochrome P450SU1 in the cytoplasm of the plant cell.

In Figures 17A to 17 D the following steps are designated with letters at the arrows:

For Figure 17A:

A 1) Hind III cut and fill in with Klenow
2) EcoRI cut

B EcoRI + HincII cut

For Figure 17C:

C EcoRI + PstI cut

D BamHI + EcoRI cut

E BamHI + PstI cut

F 3 component ligation

For Figure 17B:

G 1) BglII and fill in with Klenow
2) BamHI cut

H BamHI + HincII

I Ligate

For Figure 17D:

J Site directed mutation of P450SU1 ATG start site to ScaI site

K 1) ScaI
2) EcoRI cut

L 1) NcoI cut and fill in with Klenow
2) EcoRI cut

M Ligate

2. Construction of plasmid pSUFe1. Flow diagram is shown in Figures 18A to 18D.

Plasmids used in sequencing the genes for cytochrome P450SU1 and FeS-B were derived by exonuclease III deletion (Henikoff, Gene 28:351-359, 1984) from either end of the 2.4 kb BamHI DNA fragment that contains these genes. One of these plasmids, pSU12-2.04, has an endpoint several base pairs downstream of the stop codon of FeS-B. By site-directed mutagenesis (Kunkel, T.A. PNAS 82: 488-492, (1985)) a ScaI site was introduced at the ATG initiation codon creating the plasmid pFeSB-1.02 and changing the sequence at the translation initiation site from

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Sca I | | | |
| GAC | ATG | ACC | ATG | to | GAC | AGT | ACT | ATG. | |
| | Met | Thr | Met | | | | Thr | Met | |

A 0.24 kb ScaI-XbaI fragment, containing the FeS-B coding sequence from pFeSB-1.02 was cloned into p29593 that had been NcoI cut and the ends filled in with the Klenow fragment of DNA polymerase I(Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY(1982)) and then subcut with XbaI creating pFeSB-3. The filled in NcoI site from p29593 recreates the ATG

initiation codon for FeS-B. p29593 is a derivative of p35S(J):Cab22L CH (Harpster et al. Mol. Gen. Genet. 212: 182-190 (1988)) that has had a BglII site introduced by site-directed mutagenesis (Kunkel, PNAS 82:488-492 (1985)) approximately 190 bp upstream of the transcription start point (using the DNA sequence of R. C. Gardner et al., Nucleic Acids Res. 9:2871-2888 (1981) nucleotide 7238 G changed to a C and nucleotide 7239 C changed to a T) of the CaMV 35S promoter (J. Odell et al. Nature 313:810-813 (1985)). p29593 contains the 35S promoter of Cauliflower Mosaic Virus (CaMV) and the 3' untranslated sequence of the nopaline synthase gene (nos) from T-DNA of Agrobacterium tumefaciens (Depicker et al. J. Mol. Appl. Genet. 1:561-573 (1982)). pSU17, from above, was digested with BamHI and partially digested with XhoI forming a 2.86 kb DNA fragment containing the cytochrome P450SU1 coding sequence and the 3' untranslated region of the petunia SSU gene. This was ligated with XhoI and BamHI digested p29593 to form pSU20. The 3'-untranslated region of the SSU gene was removed from pSU20 by partial PstI digestion and blunting the ends with T4 DNA polymerase (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY(1982)) and then digesting with BamHI and filling the ends with the Klenow fragment of DNA polymerase I (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY(1982)). The resulting DNA was intramolecularly ligated forming pSU21. A 2.6kb partially BglII digested and HindIII digested DNA fragment of pSU21 was isolated that contained the CaMV 35S promoter, P450SU1 coding sequence and the nos 3' untranslated sequence. This

DNA fragment was ligated to a 0.76kb HindIII-BglII DNA fragment of pFeSB-3 containing the CaMV 35S promoter, FeS-B coding sequence and nos gene 3' untranslated sequence forming pSUFe1. pSUFe1 contains 2 CaMV 35S promoters one that transcribes the FeS-B coding sequence and nos gene 3' untranslated sequence and the other that transcribes the P450SU1 coding sequence and a nos gene 3' untranslated sequence. The plasmid pSUFe1 when transformed into plant cells drives expression of p450SU1 and FeS-B in the cytoplasm.

In Figures 18A to 18D the following steps are designated with letters at the arrows:

For Figure 18A:

A 1) XhoI partial cut
2) BamHI cut

B XhoI + BamHI cut

C Ligate

For Figure 18B:

D 1) PstI partial, T4 blunt ends
2) BamHI cut and fill in with Klenow

E Recircularize

For Figure 18C:

F 1) NcoI and fill in with Klenow
2) XbaI cut

G Site directed mutagenesis of FeS-B ATG start to ScaI site

H ScaI + XbaI cut

I Ligate

For Figure 18D:

J BglII + HindIII cut

K 1) BglII partial
2) HindIII cut

L Ligate with HindIII cut pUC118

Plasmids that direct the expre on of cytochrome P450SU1 with or without FeS-B to the chloroplasts of plant cells.

In order to express the cytochrome P450SU1 protein or FeS-B in the chloroplasts of plants, plasmids were constructed using 5' promoter regions, coding regions of the transit peptide sequences, and in some cases the mature coding sequences of genes encoding proteins normally imported into the chloroplasts of plants. The naturally chloroplast imported genes used in these plasmids were those for ribulose bisphosphate carboxylase (SSU) and the chlorophyll a/b binding protein (Cab) both from petunia. Plasmids were made that added DNA encoding for amino terminal amino acid sequence that is normally removed upon transport into chloroplasts. Other plasmids were made that added DNA encoding the chloroplast transit peptide sequence and up to 27 amino acids of the mature transported protein onto the DNA encoding cytochrome P450SU1.

1. Construction of plasmids pSSU-SU11 and pSSU-SU12. Flow diagram is shown in Figures 19A and 19B.

A 1.8kb ClaI(end blunted with Klenow fragment of DNA polymerase I)-BamHI DNA fragment from pSSU3019(Dean et al. The Plant Cell 1:201-208, (1989)) a clone that contains the 5' and 3' flanking regions but lacks the introns of the SSU301 gene was cloned into SmaI-BamHI cleaved pUC8 generating pSSU3043. The polylinker of pUC8 adds onto the SSU301 gene an EcoRI site to be used in the constructions. To make the fusion between the chloroplast transit peptide and P450SU1 an EcoRV site was introduced by site directed mutagenesis (Kunkel, PNAS 82: 488-492, (1985)) into the mature SSU301 coding sequence after amino acid 12 creating

pSSU3044. A 1.4kb EcoRI-EcoRV DNA fragment from pSSU3044 containing the SSU301 promoter and the DNA encoding the amino-terminus of the SSU301 protein was cloned into EcoRI-ScaI cleaved pSU16 creating pSSU-SU11. pSSU-SU11 encodes 12 extra amino acids in addition to the chloroplast transit peptide from the SSU301 protein added onto the DNA encoding the amino terminus of the P450SU1 protein. To create a precise fusion of the chloroplast transit peptide coding sequence with the P450SU1 coding sequence, an oligonucleotide directed site specific deletion (Kunkel, PNAS 82: 488-492, (1985)) was used to loop out the extra nucleotides between the transit peptide and the amino terminus of P450SU1. The resulting plasmid, pSSU-SU12, contains a perfect fusion between the transit peptide of the SSU301 gene and the amino terminus of P450SU1. The plasmids pSSU-SU11 and pSSU-SU12 express cytochrome P450SU1 in plant cells that is targeted to the chloroplasts.

In Figures 19A and 19B the following steps are designated with letters at the arrows:

For Figure 19A:

A 1) ClaI cut and fill in with Klenow
2) BamHI cut

B SmaI + BamHI

C Ligate

D Site directed mutagenesis creating EcoRV site at aa12 of mature SSU301 protein

For Figure 19B:

E EcoRI + EcoRV cut

F ScaI + EcoRI

G Ligate

H Oligonucleotide loopout of sequences coding for 12aa of SSU mature peptide.

2. Construction of plasmids pCab-SU11, pCab-SU12 and pCab-SU13. Flow diagram is shown in Figures 20A t 20C.

To make the chlorophyll a/b binding protein fusion to P450SU1, a 950bp BalI-SacI DNA fragment of the petunia Cab22L gene (Dunsmuir, Nucleic Acids Res. 13:2503-2518 (1985)) containing the Cab22L promoter, chloroplast transit peptide coding sequence and part of the mature Cab22L coding sequence was cloned into SmaI-SacI digested pBluescript KS+(Stratagene Inc. San Diego, CA 92121) creating pCab22LT. A ScaI site was created by site-directed mutagenesis after the codon for amino acid 14 of the mature Cab protein coding sequence of pCab22LT creating pCab22LT1. The Cab22L promoter, codons encoding the transit peptide and 14 amino acids of the mature Cab22L protein were subcloned as a 1.2kb EcoRI-ScaI fragment of pCab22LT1 into EcoRI-ScaI cleaved pSU16 creating pCab-SU11. An oligonucleotide was made that looped in 39 nucleotides (Kunkel, PNAS 82: 488-492, (1985)) encoding amino acids 15-27 of the mature Cab22L protein into the junction between the Cab22L coding sequence and the P450SU1 coding sequence creating pCab-SU13. pCab-SU13 encodes a protein that contains the Cab22L chloroplast transit sequence, 27 amino acids of the mature Cab22L protein fused to the amino terminus of the P450SU1 protein. A SmaI site was created by site-directed mutagenesis (Kunkel, PNAS 82:488-492, (1985)) at amino acid 19 of pCab22LT creating pCab22LT2. The Cab22L promoter, codons encoding the transit peptide and 19 amino acids of the mature Cab22L protein were subcloned as a 1.2kb EcoRI-SmaI DNA fragment of pCab22LT2 into EcoRI-ScaI cleaved pSU16 creating pCab-SU12. The plasmids

pCab-SU11, pCab-SU12 and pCab-SU13 express cytochrome P450SU1 in plant cells that is targeted to the chloroplasts.

In Figures 20A to 20C the following steps are designated with letters at the arrows:

For Figure 20A:

A SmaI + SacI cut

B Ligate

C Site directed mutagenesis of Cab-M at amino acid 14 to ScaI site

D Site directed mutagenesis of Cab-M at amino acid 19 to SmaI site

For Figure 20B:

E SmaI + EcoRI cut

F EcoRI + ScaI cut

G Ligate

For Figure 20C:

F EcoRI + ScaI cut

H ScaI + EcoRI cut

I Ligate

J Use oligonucleotide to loop in 39 nucelotides coding for amino acid 15-27 of Cab mature at ScaI site

3. Construction of plasmids pSUFe3 and pSUFe4. Flow diagram is shown in Figures 21A to 21D.

p29593(see construction of pSUFe1) was cleaved with NcoI and XbaI and ligated with a 1kb NcoI-XbaI DNA fragment from pFeSB-1.02(see construction of pSUFe1) that contains the FeS-B coding sequence. This forms plasmid pFenos1 and puts the 3' untranslated sequence of the nos gene from p29593 downstream of the FeS-B coding sequence. pFenos1 was cleaved with BglII removing a .75kb BglII DNA

fragment and the remaining BglII DNA fragment was recircularized creating pFenos2. A 1.4kb EcoRI-EcoRV DNA fragment from pSSU3044 (see construction of pSSU-SU11) that contains the promoter, sequences coding for the chloroplast transit sequence and the first 12 mature amino acids of the mature protein of the petunia SSU301 gene was isolated. This fragment was ligated with pSFenos2 that had been partially digested with ScaI and completely digested with EcoRI creating pSFenos1. pSFenos1 contains the petunia SSU301 gene promoter with sequences encoding the chloroplast transit peptide and the first 12 amino acids of the mature SSU301 protein added onto the beginning of the FeS-B coding sequence. The nos 3'untranslated sequences are located after the termination codon of FeS-B. The DNA sequence that encodes the twelve amino acids of the mature SSU301 protein in pSFenos1 were removed by site directed mutagenesis (Kunkel) creating pFenos2. pFenos2 was partially digested with HindIII and ligated to HindIII cut pGEM7Zf(+)(Promega Corporation, Madison, WI 53711) to put a BamHI site downstream of the nos gene 3' untranslated sequences. This plasmid is named pSFenos3. pSFenos3 was cut with EcoRI and BamHI to give an ~1.9kb BamHI-EcoRI DNA fragment containing the petunia SSU301 gene promoted FeS-B gene. A 4.25kb EcoRI-BamHI DNA fragment was isolated from pSSU-SU12(see construction of pSSU-SU12 above) that contains the petunia SSU301 promoter, DNA encoding the chloroplast transit peptide and the 3' untranslated region flanking the cytochrome P450SU1 coding sequence. These two BamHI-EcoRI DNA fragments were ligated together along with EcoRI digested

pUC118 to create pSUFe3. The same two BamHI-EcoRI DNA fragments were ligated together along with BamHI digested pUC118 to create pSUFe4.

Both pSUFe3 and pSUFe4 contain 1). the petunia SSU301 promoter such that in plants it will transcribe sequences encoding the SSU301 chloroplast transit peptide linked to the coding sequence for FeS-B and the nos gene 3''untranslated sequence and 2). a second petunia SSU301 promoter such that in plants it will transcribe sequences encoding the SSU301 chloroplast transit peptide linked to the coding sequence for cytochrome P450SU1 and the SSU301 gene 3' untranslated sequence. pSUFe3 has these two segments oriented such that the two SSU301 promoters are adjacent to one another. pSUFe4 has these two segments oriented such that the nos gene 3' untranslated sequence and the SSU301 3' untranslated sequence are adjacent to one another.

In Figures 21A to 21D the following steps are designated with letters at the arrows:

For Figure 21A:

A. NcoI + XbaI digestion of pFeSD-1.02
B. NcoI + XbaI digestion of p29593
C. 1) BglII digestion and
   2) Recircularization of pFenos1

For Figure 21B:

D. ScaI partial digestion and EcoRI digestion of pFenos2
E. EcoRI + EcoRV digestion of pSSU3044
F. Site specific oligonucleotide directed deletion of nucleotides encoding SSU mature sequence

For Figure 21C:

G. Partial HindIII digestion of pSFenos2
H. HindIII digestion of pGEM7ZF(+)

For Figure 21D:

I. EcoRI + BamHI digestion of pSFenos3
J. EcoRI + BamHI digestion of pSSU-SU12
K. EcoRI digestion of pUC118
L. BamHI digestion of pUC118
M. 3 component ligation
N. 3 component ligation

4. Construction of pSUFe2. Flow diagram shown in Figures 22A and 22B.

p29593 can be cut with BamHI and ligated to an EcoRI-BamHI adaptor (New England Biolabs Inc., Beverly, MA) with subsequent recircularization forming p29593-1. This puts an EcoRI site in p29593-1 at the position of the BamHI site in p29593. An ~2.2kb EcoRI-BglII DNA fragment of p29593-1 containing the CaMV 35 S promoter and petunia Cab 22L 5' untranslated sequence can be ligated to p29593 cut with EcoRI and partially digested with BglII forming p29593-2. p29593-2 contains two adjacent CaMV 35S promoters and petunia Cab 22L 5' untranslated sequences arranged such that transcription from the two promoters would be in opposite directions. A 1.3kb NcoI fragment from p29593-2 containing the two CaMV 35S promoters and Cab22L 5' untranslated sequences can then be ligated to pSUFe3 that has its two petunia SSU301 promoters removed by partial NcoI digestion to form pSUFe2. pSUFe2 is similar to pSUFe1 except that both the cytochrome P450SU1 and FeS-B coding sequences have sequences for the petunia SSU301 gene chloroplast transit peptide fused to them.

In Figures 22A and 22B the following steps are designated with letters at the arrows:

For Figure 22A:

A. 1) BamHI digestion and addition of BamHI-EcoRI adapters to p29593 and
2) Recircularization

B. BglII partial digestion and EcoRI digestion of p29593

C. BglII + EcoRI digestion of p29593-1

For Figure 22B:

D. NcoI digestion of p29593-2

E. NcoI partial digestion of pSUFe3

C. Introduction of constructs into T-DNA plasmids

Six of the constructs (i.e., pSU17, pSSU-SU11, pSSU-SU12, pCabSU11, pCabSU12 and pCab-SU13) containing the P450SU1 coding sequence with promoters from plant genes were digested with BamHI and inserted into the plasmid pAGS135 at its unique BamHI site. Plasmid pAGS135 was derived from pAGS112 (P. van den Elzen et al. Plant Mol. Biol. 5:149-154, 1985, herein incorporated by reference,) by removal of the XhoI site outside of the T-DNA right border following digestion of pAGS112 DNA with XhoI, treatment with the Klenow fragment of DNA polymerase I to blunt the ends and then self ligating. The plasmid pAGS112 was derived from the wide host-range vector pLAFR (Friedman et al. Gene 18:289-296, herein incorporated by reference) by insertion of an EcoRI fragment in which the T-DNA borders flank a gene for expressing kanamycin resistance in plants and multiple cloning sites. pAGS501, pAGS502 and pZS96 are similar to pAGS135 in that they are T-DNA border containing plasmids expressing kanamycin resistance in plants.

A summary of how pAGS501, pAGS502 and pZS96 were made is described below.

pAGS501 and 502 were constructed as follows. pRK290 (G. Ditta et al., Proc. Natl. Acad. Sci. U.S.A., 77:7347-7351, (1980)) was cut with EcoRI and the ends filled in with the Klenow fragment of DNA polymerase I (T. Maniatis et al., Molecular Cloning:a Laboratory Manual, Cold Spring Harbor, NY (1982)). pAGS111 (P. J. van den Elzen et al., Plant Mol. Biol. 5:149-154, (1985)) was cut with EcoRI and HindIII and the ends filled in with the Klenow fragment of DNA polymerase I (T. Maniatis et al., (1982)). The 6.7 kb DNA fragment from pAGS111 containing the left and right borders of T-DNA and the kanamycin nucleotidyl phosphotransferase gene under the control of the nopaline synthetase promoter was ligated to the cleaved pRK290 DNA creating p1881. p1881 was cut with XhoI, the ends blunted with the Klenow fragment of DNA polymerase I and circularly ligated creating p1882. p1882 was cut with BamHI and ligated to a double stranded oligonucleotide containing XbaI, HindIII, XhoI, EcoRI and HpaI sites. The ends of the double stranded oligonucleotide are such that when ligated to BamHI cut p1882 one end recreates a BamHI site while the other end does not. Plasmids pAGS501 and pAGS502 are the two possible results of such a ligation. Both plasmids contain BamHI, HindIII and EcoRI sites between the T-DNA borders that can be used as cloning sites for DNA to be mobilized into plants.

In Figures 23A and 23B, the following steps are designated with letters at the arrows:

A. 1) HindIII + EcoRI digestion of pAGS111
2) fill in of restriction endocunlease ends with the Klenow fragment of DNA polymeraseI

B. 1) EcoRI digestion of pRK290
2) fill in of restriction endonuclease ends of pRK290
3) ligation of pRK290 with the ~6.7 kb T-DNA fragment of pAGS111

C. 1) XhoI digestion of p1881
2) fill in of restriction endocunlease ends of p1881
3) intramolecular ligation of p1881

D. 1) BamHI digestion of p1882
2) ligation of HpaI, EcoRI, HindIII, XbaI, BamHI oligonucleotide with p1882.

pZS96 was constructed as follows. This plasmid utilizes the replication and stability functions of pVS1 for use in Agrobacterium (Itoh et al., Plasmid, 11:206-220 (1984)). A derivative of pVS1, pGV910 (J. Lemans et al., Gene, 19:361-364 (1982)) was cut with BamHI and SalI and the 8.0 kb HamHI-SalI DNA fragment containing the replication origin and stability functions was ligated to a 4.1 kb BamHI-SalI fragment from pBR322 (Bolivar et al., Gene, 2:95-113 (1977)) creating pZS67. pZS67 was cut with SacI and PvuII and the ends blunted with T4 DNA polymerase (T. Maniatis et al., 1982) creating the 8.6 kb plasmid pZS68. pZS68 was cut with BamHI and the ends filled in with the Klenow fragment of DNA polymerase I (T. Maniatis et al., 1982) and recircularized creating pZS69. The unique PstI site in pZS69 was removed by exchanging the 222 bp AvaII-AvaII fragment within the ampicillin resistance gene containing the PstI site

with a similar fragment from pUC19 (C. Yanisch-Peron et al. Gene 33:103-119, (1985)) that does not contain a PstI site creating pZS71. The T-DNA region of pAGS111 (P. J. can den Elzen et al., (1985)) was cut out as a 5.7 kb EcoRI-HindIII fragment and cloned into EcoRI-HindIII cleaved pZS71 creating pZS73 (12.3 kb). pZS73 was cut with EcoRI, the ends filled in withthe Klenow fragment of DNA polymerase I (T. Maniatis et al., 1982) and the plasmid recircularized to form pZS74. pZS74 was cut with HindIII, the ends filled in with the Klenow fragment of DNA polymerase I (T. Maniatis et al. 1982) and the plasmid recircularized to form pZS75. A 444 bp HaeII-HaeII DNA fragment from pUC19 containing the polylinker region whose ends had been blunted with T4 DNA polymerase (T. Maniatis et al., 1982) was cloned into pZS75 that had been cut with BamHI sites that are not within the polylinker region. This was accomplished through sequential steps of separate partial digestions with KpnI, SalI or BamHI, filling the ends with either the Klenow fragment of DNA polymerase I or T4 DNA polymerase (T. Maniatis et al, 1982) and recircularizing the plasmimd.

In Figure 24A the following steps are designated with letters at the arrows:

A. 1) BamHI + SalI digestion of pGV910 and pBR322
   2) ligation of 4.1 kb BamHI-SalI pBR322 fragment with the 8.0 kb BamHI-SalI pGV910 fragment

B. 1) PvuII + SacII digestion of pZS67
   2) blunt restriction endonuclease ends with T4 DNA polymerase
   3) intramolecular ligation.

In Figure 24B the following steps are designated with letters at the arrows.

C. 1) BamHI digestion of pZS68
2) fill in of restriction endonuclease ends with the Klenow fragment of DNA polymerase I
3) intramolecular ligation

D. AvaII digestion of pUC19

E. 1) AvaII digestion of pZS69
2) ligation of the 222 bp AvaII fragment of pUC19 with the 11.9 kb AvaII fragment of pZS69.

In Figure 24C the following steps are designated with letters at the arrows.

F. HindIII + EcoRI digestion of pAGS111

G. 1) EcoRI + HindIII digestion of pZS71
2) Ligation of the ~5.7 kb HindIII-EcoRI fragment of pAGS111 with HindIII-EcoRI cut pZS71

H. 1) HindIII digestion of pZS73 and fill in restriction ends
2) intramolecular ligation
3) EcoRI digestion of plasmid from 2)
4) intramolecular ligation.

In Figure 24D the following steps are designated with letters at the arrows.

I. 1) HaeII digestion of pUC19 and blunt ends with T4 DNA polymerase
2) BamHI digestion of pZS75 and fill in restriction ends
3) ligate digested pZS75 with ~440 bp HaeII fragment of pUC19

J. 1) KpnI digestion, blunt ends with T4 DNA polymerase
2) intramolecular ligation
3) SalI partial digestion of plasmid from 2), blunt ends with Klenow fragment of DNA polymerase I
4) intramolecular ligation
5) BamHI partial digestion of plasmid from 4), blunt ends with Klenow fragment of DNA polymerase I
6) intramolecular ligation.

These plasmids were used as follows to clone the segments of pSUFe1, pSUFe2, pSUFe3 and pSUFe4 that enable expression of cytochrome P450SU1 and FeS-B in plants. The ~3.4kb HindIII fragment of pSUFe1 containing the two CaMV 35S promoters and nos 3' untranslated sequences along with the cytochrome P450SU1 and FeS-B coding sequences was cloned into HindIII cut pAGS502 creating pSUFe11. The ~4.75kb BamHI fragment of pSUFe2 containing two CaMV 35S promoters, nos 3' untranslated sequence, SSU301 3' untranslated sequence and the coding sequences of P450SU1 and FeS-B each linked to sequences encoding the chloroplast transit peptide of the SSU301 gene was cloned into BamHI cut pAGS501 creating pSUFe21. The 6.3kb BamHI fragment of pSUFe3 containing two petunia SSU301 promoters, nos 3' untranslated sequence, SSU301 3' untranslated sequence and the coding sequences of P450SU1 and FeS-B each linked to sequences encoding the chloroplast transit peptide of the SSU301 gene was cloned into BamHI cut pZS96 DNA creating pSUFe31. The 6.3kb EcoRI fragment of pSUFe4 containing two petunia SSU301 promoters, nos 3' untranslated sequence, SSU301 3' untranslated

sequence and the coding sequences of P450SU1 and FeS-B each linked to sequences encoding the chloroplast transit peptide of the SSU301 gene was cloned into EcoRI cut pZS96 DNA creating pSUFe41. Below is a list indicating the expression constructs described above and the name for the plasmid made from each when cloned into pAGS135, pAGS501, pAGS502 or pZS96.

| P450SU1 construction | Plasmids |
|---|---|
| pSU17 | pSU18 |
| pSSU-SU11 | pSSU-SU111 |
| pSSU-SU12 | pSSU-SU121 |
| pCab-SU11 | pCab-SU111 |
| pCab-SU12 | pCab-SU121 |
| pCab-SU13 | pCab-SU131 |
| pSuFe1 | pSuFe11 |
| pSuFe2 | pSuFe21 |
| pSuFe3 | pSuFe31 |
| pSuFe4 | pSuFe41 |

D. Transfer to Agrobacterium and into tobacco

The plasmids listed above were mobilized into the Agrobacterium strain LBA4404/pAL4404(Hoekema et al. Nature 303:179-180, 1983, herein incorporated by reference) using tri-parental matings (Ruvkin and Ausubel, Nature 289:85-88, 1981, herein incorporated by reference). The resulting Agrobacterium strains were then cocultivated with protoplasts (van den Elzen et al. Plant Mol. Biol. 5:149-154) or leaf disks (Horsch et al. Science 227:1229-1231, 1985) of Nicotiana tabacum cv. Wisconsin 38 and kanamycin resistant transformants were selected.

Kanamycin resistant transformed tobacco plants were regenerated from the transformed protoplasts or leaf disks and the leaves of plants were tested for mRNA expression of the P450SU1 and FeS-B coding sequences by primer extension. Those plants that showed moderate to high levels of mRNA were allowed to flower, were self-pollinated and seed obtained from each. Several different independent transformed plants originating from each P450SU1 expression construction were isolated. The following Table 16 lists the parent construct, promoter used and additions to the P450SU1 coding sequence for each plant line used.

TABLE 16

A. Plants Expressing Cytochrome P450SU1

| Plant Line | Parent plasmid | Promoter | Amino Terminal Additions |
|---|---|---|---|
| W38 | none | none | |
| AGS112 | pAGS112 | none (no P450 gene) | |
| SU18.8 | pSU18 | CaMV | none |
| SU18.14 | " | " | " |
| SU18.15 | " | " | " |
| SSU-SU111.5 | pSSU-SU111 | SSU | SSU transit sequence + 12 extra amino acids of mature SSU |
| SSU-SU121.3 | pSSU-SU121 | SSU | SSU Transit Sequence |
| Cab-SU111.8 | pCab-SU111 | Cab | Cab transit sequence + 14 amino acids of mature Cab |

| Plant Line | Parent plasmid | Promoter | Amino Terminal Additions |
|---|---|---|---|
| Cab-SU121.5 | pCab-SU121 | Cab | Cab transit sequence + 19 amino acids of mature Cab |
| Cab-SU131.5 | pCab-SU131 | Cab | Cab transit sequence + 27 amino acids of mature Cab |

B. Plants Expressing Cytochrome P450SU1 and FeS-B

| Plant Line | Parent plasmid | Promoter | Amino Terminal Additions |
|---|---|---|---|
| SuFe11.1 | pSuFe11 | CaMv | None |
| SuFe11.3 | pSuFe11 | 35S | None |
| SuFe11.4 | pSuFe11 | 35S | None |
| SuFe11.7 | pSuFe11 | 35S | None |
| SuFe11.8 | pSuFe11 | 35S | None |
| SuFe11.1 | pSuFe11 | 35S | None |
| SuFe21.2 | pSuFe21 | 35S | SSU Transit Sequence |
| SuFe21.5 | pSuFe21 | 35S | SSU Transit Sequence |

| | | | |
|---|---|---|---|
| SuFe21.6 | pSuFe21 | 35S | SSU Transit Sequence |
| SuFe21.7 | pSuFe21 | 35S | SSU Transit Sequence |
| SuFe21.8 | pSuFe21 | 35S | SSU Transit Sequence |
| SuFe31.13 | pSuFe31 | SSU | SSU Transit Sequence |
| SuFe31.28 | pSuFe31 | SSU | SSU Transit Sequence |
| SuFe41.34 | pSuFe41 | SSU | SSU Transit Sequence |
| SuFe41.37 | pSuFe41 | SSU | SSU Transit Sequence |
| SuFe41.56 | pSuFe41 | SSU | SSU Transit Sequence |
| SuFe41.60 | pSuFe41 | SSU | SSU Transit Sequence |

EXAMPLE 20

P450 enzyme activity in tissues of transformed tobacco

A. Experimental methods:

Plants were grown from tobacco seed produced by self-pollinated transformed plants in Metro-Mix® 350 under 16 hour light (light intensity of 400 µEinsteins per second per square meter at pot level, 22°C and 80 % relative humidity) followed by 8 hours of dark (18°C and 70 to 80 % relative humidity) and watered three times daily with half strength Hoagland's solution. The presence of the cytochrome P450 protein in individual plants was confirmed by Western blot analysis before testing for sulfonylurea metabolism.

During growth, the tobacco plants produced leaves attached to the main stalk, none of which were of the same age or size. Removal of leaves attached to the main stalk of each plant forced the growth of lateral shoots which in turn produced the many leaves of similar size and age needed for the experiments. The leaves were excised under water with a scalpel. These leaves were transferred to cups with the uptake solution (20 ppm sulfonylurea in 1 mM potassium phosphate buffer, pH 7.0) and allowed to take up the solution through the cut leaf base for 2 hours in the light (200 µEinsteins per second per sq. meter) at 22°C and 86% relative humidity. At the end of the uptake period, sample leaves were either frozen in liquid nitrogen and stored at -20°C (designated '0 hour post-uptake samples') or transferred to cups with phosphate buffer alone for an additional 5 hours of incubation in the light before freezing (designated '5 hour post-uptake samples'). In the 10001 experiments some leaves were incubated for 21

hours post-uptake under continuous light (200 μEinsteins per second per square meter, 22°C, and 63% relative humidity).

Individual leaves were extracted with 30 ml acetone/water (80%/20% by volume) for 1 minute in a Sorvall® Omni-Mixer. The brei was centrifuged to remove the tissue debris. Acetone in the supernatant was removed under a stream of nitrogen. The resulting aqueous extract was acidified to pH 2 to 3 with sulfuric acid and then extracted three times with methylene chloride. The combined methylene chloride extracts were reduced to dryness by rotary evaporation at 30 to 40°C. The dry residue was dissolved in acetone for transfer to a vial. Once transferred the acetone was removed by evaporation under a stream of nitrogen. Before HPLC analysis the dry sample was redissolved in 1.0 ml of acetonitrile /water (25%/75%). HPLC separations were carried out on a Zorbax® ODS column (4.6 mm x 250 mm) at 45°C with a flow rate of 1.4 ml per minute. Separation of extract components was achieved with a 5 to 80% acetonitrile gradient (with 0.1% formic acid; the balance was water) and a run time of 25 minutes. 10015 and its metabolites were detected at 254 nm while $^{14}$C-10001 and its metabolites were detected with a Radiomatic Flo-One® detector. A diode array detector was used in separate experiments to obtain the absorbance spectra of the 10015 metabolite and an 10014 standard in the HPLC analysis of several extracts (HPLC method is described in Romesser et al., Biochem. Biophys. Res. Comm., 140:650-659 (1986)). Comparison of the metabolite absorbance spectrum with that of the 10014 confirmed the metabolite's identity.

B. Metabolism of 10015

Leaf tissues only from progeny of the transformed tobacco plants (SSU-SU111.5, SSU-SU121.3 and CAB-SU131.5) which received plasmids engineered to direct the S. griseolus cytochrome P450 (SU1) to the chloroplast N-dealkylated 10015 to 10014 (Figure 11). The results are shown in Table 17.

TABLE 17

Metabolism of 10015 to 10014 in Tobacco Leaves

| Plant Family construction | 10014 levels (µg/leaf) at 0 hour* | at 5 hour* | No. of Leaves |
|---|---|---|---|
| W38 | 0 | 0 | 6 (3 from each of 2 plants) |
| AGS112** | 0 | 0 | 4 (4 from one plant) |
| SSU-SU111.5# | 2.1 (+/- 1.4) | 1.8 (+/- 1.4) | 6 (2 from each of 3 plants) |
| SSU-SU121.3# | 1.2 (+/- 0.5) | 1.7 (+/- 1.1) | 4 (2 from each of 2 plants) |
| Cab-SU131.5# | 1.3 (+/- 1.0) | 2.9 (+/- 1.3) | 8 (4 from each of 2 plants) |
| SU18.8@ | 0 | 0 | 8 (4 from each of 2 plants) |
| SU18.15@ | 0 | 0 | 2 (2 from one plant) |

* Post-uptake incubation time

** Control (there is no P450SU1 sequence in this plasmid)

# The P450 sequence is chloroplast directed.

@ The P450 sequence is cytoplasm directed.

The metabolite (10014) was identified by its comigration with an 10014 standard on HPLC analysis (detection at 254 nm) and by comparison of the absorbance spectrum of the metabolite with that of the 10014 standard. The absorbance spectra were identical (Figures 12A and 12B). The levels of 10014 in the leaves (Table 17) represent metabolic conversion of 10 to 20 % of the 10015 loaded into the leaves during the 2 hour uptake period (based on calculations from the volume of 10015 solution taken up). The 0 and 5 hour post-uptake incubations were included to look at the kinetics of metabolism. Because of the variability in 10014 production a reaction rate constant was not calculated.

Leaf tissues from the progenitor plant type, Wisconsin 38 tobacco, and progeny of tobacco transformed with the pAGS112 plasmid (a plasmid without the bacterial cytochrome P450SU1 gene) were unable to produce any detectable 10014 from 10015 (Table 17). This showed that the transgenic tobacco's ability to metabolize 10015 to 10014 was due to the expression of the bacterial gene in the plants and not to any native ability of the tobacco.

Leaf tissues from progeny of plants transformed with the plasmid pSU18 (i.e., plant lines SU18.14 and SU18.15), also, were tested for their ability to metabolize 10015 to 10014. Under the conditions of the experiment no conversion of 10015 to 10014 was detected (Table 17).

Transit sequences from the small subunit of carboxylase (the SSU transformants) or from the chlorophyll a/b protein (the Cab transformants) were included in the engineered plasmids to direct the

cytochrome p450SU1 protein to the chloroplast after synthesis in the cytoplasm. The mature cytochrome P450SU1 protein for the SSU-SU111.5 transformant included a 14 amino acid fragment of the small subunit of RuBP carboxylase. The SSU-SU121.3 transformant's mature cytochrome had no additional amino acid additions. The mature cytochrome protein for the Cab-SU131.5 transformant had an additional 27 amino acids from the chlorophyll a/b protein. Inspection of the levels of 10014 produced in the transformants (Table 17) showed that the 14 or 27 amino acids added to the cytochrome P450SU1 did not prevent the metabolism of 10015 to 10014 in the transgenic tobacco leaf tissues.

C. Metabolism of 10001

Leaf tissues from a plant transformed with pAGS112, a plasmid not containing DNA encoding cytochrome P450SU1, metabolized 10001 to 10002 and 10003 through O-demethylation and de-esterification, respectively (Figure 13A). The metabolism of 10001 was assumed to be a native metabolic ability of tobacco. However, it complicated the assessment of the metabolic activity of 10001 of the tissues from plants transformed with cytochrome P450SU1 containing plasmids (pSU18, pSSU-SU111. pSSU-SU121, and pCab-SU131). Although a limited number of samples were tested (each time point represents the average of two leaves tested), the results (Figures 13A, 13B and 13C) showed that after 21 hours, 10001 was metabolized to a signficantly greater extent and more 10002 was produced in the leaf tissues from those plants transformed with plasmids pSSU-SU111 and pCab-SU131 than in leaf tissues from plants

transformed with pAGS112. The results indicated that the cytochrome P450SU1 was actively metabolizing 10001 in the transgenic tobacco.

Leaf tissues from progeny of the transformants, SU18.8 and SU18.14, were tested for the ability to metabolize 10001 to 10002 and 10003. Because of the native ability of the tobacco to metabolize 10001, it was difficult to determine whether there was a contribution of the bacterial enzyme to metabolism.

## EXAMPLE 21

### Metabolism of 10015 by tobacco plants transformed with P450SU1

Sulfonylurea compound 10015 exhibits low phytotoxicity to a wide variety of plant species. This compound is an excellent substrate for P450SU1, which rapidly converts it to into the highly phytotoxic compound 10014 (Figure 11). Thus, tobacco plants transformed to contain P450SU1 and sprayed with normally subtoxic rates of 10015 would be severely damaged if they contained functional P450SU1 in sufficient quantity to allow the accumulation of toxic compound 10014 within plant tissues.

Tobacco plants (Nicotiana tabacum cv. Wisconsin 38) transformed with plasmids pAGS112, pSU18, pSSU-SU111, pCab-SU121 or pCab-SU131 (singly) and accumulating the P450SU1 protein were identified by Western blot analysis. Seed arising from the self pollination of individual primary transformants designated AGS112, SU18.8, SU18.14, SSU-SU111.5, Cab-SU111.8, and Cab-SU131.5 gave rise to the plants described in this Example. The progeny of primary AGS112 and Cab-SU121.5 transformants segregated for single copy kanamycin resistance. In contrast, the

progeny of the plants transformed with plasmids containing P450SU1 (other than Cab-SU121.5) segregated for multicopy kanamycin resistance. Thus, most plants containing the gene encoding P450SU1 and sprayed with 10015 in this Example likely contained at least one, and in many cases multiple, copies of P450SU1, while very few individuals likely carried no copies of the gene encoding P450SU1.

Seed from transformed and nontransformed tobacco plants was germinated in a commercial potting mix Metro-Mix® 350 for 25 days. Individual plants were then transferred to 4 inch X 4 inch pots containing Metro Mix® 350 and were allowed to grow in the greenhouse for an additional 22 days at which time most plants contained 4-5 fully expanded leaves. A developmentally uniform population of plants was selected from among these transplants and the individual plants were then sprayed with a carrier solution of AGWT (90.2% Acetone: 4.8% Glycerol: 4.8% Water: 0.24% Tween 20 by volume) containing sulfonylurea compound 10015 at concentrations of 1,4, or 16 grams/hectare (g/ha). The sprayed plants were returned to the greenhouse and rated for injury 22 days later.

One to two days before spraying one leaf from each plant was frozen and stored at -80°C for analysis of P450SU1 content. At assay thawed leaves were ground in a buffer containing 0.1M sodium tricine pH 7.8, 10mM sodium chloride, and 5mM magnesium chloride. The extracts were clarified by centrifugation at 28,000 to 33,000 x g for one hour and protein in the supernatant fraction was concentrated. The relative P450SU1 content of the supernatant fraction from each leaf was estimated by

"immuno slot blots" where the extract from each leaf was placed in a template which deposited the extract onto individual "slots" or narrow lanes on nitrocellulose paper. The protein bound to the nitrocellulose was incubated with a P450SU1 specific antibody and subsequently treated as described for the Western Blot procedure.

Foliar application of 10015 at a rate of 1g/ha (Table 18) resulted in minimal damage to nontransformed plants (W38) and the progeny of plants transformed with plasmid pAGS112. In contrast, plants transformed with constructions directing P450SU1 to the chloroplast (SSU-SU111.5, Cab-SU111.8, Cab-SU121.5 and Cab-SU131.5) were severely injured by application of compound 10015 at 1g/ha. As shown in Figure 14 damage to the plants containing P450SU1 was indeed dramatic, resulting not only in a severe inhibition of plant growth but also in leaf chlorosis and gross morphological deformation of new leaves arising near the shoot apex. The difference in height between the progeny of untransformed (W38) plants and plants transformed with pAGS112 in Figure 14 was within the range of normal variation expected following growout of untreated, transplanted tobacco plants.

Immuno slot blot analysis confirmed that injured plants contained the P450SU1 protein at the time of spraying. Two "exceptional" plants which showed lower than expected levels of injury contained either no (Cab-SU121.5-19) or lower (Cab-SU131.5-15) levels of P450SU1 than severely injured plants in the same family. These plants may be segregants containing no or single copies, respectively, of the gene encoding P450SU1. Control

plants arising from pAGS112 primary transformants were not individually typed for kanamycin resistance. However, since these plants segregated 3 resistant : 1 sensitive for the ability to germinate on kanamycin, there is a >98% probability that at least one plant transformed with pAGS112 was sampled at the optimal discriminatory rate of 1g/ha 10015.

When this experiment was repeated at a higher application rate of 4 g/ha of compound 10015, most plants transformed with constructions directing P450 SU1 to the chloroplast were again severely injured (Table 19). Nontransformed plants and progeny plants from a primary AGS112 transformant showed some background damage at this higher application rate, but this damage was clearly less than that experienced by plants transformed to contain chloroplast localized P450SU1. In this experiment two exceptional plants containing P450SU1 (Cab-SU111.8-10; Cab- SU131.5-21) exhibited lower than expected phytotoxic damage. Given that plants transformed with these plasmids and expressing P450SU1 were severely damaged at the lower application rate of 1g/ha of compound 10015 (Table 18), it is likely that these two exceptional plants received a reduced dose of 10015 during treatment or were mistyped during immuno slot analysis.

Several transformants containing cytoplasmically directed P450SU1 (SU18.8, SU18.14) were damaged more severely than nontransformed plants, but to a lesser extent than transformants containing chloroplast localized P450SU1. This was especially evident at the treatment rate of 4g/ha of compound 10015 (Table 19). The difference in activity between cytoplasmically localized and

chloroplast localized P450SU1 does not always reflect a higher accumulation of P450SU1 in the chloroplast directed constructions (compare SU18.8, SU18.14 with Cab-SU111.8 and Cab-SU121.5, Table 19). This suggests that P450SU1 functions more efficiently in the chloroplast than in the cytoplasm.

When 10015 was applied at a concentration of 16 g/ha both transformed and non-transformed plants were extensively injured and no useful information was obtained.

These data demonstrate that P450SU1 is functionally active in the progeny of transformed plants at the whole plant level. In the case of constructions designed to direct the P450SU1 protein to the chloroplast the inclusion of amino terminal extensions to P450SU1 as described in Example 19 did not prevent the activity of P450SU1 in intact plants.

TABLE 18

Response of P450SU1 Transformed Tobacco to Sulfonylurea Compound 10015 (1 g/ha)

| Family/ Construction | Plant I.D. No. | % Damage | Protein (ug/slot) | Blot Rating |
|---|---|---|---|---|
| W38 | | 0 | | NT |
| W38 | | 0 | | NT |
| W38 | | 10 | | NT |
| AGS 112 | | 0 | 10 | (-) |
| AGS 112 | | 0 | 10 | (-) |
| AGS 112 | | 10 | | NT |
| SU18.8 | 14 | 10 | 6 | (++) |
| SU18.14 | 11 | 40 | 5 | (++) |
| SSU-SU111.5 | 10 | 95 | 11 | (++++) |
| SSU-SU111.5 | 22 | 95 | 10 | (+++) |
| SSU-SU111.5 | 24 | 95 | | NT |
| Cab-SU111.8 | 12 | 90 | 9 | (+) |
| Cab-SU111.8 | 14 | 95 | 4 | (+) |
| Cab-SU111.8 | 17 | 95 | | NT |
| Cab-SU121.5 | 11 | 85 | | NT |
| Cab-SU121.5 | 13 | 95 | 7 | (+) |
| Cab-SU121.5 | 18 | 95 | | NT |
| Cab-SU121.5 | 19 | 20 | 5 | (-) |
| Cab-SU131.5 | 15 | 50 | 4 | (+) |
| Cab-SU131.5 | 18 | 95 | 13 | (++1/2) |
| Cab-SU131.5 | 27 | 90 | 11 | (++) |

NT = Not Tested
(+), (++), (+++), (++++) = relative amount of immunologically detected P450SU1

TABLE 19

Response of P450SU1 Transformed Tobacco to Sulfonylurea Compound 10015 (4 g/ha)

| Family/ Construction | Plant I.D. No. | % Damage | Protein (ug/slot) | Blot Rating |
|---|---|---|---|---|
| W38 | | 0 | | |
| W38 | | 10 | | |
| W38 | | 20 | | NT |
| W38 | | 30 | | NT |
| AGS 112 | | 0 | | NT |
| AGS 112 | | 40 | | NT |
| SU18.8 | 11 | 60 | 6 | (++) |
| SU18.14 | 15 | 70 | 6 | (++) |
| SSU-SU111.5 | 11 | 100 | 13 | (++++) |
| SSU-SU111.5 | 12 | 100 | 2 | (+++) |
| SSU-SU111.5 | 13 | 100 | | NT |
| Cab-SU111.8 | 10 | 0 | 6 | (+) |
| Cab-SU111.8 | 15 | 99 | 5 | (+) |
| Cab-SU121.5 | 12 | 90 | 5 | (+) |
| Cab-SU121.5 | 22 | 100 | 9 | (+) |
| Cab-SU121.5 | 28 | 95 | | NT |
| Cab-SU131.5 | 21 | 40 | 12 | (++) |
| Cab-SU131.5 | 28 | 100 | 13 | (++) |
| Cab-SU131.5 | 29 | 100 | | NT |

NT = Not Tested
(+), (++), (+++), (++++) = relative amount of immulogically detected P450SU1

EXAMPLES 22-25

Tissue Culture Growth of Transformed Tobacco Seed

Growth medium was prepared from Murishige minimal organics medium (Gibco Laboratories, Grand Island, NY), 8 g/l agar, supplemented with T vitamins (50ppb biotin, 0.5ppm pyridoxine HCl, 0.5ppm thiamine HCl, 5ppm nicotinic acid, 0.5ppm folic acid, 2ppm glycine, 100ppm myo-inositol), sterilized and placed into sterile PlantCon plant tissue culture containers (Flow Laboratories, McLean, Virginia). Tobacco seed obtained from the self-pollination of the primary transformants was surface sterilized by a treatment of not more than 30 min in 20% chlorine bleach, 0.1% sodium dodecyl sulfate, followed by rinsing in distilled water, and placed on the surface of the medium in the sterile containers. Following this treatment, the seed was allowed to germinate and grow under illumination (100 microeinsteins$\cdot m^{-2} \cdot s^{-1}$), at 22°C.

Determination of the number of genetic loci of the transformed tobacco

The number of loci where t-DNA was incorporated into the genome of the tobacco was determined by segregation analysis of kanamycin resistance in the next generation of progeny. Seed from the self pollination of the primary transformants was grown as described above on medium supplemented with 200ppm kanamycin sulfate (Sigma). After 21 days, resistant (transformed) plants were unaffected compared to control plants, whereas sensitive plants were smaller, partially chlorotic, and poorly rooted. Determination of the number of genetic loci of the

primary transformants was based on the segregation of the kanamycin resistance trait.

## EXAMPLE 22

### Detection of the P-450 phenotype by sulfonylurea treatment

Because of the increased phytotoxicity of 10014 over that of 10015, plants grown in media containing 10015 should be growth inhibited if they contain an active cytochrome P450SU1. To test this, seeds of tobacco were grown in tissue culture media supplemented with 50 nM compound 10015, and the results are shown in Table 20.

Table 20

| Plant Line | Number of genetic loci[a] | Inhibition[b] |
|---|---|---|
| AGS112 (Control) | 1 | 1 |
| SU18.8 | >2 | 1 |
| SU18.14 | 2 | 2 |
| SU18.15 | >2 | 2 |
| SSU-SU111.5 | ≥3 | 3 |
| Cab-SU111.8 | >2 | 3 |
| Cab-SU121.5 | 2 | 3 |
| Cab-SU131.5 | N.D.[c] | 3 |

[a] Number of genetic loci was determined by segregation analysis of >100 seed

[b] Inhibition of growth of 6 individuals was visually rated. 1= little or no inhibition; 2= moderate inhibition; 3= severe inhibition, cotyledons expand but no growth occurs.

[c] Number of genetic loci could not be determined.

The data in this table showed that plants containing the P450 gene, especially those where the mature protein is directed to the chloroplast, were sensitive to inhibition by compound 10015. The high (>1) number of genetic loci of most of the parents of these plants insures a high liklihood that all of the six plants sampled have the P450 gene.

Sulfonylurea Selection of P450 + FeS containing Plants

The results above demonstrated that it was possible to use the P450 gene as a selectable marker by growing the plants in the presence of 50 nM of compound 10015. This technique was used to analyze the progeny of plants transformed with both the gene for SU1 and FeS-B, and the results are shown in Table 21.

Table 21

| Plant Line | Kanamycin Resistant | Copy No. | Compound 10015 Sensitive |
|---|---|---|---|
| AGS502(Control) | 6/9 | 1 | 0/9 |
| SuFe11.1 | 7/9 | >4 | 2/9 |
| SuFe11.3 | 5/10 | 1 | 0/8 |
| SuFe11.4 | 7/10 | 1 | 0/10 |
| SuFe11.7 | 9/10 | 1 | 0/9 |
| SuFe11.8 | 10/10 | 2 | 1/10 |
| SuFe11.11 | 7/8 | 2 | 0/9 |
| SuFe21.2 | 7/10 | 1 | 7/9 |
| SuFe21.5 | 8/10 | 1 | 8/9 |
| SuFe21.6 | 9/10 | 1 | 7/9 |
| SuFe21.7 | 7/10 | 1 | 5/7 |
| SuFe21.8 | 8/9 | 1 | 10/10 |

Results for kanamycin resistance and compound 10015 sensitivity are expressed as plants demonstrating result/total plants. Copy number determination is from kanamycin sensitive segregation analysis of about 100 seed.

Plants exhibiting sensitivity to compound 10015 could be rescued from this treatment by plucking the seedlings from the surface of the medium and placing them in fresh medium containing no 10015. After several weeks growth, leaf tissue from the plants was collected, homogenized and analyzed for presence of the cytochrome P450SU1 antigen by Western blot analysis. Analysis of both 10015 resistant and 10015 sensitive plants from lines SuFe21.2, SuFe21.5, SuFe21.6, SuFe21.7, and SuFe21.8 revealed that of 8 plants characterized as 10015 resistant none had detectable levels of P450SU1 on a Western blot, while 18 out of 21 plants sensitive to 10015 had Western blot detectable levels of P450SU1.

These results demonstrated that the expression of P450SU1 leads to a negative selectable phenotype. When the mature protein was targeted to the chloroplast, this selection was comparable to the positive selection by kanamycin.

## EXAMPLE 23

### Sulfonylurea Selection of Transgenic Tobacco Lines SuFe31 and SuFe41

Plants transformed with the plasmids pSuFe31 and pSuFe41 were selected for high expression of P450SU1 and FeS-B mRNA by primer extension analysis as described in Example 19, and self pollenated to produce seed. This seed was germinated on medium

containing 50 nM of compound 10015 and analyzed for sensitivity to this compound (indicating the presence of an active cytochrome P450SU1 enzyme) as in Example 22.

Table 22

| Plant Line | Number 10015 Resistant | Number 10015 Sensitive | Ratio Sensitive/ Resistant |
|---|---|---|---|
| AGS502(Control) | 32 | 0 | 0 |
| SuFe31.13 | 4 | 12 | 3.0 |
| SuFe31.28 | 5 | 10 | 2.0 |
| SuFe41.34 | 32 | 0 | 0 |
| SuFe41.37 | 8 | 24 | 3.0 |
| SuFe41.56 | 32 | 0 | 0 |
| SuFe41.60 | 15 | 0 | 0 |

Segregation of the compound 10015 sensitivity trait in SuFe31.13, SuFe31.28 and SuFe41.37 demonstrates that the sensitive plants are expressing an enzymatically active cytochrome P450SU1, and these plants are progeny of a heterozygous plant transformed at a single locus.

## EXAMPLE 24

### Transformed Tobacco Detoxification of Sulfonylurea

Tobacco seed from plants transformed with several P450SU1 and P450SU1+FeS-B constructions were tested for their ability to grow on tissue culture medium supplemented with compound 10001. Seed was placed on medium containing: 0, 5, 10, 20, and 40 nM of compound 10001. After 100 days, the plants were visually rated for their resistance (due to herbicide detoxification) to the herbicide treatment. All plants grown in the absence of herbicide and those

treated with the lowest levels (5nM and 10nM) of 10001 had grown so large that no comparative rating was possible. The severely growth inhibited plants (those grown in the presence of 20nM and 40nM of compound 10001) were scored by comparing them to AGS502 plants (transformed without P450 gene) in Table 23.

Table 23

| Plant Line | 10001 Concentration (nM) | Individual Scores |
|---|---|---|
| SuFe21.5 | 20 | 2,0,0,0,0,0 |
| SuFe21.5 | 40 | 3,3,3,2,1 |
| SuFe21.8 | 20 | 3,3,0,0,0 |
| SuFe11.8 | 20 | 0,0,0,0,0 |
| SuFe11.8 | 40 | 0,0,0,0,0,0 |
| SuFe11.4 | 20 | 3,3,2,0,0 |
| SuFe11.4 | 40 | 0,0,0,0,0,0 |
| SSU-SU111.5 | 20 | 0,0,0,0,0 |
| SSU-SU111.5 | 40 | 0,0,0,0 |

SCORING SYSTEM: Visual comparison to AGS502 plants which uniformly appeared:
20nM ~1cm tall, 6 leaves ~1cm diameter
40nM ~0.5cm tall, 6 leaves ~0.3cm diameter, chlorotic
Ratings: 0 = essentially identical to AGS 502 controls;
1 = marginally larger ($\geq$1.2x) than AGS502; 2 = clearly larger (1.2 to 2x) than AGS502; 3 = substantially larger (>2x) than AGS502.

EXAMPLE 25

Interchangeability of FeS-A and FeS-B

The ability of both FeS-A or FeS-B to transfer reducing equivalents to either P450SU1 or P450SU2 was examined. Mixtures of the purified proteins were tested to see if FeS-A and/or FeS-B carried out the transfer of electrons from NADPH and Spinach ferredoxin:NADP oxidoreductase to cytochromes P450SU1 or P450SU2. The ability for these FeS proteins to transfer reducing equivalents is a prerequisite for their involvement in the catalytic activity of the cytochromes P450.

The experiment was carried out at room temperature, in a buffer consisting of 0.1M MOPS-NaOH (pH 7.0), 0.2M NaCl, 10mM compound 10013 (chlorsulfuron), and 50nM ferredoxin:NADP oxidoreductase (purified from spinach leaves according to the method of Zanetti and Curti, Methods in Enzymology, 1980, Vol 69, pp250-255, herein incorporated by reference). To this mixture was added either FeS-A or FeS-B (as indicated in Table 24, 0.03mM NADPH, and incubated for 10 min. The absorption spectrum was measured (Hewlett-Packard Model 8450A uv/vis spectrophotometer) , P450 protein added, the sample bubbled with CO for 30 seconds, and the absorption measured again after 1 min and 5 min. The appearance of an absorption band at about 450 nm indicated the reduction of the cytochrome by the FeS protein.

Table 24

| Additions[a] | Fraction P450 Reduced 1 min | Fraction P450 Reduced 5 min |
|---|---|---|
| P450SU1 | <0.05 | <0.05 |
| P450SU1+FeS-A | 0.51 | 0.81 |
| P450SU1+FeS-B | 0.10 | 0.46 |
| P-450SU2 | <0.05 | <0.05 |
| P450SU2+FeS-A | 0.98 | 1.0 |
| P450SU2+FeS-B | 0.91 | 1.0 |

[a] Proteins were added at concentrations defined by their absorption spectra: SU1 (A418=0.017), SU2 (A418=0.027), FeS-A (A410=0.066), FeS-B (A410=0.071).

The data in Table 24 demonstrated that the FeS proteins participate in the transfer of electrons to the cytochromes P450, and that they may be used interchangeably, either FeS-A or FeS-B with SU1, or either FeS-A or FeS-B with SU2.

## EXAMPLE 26

### A negative selection system in transgenic Arabidopsis

Shoot growth of Arabidopsis seedlings that carry the cytochrome P450SU1 coding region was arrested when seeds were germinated on medium containing the sulfonylurea 10015 at concentrations which have no visible effect on seedlings lacking the coding region, thus providing a negative selection system for plants expressing the introduced gene. Plants lacking expression of the gene survived, while shoot growth of those expressing the gene was stunted.

The plasmids pSU18, pSSU-SU111, and pCab-SU111, as described in Example 19, Section C, were transformed directly into the Agrobacterium strain

LBA4404/pAL4404 (Hoekema et al. Nature 303:179-180, 1983) using the freeze-thaw method (Plant Molec. Biol. Manual, S.B. Gelvin and R.A. Schilperoot, editors, A3:1-19, 1988, herein incorporated by reference) and selected on YEP medium (Table 25) with 50 mg/l rifampicin and 5 mg/l tetracycline. The presence of the plasmid DNA in selected clones was verified by restriction digests.

Standard aseptic techniques for the manipulation of sterile media and axenic plant/bacterial cultures were followed, including the use of a laminar flow hood for all transfers. Compositions of the culture media are given in Table 25. Unless otherwise indicated, 25x100 mm petri plates were used for plant tissue cultures. Incubation of plant tissue cultures was at 23°C under constant illumination with mixed fluorescent and "Gro and Sho" plant lights (General Electric) unless otherwise noted.

The source of explants was in vitro grown plants of Arabidopsis thaliana (L.) Heynh, geographic race Wassilewskija. Seeds were sterilized for 10 min in a solution of 50% chlorine bleach with 0.1% sodium dodecylsulfate, rinsed three to five times with sterile distilled $H_2O$, dried thoroughly on sterile filter paper, and then sown on GM medium. The plates were sealed with filter tape (Carolina Biologicals, Burlington, NC USA) and incubated for seven days as described above. Seedlings were transferred to GM medium in 25x150 mm petri dishes, 36-40 per plate. Plates were sealed with filter tape and incubated for 2-3 weeks.

Prior to inoculation with Agrobacterium, root tissues were cultured on callus induction medium (MSKig) for four days. Whole root systems were harvested by pulling plantlets out of the agar using forceps, laying the roots on MSKig medium, and then cutting off the shoot with a scalpel. Petri dishes were sealed with filter tape and incubated for four days.

Cultures of Agrobacterium cells containing each of the plasmids were grown in 5 ml of YEP broth containing 2 mg/l tetracycline. The cultures were grown for approximately 17-20 hours in glass culture tubes in a New Brunswick platform shaker (225 rpm) maintained at 28°C. Pre-cultured whole roots were cut into 0.5 cm segments and placed in a 100 µm filter, made from a Tri-Pour beaker (VWR Scientific, San Francisco, CA USA) and 100 µm wire mesh, which is set in a petri dish. Root segments were inoculated for several minutes in 30-50 ml of a 1:20 dilution of an overnight Agrobacterium culture with periodic gentle mixing. Inoculated roots were transferred to sterile filter paper to draw off most of the liquid. Small bundles of roots, consisting of several root segments, were placed on MSKig medium containing 100 µM acetosyringone (3',5'-dimethoxy-4'-hydroxyaceto-phenone, Aldrich Chemical Co., Milwaukee, WI, USA). Petri plates were sealed with parafilm or filter tape and incubated for two to three days.

After inoculation, root segments were rinsed and transferred to shoot induction medium containing antibiotics. Root bundles were placed in a 100 µm filter unit (described above) and rinsed with 30-50 ml liquid MSKig medium. The filter was vigorously shaken in the solution to help remove the Agrobacterium, transferred t a clean petri dish, and

rinsed again. Roots were blotted on sterile filter paper and bundles of roots were placed on MSg medium containing 500 mg/l vancomycin with and without 50 mg/l kanamycin. Plates were sealed with filter tape and incubated for 12 to 21 days.

Green nodules and small shoot primordia were visible at about 2 weeks. The explants were either left intact or were broken into numerous pieces and placed on GM medium containing 200-300 mg/l vancomycin for further shoot development. Plates were either sealed with two pieces of tape or with filter tape. As they developed, individual shoots were isolated from the callus and were placed on MSRg medium containing 100 mg/l vancomycin. Dishes were sealed as described above and incubated for four to seven days. Shoots were then transferred to GM medium containing 100-200 mg/l vancomycin in 25x100 mm petri dishes or PlantCon containers (Flow Laboratories, McLean, VA). Many primary transformants (T1) which were transferred to individual containers set seed (T2).

T2 seed was harvested from selected putative transformants and sown on GM medium containing 50 mg/l kanamycin and that containing 5 ppb 10015. Plates were sealed with filter tape, cold treated for 2 or more days at 4°C, and then incubated for 10 to 20 days at 23°C under constant illumination as described above. Seedlings were scored as resistant (green, true leaves develop) and sensitive (no true leaves develop). Table 26 shows the number and percent of seedlings that are resistant and sensitive to kanamycin and 10015. The percent of resistant seedlings is inversely proportional with the two selections. Kanamycin is a positive selection for

those seedlings that carry the foreign DNA. Therefore, this inverse relationship indicated that 10015 negatively selected, that is arrested shoot growth of, seedling expressing the P450SU1 gene, while those seedlings that do not express the P450SU1 gene survived.

Selected T2 seedlings that were kanamycin resistant were transplanted to soil and were grown to maturity at 21°C daytime (14 hours) 18°C nighttime (10 hours) at 65-80% relative humidity. T3 seed was collected, sterilized, and germinated on GM medium containing 50 mg/l kanamycin and that containing 5 ppb 10015. Plates were sealed with filter tape, cold treated for 2 or more days at 4°C, and then incubated for 10 to 20 days at 23°C under constant illumination as described above. Seedlings were scored as resistant and sensitive and results are shown in Table 27. Two of the six plants produced seed that was 100% resistant to kanamycin and 100% sensitive to 10015; they are homozygous for the inserted DNA. The other four parents are heterozygous and show an inverse proportion of kanamycin and 10015 resistant seedlings. Thus, growth of seedlings which carry the heterologous genes was arrested when grown on medium containing 10015, while growth of seedlings which do not carry the genes was not affected. Therefore, seedlings expressing the P450SU1 gene can be negatively selected.

Selective destruction of plant tissues was exhibited in seedlings grown on medium containing 10015. Expression of the P450SU1 gene was controlled by the tissue-specific promoter from the cab gene which is expressed in the green tissues of plants. T3 seedlings grown on 5 ppb 10015, as described

above, exhibited root growth but shoot growth was inhibited. Thus, only the shoot, which expresses P450SU1 was destroyed by the application of 10015.

Germination assays of homozygous seed on various concentrations of 10015 were performed to assay the relative sensitivity to 10015 produced by the three different promoters and between independent transformants. Seed was sterilized, sown on GM medium with 0, 0.5, 1, 2, 5, 10 and 20 ppb 10015, incubated, and scored as described above. In addition, seedlings are rated on a scale of one to three for the amount of growth. Results are shown in Table 27A. These results show that independent transformants exhibit different sensitivity to 10015.

To test if seedlings that do not carry the cytochrome P450SU1 coding region or those that exhibit lower activity of the gene product can be selected, wild type seeds were placed in specified areas on GM medium containing 10 ppb 10015. Over 500 seed that are homozygous for the P450SU1 coding region were sown on the same plates. The plates were sealed, cold-treated, and incubated as previously described. Plates were observed to determine if differentiation exists between the wild type seeds, and those exhibiting activation of 10015. Wild type seedlings were unaffected by the dying transformed seedlings. In addition, when greater than 10,000 seed of a homozygous Cab-SU111 plant was sown on GM medium with 10 ppb 10015, then cold-treated and incubated as described above, eighteen seedlings were resistant to 10015 and were unaffected by neighboring seedlings.

Integration of the coding region is confirmed by Southern blot analysis of selected progeny exhibiting kanamycin resistance and 10015 sensitivity. Southern blots are performed as described in Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd edition (Cold Spring Harbor Laboratory, New York, 1989) hereby incorporated by reference. Plant DNA is digested with an enzyme appropriate for producing a DNA fragment containing DNA sequence from the introduced DNA, and an appropriate probe is used to detect this fragment.

TABLE 25

Medium Composition

YEP MEDIUM

| | Per Liter |
|---|---|
| Bacto Yeast Extract | 10.0 g |
| Bacto Peptone | 10.0 g |
| NaCl | 5.0 g |
| Agar (optional) | 15.0 g |

pH 7.0

BASIC MEDIUM

1 pkg. Murashige and Skoog Minimal Organics Medium without Sucrose (Gibco #510-3118 or Sigma # M6899)
10 ml Vitamin Supplement
0.05% MES
0.8% agar
pH 5.8

VITAMIN SUPPLEMENT - 100 X Stock
10 mg/l thiamine
50 mg/l pyridoxine
50 mg/l nicotinic acid

GM = Germination Medium
Basic Medium
1% sucrose

MSKig = Callus Induction Medium
Basic Medium
2% glucose
0.5 mg/l 2,4-D

0.3 mg/l Kinetin
5 mg/l IAA

MSg = Shoot Induction Medium

| | |
|---|---|
| Basic Medium | |
| 2% glucose | 20 g/l |
| 0.15 mg/l IAA | 0.86 uM |
| 5.0 mg/l 2iP | 24.6 uM |

MSRg = Shoot Induction Medium

| | |
|---|---|
| Basic medium | |
| 2% glucose | 20 g/l |
| 12 mg/l IBA | 58.8 uM |
| 0.1 mg/l Kinetin | 0.46 uM |

TABLE 26

Seed Germination Assay

| ID | Plasmid | Kanamycin (50 mg/l) | | | 10015 (5 ppb) | | |
|---|---|---|---|---|---|---|---|
| | | #R[a] | #S[b] | %R[c] | #R[a] | #S[b] | %R[c] |
| 12-1 | pCab-SU111 | 11 | 6 | 65 | 3 | 10 | 23 |
| 12-2 | pCab-SU111 | 0 | 12 | 0 | 13 | 0 | 100 |
| 12-4 | pCab-SU111 | 13 | 18 | 42 | 12 | 8 | 60 |
| 12-5 | pCab-SU111 | 0 | 20 | 0 | 15 | 0 | 100 |
| 12-6 | pCab-SU111 | 15 | 9 | 62 | 5 | 9 | 36 |
| 12-7 | pCab-SU111 | 21 | 7 | 75 | 9 | 13 | 40 |
| 12-8 | pCab-SU111 | 6 | 5 | 55 | 1 | 7 | 13 |
| 12-9 | pCab-SU111 | 13 | 3 | 81 | 3 | 9 | 25 |
| 12-10 | pCab-SU111 | 0 | 21 | 0 | 9 | 0 | 100 |
| 12-11 | pCab-SU111 | 0 | 9 | 0 | 4 | 0 | 100 |

[a] = number of resistant seedlings
[b] = number of sensitive seedlings
c = percent of seedlings which are resistant

TABLE 27

T3 Seed Germination Assay

| ID | Plasmid | Kanamycin (50 mg/l) | | | 10015 (5 ppb) | | |
|---|---|---|---|---|---|---|---|
| | | #R[a] | #S[b] | %R[c] | #R[a] | #S[b] | %R[c] |
| 12-1-1 | pCab-SU111 | 184 | 0 | 100 | 0 | 196 | 0 |
| 12-1-2 | pCab-SU111 | 191 | 0 | 100 | 0 | 191 | 0 |
| 12-1-3 | pCab-SU111 | 136 | 41 | 77 | 42 | 90 | 32 |
| 12-1-4 | pCab-SU111 | 98 | 39 | 72 | 80 | 129 | 37 |
| 12-1-5 | pCab-SU111 | 161 | 56 | 74 | 99 | 177 | 36 |
| 12-1-6 | pCab-SU111 | 254 | 75 | 77 | 83 | 152 | 35 |
| WT | - | 0 | 77 | 0 | 58 | 5 | 92 |

[a] = number of resistant seedlings

[b] = number of sensitive seedlings

[c] = percent of seedlings which are resistant

TABLE 27A

| ID | Construct | Visual Inhibition Ratings of Seedlings* ppb 10015 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 5 | 10 | 20 |
| Wild type | | 1 | 1 | 1 | 1 | 1 | 1 |
| 12.20.5 | Cab-SU111 | 1 | 1 | 2 | 3 | 4 | 4 |
| 12.1.1 | Cab-SU111 | 1 | 1 | 1 | 2 | 4 | 4 |
| 12.16.2 | Cab-SU111 | 1 | 1 | 1 | 1 | 1 | 1 |
| 17.74.4 | SSU-SU111 | 1 | 1 | 1 | 2 | 3 | 3 |
| 13.4.6 | SSU-SU111 | 1 | 1 | 1 | 3 | 4 | 4 |
| 17.92.3 | SSU-SU111 | 1 | 1 | 1 | 1 | 1 | 1 |
| 17.17.3 | SU18 | 1 | 1 | - | 1 | 2 | 4 |
| 13.3.1 | SU18 | 1 | 1 | 1 | 2 | 4 | 4 |
| 17.1.10 | SU18 | 1 | 1 | 1 | 3 | 4 | 4 |

*Scale of 1 to 4 with 4 = complete inhibition, and 1 = no inhibition.

## EXAMPLE 27

### Cytochrome p450SU1 metabolism of sulfonylureas supported by various FeS proteins

The purpose of this example was to define the combination of cytochrome p450SU1 and other proteins which results in optimal metabolism of sulfonylureas, based on conclusions from *in vitro* experiments. In these experiments, the rate of metabolism of 10015 to 10014, mediated by purified cytochrome p450SU1, was tested to find which FeS protein functions as the best direct electron donor.

Assays were carried out in 0.025 ml of buffer containing 0.1 M MOPS-NaOH (pH 7.0), 0.2 M NaCl, 0.2 mM 10015, 2 µM purified cytochrome p450SU1, Spinach ferredoxin:NADP oxidoreductase (FNR) as indicated in

Table 28, various FeS proteins as indicated in Table 28, and an NADPH regenerating system consisting of 5 mM glucose-6-phosphate, and 2 Units/ml Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of NADPH to a final concentration of 0.03 mM. After 15 min the reaction was terminated by the addition of 0.25 ml $H_2O$:acetonitrile:$H_3PO_4$ (80:19:1). After filtering this mixture through a 0.2 µm filter, the amount of metabolite (10014) formed was analyzed by HPLC.

TABLE 28

| FES Addition | FNR concentration (mM) | Rate (nmol 10014)•(nmol p450)-1•(min)-1 |
|---|---|---|
| 4 mM FeS-B | 0.2 | 0.3 |
| 4 mM FeS-B | 2.0 | 0.3 |
| 20 mM FeS-B | 2.0 | 0.4 |
| 4 mM FeS-A | 0.2 | 1.0 |
| 4 mM FeS-A | 2.0 | 2.6 |
| 20 mM Fes-A | 2.0 | 5.1 |
| 4 mM spinach Fd | 0.2 | 0.2 |
| 4 mM spinach Fd | 2.0 | 0.2 |
| 20 mM spinach Fd | 2.0 | 0.4 |
| none | 0.2 | <0.03 |
| none | 2.0 | 0.06 |

These results demonstrated that FeS-A, FeS-B, and spinach ferredoxin (an FeS protein) functioned as the direct reductant of P450SU1 during 10015 metabolism. Because a 10-fold increase in the FNR concentration did not increase the rate of metabolism with FeS-B or spinach ferredoxin, it was apparent that FNR was not rate limiting for those reactions, and the overall rate of metabolism was determined by FeS reduction of the P450. At 2 μM FNR, although somewhat rate limited by FNR, the rate of metabolism when 4 μM FeS-A was present was still at least 8-fold faster than with the same concentration of FeS-B.

It is not known if this differential ability of the two S. griseolus FeS proteins to support sulfonylurea metabolism was a result of some damage to FeS-B occuring during purification, or if the same differential ability occurs in vivo with the endogenous reductase proteins. Nonetheless, these in vitro results suggested that p450SU1 and FeS-A were the optimal combination for maximal p450SU1 metabolic activity, and supported claims for combinations of DNA resulting in coordinated expression of these two proteins as preferred constructions.

## EXAMPLE 28

The cytochrome p450SU1 coding region was expressed specifically in the anther tissue of tobacco plants. The promoter region derived from the tobacco TA29 gene, which is a gene expressed naturally only in the tapetal tissue of the tobacco anther, was used. The tobacco TA29 gene has been described by Goldberg in Science 240, pp. 1460-1467 (1988) and in EPA 89-344029. The TA29 promoter

fragment was prepared from the TA29 gene by isolating a 1500 bp ClaI-HindIII fragment from the TA29 gene clone, during which the ClaI end was filled in, and cloning this into the HincII (blunt) and HindIII sites of M13mp19. The sequence of DNA surrounding the translation initiation ATG was determined by sequencing in from the HindIII end of the fragment according to the method of Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977) using a U.S. Biochemical Corporation Sequenase DNA sequencing kit and following the manufacturer's protocol. It was then altered to create an NcoI site at this ATG by using site-directed mutagenesis, as described in Viitanen et al., J. Biol. Chem. 263:15000-15007 (1988). The mutagenesis was carried out using the oligonucleotide of sequence AGAAATTAGCTACCATGGTAGCTCCAAAAT that was synthesized using an Applied Biosystems DNA synthesizer and following the manufacturer's procedure. The TA29 promoter fragment containing the new NcoI site was then moved as a SmaI-HindIII fragment, the SmaI site being derived from the M13mp19 polylinker, into SmaI and HindIII digested pTZ19 (Pharmacia) creating pTZAL.

Two chimeric genes were constructed that contain the TA-29 promoter and the SU1 coding region followed by the petunia Rubisco small subunit gene untranslated and polyadenylation region ("SSU301 3'"). One chimeric gene also contained the Rubisco small subunit chloroplast transit sequence and the other did not. These chimeric genes were called A-T-SU1 and A-SU1, respectively. To construct A-SU1 a ScaI-BamHI fragment was isolated that contains the same SU1 coding region and "SSU301" polyadenylation region that was present in the clone

pSU17 which has been deposited with the ATCC and bears accession number 67995. The ScaI end of the fragment was joined to the filled in NcoI site at the 3' end of the TA29 promoter. The plasmid containing the TA29 promoter fragment was also digested with BamHI to accomodate the 3' end of the SU1 fragment. The resulting plasmid was called pTZA-SU1.

In constructing A-T+SU1 the source of the SU1 coding region adjacent to the transit sequence was the SSu-SU11 gene which has been deposited with the ATCC as pSSU-SU11 and bears the accession number 67994. An NcoI fragment containing the transit sequence and the 5' region of SU1 was purified as well as an NcoI-HindIII fragment containing the 3' region of SU1 and some polyadenylation region from the SSU301 gene. The NcoI-HindIII 3' fragment was first ligated with NcoI and HindIII cut pTZAL that was described above. Next the NcoI fragment was ligated into the NcoI site of the resulting plasmid and a clone containing this fragment in the correct orientation was identified by digestion with SphI. From the resulting clone a SmaI-DraI fragment containing the TA29 promoter, transit sequence, and part of the SU1 coding region was cloned into SmaI and DraI digested pTZA-SU1, the SmaI site in both being present in the pTZ19 polylinker. This step was carried out to place the promoter, transit, and 5' SU1 sequences adjacent to a complete SSU301 polyadenylation region. The resulting plasmid was called pTZA-T-SU1.

The SU1 chimeric genes were each isolated as Asp718-BamHI fragments, the Asp718 site coming from the polylinker of pTZ19. They were ligated into Asp718 and BamIII digested pZS96. pZS96 was prepared

as described in Example 19. The resulting plasmid with the A-SU1 chimeric gene residing in the pZS96 plasmid was pZ6A-SU1 and is shown in Figure 25A. The resulting plasmid with the A-T-SU1 chimeric gene residing in the pZS96 plasmid was called pZ6A-T-SU1 and is shown in Figure 25B.

pZ6A-SU1 and pZ6A-T-SU1 were each transformed into Agrobacterium tumefaciens strain LBA4404 by direct DNA uptake following the procedure described in Plant Molecular Biology Manual, SB Gelvin et al., eds. Kluwer Academic Press PMAN-A3/7, 1988, herein incorporated by reference. The presence of each intact vector in Agrobacterium colonies selected on minA medium with sucrose containing 100 µg/ml kanamycin and 100 µg/ml carbenicillin was verified by restriction enzyme digests of miniprep DNA. Leaf disks of Nicotiana tabacum cv. Xanthi were inoculated with Agrobacterium carrying the constructed plasmids and kanamycin resistant plants were obtained as described previously.

Twenty-one plants transformed with the A-T-SU1 gene and 15 plants transformed with the A-SU1 gene were grown to maturity. From each plant, anthers were dissected from five early developmental stage buds in which the petals had not yet separated and they were frozen in liquid nitrogen. RNA was prepared following the procedure of Verwoerd et al., Nucleic Acids Research 17:2362, 1989, and analyzed on Northern blots for the presence of messenger RNA (mRNA) produced by the chimeric SU1 gene that was introduced into the plant. Northern blots were prepared according to Rave et al. Nucleic Acids Research 6:3559-3569, 1979, and probed as described in Maniatis et al. Molecular Cloning:a Laboratory

Manual, Cold Spring Harbor, NY, each herein incorporated by reference. The probe fragment used was a PstI fragment isolated from SSU-SU114 containing part of the transit sequence and the SU1 coding region. This probe detected the A-SU1 and A-T-Su1 mRNAs as well as the Rubisco small subunit mRNA (due to homology with the transit sequence).

In several plants no SU1 mRNA was detected in the anthers and these plants were not analyzed further. Plants showing expression of the SU1 mRNA in the anthers were analyzed further to determine whether the SU1 mRNA expression was anther-specific. Leaf RNA was prepared from each plant and compared to the anther RNA isolated from the same plant on Northern blots. The levels of SU1 mRNA in the leaf and anther RNA samples from each plant were compared to distinguish those plants in which the anther SU1 mRNA was in greater abundance than the leaf SU1 mRNA, indicating anther-specific expression. "Anther-specific" as used herein means expression of the gene regulated by the anther-specific promoter is predominantly in the desired anther tissue. Out of 14 plants expressing the A-T-SU1 mRNA in the anther, 71% showed anther-specific expression. The term "anther" refers to the part of the flower that physically contains pollen. Pollen grains, at all stages of development, are considered a part of the anther. For the sake of simplicity, the term is intended to include gamete as well. By "gamete" is meant a mature gern cell capable of forming a new individual by fusion with another gamete. Out of 12 plants expressing the A-SU1 mRNA in the anther, 42% showed anther-specific expression. Thus there was some variability among plants receiving the SU1

coding region regulated by the TA29 promoter, but plants with anther-specific expression of the p450SU1 gene were created.

The resulting data was as follows:

A denotes anther, L denotes leaf

| | | |
|---|---|---|
| A-T-SU1 plants: | A>>L: | 17A, 33A, 41A, 43A, 56A |
| | A>L: | 13A, 24A, 28A, 31B, 38A |
| | A=L: | 61A, 63A, 64B |
| | A<L: | 52B |
| | no A: | 7A, 12A, 23A, 37A, 59A, 62B |
| | plant 65A produced no buds | |
| A-SU1 plants: | A>>L: | 19A, 31A, 34A |
| | A>L: | 26A, 56A |
| | A=L: | 36C, 52A, 59A |
| | A<L: | 11B, 32A, 40A, 64B |
| | no A: | 3A, 8A |
| | plant 14A produced no buds | |

Application of compound 10015 was as follows. The tapetal cells of the anther surround the developing pollen and are instrumental in supporting the development of mature pollen. Thus production of a toxin in the tapetal cells was expected to disrupt the development of normal mature pollen. The nontoxic compound 10015 was sprayed onto flowering transgenic plants that had anther-specific expression of P450SU1 mRNA. Plants were hand sprayed with rates between 4 and 128 g/hectare, which consisted of 14-25 ml of 5.3-95 µg/ml of 10015. The 10015 was first dissolved in up to 0.8 ml of .01 N ammonium hydroxide and then diluted into AGWT (90.2% Acetone:4.8% Glycerol:4.8% Water:0.24% Tween 20 by volume). Little effect was seen when rates of less

than 32 g/ha were applied. At 32 g/ha and above, effects were seen on the ability of pollen to germinate *in vitro*. Newly opened buds were collected at time intervals between four and 23 days after spraying. The anthers were removed and pollen was brushed into Brewbaker and Kwak medium (15% sucrose, 200 ppm calcium nitrate, 100 ppm boric acid). Growth of tubes from the pollen, indicating germination, was assessed after four hours of incubation by microscopic observation.

Pollen from some of the transgenic plants collected following application of 10015 showed reduced ability to germinate *in vitro*. Germination of pollen from two plants with anther-specific expression of the A-T-SU1 gene (41A and 56 A) was reduced to 0% at 7 to 11 days after application of 32 g/ha of 10015 (0.59 mg in 20 ml). The germination stayed at less than 0.1% through 18 days on one of these plants and through 13 days on the other plant. Three other plants (31B, 43A, 24A) also had reduced pollen germination rates varying between 0 and 2% for time periods of a few days to a week. Pollen from the control untransformed plant with the same application of 10015 germinated at a rate of 50% to 90% (varying between buds) over this same time period. Two other transgenic plants sprayed with 100 g/ha (1.85 mg in 24 ml) were greatly affected: one (plant 28A) had no pollen germination at 7-14 days after treatment and less than 0.1% germination at 21 days, the other (plant 38A) had no pollen germination at 7-11 days, but increased to 1-25% (varying between buds) at 14 days. Thus seven plants out of the 10 plants tested that had anther-specific expression of A-T-SU1 showed reduced pollen viability *in vitro*

following application of 10015, and three plants showed almost complete absence of pollen viability for a week or more.

The transgenic plants expressing the A-SU1 gene were not as affected by 10015 application: two plants showed only slight reduction in pollen viability.

Thus it has been demonstrated by an in vitro pollen germination assay that application of 10015 to transgenic plants, that express the p450SU1 gene from the TA29 promoter and show anther-specific expression of the p450SU1 mRNA, substantially reduced the viability of the pollen. The ability of pollen from treated plants to function in vivo is being tested by using it to cross fertilize emasculated flowers on control untreated plants. The female fertility of treated plants is being tested by cross-fertilizing emasculated flowers of these plants with pollen from untreated control plants. It is expected that the three plants that were most affected by the 10015 appliction as described above will behave as male sterile, female fertile plants in the time period when the in vitro pollen germination is lowest.

## EXAMPLE 29

## Metabolism of Non-sulfonylurea Herbicides by Bacteria Containing the Genes for p450SU1

This experiment was performed as in Example 17 with the following changes. Separate cultures (50 ml) inoculated with S. lividans C37, S. lividans transformed with pCAO200SU1-FeS-B#9, or S. griseolus ATCC11796 were cultured in sporulation broth for 18 hours at 30°C with shaking. Each cluture was then resuspended in 25 ml fresh sporulation broth and 3.0

mg herbicide added. Each culture was reincubated for 18 hours, then an aliquot of the medium was withdrawn and analyzed by HPLC. The percent conversion of herbicide was determined.

The percent conversion of herbicide is presented in Table 29. The results in Table 29 show that bacteria containing constitutively expressed P450SU1 metabolized the nonsulfonylurea herbicides 10033, 10034, 10035, and 10036.

TABLE 29

| | Percent Conversion | | | |
|---|---|---|---|---|
| Strain | 10033 | 10034 | 10035 | 10036 |
| S. griseolus ATCC11796 | 100 | 13 | 21 | 100 |
| S. lividans C37 | 5 | 10 | 22 | 8 |
| S. lividans pCAO200 SU1-FeS-B#9 | 88 | 69 | 96 | 100 |

CLAIMS

What is claimed is:

1. A fragment of DNA comprising a 2.4 kb BamHI fragment of DNA sequence encoding cytochrome P450 enzyme P450SU1 which causes metabolism of herbicide compounds when transformed into bacteria or plants.

2. A fragment of DNA comprising a 2.0 kb BamHI fragment of DNA sequence encoding cytochrome P450 enzyme P450SU2 which causes metabolism of herbicide compounds when transformed into bacteria or plants.

3. A fragment of DNA of Claim 1 comprising a 2.4 kb BamHI fragment of DNA encoding enzyme P450SU1 and iron sulfur protein FeS-B isolated from S. griseolus.

4. A fragment of DNA of Claim 3 in plasmid pUC18-SU1-BamHI as deposited with the American Type Culture Collection as deposit number 67780.

5. A fragment of DNA comprising a 2.0 kb SacI-BamHI fragment encoding enzyme P450SU1 and iron sulfur protein FeS-B isolated from the fragment of Claim 3.

6. A fragment of DNA of Claim 3 comprising a 1.8 kb DNA sequence encoding cytochrome P450 enzyme P450SU1 of 122 base pairs.

7. A DNA fragment of Claim 3 comprising the sequence encoding iron-sulfur protein FeS-B running

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in the downstream direction from base position 1369 to 1578.

8. A fragment of DNA of Claim 2 comprising a 2.0 kb BamHI fragment encoding enzyme P450SU2 and iron sulfur protein FeS-A isolated from S. griseolus mutant PH2001.

9. A fragment of DNA of Claim 8 comprising the DNA sequence encoding cytochrome P450 enzyme P450SU2 running in the downstream direction from base position 195 to base position 1406.

10. A DNA fragment of Claim 8 comprising the sequence encoding iron-sulfur protein FeS-A running in the downstream direction from base position 1452 to 1646.

11. A combination of the DNA sequences of 1) Claims 6 and 10 or 2) Claims 7 and 9.

12. A fragment of DNA of Claim 8 in plasmid pUC19-SU2-8 as deposited with the American Type Culture Collection as accession number 67781.

13. A sequence of DNA encoding cytochrome P450 enzyme P450SU1 and iron sulfur protein FeS-B of Claim 1 comprising

```
        10                  30                  50
GCGGACAGGGGGACTCCTGAAGATGTCTGATAGAGGCCGTTGCGTTCTCTACGGGGGCAA
---------+---------+---------+---------+---------+---------+
CGCCTGTCCCCCTGAGGACTTCTACAGACTATCTCCGGCAACGCAAGAGATGCCCCCGTT
```

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        70                  90                  110
GTCTATGCTCCGAAATAGAGAACATGGCGTTCTTTAAAGGTGAGAATTCTTGAATCGGAG
---------+---------+---------+---------+---------+---------+
CAGATACGAGGCTTTATCTCTTGTACCGCAAGAAATTTCCACTCTTAAGAACTTAGCCTC
                                                 EcoRI

       130                 150                 170
TGGACCGATGACCGATACCGCCACGACGCCCCAGACCACGGACGCACCCGCCTTCCCGAG
---------+---------+---------+---------+---------+---------+
ACCTGGCTACTGGCTATGGCGGTGCTGCGGGGTCTGGTGCCTGCGTGGGCGGAAGGGCTC
P450SU1MetThrAspThrAlaThrThrProGlnThrThrAspAlaProAlaPheProSe
Start

       190                 210                 230
CAACCGGAGCTGTCCCTACCAGTTACCGGACGGCTACGCCCAGCTCCGGGACACCCCCGG
---------+---------+---------+---------+---------+---------+
GTTGGCCTCGACAGGGATGGTCAATGGCCTGCCGATGCGGGTCGAGGCCCTGTGGGGGCC
rAsnArgSerCysProTyrGlnLeuProAspGlyTyrAlaGlnLeuArgAspThrProGl

       250                 270                 290
CCCCCTGCACCGGGTGACGCTCTACGACGGCCGTCAGGCGTGGGTGGTGACCAAGCACGA
---------+---------+---------+---------+---------+---------+
GGGGGACGTGGCCCACTGCGAGATGCTGCCGGCAGTCCGCACCCACCACTGGTTCGTGCT
yProLeuHisArgValThrLeuTyrAspGlyArgGlnAlaTrpValValThrLysHisGl

       310                 330                 350
GGCCGCGCGCAAACTGCTCGGCGACCCCCGGCTGTCCTCCAACCGGACGGACGACAACTT
---------+---------+---------+---------+---------+---------+
CCGGCGCGCGTTTGACGAGCCGCTGGGGGCCGACAGGAGGTTGGCCTGCCTGCTGTTGAA
uAlaAlaArgLysLeuLeuGlyAspProArgLeuSerSerAsnArgThrAspAspAsnPh

       370                 390                 410
CCCCGCCACGTCACCGCGCTTCGAGGCCGTCCGGGAGAGCCCGCAGGCGTTCATCGGCCT
---------+---------+---------+---------+---------+---------+
GGGGCGGTGCAGTGGCGCGAAGCTCCGGCAGGCCCTCTCGGGCGTCCGCAAGTAGCCGGA
eProAlaThrSerProArgPheGluAlaValArgGluSerProGlnAlaPheIleGlyLe

       430                 450                 470
GGACCCGCCCGAGCACGGCACCCGGCGGCGGATGACGATCAGCGAGTTCACCGTCAAGCG
---------+---------+---------+---------+---------+---------+
CCTGGGCGGGCTCGTGCCGTGGGCCGCCGCCTACTGCTAGTCGCTCAAGTGGCAGTTCGC
uAspProProGluHisGlyThrArgArgArgMetThrIleSerGluPheThrValLysAr

       490                 510                 530
GATCAAGGGCATGCGCCCCGAGGTCGAGGAGGTGGTGCACGGCTTCCTCGACGAGATGCT
---------+---------+---------+---------+---------+---------+
CTAGTTCCCGTACGCGGGGCTCCAGCTCCTCCACCACGTGCCGAAGGAGCTGCTCTACGA
gIleLysGlyMetArgProGluValGluGluValValHisGlyPheLeuAspGluMetLe
```

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       550                 570                 590
GGCCGCCGGCCCGACCGCCGACCTGGTCAGTCAGTTCGCGCTGCCGGTGCCCTCCATGGT
---------+---------+---------+---------+---------+---------+
CCGGCGGCCGGGCTGGCGGCTGGACCAGTCAGTCAAGCGCGACGGCCACGGGAGGTACCA
uAlaAlaGlyProThrAlaAspLeuValSerGlnPheAlaLeuProValProSerMetVa

       610                 630                 650
GATCTGCCGACTCCTCGGCGTGCCCTACGCCGACCACGAGTTCTTCCAGGACGCGAGCAA
---------+---------+---------+---------+---------+---------+
CTAGACGGCTGAGGAGCCGCACGGGATGCGGCTGGTGCTCAAGAAGGTCCTGCGCTCGTT
lIleCysArgLeuLeuGlyValProTyrAlaAspHisGluPhePheGlnAspAlaSerLy

       670                 690                 710
GCGGCTGGTGCAGTCCACGGACGCGCAGAGCGCGCTCACCGCGCGGAACGACCTCGCGGG
---------+---------+---------+---------+---------+---------+
CGCCGACCACGTCAGGTGCCTGCGCGTCTCGCGCGAGTGGCGCGCCTTGCTGGAGCGCCC
sArgLeuValGlnSerThrAspAlaGlnSerAlaLeuThrAlaArgAsnAspLeuAlaGl

       730                 750                 770
TTACCTGGACGGCCTCATCACCCAGTTCCAGACCGAACCGGGCGCGGGCCTGGTGGGCGC
---------+---------+---------+---------+---------+---------+
AATGGACCTGCCGGAGTAGTGGGTCAAGGTCTGGCTTGGCCCGCGCCCGGACCACCCGCG
yTyrLeuAspGlyLeuIleThrGlnPheGlnThrGluProGlyAlaGlyLeuValGlyAl

       790                 810                 830
TCTGGTCGCCGACCAGCTGGCCAACGGCGAGATCGACCGTGAGGAACTGATCTCCACCGC
---------+---------+---------+---------+---------+---------+
AGACCAGCGGCTGGTCGACCGGTTGCCGCTCTAGCTGGCACTCCTTGACTAGAGGTGGCG
aLeuValAlaAspGlnLeuAlaAsnGlyGluIleAspArgGluGluLeuIleSerThrAl

       850                 870                 890
GATGCTGCTCCTCATCGCCGGCCACGAGACCACGGCCTCGATGACCTCCCTCAGCGTGAT
---------+---------+---------+---------+---------+---------+
CTACGACGAGGAGTAGCGGCCGGTGCTCTGGTGCCGGAGCTACTGGAGGGAGTCGCACTA
aMetLeuLeuLeuIleAlaGlyHisGluThrThrAlaSerMetThrSerLeuSerValIl

       910                 930                 950
CACCCTGCTGGACCACCCCGAGCAGTACGCCGCCCTGCGCGCCGACCGCAGCCTCGTGCC
---------+---------+---------+---------+---------+---------+
GTGGGACGACCTGGTGGGGCTCGTCATGCGGCGGGACGCGCGGCTGGCGTCGGAGCACGG
eThrLeuLeuAspHisProGluGlnTyrAlaAlaLeuArgAlaAspArgSerLeuValPr

       970                 990                 1010
CGGCGCGGTGGAGGAACTGCTCCGCTACCTCGCCATCGCCGACATCGCGGGCGGCCGCGT
---------+---------+---------+---------+---------+---------+
GCCGCGCCACCTCCTTGACGAGGCGATGGAGCGGTAGCGGCTGTAGCGCCCGCCGGCGCA
oGlyAlaValGluGluLeuLeuArgTyrLeuAlaIleAlaAspIleAlaGlyGlyArgVa
```

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      1030                 1050                 1070
CGCCACGGCGGACATCGAGGTCGAGGGGCACCTCATCCGGGCCGGCGAGGGCGTGATCGT
---------+---------+---------+---------+---------+---------+
GCGGTGCCGCCTGTAGCTCCAGCTCCCCGTGGAGTAGGCCCGGCCGCTCCCGCACTAGCA
lAlaThrAlaAspIleGluValGluGlyHisLeuIleArgAlaGlyGluGlyValIleVa

      1090                 1110                 1130
CGTCAACTCGATAGCCAACCGGGACGGCACGGTGTACGAGGACCCGGACGCCCTCGACAT
---------+---------+---------+---------+---------+---------+
GCAGTTGAGCTATCGGTTGGCCCTGCCGTGCCACATGCTCCTGGGCCTGCGGGAGCTGTA
lValAsnSerIleAlaAsnArgAspGlyThrValTyrGluAspProAspAlaLeuAspIl

      1150                 1170                 1190
CCACCGCTCCGCGCGCCACCACCTCGCCTTCGGCTTCGGCGTGCACCAGTGCCTGGGCCA
---------+---------+---------+---------+---------+---------+
GGTGGCGAGGCGCGCGGTGGTGGAGCGGAAGCCGAAGCCGCACGTGGTCACGGACCCGGT
eHisArgSerAlaArgHisHisLeuAlaPheGlyPheGlyValHisGlnCysLeuGlyGl

      1210                 1230                 1250
GAACCTCGCCCGGCTGGAGCTGGAGGTCATCCTCAACGCCCTCATGGACCGCGTCCCGAC
---------+---------+---------+---------+---------+---------+
CTTGGAGCGGGCCGACCTCGACCTCCAGTAGGAGTTGCGGGAGTACCTGGCGCAGGGCTG
nAsnLeuAlaArgLeuGluLeuGluValIleLeuAsnAlaLeuMetAspArgValProTh

      1270                 1290                 1310
GCTGCGACTGGCCGTCCCCGTCGAGCAGTTGGTGCTGCGGCCGGGTACGACGATCCAGGG
---------+---------+---------+---------+---------+---------+
CGACGCTGACCGGCAGGGGCAGCTCGTCAACCACGACGCCGGCCCATGCTGCTAGGTCCC
rLeuArgLeuAlaValProValGluGlnLeuValLeuArgProGlyThrThrIleGlnGl

      1330                 1350                 1370
CGTCAACGAACTCCCGGTCACCTGGTGACGGGGGAGAGGGGCAAGGACATGACCATGCGG
---------+---------+---------+---------+---------+---------+
GCAGTTGCTTGAGGGCCAGTGGACCACTGCCCCCTCTCCCCGTTCCTGTACTGGTACGCC
yValAsnGluLeuProValThrTrpEnd       FeS-B Start  MetThrMetArg

      1390                 1410                 1430
GTGAGTGCGGATCGGACGGTCTGCGTCGGTGCCGGGCTGTGTGCGCTGACGGCGCCGGGC
---------+---------+---------+---------+---------+---------+
CACTCACGCCTAGCCTGCCAGACGCAGCCACGGCCCGACACACGCGACTGCCGCGGCCCG
ValSerAlaAspArgThrValCysValGlyAlaGlyLeuCysAlaLeuThrAlaProGly

      1450                 1470                 1490
GTCTTCGACCAGGACGACGACGGGATCGTCACGGTGCTGACGGCCGAACCCGCCGCCGAC
---------+---------+---------+---------+---------+---------+
CAGAAGCTGGTCCTGCTGCTGCCCTAGCAGTGCCACGACTGCCGGCTTGGGCGGCGGCTG
ValPheAspGlnAspAspAspGlyIleValThrValLeuThrAlaGluProAlaAlaAsp
```

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      1510                1530                1550
GACGACCGGCGCACCGCGCGCGAGGCCGGCCATCTCTGTCCGTCCGGTGCGGTCCGCGTC
---------+---------+---------+---------+---------+---------+
CTGCTGGCCGCGTGGCGCGCGCTCCGGCCGGTAGAGACAGGCAGGCCACGCCAGGCGCAG
AspAspArgArgThrAlaArgGluAlaGlyHisLeuCysProSerGlyAlaValArgVal

     1570                1590                1610
GTCGAGGACACGGAATAGGGTCAAGGACACGGAACAGGCGAGCGGGGATTCCGGCCGTCG
---------+---------+---------+---------+---------+---------+
CAGCTCCTGTGCCTTATCCCAGTTCCTGTGCCTTGTCCGCTCGCCCCTAAGGCCGGCAGC
ValGluAspThrGluEnd

      1630                1650                1670
GCCGGGGCGGTCTCCGGCCGACGGGCTGGGGCCGCCCGCGGTGCCGCCGCGCAGGCGAGG
---------+---------+---------+---------+---------+---------+
CGGCCCCGCCAGAGGCCGGCTGCCCGACCCCGGCGGGCGCCACGGCGGCGCGTCCGCTCC

      1690                1710                1730
CCGCCGGTGGCGCCCGGCACCCGCGGCGGCCGTCAGATCCACCCCTTCCGCGCCGCGTAC
---------+---------+---------+---------+---------+---------+
GGCGGCCACCGCGGGCCGTGGGCGCCGCCGGCAGTCTAGGTGGGGAAGGCGCGGCGCATG

      1750                1770                1790
AGAGCGAGTTGGAAACGGGTGGTGGCGTCGGCGGCGCGGTTGAGCTGCTCCAACTGGCGG
---------+---------+---------+---------+---------+---------+
TCTCGCTCAACCTTTGCCCACCACCGCAGCCGCCGCGCCAACTCGACGAGGTTGACCGCC

      1810                1830                1850
GAGAGGGTGCGTCGACTGATGCCGAGCAGTTCGGCGATGGTCTCGTCCGTGACGCCGCTC
---------+---------+---------+---------+---------+---------+
CTCTCCCACGCAGCTGACTACGGCTCGTCAAGCCGCTACCAGAGCAGGCACTGCGGCGAG

      1870
CCCAGCAGCTCCAGGATCC
---------+---------
GGGTCGTCGAGGTCCTAGG
          BamHI
```

and any mutant or derivative of said sequence which causes metabolism of herbicide compounds when transformed into bacteria or plants.

14. A sequence of DNA encoding cytochrome P450 enzyme P450SU1 of Claim 1 comprising

```
130                 150                 170
ATGACCGATACCGCCACGACGCCCCAGACCACGGACGCACCCGCCTTCCCGAG
--+---------+---------+---------+---------+---------+
TACTGGCTATGGCGGTGCTGCGGGGTCTGGTGCCTGCGTGGGCGGAAGGGCTC
MetThrAspThrAlaThrThrProGlnThrThrAspAlaProAlaPheProSe
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```
       190                 210                 230
CAACCGGAGCTGTCCCTACCAGTTACCGGACGGCTACGCCCAGCTCCGGGACACCCCCGG
---------+---------+---------+---------+---------+---------+
GTTGGCCTCGACAGGGATGGTCAATGGCCTGCCGATGCGGGTCGAGGCCCTGTGGGGGCC
rAsnArgSerCysProTyrGlnLeuProAspGlyTyrAlaGlnLeuArgAspThrProGl

       250                 270                 290
CCCCCTGCACCGGGTGACGCTCTACGACGGCCGTCAGGCGTGGGTGGTGACCAAGCACGA
---------+---------+---------+---------+---------+---------+
GGGGGACGTGGCCCACTGCGAGATGCTGCCGGCAGTCCGCACCCACCACTGGTTCGTGCT
yProLeuHisArgValThrLeuTyrAspGlyArgGlnAlaTrpValValThrLysHisGl

       310                 330                 350
GGCCGCGCGCAAACTGCTCGGCGACCCCCGGCTGTCCTCCAACCGGACGGACGACAACTT
---------+---------+---------+---------+---------+---------+
CCGGCGCGCGTTTGACGAGCCGCTGGGGGCCGACAGGAGGTTGGCCTGCCTGCTGTTGAA
uAlaAlaArgLysLeuLeuGlyAspProArgLeuSerSerAsnArgThrAspAspAsnPh

       370                 390                 410
CCCCGCCACGTCACCGCGCTTCGAGGCCGTCCGGGAGAGCCCGCAGGCGTTCATCGGCCT
---------+---------+---------+---------+---------+---------+
GGGGCGGTGCAGTGGCGCGAAGCTCCGGCAGGCCCTCTCGGGCGTCCGCAAGTAGCCGGA
eProAlaThrSerProArgPheGluAlaValArgGluSerProGlnAlaPheIleGlyLe

       430                 450                 470
GGACCCGCCCGAGCACGGCACCCGGCGGCGGATGACGATCAGCGAGTTCACCGTCAAGCG
---------+---------+---------+---------+---------+---------+
CCTGGGCGGGCTCGTGCCGTGGGCCGCCGCCTACTGCTAGTCGCTCAAGTGGCAGTTCGC
uAspProProGluHisGlyThrArgArgArgMetThrIleSerGluPheThrValLysAr

       490                 510                 530
GATCAAGGGCATGCGCCCCGAGGTCGAGGAGGTGGTGCACGGCTTCCTCGACGAGATGCT
---------+---------+---------+---------+---------+---------+
CTAGTTCCCGTACGCGGGGCTCCAGCTCCTCCACCACGTGCCGAAGGAGCTGCTCTACGA
gIleLysGlyMetArgProGluValGluGluValValHisGlyPheLeuAspGluMetLe

       550                 570                 590
GGCCGCCGGCCCGACCGCCGACCTGGTCAGTCAGTTCGCGCTGCCGGTGCCCTCCATGGT
---------+---------+---------+---------+---------+---------+
CCGGCGGCCGGGCTGGCGGCTGGACCAGTCAGTCAAGCGCGACGGCCACGGGAGGTACCA
uAlaAlaGlyProThrAlaAspLeuValSerGlnPheAlaLeuProValProSerMetVa

       610                 630                 650
GATCTGCCGACTCCTCGGCGTGCCCTACGCCGACCACGAGTTCTTCCAGGACGCGAGCAA
---------+---------+---------+---------+---------+---------+
CTAGACGGCTGAGGAGCCGCACGGGATGCGGCTGGTGCTCAAGAAGGTCCTGCGCTCGTT
lIleCysArgLeuLeuGlyValProTyrAlaAspHisGluPhePheGlnAspAlaSerLy
```

```
       670                 690                 710
GCGGCTGGTGCAGTCCACGGACGCGCAGAGCGCGCTCACCGCGCGGAACGACCTCGCGGG
---------+---------+---------+---------+---------+---------+
CGCCGACCACGTCAGGTGCCTGCGCGTCTCGCGCGAGTGGCGCGCCTTGCTGGAGCGCCC
sArgLeuValGlnSerThrAspAlaGlnSerAlaLeuThrAlaArgAsnAspLeuAlaGl

       730                 750                 770
TTACCTGGACGGCCTCATCACCCAGTTCCAGACCGAACCGGGCGCGGGCCTGGTGGGCGC
---------+---------+---------+---------+---------+---------+
AATGGACCTGCCGGAGTAGTGGGTCAAGGTCTGGCTTGGCCCGCGCCCGGACCACCCGCG
yTyrLeuAspGlyLeuIleThrGlnPheGlnThrGluProGlyAlaGlyLeuValGlyAl

       790                 810                 830
TCTGGTCGCCGACCAGCTGGCCAACGGCGAGATCGACCGTGAGGAACTGATCTCCACCGC
---------+---------+---------+---------+---------+---------+
AGACCAGCGGCTGGTCGACCGGTTGCCGCTCTAGCTGGCACTCCTTGACTAGAGGTGGCG
aLeuValAlaAspGlnLeuAlaAsnGlyGluIleAspArgGluGluLeuIleSerThrAl

       850                 870                 890
GATGCTGCTCCTCATCGCCGGCCACGAGACCACGGCCTCGATGACCTCCCTCAGCGTGAT
---------+---------+---------+---------+---------+---------+
CTACGACGAGGAGTAGCGGCCGGTGCTCTGGTGCCGGAGCTACTGGAGGGAGTCGCACTA
aMetLeuLeuLeuIleAlaGlyHisGluThrThrAlaSerMetThrSerLeuSerValIl

       910                 930                 950
CACCCTGCTGGACCACCCCGAGCAGTACGCCGCCCTGCGCGCCGACCGCAGCCTCGTGCC
---------+---------+---------+---------+---------+---------+
GTGGGACGACCTGGTGGGGCTCGTCATGCGGCGGGACGCGCGGCTGGCGTCGGAGCACGG
eThrLeuLeuAspHisProGluGlnTyrAlaAlaLeuArgAlaAspArgSerLeuValPr

       970                 990                1010
CGGCGCGGTGGAGGAACTGCTCCGCTACCTCGCCATCGCCGACATCGCGGGCGGCCGCGT
---------+---------+---------+---------+---------+---------+
GCCGCGCCACCTCCTTGACGAGGCGATGGAGCGGTAGCGGCTGTAGCGCCCGCCGGCGCA
oGlyAlaValGluGluLeuLeuArgTyrLeuAlaIleAlaAspIleAlaGlyGlyArgVa

      1030                1050                1070
CGCCACGGCGGACATCGAGGTCGAGGGGCACCTCATCCGGGCCGGCGAGGGCGTGATCGT
---------+---------+---------+---------+---------+---------+
GCGGTGCCGCCTGTAGCTCCAGCTCCCCGTGGAGTAGGCCCGGCCGCTCCCGCACTAGCA
lAlaThrAlaAspIleGluValGluGlyHisLeuIleArgAlaGlyGluGlyValIleVa

      1090                1110                1130
CGTCAACTCGATAGCCAACCGGGACGGCACGGTGTACGAGGACCCGGACGCCCTCGACAT
---------+---------+---------+---------+---------+---------+
GCAGTTGAGCTATCGGTTGGCCCTGCCGTGCCACATGCTCCTGGGCCTGCGGGAGCTGTA
lValAsnSerIleAlaAsnArgAspGlyThrValTyrGluAspProAspAlaLeuAspIl
```

```
      1150                 1170                 1190
CCACCGCTCCGCGCGCCACCACCTCGCCTTCGGCTTCGGCGTGCACCAGTGCCTGGGCCA
---------+---------+---------+---------+---------+---------+
GGTGGCGAGGCGCGCGGTGGTGGAGCGGAAGCCGAAGCCGCACGTGGTCACCGACCCGGT
eHisArgSerAlaArgHisHisLeuAlaPheGlyPheGlyValHisGlnCysLeuGlyGl

      1210                 1230                 1250
GAACCTCGCCCGGCTGGAGCTGGAGGTCATCCTCAACGCCCTCATGGACCGCGTCCCGAC
---------+---------+---------+---------+---------+---------+
CTTGGAGCGGGCCGACCTCGACCTCCAGTAGGAGTTGCGGGAGTACCTGGCGCAGGGCTG
nAsnLeuAlaArgLeuGluLeuGluValIleLeuAsnAlaLeuMetAspArgValProTh

      1270                 1290                 1310
GCTGCGACTGGCCGTCCCCGTCGAGCAGTTGGTGCTGCGGCCGGGTACGACGATCCAGGG
---------+---------+---------+---------+---------+---------+
CGACGCTGACCGGCAGGGGCAGCTCGTCAACCACGACGCCGGCCCATGCTGCTAGGTCCC
rLeuArgLeuAlaValProValGluGlnLeuValLeuArgProGlyThrThrIleGlnGl

      1330                 1350
CGTCAACGAACTCCCGGTCACCTGGTGA
---------+---------+--------
GCAGTTGCTTGAGGGCCAGTGGACCACT
yValAsnGluLeuProValThrTrpEnd
```

and any mutant or derivative of said sequence which causes metabolism of herbicide compounds when transformed into bacteria or plants.

15. A sequence of DNA encoding cytochrome P450 enzyme P450SU2 and iron sulfur protein FeS-A of Claim 2 comprising

```
       10                   30                   50
GGATCCGGCCACCGCCCGACCCGTCCGCACTCCGCCCCGCCGACCGTCGTCCATCCGCCC
---------+---------+---------+---------+---------+---------+
CCTAGGCCGGTGGCGGGCTGGGCAGGCGTGAGGCGGGGCGGCTGGCAGCAGGTAGGCGGG
BamHI

       70                   90                  110
CTGCGGCCATGCGGTTTGAGCCAACCTCGGTGCTGCCGCGATCTGCCCTTCCCTCCCCCG
---------+---------+---------+---------+---------+---------+
GACGCCGGTACGCCAAACTCGGTTGGAGCCACGACGGCGCTAGACGGGAAGGGAGGGGGC

       130                  150                  170
CCGGGCCTGCGTTAGCGTGACGACATCTTAATTACCTAAGTTAGGTAATTAGCTCACGCG
---------+---------+---------+---------+---------+---------+
GGCCCGGACGCAATCGCACTGCTGTAGAATTAATGGATTCAATCCATTAATCGAGTGCGC
```

SUBSTITUTE SHEET

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       190                 210                 230
GAAGGACCGGCCGCATGACGACCGCAGAACGCACCGCTCCCCCCGACGCCCTCACCGTCC
---------+---------+---------+---------+---------+---------+
CTTCCTGGCCGGCGTACTGCTGGCGTCTTGCGTGGCGAGGGGGGCTGCGGGAGTGGCAGG
P450SU2       MetThrThrAlaGluArgThrAlaProProAspAlaLeuThrValP
Start

       250                 270                 290
CGGCCAGCCGCGCCCCCGGCTGCCCCTTCGACCCCGCGCCCGACGTCACCGAGGCGGCCC
---------+---------+---------+---------+---------+---------+
GCCGGTCGGCGCGGGGGCCGACGGGGAAGCTGGGGCGCGGGCTGCAGTGGCTCCGCCGGG
roAlaSerArgAlaProGlyCysProPheAspProAlaProAspValThrGluAlaAlaA

       310                 330                 350
GCACCGAACCGGTCACCCGGGCCACCCTCTGGGACGGCTCCTCCTGCTGGCTGGTGACGC
---------+---------+---------+---------+---------+---------+
CGTGGCTTGGCCAGTGGGCCCGGTGGGAGACCCTGCCGAGGAGGACGACCGACCACTGCG
rgThrGluProValThrArgAlaThrLeuTrpAspGlySerSerCysTrpLeuValThrA

       370                 390                 410
GCCATCAGGACGTCCGCGCGGTCCTCGGCGACCCGCGCTTCAGCGCCGACGCCCACCGCA
---------+---------+---------+---------+---------+---------+
CGGTAGTCCTGCAGGCGCGCCAGGAGCCGCTGGGCGCGAAGTCGCGGCTGCGGGTGGCGT
rgHisGlnAspValArgAlaValLeuGlyAspProArgPheSerAlaAspAlaHisArgT

       430                 450                 470
CCGGCTTCCCCTTCCTGACCGCCGGCGGCCGCGAGATCATCGGCACCAACCCGACCTTCC
---------+---------+---------+---------+---------+---------+
GGCCGAAGGGGAAGGACTGGCGGCCGCCGGCGCTCTAGTAGCCGTGGTTGGGCTGGAAGG
hrGlyPheProPheLeuThrAlaGlyGlyArgGluIleIleGlyThrAsnProThrPheL

       490                 510                 530
TGCGCATGGACGACCCGGAGCACGCCCGACTGCGCCGGATGCTCACCGCCGACTTCATCG
---------+---------+---------+---------+---------+---------+
ACGCGTACCTGCTGGGCCTCGTGCGGGCTGACGCGGCCTACGAGTGGCGGCTGAAGTAGC
euArgMetAspAspProGluHisAlaArgLeuArgArgMetLeuThrAlaAspPheIleV

       550                 570                 590
TCAAGAAGGTCGAGGCGATGCGCCCCGAGGTGCAGCGCCTCGCCGACGACCTGGTCGACC
---------+---------+---------+---------+---------+---------+
AGTTCTTCCAGCTCCGCTACGCGGGGCTCCACGTCGCGGAGCGGCTGCTGGACCAGCTGG
alLysLysValGluAlaMetArgProGluValGlnArgLeuAlaAspAspLeuValAspA

       610                 630                 650
GGATGACCACCGGACGCACCTCCGCCGACCTGGTCACCGAGTTCGCGCTGCCGCTGCCGT
---------+---------+---------+---------+---------+---------+
CCTACTGGTGGCCTGCGTGGAGGCGGCTGGACCAGTGGCTCAAGCGCGACGGCGACGGCA
rgMetThrThrGlyArgThrSerAlaAspLeuValThrGluPheAlaLeuProLeuProS
```

```
       670                 690                 710
CCCTGGTGATCTGCCTGCTGCTCGGCGTCCCCTACGAGGACCACGCGTTCTTCCAGGAGC
---------+---------+---------+---------+---------+---------+
GGGACCACTAGACGGACGACGAGCCGCAGGGGATGCTCCTGGTGCGCAAGAAGGTCCTCG
erLeuValIleCysLeuLeuGlyValProTyrGluAspHisAlaPhePheGlnGluA

       730                 750                 770
GCAGCCGGGTCCTGCTCACCCTGCGGTCCACTCCCGAGGAAGTCCGGGCCGCCCAGGACG
---------+---------+---------+---------+---------+---------+
CGTCGGCCCAGGACGAGTGGGACGCCAGGTGAGGGCTCCTTCAGGCCCGGCGGGTCCTGC
rgSerArgValLeuLeuThrLeuArgSerThrProGluGluValArgAlaAlaGlnAspG

       790                 810                 830
AGTTGCTGGAGTACCTCGCCCGGCTCGCCCGGACCAAGCGGGAGCGGCCGGACGACGCCA
---------+---------+---------+---------+---------+---------+
TCAACGACCTCATGGAGCGGGCCGAGCGGGCCTGGTTCGCCCTCGCCGGCCTGCTGCGGT
luLeuLeuGluTyrLeuAlaArgLeuAlaArgThrLysArgGluArgProAspAspAlaI

       850                 870                 890
TCATCAGCCGCCTGGTCGCCCGCGGCGAGCTCGACGACACCCAGATCGCCACCATGGGAC
---------+---------+---------+---------+---------+---------+
AGTAGTCGGCGGACCAGCGGGCGCCGCTCGAGCTGCTGTGGGTCTAGCGGTGGTACCCTG
leIleSerArgLeuValAlaArgGlyGluLeuAspAspThrGlnIleAlaThrMetGlyA

       910                 930                 950
GCCTGTTGCTGGTCGCCGGCCACGAGACGACCGCCAACATGACCGCGCTCTCCACCCTCG
---------+---------+---------+---------+---------+---------+
CGGACAACGACCAGCGGCCGGTGCTCTGCTGGCGGTTGTACTGGCGCGAGAGGTGGGAGC
rgLeuLeuLeuValAlaGlyHisGluThrThrAlaAsnMetThrAlaLeuSerThrLeuV

       970                 990                1010
TGCTGCTGCGCAACCCCGACCAACTCGCCCGGCTGCGCGCCGAACCCGCGCTCGTCAAGG
---------+---------+---------+---------+---------+---------+
ACGACGACGCGTTGGGGCTGGTTGAGCGGGCCGACGCGCGGCTTGGGCGCGAGCAGTTCC
alLeuLeuArgAsnProAspGlnLeuAlaArgLeuArgAlaGluProAlaLeuValLysG

      1030                1050                1070
GCGCCGTCGAGGAGCTGCTGCGCTACCTGACGATCGTGCACAACGGCGTTCCCCGGATCG
---------+---------+---------+---------+---------+---------+
CGCGGCAGCTCCTCGACGACGCGATGGACTGCTAGCACGTGTTGCCGCAAGGGGCCTAGC
lyAlaValGluGluLeuLeuArgTyrLeuThrIleValHisAsnGlyValProArgIleA

      1090                1110                1130
CCACCGAGGACGTGCTCATCGGCGGCCGCACCATCGCCGCCGGCGAGGGCGTCCTGTGCA
---------+---------+---------+---------+---------+---------+
GGTGGCTCCTGCACGAGTAGCCGCCGGCGTGGTAGCGGCGGCCGCTCCCGCAGGACACGT
laThrGluAspValLeuIleGlyGlyArgThrIleAlaAlaGlyGluGlyValLeuCysM
```

```
      1150                1170                1190
TGATCAGCTCCGCCAACCGGGACGCCGAGGTGTTCCCCGGCGGCGACGACCTCGACGTGG
---------+---------+---------+---------+---------+---------+
ACTAGTCGAGGCGGTTGGCCCTGCGGCTCCACAAGGGGCCGCCGCTGCTGGAGCTGCACC
etIleSerSerAlaAsnArgAspAlaGluValPheProGlyGlyAspAspLeuAspValA

      1210                1230                1250
CCCGCGACGCCCGCCGCCACGTGGCCTTCGGCTTCGGCGTCCACCAGTGCCTGGGACAGC
---------+---------+---------+---------+---------+---------+
GGGCGCTGCGGGCGGCGGTGCACCGGAAGCCGAAGCCGCAGGTGGTCACGGACCCTGTCG
laArgAspAlaArgArgHisValAlaPheGlyPheGlyValHisGlnCysLeuGlyGlnP

      1270                1290                1310
CGTTGGCCAGGGTGGAGCTCCAGATCGCCATCGAAACGCTGCTGCGCCGCCTGCCGGACC
---------+---------+---------+---------+---------+---------+
GCAACCGGTCCCACCTCGAGGTCTAGCGGTAGCTTTGCGACGACGCGGCGGACGGCCTGG
roLeuAlaArgValGluLeuGlnIleAlaIleGluThrLeuLeuArgArgLeuProAspL

      1330                1350                1370
TGCGGCTGGCCGTGCCCCACGAGGAGATCCCGTTCCGCGGCGACATGGCGATCTACGGGG
---------+---------+---------+---------+---------+---------+
ACGCCGACCGGCACGGGGTGCTCCTCTAGGGCAAGGCGCCGCTGTACCGCTAGATGCCCC
euArgLeuAlaValProHisGluGluIleProPheArgGlyAspMetAlaIleTyrGlyV

      1390                1410                1430
TCCACTCGCTGCCGATCGCCTGGTAGCCCGGGCGCCCCCACCACCGACCACCACGCACCC
---------+---------+---------+---------+---------+---------+
AGGTGAGCGACGGCTAGCGGACCATCGGGCCCGCGGGGGTGGTGGCTGGTGGTGCGTGGG
alHisSerLeuProIleAlaTrpEnd

      1450                1470                1490
TTGGGAGCACCATGCGCATCCACGTCGACCAGGACAAGTGCTGCGGCGCCGGCAGTTGCG
---------+---------+---------+---------+---------+---------+
AACCCTCGTGGTACGCGTAGGTGCAGCTGGTCCTGTTCACGACGCCGCGGCCGTCAACGC
FeS-A      MetArgIleHisValAspGlnAspLysCysCysGlyAlaGlySerCysV
Start

      1510                1530                1550
TCCTCGCCGCGCCCGACGTCTTCGACCAGCGGGAGGAGGACGGCATCGTGGTCCTCCTCG
---------+---------+---------+---------+---------+---------+
AGGAGCGGCGCGGGCTGCAGAAGCTGGTCGCCCTCCTCCTGCCGTAGCACCAGGAGGAGC
alLeuAlaAlaProAspValPheAspGlnArgGluGluAspGlyIleValValLeuLeuA

      1570                1590                1610
ACACCGCGCCGCCCGCCGCGCTGCACGACGCGGTCCGTGAGGCGGCGACCATCTGCCCCG
---------+---------+---------+---------+---------+---------+
TGTGGCGCGGCGGGCGGCGCGACGTGCTGCGCCAGGCACTCCGCCGCTGGTAGACGGGGC
spThrAlaProProAlaAlaLeuHisAspAlaValArgGluAlaAlaThrIleCysProA
```

```
      1630                 1650                 1670
CCGCCGCGATCACGGTGACCGACTGAGCCACCGGCCGCCCCGCCCGCCCGCGCCCCGGTC
---------+---------+---------+---------+---------+---------+
GGCGGCGCTAGTGCCACTGGCTGACTCGGTGGCCGGCGGGGCGGGCGGGCGCGGGGCCAG
laAlaAlaIleThrValThrAspEnd

      1690                 1710                 1730
CCCGCATCCCCCCGCGGCCCGGGGCGCGCCCCTAACCCGCCGCCCCGCACGCCGTCGCGC
---------+---------+---------+---------+---------+---------+
GGGCGTAGGGGGGCGCCGGGCCCCGCGCGGGGATTGGGCGGCGGGGCGTGCGGCAGCGCG

      1750                 1770                 1790
GCGCCGCCAGTGCCCGCAGCGCCGCCTCGGACGACGAACCCGGCACGGCGTGGTGGGTCA
---------+---------+---------+---------+---------+---------+
CGCGGCGGTCACGGGCGTCGCGGCGGAGCCTGCTGCTTGGGCCGTGCCGCACCACCCAGT

      1810                 1830                 1850
CCAGCGTCTGCCCCGGTTCGGCCGCCACGCGCAGCGTCCCGTAGGTCAGGGTCAGCGGGC
---------+---------+---------+---------+---------+---------+
GGTCGCAGACGGGGCCAAGCCGGCGGTGCGCGTCGCAGGGCATCCAGTCCCAGTCGCCCG

      1870                 1890                 1910
CCACCACCGGGTGGTCCAGCTGCTTCGTCCCGAAACCCTTGTCCTTGATGTCGTGCCGGG
---------+---------+---------+---------+---------+---------+
GGTGGTGGCCCACCAGGTCGACGAAGCAGGGCTTTGGGAACAGGAACTACAGCACGGCCC

      1930                 1950                 1970
CCCAGAAGCGCCGGAACTCCTCGCTCTGCACGGTCAGTTCGGTGATCCGCGCGGTCAGCG
---------+---------+---------+---------+---------+---------+
GGGTCTTCGCGGCCTTGAGGAGCGAGACGTGCCAGTCAAGCCACTAGGCGCGCCAGTCGC

      1990
CGGCGTCGTCCGGGATCC
---------+--------
GCCGCAGCAGGCCCTAGG
            BamHI
```

and any mutant or derivative of said sequence which causes metabolism of herbicide compounds when transformed into bacteria or plants.

16. A sequence of DNA encoding cytochrome P450 enzyme P450SU2 of Claim 2 comprising

```
             210                  230
ATGACGACCGCAGAACGCACCGCTCCCCCCGACGCCCTCACCGTCC
-----+---------+---------+---------+---------+
TACTGCTGGCGTCTTGCGTGGCGAGGGGGGCTGCGGGAGTGGCAGG
MetThrThrAlaGluArgThrAlaProProAspAlaLeuThrValP
```

```
        250                 270                 290
    CGGCCAGCCGCGCCCCCGGCTGCCCCTTCGACCCCGCGCCCGACGTCACCGAGGCGGCCC
5   ---------+---------+---------+---------+---------+---------+
    GCCGGTCGGCGCGGGGGCCGACGGGGAAGCTGGGGCGCGGGCTGCAGTGGCTCCGCCGGG
    roAlaSerArgAlaProGlyCysProPheAspProAlaProAspValThrGluAlaAlaA

        310                 330                 350
10  GCACCGAACCGGTCACCCGGGCCACCCTCTGGGACGGCTCCTCCTGCTGGCTGGTGACGC
    ---------+---------+---------+---------+---------+---------+
    CGTGGCTTGGCCAGTGGGCCCGGTGGGAGACCCTGCCGAGGAGGACGACCGACCACTGCG
    rgThrGluProValThrArgAlaThrLeuTrpAspGlySerSerCysTrpLeuValThrA

15      370                 390                 410
    GCCATCAGGACGTCCGCGCGGTCCTCGGCGACCCGCGCTTCAGCGCCGACGCCCACCGCA
    ---------+---------+---------+---------+---------+---------+
    CGGTAGTCCTGCAGGCGCGCCAGGAGCCGCTGGGCGCGAAGTCGCGGCTGCGGGTGGCGT
    rgHisGlnAspValArgAlaValLeuGlyAspProArgPheSerAlaAspAlaHisArgT
20
        430                 450                 470
    CCGGCTTCCCCTTCCTGACCGCCGGCGGCCGCGAGATCATCGGCACCAACCCGACCTTCC
    ---------+---------+---------+---------+---------+---------+
    GGCCGAAGGGGAAGGACTGGCGGCCGCCGGCGCTCTAGTAGCCGTGGTTGGGCTGGAAGG
25  hrGlyPheProPheLeuThrAlaGlyGlyArgGluIleIleGlyThrAsnProThrPheL

        490                 510                 530
    TGCGCATGGACGACCCGGAGCACGCCCGACTGCGCCGGATGCTCACCGCCGACTTCATCG
    ---------+---------+---------+---------+---------+---------+
30  ACGCGTACCTGCTGGGCCTCGTGCGGGCTGACGCGGCCTACGAGTGGCGGCTGAAGTAGC
    euArgMetAspAspProGluHisAlaArgLeuArgArgMetLeuThrAlaAspPheIleV

        550                 570                 590
    TCAAGAAGGTCGAGGCGATGCGCCCCGAGGTGCAGCGCCTCGCCGACGACCTGGTCGACC
35  ---------+---------+---------+---------+---------+---------+
    AGTTCTTCCAGCTCCGCTACGCGGGGCTCCACGTCGCGGAGCGGCTGCTGGACCAGCTGG
    alLysLysValGluAlaMetArgProGluValGlnArgLeuAlaAspAspLeuValAspA
```

```
          610                 630                 650
GGATGACCACCGGACGCACCTCCGCCGACCTGGTCACCGAGTTCGCGCTGCCGCTGCCGT
---------+---------+---------+---------+---------+---------+
CCTACTGGTGGCCTGCGTGGAGGCGGCTGGACCAGTGGCTCAAGCGCGACGGCGACGGCA
rgMetThrThrGlyArgThrSerAlaAspLeuValThrGluPheAlaLeuProLeuProS

          670                 690                 710
CCCTGGTGATCTGCCTGCTGCTCGGCGTCCCCTACGAGGACCACGCGTTCTTCCAGGAGC
---------+---------+---------+---------+---------+---------+
GGGACCACTAGACGGACGACGAGCCGCAGGGGATGCTCCTGGTGCGCAAGAAGGTCCTCG
erLeuValIleCysLeuLeuLeuGlyValProTyrGluAspHisAlaPhePheGlnGluA

          730                 750                 770
GCAGCCGGGTCCTGCTCACCCTGCGGTCCACTCCCGAGGAAGTCCGGGCCGCCCAGGACG
---------+---------+---------+---------+---------+---------+
CGTCGGCCCAGGACGAGTGGGACGCCAGGTGAGGGCTCCTTCAGGCCCGGCGGGTCCTGC
rgSerArgValLeuLeuThrLeuArgSerThrProGluGluValArgAlaAlaGlnAspG

          790                 810                 830
AGTTGCTGGAGTACCTCGCCCGGCTCGCCCGGACCAAGCGGGAGCGGCCGGACGACGCCA
---------+---------+---------+---------+---------+---------+
TCAACGACCTCATGGAGCGGGCCGAGCGGGCCTGGTTCGCCCTCGCCGGCCTGCTGCGGT
luLeuLeuGluTyrLeuAlaArgLeuAlaArgThrLysArgGluArgProAspAspAlaI

          850                 870                 890
TCATCAGCCGCCTGGTCGCCCGCGGCGAGCTCGACGACACCCAGATCGCCACCATGGGAC
---------+---------+---------+---------+---------+---------+
AGTAGTCGGCGGACCAGCGGGCGCCGCTCGAGCTGCTGTGGGTCTAGCGGTGGTACCCTG
leIleSerArgLeuValAlaArgGlyGluLeuAspAspThrGlnIleAlaThrMetGlyA

          910                 930                 950
GCCTGTTGCTGGTCGCCGGCCACGAGACGACCGCCAACATGACCGCGCTCTCCACCCTCG
---------+---------+---------+---------+---------+---------+
CGGACAACGACCAGCGGCCGGTGCTCTGCTGGCGGTTGTACTGGCGCGAGAGGTGGGAGC
rgLeuLeuLeuValAlaGlyHisGluThrThrAlaAsnMetThrAlaLeuSerThrLeuV
```

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          970                 990                1010
      TGCTGCTGCGCAACCCCGACCAACTCGCCCGGCTGCGCGCCGAACCCGCGCTCGTCAAGG
      ---------+---------+---------+---------+---------+---------+
      ACGACGACGCGTTGGGGCTGGTTGAGCGGGCCGACGCGCGGCTTGGGCGCGAGCAGTTCC
      alLeuLeuArgAsnProAspGlnLeuAlaArgLeuArgAlaGluProAlaLeuValLysG

          1030                1050               1070
      GCGCCGTCGAGGAGCTGCTGCGCTACCTGACGATCGTGCACAACGGCGTTCCCCGGATCG
      ---------+---------+---------+---------+---------+---------+
      CGCGGCAGCTCCTCGACGACGCGATGGACTGCTAGCACGTGTTGCCGCAAGGGGCCTAGC
      lyAlaValGluGluLeuLeuArgTyrLeuThrIleValHisAsnGlyValProArgIleA

          1090                1110               1130
      CCACCGAGGACGTGCTCATCGGCGGCCGCACCATCGCCGCCGGCGAGGGCGTCCTGTGCA
      ---------+---------+---------+---------+---------+---------+
      GGTGGCTCCTGCACGAGTAGCCGCCGGCGTGGTAGCGGCGGCCGCTCCCGCAGGACACGT
      laThrGluAspValLeuIleGlyGlyArgThrIleAlaAlaGlyGluGlyValLeuCysM

          1150                1170               1190
      TGATCAGCTCCGCCAACCGGGACGCCGAGGTGTTCCCCGGCGGCGACGACCTCGACGTGG
      ---------+---------+---------+---------+---------+---------+
      ACTAGTCGAGGCGGTTGGCCCTGCGGCTCCACAAGGGGCCGCCGCTGCTGGAGCTGCACC
      etIleSerSerAlaAsnArgAspAlaGluValPheProGlyGlyAspAspLeuAspValA

          1210                1230               1250
      CCCGCGACGCCCGCCGCCACGTGGCCTTCGGCTTCGGCGTCCACCAGTGCCTGGGACAGC
      ---------+---------+---------+---------+---------+---------+
      GGGCGCTGCGGGCGGCGGTGCACCGGAAGCCGAAGCCGCAGGTGGTCACGGACCCTGTCG
      laArgAspAlaArgArgHisValAlaPheGlyPheGlyValHisGlnCysLeuGlyGlnP

          1270                1290               1310
      CGTTGGCCAGGGTGGAGCTCCAGATCGCCATCGAAACGCTGCTGCGCCGCCTGCCGGACC
      ---------+---------+---------+---------+---------+---------+
      GCAACCGGTCCCACCTCGAGGTCTAGCGGTAGCTTTGCGACGACGCGGCGGACGGCCTGG
      roLeuAlaArgValGluLeuGlnIleAlaIleGluThrLeuLeuArgArgLeuProAspL
```

```
      1330                1350                1370
TGCGGCTGGCCGTGCCCCACGAGGAGATCCCGTTCCGCGGCGACATGGCGATCTACGGGG
---------+---------+---------+---------+---------+---------+
ACGCCGACCGGCACGGGGTGCTCCTCTAGGGCAAGGCGCCGCTGTACCGCTAGATGCCCC
euArgLeuAlaValProHisGluGluIleProPheArgGlyAspMetAlaIleTyrGlyV

      1390
TCCACTCGCTGCCGATCGCCTGGTAG
---------+---------+------
AGGTGAGCGACGGCTAGCGGACCATC
alHisSerLeuProIleAlaTrpEnd
```

and any mutant or derivative of said sequence which causes metabolism of herbicide compounds when transformed into bacteria or plants.

17. A recombinant plasmid comprising a DNA fragment of Claim 3, 5, 6 or 8 and a DNA sequence for a selected promoter and transcription signal from the genus Streptomyces or S. griseolus operably linked to said fragment which transforms bacteria of the genus Streptomyces to constitutively express cytochrome P450 enzyme P450SU1.

18. A recombinant plasmid comprising A) one or more segments of the DNA fragment of Claim 3, 5, 6, 7, 8, 9, 10 or 11, B) one or more segments of the DNA sequence of a plant promoter upstream and operably linked to said fragment, C) one or more of a 5'-untranslated sequence including a ribosomal binding site upstream and operably linked to said fragment, and D) one or more of a DNA sequence downstream and operably linked to said fragments of a 3'-untranslated sequence which enables the mRNA transcribed from the plasmids to be polyadenylated on its 3' end.

19. A plasmid of Claim 18 wherein the promoter and 5'-untranslated sequences are those of the 35S promoter from the Cauliflower Mosaic Virus, the promoter from the SSU301 gene from petunia, or the promoter from the Cab22L gene from petunia and the 3'-untranslated sequences are those of the SSU301 gene from petunia, or of the gene for nopaline synthetase derived from T-DNA of Agrobacterium tumefaciens.

20. A plasmid of Claim 18 wherein the promoter is a plant tissue specific promoter.

21. A recombinant plasmid comprising A) one or more segments of the DNA fragment of Claim 3, 5, 6, 7, 8, 9, 10 or 11; B) one or more segments of the DNA sequence of a plant promoter operably linked to said fragment in the upstream position; C) one or more of a 5'-untranslated sequence including a ribosomal binding site operably linked to said fragment in the upstream position; D) one or more of a DNA sequence operably linked to said fragment in the downstream position of a 3'-untranslated sequence which enables the mRNA transcribed from the plasmids to be polyadenylated on its 3' end; and E) one or more of a transit peptide coding sequence or a transit peptide encoding sequence and additionally mature coding sequence of nuclear genes that encode proteins that are normally imported into the chloroplasts of plants operably linked to the DNA encoding for the amino terminus of the cytochrome P450, or to the DNA encoding for the amino terminae of the cytochrome P450 and the FeS protein, and downstream from the promoter and ribosomal binding site.

22. A plasmid of Claim 21 wherein the promoter is a plant tissue specific promoter.

23. A plasmid of Claim 21 wherein the promoter and 5'-untranslated sequences are of the 35S promoter from Cauliflower Mosaic Virus, the promoter from the SSU301 gene from petunia, or the promoter from the Cab22L gene from petunia; the 3'-untranslated sequences are those of the SSU301 gene from petunia or the gene for nopaline synthetase derived from T-DNA of Agrobacterium tumefaciens; and the transit peptide coding sequence is that from ribulose bisphosphate carboxylase gene of petunia or that from the chlorophyll a/b binding protein gene of petunia.

24. A plasmid pSU18 comprising one of the following combinations: 1) the plasmid pSU17 and plasmid pAGS135, capable of transforming plant cells to express cytochrome P450 enzyme P450SU1 in the cytoplasm, 2) the plasmid pSSU-SU11 and plasmid pAGS135, capable of transforming plant cells to express cytochrome P450 enzyme P450SU1 targeted to the chloroplast; 3) the plasmid pSSU-SU12 and plasmid pAGS135, capable of transforming plant cells to express cytochrome P450 enzyme P450SU1 targeted to the chloroplast; 4) the plasmid pTab-SU11 and plasmid pAGS135, capable of transforming plant cells to express cytochrome P450 enzyme P450SU1 targeted to the chloroplast; 5) the plasmid of pTab-SU12 and plasmid pAGS135, capable of transforming plant cells to express cytochrome P450 enzyme P450SU1 targeted to the chloroplast; or 6) the plasmid pTab-SU13 and plasmid pAGS135, capable of transforming plant cells

to express cytochrome P450 enzyme P450SU1 targeted to the chloroplast.

25. A plasmid pSuFe11 comprising the combination of the plasmid pSUFe1 and plasmid pAGS502, capable of transforming plant cells to express cytochrome P450 enzyme P450SU1 and FeS-B proteins in the cytoplasm.

26. A plasmid pSuFe21 comprising the combination of the plasmid pSUFe2 and plasmid pAGS501, capable of transforming plant cells to express cytochrome P450 enzyme P450SU1 and FeS-B proteins targeted to the chloroplast.

27. A plasmid comprising one of the following combinations 1) the plasmid pSUFe3 and plasmid pZS96, capable of transforming plant cells to express cytochrome P450 enzyme P450SU1 and FeS-B proteins targeted to the chloroplast, or 2) the plasmid pSUFe4 and plasmid pZS96, capable of transforming plant cells to express cytochrome P450 enzyme P450SU1 and FeS-B proteins targeted to the chloroplast.

28. A transformed bacterium of the genus Streptomyces containing the fragment of Claim 3, 5, 6, 7, 8, 9, 10 or 11.

29. A transformed bacterium of the genus Streptomyces containing a plasmid of Claim 17.

30. A seed coated with transformed bacteria containing the DNA sequence of Claim 3, 5, 6, 7, 8, 9, 10 or 11.

31. A seed coated with transformed bacteria containing a plasmid of Claim 17.

32. A transformed plant containing the DNA sequence of Claim 3, 5, 7, 8, 9, 10, 11, 14 or 16.

33. A transformed plant containing a plasmid of Claim 18, 20, 21 or 22.

34. Seed and progeny from a plant of Claim 32 or 33.

35. A method of preparation of metabolites of a herbicide compound comprising incubating said herbicide compound with the transformed bacteria of Claim 28 or 29.

36. A method of protecting plants in soil containing herbicide compounds comprising soaking a plant seedling in the transformed bacteria of Claim 28 or 29 prior to planting in said soil.

37. A method of transforming a plant to metabolize herbicide compounds comprising introducing into the plant a plasmid of Claim 18 or 21.

38. A method of introducing the cytochrome P450 with or without FeS protein into plants comprising

A) obtaining a bacterial cytochrome P450 gene;

B) forming a plasmid comprising elements (a) one or more segments of the DNA fragments of a cytochrome P450 enzyme; (b) one or

more of segments of a DNA sequence of a plant promoter operably linked to said fragment in the upstream position; (c) one or more of a 5'-untranslated sequence including a ribosomal binding site operably linked to said fragment in the upstream position; (d) one or more of a DNA sequence operably linked to said fragments in the downstream position of a 3'-untranslated sequence which enables the mRNA transcribed from the plasmids to be polyadenylated on its 3' end; and

C) introducing said plasmid into plants.

39. A method of introducing cytochrome P450 with or without FeS protein into plants comprising

A) obtaining a bacterial cytochrome P450 gene;

B) forming a plasmid comprising elements (a) one or more segments of a DNA fragment of a cytochrome P450 enzyme; (b) one or more segments of a DNA sequence of a plant promoter operably linked to said fragment in the upstream position; (c) one or more of a 5'-untranslated sequence including a ribosomal binding site operably linked to said fragment in the upstream position; (d) one or more of a DNA sequence operably linked to said fragments in the downstream position of a 3'-untranslated sequence which enables the mRNA transcribed from the plasmids to be polyadenylated on its 3' end; (e) one or more of a transit peptide coding sequence

or a transit peptide encoding sequence and additionally mature coding sequence of nuclear genes encoding proteins that are normally imported into the chloroplasts of plants operably linked to the DNA encoding for the amino terminus of the cytochrome P450 enzyme, or to the DNA encoding for the amino terminae of the cytochrome P450 and the FeS protein, and downstream from the promoter and ribosomal binding site; and

C) introducing said plasmid into plants.

40. The method of Claim 39 wherein the transit encoding sequence is from the ribulose bisphosphate carboxylase of petunia or from the chlorophyll a/b binding protein of petunia.

41. A method for the selective destruction of plant tissues comprising contacting a transformed plant containing a plasmid of Claim 18 or 21 wherein the promoter is a 5' upstream tissue specific promoter with a sulfonylurea compound; said compound then being metabolized to a distinct phytotoxic compound by the enzyme P450SU1, said phytotoxic compound then destroying the tissue containing the enzyme P450SU1.

42. The method of Claim 41 wherein the tissue is plant anther cells.

43. A method for producing plants having nongerminable pollen comprising contacting a transformed plant containing a plasmid of Claim 21

wherein the promoter is a 5' upstream anther tissue specific promoter with a sulfonylurea compound.

44. A transformed plant having nongerminable pollen prepared according to the method of Claim 41 or 43.

45. A method for producing male sterility in plants comprising contacting a sulfonylurea compound with a transformed plant containing a plasmid of Claim 21 wherein the promoter is a 5' upstream anther tissue specific promoter.

46. A transformed plant which is male sterile prepared according to the method of Claim 45.

47. A method to reduce herbicide residues in plants, progeny, and seeds comprising transforming the parent plants with the plasmid of Claim 18 or 21 to produce progeny plants expressing P450SU1 enzyme which metabolizes said herbicide compounds.

48. A transformed plant containing one or more of the DNA sequences of Claim 7, 10, 14 or 16 which is resistant to herbicides.

49. From a group of plants or cells thereof transformed with a plasmid of Claim 18 or 21, a method to select plants or cells thereof that do not express or contain a complete cytochrome P450 coding sequence comprising contacting said transformed plants or cells thereof with a selected sulfonylurea compound.

50. A method to kill, or arrest the growth of, plants or cells thereof which contain a plasmid of Claim 18 or 21 and express an introduced cytochrome P450 coding sequence, without harm to plants or cells thereof that do not express or contain an introduced cytochrome P450 coding sequence, comprising contacting said plants or cells with a selected sulfonylurea compound.

FIG. 1

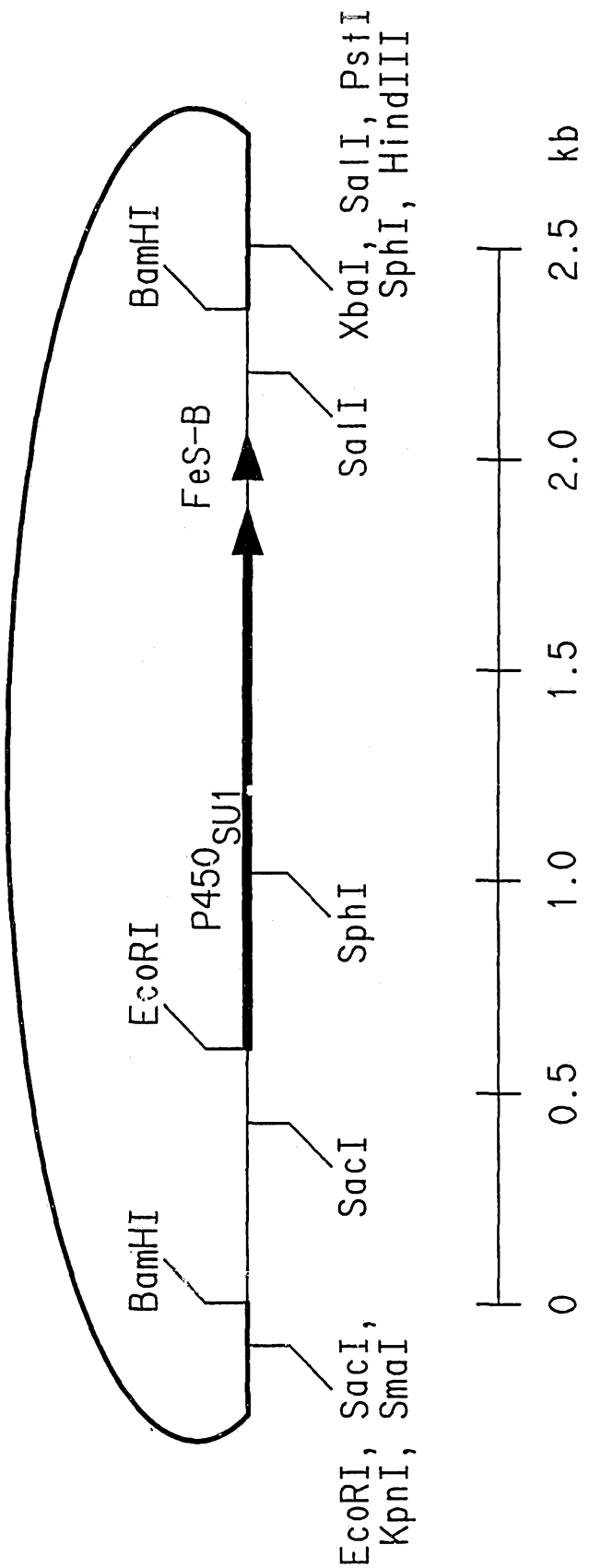

BamHI
XbaI, SalI, PstI
SphI, HindIII
SalI
FeS-B
P450SU1
EcoRI
SphI
SacI
BamHI
EcoRI, SacI,
KpnI, SmaI
0
0.5
1.0
1.5
2.0
2.5
kb

FIG.2A

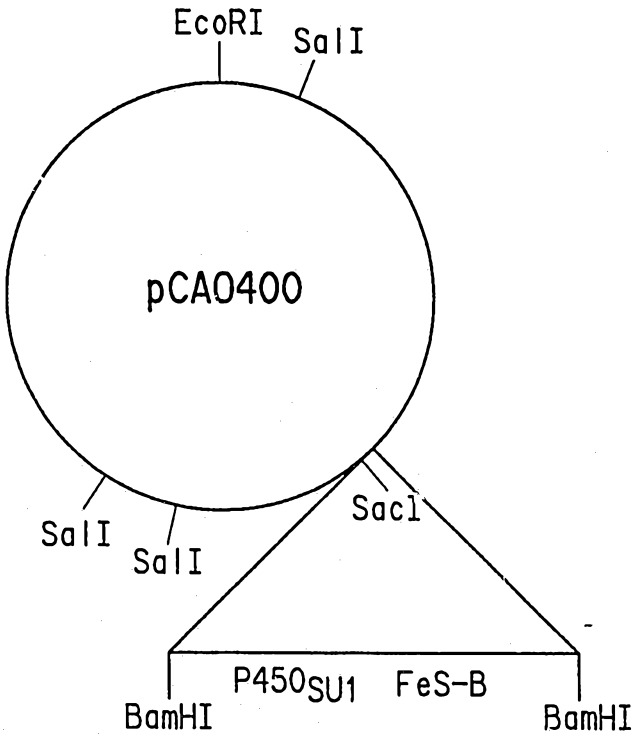

EcoRI
SalI
pCA0400
SalI
SalI
SacI
P450SU1
FeS-B
BamHI
BamHI

FIG.2B

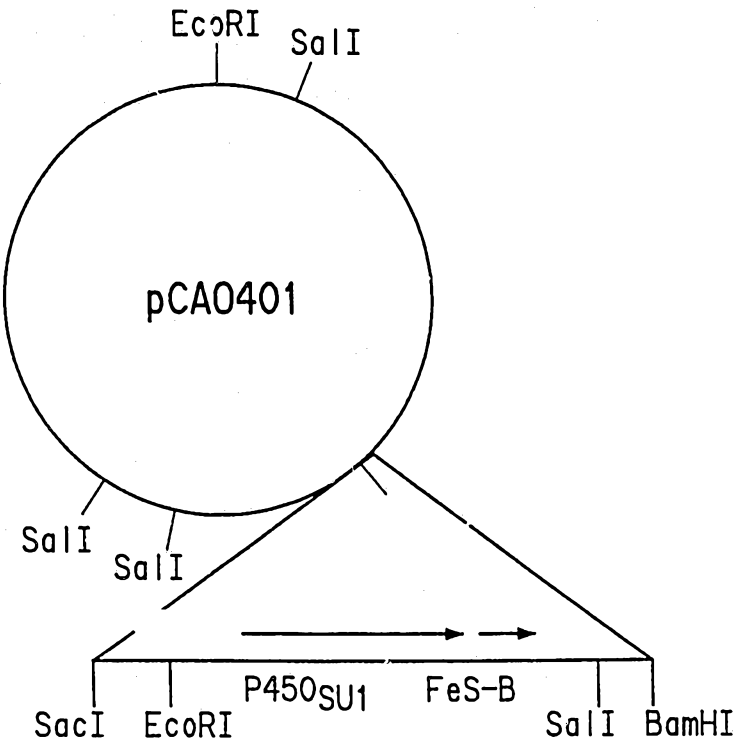

EcoRI
SalI
pCA0401
SalI
SalI
P450SU1
FeS-B
SacI
EcoRI
SalI
BamHI

FIG.3A

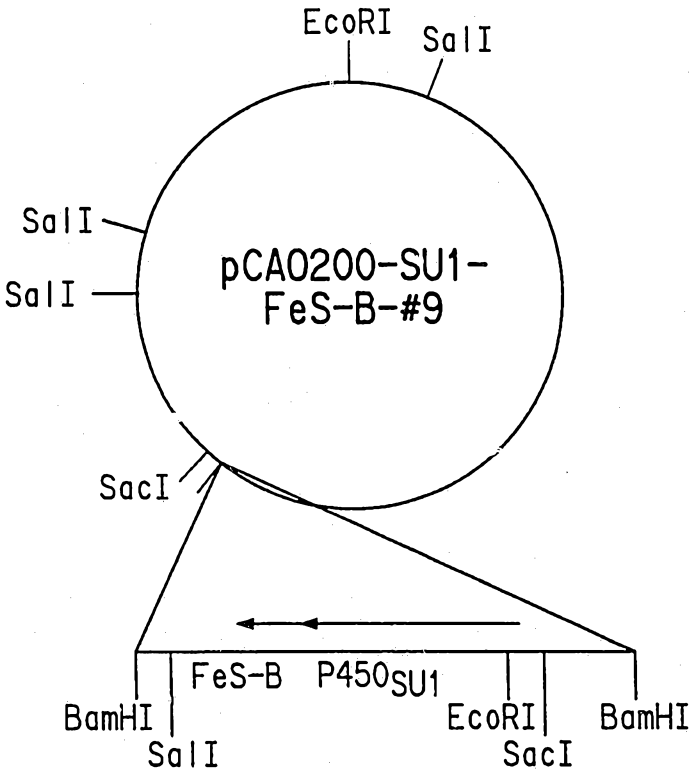

EcoRI
SalI
SalI
SalI
pCA0200-SU1-
FeS-B-#9
SacI
FeS-B
P450SU1
BamHI
SalI
EcoRI
SacI
BamHI

FIG.3B

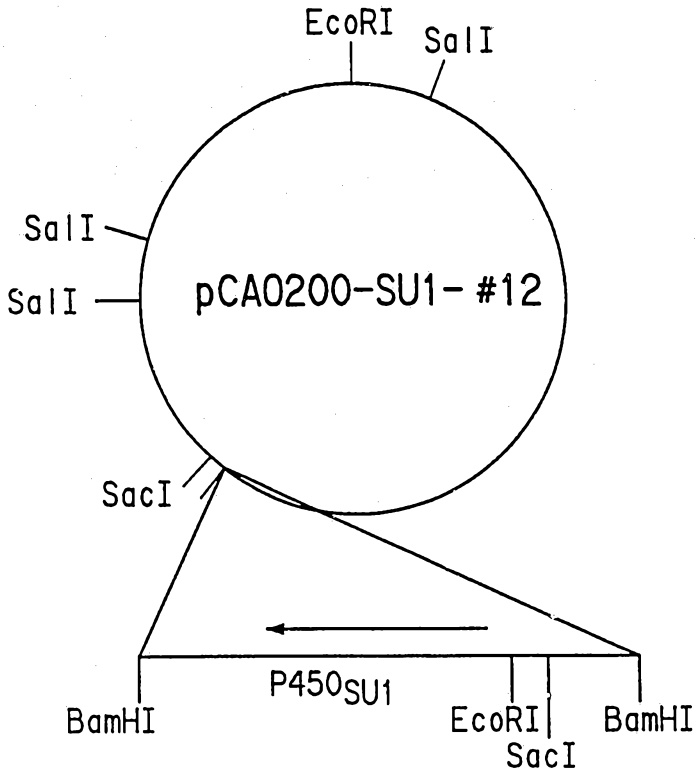

EcoRI
SalI
SalI
SalI
pCA0200-SU1- #12
SacI
P450SU1
BamHI
EcoRI
SacI
BamHI

FIG. 4

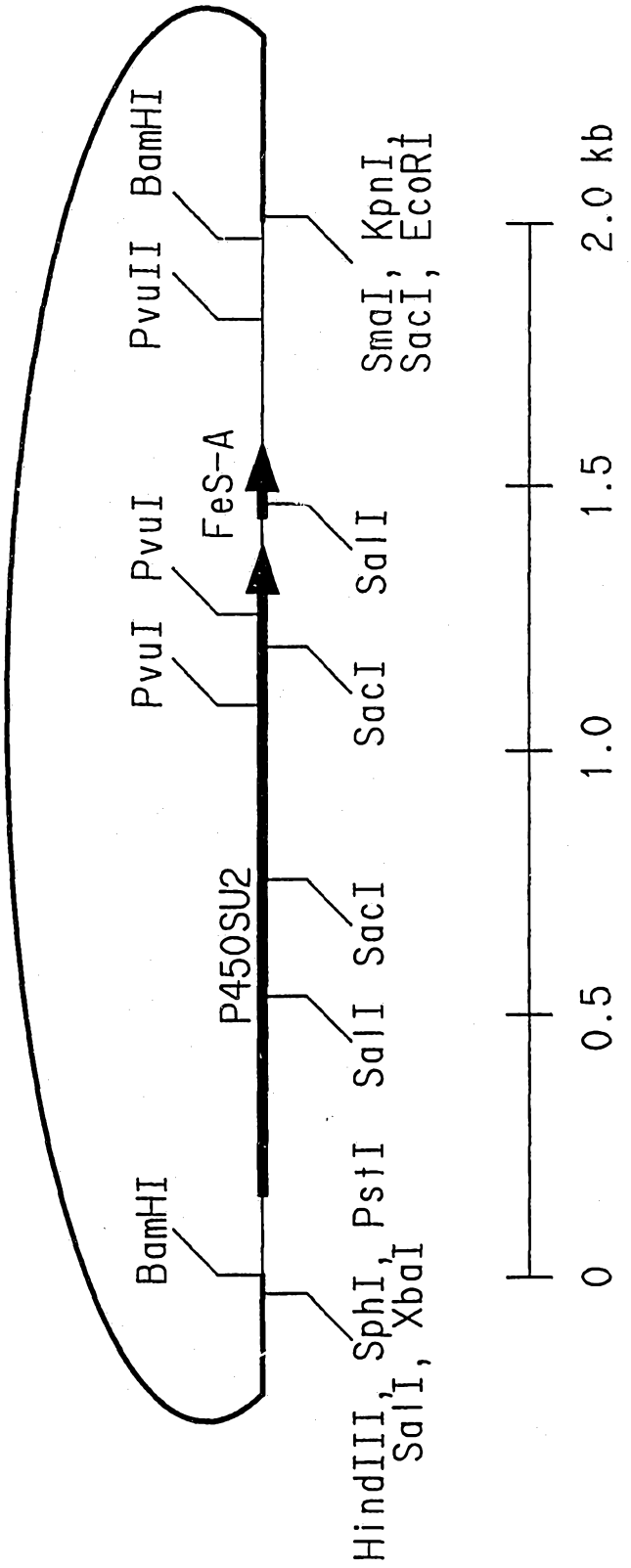

HindIII, SphI, PstI
SalI, XbaI
BamHI
P450SU2
SalI
SacI
PvuI
PvuI
SacI
SalI
FeS-A
PvuII
BamHI
SmaI, KpnI,
SacI, EcoRI
0
0.5
1.0
1.5
2.0 kb

FIG.5

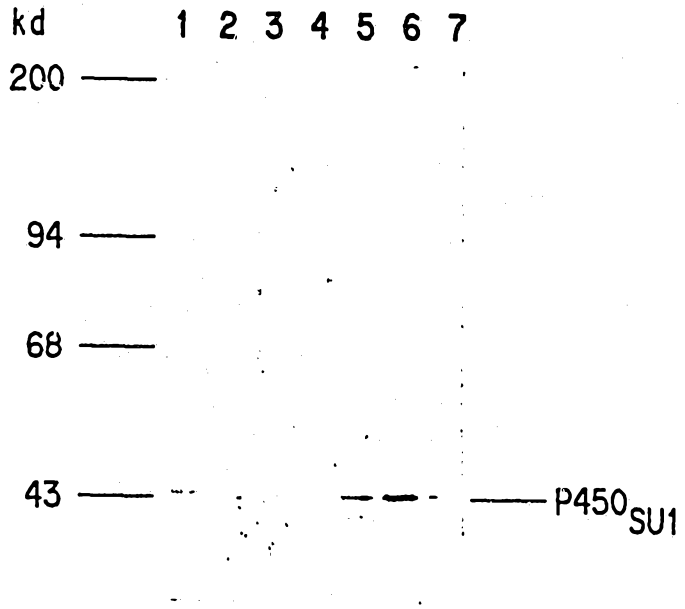


FIG.6

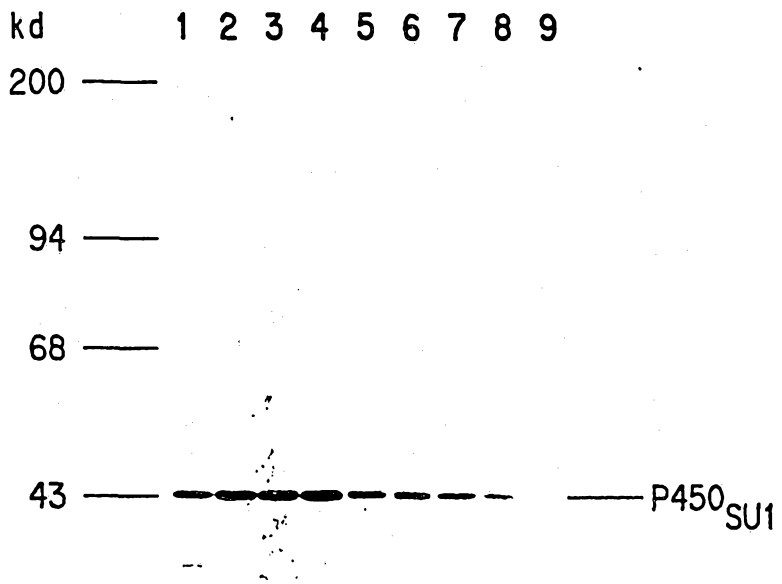


FIG.7

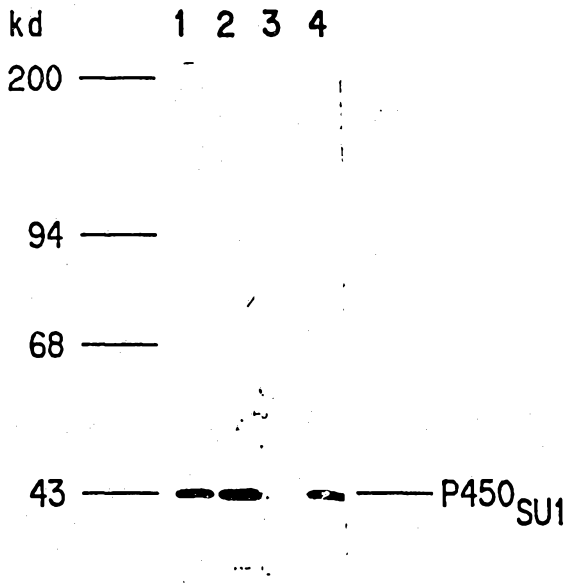


FIG.8

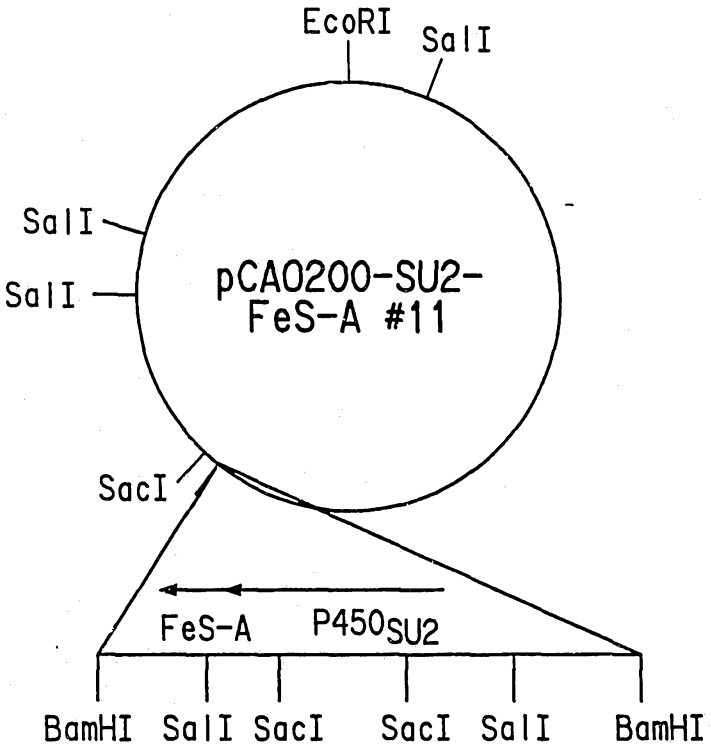

EcoRI
SalI
SalI
SalI
pCA0200-SU2-
FeS-A #11
SacI
FeS-A
P450SU2
BamHI
SalI
SacI
SacI
SalI
BamHI

FIG.9

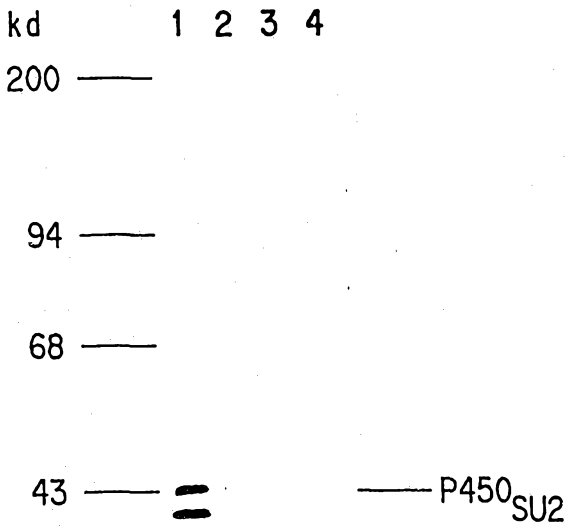


FIG. 10A

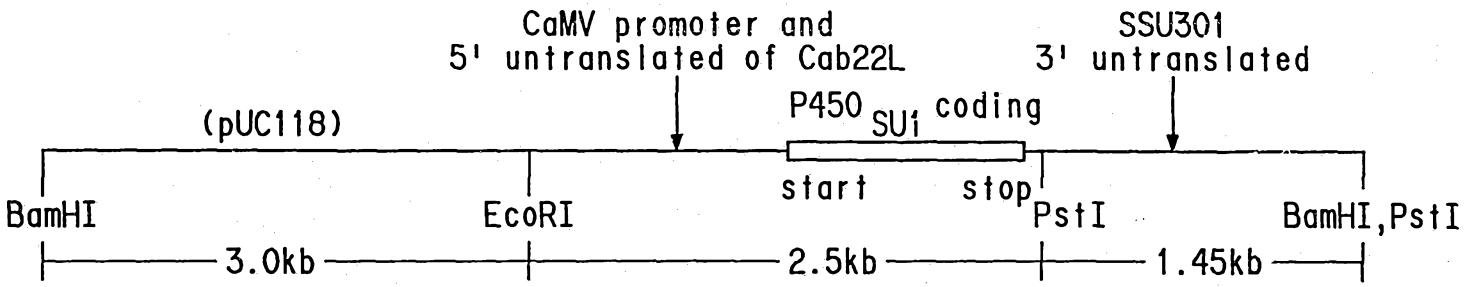



FIG. 10B

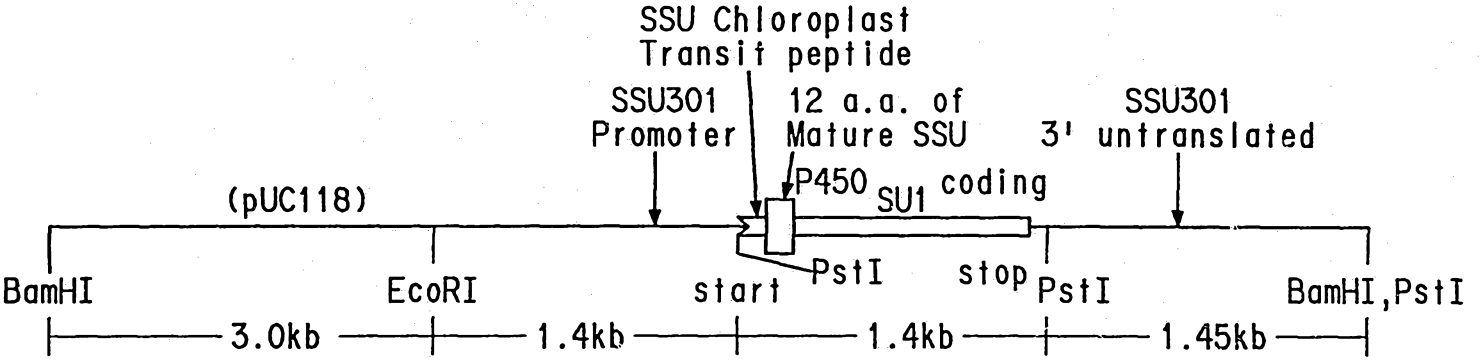



FIG. 10C

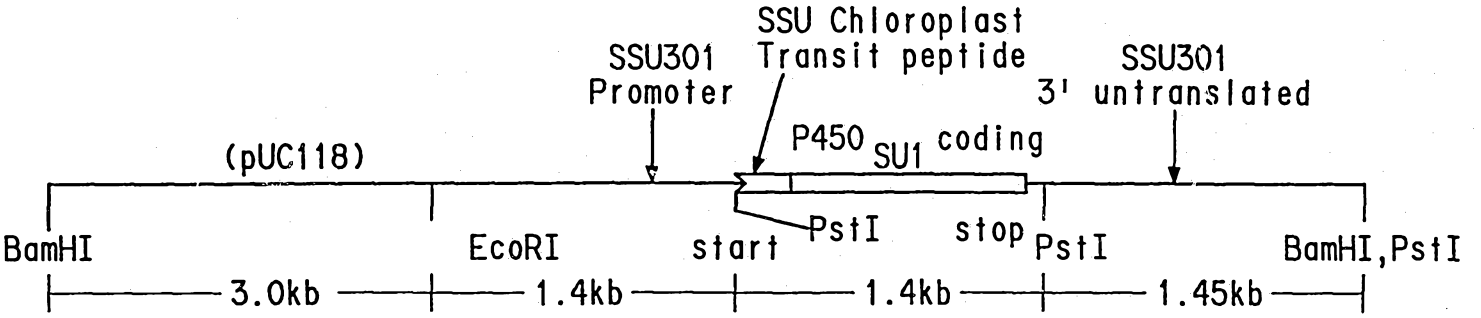


FIG.10D

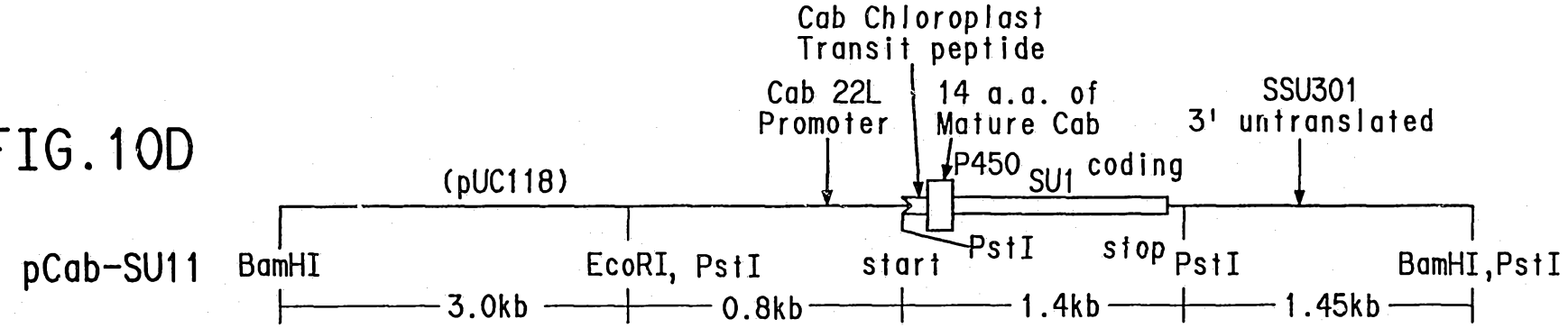



FIG.10E

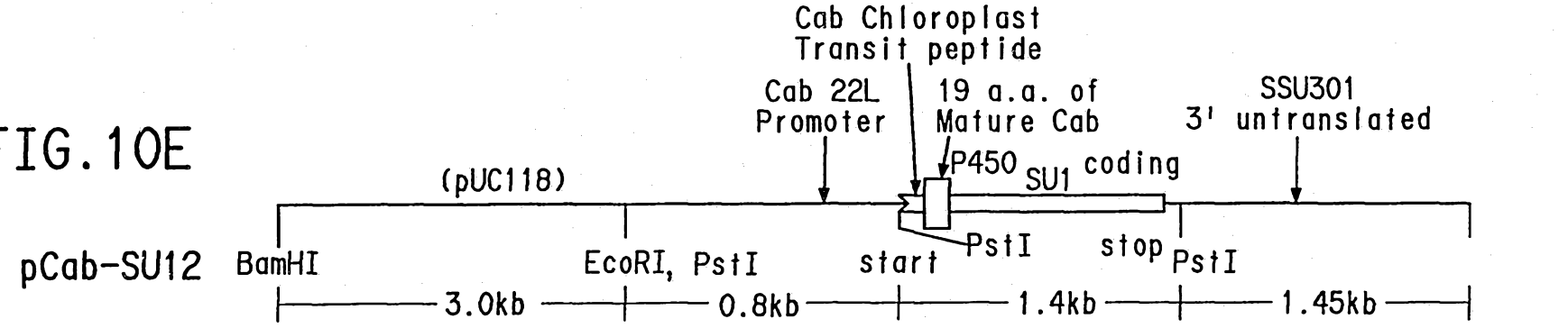



FIG.10F

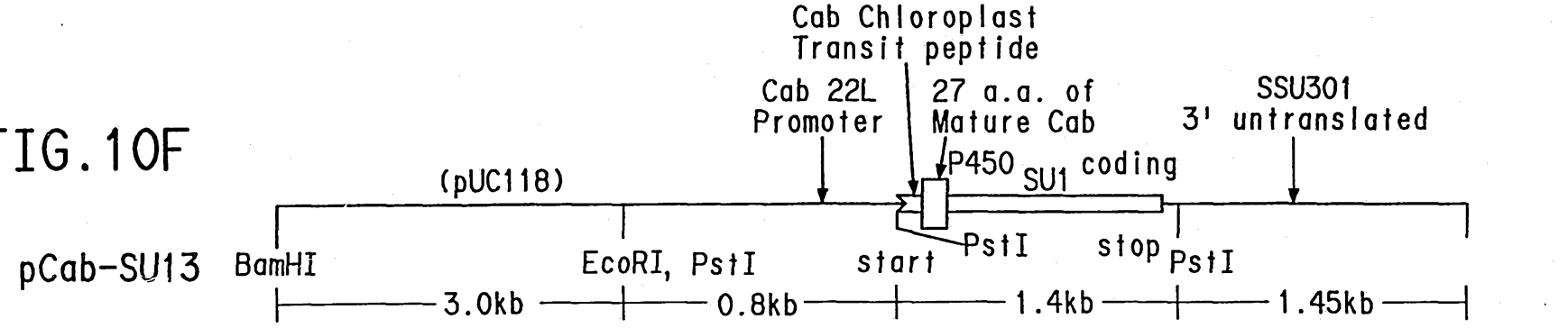


FIG.11

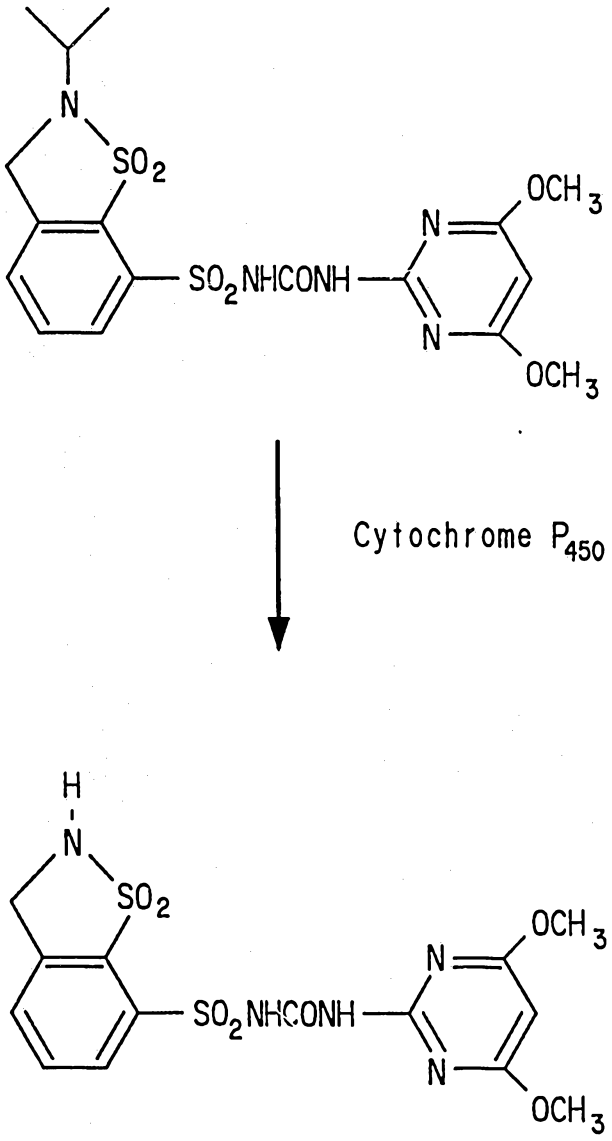

N
SO2
SO2NHCONH
N
N
OCH3
OCH3
Cytochrome P450
H
N
SO2
SO2NHCONH
N
N
OCH3
OCH3

FIG. 12A

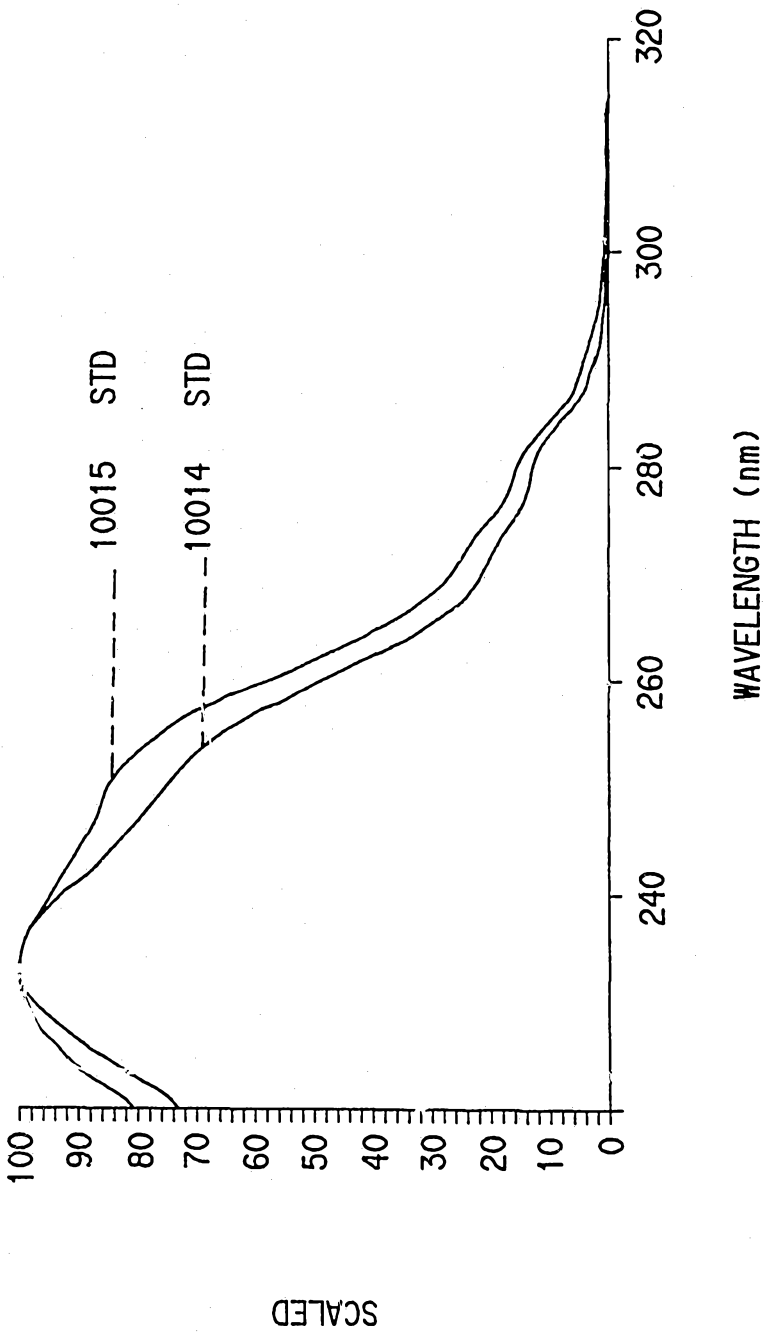

STD
10015
STD
10014
320
300
280
260
240
WAVELENGTH (nm)
100
90
80
70
60
50
40
30
20
10
0
SCALED

FIG. 12B

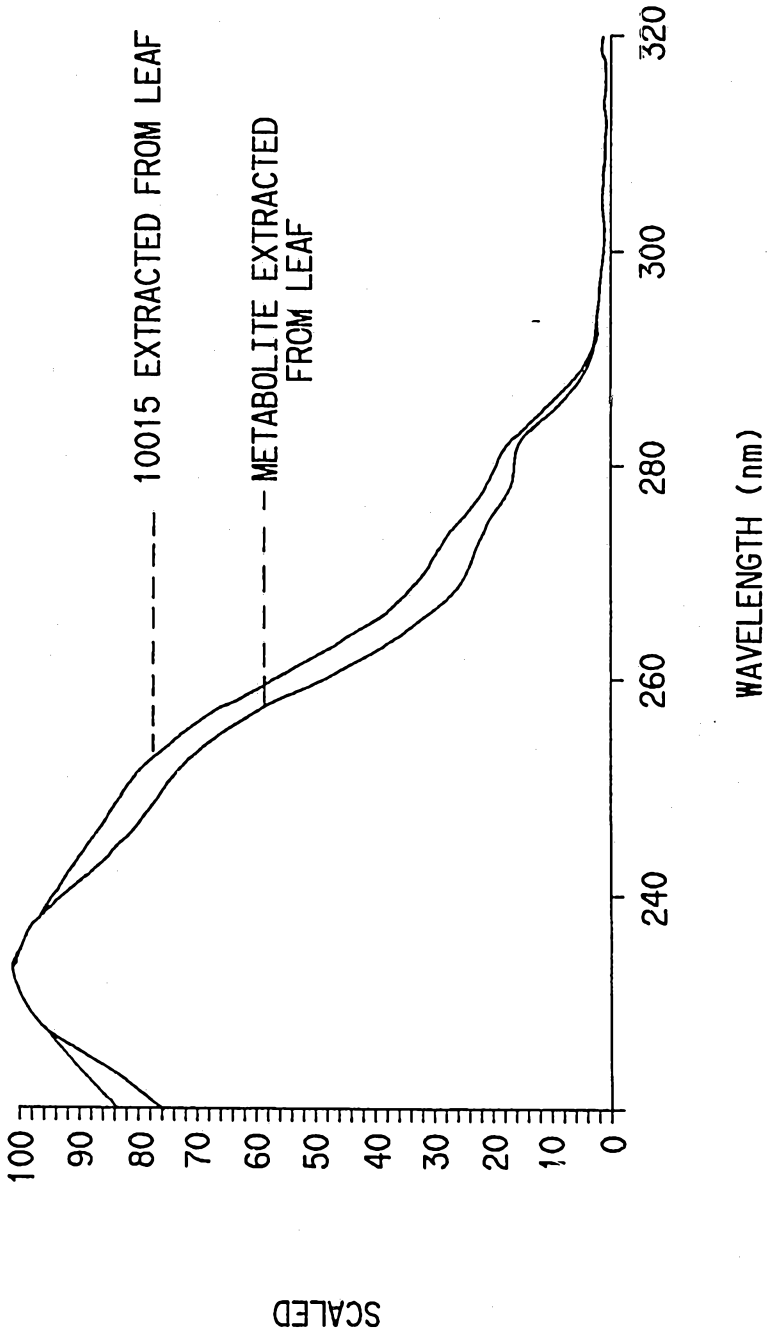

10015 EXTRACTED FROM LEAF
METABOLITE EXTRACTED FROM LEAF
100
90
80
70
60
50
40
30
20
10
0
SCALED
240
260
280
300
320
WAVELENGTH (nm)

FIG.13B

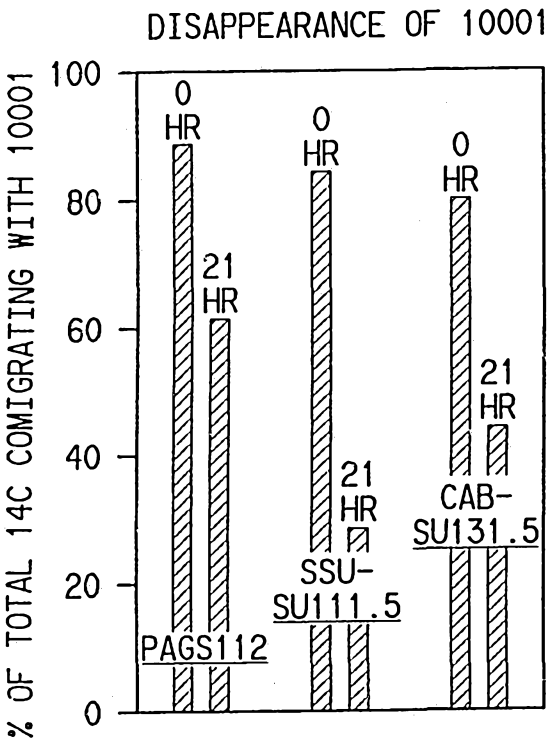



FIG.13C

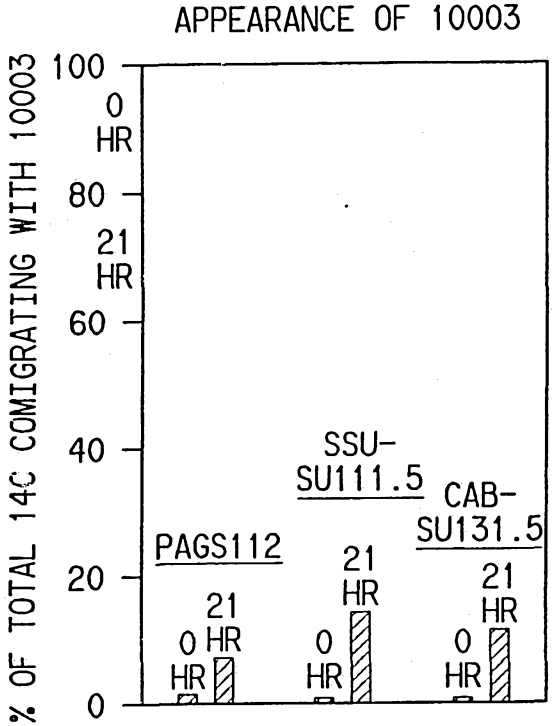



FIG.13D

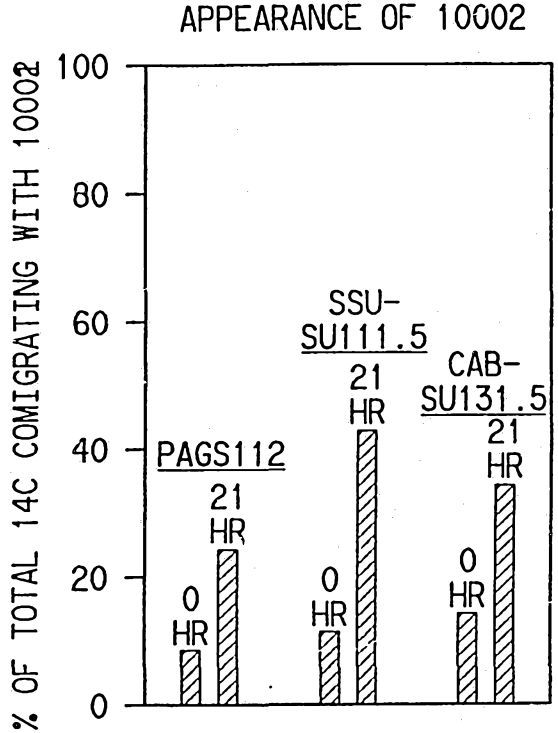



FIG.13A

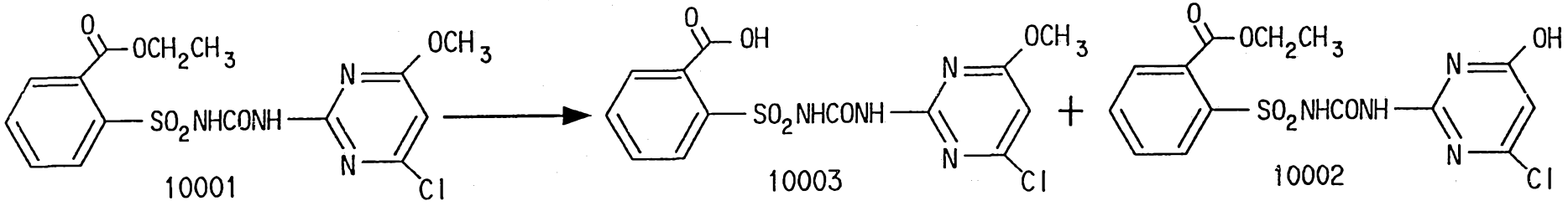


FIG. 14

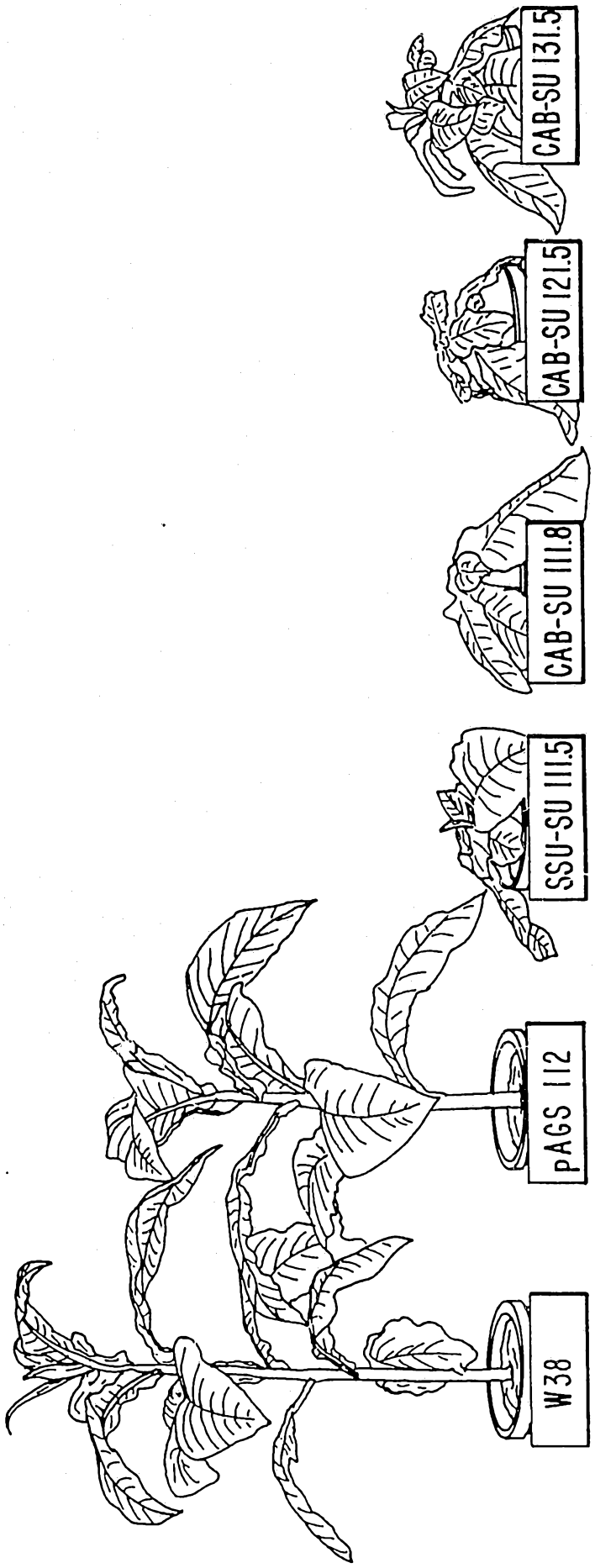


FIG.15A

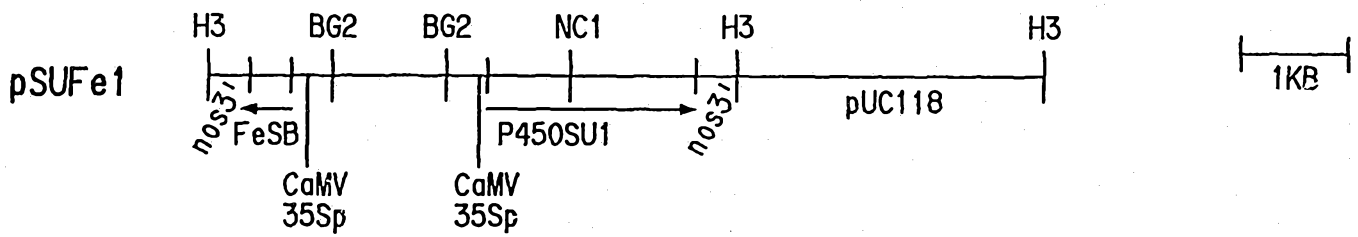



FIG.15B

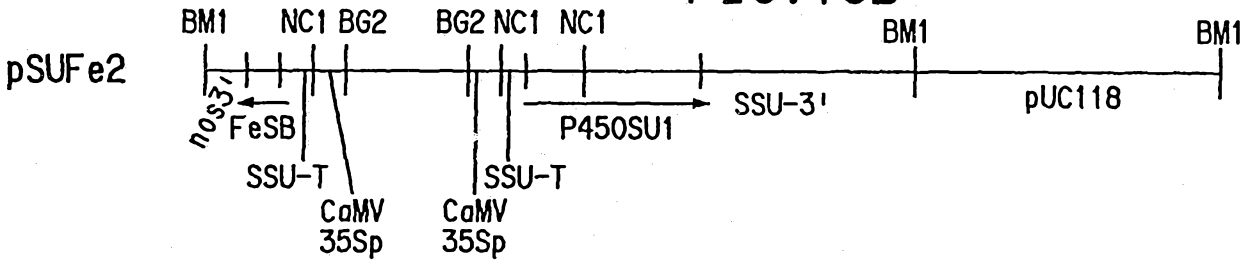



FIG.15C

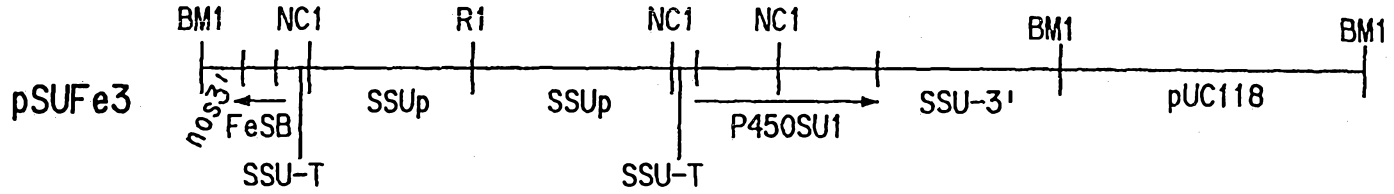



FIG.15D

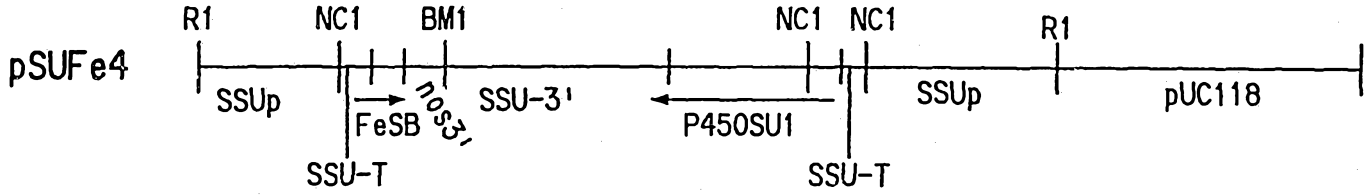


FIG.16A

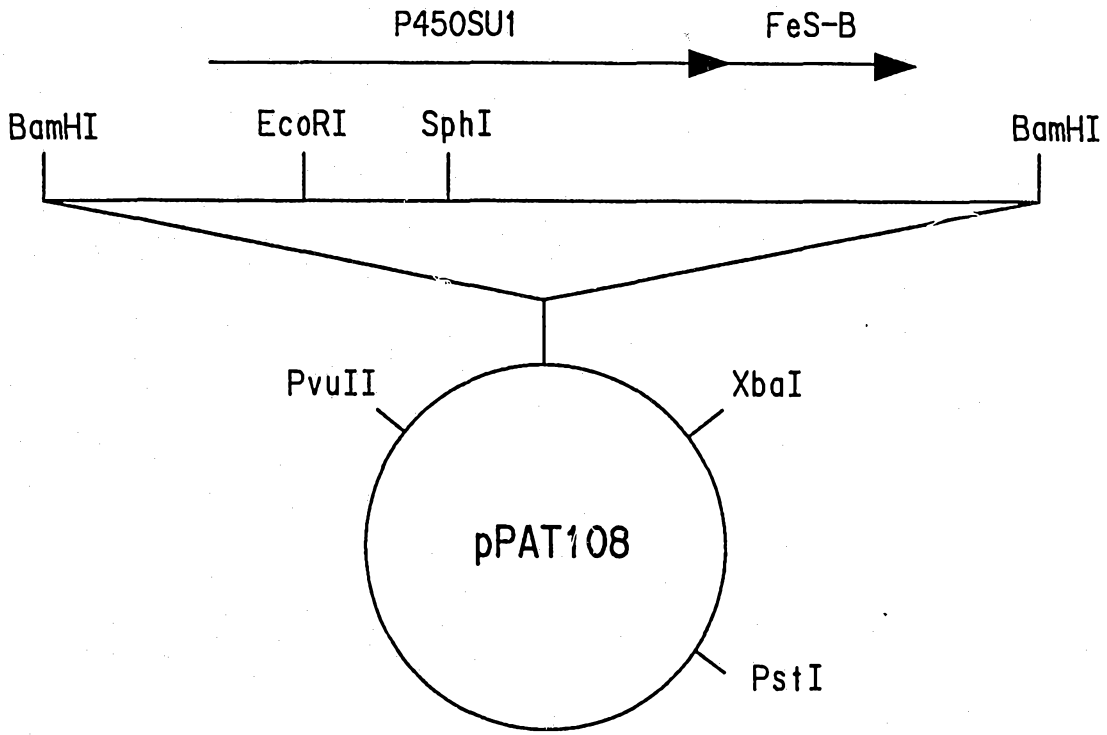



FIG.16B

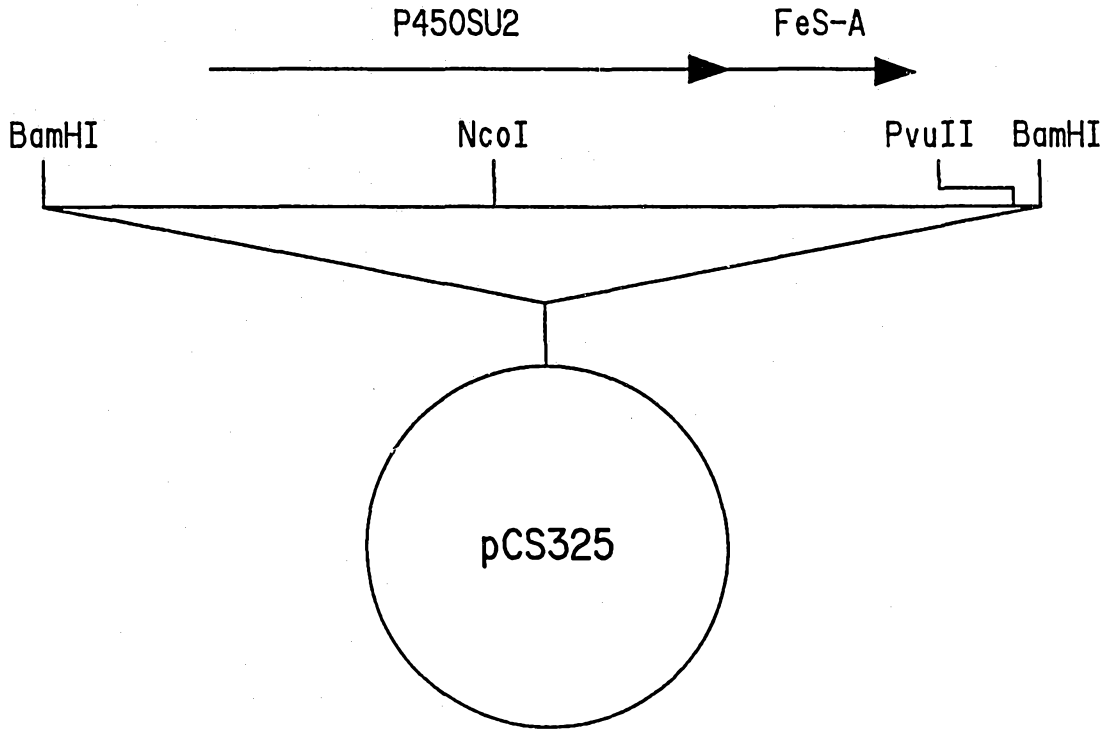


FIG. 17A

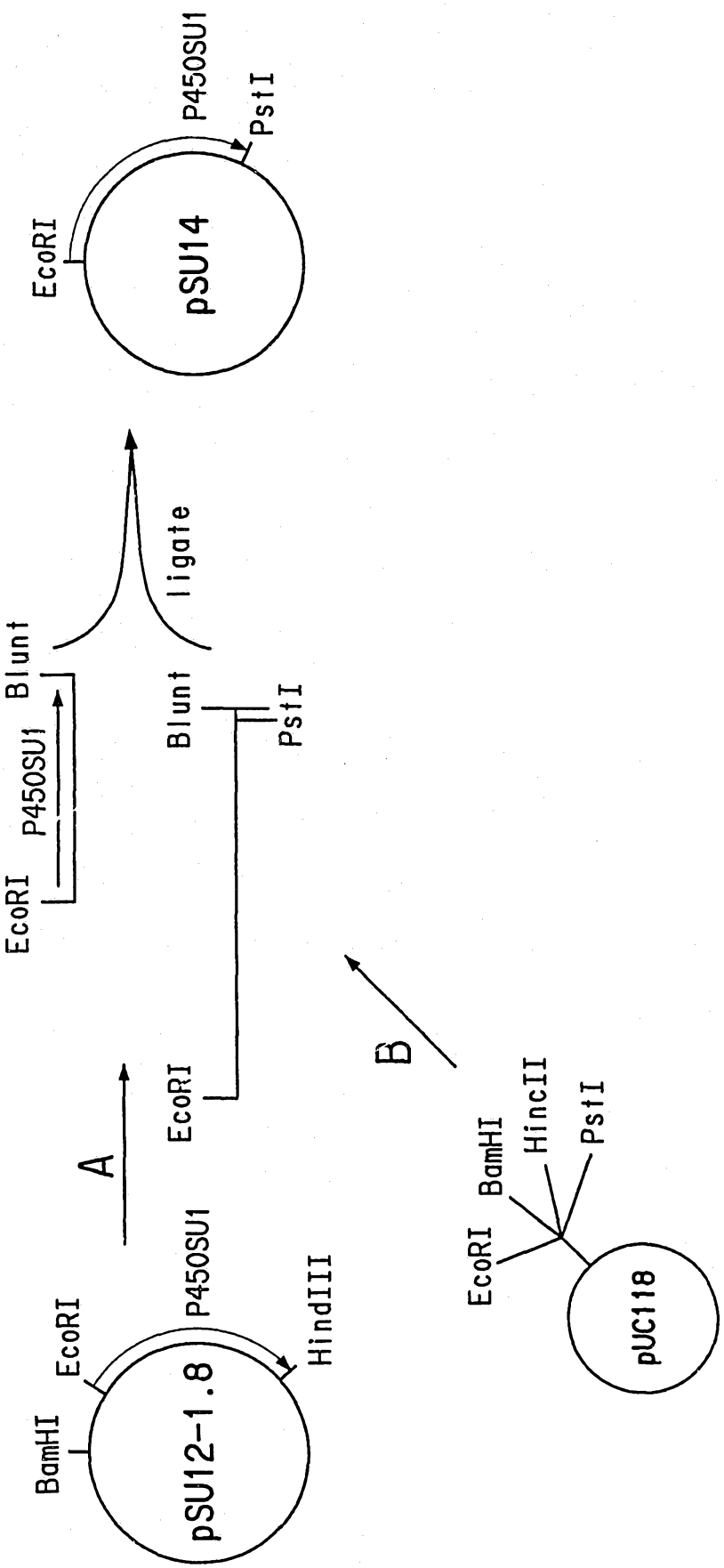

pSU12-1.8
BamHI
EcoRI
P450SU1
HindIII
A
EcoRI
P450SU1
Blunt
PstI
B
EcoRI
BamHI
HincII
PstI
pUC118
EcoRI
P450SU1
PstI
pSU14
ligate
EcoRI
P450SU1
Blunt

FIG. 17B

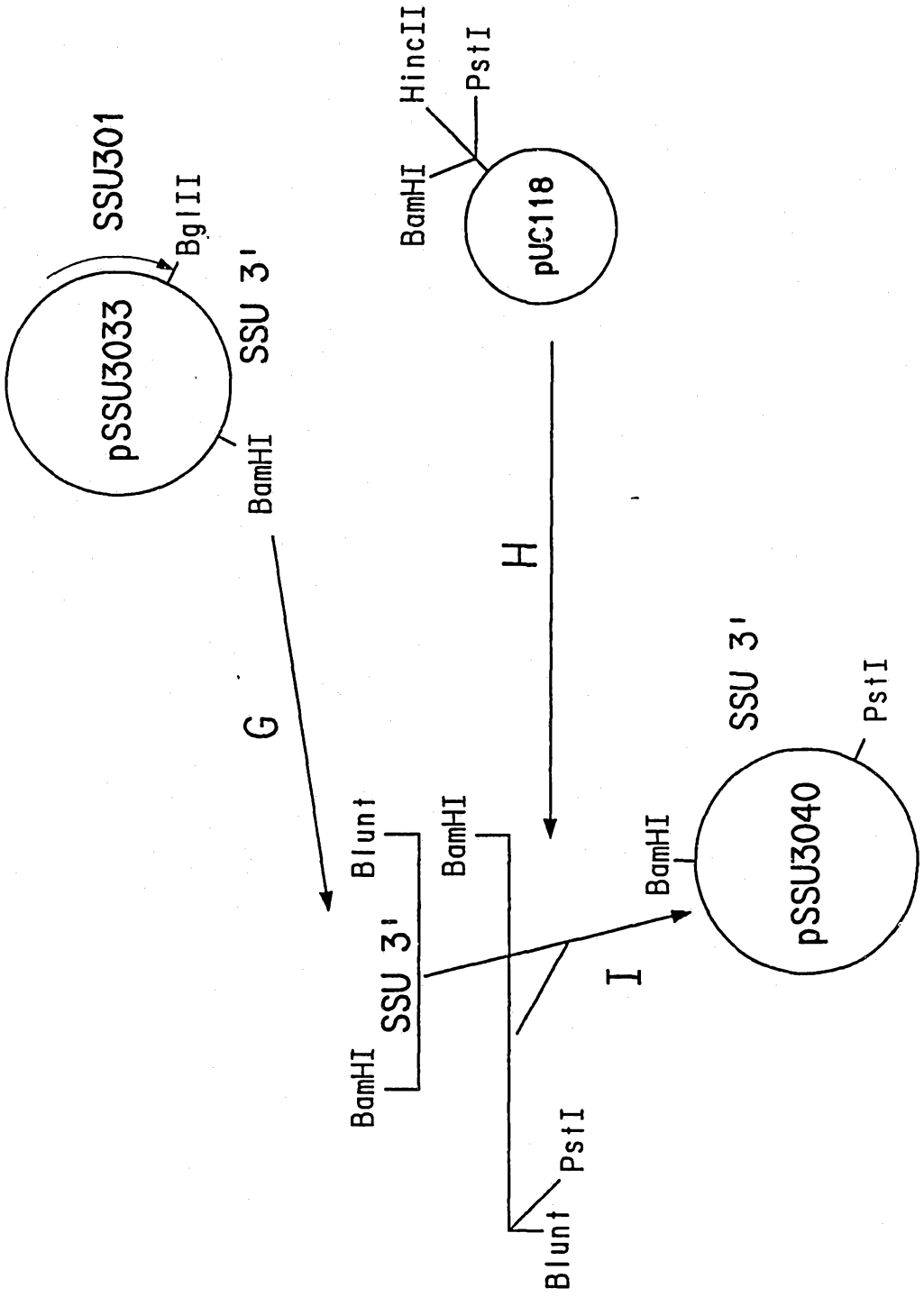

SSU301
BglII
pSSU3033
SSU 3'
BamHI
G
HincII
PstI
BamHI
pUC118
H
Blunt
BamHI
SSU 3'
BamHI
PstI
Blunt
I
SSU 3'
PstI
BamHI
pSSU3040

FIG.17C

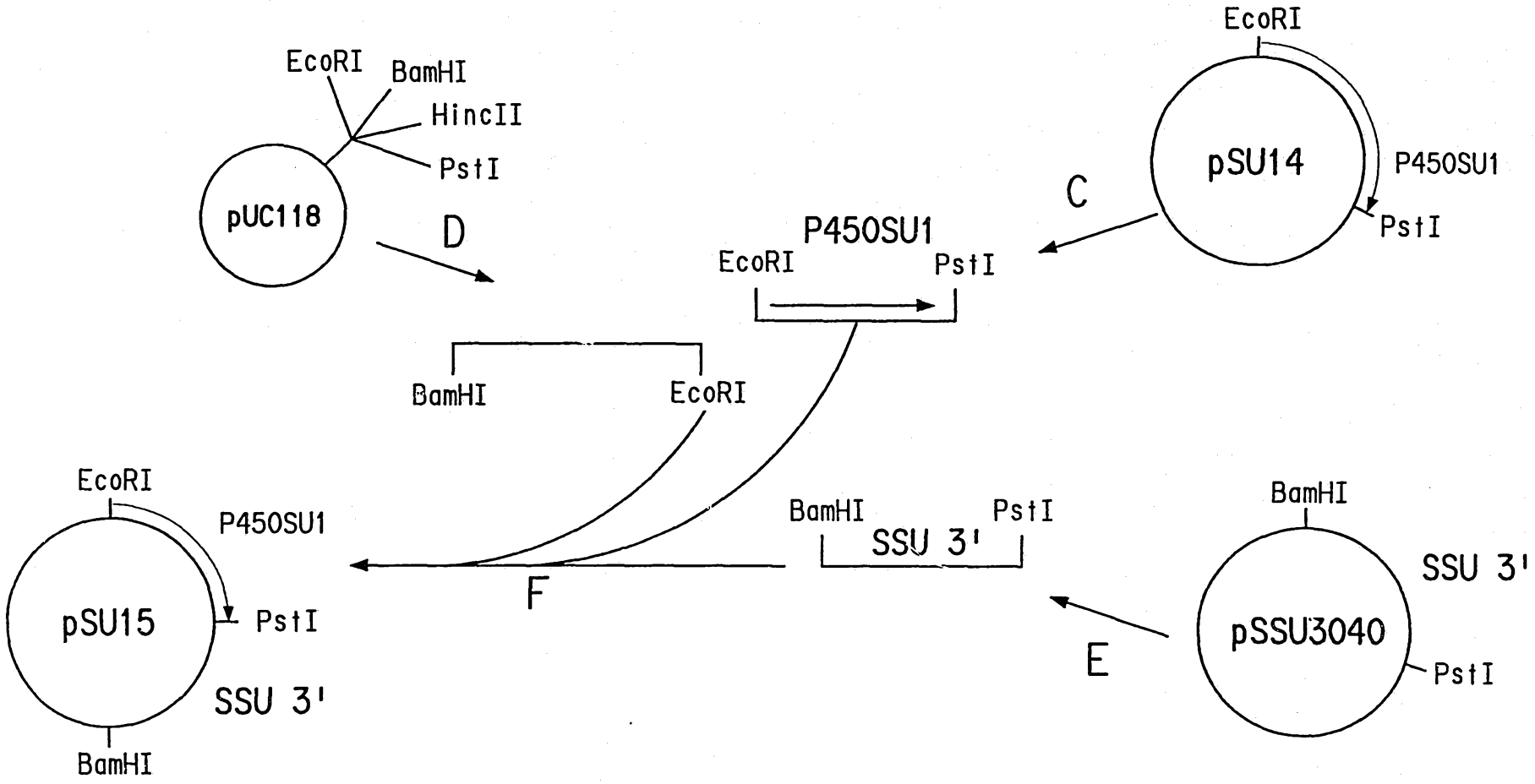

EcoRI
BamHI
HincII
PstI
pUC118
D
P450SU1
EcoRI
PstI
C
EcoRI
pSU14
P450SU1
PstI
BamHI
EcoRI
EcoRI
P450SU1
pSU15
PstI
SSU 3'
BamHI
F
BamHI
PstI
SSU 3'
E
BamHI
SSU 3'
pSSU3040
PstI

FIG.17D

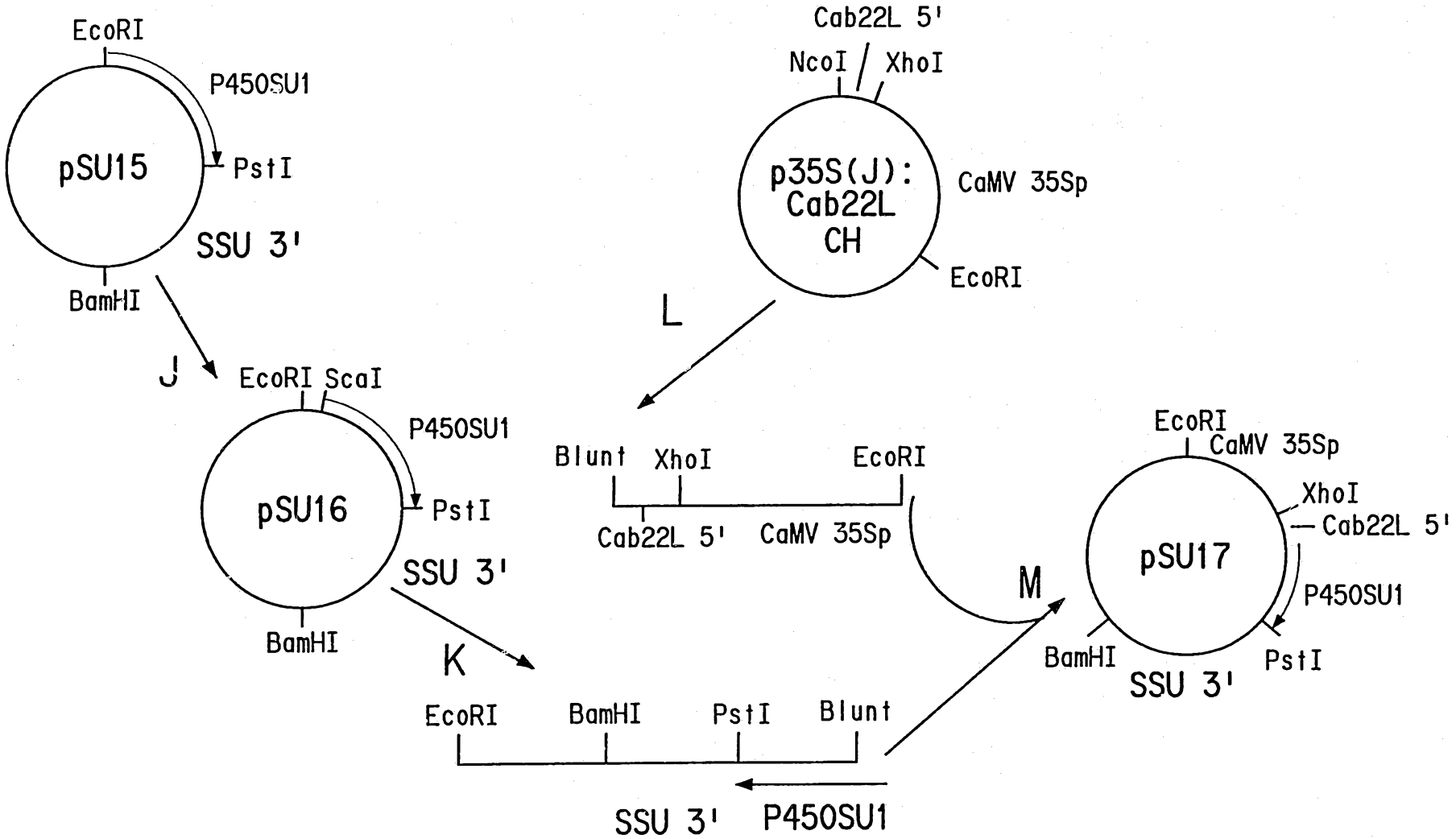

EcoRI
P450SU1
pSU15
PstI
SSU 3'
BamHI
J
EcoRI ScaI
P450SU1
pSU16
PstI
SSU 3'
BamHI
K
Cab22L 5'
NcoI
XhoI
p35S(J):
Cab22L
CH
CaMV 35Sp
EcoRI
L
Blunt
XhoI
EcoRI
Cab22L 5'
CaMV 35Sp
M
EcoRI
CaMV 35Sp
XhoI
Cab22L 5'
pSU17
P450SU1
BamHI
PstI
SSU 3'
EcoRI
BamHI
PstI
Blunt
SSU 3'
P450SU1

FIG. 18A

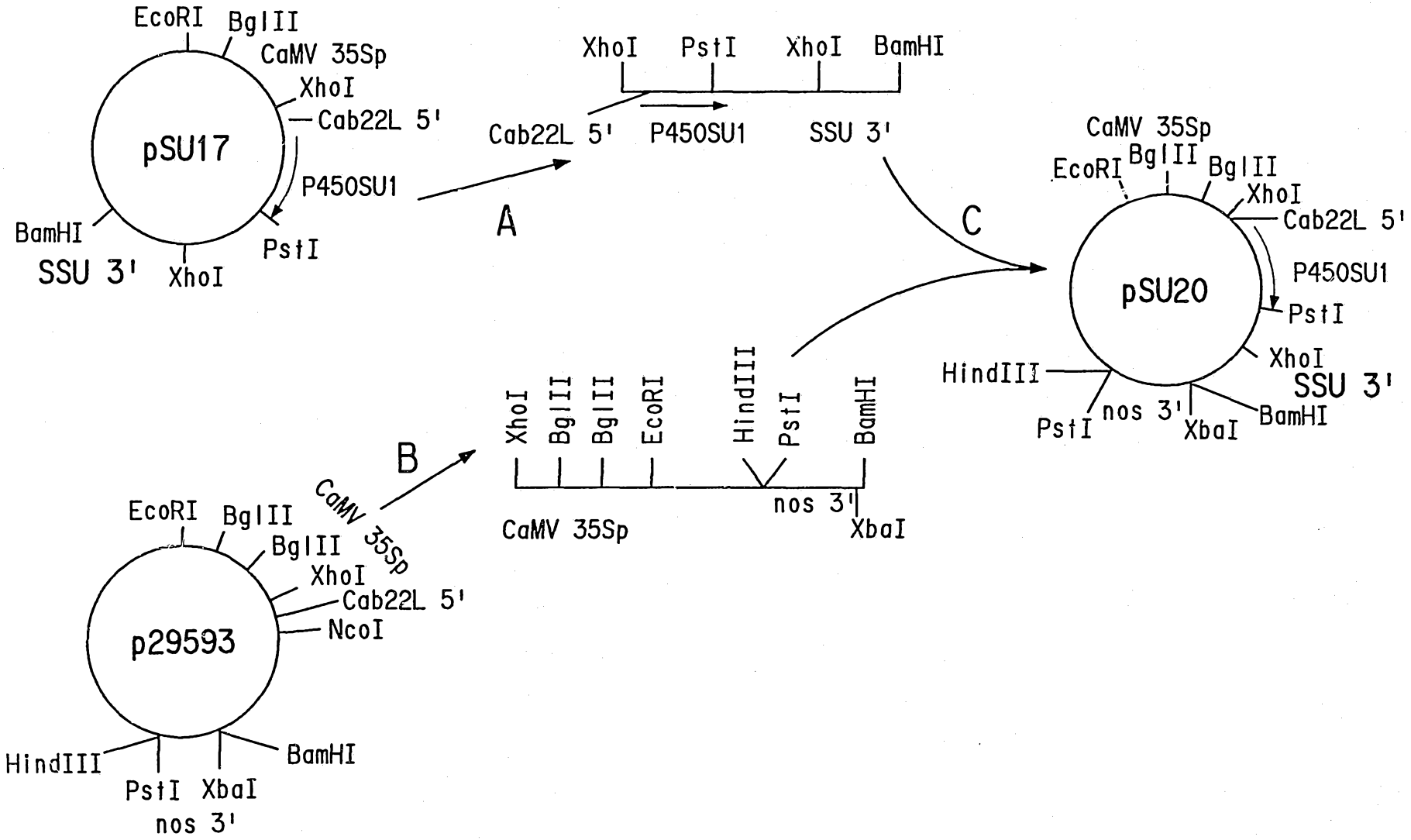

EcoRI
BglII
CaMV 35Sp
XhoI
Cab22L 5'
pSU17
P450SU1
BamHI
SSU 3'
XhoI
PstI
A
XhoI
PstI
XhoI
BamHI
Cab22L 5'
P450SU1
SSU 3'
C
CaMV 35Sp
EcoRI
BglII
BglII
XhoI
Cab22L 5'
pSU20
P450SU1
PstI
XhoI
SSU 3'
HindIII
PstI
nos 3'
XbaI
BamHI
B
CaMV 35Sp
XhoI
BglII
BglII
EcoRI
HindIII
PstI
BamHI
nos 3'
CaMV 35Sp
XbaI
EcoRI
BglII
BglII
XhoI
Cab22L 5'
NcoI
p29593
HindIII
PstI
XbaI
nos 3'
BamHI

FIG.18B

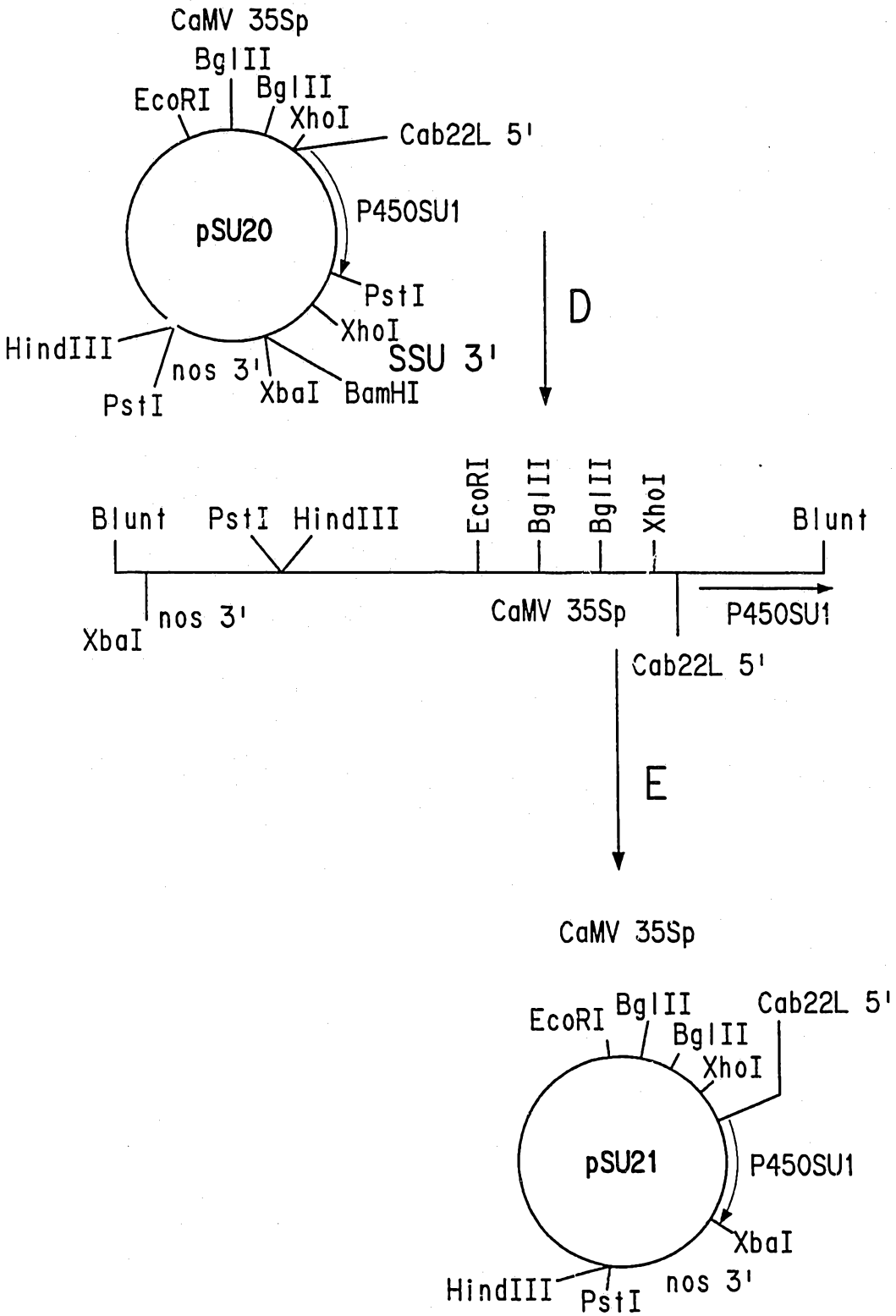

CaMV 35Sp
BglII
EcoRI
BglII
XhoI
Cab22L 5'
pSU20
P450SU1
PstI
XhoI
HindIII
nos 3'
SSU 3'
PstI
XbaI
BamHI
D
Blunt
PstI
HindIII
EcoRI
BglII
BglII
XhoI
Blunt
XbaI
nos 3'
CaMV 35Sp
P450SU1
Cab22L 5'
E
CaMV 35Sp
EcoRI
BglII
BglII
XhoI
Cab22L 5'
pSU21
P450SU1
XbaI
HindIII
PstI
nos 3'

FIG.18C

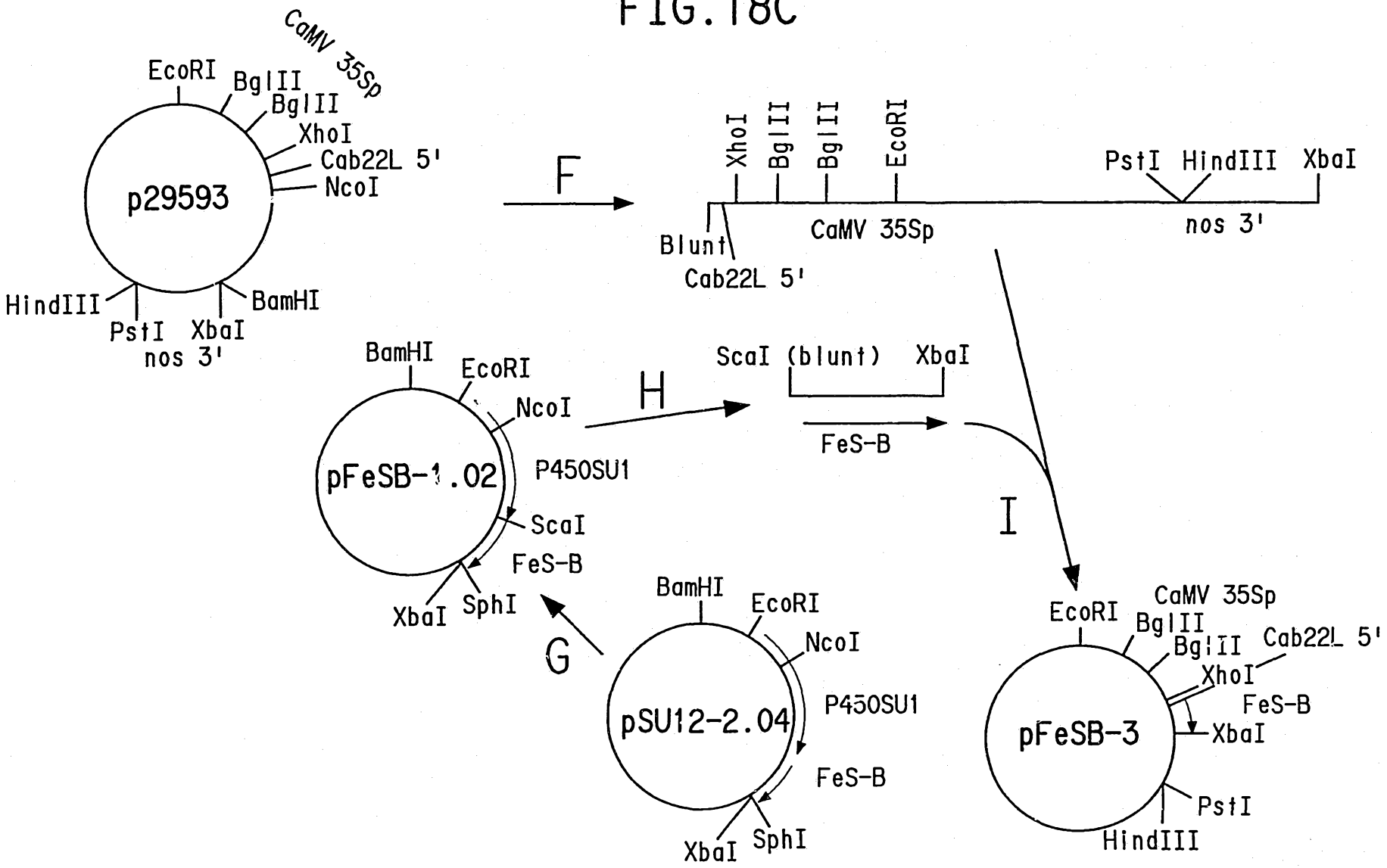

CaMV 35Sp
EcoRI
BglII
BglII
XhoI
Cab22L 5'
NcoI
p29593
HindIII
PstI
XbaI
nos 3'
BamHI
F
XhoI
BglII
BglII
EcoRI
PstI
HindIII
XbaI
Blunt
Cab22L 5'
CaMV 35Sp
nos 3'
BamHI
EcoRI
NcoI
pFeSB-1.02
P450SU1
ScaI
FeS-B
XbaI
SphI
H
ScaI (blunt)
XbaI
FeS-B
I
G
BamHI
EcoRI
NcoI
pSU12-2.04
P450SU1
FeS-B
XbaI
SphI
EcoRI
CaMV 35Sp
BglII
BglII
Cab22L 5'
XhoI
FeS-B
pFeSB-3
XbaI
PstI
HindIII

FIG.18D

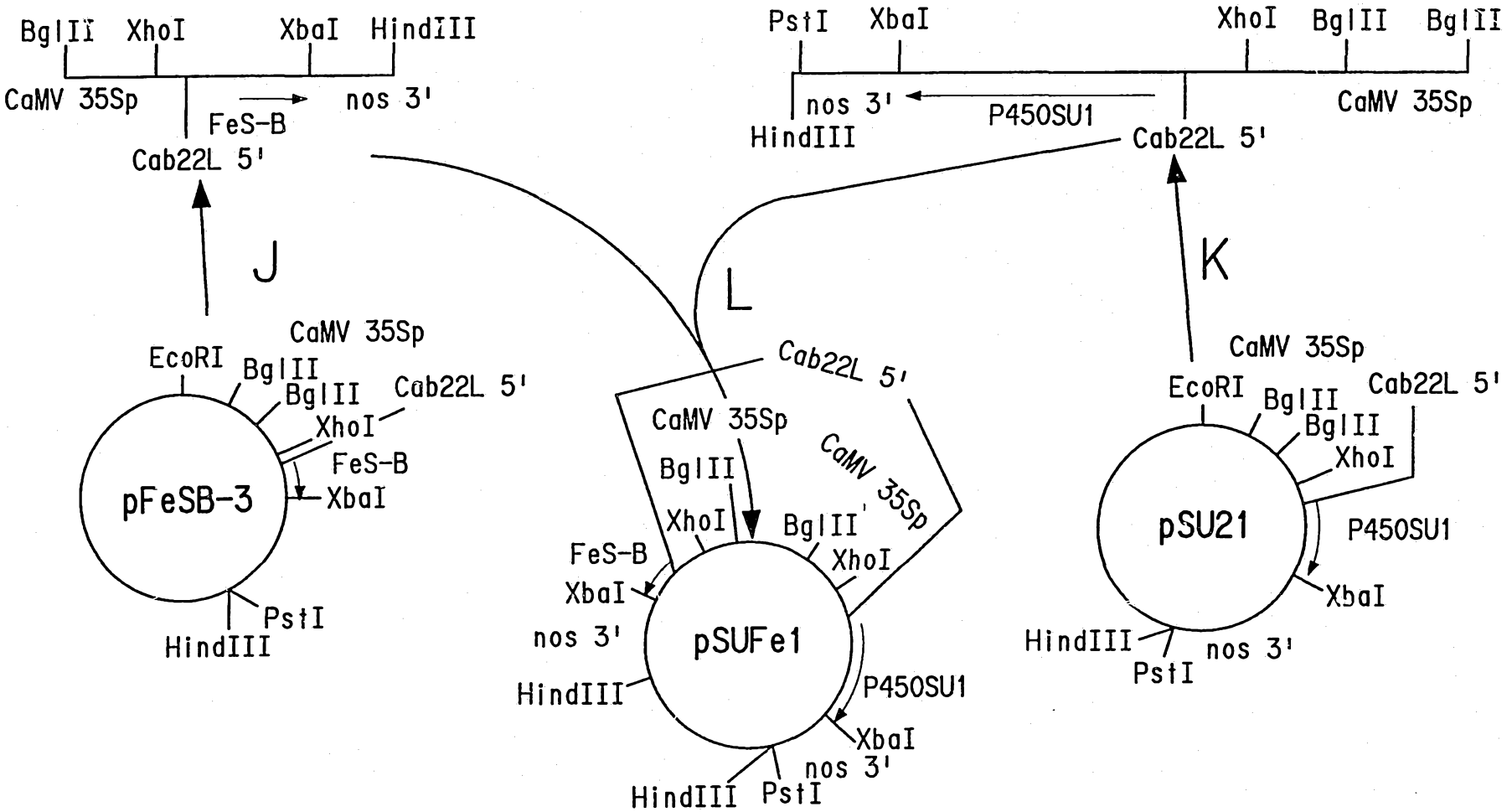

BglII XhoI XbaI HindIII
CaMV 35Sp
FeS-B
nos 3'
Cab22L 5'
J
EcoRI BglII
CaMV 35Sp
BglII Cab22L 5'
XhoI
FeS-B
pFeSB-3
XbaI
PstI
HindIII
PstI XbaI
XhoI BglII BglII
nos 3'
P450SU1
CaMV 35Sp
HindIII
Cab22L 5'
K
L
Cab22L 5'
CaMV 35Sp
CaMV 35Sp
BglII
XhoI
BglII
XhoI
FeS-B
XbaI
pSUFe1
nos 3'
HindIII
P450SU1
XbaI
nos 3'
HindIII PstI
CaMV 35Sp
EcoRI BglII
Cab22L 5'
BglII
XhoI
pSU21
P450SU1
XbaI
HindIII
nos 3'
PstI

FIG. 19A

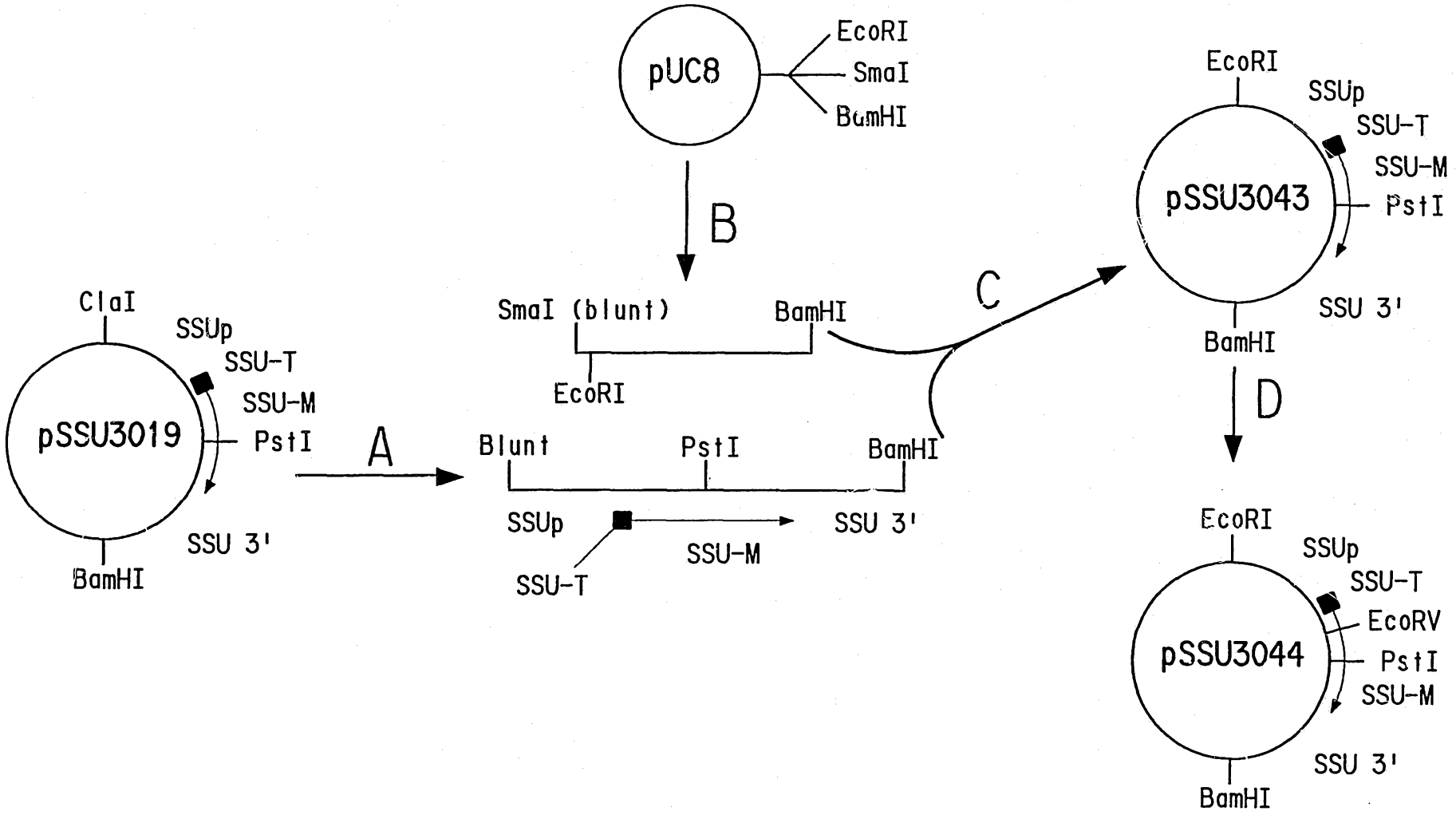

pUC8
EcoRI
SmaI
BamHI
B
ClaI
SSUp
SSU-T
SSU-M
pSSU3019
PstI
A
SSU 3'
BamHI
SmaI (blunt)
BamHI
EcoRI
C
Blunt
PstI
BamHI
SSUp
SSU 3'
SSU-M
SSU-T
EcoRI
SSUp
SSU-T
SSU-M
pSSU3043
PstI
SSU 3'
BamHI
D
EcoRI
SSUp
SSU-T
EcoRV
pSSU3044
PstI
SSU-M
SSU 3'
BamHI

FIG.19B

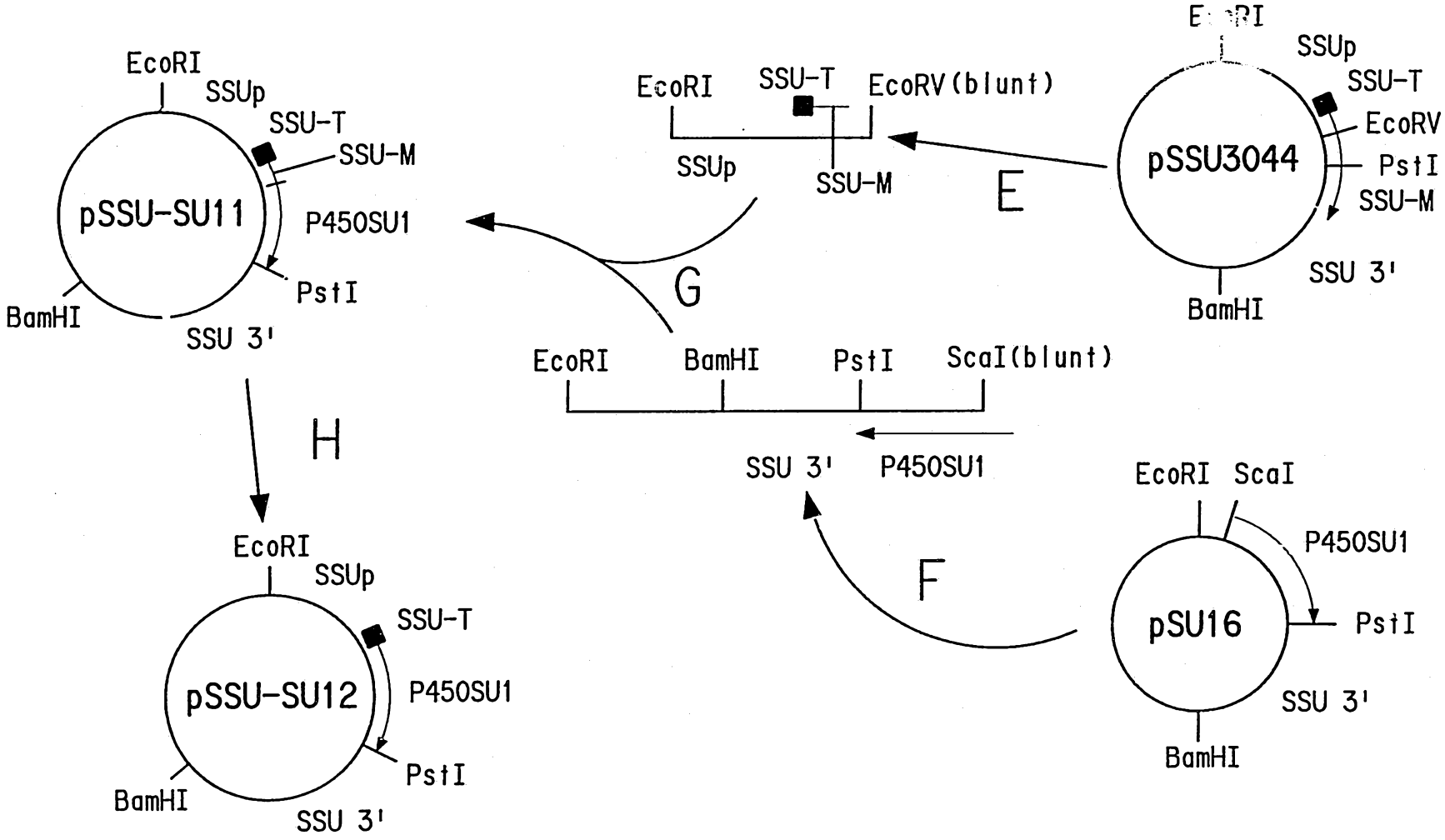

EcoRI
SSUp
SSU-T
SSU-M
pSSU-SU11
P450SU1
PstI
BamHI
SSU 3'
H
EcoRI
SSUp
SSU-T
pSSU-SU12
P450SU1
PstI
BamHI
SSU 3'
G
EcoRI
SSU-T
EcoRV(blunt)
SSUp
SSU-M
E
EcoRI
SSUp
SSU-T
EcoRV
PstI
SSU-M
pSSU3044
SSU 3'
BamHI
EcoRI
BamHI
PstI
ScaI(blunt)
SSU 3'
P450SU1
F
EcoRI
ScaI
P450SU1
PstI
pSU16
SSU 3'
BamHI

FIG.20A

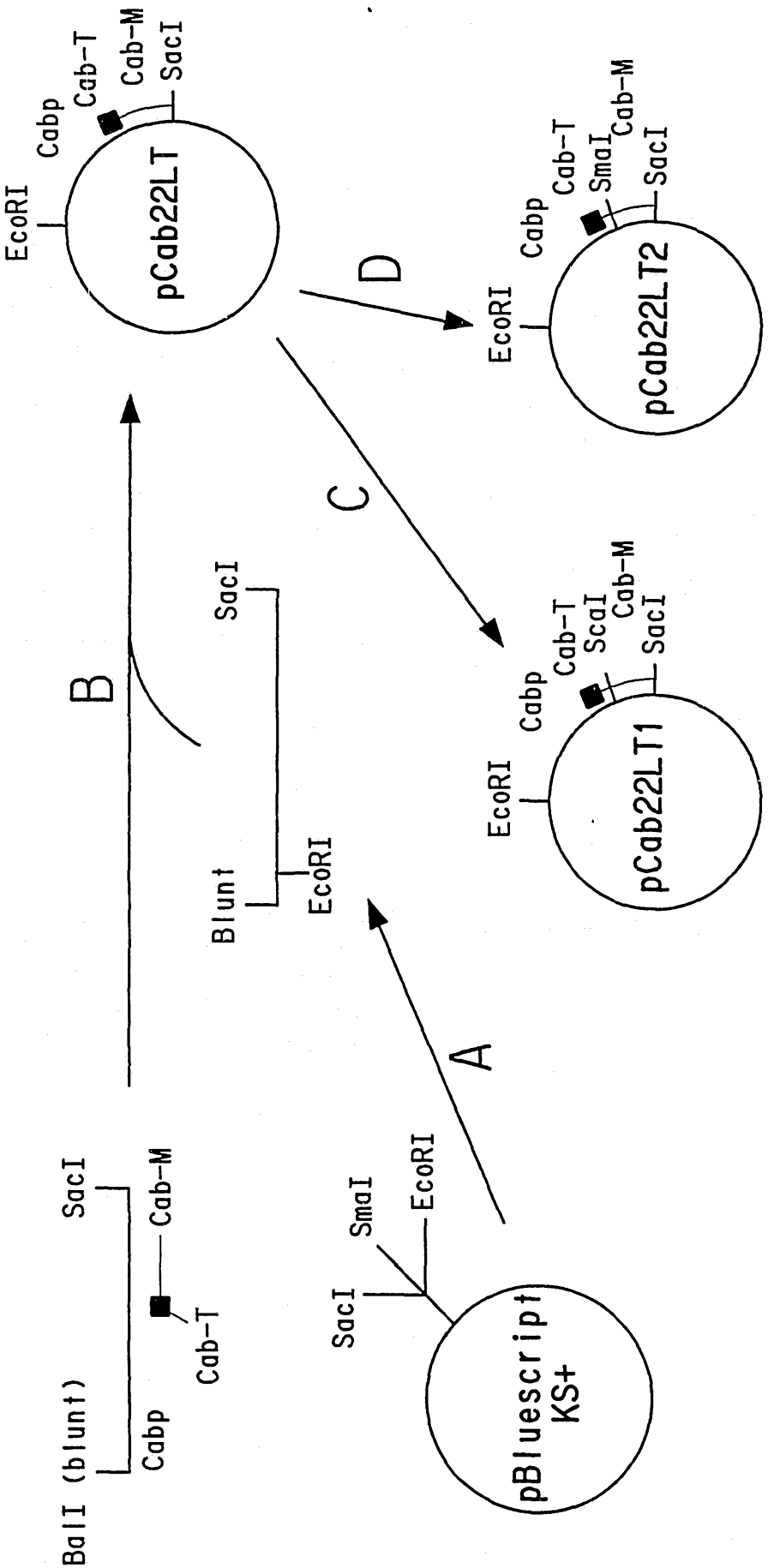

pBluescript KS+
SacI
SmaI
EcoRI
A
pCab22LT1
EcoRI
Cabp
Cab-T
ScaI
Cab-M
SacI
B
Blunt
EcoRI
SacI
C
pCab22LT
EcoRI
Cabp
Cab-T
Cab-M
SacI
D
pCab22LT2
EcoRI
Cabp
Cab-T
SmaI
Cab-M
SacI
BalI (blunt)
Cabp
Cab-T
Cab-M
SacI

FIG.20B

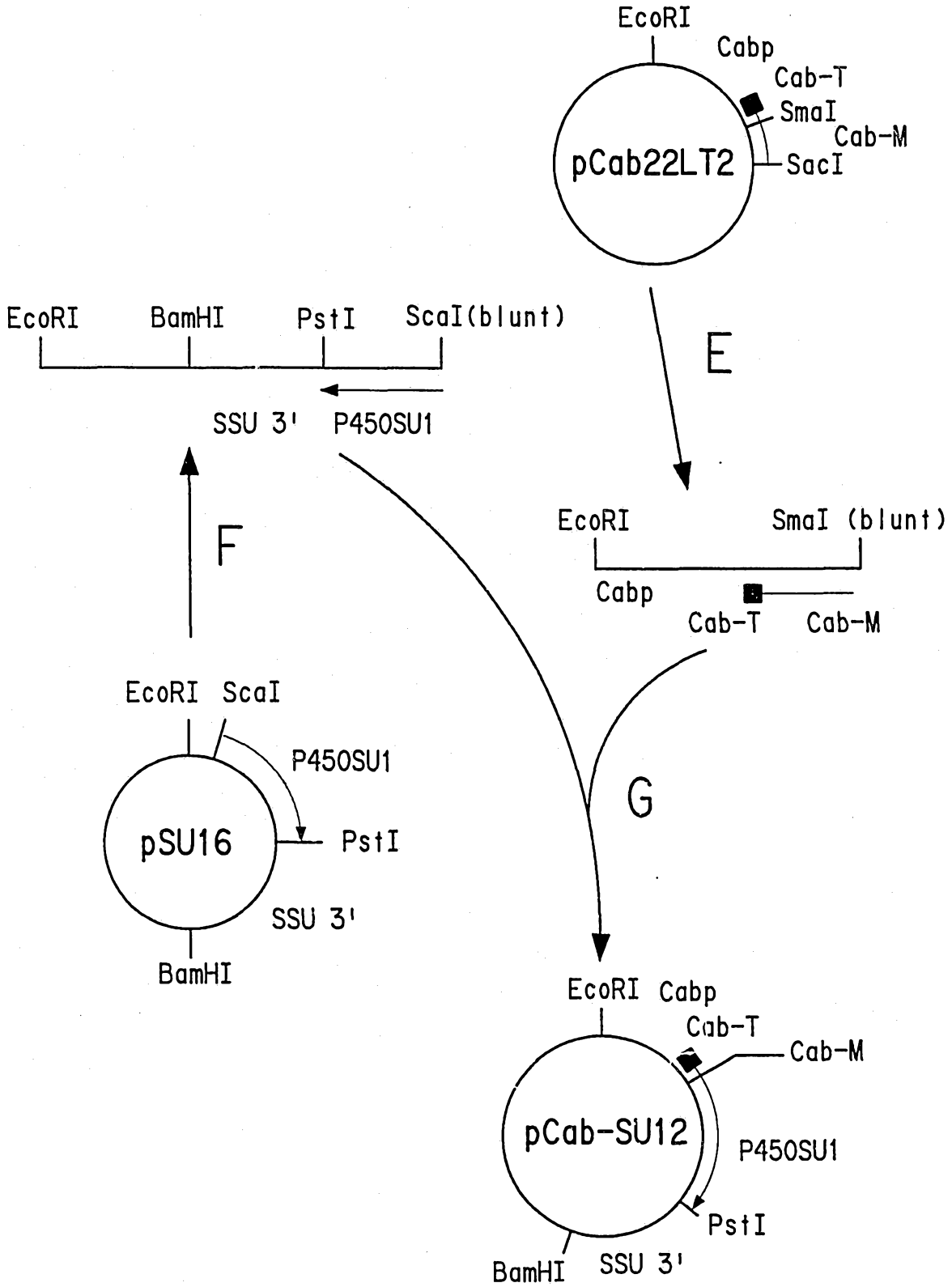

EcoRI
Cabp
Cab-T
SmaI
Cab-M
SacI
pCab22LT2
E
EcoRI BamHI PstI ScaI(blunt)
SSU 3'
P450SU1
EcoRI SmaI (blunt)
Cabp
Cab-T
Cab-M
F
G
EcoRI ScaI
P450SU1
pSU16
PstI
SSU 3'
BamHI
EcoRI Cabp
Cab-T
Cab-M
pCab-SU12
P450SU1
PstI
BamHI
SSU 3'

FIG.20C

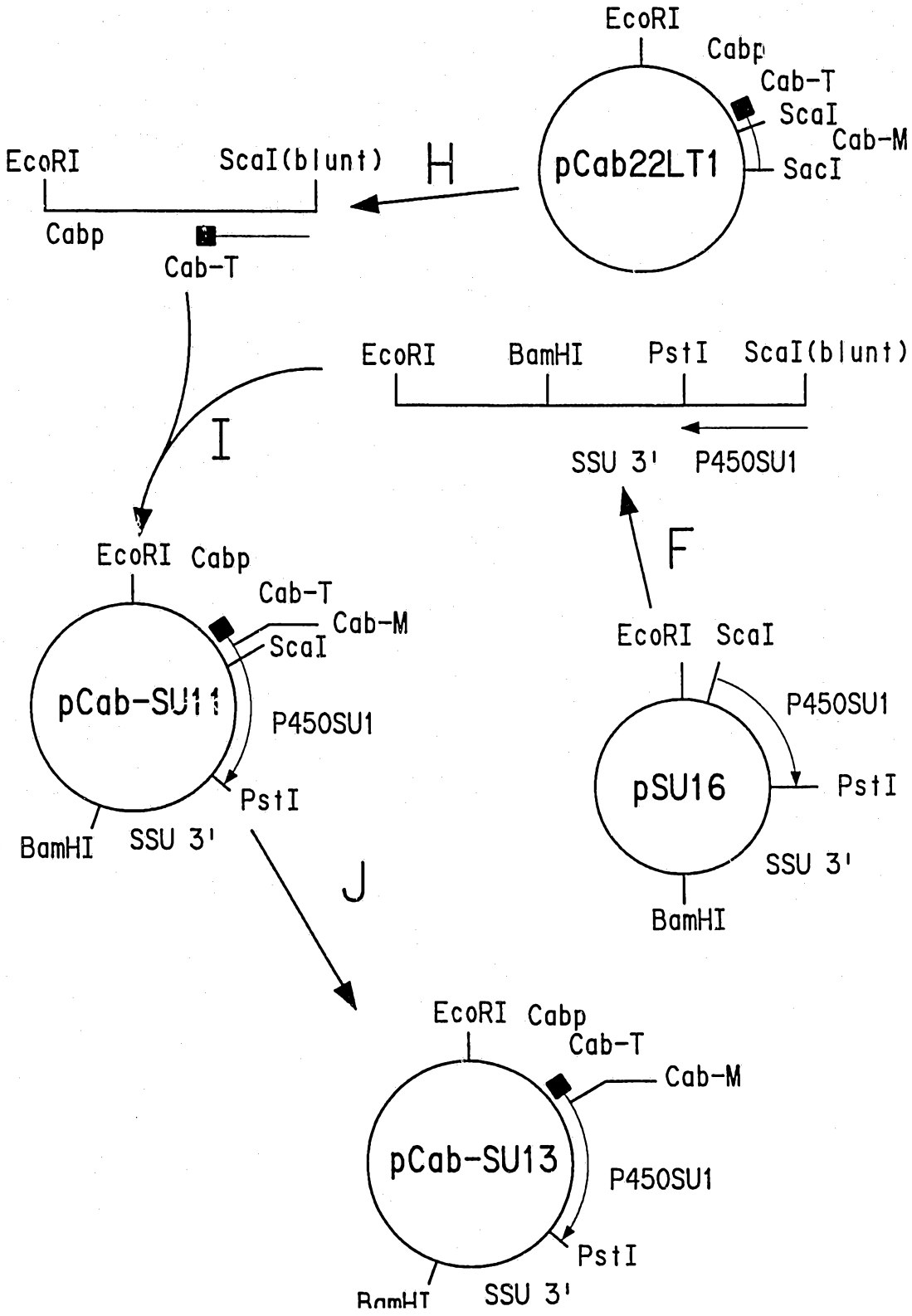

EcoRI
Cabp
Cab-T
ScaI
Cab-M
SacI
pCab22LT1
H
EcoRI
ScaI(blunt)
Cabp
Cab-T
I
EcoRI
BamHI
PstI
ScaI(blunt)
SSU 3'
P450SU1
F
EcoRI Cabp
Cab-T
Cab-M
ScaI
pCab-SU11
P450SU1
PstI
BamHI
SSU 3'
EcoRI ScaI
P450SU1
pSU16
PstI
SSU 3'
BamHI
J
EcoRI Cabp
Cab-T
Cab-M
pCab-SU13
P450SU1
PstI
BamHI
SSU 3'

FIG.21A

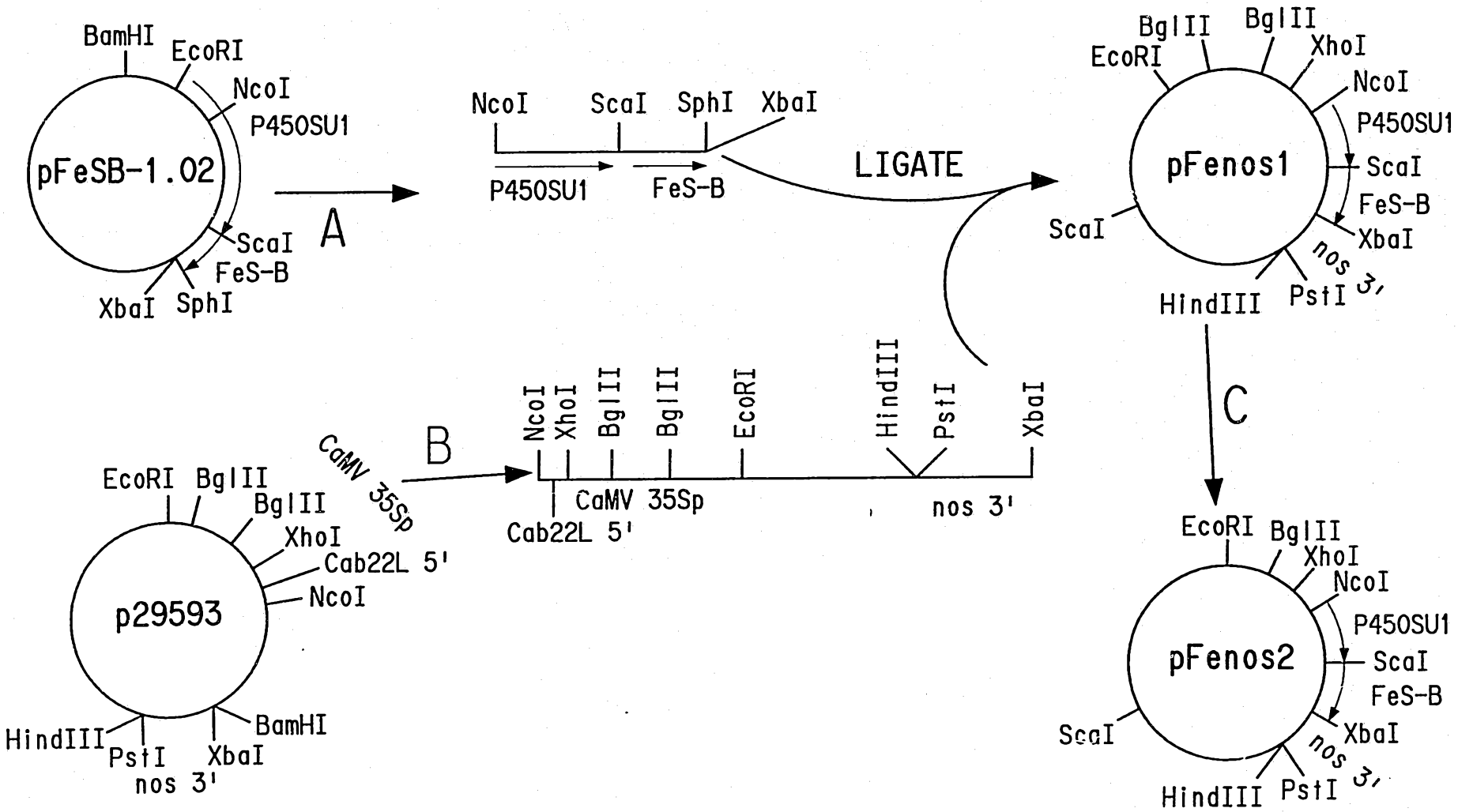

BamHI
EcoRI
NcoI
P450SU1
pFeSB-1.02
ScaI
FeS-B
XbaI
SphI
A
NcoI
ScaI
SphI
XbaI
P450SU1
FeS-B
LIGATE
BglII
BglII
EcoRI
XhoI
NcoI
P450SU1
pFenos1
ScaI
FeS-B
XbaI
ScaI
nos 3'
HindIII
PstI
B
CaMV 35Sp
NcoI
XhoI
BglII
BglII
EcoRI
HindIII
PstI
XbaI
CaMV 35Sp
Cab22L 5'
nos 3'
C
EcoRI
BglII
BglII
XhoI
Cab22L 5'
NcoI
p29593
HindIII
PstI
XbaI
BamHI
nos 3'
EcoRI
BglII
XhoI
NcoI
P450SU1
pFenos2
ScaI
FeS-B
XbaI
ScaI
nos 3'
HindIII
PstI

FIG.21B

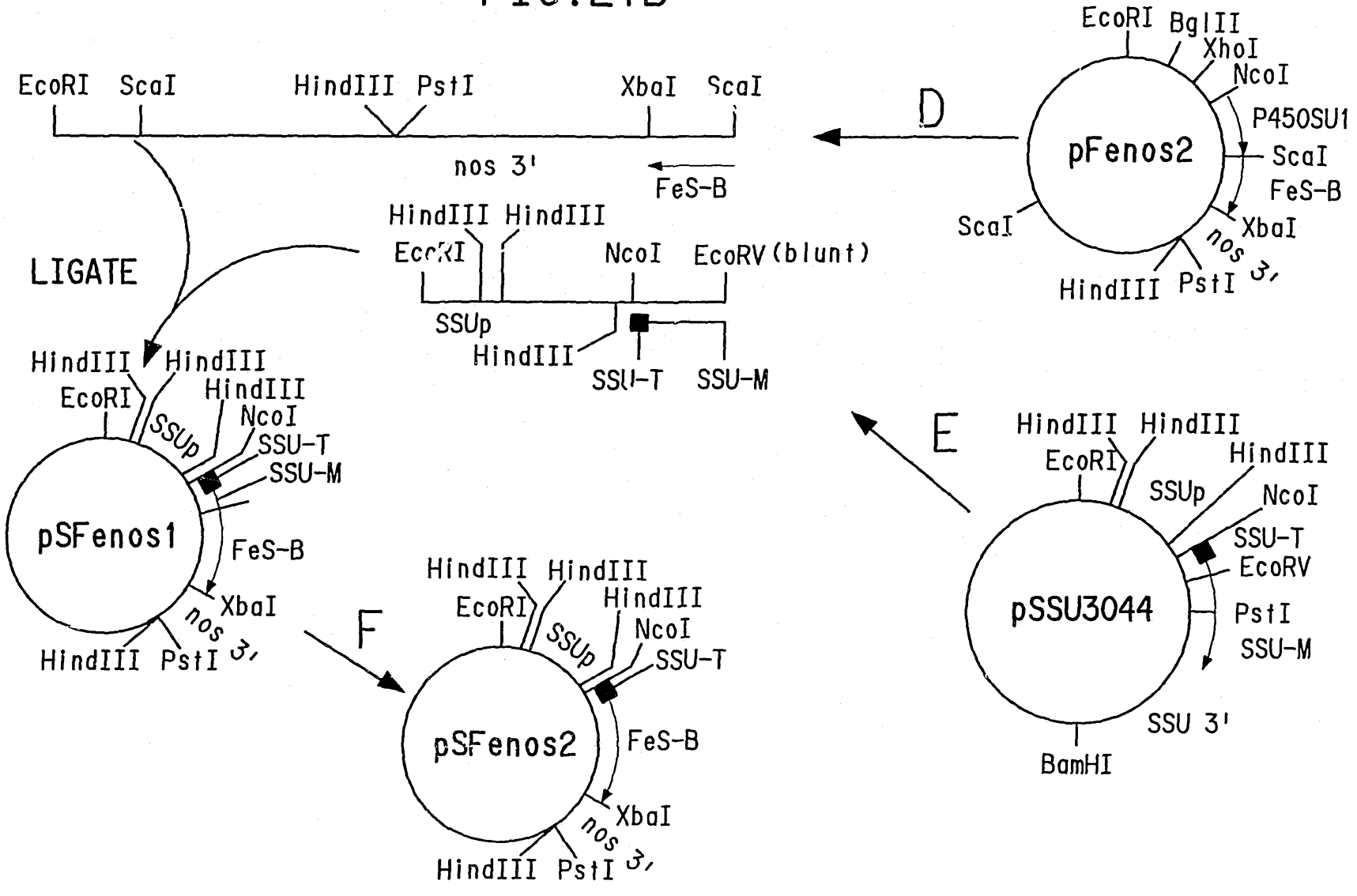

EcoRI ScaI HindIII PstI XbaI ScaI
nos 3'
FeS-B
D
EcoRI BglII XhoI NcoI P450SU1 ScaI FeS-B XbaI nos 3' HindIII PstI ScaI
pFenos2
HindIII HindIII EcoRI NcoI EcoRV(blunt)
SSUp HindIII SSU-T SSU-M
LIGATE
HindIII HindIII EcoRI HindIII NcoI SSUp SSU-T SSU-M FeS-B XbaI nos 3' HindIII PstI
pSFenos1
E
HindIII HindIII EcoRI HindIII SSUp NcoI SSU-T EcoRV PstI SSU-M SSU 3' BamHI
pSSU3044
F
HindIII HindIII EcoRI HindIII SSUp NcoI SSU-T FeS-B XbaI nos 3' HindIII PstI
pSFenos2

FIG.21C

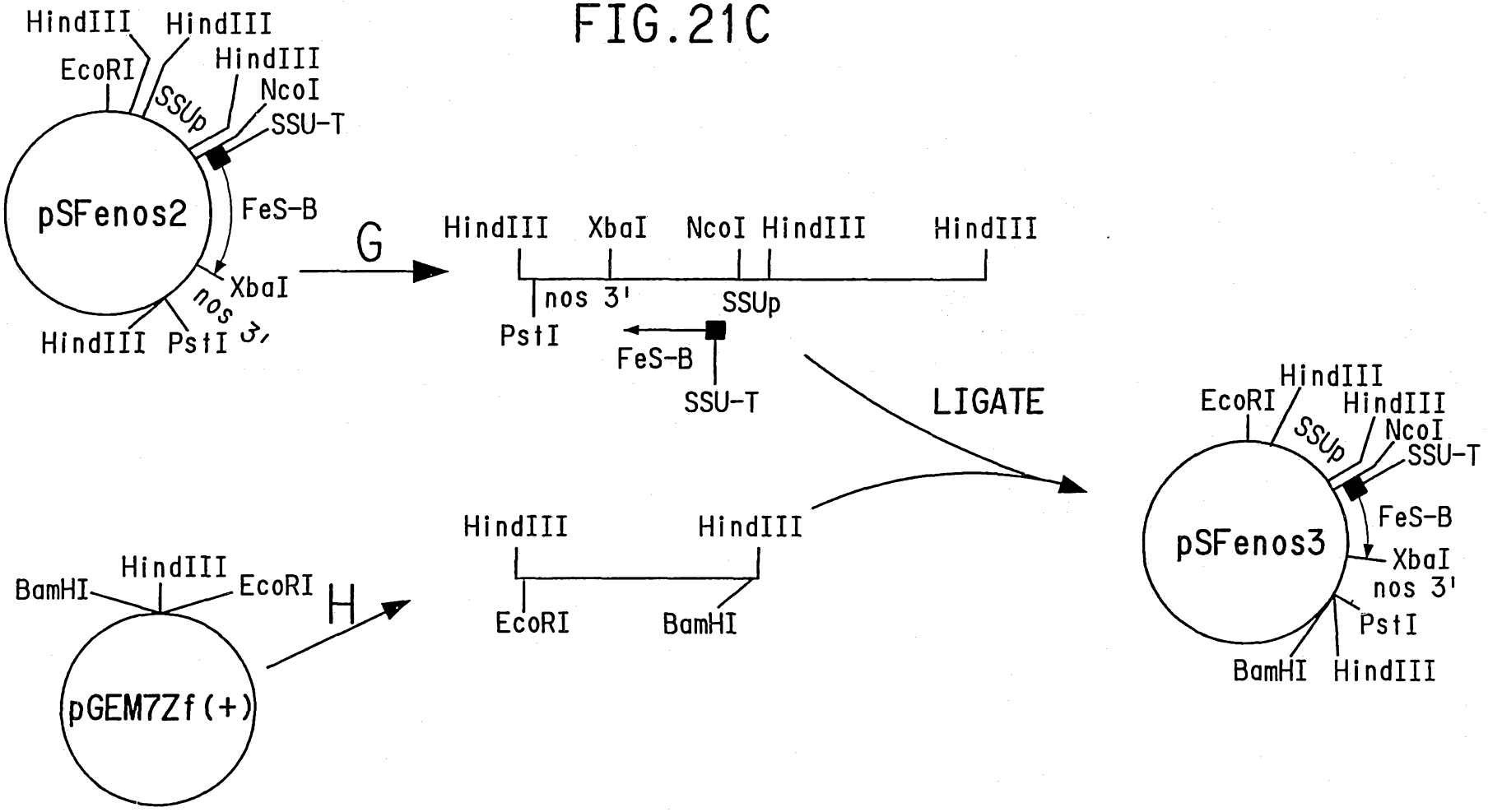

HindIII
HindIII
EcoRI
HindIII
NcoI
SSUp
SSU-T
pSFenos2
FeS-B
XbaI
nos 3'
HindIII
PstI
G
HindIII
XbaI
NcoI
HindIII
HindIII
nos 3'
SSUp
PstI
FeS-B
SSU-T
LIGATE
BamHI
HindIII
EcoRI
pGEM7Zf(+)
H
HindIII
HindIII
EcoRI
BamHI
HindIII
EcoRI
HindIII
NcoI
SSUp
SSU-T
FeS-B
pSFenos3
XbaI
nos 3'
PstI
BamHI
HindIII

FIG.21D

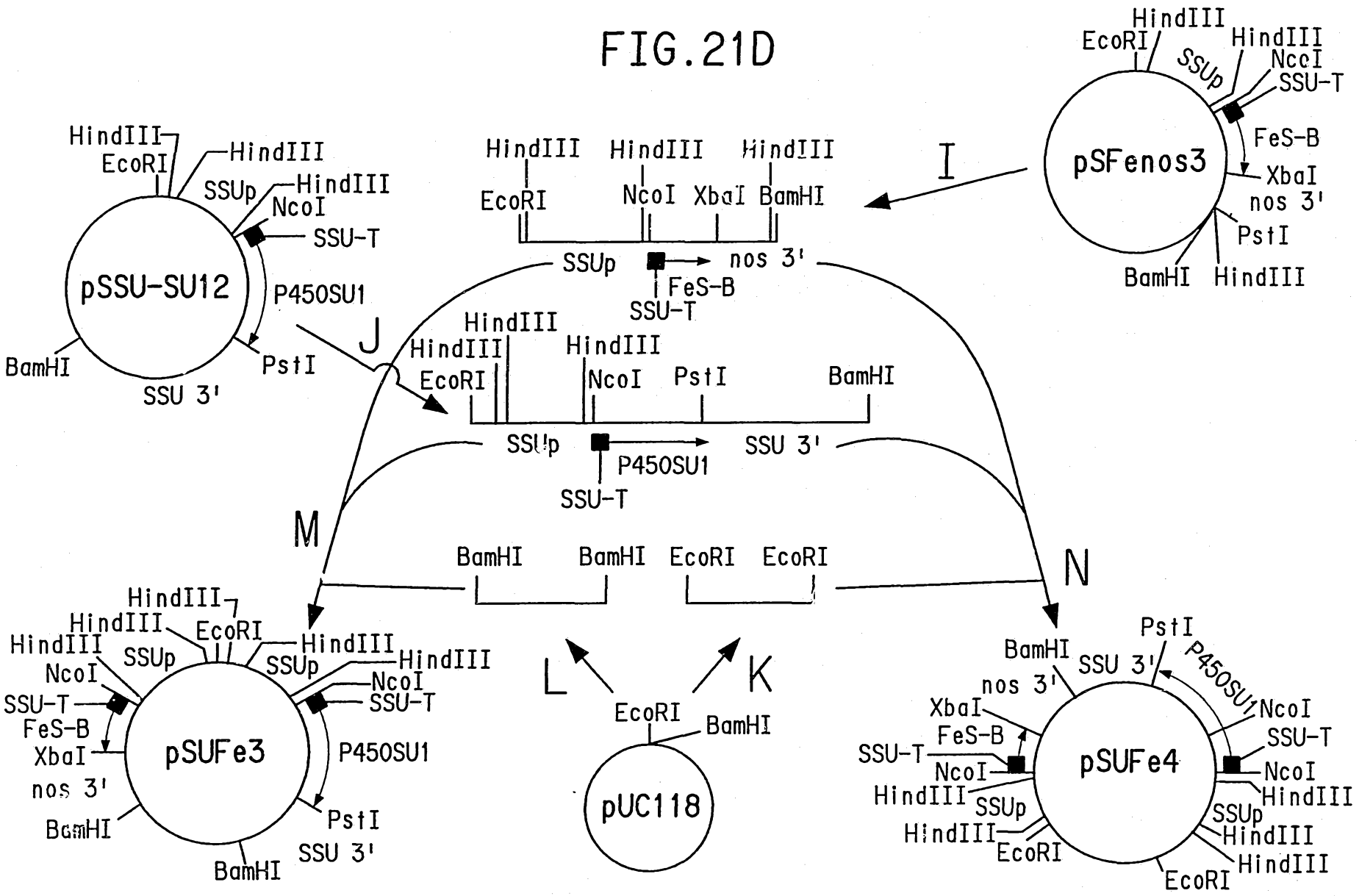

pSFenos3
HindIII
EcoRI
SSUp
HindIII
NcoI
SSU-T
FeS-B
XbaI
nos 3'
PstI
BamHI
HindIII
I
pSSU-SU12
HindIII
EcoRI
HindIII
SSUp
HindIII
NcoI
SSU-T
P450SU1
BamHI
PstI
SSU 3'
J
HindIII HindIII HindIII
EcoRI NcoI XbaI BamHI
SSUp
nos 3'
FeS-B
SSU-T
HindIII
HindIII
HindIII
EcoRI
NcoI
PstI
BamHI
SSUp
P450SU1
SSU 3'
SSU-T
M
N
BamHI BamHI EcoRI EcoRI
HindIII
HindIII
HindIII
EcoRI
HindIII
SSUp
SSUp
HindIII
NcoI
NcoI
SSU-T
SSU-T
FeS-B
XbaI
nos 3'
BamHI
pSUFe3
P450SU1
PstI
SSU 3'
BamHI
L
K
EcoRI
BamHI
pUC118
PstI
BamHI
SSU 3'
P450SU1
nos 3'
XbaI
FeS-B
SSU-T
NcoI
HindIII
SSUp
HindIII
EcoRI
pSUFe4
NcoI
SSU-T
NcoI
HindIII
SSUp
HindIII
HindIII
EcoRI

FIG.22A

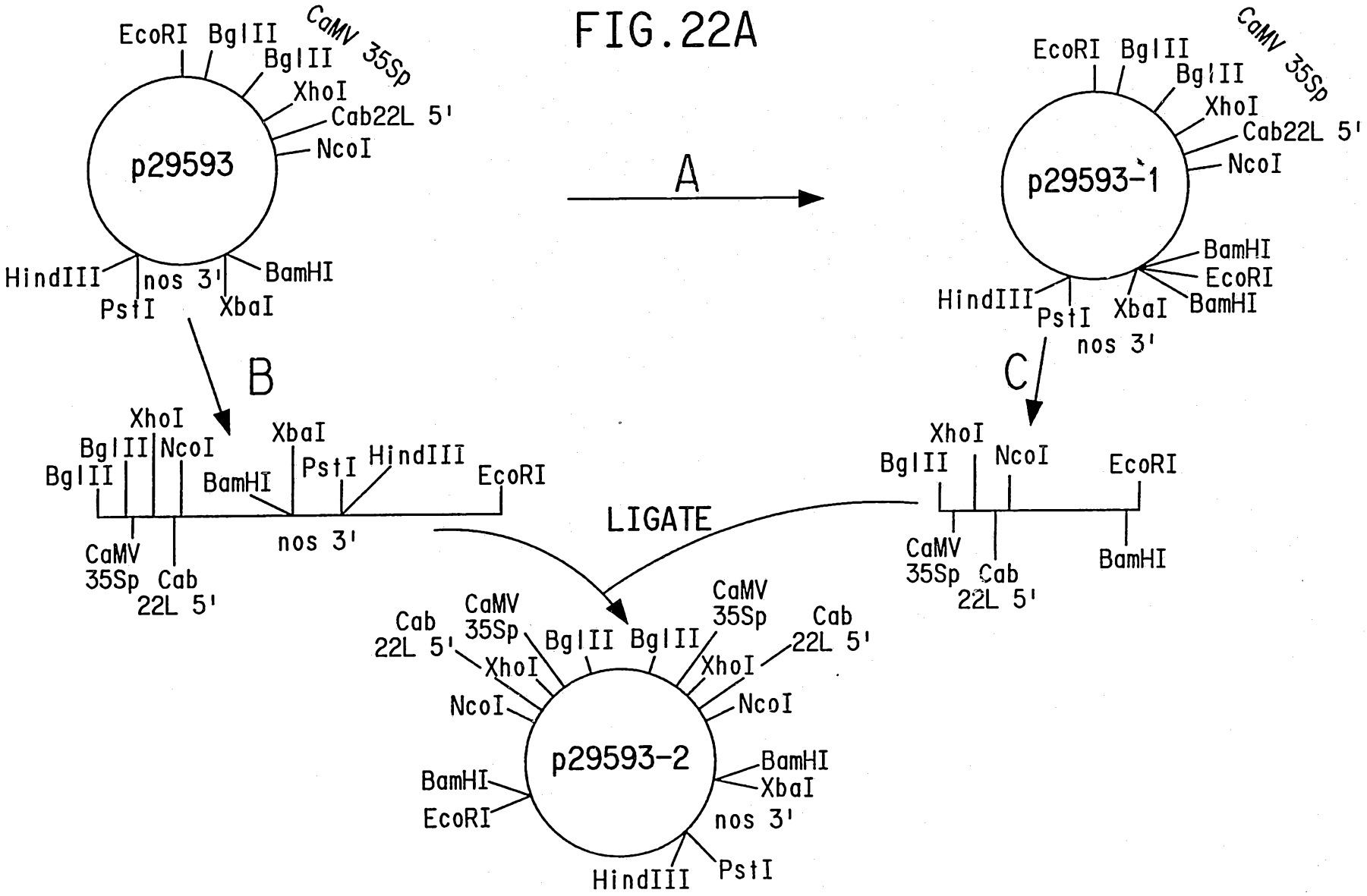

EcoRI
BglII
BglII
CaMV 35Sp
XhoI
Cab22L 5'
NcoI
p29593
HindIII
PstI
nos 3'
XbaI
BamHI
A
p29593-1
B
C
XhoI
BglII
NcoI
XbaI
PstI
HindIII
EcoRI
BamHI
CaMV 35Sp
Cab 22L 5'
LIGATE
p29593-2

FIG.22B

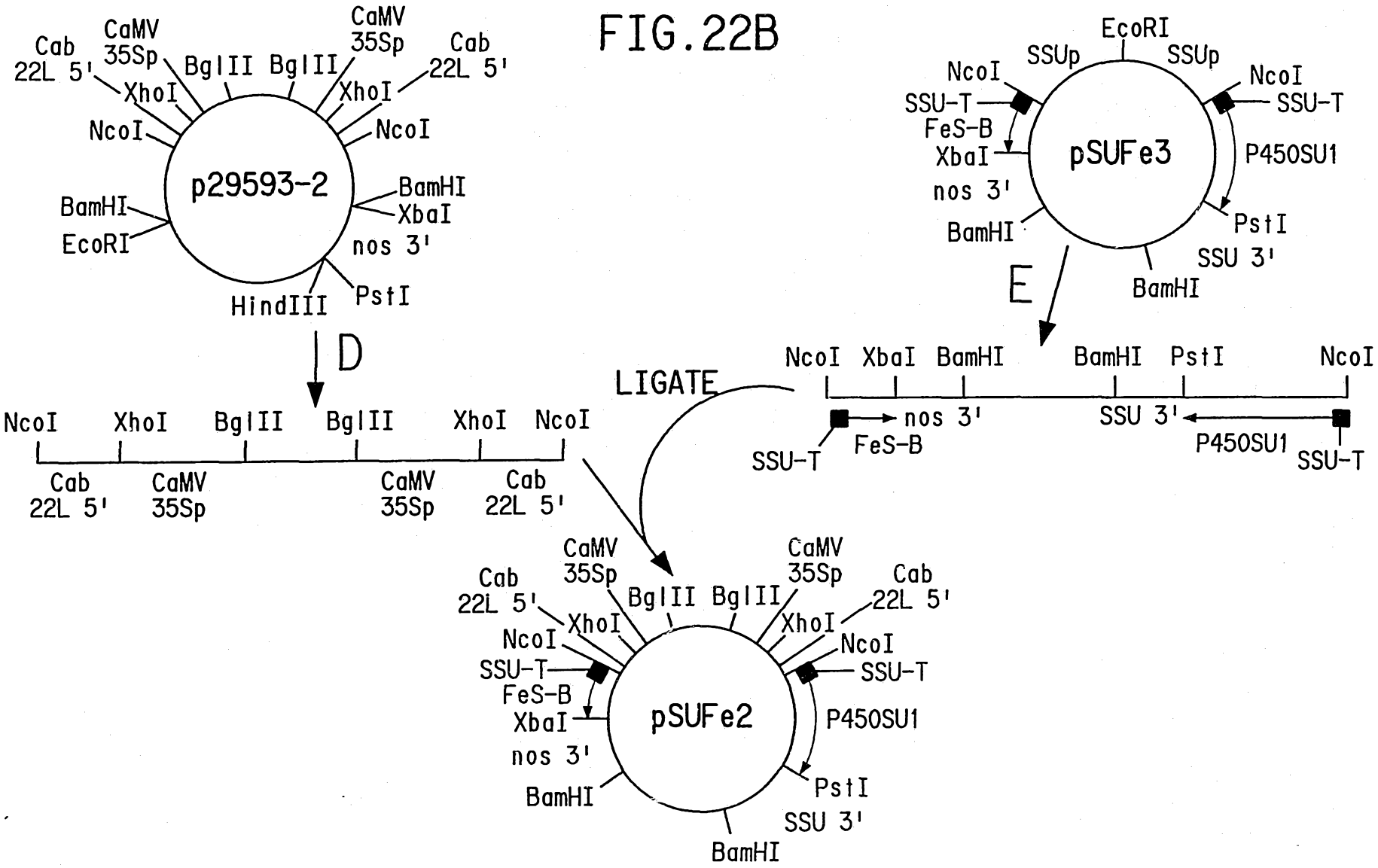

p29593-2
Cab 22L 5'
CaMV 35Sp
XhoI
BglII
NcoI
BamHI
EcoRI
HindIII
PstI
XbaI
nos 3'
D
NcoI
XhoI
BglII
Cab 22L 5'
CaMV 35Sp
LIGATE
pSUFe3
EcoRI
SSUp
SSU-T
FeS-B
P450SU1
SSU 3'
E
pSUFe2

FIG.23A

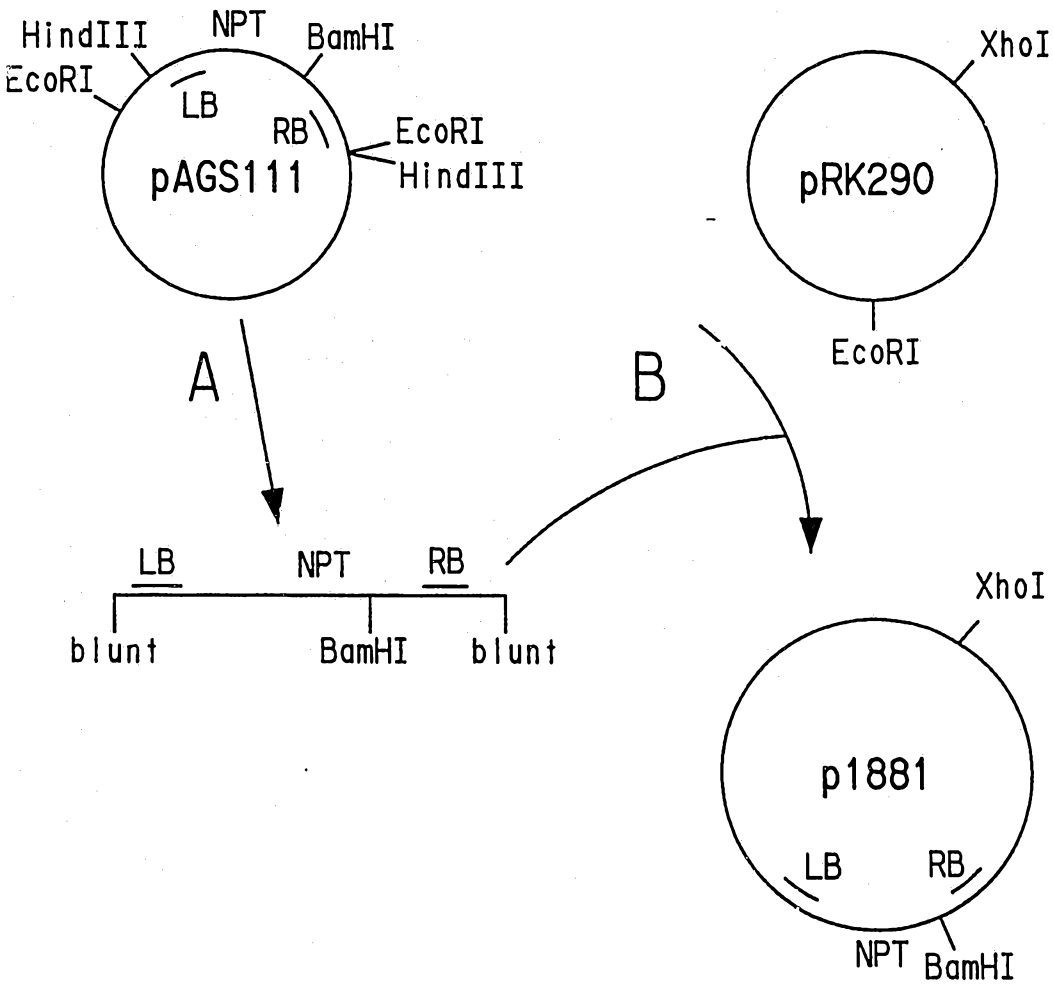

HindIII
NPT
BamHI
EcoRI
LB
RB
EcoRI
HindIII
pAGS111
XhoI
pRK290
EcoRI
A
B
LB
NPT
RB
blunt
BamHI
blunt
XhoI
p1881
LB
RB
NPT
BamHI

FIG.23B

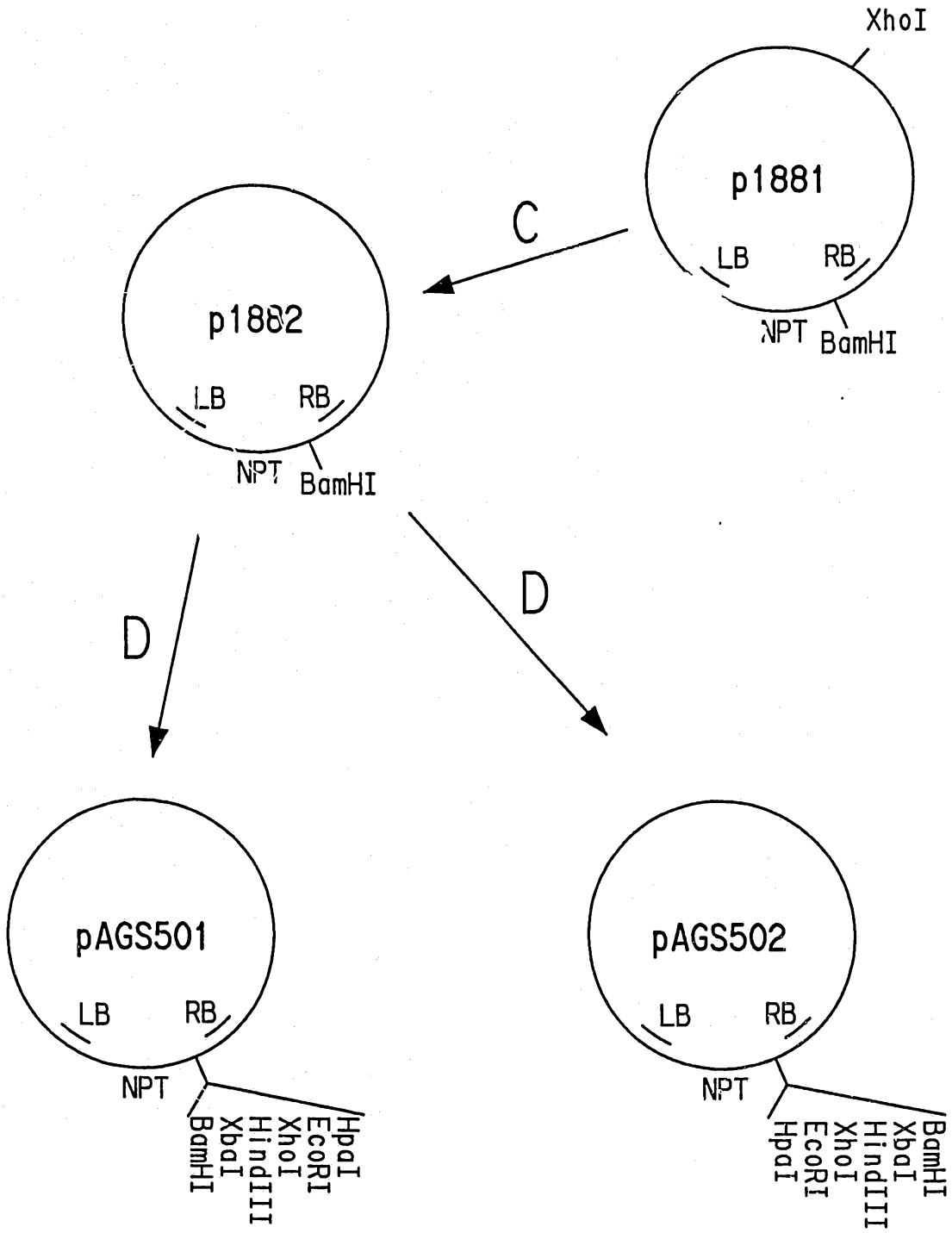

XhoI
p1881
LB
RB
NPT
BamHI
C
p1882
LB
RB
NPT
BamHI
D
D
pAGS501
LB
RB
NPT
BamHI
XbaI
HindIII
XhoI
EcoRI
HpaI
pAGS502
LB
RB
NPT
HpaI
EcoRI
XhoI
HindIII
XbaI
BamHI

FIG.24A

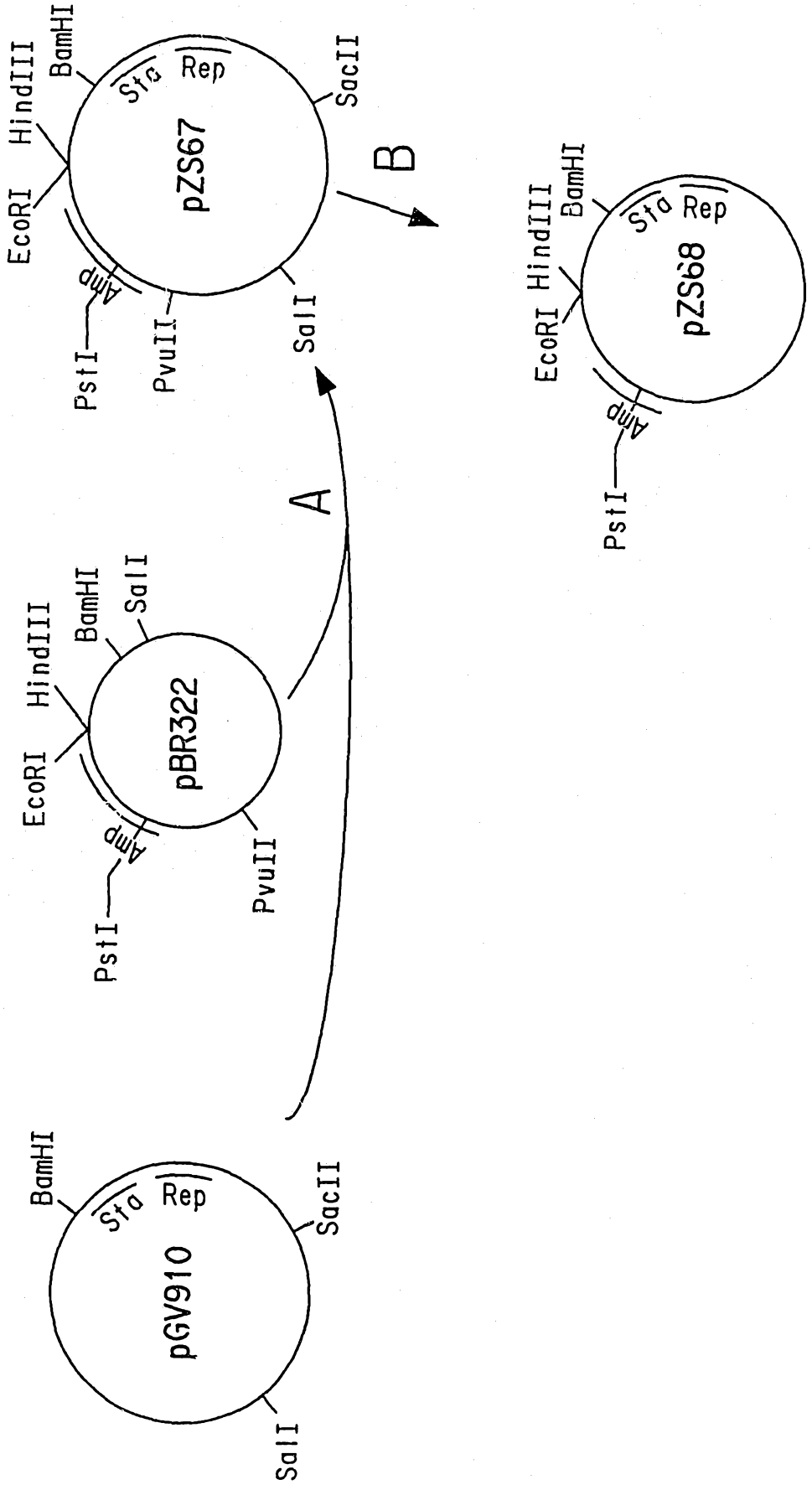

pZS67
BamHI
Sta
Rep
SacII
HindIII
EcoRI
Amp
PstI
PvuII
SalI
B
pZS68
BamHI
Sta
Rep
HindIII
EcoRI
Amp
PstI
A
pBR322
BamHI
SalI
HindIII
EcoRI
Amp
PstI
PvuII
pGV910
BamHI
Sta
Rep
SacII
SalI

FIG. 24B

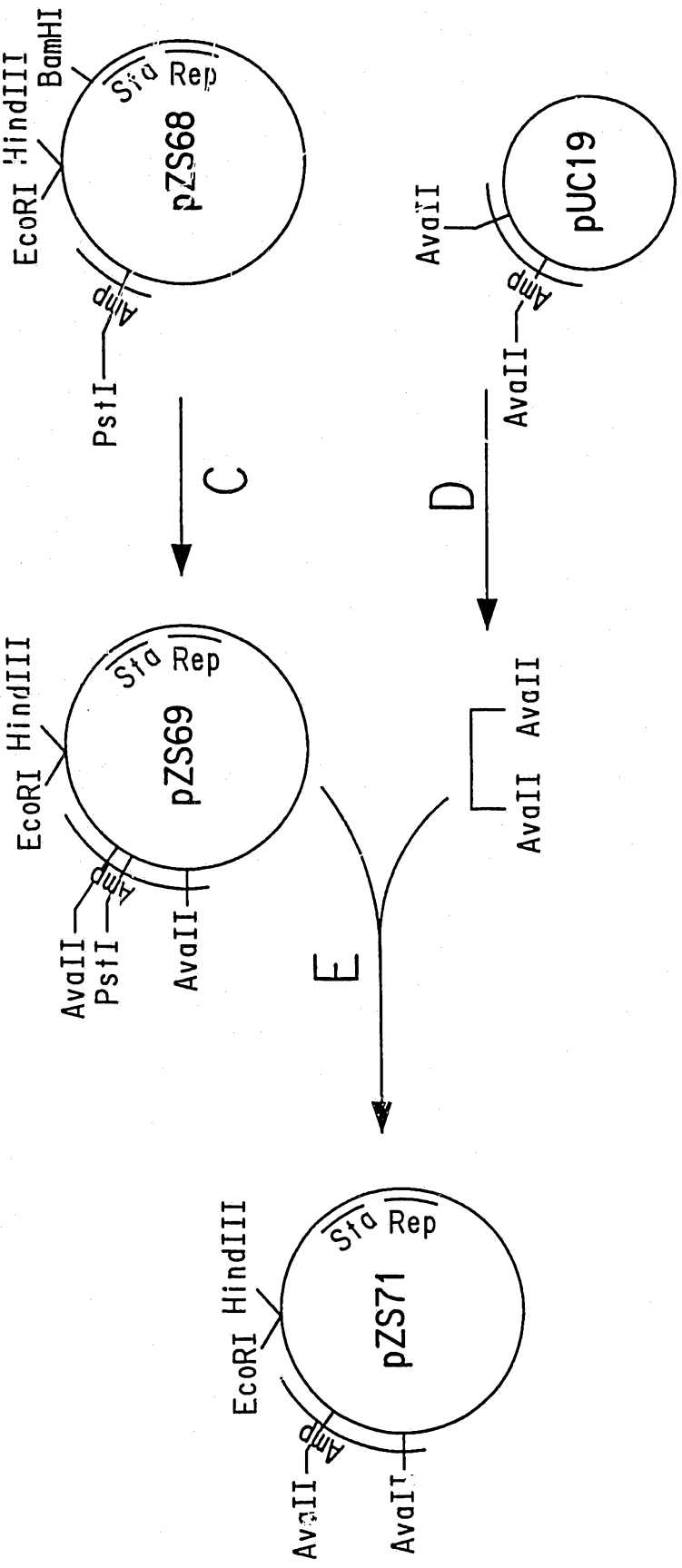
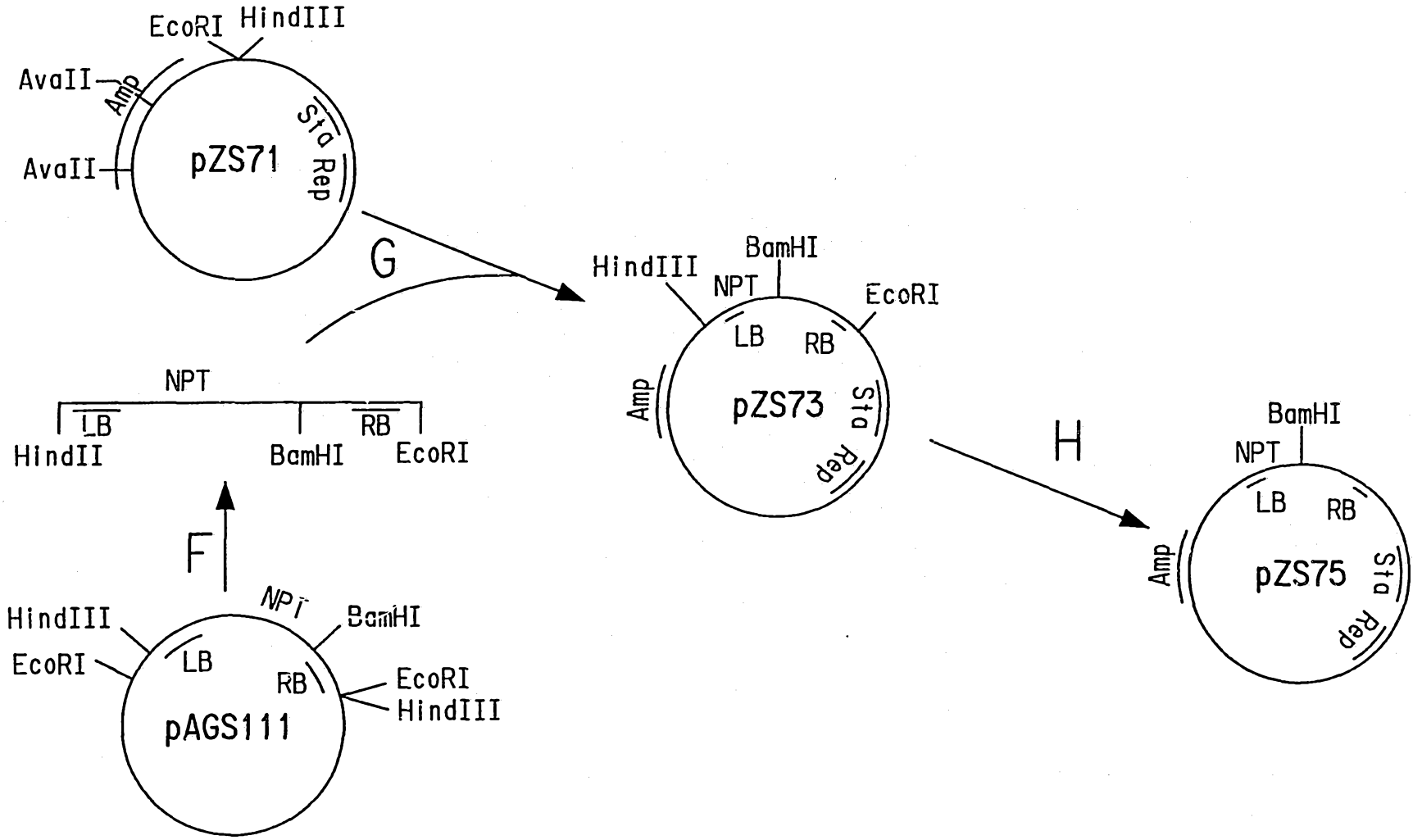

EcoRI
HindIII
BamHI
Sta
Rep
pZS68
Amp
PstI
C
AvaII
pUC19
Amp
AvaII
D
EcoRI
HindIII
Sta
Rep
pZS69
Amp
AvaII
PstI
AvaII
E
AvaII
AvaII
EcoRI
HindIII
Sta
Rep
pZS71
Amp
AvaII
AvaII

FIG.24C


EcoRI
HindIII
AvaII
Amp
AvaII
pZS71
Sta Rep
G
HindIII
BamHI
NPT
EcoRI
LB
RB
Amp
pZS73
Sta
Rep
H
BamHI
NPT
LB
RB
Amp
pZS75
Sta
Rep
NPT
LB
RB
HindII
BamHI
EcoRI
F
NPT
HindIII
BamHI
EcoRI
LB
RB
EcoRI
HindIII
pAGS111

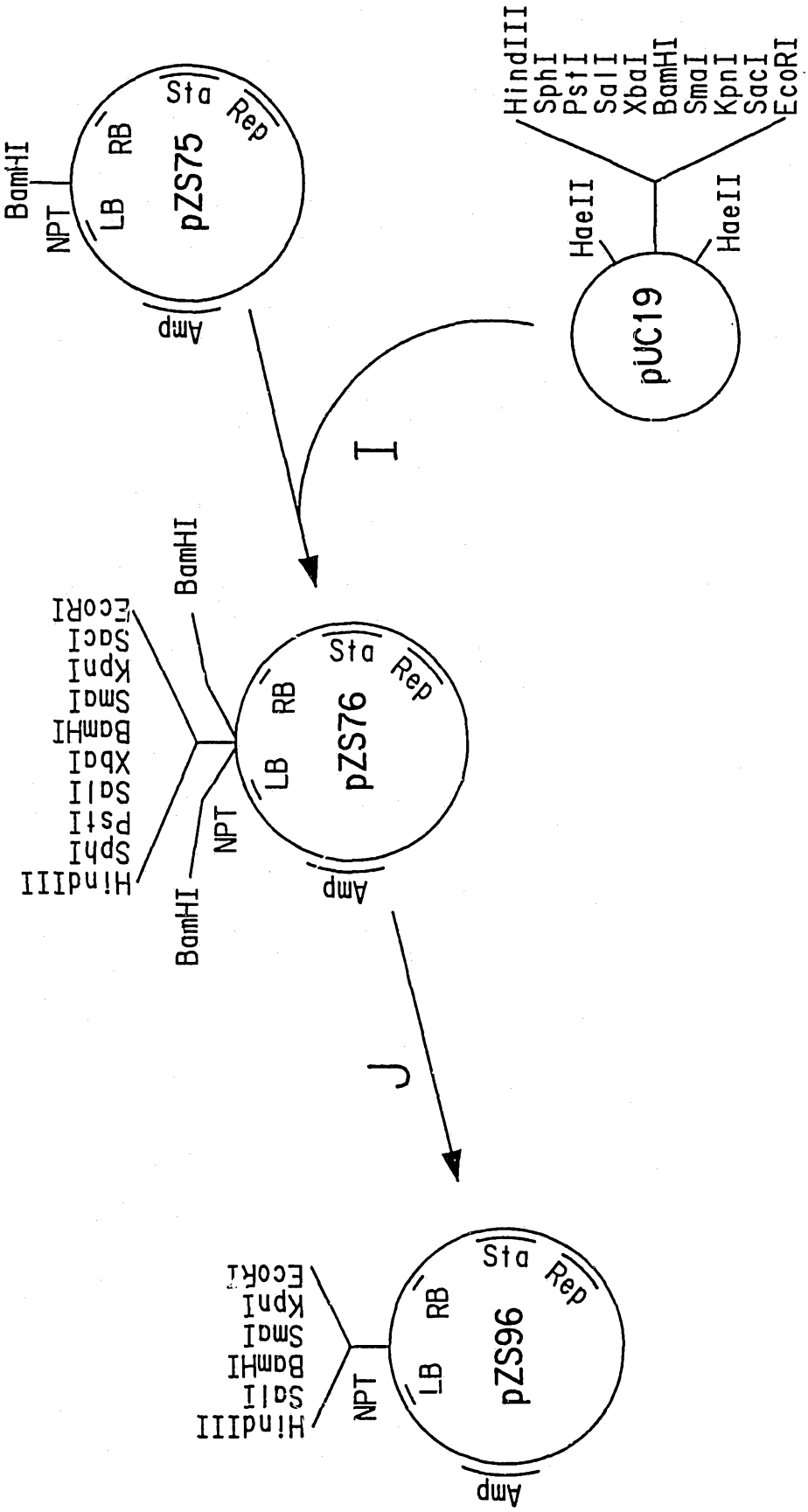

pZS75
Sta
Rep
RB
LB
NPT
BamHI
Amp
HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
HaeII
HaeII
pUC19
I
pZS76
BamHI
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
J
pZS96
HindIII
SalI
BamHI
SmaI
KpnI
EcoRI

FIG.24D

FIG.25A

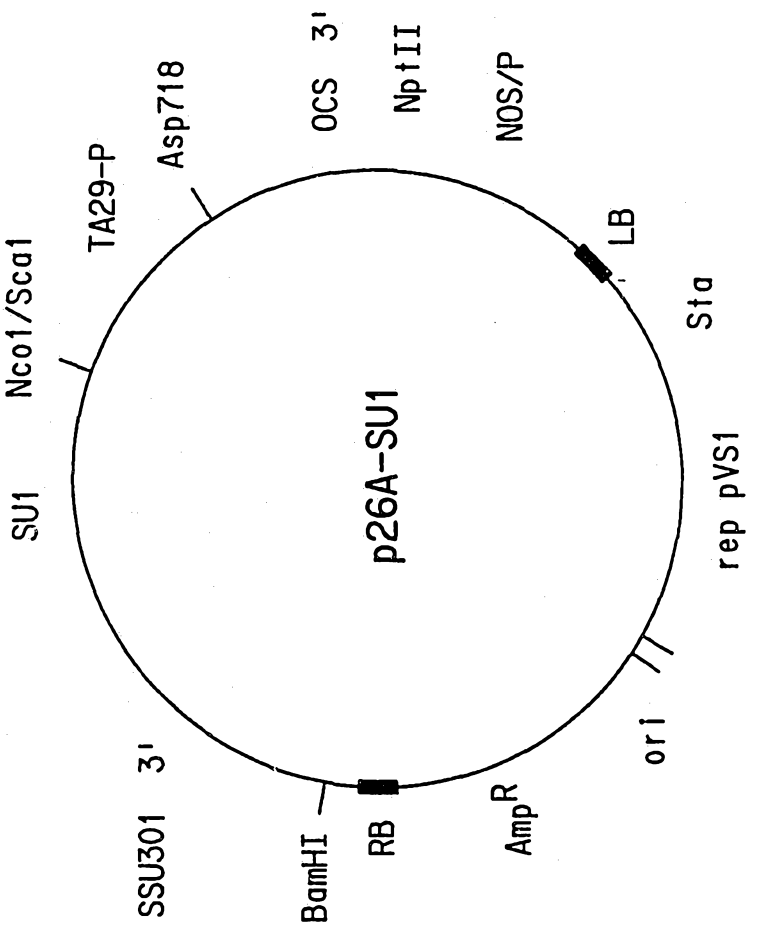
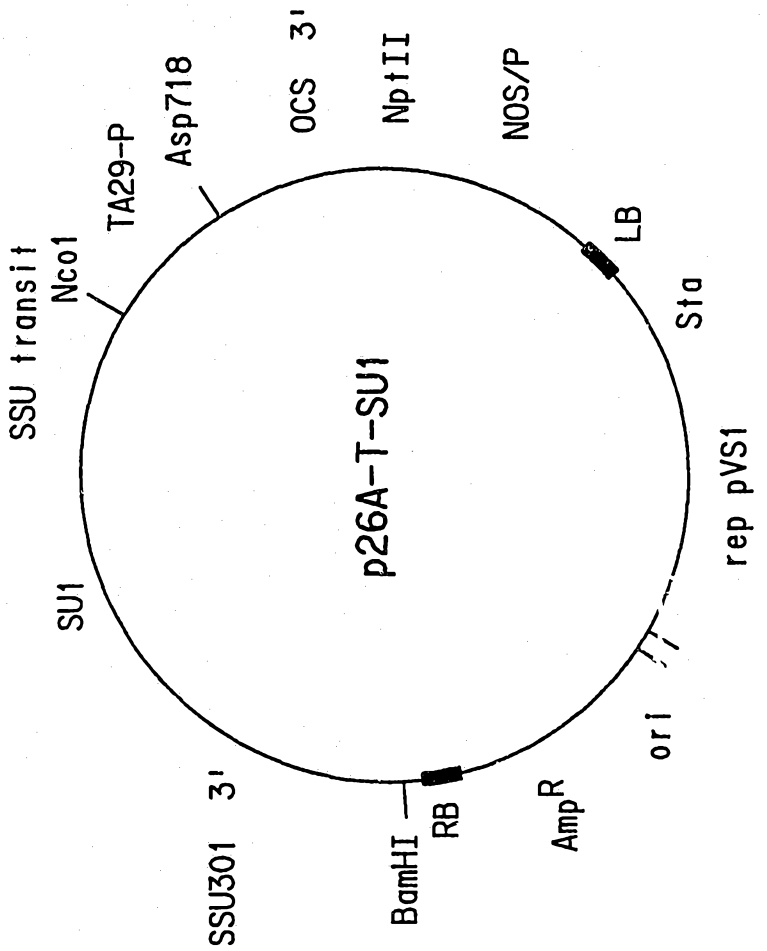

p26A-SU1
SU1
Nco1/Sca1
TA29-P
Asp718
OCS 3'
NptII
NOS/P
LB
Sta
rep pVS1
ori
AmpR
RB
BamHI
SSU301 3'

FIG.25B


p26A-T-SU1
SSU transit
Nco1
TA29-P
Asp718
OCS 3'
NptII
NOS/P
LB
Sta
rep pVS1
ori
AmpR
RB
BamHI
SSU301 3'
SU1

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/04785

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/53, C 12 N 15/82, A 01 H 5/00, C 12 N 9/02, C 12 N 1/21// (C 12 N 1/21, C 12 R 1:465

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| IPC[5] | C 12 N |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category [10] | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| P,X | Journal of Bacteriology, volume 172, no. 6, June 1990, American Society for Microbiology, (Baltimore, US), C.A. Omer et al.: "Genes for two herbicide-inducible cytochromes P-450 from streptomyces griseolus", pages 3335-3345 see the whole article -- | 1-2,14,16 |
| Y | Recent Advances in Phytochemistry, volume 21, 1987, (Amsterdam, NL) D.P. O'KEEFE et al.: " Plant and bacterial cytochromes P-450: involvement in herbicide metabolism", pages 151-173 see pages 160-166; figures 3,6 (cited in the application) -- | 1,2,14,16 |

./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 30th November 1990 | 20. 12. 90 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | R.J. Eernisse |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| Y | Patent Abstracts of Japan, volume 13, no. 171 (C-588)(3519) 24 April 1989 & JP, A, 642585 (SANKYO CO. LTD.) 6 January 1989 see the Abstract | 1-2,14,16 |
| Y | Journal of Biological Chemistry, volume 261, no. 3, January 1986, The American Society of Biological Chemists, Inc., (Baltimore, US), B.P. Unger et al.: "Nucleotide sequence of the pseudomonas putida cytochrome $P\text{-}450_{cam}$ gene and its expression in escherichia coli", pages 1158-1163 see the whole article (cited in the application) | 1-2,14,16 |
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# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9004785 SA 39648

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/12/90
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