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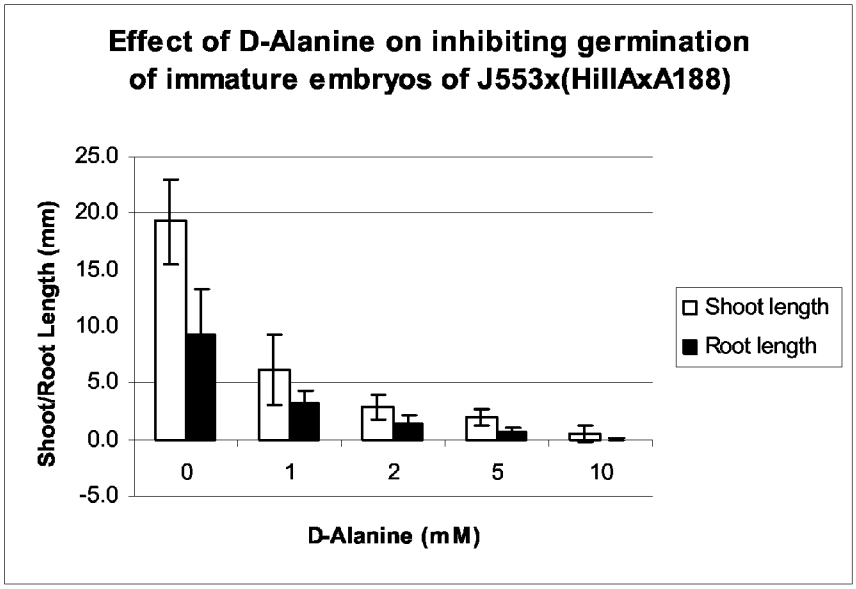
(54) **Title:** SELECTION SYSTEM FOR MAIZE



(57) **Abstract:** The present invention relates to improved methods for the incorporation of DNA into the genome of a *Zea mays* plant based on a D-alanine or D-serine selection. Preferably the transformation is mediated by Agrobacterium.

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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.

Selection system for maize

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to improved methods for the incorporation of DNA into the genome of a *Zea mays* plant based on a D-alanine or D-serine selection. Preferably the transformation is mediated by Agrobacterium.

Description of the Related Art

During the past decade, it has become possible to transfer genes from a wide range of organisms to crop plants by recombinant DNA technology. This advance has provided enormous opportunities to improve plant resistance to pests, diseases and herbicides, and to modify biosynthetic processes to change the quality of plant products. There have been many methods attempted for the transformation of monocotyledonous plants. "Biolistics" is one of the most widely used transformation methods. In the "biolistics" (microprojectile-mediated DNA delivery) method microprojectile particles are coated with DNA and accelerated by a mechanical device to a speed high enough to penetrate the plant cell wall and nucleus (WO 91/02071). The foreign DNA gets incorporated into the host DNA and results in a transformed cell. There are many variations on the "biolistics" method (Sanford 1990; Fromm 1990; Christou 1988; Sautter 1991).

While widely useful in dicotyledonous plants, *Agrobacterium*-mediated gene transfer has long been disappointing when adapted to use in monocots. Attempts by Hiei *et al.* (1994) suggested that transgenic rice plants could be obtained following *Agrobacterium*-mediated transformation, but the particular bacterial strains used and the choice of bacterial vectors were critical for successfully obtaining transgenics. A paper by Ishida *et al.* (1996) indicated that high-efficiency transformation of maize was possible by co-culture of immature embryos with A. tumefaciens. In both reports on rice and maize transformation, a super-binary vector pTOK233 containing additional copies of the virB, virC and virG genes was used to achieve high-efficiency transformation. WO 95/06722 and EP-A1 672 752 disclose a method of transforming monocotyledons using scutellum of immature embryos with A. tumefaciens. EP-A1 0 709 462 describes a method for transforming monocotyledonous plants, wherein the improvement is pointed out to include a recovery period after the co-cultivation step without a selection device for one day. A number of other methods have been reported for the transformation of monocotyledonous plants including, for example, the "pollen tube method" (WO 93/18168; Luo 1988), macro-injection of DNA into floral tillers (Du 1989; De la Pena 1987), injection of *Agrobacterium* into developing caryopses (WO 00/63398), and tissue incubation of seeds in DNA solutions (Töpfer 1989). Direct injection of exogenous DNA into the fertilized plant ovule at the onset of embryogenesis was disclosed in WO 94/00583.

All transformation methods for maize are inefficient and require the presence of a selection marker to distinguish transformed cells and plants form non-transformed. Mainly negative selection markers are employed, which confer a resistance against a phytotoxic agent (such as an herbicide or antibiotic). The negative selection marker in maize employed so far are mainly limited to phosphinothricin acetyltransferases (PAT; also named Bialophos® resistance; bar; de Block 1987; EP 0 333 033; US 4,975,374), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; conferring resistance to Gly-

phosate® (N-(phosphonomethyl)glycine); Shah 1986), and sulfonylurea- and imidazolinone-inactivating acetolactate synthases (e.g., the XI12 *ahas2* gene, US 6,653,529; the mutated XA17 *ahas* gene, Bernnasconi 1995, US 4,761,373; US 5,304,732; Anderson & Gregeson 1989; Currie 1995; Newhouse 1991).

Multiple subsequent transformations of maize plants 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 hygromycin or kanamycin resistance) become less viable options as a result of tightened regulatory requirements and environmental concerns. Alternative selection marker systems, such as a system based on D-amino acid metabolizing enzymes (e.g., D-amino acid dehydratases or oxidases), has been recently described on a general basis (WO 03/060133). However, no adoption and/or optimization of such a system for use in maize has been described so far. Accordingly, the object of the present invention is to provide an improved, efficient method for transforming *Zea mays* plants based on D-amino acid selection. This objective is achieved by the present invention.

SUMMARY OF THE INVENTION

A first embodiment of the invention relates to a method for generating a transgenic *Zea mays* plant comprising the steps of

a. introducing into a *Zea mays* cell or tissue a DNA construct comprising
   i) at least one first expression construct comprising a ubiquitin (constitutive?) promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,
   ii) at least one second expression construct conferring to said *Zea mays* plant an agronomically valuable trait, and
b. incubating said *Zea mays* cell or tissue of step a) on a selection medium comprising D-alanine and/or D-serine and/or a derivative thereof in a total concentration of about 1 mM to 100 mM for a time period of at least 5 days, and
c. transferring said *Zea mays* cell or tissue of step b) to a regeneration medium and regenerating and selecting *Zea mays* plants comprising said DNA construct.

Preferably, the enzyme capable to metabolize D-alanine or D-serine is selected from the group consisting of D-serine ammonia-lyases (EC 4.3.1.18), D-Amino acid oxidases (EC 1.4.3.3), and D-Alanine transaminases (EC 2.6.1.21). More preferably the enzyme capable to metabolize D-alanine or D-serine is selected from the group consisting of D-serine ammonia-lyases (EC 4.3.1.18), and D-Amino acid oxidases (EC 1.4.3.3). Even more preferably for the method of the invention, the enzyme capable to metabolize D-serine is selected from the group consisting of

i) the *E.coli* D-serine ammonia-lyase as encoded by SEQ ID NO: 2, and
ii) enzymes having the same enzymatic activity and an identity of at least 80% to the sequence as encoded by SEQ ID NO: 2, and
ii) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence described by SEQ ID NO: 1,

and wherein selection is done on a medium comprising D-serine in a concentration from about 1 mM to 100 mM.

Also more preferably for the method of the invention, the enzyme capable to metabolize D-serine and D-alanine is selected from the group consisting of

i) the *Rhodotorula gracilis* D-amino acid oxidase as encoded by SEQ ID NO: 4, and

ii) enzymes having the same enzymatic activity and an identity of at least 80% to the sequence as encoded by SEQ ID NO: 4, and

iii) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence described by SEQ ID NO: 3,

and wherein selection is done on a medium comprising D-alanine and/or D-serine in a total concentration from about 1 mM to 100 mM.

The promoter operably linked to the enzyme capable to metabolize D-alanine or D-serine is an important feature of the invention. Preferably the ubiquitin promoter is a monocot ubiquitin promoter, more preferably a *Zea mays* promoter. Even more preferably, the ubiquitin promoter is selected from the group consisting of

a) sequences comprising the sequence as described by SEQ ID NO: 5, and

b) sequences comprising at least one fragment of at least 50 consecutive base pairs of the sequence as described by SEQ ID NO: 5, and having promoter activity in *Zea mays*,

c) sequences comprising a sequence having at least 60% identity to the sequence as described by SEQ ID NO: 5, and having promoter activity in *Zea mays*,

d) sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 5, and having promoter activity in *Zea mays*.

The sequence described by SEQ ID NO: 5 is the core promoter of the Zea mays ubiquitin promoter. In one preferred embodiment not only the promoter region is employed as a transcription regulating sequence but also a 5'-untranslated region and/or an intron. More preferably the region spanning the promoter, the 5'-untranslated region and the first intron of the Zea mays ubiquitin gene are used, even more preferably the region described by SEQ ID NO: 6. Accordingly in another preferred embodiment the ubiquitin promoter utilized in the method of the invention is selected from the group consisting of

a) sequences comprising the sequence as described by SEQ ID NO: 6, and

b) sequences comprising at least one fragment of at least 50 consecutive base pairs of the sequence as described by SEQ ID NO: 6, and having promoter activity in *Zea mays*,

c) sequences comprising a sequence having at least 60% identity to the sequence as described by SEQ ID NO: 6, and having promoter activity in *Zea mays*,

d) sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 6, and having promoter activity in *Zea mays*.

In one preferred embodiment of the invention the selection of step b) is done using about 3 to about 15 mM D-alanine or about 7 to about 30 mM D-serine. The total selection time under dedifferentiating conditions is from about 3 to 4 weeks.

More preferably, the selection of step b) is done in two steps, using a first selection step for about 5 to 20 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 5 to 20 days.

Various methods can be employed to introduce the DNA constructs of the invention into maize plants. Preferably, introduction of said DNA construct is mediated by a method selected from the group consisting of *Rhizobiaceae* mediated transformation and particle bombardment mediated transformation. More preferably, transformation is mediated by a *Rhizobiaceae* bacterium selected from the group of disarmed *Agrobacterium tumefaciens* or A*grobacterium rhizogenes* bacterium strains. In another preferred embodiment the soil-borne bacterium is a disarmed strain variant of *Agrobacterium rhizogenes* strain K599 (NCPPB 2659). Such strains are described in US provisional patent application No. 60/606789, filed September 2nd, 2004, hereby incorporated entirely by reference.

In one preferred embodiment of the invention the method of the invention comprises the following steps

a. isolating an immature embryo of a *Zea mays* plant, and
b. co-cultivating said isolated immature embryo, which has not been subjected to a dedifferentiation treatment, with a bacterium belonging to genus *Rhizobiaceae* comprising at least one transgenic T-DNA, said T-DNA comprising
   i) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,
   ii) at least one second expression construct conferring to said *Zea mays* plant an agronomically valuable trait, and
c. transferring the co-cultivated immature embryos to a recovering medium, said recovery medium lacking a phytotoxic effective amount of D-serine or D-alanine, and
d. inducing formation of embryogenic callus and selecting transgenic callus on a medium comprising,
   i. an effective amount of at least one auxin compound, and
   ii. D-alanine and/or D-serine in a total concentration from about 1 mM to 100 mM, and
e. regenerating and selecting plants containing the transgenic T-DNA from the said transgenic callus.

In said preferred method the selection of step d) is done using about 3 to about 15 mM D-alanine or about 7 to about 30 mM D-serine. More preferably, the selection of step d) is done in two steps, using a first selection step for about 5 to 20 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 5 to 20 days.

In said preferred method the recovery medium of step c) comprises preferably

i. an effective amount of at least one antibiotic that inhibits or suppresses the growth of the soil-borne bacteria, and
ii. L-proline in a concentration from about 1 g/l to about 10g/l, and
iii. silver nitrate in a concentration from about 1 µM to about 50 µM,
iv. an effective amount of at least one auxin compound.

In said preferred recovery medium of step c) the effective amount of the auxin compound is preferably equivalent to a concentration of about 0.2 mg/l to about 6 mg/l 2,4-D. Preferably, the medium employed during co-cultivation comprises from about 1 µM to about 10 µM of silver nitrate and/or (preferably "and") from about 50 mg/L to about 1,000 mg/L of L-Cysteine.

Virtually any Zea mays plant can function as a source for the target material for the transformation. Preferably, said *Zea mays* plant, immature embryo, cell or tissue is selected from the group of *Zea mays* plants consisting of inbreds, hybrids, F1 between inbreds, F1 between an inbred and a hybrid, F1 between an inbred and a naturally-pollinated variety, commercial F1 varieties, any F2 crossing or self-pollination between the before mentioned varieties and the progeny of any of the before mentioned. More preferably, said Zea mays cell or tissue or said immature embryo is isolated from a cross of a (HiIIA x A188) hybrid with an inbred-line selected from the group of which representative seed having been deposited with the American Type Culture Collection under the Patent Deposit Designation PTA-6170 and PTA-6171.

The method of the invention, especially when used with D-Amino acid oxidases, can be advantageously combined with marker excision technology making use of the dual-function properties the D-amino acid oxidase. Thus, one embodiment of the invention relates to a method comprising the steps of:

i) transforming a *Zea mays* plant cell with a first DNA construct comprising
   a) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an D-amino acid oxidase enzyme, wherein said first expression cassette is flanked by sequences which allow for specific deletion of said first expression cassette, and
   b) at least one second expression cassette suitable for conferring to said plant an agronomically valuable trait, wherein said second expression cassette is not localized between said sequences which allow for specific deletion of said first expression cassette, and
ii) treating said transformed *Zea mays* plant cells of step i) with a first compound selected from the group consisting of D-alanine, D-serine or derivatives thereof in a phytotoxic concentration and selecting plant cells comprising in their genome said first DNA construct, conferring resistance to said transformed plant cells against said first compound by expression of said D-amino acid oxidase, and
iii) inducing deletion of said first expression cassette from the genome of said transformed plant cells and treating said plant cells with a second compound selected from the group consisting of D-isoleucine, D-valine and derivatives thereof in a concentration toxic to plant cells still comprising said first expression cassette, thereby selecting plant cells comprising said second expression cassette but lacking said first expression cassette.

Preferably, the ubiquitin promoter and/or the D-amino acid oxidase are defined as above.

Another embodiment of the invention relates to a recombinant expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, wherein said pro-

moter is heterologous in relation to said enzyme encoding sequence. Preferably, the ubiquitin promoter and/or the D-amino acid oxidase are defined as above.

Yet another embodiment of the invention relates to a DNA construct comprising

i) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,

ii) at least one second expression construct conferring to said *Zea mays* plant an agronomically valuable trait.

Preferably, the ubiquitin promoter and/or the D-amino acid oxidase is defined as above. Preferably said DNA construct is comprising features to allow marker deletion, preferably said construct is comprising

a) a first expression cassette comprising a nucleic acid sequence encoding a D-amino acid oxidase operably linked with a ubiquitin promoter, wherein said first expression cassette is flanked by sequences which allow for specific deletion of said first expression cassette, and

b) at least one second expression cassette suitable for conferring to said plant an agronomically valuable trait, wherein said second expression cassette is not localized between said sequences which allow for specific deletion of said first expression cassette.

More preferably said sequences which allow for specific deletion of said first expression cassette are selected from the group of sequences consisting of

a) recombination sites for a sequences-specific recombinase arranged in a way that recombination between said flanking recombination sites results in deletion of the sequences in-between from the genome, and

b) homology sequences A and A' having a sufficient length and homology in order to ensure homologous recombination between A and A', and having an orientation which – upon recombination between A and A' – will result in deletion of the sequences in-between from the genome.

Even more preferably, said construct (for marker deletion) comprises at least one recognition site for a sequence specific nuclease localized between said sequences which allow for specific deletion of said first expression cassette.

Other embodiments of the invention relate toa vector comprising an expression construct or a DNA construct of any of the invention, a transgenic cell or non-human organism comprising an expression construct, a DNA construct, or a vector of the invention. Preferably said transgenic cell or non-human organism is a plant cell and/or said organism is a plant, more preferably a *Zea mays* plant cell and/or a *Zea mays* plant.

Thus another embodiment of the invention relates to a transgenic, fertile *Zea mays* plant comprising stably integrated into its genome a DNA construct comprising

a) at least one first expression construct comprising a promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine,

b) at least one second expression construct conferring to said *Zea mays* plant an agronomically valuable trait.

Preferably the maize plant employed for transformation is obtained by crossing a (HiIIA x A188) hybrid with an inbred-line selected from the group of which representative seed having been deposited with the American Type Culture Collection under the Patent Deposit Designation PTA-6170 and PTA-6171. Preferably, the ubiquitin promoter and/or the D-amino acid oxidase is defined as above. Further embodiments of the invention related to descendant plants of a maize plant of the invention, hybrid and inbred plants produced from any of the above mentioned maize plants of the invention, and parts of said maize plants. Preferred parts are selected from the group consisting of tissue, cells, pollen, ovule, roots, leaves, seeds, microspores, and vegetative parts.

The methods and compositions of the invention can advantageously be employed in gene stacking approaches (i.e. for subsequent multiple transformations). Thus another embodiment of the inventions relates to a method for subsequent transformation of at least two DNA constructs into a Zea mays plant comprising the steps of:

a) a transformation with a first construct said construct comprising at least one expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, and
b) a transformation with a second construct said construct comprising a second selection marker gene, which is not conferring resistance against D-alanine or D-serine.

Preferably said second marker gene is conferring resistance against at least one compound select from the group consisting of phosphinotricin, glyphosate, sulfonylurea- and imidazolinone-type herbicides. More preferably, the marker gene is selected from the group of XI12 ahas mutant genes and XA17 ahas mutant genes.

Comprised are also the maize plants provided by such method. Thus another embodiment relates to a maize plant comprising

a) a first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, and
b) a second expression construct for a selection marker gene, which is not conferring resistance against D-alanine or D-serine.

Furthermore, the dsdA and dao gene provided hereunder can also be employed in subsequent transformations. Accordingly another embodiment of the invention relates to a method for subsequent transformation of at least two DNA constructs into a Zea mays plant comprising the steps of:

a) a transformation with a first construct said construct comprising a expression construct comprising a plant promoter and operably linked thereto a nucleic acid sequence encoding an dsdA enzyme and selecting with D-serine, and
b) a transformation with a second construct said construct comprising a expression construct comprising a plant promoter and operably linked thereto a nucleic acid sequence encoding an dao enzyme and selecting with D-alanine.

Additional object of the invention relate to descendant plants of a maize plant of the invention, hybrid plants and inbred plants produced from said descendent plants, and part of the before mentioned maize plants. Preferred parts are selected from the group

consisting of tissue, cells, pollen, ovule, roots, leaves, seeds, microspores, and vegetative parts.

DESCRIPTION OF THE DRAWINGS

Fig. 1A Effect of D-Alanine on inhibiting germination of dissected corn immature embryos.

Fig. 1B Effect of D-Serine on inhibiting germination of dissected corn immature embryos.

GENERAL DEFINITIONS

The teachings, methods, sequences etc. employed and described in the international patent applications WO 03/004659 (RECOMBINATION SYSTEMS AND A METHOD FOR REMOVING NUCLEIC ACID SEQUENCES FROM THE GENOME OF EUKARYOTIC ORGANISMS), WO 03/060133 (SELECTIVE PLANT GROWTH USING D-AMINO ACIDS), international patent application PCT/EP 2005/002735, international patent application PCT/EP 2005/002734, US provisional patent application No. 60/612,432 filed 23.09.2004 are hereby incorporated by reference.

It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, plant species or genera, constructs, and reagents described as such. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims. It must be noted that as used herein and in the appended claims, the singular forms "a," "and," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a vector" is a reference to one or more vectors and includes equivalents thereof known to those skilled in the art, and so forth.

The term "about" is used herein 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 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).

As used herein, the word "or" means any one member of a particular list and also includes any combination of members of that list.

"Agronomically valuable trait" include any phenotype in a plant organism that is useful or advantageous for food production or food products, including plant parts and plant products. Non-food agricultural products such as paper, etc. are also included. A partial list of agronomically valuable traits includes pest resistance, vigor, development time (time to harvest), enhanced nutrient content, novel growth patterns, flavors or colors, salt, heat, drought and cold tolerance, and the like. Preferably, agronomically valuable traits do not include selectable marker genes (e. g., genes encoding herbicide or antibiotic resistance used only to facilitate detection or selection of transformed cells), hormone biosynthesis genes leading to the production of a plant hormone (e.g., auxins,

gibberllins, cytokinins, abscisic acid and ethylene that are used only for selection), or reporter genes (e.g. luciferase, glucuronidase, chloramphenicol acetyl transferase (CAT, etc.). Such agronomically valuable important traits may include improvement of pest resistance (e.g., Melchers 2000), vigor, development time (time to harvest), enhanced nutrient content, novel growth patterns, flavors or colors, salt, heat, drought, and cold tolerance (e.g., Sakamoto 2000; Saijo 2000; Yeo 2000; Cushman 2000), and the like. Those of skill will recognize that there are numerous polynucleotides from which to choose to confer these and other agronomically valuable traits.

As used herein, the term "amino acid sequence" refers to a list of abbreviations, letters, characters or words representing amino acid residues. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes. The abbreviations used herein are conventional one letter codes for the amino acids: A, alanine; B, asparagine or aspartic acid; C, cysteine; D aspartic acid; E, glutamate, glutamic acid; F, phenylalanine; G, glycine; H histidine; I isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine ; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; Z, glutamine or glutamic acid (see L. Stryer, Biochemistry, 1988, W. H. Freeman and Company, New York. The letter "x" as used herein within an amino acid sequence can stand for any amino acid residue.

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

The phrase "nucleic acid sequence" as used herein refers to a consecutive list of abbreviations, letters, characters or words, which represent nucleotides. In one embodiment, a nucleic acid can be a "probe" which is a relatively short nucleic acid, usually less than 100 nucleotides in length. Often a nucleic acid probe is from about 50 nucleotides in length to about 10 nucleotides in length. A "target region" of a nucleic acid is a portion of a nucleic acid that is identified to be of interest. A "coding region" of a 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. The coding region is said to encode such a polypeptide or protein. 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, "mRNA", "oligonucleotide," and "polynucleotide".

The term "nucleotide sequence of interest" refers to any nucleotide sequence, the manipulation of which may be deemed desirable for any reason (*e.g.*, confer improved qualities), by one of ordinary skill in the art. Such nucleotide sequences include, but are not limited to, coding sequences of structural genes (*e.g.*, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*), and non-coding regulatory sequences which do not encode an mRNA or protein product, (*e.g.*, promoter sequence, polyadenylation sequence, termination sequence, enhancer sequence, *etc.*). A nucleic acid sequence of interest may preferably encode for an

agronomically valuable trait.

The term "antisense" is understood to mean a nucleic acid having a sequence complementary to a target sequence, for example a messenger RNA (mRNA) sequence the blocking of whose expression is sought to be initiated by hybridization with the target sequence.

The term "sense" is understood to mean a nucleic acid having a sequence which is homologous or identical to a target sequence, for example a sequence which binds to a protein transcription factor and which is involved in the expression of a given gene. According to a preferred embodiment, the nucleic acid comprises a gene of interest and elements allowing the expression of the said gene of interest.

As used herein, the terms "complementary" or "complementarity" are used in reference to nucleotide sequences related by the base-pairing rules. For example, the sequence 5'-AGT-3' is complementary to the sequence 5'-ACT-3'. Complementarity can be "partial" or "total." "Partial" complementarity is where one or more nucleic acid bases is not matched according to the base pairing rules. "Total" or "complete" complementarity between nucleic acids is where each and every nucleic acid base is matched with another base under the base pairing rules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. A "complement" of a nucleic acid sequence as used herein refers to a nucleotide sequence whose nucleic acids show total complementarity to the nucleic acids of the nucleic acid sequence.

The term “genome” or “genomic DNA” is referring to the heritable genetic information of a host organism. Said genomic DNA comprises the DNA of the nucleus (also referred to as chromosomal DNA) but also the DNA of the plastids (e.g., chloroplasts) and other cellular organelles (e.g., mitochondria). Preferably the terms genome or genomic DNA is referring to the chromosomal DNA of the nucleus.

The term “chromosomal DNA” or "chromosomal DNA-sequence" is to be understood as the genomic DNA of the cellular nucleus independent from the cell cycle status. Chromosomal DNA might therefore be organized in chromosomes or chromatids, they might be condensed or uncoiled. An insertion into the chromosomal DNA can be demonstrated and analyzed by various methods known in the art like *e.g.*, polymerase chain reaction (PCR) analysis, Southern blot analysis, fluorescence *in situ* hybridization (FISH), and *in situ* PCR.

Preferably, the term "isolated" when used in relation to a nucleic acid, as in "an isolated nucleic acid sequence" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is nucleic acid present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids are nucleic acids such as DNA and RNA, which are found in the state they exist in nature. For example, a given DNA sequence (*e.g.,* a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs, which encode a multitude of proteins. However, an isolated

nucleic acid sequence comprising SEQ ID NO:1 includes, by way of example, such nucleic acid sequences in cells which ordinarily contain SEQ ID NO:1 where the nucleic acid sequence is in a chromosomal or extrachromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid sequence may be present in single-stranded or double-stranded form. When an isolated nucleic acid sequence is to be utilized to express a protein, the nucleic acid sequence will contain at a minimum at least a portion of the sense or coding strand (*i.e.,* the nucleic acid sequence may be single-stranded). Alternatively, it may contain both the sense and anti-sense strands (*i.e.,* the nucleic acid sequence may be double-stranded).

As used herein, the term "purified" refers to molecules, either nucleic or amino acid sequences that are removed from their natural environment, isolated or separated. An "isolated nucleic acid sequence" is therefore a purified nucleic acid sequence. "Substantially purified" molecules are at least 60% free, preferably at least 75% free, and more preferably at least 90% free from other components with which they are naturally associated.

A "polynucleotide construct" refers to a nucleic acid at least partly created by recombinant methods. The term “DNA construct” is referring to a polynucleotide construct consisting of deoxyribonucleotides. 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 one of a DNA construct. Constructs can be prepared by means of customary recombination and cloning techniques as are described, for example, in Maniatis 1989, Silhavy 1984, and in Ausubel 1987.

The term "wild-type", "natural" or of "natural origin" means with respect to an organism, polypeptide, or nucleic acid sequence, that said organism is naturally occurring or available in at least one naturally occurring organism which is not changed, mutated, or otherwise manipulated by man.

The term "foreign gene" refers to any nucleic acid (*e.g.*, gene sequence) which is introduced into the genome of a cell by experimental manipulations and may include gene sequences found in that cell so long as the introduced gene contains some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, *etc.*) relative to the naturally-occurring gene.

The terms "heterologous nucleic acid sequence" or "heterologous DNA" are used interchangeably to refer to a nucleotide sequence, which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Generally, although not necessarily, such heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is expressed. A promoter, transcription regulating sequence or other genetic element is considered to be “heterologous” in relation to another sequence (e.g., encoding a marker sequence or am agronomically relevant trait) if said two sequences are not combined or differently operably linked their natural environment. Preferably, said sequences are not operably linked in their natural environment (i.e. come from different genes). Most preferably, said regulatory

sequence is covalently joined and adjacent to a nucleic acid to which it is not adjacent in its natural environment.

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 copy number is increased or decreased). A transgene may be an "endogenous DNA sequence", "an "exogenous DNA sequence" (*e.g.*, a foreign gene), or a "heterologous DNA sequence". The term "endogenous DNA sequence" refers to a nucleotide sequence, which is naturally found in the cell into which it is introduced so long as it does not contain some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, *etc.*) relative to the naturally-occurring sequence.

The term "transgenic" or "recombinant" when used in reference to a cell or an organism (e.g., with regard to a *Zea mays* plant or plant cell) refers to a cell or organism which contains a transgene, or whose genome has been altered by the introduction of 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).

A "recombinant polypeptide" is a non-naturally occurring polypeptide that differs in sequence from a naturally occurring polypeptide by at least one amino acid residue. Preferred methods for producing said recombinant polypeptide and/or nucleic acid may comprise directed or non-directed mutagenesis, DNA shuffling or other methods of recursive recombination.

The terms "homology" or "identity" when used in relation to nucleic acids refers to a degree of complementarity. Homology or identity between two nucleic acids is understood as meaning the identity of the nucleic acid sequence over in each case the entire length of the sequence, which is calculated by comparison with the aid of the program algorithm GAP (Wisconsin Package Version 10.0, University of Wisconsin, Genetics Computer Group (GCG), Madison, USA) with the parameters being set as follows:

Gap Weight: 12	Length Weight: 4
Average Match: 2,912	Average Mismatch:-2,003

For example, a sequence with at least 95% homology (or identity) to the sequence SEQ ID NO: 1 at the nucleic acid level is understood as meaning the sequence which, upon comparison with the sequence SEQ ID NO: 1 by the above program algorithm with the above parameter set, has at least 95% homology. There may be partial homology (*i.e.*, partial identity of less then 100%) or complete homology (*i.e.*, complete identity of 100%).

The term "hybridization" as used herein includes "any process by which a strand of nucleic acid joins with a complementary strand through base pairing." (Coombs 1994).

Hybridization and the strength of hybridization (*i.e.,* the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementarity between the nucleic acids, stringency of the conditions involved, the Tm of the formed hybrid, and the G:C ratio within the nucleic acids. As used herein, the term "Tm" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the Tm of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the Tm value may be calculated by the equation: Tm=81.5+0.41(% G+C), when a nucleic acid is in aqueous solution at 1 M NaCl [see *e.g.*, Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985)]. Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of Tm.

An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2 X SSC wash at 65°C for 15 minutes (see, Sambrook, infra, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1 X SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4 to 6 X SSC at 40°C for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.5 M, more preferably about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C and at least about 60°C for long probes (e.g., >50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2 X (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the proteins that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of highly stringent conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or Northern blot is 50% formamide, e.g., hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1 x SSC at 60 to 65°C. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20 X SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5 X to 1 X SSC at 55 to 60°C.

The term "equivalent" when made in reference to a hybridization condition as it relates to a hybridization condition of interest means that the hybridization condition and the hybridization condition of interest result in hybridization of nucleic acid sequences which have the same range of percent (%) homology. For example, if a hybridization condition of interest results in hybridization of a first nucleic acid sequence with other

nucleic acid sequences that have from 80% to 90% homology to the first nucleic acid sequence, then another hybridization condition is said to be equivalent to the hybridization condition of interest if this other hybridization condition also results in hybridization of the first nucleic acid sequence with the other nucleic acid sequences that have from 80% to 90% homology to the first nucleic acid sequence.

When used in reference to nucleic acid hybridization the art knows well that numerous equivalent conditions may be employed to comprise either low or high stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, *etc.*) and the concentration of the salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency hybridization different from, but equivalent to, the above-listed conditions. Those skilled in the art know that whereas higher stringencies may be preferred to reduce or eliminate non-specific binding, lower stringencies may be preferred to detect a larger number of nucleic acid sequences having different homologies.

The term "gene" refers to a coding region operably joined to appropriate regulatory sequences capable of regulating the expression of the polypeptide in some manner. A gene includes untranslated regulatory regions of DNA (e. g., promoters, enhancers, repressors, etc.) preceding (upstream) and following (downstream) the coding region (open reading frame, ORF) as well as, where applicable, intervening sequences (i.e., introns) between individual coding regions (i.e., exons). The term "structural gene" as used herein is intended to mean a DNA sequence that is transcribed into mRNA which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

As used herein the term "coding region" when used in reference to a structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded, in eukaryotes, on the 5'side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3'-side by one of the three triplets which specify stop codons (i.e., TAA, TAG, TGA). In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5'- and 3'-end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5'-flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the gene. The 3'-flanking region may contain sequences which direct the termination of transcription, posttranscriptional cleavage and polyadenylation.

The terms "polypeptide", "peptide", "oligopeptide", "polypeptide", "gene product", "expression product" and "protein" are used interchangeably herein to refer to a polymer or oligomer of consecutive amino acid residues.

The term "isolated" as used herein means that a material has been removed from its original environment. For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide,

separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides can be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and would be isolated in that such a vector or composition is not part of its original environment.

The term "genetically-modified organism" or "GMO" refers to any organism that comprises transgene DNA. Exemplary organisms include plants, animals and microorganisms.

The term "cell" or "plant cell" as used herein refers to a single cell. The term "cells" refers to a population of cells. The population may be a pure population comprising one cell type. Likewise, the population may comprise more than one cell type. In the present invention, there is no limit on the number of cell types that a cell population may comprise. The cells may be synchronized or not synchronized. A plant cell within the meaning of this invention may be isolated (e.g., in suspension culture) or comprised in a plant tissue, plant organ or plant at any developmental stage.

The term "organ" with respect to a plant (or "plant organ") means parts of a plant and may include (but shall not limited to) for example roots, fruits, shoots, stem, leaves, anthers, sepals, petals, pollen, seeds, *etc.*

The term "tissue" with respect to a plant (or "plant tissue") means arrangement of multiple plant cells including differentiated and undifferentiated tissues of plants. Plant tissues may constitute part of a plant organ (*e.g.,* the epidermis of a plant leaf) but may also constitute tumor tissues (e.g., callus tissue) and various types of cells in culture (*e.g.*, single cells, protoplasts, embryos, calli, protocorm-like bodies, *etc.*). Plant tissue may be *in planta*, in organ culture, tissue culture, or cell culture.

The term "plant" as used herein refers to a plurality of plant cells which are largely differentiated into a structure that is present at any stage of a plant's development. Such structures include one or more plant organs including, but are not limited to, fruit, shoot, stem, leaf, flower petal, *etc.*

The term "chromosomal DNA" or "chromosomal DNA-sequence" is to be understood as the genomic DNA of the cellular nucleus independent from the cell cycle status. Chromosomal DNA might therefore be organized in chromosomes or chromatids, they might be condensed or uncoiled. An insertion into the chromosomal DNA can be demonstrated and analyzed by various methods known in the art like e.g., PCR analysis, Southern blot analysis, fluorescence in situ hybridization (FISH), and in situ PCR.

The term "structural gene" as used herein is intended to mean a DNA sequence that is transcribed into mRNA which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

The term "expression" refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and - optionally - the subsequent translation of mRNA into one or more polypeptides.

The term "expression cassette" or "expression construct" as used herein is intended to mean the combination of any nucleic acid sequence to be expressed in operable linkage with a promoter sequence and - optionally – additional elements (like e.g., terminator and/or polyadenylation sequences) which facilitate expression of said nucleic acid sequence.

„Promoter", "promoter element," or "promoter sequence" as used herein, refers to the nucleotide sequences at the 5' end of a nucleotide sequence which direct the initiation of transcription (i.e., is capable of controlling the transcription of the nucleotide sequence into mRNA). A promoter is typically, though not necessarily, located 5' (*i.e.*, upstream) of a nucleotide sequence of interest (*e.g.*, proximal to the transcriptional start site of a structural gene) whose transcription into mRNA it controls, and provides a site for specific binding by RNA polymerase and other transcription factors for initiation of transcription. Promoter sequences are necessary, but not always sufficient, to drive the expression of a downstream gene. In general, eukaryotic promoters include a characteristic DNA sequence homologous to the consensus 5'-TATAAT-3' (TATA) box about 10-30 bp 5' to the transcription start (cap) site, which, by convention, is numbered +1. Bases 3' to the cap site are given positive numbers, whereas bases 5' to the cap site receive negative numbers, reflecting their distance from the cap site. Another promoter component, the CAAT box, is often found about 30 to 70 bp 5' to the TATA box and has homology to the canonical form 5'-CCAAT-3' (Breathnach 1981). In plants the CAAT box is sometimes replaced by a sequence known as the AGGA box, a region having adenine residues symmetrically flanking the triplet G(orT)NG (Messing 1983). Other sequences conferring regulatory influences on transcription can be found within the promoter region and extending as far as 1000 bp or more 5' from the cap site. The term "constitutive" when made in reference to a promoter means that the promoter is capable of directing transcription of an operably linked nucleic acid sequence in the absence of a stimulus (*e.g.*, heat shock, chemicals, light, *etc.*). Typically, constitutive promoters are capable of directing expression of a transgene in substantially any cell and any tissue.

Regulatory Control refers to the modulation of gene expression induced by DNA sequence elements located primarily, but not exclusively, upstream of (5' to) the transcription start site. Regulation may result in an all-or-nothing response to environmental stimuli, or it may result in variations in the level of gene expression. In this invention, the heat shock regulatory elements function to enhance transiently the level of downstream gene expression in response to sudden temperature elevation.

Polyadenylation signal refers to any nucleic acid sequence capable of effecting mRNA processing, usually characterized by the addition of polyadenylic acid tracts to the 3'-ends of the mRNA precursors. The polyadenylation signal DNA segment may itself be a composite of segments derived from several sources, naturally occurring or synthetic, and may be from a genomic DNA or an RNA-derived cDNA. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5'-AATAA-3', although variation of distance, partial "readthrough", and multiple tandem canonical sequences are not uncommon (Messing 1983). It should be recognized that a canonical "polyadenylation signal" may in fact cause transcriptional termination and not polyadenylation per se (Montell 1983).

Heat shock elements refer to DNA sequences that regulate gene expression in response to the stress of sudden temperature elevations. The response is seen as an immediate albeit transitory enhancement in level of expression of a downstream gene. The original work on heat shock genes was done with Drosophila but many other species including plants (Barnett 1980) exhibited analogous responses to stress. The essential primary component of the heat shock element was described in Drosophila to have the consensus sequence 5'-CTGGAATNTTCTAGA-3' (where N=A, T, C, or G) and to be located in the region between residues -66 through -47 bp upstream to the transcriptional start site (Pelham 1982). A chemically synthesized oligonucleotide copy of this consensus sequence can replace the natural sequence in conferring heat shock inducibility.

Leader sequence refers to a DNA sequence comprising about 100 nucleotides located between the transcription start site and the translation start site. Embodied within the leader sequence is a region that specifies the ribosome binding site.

Introns or intervening sequences refer in this work to those regions of DNA sequence that are transcribed along with the coding sequences (exons) but are then removed in the formation of the mature mRNA. Introns may occur anywhere within a transcribed sequence--between coding sequences of the same or different genes, within the coding sequence of a gene, interrupting and splitting its amino acid sequences, and within the promoter region (5' to the translation start site). Introns in the primary transcript are excised and the coding sequences are simultaneously and precisely ligated to form the mature mRNA. The junctions of introns and exons form the splice sites. The base sequence of an intron begins with GU and ends with AG. The same splicing signal is found in many higher eukaryotes.

The term "operable linkage" or "operably linked" is to be understood as meaning, for example, the sequential arrangement of a regulatory element (e.g. a promoter) with a nucleic acid sequence to be expressed and, if appropriate, further regulatory elements (such as e.g., a terminator) in such a way that each of the regulatory elements can fulfill its intended function to allow, modify, facilitate or otherwise influence expression of said nucleic acid sequence. The expression may result depending on the arrangement of the nucleic acid sequences in relation to sense or antisense RNA. To this end, direct linkage in the chemical sense is not necessarily required. Genetic control sequences such as, for example, enhancer sequences, can also exert their function on the target sequence from positions which are further away, or indeed from other DNA molecules. Preferred arrangements are those in which the nucleic acid sequence to be expressed recombinantly is positioned behind the sequence acting as promoter, so that the two sequences are linked covalently to each other. The distance between the promoter sequence and the nucleic acid sequence to be expressed recombinantly is preferably less than 200 base pairs, especially preferably less than 100 base pairs, very especially preferably less than 50 base pairs. Operable linkage, and an expression cassette, can be generated by means of customary recombination and cloning techniques as described (e.g., in Maniatis 1989; Silhavy 1984; Ausubel 1987; Gelvin 1990). However, further sequences which, for example, act as a linker with specific cleavage sites for restriction enzymes, or as a signal peptide, may also be positioned between the two sequences. The insertion of sequences may also lead to the expression of fusion proteins. Preferably, the expression cassette, consisting of a linkage of promoter and nu-

cleic acid sequence to be expressed, can exist in a vector-integrated form and be inserted into a plant genome, for example by transformation.

The term "transformation" as used herein refers to the introduction of genetic material (*e.g.*, a transgene) into a cell. Transformation of a cell may be stable or transient. The term "transient transformation" or "transiently transformed" refers to the introduction of one or more transgenes into a cell in the absence of integration of the transgene into the host cell's genome. Transient transformation may be detected by, for example, enzyme-linked immunosorbent assay (ELISA) which detects the presence of a polypeptide encoded by one or more of the transgenes. Alternatively, transient transformation may be detected by detecting the activity of the protein (*e.g.*, β-glucuronidase) encoded by the transgene (*e.g.*, the uid A gene) as demonstrated herein [*e.g.*, histochemical assay of GUS enzyme activity by staining with X-gluc which gives a blue precipitate in the presence of the GUS enzyme; and a chemiluminescent assay of GUS enzyme activity using the GUS-Light kit (Tropix)]. The term "transient transformant" refers to a cell which has transiently incorporated one or more transgenes. In contrast, the term "stable transformation" or "stably transformed" refers to the introduction and integration of one or more transgenes into the genome of a cell, preferably resulting in chromosomal integration and stable heritability through meiosis. Stable transformation of a cell may be detected by Southern blot hybridization of genomic DNA of the cell with nucleic acid sequences which are capable of binding to one or more of the transgenes. Alternatively, stable transformation of a cell may also be detected by the polymerase chain reaction of genomic DNA of the cell to amplify transgene sequences. The term "stable transformant" refers to a cell which has stably integrated one or more transgenes into the genomic DNA (including the DNA of the plastids and the nucleus), preferably integration into the chromosomal DNA of the nucleus. Thus, a stable transformant is distinguished from a transient transformant in that, whereas genomic DNA from the stable transformant contains one or more transgenes, genomic DNA from the transient transformant does not contain a transgene. Transformation also includes introduction of genetic material into plant cells in the form of plant viral vectors involving epichromosomal replication and gene expression which may exhibit variable properties with respect to meiotic stability. Transformation also includes introduction of genetic material into plant cells in the form of plant viral vectors involving epichromosomal replication and gene expression which may exhibit variable properties with respect to meiotic stability. Preferably, the term "transformation" includes introduction of genetic material into plant cells resulting in chromosomal integration and stable heritability through meiosis.

The terms "infecting" and "infection" with a bacterium refer to co-incubation of a target biological sample, (*e.g.*, cell, tissue, *etc.*) with the bacterium under conditions such that nucleic acid sequences contained within the bacterium are introduced into one or more cells of the target biological sample.

The term "*Agrobacterium*" refers to a soil-borne, Gram-negative, rod-shaped phytopathogenic bacterium which causes crown gall. The term "*Agrobacterium*" includes, but is not limited to, the strains *Agrobacterium tumefaciens*, (which typically causes crown gall in infected plants), and *Agrobacterium rhizogenes* (which causes hairy root disease in infected host plants). Infection of a plant cell with *Agrobacterium* generally results in the production of opines (*e.g.*, nopaline, agropine, octopine *etc.*) by the infected cell. Thus, *Agrobacterium* strains which cause production of nopaline (*e.g.*, strain LBA4301,

C58, A208) are referred to as "nopaline-type" *Agrobacteria*; *Agrobacterium* strains which cause production of octopine (*e.g.*, strain LBA4404, Ach5, B6) are referred to as "octopine-type" *Agrobacteria*; and *Agrobacterium* strains which cause production of agropine (*e.g.*, strain EHA105, EHA101, A281) are referred to as "agropine-type" *Agrobacteria*.

The terms "bombarding, "bombardment," and "biolistic bombardment" refer to the process of accelerating particles towards a target biological sample (*e.g.*, cell, tissue, *etc.*) to effect wounding of the cell membrane of a cell in the target biological sample and/or entry of the particles into the target biological sample. Methods for biolistic bombardment are known in the art (*e.g.*, US 5,584,807, the contents of which are herein incorporated by reference), and are commercially available (*e.g.*, the helium gas-driven microprojectile accelerator (PDS-1000/He) (BioRad).

The term "microwounding" when made in reference to plant tissue refers to the introduction of microscopic wounds in that tissue. Microwounding may be achieved by, for example, particle bombardment as described herein.

The "efficiency of transformation" or "frequency of transformation" as used herein can be measured by the number of transformed cells (or transgenic organisms grown from individual transformed cells) that are recovered under standard experimental conditions (i.e. standardized or normalized with respect to amount of cells contacted with foreign DNA, amount of delivered DNA, type and conditions of DNA delivery, general culture conditions etc.) For example, when isolated immature embryos are used as starting material for transformation, the frequency of transformation can be expressed as the number of transgenic plant lines obtained per 100 isolated immature embryos transformed.

DETAILED DESCRIPTION OF THE INVENTION

A first embodiment of the invention relates to a method for generating a transgenic *Zea mays* plant comprising the steps of

a. introducing into a *Zea mays* cell or tissue a DNA construct comprising
   i) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,
   ii) at least one second expression construct conferring to said *Zea mays* plant an agronomically valuable trait, and
b. incubating said *Zea mays* cell or tissue of step a) on a selection medium comprising D-alanine and/or D-serine and/or a derivative thereof in a total concentration from about 1 mM to 100 mM for a time period of at least 5 days, and
c. transferring said *Zea mays* cell or tissue of step b) to a regeneration medium and regenerating and selecting *Zea mays* plants comprising said DNA construct.

It is an important feature of the invention that ubiquitin promoter, preferably the *Zea mays* ubiquitin promoter is effective in driving the expression of both the D-alanine and/or D-serine metabolizing enzyme genes. Surprisingly use of the ubiquitin promoter results in a consistently higher transformation efficiency than for other promoters normally used in monocotyledonous plants, such as the *Zea mays ahas* promoter (U.S. Pat. No. 5,750,866) or the ScBV promoter (U.S. Patent Number 6,489,462). In com-

parison with these promoters, transformation efficiency with the ubiquitin promoter was higher (in some cases by at least 100%) and/or the applicability was broader, although these promoters are described as strong constitutive promoters in the art. The reasons for this surprisingly superior performance of the ubiquitin promoter are not known. However, it is known that optimal selection needs expression of the selection marker in the relevant cells of the target tissue (which later dedifferentiate and regenerate into the transgenic plants), at the right time and to the right concentration (high enough to ensure efficient selection but not too high to prevent potential negative effects to the cells). The superior function and the effectiveness of maize ubiquitin promoter particularly, may also indicate the need for maize transgenic cells to have sufficient quantity of the D-alanine and/or D-serine metabolizing enzyme (e.g., the DSDA or DAO proteins) that are exogenous (non-native) to maize, in order to survive the selection pressure imposed on them. These effects may be promoter and/or marker dependent, so that certain combinations of promoters and markers outperform others.

Furthermore, the ubiquitin promoter seems to have a higher flexibility for the D-amino acid metabolizing marker gene sequence. Other promoters are sometime functioning quite well with one specific marker but much less with another. The ubiquitin promoter thus can be employed as a standard promoter to drive expression of D-amino acid metabolizing enzymes in maize. By using the *Zea mays* promoters high transformation efficiencies can be achieved, which are comparable to other established selection systems such as the *ahas*-selection system.

The markers utilized herein after sequences from bacteria or yeast, which are commonly found in human and animal food or feed. In a preferred embodiment the markers and method provided herein allow for easy removal of the marker sequence. Furthermore, a detailed optimized transformation protocol for maize is provided herein which allows for efficient transformation on an industrial scale. The plants obtained by the method of the invention were fertile, and phenotypically normal. Transformation worked well in both hybrid and inbred lines. We demonstrated the ability to stack selection markers dsdA (primary) and dao1 (secondary), or *ahas* (primary) and dsdA (secondary), indicating the compatibility the *ahas* system with D-amino acid selection system.

1. The DNA construct of the invention

Another embodiment of the invention relates to a DNA construct comprising

i) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,

ii) at least one second expression construct conferring to said *Zea mays* plant an agronomically valuable trait.

The ubiquitin promoter and/or the enzyme capable to metabolize D-alanine or D-serine are defined below in detail.

1.1 The first expression construct of the invention

One embodiment of the invention relates to a recombinant expression construct comprising a promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, wherein said promoter is heterologous in relation to said enzyme encoding sequence.

The ubiquitin promoter and/or the enzyme capable to metabolize D-alanine or D-serine are defined below in detail.

1.1 .1 The enzyme capable to metabolize D-alanine or D-serine

The person skilled in the art is aware of numerous sequences suitable to metabolize D-alanine and/or D-serine. The term "enzyme capable to metabolize D-alanine or D-serine" means preferably an enzyme, which converts and/or metabolizes D-alanine and/or D-serine with an activity that is at least two times (at least 100% higher), preferably at least three times, more preferably at least five times, even more preferably at least 10 times, most preferably at least 50 times or 100 times the activity for the conversion of the corresponding L-amino acid (i.e., D-alanine and/or D-serine) and – more preferably – also of any other D- and/or L- or achiral amino acid.

Preferably, the enzyme capable to metabolize D-alanine or D-serine is selected from the group consisting of D-serine ammonia-lyase (D-Serine dehydratases; EC 4.3.1.18; formerly EC 4. 2.1.14), D-Amino acid oxidases (EC 1.4.3.3), and D-Alanine transaminases (EC 2.6.1.21). More preferably, the enzyme capable to metabolize D-alanine or D-serine is selected from the group consisting of D-serine ammonia-lyase (D-Serine dehydratases; EC 4.3.1.18; formerly EC 4. 2.1.14), and D-Amino acid oxidases (EC 1.4.3.3).

The term " D-serine ammonia-lyase" (D-Serine dehydratases; EC 4.3.1.18; formerly EC 4. 2.1.14) means enzymes catalyzing the conversion of D-serine to pyruvate and ammonia. The reaction catalyzed probably involves initial elimination of water (hence the enzyme's original classification as EC 4.2.1.14), followed by isomerization and hydrolysis of the product with C-N bond breakage. For examples of suitable enzyme see http://www.expasy.org/enzyme/4.3.1.18.

The term "D-Alanine transaminases" (EC 2.6.1.21).means enzymes catalyzing the reaction of D-Alanine with 2-oxoglutarate to pyruvate and D-glutamate. D-glutamate is much less toxic to plants than D-Alanine. http://www.expasy.org/enzyme/2.6.1.21.

The term D-amino acid oxidase (EC 1.4.3.3; abbreviated DAAO, DAMOX, or DAO) is referring to the enzyme converting a D-amino acid into a 2-oxo acid, by - preferably - employing Oxygen (O_2) as a substrate and producing hydrogen peroxide (H_2O_2) as a co-product (Dixon 1965a,b,c; Massey 1961; Meister 1963). DAAO can be described by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) with the EC (Enzyme Commission) number EC 1.4.3.3. Generally an DAAO enzyme of the EC 1.4.3.3. class is an FAD flavoenzyme that catalyzes the oxidation of neutral and basic D-amino acids into their corresponding keto acids. DAAOs have been characterized and sequenced in fungi and vertebrates where they are known to be located in the peroxisomes. In DAAO, a conserved histidine has been shown (Miyano 1991) to be important for the enzyme's catalytic activity. In a preferred embodiment of the invention a DAAO is referring to a protein comprising the following consensus motive:

[LIVM]-[LIVM]-H*-[NHA]-Y-G-x-[GSA]-[GSA]-x-G-x_5-G-x-A

wherein amino acid residues given in brackets represent alternative residues for the

respective position, x represents any amino acid residue, and indices numbers indicate the respective number of consecutive amino acid residues. The abbreviation for the individual amino acid residues have their standard IUPAC meaning as defined above. D-Amino acid oxidase (EC-number 1.4.3.3) can be isolated from various organisms, including but not limited to pig, human, rat, yeast, bacteria or fungi. Example organisms are Candida tropicalis, Trigonopsis variabilis, Neurospora crassa, Chlorella vulgaris, and Rhodotorula gracilis. A suitable D-amino acid metabolising polypeptide may be an eukaryotic enzyme, for example from a yeast (e.g. *Rhodotorula gracilis),* fungus, or animal or it may be a prokaryotic enzyme, for example, from a bacterium such as *Escherichia coli.* For examples of suitable enzyme see http://www.expasy.org/enzyme/1.4.3.3.

Examples of suitable polypeptides which metabolise D-amino acids are shown in Table 1. The nucleic acid sequences encoding said enzymes are available form databases (e.g., under Genbank Acc.-No. U60066, A56901, AF003339, Z71657, AF003340, U63139, D00809, Z50019, NC_003421, AL939129, AB042032). As demonstrated above, DAAO from several different species have been characterized and shown to differ slightly in substrate affinities (Gabler 2000), but in general they display broad substrate specificity, oxidatively deaminating all D-amino acids.

Table 1: Enzymes suitable for metabolizing D-serine and/or D-alanine. Especially preferred enzymes are presented in bold letters

Enzyme	EC number	Example	Source organism	Substrate
D-Serine dehydratase (D-Serine ammonia lyase, D-Serine deaminiase)	EC 4.3.1.18 (originally EC 4.2.1.14)	P54555	Bacillus subtilis	**D-Ser** D-Thr D-allothreonine
		P00926	**Escherichia coli. DSDA**	
		Q9KL72	Vibrio cholera. VCA0875	
		Q9KC12	Bacillus halodurans.	
D-Amino acid oxidase	EC 1.4.3.3	JX0152	Fusarium solani	Most D-amino acid
		O01739	Caenorhabditis elegans.	
		O33145	Mycobacterium leprae. AAO.	
		O35078	Rattus norvegicus (Rat)	
		O45307	Caenorhabditis elegans	
		P00371	Sus scrofa (Pig)	
		P14920	Homo sapiens (Human)	
		P14920	Homo sapiens (Human)	
		P18894	Mus musculus (Mouse)	
		P22942	Oryctolagus cuniculus (Rabbit)	
		P24552	Fusarium solani (subsp. pisi) (Nectria haematococca)	
		P80324	**Rhodosporidium toruloides (Yeast) (Rhodotorula gracilis)**	
		Q19564	Caenorhabditis elegans	
		Q28382	Sus scrofa (pig)	
		Q7SFW4	Neurospora crassa	
		Q7Z312	Homo sapiens (Human)	
		Q82MI8	Streptomyces avermitilis	
		Q8P4M9	Xanthomonas campestris	
		Q8PG95	Xanthomonas axonopodis	
		Q8R2R2	Mus musculus (Mouse)	
		Q8SZN5	Drosophila melanogaster (Fruit fly)	
		Q8VCW7	Mus musculus (Mouse)	
		Q921M5	Cavia parcellus (Guinea pig)	
		Q95XG9	Caenorhabditis elegans	

Enzyme	EC number	Example	Source organism	Substrate
D-Amino acid oxidase	EC 1.4.3.3	Q99042	Trigonopsis variabilis	
		Q9C1L2	Neurospora crassa	
		Q9JXF8	Neisseria meningitidis (sero-group B) NMB2068	
		Q9V5P1	Drosophila melanogaster (Fruit fly)	
		Q9VM80	Drosophila melanogaster (Fruit fly)	
		Q9X7P6	Streptomyces coelicolor	
		Q9Y7N4	Schizosaccharomyces pombe (Fission yeast) SPCC1450	
		Q9Z1M5	Cavia porcellus (Guinea pig)	
		Q9Z302	Cricetulus griseus	
		U60066	**Rhodosporidium toruloides, (Rhodotorula gracilis) strain TCC 26217**	
D-Alanine transaminase	EC-number 2.6.1.21	P54692	Bacillus licheniformis	**D-Ala**
		P54693	Bacillus sphaericus	D-Arg
		P19938	Bacillus sp. (strain YM-1)	D-Asp
		007597	Bacillus subtilis	D-Glu
		085046	Listeria monocytogenes	D-Leu
		P54694	Staphylococcus haemolyticus	D-Lys
				D-Met
				D-Phe
				D-Norvaline

Especially preferred in this context are the *dao*1 gene (EC: 1.4. 3.3 : GenBank Acc.-No.: U60066) from the yeast *Rhodotorula gracilis* (*Rhodosporidium toruloides*) and the *E. coli* gene *dsd*A (D-serine dehydratase (D-serine deaminase) [EC: 4.3. 1.18; GenBank Acc.-No.: J01603). The dao1 gene is of special advantage since it can be employed as a dual function marker (see international patent application PCT/EP 2005/002734).

Suitable D-amino acid metabolizing enzymes also include 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 the D-amino acid metabolizing enzyme as defined above. Changes to a sequence, to produce a mutant, variant or derivative, may be by one or more of 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 difference to the encoded amino acid sequence are included.

More preferably for the method of the invention, the enzyme capable to metabolize D-serine is selected from the group consisting of

i) the *E.coli* D-serine ammonia-lyase as encoded by SEQ ID NO: 2, and

ii) enzymes having the same enzymatic activity and an identity of at least 80% (preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, most preferably at least 98%) to the sequence as encoded by SEQ ID NO: 2, and

iii) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence described by SEQ ID NO: 1,

and wherein selection is done on a medium comprising D-serine in a concentration from about 1 mM to 100 mM (more preferably from about 5 mM to about 50 mM, even more preferably from about 7 mM to about 30 mM, most preferably about 10 to 20 mM).

"Same activity" in the context of a D-serine ammonia-lyase means the capability to metabolize D-serine, preferably as the most preferred substrate. Metabolization means the lyase reaction specified above. Hybridization under iii) means preferably hybridization under low stringency conditions (with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X to 2X SSC at 50 to 55°C), more preferably moderate stringency conditions (in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5 X to 1 X SSC at 55 to 60°C), and most preferably under very stringent conditions (in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1 x SSC at 60 to 65°C).

Also more preferably for the method of the invention, the enzyme capable to metabolize D-serine and D-alanine is selected from the group consisting of

i) the *Rhodotorula gracilis* D-amino acid oxidase as encoded by SEQ ID NO: 4, and

ii) enzymes having the same enzymatic activity and an identity of at least 80% (preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, most preferably at least 98%) to the sequence as encoded by SEQ ID NO: 4, and

iii) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence described by SEQ ID NO: 3,

and wherein selection is done on a medium comprising D-alanine and/or D-serine in a total concentration from about 1 mM to 100 mM (more preferably from about 2 mM to about 50 mM, even more preferably from about 3 mM to about 20 mM, most preferably about 5 to 15 mM).

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 comprise the metabolizing activity regarding D-serine and or D-alanine. Such sequences and proteins also encompass, sequences and protein derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different coding sequences can be manipulated to create a new polypeptide possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined in vitro or in vivo. Polynucleotides encoding a candidate enzyme can, for example, be modulated with DNA shuffling protocols. DNA shuffling is a method to rapidly, easily and efficiently introduce mutations or rearrangements, preferably randomly, in a DNA molecule or to generate exchanges of DNA sequences between two or more DNA molecules, preferably randomly. The DNA molecule resulting from DNA shuffling is a shuffled DNA molecule that is a non-naturally occurring DNA molecule derived from at least one template DNA molecule. The shuffled DNA encodes an enzyme modified with respect to the enzyme encoded by the template DNA, and preferably has an altered

biological activity with respect to the enzyme encoded by the template DNA. DNA shuffling can be based on a process of recursive recombination and mutation, performed by random fragmentation of a pool of related genes, followed by reassembly of the fragments by a polymerase chain reaction-like process. See, e.g., Stemmer 1994 a,b; Crameri 1997; Moore 1997; Zhang 1997; Crameri 1998; US 5,605,793, US 5,837,458, US 5,830,721 and US 5, 811,238. The resulting dsdA- or dao-like enzyme encoded by the shuffled DNA may possess different amino acid sequences from the original version of enzyme. Exemplary ranges for sequence identity are specified above.

"Same activity" in the context of a D-amino acid oxidase means the capability to metabolize a broad spectrum of D-amino acids (preferably at least D-serine and/or D-alanine). Metabolization means the oxidase reaction specified above. Hybridization under iii) means preferably hybridization under low stringency conditions (with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X to 2X SSC at 50 to 55°C), more preferably moderate stringency conditions (in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5 X to 1 X SSC at 55 to 60°C), and most preferably under very stringent conditions (in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1 x SSC at 60 to 65°C).

Preferably, concentrations and times for the selection are specified in detail below. Preferably the selection is done using about 3 to about 15 mM D-alanine or about 7 to about 30 mM D-serine. The total selection time under dedifferentiating conditions is preferably from about 3 to 4 weeks.

The D-amino acid metabolizing enzyme of the invention may be expressed in the cytosol, peroxisome, or other intracellular compartment of the plant cell. Compartmentalisation of the D-amino acid metabolizing enzyme may be achieved by fusing the nucleic acid sequence encoding the DAAO polypeptide to a sequence encoding a transit peptide to generate a fusion protein. Gene products expressed without such transit peptides generally accumulate in the cytosol.

1.1.2 The ubiquitin promoter

The D-alanine and/or D-serine metabolizing enzyme is coupled to a ubiquitin promoter, preferably a plant ubiquitin promoter, more preferably a monocotyledonous plant ubiquitin promoter, even more preferably a *Zea mays* ubiquitin promoter.

The term "ubiquitin promoter" as used herein means the region of genomic DNA up to 5000 base pairs (bp) upstream from either the start codon, or a mapped transcriptional start site, of a ubiquitin, or ubiquitin-like, gene. Ubiquitin is an abundant 76 amino acid polypeptide found in all eukaryotic cells. There are several different genes that encode ubiquitin and their homology at the amino acid level is quite high. For example, human and mouse have many different genes encoding ubiquitin, each located at a different chromosomal locus. Functionally, all ubiquitin genes are critical players in the ubiquitin-dependent proteolytic machinery of the cell. Each ubiquitin gene is associated with a promoter that drives its expression. A ubiquitin promoter is the region of genomic DNA up to 5,000 bp upstream from either the start codon, or a mapped transcriptional start site, of a ubiquitin, or ubiquitin-like, gene.

The term "plant ubiquitin regulatory system" refers to the approximately 2 kb nucleotide sequence 5' to the translation start site of a plant (preferably the maize) ubiquitin gene and comprises sequences that direct initiation of transcription, regulation of transcription, control of expression level, induction of stress genes and enhancement of expression in response to stress. The regulatory system, comprising both promoter and regulatory functions, is the DNA sequence providing regulatory control or modulation of gene expression.

Various plant ubiquitin genes and their promoters are described (Callis 1989, 1990). Described are promoters from dicotyledonous plants, such as for potato (Garbarino 1992), tobacco (Genschick 1994), tomato (Hoffman 1991), parsely (Kawalleck 1993; WO03/102198, herein incorporated by reference), Arabidopsis (Callis 1990; Holtorf 1995;UBQ8, GenBank Acc.- No: NM_111814; UBQ1, GenBank Acc.- No: NM_115119; UBQ5, GenBank Acc.- No: NM_116090).

Accordingly the ubiquitin promoter of the invention is a DNA fragment (preferably approximately 2 kb in length), said DNA fragment comprising a plant ubiquitin regulatory system, wherein said regulatory system contains a promoter comprising a transcription start site, and - preferably - one or more heat shock elements positioned 5' to said transcription start site, and – preferably- an intron positioned 3' to said transcription start site, wherein said regulatory system is capable of regulating expression in maize. Preferably the expression is a constitutive and inducible gene expression such that the level of said constitutive gene expression in monocots is about one-third that obtained in said inducible gene expression in monocots.

Preferred are ubiquitin promoters from monocotyledonous plants. Such promoters are described for maize (Christensen 1992, 1996) Transgenic Res 5:213-218), rice (RUBQ1, RUBQ2, RUBQ3, and RUBQ4; promoters from RUBQ1 and RUBQ2 are suitable for constitutive expression; US 6,528,701).

Most preferred is the ubiquitin promoter from Zea mays as described in U.S. Pat. Nos. 5,614,399, 5,510,474, 6,020,190, 6,054,574, and 6,068,994. The promoter regulates expression of a maize polyubiquitin gene containing 7 tandem repeats. Expression of this maize ubiquitin gene was constitutive at 25° C, and was induced by heat shock at 42°C. The promoter was successfully used in several monocot plants (Christensen 1996). In the maize ubil promoter region, a TATA box was found at position of -30, and two overlapping heat shock sequences, 5'-CTGGTCCCCTCCGA-3' and CTCGA-GATTCCGCT-3', were found at positions -214 and -204. The canonical CCAAT and the GC boxes were not found in the promoter region, but the sequence 5-CACGGCA-3' (function unknown) occurred four times, at positions -236, -122, -96, and -91 of the promoter region (Christensen 1992). Promoters and their expression pattern are described for Ubi-1 and Ubi-2 of Zea mays (US 6,054574; Christensen 1992).

More preferably the ubiquitin promoter is selected from the group consisting of

a) sequences comprising the sequence as described by SEQ ID NO: 5, and

b) sequences comprising at least one fragment of at least 50 (preferably at least 100, more preferably at least 250, even more preferably at least 500, most preferably at

least 1000) consecutive base pairs of the sequence as described by SEQ ID NO: 5, and having promoter activity in *Zea mays*,

c) sequences comprising a sequence having at least 60% (preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95%) identity to the sequence as described by SEQ ID NO: 5, and having promoter activity in *Zea mays*,

d) sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 5, and having promoter activity in *Zea mays*.

"Promoter activity" in Zea mays means the capability to realized transcription of an operably linked nucleic acid sequence in at least one cell or tissue of a *Zea mays* plant or derived from a *Zea mays* plant. Preferably it means a constitutive transcription activity allowing for expression in most tissues and most developmental stages. The heat shock element related activity of the *Zea mays* ubiquitin promoter may be present but is not required.

Hybridization under d) means preferably hybridization under low stringency conditions (with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X to 2X SSC at 50 to 55°C), more preferably moderate stringency conditions (in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5 X to 1 X SSC at 55 to 60°C), and most preferably under very stringent conditions (in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1 x SSC at 60 to 65°C).

The sequence described by SEQ ID NO: 5 is the core promoter of the *Zea mays* ubiquitin promoter. In one preferred embodiment not only the promoter region is employed as a transcription regulating sequence but also a 5'-untranslated region and/or an intron. The ubiquitin promoter is preferably employed in combination with an intron, more preferably with an expression enhancing intron. Such an intron can be the natural intron 1 of the ubil gene (MubG1 contains a 1004-base pair (bp) intron in its 5' untranslated region; Liu 1995). More preferably the ubiquitin promoter system is characterized by a length of approximately 2 kb, further comprising, in the following order beginning with the 5' most element and proceeding toward the 3' terminus of said DNA fragment:
(a) one or more heat shock elements, which elements may or may not be overlapping;
(b) a promoter comprising a transcription start site; and
(c) an intron of about 1 kb in length.

More preferably the region spanning the promoter, the 5'-untranslated region and the first intron of the Zea mays ubiquitin gene are used, even more preferably the region described by SEQ ID NO: 6. Accordingly in another preferred embodiment the ubiquitin promoter utilized in the method of the invention is selected from the group consisting of

a) sequences comprising the sequence as described by SEQ ID NO: 6, and

b) sequences comprising at least one fragment of at least 50 (preferably at least 100, more preferably at least 250, even more preferably at least 500, most preferably at least 1000) consecutive base pairs of the sequence as described by SEQ ID NO: 6, and having promoter activity in *Zea mays*,

c) sequences comprising a sequence having at least 60% (preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95%) identity to the sequence as described by SEQ ID NO: 6, and having promoter activity in *Zea mays*,

d) sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 6, and having promoter activity in *Zea mays*.

Hybridization under d) means preferably hybridization under low stringency conditions (with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X to 2X SSC at 50 to 55°C), more preferably moderate stringency conditions (in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5 X to 1 X SSC at 55 to 60°C), and most preferably under very stringent conditions (in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1 x SSC at 60 to 65°C).

Accordingly the ubiquitin promoter utilized of the invention may also be a fragment of the promoter described by SEQ ID NO: 5 or 6 or a derivative thereof. Fragments may include truncated versions of the promoter as described by SEQ ID NO: 5 or 6, wherein un-essential sequences have been removed. Shortened promoter sequences are of high advantage since they are easier to handle and sometime optimized in their gene expression profile. One efficient, targeted means for preparing shortened or truncated promoters relies upon the identification of putative regulatory elements within the promoter sequence. This can be initiated by comparison with promoter sequences known to be expressed in similar tissue-specific or developmentally unique manner. Sequences, which are shared among promoters with similar expression patterns, are likely candidates for the binding of transcription factors and are thus likely elements that confer expression patterns. Confirmation of these putative regulatory elements can be achieved by deletion analysis of each putative regulatory region followed by functional analysis of each deletion construct by assay of a reporter gene, which is functionally attached to each construct. As such, once a starting promoter sequence is provided, any of a number of different deletion mutants of the starting promoter could be readily prepared.

Functionally equivalent fragments of an ubiquitin promoter (e.g., as described by SEQ ID NO: 5 or 6) can also be obtained by removing or deleting non-essential sequences without deleting the essential one. Narrowing the transcription regulating nucleotide sequence to its essential, transcription mediating elements can be realized in vitro by trial-and-arrow deletion mutations, or *in silico* using promoter element search routines. Regions essential for promoter activity often demonstrate clusters of certain, known promoter elements. Such analysis can be performed using available computer algorithms such as PLACE ("Plant Cis-acting Regulatory DNA Elements"; Higo 1999), the BIOBASE database "Transfac" (Biologische Datenbanken GmbH, Braunschweig; Wingender 2001) or the database PlantCARE (Lescot 2002). Preferably, functional equivalent fragments of one of the transcription regulating nucleotide sequences of the invention comprises at least 100 base pairs, preferably, at least 200 base pairs, more preferably at least 500 base pairs of a transcription regulating nucleotide sequence as described by SEQ ID NO: 5 or 6. More preferably this fragment is starting from the 3'-end of the indicated sequences.

Especially preferred are equivalent fragments of transcription regulating nucleotide sequences, which are obtained by deleting the region encoding the 5'-untranslated region of the mRNA, thus only providing the (untranscribed) promoter region. The 5'-untranslated region can be easily determined by methods known in the art (such as 5'-RACE analysis). Thus, the core promoter region as described by SEQ ID NO: 5 is a fragment of the sequence described by SEQ ID NO: 6, which still comprises the 5'-untranslated region and the intron.

Derivatives may include for example also modified Zea mays promoter sequences, which – for example - do not include two overlapping heat shock elements. Such sequences are for example described in US Pat. Appl. 20030066108 (W0 01/18220).

1.1.3 Additional elements

The expression cassettes of the invention (or the vectors in which these are comprised) may comprise further functional elements and genetic control sequences in addition to the ubiquitin promoter. 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 expression cassette according to the invention. For example, genetic control sequences modify the transcription and translation. Genetic control sequences are described (e.g., Goeddel 1990; Gruber 1993 and the references cited therein).

Preferably, the expression cassettes according to the invention encompass a ubiquitin promoter functional in plants 5'-upstream of the nucleic acid sequence (e.g., encoding the D-amino acid metabolizing enzyme), and 3'-downstream a terminator sequence and polyadenylation signals and, if appropriate, further customary regulatory elements, in each case linked operably to the nucleic acid sequence to be expressed.

Genetic control sequences and functional elements furthermore also encompass the 5'-untranslated regions, introns or noncoding 3'-region of genes, such as, for example, the actin-1 intron, or the Adh1-S introns 1, 2 and 6 (general reference: The Maize Handbook, Chapter 116, Freeling and Walbot, Eds., Springer, New York (1994)). It has been demonstrated that they may play a significant role in the regulation of gene expression. Thus, it has been demonstrated that 5'-untranslated sequences can enhance the transient expression of heterologous genes. Examples of translation enhancers which may be mentioned are the tobacco mosaic virus 5' leader sequence (Gallie 1987) and the like. Furthermore, they may promote tissue specificity (Rouster 1998).

Polyadenylation signals which are suitable as genetic control sequences are plant polyadenylation signals, preferably those which correspond essentially to T-DNA polyadenylation signals from *Agrobacterium tumefaciens*. Examples of particularly suitable terminator sequences are the OCS (octopine synthase) terminator and the NOS (nopaline synthase) terminator.

Functional elements which may be comprised in a vector of the invention include

i) Origins of replication which ensure replication of the expression cassettes or vectors 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 (Sam-

brook 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,

iii) Sequences which make possible homologous recombination, marker deletion, or insertion into the genome of a host organism. Methods based on the cre/lox (Sauer 1998; Odell 1990; Dale 1991), FLP/FRT (Lysnik 1993), or Ac/Ds system (Wader 1987; US 5,225,341; Baker 1987; Lawson 1994) permit a - if appropriate tissue-specific and/or inducible - removal of a specific DNA sequence from the genome of the host organism. Control sequences may in this context mean the specific flanking sequences (e.g., lox sequences), which later allow removal (e.g., by means of cre recombinase) (see also see international patent application PCT/EP 2005/002734),

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.

1.2. The second expression cassette

Preferably, the DNA construct inserted into the genome of the target plant comprises at least one second expression cassette, which confers to the Zea mays plant an agronomically relevant 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 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; Dunwell 2000), 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. Advantageous properties may be conferred either by overexpressing proteins or by decreasing expression of endogenous proteins by e.g., expressing a corresponding antisense (Sheehy 1988; US 4,801,340; Mol 1990) or double-stranded RNA (Matzke 2000; Fire 1998; Waterhouse 1998; WO 99/32619; WO 99/53050; WO 00/68374; WO 00/44914; WO 00/44895; WO 00/49035; WO 00/63364).

For expression of these sequences all promoters suitable for expression of genes in maize can be employed. Preferably, said second expression construct is not comprising a promoter which is identical to the promoter used to express the D-amino acid metabolizing enzyme. Expression can be, for example, constitutive, inducible or development-dependent. Various promoters are known for expression in monocots like maize, such as the rice actin promoter (McElroy 1990), maize H3 histone promoter (Lepetit 1992; Atanassova 1992), the promoter of a proline-rich protein from wheat (WO 91/13991). Promoters which are furthermore preferred are those which permit a seed-specific expression in monocots such as the promoters described in WO 99/16890 (promoters of the hordein gene, the glutelin gene, the oryzin gene, the prolamin gene, the gliadin gene, the glutelin gene, the zein gene, the casirin gene or the secalin gene).

2. The transformation and selection method of the invention

2.1 Source and preparation of the plant material

Various plant material can be employed for the transformation procedure disclosed herein. Such plant material may include but is not limited to for example leaf, root or stalk sections, immature embryos, pollen, but also callus, protoplasts or suspensions of plant cells. Preferably, the plant material is an immature embryo. 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 immature embryo) can be obtained or isolated from virtually any *Zea mays* variety or plant. Preferably the *Zea mays* plant utilized as a source for the transformation material is from the group consisting of in-breds, hybrids, F1 between (preferably different) inbred lines, F1 between an inbred and a hybrid, F1 between an inbred and a naturally-pollinated variety, commercial F1 varieties, any F2 crossing or self-pollination between the before mentioned varieties and the progeny of any of the before mentioned. All combinations of male and female parents for the before mentioned lines and crossings are included.

Suitable *Zea mays* varieties include but are not limited to P3732, A188, H84, B37Ht, Mo17Ht, W117Ht, Oh43, H99, W64A Ht rhm, F1 (A188 x Black Mexican Sweet), F1 (A188 x B73Ht), F1 (B73Ht x A188), F1 (H84 x A188), F1 (Mo17Ht x A188) and F1 (C103 x A188). Such varieties are available as seeds from deposits such as American Type Culture Collection (ATCC) and other deposits for seed material known in the art. More preferably, the immature embryo is isolated from a cross of a F1 or F2 (HiIIA x A188) plants with an inbred-line. F1 seeds of corn genotype HiIIAxA188 can be preferably produced by crossing HiIIA (female parent) with inbred line A188 (male), and planted in the greenhouse as pollen donor. F2 seeds of (HiIIAxA188) are produced by self-pollination of F1 (HiIIAxA188) plants either in the greenhouse or in the field, and planted in the greenhouse as the pollen donor.

Most preferred as inbred lines for the crossing with a F1 or F2 (HiIIA x A188) plants are lines selected from group of lines selected from the group of which representative seed having been deposited under the Budapest Treaty with the American Type Culture Collection (Manasses, VA 20110-2209, USA) under the Patent Deposit Designation PTA-6170 (for seeds of line BPS553), and PTA-6171 (for seeds of line BPS631).

Hybrid plants (or callus, tissue, immature embryos or other plant material thereof) of BPS553x(HiIIAxA188) or BPS631x(HiIIAxA188) are preferably produced using inbred line BPS553 or BPS631 as the female parents, and either F1 or F2 (HiIIAxA188) plants as the male parent in the greenhouse. These hybrid immature embryos have demonstrated extraordinary high transformability in comparison with (HiIIA x A188) immature embryos alone, known in the art as one of the best transformable *Zea mays* material (Ishida et al. 1996, Frame et al. 2002). The transformability of a hybrid immature embryo from a cross between a (HiIIA x A188) hybrid the BPS553 lines is at least twice the efficiency as for a (HiIIA x A188) embryo (for a comparison see Example 7 below). In consequence the above mentioned crosses are superior material for Zea mays transformation. Said inbred-lines have been deposited under the Budapest Treaty with the American Type Culture Collection (Manasses, VA 20110-2209, USA) under the

Patent Deposit Designation PTA-6170 (for seeds of line BPS553), and PTA-6171 (for seeds of line BPS631).

Other objects of the invention relate to descendants of said maize plant (such as for example inbred lines), inbreds or hybrid plants produced from said descendants, and parts of the before mentioned plants. Such parts may include but are not limited to tissue, cells, pollen, ovule, roots, leaves, seeds, microspores, and vegetative parts.

Zea mays plants for isolation of immature embryos are grown and pollinated as known in the art, preferably as described below in the examples.

In one preferred embodiment of the invention the method is comprising the following steps

a. isolating an immature embryo of a *Zea mays* plant, and
b. co-cultivating said isolated immature embryo, which has not been subjected to a dedifferentiation treatment, with a bacterium belonging to genus *Rhizobiaceae* comprising at least one transgenic T-DNA, said T-DNA comprising
   i) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,
   ii) at least one second expression construct conferring to said *Zea mays* plant an agronomically valuable trait, and
c. transferring the co-cultivated immature embryos to a recovering medium, said recovery medium lacking a phytotoxic effective amount of D-serine or D-alanine, and
d. inducing formation of embryogenic callus and selecting transgenic callus on a medium comprising,
   i. an effective amount of at least one auxin compound, and
   ii. D-alanine and/or D-serine in a total concentration from about 1 mM to 100 mM , and
e. regenerating and selecting plants containing the transgenic T-DNA from the said transgenic callus.

The term "immature embryo" as used herein means the embryo of an immature seed which is in the stage of early development and maturation after pollination. The developmental stage of the immature embryos to be treated by the method of the present invention are not restricted and the collected embryos may be in any stage after pollination. Preferred embryos are those collected on not less than 2 days after their fertilization. Also preferred are scutella of immature embryos capable of inducing dedifferentiated calli having an ability to regenerate normal plants after having been transformed by the method mentioned below.

In a preferred embodiment the immature embryo is one in the stage of not less than 2 days after pollination. More preferably, immature embryos are isolated from ears from corn plants (preferably the first ear that comes out) harvested 7 to 14 days (preferably 8 to 11 days) after pollination (DAP). Exact timing of harvest varies depending on growth conditions and maize variety. The size of immature embryos is a good indication of their stage of development. The optimal length of immature embryos for transformation is about 1 to 1.6 mm, including the length of the scutellum. The embryo should be translucent, not opaque.

In a preferred embodiment of the invention, the immature embryos are isolated and directly placed on the surface a solidified co-cultivation medium without additional washing steps. While the methods described in the art all include several preparation and washing steps all these are omitted in said improvement saving significant time and costs. With the present invention, the *Agrobacterium* infection step takes place on the co-cultivation medium, instead of in a tube containing *Agrobacterium* suspension cells, known to the art.

Preferably, the immature embryo is subjected to transformation (co-cultivation) without dedifferentiating pretreatment. Treatment of the immature embryos with a cell wall degrading enzyme or injuring (e.g., cutting with scalpels or perforation with needles) is optional. However, this degradation or injury step is not necessary and is omitted in a preferred embodiment of the invention.

The term "dedifferentiation", "dedifferentiation treatment" or "dedifferentiation pretreatment" means a process of obtaining cell clusters, such as callus, that show unorganized growth by culturing differentiated cells of plant tissues on a dedifferentiation medium. More specifically, the term "dedifferentiation" as used herein is intended to mean the process of formation of rapidly dividing cells without particular function in the scope of the plant body. These cells often possess an increased potency with regard to its ability to develop into various plant tissues. Preferably the term is intended to mean the reversion of a differentiated or specialized tissues to a more pluripotent or totipotent (e.g., embryonic) form. Dedifferentiation may lead to reprogramming of a plant tissue (revert first to undifferentiated, non-specialized cells. then to new and different paths). The term "totipotency" as used herein is intended to mean a plant cell containing all the genetic and/or cellular information required to form an entire plant. Dedifferentiation can be initiated by certain plant growth regulators (e.g., auxin and/or cytokinin compounds), especially by certain combinations and/or concentrations thereof.

2.2 Transformation Procedures

2.2.1 General Techniques

A DNA construct according to the invention may advantageously be introduced into cells using vectors into which said DNA construct is inserted. Examples of vectors may be plasmids, cosmids, phages, viruses, retroviruses or *Agrobacteria*. In an advantageous embodiment, the expression cassette is introduced by means of plasmid vectors. Preferred vectors are those, which enable the stable integration of the expression cassette into the host genome.

The DNA construct can be introduced into the target plant cells and/or organisms by any of the several means known to those of skill in the art, a procedure which is termed transformation (see also Keown 1990). Various transformation procedures suitable for *Zea mays* have been described.

For example, the DNA constructs can be introduced directly to plant cells using ballistic methods, such as DNA particle bombardment, or the DNA construct can be introduced using techniques such as electroporation and microinjection of a cell. Particle-mediated transformation techniques (also known as "biolistics") are described in, e.g., EP-A1

270,356; US 5,100,792, EP-A-444 882, EP-A-434 616; Klein 1987; Vasil 1993; and Becker 1994). These methods involve penetration of cells by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface. The biolistic PDS-1000 Gene Gun (Biorad, Hercules, CA) uses helium pressure to accelerate DNA-coated gold or tungsten microcarriers toward target cells. The process is applicable to a wide range of tissues and cells from organisms, including plants. Other transformation methods are also known to those of skill in the art.

Other techniques include microinjection (WO 92/09696, WO 94/00583, EP-A 331 083, EP-A 175 966, Green 1987), polyethylene glycol (PEG) mediated transformation (Paszkowski 1984; Lazzeri 1995), liposome-based gene delivery (WO 93/24640; Freeman 1984), electroporation (EP-A 290 395, WO 87/06614; Fromm 1985; Shimamoto 1992).

In the case of injection or electroporation of DNA into plant cells, the DNA construct to be transformed not need to meet any particular requirement (in fact the „naked" expression cassettes can be utilized). Simple plasmids such as those of the pUC series may be used.

In addition and preferred to these "direct" transformation techniques, transformation can also be carried out by bacterial infection by means of soil born bacteria such as *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. These strains contain a plasmid (Ti or Ri plasmid). Part of this plasmid, termed T-DNA (transferred DNA), is transferred to the plant following Agrobacterium infection and integrated into the genome of the plant cell. Although originally developed for dicotyledonous plants, *Agrobacterium* mediated transformation is employed for transformation methods of monocots (Hiei 1994). Transformation is described e.g., for rice, maize, wheat, oat, and barley (reviewed in Shimamoto1994; Vasil et al. 1992; Vain 1995; Vasil 1996; Wan & Lemaux 1994).

For Agrobacterium-mediated transformation of plants, the DNA construct of the invention may be combined with suitable T-DNA flanking regions and introduced into a conventional Agrobacterium tumefaciens host vector. The virulence functions of the A. tumefaciens host will direct the insertion of a transgene and adjacent marker gene(s) (if present) into the plant cell DNA when the cell is infected by the bacteria. Thus, the DNA construct of the invention is preferably integrated into specific plasmids suitable for Agrobacterium mediated transformation, either into a shuttle, or intermediate, vector or into a binary vector). If, for example, a Ti or Ri plasmid is to be used for the transformation, at least the right border, but in most cases the right and the left border, of the Ti or Ri plasmid T-DNA is linked with the expression cassette to be introduced as a flanking region. Binary vectors, capable of replication both in E. coli and in Agrobacterium, are preferably used. They can be transformed directly into Agrobacterium (Holsters 1978).

2.2.2 Agrobacterium mediated transformation (co-cultivation)

The soil-borne bacterium employed for transfer of an T-DNA into the immature embryo can be any specie of the *Rhizobiaceae* family. The *Rhizobiaceae* family comprises the genera *Agrobacterium*, *Rhizobium*, *Sinorhizobium,* and *Allorhizobium* are genera within

the bacterial family and has been included in the alpha-2 subclass of Proteobacteria on the basis of ribosomal characteristics. Members of this family are aerobic, Gram-negative. The cells are normally rod-shaped (0.6-1.0 µm by 1.5-3.0 µm), occur singly or in pairs, without endospore, and are motile by one to six peritrichous flagella. Considerable extracellular polysaccharide slime is usually produced during growth on carbohydrate-containing media. Especially preferred are *Rhizobiaceae* such as *Sinorhizobium meliloti, Sinorhizobium medicae, Sinorhizobium fredii, Rhizobium sp.* NGR234, *Rhizobium sp.* BR816, *Rhizobium sp.* N33, *Rhizobium sp.* GRH2, *Sinorhizobium saheli, Sinorhizobium terangae, Rhizobium leguminosarum biovar trifolii, Rhizobium leguminosarum biovar viciae, Rhizobium leguminosarum biovar phaseoli, Rhizobium tropici, Rhizobium etli, Rhizobium galegae, Rhizobium gallicum, Rhizobium giardinii, Rhizobium hainanense, Rhizobium mongolense, Rhizobium lupini, Mesorhizobium loti, Mesorhizobium huakuii, Mesorhizobium ciceri, Mesorhizobium mediterraneium, Mesorhizobium tianshanense, Bradyrhizobium elkanni, Bradyrhizobium japonicum, Bradyrhizobium liaoningense, Azorhizobium caulinodans, Allobacterium undicola, Phyllobacterium myrsinacearum, Agrobacterium tumefaciens, Agrobacterium radiobacter, Agrobacterium rhizogenes, Agrobacterium vitis, and Agrobacterium rubi.* Preferred are also the strains and method described in Broothaerts W et al. (2005) Nature 433:629-633.

The monophyletic nature of *Agrobacterium*, *Allorhizobium* and *Rhizobium* and their common phenotypic generic circumscription support their amalgamation into a single genus, *Rhizobium*. The classification and characterization of *Agrobacterium* strains including differentiation of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* and their various opine-type classes is a practice well known in the art (see for example Laboratory guide for identification of plant pathogenic bacteria, 3rd edition. (2001) Schaad, Jones, and Chun (eds.) ISBN 0890542635; for example the article of Moore *et al.* published therein). Recent analyses demonstrate that classification by its plant-pathogenic properties may not be justified. Accordingly more advanced methods based on genome analysis and comparison (such as 16S rRNA sequencing; RFLP, Rep-PCR, *etc.*) are employed to elucidate the relationship of the various strains (see for example Young 2003, Farrand 2003, de Bruijn 1996, Vinuesa 1998). The phylogenetic relationships of members of the genus *Agrobacterium* by two methods demonstrating the relationship of *Agrobacterium* strains K599 are presented in Llob 2003 (figure 2).

It is known in the art that not only Agrobacterium but also other soil-borne bacteria are capable to mediate T-DNA transfer provided that they the relevant functional elements for the T-DNA transfer of an Ti- or Ri-plasmid (Klein & Klein 1953; Hooykaas 1977; van Veen 1988).

Preferably, the soil-born bacterium is of the genus *Agrobacterium*. The term "*Agrobacterium*" as used herein refers to a soil-borne, Gram-negative, rod-shaped phytopathogenic bacterium. The species of *Agrobacterium*, *Agrobacterium tumefaciens* (syn. *Agrobacterium radiobacter*), *Agrobacterium rhizogenes*, *Agrobacterium rubi* and *Agrobacterium vitis*, together with *Allorhizobium undicola*, form a monophyletic group with all *Rhizobium* species, based on comparative 16S rDNA analyses (Sawada 1993, Young 2003). *Agrobacterium* is an artificial genus comprising plant-pathogenic species.

The term Ti-plasmid as used herein is referring to a plasmid, which is replicable in

Agrobacterium and is in its natural, "armed" form mediating crown gall in *Agrobacterium* infected plants. Infection of a plant cell with a natural, "armed" form of a Ti-plasmid of *Agrobacterium* generally results in the production of opines (*e.g.*, nopaline, agropine, octopine *etc.*) by the infected cell. Thus, *Agrobacterium* strains which cause production of nopaline (*e.g.*, strain LBA4301, C58, A208) are referred to as "nopaline-type" *Agrobacteria*; *Agrobacterium* strains which cause production of octopine (*e.g.*, strain LBA4404, Ach5, B6) are referred to as "octopine-type" *Agrobacteria*; and *Agrobacterium* strains which cause production of agropine (*e.g.*, strain EHA105, EHA101, A281) are referred to as "agropine-type" *Agrobacteria*. A disarmed Ti-plasmid is understood as a Ti-plasmid lacking its crown gall mediating properties but otherwise providing the functions for plant infection. Preferably, the T-DNA region of said "disarmed" plasmid was modified in a way, that beside the border sequences no functional internal Ti-sequences can be transferred into the plant genome. In a preferred embodiment – when used with a binary vector system – the entire T-DNA region (including the T-DNA borders) is deleted.

The term Ri-plasmid as used herein is referring to a plasmid which is replicable in *Agrobacterium* and is in its natural, "armed" form mediating hairy-root disease in *Agrobacterium* infected plants. Infection of a plant cell with a natural, "armed" form of an Ri-plasmid of *Agrobacterium* generally results in the production of opines (specific amino sugar derivatives produced in transformed plant cells such as *e.g.*, agropine, cucumopine, octopine, mikimopine *etc.*) by the infected cell. *Agrobacterium rhizogenes* strains are traditionally distinguished into subclasses in the same way *A. tumefaciens* strains are. The most common strains are agropine-type strains (*e.g.*, characterized by the Ri-plasmid pRi-A4), mannopine-type strains (*e.g.*, characterized by the Ri-plasmid pRi8196) and cucumopine-type strains (*e.g.*, characterized by the Ri-plasmid pRi2659). Some other strains are of the mikimopine-type (*e.g.*, characterized by the Ri-plasmid pRi1723). Mikimopine and cucumopine are stereo isomers but no homology was found between the pRi plasmids on the nucleotide level (Suzuki 2001). A disarmed Ri-plasmid is understood as a Ri-plasmid lacking its hairy-root disease mediating properties but otherwise providing the functions for plant infection. Preferably, the T-DNA region of said "disarmed" Ri plasmid was modified in a way, that beside the border sequences no functional internal Ri-sequences can be transferred into the plant genome. In a preferred embodiment – when used with a binary vector system – the entire T-DNA region (including the T-DNA borders) is deleted.

The Ti and Ri plasmids of A. tumefaciens and A. rhizogenes, respectively, carry genes responsible for genetic transformation of the plant (Kado 1991). Vectors are based on the *Agrobacterium* Ti- or Ri-plasmid and utilize a natural system of DNA transfer into the plant genome. As part of this highly developed parasitism *Agrobacterium* transfers a defined part of its genomic information (the T-DNA; flanked by about 25 bp repeats, named left and right border) into the chromosomal DNA of the plant cell (Zupan 2000). By combined action of the so called vir genes (part of the original Ti-plasmids) said DNA-transfer is mediated. For utilization of this natural system, Ti-plasmids were developed which lack the original tumor inducing genes ("disarmed vectors"). In a further improvement, the so called "binary vector systems", the T-DNA was physically separated from the other functional elements of the Ti-plasmid (e.g., the vir genes), by being incorporated into a shuttle vector, which allowed easier handling (EP-A 120 516; US 4,940,838). These binary vectors comprise (beside the disarmed T-DNA with its border

sequences), prokaryotic sequences for replication both in *Agrobacterium* and E. coli. It is an advantage of *Agrobacterium*-mediated transformation that in general only the DNA flanked by the borders is transferred into the genome and that preferentially only one copy is inserted. Descriptions of *Agrobacterium* vector systems and methods for *Agrobacterium*-mediated gene transfer are known in the art (Miki 1993; Gruber 1993; Moloney 1989).

Hence, for *Agrobacteria*-mediated transformation the genetic composition (e.g., comprising an expression cassette) is integrated into specific plasmids, either into a shuttle or intermediate vector, or into a binary vector. If a Ti or Ri plasmid is to be used for the transformation, at least the right border, but in most cases the right and left border, of the Ti or Ri plasmid T-DNA is linked to the expression cassette to be introduced in the form of a flanking region. Binary vectors are preferably used. Binary vectors are capable of replication both in E.coli and in *Agrobacterium*. They may comprise a selection marker gene and a linker or polylinker (for insertion of e.g. the expression cassette to be transferred) flanked by the right and left T-DNA border sequence. They can be transferred directly into *Agrobacterium* (Holsters 1978). The selection marker gene permits the selection of transformed *Agrobacteria* and is, for example, the nptII gene, which confers resistance to kanamycin. The *Agrobacterium* which acts as the host organism in this case should already contain a plasmid with the vir region. The latter is required for transferring the T-DNA to the plant cell. An *Agrobacterium* transformed in this way can be used for transforming plant cells. The use of T-DNA for transforming plant cells has been studied and described intensively (EP 120 516; Hoekema 1985; An 1985).

Common binary vectors are based on "broad host range"-plasmids like pRK252 (Bevan 1984) or pTJS75 (Watson 1985) derived from the P-type plasmid RK2. Most of these vetors are derivatives of pBIN19 (Bevan 1984). Various binary vectors are known, some of which are commercially available such as, for example, pBI101.2 or pBIN19 (Clontech Laboratories, Inc. USA). Additional vectors were improved with regard to size and handling (e.g. pPZP; Hajdukiewicz 1994). Improved vector systems are described also in WO 02/00900.

Preferably the soil-borne bacterium is a bacterium belonging to family *Agrobacterium*, more preferably a disarmed *Agrobacterium tumefaciens* or *rhizogenes* strain. In a preferred embodiment, *Agrobacterium* strains for use in the practice of the invention include octopine strains, e.g., LBA4404 or agropine strains, e.g., EHA101 or EHA105. Suitable strains of A. tumefaciens for DNA transfer are for example EHA101pEHA101 (Hood 1986), EHA105[pEHA105] (Li 1992), LBA4404[pAL4404] (Hoekema 1983), C58C1[pMP90] (Koncz & Schell 1986), and C58C1[pGV2260] (Deblaere 1985). Other suitable strains are *Agrobacterium* tumefaciens C58, a nopaline strain. Other suitable strains are A. tumefaciens C58C1 (Van Larebeke 1974), A136 (Watson 1975) or LBA4011 (Klapwijk 1980). In another preferred embodiment the soil-borne bacterium is a disarmed strain variant of *Agrobacterium rhizogenes* strain K599 (NCPPB 2659). Such strains are described in US provisional application Application No. 60/606,789, filed September 2nd, 2004, hereby incorporated entirely by reference.

Preferably, these strains are comprising a disarmed plasmid variant of a Ti- or Ri-plasmid providing the functions required for T-DNA transfer into plant cells (e.g., the vir

genes). In a preferred embodiment, the *Agrobacterium* strain used to transform the plant tissue pre-cultured with the plant phenolic compound contains a L,L-succinamopine type Ti-plasmid, preferably disarmed, such as pEHA101. In another preferred embodiment, the *Agrobacterium* strain used to transform the plant tissue pre-cultured with the plant phenolic compound contains an octopine-type Ti-plasmid, preferably disarmed, such as pAL4404. Generally, when using octopine-type Ti-plasmids or helper plasmids, it is preferred that the virF gene be deleted or inactivated (Jarschow 1991).

The method of the invention can also be used in combination with particular *Agrobacterium* strains, to further increase the transformation efficiency, such as *Agrobacterium* strains wherein the vir gene expression and/or induction thereof is altered due to the presence of mutant or chimeric virA or virG genes (e.g. Hansen 1994; Chen and Winans 1991; Scheeren-Groot , 1994). Preferred are further combinations of *Agrobacterium* tumefaciens strain LBA4404 (Hiei 1994) with super-virulent plasmids. These are preferably pTOK246-based vectors (Ishida 1996).

A binary vector or any other vector can be modified by common DNA recombination techniques, multiplied in E. coli, and introduced into *Agrobacterium* by e.g., electroporation or other transformation techniques (Mozo 1991).

Agrobacterium is preferably grown and used in a manner similar to that described in Ishida (Ishida 1996). The vector comprising *Agrobacterium* strain may, for example, be grown for 3 days on YP medium (5 g/l yeast extract, 10 g/l peptone, 5 g/l NaCl, 15 g/l agar, pH 6.8) supplemented with the appropriate antibiotic (e.g., 50 mg/l spectinomycin). Bacteria are collected with a loop from the solid medium and resuspended. In a preferred embodiment of the invention, *Agrobacterium* cultures are started by use of aliquots frozen at -80°C.

The transformation of the immature embryos by the *Agrobacterium* may be carried out by merely contacting the immature embryos with the *Agrobacterium*. The concentration of *Agrobacterium* used for infection and co-cultivation may need to be varied. For example, a cell suspension of the *Agrobacterium* having a population density of approximately from 10^5 to 10^{11}, preferably 10^6 to 10^{10}, more preferably about 10^8 cells or cfu / ml is prepared and the immature embryos are immersed in this suspension for about 3 to 10 minutes. The resulting immature embryos are then cultured on a solid medium for several days together with the *Agrobacterium*.

In another preferred embodiment for the infection and co-cultivation step a suspension of the soil-borne bacterium (e.g., *Agrobacteria*) in the co-cultivation or infection medium is directly applied to each embryo, and excess amount of liquid covering the embryo is removed. Removal can be done by various means, preferably through either air-drying or absorbing. This is saving labor and time and is reducing unintended Agrobacterium-mediated damage by excess Agrobacterium usage. In a preferred embodiment from about 1 to about 10 µl of a suspension of the soil-borne bacterium (e.g., *Agrobacteria*) are employed. Preferably, the immature embryo is infected with *Agrobacterium* directly on the co-cultivation medium. Preferably, the bacterium is employed in concentration of 10^6 to 10^{11} cfu/ml.

For *Agrobacterium* treatment of isolated immature embryos, the bacteria are resuspended in a plant compatible co-cultivation medium. Supplementation of the co-culture medium with ethylene inhibitors (e.g., silver nitrate), phenol-absorbing compounds (like polyvinylpyrrolidone, Perl 1996) or antioxidants (such as thiol compounds, e.g., dithiothreitol, L-cysteine, Olhoft 2001) which can decrease tissue necrosis due to plant defense responses (like phenolic oxidation) may further improve the efficiency of *Agrobacterium*-mediated transformation. In another preferred embodiment, the co-cultivation medium of comprises least one thiol compound, preferably selected from the group consisting of sodium thiolsulfate, dithiotrietol (DTT) and cysteine. Preferably the concentration is between about 1 mM and 10mM of L-Cysteine, 0.1 mM to 5 mM DTT, and/or 0.1 mM to 5 mM sodium thiolsulfate. Preferably, the medium employed during co-cultivation comprises from about 1 µM to about 10 µM of silver nitrate and/or (preferably "and") from about 50 mg/L to about 1,000 mg/L of L-Cysteine. This results in a highly reduced vulnerability of the immature embryo against Agrobacterium-mediated damage (such as induced necrosis) and highly improves overall transformation efficiency.

A range of co-cultivation periods from a few hours to 7 days may be employed. The co-cultivation of *Agrobacterium* with the isolated immature embryos is in general carried out for about 12 hours to about five days, preferably about 1 day to about 3 days.

In an improved embodiment of the invention the isolated immature embryos and/or the *Agrobacteria* may be treated with a phenolic compound prior to or during the *Agrobacterium* co-cultivation. "Plant phenolic compounds" or "plant phenolics" suitable within the scope of the invention are those isolated substituted phenolic molecules which are capable to induce a positive chemotactic response, particularly those who are capable to induce increased vir gene expression in a Ti-plasmid containing *Agrobacterium* sp., particularly a Ti-plasmid containing *Agrobacterium* tumefaciens. Methods to measure chemotactic responses towards plant phenolic compounds have been like e.g., described (Ashby 1988) and methods to measure induction of vir gene expression are also well known (Stachel 1985; Bolton 1986). The pre-treatment and/or treatment during *Agrobacterium* co-cultivation has at least two beneficial effects: Induction of the vir genes of Ti plasmids or helper plasmids (Van Wordragen 1992; Jacq 1993; James 1993; Guivarc'h 1993), and enhancement of the competence for incorporation of foreign DNA into the genome of the plant cell.

Preferred plant phenolic compounds are those found in wound exudates of plant cells. One of the best known plant phenolic compounds is acetosyringone, which is present in a number of wounded and intact cells of various plants, albeit in different concentrations. However, acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) is not the only plant phenolic which can induce the expression of vir genes. Other examples are α–hydroxy-acetosyringone, sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), syringic acid (4-hydroxy-3,5 dimethoxybenzoic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), catechol (1,2-dihydroxybenzene), p-hydroxybenzoic acid (4-hydroxybenzoic acid), β-resorcylic acid (2,4-dihydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), pyrrogallic acid (2,3,4-trihydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid) and vanillin (3-methoxy-4-hydroxybenzaldehyde), and these phenolic compounds are known or expected to be able to replace acetosyringone in the cultivation media with similar results. As used herein, the mentioned

molecules are referred to as plant phenolic compounds.

Plant phenolic compounds can be added to the plant culture medium either alone or in combination with other plant phenolic compounds. A particularly preferred combination of plant phenolic compounds comprises at least acetosyringone and p-hydroxybenzoic acid, but it is expected that other combinations of two, or more, plant phenolic compounds will also act synergistically in enhancing the transformation efficiency.

Moreover, certain compounds, such as osmoprotectants (e.g. L-proline preferably at a concentration of about 700 mg/L or betaine), phytohormes (inter alia NAA), opines, or sugars, act synergistically when added in combination with plant phenolic compounds.

In one embodiment of the invention, it is preferred that the plant phenolic compound, particularly acetosyringone is added to the medium prior to contacting the isolated immature embryos with *Agrobacteria* (for e.g., several hours to one day). The exact period in which the cultured cells are incubated in the medium containing the plant phenolic compound such as acetosyringone, is believed not to be critical and only limited by the time the immature embryos start to differentiate.

The concentration of the plant phenolic compound in the medium is also believed to have an effect on the development of competence for integrative transformation. The optimal concentration range of plant phenolic compounds in the medium may vary depending on the *Zea mays* variety from which the immature embryos derived, but it is expected that about 100 µM to 700 µM is a suitable concentration for many purposes. However, concentrations as low as approximately 25 µM can be used to obtain a good effect on transformation efficiency. Likewise, it is expected that higher concentrations up to approximately 1000 µM will yield similar effects. Comparable concentrations apply to other plant phenolic compounds, and optimal concentrations can be established easily by experimentation in accordance with this invention.

Agrobacteria to be co-cultivated with the isolated immature embryos can be either preincubated with acetosyringone or another plant phenolic compound, as known by the person skilled in the art, or used directly after isolation from their culture medium. Particularly suited induction conditions for *Agrobacterium* tumefaciens have been described by Vernade *et al.* (1988). Efficiency of transformation with *Agrobacterium* can be enhanced by numerous other methods known in the art like for example vacuum infiltration (WO 00/58484), heat shock and/or centrifugation, addition of silver nitrate, sonication etc.

It has been observed within this invention that transformation efficacy of the isolated immature embryos by *Agrobacterium* can be significantly improved by keeping the pH of the co-cultivation medium in a range from 5.4 to 6.4, preferably 5.6 to 6.2, especially preferably 5.8 to 6.0. In an improved embodiment of the invention stabilization pf the pH in this range is mediated by a combination of MES and potassium hydrogenphosphate buffers.

2.3 Recovery

Transformed cells, i.e. those which comprise the DNA integrated into the DNA of the host cell, can be selected from untransformed cells preferably using the selection method of the invention.

Prior to a transfer to a recovery and/or selection medium, especially in case of Agrobacterium-mediated transformation, certain other intermediate steps may be employed. 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 recovery and/ or selection medium preferably comprises a bacteriocide (antibiotic) suitable to prevent *Agrobacterium* growth. Preferred bactericidal antibiotics to be employed are e.g., carbenicillin (500 mg/L) 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 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).

It is preferred that the step directly following the transformation procedure (e.g., co-cultivation) is not comprising an effective, phytotoxic amount of D-alanine and/or D-serine or derivatives thereof (which are subsequently used for transformation). Thus, this step is intended to allow for regeneration of the transformed tissue, to promote initiation of embryogenic callus formation in the *Agrobacterium*-infected embryo, and kill the remaining *Agrobacterium* cells. Accordingly, in a preferred embodiment the method of the invention comprises the step of transferring the transformed target tissue (e.g., the co-cultivated immature embryos) to a recovering medium (used in step c) comprising

i. an effective amount of at least one antibiotic that inhibits or suppresses the growth of the soil-borne bacteria, and/or (preferably "and")
ii. L-proline in a concentration from about 1 g/l to about 10g/l, and/or (preferably "and")
iii. silver nitrate in a concentration from about 1 µM to about 50 µM.

Preferably said recovery medium does not comprise an effective, phytotoxic amount of D-alanine and/or D-serine or a derivative thereof. The recovery medium may further comprise an effective amount of at least one plant growth regulator (e.g., an effective amount of at least one auxin compound). Thus the recovery medium of step c) preferably comprises

i. an effective amount of at least one antibiotic that inhibits or suppresses the growth of the soil-borne bacteria, and
ii. L-proline in a concentration from about 1 g/l to about 10g/l, and
iii. silver nitrate in a concentration from about 1 µM to about 50 µM,
iv. an effective amount of at least one auxin compound.

Examples for preferred recovery media are given below in the Examples (A-4 or A-5). The recovery period may last for about 1 day to about 14 days, preferably about 5 days to about 8 days. Preferably, the scutellum side is kept up during this time and do not embedded into the media.

2.4 Selection

After the recovery step the target tissue (e.g., the immature embryos) are transferred to and incubated on a selection medium. The selection medium comprises D-alanine and/or D-serine or a derivative thereof in a phytotoxic concentration (i.e., in a concentration which either terminates or at least retard the growth of the non-transformed cells). The term "phytotoxic", "phytotoxicity" or "phytotoxic effect" as used herein is intended to mean any measurable, negative effect on the physiology of a plant or plant cell resulting in symptoms including (but not limited to) for example reduced or impaired growth, reduced or impaired photosynthesis, reduced or impaired cell division, reduced or impaired regeneration (e.g., of a mature plant from a cell culture, callus, or shoot etc.), reduced or impaired fertility etc. Phytotoxicity may further include effects like e.g., necrosis or apoptosis. In a preferred 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 specific compound employed for selection is chosen depending on which marker protein is expressed. For example in cases where the *E.coli* D-serine ammonia-lyase is employed, selection is done on a medium comprising D-serine. In cases where the *Rhodotorula gracilis* D-amino acid oxidase is employed, selection is done on a medium comprising D-alanine and/or D-serine.

The fact that D-amino acids are employed does not rule out the presence of L-amino acid structures or L-amino acids. For some applications it may be preferred (e.g., for cost reasons) to apply a racemic mixture of D- and L-amino acids (or a mixture with enriched content of D-amino acids). Preferably, the ratio of the D-amino acid to the corresponding L-enantiomer is at least 1:1, preferably 2:1, more preferably 5:1, most preferably 10:1 or 100:1. The use of D-alanine has the advantage that racemic mixtures of D- and L-alanine can be applied without disturbing or detrimental effects of the L-enantiomer. Therefore, in an improved embodiment a racemic mixture of D/L-alanine is employed as compound

The term "derivative" with respect to D-alanine or D-serine means chemical compound which are comprising the respective D-amino acid structure of D-alanine or D-serine, but are chemically modified. As used herein the term a "D-amino acid structure" (such as a "D-serine structure") is intended to include the D-amino acid, as well as analogues, derivatives and mimetics of the D-amino acid that maintain the functional activity of the compound. As used herein, a "derivative" also refers to a form of D-serine or D-alanine in which one or more reaction groups on the compound have been derivatized with a substituent group. The D-amino acid employed may be modified by an amino-terminal or a carboxy-terminal modifying group or by modification of the side-chain. The amino-terminal modifying group may be – for example - selected from the group consisting of phenylacetyl, diphenylacetyl, triphenylacetyl, butanoyl, isobutanoyl hexanoyl, propionyl, 3-hydroxybutanoyl, 4-hydroxybutanoyl, 3-hydroxypropionoyl, 2,4-dihydroxybutyroyl, 1-Adamantanecarbonyl, 4-methylvaleryl, 2-hydroxyphenylacetyl, 3-hydroxyphenylacetyl, 4-hydroxyphenylacetyl, 3,5-dihydroxy-2-naphthoyl, 3,7-dihydroxy-2-napthoyl, 2-hydroxycinnamoyl, 3-hydroxycinnamoyl, 4-hydroxycinnamoyl, hydrocinnamoyl, 4-formylcinnamoyl, 3-hydroxy-4-methoxycinnamoyl, 4-hydroxy-3-methoxycinnamoyl, 2-carboxycinnamoyl, 3,4,-dihydroxyhydrocinnamoyl, 3,4-dihydroxycinnamoyl, trans-Cinnamoyl, (±)-mandelyl, (±)-mandelyl-(±)-mandelyl, glyco-

lyl, 3-formylbenzoyl, 4-formylbenzoyl, 2-formylphenoxyacetyl, 8-formyl-1-napthoyl, 4-(hydroxymethyl)benzoyl, 3-hydroxybenzoyl, 4-hydroxybenzoyl, 5-hydantoinacetyl, L-hydroorotyl, 2,4-dihydroxybenzoyl, 3-benzoylpropanoyl, (±)-2,4-dihydroxy-3,3-dimethylbutanoyl, DL-3-(4-hydroxyphenyl)lactyl, 3-(2-hydroxyphenyl)propionyl, 4-(2-hydroxyphenyl)propionyl, D-3-phenyllactyl, 3-(4-hydroxyphenyl)propionyl, L-3-phenyllactyl, 3-pyridylacetyl, 4-pyridylacetyl, isonicotinoyl, 4-quinolinecarboxyl, 1-isoquinolinecarboxyl and 3-isoquinolinecarboxyl. The carboxy-terminal modifying group may be – for example - selected from the group consisting of an amide group, an alkyl amide group, an aryl amide group and a hydroxy group. The "derivative" as used herein are intended to include molecules which mimic the chemical structure of a respective D-amino acid structure and retain the functional properties of the D-amino acid structure. Approaches to designing amino acid or peptide analogs, derivatives and mimetics are known in the art (e.g., see Farmer 1980; Ball 1990; Morgan 1989; Freidinger 1989; Sawyer 1995; Smith 1995; Smith 1994; Hirschman 1993). Other possible modifications include N-alkyl (or aryl) substitutions, or backbone crosslinking to construct lactams and other cyclic structures. Other derivatives include C-terminal hydroxymethyl derivatives, O-modified derivatives (e.g., C-terminal hydroxymethyl benzyl ether), N-terminally modified derivatives including substituted amides such as alkylamides and hydrazides. Furthermore, D-amino acid structure comprising herbicidal compounds may be employed. Such compounds are for example described in US 5,059,239, and may include (but shall not be limited to) N-benzoyl-N-(3-chloro-4-fluorophenyl)-DL-alanine, N-benzoyl-N-(3-chloro-4-fluorophenyl) -DL-alanine methyl ester, N-benzoyl-N-(3-chloro-4-fluorophenyl)-DL-alanine ethyl ester, N-benzoyl-N-(3-chloro-4-fluorophenyl)-*D-alanine,* N-benzoyl-N-(3-chloro-4-fluorophenyl)-*D-alanine* methyl ester, or N-benzoyl-N-(3-chloro-4-fluorophenyl)-*D-alanine* isopropyl ester.

The selection compound may be used in combination with other substances. For the purpose of application, the selection compound may also be used together with the adjuvants conventionally employed in the art of formulation, and are therefore formulated in known manner, e.g. into emulsifiable concentrates, coatable pastes, directly sprayable or dilutable solutions, dilute emulsions, wettable powders, soluble powders, dusts, granulates, and also encapsulations in e.g. polymer substances. As with the nature of the compositions to be used, the methods of application, such as spraying, atomising, dusting, scattering, coating or pouring, are chosen in accordance with the intended objectives and the prevailing circumstances. However, more preferably the selection compound is directly applied to the medium. It is an advantage that stock solutions of the selection compound can be made and stored at room temperature for an extended period without a loss of selection efficiency.

The optimal concentration of the selection compound (i.e. D-alanine, D-serine, derivatives thereof or any combination thereof) may vary depending on the target tissue employed for transformation but in general (and preferably for immature embryo transformation) the total concentration (i.e. the sum in case of a mixture) of D-alanine, D-serine or derivatives thereof is in the range from about 1 mM to about 100. For example in cases where the *E.coli* D-serine ammonia-lyase is employed, selection is done on a medium comprising D-serine (e.g., incorporated into agar-solidified MS media plates), preferably in a concentration from about 1 mM to about 100 mM, more preferably from about 5 mM to about 50 mM, even more preferably from about 7 mM to about 30 mM, most preferably about 10 to 20 mM. In cases where the *Rhodotorula gracilis* D-amino

acid oxidase is employed, selection is done on a medium comprising D-alanine and/or D-serine (e.g., incorporated into agar-solidified MS media plates), preferably in a total concentration from about 1 mM to 100 mM, more preferably from about 2 mM to about 50 mM, even more preferably from about 3 mM to about 20 mM, most preferably about 5 to 15 mM.

Also the selection time may vary depending on the target tissue used and the regeneration protocol employed. In general a selection time is at least 5 days. More specifically the total selection time under dedifferentiating conditions (i.e., callus induction) is from about 1 to about 10 weeks, preferably, 3 to 7 weeks, more preferably 3 to 4 weeks. However, it is preferred that the selection under the dedifferentiating conditions is employed for not longer than 70 days. In between the selection period the callus may be transferred to fresh selection medium one or more times. For the specific protocol provided herein it is preferred that two selection medium steps (e.g., one transfer to new selection medium) is employed. Preferably, the selection of step is done in two steps, using a first selection step for about 5 to 20 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 5 to 20 days. However, it is also possible to apply a single step selection.

Preferably said selection medium is 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 embryogenic 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).

Preferably, when used as the sole auxin compound, 2,4-D in a concentration of about 0.2 mg/l to about 6 mg/l, more preferably about 0.3 to about 2 mg/l, most preferably about 1.5 mg/l is employed. In case other auxin compounds or combinations thereof are employed, their preferred combinations is chosen in a way that the dedifferentiating effect is equivalent to the effect achieved with the above specified concentrations of

2,4-D when used as the sole auxin compound. Thus, the effective amount of the auxin compound is preferably equivalent to a concentration of about 0.2 mg/l to about 6 mg/l (more preferably about 0.3 to about 2 mg/l, most preferably about 1.5 mg/l) of 2,4-D.

Furthermore, combination of different auxins can be employed, for example a combination of 2,4-D and Picloram. Preferably, 2,4-D in a concentration of about 0.5 mg/l can be combined with one or more other types of auxin compounds e.g. Picloram in a concentration of about 1 to about 2 mg/l for improving quality/quantity of embryogenic callus formation.

The medium may be optionally further supplemented with one or more additional plant growth regulator, like e.g., cytokinin compounds (e.g., 6-benzylaminopurine) and/or other auxin compounds. Such compounds include, but are not limited to, IAA, NAA, IBA, cytokinins, auxins, kinetins, glyphosate, and thiadiazorun. Cytokinin compounds comprise, for example, 6-isopentenyladenine (IPA) and 6-benzyladenine/6-benzylaminopurine (BAP).

The presence of the D-amino acid metabolizing enzymes does not rule out that additional markers are employed.

The selection (application of the selection compound) may end after the dedifferentiation and selection period. However, it is preferred to apply selection also during the subsequent regeneration period (in part or throughout), and even during rooting.

2.5 Regeneration

The formation of shoot and root from dedifferentiated cells can be induced in the known fashion. The shoots obtained can be planted and cultured. Transformed plant cells, derived by any of the above transformation techniques, can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium. Plant regeneration from cultured protoplasts is described (e.g., in Evans 1983; Binding 1985). Regeneration can also be obtained from plant callus, explants, somatic embryos (Dandekar 1989; McGranahan 1990), organs, or parts thereof. Such regeneration techniques are described generally (e.g., in Klee 1987). Other available regeneration techniques are reviewed in Vasil 1984, and Weissbach 1989.

After the dedifferentiation and selection period (as described above) the resulting cells (e.g., maturing embryogenic callus) are transferred to a medium allowing conversion of transgenic plantlets. Preferably such medium does not comprise auxins such as 2,4-D in a concentration leading to dedifferentiation. In a preferred embodiment such medium may comprise one or more compounds selected from the group consisting of:

i) cytokinins such as for example zeatin, preferably in a concentration from about 0.5 to about 10 mg/L, more preferably from about 1.5 to about 5 mg/L,

ii) an effective amount of at least one antibiotic that inhibits or suppresses the growth of the soil-borne bacteria (as defined above), and

iii) an effective amount of a selection agent (e.g., D-alanine, D-serine, or derivatives thereof) allowing for selection of transgenic cells(e.g., comprising the transgenic T-DNA).

The embryogenic callus is preferably incubated on this medium until shoots are formed and then transferred to a rooting medium. Such incubation may take from 1 to 5, preferably from 2 to 3 weeks. Regenerated shoots or plantlets (i.e., shoots with roots) are transferred to Phytatray or Magenta boxes containing rooting medium (such as the medium described by recipe A-8) and incubate until rooted plantlets have developed (usually 1 to 4 weeks, preferably 2 weeks). The rooted seedlings are transferred to Metromix soil and grown to mature plants as described in the art (see examples). In a preferred embodiment of the invention an improved procedure is employed and plantlets regenerated on plates are directly transplanted to MetroMix in the greenhouse, omitting the step in the rooting box, thereby saving time and labor.

The resulting plants can be bred and hybridized in the customary fashion. Two or more generations should be grown in order to ensure that the genomic integration is stable and hereditary. For example, at the flowering stage, the tassels of transgenic plants are bagged with brown paper bags to prevent pollen escape. Pollination is performed on the transgenic plants. It is best to do self-pollination on the transgenic plants. If silking and anthesis are not synchronized, a wild-type pollen donor or recipient plant with same genetic background as the transgenic T_0 plant should be available for performing cross-pollination. T_1 seeds are harvested, dried and stored properly with adequate label on the seed bag. After harvesting the transgenic T_1 seeds, T_0 plants including the soil and pot should be bagged in autoclave bags and autoclaved (double bagging). Other important aspects of the invention include the progeny of the transgenic plants prepared by the disclosed methods, as well as the cells derived from such progeny, and the seeds obtained from such progeny.

2.6 Generation of descendants

After transformation, selection and regeneration of a transgenic plant (comprising the DNA construct of the invention) descendants are generated, which – because of the activity of the excision promoter – underwent excision and do not comprise the marker sequence(s) and expression cassette for the endonuclease.

Descendants can be generated by sexual or non-sexual propagation. Non-sexual propagation can be realized by introduction of somatic embryogenesis by techniques well known in the art. Preferably, descendants are generated by sexual propagation / fertilization. Fertilization can be realized either by selfing (self-pollination) or crossing with other transgenic or non-transgenic plants. The transgenic plant of the invention can herein function either as maternal or paternal plant.

After the fertilization process, seeds are harvested, germinated and grown into mature plants. Isolation and identification of descendants which underwent the excision process can be done at any stage of plant development. Methods for said identification are well known in the art and may comprise – for example – PCR analysis, Northern blot, Southern blot, or phenotypic screening (e.g., for an negative selection marker).

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 gene.

Also in accordance with the invention are cells, cell cultures, parts – such as, for example, in the case of transgenic plant organisms, roots, leaves and the like – derived from the above-described transgenic organisms, and transgenic propagation material (such as seeds or fruits).

Genetically modified plants according to the invention which can be consumed by humans or animals can also be used as food or feedstuffs, for example directly or following processing known per se. Here, the deletion of, for example, resistances to antibiotics and/or herbicides, as are frequently introduced when generating the transgenic plants, makes sense for reasons of customer acceptance, but also product safety.

A further subject matter of the invention relates to the use of the above-described transgenic organisms according to the invention and the cells, cell cultures, parts – such as, for example, in the case of transgenic plant organisms, roots, leaves and the like – derived from them, and transgenic propagation material such as seeds or fruits, for the production of food or feedstuffs, pharmaceuticals or fine chemicals. Here again, the deletion of, for example, resistances to antibiotics and/or herbicides is advantageous for reasons of customer acceptance, but also product safety.

Fine chemicals is understood as meaning enzymes, vitamins, amino acids, sugars, fatty acids, natural and synthetic flavors, aromas and colorants. Especially preferred is the production of tocopherols and tocotrienols, and of carotenoids. Culturing the transformed host organisms, and isolation from the host organisms or from the culture medium, is performed by methods known to the skilled worker. The production of pharmaceuticals such as, for example, antibodies or vaccines, is described (e.g., by Hood 1999; Ma 1999).

3. Further modifications

3.1 Counter selection and subsequent marker deletion

The first expression construct for the D-amino acid metabolizing enzyme can be preferably constructed in a way to allow for subsequent marker deletion, especially when said enzyme is a D-amino acid oxidase, which can be employed both for negative selection and counter selection (i.e. as a dual-function marker). Such methods are in detail described in (ADD) hereby incorporated entirely by reference.

For this purpose the first expression cassette is preferably flanked by sequences, which allow for specific deletion of said first expression cassette. This embodiment of the invention makes use of the property of D-amino oxidase (DAAO) to function as dual-function markers, i.e., as markers which both allow (depending on the used substrate) as negative selection marker and counter selection marker. In contrast to D-amino acids like D-serine and D-alanine (which are highly phytoptoxic to plants and are "detoxified" by the D-amino acid oxidase), D-valine and D-isoleucine are not toxic to wild-type plants but are converted to toxic compounds by plants expressing the D-amino acid oxidase DAAO. The findings that DAAO expression mitigated the toxicity of

D-serine and D-alanine, but induced metabolic changes that made D-isoleucine and D-valine toxic, demonstrate that the enzyme could provide a substrate-dependent, dual-function, selectable marker in plants.

Accordingly, another embodiment of the invention relates to a method for producing a transgenic *Zea mays* plant comprising:

i) transforming a *Zea mays* plant cell with a first DNA construct comprising
   a) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an D-amino acid oxidase enzyme, wherein said first expression cassette is flanked by sequences which allow for specific deletion of said first expression cassette, and
   b) at least one second expression cassette suitable for conferring to said plant an agronomically valuable trait, wherein said second expression cassette is not localized between said sequences which allow for specific deletion of said first expression cassette, and
ii) treating said transformed *Zea mays* plant cells of step i) with a first compound selected from the group consisting of D-alanine, D-serine or derivatives thereof in a phytotoxic concentration and selecting plant cells comprising in their genome said first DNA construct, conferring resistance to said transformed plant cells against said first compound by expression of said D-amino acid oxidase, and
iii) inducing deletion of said first expression cassette from the genome of said transformed plant cells and treating said plant cells with a second compound selected from the group consisting of D-isoleucine, D-valine and derivatives thereof in a concentration toxic to plant cells still comprising said first expression cassette, thereby selecting plant cells comprising said second expression cassette but lacking said first expression cassette.

Preferred ubiquitin promoters and D-amino acid oxidase sequences are described above.

Preferably, deletion of the first expression cassette can be realized by various means known in the art, including but not limited to one or more of the following methods:

a) recombination induced by a sequence specific recombinase, wherein said first expression cassette is flanked by corresponding recombination sites in a way that recombination between said flanking recombination sites results in deletion of the sequences in-between from the genome,
b) homologous recombination between homology sequences A and A' flanking said first expression cassette, preferably induced by a sequence-specific double-strand break between said homology sequences caused by a sequence specific endonuclease, wherein said homology sequences A and A' have sufficient length and homology in order to ensure homologous recombination between A and A', and having an orientation which – upon recombination between A and A' – will lead to excision of said first expression cassette from the genome of said plant.

Various means are available for the person skilled in art to combine the deletion/excision inducing mechanism with the DNA construct of the invention comprising the D-amino acid oxidase dual-function selection marker. Preferably, a recombinase or endonuclease employable in the method of the invention can be expressed by a method selected from the group consisting of:

a) incorporation of a second expression cassette for expression of the recombinase or sequence-specific endonuclease operably linked to a plant promoter into said DNA construct, preferably together with said first expression cassette flanked by said sequences which allow for specific deletion,
b) incorporation of a second expression cassette for expression of the recombinase or sequence-specific endonuclease operably linked to a plant promoter into the plant cells or plants used as target material for the transformation thereby generating master cell lines or cells,
c) incorporation of a second expression cassette for expression of the recombinase or sequence-specific endonuclease operably linked to a plant promoter into a separate DNA construct, which is transformed by way of co-transformation with said first DNA construct into said plant cells,
d) incorporation of a second expression cassette for expression of the recombinase or sequence-specific endonuclease operably linked to a plant promoter into the plant cells or plants which are subsequently crossed with plants comprising the DNA construct of the invention.

In another preferred embodiment the mechanism of deletion/excision can be induced or activated in a way to prevent pre-mature deletion/excision of the dual-function marker. Preferably, thus expression and/or activity of an preferably employed sequence-specific recombinase or endonuclease can be induced and/or activated, preferably by a method selected from the group consisting of

a) inducible expression by operably linking the sequence encoding said recombinase or endonuclease to an inducible promoter,
b) inducible activation, by employing a modified recombinase or endonuclease comprising a ligand-binding-domain, wherein activity of said modified recombinase or endonuclease can by modified by treatment of a compound having binding activity to said ligand-binding-domain.

Preferably, thus the method of the inventions results in a plant cell or plant which is selection marker-free.

Another subject matter of the invention relates to DNA constructs, which are suitable for employing in the method of the invention. A DNA construct suitable for use within the present invention is preferably comprising

a) a first expression cassette comprising a nucleic acid sequence encoding a D-amino acid oxidase operably linked with a ubiquitin promoter, wherein said first expression cassette is flanked by sequences which allow for specific deletion of said first expression cassette, and
b) at least one second expression cassette suitable for conferring to said plant an agronomically valuable trait, wherein said second expression cassette is not localized between said sequences which allow for specific deletion of said first expression cassette.

Preferred ubiquitin promoters and D-amino acid oxidase sequences are described above.

For ensuring marker deletion / excision the expression cassette for the D-amino acid oxidase (the first expression construct) comprised in the DNA construct of the invention

is flanked by recombination sites for a sequence specific recombinase in a way the recombination induced between said flanking recombination sites results in deletion of the said first expression cassette from the genome. Preferably said sequences which allow for specific deletion of said first expression cassette are selected from the group of sequences consisting of

a) recombination sites for a sequences-specific recombinase arranged in a way that recombination between said flanking recombination sites results in deletion of the sequences in-between from the genome, and
b) homology sequences A and A' having a sufficient length and homology in order to ensure homologous recombination between A and A', and having an orientation which – upon recombination between A and A' – results in deletion of the sequences in-between from the genome.

Preferably, the construct comprises at least one recognition site for a sequence specific nuclease localized between said sequences which allow for specific deletion of said first expression cassette (especially for variant b above).

There are various recombination sites and corresponding sequence specific recombinases known in the art, which can be employed for the purpose of the invention. The person skilled in the art is familiar with a variety of systems for the site-directed removal of recombinantly introduced nucleic acid sequences. They are mainly based on the use of sequence specific recombinases. Various sequence-specific recombination systems are described, such as the Cre/lox system of the bacteriophage P1 (Dale1991; Russell 1992; Osborne 1995), the yeast FLP/FRT system (Kilby 1995; Lyznik 1996), the Mu phage Gin recombinase, the E. coli Pin recombinase or the R/RS system of the plasmid pSR1 (Onouchi 1995; Sugita 2000). Also a system based on attP sites and bacteriophage Lambda recombinase can be employed (Zubko 2000). Further methods suitable for combination with the methods described herein are described in WO 97/037012 and WO 02/10415.

In a preferred embodiment, deletion / excision of the dual-marker sequence is deleted by homologous recombination induced by a sequence-specific double-strand break. The basic principles are disclosed in WO 03/004659, hereby incorporated by reference. For this purpose the first expression construct (encoding for the dual-function marker) is flanked by homology sequences A and A', wherein said homology sequences have sufficient length and homology in order to ensure homologous recombination between A and A', and having an orientation which – upon recombination between A and A' – will lead to an excision of first expression cassette from the genome. Furthermore, the sequence flanked by said homology sequences further comprises at least one recognition sequence of at least 10 base pairs for the site-directed induction of DNA double-strand breaks by a sequence specific DNA double-strand break inducing enzyme, preferably a sequence-specific DNA-endonuclease, more preferably a homing-endonuclease, most preferably an endonuclease selected from the group consisting of I-SceI, I-CeuI, I-CpaI, I-CpaII, I-CreI and I-ChuI or chimeras thereof with ligand-binding domains.

The expression cassette for the endonuclease or recombinase (comprising a sequence-specific recombinase or endonuclease operably linked to a plant promote) may be included in the DNA construct of the invention. Preferably, said second expression

cassette is together with said first expression cassette flanked by said sequences which allow for specific deletion.

In another preferred embodiment, the expression and/or activity of said sequence-specific recombinase or endonuclease can be induced and/or activated for avoiding premature deletion / excision of the dual-function marker during a period where its action as a negative selection marker is still required. Preferably induction / activation can be realized by a method selected from the group consisting of

a) inducible expression by operably linking the sequence encoding said recombinase or endonuclease to an inducible promoter,
b) inducible activation, by employing a modified recombinase or endonuclease comprising a ligand-binding-domain, wherein activity of said modified recombinase or endonuclease can by modified by treatment of a compound having binding activity to said ligand-binding-domain.

Further embodiments of the inventions are related to transgenic vectors comprising a DNA construct of the invention. Transgenic cells or non-human organisms comprising a DNA construct or vector of the invention. Preferably said cells or non-human organisms are plant cells or plants, preferably plants which are of agronomical use.

The present invention enables generation of marker-free transgenic cells and organisms, preferably plants, an accurately predictable manner with high efficiency.

The preferences for the counter selection step (ii) with regard to choice of compound, concentration, mode of application for D-alanine, D-serine, or derivatives thereof are described above in the context of the general selection scheme.

For the counter selection step (iii) the compound is selected from the group of compounds comprising a D-isoleucine or D-valine structure. More preferably the compound is selected from the group consisting of D-isoleucine and D-valine. Most preferably the compound or composition used for counter selection comprises D-isoleucine.

When applied via the cell culture medium (e.g., incorporated into agar-solidified MS media plates), D-isoleucine can be employed in concentrations of about 0.1 mM to about 100 mM, preferably about 1 mM to about 50 mM, more preferably about 10 mM to about 30 mM. When applied via the cell culture medium (e.g., incorporated into agar-solidified MS media plates), D-valine can be employed in concentrations of about 1 to about 100 mM, preferably about 5 to 50 mM, more preferably about 15 mM to about 30 mM.

Thus, using the above described method it becomes possible to create a *Zea mays* plant which is marker-free. The terms "marker-free" or "selection marker free" as used herein with respect to a cell or an organisms are intended to mean a cell or an organism which is not able to express a functional selection marker protein (encoded by expression cassette b; as defined above) which was inserted into said cell or organism in combination with the gene encoding for the agronomically valuable trait. The sequence encoding said selection marker protein may be absent in part or –preferably – entirely. Furthermore the promoter operably linked thereto may be dysfunctional by being absent in part or entirely. The resulting plant may however comprise other sequences

which may function as a selection marker. For example the plant may comprise as a agronomically valuable trait a herbicide resistance conferring gene. However, it is most preferred that the resulting plant does not comprise any selection marker.

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.

3.2 Gene Stacking

The methods and compositions of the invention allow for subsequent transformation. The D-serine and/or D-alanine metabolizing enzymes are 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 constructs of the invention or to subsequently transform the plants obtained by the method of the invention (and comprising the expression constructs for the D-serine and/or D-alanine metabolizing enzyme) with another marker. This, another embodiment of the invention relates to a method for subsequent transformation of at least two DNA constructs into a *Zea mays* plant comprising the steps of:

a) a transformation with a first construct said construct comprising at least one expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, and
b) a transformation with a second construct said construct comprising a second selection marker gene, which is not conferring resistance against D-alanine or D-serine.

Preferably said second marker gene is a negative selection markers conferring a resistance to a biocidal compound such as a (non-D-amino acid) metabolic inhibitor (e.g., 2-deoxyglucose-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; de Block 1987; Vasil 1992, 1993; Weeks 1993; Becker 1994; Nehra 1994; Wan & Lemaux 1994; EP 0 333 033; US 4,975,374)
- 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) conferring resistance to Glyphosate® (N-(phosphonomethyl)glycine) (Shah 1986; Della-Cioppa 1987)
- Glyphosate® degrading enzymes (Glyphosate® oxidoreductase; gox),
- Dalapon® inactivating dehalogenases (deh)
- sulfonylurea- and/or imidazolinone-inactivating acetolactate synthases (*ahas* or ALS; for example mutated ahas/ALS variants with, for example, the S4, XI12, XA17, and/or Hra mutation
- Bromoxynil® degrading nitrilases (bxn)
- Kanamycin- or. geneticin (G418) resistance genes (NPTII; NPTI) coding e.g., for neomycin phosphotransferases (Fraley 1983; Nehra 1994)
- hygromycin phosphotransferase (HPT), which mediates resistance to hygromycin (Vanden Elzen 1985).
- dihydrofolate reductase (Eichholtz 1987)

Various time schemes can be employed for the various negative selection marker genes. In case of resistance genes (e.g., against herbicides) selection is preferably applied throughout callus induction phase for about 4 weeks and beyond at least 4 weeks into regeneration. Such a selection scheme can be applied for all selection regimes. It is furthermore possible (although not explicitly preferred) to remain the selection also throughout the entire regeneration scheme including rooting. For example, with the phosphinotricin resistance gene (bar) as the selective marker, phosphinotricin at a concentration of from about 1 to 50 mg/l may be included in the medium. For example, with the mutated ahas genes as the selective marker, PURSUIT™ at a concentration of from about 100 to about 1500 nM may be included in the medium. Typical concentrations for selection are about 500 to about 1000 nM.

In a preferred embodiment of the invention the negative selection marker is an *ahas* genes conferring resistance against sulfonylurea- and/or imidazolinone-type herbicides. Said *ahas* genes may be the XI12 mutant ahas2 gene. XI12 mutant maize lines with the mutated *ahas2* gene demonstrate to be highly resistant to imazethapyr (PURSUIT™), but sensitive to imazaquin (SCEPTER™) and susceptible to sulfonylurea herbicides. XI12 *ahas2* gene isolated from the mutant maize line coupled with selection on imidazolinone herbicide has been used successfully in the art for transformation of corn, rice and wheat (US 6,653,529). Selection for constructs comprising a XI12 mutant *ahas* selection marker can be carried out for example with PURSUIT™. Another suitable ahas gene is the XA17 *ahas* mutant gene. Maize XA17 mutants demonstrates to be highly resistant to both imazethapyr and imazaquin (SCEPTER™), and slightly tolerant to sulfonylurea herbicides. As the maize XA17 gene confers differential tolerance to different imidazolinone compounds and the sulfonylurea herbicides, it can be used as a selectable marker in plant transformation with the choice of using imazethapyr, imazaquin or sulfonylurea as selective reagent. The mutated XA17 *ahas* gene and its promoter can be isolated from XA17 mutant line. The sequence was isolated and the gene characterized (Bernnasconi 1995). The XA17 mutant and its phenotype has be described (US 4,761,373; US 5,304,732; Anderson & Gregeson 1989; Currie 1995; Newhouse 1991). Selection can be carried out with the SCEPTER™ herbicide or sulfonylurea compound for selection. In consequence the combination of the various *ahas* mutants allows for efficient gene stacking providing a mechanism for double transformation.

Preferably said second marker is conferring resistance against at least one compound select from the group consisting of phosphinotricin, glyphosate, sulfonylurea- and imidazolinone-type herbicides. More preferably, said second marker gene is an ahas resistance gene, most preferably conferring resistance against a compound selected from the group of XI12 ahas mutant genes and XA17 ahas mutant genes.

Another embodiment of the invention relates to a maize plant comprising

a) a first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, and
b) a second expression construct for a selection marker gene, which is not conferring resistance against D-alanine or D-serine.

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, sulfonylurea- and imidazolinone-type herbicides.

The following combinations are especially preferred:

1) A first transformation with an ahas selection marker gene followed by a second transformation with a dsdA selections marker gene;
2) A first transformation with an ahas selection marker gene followed by a second transformation with a dao1 selection marker gene;
3) A first transformation with a dsdA selection marker gene followed by a second transformation with an ahas selection marker gene;
4) A first transformation with a dao1 followed by a second transformation with an ahas selection marker gene;

Beside the stacking with a second expression construct for a selection marker gene, which is not conferring resistance against D-alanine or D-serine, also the dsdA and dao1 genes can be stacked. For example a first selection can be made using the dsdA gene and D-serine as a selection agent and a second selection can be subsequently made by using dao1 gene and D-alanine as selection agent. Thus another embodiment of the invention relates to a method for subsequent transformation of at least two DNA constructs into a Zea mays plant comprising the steps of:

a) a transformation with a first construct said construct comprising an expression construct comprising a plant promoter (preferably a ubiquitin promoter as defined above) and operably linked thereto a nucleic acid sequence encoding a dsdA enzyme and selecting with D-serine, and
b) a transformation with a second construct said construct comprising an expression construct comprising a plant promoter (preferably a ubiquitin promoter as defined above) and operably linked thereto a nucleic acid sequence encoding a dao enzyme and selecting with D-alanine.

Sequences

1. SEQ ID NO: 1	Nucleic acid sequence encoding E.coli D-serine dehydratase [dsdA] gene
2. SEQ ID NO: 2	Amino acid sequence encoding E.coli D-serine dehydratase [dsdA]
3. SEQ ID NO: 3	Nucleic acid sequence encoding Rhodosporidium toruloides D-amino acid oxidase gene
4. SEQ ID NO: 4	Amino acid sequence encoding Rhodosporidium toruloides D-amino acid oxidase
5. SEQ ID NO: 5	Nucleic acid sequence encoding Zea mays ubiquitin core promoter region
6. SEQ ID NO: 6	Nucleic acid sequence encoding Zea mays ubiquitin promoter further comprising 5'-untranslated region and first intron

7. SEQ ID NO: 7 Nucleic acid sequence encoding Sugarcane bacilliform virus 275 bp core promoter

8. SEQ ID NO: 8 Nucleic acid sequence encoding Sugarcane bacilliform virus promoter

9. SEQ ID NO: 9 Nucleic acid sequence encoding T-DNA region of construct RLM175

10. SEQ ID NO: 10 Nucleic acid sequence encoding T-DNA region of construct RLM151

11. SEQ ID NO: 11 Amino acid sequence encoding E.coli D-serine dehydratase [dsdA]

12. SEQ ID NO: 12 Nucleic acid sequence encoding T-DNA region of construct RLM255

13. SEQ ID NO: 13 Amino acid sequence encoding Rhodosporidium toruloides D-amino acid oxidase

14. SEQ ID NO: 14 Nucleic acid sequence encoding T-DNA region of construct RLM220

15. SEQ ID NO: 15 Nucleic acid sequence encoding T-DNA region of construct RLM242

16. SEQ ID NO: 16 Amino acid sequence encoding Rhodosporidium toruloides D-amino acid oxidase

17. SEQ ID NO: 17 Nucleic acid sequence encoding T-DNA region of construct RLM241

18. SEQ ID NO: 18 Amino acid sequence encoding Rhodosporidium toruloides D-amino acid oxidase

19. SEQ ID NO: 19 Nucleic acid sequence enconding T-DNA region of construct RLM240

20. SEQ ID NO: 20 Amino acid sequence encoding E.coli D-serine dehydratase [dsdA]

21. SEQ ID NO: 21 Nucleic acid sequence encoding T-DNA region of construct RLM239

22. SEQ ID NO: 22 Amino acid sequence encoding E.coli D-serine dehydratase [dsdA]

Deposit under the Budapest Treaty

This invention makes reference to a deposit made under the Budapest Treaty in relation to US Provisional Patent Application No. 60/693,321 filed 23.06.2005, herein incorporated by reference, for the following material:

1. Seed of Zea mays line BPS553; Patent Deposit Designation PTA-6170.
2. Seed of Zea mays line BPS631; Patent Deposit Designation PTA-6171.

The deposit was made with the American Type Culture Collection (ATCC), Manassas, VA 20110-2209 USA on August 26, 2004.

Examples

General methods:

Unless indicated otherwise, chemicals and reagents in the Examples were obtained from Sigma Chemical Company (St. Louis, MO). Materials for cell culture media were obtained from Gibco/BRL (Gaithersburg, MD) or DIFCO (Detroit, MI). 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.

Media Recipes

A-1. Maize YP Media (for growing *Agrobacterium*)

Media Components	Final Concentration	500 mL	1 L	2 L
Yeast extract	5 g/L	2.5 g	5 g	10 g
Peptone (from meat)	10 g/L	5 g	10 g	20 g
NaCl	5 g/L	2.5 g	5 g	10 g

Adjust pH to 6.8 with 1 M NaOH. For solid medium add 3 g agar (EM Science) per 250 mL bottle. Aliquot 100 mL media to each 250 mL bottle, autoclave, let cool and solidify in bottles. For plate preparation, medium in bottle is melted in microwave oven, and the bottle is placed in water bath and cool to 55°C. When cooled, add spectinomycin (Sigma S-4014) to a final concentration of 50 mg/L mix well and pour the plates.

A-2. Maize LS-inf Medium

Media Components	Final Concentration	500 mL	1 L	2 L
MS (Murashige and Skoog basal media) M-5524	4.3 g/L	2.15 g	4.3 g	8.6 g
Vitamin assay casamino acids (Difco)	1.0 g/L	500 mg	1.0 g	2 g
Glucose	36 g/L	18 g	36 g	72 g
Sucrose	68.5 g/L	34.25 g	68.5 g	137 g
2,4-D (stock at 0.5 mg/mL)	1.5 mg/L	1.5 mL	3 mL	6 mL
Nicotinic acid (stock 0.5 mg/mL) sterile	0.5 mg/L	0.5 mL	1 mL	2 mL
Pyridoxine HCl (0.5 mg/mL) sterile	0.5 mg/L	0.5 mL	1 mL	2 mL
Thiamine HCl (1.0 mg/mL) sterile	1.0 mg/L	0.5 mL	1 mL	2 mL
Myo-inositol (100 mg/mL) sterile	100 mg/L	0.5 mL	1 mL	2 mL

Adjust pH to 5.2 with 1 M HCl, filter sterilize, dispense in 100 mL aliquots, add acetosyringone (100 µM) to the medium right before used for *Agrobacterium* infection (50 µL to 100 mL media – 200 mM stock).

A-3. Maize 1.5LSAs Medium (for co-cultivation)

Media Components	**Final Concentration**	**500 mL**	**1 L**	**2 L**
MS (Murashige and Skoog basal media) M-5524	4.3 g/L	2.15 g	4.3 g	8.6 g
Glucose	10 g/L	5 g	10 g	20 g
Sucrose	20 g/L	10 g	20 g	40 g
2,4-D (stock at 0.5 mg/mL)	1.5 mg/L	1.5 mL	3 mL	6 mL
Nicotinic acid (stock 0.5 mg/mL) sterile	0.5 mg/L	0.5 mL	1 mL	2 mL
Pyridoxine HCl (0.5 mg/mL) sterile	0.5 mg/L	0.5 mL	1 mL	2 mL
Thiamine HCl (1.0 mg/mL) sterile	1.0 mg/L	0.5 mL	1 mL	2 mL
Myo-inositol (100 mg/mL) sterile	100 mg/L	0.5 mL	1 mL	2 mL
L-proline (stock 350 mg/mL)	700 mg/L	1 mL	2 mL	4 mL
MES (stock 250 mg/mL)	500 mg/L	1 mL	2 mL	4 mL

Adjust pH media to 5.8 with 1 M NaOH, weigh 4 g Sigma Purified Agar per bottle (8g/L) and dispense 500 mL media per bottle, autoclave. When cooled add:

Media Components	**Final Concentration**	**500 mL**
Acetosyringone (stock at 200 mM)	100 µM	250 µL
$AgNO_3$* (stock at 15 mM)	15 µM	500 µL
L-cysteine* (stock at 150 mg/mL)	300 mg/L	1 mL

*optional for BPS553x(HiIIAxA188) genotypes.

Pour the resulting medium into 100 x 20 mm Petri plates (medium containing acetosyringone should be used freshly without long term storage).

A-4. Maize Recovery Medium – 1 : IM medium

Media Components	**Final Conc.**	**500ml**	**1L**	**2L**	**5L**
MS (Murashige and Skoog basal media) M-5524(SIGMA)	4.3g/L	2.15g	4.3g	8.6g	21.5g
Sucrose	30g/L	15g	30g	60g	150g
2,4D(stock 0.5mg/mL)	1.5mg/mL	1.mL	3mL	6mL	15mL
Casein hydrolysate	100mg/L	50/mg	100mg	200mg	500mg
Proline	2.9g/L	1.45g	2.9g	5.8g	14.5g

Measure approx. ¾ of the total volume ddH_2O desired, add sucrose and salts, stirring until dissolved. After all ingredients are dissolved, bring up to the final volume and adjust to pH 5.8 using 1M KOH. Aliquot to 500 mL/1L bottle, add 0.9 g gelrite to each bottle of liquid and label properly. Autoclave for 20 minutes on liquid cycle. After autoclaving place bottles into a water bath to cool to 55°C , and add the following components:

Post autoclaving components	Final Concentration	Volume added to 500 mL medium
MS Vitamins- Sigma	1.0mg/mL	0.5 mL
15 μM silver nitrate (stock 15mM)	15 μM	0.5 mL
Timentin (stock at 200 mg/mL)	150 mg/L	0.5 mL

Pour the resulting media into 100 X 20 mm petri plates under a laminar flow hood and allow the media to remain under the hood overnight to prevent excess condensation.

A-6. Selection Media

Media Components	Final Concentration	500 mL	1 L	2 L
MS (Murashige and Skoog basal media) M-5524	4.3 g/L	2.15 g	4.3 g	8.6 g
Sucrose	20 g/L	10 g	20 g	40 g
2,4-D (stock at 2.0 mg/mL)	0.5 mg/L	0.125 mL	0.25 mL	0.5 mL
Nicotinic acid (stock 0.5 mg/mL) sterile	0.5 mg/L	0.5 mL	1 mL	2 mL
Pyridoxine HCl (0.5 mg/mL) sterile	0.5 mg/L	0.5 mL	1 mL	2 mL
Thiamine HCl (1.0 mg/mL) sterile	1.0 mg/L	0.5 mL	1 mL	2 mL
Myo-inositol (100 mg/mL) sterile	100 mg/L	0.5 mL	1 mL	2 mL
L-proline (stock 350 mg/mL)	700 mg/L	1 mL	2 mL	4 mL
MES (stock 250 mg/mL)	500 mg/L	1 mL	2 mL	4 mL

Adjust pH of media to pH 5.8 with 1 M NaOH. Add Sigma Purified Agar (8g/L), dispense 500 mL media per 1L bottle, autoclave, when cooled add:

Medium type	Post autoclaving components	Final Concentration	500 mL	1 L
S-DS (Selection with D-Serine)	Timentin (stock at 200 mg/ml)	150 mg/L	0.5	1 ml
	Picloram (2 mg/ml)	2 mg/l	1 ml	2 ml
	D-serine (stock at 1 M)	5 mM	2.5	5 ml
		10	5	10
		15	7.5	15
		20	10	20
S-DA (Selection with D-Alanine)	Timentin (stock at 200 mg/ml)	150 mg/l	0.5	1 ml
	Picloram (2 mg/ml)	2 mg/l	1 ml	2 ml
	D-Alanine (stock at 1 M)	2.5 mM	1.25 ml	2 – 10 ml
		5 mM	2.5	5
		10 mM	5	10
		15 mM	7.5	15

A-7. Maize Regeneration Media

Media Components	**Final Concentration**	**500 ml**	**1 L**	**2 L**
MS (Murashige and Skoog basal media) M-5524	4.3 g/L	2.15 g	4.3 g	8.6 g
Sucrose	20 g/L	10 g	20 g	40 g
Nicotinic acid (stock 0.5 mg/mL) sterile	0.5 mg/L	0.5 mL	1 mL	2 mL
Pyridoxine HCl (0.5 mg/mL) sterile	0.5 mg/L	0.5 mL	1 mL	2 mL
Thiamine HCl (1.0 mg/mL) sterile	1.0 mg/L	0.5 mL	1 mL	2 mL
Myo-inositol (100 mg/mL) sterile	100 mg/L	0.5 mL	1 mL	2 mL
L-proline (stock 350 mg/mL)	700 mg/L	1 mL	2 mL	4 mL
MES (stock 250 mg/mL)	500 mg/L	1 mL	2 mL	4 mL

Adjust pH media to 5.8 with 1 M NaOH. Weigh 4 g Sigma Purified Agar per bottle (8g/L). Dispense 500 mL media per bottle, autoclave and let solidify in bottles. For use, microwave to melt media, when cooled, add

Medium type	**Post autoclaving components**	**Final Concentration**	**500 ml**	**1 L**	**2 L**
R-DS (Regeneration with D-Serine)	Timentin (200 mg/mL)	150 mg/L	0.5 mL	1 mL	2 mL
	D-Serine (stock at 1M)	5 mM	2.5 mL	5.0 mL	10 mL
		10 mM	5.0 mL	10.0 mL	20.0 mL
		15 mM	7.5 mL	15.0 mL	30.0 mL
	Zeatin (stock at 5 mg/mL)	2.5 mg/L	0.25 mL	0.5 mL	1 mL
R-DA (Regeneration with D-Alanine)	Timentin (200 mg/mL)	150 mg/L	0.5 mL	1 mL	2 mL
	D-Alanine (stock at 1M)	2 mM	1 mL	2.0 mL	4.0 mL
		5 mM	2.5 mL	5.0 mL	10.0 mL
		10 mM	5.0 mL	10.0 mL	20.0 mL
	Zeatin (stock at 5 mg/mL)	2.5 mg/L	0.25 mL	0.5 mL	1 mL

Pour into 100 x 20 mm Petri plates

A-8. Maize Rooting Media (rooting)

Media Components	**Final Concentration**	**500 mL**	**1 L**	**2 L**
½ MS (Murashige and Skoog basal media) M-5524	2.15g/L	1.08 g	2.15 g	4.3 g
Sucrose	20 g/L	10 g	20 g	40 g
Nicotinic acid (stock 0.5 mg/mL) sterile	0.5 mg/L	0.5 mL	1 mL	2 mL
Pyridoxine HCl (0.5 mg/mL) sterile	0.5 mg/L	0.5 mL	1 mL	2 mL
Thiamine HCl (1.0 mg/mL) sterile	1.0 mg/L	0.5 mL	1 mL	2 mL
Myo-inositol (100 mg/mL) sterile	100 mg/L	0.5 mL	1 mL	2 mL
L-proline (stock 350 mg/mL)	700 mg/L	1 mL	2 mL	4 mL
MES (stock 250 mg/mL)	500 mg/L	1 mL	2 mL	4 mL

Adjust pH of media to pH 5.8 with 1 M NaOH, add 1 g Gelrite per bottle (2g/L), dispense 500 mL media per bottle, autoclave, pour into disposable Phyatrays.

Medium type	Post autoclaving components	Final Concentration	500ml	1 L	2 L
Rt-DS (Rooting with D-Serine)	Timentin (200 mg/mL)	150 mg/L	0.5 mL	1 mL	2 mL
	D-Serine (stock at 1M)	5 mM	2.5 mL	5.0 mL	10 mL
		10 mM	5.0 mL	10.0 mL	20.0 mL
		15 mM	7.5 mL	15.0 mL	30.0 mL
Rt-DA (Rooting with D-Alanine)	Timentin (200 mg/mL)	150 mg/L	0.5 mL	1 mL	2 mL
	D-Alanine (stock at 1M)	2 mM	1 mL	2.0 mL	4.0 mL
		5 mM	2.5 mL	5.0 mL	10.0 mL
		10 mM	5.0 mL	10.0 mL	20.0 mL

A-9. D-Serine stock solution (1 M): 1 M D-Serine (store at room temperature)

A-10. D-Alanine stock solution (1 M): 1 M D-Alanine (store at room temperature)

A-11. Acetosyringone stock (200 mM in DMSO), store at –20C.

2. Summary of the Protocol

This protocol works for both hybrid lines and inbred lines.

Table 2: Summary of tranformation protocol

Transformation phases	Methods	Media used	Conditions
1. Agrobacterium inoculation	1.1 Modified "Tube"	LS-Inf liquid with 100-200 nM Acetosyringone	Dissect immature embryos directly into *Agrobacterium* suspension
	1.2 The "Drop" method	LS-Inf + 100-200 nM Acetosyringone for preparing agrobacterium cell suspension	Dissect immature embryos directly onto agar co-cultivation medium, and apply a drop (ca. 5 µL) of *Agrobacterium* cell suspension (OD600 = 0.5 – 2.0).
2. Co-cultivation		1.5LSAs Medium with 15 mM AgNO3, and 300 mg/L L-cysteine	Incubate culture at 22C in the dark for 1-3 days, typically 2-3 days.
3. Recovery	3.1	MS medium with 150 mg/L timentin, and 15 uM AgNO3	Incubate cultures at 25-27°C in the dark for 5-7 days
	3.2	IM medium (MS medium with 15 uM AgNO3, 2.7 g proline, 150 mg/L Timentin	Incubate cultures at 25-27°C in dark for 5-7 days
Selection	1st selection	MS medium with 2-15 mM D-Alanine /or 5-20 mM D-Serine, 150 mg/L Timentin, 0.5 mg/L 2,4-D and 2 mg/L Picloram	Incubate cultures at 25-27°C in dark for 14 days
	2nd selection	Same as 1st selection	Incubate cultures at 25-27°C in dark for 14 days
Regeneration		MS medium with 5 -10 mM D-Alanine /or 15 mM D-Serine, 2.5 mg/L Zeatin and 150 mg/L Timentin	Incubate cultures at 25-27C in light for 14 days
Rooting		1/2MS medium with 5 -10 mM D-Alanine /or 15 mM D-Serine	Incubate cultures at 25-27C in light for 14 days

Example 1: Basic Transformation Protocol

1.1 Preparation of hybrid donor plants

The method of the inventions works equally for inbred and hybrid lines and varieties of various genotypes of Zea mays. The following *Zea mays* inbred lines are employed for the following steps:

1. HiIIA: Hill parent A; deposit No.:T0940A, Maize Genetics and Genomics Database), available from Maize Genetics Cooperation - Stock Center USDA/ARS & Crop Sci/UIUC, S-123 Turner Hall, 1102 S. Goodwin Avenue, Urbana IL USA 61801-4798; http://www.maizegdb.org/stock.php.
2. A188: Agronomy & Plant Genetics, 411 Borlaug Hall, Univ of Minnesota, Saint Paul MN 55108.
3. BPS533 (ATCC Patent Deposit Designation PTA-6170)
4. BPS631 (ATCC Patent Deposit Designation PTA-6171)

F1 seeds of corn genotype HiIIAxA188 are produced by crossing HiIIA (female parent) with inbred line A188 (male), and planted in the greenhouse as pollen donor. F2 seeds of (HiIIAxA188) are produced by self-pollination of F1 (HiIIAxA188) plants either in the greenhouse or in the field, and planted in the greenhouse as the pollen donor. Hybrid immature embryos of BPS553x(HiIIAxA188) or BPS631x(HiIIAxA188) are produced using inbred line BPS553 (ATCC Patent Deposit Designation PTA-6170) or BPS631 (ATCC Patent Deposit Designation PTA-6171) as the female parents, and either F1 or F2 (HiIIAxA188) plants as the male parent in the greenhouse.

Two seeds are sowed in pots containing Metromix. Once the seeds become germinated and rooted, one seedling/pot is maintained for immature embryo production, and the second seedling is discarded; Alternatively seeds are started in a 4x4 inch pots, and seedlings are transplanted to 10-inch pots two weeks after sowing the seeds. Approximately one tablespoon of Osmocote 14-14-14 (a type of slow releasing fertilizer) is added to the surface of each pot. The temperature in the greenhouse is maintained at 24°C night and 28°C day. Watering is done automatically, but is supplemented daily mannually as needed. Twice a week, the plants are watered with a 1:15 dilution of Peters 20-20-20 fertilizer. Routine insect and disease managements is performed.

1.1.1 Preparation of inbred donor plants

Seeds of inbred lines BPS553 or BPS631 are sown either directly in 4-inch pots, and the seedlings are transplanted to 10-inch pots two weeks after sowing the seeds. Alternatively, seeds are directly sown into 10-inch pots. Self- or sib-pollination is performed. The growing conditions are same as above for the hybrid line.

1.1.2 Hand-Pollination

Every corn plant is monitored for ear shoots, and when appeared, they are covered with a small white ear shoot bag (Lawson). Once the ear shoots have started to produce silks, the silks are cut and covered again with the ear shoot bag. The tassel of the same plant is bagged with a brown paper bag (providing that the tassel has entered anthesis). The next morning, the tassel is shaken to remove pollen and anthers into the bag. The bag is then removed and pollen is shaken over the silks of the ear shoot. Pollinating is done between 8 and 10 a.m. in the morning. Secure the brown paper bag

over the ear shoot and around the corn stalk. After pollination, the tassel is removed from the plant to reduce pollen (allergens to many people) in the greenhouse.

To ensure synchronized pollinations for the same genotypes, and hence to avoid week-end harvesting/transformation, ear shoots of those early flowering plants are cut back again. A group of plants, e.g. > 5 to 10 plants are then pollinated on the same day. For example, if the planned transformation date is August 19, 2002, the ideal pollination date, therefore, is around August 9 – 10, 2002. Ear shoots that are ready before August 9, 2002, (e.g. August 7, or 8) should be cut back. However, this practice is dependent on the quality/quantity of pollens on a plant. Sib-pollination is needed for the inbred lines. For instance either BPS553 or BPS631 can be either selfed or sib-pollinated between the same genotype).

1.1.3 Harvest and Pre-treat Ears

Ears from corn plants (the first ear that comes out is the best) are harvested 8 to 14 (average 10) days after pollination (DAP). Timing of harvest varies depending on growth conditions and maize variety. The size of immature embryos is a good indication of their stage of development. The optimal length of immature embryos for transformation is about 1 to 1.5 mm, including the length of the scutellum. The embryo should be translucent, not opaque. Immature embryos with size larger than 2.0 mm should preferably not be used in Hill genotypes. If the ear is ready, but can not be used for transformation that day, the ear can be harvested, put in the pollination bag, and stored in a plastic bag in a 4°C refrigerator for 1 to 3 days.)

1.2 Preparation of *Agrobacterium*

Agrobacterium glycerol stock is stored at –80°C. Inoculums of *Agrobacterium* are streaked from glycerol stocks onto YP agar medium (A-1) containing appropriate antibiotics (e.g. 50 mg/L spectinomycin and/or 10 mg/l tetracycline). The bacterial cultures are incubated in the dark at 28°C for 1 to 3 days, or until single colonies are visible (it normally take 2 days to grow agro cultures directly from –80C freezer). The obtained plate can be stored at 4°C for 1 month and used as a master plate to streak out fresh cells. Fresh cells should be streaked onto YP agar with the appropriate antibiotic from a single colony on the master plate, at least 2 days in advance of transformation. These bacterial cultures can be incubated in the dark at 28°C for 1 to 3 days.

Alternatively frozen *Agrobacterium* stock can be prepared: Streak *Agrobacterium* cells from frozen stock either to a plate B-YP-002 (YP+50 mg/l spectinomycin + 10 mg/l tetracycline) or to a plate YP/or LB medium with 50 to 100 mg/l kanamycin, depending on the bacterial selection marker genes on the plasmid. Grow at 28°C for 2 to 3 days. Save it as master plate and store at 4C for up to a month. From the master plate, streak a loop of agro cells into a flask containing 25 ml liquid B-YP-000 medium supplemented with 50 mg/L Spectinomycin plus 10 mg/L tetracycline or 50-100 mg/L kanamycin, respectively. Grow on a shaker set at 300 rpm and 28°C 2 to 3 days. Prepare frozen agro stock by mixing 1 part of the above agro culture with 1 part of sterile 30% glycerol. Vortex to mix well and dispense 10 µL the *Agrobacterium*/glycerol mixture to a 50 µL Eppendorf tube. Store at –80°C.

One to two loops full (2 mm in diameter) of bacterial culture is suspended in 1.0 to 1.8 ml LS-inf medium supplemented with 100 µM acetosyringone. This yields a bacterial suspension with approximate optical density (OD_{600}) between 0.5 to 2.0. Vortex for 0.5 to 3 hours. Vortexing is performed by fixing (e.g. with tape) the microfuge tube horizontally (instead of vertically) on the platform of a vortexer to ensure better disperse *Agrobacterium* cells into the solution. Mix 100 µl of *Agrobacterium* cell suspension with 900 uLof LS-inf solution in a cuvet, and measure OD_{600}. Adjust OD of original *Agrobacterium* solution to 0.6 to 2.0 with LS-Inf (with 100 µM acetosyringone) solution. The *Agrobacterium* suspension is preferably vortexed in the LS-inf + acetosyringone media for at least 0.5 to 3 hours prior to infection. Prepare this suspension before starting harvesting embryos.

Alternatively *Agrobacterium* suspensions for corn transformation can be prepared as follows: Two days before transformation, from –80°C stock, streak *Agrobacteria* from one tube to a plate containing B-YP-002 (solidified YP+50 mg/L spectinomycin plus 10 mg/l tetracycline, or 50-100 mg/L kanamycin, respectively, depending the bacterial selection marker used) and grow at 28°C in the dark for two days. About 1 to 4 hrs before transformation, place one scoop of bacterial cells to 1.5 ml M-LS-002 medium (LSinf + 200 µM acetosyrigone) in a 2 mL Eppendorf tube. Vortex the tube to dispense the bacterial cells to solution and shake the tube at 1,000 rpm for 1 to 4 hrs. The OD_{600} should be in the range of 0.6 to 1.0 or about 10^8cfu/mL.

For the purpose of the following examples Agrobacterium tumefaciens strain LBA4404 or disarmed Agrobacterium strain K599 (NCPPB 2659)) transformed with binary vector plasmid pBPSMM232 were employed. pBPSMM232 contains the *ahas* gene (as selection marker) and the gus reporter gene.

1.3 Isolation of immature embryos

1.3.1 Surface sterilization

The ears are harvested from the greenhouse 8 to12 days after pollination. All husk and silks are removed and ears are transported in the brown pollination bag back to the tissue culture lab. The cob is moved into the sterile hood. A large pair of forceps is inserted into the basal end of the ear and the forceps are used as a handle for handling the cob. Optionally, when insects/fungus are present on the ear, the ear should be first sterilized with 20% commercial bleach for 10 min (alternatively 30% Clorox solution for 15 min), and then rinsed with sterilized water three times. While holding the cob by the forceps, the ear is completely sprayed with 70% ethanol and then rinsed with sterile ddH_2O.

1.3.2 Preparation and *Agrobacterium* inoculation of immature embryos

1.3.2.1 Method-1: The Modified "Tube" method

The cob with the forceps handle is placed in a large Petri plate. A dissecting scope may be used. The top portion (2/3's) of kernels are cut off and removed with a #10 scalpel (for safety consideration, the cut on the kernels is made by cutting away from your hand that holds the handle of the forceps). The immature embryos are then excised from the kernels on the cob with a scalpel (#11 or #15 scalpel): the scalpel blade is inserted on an angle into one end of the kernel. The endosperm is lifted upwards; the

embryo is lying underneath the endosperm. The excised embryos are collected in a microfuge tube (or a small Petri plate) containing roughly 1.5 to 1.8 ml of *Agrobacterium* suspension in LS-inf liquid medium containing acetosyrigone (see above; A-2). Each tube can contain up to 100 embryos. The tube containing embryos is hand-mixed several times, and let the tube/plate stand at room temperature (20 to 25°C) for 30 min. Remove excess bacterial suspension from the tube/plate with a pipette. Transfer the immature embryos and bacteria in the residue LS-inf medium to a Petri plate containing co-cultivation agar medium. Transfer any immature embryos that remain in the microfuge tube by a sterile loop. Remove excess bacterial suspension with a pipette. Place the immature embryos on the co-cultivation medium with the flat side down (scutellum upward). Do not embed the embryos into medium. Leave the plate cover open in the sterile hood for about 15 min for evaporating excess moisture covering immature embryos (air-drying). Seal the Petri dishes with 3M micropore tape. About 100 embryos can be placed on a Petri plate for co-cultivation. Seal the plate and wrap with a sheet of aluminum foil. Incubate the plates in the dark at 22°C for 2 to 3 days. Take 3 to 5 immature embryos for GUS staining if a GUS construct is used to assess transient GUS expression.

1.3.2.2 Method-2: The "Drop" method

Excised immature embryos are directly put on the co-cultivation medium (see medium A-3) with the flat side down (scutellum upward). Each plate (20x100 mm plate) can hold up to 100 immature embryos. Apply 5 µl of diluted *Agrobacterium* cell suspension to each immature embryo with a repeat pipettor. Remove excess moisture covering immature embryos by leaving the plate cover open in the hood for about 15 min. Seal the plate with 3M micropore tape and wrap with aluminum foil. Incubate the plate in the dark at 22°C for 2 to 3 days. Take 3-5 immature embryos for GUS staining if a GUS construct is used to assess transient GUS expression.

1.4 Recovery

After co-cultivation, transfer the embryos to recovery media (A-4 or A-5) and incubate the plates in dark at 27°C for about 5 to 7 days. Keep scutellum side up and do not embed into the media.

1.5 Selection

Transfer immature embryos to 1st selection media (A-6). Roughly 25 to 50 immature embryos can be placed on each plate. Be careful to maintain the same orientation of the embryos (scutellum up). Do not embed the embryos in the media. Seal the Petri plates with 3M micropore tape. Incubate in the dark at 27°C for 10 to14 days (First selection). Subculture all immature embryos that produce variable calli to 2nd selection media (A-6). Try to avoid transferring slimy or soft calli. At this stage, use scissors to remove any shoots that have formed (try to remove the entire embryo from the scutellum if possible and discard it). Firmly place the callus on the media – do not embed into the media. Wrap the plates in 3M Micropore tape and put in the dark at 27°C. Incubate for 2 weeks under the same conditions for the first selection (Second selection). Using 2 pairs of fine forceps, excise the regenerable calli from the scutellum under a stereoscopic microscope. The regenerable calli is whitish/yellowish in color, compact, not slimy and may have some embryo-like structures. Transfer calli to fresh 2nd selection media (A-6), wrap in 3M Micropore tape and incubate in the dark at 27°C for 2 weeks.

Firmly place the callus on the media – do not embed into the media. Be careful to group and mark the calli pieces that came from the same embryo.

1.6 Regeneration of transformed plants

Excise the proliferated calli (whitish with embryonic structures forming), in the same manner as for 2nd selection and transfer to regeneration media (A-7) in 25x100 mm plates. Firmly place the callus on the media – do not embed into the media. Wrap the plates in 3M Micropore tape and put in the light at 25 or 27°C. Be careful to group the calli pieces that came from the same embryo and number them by embryo.

Incubate under light (ca. 2,000 lux; 14/10hr light/dark) at 25 or 27°C for 2 to 3 weeks, or until shoot-like structures are visible. Transfer to fresh regeneration media if necessary. Transfer calli sections with regenerated shoots or shoot-like structures to a Phytatray or Magenta boxes containing rooting medium (A-8) and incubate for 2 weeks under the same condition for the above step, or until rooted plantlets have developed. After 2 to 4 weeks on rooting media, transfer calli that still have green regions (but which have not regenerated seedlings) to fresh rooting Phytotrays. Seedling samples are taken for TaqMan analysis to determine the T-DNA insertion numbers.

1.7 Transgenic plants in the greenhouse

Transfer rooted seedlings to Metromix soil in greenhouse and cover each with plastic dome for at least 1 week, until seedlings have established. Maintain the plants with daily watering, and supplementing liquid fertilizer twice a week. When plants reach the 3 to 4 leaf-stages, they are fertilized with Osmocote. Survived plants are transplanted into 10" pots with MetroMix and 1 teaspoon Osmocote™.

At the flowering stage, the tassels of transgenic plants are bagged with brown paper bags to prevent pollen escape. Pollination is performed on the transgenic plants. It is best to do self-pollination on the transgenic plants. If silking and anthesis are not synchronized, a wild-type pollen donor or recipient plant with same genetic background as the transgenic T_0 plant should be available for performing cross-pollination. T_1 seeds are harvested, dried and stored properly with adequate label on the seed bag. After harvesting the transgenic T_1 seeds, T_0 plants including the soil and pot should be bagged in autoclave bags and autoclaved (double bagging).

Example 2: Transformation vectors used for evaluating dsdA and dao1 genes

Several transformation vectors were made containing either dsdA or dao 1 gene. A construct comprising the *ahas* selection marker was used for comparison purpose in the transformation experiments (Table 3).

Table 3 Description of transformation vectors used for the experiments in establishing transformation with *dsdA* and *dao1* genes as the selection marker. (EcdsdA = E.coli dsdA; dao1 = D-Amino acid oxydase gene; p-ScBV = ScBV promoter; p-Ubi = maize ubi promoter; t-OCS3' = OCS3' terminator; t-NOS = nos terminator; PsFed1 = translational leader sequence)

Vector	LB-Selection marker	Reporter/Selection marker-RB
MM232	p-Ubi::*ahas*::ahas-3'	p-Ubi::gus::t-NOS
LM151	p-Ubi::*EcdsdA*:: t-OCS3'	
LM166	p-Ubi::*EcdsdA*::t-OCS3'	p-ScBV-p::*gus(I-PIV2)*::t-NOS
LM179	p-ahas::ahas::ahas-3'	p-Ubi::*EcdsdA*::t-OCS3
LM198	p-Ubi::*dao1*/ko:: t-OCS3'	p-ScBV::*ZsGreen*::t-NOS
LM199	p-Ubi::PsFed1::*dao1*/ko::t-OCS3'	p-ScBV::*ZsGreen*::t-NOS
LM205	p-Ubi::*dao1*::t-OCS3'	p-ScBV::*gus*::t-NOS
LM221	p-Ubi:I-PsFed1:*EcdsdA*:: t-OCS3'	p-ScBV::*gus*::t-NOS
LM222	p-Ubi::*EcdsdA*/KO:: t-OCS3'	p-ScBV::*gus*::t-NOS
LM223	p-Ubi:I-PsFed1:*EcdsdA*/KO:: t-OCS3'	p-ScBV::*gus*::t-NOS
LM224	p-Ubi:I-PsFed1:*dao1*:: t-OCS3'	p-ScBV::*gus*::t-NOS
LM225	p-Ubi::*dao1*/KO:: t-OCS3'	p-ScBV::*gus*::t-NOS
LM226	p-Ubi:I-PsFed1:*dao1*/KO:: t-OCS3'	p-ScBV::*gus*::t-NOS
LM227	p-Ubi::*EcdsdA*::t-OCS3'	p-ScBV::*DsRed2*::t-NOS
LM228	p-Ubi::*dao1*::t-OCS3'	p-ScBV::*DsRed2*::t-NOS
LM239	p-ahas:: *EcdsdA*::t-NOS	
LM240	p-ScBV:: *EcdsdA*::t-NOS	
LM241	p-ahas:: *dao1*::t-NOS	
LM242	p-ScBV::*dao1*::t-NOS	
LM255	p-Ubi::*dao1*:: t-OCS3'	

Example 3: Establishing kill curves with D-Serine and D-Alanine

In order to establish effective concentrations of D-Serine and D-Alanine on inhibiting growth of tissue cultured corn cells, a bioassay system using immature embryos was applied. Immature embryos 2 mm in length were dissected onto germination medium with the selection agents, and incubated at 27°C in light. The lengths of the shoot and primary root of a germinated seedling were measured 7 days after embryo dissection (Figure 1A and 1B).

In the immature embryo germination inhibition experiment, significant growth inhibition occurs when the concentrations of D-Alanine higher than 2 mM, especially reaching 10 mM (Fig. 1A). Similar results point out that concentration of D-Serine higher than 9 mM cause pronounced growth inhibition (Figure 1B). Further testing of growth inhibition with both D-Serine and D-Alanine in culture callus tissue confirmed the effective inhibitory concentrations of these D-amino acids (Data not shown).

Example 4: Effect of D-Serine concentrations in selection media on the transformation

The effect of D-Serine concentrations in the selection media on transformation efficiencies was determined using transformation vector LM179 (see Table 3). Transformation efficiencies of above 20% were obtained with D-Serine concentration ranges between 5 to 15 mM (Table 3.1). Without adding D-Serine in the selection medium (0 mM D-Serine), every piece of calli in culture was transferred to the regeneration medium with 15 mM D-Serine selection, and therefore, the non-transgenic calli were killed at this step. With 20 mM D-Ser in the selection medium, transformation efficiency appears to

be reduced (with one replicate) to 2% (Table 3.1). Therefore, D-Ser concentrations ranging from 5 to 15 were applied in our transformation protocol.

Table 4 Effect of D-Serine (D-Ser) concentrations in the selection media on the transformation efficiencies in the maize genotype J553x(HiIIAxA188). The vector LM179 was used in the experiments. Data were derived from four different experiments. The putative transgenic calli from 0 mM D-Ser control were transferred to the regeneration medium with 10 mM D-Ser, and the survived plants were confirmed by Taqman assay.

D-Ser (mM)	# Replicates	# Ies	TE (%) ± Std Error
0	1	41	22.0
5	3	125	20.8±17.8
10	4	196	33.5±9.1
15	2	84	26.5
20	1	45	2.2

Example 5: Transformation comparison with *ahas* and *dsdA* as the selection markers

Similar transformation efficiencies were obtained with two selection regimes: *dsdA*/10 mM D-Serine and *ahas*/750 nM Pursuit with both LM179 and MM232 vectors (Table 4). The vector LM179 contains both *ahas* and *dsdA* selection markers while MM232 contains only *ahas* marker (Table 3). Transformation results indicate that dsdA selection marker provides the same transformation efficiency as the ahas selection marker.

Table 5 Comparison of transformation efficiencies with two selection systems: *dsdA*/D-Serine and ahas/Pursuit with the vector LM179 (Table 3) in J553x(HiIIAxA188) genotype, using MM232 as the control transformation vector.

Vector	Selection	# Rep	# IEs	TE (%) ± Std Error
MM232	750 nM Pursuit	8	364	41.7± 18.1
LM179	10 mM D-Ser	9	466	40.7±14.6
LM179	750 nM Pursuit	6	331	44.5±11.1

Example 6: Establishing transformation with *dao1* gene and the effect of translation leader sequence (PsFed1) on transformation

Similar transformation efficiencies were obtained with LM198 and LM199 (see Table 3) vectors, indicating no significant effect of the translation leader sequence in front of the coding sequence observed in the genotype J553x(HiIIAxA188) (Table 6 ~~5~~). There was a tight selection with 10 mM D-Ala in the selection, regeneration and rooting media since there was no escape when immature embryos were infected with *Agrobacterium* strain containing MM232 and selected on 10 mM D-Ala (Table 6). Furthermore, combination of codon-optimized *dao1* gene and the translational leader sequence (PsFed1, vector LM226) did not yield higher transformation efficiency in the maize model line (Table 7). On the other hand, there are no significant differences in the transformation efficiencies using vectors containing non-optimized dao1 with translational leader (vector LM224) or codon-optimized dao1 without the translational leader (vector LM225). Therefore, the results indicate that codon-optimization of the dsdA and dao1 genes does not provide further enhancement of transformation in the model genotype.

Table 6 The effect of translational leader sequence (PsFed) on the transformation efficiency with *dao1* gene as the selection marker in J553x(HiIIAxA188). Vector LM199 contains codon-optimized *dao1* gene and the translational leader sequence. Vector LM198 contains codon-optimized *dao1* gene without the leader sequence.

Vector	Selection	# Rep	# IEs	TE (%) + Std Error
LM198	10 mM D-Ala	3	179	29.3 + 9.0
LM199	10 mM D-Ala	3	168	29.2 + 5.6
MM232	10 mM D-Ala	1	75	0
MM232	750 nM Pursuit	1	84	53.6

Table 7 Effect of codon-optimization and translational leader sequence on the transformation efficiencies with dao1 gene in J553x(HiIIAxA188). Transformation experiments using vector LM226 containing the codon-optimized dao1 gene with translational leader sequence had the relatively lower efficiency than other two vectors.

Vector	# Reps	# IEs/rep (AVG)	TE%
LM224	7	67	27.8
LM225	7	67	22.6
LM226	7	66	16

Example 7: Effect of two different D-Amino acids, D-Ser and D-Ala on the transformation with *dao1* gene as the selection marker.

The DAO1 protein has a broader range of D-Amino acids, e.g. both D-Ser and D-Ala as the substrates, in contrast to DSDA protein that uses the D-Serine only as the substrate. A comparison experiment, therefore, was conducted to determine the effect of D-Ser and D-Ala on the transformation. Transgenic plants were generated with both D-Ser and D-Ala as the selection agents with similar transformation efficiencies (Table 8). The result indicates that both D-Amino acids work equally well as the selection agents for *dao1* gene constructs in the model genotype.

Table 8 Comparison of using two different D-Amino acids, D-Serine and D-Alanine as ther selection agents on the transformation efficiencies in J553x(HiIIAxA188). Two transformation vectors, LM198 and LM199 were used in this experiment.

Vector	Selection	# IEs	# Events	TE %
LM198	10 mM D-Ala	80	27	33.8
LM198	10 mM D-Ser	84	20	23.8
LM199	10 mM D-Ala	88	21	23.9
LM199	10 mM D-Ser	83	30	36.1

Example 8: Comparison of three selectable markers: *ahas*, *dsdA* and *dao1*

Transformation experiments were conducted to compare transformation efficiencies with three selectable marker/selection agent systems, namely *ahas*/Pursuit; *dsdA*/D-Serine and *dao1*/D-Ala. The average transformation efficiencies of 31%, 42% and 31% were obtained with vectors of MM232/Pursuit, LM166/D-Ser and LM205/D-Ala, respectively. Both D-Ser and D-Ala generated very tight selection since there were no escapes when immature embryos were infected with an Agrobacterium strain containing MM232 vector, and selected either on 10 mM D-Ser or 10 mM D-Ala (Table 9).

Table 9 Comparison of transformation efficiencies with three selection regimes: (1) ahas/750 nM Pursuit; (2) dsdA/10 mM D-Alanine, and (3) dao1/10 mM D-serine.

Construct	Selection	# Rep	# IEs	TE (%) ± Std Error
MM232	750 nM Pursuit	3	71	31.3 ± 4.5
MM232	10 mM D-Ala	3	69	0
MM232	10 mM D-Ser	3	67	0
LM166	10 mM D-Ser	3	165	41.6 ± 15.3
LM205	10 mM D-Ala	3	171	30.8 ± 11.6

Example 9: Re-transformation (Gene stacking) with both dsdA and dao1 genes

Due to the substrate specificity of the dsdA protein (mainly D-Serine), and the broader range of substrates utilized by the dao1 protein, the primary transgenic plants containing the dsdA gene are sensitive to D-alanine, and able to be re-transformed with the dao1 gene using D-Alanine as the selection agent. An experiment of re-transformation was conducted by re-transforming dsdA primary T1 transgenic immature embryos containing LM179 (see Table 3) T-DNA with the construct LM205 (see Table 3) containing dao1 and gus (Table 10).

Because the primary transgenic plants contain both the dsdA and ahas genes, the non-infected immature embryos were able to survive D-serine (Treatment 1) and Pursuit (Treatment 2) selections, but not to survive D-alanine selection (Treatment 3). After infection with an agrobacterium strain containing LM205 plasmid, a positive transgenic event containing both dsdA and dao1 genes was generated, and molecularly confirmed (Table 10 , Treatment 4).

Table 10 Re-transformation experiment for stacking dsdA and dao1 genes. Primary transgenic plants containing LM179 T-DNA (p-Ubi::ahas::ahas

Treatment	Infection with agrobacterium containing LM205	Selection regime	# Immature embryos	# Events survived on selection	Molecularly (GUS staining, PCR) confirmed for
1	No	10 - 15 mM D-Serine	25	17	ahas (+) dsdA(+) GUS (-)
2	No	750 nM Pursuit	25	16	Ahas (+) DsdA (+) GUS (-)
3	No	5 – 10 mM D-Alanine	27	0	NA
4	Yes	5 – 10 mM D-Alanine	73	1	ahas (+) dsdA (+) dao1(+) GUS (+)

Eample 10: Re-transformation of primary transgenic plants containing *ahas* with construct containing *dsdA* or *dao1*

To test the ability of stacking *ahas* with either *dsdA* or *dao1* genes, primary transgenic plants containing *ahas* gene was re-transformed with construct containing dsdA gene. Confirmed transgenic events containing both dsdA and ahas were generated, indicating dsdA and ahas selection markers can be stacked with two selection agents, Pursuit and D-serine.

Gene stacking experiments with ahas (primary events) and dao1 (secondary transformation), and dsdA (primary events)/or dao1 (primary events) with ahas (secondary transformation are in progress at this time.

Table 11 Selection marker stacking experiments between *ahas* and *dsdA.* Selection was performed by using both 750 nM pursuit and 15 mM D-serine (for dsdA constructs).

Experiment ID	**Primary transgenic plants**	**Re-transformation Construct (selection marker)**	**# Immature embryos**	**# Confirmed events**	**Transformation efficiency**
LF030405B	*ahas*	LM166 (dsdA)	66	4	6.1
LF030405D	*ahas*	LM166 (dsdA)	40	5	12.5

Table 12 Evaluation of gene stacking with constructs containing *ahas* and *dao1* selection markers. The primary transgenic material used for the gene stacking experiments were homozygous T2 immature embryos, and contained the mutated *ahas* marker, and re-transformed with a *dao1* construct, LM255 using either D-Serine and/or D-Alanine as the selection agent.

Selection condition	# Experiment	# IEs Infected	# Confirmed dao1 events	TE%
10 mM D-Alanine	2	157	33	21
10 mM D-Serine	1	48	10	21
10 mM D-serine + 4 mM D-Alanine	1	52	10	19

Example 11: D-Serine and D-Alanine spray test in the greenhouse

Experiments were conducted by spraying wild-type corn seedlings (genotype J553x(HillAxA188) with either D-serine or D-alanine in the greenhouse. Four spray dosages of D-serine or D-alanine were tested: 0, 100, 1,000, and 10,000 g/Ac. There were no injury symptoms observed on the plants even with the highest concentration, 10,000 g/ac evaluated (data not shown).

Example 12: Evaluate effect of different promoters on using dsdA or dao1 genes as selection markers in transformation of hybrid (BPS553 x (HillAxA188) and an inbred (BPS553) lines.

Transformation data suggest that the dsdA gene driven by the maize ubiquitin promoter (construct LM151) works more effectively in maize tissue culture, and offers higher transformation efficiency in the hybrid line. There is no transformation efficiency difference observed when dao1 gene is driving either by ScBV (LM242) or maize ubiquitin (LM255) promoter in both the hybrid and inbred lines. Both dsdA and dao1 genes combined with maize ahas promoter (LM239 and LM241) do not yield transgenic events.

Table 13 Summary of transformation experiments conducted on evaluating constructs with different promoters driving dsdA and dao1 genes. Experimental data are pooled based on genotype and constructs.

Construct	Construct description	Genotype*	# IEs**	#Events	TE (%) STDEV**
LM151	p-Ubi::*EcdsdA*::t-OCS3'	A	694	207	30 ± 16.6
LM239	p-ahas::*EcdsdA*::t-NOS	A	398	0	0
LM240	p-ScBV::*EcdsdA*::t-NOS	A	392	0	0
LM241	p-ahas:: *dao1*::t-NOS	A	467	0	0
LM242	p-ScBV::*dao1*::t-NOS	A	489	87	17.8 ± 18.0
LM255	p-Ubi::*dao1*:: t-OCS3'	A	934	122	13.1 ± 8.9
LM151	p-Ubi::*EcdsdA*::t-OCS3'	B	510	11	2.2 ± 2.8
LM239	p-ahas::*EcdsdA*::t-NOS	B	413	0	0
LM240	p-ScBV::*EcdsdA*::t-NOS	B	548	0	0
LM241	p-ahas:: *dao1*::t-NOS	B	691	0	0
LM242	p-ScBV::*dao1*::t-NOS	B	764	24	3.2 ± 6.8
LM255	p-Ubi::*dao1*:: t-OCS3'	B	872	36	4.1 ± 4.1

*Genotype A = BPS553x(HiIIAxA188); B = BPS553. ** # IEs = number of immature embryos infected; TE (%) = transformation efficiency (%); STDEV = standard error of mean.

Example 13. Improve Enzymatic Efficiency of DSDA and DAO1 Proteins

DSDA and DAO1 proteins are modified for improved enzymatic efficiencies through gene shuffling. Synthetic DSDA and/or DAO1 proteins are produced by randomly combining domains derived from DSDA and/or DAO1 DNA sequences with potentially functional DNA fragments from other proteins genes. The resulting chimerical DNA sequences are expressed in the microbial system, e.g. *E. coli* and the proteins are assayed for the enzymatic kinetics.

REFERENCES

The references listed below and all references cited herein are incorporated herein by reference to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

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Claims

1. A method for generating a transgenic *Zea mays* plant comprising the steps of
   a. introducing into a *Zea mays* cell or tissue a DNA construct comprising
      i) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,
      ii) at least one second expression construct conferring to said *Zea mays* plant an agronomically valuable trait, and
   b. incubating said *Zea mays* cell or tissue of step a) on a selection medium comprising D-alanine and/or D-serine and/or a derivative thereof in a total concentration from about 1 mM to 100 mM for a time period of at least 5 days, and
   c. transferring said *Zea mays* cell or tissue of step b) to a regeneration medium and regenerating and selecting *Zea mays* plants comprising said DNA construct.

2. The method of claim 1, wherein the method is comprising the following steps
   a. isolating an immature embryo of a *Zea mays* plant, and
   b. co-cultivating said isolated immature embryo, which has not been subjected to a dedifferentiation treatment, with a bacterium belonging to genus *Rhizobiaceae* comprising at least one transgenic T-DNA, said T-DNA comprising
      i) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,
      ii) at least one second expression construct conferring to said *Zea mays* plant an agronomically valuable trait, and
   c. transferring the co-cultivated immature embryos to a recovering medium, said recovery medium lacking a phytotoxic effective amount of D-serine or D-alanine, and
   d. inducing formation of embryogenic callus and selecting transgenic callus on a medium comprising,
      i. an effective amount of at least one auxin compound, and
      ii. D-alanine and/or D-serine in a total concentration from about 1 mM to 100 mM , and
   e. regenerating and selecting plants containing the transgenic T-DNA from the said transgenic callus.

3. The method of claim 2, wherein the recovery medium of step c) comprises
   i. an effective amount of at least one antibiotic that inhibits or suppresses the growth of the soil-borne bacteria, and
   ii. L-proline in a concentration from about 1 g/l to about 10 g/l, and
   iii. silver nitrate in a concentration from about 1 µM to about 50 µM,
   iv. an effective amount of at least one auxin compound.

4. The method of claim 2 or 3, wherein the effective amount of the auxin compound is equivalent to a concentration of about 0.2 mg/l to about 6 mg/l 2,4-D.

5. The method of Claim 2, wherein the medium employed during co-cultivation comprises from about 1 µM to about 10 µM of silver nitrate and/or from about 50 mg/L to about 1,000 mg/L of L-Cysteine.

6. The method of any of claim 1 to 5, wherein the enzyme capable to metabolize D-alanine or D-serine is selected from the group consisting of D-serine ammonia-lyases (EC 4.3.1.18), D-Amino acid oxidases (EC 1.4.3.3), and D-Alanine transaminases (EC 2.6.1.21).

7. The method of any of claim 1 to 6, wherein the enzyme capable to metabolize D-serine is selected from the group consisting of

i) the *E.coli* D-serine ammonia-lyase as encoded by SEQ ID NO: 2, and

ii) enzymes having the same enzymatic activity and an identity of at least 80% to the sequence as encoded by SEQ ID NO: 2, and

ii) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence described by SEQ ID NO: 1,

and wherein selection is done on a medium comprising D-serine in a concentration from about 1 mM to 100 mM.

8. The method of any of claim 1 to 7, wherein the enzyme capable to metabolize D-serine and D-alanine is selected from the group consisting of

i) the *Rhodotorula gracilis* D-amino acid oxidase as encoded by SEQ ID NO: 4, and

ii) enzymes having the same enzymatic activity and an identity of at least 80% to the sequence as encoded by SEQ ID NO: 4, and

iii) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence described by SEQ ID NO: 3,

and wherein selection is done on a medium comprising D-alanine and/or D-serine in a total concentration from about 1 mM to 100 mM.

9. The method of any of claim 1 to 8, wherein the ubiquitin promoter is the maize ubiquitin promoter.

10. The method of any of claim 1 to 9, wherein the ubiquitin promoter is selected from the group consisting of

a) sequences comprising the sequence as described by SEQ ID NO: 5, and

b) sequences comprising at least one fragment of at least 50 consecutive base pairs of the sequence as described by SEQ ID NO: 5, and having promoter activity in *Zea mays*,

c) sequences comprising a sequence having at least 60% identity to the sequence as described by SEQ ID NO: 5, and having promoter activity in *Zea mays*,

d) sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 5, and having promoter activity in *Zea mays*.

11. The method of any of claim 1 to 10, wherein the ubiquitin promoter is selected from the group consisting of

    a) sequences comprising the sequence as described by SEQ ID NO: 6, and

    b) sequences comprising at least one fragment of at least 50 consecutive base pairs of the sequence as described by SEQ ID NO: 6, and having promoter activity in *Zea mays*,

    c) sequences comprising a sequence having at least 60% identity to the sequence as described by SEQ ID NO: 6, and having promoter activity in *Zea mays*,

    d) sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 6, and having promoter activity in *Zea mays*.

12. The method of claim 1 or 2, wherein the selection of step b) of claim1 or step d) of claim 2 is done using about 3 to about 15 mM D-alanine or about 7 to about 30 mM D-serine.

13. The method of claim 1, 2, or 12, wherein the total selection time under dedifferentiating conditions is from about 3 to 4 weeks.

14. The method of claim 1 or 2, wherein the selection of step b) of claim1 or step d) of claim 2 is done in two steps, using a first selection step for about 5 to 20 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 5 to 20 days.

15. The method of any of claim 1 to 14, wherein introduction of said DNA construct is mediated by a method selected from the group consisting of *Rhizobiaceae* mediated transformation and particle bombardment mediated transformation.

16 The method of claim 15, wherein the *Rhizobiaceae* bacterium is a disarmed *Agrobacterium tumefaciens* or A*grobacterium rhizogenes* bacterium.

17. The method of any of Claim 1 to 16, wherein said *Zea mays* plant, immature embryo, cell or tissue is selected from the group of *Zea mays* plants consisting of inbreds, hybrids, F1 between inbred lines, F1 between an inbred and a hybrid, F1 between an inbred and a naturally-pollinated variety, commercial F1 varieties, any F2 crossing or self-pollination between the before mentioned varieties and the progeny of any of the before mentioned.

18. The method of Claim 17, wherein said Zea mays cell or tissue or said immature embryo is isolated from a cross of a (HiIIA x A188) hybrid with an inbred line selected from the group of which representative seed having been deposited with the American Type Culture Collection under the Patent Deposit Designation PTA-6170 and PTA-6171.

19. The method of claim 1, wherein said method comprises the steps of:
    i) transforming a *Zea mays* plant cell with a first DNA construct comprising
        a) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding a D-amino acid oxidase enzyme, wherein said first expression cassette is flanked by sequences which allow for specific deletion of said first expression cassette, and
        b) at least one second expression cassette suitable for conferring to said plant an agronomically valuable trait, wherein said second expression cassette is not localized between said sequences which allow for specific deletion of said first expression cassette, and
    ii) treating said transformed *Zea mays* plant cells of step i) with a first compound selected from the group consisting of D-alanine, D-serine or derivatives thereof in a phytotoxic concentration and selecting plant cells comprising in their genome said first DNA construct, conferring resistance to said transformed plant cells against said first compound by expression of said D-amino acid oxidase, and
    iii) inducing deletion of said first expression cassette from the genome of said transformed plant cells and treating said plant cells with a second compound selected from the group consisting of D-isoleucine, D-valine and derivatives thereof in a concentration toxic to plant cells still comprising said first expression cassette, thereby selecting plant cells comprising said second expression cassette but lacking said first expression cassette.

20. The method of claim 19, wherein
    a) the ubiquitin promoter is defined as in any of claim 9 to 11, and/or
    b) D-amino oxid oxidases is defined as in claim 6 or 8.

21. A recombinant expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, wherein said promoter is heterologous in relation to said enzyme encoding sequence.

22. The recombinant expression construct of claim 21, wherein
    a) the ubiquitin promoter is defined as in any of claim 9 to 11, and/or
    b) enzyme capable to metabolize D-alanine or D-serine is defined as in any of claim 6 to 8.

23. A DNA construct comprising
    i) at least one first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,
    ii) at least one second expression construct conferring to said *Zea mays* plant an agronomically valuable trait.

24. The DNA construct of claim 23 comprising
    a) a first expression cassette comprising a nucleic acid sequence encoding a D-amino acid oxidase operably linked with a ubiquitin promoter, wherein said first expression cassette is flanked by sequences which allow for specific deletion of said first expression cassette, and
    b) at least one second expression cassette suitable for conferring to said plant an agronomically valuable trait, wherein said second expression cassette is not localized between said sequences which allow for specific deletion of said first expression cassette.

25. The DNA construct of claim 24, wherein said sequences which allow for specific deletion of said first expression cassette are selected from the group of sequences consisting of
    a) recombination sites for a sequences-specific recombinase arranged in a way that recombination between said flanking recombination sites results in deletion of the sequences in-between from the genome, and
    b) homology sequences A and A' having a sufficient length and homology in order to ensure homologous recombination between A and A', and having an orientation which – upon recombination between A and A' – will result in deletion of the sequences in-between from the genome.

26. The DNA construct of claim 24 or 25, wherein said construct comprises at least one recognition site for a sequence specific nuclease localized between said sequences which allow for specific deletion of said first expression cassette.

27. A vector comprising an expression construct of claim 21 or 22, or a DNA construct of any of claim 23 to 26.

28. A transgenic cell or non-human organism comprising an expression construct of claim 21 or 22, a DNA construct of any of claim 23 to 26, or a vector of claim 27.

29. The transgenic cell or non-human organism of claim 28, wherein said cell is a plant cell and/or said organism is a plant.

30. The transgenic cell or non-human organism of claim 28 or 29, wherein said cell is a *Zea mays* plant cell and/or said organism is a *Zea mays* plant.

31. The maize plant of claim 30, wherein said plant was obtained by crossing a (HiIIA x A188) hybrid with an inbred-line selected from the group of which representative seed having been deposited with the American Type Culture Collection under the Patent Deposit Designation PTA-6170 and PTA-6171.

32. A descendant plant of a maize plant of Claim 30 or 31.

33. A hybrid plant produced from a maize plant of any of claim 30 to 32.

34. An inbred plant produced from a maize plant of any of claim 30 to 32..

35. A part of a maize plant of claim 30 to 34.

36. A method for subsequent transformation of at least two DNA constructs into a Zea mays plant comprising the steps of:
    a) a transformation with a first construct said construct comprising at least one expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, and
    b) a transformation with a second construct said construct comprising a second selection marker gene, which is not conferring resistance against D-alanine or D-serine.

37. The method of claim 36, wherein said second marker gene is conferring resistance against at least one compound select from the group consisting of phosphinotricin, glyphosate, sulfonylurea- and imidazolinone-type herbicides.

38. The method of claim 36 or 37, wherein the marker gene is selected from the group of XI12 ahas mutant genes and XA17 ahas mutant genes.

39. The maize plant comprising
    a) a first expression construct comprising a ubiquitin promoter and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, and
    b) a second expression construct for a selection marker gene, which is not conferring resistance against D-alanine or D-serine.

40. A method for subsequent transformation of at least two DNA constructs into a Zea mays plant comprising the steps of:
    a) a transformation with a first construct said construct comprising a expression construct comprising a plant promoter and operably linked thereto a nucleic acid sequence encoding an dsdA enzyme and selecting with D-serine, and
    b) a transformation with a second construct said construct comprising a expression construct comprising a plant promoter and operably linked thereto a nucleic acid sequence encoding a dao enzyme and selecting with D-alanine.

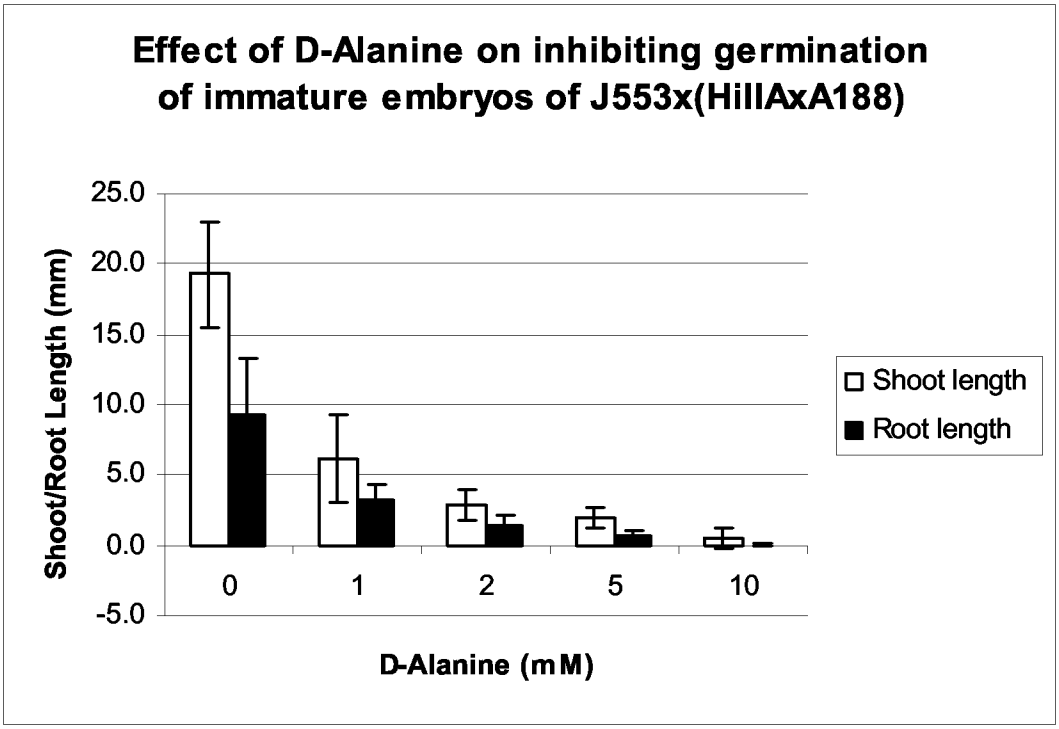
Effect of D-Alanine on inhibiting germination of immature embryos of J553x(HillAxA188)
Shoot/Root Length (mm)
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
0
1
2
5
10
D-Alanine (mM)
Shoot length
Root length

Fig. 1

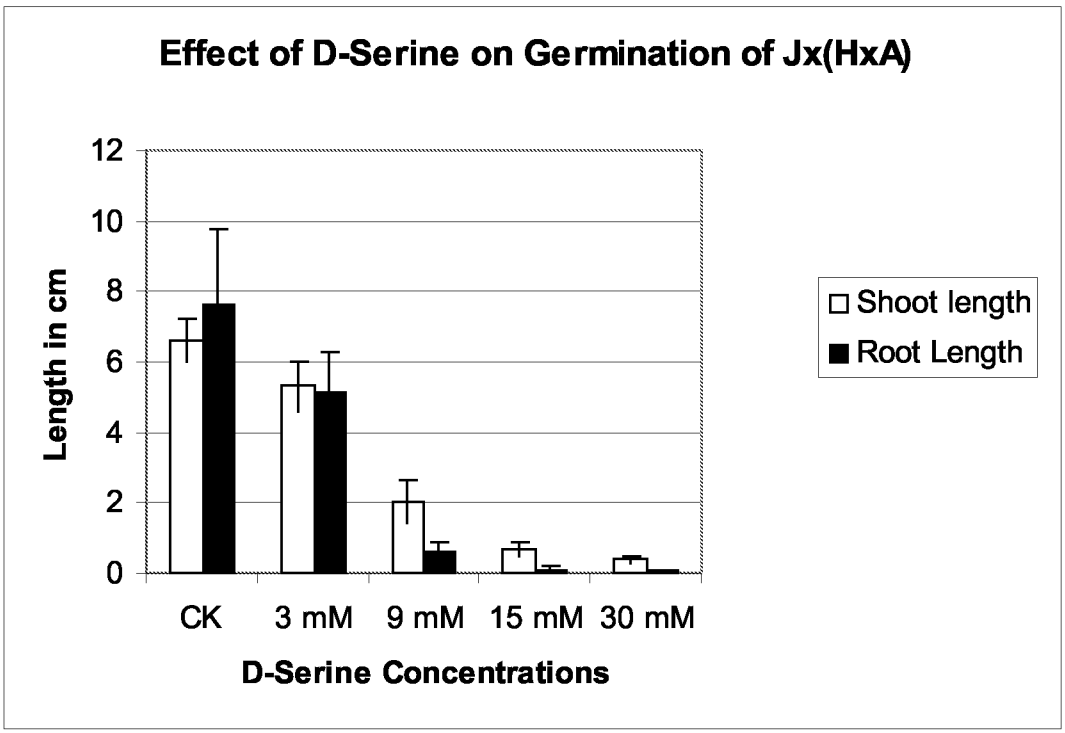
Effect of D-Serine on Germination of Jx(HxA)
Length in cm
12
10
8
6
4
2
0
CK
3 mM
9 mM
15 mM
30 mM
D-Serine Concentrations
Shoot length
Root Length

Fig. 2