(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 April 2001 (26.04.2001)

PCT

(10) International Publication Number
WO 01/29237 A2

(51) International Patent Classification[7]: C12N 15/82, A01H 1/02, 5/00

(21) International Application Number: PCT/EP00/10281

(22) International Filing Date: 13 October 2000 (13.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/418,817 15 October 1999 (15.10.1999) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *Without international search report and to be republished upon receipt of that report.*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 01/29237 A2

(54) Title: CONDITIONAL STERILITY IN WHEAT

(57) Abstract: The invention relates to the use of a deacetylase coding sequence for obtaining conditional sterility in wheat. The invention relates to vectors comprising a deacetylase coding sequence under control of promoters which direct stamen-selective expression in wheat, which are particularly suited for the production of wheat plants which can be made male-sterile upon application of an acetylated toxin.

# CONDITIONAL STERILITY IN WHEAT

## FIELD OF THE INVENTION

The invention relates to the production of hybrid seed in wheat by using a deacetylase gene to obtain conditional sterility. Vectors are described comprising a deacetylase gene under the control of promoters directing expression selectively in stamen cells in wheat, which are particularly suited for the production of wheat plants which can be made male-sterile upon application of N-acetyl-PPT.

All documents cited herein are included herein by reference.

## BACKGROUND OF THE INVENTION

Deacetylases are capable of deacetylating acetylated toxins, such as N-acetyl-phosphinothricin (N-Ac-PTC or N-Ac-PPT), intracellularly, whereupon the cytotoxic activity of the toxins is restored (formation of PTC or PPT). US 5,650,310, US 5,668,297, US 5,767,370, and US 5,767,371, describe deacetylase genes, for the production of phosphinothricin or phosphinothricyl-alanyl-alanine, as well as processes for their isolation and use. Furthermore deacetylase genes are disclosed therein which are isolated from *Streptomyces viridochromogenes* (dea) and from *E. coli* (ArgE). The use of such genes for the production of plants with selectively destroyable plant parts is described therein. More specifically, a method is disclosed for the production of conditionally male-sterile plants. This is achieved by introducing a deacetylase gene, under the control of a tapetum-specific promoter into the genome of a plant. Upon application of N-Ac-PPT to the plant, in the tapetum cells, where the deacetylase is expressed, N-Ac-PPT is converted into PPT, which is toxic to the cells. The cytotoxic activity in the tapetum cells inhibits the development of microspores and renders the plant male-sterile.

CONFIRMATION COPY

WO 98/13504 describes the use of deacetylase genes for the production of female-sterile plants. WO 98/39462 describes a method of hybrid seed production using conditional female sterility, whereby a plant is made conditionally female-sterile by transformation with the ArgE gene linked to a female-preferential promoter.

WO 98/27201 describes novel genes encoding amino acid deacetylases isolated from *Stenotrophomonas sp.* (deac) and from *Comamonas acidovorans* (ama) and their use in the production of transgenic plants.

The high production cost of hybrid wheat has limited the use of commercial hybrids. The economic relationship between the additional costs of male sterilization and seed production are up to now clearly unfavorable in breeding and agriculture. In contrast to crops such as maize, the hybrid seed production in wheat has been subject to considerable difficulties. The reproductive system of wheat, a self-fertilizer, is poorly adapted to cross-fertilization, which has led to poor hybrid seed set and consequently low yields. This has resulted in a high price for the hybrid seed on farm-level. In comparison to line varieties, the yield advantage of hybrids in wheat is up to now not sufficient enough to compensate high seed cost. (Picket, 1993, In: Hybrid Wheat-Results and Problems, Advances in Plant Breeding, Paul Parey Scientific Publishers, Berlin and Hamburg).

In the last few years, mainly due to an improvement in agricultural production steps, interest in hybrid wheat has resurfaced, and first hybrid wheat varieties have been marketed.

Traditional large-scale hybrid seed production is accomplished by planting separate rows or blocks of female parent lines and male parent lines as pollinators. Only the seed produced on the female parent rows or blocks is harvested. To ensure that this seed is hybrid seed, uncontaminated with selfed seed, pollination control must be implemented. Known pollination control methods are generally chemical, using a hybridizing agent, or genetic. Genetic methods have been developed using either natural or molecular genes,

based on either cytoplasmic or nuclear sterility factors. Neither the cytoplasmic male-sterility nor the chemical male-sterility are ideal and both show important economical, practical and/or environmental drawbacks.

The successful use of cytoplasmic male-sterility for commercial hybrid seed production requires a stable male cytoplasm and the availability of reliable restorer genes which is a limiting factor in the wheat system presently used based on the *T. timopheevi* system (Wilson, J.A. and Ross, W.M. (1962). Wheat Information Service, Kyoto University 14: 29-30; Wilson, J.A. and Ross, W.M. (1962b). Crop Science 2: 415-417). Furthermore such a system of cytoplasmic male-sterility requires three lines to produce a single crossed hybrid: the A line (male-sterile), the B line (male-fertile maintainer), and an R line (male-fertile with restorer genes). Three-way crosses produced with cytoplasmic-genetic male sterility involve maintenance and production of four lines, the A and B lines of one inbred and male-fertile inbreds of the other two.

Alternatively, the production of hybrid seed can be achieved using chemicals that block or kill viable pollen formation. These chemicals, called gametocides, are used to impart a transitory male-sterility. The expense and the availability of the chemical, the reliability of the applications under different environmental conditions and the toxicity of the chemical to the environment however, limit its use in the production of hybrid seed.

Molecular methods for hybrid seed production offer a new promising alternative. Male-sterile wheat plants were obtained by introducing the barnase gene under the control of tapetum-specific promoters from corn and rice (De Block et al., 1997, Theor. Appl. Genet. 95:125-131). Such transformed plants are functionally male-sterile and can be used for the production of hybrid seed by crossing with male-fertile pollinator plants, containing corresponding restorer genes (to ensure restoration of fertility in the hybrid; as described in EP 0 344 029, US 5,689,041, US 5,792,929, US 5,723,763). The genetically engineered male-sterility, whether it works through anti-sense or RNAse, can only be maintained in a heterozygous state. The heterozygous female parent, of which only 50% will be male-sterile, must be planted in rows next to the pollen donor (male) parent and the other 50% of fertile female parent have to be removed. By genetic engineering it is

possible to link the male-sterility gene to a herbicide resistance gene and remove the fertile plants by spraying the corresponding herbicide. Still this system has economical drawbacks. Due to the fact that only 50% of the female plants can be used for hybrid seed production, the female parent rows must be planted at double density in order to obtain the same yield per acre. This will cause yield loss because of competition between plants. The herbicide spray also induces yield loss, as resistant plants are never 100% resistant to the herbicide. The considerable costs for the chemical and for the "restorer" breeding program makes that this hybrid seed production system is economically not ideal for wheat. To achieve a more economical hybrid seed production system for wheat and potentially other cereal crops, the use of a conditional male-sterility system, whereby plants can be maintained in their male fertile form and no restorer genes are needed would be desirable. It remains to be described how such a conditional male-sterility system can optimally be developed for wheat and other cereal crops like barley, rye and oats.

## SUMMARY OF THE INVENTION

The invention relates to a method for producing conditionally male-sterile wheat plants, which method comprises 1) introducing into the genome of a wheat cell or tissue a foreign DNA comprising a chimeric gene which comprises a DNA molecule encoding a deacetylase, more specifically a deacetylase from *Stenotrophomonas sp.*, under the control of a promoter directing expression selectively in stamen cells in wheat, and 2) regenerating a plant from the cell or tissue, which plant is conditionally male-sterile.

The invention further relates to a conditionally male-sterile wheat plant comprising, integrated into its genome, a foreign DNA comprising a chimeric gene which comprises:
a) a DNA molecule encoding a deacetylase, more specifically a deacetylase from *Stenotrophomonas sp.*, under the control of
b) a promoter directing expression selectively in stamen cells in wheat, such as the CA55 promoter, the T72 promoter or the E1 promoter;

whereby the wheat plant can be made male-sterile by application of an acetylated toxin, such as N-Acetyl-PPT, to the plant.

The invention further relates to a conditionally male-sterile wheat plant comprising, integrated into its genome, a foreign DNA comprising a chimeric gene which comprises a deacetylase coding sequence isolated from *Stenotrophomonas sp.*, preferably from the organism deposited under accession No. DSM 9734 or a biologically active fragment or variant thereof; additionally or alternatively according to the present invention, the deacetylase coding sequence encodes the deacetylase corresponding to SEQ ID No. 10 described herein or a biologically active fragment or variant thereof; additionally or alternatively the DNA encoding a deacetylase of the invention comprises the sequence of SEQ ID No. 11, or a fragment or variant thereof and/or corresponds to a DNA sequence which hybridizes to SEQ ID No. 11 under standard stringent conditions; additionally or alternatively the chimeric gene of the invention comprises SEQ ID No. 12, SEQ ID No. 13 or SEQ ID No 14.

The invention further relates to a method for producing composite hybrid seed in wheat, which comprises:

- producing seeds capable of growing into conditionally male-sterile plants, comprising, integrated into their genome, a foreign DNA comprising a chimeric gene which comprises a DNA encoding a deacetylase from *Stenotrophomonas sp.* under control of a promoter directing expression selectively in stamen cells in wheat,
- interplanting these seeds with seeds capable of growing into male fertile plants,
- inducing male-sterility in the conditionally male-sterile plants by applying an acetylated toxin, such as N-acetyl PPT to the conditionally male-sterile plants; and
- harvesting seed from both conditionally male-sterile plants and male fertile plants, which is composite hybrid seed.

The invention further relates to a method for producing pure hybrid seed in wheat by planting the above-described seeds capable of growing into conditionally male-sterile

plants and seeds capable of growing into male-fertile plants in separate rows or blocks, and harvesting seed only from the pollinated conditionally male-sterile plants.

The invention further relates to a method for producing pure hybrid wheat seed by crossing conditionally male-sterile wheat plants, comprising integrated into their genome a DNA encoding a deacetylase from *Stenotrophomonas sp.* under control of a stamen selective promoter, with female sterile plants as described in US 5,633,441 or US 5,767,374 or conditionally female-sterile plants as described in WO 98/13504, which are male-fertile, and harvesting hybrid seed from the pollinated conditionally male-sterile plants.

The invention further relates to a conditionally male-sterile wheat plant comprising, integrated into its genome, a foreign DNA comprising chimeric gene which comprises a DNA molecule encoding a deacetylase from *Stenotrophomonas sp.*, under control of a stamen-selective promoter, and which further comprises a second chimeric gene which confers on the plant resistance to a herbicide.

The invention further relates to a conditionally male-sterile wheat plant, comprising the chimeric gene of the invention comprising a DNA encoding a deacetylase and further comprising a second chimeric gene which confers on the plant resistance to glyphosate. More specifically, said second chimeric gene comprises a gene, such as the EPSP gene, under control of a constitutive promoter, conferring resistance to glyphosate to the plant.

The invention further relates to a conditionally male-sterile wheat plant, comprising the chimeric gene of the invention comprising a DNA molecule encoding a deacetylase and further comprising a second chimeric gene which confers on the plant resistance to PPT. More specifically said second chimeric gene comprises a gene, such as the *pat* or *bar* gene, capable of converting PPT into N-acetyl-PPT. The N-acetyl-PPT so produced can be transported through the plant to the stamen cells in which the deacetylase is expressed, where it can be converted to PPT. It is understood that in this embodiment of the

invention, specific destruction of the stamen cells can be induced by treatment of the plant with N-acetyl PPT or PPT.

The invention further relates to a method for producing pure hybrid seed in wheat, which comprises:

- producing seeds capable of growing into conditionally male-sterile plants comprising, integrated in their genome, a foreign DNA comprising

a) a chimeric gene comprising a DNA molecule encoding a deacetylase from *Stenotrophomonas sp.* under control of a promoter that directs stamen selective expression in wheat, and further comprising

b) a chimeric gene which confers on the plants resistance to a herbicide,

- interplanting seeds capable of growing into conditionally male-sterile plants with seeds capable of growing into male fertile plants, that are not resistant to the herbicide,
- inducing male-sterility in the conditionally male-sterile plants by applying an acetylated toxin, such as N-acetyl PPT, to the conditionally male-sterile plants;
- allowing pollination of the male-sterile plants by the male fertile plants;
- applying the herbicide to eliminate the male fertile plants; and
- harvesting seed from the conditionally male-sterile plants.

The present invention also demonstrates that particular stamen selective promoters, particularly the CA55 promoter, the PE1 promoter, and the T72 promoter are particularly useful, when linked to a deacetylase gene, for the generation of conditionally male-sterile plants according to the invention.

The present invention further demonstrates that the deac system is particularly suited for obtaining inducible male-sterility in cereal plants, such as barley, oats, rye and most particularly in wheat.

It will be understood that particular embodiments of the invention are described by the dependent claims cited herein.

DETAILED DESCRIPTION

The invention relates to a conditionally male-sterile wheat plant, comprising a deacetylase coding sequence placed under the control of a promoter directing stamen-selective expression in wheat. The deacetylase of the invention, more specifically the deacetylase encoded by the deac gene from *Stenotrophomonas sp.*, is capable of deacetylating an acetylated toxin in wheat cells, rendering it toxic to the wheat plant cell in which it is expressed. As described herein, the acetylated toxin, more specifically N-acetyl-PPT, when applied to a wheat plant, is transported to the stamen cells, where in the presence of a deacetylase, it is converted to a toxin, more specifically PPT, which is toxic to the cells.

The term "gene" as used herein refers to any DNA sequence comprising several operably linked DNA fragments such as a promoter and a 5' regulatory region (which together form the promoter region), a coding sequence, and an untranslated 3' region comprising a polyadenylation site.

The term "chimeric" when referring to a gene or DNA sequence is used to refer to the fact that in nature, the coding sequence is not associated with the promoter or with at least one other regulatory region of the DNA in the gene.

A "chimeric gene" as used herein may comprise one or more genes or DNA sequences as described above. "Foreign" when referring to a gene or a DNA sequence with respect to a plant species is used to indicate that the gene or DNA sequence is not naturally found in that plant species or is naturally located in a different locus in the genome of that plant species.

A genome of a plant, plant tissue or plant cell, as used herein, refers to any and all genetic material in the plant, plant tissue or plant cell, and includes both the nuclear and the plastid (chloroplast) and mitochondrial genome.

A DNA molecule or coding sequence encoding a deacetylase as used herein refers to a DNA sequence encoding a protein having the activity of a deacetylase. A deacetylase is a molecule which is capable of deacetylating an acetylated toxin (which in itself –i.e. in the

absence of a deacetylase- is not toxic to the plant or its plant cells) to produce the active toxin. Preferably, the deacetylase is capable of deacetylating N-acetyl PPT, to produce PPT which is cytotoxic. Deacetylase activity can be measured by radioactive or non-radioactive assays as described in WO 98/27201 and US 5,668,297.

According to the present invention, a preferred embodiment of the DNA molecule encoding a deacetylase, also referred to herein as a "deac" coding sequence, is a DNA encoding a deacetylase from *Stenotrophomonas sp.*, such as can be isolated from the micro-organism deposited under DSM 9734, or a biologically active fragment or variant thereof. A "fragment" of a sequence means a truncated sequence of the original sequence (nucleic acid or amino acid) referred to, which is sufficient to ensure the (encoded) protein to be biologically active.

A variant of a sequence (nucleic acid or amino acid) refers to a sequence which is essentially similar, meaning that when two sequences are aligned, the percent sequence identity, i.e. the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the sequences, is higher than 80%, preferably higher than 90%. The alignment of two nucleotide sequences is performed by the algorithm as described by Wilbur and Lipmann (1983, Proc. Natl. Acad. Sci. U.S.A. 80:726) using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4. Preferably a variant of a DNA molecule encoding a deacetylase as used herein is a variant of a deacetylase gene from *Stenotrophomonas sp.*, which has been adapted for preferred codon usage in monocots, particularly wheat.

In a preferred embodiment of the invention, the DNA molecule encoding a deacetylase used in the context of the present invention, is a DNA molecule which can be isolated from the deposit with deposit No. DSM 9734 (as described in WO 98/27201). More specifically such a DNA is a DNA encoding a deacetylase having the sequence of SEQ ID No. 10, or a biologically active fragment thereof. Additionally or alternatively such a DNA can comprise the DNA sequence of SEQ ID No. 11, or a variant or fragment thereof, or can correspond to a DNA sequence which hybridizes with the sequence of SEQ ID No. 11 under standard stringent conditions. Standard stringent conditions as used

herein refers to the conventional hybridizing conditions as described by Sambrook et al. (1989) (Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, NY) which for instance can comprise the following steps: 1) immobilizing plant genomic DNA fragments on a filter, 2) prehybridizing the filter for 1 to 2 hours at 42°C in 50% formamide, 5 X SSPE, 2 X Denhardt's reagent and 0.1% SDS, or for 1 to 2 hours at 68°C in 6 X SSC, 2 X Denhardt's reagent and 0.1% SDS, 3) adding the hybridization probe which has been labeled, 4) incubating for 16 to 24 hours, 5) washing the filter for 20 min. at room temperature in 1X SSC, 0.1 %SDS, 6) washing the filter three times for 20 min. each at 68°C in 0.2 X SSC, 0.1 %SDS, and 7) exposing the filter for 24 to 48 hours to X-ray film at -70°C with an intensifying screen.

Other nucleotide sequences encoding deacetylases that can be used in the context of the present invention have been isolated, such as the dea gene from *Streptomyces viridochromogenes* and the ArgE gene from *E. coli* (US 5,767,371), and the ama gene from *Comamonas acidovorans* (WO 98/27201).

As used herein "N-acetyl-PPT" refers to N-acetyl-phosphinothricin, or a composite structure thereof, such as, but not limited to N-acetyl-phosphinothricyl-alanyl-alanine (N-acetyl-PTT), which in itself is not toxic to cells but is converted to a toxin by a deacetylase. Thus, the term N-acetyl-PPT refers to any formulation of N-acetyl-phosphinothricin, or derivatives or related compounds, such as acetylated forms of desmethylphosphinothricine (2-amino-4-hydroxyphosphinobutyric acid), acetylated forms of bialaphos (phosphinothricyl-alanyl-alanine), acetylated forms of desmethylbialaphos and the like, their salts, racemic mixtures and active enantiomers, which can be used in the context of the present invention.

According to the present invention, a "stamen-selective promoter" or a promoter that directs expression selectively in stamen cells is a promoter directing expression in selective stamen cells (including anther cells and pollen) in wheat, preferably directing expression essentially only in stamen cells in wheat. More specifically, the stamen-selective promoter is a tapetum-specific promoter directing expression specifically in

tapetum cells in wheat. More specifically, the stamen-selective promoter as used herein refers to a promoter selected from the CA55 promoter isolated form corn (WO 92/00275, US 5,589,610), the T72 or the E1 promoter isolated from rice (US 5,639,948), which were shown to cause male-sterility when linked to a barnase coding sequence in wheat (De Block et al, supra). Other stamen specific promoters which have been isolated and can be used in the context of the present invention are, for example but not limited to, the stamen-specific promoters TA29 (US 5,652,354), SGBG (US 5,837,850), and promoter 5126 (US 5,689,049). Additionally, it can be envisaged that promoters of genes involved in the early development of the stamen can be used in the context of the present invention.

A "male-sterile plant" as used herein refers to a plant that is not capable of producing viable, fertile pollen. In the context of the present invention, a male-sterile plant as used herein is female fertile (and can thus function as a female parent to produce seed when pollinated by a male-fertile plant). A "conditionally male-sterile plant" refers to a plant which, under normal growing conditions, is male fertile, i.e. produces viable, fertile pollen, and which can be made male-sterile under specific conditions. More specifically, these conditions are understood to refer to the treatment of the plant with an acetylated toxin, which normally is not toxic to the plant or plant cells, but in the presence of a deacetylase in the plant cell is converted into a substance that is cytotoxic.

"Treatment" of the plant can refer to administering the substance in any way, such as, but not limited to manual treatment, or spraying of the plant, plant part (or of the field or greenhouse wherein the plant is grown), or through other forms of irrigation, which can lead to uptake of the substance by leaves, roots, or other tissues.
It is described herein that treatment of the leaf cells of a wheat plant with N-acetyl-PPT will result in uptake of N-acetyl-PPT by the leaf cells and transportation to different plant organs. Thus, treatment of a wheat plant which comprises a chimeric gene comprising a deacetylase coding sequence expressed in specific cells of the plant, will result in the conversion of N-acetyl-PPT to PPT in those cells, causing these cells to die.

In a specific embodiment of the invention, the transgenic plant, comprising in its genome the DNA molecule of the invention encoding a protein having the biological activity of a deacetylase, additionally comprises a chimeric gene conferring on said plants resistance to PPT, such as, but not limited to the herbicide resistance genes, *pat* or *bar* described in EP 0 242 236, EP 0 242 246, EP 0 257 542, EP 0 531 716. These genes encode enzymes which are capable of converting PPT into N-acetyl-PPT. This N-acetyl-PPT can be transported throughout the plant and act as the substrate for a deacetylase specifically expressed in certain plant cells or tissues. It is understood that according to this specific embodiment, induction of tissue-specific cytotoxicity will also occur upon treatment of the plant with PPT.

A "female-sterile plant" as used herein refers to a plant that cannot produce viable seed. In the context of the present invention a female-sterile plant is male fertile, i.e. capable of producing viable pollen (and can thus function as the male-parent or pollinator). Female sterility can be the result of breeding selection or due to the presence of a transgene (such as, but not limited to those described in US 5,633,441, US 5,767,374). A conditionally female-sterile plant refers to a plant which under normal growing conditions is female fertile and which can be made female-sterile under specific conditions. Examples of conditionally female-sterile plants that are made sterile by application of an N-acetyl-PPT, are described in WO 98/13504.

"Interplanting" as used herein refers to a method of planting seeds or plants in the field that ensures adequate cross-pollination of the conditionally male-sterile plants by the male-fertile plants. This can be achieved by either random mixing of female and male parent seed in different blends (80/20; 90/10; etc.) before planting or by planting in specific field patterns, whereby different seeds are alternated. When separate harvesting from different plants is required, planting in alternating blocks or rows are preferred.

A "chimeric marker gene" as used herein is a gene comprising a marker DNA under the control of a promoter that is active in plant cells. The marker DNA encodes an RNA, protein or polypeptide, which, when expressed in a plant or plant cell, allows such plants

or plant cells to be distinguished and separated from plants or plant cells not expressing the marker DNA. Examples of marker genes are genes that provide a specific color to the cells (such as the A1 gene, Meyer et al. 1987, Nature 330:667) or genes that encode a particular resistance to the plant cells or the plant such as antibiotic resistance (e.g. the aac(6') gene, encoding resistance to gentamycin, WO 94/01560).

Preferred marker genes are herbicide resistance genes. A herbicide resistance gene as used herein is a gene conferring to the plant or plant cell resistance to a herbicide. In the context of the present invention, the herbicide resistance gene can also be used to eliminate wheat plants that do not comprise the herbicide resistance gene in their genome. Particularly it can be used in the production of hybrid wheat seed for the elimination of male-fertile wheat plants (that are female fertile) after pollination of the male-sterile plants has occurred, to avoid production of selfed seed on the male-fertile plants, which contaminate the hybrid seed during harvest. Examples of herbicide resistance genes are the genes encoding resistance to phenmedipham (such as the pmph gene, US 5,347,047; US 5,543,306), the genes encoding resistance to glyphosate (such as the EPSPS genes, US 5,510,471), genes encoding bromoxynyl resistance (such as described in US 4,810,648) genes encoding resistance to sulfonylurea (such as described in EP 0 360 750), genes encoding resistance to the herbicide dalapon (such as described in WO 99/27116), and genes encoding resistance to cyanamide (such as described in WO 98/48023 and WO 98/56238) and genes encoding resistance to glutamine synthetase inhibitors, such as PPT (such as described in EP 0 242 236, EP 0 242 246, EP 0 257 542). Preferably, the herbicide resistance gene is a gene which also confers resistance to a herbicide which is particularly useful for wheat production, i.e. a herbicide which is capable of controlling the major weeds of wheat, such as annual grassy weeds including wild oats, green and yellow foxtail (wild millet), broadleaf weeds including annual sunflower, burdock, chickweed, cocklebur, corn spurry, cow cockle, field horsetail, flixweed, green smartweed, hemp-nettle, hoary cress, kochia, lady's- thumb, lamb's-quarters, mustards (except dog and green tansy), plantain, prickly lettuce, ragweeds, redroot pigweed, Russian pigweed, Russian thistle, shepherd's-purse, stinkweed, vetch, volunteer canola (rapeseed), wild buckwheat, and wild radish.

For specific applications of the present invention it can be desirable to remove the marker gene from the plant (i.e. to avoid stacking of marker genes). This can be achieved by site-specific homologous recombination also referred to as the "Kick/Out-system". The marker gene to be kicked out is flanked by excision sites in the transformation construct, which are specifically recognized by a recombinase. Crossing of a plant carrying the marker gene flanked by specific excision sites with a plant carrying a gene encoding the corresponding recombinase, results in excision of the marker gene in the progeny. Examples of such site-specific homologous recombination systems are the flp/frt system (Lyznik et al., 1993, Nucleic Acids Res. 21:969-975; Lyznik et al., Nucleic Acids Res. 1996, 24:3784-3789), and the Cre/Lox system (Bayley, C.C. et al., 1992, Plant Mol. Biol. 18, 353-361).

The cytotoxic effect of certain toxins may be enhanced by targeting to the specific parts of the cell, such as the chloroplast or mitochondria. Phosphinothricin is a glutamine synthase inhibitor. In plants, glutamine synthase exists in multiple isozymic forms that can be localized within the cell in the cytosol and plastids. Thus, it is contemplated that targeting of the deacetylase to the chloroplast, whereby N-acetyl-PPT is converted to PPT in the chloroplast, increases cytotoxic efficiency. Thereto, the foreign DNA comprising the deacetylase coding sequence can comprise an additional foreign DNA encoding a transit peptide. The additional DNA is preferably located between the promoter and the deacetylase coding sequence. Alternatively, it can be desired that the marker protein or polypeptide is transported from the cytoplasm into chloroplasts or mitochondria of the transformed plant cells. Thereto, an additional DNA encoding a transit peptide is located between the marker DNA and its promoter. By transit peptide is meant a polypeptide fragment which is normally associated with a chloroplast or mitochondrial protein or subunit of the protein and is produced in a cell as a precursor protein encoded by the DNA of the cell. The transit peptide is responsible for the translocation process of the nuclear-encoded chloroplast or mitochondrial protein or subunit into the chloroplasts or mitochondria, and during such a process, the transit peptide is separated or proteolytically removed from the chloroplast or mitochondrial protein or subunit. One or more of such

additional DNA's can be provided in the foreign DNAs of this invention as generally described and exemplified in European patent publication EP 0 189 707 and EP 0 344 029. Alternatively, the transit peptide can be an artificially engineered sequence which has been designed and/or selected to be able to function as such.

Transformation of the plants of the invention can occur in a number of ways described in the art. Such methods include, but are not limited to Agrobacterium-mediated transformation (EP 0 116 718, De Blaere et al., 1987, Meth. Enzymol. 153:277-293), or direct gene transfer (US 5,712,135; Pazkowski et al., 1984, EMBO J. 3:2717-2722; Crossway et al., 1986, Mol. Gen. Genet. 102:179; Riggs et al., 1986, Proc. Natl. Acad. Sci. USA 83:5602-5606; WO92/09696; Potrykus et al. 1991, Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:205-225); see also Christou P. 1994, Agro-Food Industry Hi-Tech 2:17; Langridge et al. 1992, Plant J. 2:631; Vasil , 1994, Plant Mol. Biol. 25:925; Chen D., 1998, Methods Mol. Biol. 81:373.

Alternatively, the foreign DNA comprising the DNA encoding a deacetylase of the invention can be inserted into the plastid genome of the cell or cells of a plant. Gene transfer can be carried out, for example, by particle gun bombardment (as described by Svab et al., 1990, Proc. Natl. Acad. Sci. USA 87:8526-8530) or by Agrobacterium-mediated transformation (De Block et al., 1985, EMBO J. 4:1367). It is understood that in this case, suitable promoters, i.e. that are active in plastids, need to be used.

The present invention relates particularly to cereal crops, such as barley, rye, oats, and most particularly wheat. Most cultivated wheat plants belong to the *Triticum aestivum* species, but other cultivated or wild forms may also be used.

As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be

embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA sequence which is functionally or structurally defined, may comprise additional DNA sequences, etc.

Unless otherwise stated, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbour Laboratory Press, NY and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

Sequence Listing:

SEQ ID No. 1: sequence of the NotI fragment of pCDDUG+

SEQ ID No. 2: sequence of the NotI fragment of pADP72

SEQ ID No. 3: sequence of the NotI fragment of pADP73

SEQ ID No. 4: primer GAT1

SEQ ID No. 5: primer GAT2

SEQ ID No. 6: primer CA6

SEQ ID No. 7: primer PA7

SEQ ID No. 8: primer CA1

SEQ ID No. 9: primer CA2

SEQ ID No. 10: amino acid sequence of a deacetylase from *Stenotrophomonas sp.*

SEQ ID No. 11: sequence encoding a deacetylase from *Stenotrophomonas sp.*

SEQ ID No. 12: chimeric gene comprising a deacetylase coding sequence under control of a CA55 promoter

SEQ ID No. 13: chimeric gene comprising a deacetylase coding sequence under control of a T72 promoter

SEQ ID No. 14: chimeric gene comprising a deacetylase coding sequence under control of a E1 promoter

# EXAMPLES

Example 1: Foliar uptake and distribution of 14C-labeled N-Acetyl-PPT in wheat

*Triticum aestivum* plants were cultivated in outdoor conditions and treated in stage 32 (BBCH scale, = 2nd node detectable, ear length about 2.5 cm) with the test compound dinatrium L-2-acetamido-4-methylphosphinato-butyrate-[$3^{14C}$, $4^{14C}$] (spec. activity 6777 MBq/g).

The concentration of the test compound was 0.1% active ingredient in application solution, which corresponded to the aqueous solution of Basta®.

*Plant treatment*: The application solution of the formulated test compound was applied in the form of microdroplets by syringe to the blades (adaxial surface) of the fully developed leaves. A volume of 20 µl each was applied to the 3rd or 4th leaf of the main shoot and of two tillers per plant (= a total of 60 µl application solution per plant.). Plants were kept under outdoor conditions and uptake and translocation of the test substance was analyzed at different stages.

*Sampling of leaf tissue* was carried out at the following time points:

1) 1 week after treatment
2) 3 weeks after treatment
3) heading stage
4) grain maturity

*Processing of collected samples for quantitative determinations:*

For the sampling done at time points 1, 2 and 3, the treated leaf blades were cut off and the non-absorbed test compound was washed from the treated leaf blades by two consecutive short washes with water. Subsequently plants were dissected further, resulting in the following parts:

- treated leaf blades

- ear ( sampling time point 3 only)
- rest of shoot
- root

Radioactivity was quantified in the different plant parts and in the washing solutions and was calculated as equivalents of the labeled compounds. The chemical nature of the radioactive residue is identified in the ears.

For the sampling time point 4, radioactivity is determined in the grains only and is calculated as equivalents of the labeled test compound. The chemical nature of the radioactive residue is identified if the residue level permits.

In addition to the quantitative radioactivity measurements the distribution of radioactivity is determined by autoradiography in plants of the sampling time points 1 and 2.

Based on measurements of radioactivity in the samples taken at different time points as well as on the autoradiography of plant material taken at time points 1 and 2, it can be concluded that there is good foliar uptake of N-acetyl-PPT in wheat tissue and there is translocation through the phloem to the ears both in the main shoot and the tillers.

Example 2: Construction of vectors for the expression of the deacetylase gene in wheat

*a) vector comprising the deac coding sequence under control of the 35S promoter*

The vector pADP62 was made by introducing the following structural elements into pUC18:

- a marker gene cassette comprising the Ubiquitin promoter from maize containing its first exon and first intron, linked to the 5' untranslated leader sequence of the cab22L gene from pea, operably linked to the gentamycin acetyltransferase (GAT) coding sequence and the 3' untranslated region from the CaMV 35S transcript. The whole marker gene cassette is flanked by frt1/frt2 sequences (described by Lyznik et al., 1993, 1996, above) as part of the frt/flp excision system.

- a deac gene expression cassette comprising the 35S promoter from Cauliflower Mosaic virus operably linked to the deac coding sequence (WO 98/27201) and the 3' untranslated region from the CaMV 35S transcript.

The complete insert is flanked by left and right T-DNA border sequences from Ti-plasmid pTIT37 of *Agrobacterium tumefaciens* (Caplan et al., 1983, Science 222:815-820; Depicker et al. 1982, J. Mol. Appl. Genet. 1:561-573).
The coordinates of the described genetic elements are given in Table 1.

Table 1. T-DNA of pADP62

| Position | Description |
|---|---|
| 14-37 | Left T-DNA border from pTIT37 (counterclockwise) |
| 79-132 | Target sequence for frt/flp excision system |
| 159-1063 | Ubiquitin promoter from maize |
| 1064-1144 | 1st exon of ubi gene |
| 1145-2154 | 1st intron of ubi gene |
| 2176-2242 | 5' untranslated leader sequence from cab22 gene from pea |
| 2244-2855 | Gentamycin acetyltransferase coding sequence |
| 2858-3092 | 3' untranslated region from CaMV 35S transcript |
| 3125-3178 | Target sequence for frt/flp excision system |
| 3220-3750 | 35S Promoter |
| 3773-5092 | Deac coding sequence from *Stenotrophomonas sp.* |
| 5160-5355 | 3' untranslated region from CaMV 35S transcript |
| 5410-5387 | Right T-DNA border from pTIT37 |

b) *vectors comprising the deac coding sequence under control of the CA55 promoter*

The vector pCDUG+ was made by introducing the following structural elements into pUC18:

- a marker gene cassette comprising the Ubiquitin promoter from maize containing its first exon and first intron, linked to the 5' untranslated leader sequence of the cab22L

gene from pea, operably linked to the gentamycin acetyltransferase (GAT) coding sequence and the 3' untranslated region from the CaMV 35S transcript.

- a deac gene expression cassette comprising the tapetum-specific promoter CA55 from maize (WO 92/13956) operably linked to the deac coding sequence (WO 98/27201) and the 3' untranslated region from the CaMV 35S transcript.

The complete insert is flanked by left and right T-DNA border sequences from Ti-plasmid pTIT37 of *Agrobacterium tumefaciens* (above).

The coordinates of the described genetic elements are given in Table 2. The complete insert comprising marker and deac cassette was isolated as a 6215 bp NotI fragment by HPLC and used for plant transformation. The DNA sequence of this fragment is provided in SEQ ID NO. 1.

Table 2. NotI fragment of pCDUG+

| Position | Description |
|---|---|
| 9-36 | Left T-DNA border from pTIT37 |
| 116-1020 | Ubiquitin promoter from maize |
| 1021-1101 | 1st exon of ubi gene |
| 1102-2111 | 1st intron of ubi gene |
| 2133-2199 | 5' untranslated leader sequence from cab22 gene from pea |
| 2201-2812 | Gentamycin acetyltransferase coding sequence |
| 2815-3049 | 3' untranslated region from CaMV 35S transcript |
| 3071-4248 | CA55 promoter from maize |
| 4261-5580 | Deac coding sequence from *Stenotrophomonas sp.* |
| 5648-5843 | 3' untranslated region from CaMV 35S transcript |
| 6096-6120 | Right T-DNA border from pTIT37 |

Alternative constructs were made to allow for the possibility of removing the marker gene cassette. In these alternative constructs, the whole marker gene cassette is flanked by frt1/frt2 sequences as part of the frt/flp excision system (described above).

c) *vectors comprising the deac coding sequence under control of the T72 or E1 promoters*

The vector pADP72 was made by introducing the following structural elements into pUC18:

- a marker gene cassette comprising the Ubiquitin promoter from maize containing its first exon and first intron, linked to the 5' untranslated leader sequence of the cab22L gene from pea, operably linked to the gentamycin acetyltransferase (GAT) coding sequence and the 3' untranslated region from the CaMV 35S transcript. The whole marker gene cassette is flanked by frt1/frt2 sequences (described above) as part of the flp/frt excision system.
- a deac gene expression cassette comprising the tapetum-specific promoter T72 from rice (US 5,639,948) operably linked to the deac coding sequence and the 3' untranslated region from the CaMV 35S transcript (as described above).

The complete insert is flanked by left and right T-DNA border sequences from Ti-plasmid pTIT37 of *Agrobacterium tumefaciens* (as described above).

The coordinates of the described genetic elements are given in Table 3. The complete insert comprising marker and deac cassette was isolated as a 6581 bp NotI fragment and used for plant transformation. The DNA sequence of this fragment is provided in SEQ ID NO. 2.

Table 3. NotI fragment of pADP72

| Position | Description |
|---|---|
| 12-35 | Left T-DNA border from pTIT37 (counterclockwise) |
| 77-130 | Target sequence for frt/flp excision system |
| 157-1061 | Ubiquitin promoter from maize |
| 106-1142 | 1$^{st}$ exon of ubi gene |
| 1143-2152 | 1$^{st}$ intron of ubi gene |
| 2174-2240 | 5' untranslated leader sequence from cab22 gene from pea |
| 2242-2853 | Gentamycin acetyltransferase coding sequence |

| | |
|---|---|
| 2856-3090 | 3' untranslated region from CaMV 35S transcript |
| 3123-3176 | Target sequence for frt/flp excision system |
| 3238-4835 | PT72 promoter from rice |
| 4850-6169 | Deac coding sequence from *Stenotrophomonas sp.* |
| 6237-6432 | 3' untranslated region from CaMV 35S transcript |
| 6460-6483 | Right T-DNA border from pTIT37 (counterclockwise) |

The vector pADP73 was made by introducing the following structural elements into pUC18:

- a marker gene cassette comprising the Ubiquitin promoter from maize containing its first exon and first intron, linked to the 5' untranslated leader sequence of the cab22L gene from pea, operably linked to the gentamycin acetyltransferase (GAT) coding sequence and the 3' untranslated region from the CaMV 35S transcript. The whole marker gene cassette is flanked by frt1/frt2 sequences (described above) as part of the flp/frt excision system.
- a deac gene expression cassette comprising the tapetum-specific promoter E1 from rice (US 5639948) operably linked to the deac coding sequence and the 3' untranslated region from the CaMV 35S transcript as described above.

The complete insert is flanked by left and right T-DNA border sequences from Ti-plasmid pTIT37 of *Agrobacterium tumefaciens* (as described above).

The coordinates of the described genetic elements are given in Table 4. The complete insert comprising marker and deac cassette was isolated as a 6667 bp NotI fragment by HPLC and used for plant transformation. The DNA sequence of this fragment is provided in SEQ ID NO. 3.

Table 4. NotI fragment of pADP73

| Position | Description |
|---|---|
| 12-35 | Left T-DNA border from pTIT37 (counterclockwise) |
| 77-130 | Target sequence for frt/flp excision system |
| 157-1061 | Ubiquitin promoter from maize |

| | |
|---|---|
| 1062-1142 | 1st exon of ubi gene |
| 1143-2152 | 1st intron of ubi gene |
| 2174-2240 | 5' untranslated leader sequence from cab22 gene from pea |
| 2242-2853 | Gentamycin acetyltransferase coding sequence |
| 2856-3090 | 3' untranslated region from CaMV 35S transcript |
| 3123-3176 | Target sequence for frt/flp excision system |
| 3237-4923 | PE1 promoter from rice |
| 4938-6257 | Deac coding sequence from *Stenotrophomonas sp.* |
| 6325-6520 | 3' untranslated region from CaMV 35S transcript |
| 6571-6548 | Right T-DNA border from pTIT37 (counterclockwise) |

Alternatively, constructs were made similar to those present in pCDUG+, pCDUGFRT, pADP72 and pADP73 as described above, but comprising the deacetylase coding region of *Stenotrophomonas sp.* (as described in SEQ ID. No. 11), directly flanked by the 3' untranslated region from the CaMV 35S transcript.

Example 3: Transformation of *Triticum aestivum* (wheat)

A number of different cultivars of spring (sw) and winter wheat (ww) were used to establish optimal transformation and regeneration conditions.

Seeds were grown in soil and cultured in growth chambers or in the greenhouse until flowering. The maturity status of the kernels was checked regularly to determine which spikes could be collected for transformation purposes.

After collection of the spikes, the immature kernels were isolated by separating them from the glumes surrounding them. The kernels were then surface-sterilized for 1 min. in 70% ethanol and for 15 min. in a sodium hypochlorite solution with about 1.3% active chlorine. Finally, the immature kernels were rinsed 3 times in sterile water.

Wheat tissue was transformed with the purified DNA from the plasmids (constructs described in Example 2) using direct gene transfer and regenerated to plants on appropriate media.

The small plantlets initiated on the selective regeneration media were transferred to hormone-free medium for elongation. Well elongated plantlets were then transferred to the growth chambers or to the greenhouses.

Example 4: Selection of transformants

Primary transformants (T0) with constructs were tested for correct transformation by PCR and southern blot analysis as described below.

**PCR**

Presence of the transgene was checked with a PCR using specific primers for the GAT gene and specific primers for the deac coding sequence and the CA55, T72 and E1 promoters.

Genomic DNA was isolated from plant tissue according to the protocol described for the DNeasy Plant Mini Kit (Qiagen, Catalog Nr. 69103).

The primers used for identification of the plants transformed with pCDUG+ or pCDUGFRT were as follows:

a) GAT specific primers:

| | sequence (5'→3') | position in pCDUG+ |
|---|---|---|
| GAT1: | GCACCGATTCCGTCACACTGC (SEQ ID No 4) | 2268 → 2286 |
| GAT2: | GTACACGGCTGGACCATCTGG (SEQ ID No 5) | 2744 ← 2764 |

b) deac-specific primers

| | sequence (5'→ 3') | position in pCDUG+ |
|---|---|---|
| CA6: | GTTACGAGCACGTAGTTGGCG (SEQ ID No 6) | 4054 → 4074 |
| 7PA: | GCTCATGGGAGGTCCTTCTTC (SEQ ID No 7) | 5607 ← 5627 |

c) CA55-specific primers:

| | sequence (5'→ 3') | position in pCDUG+ |
|---|---|---|
| CA1: | TACGCCCCGGGTGGTATGCATCAATAGAGCCG (SEQ ID No 8) | 3071 → 3092 |
| CA2: | TACGCGGATCCGCTGCAGCTAGTTAGCTCGAT (SEQ ID No 9) | 4227 ← 4248 |

For the identification of plants transformed with the pADP62, pADP72 and pADP73 constructs, the primers described under a) and b) above were used.

Standard PCR's were carried out in 50 µl assays containing 10 ng of genomic plant DNA as template and 0.2 µM of each primer.

In reactions containing deac-specific primers 6.7 % glycerol and 5 % DMSO was included in the assay.

| | | |
|---|---|---|
| Cycling parameters: | denaturation | 5 min. at 94°C |
| | 25 cycles: | 1 min. at 94°C |
| | | 1 min. at 60°C |
| | | 2 min. at 72°C |

final extension : 10 min. at 72°C

10 µl of the reactions were analyzed on 1 % agarose gels in order to identify the correct amplification products.

Plant samples were considered as positive, when the correct DNA bands were produced when tested with at least two primer combinations. For instance, primary transformants with pCDUG+, 4WH21, 5WH22, 6WH23, 8WH32, 9WH21, 10WH23, and 12WH23, were positive when tested with the primer combinations GAT1/GAT2 and CA6/7PA, while 7WH26 and 11WH26 tested negative

**Southern blotting**

Presence of the transgene was checked by standard Southern blot analysis using the DNeasy Plant Mini Kit (Quiagen, Catalog Nr. 69103). 10 µg of DNA were digested with BamHI, EcoRI, HindIII, and SalI, respectively.

Southern blots are prepared using standard protocols as described by Maniatis et al. (above). For the pCDUG transformants, a 1.4-kb BamHI/SalI fragment from plasmid pCDUG, carrying the deac structural gene, is isolated and labeled with alpha-[32P]-dCTP by random primer labelling using the Megaprime kit from Amersham. Similar fragments are isolated from the pCDUGFRT, pADP62, pADP72 and pADP73 vectors.

The fragments are used as probes for Southern hybridization in 50 % formamide at 42°C. Conditions for hybridization, filter washing and X-ray film exposure are as described by Maniatis et al., 1989.

Example 5 : Testing of deacetylase activity in plants

$T_0$ plants were screened for deacetylase activity in the leaves or anthers as described below to identify positive transformation events. $T_0$ plants were then selfed for the production of $T_1$ progeny. These plants were sprayed with acetyl PPT (1-3 sprays of 5mg/ml of N-acetyl-PPT 2-3 weeks before inflorescence formation) and plants were evaluated for general appearance (constitutive constructs) and/or for male sterility of flowers (stamen-specific constructs).

**Deacetylase assay with [$^{14}$C]-N-acetyl-PPT**

Method A:

- Anthers or leaves were harvested from young inflorescences and homogenized with a metal pistil in an Eppendorf tube in 100µl 20 mM Tris/HCl, pH=8.0.
- Cell debris is then pelleted in a microcentrifuge and the supernatant was then either
  a) dialyzed for 2 h against uptake buffer (10 mM NaCl, 10 mM NaPhosphate, pH=7.5); or
  b) concentrated 3-5x (end volume 20-30µl) and dialyzed against uptake buffer simultaneously by 2 successive ultrafiltrations through Microcon 30 Membranes (Amicon).
- Protein concentrations were determined by Bradford assay.
- The dialyzed crude extract (18µl) were incubated with 2µl [$^{14}$C]-N-acetyl-PPT (end concentration of 0.1mM) overnight at 37°C. The following controls were included in the assay: (1) purified recombinant deac protein (final concentration 0.3 µg/µl) in uptake buffer, (2) crude extract from untransformed wheat lines, (3) deac protein in crude extract of untransformed wheat lines, and (4) [$^{14}$C]-N-acetyl-PPT in uptake buffer (substrate control).

- Samples were diluted 1:5 in HPLC buffer (2mM $CuSO_4$, 10% methanol) and analyzed by radio HPLC on a Chirex (D) penicillamine column (Phenomex) with a flow rate of 0.5 ml/min.

Under the conditions described, L-PPT eluted at 13.9 min. and N-acetyl-PPT at 50.3 min. Substrate and product peaks were quantified by integration and deac activity was determined as relative conversion of substrate N-acetyl-PPT into product L-PPT.

Method B:

- Anthers or leaves are harvested from 2 flowers and homogenized in an Eppendorf tube in 50µl uptake buffer (10 mM NaCl, 10 mM NaPhosphate, pH=7.5).
- Cell debris is then pelleted in a microcentrifuge and the supernatant is dialyzed for 2 h against uptake buffer.
- The dialyzed crude extract (10µl) is incubated with 1µl [$^{14}$C]-N-acetyl-PPT (at a 1:40 dilution from the stock solution of 1mM) overnight at 37°C. Remaining crude extracts can be stored in the freezer and used for determination of protein content.
- The assay solution is loaded (5µl) onto TLC-plates (Merck, HPTLC-cellulose, ref. 15035), which are developed in a 3:2 solution of 2x n-propanol:25% ammonia. Control samples loaded are [$^{14}$C]-N-acetyl-PPT and [$^{14}$C]-PPT.
- Kodak XAR5 film is exposed to the TLC plates for 2-3 days.

$T_0$ plants transformed with plasmid pADP62 (constitutive construct) and their $T_1$ descendants were tested for presence of the transgene by PCR as described above.

Leaves from 3 PCR positive $T_1$ plants were analyzed for deac activity using method A. Crude extracts were prepared according to the protocol under (b). The results are summarized in Table 5.

Table 5: Deacetylase activity in leaves of wheat transformants

| Wheat transformant (T1) | PCR result | Protein concentration of crude extract [mg/ml] | L-PPT [%]* | N-acetyl-PPT [%]* |
|---|---|---|---|---|
| WH83T2 | + | 3.0 | 46.9 | 53.1 |
| WH85T1 | + | 9.0 | 39.6 | 60.4 |
| WH85T2 | + | 6.0 | 7.9 | 92.1 |
| Control (1) | | 0.3 (*deac*) | 90.3 | 9.7 |
| Control (2) | | 8.0 (wildtype) | 0 | 100 |
| Control (3) | | 8.3 (*deac* + wildtype) | 83.1 | 16.2 |
| Control (4) | | - (substrate without enzyme) | 0 | 100 |

*: percentage of total radioactivity applied

All 3 PCR positive deac transformants expressed deac activity in leaves, though activity in WH85T2 was significantly lower compared to the 2 other transformants.

These findings were in agreement with the observed phenotype after treatment of leaves with 5 mg/ml N-acetyl-PPT solution. Transformants WH83T2 and WH85T1 showed herbicidal symptoms, while WH85T2 remained unaffected and looked like the untransformed controls.

$T_1$ descendant plants from primary transformants with constructs pCDUG+ (cultivar PAVON) and pCDUGFRT (cultivar Combi) were tested for the presence of the transgene by PCR as described above, and then analysed for deacetylase activity using method A (supernatants were treated as in (a) for the the pCDUGFRT transformants and (b) for the pCDUG+ transformants). The results are described in Table 6.

Table 6: Deacetylase activity in anthers of wheat transformants

| Wheat transformant (T1) | Construct | PCR result | Protein concentration of crude extract [mg/ml] | L-PPT [%]* | N-acetyl-PPT [%]* |
|---|---|---|---|---|---|
| WH26T4.3.4 | PCDUGFRT | + | 0.45 | 0 | 100 |
| WH26T4.1.1 | | + | 0.89 | 0 | 100 |

| | | | | | |
|---|---|---|---|---|---|
| WH22T1.11.3 | | - | 0.95 | 0 | 100 |
| WH23T5.5.4 | | + | 1.22 | 16.7 | 83.3 |
| WH23T5.2.1 | | + | 0.89 | 6.2 | 93.8 |
| WH32T6.3.4 | | + | 0.82 | 2.8 | 97.2 |
| WH23T1.11.1 | | + | 0.65 | 0 | 100 |
| WH32T6.3.3 | | - | 0.70 | 0 | 100 |
| WH32T6.2.1 | | + | 0.89 | 5.2 | 94.8 |
| TA009-01-10-8 | PCDUG+ | + | 11.6 | 11.7 | 88.3 |
| TA009-01-10-1 | | + | 11.0 | 18.3 | 81.7 |
| TA009-01-18-2 | | + | 12.4 | 18.5 | 81.5 |
| TA009-01-12-3 | | + | 12.3 | 8.2 | 91.8 |
| TA009-01-12-1 | | + | 11.2 | 17.6 | 82.4 |
| TA009-01-17-3 | | + | 10.8 | 8.0 | 92.0 |
| TA009-01-10-7 | | + | 12.6 | 15.1 | 84.9 |
| TA009-01-12-7 | | + | 11.2 | 7.2 | 82.8 |
| TA009-01-17-1 | | + | 11.0 | 8.3 | 91.7 |
| TA009-01-18-4 | | + | 13.2 | 12.1 | 87.9 |
| Control (1) | - | NA | 0.3 (*deac*) | 90.3 | 9.7 |
| Control (2) | - | NA | 11.5 (Combi) | 0 | 100 |
| Control (3) | - | NA | 11.8 (*deac* + Combi) | 61.1 | 38.9 |
| Control (4) | - | NA | - (substrate without enzyme) | 0 | 100 |

*: percentage of total radioactivity applied

Most of the PCR positive transformants expressed a significant deacetylase activity in young anthers with conversion rates between 2.8 and 18.5 %. No deacetylase activity was detectable in anthers of PCR negative plants. The in average higher activities in the second group of transformants (TA009-01) are probably due to the higher protein content of the samples as a consequence of the applied filter concentration method for the crude extracts.

The untransformed cultivar Combi did not express a detectable deacetylase activity [Control (2)]. The substrate N-acetyl-PPT was completely stable under the given reaction conditions [Control (4)].

Example 6: Production of conditionally male-sterile wheat plants comprising a herbicide resistance gene.

A plasmid for transformation of wheat plant cells is constructed by assembling the following well-known DNA fragments:

- a marker gene cassette comprising the Ubiquitin promoter from maize containing its first exon and first intron, linked to the 5' untranslated leader sequence of the cab22 gene from pea, operably linked to the gentamycin acetyltransferase (GAT) coding sequence with the 3' untranslated region from the CaMV 35S transcript. This cassette is flanked by frt1/frt2 sequences as part of the flp/frt excision system (Lyznik et al., 1993, above).
- a deac gene expression cassette comprising the tapetum-specific promoter CA55 from maize (WO 92/13956) operably linked to the deac coding sequence (WO 98/27201 – SEQ ID No. 11) with the 3' untranslated region from the CaMV 35S transcript.
- a chimeric sequence comprising the phenmedipham hydrolase (pmph) coding sequence (Streber et al. Plant Mol. Biol. 25:977-987, 1994; US 5,347,047; US 5,543,306) as a herbicide resistance marker under control of the constitutive 35S promoter from Cauliflower Mosaic Virus (Franck A. et al., Cell 21:285-294, 1980; Pietrzak M. et al., Nucl. Acids Res. 4:5857-5868, 1986).

Alternatively, the chimeric sequence contains a gene conferring glyphosate resistance (such as the EPSPS gene as described in, for example, US 5,510,471) as a herbicide resistance marker, under control of a constitutive promoter (plasmid pDP140, Figure 6):

- a chimeric sequence comprising coding sequence of the double mutated EPSP synthase gene of corn ("EPSPi+s"; Lebrun et al. 1997; WO 97/04103) under control of the promoter and intron of the rice actin gene ("PactOs", "int";

McElroy and Wu, US 5641876) and linked to the 3' untranslated end of the nopaline synthase gene (Bevan et al. , 1983, Nucleic Acids Res. 11(2):369-385); This chimeric sequence is preceded by the sequence of an optimized transit peptide (Capellades et al., EP 0 652 286) for transfer into the chloroplast ("OTP").

- a deac gene expression cassette comprising the tapetum-specific promoter CA55 from maize ("Pca55"; WO 92/13956) operably linked to the deac coding sequence ("deac"; WO 98/27201) with the 3' untranslated region from the CaMV 35S transcript ("3'35S", Sanfaçon et al., 1990, Genes and Development 5:141-149).

The complete insert is flanked by left and right T-DNA border sequences from Ti-plasmid pTIT37 of *Agrobacterium tumefaciens* (as described above in Example 2). The plasmid contains a β-lactamase selection marker gene outside the NotI-cassette.

The construct can also contain the frt-excision sites of the flp/frt kick-out system, which allows for an optional excision of the marker gene by a recombinase. On the other hand the herbicide resistance marker can be maintained in the male-sterile line, in order to eliminate the pollinator plants by herbicide treatment on the production field under mixed planting conditions.

Primary transformants (T0) are tested for correct transformation by PCR and Southern blot analysis. T0 plants are screened for deacetylase activity as described above to identify positive transformation events and are tested for tolerance to the herbicide (phenmedipham or glyphosate). T0 plants are then selfed for the production of T1 progeny and developed as described above, whereby successive generations are tested for tolerance to the herbicide.

Example 7: Production of hybrid seed of wheat using conditionally male-sterile plants

Conditional male-sterility can be used in wheat breeding according to the following schemes.

a) *Production of composite hybrid seed*

Seed comprising the deac gene under control of a stamen-selective promoter is obtained as described in example 2-3 above and is interplanted with wild-type plants (male parent) at a ratio which ensures adequate pollen transfer with a minimum of male parent plants (e.g., 80:20). Fields are sprayed with N-acetyl-PPT to induce male-sterility in the conditionally male-sterile plants. Seed harvested from the field optimally comprises about 80% hybrid seed (harvested from the conditionally male-sterile plants) and at least 20% selfed seed (harvested from the male parent).

b) *Production of pure hybrid seed*

Plants are obtained according to the principles of example 6 above, whereby a herbicide resistance gene is introduced together with the deac gene. Seeds comprising this construct are planted in a mixture with seeds of a wheat inbred line as male parent (polinator), that are not resistant to the herbicide, at a ratio which ensures adequate pollen transfer with a minimum of male parent plants (e.g., 80:20). Fields are sprayed with N-acetyl-PPT to induce male-sterility in the conditionally male-sterile plants. After pollination, fields are sprayed with the herbicide to remove the male parent plants. Seed harvested from the field is pure (about 100%) hybrid seed.

c) *production of pure hybrid seed using conditionally female sterile plants*

Seed capable of growing into conditionally male-sterile plants are obtained according to examples 2-3. Additionally, seeds capable of growing into conditionally female-sterile plants are obtained according to WO 98/13504. Seeds comprising the gene for conditional male-sterility (female parent) are planted in a mixture with seeds of a homozygous inbred line comprising the gene for conditional female sterility (male parent or polinator) at a ratio which ensures adequate pollen transfer with a minimum of male parent plants (e.g., 80:20). Fields are sprayed with N-acetyl-PPT to induce male-sterility in the conditionally male-sterile plants and female-sterility in the conditionally female-sterile plants. Seed harvested from the field is pure hybrid seed (about 100%).

d) *production of pure hybrid seed using female sterile plants*

Seed capable of growing into conditionally male-sterile plants are obtained according to examples 2-3. These are planted in a mixture with seeds capable of growing into plants which are male fertile (male parent) but female-sterile (obtained either through inbreeding or by use of a transgene as described in US 5,663,441 or US 5,767,374) at a ratio which ensures adequate pollen transfer with a minimum of male parent plants (e.g., 80:20). Fields are sprayed with N-acetyl-PPT to induce male-sterility in the conditionally male-sterile plants. Seed harvested from the field is real hybrid seed (about 100%).

The above description of the invention is intended to be illustrative and not limiting. Various changes or modifications in the embodiments described may occur to those skilled in the art. These can be made without departing from the spirit or scope of the invention.

# Claims

1. A wheat plant, having a chimeric gene integrated into its genome, the chimeric gene comprising:

   a) a DNA molecule encoding a deacetylase from *Stenotrophomonas sp.*, or a biologically active fragment thereof; and

   b) a promoter directing stamen-selective expression in wheat

   wherein the DNA molecule encoding the deacetylase is in the same transcriptional unit and under the control of the stamen-selective promoter.

2. The wheat plant of claim 1, wherein said DNA molecule encodes a biologically active fragment or a variant of the deacetylase comprising the amino acid sequence of SEQ ID No. 10.

3. The wheat plant of claim 1, wherein said DNA molecule encodes a deacetylase having the amino acid sequence of SEQ ID No. 10.

4. The wheat plant of claim 1, wherein said DNA molecule comprises the sequence of SEQ ID No. 11, or a sequence capable of hybridizing to SEQ ID No. 11 under standard stringent conditions.

5. The wheat plant of claim 1, wherein said stamen-selective promoter is a CA55 promoter.

6. The wheat plant of claim 1, wherein said stamen-selective promoter is a T72 promoter.

7. The wheat plant of claim 1, wherein said stamen-selective promoter is an E1 promoter.

8. The wheat plant of claim 1, wherein said chimeric gene comprises the sequence selected from the group of SEQ ID No. 12, SEQ ID No. 13 and SEQ ID No. 14.

9. The wheat plant of claim 1, wherein said chimeric gene further comprises a herbicide resistance gene encoding an RNA, protein or polypeptide that confers herbicide resistance on the wheat plant.

10. A process for producing hybrid wheat seed, said process comprising
    i) producing seeds capable of growing into conditionally male-sterile wheat plants, said seeds having a chimeric gene integrated in their genome, the chimeric gene comprising:
    a) a DNA molecule encoding a deacetylase from *Stenotrophomonas sp.*, and
    b) a stamen-selective promoter
    wherein the DNA molecule encoding the deacetylase is in the same transcriptional unit and under the control of the stamen-selective promoter;
    ii) interplanting said seeds capable of growing into conditionally male-sterile wheat plants with seeds capable of growing into male fertile wheat plants;
    iii) inducing male-sterility in said conditionally male-sterile plants by applying an acetylated toxin, which in itself is not toxic to the plants or plant cells; and
    iv) harvesting hybrid seed.

11. The process of claim 10, wherein said DNA molecule encodes a biologically active fragment or a variant of the deacetylase comprising the amino acid sequence of SEQ ID No. 10.

12. The process of claim 10, wherein said DNA molecule encodes the deacetylase comprising the amino acid sequence of SEQ ID No. 10.

13. The process of claim 10, wherein said DNA molecule comprises the sequence of SEQ ID No. 11, or a sequence capable of hybridizing to SEQ ID No. 11 under standard stingent conditions.

14. The process of claim 10, wherein said stamen-selective promoter is a CA55 promoter.

15. The process of claim 10, wherein said stamen-selective promoter is a T72 promoter.

16. The process of claim 10, wherein said stamen-selective promoter is an E1 promoter.

17. The process of claim 10, wherein said chimeric gene comprises the sequence selected from the group of SEQ ID No. 12, SEQ ID No. 13 and SEQ ID No. 14.

18. The process of claim 10, wherein said acetylated toxin is N-acetyl-PPT .

19. The process of claim 10, wherein said male fertile plants are female-sterile.

20. A process for producing pure hybrid wheat seed, said process comprising:
    i) producing seeds capable of growing into conditionally male-sterile wheat plants, said seeds having a chimeric gene integrated in their genome, the chimeric gene comprising:
    a) a DNA molecule encoding a deacetylase from *Stenotrophomonas sp.*
    b) a stamen-selective promoter
    wherein the DNA molecule encoding the deacetylase is in the same transcriptional unit and under the control of the stamen-selective promoter; which chimeric gene further comprises a herbicide resistance gene, which confers on said wheat plant resistance to a herbicide,
    ii) interplanting said seeds capable of growing into conditionally male-sterile wheat plants with seeds capable of growing into male fertile wheat plants, which do not comprise said herbicide resistance gene;

iii) inducing male-sterility in the conditionally male-sterile plants by applying an acetylated toxin, which is in itself not toxic to the plants or plant cells,

iv) after pollination, applying said herbicide, eliminating said male fertile plants, and

v) harvesting pure hybrid seed.

21. The process of claim 20, wherein said DNA molecule encodes a biologically active fragment or a variant of the deacetylase having the amino acid sequence of SEQ ID No. 10.

22. The process of claim 20, wherein said DNA molecule encodes the deacetylase having the amino acid sequence of SEQ ID No. 10.

23. The process of claim 20, wherein said DNA molecule comprises the sequence of SEQ ID No. 11 or a sequence capable of hybridizing with SEQ ID No. 11.

24. The process of claim 20, wherein said stamen-selective promoter is a CA55 promoter.

25. The process of claim 20, wherein said stamen-selective promoter is a T72 promoter.

26. The process of claim 20, wherein said chimeric gene comprises the sequence selected from the group of SEQ ID No. 12, SEQ ID No. 13 and SEQ ID No. 14.

27. The process of claim 20, wherein said acetylated toxin is N-acetyl-PPT.

28. The process of claim 20, wherein said herbicide resistance gene is a gene conferring on said plant resistance to glyphosate.

29. The process of claim 28, wherein said herbicide resistance gene is an EPSPS gene.

30. A process for producing a conditionally male-sterile wheat plant, said process comprising
    i) transforming a wheat plant cell or tissue with a chimeric gene which comprises:
        a) a DNA molecule encoding a deacetylase from *Stenotrophomonas sp.*, and
        b) a stamen-selective promoter
        wherein the DNA molecule encoding the deacetylase is in the same transcriptional unit and under the control of said stamen-selective promoter;
    ii) regenerating said conditionally male-sterile plant from said cell or tissue; and optionally,
    iii) applying an acetylated toxin to said conditionally male-sterile plant, which is in itself not toxic to the plant or plant cells to make said plant male-sterile.

31. The process of claim 30, wherein said DNA molecule encodes a biologically active fragment or a variant of the deacetylase encoded by SEQ ID No. 10.

32. The process of claim 30, wherein said DNA molecule encodes the deacetylase of SEQ ID No. 10.

33. The process of claim 30, wherein said DNA molecule comprises the sequence of SEQ ID No. 11, or a sequence capable of hybridizing to SEQ ID No. 11 under standard stringent conditions.

34. The process of claim 30, wherein said stamen-selective promoter is a CA55 promoter.

35. The process of claim 30, wherein said stamen-selective promoter is a T72 promoter.

36. The process of claim 30, wherein said stamen-selective promoter is an E1 promoter.

37. The process of claim 30, wherein said chimeric gene comprises the sequence selected from the group of SEQ ID No. 12, SEQ ID No. 13 and SEQ ID No. 14.

38. The process of claim 30, wherein said acetylated toxin is N-acetyl-PPT.

39. The process of claim 30, wherein said foreign DNA additionally comprises a herbicide resistance gene, which confers on said wheat plant resistance to a herbicide.

40. The process of claim 30, wherein said herbicide resistance gene is a gene conferring on said plant resistance to glyphosate.

41. The process of claim 40, wherein said herbicide resistance gene is an EPSPS gene.

42. A conditionally male-sterile wheat plant obtained by the process of claim 30.

42. A process for generating male-sterility in wheat plants, said process comprising
i) obtaining a conditionally male-sterile wheat plant by transforming a wheat plant cell or tissue with a chimeric gene which comprises:
a) a DNA molecule encoding a deacetylase from *Stenotrophomonas sp.*, and
b) a stamen-selective promoter
wherein said DNA molecule encoding the deacetylase is in the same transcriptional unit and under the control of said stamen-selective promoter, and regenerating said conditionally male-sterile plant from said cell or tissue; and optionally obtaining conditionally male-sterile progeny from said plant

ii) applying an acetylated toxin to said conditionally male-sterile plant or its conditionally male-sterile progeny, which is in itself not toxic to the plant or plant cells to make said plant male-sterile.

43. A male sterile wheat plant, or cells or tissues thereof, obtained by the process of claim 42.

44. Wheat plant cells, tissues and seed transformed with the chimeric DNA of claim 1.

45. A process for producing pure hybrid wheat seed, said process comprising the steps of:

i) producing seeds capable of growing into conditionally male-sterile wheat plants, said seeds having,

a) a chimeric gene integrated in their genome the chimeric gene comprising:

1) a DNA molecule encoding a deacetylase from *Stenotrophomonas sp.* and

2) a stamen-selective promoter

wherein the DNA molecule encoding the deacetylase is in the same transcriptional unit and under the control of the stamen-selective promter, and

b) a second chimeric gene, integrated in the genome, comprising a herbicide resistance gene, which confers resistance to a herbicide on said wheat plant;

ii) interplanting said seeds capable of growing into conditionally male-sterile wheat plants with seeds capable of growing into male fertile wheat plants, which do not comprise said herbicide resistance gene;

iii) inducing male-sterility in the conditionally male-sterile plants by applying an acetylated toxin, which is in itself not toxic to the plants or plant cells

iv) after pollination, applying said herbicide, eliminating said male fertile plants; and

v) harvesting pure hybrid seed.

46. The wheat plant of claim 1, wherein said DNA molecule comprises a sequence which can be obtained from *Stenotrophomonas sp.*, deposited as DSM No. 9734, or a sequence capable of hybridizing therewith under standard stringent conditions.

47. The process of claim 10, wherein said DNA molecule comprises a sequence which can be obtained from *Stenotrophomonas sp.*, deposited as DSM No. 9734, or a sequence capable of hybridizing therewith under standard stringent conditions.

48. The process of claim 20, wherein said DNA molecule comprises a sequence which can be obtained from *Stenotrophomonas sp.*, deposited as DSM No. 9734, or a sequence capable of hybridizing therewith under standard stringent conditions.

49. The process of claim 30, wherein said DNA molecule comprises a sequence which can be obtained from *Stenotrophomonas sp.*, deposited as DSM No. 9734, or a sequence capable of hybridizing therewith under standard stringent conditions.

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<210> 6
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35 40 45

His Gln His Pro Glu Leu Ser Asn Arg Glu Val Arg Thr Ala Ala Lys
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Val Ala Glu Arg Leu Arg Ala Met Gly Leu Gln Pro Gln Thr Gly Val
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Ala Val His Gly Val Val Ala Ile Ile Lys Gly Ala Leu Pro Gly Pro
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Lys Ile Ala Leu Arg Ala Asp Met Asp Ala Leu Pro Val Thr Glu Gln
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Thr Gly Leu Pro Phe Ala Ser Thr Ala Thr Ala Glu Tyr Arg Gly Glu
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Lys Val Gly Val Met His Ala Cys Gly His Asp Ala His Thr Ala Thr
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Glu Phe Lys Pro Glu Ala Val Phe Gly Leu His Val Phe Ser Ser Val
        195                 200                 205

Gln Ala Gly Gln Ile Ala Val Arg Gly Gly Pro Leu Met Ala Ala Ser
    210                 215                 220

Asp Arg Phe Ala Ile Thr Val Asn Gly Arg Gln Thr His Gly Ser Ala
225                 230                 235                 240

Pro Trp Asn Gly Ile Asp Pro Ile Val Ala Ala Ser Asp Leu Ile Gly
                245                 250                 255

Thr Ala Gln Thr Ile Val Ser Arg Arg Ala Asn Leu Ser Lys Gln Pro
            260                 265                 270

Ala Val Leu Thr Phe Gly Ala Ile Lys Gly Gly Ile Arg Tyr Asn Ile
        275                 280                 285

Ile Pro Asp Ser Val Glu Met Val Gly Thr Ile Arg Thr Phe Asp Pro
    290                 295                 300

Asp Met Arg Lys Gln Ile Phe Ala Asp Leu Arg Asn Val Ala Glu His
305                 310                 315                 320

Thr Ala Ala Ala His Gly Ala Thr Ala Thr Thr Asp Ile Tyr Glu Lys
                325                 330                 335

Asp Gly Asn Pro Ala Thr Val Asn Asp Pro Ala Leu Thr Ala Arg Met
            340                 345                 350

Leu Pro Ser Leu Gln Ala Val Val Gly Lys Asp Asn Val Tyr Glu Pro
        355                 360                 365

Pro Leu Gln Met Gly Ser Glu Asp Phe Ser Leu Tyr Ala Gln Gln Val
    370                 375                 380

Pro Ala Met Phe Phe Phe Val Gly Ser Thr Gly Ala Gly Ile Asp Pro
385                 390                 395                 400
```

Ala Thr Ala Pro Ser Asn His Ser Pro Lys Phe Leu Leu Asp Glu Lys
405 410 415

Ala Leu Asp Val Gly Leu Arg Ala Leu Leu Gln Val Ser Leu Asp Tyr
420 425 430

Leu His Gly Gly Lys Ala Gly
435

<210> 11
<211> 1320
<212> DNA
<213> Stenotrophomonas sp.

<400> 11
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accgccgccg aaggccagcg gcccgaggtg gaggccgccg ccgcgcgcct gcagcggcag 120
gtggtggagt ggcgccgcga tttccaccag catccggagc tgtccaaccg cgaggtgcgc 180
accgccgcca aggtggccga gcgcctgcgc gcgatgggcc tgcaaccgca gaccggcgtc 240
gccgtgcacg gcgtggtggc gatcatcaag ggcgccctgc cggggccgaa gatcgccctg 300
cgcgcggaca tggacgcgct gccggtgacc gaacagaccg ggctgccgtt cgcctccacc 360
gccacggccg agtaccgcgg cgagaaggtc ggggtgatgc atgcctgcgg ccacgacgcc 420
cataccgcca ccctgctcgg cgtggccgac gcgctggtgg ccatgcgcga cacgctgccc 480
ggcgaagtaa tgctgatctt ccagccggcc gaggaaggcg cgccgccgcc ggagcagggc 540
ggtgccgagc tgatgctcaa ggaggggctg ttcaaggagt tcaagccgga ggcggtgttc 600
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ccctggaacg gcatcgatcc gattgtcgcc gcctccgacc tgatcggcac cgcgcagacc 780
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accttcgacc cggacatgcg caagcagatc ttcgccgact tgcgcaacgt cgccgagcac 960
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gccaccgcgc ccagcaacca ctcgccgaag ttcctgctcg acgagaaggc gctggacgtg 1260
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<210> 12
<211> 2510
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CA55 promoter
- coding sequence of deac gene

<400> 12
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gaacttgaca tctgaataga tattctccct tgtccctctg gtaaaaaaaa ctgttgtcac 120
atttgccttc gctgtgactt ggatgtatca tgtatatctt tgaccattga tatcttggtt 180
aatcagacgg tgcattacaa tcatggcctc attcatatag ggtttagggt taccacgatt 240
ggtttgcata agtagtaccc ctccgtttca aattatgtcg tattttgatt ttttagatac 300
actttttata taatttttta ttttaaatta ggtgttttat ataatacgta tctaagtgta 360
taataaaata tatgtatcta aaagctgtaa tttagtataa attagaatgg tgtatatctt 420
caatgtatga caaataattt gaaatggagg agggtatgaa aagccaaaac ctcctagaat 480
atggaatgga gggaatacat acaaattctt tgcttcagtt aaaagaaacg agaaaaggag 540
gggaatgggg aatcgtactt cagtttttac gagttttcat caaacatgta tgcacgtctt 600
cccttggttg atgcatcttt ttggcaaatc ttcgtttaat tgcggcttct tttttatacc 660
gttcgaaggt tttcgtcgtc aatgctgaaa ctccactttc accaccttcg gttgcatctg 720
cttgctttca attcacctct aattagtcca agtgtttcat tggacgaagg tccaagtcct 780
tcagatcatc tcaattttct ttgatctgaa acaacaattt aaaactgatt ttgttacctt 840
gacctgtcga agaccttcga acgaacggta ctgtaaaaat actgtacctc agatttgtga 900
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gttgcattac gctcactcga tcggttacga gcacgtagtt ggcgcctgtc acccaaccaa 1020
accagtagtt gaggcacgcc ctgtttgctc acgatcacga acgtacagca ctataaaaca 1080
cgcagggact ggaaagcgag atttcacagc tcaaagcagc caaaacgcag aagctgcact 1140
gcatatacag aagatacatc gagctaacta gctgcagcgg atccttgagg atgatccgca 1200
agaccgttct gttgactgcg ctgtcctgcg ccctggcctc cgcgacagcc accgccgccg 1260
aaggccagcg gcccgaggtg gaggccgccg ccgcgcgcct gcagcggcag gtggtggagt 1320
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ccagcaacca ctcgccgaag ttcctgctcg acgagaaggc gctggacgtg ggcctgcgcg 2460
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<210> 13
<211> 2932
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: T72 promoter - coding region of deac gene

<400> 13
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ggatacggaa ctcttaacac atgaaaatct aaacattttc aaccaatcag aactactaga 180
aagataatct aactacttca aaatttaaaa tttgacaaat aaaataacta gttttttcta 240
aagctatctt cactggacaa cttatgaata tttatattta tgaagcgagt actctcctag 300
tacatattac atatatattc ttcttctcat gaaaaattaa cttctcgcta taaatccgaa 360
catatattat gcgtagcaag ttgtttttt taacgggtgg agtaatatta gagtatttaa 420
attccttcaa attgccatcc ctctgggact ttgctgctgt tgttcttcca cggttgctgt 480
cagtgtcacc cagatttgca tcctttccag ctcgtagcta ctgttctgca tgtattggac 540
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acatctctgc tacagtgttt ttgctgcagt accacttcat atcctggcct tccgttctag 720
atcatgtgat gtacatgttt ttttgaaaca acccgcacaa gacattgata gagtaggaaa 780
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catagccgtg gaataagttc tgcacaaaag ttgccattcg agatcaacta ctggtagtag 1080
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cccggacatg cgcaagcaga tcttcgccga cttgcgcaac gtcgccgagc acaccgctgc 2580
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caacgacccg gcgctgaccg cgcgcatgct gcccagcctg caggccgtgg tcggcaagga 2700
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gcccagcaac cactcgccga agttcctgct cgacgagaag gcgctggacg tgggcctgcg 2880
cgcgctgctg caggtgtcgc tggactatct gcacggtggc aaggcggggt ga 2932

<210> 14
<211> 1687
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: E1 promoter-
coding sequence of deac gene

<400> 14
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atatagtaga cttgtcaagc tgcaagaact tccaatcgcc gacaatacca atagagatcc 120
aaccacctta atatcataaa caatctgatt gttagtccag aactatattg agtagtgaac 180
aacaatagca cattaacatt atgaggatta ttggctaact ctgcaattca atattctgat 240
gcgtctaatc tggtcaattt tagcgctcca gaaagaattg cacaatcctt ggacaatgtt 300
ggcactggaa ctgttgcatg tttttacatc tcttattaac gtagcaaagg agtagattat 360
tatgtaccag gagaaatctc ttcagatcct ttccacatgc aatgtcgtaa agaacagata 420
cagtgtacgt tagtttgtaa tggacggtca atgccatttc tctgaaggca tgttcagaga 480
tgatgatttc tgggatcctt ggaggggccc tgaaattcgg aaacagttag ttgagtttta 540
gtacctaatg tcttgcgtta tactacgtga aatgccattt ctgtaagctg agttttctac 600
catctccaca ggaaataaag ctaatacctg tccaagagtg gtgcggcatt tgaccaaatg 660
aagatcacaa gcatggcaag aatggcaatc tggcaaagga gcggaattat attgtattct 720
actacatcga acaggaacca tatcaatgtt gccccagcaa ggacccccgc agataagttc 780
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acgcacgccg gccacgataa cccatcctag catcccggtg tccagcaaga gatccatcaa 1680
gccgtcg 1687