IDS copy

9608249-0

H1-247

23 FEB 1996

HOECHST AKTIENGESELLSCHAFT HOE 91/F 250 Dr.LP/AL

Description

Deacetylase genes for the production of phosphinothricin or phosphinothricyl-alanyl-alanine, processes for their isolation, and their use

The invention relates to deacetylase genes, to processes for their isolation, and to their use, in particular for the production of transgenic plants using tissue-specific promoters. In these plants, the development of certain parts can be prevented in a targeted fashion. With the aid of deacetylase genes, it is also possible to identify and isolate tissue-specific promoters in transgenic plants.

Phosphinothricin (PTC, 2-amino-4-methylphosphinobutyric acid) is a glutamine synthetase (GS) inhibitor. PTC is a "building block" of the antibiotic phosphinothricyl-alanyl-alanine. This tripeptide (PTT) is active against Gram-positive and Gram-negative bacteria and also against the fungus Botrytis cinerea. PTT is produced by the strain Streptomyces viridochromogenes Tü494 which has been deposited at the Deutsche Sammlung für Mikroorganismen [German Collection of Microorganisms], from where it can be obtained; Deposit Nos. DSM 40736 and DSM 4112.

German Patent 2,717,440 discloses that PTC acts as a total herbicide. The published application (EP-A-0,257,542) describes how herbicide-resistant plants are produced with the aid of a phosphinothricin-N-acetyltransferase (pat) gene. The phosphinothricin-N-acetyltransferase encoded by the pat gene modifies the intracellular PTC and detoxifies the herbicide.

This Sheet Has Been Replaced

THIS SHEET HAS BEEN REPLACED

The present invention describes deacetylase genes (dea), whose expression products are capable of deacetylating N-acetyl-phosphinothricin (N-Ac-PTC), or N-Ac-PTT, intracellularly, whereupon the antibiotic activity of these compounds is restored.

An N-acetyl-phosphinothricin tripeptide deacetylase gene according to the invention can be isolated from S. viridochromogenes Tü494. The dea gene is located downstream of the pat gene on the 4.0 kb BamHI fragment, which has already been disclosed (EP-A-0,257,542). This gene is located on a BglII-BamHI fragment and is specified in detail by the sequence (Fig. 1 and Table 1). The protein sequence is defined by the DNA sequence. An ATG codon which is recognised in bacteria and in plants acts as the translation start codon; the Shine-Dalgarno sequence is emphasised by underlining. This gene codes for the last step in PTT biosynthesis, namely the deacetylation of inactive N-acetyl-phosphinothricin tripeptide to give the active PTT.

Table 1:

BglII RBS ↓Met

```
AGATCTGAGC GGAGAGCGCA TGGCATCGTC GGAGTTGGAG CTGGTGCGGC AACTGATCGG
GCTCAACTGG CACACCCGCA ACGGCGATGT GGAGCCACGC CGGGTGGCCT ACGACCGAGC
CCAGGAGGCC TTCGGGCACC TGGGCCTGCC CCCCGGCGAG ACCGTCGTGA TCGGCGACTG
CTCGGCGGAG TGGGTACGGC CCGCCCAGGA GGACGGCAGG ACCCTGCTGT ACCTGCACGG
CGGTTCGTAC GCCCTCGGAT CGCCGCAGTC GCACCGCCAT CTGTCCAGCG CGCTGGGCGC
GGCGGCCGGG GCGGCGGTGC TCGCCCTGCA CTACCGCAGG CCGCCCGAGT CTCCCTTCCC
GGCGGCGGTG GAGGACGCCG TGGCGGCCTA CCGGATGCTG CGGGAGCGGG GCCTGCCGCC
GGGGCGGATC ACCTTCGCCG GTGACTCGGC CGGCGCGGGC CTCGCCGTCG CCGCCCTCCA
GGTGCTGCGC GACGCCGGGG ACCCGCTGCC GGCCGCCGCG GTGTGCATCT CGCCCTGGGC
CGACCTGGCC TGCGAGGGCG CCTCGCACGT CACCCGCAAG GAGCGCGAGA TCCTCCTGGA
CACCGAGGAC CTGCTCCGCA TGGCGGGGCG CTACCTGGCC GGCACCGATC CCAGGAACCC
CCTGGCCTCG CCCGCCCACG GCGATCTGAC CGGTCTGCCG CCGCTGCTCA TCCAGGTCGG
TTCCGAGGAA GTCCTGTACG ACGACGCCCG GGCGCTGGAA CAGGCGGCGC TCAAGGCGGG
CGTACCGGTC ACCTTCGACG AGTGGCCGGA GATGTTCCAC GTCTGGCACT GGTACCACCC
GGTGCTCCCC GAGGGGCGTG CGCCGTCGAG ACGGCGGGCG TGTTCCTGCG CCGCGCCACC
GAGGAGGGCG AGCGGTGACC GACTGGATCC T
```

Stop BamHI

This Sheet Has Been Replaced

THIS SHEET HAS BEEN REPLACED

It is known of many enzymes that their specificity is not limited to one substrate. For example, the phosphinothricin-N-acetyl transferase, which is encoded by the pat gene, is actually used in PTT biosynthesis for the acetylation of desmethyl-PTC and can be used for the detoxification of PTC due to its non-specificity. Superexpression of the dea gene (with the aid of suitable promoters or by cloning onto high-copy vectors) it is now possible to use an N-acetyl-PTT-deacetylase of insufficient specificity for activating N-acetyl-phosphinothricin.

Another dea gene can be obtained from E. coli. In fact, it has been found that, in contrast with other bacteria (for example rhizobia and streptomycetes), no activity can be detected in the so-called PAT assay (Ph.D. thesis Inge Broer, Faculty of Biology, University of Bielefeld, Expression des Phosphinthricin-N-Acetyltransferase-Gens aus Streptomyces viridochromogenes in Nicotiana tabacum [Expression of the phosphinothricin-N-acetyltransferase gene from Streptomyces viridochromogenes in Nicotiana tabacum], p. 42-43, 1989) after cloning the pat gene into suitable expression vectors (Strauch et al., Gene, 63, 65-74, 1988; Wohlleben et al., Gene, 70, 25-37, 1988). Moreover, a low number of copies of the pat gene in E. coli is incapable of imparting PTT resistance since the endogenic deacetylase compensates for the action of the phosphinothricin-N-acetyltransferase. Finally, this deacetylase activity can be detected directly by the effective inhibition of the GS activity after an addition of N-acetyl-phosphinothricin. N-Ac-PTC is reacted by the deacetylase to give PTC, which then inhibits the GS in the known manner, which can be measured in the γ-glutamyl transferase assay (Bender et al., J. Bacteriol. 129, 1001-1009, 1977). This is due to an endogenic deacetylase activity of E. coli.

This Sheet Has Been Replaced



THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

It should be assumed that this activity cannot be found in the argE mutant, which is known from the literature (Baumberg, Molec. Gen. Genetics 106, 162-173, 1970). Other E. coli deacetylase mutants can be selected easily: following traditional (Delić et al., Mut. Res. 9, 167-182, 1970; Drake and Baltz, Ann. Rev. Biochem. 45, 11-38, 1976) or Tn5 mutagenesis (Kleckner, Ann. Rev. Genet. 15, 341-404, 1981), such mutants can be recognised on PTT-supplemented minimal medium by the fact that they can only grow after transformation with a pat gene cloned into a low-copy vector.

Accordingly, the deacetylase gene can be isolated from E. coli by producing a gene bank, for example in the argE mutant of E. coli, or in a recently isolated mutant, using conventional processes (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982).

Methods for isolating further deacetylase genes result from the above text: for example isolation of novel organisms which are PTT-sensitive despite the presence of a pat gene on a low-copy vector, followed by isolation of a deacetylase gene.

In a further aspect of the invention, pat genes and dea genes can be used together with tissue-specific promoters to prevent the development of certain plant tissues in a targeted fashion. A specific use is, for example, the production of male-sterile plants.

The production of hybrid seed in plant breeding depends on the guaranteed avoidance of selfing of the mother plant. In many plant species, male-sterile mutants occur naturally, and these are used in breeding. The molecular mechanism of cytoplasmatic male sterility (cms) remains insufficiently explained as yet. Moreover, no cms variants exist in a large number of cultivated varieties such

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

as, for example, Beta vulgaris. It is therefore of great interest for agriculture to produce defined cms mutants of all important cultured varieties by way of molecular genetics. The company PGS/Belgium has proposed such a method in Patent Application PCT/EP 89/00495. It is based on the destruction of the tissue surrounding the pollen mother cells (tapetum). To this end, an RNAase gene is fused with a tapetum-specific promoter (Mariani et al.; Nature 347, 737-741, 1990). The exclusive expression of the gene in tapetum cells provides the selective destruction of tissue and thus prevents the formation of mature pollen. A plant carrying this gene should only be able to produce seeds after cross-fertilization. An essential shortcoming of this system is the fact that progeny of this plant are also male-sterile and can therefore not form seeds in the field, where they depend on selfing. This is only successful when the male partner of the crops carries a gene which can compensate for the action of the RNAase in the progeny. According to the above-mentioned disclosed patent application, this is supposed to be effected by the barstar gene. The fact remains that only genetically modified, i.e. transgenic, partners can be used in the cross.

The text hereinafter proposes methods for the production of cms plants which allow transgenic mother plants to be crossed with any partners of the same species. This is achieved by combining a dea gene which is under the control of, for example, a tapetum promoter, in connection with a constitutively expressed pat gene. Application of PTC, or PTT, results in a targeted inhibition of the glutamine synthethase in the tapetum cells, causing their death. An even simpler system consists in the production of transgenic plants which contain only a single foreign gene, namely a dea gene under the control of a tissue-specific promoter, in this case a tapetum promoter, and application of N-Ac-PTC, or N-Ac-PTT, to the plant.

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

Generally speaking, the invention accordingly comprises the following methods for the tissue-specific inhibition with the aid of a deacetylase gene, preferably the abovementioned dea gene from E. coli or S. viridochromogenes Tü 494:

1) Plants which are resistant to PTT or PTC by pat activity (for example produced as described in EP 0,257,542) are transformed with the deacetylase gene from streptomycetes under the control of a plant-tissue specific promoter. Application of PTT or PTC leads to the expression of the deacetylase gene for compensating for the phosphinothricin-N-acetyltransferase activity in the respective tissues. These are then destroyed selectively, while the remaining plant is resistant.

2) PTT- or PTC-resistant plants are transformed with the E. coli deacetylase gene under the control of a tissue-specific promoter. Application of PTT or PTC leads to the expression of the deacetylase gene for compensating for the phosphinothricin-N-acetyltransferase activity in the respective tissues. These are then destroyed selectively, while the remaining plant is resistant.

The use of N-acetyl-phosphinothricin, or N-acetyl-phosphinothricin tripeptide, can simplify this system. Both substances are not active as herbicides, but are taken up by plants, translocated and not degraded immediately. Deacetylase activity for N-acetyl-phosphinothricin and N-acetyl-phosphinothricin tripeptide has not been detected in plants as yet. The above-described 2-gene system can therefore be reduced to a 1-gene system and thus greatly simplified as illustrated further below:

3) Any plants are transformed with a deacetylase gene from streptomycetes under the control of a tissue-specific promoter. After application of N-acetyl-phosphinothricin or N-acetyl-phosphinothricin tripeptide, the

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

tissue-specific expression leads to the immediate death of the respective tissue.

4) Any plants are transformed with a deacetylase gene from E. coli under the control of a tissue-specific promoter. After application of N-acetyl-phosphinothricin or N-acetyl-phosphinothricin tripeptide, the tissue-specific expression leads to the immediate death of the respective tissue.

Since the specificity of the deacetylase from streptomycetes for N-acetyl-phosphinothricin tripeptide is higher, it will be preferred to use N-acetyl-phosphinothricin tripeptide in case 3) and N-acetyl-phosphinothricin in case 4) if high activities are required. Tissue-specific promoters which can be used are all described promoters where selective expression in certain tissues has been detected (for example Koltunow et al., The Plant Cell., Vol. 2, 1201-1224, 1990). All newly-isolated promoters with similar properties are of course, also suitable. Other promoters which are suitable in addition to tissue-specific promoters are those which are subject to a different type of regulation (for example time-dependent, stress-dependent, environment-dependent) and which is tissue-specific.

These methods furthermore allow analysis of the differentiation of cell regulation and the production of plants in which the development of certain parts was inhibited in a targeted fashion, preferably the production of male-sterile plants.

A further application is the use of a dea gene for the identification of selectively expressed promoters. If DNA fragments with promoter activity are cloned upstream of dea genes, then the selective disappearance of parts of tissue after application of N-acetyl-phosphinothricin or N-acetyl-phosphinothricin tripeptide indicates the

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

specificity of the promoter.

Finally, the invention relates to positive selection systems. Those cells in which the dea gene has been inactivated can be selected either in combination with the pat gene and PTT (or PTC) together with a dea gene or with N-acetyl-phosphinothricin (or N-acetyl-phosphinothricin tripeptide) and a dea gene alone. This allows successful cloning (insertion inactivation), but also rare events (for example transposition), to be selected directly. Other aspects of the invention are mentioned in the examples.

Example 1: Fusion of the deacetylase encoding region with eucaryotic transcription signals

The plasmid pPRI (see EP-0,257,542) was isolated from an E. coli strain and cleaved with BamHI and BgIII. The digested DNA was separated on an agarose gel, and an 0.9 kb fragment was isolated from the gel. The vector pROKI (Baulcombe et al., Nature 321, 446-449, 1986) was also restricted with BamHI. The two batches were combined and ligated. The ligation mixture was transformed into E. coli S17.1 (Simon et al., Bio/Technology 1, 784-791, 1983). Colonies growing on kanamycin-containing media were transferred to nitrocellulose filters, incubated for 12 hours at 37°C and then lysed. The DNA of the bacteria was fixed on the filter. The 0.9 kb fragment isolated from the agarose gel was made single-stranded by incubation at 100°C. The missing strand was then synthesized onto the existing strand using Klenow polymerase and digoxigenin-labeled nucleotides. The labeled strand was used as a sample for hybridizing with the bacterial DNA bound to the filter. Hybridizing clones could be detected with the aid of an antibody reaction. The DNA of the positive clones were isolated by means of Qiagen lysis and digested with BamHI/EcoRI as well as BamHI/HindIII. This restriction allows the orientation of the inserted

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

0.9 kb fragment to be determined. The plasmid in orientation I was designated pIB17.1, that of orientation II as pIB17.2 (see Fig. 2).

Example 2: Proof of the deacetylation of N-acetyl-PTC and N-acetyl-PTT by the deacetylase gene

It was possible to demonstrate that the eucaryotic transcription signals cloned in vector pROKI also allow expression in R. meliloti, A. tumefaciens and E. coli.

The plasmids pIB17.1 and pIB17.2 were therefore transferred into Rhizobium meliloti strain 2011 by means of a 2-factorial cross. By incubation of R. meliloti wild type strains with radiolabeled N-acetyl-PTC, it was possible to demonstrate that this strain does not deacetylate N-acetyl-PTC. (After incubation of PIB17.1-carrying strains with N-acetyl-PTC and N-acetyl-PTT, deacetylation can be detected by thin-layer chromatography). It was also possible to demonstrate that R. meliloti reacts highly sensitively to PTC and PTT. Deacetylation can therefore also be detected via inhibition of the R. meliloti glutamine synthesase, by the PTC which is liberated.

Example 3: Transfer of the modified deacetylase gene into Nicotiana tabacum

The deacetylase gene modified as in Example 1 was transferred into A. tumefaciens LBA4404 by means of a 2-factorial cross. The resulting strains LBA4404/17.1 and LBA4404/17.2 were used for incubating leaf discs of Nicotiana tabacum, which were transferred after 3 days to a kanamycin-containing shoot induction medium. Regenerating kanamycin-resistant shoots can be tested for the presence of the deacetylase gene by Southern hybridization. After treatment with N-acetyl-PTC or N-acetyl-PTT, the plants are then destroyed by the PTC, or PTT, which is liberated.

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

Example 4: Construction of a vector for the transient expression of the modified deacetylase gene in E. coli and tobacco protoplasts

The modified deacetylase gene from pIB17.1 and pIB17.2 was cut out of the plasmids by digestion with EcoRI/HindIII. The restricted DNA was separated in an agarose gel and an 0.9kb fragment was isolated in each case. The vector pSVB28 (Arnold and Pühler, Gene 70, 171-179, 1988) was also digested with EcoRI/HindIII. The two batches were combined and ligated. After transformation into the β-galactosidase-negative E. coli strain JM83, all clones which carried the vector turned blue, while clones which carried a vector into which the deacetylase gene had been inserted remained white. The DNA was isolated from the clones which had been identified in this way and digested with EcoRI/HindIII. The clones which contained the modified deacetylase gene could be recognized on the basis of the restriction pattern. The vectors which had been constructed are termed pIB27.1 and pIB27.2 (see Fig. 2). They exist in E. coli in a large number of copies.

Example 5: Transient expression of the modified deacetylase gene in tobacco protoplasts

The plasmid DNA was isolated from the E. coli strains constructed in Example 4. Young tobacco leaves were incubated with digestion enzymes for 20 h. The protoplasts which get disengaged from the leaf skeleton were purified and incubated with polyethylene glycol (PEG) and the isolated DNA in a transfer buffer. The protoplasts were then washed and taken up in a culture liquid (K3 medium). After incubation for 3 days under weak illumination, the regenerating protoplasts were lysed and the crude extracts were incubated with radiolabeled N-acetyl-PTC and N-acetyl-PTT. The deacetylated PTC or PTT can be detected by thin-layer chromatography.

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

Example 6: Method for the production of male-sterile crop plants using the deacetylase gene from S. viridochromogenes under the control of a tapetum-specific promoter.

The deacetylase gene from Streptomyces viridochromogenes is fused with a tapetum-specific promoter from Nicotiana tabacum and introduced into tobacco cells by means of agrobacteria-mediated leaf disc transformation. The plants regenerating from these cells are sprayed with N-acetyl-PTC or N-acetyl-PTT at any desired point in time before anthesis. It can be shown that N-acetyl-PTC is stable in the plant cell and transported into all cells. None of the two substances has noticeable negative consequences for the wild type plant. As soon as the first tapetum cells are formed, they start to express the deacetylase gene. The N-acetyl-PTC or N-acetyl-PTT stored in the cell is deacetylated by the enzyme and so converted into its active form. It inhibits the glutamine synthetase of the cells and so results in rapid destruction. Mature pollen can no longer be formed. In addition, the formation of deacetylase is also interrupted. Cells in the vicinity should not be affected. If the plant is not treated with N-acetyl-PTC or N-acetyl-PTT, it is fully fertile. This makes compensation for the cms by a gene of the male crossing partner unnecessary. At the same time, there exists an accurately defined mutation which has no consequences on the vitality and usefulness of the plant.

Example 7: Identification of tissue-specific promoters in transgenic plants

Tissue-specific promoters can be identified directly in the plant with the aid of the deacetylase gene from Streptomyces viridochromogenes.

The deacetylase gene is cloned, without a promoter, to the right or left end of a disarmed T-DNA in such a way

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

that a promoter which is located at the insertion site of the T-DNA in the plant genome can read into the gene and so bring about its expression. Transgenic plants are cloned via the propagation of cuttings. One clone is treated with N-acetyl-PTC or N-acetyl-PTT and examined for tissue which may be in the process of dying. Using reverse PCR, the gene which has been affected can be multiplied from a clone which has not been treated with N-acetyl-PTC or N-acetyl-PTT and isolated (Kahl and Weising, Gentransfer bei Pflanzen [Gene Transfer in Plants], Biologie in unserer Zeit, No. 6, p. 181, 1988).

Example 8: Detection of N-acetyl-phosphinothricin (PPT)-deacetylase activity in soil samples

Soil samples of 500 mg each (sandy loam, Schwanheimer Düne) were adjusted to 40% of their maximum water capacity and treated with 5 µl of a 15 mM stock solution of ^{14}[C]-L-N-acetyl-PPT. The test samples were incubated at 28°C for various periods of time (0 hours, 4, 7, 11 and 14 days) and subsequently worked up by extraction with 1 × 500 µl and 1 × 250 µl of water. 14 µl aliquots from the combined aqueous supernatants were applied to thin-layer chromatography plates (HPTLC cellulose, Merck) and developed 2 × in n-propanol: 25% ammonia = 3 : 2 as the mobile phase. The assays were evaluated by autoradiography. It was possible to identify N-acetyl-PPT and PPT by comparing the R_f values of the radioactive spots with the corresponding reference substances. It emerged that N-acetyl-PPT in the soil is metabolized within 14 days almost completely to give PPT. In contrast, in a control assay with sterile soil samples (soil 4 hours at 200°C), the substance proved to be completely stable.

Example 9: Isolation and identification of soil microorganisms having an N-acetyl-PPT-specific deacetylase activity

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

1 g samples of soil were extracted for 1 hour at room temperature using 10 ml 10 mM NaCl, 10 mM sodium phosphate buffer, pH = 7.0. To select various groups of microorganisms, the soil supernatants were plated onto the following agar media and used for inoculating enrichment cultures in Erlenmeyer flasks with the corresponding liquid media:

(1) MS1 medium (for eubacteria):

5 mM glucose
5 mM succinate
10 mM glycerol
1 g/l NH_4Cl
50 ml/l solution A
25 ml/l solution B

Solution A: 50 g/l K_2HPO_4

Solution B: 2.5 g/l $MgSO_4$
0.5 g/l NaCl
25 ml/l trace elements

(2) Chitin medium (for actinomycetes and streptomycetes as well as chitinovorous bacteria):

10 g/l crab chitin
1 g/l $(NH_4)_2SO_4$
0.5 g/l $MgSO_4$
50 ml/l solution A
1 ml/l trace elements

(3) Antibiotics medium (for higher fungi):

20 g/l malt extract
10 g/l glucose
2 g/l yeast extract
0.5 g/l $(NH_4)_2SO_4$
50 µg/ml tetracyclin

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

All media contained 5 mM N-acetyl-PPT. The agar plates and the liquid cultures were incubated for 3-5 days at 28°C.

20 individual colonies were isolated from each of the selective agar plates and transferred into 5 ml liquid cultures with the corresponding medium. The cells were allowed to grow for 3-5 days and then centrifuged at 10,000 rpm, and the supernatants were examined in the aminoacid analyzer (Biotronic LC 5001) for the formation of PPT. In this manner, a PPT-positive culture (CB 10) was isolated from a selection with chitin medium.

The deacetylase activity of the cells of this culture were subsequently additionally tested by biotransformation with ^{14}[C]-L-N-acetyl-PPT as the substrate. To do this, 1.5 ml of the culture were centrifuged as above, the cell pellet was washed 1 x in 10 mM NaCl, 10 mM sodium phosphate buffer, pH = 7.0, and resuspended in 100 µl of the same buffer. 10 µl of the suspension were treated with 10 µl of an 0.25 mM solution of ^{14}[C]-L-N-acetyl-PT and the mixture was incubated for 15 hours at 28°C. The bacteria were then centrifuged off, and 7 µl of the supernatant were analyzed by thin-layer chromatography and autoradiography as described in Example 1. A virtually quantitative reaction of N-acetyl-PPT into PPT could be observed. In addition, the assay showed that the deacetylase found accepts the L enantiomer of the acetylated PPT as substrate.

To further purify the strain with the desired deacetylase activity, the culture CB 10 was plated onto LB agar (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar) and incubated for 2 days at 28°C. 10 individual colonies were isolated from the plate, transferred to chitin liquid medium, and the cultures were tested for N-acetyl-PPT deacetylase activity as described above. The deacetylase-positive isolates were replated to check for uniformity of the culture. The active strains CB 10 3496

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

and CB 10 34397 were identified as .... (DSM deposit No. ...) and .... (DSM deposit No. ....).

The enrichment cultures in the soil samples in the various liquid media were tested for deacetylation of N-acetyl-PPT as described above. Only the chitin medium cultures proved to be deacetylase-positive. After these cultures were plated onto chitin agar, a total of 40 individual colonies was isolated, grown in chitin liquid medium and subsequently tested for deacetylase activity. Six positive isolates were found (BoK1, BoK9, BCÜ1, BCÜ2, BCÜ3), from which the active pure cultures were obtained by replating onto agar plates and culturing further on individual colonies (see above). The strains were ....., ...(DSM deposit Nos. ...., ...).

Example 10: N-Acetyl-PPT deacetylase enzyme assays with the isolated microorganisms

5 ml precultures of strains BoK1 and BoK5 were grown in LB medium overnight at 28°C, and 0.5 ml aliquots were transferred to 20 ml of LB medium or 20 ml of chitin medium containing 1 mM N-acetyl-PPT. The LB cultures were incubated for 15 hours and the chitin cultures for 4 days in 100 ml Erlenmeyer flasks at 28°C and 150 rpm. The cells were subsequently harvested by centrifugation for 10 minutes at 10,000 rpm, the cell pellets were washed 1 x in 10 ml mM NaCl, 10 mM sodium phosphate buffer, pH = 7.0, weighed and resuspended in 100 mM tris/HCl, pH = 8.0 at c = 100 mg/ml. The suspensions were mixed with 1 volume of 100 mM N-acetyl-PPT and incubated in 50 ml Erlenmeyer flasks for 24 hours at 28°C and 220 rpm. The cells were separated by centrifugation for 10 minutes at 5000 rpm, and the PPT content in the supernatants was then determined in the aminoacid analyzer (see Example 2). The results are compiled in Table 1.

THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

Table 1: N-Acetyl-PPT deacetylase assays with soil microorganisms

Strain:	Medium:	Concentration of PPT in the supernatant [mM]:	[%]*:
BoK1	LB	0.7	2.7
BoK1	Chitin	13.9	55.5
BoK5	LB	6.0	23.9
BoK5	Chitin	14.3	57.2

*: based on the L-enantiomer in the N-acetyl-PPT racemate.

Example 11: N-Acetyl-PPT deacetylase enzyme assays with actinomycetes

N-Acetyl-PPT-specific deacetylase activities were also found during fermentation tests with the two actinomycetes strains Actinoplanes liguriae (IFO No. 13997) and Actinoplanes sp. (Strain Collection Zentralforschung No. A 1015) in the presence of N-acetyl-PPT and by biotransformation with ^{14}[C]-L-N-acetyl-PPT as the substrate.

To determine the conversion rates, biotransformations were carried out on the two strains as described in Example 3. The following media were used:

Medium A: 0.2% yeast extract
0.2% meat extract
0.4% polypeptone (from soya meal)
1% glucose

Medium B: 20 g/l oat flakes
1 ml/l trace elements

The results are compiled in Table 2.

THIS SHEET HAS BEEN REPLACED

Table 2: N-Acetyl-PPT deacetylase assays with actinomycetes

Strain:	Medium:	Concentration of PPT in the supernatant [mM]:	[%]*:
Actinoplanes liguriae (IFO No. 13997)	A	3.3	13.2
Actinoplanes liguriae (IFO No. 13997)	B	7.6	30.4
Actinoplanes sp. (No. A 1015)	A	11.0	44.0
Actinoplanes sp. (No. A 1015)	B	2.7	10.8

*: based on the L enantiomer in the N-acetyl-PPT racemate

This Sheet Has Been Replaced



THIS SHEET HAS BEEN REPLACED

This Sheet Has Been Replaced

Patent Claims:

1. A process for isolating deacetylase genes from microorganisms, which comprises establishing a gene bank in a deacetylase mutant which has been provided with a pat gene, and identifying deacetylase-carrying clones by means of PTT- or PTC-sensitivity.

2. The deacetylase gene from E. coli, obtainable by the process as claimed in claim 1.

3. The deacetylase gene from S. viridochromogenes, comprising an amino-acid sequence encoded by the nucleotide sequence of Table 1 and obtainable by the process as claimed in claim 1.

4. A process for the production of transgenic plants with selectively destroyable parts, which comprises bringing a deacetylase gene under the control of a tissue-specific promoter and causing death of the tissue portions concerned by suitable timely treatment with N-acetyl-PTC or N-acetyl-PTT.

5. A process for the production of transgenic plants with selectively destroyable parts, wherein the plant has PTC resistance and additionally contains a deacetylase gene under the control of a tissue-specific promoter and causing death of the tissue portions concerned by suitable timely treatment with PTC or PTT.

6. The process as claimed in claim 4 or 5, wherein the deacetylase gene originates from E. coli and the plant is treated with N-acetyl-PTC or with PTC.

7. The process as claimed in claim 4 or 5, wherein the deacetylase gene originates from Streptomyces viridochromogenes and the plant is treated with N-acetyl-PTT or

* C L M S *

THIS SHEET HAS BEEN REPLACED

with PTT.

8. The process as claimed in claim 4, 5, 6 or 7, wherein male-sterile plants are produced.

9. The process as claimed in claim 8, wherein the deacetylase gene is under the control of the tapetum promoter.

10. A process for detecting tissue-specific promoter activity, which comprises placing a deacetylase gene under the control of a DNA sequence which probably contains a tissue-specific promoter sequence and the promoter activity is recognized indirectly by the death of certain tissues.

11. A positive selection system for detecting clones or transpositions by insertion inactivation, which comprises selecting those cells in which the dea gene had been inactivated, in connection with a pat gene and PTT or PTC and the dea gene or with N-acetyl-PTC or N-acetyl-PTT and the dea gene alone.

12. The use of a deacetylase gene for the targeted prevention of specific parts of the plant from functioning.

This Sheet Has Been Replaced





* C L M S *

THIS SHEET HAS BEEN REPLACED

Abstract of the disclosure:

Deacetylase genes for the production of phosphinothricin or phosphinothricyl-alanyl-alanine, processes for their isolation, and their use

The invention relates to deacetylase genes, to processes for their isolation, and to their use, in particular for the production of transgenic plants using tissue-specific promoters. It is possible to prevent the development of certain parts in these plants in a targeted manner. With the aid of deacetylase genes, it is furthermore possible to identify and isolate tissue-specific promoters in transgenic plants.

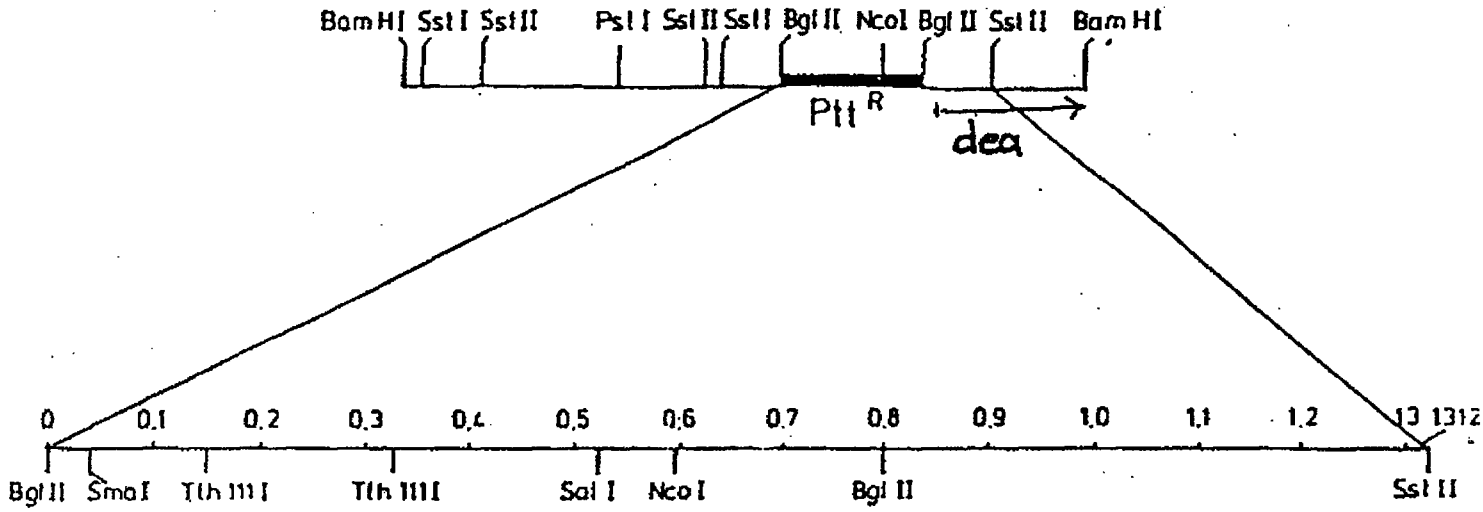
This Sheet Has Been Replaced



0-093809?

THIS SHEET HAS BEEN REPLACED

Figure 1



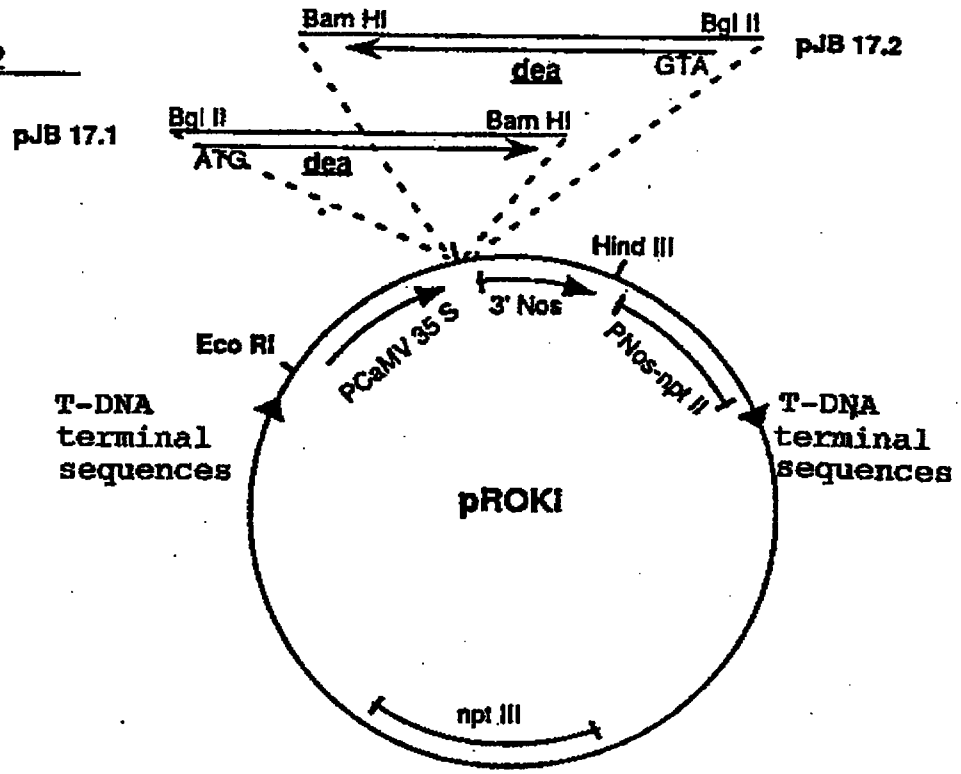
This Sheet Has Been Replaced



THIS SHEET HAS BEEN REPLACED

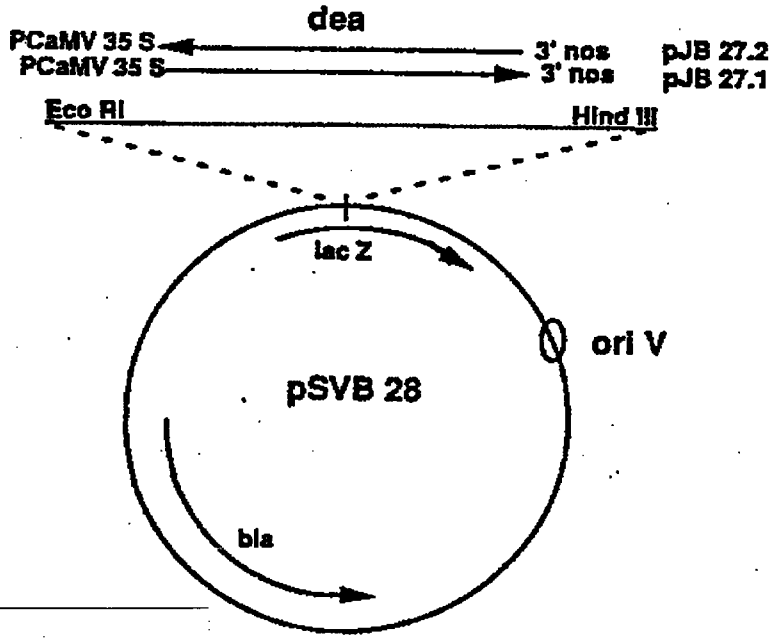
Figure 2

pJB 17.1 or pJB 17.2



npt II: Neo - resistance gene from TN5
npt III: Neo - resistance gene from S. faecalis

pJB 27.1 or pJB 27.2
3.6 kb



This Sheet Has Been Replaced



THIS SHEET HAS BEEN REPLACED

9608249-0
25 FEB 1997
REPLACEMENT SHEET
25 FEB 1997

DEACETYLASE GENES FOR THE PRODUCTION OF PHOSPHINOTHRICIN OR PHOSPHINOTHRICYL-ALANYL-ALANINE, PROCESSES FOR THEIR ISOLATION, AND THEIR USE

The invention relates to deacetylase genes, to processes for their isolation, and to their use, in particular for the production of transgenic plants using tissue-specific promoters. In these plants, the development of certain parts can be prevented in a targeted fashion. With the aid of deacetylase genes, it is also possible to identify and isolate tissue-specific promoters in transgenic plants.

Phosphinothricin (PTC, 2-amino-4-methylphosphinobutyric acid) is a glutamine synthetase (GS) inhibitor. PTC is a "building block" of the antibiotic phosphinothricyl-alanyl-alanine. This tripeptide (PTT) is active against Gram-positive and Gram-negative bacteria and also against the fungus Botrytis cinerea. PTT is produced by the strain Streptomyces viridochromogenes Tü494 which has been deposited at the Deutsche Sammlung für Mikroorganismen [German Collection of Microorganisms], from where it can be obtained; Deposit Nos. DSM 40736 and DSM 4112.

German Patent 2,717,440 discloses that PTC acts as a total herbicide. The published application (EP-A-0,257,542) describes how herbicide-resistant plants are produced with the aid of a phosphinothricin-N-acetyltransferase (pat) gene. The phosphinothricin-N-acetyltransferase encoded by the pat gene modifies the intracellular PTC and detoxifies the herbicide.

REPLACEMENT SHEET

25 FEB 1997

The present invention describes deacetylase genes (dea), whose expression products are capable of deacetylating N-acetyl-phosphinothricin (N-Ac-PTC), or N-Ac-PTT, intracellularly, whereupon the antibiotic activity of these compounds is restored.

An N-acetyl-phosphinothricin tripeptide deacetylase gene according to the invention can be isolated from S. viridochromogenes Tü494. The dea gene is located downstream of the pat gene on the 4.0 kb BamHI fragment, which has already been disclosed (EP-A-0,257,542). This gene is located on a BgIII-BamHI fragment and is specified in detail by the sequence (Fig. 1 and Table 1). The protein sequence is defined by the DNA sequence. An ATG codon which is recognised in bacteria and in plants acts as the translation start codon; the Shine-Dalgarno sequence is emphasised by underlining. This gene codes for the last step in PTT biosynthesis, namely the deacetylation of inactive N-acetyl-phosphinothricin tripeptide to give the active PTT.

Table 1:

BglII RBS ↓Met

```
AGATCTGAGC GGAGAGCGCA TGGCATCGTC GGAGTTGGAG CTGGTGCGGG AACTGATCGG
GCTCAACTGG CACAGCCGCA ACGGCGATGT GGAGCCACGC CGGGTGGCCT ACGACCGAGC
CCAGGAGGCC TTCGGGCAGC TGGGCCTGCC GCCCGGCGAG ACCGTCGTGA TCGGCGACTG
CTCGGCGGAG TGGGTACGGC CCGCCCAGGA GGACGGCAGG ACCCTGCTGT ACCTGCACGG
CGGTTCGTAC GCCCTCGGAT CGCCGCAGTC GCACCGCCAT CTGTCCAGCG CGCTGGGCGC
GGCGGCCGGG GCGGCGGTGC TCGGCGTGCA CTACCGCAGG CCGCCGGAGT CTCCCTTCCC
CGCCGCCGGTG GAGGACGCCG TGGCGGCCTA CCGGATGCTG CGGGAGCGGG GCCTGCCGCC
GGGGCGGATC ACCTTCGCCG GTGACTCGGC CGGCGCGGGC CTCGCCGTCG CCGCCCTCCA
GGTGCTGCGC GACGCCGGGG ACCCGCTGCC GGCCGCCGCG GTGTGCATCT CGCCCTGGGC
CGACCTGGCC TGCGAGGGCG CCTCGCACGT CACCCGCAAG GAGCGCGAGA TCCTCCTGGA
CACCGAGGAC CTGCTCCGCA TGGCGGGGCG CTACCTGGCC GGCACGGATC CCAGGAACCC
CCTGGCCTCG CCCGCCCACG GCGATCTGAC CGGTCTGCCG CCGCTGCTCA TCCAGGTCGG
TTCCGAGGAA GTCCTGTACG ACGACGCCCG GGCGCTGGAA CAGGCGGCGC TCAAGGCGGG
CGTACCGGTC ACCTTCGACG AGTGGCCGGA GATGTTCCAC GTCTGGCACT GGTACCACCC
GGTGCTCCCC GAGGGGCGTGC CGCCGTCGAG ACGGCGGGCG TGTTCCTGCG CCGCGCCACC
GAGGAGGCCG AGCGGTGACC GACTGGATCC T
```

Stop BamHI

REPLACEMENT SHEET
25 FEB 1997

It is known of many enzymes that their specificity is not limited to one substrate. For example, the phosphinothricin-N-acetyl transferase, which is encoded by the pat gene, is actually used in PTT biosynthesis for the acetylation of desmethyl-PTC and can be used for the detoxification of PTC due to its non-specificity. Superexpression of the dea gene (with the aid of suitable promoters or by cloning onto high-copy vectors) it is now possible to use an N-acetyl-PTT-deacetylase of insufficient specificity for activating N-acetyl-phosphinothricin.

Another dea gene can be obtained from E. coli. In fact, it has been found that, in contrast with other bacteria (for example rhizobia and streptomycetes), no activity can be detected in the so-called PAT assay (Ph.D. thesis Inge Broer, Faculty of Biology, University of Bielefeld, Expression des Phosphinthricin-N-Acetyltransferase-Gens aus Streptomyces viridochromogenes in Nicotiana tabacum [Expression of the phosphinothricin-N-acetyltransferase gene from Streptomyces viridochromogenes in Nicotiana tabacum], p. 42-43, 1989) after cloning the pat gene into suitable expression vectors (Strauch et al., Gene, 63, 65-74, 1988; Wohlleben et al., Gene, 70, 25-37, 1988). Moreover, a low number of copies of the pat gene in E. coli is incapable of imparting PTT resistance since the endogenic deacetylase compensates for the action of the phosphinothricin-N-acetyltransferase. Finally, this deacetylase activity can be detected directly by the effective inhibition of the GS activity after an addition of N-acetyl-phosphinothricin. N-Ac-PTC is reacted by the deacetylase to give PTC, which then inhibits the GS in the known manner, which can be measured in the γ-glutamyl transferase assay (Bender et al., J. Bacteriol. 129, 1001-1009, 1977). This is due to an endogenic deacetylase activity of E. coli.

REPLACEMENT SHEET
25 FEB 1997

It should be assumed that this activity cannot be found in the argE mutant, which is known from the literature (Baumberg, Molec. Gen. Genetics 106, 162-173, 1970). Other E. coli deacetylase mutants can be selected easily: following traditional (Delić et al., Mut. Res. 9, 167-182, 1970; Drake and Baltz, Ann. Rev. Biochem. 45, 11-38, 1976) or Tn5 mutagenesis (Kleckner, Ann. Rev. Genet. 15, 341-404, 1981), such mutants can be recognised on PTT-supplemented minimal medium by the fact that they can only grow after transformation with a pat gene cloned into a low-copy vector.

Accordingly, the deacetylase gene can be isolated from E. coli by producing a gene bank, for example in the argE mutant of E. coli, or in a recently isolated mutant, using conventional processes (Maniatis et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982).

Methods for isolating further deacetylase genes result from the above text: for example isolation of novel organisms which are PTT-sensitive despite the presence of a pat gene on a low-copy vector, followed by isolation of a deacetylase gene.

In a further aspect of the invention, pat genes and dea genes can be used together with tissue-specific promoters to prevent the development of certain plant tissues in a targeted fashion. A specific use is, for example, the production of male-sterile plants.

The production of hybrid seed in plant breeding depends on the guaranteed avoidance of selfing of the mother plant. In many plant species, male-sterile mutants occur naturally, and these are used in breeding. The molecular mechanism of cytoplasmatic male sterility (cms) remains insufficiently explained as yet. Moreover, no cms variants exist in a large number of cultivated varieties such

REPLACEMENT SHEET
25 FEB 1997

as, for example, Beta vulgaris. It is therefore of great interest for agriculture to produce defined cms mutants of all important cultured varieties by way of molecular genetics. The company PGS/Belgium has proposed such a method in Patent Application PCT/EP 89/00495. It is based on the destruction of the tissue surrounding the pollen mother cells (tapetum). To this end, an RNAase gene is fused with a tapetum-specific promoter (Mariani et al.; Nature 347, 737-741, 1990). The exclusive expression of the gene in tapetum cells provides the selective destruction of tissue and thus prevents the formation of mature pollen. A plant carrying this gene should only be able to produce seeds after cross-fertilization. An essential shortcoming of this system is the fact that progeny of this plant are also male-sterile and can therefore not form seeds in the field, where they depend on selfing. This is only successful when the male partner of the crops carries a gene which can compensate for the action of the RNAase in the progeny. According to the above-mentioned disclosed patent application, this is supposed to be effected by the barstar gene. The fact remains that only genetically modified, i.e. transgenic, partners can be used in the cross.

The text hereinafter proposes methods for the production of cms plants which allow transgenic mother plants to be crossed with any partners of the same species. This is achieved by combining a dea gene which is under the control of, for example, a tapetum promoter, in connection with a constitutively expressed pat gene. Application of PTC, or PTT, results in a targeted inhibition of the glutamine synthethase in the tapetum cells, causing their death. An even simpler system consists in the production of transgenic plants which contain only a single foreign gene, namely a dea gene under the control of a tissue-specific promoter, in this case a tapetum promoter, and application of N-Ac-PTC, or N-Ac-PTT, to the plant.

REPLACEMENT SHEET
25 FEB 1997

Generally speaking, the invention accordingly comprises the following methods for the tissue-specific inhibition with the aid of a deacetylase gene, preferably the abovementioned dea gene from E. coli or S. viridochromogenes Tü 494:

1) Plants which are resistant to PTT or PTC by pat activity (for example produced as described in EP 0,257,542) are transformed with the deacetylase gene from streptomycetes under the control of a plant-tissue specific promoter. Application of PTT or PTC leads to the expression of the deacetylase gene for compensating for the phosphinothricin-N-acetyltransferase activity in the respective tissues. These are then destroyed selectively, while the remaining plant is resistant.

2) PTT- or PTC-resistant plants are transformed with the E. coli deacetylase gene under the control of a tissue-specific promoter. Application of PTT or PTC leads to the expression of the deacetylase gene for compensating for the phosphinothricin-N-acetyltransferase activity in the respective tissues. These are then destroyed selectively, while the remaining plant is resistant.

The use of N-acetyl-phosphinothricin, or N-acetyl-phosphinothricin tripeptide, can simplify this system. Both substances are not active as herbicides, but are taken up by plants, translocated and not degraded immediately. Deacetylase activity for N-acetyl-phosphinothricin and N-acetyl-phosphinothricin tripeptide has not been detected in plants as yet. The above-described 2-gene system can therefore be reduced to a 1-gene system and thus greatly simplified as illustrated further below:

3) Any plants are transformed with a deacetylase gene from streptomycetes under the control of a tissue-specific promoter. After application of N-acetyl-phosphinothricin or N-acetyl-phosphinothricin tripeptide, the

REPLACEMENT SHEET
25 FEB 1997

tissue-specific expression leads to the immediate death of the respective tissue.

4) Any plants are transformed with a deacetylase gene from E. coli under the control of a tissue-specific promoter. After application of N-acetyl-phosphinothricin or N-acetyl-phosphinothricin tripeptide, the tissue-specific expression leads to the immediate death of the respective tissue.

Since the specificity of the deacetylase from streptomycetes for N-acetyl-phosphinothricin tripeptide is higher, it will be preferred to use N-acetyl-phosphinothricin tripeptide in case 3) and N-acetyl-phosphinothricin in case 4) if high activities are required. Tissue-specific promoters which can be used are all described promoters where selective expression in certain tissues has been detected (for example Koltunow et al., The Plant Cell., Vol. 2, 1201-1224, 1990). All newly-isolated promoters with similar properties are of course, also suitable. Other promoters which are suitable in addition to tissue-specific promoters are those which are subject to a different type of regulation (for example time-dependent, stress-dependent, environment-dependent) and which is tissue-specific.

These methods furthermore allow analysis of the differentiation of cell regulation and the production of plants in which the development of certain parts was inhibited in a targeted fashion, preferably the production of male-sterile plants.

A further application is the use of a dea gene for the identification of selectively expressed promoters. If DNA fragments with promoter activity are cloned upstream of dea genes, then the selective disappearance of parts of tissue after application of N-acetyl-phosphinothricin or N-acetyl-phosphinothricin tripeptide indicates the

REPLACEMENT SHEET
25 FEB 1997

specificity of the promoter.

Finally, the invention relates to positive selection systems. Those cells in which the dea gene has been inactivated can be selected either in combination with the pat gene and PTT (or PTC) together with a dea gene or with N-acetyl-phosphinothricin (or N-acetyl-phosphinothricin tripeptide) and a dea gene alone. This allows successful cloning (insertion inactivation), but also rare events (for example transposition), to be selected directly. Other aspects of the invention are mentioned in the examples.

Example 1: Fusion of the deacetylase encoding region with eucaryotic transcription signals

The plasmid pPRI (see EP-0,257,542) was isolated from an E. coli strain and cleaved with BamHI and BglII. The digested DNA was separated on an agarose gel, and an 0.9 kb fragment was isolated from the gel. The vector pROKI (Baulcombe et al., Nature 321, 446-449, 1986) was also restricted with BamHI. The two batches were combined and ligated. The ligation mixture was transformed into E. coli S17.1 (Simon et al., Bio/Technology 1, 784-791, 1983). Colonies growing on kanamycin-containing media were transferred to nitrocellulose filters, incubated for 12 hours at 37°C and then lysed. The DNA of the bacteria was fixed on the filter. The 0.9 kb fragment isolated from the agarose gel was made single-stranded by incubation at 100°C. The missing strand was then synthesized onto the existing strand using Klenow polymerase and digoxigenin-labeled nucleotides. The labeled strand was used as a sample for hybridizing with the bacterial DNA bound to the filter. Hybridizing clones could be detected with the aid of an antibody reaction. The DNA of the positive clones were isolated by means of Qiagen lysis and digested with BamHI/EcoRI as well as BamHI/HindIII. This restriction allows the orientation of the inserted

REPLACEMENT SHEET
25 FEB 1997

0.9 kb fragment to be determined. The plasmid in orientation I was designated pIB17.1, that of orientation II as pIB17.2 (see Fig. 2).

Example 2: Proof of the deacetylation of N-acetyl-PTC and N-acetyl-PTT by the deacetylase gene

It was possible to demonstrate that the eucaryotic transcription signals cloned in vector pROKI also allow expression in R. meliloti, A. tumefaciens and E. coli.

The plasmids pIB17.1 and pIB17.2 were therefore transferred into Rhizobium meliloti strain 2011 by means of a 2-factorial cross. By incubation of R. meliloti wild type strains with radiolabeled N-acetyl-PTC, it was possible to demonstrate that this strain does not deacetylate N-acetyl-PTC. (After incubation of PIB17.1-carrying strains with N-acetyl-PTC and N-acetyl-PTT, deacetylation can be detected by thin-layer chromatography). It was also possible to demonstrate that R. meliloti reacts highly sensitively to PTC and PTT. Deacetylation can therefore also be detected via inhibition of the R. meliloti glutamine synthesase, by the PTC which is liberated.

Example 3: Transfer of the modified deacetylase gene into Nicotiana tabacum

The deacetylase gene modified as in Example 1 was transferred into A. tumefaciens LBA4404 by means of a 2-factorial cross. The resulting strains LBA4404/17.1 and LBA4404/17.2 were used for incubating leaf discs of Nicotiana tabacum, which were transferred after 3 days to a kanamycin-containing shoot induction medium. Regenerating kanamycin-resistant shoots can be tested for the presence of the deacetylase gene by Southern hybridization. After treatment with N-acetyl-PTC or N-acetyl-PTT, the plants are then destroyed by the PTC, or PTT, which is liberated.

REPLACEMENT SHEET
25 FEB 1997

Example 4: Construction of a vector for the transient expression of the modified deacetylase gene in E. coli and tobacco protoplasts

The modified deacetylase gene from pIB17.1 and pIB17.2 was cut out of the plasmids by digestion with EcoRI/HindIII. The restricted DNA was separated in an agarose gel and an 0.9kb fragment was isolated in each case. The vector pSVB28 (Arnold and Pühler, Gene 70, 171-179, 1988) was also digested with EcoRI/HindIII. The two batches were combined and ligated. After transformation into the β-galactosidase-negative E. coli strain JM83, all clones which carried the vector turned blue, while clones which carried a vector into which the deacetylase gene had been inserted remained white. The DNA was isolated from the clones which had been identified in this way and digested with EcoRI/HindIII. The clones which contained the modified deacetylase gene could be recognized on the basis of the restriction pattern. The vectors which had been constructed are termed pIB27.1 and pIB27.2 (see Fig. 2). They exist in E. coli in a large number of copies.

Example 5: Transient expression of the modified deacetylase gene in tobacco protoplasts

The plasmid DNA was isolated from the E. coli strains constructed in Example 4. Young tobacco leaves were incubated with digestion enzymes for 20 h. The protoplasts which get disengaged from the leaf skeleton were purified and incubated with polyethylene glycol (PEG) and the isolated DNA in a transfer buffer. The protoplasts were then washed and taken up in a culture liquid (K3 medium). After incubation for 3 days under weak illumination, the regenerating protoplasts were lysed and the crude extracts were incubated with radiolabeled N-acetyl-PTC and N-acetyl-PTT. The deacetylated PTC or PTT can be detected by thin-layer chromatography.

REPLACEMENT SHEET
25 FEB 1997

Example 6: Method for the production of male-sterile crop plants using the deacetylase gene from S. viridochromogenes under the control of a tapetum-specific promoter.

The deacetylase gene from Streptomyces viridochromogenes is fused with a tapetum-specific promoter from Nicotiana tabacum and introduced into tobacco cells by means of agrobacteria-mediated leaf disc transformation. The plants regenerating from these cells are sprayed with N-acetyl-PTC or N-acetyl-PTT at any desired point in time before anthesis. It can be shown that N-acetyl-PTC is stable in the plant cell and transported into all cells. None of the two substances has noticeable negative consequences for the wild type plant. As soon as the first tapetum cells are formed, they start to express the deacetylase gene. The N-acetyl-PTC or N-acetyl-PTT stored in the cell is deacetylated by the enzyme and so converted into its active form. It inhibits the glutamine synthetase of the cells and so results in rapid destruction. Mature pollen can no longer be formed. In addition, the formation of deacetylase is also interrupted. Cells in the vicinity should not be affected. If the plant is not treated with N-acetyl-PTC or N-acetyl-PTT, it is fully fertile. This makes compensation for the cms by a gene of the male crossing partner unnecessary. At the same time, there exists an accurately defined mutation which has no consequences on the vitality and usefulness of the plant.

Example 7: Identification of tissue-specific promoters in transgenic plants

Tissue-specific promoters can be identified directly in the plant with the aid of the deacetylase gene from Streptomyces viridochromogenes.

The deacetylase gene is cloned, without a promoter, to the right or left end of a disarmed T-DNA in such a way

REPLACEMENT SHEET

25 FEB 1997

that a promoter which is located at the insertion site of the T-DNA in the plant genome can read into the gene and so bring about its expression. Transgenic plants are cloned via the propagation of cuttings. One clone is treated with N-acetyl-PTC or N-acetyl-PTT and examined for tissue which may be in the process of dying. Using reverse PCR, the gene which has been affected can be multiplied from a clone which has not been treated with N-acetyl-PTC or N-acetyl-PTT and isolated (Kahl and Weising, Gentransfer bei Pflanzen [Gene Transfer in Plants], Biologie in unserer Zeit, No. 6, p. 181, 1988).

Example 8: Detection of N-acetyl-phosphinothricin (PPT)-deacetylase activity in soil samples

Soil samples of 500 mg each (sandy loam, Schwanheimer Düne) were adjusted to 40% of their maximum water capacity and treated with 5 µl of a 15 mM stock solution of ^{14}[C]-L-N-acetyl-PPT. The test samples were incubated at 28°C for various periods of time (0 hours, 4, 7, 11 and 14 days) and subsequently worked up by extraction with 1 x 500 µl and 1 x 250 µl of water. 14 µl aliquots from the combined aqueous supernatants were applied to thin-layer chromatography plates (HPTLC cellulose, Merck) and developed 2 x in n-propanol: 25% ammonia = 3 : 2 as the mobile phase. The assays were evaluated by autoradiography. It was possible to identify N-acetyl-PPT and PPT by comparing the R_f values of the radioactive spots with the corresponding reference substances. It emerged that N-acetyl-PPT in the soil is metabolized within 14 days almost completely to give PPT. In contrast, in a control assay with sterile soil samples (soil 4 hours at 200°C), the substance proved to be completely stable.

Example 9: Isolation and identification of soil microorganisms having an N-acetyl-PPT-specific deacetylase activity

REPLACEMENT SHEET
25 FEB 1997

1 g samples of soil were extracted for 1 hour at room temperature using 10 ml 10 mM NaCl, 10 mM sodium phosphate buffer, pH = 7.0. To select various groups of microorganisms, the soil supernatants were plated onto the following agar media and used for inoculating enrichment cultures in Erlenmeyer flasks with the corresponding liquid media:

(1) MS1 medium (for eubacteria):

5 mM glucose
5 mM succinate
10 mM glycerol
1 g/l NH_4Cl
50 ml/l solution A
25 ml/l solution B

Solution A: 50 g/l K_2HPO_4
Solution B: 2.5 g/l $MgSO_4$
0.5 g/l NaCl
25 ml/l trace elements

(2) Chitin medium (for actinomycetes and streptomycetes as well as chitinovorous bacteria):

10 g/l crab chitin
1 g/l $(NH_4)_2SO_4$
0.5 g/l $MgSO_4$
50 ml/l solution A
1 ml/l trace elements

(3) Antibiotics medium (for higher fungi):

20 g/l malt extract
10 g/l glucose
2 g/l yeast extract
0.5 g/l $(NH_4)_2SO_4$
50 µg/ml tetracyclin

REPLACEMENT SHEET
25 FEB 1997

All media contained 5 mM N-acetyl-PPT. The agar plates and the liquid cultures were incubated for 3-5 days at 28°C.

20 individual colonies were isolated from each of the selective agar plates and transferred into 5 ml liquid cultures with the corresponding medium. The cells were allowed to grow for 3-5 days and then centrifuged at 10,000 rpm, and the supernatants were examined in the aminoacid analyzer (Biotronic LC 5001) for the formation of PPT. In this manner, a PPT-positive culture (CB 10) was isolated from a selection with chitin medium.

The deacetylase activity of the cells of this culture were subsequently additionally tested by biotransformation with ^{14}[C]-L-N-acetyl-PPT as the substrate. To do this, 1.5 ml of the culture were centrifuged as above, the cell pellet was washed 1 x in 10 mM NaCl, 10 mM sodium phosphate buffer, pH = 7.0, and resuspended in 100 µl of the same buffer. 10 µl of the suspension were treated with 10 µl of an 0.25 mM solution of ^{14}[C]-L-N-acetyl-PT and the mixture was incubated for 15 hours at 28°C. The bacteria were then centrifuged off, and 7 µl of the supernatant were analyzed by thin-layer chromatography and autoradiography as described in Example 1. A virtually quantitative reaction of N-acetyl-PPT into PPT could be observed. In addition, the assay showed that the deacetylase found accepts the L enantiomer of the acetylated PPT as substrate.

To further purify the strain with the desired deacetylase activity, the culture CB 10 was plated onto LB agar (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar) and incubated for 2 days at 28°C. 10 individual colonies were isolated from the plate, transferred to chitin liquid medium, and the cultures were tested for N-acetyl-PPT deacetylase activity as described above. The deacetylase-positive isolates were replated to check for uniformity of the culture. The active strains CB 10 3496

REPLACEMENT SHEET
25 FEB 1997

and CB 10 34397 were identified as .... (DSM deposit No. ...) and .... (DSM deposit No. ....).

The enrichment cultures in the soil samples in the various liquid media were tested for deacetylation of N-acetyl-PPT as described above. Only the chitin medium cultures proved to be deacetylase-positive. After these cultures were plated onto chitin agar, a total of 40 individual colonies was isolated, grown in chitin liquid medium and subsequently tested for deacetylase activity. Six positive isolates were found (BoK1, BoK9, BCÜ1, BCÜ2, BCÜ3), from which the active pure cultures were obtained by replating onto agar plates and culturing further on individual colonies (see above). The strains were ....., ...(DSM deposit Nos. ....., ...).

Example 10: N-Acetyl-PPT deacetylase enzyme assays with the isolated microorganisms

5 ml precultures of strains BoK1 and BoK5 were grown in LB medium overnight at 28°C, and 0.5 ml aliquots were transferred to 20 ml of LB medium or 20 ml of chitin medium containing 1 mM N-acetyl-PPT. The LB cultures were incubated for 15 hours and the chitin cultures for 4 days in 100 ml Erlenmeyer flasks at 28°C and 150 rpm. The cells were subsequently harvested by centrifugation for 10 minutes at 10,000 rpm, the cell pellets were washed 1 x in 10 ml mM NaCl, 10 mM sodium phosphate buffer, pH = 7.0, weighed and resuspended in 100 mM tris/HCl, pH = 8.0 at c = 100 mg/ml. The suspensions were mixed with 1 volume of 100 mM N-acetyl-PPT and incubated in 50 ml Erlenmeyer flasks for 24 hours at 28°C and 220 rpm. The cells were separated by centrifugation for 10 minutes at 5000 rpm, and the PPT content in the supernatants was then determined in the aminoacid analyzer (see Example 2). The results are compiled in Table 1.

REPLACEMENT SHEET
25 FEB 1997

Table 1: N-Acetyl-PPT deacetylase assays with soil microorganisms

Strain:	Medium:	Concentration of PPT in the supernatant [mM]:	[%]*:
BoK1	LB	0.7	2.7
BoK1	Chitin	13.9	55.5
BoK5	LB	6.0	23.9
BoK5	Chitin	14.3	57.2

*: based on the L-enantiomer in the N-acetyl-PPT racemate.

Example 11: N-Acetyl-PPT deacetylase enzyme assays with actinomycetes

N-Acetyl-PPT-specific deacetylase activities were also found during fermentation tests with the two actinomycetes strains Actinoplanes liguriae (IFO No. 13997) and Actinoplanes sp. (Strain Collection Zentralforschung No. A 1015) in the presence of N-acetyl-PPT and by biotransformation with ^{14}[C]-L-N-acetyl-PPT as the substrate.

To determine the conversion rates, biotransformations were carried out on the two strains as described in Example 3. The following media were used:

Medium A: 0.2% yeast extract
0.2% meat extract
0.4% polypeptone (from soya meal)
1% glucose

Medium B: 20 g/l oat flakes
1 ml/l trace elements

The results are compiled in Table 2.

REPLACEMENT SHEET
25 FEB 1997

Table 2: N-Acetyl-PPT deacetylase assays with actinomycetes

Strain:	Medium:	Concentration of PPT in the supernatant [mM]:	[%]*:
Actinoplanes liguriae (IFO No. 13997)	A	3.3	13.2
Actinoplanes liguriae (IFO No. 13997)	B	7.6	30.4
Actinoplanes sp. (No. A 1015)	A	11.0	44.0
Actinoplanes sp. (No. A 1015)	B	2.7	10.8

*: based on the L enantiomer in the N-acetyl-PPT racemate

REPLACEMENT SHEET
25 FEB 1997

Patent Claims:

1. A process for isolating deacetylase genes from micro-organisms, which comprises establishing a gene bank in a deacetylase mutant which has been provided with a pat gene, and identifying deacetylase-carrying clones by means of PTT- or PTC-sensitivity.

2. The deacetylase gene from E. coli, obtainable by the process as claimed in claim 1.

3. The deacetylase gene from S. viridochromogenes, comprising an amino-acid sequence encoded by the nucleotide sequence of Table 1 and obtainable by the process as claimed in claim 1.

4. A process for the production of transgenic plants with selectively destroyable parts, which comprises bringing a deacetylase gene under the control of a tissue-specific promoter and causing death of the tissue portions concerned by suitable timely treatment with N-acetyl-PTC or N-acetyl-PTT.

5. A process for the production of transgenic plants with selectively destroyable parts, wherein the plant has PTC resistance and additionally contains a deacetylase gene under the control of a tissue-specific promoter and causing death of the tissue portions concerned by suitable timely treatment with PTC or PTT.

6. The process as claimed in claim 4 or 5, wherein the deacetylase gene originates from E. coli and the plant is treated with N-acetyl-PTC or with PTC.

7. The process as claimed in claim 4 or 5, wherein the deacetylase gene originates from Streptomyces viridochromogenes and the plant is treated with N-acetyl-PTT or

* C L M S *

REPLACEMENT SHEET
25 FEB 1997

with PTT.

8. The process as claimed in claim 4, 5, 6 or 7, wherein male-sterile plants are produced.

9. The process as claimed in claim 8, wherein the deacetylase gene is under the control of the tapetum promoter.

10. A process for detecting tissue-specific promoter activity, which comprises placing a deacetylase gene under the control of a DNA sequence which probably contains a tissue-specific promoter sequence and the promoter activity is recognized indirectly by the death of certain tissues.

11. A positive selection system for detecting clones or transpositions by insertion inactivation, which comprises selecting those cells in which the dea gene had been inactivated, in connection with a pat gene and PTT or PTC and the dea gene or with N-acetyl-PTC or N-acetyl-PTT and the dea gene alone.

12. The use of a deacetylase gene for the targeted prevention of specific parts of the plant from functioning.

This Sheet Has Been Replaced





THIS SHEET HAS BEEN REPLACED

REPLACEMENT SHEET
25 FEB 1997

16. The use of a deacetylase gene for the targeted prevention of specific parts of the plant from functioning.

This Sheet Has Been Replaced





THIS SHEET HAS BEEN REPLACED

REPLACEMENT SHEET
25 FEB 1997

ABSTRACT

DEACETYLASE GENES FOR THE PRODUCTION OF PHOSPHINOTHRICIN OR PHOSPHINOTHRICYL-ALANYL-ALANINE, PROCESSES FOR THEIR ISOLATION, AND THEIR USE

The invention relates to deacetylase genes, to processes for their isolation, and to their use, in particular for the production of transgenic plants using tissue-specific promoters. It is possible to prevent the development of certain parts in these plants in a targeted manner. With the aid of deacetylase genes, it is furthermore possible to identify and isolate tissue-specific promoters in transgenic plants.

FIGURE 1

This Sheet Has Been Replaced



THIS SHEET HAS BEEN REPLACED

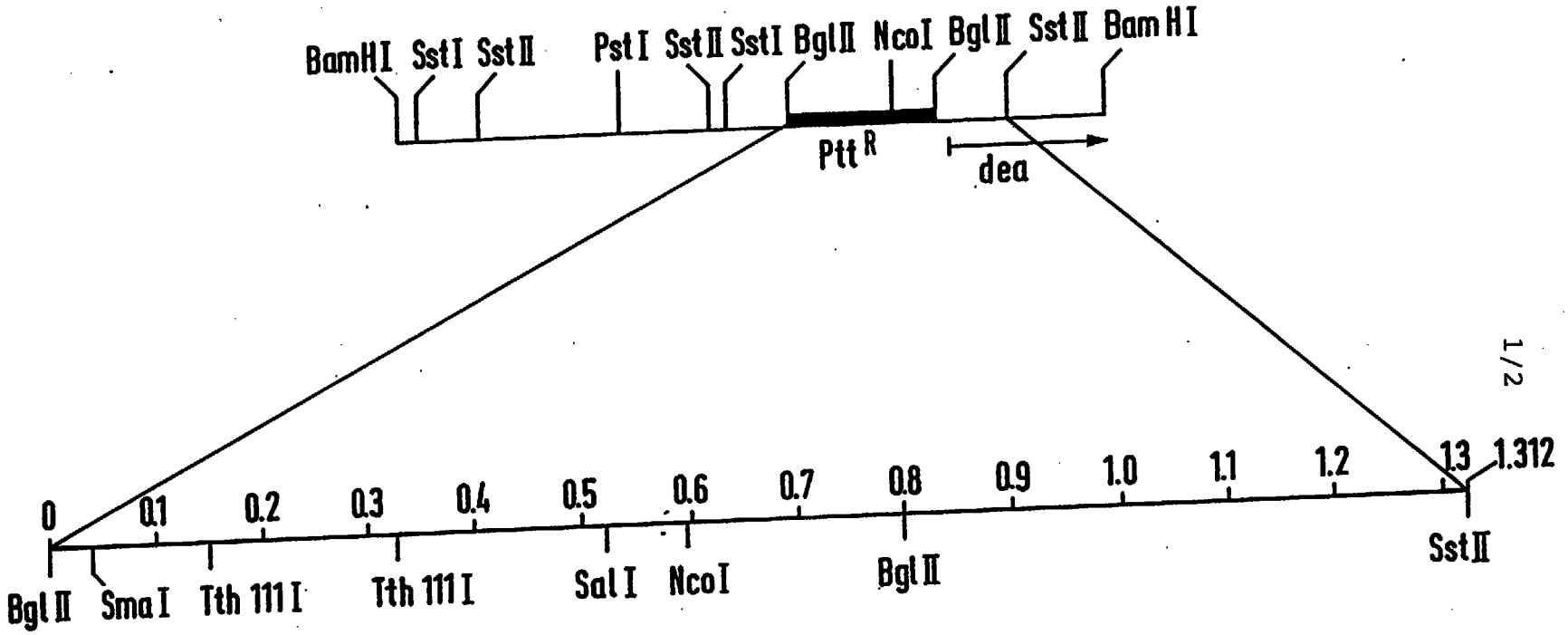
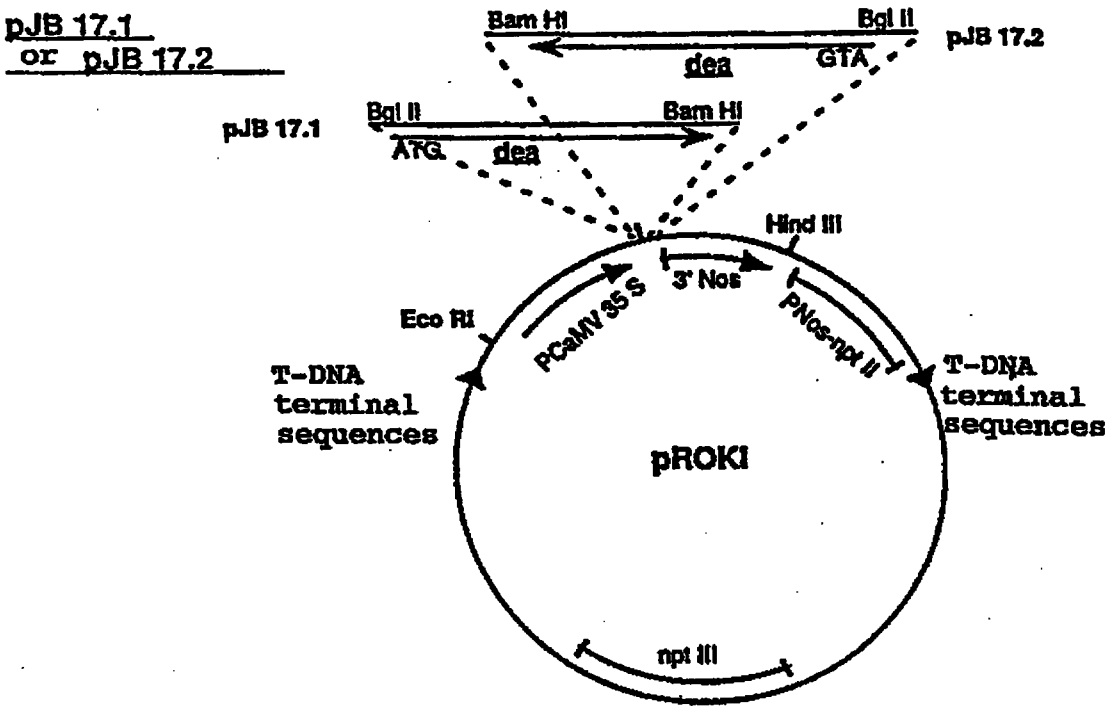

BamHI SstI SstII
PstI SstII SstI BglII NcoI BglII SstII BamHI
Ptt R
dea
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.312
BglII SmaI Tth 111 I
Tth 111 I
SalI NcoI
BglII
SstII

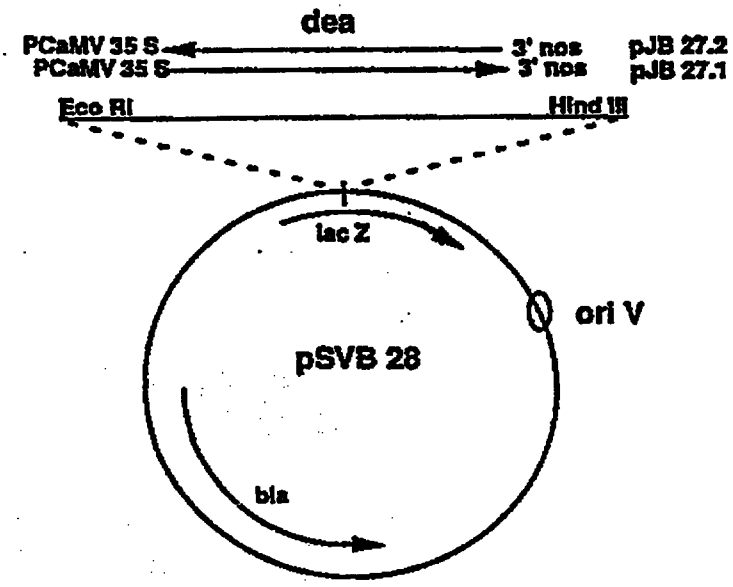
Fig. 1

Figure 2



npt II: Neo - resistance gene from TN5
npt III: Neo - resistance gene from S. faecalis

pJB 27.1 or pJB 27.2
3.6 kb



REPLACEMENT SHEET
04 DEC 1997

ABSTRACT

DEACETYLASE GENES FOR THE PRODUCTION OF PHOSPHINOTHRICIN OR PHOSPHINOTHRICYL-ALANYL-ALANINE, PROCESSES FOR THEIR ISOLATION, AND THEIR USE

The invention relates to deacetylase genes, to processes for their isolation, and to their use, in particular for the production of transgenic plants using tissue-specific promoters. It is possible to prevent the development of certain parts in these plants in a targeted manner. With the aid of deacetylase genes, it is furthermore possible to identify and isolate tissue-specific promoters in transgenic plants.

FIGURE 1

This Sheet Has Been Replaced



THIS SHEET HAS BEEN REPLACED

REPLACEMENT SHEET
22 JUL 1999

with PTT.

8. The process as claimed in claim 4, 5, 6 or 7, wherein male-sterile plants are produced.

9. The process as claimed in claim 8, wherein the deacetylase gene is under the control of the tapetum promoter.

10. A process for detecting tissue-specific promoter activity, which comprises placing a deacetylase gene under the control of a DNA sequence which probably contains a tissue-specific promoter sequence and the promoter activity is recognized indirectly by the death of certain tissues.

11. A positive selection system for detecting clones or transpositions by insertion inactivation, which comprises selecting those cells in which the dea gene had been inactivated, in connection with a pat gene and PTT or PTC and the dea gene or with N-acetyl-PTC or N-acetyl-PTT and the dea gene alone.

12. The use of a deacetylase gene for the targeted prevention of specific parts of the plant from functioning.

13. A transgenic plant with selectively destroyable parts, which comprises a deacetylase gene under the control of a tissue-specific promoter, whereby death of said plant parts can be obtained by suitable timely treatment with N-acetyl-PTC or N-acetyl-PPT.

14. A transgenic plant which comprises a deacetylase gene under the control of a tapetum-specific promoter, whereby said plant can be made male-sterile by suitable timely treatment with N-acetyl-PTC or N-acetyl PPT.

* C L M S *

REPLACEMENT SHEET
22 JUL 1999

15. A seed from the plant of claim 13 or 14.

16. The seed of claim 15, that is a hybrid seed.

17. A plant cell of the plant of claim 13 or 14.

18. A plant grown from the seed of claim 15.

* C L M S *

REPLACEMENT SHEET
22 JUL 1999

ABSTRACT

DEACETYLASE GENES FOR THE PRODUCTION OF PHOSPHINOTHRICIN OR PHOSPHINOTHRICYL-ALANYL-ALANINE, PROCESSES FOR THEIR ISOLATION, AND THEIR USE

The invention relates to deacetylase genes, to processes for their isolation, and to their use, in particular for the production of transgenic plants using tissue-specific promoters. It is possible to prevent the development of certain parts in these plants in a targeted manner. With the aid of deacetylase genes, it is furthermore possible to identify and isolate tissue-specific promoters in transgenic plants.

FIGURE 1