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(58) Field of Search:
INT CL **C12N**
Other:

(54) Abstract Title: **Linum transformation method using acetohydroxyacid synthase gene selection marker**

(57) A method for transforming a plant of the genus Linum such as Linseed (L. usitatissium) wherein the selectable marker is an acetohydroxyacid synthase gene. Preferably the transformation method uses hypocotyls expants and is mediated by Rhzobiaceae bacteria such as Agrobacterium. The acetohydroxyacid synthase gene may be isolated from Arabidopsis and be under the control of its own promoter. Constructs comprising the acetohydroxyacid synthase gene and its promoter are claimed, as are methods of multiple rounds of plant transformation.

Figure 1

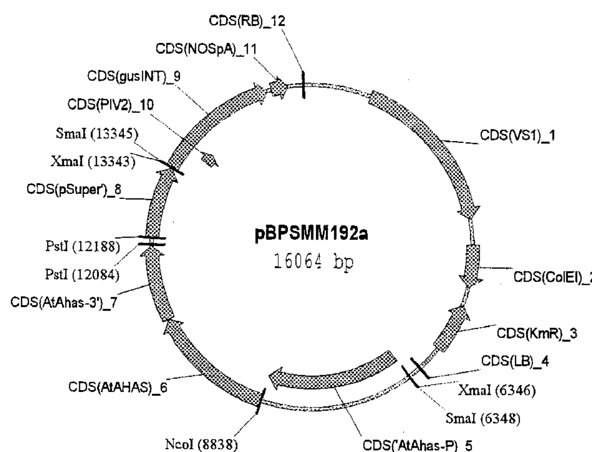

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Figure 1

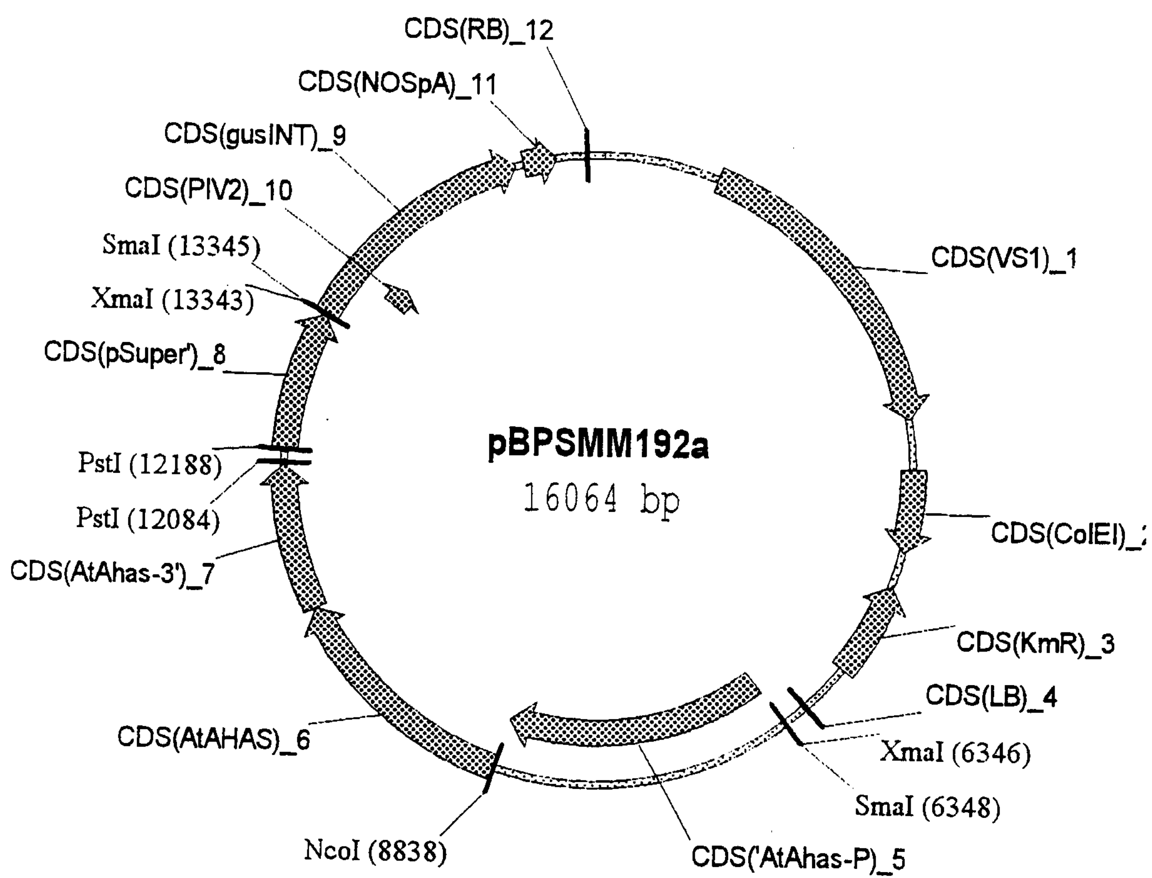

Figure 2

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Figure 3

5

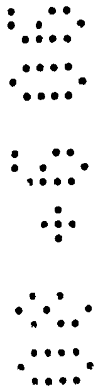

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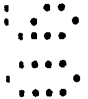
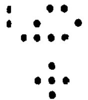
Figure 4





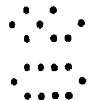


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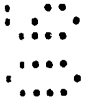
Figure 5



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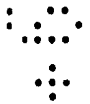

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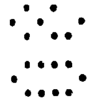
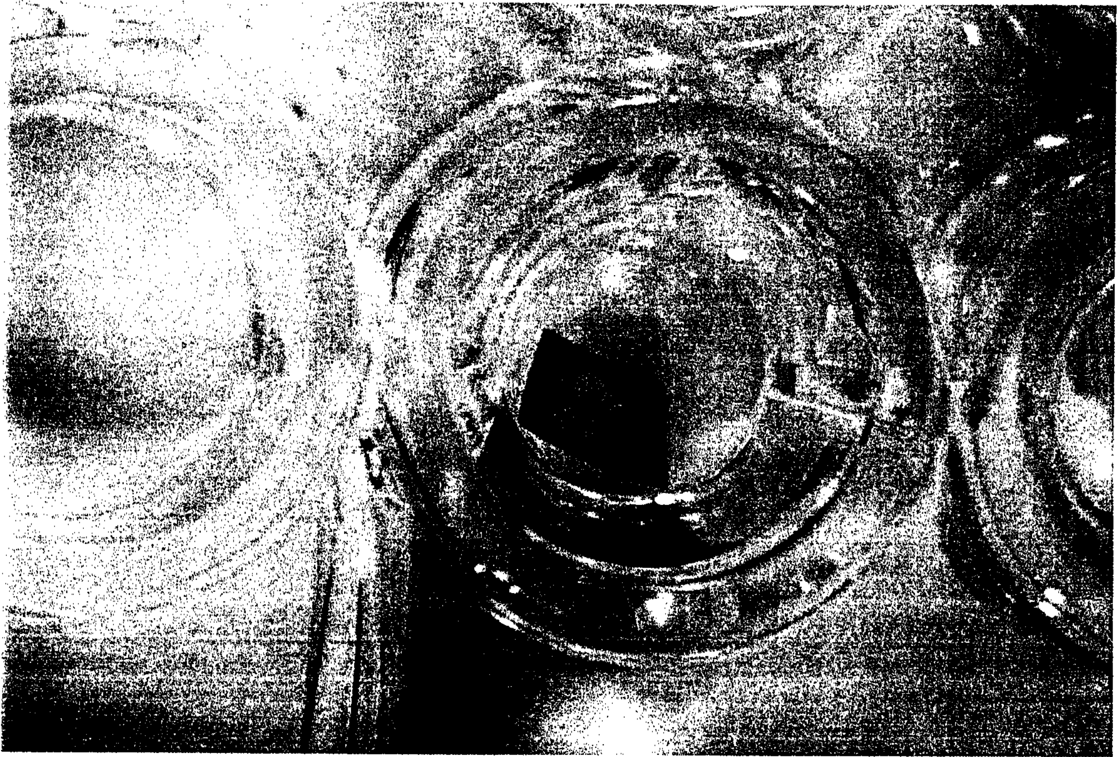
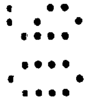


Figure 6





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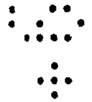


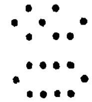
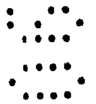


Figure 7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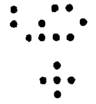
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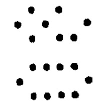
15

Acetohydroxyacid synthase inhibiting herbicide as a selectable marker for the introduction of a nucleic acid sequence into the genome of a plant of the genus Linum

5 The present invention relates to improved methods for the introduction of a nucleic acid sequence into the genome of a plant of the genus Linum, preferably linum usitatissimum based on selection with an acetohydroxyacid synthase inhibiting herbicide. Preferably, the introduction, e.g. transformation, is mediated by Agrobacterium.

10

The selection of transgenic cells and later on transgenic tissues and plants by employing adequate selection system is an essential step in a successful transformation experiment. The selection system furthermore has to be suitable for the particular crop. Moreover the selection system has to have public acceptance, as it is
15 employed in food production.

Although several different selection systems are known, for linseed only the selectable marker systems based on antibiotic resistance is used, for a survey see e.g. WO 01/05221.

20

Transgenic linseed plants were selected under antibiotic selection pressure even for the introduction of herbicide resistence genes, see e.g. Mc Hughen, development and preliminary field testing a glucosinate-ammonium tolerant transgenic flax, Can. J. Plant Science 75 (1995), 117-120.

25

Multiple subsequent transformations of a plant of the genus Linum with more than one construct (necessary for some of the more complicated high-value traits and for gene stacking) is complicated due to the limited availability of suitable selection markers. This situation is becoming compounded as antibiotic resistance markers (such as
30 hygromycin or kanamycin resistance) become less viable options as a result of tightened regulatory requirements and environmental concerns.

Accordingly, the object of the present invention is to provide an improved, reliable, efficient method for regeneration and selection of stably transformed plants of the
35 genus Linum, preferably linum usitatissimum. This objective is achieved by the present invention.

The present inventions provides a method for generating a transgenic plant of the genus Linum comprising the following steps

40 a. introducing into a cell of a plant of the genus linum a construct comprising
   i) a first nucleic acid sequence encoding an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide operably linked to a promoter active in said cell and
   ii) optionally a second nucleic acid sequence, preferably conferring to said plant
45 an agronomically valuable trait operably linked to a promoter active in said plant, and

b. incubating the cell of step a) on a selection medium comprising an acetohydroxyacid synthase inhibiting herbicide in a concentration of 0.1 µM or more for a time period of 5 days or more, and

50 c. transferring the cell of step b) to a regeneration medium and regenerating and optionally selecting the plant comprising said construct.

The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides or polymers or hybrids thereof in either single-or double-stranded, sense or antisense form.

A nucleic acid sequence refers to a consecutive list of abbreviations, letters, characters or words, which represent nucleotides. The coding region or open reading frame of said nucleic acid is the portion of the nucleic acid, which is transcribed and translated in a sequence-specific manner to produce into a particular polypeptide or protein when placed under the control of appropriate regulatory sequences, such as a promoter. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e. g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The term "nucleic acid" is used interchangeably herein with "gene", "cDNA and "mRNA".

A construct refers to a nucleic acid sequence at least partly created by recombinant methods. The term construct is referring to a RNA or DNA. The construct may be single- or – preferably - double stranded. The construct may be circular or linear. The skilled worker is familiar with a variety of ways to obtain the construct of the present invention.

The person skilled in the art is aware of numerous nucleic acid sequences encoding an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide. The term "acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide" means preferably an enzyme, whose catalytic activity is relatively more resistant to the presence of an acetohydroxyacid synthase inhibiting herbicide compared to a wild type acetohydroxyacid synthase.

The acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide of the present invention need not have the total catalytic activity necessary to maintain the viability of the cell of a plant of the genus linum, but must have some catalytic activity in an amount, alone or in combination with the catalytic activity of additional copies of the same acetohydroxyacid synthase to maintain the viability of a cell of a plant of the genus linum if exposed to an acetohydroxyacid synthase inhibiting herbicide. For example, catalytic activity may be increased to minimum acceptable levels for resisitance to acetohydroxyacid synthase inhibiting herbicides by introducing multiple copies of a nucleic acid sequence encoding a acetohydroxyacid synthase resistant to acetohydroxyacid synthase inhibiting herbicides into the cell or by introducing said nucleic acid sequence operably linked to a strong promoter to enhance the production of the acetohydroxyacid synthase resistant to acetohydroxyacid a synthase inhibiting herbicide.

More resistant means that the catalytic activity of the variant is diminished by the acetohydroxyacid synthase inhibiting herbicide, if at all, to a lesser degree than the wild-type, e.g. by 5 %, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% less than the wild-type when measured as described in US 5,928,937 (structure-based designed herbicide resistant products), column 13, lines 22 to 30, which is incorporated herewith by reference. Preferably the catalytic activity of an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide of the present invention retains sufficient to maintain the viability of a cell of a plant of the genus linum wherein at the same concentration of the same herbicide, wild-type acetohydroxyacid synthase would

not retain sufficient catalytic activity to maintain the viability of the cell. Preferably the enzymatic properties are measured as described in US 5,928,937 (structure-based designed herbicide resistant products), column 13, lines 19 to 52, which is incorporated herewith by reference.

5

Preferably, the acetohydroxyacid synthase (E.C. 4.1.3.18) resistant to acetohydroxyacid synthase inhibiting herbicides is as disclosed by G.W Haughn, J. Smith, B.J. Mazur, C. Somerville, Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistance to sulfonylurea herbicides. Mol.

10 Gen. Genet 204 (1986) 430-434 or B. J. Mazur, C.F. Chui , J.K. Smith, isolation and characterization of plant gene for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiology 85 (1987) 1110-111, which are incorporated herewith by reference. Especially preferred is the nucleic acid sequence as described by SEQ ID NO: 9.

15

A suitable acetohydroxyacid synthase resistant to acetohydroxyacid synthase inhibiting herbicides also includes fragments, mutants, derivatives, variants and alleles of the polypeptides exemplified above. Suitable fragments, mutants, derivatives, variants and alleles are those, which retain the functional characteristics of acetohydroxyacid

20 synthase resistant to acetohydroxyacid synthase inhibiting herbicides as defined above. Changes to a sequence, to produce a mutant, variant or derivative, may due to addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded polypeptide. Of course, changes to the nucleic acid that make no

25 difference to the encoded amino acid sequence are included.

Mutants and derivatives of the specified sequences can also comprise enzymes, which are improved in one or more characteristics (Ki, substrate specificity etc.) but still are resistant to acetohydroxyacid synthase inhibiting herbicides.

30

More preferably for the method of the invention, the acetohydroxyacid synthase is selected from the group consisting of

i) an acetohydroxyacid synthase with an amino acid sequence as described by SEQ ID NO: 10,

35 ii) an acetohydroxyacid synthase having an amino acid sequence identity of 80%, preferably 85%, more preferably 90%, even more preferably 91%, 92%, 93%, 94% or 95%, most preferably 96%, 97%, 98% or 99% or more to the amino acid sequence as described by SEQ ID NO: 10, and

iii) an acetohydroxyacid synthase encoded by a nucleic acid sequence capable to

40 hybridize to the nucleic acid sequence as described by SEQ ID NO: 9 or its complement.

Even more preferably for the method of the invention, the acetohydroxyacid synthase is selected from the group consisting of

45 i) an acetohydroxyacid synthase with an amino acid sequence as described by SEQ ID NO: 10,

ii) an acetohydroxyacid synthase having an amino acid sequence identity of 80%, preferably 85%, more preferably 90%, even more preferably 91%, 92%, 93%, 94% or 95%, most preferably 96%, 97%, 98% or 99% or more to the amino acid

50 sequence as described by SEQ ID NO: 10, and

iii) an acetohydroxyacid synthase encoded by a nucleic acid sequence capable to hybridize to the nucleic acid sequence as described by SEQ ID NO: 9 or its complement

and selection is done on a medium comprising an acetohydroxyacid synthase inhibiting herbicide in a concentration from 0.25 to 2 µM, more preferably from 0.5 to 1,75 µmM, even more preferably from 0.75 to 1,5 µM, most preferably from 1 to 1,5 µM and further most proferably from about 1 to 1,25 µM.

For the purposes of the invention hybridzation means preferably hybridization under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 2 X SSC, 0. 1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 1 X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 0.5 X SSC, 0. 1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO4, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 65°C to a nucleic acid comprising 50 to 200 or more consecutive nucleotides.

For the purposes of the invention, the percent sequence identity between two nucleic acid or polypeptide sequences is determined using the Vector NTI 7.0 (PC) software package (InforMax, 7600 Wisconsin Ave., Bethesda, MD 20814). A gap-opening penalty of 15 and a gap extension penalty of 6.66 are used for determining the percent identity of two nucleic acids. A gap-opening penalty of 10 and a gap extension penalty of 0.1 are used for determining the percent identity of two polypeptides. All other parameters are set at the default settings. For purposes of a multiple alignment (Clustal W algorithm), the gap-opening penalty is 10, and the gap extension penalty is 0.05 with blosum62 matrix. It is to be understood that for the purposes of determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymidine nucleotide sequence is equivalent to an uracil nucleotide.

The acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide of the invention may be expressed in the cytosol, peroxisome, or other intracellular compartment of the plant cell.

The term "promoter" as used herein is intended to mean a DNA sequence that directs the transcription of a DNA sequence (e.g., a structural gene). Typically, a promoter is located in the 5' region of a gene, proximal to the transcriptional start site of a structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Also, the promoter may be regulated in a tissue-specific or tissue preferred manner such that it is only active in transcribing the associated coding region in a specific tissue type(s) such as leaves, roots or meristem.

The term "promoter active in a cell of a plant of the genus Linum" means any promoter, whether plant derived or not, which is capable to induce transcription of an operably linked nucleotide sequence in at least one cell, tissue, organ or plant of the genus Linum at at least one time point in development or under dedifferentiated conditions. Such promoter may be a non-plant promoter (e.g., derived from a plant

virus or agrobacterium) or a plant promoter, perferably a dicotyledonous plant promoter.

The person skilled in the art is aware of several promoter which might be suitable for use in a plant of the genus Linum. In this context, expression can be constitutive, inducible or development-dependent.

The promoter of step i) of claim 1 and the promoter of step ii) of claim 1 can be the same or different. The promoter of step i) of claim 1 and the promoter of step ii) of claim 1 are preferably different. This applies also for the other embodiments of the present invention.

The promoter operably linked to the agronomically valuable trait of the present invention is preferably active in the tissue of the plant of the genus linum where the agronomically valuable trait is expressed.

Preferred are constitutive promoters. Most preferred is the Arabidopsis thaliana AHAS acetohydroxyacid synthase promoter.

The Arabidopsis thaliana AHAS acetohydroxyacid synthase promoter is preferably selected from the group consisting of

a) a nucleic acid sequence comprising the nucleic acid sequence as described by SEQ ID NO: 8, and
b) a nucleic acid sequence having promoter activity in a cell of a plant of the genus linum comprising at least one fragment of at least 50, 75, 100, 125, 150, 175, 200, 225 or 250 consecutive base pairs of the nucleic acid sequence as described by SEQ ID NO: 8, and,
c) a nucleic acid sequence having promoter activity in a cell of a plant of the genus linum comprising a nucleic acid sequence having at least 60%, especially 80%, preferably 85%, more preferably 90%, even more preferably 91%, 92%, 93%, 94% or 95%, most preferably 96%, 97%, 98% or 99% or more identity to the nucleic acid sequence as described by SEQ ID NO: 8, and,
d) a nucleic acid sequence having promoter activity in a cell of a plant of the genus linum comprising a nucleic acid sequence hybridizing to the nucleic acid sequence as described by SEQ ID NO: 8 or its complement.

The construct of the invention (or the vectors in which these are comprised) may comprise further functional elements and genetic control sequences in addition to the promoter active in plants of the genus Linum. The terms "functional elements" or "genetic control sequences" are to be understood in the broad sense and refer to all those sequences, which have an effect on the materialization or the function of the nucleic acid sequence according to the invention. For example, genetic control sequences modify transcription and translation of a gene. Genetic control sequences are described for example by Gruber (1993) "Vectors for Plant Transformation," in METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY; CRC Press, Boca Raton, Florida, eds.: Glick and Thompson, Chapter 7, pp.89-119 and the references cited therein.
Functional elements which may be comprised in a vector of the invention include
i) Origins of replication which ensure replication of the construct according to the invention in, for example, E. coli. Examples which may be mentioned are ORI (origin of DNA replication), the pBR322 ori or the P15A ori (Sambrook et al.:

Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989),

ii) Multiple cloning sites (MCS) to enable and facilitate the insertion of one or more nucleic acid sequences,

5 iii) Sequences which make possible homologous recombination, marker deletion, or insertion into the genome of a host organism. Available marker deletion methods are shown for example on p. 3, l. 1 to 27 of the international patent application WO 2005/090581 (Improved constructs for marker excision based on dual-fnction selection marker), which is incorporated herewith in its entirety,

10 iv) Elements, for example border sequences, which make possible the Agrobacterium-mediated transfer in plant cells for the transfer and integration into the plant genome, such as, for example, the right or left border of the T-DNA or the vir region.

15 The term "transgene" as used herein refers to any nucleic acid sequence, which is introduced into the genome of a cell or which has been manipulated by experimental manipulations by man. Preferably, said sequence is resulting in a genome which is different from a naturally occurring organism (*e.g.*, said sequence, if endogenous to said organism, is introduced into a location different from its natural location, or its 20 copy number is increased or decreased).

The term "transgenic" or "recombinant" when used in reference to a cell or an organism (e.g., with regard to a barley plant or plant cell) refers to a cell or organism which contains a transgene, or whose genome has been altered by the introduction of 25 a transgene. A transgenic organism or tissue may comprise one or more transgenic cells. Preferably, the organism or tissue is substantially consisting of transgenic cells (i.e., more than 80%, preferably 90%, more preferably 95%, most preferably 99% of the cells in said organism or tissue are transgenic).

30 A further object of the present invention are the above and below described constructs. The constructs of the present invention comprise

a) a first nucleic acid sequence encoding an enzyme as defined in claim 5 linked to the promoter as defined in claim 10 or 11 and

b) optionally a second nucleic acid sequence conferring to a plant of the genus linum 35 an agronomically valuable trait operably linked to a promoter active in a plant of the genus linum.

A further embodiment of the present invention is a plant or a part of a plant comprising a construct comprising

40 a) a first nucleic acid sequence encoding an enzyme as defined in claim 5 linked to the promoter as defined in claim 10 or 11 and

b) optionally a second nucleic acid sequence conferring to said plant an agronomically valuable trait operably linked to a promoter active in a cell of a plant of the genus linum.

45

Preferably, the construct inserted into the genome of the target plant comprises at least one second construct, which confers to a plant of the genus Linum an agronomically valuable trait. This can be achieved by expression of selection markers, trait genes, antisense RNA or double-stranded RNA. The person skilled in the art is 50 aware of numerous sequences which may be utilized in this context, e.g. to increase quality of food and feed, to produce chemicals, fine chemicals or pharmaceuticals e.g.,

vitamins, oils, carbohydrates, conferring resistance to herbicides, or conferring male sterility. Furthermore, growth, yield, and resistance against abiotic and biotic stress factors (like e.g., fungi, viruses or insects) may be enhanced.

5 A construct according to the invention may advantageously be introduced into cells using vectors into which said construct is inserted. Examples of vectors may be plasmids, cosmids, phages, viruses, retroviruses or Agrobacteria. In an advantageous embodiment, the construct is introduced by means of plasmid vectors. Preferred vectors are those, which enable the stable integration of the construct into the host
10 genome, i.e. the binary vector pBPSMM192a (Fig. 1.) as described by SEQ ID NO: 1.

The construct can be introduced into the target plant cells or organisms by any of the several means known to those of skill in the art. Various transformation procedures suitable for a plant of the genus Linum have been described, eg. WO 01/05221 A1,
15 page 6 to 7 (Novel methods for the generation and selection of transgenic linseed/flax plants) which is incorporated herewith by reference.

Especially preferred is the introduction of the construct with Agrobacterium. It is known in the art that not only Agrobacterium but also other soil-borne bacteria are
20 capable to mediate T-DNA transfer provided that the relevant functional elements for the T-DNA transfer of an Ti- or Ri-plasmid (van Veen RJM et al. (1988) Mol Plant Microb Interact 1(6):231-234) are available. More preferably, transformation is mediated by a Rhizobiaceae bacterium selected from the group of disarmed Agrobacterium tumefaciens or Agrobacterium rhizogenes bacterium strains. Especially
25 preferred is Agrobacterium tumefaciens. Most preferred is the Agrobacterium strain LBA4404.

Another preferred embodiment of the present method for generating a transgenic plant of the genus Linum, preferably linum usitatissimum, is comprising the steps of
30
a. a. isolating a hypocotyl segment of a plant of the genus linum and
b. introducing the construct of step a) of claim 1 with the aid of a bacterium of the genus Rhizobiaceae into said hypocotyl segment by co-cultivating said hypocotyl segment with said bacterium and,
c. optionally transferring the co-cultivated hypocotyl segment to a regeneration
35 medium, said regeneration medium lacking a phytotoxic effective amount of an acetohydroxyacid synthase inhibiting herbicide prior to selection,
d. inducing formation of a callus and selecting transgenic callus on a medium comprising,
   i. an effective amount of at least one auxin compound, and
40 ii. an acetohydroxyacid synthase inhibiting herbicide in a concentration of 0.1 µM or more, and
e. regenerating and optionally selecting plants containing the construct from the said transgenic callus..

45 Another embodiment is the above described method, wherein the construct of step a) of claim 1 is part of a T-DNA.

For preperation of the Agrobacterium inoculum preferably two days prior to inoculation Agrobacterium is taken from a frozen glycerol stock onto about 5 ml of LB liquid
50 medium containing about 100mg/l rifampicillin and about 50mg/l kanamycin and

placed on a shaker (250 rpm) overnight at 28 °C. One Day before inoculation the Agro bacterium culture is preferably subcultured by placing 1 ml in a tube containing 4 ml of fresh LB medium with antibiotics which is placed again on a shaker (250 rpm) at 28 °C overnight. On the day of transformation Agrobacterium is preferably spin down (10 min./ 5000 rpm) and pellet is re-suspended in 10 ml MS liquid medium (Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiologa Plantarum, 15 (1962) 473-479) to an O.D. 660 of 0.5.

The cell of a plant of the genus linum can be a differentiated cell or an undifferentiated cell. The cell of a plant of the genus linum can be isolated, in the form of tissue or in plantae. Various tissue can be employed for the transformation procedure disclosed herein. Such plant material may include but is not limited to for example leaf, root, immature and mature embryos, pollen, meristematic tissues, hypocotyl or inflorescences but also callus, protoplasts or suspensions of plant cells. Preferably, the plant material is hypocotyl. The material can be pre-treated (e.g., by inducing dedifferentiation prior to transformation) or not pre-treated.

The plant material for transformation (e.g., the hypocotyl explants) can be obtained or isolated from virtually any variety or plant of the genus Linum. Especially preferred are all linum species, e.g. linum usitatissiumum, linum sulcatum, linum perenne, linum narbonense, linum grundiflorum, linum flavum, linum elegans, linem catharticum, linum bienne, linum austriacum, linum arboreum especially of the linaceae family more especially preferred is linum usitatissimum. The method of the invention can be preferably used to produce transgenic plants from linum usitatissimum varieties such as the commercial varieties Flanders, SW0144403 (Svalöf Weibull), Gemini, McGregor, Ed 45 or Solin and especially preferred with Flanders SW 00-4402.

However, it should be pointed out, that the method of the invention is not limited to certain varieties but is highly genotype-independent. Plants of the genus Linum for isolation of hypocotyl are grown as known in the art, preferably as described below in the examples.

Hypocotyl is defined as a part of a germinating seedling of a seed plant. As the plant embryo grows at germination, it sends out a shoot called a radicle that becomes the primary root and penetrates down into the soil. After emergence of the radicle, the hypocotyl emerges and lifts the growing tip above the ground, bearing the embryonic leaves (cotyledons) and the plumule that gives rise to the first true leaves. The hypocotyl is the primary organ of extension of the young plant and develops into the stem. Especially preferred are segments of the hypocotyl of the size of about 2 to 4 mm.

Preferably hypocotyl segments for transformation are produced by the following procedure. Seeds of the genus linum are preferably rinsed with 70% ethanol for about 1 min followed by about 20 min sterilisation using 25% Klorin® with one drop of Tween 80, rinsing four times in sterile deionised water. The germination medium preferably contains MS macro and microelements including vitamins, 3% sucrose and 0.8% agar. Seeds are preferably sown in Petri dishes and germinated in a vertical position in a growth chamber at about 24 °C and about 16 h day/night photoperiod. Seed material coming from field is preferably sterilised with about 0.02% $HgCl_2$ instead of Klorin®. Preferably, hypocotyl segments with a size of about 2-4 mm of about four days old linseed seedlings are used.

The term "about" is used for the purposes of this invention to mean approximately, roughly, around, or in the region of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and
5 below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20 percent, preferably 10 percent, more preferably 5 percent up or down (higher or lower).

10 The inoculation of the hypocotyl explants with Agrobacterium ranges preferably from about 45 to 140, more preferably for about 120 minutes preferably on MS-liquid medium. The inoculated explants are generally placed on a plant compatible co-cultivation medium preferably containing MS medium with vitamins, about 1.5% sucrose, supplemented with about 40 mM acetosyringone and about 1 mg/l 2,4-
15 Dichlorophenoxyacetic acid.

A range of co-cultivation periods from a few hours to 10 days may be employed. The co-cultivation of Agrobacterium with hypocotyl is in general carried out for about 12 hours to about 7 days, preferably about 3 days to about 4 days at about 20°C to about
20 26°C, preferably at about 22 °C to about 24 °C. The Co-culture plates preferably contains MS medium with vitamins, about about 40 mM acetosyringone, about 3% sucrose, about pH 5.4, solidified with about 0.8% agar and plates are preferably incubated at about 24 °C in dark.

25 Transformed cells can be selected from untransformed cells preferably using the selection method of the invention.

Prior to a transfer to a regeneration or a selection medium, especially in case of Agrobacterium-mediated transformation, certain other intermediate steps may be
30 employed. Selection medium is defined as medium suitable for regeneration and selection of a transformed plant or part of a plant of the genus linum. For example, any Agrobacteria remaining from the co-cultivation step may be removed e.g., by a washing step. To prevent re-growth of said bacteria, the subsequently employed regeneration and/ or selection medium preferably comprises a bacteriocide (antibiotic)
35 suitable to prevent Agrobacterium growth. Preferred bactericidal antibiotics to be employed are e.g., carbenicillin, cefotaxime or Timentin™ (GlaxoSmithKline; a mixture of ticarcillin disodium and clavulanate potassium; 0.8 g Timentin™ contains 50 mg clavulanic acid with 750 mg ticarcillin. Chemically, ticarcillin disodium is N-(2-Carboxy-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-6-yl)-3-thio-phenemalonamic acid
40 disodium salt. Chemically, clavulanate potassium is potassium (Z)-(2R, 5R)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0] heptane-2-carboxylate).

Preferably, after the co-culture period, the hypocotyl segments are preferably washed two times about 15 minutes with sterile water.
45

In a further step the inoculated and washed hypocotyl explants can be cultived without selection pressure before including selection pressure. Preferably the inoculated and washed hypocotyl explants are cultivated on callus inducing and shoot regeneration medium including selection pressure of phytotoxic amount of an acetohydroxyacid
50 synthase inhibiting herbicide, preferably about 1.5 µM imidazolinone, preferably Imazamox. These plates preferably contain MS medium with vitamins, about 2 mg/l

BAP and about 0.1mg/l NAA, about 200 mg/l cefotaxime, about 3% sucrose, pH 5.4-5.6, solidified with 0.8% agar and plates are incubated preferably at about 24 °C at a about16/8h photoperiod with light intensity of about 40 $\mu Em^{-2}.s^{-1}$.

5 The term "phytotoxic" as used herein is intended to mean any measurable, negative effect on the physiology of a plant or plant cell resulting in symptoms including reduced growth, reduced photosynthesis, reduced cell division, reduced regeneration (e.g., of a mature plant from a cell culture, callus, or shoot etc.), reduced fertility. Phytotoxicity may further include effects like e.g., necrosis or apoptosis. A preferred 10 embodiment results in an reduction of growth or regenerability of at least 50%, preferably at least 80%, more preferably at least 90% in comparison with a plant which was not treated with said phytotoxic compound.

The specificacetohydroxyacid synthase inhibiting herbicide employed for selection is 15 chosen depending on which marker protein is expressed. The an acetohydroxyacid synthase inhibiting herbicide is generally present in a phytotoxic concentration.

In a preferred embodiment acetohydroxyacid synthase inhibiting herbicide is selected from the group consisting of imidazolinones, sulfonylureas, triazolopyrimidine 20 sulfonamides, pyrimidyloxy-benzoic acids, sulfamoylureas, sulfonylcarboximides and combinations thereof.

The optimal concentration of the acetohydroxyacid synthase inhibiting herbicide, may vary depending on the target tissue employed for transformation but in general the 25 total concentration (i.e. the sum in case of a mixture) of the acetohydroxyacid synthase inhibiting herbicide ranges from 0,1 µM or more, preferably 0,25 µM or more and most preferably 1 µM or more.

Preferably the acetohydroxyacid synthase inhibiting herbicide is present in a 30 concentration from 0.25 to 2 µM, more preferably from 0.5 to 1,75 µmM, even more preferably from 0.75 to 1,5 µM, most preferably from 1 to 1,5 µM and further most proferably from 1 to 1,25 µM.

Also the selection time may vary depending on the target tissue used and the 35 regeneration protocol employed. In general a selection time is at least 5, preferably at least 10 days. More specifically the total selection time is from 1 to 30 weeks, preferably, 3 to 25 weeks, more preferably 6 to 20 weeks.

In between the selection period the callus may be transferred to fresh selection 40 medium one or more times. Preferably selection is done in several steps, both during callus growth and during shoots regeneration and even under rooting conditions.

In an even more preferred embodiment, the selection of step b) of claim 1 of the present method is done in two steps, using a first selection step for a time period of 1 45 to 20, preferably for 1 to 16 and most preferably from 6 to 16 weeks and in a further embodiment from 15 to 21 days then transferring the surviving cells or tissue to a second selection medium with essentially the same composition than the first selection medium for additional from 2 to 7, more preferable from 3 to 4 weeks.

50 Preferably said selection medium is – for part of the selection period - also a dedifferentiation medium comprising at least one suitable plant growth regulator for

induction of embryogenic callus formation. The term "plant growth regulator" (PGR) as used herein means naturally occurring or synthetic (not naturally occurring) compounds that can regulate plant growth and development. PGRs may act singly or in consort with one another or with other compounds (e.g., sugars, amino acids). More specifically the medium employed for embryogenic callus induction and selection comprises

i. an effective amount of at least one auxin compound, and
ii. an effective amount of a selection agent allowing for selection of cells comprising the transgenic.

Furthermore the callus induction medium may optionally comprise an effective amount of at least one antibiotic that inhibits or suppresses the growth of the soil-borne bacteria (as defined above).

The term "auxin" or "auxin compounds" comprises compounds which stimulate cellular elongation and division, differentiation of vascular tissue, fruit development, formation of adventitious roots, production of ethylene, and - in high concentrations - induce dedifferentiation (callus formation). The most common naturally occurring auxin is indoleacetic acid (IAA), which is transported polarly in roots and stems. Synthetic auxins are used extensively in modern agriculture. Synthetic auxin compounds comprise indole-3-butyric acid (IBA), naphthylacetic acid (NAA), and 2,4-dichlorphenoxyacetic acid (2,4-D), Dicamba, especially preferred is NAA. Furthermore, combination of different auxins can be employed, for example a combination of 2,4-D and Picloram or Dicamba.

Preferably, in one embodiment when used as the sole auxin compound, NAA in a concentration of about 0.05 mg/l to about 6 mg/l, more preferably about 0.1 to about 0.5 mg/l , most preferably about 0.1 mg/l is employed.

The medium may be optionally further supplemented with one or more additional plant growth regulator, like e.g., cytokinin compounds (e.g., 6-benzylaminopurine) or other auxin compounds. Such compounds include, but are not limited to, IAA, NAA, IBA, cytokinins, auxins, kinetins, and thidiazuron. Cytokinin compounds comprise, for example zeatin, 6-isopentenyladenine (IPA) and 6-benzyladenine/6-benzylaminopurine (BAP). Especially preferred is BAP. BAP is generally applied in concentrations in the range from 0,1 to 10 mg/l, preferably from 0,5 to 6 mg/l and even more preferred from 1 to 4 mg/l and most preferred of about 2 mg/l.

In an most preferred embodiment the selection medium during callus growth is MS medium with vitamins, preferably Gamborg B5 vitamins, BAP preferably in a concentration from about 0.5 to about 10 mg/L, more preferably from about 1.0 to about 5 mg/L, even more preferred about 2 mg/l and about 0.1mg/l NAA, about 150 mg/l cefotaxime, about 3% sucrose, pH 5.8, solidified with about 0.8% agar and supplemented with a selective agent, e.g. imidazolinone in a concentration of about 0,5 to 1,5 µM depending on the germplasm used within transformation for a time period of about 150 days.

The callus tissue is preferably isolated after about 14 to 21 days and further subcultured onto fresh MS medium supplemented with preferably supplemented with about 2 mg/l BAP and about 0.1mg/l NAA, about 150 mg/l cefotaxime, about 3% sucrose, pH 5.8, solidified with about 0.8% agar and supplemented with imidazolinone

in a concentration of about 0,5 to 1,5 µM depending on the germplasm used within transformation for a time period of about 150 days.

Green shoots of the above mentioned callus tissue are preferably placed onto
5 elongation medium carrying out the second selection step (MS micro & macro elements with vitamins, about 250 mg/l timentin, about 1% sucrose, pH 5.8, solidified with about 0.8% agar and supplemented with the selective agent, preferably imidzolinone, preferably in a concentration of 1 to 5 µm at about 23 °C under a photoperiod of about 16 hours light intensity about 40 $\mu Em^{-2}.s^{-1}$ for about 3 to 4 weeks.
10

In a further step shoots, which were elongated as described above and remained green, are generally further cultured on root inducing medium preferably at about 24 °C under about 16/8 h photoperiod with light intensity of about 40 $\mu Em^{-2}.s^{-1}$. Preferably the rood inducing medium is MS hormone free und comprises about 150 mg/l
15 Cefotaxime, about 3% sucrose, pH about 5.4- 5.6, 0.8% agar, and 0,5 to 1,5, preferably 0,75 –1,25.0 µM of the acetohydroxyacid synthase inhibiting herbicide, preferably Imazamox. At this step a plant sample can be taken to determine the presence of the T-DNA, e.g. by applying Tagman analyses. Methods for said identification are well known in the art, e.g. PCR analysis, Northern blot, Southern blot,
20 or phenotypic screening.

Generally depending on the germplasm after about 3 to 4 weeks the first small roots can be observed. Rooted shoots are generally transferred into soil and kept moistened, covered and shaded until acclimatized. Transgenic plants after acclimatisation are
25 generally growing for about 12 weeks in the greenhouse (19-21 °C) till ripe seeds can be collected. Two or more generations should be grown in order to ensure that the genomic integration is stable and hereditary For example transgenic events in T1 or T2 generations could be involved in pre breeding hybridization program for combining different transgenes (gene stucking).
30

The presence of acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide does not rule out that additional markers are employed.

Other important aspects of the invention include the progeny of the transgenic plants
35 prepared by the disclosed methods, as well as the cells derived from such progeny, and the seeds obtained from such progeny.

Descendants may comprise one or more copies of the agronomically valuable trait gene. Preferably, descendants are isolated which only comprise one copy of said trait
40 gene.

Other embodiments of the invention relate to parts, organs, cells, fruits, and other reproduction material of a plant of the genus Linum, preferably linum usitatissimum of the invention. Preferred parts are selected from the group consisting of tissue, cells,
45 pollen, ovule, roots, leaves, seeds, microspores, and vegetative parts.

Another object of the present invention is the use of the plants according to the present invention for the production of food, pharmaceuticals or fine chemicals.

50 Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure. All documents mentioned in

this specification are incorporated herein in their entirety by reference. Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figure described below.

5 The methods and compositions of the invention allow for subsequent transformation. The selection with an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide of the present invention is compatible and does not interfere with other selection marker and selection systems. It is therefore possible to transform existing transgenic plants comprising another selection marker with the
10 constructs of the invention or to subsequently transform the plants obtained by the method of the invention (and comprising the expression constructs for acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide) with another marker. Thus, another embodiment of the invention relates to a method for subsequently introducing at least two constructs into a plant of the genus
15 Linum, preferably linum usitatissimum comprising the steps of

a) introducing a first construct comprising at least one nucleic acid encoding an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide operably linked to a promoter active in said cell, and
b) introducing a second construct comprising a second nucleic acid sequence
20 encoding a marker protein which is not conferring resistance against imidazolinone operably linked to a promoter active in said cell.

Preferably said second marker gene is a negative selection marker conferring a resistance to a biocidal compound such as a metabolic inhibitor (e.g., 2-deoxyglucose-
25 6-phosphate, WO 98/45456), antibiotics (e.g., kanamycin, G 418, bleomycin or hygromycin) or herbicides (e.g., phosphinothricin or glyphosate). Examples are:

- Phosphinothricin acetyltransferases (PAT; also named Bialophos ®resistance; bar; US 4,975,374)
- 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) conferring resistance to
30 Glyphosate® (N-(phosphonomethyl)glycine) (Shah et al. (1986) Science 233: 478)
- Glyphosate® degrading enzymes (Glyphosate® oxidoreductase; gox),
- Dalapon® inactivating dehalogenases (deh)
- Bromoxynil® degrading nitrilases (bxn)
- Kanamycin- or. geneticin (G418) resistance genes (NPTII; NPTI) coding e.g., for
35 neomycin phosphotransferases (Fraley 1983; Nehra 1994)
- hygromycin phosphotransferase (HPT), which mediates resistance to hygromycin (Vanden Elzen et al. (1985) Plant Mol Biol. 5:299).
- dihydrofolate reductase (Eichholtz et al. (1987) Somatic Cell and Molecular Genetics 13: 67-76)

40

Preferably, said second marker gene is defined as above and is most preferably conferring resistance against at least one compound select from the group consisting of phosphinotricin, glyphosate, phosphinotricin, glyphosate-type herbicides.

45 A further object of the present inventions relates to a plant or a part of a plant of the genus linum comprising

a) a nucleic acid sequence encoding an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide operably linked to a promoter active in said plant and optionally
50 b) a second nucleic acid sequence conferring to said plant of the genus linum an agronomically valuable trait operably linked a promoter active in said plant.

The present invention relates furthermore to a plant or a part of a plant of the genus Linum, preferably linum usitatissimum comprising

a) a first nucleic acid sequence encoding an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide operably linked to a promoter active in said plant, and
b) a second nucleic acid sequence encoding a selection marker protein, which is not conferring resistance against imidazolinone.

The invention also relates to a plant or the part of a plant of claim 16, wherein the acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide is defined by claims 5 or 6.

The invention also relates to a plant or the part of a plant comprising claim 13.

The present invention provides a further improved, reliable, efficient, publicly acceptable method for the generation and selection of stably transforming a plant of the genus Linum. It offers a minimized escape rate without interfering with hypocotyl callus formation and high number of transgenic shoots regeneration of plants of the genus Linum..

Sequences

SEQ ID NO: 1	binary vector containing AtAHAS expression cassette and GUS cassette
SEQ ID NO: 8	Arabidopsis thaliana acetohydroxyacid synthase promoter
SEQ ID NO: 9	Arabidopsis thaliana acetohydroxyacid synthase large subunit gene
SEQ ID NO: 10	Arabidopsis thaliana acetohydroxyacid synthase large subunit

Figures

Fig.1. Map of the binary vector pBPSMM192a.
Fig. 2. Nucleic acid sequence encoding an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide
Fig. 3. Linseed hypostyles *in vitro* culture.
Fig. 4. Shoots formation from callus tissues.
Fig. 5. Gus assay on leaves material from plants in the greenhouse.
Fig. 6. Gus assay on leaves material from *in vitro* plants.
Fig. 7. Transgenic linseed plants in greenhouse.

Examples

Unless indicated otherwise, chemicals and reagents in the Examples were obtained from Duchefa or Sigma–Aldrich AB, . The cloning steps carried out for the purposes of the present invention, such as, for example, transformation of E. coli cells, growing bacteria, multiplying phages and sequence analysis of recombinant DNA, are carried out as described by Sambrook (1989). The following examples are offered by way of illustration and not by way of limitation.

Abbreviations

MS	Murashige and Skoog micro and macro elements incl. vitamins
O.D.	Optical Density
BAP	6-benzyladenine/6-benzylaminopurine
NAA	naphthylacetic acid

1. Plant material

Linseed seeds variety Flanders (batch n. 00-44002 Swalöf Weibull, AB) were rinsed with 70% ethanol for 1 min following by 20 min sterilisation using 25% Klorin® with one drop of Tween 80, rinsing four times in sterile deionised water. The germination medium contained MS macro and microelements including vitamins, 3% sucrose and 0.8% agar. Seeds were sown in Petri dishes and germinated in a vertical position in a growth chamber at 24 °C and 16 h day/night photoperiod. Seed material coming from field was sterilised with 0.02% $HgCl_2$ instead of Klorin®, following the same procedure.

2. Preparation of Agrobacterium Inoculum

Two days prior to inoculation one loop of Agrobacterium was taken from a frozen glycerol stock onto 5 ml of LB liquid medium containing 100mg/l rifampicillin and 50mg/l kanamycin and placed on a shaker (250 rpm) overnight at 28 °C. Day before inoculation the Agro bacterium culture is subculture by placing 1 ml in a tube containing 4 ml of fresh LB medium with antibiotics which was placed again on a shaker (250 rpm) at 28 °C overnight. On the day of transformation Agrobacterium was spin down (10 min./ 5000 rpm) and pellet was re-suspended in 10 ml MS liquid medium (47) to an O.D. 660 of 0.5.
Transformation was performed using the bacterial strain LBA4404 carried constructs pBPSMM192a (Fig. 1.) harbouring mutate *Arabidopsis thaliana* gene (G.W Haughn, J. Smith, B.J. Mazur, C. Somerville, Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistance to sulfonylurea herbicides. Mol. Gen. Genet 204 (1986) 430-434, B. J. Mazur, C.F. Chui , J.K. Smith, isolation and characterization of plant gene for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiology 85 (1987) 1110-111).

3. Inoculation

Hypocotyls segments (2-4 mm) of four days old hypocotyls linseed seedlings were cut using sterile scalpel under the surface of 5 ml MS medium which was later replaced by 24 hour grown culture of Agrobacterium for 2 h. After two-hour infection period the Agrobacterium solution was aspirated off the hypocotyls segments. The segments were

then blotted dry between two pieces of sterile filter paper and transferred to co-culture plates. Co-culture plates containing MS medium with vitamins, 1 mg/l 2,4-D, 40 mM acetosyringone, 3% sucrose, pH 5.4, solidified with 0.8% agar and plates were incubated at 24 °C in dark.

5

4. Tissue Culture and Regeneration

After three days hypocotyl segments were rinsed three times with sterile distilled water and transferred to plates with regeneration medium. These plates contained MS
10 medium with vitamins, 2 mg/l BAP and 0.1mg/l NAA, 200 mg/l cefotaxime 3% sucrose, pH 5.4-5.6, solidified with 0.8% agar and plates are incubated at 24 °C 16/8h photoperiod with light intensity 40 $\mu Em^{-2}.s^{-1}$.

5. Callus growth and shoots regeneration under selection

15

After sixteen days the hypocotyls segments were moved onto MS medium with 2 mg/l BAP and 0.1mg/l NAA, 150 mg/l cefotaxime 3% sucrose, 0.8% agar or 0.1 mg/l Kinetin and 1.5 µM Imazamox 14 to 21 days. The response of transgenic plant tissues on imidazolinones was visible, callus was growing and expanding rapidly and green
20 shoots were developed. Green shoots were then placed in rooting MS hormone free medium with 150 mg/l Cefotaxime, 3% sucrose, pH 5.4- 5.6, 0.8% agar, and carried out the Second Selection Step 1.0 µM Imazamox at 24 °C under 16/8 h photoperiod with light intensity 40 $\mu Em^{-2}.s^{-1}$ for 21 days.

25 Leaves from *in vitro* regenerated plants are screened for the Gus expression (R.A. Jefferson, K.J. Wilson, The Gus gene fusion system. Plant Mol. Biol. (1991) B14: 1-33) and dark blue reaction in the leaf tissues showed transgenic origin of the regenerated shoots (Fig. 4). Later on the same test was performed with transgenic plants adapted and growing *in vivo* in greenhouse.

30

Rooted shoots were placed in moistened Jiffy pots and kept enclose plastic boxes until acclimatized at 20 °C, 80% humidity and with 16/8 photoperiod with 60 $\mu Em^{-2}.s^{-1}$light intensity. Shoots were hardened off 5-10 days and they were growing 2 weeks under the following conditions 24 °C, with 16/8 photoperiod with 60 $\mu Em^{-2}.s^{-1}$light intensity.

35

The presence of T-DNA introduced through the transformation process is determined by polymerase chain reaction (PCR). PCR was carried out with AHAS primers and confirmed transgenic origin of tested plants. Determination of copy number of transgene was done by Taqman and these results indicated the integration of one or
40 two copies of the nucleic acid sequence as described in figure 2 into the original plant genome. Transgenic plants after acclimatisation have been grown for 12 weeks in greenhouse (24 °C, with 16/8 photoperiod) till maturity when seeds were collected.

Figures 3 to 7 show the results of different stages of the regeneration and selection
45 process according to the present invention as well as the transgenic linseed plants obtainable by the process of the present invention.

pbpsmm192a.st25.txt
SEQUENCE LISTING

<110> BASF Plant Science GmbH

<120> Acetohydroxyacid synthase inhibiting herbicide as a selectable marker for the introduction of a nucleic acid sequence into the genome of a plant of the genus Linum

<130> PF 57661

<140> AE 20041075

<141> 2006-04-19

<160> 10

<170> PatentIn version 3.3

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<223> CDS of GUS including potato ST-LS1 intron [PIV-2]

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Met Glu Ile His Gln Ala Leu Thr Arg Ser Ser Ser Ile Arg Asn Val
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Leu Gly Asp Pro Ala Gln Glu Asp Glu Ile Phe Pro Asn Met Leu Leu
        590                 595                 600

ttt gca gca gct tgc ggg att cca gcg gcg agg gtg aca aag aaa gca      10695
Phe Ala Ala Ala Cys Gly Ile Pro Ala Ala Arg Val Thr Lys Lys Ala
    605                 610                 615

gat ctc cga gaa gct att cag aca atg ctg gat aca cca gga cct tac      10743
Asp Leu Arg Glu Ala Ile Gln Thr Met Leu Asp Thr Pro Gly Pro Tyr
620                 625                 630                 635

ctg ttg gat gtg att tgt ccg cac caa gaa cat gtg ttg ccg atg atc      10791
Leu Leu Asp Val Ile Cys Pro His Gln Glu His Val Leu Pro Met Ile
                640                 645                 650

ccg aat ggt ggc act ttc aac gat gtc ata acg gaa gga gat ggc cgg      10839
Pro Asn Gly Gly Thr Phe Asn Asp Val Ile Thr Glu Gly Asp Gly Arg
            655                 660                 665

att aaa tac tga gagatgaaac cggtgattat cagaaccttt tatggtcttt          10891
Ile Lys Tyr
        670

gtatgcatat ggtaaaaaaa cttagtttgc aatttcctgt ttgttttggt aatttgagtt   10951
tcttttagtt gttgatctgc ctgcttttttg gtttacgtca gactactact gctgttgttg  11011
tttggtttcc tttctttcat tttataaata aataatccgg ttcggtttac tccttgtgac   11071
tggctcagtt tggttattgc gaaatgcgaa tggtaaattg agtaattgaa attcgttatt   11131
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aagaagctaa aaacatagag aactcttgta aattgaagta tgaaaatata ctgaattggg   11551
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aagcttttaa aaaatagaaa agaaactagt attaaagttg taaatttaat aaacaaaaga   11731
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actagcatct gaatttcata accaatctcg atacaccaaa tcgaagatct cccgggtggt   13351
cagtcccttt atg tta cgt cct gta gaa acc cca acc cgt gaa atc aaa aaa   13402
           Met Leu Arg Pro Val Glu Thr Pro Thr Arg Glu Ile Lys Lys
                           675                 680

ctc gac ggc ctg tgg gca ttc agt ctg gat cgc gaa aac tgt gga att     13450
Leu Asp Gly Leu Trp Ala Phe Ser Leu Asp Arg Glu Asn Cys Gly Ile
685                 690                 695                 700

gat cag cgt tgg tgg gaa agc gcg tta caa gaa agc cgg gca att gct     13498
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Asp Gln Arg Trp Trp Glu Ser Ala Leu Gln Glu Ser Arg Ala Ile Ala
                705                 710                 715

gtg cca ggc agt ttt aac gat cag ttc gcc gat gca gat att cgt aat      13546
Val Pro Gly Ser Phe Asn Asp Gln Phe Ala Asp Ala Asp Ile Arg Asn
            720                 725                 730

tat gcg ggc aac gtc tgg tat cag cgc gaa gtc ttt ata ccg aaa ggt      13594
Tyr Ala Gly Asn Val Trp Tyr Gln Arg Glu Val Phe Ile Pro Lys Gly
        735                 740                 745

tgg gca ggc cag cgt atc gtg ctg cgt ttc gat gcg gtc act cat tac      13642
Trp Ala Gly Gln Arg Ile Val Leu Arg Phe Asp Ala Val Thr His Tyr
    750                 755                 760

ggc aaa gtg tgg gtc aat aat cag gaa gtg atg gag cat cag ggc ggc      13690
Gly Lys Val Trp Val Asn Asn Gln Glu Val Met Glu His Gln Gly Gly
765                 770                 775                 780

tat acg cca ttt gaa gcc gat gtc acg ccg tat gtt att gcc ggg aaa      13738
Tyr Thr Pro Phe Glu Ala Asp Val Thr Pro Tyr Val Ile Ala Gly Lys
                785                 790                 795

agt gta cgt aag ttt ctg ctt cta cct ttg ata tat ata taa taa tta      13786
Ser Val Arg Lys Phe Leu Leu Leu Pro Leu Ile Tyr Ile         Leu
            800                 805                                 810

tca tta att agt agt aat ata ata ttt caa ata ttt ttt tca aaa taa      13834
Ser Leu Ile Ser Ser Asn Ile Ile Phe Gln Ile Phe Phe Ser Lys
                815                 820                 825

aag aat gta gta tat agc aat tgc ttt tct gta gtt tat aag tgt gta      13882
Lys Asn Val Val Tyr Ser Asn Cys Phe Ser Val Val Tyr Lys Cys Val
                830                 835                 840

tat ttt aat tta taa ctt ttc taa tat atg acc aaa att tgt tga tgt      13930
Tyr Phe Asn Leu     Leu Phe     Tyr Met Thr Lys Ile Cys     Cys
            845                         850

gca ggt atc acc gtt tgt gtg aac aac gaa ctg aac tgg cag act atc      13978
Ala Gly Ile Thr Val Cys Val Asn Asn Glu Leu Asn Trp Gln Thr Ile
855                 860                 865                 870

ccg ccg gga atg gtg att acc gac gaa aac ggc aag aaa aag cag tct      14026
Pro Pro Gly Met Val Ile Thr Asp Glu Asn Gly Lys Lys Lys Gln Ser
                875                 880                 885

tac ttc cat gat ttc ttt aac tat gcc gga atc cat cgc agc gta atg      14074
Tyr Phe His Asp Phe Phe Asn Tyr Ala Gly Ile His Arg Ser Val Met
            890                 895                 900

ctc tac acc acg ccg aac acc tgg gtg gac gat atc acc gtg gtg acg      14122
Leu Tyr Thr Thr Pro Asn Thr Trp Val Asp Asp Ile Thr Val Val Thr
        905                 910                 915

cat gtc gcg caa gac tgt aac cac gcg tct gtt gac tgg cag gtg gtg      14170
His Val Ala Gln Asp Cys Asn His Ala Ser Val Asp Trp Gln Val Val
    920                 925                 930

gcc aat ggt gat gtc agc gtt gaa ctg cgt gat gcg gat caa cag gtg      14218
Ala Asn Gly Asp Val Ser Val Glu Leu Arg Asp Ala Asp Gln Gln Val
935                 940                 945                 950

gtt gca act gga caa ggc act agc ggg act ttg caa gtg gtg aat ccg      14266
Val Ala Thr Gly Gln Gly Thr Ser Gly Thr Leu Gln Val Val Asn Pro
                955                 960                 965

cac ctc tgg caa ccg ggt gaa ggt tat ctc tat gaa ctg tgc gtc aca      14314
His Leu Trp Gln Pro Gly Glu Gly Tyr Leu Tyr Glu Leu Cys Val Thr
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            970                                    975                      980

gcc aaa agc cag aca gag tgt gat atc tac ccg ctt cgc gtc ggc atc      14362
Ala Lys Ser Gln Thr Glu Cys Asp Ile Tyr Pro Leu Arg Val Gly Ile
        985                     990                     995

cgg tca  gtg gca gtg aag ggc  caa cag ttc ctg att  aac cac aaa       14407
Arg Ser  Val Ala Val Lys Gly  Gln Gln Phe Leu Ile  Asn His Lys
    1000                 1005                 1010

ccg ttc  tac ttt act ggc ttt  ggt cgt cat gaa gat  gcg gac tta       14452
Pro Phe  Tyr Phe Thr Gly Phe  Gly Arg His Glu Asp  Ala Asp Leu
    1015                 1020                 1025

cgt ggc  aaa gga ttc gat aac  gtg ctg atg gtg cac  gac cac gca       14497
Arg Gly  Lys Gly Phe Asp Asn  Val Leu Met Val His  Asp His Ala
    1030                 1035                 1040

tta atg  gac tgg att ggg gcc  aac tcc tac cgt acc  tcg cat tac       14542
Leu Met  Asp Trp Ile Gly Ala  Asn Ser Tyr Arg Thr  Ser His Tyr
    1045                 1050                 1055

cct tac  gct gaa gag atg ctc  gac tgg gca gat gaa  cat ggc atc       14587
Pro Tyr  Ala Glu Glu Met Leu  Asp Trp Ala Asp Glu  His Gly Ile
    1060                 1065                 1070

gtg gtg  att gat gaa act gct  gct gtc ggc ttt aac  ctc tct tta       14632
Val Val  Ile Asp Glu Thr Ala  Ala Val Gly Phe Asn  Leu Ser Leu
    1075                 1080                 1085

ggc att  ggt ttc gaa gcg ggc  aac aag ccg aaa gaa  ctg tac agc       14677
Gly Ile  Gly Phe Glu Ala Gly  Asn Lys Pro Lys Glu  Leu Tyr Ser
    1090                 1095                 1100

gaa gag  gca gtc aac ggg gaa  act cag caa gcg cac  tta cag gcg       14722
Glu Glu  Ala Val Asn Gly Glu  Thr Gln Gln Ala His  Leu Gln Ala
    1105                 1110                 1115

att aaa  gag ctg ata gcg cgt  gac aaa aac cac cca  agc gtg gtg       14767
Ile Lys  Glu Leu Ile Ala Arg  Asp Lys Asn His Pro  Ser Val Val
    1120                 1125                 1130

atg tgg  agt att gcc aac gaa  ccg gat acc cgt ccg  caa gtg cac       14812
Met Trp  Ser Ile Ala Asn Glu  Pro Asp Thr Arg Pro  Gln Val His
    1135                 1140                 1145

ggg aat  att tcg cca ctg gcg  gaa gca acg cgt aaa  ctc gac ccg       14857
Gly Asn  Ile Ser Pro Leu Ala  Glu Ala Thr Arg Lys  Leu Asp Pro
    1150                 1155                 1160

acg cgt  ccg atc acc tgc gtc  aat gta atg ttc tgc  gac gct cac       14902
Thr Arg  Pro Ile Thr Cys Val  Asn Val Met Phe Cys  Asp Ala His
    1165                 1170                 1175

acc gat  acc atc agc gat ctc  ttt gat gtg ctg tgc  ctg aac cgt       14947
Thr Asp  Thr Ile Ser Asp Leu  Phe Asp Val Leu Cys  Leu Asn Arg
    1180                 1185                 1190

tat tac  gga tgg tat gtc caa  agc ggc gat ttg gaa  acg gca gag       14992
Tyr Tyr  Gly Trp Tyr Val Gln  Ser Gly Asp Leu Glu  Thr Ala Glu
    1195                 1200                 1205

aag gta  ctg gaa aaa gaa ctt  ctg gcc tgg cag gag  aaa ctg cat       15037
Lys Val  Leu Glu Lys Glu Leu  Leu Ala Trp Gln Glu  Lys Leu His
    1210                 1215                 1220

cag ccg  att atc atc acc gaa  tac ggc gtg gat acg  tta gcc ggg       15082
Gln Pro  Ile Ile Ile Thr Glu  Tyr Gly Val Asp Thr  Leu Ala Gly
    1225                 1230                 1235
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ctg cac  tca atg tac acc gac  atg tgg agt gaa gag  tat cag tgt      15127
Leu His  Ser Met Tyr Thr Asp  Met Trp Ser Glu Glu  Tyr Gln Cys
    1240                 1245                 1250

gca tgg  ctg gat atg tat cac  cgc gtc ttt gat cgc  gtc agc gcc      15172
Ala Trp  Leu Asp Met Tyr His  Arg Val Phe Asp Arg  Val Ser Ala
    1255                 1260                 1265

gtc gtc  ggt gaa cag gta tgg  aat ttc gcc gat ttt  gcg acc tcg      15217
Val Val  Gly Glu Gln Val Trp  Asn Phe Ala Asp Phe  Ala Thr Ser
    1270                 1275                 1280

caa ggc  ata ttg cgc gtt ggc  ggt aac aag aaa ggg  atc ttc act      15262
Gln Gly  Ile Leu Arg Val Gly  Gly Asn Lys Lys Gly  Ile Phe Thr
    1285                 1290                 1295

cgc gac  cgc aaa ccg aag tcg  gcg gct ttt ctg ctg  caa aaa cgc      15307
Arg Asp  Arg Lys Pro Lys Ser  Ala Ala Phe Leu Leu  Gln Lys Arg
    1300                 1305                 1310

tgg act  ggc atg aac ttc ggt  gaa aaa ccg cag cag  gga ggc aaa      15352
Trp Thr  Gly Met Asn Phe Gly  Glu Lys Pro Gln Gln  Gly Gly Lys
    1315                 1320                 1325

caa tga atcaacaact ctcctggcgc accatcgtcg gctacagcct cgggaattgc     15408
Gln

taccgagctc gaatttcccc gatcgttcaa acatttggca ataaagtttc ttaagattga  15468

atcctgttgc cggtcttgcg atgattatca tataatttct gttgaattac gttaagcatg  15528

taataattaa catgtaatgc atgacgttat ttatgagatg ggtttttatg attagagtcc  15588

cgcaattata catttaatac gcgatagaaa acaaaatata gcgcgcaaac taggataaat  15648

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Ser Thr Lys Pro Ser Pro Ser Ser Ser Lys Ser Pro Leu Pro Ile Ser
            20                  25                  30
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Arg Phe Ser Leu Pro Phe Ser Leu Asn Pro Asn Lys Ser Ser Ser Ser
        35                  40                  45

Ser Arg Arg Arg Gly Ile Lys Ser Ser Ser Pro Ser Ser Ile Ser Ala
    50                  55                  60

Val Leu Asn Thr Thr Thr Asn Val Thr Thr Thr Pro Ser Pro Thr Lys
65                  70                  75                  80

Pro Thr Lys Pro Glu Thr Phe Ile Ser Arg Phe Ala Pro Asp Gln Pro
                85                  90                  95

Arg Lys Gly Ala Asp Ile Leu Val Glu Ala Leu Glu Arg Gln Gly Val
            100                 105                 110

Glu Thr Val Phe Ala Tyr Pro Gly Gly Ala Ser Met Glu Ile His Gln
        115                 120                 125

Ala Leu Thr Arg Ser Ser Ser Ile Arg Asn Val Leu Pro Arg His Glu
    130                 135                 140

Gln Gly Gly Val Phe Ala Ala Glu Gly Tyr Ala Arg Ser Ser Gly Lys
145                 150                 155                 160

Pro Gly Ile Cys Ile Ala Thr Ser Gly Pro Gly Ala Thr Asn Leu Val
                165                 170                 175

Ser Gly Leu Ala Asp Ala Leu Leu Asp Ser Val Pro Leu Val Ala Ile
            180                 185                 190

Thr Gly Gln Val Pro Arg Arg Met Ile Gly Thr Asp Ala Phe Gln Glu
        195                 200                 205

Thr Pro Ile Val Glu Val Thr Arg Ser Ile Thr Lys His Asn Tyr Leu
    210                 215                 220

Val Met Asp Val Glu Asp Ile Pro Arg Ile Ile Glu Glu Ala Phe Phe
225                 230                 235                 240

Leu Ala Thr Ser Gly Arg Pro Gly Pro Val Leu Val Asp Val Pro Lys
                245                 250                 255

Asp Ile Gln Gln Gln Leu Ala Ile Pro Asn Trp Glu Gln Ala Met Arg
            260                 265                 270

Leu Pro Gly Tyr Met Ser Arg Met Pro Lys Pro Pro Glu Asp Ser His
        275                 280                 285

Leu Glu Gln Ile Val Arg Leu Ile Ser Glu Ser Lys Lys Pro Val Leu
    290                 295                 300
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Tyr Val Gly Gly Gly Cys Leu Asn Ser Ser Asp Glu Leu Gly Arg Phe
305                 310                 315                 320

Val Glu Leu Thr Gly Ile Pro Val Ala Ser Thr Leu Met Gly Leu Gly
                325                 330                 335

Ser Tyr Pro Cys Asp Asp Glu Leu Ser Leu His Met Leu Gly Met His
            340                 345                 350

Gly Thr Val Tyr Ala Asn Tyr Ala Val Glu His Ser Asp Leu Leu Leu
        355                 360                 365

Ala Phe Gly Val Arg Phe Asp Asp Arg Val Thr Gly Lys Leu Glu Ala
    370                 375                 380

Phe Ala Ser Arg Ala Lys Ile Val His Ile Asp Ile Asp Ser Ala Glu
385                 390                 395                 400

Ile Gly Lys Asn Lys Thr Pro His Val Ser Val Cys Gly Asp Val Lys
                405                 410                 415

Leu Ala Leu Gln Gly Met Asn Lys Val Leu Glu Asn Arg Ala Glu Glu
            420                 425                 430

Leu Lys Leu Asp Phe Gly Val Trp Arg Asn Glu Leu Asn Val Gln Lys
        435                 440                 445

Gln Lys Phe Pro Leu Ser Phe Lys Thr Phe Gly Glu Ala Ile Pro Pro
    450                 455                 460

Gln Tyr Ala Ile Lys Val Leu Asp Glu Leu Thr Asp Gly Lys Ala Ile
465                 470                 475                 480

Ile Ser Thr Gly Val Gly Gln His Gln Met Trp Ala Ala Gln Phe Tyr
                485                 490                 495

Asn Tyr Lys Lys Pro Arg Gln Trp Leu Ser Ser Gly Gly Leu Gly Ala
            500                 505                 510

Met Gly Phe Gly Leu Pro Ala Ala Ile Gly Ala Ser Val Ala Asn Pro
        515                 520                 525

Asp Ala Ile Val Val Asp Ile Asp Gly Asp Gly Ser Phe Ile Met Asn
    530                 535                 540

Val Gln Glu Leu Ala Thr Ile Arg Val Glu Asn Leu Pro Val Lys Val
545                 550                 555                 560

Leu Leu Leu Asn Asn Gln His Leu Gly Met Val Met Gln Trp Glu Asp
                565                 570                 575

Arg Phe Tyr Lys Ala Asn Arg Ala His Thr Phe Leu Gly Asp Pro Ala
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580 585 590

Gln Glu Asp Glu Ile Phe Pro Asn Met Leu Leu Phe Ala Ala Ala Cys
595 600 605

Gly Ile Pro Ala Ala Arg Val Thr Lys Lys Ala Asp Leu Arg Glu Ala
610 615 620

Ile Gln Thr Met Leu Asp Thr Pro Gly Pro Tyr Leu Leu Asp Val Ile
625 630 635 640

Cys Pro His Gln Glu His Val Leu Pro Met Ile Pro Asn Gly Gly Thr
645 650 655

Phe Asn Asp Val Ile Thr Glu Gly Asp Gly Arg Ile Lys Tyr
660 665 670

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Gly Leu Trp Ala Phe Ser Leu Asp Arg Glu Asn Cys Gly Ile Asp Gln
20 25 30

Arg Trp Trp Glu Ser Ala Leu Gln Glu Ser Arg Ala Ile Ala Val Pro
35 40 45

Gly Ser Phe Asn Asp Gln Phe Ala Asp Ala Asp Ile Arg Asn Tyr Ala
50 55 60

Gly Asn Val Trp Tyr Gln Arg Glu Val Phe Ile Pro Lys Gly Trp Ala
65 70 75 80

Gly Gln Arg Ile Val Leu Arg Phe Asp Ala Val Thr His Tyr Gly Lys
85 90 95

Val Trp Val Asn Asn Gln Glu Val Met Glu His Gln Gly Gly Tyr Thr
100 105 110

Pro Phe Glu Ala Asp Val Thr Pro Tyr Val Ile Ala Gly Lys Ser Val
115 120 125

Arg Lys Phe Leu Leu Leu Pro Leu Ile Tyr Ile
130 135

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Leu Ser Leu Ile Ser Ser Asn Ile Ile Phe Gln Ile Phe Phe Ser Lys
1 5 10 15

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Lys Asn Val Val Tyr Ser Asn Cys Phe Ser Val Val Tyr Lys Cys Val
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Tyr Phe Asn Leu
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Tyr Met Thr Lys Ile Cys
1 5

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Cys Ala Gly Ile Thr Val Cys Val Asn Asn Glu Leu Asn Trp Gln Thr
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Ile Pro Pro Gly Met Val Ile Thr Asp Glu Asn Gly Lys Lys Lys Gln
20 25 30

Ser Tyr Phe His Asp Phe Phe Asn Tyr Ala Gly Ile His Arg Ser Val
35 40 45

Met Leu Tyr Thr Thr Pro Asn Thr Trp Val Asp Asp Ile Thr Val Val

50 55 60

Thr His Val Ala Gln Asp Cys Asn His Ala Ser Val Asp Trp Gln Val
65 70 75 80

Val Ala Asn Gly Asp Val Ser Val Glu Leu Arg Asp Ala Asp Gln Gln
85 90 95

Val Val Ala Thr Gly Gln Gly Thr Ser Gly Thr Leu Gln Val Val Asn
100 105 110

Pro His Leu Trp Gln Pro Gly Glu Gly Tyr Leu Tyr Glu Leu Cys Val
115 120 125

Thr Ala Lys Ser Gln Thr Glu Cys Asp Ile Tyr Pro Leu Arg Val Gly
130 135 140

Ile Arg Ser Val Ala Val Lys Gly Gln Gln Phe Leu Ile Asn His Lys
145 150 155 160

Pro Phe Tyr Phe Thr Gly Phe Gly Arg His Glu Asp Ala Asp Leu Arg
165 170 175

Gly Lys Gly Phe Asp Asn Val Leu Met Val His Asp His Ala Leu Met
180 185 190

Asp Trp Ile Gly Ala Asn Ser Tyr Arg Thr Ser His Tyr Pro Tyr Ala
195 200 205

Glu Glu Met Leu Asp Trp Ala Asp Glu His Gly Ile Val Val Ile Asp
210 215 220

Glu Thr Ala Ala Val Gly Phe Asn Leu Ser Leu Gly Ile Gly Phe Glu
225 230 235 240

Ala Gly Asn Lys Pro Lys Glu Leu Tyr Ser Glu Glu Ala Val Asn Gly
245 250 255

Glu Thr Gln Gln Ala His Leu Gln Ala Ile Lys Glu Leu Ile Ala Arg
260 265 270

Asp Lys Asn His Pro Ser Val Val Met Trp Ser Ile Ala Asn Glu Pro
275 280 285

Asp Thr Arg Pro Gln Val His Gly Asn Ile Ser Pro Leu Ala Glu Ala
290 295 300

Thr Arg Lys Leu Asp Pro Thr Arg Pro Ile Thr Cys Val Asn Val Met
305 310 315 320

Phe Cys Asp Ala His Thr Asp Thr Ile Ser Asp Leu Phe Asp Val Leu
325 330 335

Cys Leu Asn Arg Tyr Tyr Gly Trp Tyr Val Gln Ser Gly Asp Leu Glu
340 345 350

Thr Ala Glu Lys Val Leu Glu Lys Glu Leu Leu Ala Trp Gln Glu Lys
355 360 365

Leu His Gln Pro Ile Ile Ile Thr Glu Tyr Gly Val Asp Thr Leu Ala
370 375 380

Gly Leu His Ser Met Tyr Thr Asp Met Trp Ser Glu Glu Tyr Gln Cys
385 390 395 400

Ala Trp Leu Asp Met Tyr His Arg Val Phe Asp Arg Val Ser Ala Val
405 410 415

Val Gly Glu Gln Val Trp Asn Phe Ala Asp Phe Ala Thr Ser Gln Gly
420 425 430

Ile Leu Arg Val Gly Gly Asn Lys Lys Gly Ile Phe Thr Arg Asp Arg
435 440 445

Lys Pro Lys Ser Ala Ala Phe Leu Leu Gln Lys Arg Trp Thr Gly Met
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Asn Phe Gly Glu Lys Pro Gln Gln Gly Gly Lys Gln
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putative TATA box: 89-7-911

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acgcttctgt ctccttgtat ctttattata tctattcgtc ccatgagttt tgtttgttct 360

gtattcgttc gctctggtgt catggatgga gtctctgttc catgtttctg taatgcatgt 420

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aaaaaagaaa gaaagatcaa tttgataaat ttctcagcca caaattctac atttaggttt      840

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gagttttatc tataaataaa ggaccaaaaa tcaaatcccg agggcatttt cgtaatccaa      960

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Ile Xaa Xaa Met Ala Ala Ala Thr Thr Thr Thr Thr Thr Ser Ser Ser
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atc tcc ttc tcc acc aaa cca tct cct tcc tcc tcc aaa tca cca tta        96
Ile Ser Phe Ser Thr Lys Pro Ser Pro Ser Ser Ser Lys Ser Pro Leu
            20                  25                  30

cca atc tcc aga ttc tcc ctc cca ttc tcc cta aac ccc aac aaa tca       144
Pro Ile Ser Arg Phe Ser Leu Pro Phe Ser Leu Asn Pro Asn Lys Ser
        35                  40                  45

tcc tcc tcc tcc cgc cgc cgc ggt atc aaa tcc agc tct ccc tcc tcc       192
Ser Ser Ser Ser Arg Arg Arg Gly Ile Lys Ser Ser Ser Pro Ser Ser
    50                  55                  60

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65                  70                  75                  80

cca acc aaa cct acc aaa ccc gaa aca ttc atc tcc cga ttc gct cca       288
Pro Thr Lys Pro Thr Lys Pro Glu Thr Phe Ile Ser Arg Phe Ala Pro
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gat caa ccc cgc aaa ggc gct gat atc ctc gtc gaa gct tta gaa cgt       336
Asp Gln Pro Arg Lys Gly Ala Asp Ile Leu Val Glu Ala Leu Glu Arg
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caa ggc gta gaa acc gta ttc gct tac cct gga ggt gca tca atg gag       384
Gln Gly Val Glu Thr Val Phe Ala Tyr Pro Gly Gly Ala Ser Met Glu
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att cac caa gcc tta acc cgc tct tcc tca atc cgt aac gtc ctt cct       432
Ile His Gln Ala Leu Thr Arg Ser Ser Ser Ile Arg Asn Val Leu Pro
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Ser Gly Lys Pro Gly Ile Cys Ile Ala Thr Ser Gly Pro Gly Ala Thr
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aat ctc gtt agc gga tta gcc gat gcg ttg tta gat agt gtt cct ctt        576
Asn Leu Val Ser Gly Leu Ala Asp Ala Leu Leu Asp Ser Val Pro Leu
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gta gca atc aca gga caa gtc cct cgt cgt atg att ggt aca gat gcg        624
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Phe Gln Glu Thr Pro Ile Val Glu Val Thr Arg Ser Ile Thr Lys His
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Asn Tyr Leu Val Met Asp Val Glu Asp Ile Pro Arg Ile Ile Glu Glu
225                 230                 235                 240

gct ttc ttt tta gct act tct ggt aga cct gga cct gtt ttg gtt gat        768
Ala Phe Phe Leu Ala Thr Ser Gly Arg Pro Gly Pro Val Leu Val Asp
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gtt cct aaa gat att caa caa cag ctt gcg att cct aat tgg gaa cag        816
Val Pro Lys Asp Ile Gln Gln Gln Leu Ala Ile Pro Asn Trp Glu Gln
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gct atg aga tta cct ggt tat atg tct agg atg cct aaa cct ccg gaa        864
Ala Met Arg Leu Pro Gly Tyr Met Ser Arg Met Pro Lys Pro Pro Glu
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gat tct cat ttg gag cag att gtt agg ttg att tct gag tct aag aag        912
Asp Ser His Leu Glu Gln Ile Val Arg Leu Ile Ser Glu Ser Lys Lys
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cct gtg ttg tat gtt ggt ggt ggt tgt ttg aat tct agc gat gaa ttg        960
Pro Val Leu Tyr Val Gly Gly Gly Cys Leu Asn Ser Ser Asp Glu Leu
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ggt agg ttt gtt gag ctt acg ggg atc cct gtt gcg agt acg ttg atg       1008
Gly Arg Phe Val Glu Leu Thr Gly Ile Pro Val Ala Ser Thr Leu Met
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ggg ctg gga tct tat cct tgt gat gat gag ttg tcg tta cat atg ctt       1056
Gly Leu Gly Ser Tyr Pro Cys Asp Asp Glu Leu Ser Leu His Met Leu
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gga atg cat ggg act gtg tat gca aat tac gct gtg gag cat agt gat       1104
Gly Met His Gly Thr Val Tyr Ala Asn Tyr Ala Val Glu His Ser Asp
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ctt gag gct ttt gct agt agg gct aag att gtt cat att gat att gac       1200
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Ser Ala Glu Ile Gly Lys Asn Lys Thr Pro His Val Ser Val Cys Gly
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cga gaa gct att cag aca atg ctg gat aca cca gga cct tac ctg ttg        1920
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ggt ggc act ttc aac gat gtc ata acg gaa gga gat ggc cgg att aaa        2016
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Tyr
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Asp Pro Ala Gln Glu Asp Glu Ile Phe Pro Asn Met Leu Leu Phe Ala
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Gly Gly Thr Phe Asn Asp Val Ile Thr Glu Gly Asp Gly Arg Ile Lys
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Tyr

Claims

1. A method for generating a transgenic plant of the genus Linum comprising the following steps
   a. introducing into a cell of a plant of the genus linum a construct comprising
      i) a first nucleic acid sequence encoding an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide operably linked to a promoter active in said cell and
      ii) optionally a second nucleic acid sequence operably linked to a promoter active in said plant, and
   b. incubating the cell of step a) on a selection medium comprising an acetohydroxyacid synthase inhibiting herbicide in a concentration of 0.1 μM or more for a time period of 5 days or more, and
   c. transferring the cell of step b) to a regeneration medium and regenerating and optionally selecting the plant comprising said construct.

2. The method of claim 1 comprising the steps of
   a. isolating a hypocotyl segment of a plant of the genus linum and
   b. introducing the construct of step a) of claim 1 with the aid of a bacterium of the genus *Rhizobiaceae* into said hypocotyl segment by co-cultivating said hypocotyl segment with said bacterium and,
   c. optionally transferring the co-cultivated hypocotyl segment to a regeneration medium, said regeneration medium lacking a phytotoxic effective amount of an acetohydroxyacid synthase inhibiting herbicide prior to selection,
   d. inducing formation of a callus and selecting transgenic callus on a medium comprising,
      i. an effective amount of at least one auxin compound, and
      ii. an acetohydroxyacid synthase inhibiting herbicide in a concentration of 0.1 μM or more, and
   e. regenerating and optionally selecting plants containing the construct from the said transgenic callus.

3. The method of claim 1 or 2, wherein the construct of step a of claim 1 is part of a T-DNA.

4. The method of any of claims 1 to 3, wherein the acetohydroxyacid synthase inhibiting herbicide is selected from the group consisting of imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyloxy-benzoic acids, sulfamoylureas, sulfonylcarboximides and combinations thereof.

5. The method of any of claims 1 to 4, wherein the acetohydroxyacid synthase is selected from the group consisting of
   i) an acetohydroxyacid synthase with an amino acid sequence as described by SEQ ID NO: 10,
   ii) an acetohydroxyacid synthase having an amino acid sequence identity of 80% or more to the amino acid sequence as described by SEQ ID NO: 10, and
   iii) an acetohydroxyacid synthase encoded by a nucleic acid sequence capable to hybridize to the nucleic acid sequence as described by SEQ ID NO: 9 or its complement.

6. The method of any of claims 1 to 5, wherein the selection of step b) of claim 1 or step d) of claim 2 is done in the presence of 1 to 1,5 μM imidazolinone.

7. The method of any of claims 1 to 6, wherein the total selection time period is from 6 to 20 weeks.

8. The method of any of claims 1 to 7, wherein the selection of step b) of claim 1 or step d) of claim 2 is done in a two step process, using a first selection step for a time period of 1 to 20 weeks then transferring the surviving cells or tissue to a second selection medium for additional 2 to 7 weeks.

9. The method of any of claim 1 to 8, wherein said plant of the genus linum is linum usitatissimum.

10. The method of any of claims 1 to 9, wherein said promoter active in said cell of a plant of the genus linum is an Arabidopsis thaliana acetohydroxyacid synthase promoter.

11. The method of any of claims 1 to 10, wherein the acetohydroxyacid synthase promoter is selected from the group consisting of
    a) a nucleic acid sequence comprising the nucleic acid sequence as described by SEQ ID NO: 8, and
    b) a nucleic acid sequence having promoter activity in a cell of a plant of the genus linum comprising at least one fragment of at least 50 consecutive base pairs of the nucleic acid sequence as described by SEQ ID NO: 8, and,
    c) a nucleic acid sequence having promoter activity in a cell of a plant of the genus linum comprising a nucleic acid sequence having at least 60% identity to the nucleic acid sequence as described by SEQ ID NO: 8, and,
    d) a nucleic acid sequence having promoter activity in a cell of a plant of the genus linum comprising a nucleic acid sequence hybridizing to the nucleic acid sequence as described by SEQ ID NO: 8 or its complement.

12. Construct comprising
    a) a first nucleic acid sequence encoding an acetohydroxyacid synthase as defined in claim 5 linked to the promoter as defined in claim 10 or 11 and
    b) optionally a second nucleic acid sequence conferring to a plant of the genus linum an agronomically valuable trait operably linked to a promoter active in a plant of the genus linum.

13. A plant or a part of a plant of the genus linum comprising
    a) a nucleic acid sequence encoding an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide operably linked to a promoter active in said plant and optionally
    b) a second nucleic acid sequence conferring to said plant of the genus linum an agronomically valuable trait operably linked a promoter active in said plant.

14. A method for subsequently introducing at least two constructs into a cell of a plant of the genus linum comprising the steps of

a) introducing a first construct comprising at least one nucleic acid encoding an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide operably linked to a promoter active in said cell, and

b) introducing a second construct comprising a second nucleic acid sequence encoding a marker protein which is not conferring resistance against imidazolinone operably linked to a promoter active in said cell.

15. A plant or a part of a plant of the genus linum comprising

a) a first nucleic acid sequence encoding an acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide operably linked to a promoter active in said plant, and

b) a second nucleic acid sequence encoding a selection marker protein, which is not conferring resistance against imidazolinone.

16. The plant or the part of a plant of claim 15, wherein the acetohydroxyacid synthase resistant to an acetohydroxyacid synthase inhibiting herbicide is defined by claim 5.

17. A plant or a part of a plant comprising the construct of claim 12.

For Innovation

45

Application No: GB0607937.0 **Examiner:** Dr Patrick Purcell
Claims searched: 1-17 **Date of search:** 10 August 2006

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X,Y	X:12, 17 Y:1-11, 13-16	WO2004/005516 A (BASF PLANT SCIENCE GMBH) see whole document, esp. page 15, lines 31-41 and SEQ ID NO: 1, Fig. 1
X,Y	X: 12, 17 Y:1-11, 13-16	WO2005/121345 A (BASF PLANT SCIENCE GMBH) see whole document, esp. page 33, SEQ ID NO: 2 and Example 13
Y	1-11, 13-16	WO01/05221 A (GES. FUR ERWERB UND VERWERTUNG VON SCHUTZRECHTEN-GVS MBH) see whole document, esp. page 2, line 27-page 3, line 4 and Example 2
Y	1-11, 13-16	J Draper et al, "Plant Genetic Transformation and Gene Expression", published 1988, Blackwell Scientific Publications Chapter 2, pages 106-115
Y	1-11, 13-16	Euphytica, Vol 85, 1995, GG Rowland et al, "The application of chemical mutagenesis and biotechnology to the modification of linseed (Linum usitatissimum L.), 317-321 see page 319-320
Y	1-11, 13-16	Plant Cell Reports, Vol 13, 1994, L Mlynarova et al, "High efficiency Agrobacterium mediated gene transfer to flax", 282-285 see whole document
Y	1-17	WO2006/007373 A (BASF PLANT SCIENCE GMBH) see whole document, esp. page 9, lines 9-22, page 32, line 20-page 33, line 24, page 34, line 5-page 36, line 20
Y	1-17	WO03/012115 A (SYNGENTA PARTICIPATIONS AG) see whole document, esp. page 2, lines 8-21, page 3, lines 3-5,page 26, line 22-page 31, line 24
Y	1-17	WO01/85970 A (BOARD OF SUPERVISORS OR LOUISIANA STATE UNIVERSITY AND AGRICULTURAL AND MECHANICAL COLLEGE) see whole document, esp. page 10, lines 1-27

For Innovation

46

Y	1-17	EP0360750 A (CIBA-GEIGY AG) see whole document, esp. page 2, lines 2-50, page 11, line 20-page 14, line 60
Y	12, 17	WO2006/024509 A (BASF PLANT SCIENCE GMBH) see whole document, esp. page 54, SEQ ID NO: 16 and Example 4

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^{X} :

Worldwide search of patent documents classified in the following areas of the IPC

C12N

The following online and other databases have been used in the preparation of this search report

ONLINE: EPODOC, WPI, BIOSIS, MEDLINE, CAPLUS, BIOTECHNO, AGRICOLA