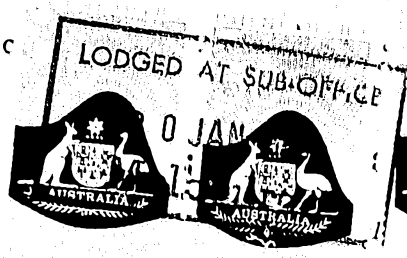
(CONVENTION. By c



Form 4

CONVENTION APPLICATION FOR A PATENT

FEE STAMP TO VALUE OF $ 110 ATTACHED
MAIL OFFICER

(1) Here insert (in full) Name or Names of Applicant or Applicants, followed by Address (es).

~~I~~/We (1) HOECHST AKTIENGESELLSCHAFT

of 45 Bruningstrasse, D6230 Frankfurt/Main 80,

Federal Republic of Germany

(2) Here insert Title of Invention.

hereby apply for the grant of a Patent for an invention entitled: (2)

PHOSPHINOTHRICIN-RESISTANCE GENE ACTIVE IN PLANTS, AND ITS USE

(3) Here insert number(s) of basic application(s)

which is described in the accompanying complete specification. This application is a Convention application and is based on the application numbered (3)

P37 01 624.5 and P37 37 918.6

(4) Here insert Name of basic Country or Countries, and basic date or dates

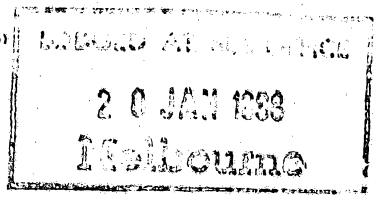
for a patent or similar protection made in (4) Federal Republic of Germany on 21st January 1987 and 7th November 1987

APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 25-1-91

~~My~~/Our address for service is Messrs. Edwd. Waters & Sons, Patent Attorneys, 50 Queen Street, Melbourne, Victoria, Australia.

DATED this 19th day of January 1988.

(5) Signature (s) of Applicant (s) or Seal of Company and Signatures of its Officers as prescribed by its Articles of



HOECHST AKTIENGESELLSCHAFT

by

* Hofheim am Taunus, a) - j) Federal Republic of Germany Form 7

COMMONWEALTH OF AUSTRALIA

Patents Act 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION UNDER PART XVI. FOR A PATENT.

In support of the Convention application made under Part XVI. of the Patents Act 1952 by HOECHST AKTIENGESELLSCHAFT of 45, Brüningstrasse, D-6230 Frankfurt/Main 80, Federal Republic of Germany for a patent for an invention entitled:

PHOSPHINOTHRICIN-RESISTANCE GENE ACTIVE IN PLANTS, AND ITS USE

We, Bernhard Beck, 4 Drosselweg, D-6246 Glashütten/Taunus, Franz Lapice, 2 Sandweg, D-6233 Kelkheim (Taunus), Federal Republic of Germany

do solemnly and sincerely declare as follows:

1. **We are authorized by HOECHST AKTIENGESELLSCHAFT the applicant for the patent to make this declaration on its behalf.**

2. **The basic application as defined by Section 141 of the Act was made in the Federal Republic of Germany under No.** P 37 01 624.5 **on** January 21, 1987 under No. P 37 37 918.6 on November 7, 1987 by HOECHST AKTIENGESELLSCHAFT

3. a) Eckhard Strauch, 2 Rosenheide, D-4800 Bielefeld, b) Walter Arnold, 25 Am Gottesberg, D-4800 Bielefeld, c) Renate Alijah, 14 Kösterkamp, D-4800 Bielefeld, d) Wolfgang Wohlleben, 1 Menzelstraße, D-4800 Bielefeld, e) Alfred Pühler, 2 Am Waldschlößchen, D-4800 Bielefeld, f) Peter Eckes, 18 Am Flachsland, D-6233 Kelkheim (Taunus), g) Günter Donn, 35 Sachsenring, D-6238 Hofheim am Taunus, h) Eugen Uhlmann, 31 Zum Talblick, D-6246 Glashütten/Taunus, i) Friedrich Hein, 40 Erlesring, D-6234 Hattersheim am Main, j) Friedrich Wengenmayer, 38 Am Seyenbach, D-6238*
is/are the actual inventor(s) of the invention and the facts upon which HOECHST AKTIENGESELLSCHAFT

is entitled to make the application are as follows:

The said HOECHST AKTIENGESELLSCHAFT

is the assignee of the said Eckhard Strauch, Walter Arnold, Renate Alijah, Wolfgang Wohlleben, Alfred Pühler, Peter Eckes, Günter Donn, Eugen Uhlmann, Friedrich Hein, Friedrich Wengenmayer

4. **The basic applications referred to in paragraph 2 of this Declaration were the first application made in a Convention country in respect of the invention the subject of the application.**

DECLARED at Frankfurt/Main, Federal Republic of Germany

this 5th day of January 1988.

To the Commissioner of Patents

HOECHST AKTIENGESELLSCHAFT

ppa. Beck Prokurist i.V. Lapice Authorized Signatory

PAT 510

(12) PATENT ABRIDGMENT (11) Document No. AU-B-10619/88
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 609082

(54) Title
PHOSPHINOTHRICIN-RESISTANCE GENE ACTIVE IN PLANTS, AND ITS USE

International Patent Classification(s)

(51)[4]	C12N 015/00	A01H 001/00	A01H 005/00	C07G 017/00
	C07H 021/04	C12N 001/20	C12N 001/21	C12N 005/00
	C12N 005/10	C12N 015/52	C12P 019/34	C12P 021/00

(21) Application No. : 10619/88 (22) Application Date : 20.01.88

(30) Priority Data

(31) Number	(32) Date	(33) Country
3701624	21.01.87	DE FEDERAL REPUBLIC OF GERMANY
3737918	07.11.87	DE FEDERAL REPUBLIC OF GERMANY

(43) Publication Date : 28.07.88

(44) Publication Date of Accepted Application : 26.04.91

(71) Applicant(s)
HOECHST AKTIENGESELLSCHAFT

(72) Inventor(s)
ECKHARD STRAUCH; WALTER ARNOLD; RENATE ALIJAH; WOLFGANG WOHLLEBEN; ALFRED PUHLER; PETER ECKES; GUNTER DONN; EUGEN UHLMANN; FRIEDRICH HEIN; FRIEDRICH WENGENMAYER

(74) Attorney or Agent
WATERMARK PATENT & TRADEMARK ATTORNEYS, Locked Bag 5, HAWTHORN VIC 3122

(56) Prior Art Documents
AU 77318/87 C07H 21/00
AU 16146/88 C12N 15/00
AU 18605/88 C07H 21/04

(57) The invention relates to a modification of the resistance gene which is proposed in German Patent Application P 36 28 747.4 and the additional application P 36 42 829.9, namely an adaptation to the codon usage in plants. The corresponding amino acid sequence is depicted in the annex.

.../2

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      MET SER PRO GLU ARG ARG PRO VAL GLU ILE ARG PRO ALA THR ALA ALA ASP MET ALA ALA VAL CYS ASP ILE VAL ASN HIS TYR
TC GAC ATG TCT CCG GAG AGG AGA CCA GTT GAG ATT AGG CCA GCT ACA GCA GCT GAT ATG GCC GCG GTT TGT GAT ATC GTT AAC CAT TAC
    G TAC AGA GGC CTC TCC TCT GGT CAA CTC TAA TCC GGT CGA TGT CGT CGA CTA TAC CGG CGC CAA ACA CTA TAG CAA TTG GTA ATG

1

ILE GLU THR SER THR VAL ASN PHE ARG THR GLU PRO GLN THR PRO GLN GLU TRP ILE ASP ASP LEU GLU ARG LEU GLN ASP ARG TYR PRO
ATT GAG ACG TCT ACA GTG AAC TTT AGG ACA GAG CCA CAA ACA CCA CAA GAG TGG ATT GAT GAT CTA GAG AGG TTG CAA GAT AGA TAC CCT
TAA CTC TGC AGA TGT CAC TTG AAA TCC TGT CTC GGT GTT TGT GGT GTT CTC ACC TAA CTA CTA GAT CTC TCC AAC GTT CTA TCT ATG GGA

100

TRP LEU VAL ALA GLU VAL GLU GLY VAL VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA ARG ASN ALA TYR ASP TRP THR VAL GLU
TGG TTG GTT GCT GAG GTT GAG GGT GTT GTG GCT GGT ATT GCT TAC GCT GGG CCC TGG AAG GCT AGG AAC GCT TAC GAT TGG ACA GTT GAG
ACC AAC CAA CGA CTC CAA CTC CCA CAA CAC CGA CCA TAA CGA ATG CGA CCC GGG ACC TTC CGA TCC TTG CGA ATG CTA ACC TGT CAA CTC

200

SER THR VAL TYR VAL SER HIS ARG HIS GLN ARG LEU GLY LEU GLY SER THR LEU TYR THR HIS LEU LEU LYS SER MET GLU ALA GLN GLY
AGT ACT GTT TAC GTG TCA CAT AGG CAT CAA AGG TTG GGC CTA GGA TCC ACA TTG TAC ACA CAT TTG CTT AAG TCT ATG GAG GCG CAA GGT
TCA TGA CAA ATG CAC AGT GTA TCC GTA GTT TCC AAC CCG GAT CCT AGG TGT AAC ATG TGT GTA AAC GAA TTC AGA TAC CTC CGC GTT CCA

300

PHE LYS SER VAL VAL ALA VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG LEU HIS GLU ALA LEU GLY TYR THR ALA ARG GLY THR LEU
TTT AAG TCT GTG GTT GCT GTT ATA GGC CTT CCA AAC GAT CCA TCT GTT AGG TTG CAT GAG GCT TTG GGA TAC ACA GCC CGG GGT ACA TTG
AAA TTC AGA CAC CAA CGA CAA TAT CCG GAA GGT TTG CTA GGT AGA CAA TCC AAC GTA CTC CGA AAC CCT ATG TGT CGG GCC CCA TGT AAC

400

ARG ALA ALA GLY TYR LYS HIS GLY GLY TRP HIS ASP VAL GLY PHE TRP GLN ARG ASP PHE GLU LEU PRO ALA PRO PRO ARG PRO VAL ARG
CGC GCA GCT GGA TAC AAG CAT GGT GGA TGG CAT GAT GTT GGT TTT TGG CAA AGG GAT TTT GAG TTG CCA GCT CCT CCA AGG CCA GTT AGG
GCG CGT CGA CCT ATG TTC GTA CCA CCT ACC GTA CTA CAA CCA AAA ACC GTT TCC CTA AAA CTC AAC GGT CGA GGA GGT TCC GGT CAA TCC

500

PRO VAL THR GLN ILE ---
CCA GTT ACC CAG ATC TGA G
GGT CAA TGG GTC TAG ACT CAG CT
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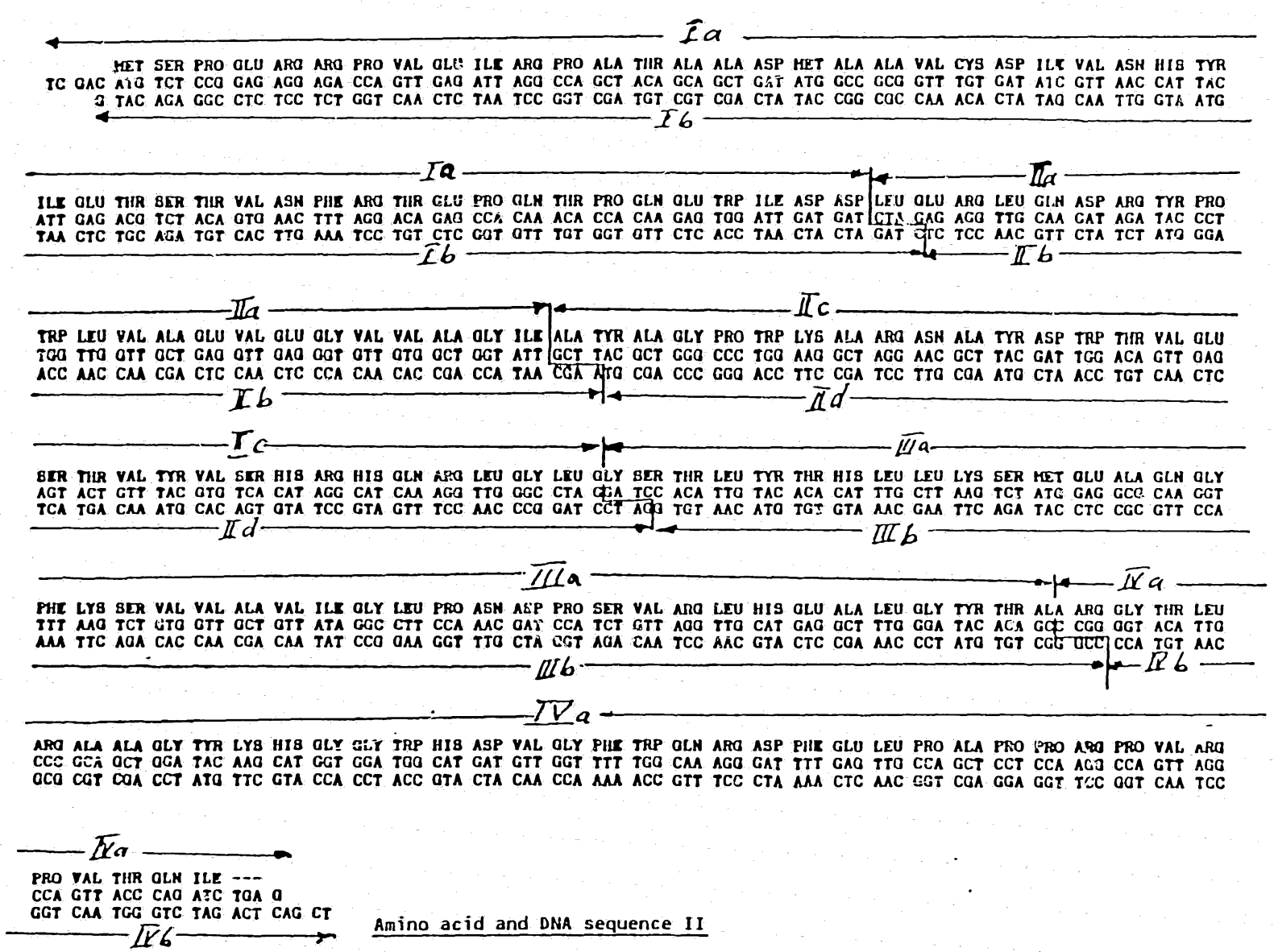
Amino acid and DNA sequence I



Amino acid and DNA sequence II

CLAIM

1. A resistance gene coding for the protein of amino acid sequence I as herein defined, which gene is adapted to the codon usage in plants.

6. Vectors containing one or more of gene fragments I - IV as herein defined.

COMMONWEALTH OF AUSTRALIA 609082 Form 10

PATENTS ACT 1952-69

COMPLETE SPECIFICATION

(ORIGINAL)

Class Int. Class

Application Number:
Lodged:

Complete Specification Lodged:
Accepted:
Published:

Priority :

Related Art :

Name of Applicant : HOECHST AKTIENGESELLSCHAFT

Address of Applicant : 45 Bruningstrasse, D6230 Frankfurt/Main 80, Federal Republic of Germany

Actual Inventor: ECKHARD STRAUCH, WALTER ARNOLD, RENATE ALIJAH, WOLFGANG WOHLLEBEN, ALFREDPUHLER, PETER ECKES, GUNTER DONN, EUGEN UHLMANN, FRIEDRICH HEIN, FRIEDRICH WENGENMAYER

Address for Service : EDWD. WATERS & SONS,
50 QUEEN STREET, MELBOURNE, AUSTRALIA, 3000.

Complete Specification for the invention entitled:

PHOSPHINOTHRICIN-RESISTANCE GENE ACTIVE IN PLANTS, AND ITS USE

The following statement is a full description of this invention, including the best method of performing it known to :- us

HOECHST AKTIENGESELLSCHAFT HOE 87/F 333 J Dr. KL/mü

Specification

Phosphinothricin-resistance gene active in plants, and its use

Non prior-published German Patent Application P 36 28 747.4 ("main application") proposes a phosphinothricin (PTC)-resistance gene which can be obtained from the total DNA of Streptomyces viridochromogenes DSM 40736 (general collection) or DSM 4112 (deposition under the Budapest Treaty), which has been selected for phosphinothricyl-alanyl-alanine (PTT)-resistance, by cutting with BamHI, cloning of a fragment 4.0 kb in size, and selection for PTT-resistance, as well as the use of this gene for the production of PTC-resistant plants, as PTT-resistance marker in bacteria and PTC-resistance marker in plant cells. The BamHI fragment which is 4 kb in size and on which the resistance gene is located is defined in detail by a restriction map (Figure 1).

The position of the coding region has been located more accurately by cloning part-regions of this 4 kb fragment. It emerged from this that the resistance gene is located on the 1.6 kb SstII-SstI fragment (positions 0.55 to 2.15 in Fig. 1 of the main application). Digestion with BglII results in a fragment 0.8 kb in size which confers PTT-resistance after incorporation in a plasmid and transformation of S. lividans. This resistance depends on the N-acetylation of PTC. Hence the resistance gene codes for an acetyltransferase.

The DNA sequence of the abovementioned 0.8 kb fragment is reproduced in the German application for a patent of addition P 36 42 829.9.

It is possible to determine from the sequence the start codon and the open reading frame of the gene sequence. The last nucleotide is part of the stop codon TGA.

Genes from Streptomycetes have a very high proportion of G + C, the adenine (A) + thymine (T) : guanine (G) +

cytosine (C) ratio being about 30 : 70. The proportion of GC in plant genes is far lower, being about 50%. For this reason, in a further development of the inventive idea, the DNA sequence of the resistance gene has been optimized, by de novo synthesis, to a codon usage favorable for plant RNA polymerase II.

The invention relates to a modification of the resistance gene which is proposed in German Patent Application P 36 28 747.4 and the additional application P 36 42 829.9, namely an adaptation to the codon usage in plants. The corresponding amino acid sequence is depicted in the annex. Further embodiments of the invention are defined in the patent claims or are explained hereinafter.

As is known, the genetics code is degenerate, i.e. only 2 amino acids are coded for by a single triplet, whereas the remaining 18 genetically codable amino acids are assigned to 2 to 6 triplets. Thus, theoretically, a wide variety of codon combinations can be chosen for the synthesis of the gene. Since the said relative proportion of the individual nucleotides in the total DNA sequence exerts an influence, it was used as one of the criteria on which the sequence optimization was based.

The following modifications were made to the sequenced gene:

1. The Streptomycetes gene start codon GTG (position 258-260 in the sequence in the additional application) was replaced by the start codon ATG which is used by plant RNA polymerase II.
2. Within the gene, the Streptomycetes gene codons were changed in such a way that they resulted in codons suitable in plant genes (G/C ratio).
3. The TGA stop codon was placed at the end of the sequence to terminate the translation process.
4. The beginning and end of the gene sequence were provided with protruding ends of restriction sites in order to be able to amplify the gene and ligate

it between plant regulation sequences.

5. Palindromic sequences were reduced to a minimum.

The DNA sequence I according to the invention (with the corresponding amino acid sequence) is depicted in the annex.

Three internal unique cleavage sites for the restriction enzymes XbaI (position 152), BamHI (312) and XmaI (436) make possible the subcloning of part-sequences which can be incorporated in well-investigated cloning vectors such as, for example, pUC18 or pUC19. In addition, a number of other unique recognition sequences for restriction enzymes were incorporated within the gene, and these, on the one hand, provide access to part-sequences of acetyltransferase and, on the other hand, allow modifications to be made:

Restriction enzyme	Cut after nucleotide No. (codings strand)
BspMII	11
SacII	64
EcoRV	74
HpaI	80
AatII	99
BstXI	139
ApaI	232
ScaI	272
AvrII	308
AflII	336
StuI	385
BssHII	449
FokI	487
BglI	536
BglII	550

The construction of part-sequences by chemical synthesis and enzymatic ligation reactions is carried out in a manner known per se (EP-A 0,133,282, 0,136,472, 0,155,590,

0,161,504, 0,163,249, 0,171,024, 0,173,149 or 0,177,827). Details, such as restriction analyses, ligation of DNA fragments and transformation of plasmids in E. coli, are described at length in the textbook of Maniatis (Molecular Cloning, Maniatis et al., Cold Spring Harbor, 1982).

The gene sequence which has been cloned in this way is then introduced into plants, under the control of plant regulation signals, and its expression is induced. EP-A 0,122,791 reviews known methods. In this way are obtained PTC-resistant plant cells (i.e. a selection feature for transformed cells is available), plants or parts of plants and seeds.

Some embodiments of the invention are explained in detail in the examples which follow. Unless otherwise indicated, percentage data therein relate to weight.

Examples

The following media were used:

a) for bacteria:

YT medium: 0.5% yeast extract, 0.8% Bacto tryptone, 0.5% NaCl

LB medium: 0.5% yeast extract, 1% Bacto tryptone, 1% NaCl

as solid medium: addition of 1.5% agar to each

b) for plants:

M+S medium: see Murashige and Skoog, Physiologica Plantarum 15 (1962) 473

2MS medium: M+S medium containing 2% sucrose

MSC10 medium: M+S medium containing 2% sucrose, 500 mg/l cefotaxime, 0.1 mg/l naphthylacetic acid (NAA), 1 mg/l benzylaminopurine (BAP), 100 mg/l kanamycin

MSC15 medium: M+S medium containing 2% sucrose,

500 mg/l cefotaxime, 100 mg/l kanamycin.

1. Chemical synthesis of a single-stranded oligonucleotide

The synthesis of fragment II, one of the four part-fragments I - IV, started from the terminal oligonucleotide IIc (nucleotide No. 219 to 312 in the coding strand of DNA sequence I). For the solid-phase synthesis, the nucleoside at the 3' end, that is to say guanosine (nucleotide No. 312) in the present case, is covalently bonded via the 3'-hydroxyl group to a support. The support material is CPG (controlled pore glass) functionalized with long-chain aminoalkyl radicals. Otherwise, the synthesis follows the known (from the said EP-As) methods.

The plan of synthesis is indicated in DNA sequence II (annex), which otherwise corresponds to DNA sequence I.

2. Enzymatic linkage of the single-stranded oligonucleotides to give gene fragment II

For the phosphorylation of the oligonucleotides at the 5' end, 1 nmol of each of oligonucleotides IIb and IIc was treated with 5 nmol of adenosine triphosphate and 4 units of T4 polynucleotide kinase in 20 µl of 50 mM tris-HCl buffer (pH 7.6), 10 mM magnesium chloride and 10 mM dithiothreitol (DTT) at 37°C for 30 minutes. The enzyme is inactivated by heating at 95°C for 5 minutes. Oligonucleotides IIa and IId, which form the "protruding" sequence in DNA fragment II, are not phosphorylated. This prevents the formation, during the subsequent ligation, of larger subfragments than correspond to DNA fragment II.

Oligonucleotides II (a-d) are ligated to give subfragment II as follows: 1 nmol of each of oligonucleotides IIa and IId and the 5'-phosphates of IIb and IIc are together dissolved in 45 µl of buffer containing 50 mM tris-HCl (pH 7.6), 20 mM magnesium chloride, 25 mM potassium chloride and 10 mM DTT. For the annealing of the oligonucleotides corresponding to DNA fragment II the solution of the oligonucleotides is heated at 95°C for 2 minutes and then slowly cooled (2-3 hours) to 20°C. Then, for the enzymatic linkage, 2 µl of 0.1 M DTT, 8 µl of 2.5 mM adenosine triphosphate (pH 7) and 5 µl of T4 DNA ligase (2000 units) are added, and the mixture is incubated at 22°C for 16 hours.

The gene fragment II is purified by gel electrophoresis on a 10% polyacrylamide gel (without addition of urea, 20 x 40 cm, 1 mm thick), the marker substance used being ØX 174 DNA (from BRL) cut with HinfI, or pBR322 cut with HaeIII.

Gene fragments I, III and IV are prepared analogously, although the "protruding" sequences are, before the annealing, converted into the 5'-phosphates because no ligation step is necessary.

3. Preparation of hybrid plasmids containing gene fragments I, II, III and IV.

a) Incorporation of gene fragment I in pUC18

The commercially available plasmid pUC18 is opened in a known manner using the restriction endo nucleases SalI and XbaI in accordance with the manufacturers' instructions. The digestion mixture is fractionated by electrophoresis in a known manner on a 1% agarose gel, and the fragments are visualized by staining with ethidium bromide. The plasmid band (about 2.6 kb) is then cut out of the agarose

gel and removed from the agarose by electro-elution.

1 µg of plasmid, opened with XbaI and SalI, is then ligated with 10 ng of DNA fragment I at 16°C overnight.

b) Incorporation of gene fragment II in pUC18.

In analogy to a), pUC18 is cut open with XbaI and BamHI and ligated with gene fragment II which has previously been phosphorylated at the protruding ends as described in Example 2.

c) Incorporation of gene fragment III in pUC18

In analogy to a), pUC18 is cut open with BamHI and XmaIII and ligated with gene fragment III.

d) Incorporation of gene fragment IV in pUC18

In analogy to a), pUC18 is cut with XmaIII and SalI and ligated with gene fragment IV.

4. Construction of the complete gene and cloning in a pUC plasmid

a) Transformation and amplification of gene fragments I - IV

The hybrid plasmids obtained in this way are transformed into E. coli. For this purpose, the strain E. coli K 12 is made competent by treatment with a 70 mM calcium chloride solution, and the suspension of the hybrid plasmid in 10 mM tris-HCl buffer (pH 7.5), which is 70 mM in calcium chloride, is added. The transformed strains are selected as is customary, utilizing the antibiotic resistances or sensitivities conferred by

the plasmid, and the hybrid vectors are amplified. After the cells have been killed, the hybrid plasmids are isolated and cut open with the restriction enzymes originally used, and gene fragments I, II, III and IV are isolated by gel electrophoresis.

b) Linkage of gene fragments I, II, III and IV to give the total gene

Subfragments I and II obtained by amplification are linked as follows. 100 ng of each of the isolated fragments I and II are dissolved together in 10 µl of buffer containing 50 mM tris-HCl (pH 7.6), 20 mM magnesium chloride and 10 mM DTT, and this solution is heated at 57°C for 5 minutes. After the solution has cooled to room temperature, 1 µl of 10 mM adenosine triphosphate (pH 7) and 1 µl of T4 ligase (400 units) are added, and the mixture is incubated at room temperature for 16 hours. After subsequent cutting with the restriction enzymes SalI and BamHI, the desired 312 bp fragment (nucleotides 1-312, SalI-BamHI) is purified by gel electrophoresis on an 8% polyacrylamide gel, the marker substance used being ØX 174 RF DNA (from BRL) cut with the restriction enzyme HaeIII.

Gene fragments III and IV are linked together in the same way, there being obtained after purification a 246 bp fragment (nucleotides 313-558, BamHI-SalI). The marker used for the gel electrophoresis is pBR322 cut with the restriction enzyme MspI.

To contruct the total gene (DNA sequence I), 15 ng of the 312 bp fragment and 12 ng of the 246 bp fragment are ligated, as described above, with 1 µg of the commercially available plasmid pUC18

which has previously been cut open with the restriction enzyme SalI and enzymatically dephosphorylated at the ends. After transformation and amplification (as described in Example 4a), the correct clone having the 558 bp fragment corresponding to DNA sequence I is identified by SalI digestion.

5. Transformation of the hybrid plasmids

Competent E. coli cells are transformed with 0.1 to 1 µg of the hybrid plasmid containing DNA sequence I, and are plated out on amplicillin-containing agar plates. It is then possible to identify clones which contain the correctly integrated sequences in the plasmid by rapid DNA analysis (Maniatis loc. cit.).

6. Fusion of the synthetic gene to regulation signals which are recognized in plants.

The optimized resistance gene which had been provided at the ends with SalI cleavage sites was ligated in the SalI cleavage site of the polylinker sequence of the plasmid pDH51 (Pietrzak et al., Nucleic Acids Res. 14 (1986) 5857). The promoter and terminator of the 35S transcript from cauliflower mosaic virus, which are recognized by the plant transcription apparatus, are located on this plasmid. The ligation of the resistance gene resulted in it being inserted downstream of the promoter and upstream of the terminator of the 35S transcript. The correct orientation of the gene was confirmed by restriction analyses.

The promoter of the ST-LS1 gene from Solanum tuberosum (Eckes et al., Mol. Gen. Genet. 205 (1986) 14) was likewise used for the expression of the optimized acetyltransferase gene in plants.

7. Insertion of the resistance gene having the regulation sequences into Agrobacterium tumefaciens

a) Cointegrate method

The entire transcription unit comprising promoter, optimized resistance gene and terminator (Example 6) was cut out with the restriction enzyme EcoRI and ligated in the EcoRI cleavage site of the intermediary E. coli vector pMPK110 (Peter Eckes, Thesis, Univ. Cologne, 1985, pages 91 et seq.). This intermediary vector was necessary for the transfer of the resistance gene with its regulation sequences into the Ti plasmid of Agrobacterium tumefaciens. This so-called conjugation was carried out by the method described by Van Haute et al. (EMBO J. 2 (1983) 411). This entailed the gene with its regulation signals being integrated in the Ti plasmid by homologous recombination via the sequences of the standard vector pBR322 which are present in the pMPK110 vector and in the Ti plasmid pGV3850kanR (Jones et al., EMBO J. 4 (1985) 2411).

For this purpose, 50 µl of fresh liquid cultures of each of the E. coli strains DH1 (host strain of the pMPK110 derivative) and GJ23 (Van Haute et al., Nucleic Acids Res. 14 (1986) 5857) were mixed on a dry YT-agar plate and incubated at 37°C for one hour. The bacteria were resuspended in 3 ml of 10 mM $MgSO_4$ and plated out on antibiotic-agar plates (spectinomycin 50 µg/ml: selection for pMPK110; tetracycline 10 µg/ml: selection for R64drd11; kanamycin 50 µg/ml: selection for pGJ28). The bacteria growing on the selective agar plates contained the three plasmids and were grown for the conjugation with Agrobacterium tumefaciens in YT liquid medium at 37°C. The Agrobacteria were cultivated in LB medium at 28°C.

50 µl of each bacterium suspension were mixed on a dry YT-agar plate and incubated at 28°C for 12 to 16 hours. The bacteria were resuspended in 3 ml of 10 mM $MgSO_4$ and plated out on antibiotic plates (erythromycin 0.05 g/l, chloramphenicol 0.025 g/l: selection for the Agrobacterium strain; streptomycin 0.03 g/l and spectinomycin 0.1 g/l: selection for integration of pMPK110 in the Ti plasmid). Only Agrobacteria in which the pMPK110 derivative has been integrated into the bacterial Ti plasmid by homologous recombination are able to grow on these selected plates.

Besides the gene for resistance to the antibiotic kanamycin, which is active in plants and was already present from the outset, the PTC-resistance gene was now also located on the Ti plasmid pGV3850kanR. Before these Agrobacterium clones were used for transformation, a Southern blot experiment was carried out to check whether the desired integration had taken place.

b) Binary vector method

The binary vector system described by Koncz et al. (Mol. Gen. Genet. 204 (1986) 383) was used. The vector pPCV701 described by Koncz et al. (PNAS 84 (1987) 131) was modified in the following way: the restriction enzymes BamHI and HindIII were used to delete from the vector a fragment on which are located, inter alia, the TR1 and TR2 promoters. The resulting plasmid was recircularized. Into the EcoRI cleavage site present on this vector was inserted a fragment from the vector pDH51 which is about 800 base-pairs in length and on which were located the promoter and terminator of the 35S transcript from cauliflower mosaic virus (Pietrzak et al., Nucleic Acids Res. 14 (1986) 5858). The resulting plasmid pPCV801

had a unique SalI cleavage site between the 35S promoter and terminator. The optimized PTC-resistance gene was inserted into this cleavage site. Its expression was now under the control of the 35S transcript regulation sequences.

This plasmid (pPCV801Ac) was transformed into the E. coli strain SM10 (Simon et al., Bio/Technology 1 (1983) 784). For the transfer of the plasmid pPCV801Ac into Agrobacterium tumefaciens, 50 µl of both the SM10 culture and a C58 Agrobacterium culture (GV3101, Van Larebeke et al., Nature 252 (1974) 169) were mixed with the Ti plasmid pMP90RK (Koncz et.al.Mol.Grn.Genet.204(1986)383 as helper plasmid on a dry YT-agar plate, and the mixture was incubated at 28°C for about 16 hours. The bacteria were then resuspended in 3 ml of 1 mM $MgSO_4$ and plated out on antibiotic plates (rifampicin 0.1 g/l: selection for GV3101, kanamycin 0.025 g/l: selection for pMP90RK, carbenicillin 0.1 g/l: selection for pPCV801Ac). Only Agrobacteria which contained both plasmids (pMP90RK and pPCV801Ac) are able to grow on these plates. Before these Agrobacteria were used for the plant transformation, Southern blotting was carried out to check that the plasmid pPCV801Ac is present in its correct form in the Agrobacteria.

8. Transformation of Nicotina tabacum by Agrobacterium tumefaciens

The optimized resistance gene was transferred into tobacco plants using the so-called leaf disk transformation method.

The Agrobacteria were cultured in 30 ml of LB medium containing the appropriate antibiotics at 28°C, shaking continuously (about 3 days). The bacteria were then sedimented by centrifugation at 7000 rpm in a

Christ centrifuge for 10 minutes, and were washed once with 20 ml of 10 mM $MgSO_4$. After a further centrifugation, the bacteria were suspended in 20 ml of 10 mM $MgSO_4$ and transferred into a Petri dish. Leaves of Wisconsin 38 tobacco plants growing on 2MS medium in sterile culture were used for the leaf disk infection. All the sterile cultures were maintained at 25 to 27°C in a 16 hours light/8 hours dark rhythm under white light.

Tobacco leaves were cut off, and the leaf surfaces were lacerated with sandpaper. After the laceration, the leaves were cut into smaller pieces and dipped in the bacterium culture. The leaf pieces were then transferred to M+S medium and maintained under normal culture conditions for two days. After the 2-day infection with the bacteria, the leaf pieces were washed in liquid M+S medium and transferred to MSC10-agar plates. Transformed shoots were selected on the basis of the resistance to the antibiotic kanamycin which had also been transferred. The first shoots became visible 3 to 6 weeks later. Individual shoots were further cultivated on MSC15 medium in glass jars. In the weeks which followed, some of the shoots which had been cut off developed roots at the site of the cut.

It was also possible to select transformed plants directly on PTC-containing plant media. The presence and the expression of the PTC-resistance gene was demonstrated by DNA analysis (Southern blotting) and RNA analysis (Northern blotting) of the transformed plants.

9. Demonstration of the PTC-resistance of the transformed plants

To check the functioning of the resistance gene in transformed plants, leaf fragments from transformed

and non-transformed plants were transferred to M+S nutrient media containing 1 x 10^{-4} M L-PTC. The fragments from non-transformed plants died, while the fragments from transformed plants were able to regenerate new shoots. Transformed shoots took root and grew without difficulty on M+S nutrient media containing 1 x 10^{-3} M L-PTC. Transformed plants were, from sterile conditions, potted in soil and sprayed with 2 kg/ha and 5 kg/ha PTC. Whereas non-transformed plants did not survive this herbicide treatment, transformed plants showed no damage brought about by the herbicide. The appearance and growth behavior of the sprayed transformed plants was at least as good as that of unsprayed control plants.

10. Acetyltransferase assay to demonstrate acetylation of PTC in transgenic PTC-resistant plants

About 100 mg of leaf tissue from transgenic PTC-resistant tobacco plants or from non-transformed tobacco plants were homogenized in a buffer composed of: 50 mM tris-HCl, pH 7.5; 2 mM EDTA; 0.1 mg/ml leupeptin; 0.3 mg/ml bovine serum albumin; 0.3 mg/ml DTT; 0.15 mg/ml phenylmethylsulfonyl fluoride (PMSF).

After subsequent centrifugation, 20 µl of the clear supernatant were incubated with 1 µl of 10 mM radio-labelled D,L-PTC and 1 µl of 100 mM acetyl-CoA at 37°C for 20 minutes. 25 µl of 12% trichloroacetic acid were then added to the reaction mixture, followed by centrifugation. 7 µl of the supernatant were transferred to a thin-layer chromatography plate and subjected to ascending development twice in a mixture of pyridine : n-butanol : acetic acid : water (50 : 75 : 15 : 60 parts by volume). PTC and acetyl-PTC were separated from one another in this way, and could be detected by autoradiography. Non-transformed plants exhibited no conversion of PTC into acetyl-PTC, whereas transgenic resistant plants were capable of this.

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      MET SER PRO GLU ARG ARG PRO VAL GLU ILE ARG PRO ALA THR ALA ALA ASP MET ALA ALA VAL CYS ASP ILE VAL ASN HIS TYR
TC GAC ATG TCT CCG GAG AGG AGA CCA GTT GAG ATT AGG CCA GCT ACA GCA GCT GAT ATG GCC GCG GTT TGT GAT ATC GTT AAC CAT TAC
    G TAC AGA GGC CTC TCC TCT GGT CAA CTC TAA TCC GGT CGA TGT CGT CGA CTA TAC CGG CGC CAA ACA CTA TAG CAA TTG GTA ATG

1

ILE GLU THR SER THR VAL ASN PHE ARG THR GLU PRO GLN THR PRO GLN GLU TRP ILE ASP ASP LEU GLU ARG LEU GLN ASP ARG TYR PRO
ATT GAG ACG TCT ACA GTG AAC TTT AGG ACA GAG CCA CAA ACA CCA CAA GAG TGG ATT GAT GAT CTA GAG AGG TTG CAA GAT AGA TAC CCT
TAA CTC TGC AGA TGT CAC TTG AAA TCC TGT CTC GGT GTT TGT GGT GTT CTC ACC TAA CTA CTA GAT CTC TCC AAC GTT CTA TCT ATG GGA

100

TRP LEU VAL ALA GLU VAL GLU GLY VAL VAL ALA GLY ILE ALA TYR ALA GLY PRO TRP LYS ALA ARG ASN ALA TYR ASP TRP THR VAL GLU
TGG TTG GTT GCT GAG GTT GAG GGT GTT GTG GCT GGT ATT GCT TAC GCT GGG CCC TGG AAG GCT AGG AAC GCT TAC GAT TGG ACA GTT GAG
ACC AAC CAA CGA CTC CAA CTC CCA CAA CAC CGA CCA TAA CGA ATG CGA CCC GGG ACC TTC CGA TCC TTG CGA ATG CTA ACC TGT CAA CTC

200

SER THR VAL TYR VAL SER HIS ARG HIS GLN ARG LEU GLY LEU GLY SER THR LEU TYR THR HIS LEU LEU LYS SER MET GLU ALA GLN GLY
AGT ACT GTT TAC GTG TCA CAT AGG CAT CAA AGG TTG GGC CTA GGA TCC ACA TTG TAC ACA CAT TTG CTT AAG TCT ATG GAG GCG CAA GGT
TCA TGA CAA ATG CAC AGT GTA TCC GTA GTT TCC AAC CCG GAT CCT AGG TGT AAC ATG TGT GTA AAC GAA TTC AGA TAC CTC CGC GTT CCA

300

PHE LYS SER VAL VAL ALA VAL ILE GLY LEU PRO ASN ASP PRO SER VAL ARG LEU HIS GLU ALA LEU GLY TYR THR ALA ARG GLY THR LEU
TTT AAG TCT GTG GTT GCT GTT ATA GGC CTT CCA AAC GAT CCA TCT GTT AGG TTG CAT GAG GCT TTG GGA TAC ACA GCC CGG GGT ACA TTG
AAA TTC AGA CAC CAA CGA CAA TAT CCG GAA GGT TTG CTA GGT AGA CAA TCC AAC GTA CTC CGA AAC CCT ATG TGT CGG GCC CCA TGT AAC

400

ARG ALA ALA GLY TYR LYS HIS GLY GLY TRP HIS ASP VAL GLY PHE TRP GLN ARG ASP PHE GLU LEU PRO ALA PRO PRO ARG PRO VAL ARG
CGC GCA GCT GGA TAC AAG CAT GGT GGA TGG CAT GAT GTT GGT TTT TGG CAA AGG GAT TTT GAG TTG CCA GCT CCT CCA AGG CCA GTT AGG
GCG CGT CGA CCT ATG TTC GTA CCA CCT ACC GTA CTA CAA CCA AAA ACC GTT TCC CTA AAA CTC AAC GGT CGA GGA GGT TCC GGT CAA TCC

500

PRO VAL THR GLN ILE ---
CCA GTT ACC CAG ATC TGA G
GGT CAA TGG GTC TAG ACT CAG CT
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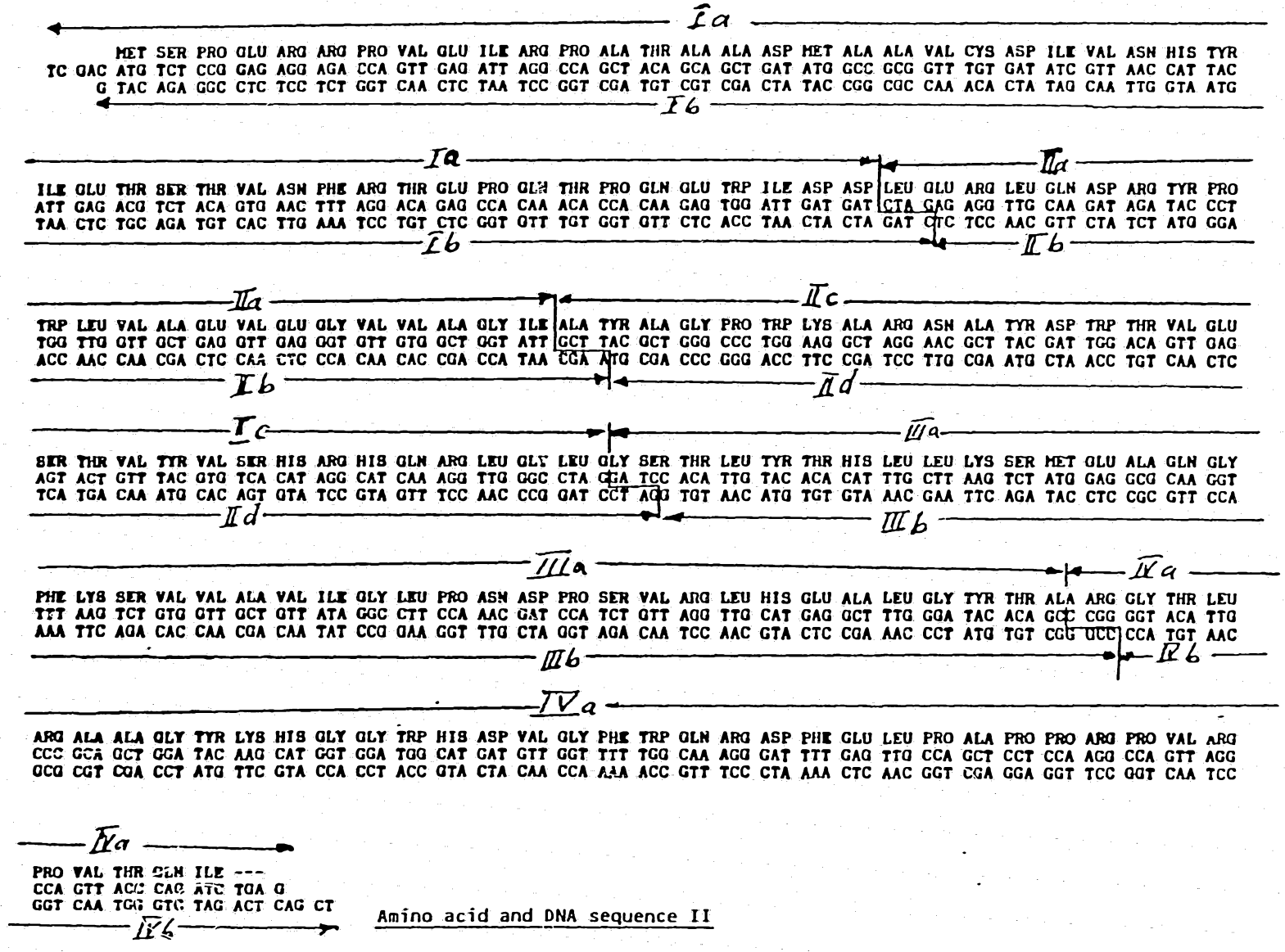
Amino acid and DNA sequence I



Amino acid and DNA sequence II

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A resistance gene coding for the protein of amino acid sequence I as herein defined, which gene is adapted to the codon usage in plants.

2. The resistance gene as claimed in claim 1, having DNA sequence I, nucleotide positions 9-554.

3. A gene structure having DNA sequence I as herein defined coupled to regulation and expression signals active in plants.

4. A vector containing the resistance gene as claimed in claim 1 or 2.

5. A vector containing a gene structure as claimed in claim 3.

6. Vectors containing one or more of gene fragments I - IV as herein defined.

7. A host cell containing a vector as claimed in claim 4, 5 or 6.

8. A plant cell containing a gene as claimed in claim 1, 2 or 3.

9. Plants, their parts and seeds containing a gene as claimed in claim 1, 2 or 3.

10. The use of the gene as claimed in claim 1 or 2 or of the gene structure as claimed in claim 3 for generating phosphinothricin-resistant plant cells, parts of plants, plants and seeds.

DATED this 24th day of December, 1990

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