

German Patent Application P 36 28 747.4 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 Streptomyces 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
5 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 herein-
10 after.

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
15 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.

20 The following modifications were made to the sequenced gene:

1. The Streptomyces 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
25 plant RNA polymerase II.
2. Within the gene, the Streptomyces 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
30 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.
- 35 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.

5 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
10 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:

15	Restriction enzyme	Cut after nucleotide No. (codings strand)
	BspMII	11
	SacII	64
	EcoRV	74
20	HpaI	80
	AatII	99
	BstXI	139
	ApaI	232
	ScaI	272
25	AvrII	308
	AflII	336
	StuI	385
	BssHII	449
	FokI	487
30	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
5 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.
10 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
15 in the examples which follow. Unless otherwise indicated, percentage data therein relate to weight.

Examples

The following media were used:

a) for bacteria:

20 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

25 b) for plants:

M+S medium: see Murashige and Skoog, Physiologia Plantarum 15 (1962) 473
2MS medium: M+S medium containing 2% sucrose
MSC10 medium: M+S medium containing 2% sucrose,
30 500 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.

5 1. Chemical synthesis of a single-stranded oligonucleotide

10 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 page 4, lines 3 - 4 of the said EP-A) methods.

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

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

25 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 μ l 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.

5 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 μ l of buffer
10 containing 50 mM tris-HCl (pH 7.6), 20 mM magnesium
chloride, 25 mM potassium chloride and 10 mM DDT. 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 μ l of 0.1 M DTT, 8 μ l of 2.5 mM adenosine
triphosphate (pH 7) and 5 μ l of T4 DNA ligase
(2000 units) are added, and the mixture is incubated
at 22°C for 16 hours.

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

20 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.

25 3. Preparation of hybrid plasmids containing gene frag-
ments I, II, III and IV.

a) Incorporation of gene fragment I in pUC 18

30 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 electroelution.

5 1 μ g 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.

10 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.

15 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

20 a) Transformation and amplification of gene fragments I - IV

25 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 μ l 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 μ l of 10 mM adenosine triphosphate (pH 7) and 1 μ l 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 ϕ X 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 construct the total gene (DNA sequence I), 15 ng of the 312 bp fragment and 12 ng of the 246 bp

5 fragment are ligated, as described above, with 1 μ g 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

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

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

20 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,
25 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.

30 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

5 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.).
10 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.
15 (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
20 pGV3850kanR (Jones et al., EMBO J. 4 (1985) 2411).

For this purpose, 50 μ l 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
25 dry YT-agar plate and incubated at 37°C for one hour. The bacteria were resuspended in 3 ml of 10 mM MgSO₄ and plated out on antibiotic-agar plates (spectinomycin 50 μ g/ml: selection for pMPK110; tetracycline 10 μ g/ml: selection for R64drd11;
30 kanamycin 50 μ g/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
35 cultivated in LB medium at 28°C. 50 μ l 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 MgSO₄ 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.

20 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.

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10 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 μ l 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. Gen. 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 MgSO₄ 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.

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8. Transformation of Nicotiana tabacum by Agrobacterium tumefaciens

30 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 5 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 MS10-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

5 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 1×10^{-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 1×10^{-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 nontransformed 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

25 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).

30 After subsequent centrifugation, 20 μ l of the clear supernatant were incubated with 1 μ l of 10 mM radio-labelled D,L-PTC and 1 μ l of 100 mM acetyl-CoA at 37°C for 20 minutes. 25 μ l of 12% trichloroacetic acid were then added to the reaction mixture, followed by centrifugation. 7 μ l of the supernatant were transferred to a thin-layer chromatography

5 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.



Patent claims:

1. A resistance gene coding for the protein of amino acid sequence I (annex), in that ATG is used as start codon and TGA is used as stop codon, and the GC content of the gene is adapted to that in plants.
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2. The resistance gene as claimed in claim 1, having DNA sequence I (annex, nucleotide position 9-554).
3. A gene structure having DNA sequence I (annex) coupled to regulation and expression signals active in plants.
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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.
- 15 6. Vectors containing one or more DNA sequences (annex) from
Group I (nucleotide No. 1 to 152),
Group II (nucleotide No. 153 to 312),
Group III (nucleotide No. 313 to 436) or
20 Group IV (nucleotide No. 437 to 558).
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.
- 25 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



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generating phosphinothricin-resistant plant cells,
parts of plants, plants and seeds.