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(54) Title: A NEW SELECTION SYSTEM FOR WHEAT

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

WO 2007/031493 A2

A new selection system for wheat

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to improved methods for the incorporation of DNA
5 into the genome of a wheat 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
10 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
15 methods. In the "biolistics" (microprojectile-mediated DNA delivery) method microprojectil 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
20 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 but has recently been adopted to monocot plants (Ishida et al. 1996; WO 95/06722; EP-A1 672 752;
25 EP-A1 0 709 462).

For wheat transformation using microprojectil bombardment has been reported over the decades by several authors (Vasil et al. 1992, 1993, Weeks et al.1993; Becker et al. 1994, Nehra et al.1994, Zhou et al.1995, Altpeter et al. 1996, Ortiz et
30 al.1996, Lazzeri et al. 1997, Barro et al.1998, Witrzens et al.1998, Cheng et al.1998, Bliffeld et al. 1999, Uze et al. 1999, Srivastava et al. 1999, Rasco-Gaunt et al. 2001, Varshney and Altpeter 2001, Huber et al.2002, Pellegrineshi et al. 2002, Rasco-Grunt et al. 2003, Patniaik and Khurana 2004). In all these experiments selection based on herbicides was employed.

Mainly two genes *pat* and *bar* encoding the enzyme phosphinothricin- N-cetyltransferase (PAT) have been used to give tolerance to PPT (glufosinate ammonium) in transgenic wheat plants. The *bar* and *pat* genes confers resistance to the herbicides bialaphos and Basta (Thomson et al.1987; Wohlleben et al. 1988).

5 Two other herbicide resistance genes are CP4 and GOX (encoding glyphosate oxidoreductase from *Achrobacter* sp.; Barry et al.1992 and Kishore et al. 1992). Both CP4 and GOX detoxify glyphosate by converting it to aminomethyl phosphonic acid which is non-toxic to plant cells. These two genes are conferring resistance to glyphosate an active compound in herbicide like Roundup (US 6,689,880)

10 and they have been also used for selecting transgenic wheat plants (Zhou et al.1995).

All of the genes effective as sources of antibiotic resistance and used as selectable marker genes for transgenic plants have got an bacterial origin *Escherichia coli* Tn5 transposon (Fraley et al. 1983, Waldron et al.1985). Aminoglycoside antibiotics are including a number of molecules (e.g. kanamycin, neomycin, gentamycin, derivative G418 and paromycin) which are toxic to plant, fungal and animal cells (Nap et al.1992). Bacterial neomycin phosphotransferase II (*npt II*) was shown to be effective as selectable marker gene in petunia and tobacco (Bevan et al. 1983, Herrera-

15 Estrella et al.1983) and first wheat transgenic plants selected with geneticin G418 were obtained by Nehra et al. (1994). The *E. coli* *hpt* gene (encoding hygromycin phosphotransferase) was efficiently used as selectable marker gene for wheat stable transformation (Ortiz et al. 1996). Transformation of bread wheat (*Triticum aestivum* L.) and pasta wheat (*T. turgidum* ssp.*durum* Desf.) with *npt II* and *bar* gene

25 and application of antibiotic and herbicide selection were published recently (Goodwin et al. 2005). A modified sulphonamide (DHPS) resistance gene as a selectable marker in wheat was used by Freeman and Bowden (1998). Another selection agent used in wheat transformation is methotrexate, which targets the enzyme dihydrofolate reductase (DHFR). The *dhfr* gene producing resistance to meth-

30 otrexate has been identified and used as selectable marker gene in wheat transformation experiments (Kemper et al.1992). A cyanamid hydratase (a gene from the soil fungus *Myrothecium verrucaria*, which converts cyanamide into urea, confers resistance to cyanamide in transgenic wheat cells (Weeks 2005; US 6,268,547).

Several attempts to transform wheat via *Agrobacterium*-mediated transformation methods were published in early 1990s using spikelets (Hess et al. 1990), bases of leaves sheath (Deng et al. 1990) or immature embryos (Mooney et al. 1991) but only several kanamycin-resistant calli colonies were obtained. A protocol, which resulted in transgenic wheat plants production, was published by Cheng et al. (1997) and npt II was as selection marker gene. Transgenic wheat plants producing high levels of the osmoprotectant proline were selected on kanamycin (Sawahel & Hassan 2002) and Przetakiewicz et al. (2004), used kanamycin as well as on bar selection with PPT as selection compound. Improved transformation protocol employing some physical factors as desiccation of plant tissue post-*Agrobacterium* infection was disclosed (Cheng et al. 2003). Herbicide selection based on introducing CP4 gene and bar gene via *Agrobacterium* mediated transformations were applied and selecting substrates like glyphosate (Hu et al. 2003) or PPT (Iser et al. 1999, Khana & Daggar 2003, Wu et al. 2003) have been used for selecting transgenic wheat plants. Wheat plants resistant to the imidazolinone herbicide using the maize XI12 mutants of ahas gene was described (U.S. Pat. 6,653,529). Several factors influencing *Agrobacterium* mediated transformation were evaluated (Cheng et al. 2004). Recent developments in wheat transformation are reviewed in Sahrawat et al. 2003 and Jones (2005).

20

Recently a new selection system based on D-amino acids was reported and demonstrated to be effective in *Arabidopsis* (WO 03/060133; Erikson et al. 2004). No use or adoption of this system in monocotyledonous plants such as wheat has been described so far.

25

Multiple subsequent transformations of wheat 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. Thus, selection systems for wheat are essentially restricted to the bar selection system.

30

Accordingly, the object of the present invention is to provide an improved, efficient method for transforming wheat plants based on D-amino acid selection. This objective is achieved by the present invention.

5 SUMMARY OF THE INVENTION

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

- a. introducing into a wheat cell or tissue a DNA construct comprising at least one first expression construct comprising a promoter active in said wheat plant and operable linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine, and
- 10 b. incubating said wheat 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 3 mM to about 100 mM for a time period of at least 5 days
- 15 (preferably at least 14 days), and
- c. transferring said wheat cell or tissue of step b) to a regeneration medium and regenerating and selecting wheat plants comprising said DNA construct.

In one preferred embodiment the DNA construct introducing into said wheat cell or tissue further comprises at least one second expression construct conferring to said wheat plant an agronomic valuable trait. However also other genes (e.g., reporter genes) can be transformed into the wheat plant in combination with the expression cassette for the enzyme capable to metabolize D-alanine and/or D-serine (i.e., the selectable marker).

25

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

- 30 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,
- 5 and wherein selection is done on a medium comprising D-serine in a concentration from about 3 mM to about 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

- 10 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
- 15 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 3 mM to about 100 mM.

The promoter active in said wheat plant is preferably an ubiquitin promoter, more preferably a monocot ubiquitin promoter, most preferably a maize ubiquitin 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
- 25 pairs of the sequence as described by SEQ ID NO: 5, and having promoter activity in wheat,
- 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 wheat,
 - d) sequences comprising a sequence hybridizing to the sequence as described
- 30 by SEQ ID NO: 5, and having promoter activity in wheat.

The sequence described by SEQ ID NO: 5 is the core promoter of the maize 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 maize 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
- 5 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 wheat,
 - 10 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 wheat,
 - d) sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 6, and having promoter activity in wheat.
- 15 In one preferred embodiment of the invention the selection of step b) is done using about 3 mM to about 15 mM D-alanine or about 3mM to about 30 mM D-Serine. The total selection time under dedifferentiating conditions is from about 3 to 4 weeks.
- 20 More preferably, the selection of step b) is done in two steps, using a first selection step for about 14 to about 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 about 14 to about 20 days.
- Various methods can be employed to introduce the DNA constructs of the invention
- 25 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 *Agrobacterium rhizogenes* bacterium strains.
- 30 In another preferred embodiment the soil-borne bacterium is a disarmed strain variant of *Agrobacterium rhizogenes* strain K599 (NCPBP 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 wheat plant, and
 - b. co-cultivating said isolated immature embryo, which has not been subjected to a dedifferentiate treatment, with a bacterium belonging to genus *Rhizobium* comprising at least one transgenic T-DNA, said T-DNA comprising at least one first expression construct comprising a promoter active in said wheat plant and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,
 - 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 for 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 3 mM to 100 mM, and
 - e. regenerating and selecting plants containing the transgenic T-DNA from the said transgenic callus.
- In one preferred embodiment the T-DNA further comprises at least one second expression construct conferring to said wheat plant an agronomic valuable trait.

In said preferred method the selection of step d) is done using about 3 mM to about 15 mM D-alanine or about 3 mM 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 14 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 about 14 to about 20 days.

- The medium employed in the regeneration step e) is preferably comprising:
- i) an effective amount of at least one cytokinin compound, and/or
 - ii) D-alanine and/or D-serine in a total concentration from about 3 mM to 100 mM.

In said preferred recovery medium of step c) the effective amount of the auxin com-

compound is preferably equivalent to a concentration of about 0.2 mg/l to about 6 mg/l 2,4-D.

Virtually any wheat plant can function as a source for the target material for the transformation. Preferably, said wheat plant, immature embryo, cell or tissue is from a plant selected from the Triticum family group of plants. More preferably, said wheat cell or tissue or said immature embryo is (e.g., isolated) from a plant specie of the group consisting of Triticum spp.: common (T. aestivum), durum (T. durum), spelt (T. spelta), Triticum dicoccum (Emmer wheat), Triticum turgidum, and Triticum monococcum (Einkorn wheat).

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 wheat plant cell with a first DNA construct comprising
 - a) at least one first expression construct comprising a promoter active in said wheat plant 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 wheat 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.

5 The promoter active in wheat plants and/or the D-amino acid oxidase are defined as above.

Another embodiment of the invention relates to a wheat plant or cell comprising a promoter active in said wheat plants or cells 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. Preferably, the promoter and/or the enzyme capable to metabolize D-alanine or D-serine is defined as above. More preferably the wheat plant is further comprising at least one second expression construct conferring to said wheat plant an agronomically valuable trait. In one preferred embodiment the wheat plant selected from the Triticum family group of plants. More preferably from a plant species of the group consisting of Triticum spp.: common (T. aestivum), durum (T. durum), spelt (T. spelta), Triticum dicoccum (Emmer wheat), Triticum turgidum, and Triticum monococcum (Einkorn wheat), most preferably from a variety of Triticum aestivum. Other embodiments of the invention relate to parts, organs, cells, fruits, and other reproduction material of a wheat plant of the invention. Preferred parts are selected from the group consisting of tissue, cells, pollen, ovule, roots, leaves, seeds, microspores, and vegetative parts.

25 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 wheat plant comprising the steps of:

- a) a transformation with a first construct said construct comprising at least one expression construct comprising a promoter active in said wheat plants and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, and
- 30 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. The promoter active in wheat plants and/or the D-amino acid oxidase are defined as above.

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

- a) a first expression construct comprising a promoter active in said wheat plant 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 wheat plant comprising the steps of:

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

The promoter active in wheat plants and/or the D-amino acid oxidase are defined as above. Additional object of the invention relate to the model and the elite varieties of spring, winter and alternative type of wheat. Preferred parts are selected from the group consisting of tissue, cells, pollen, ovule, microspores, inflorescence, roots, leaves, seeds, and meristematic tissues.

DESCRIPTION OF THE DRAWINGS

- Fig. 1 Construct containing dsdA gene: pRLM167.
- Fig. 2 Constructs containing dsdA gene:
- 5 I) pRLM166
M) pRLM 179
III) pRLM 151
- Fig. 3 Constructs containing Dao1 genes:
- 10 I) pRLM205 dao1 original
II) pRLM226 dao1/ko
- Fig. 4 Expression of dsdA gene measured as germination of T1 immature embryos on D-serine containing medium:
- 15 I) Segregation of T1 transgenic and non-transgenic in vitro seedlings
II) Rooted of T1 dsdA transgenic seedlings on selection. Non-transgenic segregants are circled in white in comparison to the transgenic plants
- 20 Fig. 5 Transgenic plants with dsdA/PAT genes have normal phenotype, vigorous growth and full seed set
- Fig. 6 A-B Regeneration on different concentrations D-serine or D-alanine containing medium (Killing curves):
- 25 I) Canon on D-serine
II) BW-56 on D-serine
III) BW-56 on D - alanine
- Fig. 7 Rooting and growth of Canon plants on D-serine containing medium (Killing curves)
- 30 Fig. 8 Germination of non-transgenic immature embryos on D-serine containing medium (Killing curves)

- Fig. 9 Transgenic plant rooted under constant selection pressure (5mM D-serine)
- Fig. 10 Expression of gusINT gene driven by pScBV promoter in endosperm,
5 immature embryos, leaves, roots and anthers
- Fig. 11 Negative selection effect of D-isoleucine on T1 dao1 transgenic seedlings and non-transgenic plants
- 10 Fig. 12 Southern blot analyses of transgenic wheat plants with dsdA gene:
I) Transformants with pRLM166. Genomic DNA was digested with EcoRV and hybridized with 847 bp fragment of dsdA gene. Lines: (1- 3) transgenic TO and T1 event 4; (4-5) transgenic TO and T1 event 5; (6-8) transgenic TO and T1 event 6; (9-11) transgenic TO plants 314,313,315;
15 (12) transgenic TO plant 242; (13) transgenic TO plant 244; (14)) transgenic TO plant 250; (C) Canon non transgenic plant; (M) λ^{HindMI}
- II) Transformants with pRLM151. Genomic DNA was digested with BamHI and hybridized with 847 bp fragment of dsdA gene. Lines: (M) $\lambda^{HindIII}$; (i_2) transgenic TO and T1 event 385; (3-4) transgenic TO and
20 T1 event 406; (C) Canon non transgenic plant
- III) Transformants with pRLM179 and selected on D-serine. Genomic DNA was digested with BamHI and hybridized with 847 bp fragment of dsdA gene. Lines: (M) $\lambda^{HindIII}$; (1-5) transgenic TO and T1 event 2258;
25 (6-10) transgenic TO and T1 event 2256; (11-15) transgenic TO and T1 event 2263 (C) Canon non transgenic plant
- Fig. 13 Southern blot analyses of transgenic wheat plants with dao1 :
30 Transformants with pRLM205. Genomic DNA was digested with BamHI and hybridized with 1156 bp fragment of gus gene. Lines: (M) $\lambda^{HindIII}$; (1-3) transgenic TO and T1 event 51; (5-6) transgenic TO and T1 event 52; (7-9) transgenic TO and T1 event 91; (10-12) transgenic TO and T1 event 26; (13-14) transgenic T1 event 9; (C) Canon non transgenic plant

Fig. 14 D-serine deaminase activity in 19 transgenic lines. DSD activity is defined as Pyrovate produced mM /mg/h. As controls: C - Canon; VC05 - Vector control bar tranagenic plant and DAO1 - transgenic plant with dao1 gene

Fig. 15 Effect of D-serine on transgenic and non transgenic plants grown in hydroponics measured as dray wheat (DW) of 14 days old seedlings:
 I) Transgenic dsdA/ahas T2 progenies from events: 2256, 2258 and 2263;
 II) Transgenic dsdA/gus and dsdA/pat T2 progenies from events: (1) Canon, (2) Event 1; (3) Event 3; (4) Event 4 ; (5) Event 15

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).

5

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, gibberellins, 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", "imRNA", "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 imRNA or protein product, (e.g., promoter sequence, polyadenylation sequence, termination se-

quence, enhancer sequence, etc.). A nucleic acid sequence of interest may preferably encode for an agronomically valuable trait.

5 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.

10 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.

15 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"
20 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
25 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.,
30 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.

10

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.

15

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.

20

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 an agronomically relevant trait) if said two sequences are not combined or differently operably linked in 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

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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 wheat 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 be-

ing set as follows:

Gap Weight: 12

Length Weight: 4

5 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
10 algorithm with the above parameter set, has at least 95% homology. There may be partial homology (i.e., partial identity of less than 100%) or complete homology (i.e., complete identity of 100%).

The term "hybridization" as used herein includes "any process by which a strand of
15 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 T_m of the formed hybrid, and the G:C ratio within the nucleic acids. As used
20 herein, the term " T_m " 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 T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation:
25 $T_m = 81.5 + 0.41 (\% \text{ G+C})$, when a nucleic acid is in aqueous solution at 1 M NaCl [see e.g., Anderson and Young, 1985]. Other references include more sophisticated computations, which take structural as well as sequence characteristics into account for the calculation of T_m .

30 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, Maniatis, *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
5 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
10 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.

15

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,
20 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,
25 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
30 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 condi-

tion 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.

- 5 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, 10 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. 20 25

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 30

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.

20

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.

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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.

5 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
10 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,
15 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
20 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.

25 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
30 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 in-

tended 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.

5

„ 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.

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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 envi-

ronmental 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.

5

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-S' (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 se-

quence that are transcribed along with the coding sequences (exons) but are then removed in the formation of the mature imRNA. 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 imRNA. 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.

10

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 nucleic acid sequence to be expressed, can exist in a vector-integrated form

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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 ex-

hibit 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.

- 5 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.
- 10 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
- 15 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*.
- 20

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).

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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.

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DETAILED DESCRIPTION OF THE INVENTION

A first embodiment of the invention relates to a method for generating a transgenic plant:

- a. introducing into a wheat cell or tissue a DNA construct comprising at least one first expression construct comprising a promoter active in said wheat plant and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine, and
- b. incubating said wheat 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 3 mM to 100 mM for a time period of at least 5 days (preferably at least 14 days), and
- c. transferring said wheat cell or tissue of step b) to a regeneration medium and regenerating and selecting wheat plants comprising said DNA construct.

In one preferred embodiment the DNA construct introducing into said wheat cell or tissue further comprises at least one second expression construct conferring to said wheat plant an agronomic valuable trait. However also other genes (e.g., reporter genes) can be transformed into the wheat plant in combination with the expression cassette for the enzyme capable to metabolize D-alanine and/or D-serine (i.e., the selectable marker).

The invention provides a new selection system for wheat, which offers a minimized escape rate without interfering with embryogenic callus formation and high number of transgenic shoots regeneration in wheat. In addition the selection has a potential

advantage as a selective marker compare to the previously described antibiotic and/or herbicide based systems:

- Defined phenotype of toxicity in in vitro.
 - No toxic for other organisms
- 5 - No selective advantage for transgenic plants in the nature.
- Naturally occurring in bacteria, fungi and animals.

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 wheat is provided herein which allows for efficient *Agrobacterium* - mediated transformation.

10 The plants obtained by the method of the invention were fertile with normal phenotype.

15 Further requirements of the method of the invention are described below. Accordingly, in one embodiment, the method of the invention comprises the introduction of a DNA construct as defined below, further comprises the selection as defined below and/or comprises furthermore the regeneration as defined below.

20 1. The DNA construct

In another embodiment of the invention the DNA construct comprises at least one first expression cassette comprising a promoter active in wheat plants and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine.

25

In one embodiment, the method of the invention comprises the introduction of a second expression cassette, e.g. comprised in the first or in a second DNA construct. Thus, the second expression cassette can be introduced into said cells or tissues as part of a separate DNA construct, e.g. via co-transformation or e.g. a breeding or a cell fusion step.

30

In one preferred embodiment the DNA construct introduced according to the method of the invention into said wheat cell or tissue further comprises at least one second expression construct conferring to said wheat plant an agronomic valuable

trait. However also other genes (e.g., reporter genes) can be transformed into the wheat plant in combination with the expression cassette for the enzyme capable to metabolize D-alanine and/or D-serine (i.e., the selectable marker). In one embodiment the DNA construct is a T-DNA, more preferably a disarmed T-DNA (e.g.,
 5 without neoplastic growth inducing properties).

The promoter active in wheat plants and/or the D-amino acid oxidase are defined below in detail.

10 1.1 The first expression construct

One embodiment of the invention the recombinant expression construct comprises a promoter active in wheat plants 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
 15 promoter active in wheat plants and/or the D-amino acid oxidase 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
 20 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
 30

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>.

10 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>.

15 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 (O₂) as a substrate and producing hydrogen peroxide (H₂O₂) 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
20 fungi and vertebrates where they are known to be located in the peroxisomes. In
25 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-X₅-G-X-A

30

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
- 5 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>.
- 10 Examples of suitable polypeptides which metabolize D-amino acids are shown in Table 1. The nucleic acid sequences encoding said enzymes are available from 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 character-
- 15 ized 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 as well as preferred substrates are presented in bold letters

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

Enzyme	EC number	Example Source organism	Substrate
D-Amino acid oxidase	EC 1.4.3.3	Q8PG95 <i>Xanthomonas axonopodis</i> Q8R2R2 <i>Mus musculus</i> (Mouse) Q8SZN5 <i>Drosophila melanogaster</i> Q8VCW7 <i>Mus musculus</i> (Mouse) Q921M5 <i>Cavia parcellus</i> (Guinea pig) Q95XG9 <i>Caenorhabditis elegans</i> Q99042 <i>Trigonopsis variabilis</i> Q9C1L2 <i>Neurospora crassa</i> Q9JXF8 <i>Neisseria meningitidis</i> (serogroup B) NMB2068 Q9V5P1 <i>Drosophila melanogaster</i> (Fruit fly) Q9VM80 <i>Drosophila melanogaster</i> (Fruit fly) Q9X7P6 <i>Streptomyces coelicolor</i> Q9Y7N4 <i>Schizosaccharomyces pombe</i> (Fission yeast) SPCC1450 Q9Z1M5 <i>Cavia porcellus</i> (Guinea pig) Q9Z302 <i>Cricetulus griseus</i> U60066 <i>Rhodospiridium toruloides</i>, (<i>Rhodotorula gracilis</i>) strain TCC 26217	
D-Alanine transaminase	EC-number 2.6.1.21	P54692 <i>Bacillus licheniformis</i> P54693 <i>Bacillus sphaericus</i> P19938 <i>Bacillus</i> sp. (strain YM-1) 007597 <i>Bacillus subtilis</i>	D-Ala D-Arg D-Asp D-Glu

Enzyme	EC number	Example Source organism	Substrate
		085046 <i>Listeria monocytogenes</i>	D-Leu
		P54694 <i>Staphylococcus haemolyticus</i>	D-Lys
			D-Met
			D-Phe
			D-Norvaline

Especially preferred in this context are the *dao1* gene (EC: 1.4. 3.3 : GenBank Acc.-No.: U60066) from the yeast *Rhodotorula gracilis* (*Rhodospiridium toruloides*) and the *E. coli* gene *dsdA* (D-serine dehydratase (D-serine deaminase) [EC: 4.3. 1.18; GenBank Acc.-No.: J01603). The *dao1* gene is of special advantage since it

5 can be employed as a dual function marker (see international patent application PCT/EP 2005/002734).

In a preferred embodiment, the method of the invention comprises the use of the above mentioned preferred enzymes, in particular of the especially preferred en-

10 zymes together with the substrates indicated as preferred substrates.

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.

15 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.

20

For the method of the invention, the enzyme capable to metabolize D-alanine is selected from the group consisting of

- i) the D-Alanine transaminase as shown in Table 1, and
- 25 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 an amino acid sequence of a D-Alanine transaminase as shown in Table 1;

iii) enzymes having the same enzymatic activity and an identity of the encoding nucleic acid sequence 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 a nucleic acid sequence of a D-Alanine transaminase as shown in

5 Table I, and

iv) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence encoding the D-Alanine transaminase as shown in Table I,

and wherein selection is done on a medium comprising D-alanine and/or D-serine
10 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)

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

i) the D-serine ammonia-lyase as shown in Table I, 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 an amino acid sequence of a D-serine ammonia-lyase as shown in Table I;
20

iii) enzymes having the same enzymatic activity and an identity of the encoding nucleic acid sequence 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 a nucleic acid sequence of a D-serine ammonia-lyase as shown in
25 Table I, and

iv) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence encoding the D-serine ammonia-lyase as shown in Table I,

and wherein selection is done on a medium comprising D-serine in a concentration
30 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).

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%
5 (preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, most preferably at least 98%) to the amino acid sequence as shown by SEQ ID NO: 2, and
 - iii) enzymes having the same enzymatic activity and an identity of the encoding
10 nucleic acid sequence 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 nucleic acid sequence as shown by SEQ ID NO: 1, and
 - iv) 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
15 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
20 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,
25 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 me-
30 tabolize D-serine is selected from the group consisting of

- i) the D-amino acid oxidase as shown in Table I, 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 an amino acid sequence of a D-amino acid oxidase as shown in Table 1;

iii) enzymes having the same enzymatic activity and an identity of the encoding nucleic acid sequence 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 a nucleic acid sequence of a D-amino acid oxidase as shown in Table 1, and

iv) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence encoding the D-amino acid oxidase as shown in Table 1,

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)

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 shown by SEQ ID NO: 4,

iii) enzymes having the same enzymatic activity and an identity of the encoding nucleic acid sequence 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 nucleic acid sequence as shown by SEQ ID NO: 3, and

iiv 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 (K_i, 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; Cramer 1997; Moore 1997; Zhang 1997; Cramer 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).

- 5 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 7mM to about 30 mM D-serine. The total selection time under dedifferentiating conditions is preferably from about 3 to 4 weeks.
- 10 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
- 15 such transit peptides generally accumulate in the cytosol.

In one embodiment, the D-amino acid metabolizing enzyme is functionally linked to a promoter, in particular to a promoter which confers - in combination with corresponding further expression regulation signals - expression of the accordingly controlled gene in wheat plants. Such a promoter can be for example a constitutive

20 promoter, a promoter which is regulated or a promoter which is active in a suitable tissue or organ.

1.1.2 Promoters for wheat plants

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

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

The person skilled in the art is aware of several promoter which might be suitable for use in wheat plants. In this context, expression can be, for example, constitutive, inducible or development-dependent. The following promoters are preferred:
10

a) Constitutive promoters

" Constitutive" promoters refers to those promoters which ensure expression in a large number of, preferably all, tissues over a substantial period of plant development, preferably at all times during plant development. Preferred are:
15 the promoter of the CaMV (cauliflower mosaic virus) 35S transcript (Franck 1980; Odell 1985; Shewmaker 1985; Gardner 1986), the 19S CaMV promoter (US 5,352,605; WO 84/02913; Benfey 1989) are especially preferred, the rice actin promoter (McElroy 1990), the Rubisco small subunit (SSU) promoter (US
20 4,962,028), the promoter of the nopaline synthase from Agrobacterium, the OCS (octopine synthase) promoter from Agrobacterium, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (US 5,683,439), the promoters of the vacuolar ATPase subunits, the pEMU promoter (Last 1991); the MAS promoter (Velten 1984) and maize H3 histone promoter (Lepetit 1992;
25 Atanassova 1992), the maize ahas promoter (US 5,750,866) or the ScBV promoter (US 6,489,462).

b) Tissue-specific or tissue-preferred promoters

Promoters which are furthermore preferred are those which permit a seed-specific expression in monocots such as maize, barley, wheat, rye, rice and
30 the like. The promoter of the *lpt2* or *lpt1* gene (WO 95/15389, WO 95/23230) or 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) can advance

tageously be employed. Further preferred are a leaf-specific and light-induced promoter such as that from cab or rubisco (Simpson 1985; Timko 1985); an anther-specific promoter such as that from LAT52 (Twell 1989b); a pollen-specific promoter such as that from Zml3 (Guerrero 1993); and a microspore-preferred promoter such as that from apg (Twell 1993).

c) Chemically inducible promoters

The expression cassettes may also contain a chemically inducible promoter (review article: Gatz 1997), by means of which the expression of the exogenous gene in the plant can be controlled at a particular point in time. Such promoters such as, for example, the PRP1 promoter (Ward 1993), a salicylic acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP 0 388 186), a tetracyclin-inducible promoter (Gatz 1991, 1992), an abscisic acid-inducible promoter (EP 0 335 528) or an ethanol-cyclohexanone-inducible promoter (WO 93/21334) can likewise be used. Also suitable is the promoter of the glutathione-S transferase isoform II gene (GST-II-27), which can be activated by exogenously applied safeners such as, for example, N,N-diallyl-2,2-dichloroacetamide (WO 93/01294) and which is operable in a large number of tissues of both monocots and dicots. Further exemplary inducible promoters that can be utilized in the instant invention include that from the ACE1 system which responds to copper (Mett 1993); or the In2 promoter from maize which responds to benzenesulfonamide herbicide safeners (Hershey 1991; Gatz 1994). A promoter that responds to an inducing agent to which plants do not normally respond can be utilized. An exemplary inducible promoter is the inducible promoter from a steroid hormone gene, the transcriptional activity of which is induced by a glucocorticosteroid hormone (Schena 1991).

Particularly preferred are constitutive promoters. Most preferred are ubiquitin promoters (see below in detail) such as the ubiquitin promoter (Holtorf 1995), and the ubiquitin 1 promoter (Christensen 1989, 1992; Bruce 1989).

1.1.2.1 The ubiquitin promoter

It one preferred embodiment of the invention the promoter functional in wheat plants is an ubiquitin promoter, preferably a ubiquitin promoter derived from a monocotyl plant, e.g. the Zea maize ubiquitin promoter. The use of the ubiquitin promoter results in a consistently high transformation efficiency. The reasons for the 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 wheat 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 wheat, 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. The ubiquitin promoter thus can be employed as a standard promoter to drive expression of D-amino acid metabolizing enzymes in wheat.

Thus, in a preferred embodiment of the invention 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 ei-

ther 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
5 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
10 control or modulation of gene expression.

Various plant ubiquitin genes and their promoters are described (CaIns 1989, 1990). Described are promoters from dicotyledonous plants, such as for potato (Garbarino 1992), tobacco (Genschick 1994), tomato (Hoffman 1991), parsley
15 (Kawalleck 1993; WO03/102198, herein incorporated by reference), Arabidopsis (CaIns 1990; Holtorf 1995;UBQ8, GenBank Ace- No: NM_1 11814; UBQ1, GenBank Ace- No: NMJ 151 19; UBQ5, GenBank Ace- No: NM_1 16090).

Accordingly the ubiquitin promoter of the invention is a DNA fragment (preferably
20 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
25 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
30 are described for maize (Christensen 1992, 1996), 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 maize 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 regu-

lates 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 CTCGAGATTCCGCT-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 wheat (US 6,054,574; 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
 - 15 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 wheat,
 - 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 wheat,
 - 20 d) sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 5, and having promoter activity in wheat.

25

" Promoter activity" in wheat means the capability to realized transcription of an operably linked nucleic acid sequence in at least one cell or tissue of a wheat plant or derived from a wheat 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 maize ubiquitin promoter may be present but is not required.

30

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
5 60 to 65°C).

The sequence described by SEQ ID NO: 5 is the core promoter of the maize ubiquitin promoter. In one preferred embodiment not only the promoter region is employed as a transcription regulating sequence but also a 5' -untranslated region
10 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
15 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
20 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 maize ubiquitin gene are used, even more preferably the region described by SEQ ID NO: 6. Accordingly in another preferred embodiment the
25 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
30 preferably at least 1000) consecutive base pairs of the sequence as described by SEQ ID NO: 6, and having promoter activity in wheat,
- 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 wheat,

- d. sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 6, and having promoter activity in wheat.

5

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 10 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 15 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 20 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 25 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 30 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 regulat-

ing nucleotide sequence to its essential, transcription mediating elements can be realized in vitro by trial-and-error 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 wheat promoter sequences, which - for example - do not include two overlapping heat shock elements. Such sequences are for example described in U.S. Pat. Appl. 20030066108 (WO 01/18220).

1.1.3 Additional elements

The expression cassettes (or the vectors in which these are comprised) may comprise further functional elements and genetic control sequences in addition to the promoter active in wheat plants (e.g., 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).

- 5 Preferably, the expression cassettes encompass a promoter active in wheat plants (e.g, the ubiquitin promoter) 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
10 expressed.

- Genetic control sequences and functional elements furthermore also encompass the 5' -untranslated regions, introns or non coding 3' -region of genes, such as, for example, the actin-1 intron, or the AdM-S introns 1, 2 and 6 (general reference:
15 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'
20 leader sequence (GalNe 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
25 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 include

- 30 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 (Maniatis, 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 wheat 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 over-expressing 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 wheat 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
5 in monocots like wheat (see above for details), 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
10 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

15 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, immature and mature embryos, pollen, meristematic tissues, inflorescences, 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
20 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 wheat variety or plant. Especially preferred are all wheat species especially of the Triticum family (including winter, spring and alter-
25 native type wheat), more especially Triticum spp.: common (T. aestivum), durum (T. durum), spelt (T. spelta), Triticum dicoccum (Emmer wheat), Triticum turgidum, and Triticum monococcum (Einkorn wheat), with T. aestivum being particularly preferred. The method of the invention can be used to produce transgenic plants from spring wheat varieties, such as, for example, Bobwhite, and Canon, as well as
30 from winter wheat varieties, such as, for example, Florida as well as from alternative wheat varieties such as, for example Corinto. However, it should be pointed out, that the method of the invention is not limited to certain varieties but is highly genotype-independent. Wheat plants for isolation of immature embryos are grown

and pollinated as known in the art, preferably as described below in the examples. Donor plants are preferably prepared for transformation by reducing the tillers.

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

- a. isolating an immature embryo of a wheat 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 at least one first expression construct comprising a promoter active in said wheat plant and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine,
- 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 3 mM to 100 mM, and
- e. regenerating and selecting plants containing the transgenic T-DNA from the said transgenic callus.

In one preferred embodiment the T-DNA further comprises at least one second expression construct conferring to said wheat plant an agronomic valuable trait. However also other genes (e.g., reporter genes) can be transformed into the wheat plant in combination with the expression cassette for the enzyme capable to metabolize D-alanine and/or D-serine (i.e., the selectable marker).

30

Thus, in one embodiment, the present invention relates also to a cell culture comprising one or more embryogenic calli derived from immature wheat embryo, at least one auxin, preferably in a concentration as described below, and D-alanine and/or D-serine in a total concentration from about 3 mM to 100 mM. In one em-

bodiment, the cell culture also comprises a bacterium belonging to genus Rhizobiaceae

5 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
10 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 10 days after pollination. More preferably, immature embryos are isolated from
15 spikes 12 to 14 days after pollination (DAP). Exact timing of harvest varies depending on growth conditions and wheat 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 1.2 mm, including the length of the scutellum. The embryo should be translucent, not opaque.

20

In this invention, the immature embryos may be isolated in liquid infection medium, washed twice with the same media to clean the surface of the embryos and to prepare cells for Agrobacterium infection. After the infection, explants are placed with a scutellum side up for co-cultivation. However, infection can also be done by various
25 other means known to the person skilled in the art such as, for example, directly inoculating isolated embryos, which are placed on the surface of a solidified co-cultivation medium, with a small amount of Agrobacterium suspension.

Preferably, the immature embryo is subjected to transformation (co-cultivation)
30 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 pre-treatment" 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 may according to the invention 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 wheat 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 de-

scribed 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).

15

In the case of injection or electroporation of DNA into plant cells, the DNA construct to be transformed need not 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.

20

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 Shimamoto 1994; Vasil 1992, 1996; Vain 1995; Wan & Lemaux 1994).

30

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 teranga*, *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 mediterraneum*, *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 (2005).

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.

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.

30

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).

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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 vectors 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 EHA101 [pEHA101] (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 LBA401.1 (Klapwijk 1980). In another preferred embodiment the soil-borne bacterium is a disarmed strain variant of *Agrobacterium rhizogenes* strain K599 (NCPBP 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 L₁L-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).

5

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.

25

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 minutes to 5 hours, preferably for about 1 hour at 26°C. The resulting immature embryos are then cultured on a solid medium for several days together with the Agrobacterium (co-cultivation).

30

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 μ l 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 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 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 10 mM 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 μ M to about 10 μ M 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 10 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 7 days, preferably about 5 days to about 6 days at 26°C (more preferably in medium PAW-1 as described below in the Examples).

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.

Accordingly, in one embodiment, the present invention relates also to a cell culture comprising one or more embryogenic calli derived from immature wheat embryo, at least one auxin, preferably in a concentration as described below, D-alanine and/or D-serine in a total concentration from about 3 mM to 100 mM and at least one plant phenolic compound, e.g. one or more plant phenolic compounds listed below. In one embodiment, the cell culture also comprises a bacterium belonging to genus Rhizobiaceae

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 4-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), p-resorcylic acid (2,4-

dihydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), pyrogalllic 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 200-1000 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* (preferably for about 1 hour to about 24 hours). 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 wheat variety from which the immature embryos derived, but it is expected that about 100 μ M to 700 μ M is a suitable concentration for many purposes. However, concentrations as low as approximately 25 μ M can be used to obtain a good effect on transformation efficiency. Likewise, it is expected that

higher concentrations up to approximately 1000 μ M 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.

5

Agrobacteria to be co-cultivated with the isolated immature embryos can be either pre-incubated 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
10 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.

15 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 of the pH in this range is mediated by a combination of MES and potassium hydrogenphosphate buffers.
20

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.
25

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
30 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 bactericide (antibiotic) suitable to prevent *Agrobacterium* growth. Preferred bactericidal antibiotics to be employed are e.g., cefotaxime (e.g., in a concentration of about 500 mg/l) or TimentinTM (e.g., in a concentration of about 160 mg/l mg/L;

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
5 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).
10 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
20 " and")
- iii. silver nitrate in a concentration from about 1 μ M to about 50 μ M.

Thus, in one embodiment, the present invention relates to a recovery medium comprising an effective amount of at least one antibiotic that inhibits or suppresses
25 the growth of the soil-borne bacteria, and/or (preferably " and") L-proline in a concentration from about 1 g/l to about **10g/l**, and/or (preferably " and") silver nitrate in a concentration from about 0 μ M to about 50 μ M. Preferably, the medium comprises further the transformed target tissue (e.g., the co-cultivated immature embryos).

30

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
- 5 ii. L-proline in a concentration from about 1 g/l to about 10g/l, and
- iii. silver nitrate in a concentration from about 0 μ M to about 50 μ M, preferably no silver nitrate is used;
- iv. an effective amount of at least one auxin compound.

10 Examples for preferred recovery media are given below in the Examples (A-4 or A-5). The recovery period (i.e. the period under defifferentiating conditions without a selection pressure by a phytotoxic amount of D-alanine and/or D-serine) may last for about 1 day to about 30 days, preferably about 5 days to about 20 days, more preferably about 14 days. Preferably, the recovery period (callus initiation and prolifera-
15 tion) is for about seven days in the dark and about additional seven days in semi light $13.2 \mu \text{mol/m}^{-2}/\text{sec}^{-1}$. A medium such as PAW-2 (see Examples) can be employed for this purpose. Preferably, the scutellum side is kept up during this time and do not embedded into the media.

20 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-
25 transformed cells). The term "phytotoxic", "phototoxicity" 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
30 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.

Thus, in one embodiment, the present invention relates to an selection medium comprising the target tissue (e.g., embryonic wheat calli, i.e. the transformed and regenerated wheat immature embryos described above) and D-alanine and/or D-serine or a derivative thereof in a phytotoxic concentration as described below.

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-naphthoyl, 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, glycolyl, 3-formyl benzoyl, 4-formyl benzoyl, 2-formylphenoxyacetyl, 8-formyl-1-naphthoyl, 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
- 5 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 ac-
- 10 cordance 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.
- 15 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 3 mM to about
- 20 100 mM. 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 3 mM to about 100 mM, more preferably from about 4 mM to about 50 mM, even more preferably from about 4.5 mM to about 30 mM, most preferably about 5 mM to about 10
- 25 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 3 mM to 100 mM, more preferably from about 4 mM to about 50 mM, even more preferably from about 4.5 mM to about 20 mM, most preferably about 5 mM
- 30 to about 10 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 about 5 days, preferably at least about 14 days. More specifically the total selection time under

dedifferentiating conditions (i.e., callus induction) is from about 1 to about 10 weeks, preferably, about 3 to 7 weeks, more preferably about 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
5 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 14 to 20 days, then transferring the surviving cells or tissue to a second selection medium with essentially the same
10 composition than the first selection medium for additional 14 to 20 days. However, it is also possible to apply a single step selection.

Preferably said selection medium is - for part of the selection period - also a dedifferentiation medium comprising at least one suitable plant growth regulator for induction of embryogenic callus formation. The term "plant growth regulator" (PGR)
15 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
20

- 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.
- 25 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,
30 formation of adventitious roots, production of ethylene, and - in high concentrations - induce dedifferentiate (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 com-

pounds comprise indole-3-butyric acid (IBA), naphthylacetic acid (NAA), and 2,4-dichlorophenoxyacetic 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 5 mg/l, most preferably about 2mg/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 4 mg/l, most preferably about 2 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 to 2 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 2.5 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 thiadiazuron. Cytokinin compounds comprise, for example zeatin, 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. In one typical selection scheme the following conditions may be applied:

- Selection I: Selection under dedifferentiation conditions (callus proliferation) for about 7 to about 50 days, preferably from about 14 to about 21 days. Selection can be preferably done under light with a medium such as PAW-2 sel (see Examples).
- 5 Selection II: Selection under regeneration conditions (see below) for about 7 to about 50 days, preferably for about 3 weeks (21 days). Regenerations can be done with a medium such as PAW-4 sel (see Examples).
- 10 Selection III Selection under shoot elongation conditions for about 7 to about 50 days, preferably for about 3 weeks (21 days). Shoot elongation can be done with a medium such as PAW-5 selection in plates (see Examples).
- 15 Selection IV Selection under shoots growth and rooting conditions for about 7 to about 50 days, preferably for about 3 weeks (21 days). Shoots growth and rooting can be done with a medium such as PAW 5 selection in boxes (see Examples).

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 wheat plant cells, preferably wheat embryogenic callus, 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 con-

version 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 regeneration medium may comprise one or more
5 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
- 10 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).

More preferably, the medium employed in the regeneration step e) is preferably
15 comprising:

- i) an effective amount of at least one cytokinin compound, and/or
- ii) D-alanine and/or D-serine in a total concentration from about 3 mM to 100 mM.

20 The embryogenic callus is preferably incubated on this medium until shoots are formed and then transferred to a (preferably hormone free) elongation 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, Magenta boxes or Sky-Light plastic boxes containing rooting medium (such as the medium
25 described in PAW-5) and incubate until rooted plantlets have developed (usually 1 to 4 weeks, preferably 2 weeks). The rooted seedlings are transferred to Jiffy for acclimatisation (usually for 10days). After analyses the transgenic plants are transferred to sil K-Jord and grown to mature plants as described in the art (see examples).

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The resulting transgenic plants are self pollinated by bagging all spikes individually while they are emerging from the flag leaf. T1 seeds are spikewise harvested, dried and stored properly with adequate label on the seed bags. Two or more generations should be grown in order to ensure that the genomic integration is stable

and hereditary. For example, transgenic events in T1 or T2 generations could be involved in a pre-breeding hybridization program for combining different transgenes (gene stacking).

- 5 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

- 10 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.
- 15 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 trans-
- 20 genic 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
- 25 analysis, Northern blot, Southern blot, or phenotypic screening (e.g., for a 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
- 30 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.

10

A further subject matter of the invention relates to the use of the above-described transgenic organisms the cells, cell cultures, and/or 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.

15

A further subject matter of the invention relates to a composition for selection, regeneration, growing, cultivation or maintaining of transgenic wheat plant cells, transgenic wheat plant tissue, transgenic wheat plant organs or transgenic wheat plants or a part thereof comprising an effective amount of D-alanine, D-serine, or a derivative thereof allowing for selection of transgenic wheat plant cells, transgenic wheat plant tissue, transgenic wheat plant organs or transgenic wheat plants or a part thereof and the above-described transgenic wheat organisms, the transgenic wheat cells, transgenic wheat cell cultures, transgenic wheat plants and/or parts thereof - such as, for example, in the case of transgenic plant organisms roots, leaves and the like - derived from them.

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Another embodiment of the invention relates to a wheat plant or cell comprising a promoter active in said wheat plants or cells 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. Preferably, the the promoter and/or the enzyme capable to metabolize D-alanine or D-serine is defined as above. More preferably the wheat plant is further comprising at least one second expression construct conferring to said wheat plant

an agronomically valuable trait. In one preferred embodiment the wheat plant selected from the Triticum family group of plants, more preferably from a plant species of the group consisting of Triticum spp.: common (T. aestivum), durum (T. durum), spelt (T. spelta), Triticum dicoccum (Emmer wheat), Triticum turgidum, and Triticum monococcum (Einkorn wheat), most preferably from a variety of Triticum aestivum. Other embodiments of the invention relate to parts, organs, cells, fruits, and other reproduction material of a wheat plant of the invention. Preferred parts are selected from the group consisting of tissue, cells, pollen, ovule, roots, leaves, seeds, microspores, and vegetative parts.

10

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).

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3. Further modifications

20 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 PCT/EP 2005/002734, hereby incorporated entirely by reference.

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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 phytotoxic to plants and are "detoxified" by the D-amino acid oxidase), D-valine and D-isoleucine are

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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 wheat plant comprising:

- i) transforming a wheat plant cell with a first DNA construct comprising
 - a) at least one first expression construct comprising a promoter active in said wheat plant 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 wheat 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 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:

- 5 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,
- 10 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
15 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:

- 20 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,
25 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,
- 30 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.

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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

10 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 be modified by treatment of a compound having
- 15 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.

20

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 promoter active in wheat plants (as defined above; preferably an ubiquitin promoter), wherein said first expression cassette is flanked by sequences which allow for specific deletion of said first expression cassette, and
- 25 b) at least one second expression cassette suitable for conferring to said plant an agronomically valuable trait, wherein said second expression cassette is not
- 30 localized between said sequences which allow for specific deletion of said first expression cassette.

Preferred 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 above mentioned DNA construct 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).

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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 (Dalel 1991 ; 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.

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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 promoter) 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.

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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 be modified by treatment of a compound having binding activity to said ligand-binding-domain.

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Further embodiments of the inventions are related to transgenic vectors comprising a DNA construct of the invention. Transgenic cells or non-human organisms com-

prising 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.

- 5 The present invention enables generation of marker-free transgenic cells and organisms, preferably plants, in 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
10 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
15 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 0.5 mM to
20 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.

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Thus, using the above described method it becomes possible to create a wheat plant which is marker-free. The terms "marker-free" or "selection marker free" as used herein with respect to a cell or an organism are intended to mean a cell or an organism which is not able to express a functional selection marker protein (en-
30 coded 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.

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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.

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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 wheat plant comprising the steps of:

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- a) a transformation with a first construct said construct comprising at least one expression construct comprising a promoter active in said wheat plants 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.

30

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)
- sulfonyleurea- and/or imidazolinone-inactivating acetolactate synthases (ahas or ALS; for example mutated ahas/ALS variants with, for example, the S4, XI12, XA1 7, 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 phosphinothricin resistance gene (bar, PAT) as the selective marker, phosphinothricin or bialaphos at a concentration of from about 1 to 50 mg/l may be included in the medium.

Preferably said second marker is conferring resistance against at least one compound select from the group consisting of phosphinothricin, glyphosate, sulfonyleurea- and imidazolinone-type herbicides.

Another embodiment of the invention relates to a wheat plant comprising

- a) a transformation with a first construct said construct comprising at least one expression construct comprising a promoter active in said wheat plants (pref-

erably a ubiquitin promoter as defined above) and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine or D-serine, and

- 5 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 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:

- 15 - A first transformation with a selection marker conferring resistance against phosphinothricin followed by a second transformation with a dsdA selection marker gene;
- A first transformation with a selection marker conferring resistance against phosphinothricin followed by a second transformation with a dao1 selection marker gene;
- 20 - A first transformation with a dsdA selection marker gene followed by a second transformation with a selection marker conferring resistance against phosphinothricin ;
- A first transformation with a dao1 followed by a second transformation with a selection marker conferring resistance against phosphinothricin;

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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 wheat plant comprising the steps of:

- 30 a) a transformation with a first construct said construct comprising an expression construct comprising a promoter active in said wheat plants (preferably a ubiq-

- 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 promoter active in said wheat plants and operably linked thereto a nucleic acid sequence encoding a dao enzyme and selecting with D-alanine.

Another embodiment of the invention relates to the wheat plants generated with this method. Thus, the invention also relates to a wheat plant comprising

- a) a first construct said construct comprising an expression construct comprising a promoter active in said wheat plants and operably linked thereto a nucleic acid sequence encoding an dsdA enzyme, and
- b) a second construct said construct comprising an expression construct comprising promoter active in said wheat plants and operably linked thereto a nucleic acid sequence encoding a dao enzyme.

In the above-mentioned constructs comprising two expression cassettes it is preferred that the two promoters active in wheat plants are not identical. Preferably one promoter (e.g., the promoter for expression of the D-alanine and/or D-serine metabolizing enzyme) is an ubiquitin promoter as defined above), while the other promoter is a different promoter (e.g., the ScBV promoter or the ahas promoter).

Sequences

- | | | |
|----|------------------|--|
| 5 | 1. SEQ ID NO: 1 | Nucleic acid sequence encoding E.coli D-serine dehydratase [dsdA] gene |
| 10 | 2. SEQ ID NO: 2 | Amino acid sequence encoding E.coli D-serine dehydratase [dsdA] |
| 15 | 3. SEQ ID NO: 3 | Nucleic acid sequence encoding Rhodosporidium toruloides D-amino acid oxidase gene |
| 20 | 4. SEQ ID NO: 4 | Amino acid sequence encoding Rhodosporidium toruloides D-amino acid oxidase |
| 25 | 5. SEQ ID NO: 5 | Nucleic acid sequence encoding maize ubiquitin core promoter region |
| 30 | 6. SEQ ID NO: 6 | Nucleic acid sequence encoding maize ubiquitin promoter further comprising 5' -untranslated region and first intron |
| | 7. SEQ ID NO: 7 | Nucleic acid sequence encoding sugarcane bacilliform virus core promoter region |
| | 8. SEQ ID NO: 8 | Nucleic acid sequence encoding sugarcane bacilliform virus promoter further comprising 5' -untranslated region |
| | 9. SEQ ID NO:9 | Nucleic acid sequence encoding pRLM175, a kanamycin resistant pSB1 1-type binary vector. |
| | 10. SEQ ID NO:10 | Nucleic acid sequence encoding T-DNA region of pRLM166, a pRLM175 derived binary vector containing p-ZmUBI+I::c-dsdA::t-OCS and p-ScBV::c-gusINT::t-NOS cassettes. |

11. SEQ ID NO:11 Nucleic acid sequence encoding T-DNA region of pRLM167, a pRLM175 derived binary vector containing p-ZmUBI+l::c-dsdA::t-OCS and p-ZmUBI+l::c-PAT::t-OCS cassettes.
- 5
12. SEQ ID NO:12 Nucleic acid sequence encoding T-DNA region of pRLM179, a pRLM175 derived binary vector containing ZmAHAS L2/Xi12 and p-ZmUBI+l::c-dsdA::t-OCS cassettes.
- 10
13. SEQ ID NO:13 Nucleic acid sequence encoding T-DNA region of pRLM205, a pRLM175 derived binary vector containing p-ZmUBI+l::c-dao1 ::t-OCS and p-ScBV::c-gusINT::t-NOS cassettes.
- 15
14. SEQ ID NO: 14 Nucleic acid sequence encoding T-DNA region of pRLM226, a pRLM175 derived binary vector containing p-ZmUBI+l::l-PsFed1 ::c-dao1/ko::t-OCS and p-ScBV::c-gusINT::t-NOS cassettes.
- 20
15. SEQ ID NO: 15 Nucleic acid sequence encoding a Zea mays codon optimize Rhodosporidium toruloides D-amino acid oxidase CDS
- 25
16. SEQ ID NO: 16 Amino acid sequence encoding Rhodosporidium toruloides D-amino acid oxidase
17. SEQ ID NO:17 Nucleic acid sequence encoding qPCR primer GUSCommon-341 F: 5' CCGGGTGAAG GTTATCTCTA TGA 3'
- 30
18. SEQ ID NO:18 Nucleic acid sequence encoding qPCR primer GUSCommon-414 R: 5' CGAAGCGGGT AGATATCACA CTCT 3'
19. SEQ ID NO:19 Nucleic acid sequence encoding qPCR probe GUSCommon-366FAM: 5' TGTGCGTCAC AGCCAAAAGC CAGA 3'

20. SEQ ID NO:20 Nucleic acid sequence encoding qPCR primer EcdsdA-860F:
5' TCGCATTCGG GCTTAACTG 3'
- 5
21. SEQ ID NO: 21 Nucleic acid sequence encoding qPCR primer EcdsdA-922R:
5' GCGTTGGTTC GGCAAAAA 3'
- 10
22. SEQ ID NO: 22 Nucleic acid sequence encoding qPCR probe EcdsdA-883FAM:
5' TTTGGCGATC ATGTTCCTG C 3'
- 15
23. SEQ ID NO: 23 Nucleic acid sequence encoding qPCR primer TaGBSS:!
F:
5' TTCTGCATCC ACAACATCTC GTA 3'
- 20
24. SEQ ID NO: 24 Nucleic acid sequence encoding qPCR primer TaGBSS:!
R :
5' TAGCCGTCGA TGAAGTCGAA 3'
- 25
25. SEQ ID NO: 25 Nucleic acid sequence encoding qPCR probe TaGBSS:1-TET:
5' CGACGACTTC GCGCAGCTCA AC 3'
- 30
26. SEQ ID NO: 26 Nucleic acid sequence encoding qPCR primer dao1/pa-285F:
5' GTTCGCGCAG AACGAAGAC 3'
27. SEQ ID NO: 27 Nucleic acid sequence encoding qPCR primer dao1/pa-349R:
5' GGCGGTAATT TGGCGTGA 3'

28. SEQ ID NO: 28 Nucleic acid sequence encoding qPCR probe dao1/pa-308FAM: 5' TCCTTGTTACC AGTGCCCGAG CA 3'
- 5 29. SEQ ID NO: 29 Nucleic acid sequence encoding forward PCR primer for gusINT gene: 5'-ACCGTTTGTG TGAACAACGA -3'
30. SEQ ID NO: 30 Nucleic acid sequence encoding reverse PCR primer for gusINT gene: 5' -GGCACAGCAC ATCAAAGAGA- 3'
- 10 31. SEQ ID NO: 31 Nucleic acid sequence encoding forward PCR primer for dsdA gene: 5'-GCTTTTTGTT CGCTTGTTG TG -3'
32. SEQ ID NO: 32 Nucleic acid sequence encoding reverse PCR primer for dsdA gene: 5'-TCAATAATCC CCCCAGTGGC- 3'
- 15 33. SEQ ID NO: 33 Nucleic acid sequence encoding forward PCR primer for dao1 gene: 5'-GACAAGCAAA ATGGGAAGAA TC -3'
- 34 SEQ ID NO: 34 Nucleic acid sequence encoding reverse PCR primer for dao1 gene: 5'-TCGGGGAATG ATGTAGGC - 3'
- 20 35. SEQ ID NO: 35 Nucleic acid sequence encoding forward PCR primer for dao1/ko gene: 5'-AAGCAGGCCT TCTCACA CTT GA -3'
- 25 36. SEQ ID NO: 36 Nucleic acid sequence encoding reverse PCR primer for dao1/ko gene: 5'-TTCCAACAAA GCCCGATGCG - 3'
37. SEQ ID NO: 37 Nucleic acid sequence encoding forward PCR primer for PAT gene: 5' -
- 30 ATGTCTCCGGAGAGGAGACCAGTTGAGAT-S'
38. SEQ ID NO: 38 Nucleic acid sequence encoding reverse PCR primer for PAT gene: 5' -GCCAAAAACCAACATCATGCCATCCA-3'

39. SEQ ID NO: 39 Nucleic acid sequence encoding T-DNA region of pRLM 151 , a pRLM175 derived binary vector containing p-ZmUBI+l::c-dsdA::t-OCS.
- 5 40. SEQ ID NO: 40 Synthetic Construct E. coli D-serine deaminase [dsdA] CDS
41. SEQ ID NO: 41 Nucleic acid sequence encoding forward PCR primer for ahas gene: F:5' -TGACTTTGG CTCAR GGA ACG-3'
- 10 42. SEQ ID NO: 42 Nucleic acid sequence encoding reverse PCR primer for ahas gene: R: 5' -ATCTCACTTT CATTCTCTGGGTTT-3'

Examples

General methods

- 15 Unless indicated otherwise, chemicals and reagents in the Examples were obtained from Sigma- Aldrich AB, Sweden Materials for cell culture media were obtained from GIBCO Invitrogene AB Sweden, Duchefa SAVEEN Sweden or DIFCO NordicaBiolabs, Sweden. The cloning steps carried out for the purposes of the present invention, such as, for example, transformation of E. coli cells, growing bacteria,
- 20 multiplying phages and sequence analysis of recombinant DNA, are carried out as described by Maniatis (1989). The following examples are offered by way of illustration and not by way of limitation.

Medium for transformation

- 25 PAW-Inf.

Ingredient	Concentration mg/l	Stock solution	Supplier
MS micro, macro salts	4300		Duchefa M0221
Nicotinic acid	0.5	100x	Sigma N4126
Pyridoxine HCl	0.5		Sigma P9755
Thiamine HCl	1.0		Sigma T4625
Myo-inositol	100		Duchefa I0609
Casamino acid	1000		Difco 228820

2,4-D	2.0	0.1mg/ml	Duchefa D0911
Sucrose	68460 (0.2M)		Duchefa S0809
Glucose	39630 (0.2M)		Duchefa M0811

pH=5.2; Compound added before use: Acetosyringone (300µM).

PAW-1 (Co-cultivation Medium)

Ingredient	Concentration mg/l	Conc. Stock solution	Supplier
MS micro, macro salts	4300		Duchefa M0221
Nicotinic acid	0.5	10ml	Sigma N4126
Pyridoxine HCl	0.5		Sigma P9755
Thiamine HCl	1.0		Sigma T4625
Myo-inositol	100		Duchefa I0609
Glutamine	500		Duchefa G0708
Casein hydrolysate	100		Duchefa C1301
Ascorbic acid	100		Duchefa A0602
CuSO ₄ ·5H ₂ O	0.5	1mg/ml	Duchefa C0508
MES	500		Duchefa M1503
2.4D	2.0	0.1mg/ml	Duchefa D0911
Sucrose	20000		Duchefa S0809
Maltose	10000		Duchefa M0811
Glucose	10000		Duchefa G0802
Gelrite	2500		Duchefa G1101

pH=5.65; Compound added after autoclaving: Acetosyringone (300µM).

5 PAW-2 (Callus Induction - Recovery Medium)

Ingredient	Concentration mg/l	Conc. Stock solution	Supplier
MS macro, micro salts	4300		Duchefa M0221
Nicotinic acid	0.5	10ml	Sigma N4126
Pyridoxine HCl	0.5		Sigma P9755
Thiamine HCl	1.0		Sigma T4625
Myo-inositol	100		Duchefa I0609

Glutamine	500		Duchefa G0708
Casein hydrolysate	100		Duchefa C1301
Ascorbic acid	100		Duchefa A0602
CuSO ₄ ·5H ₂ O	0.5	1mg/ml	Duchefa C0508
MES	500		Duchefa M1503
2,4-D	2.0	0.1mg/ml	Duchefa D0911
Sucrose	20000		Duchefa S0809
Maltose	10000		Duchefa M0811
Gelrite	2500		Duchefa G1101

pH=5.65; Compound added after autoclaving : Timentin (160 mg/l).

PAW-2 (Callus Proliferation - Selection Medium)

Ingredient	Concentration mg/l	Conc. Stock solution	Supplier
MS macro, micro salts	4300		Duchefa M0221
Nicotinic acid	0.5	10ml	Sigma N4126
Pyridoxine HCl	0.5		Sigma P9755
Thiamine HCl	1.0		Sigma T4625
Myo-inositol	100		Duchefa I0609
Glutamine	500		Duchefa G0708
Casein hydrolysate	100		Duchefa C1301
Ascorbic acid	100		Duchefa A0602
CuSO ₄ ·5H ₂ O	0.5	1mg/ml	Duchefa C0508
MES	500		Duchefa M1503
2,4-D	2.0	0.1mg/ml	Duchefa D0911
Sucrose	20000		Duchefa S0809
Maltose	10000		Duchefa M0811
Gelrite	2500		Duchefa G1101

pH=5.65; Compounds added after autoclaving: Timentin (160 mg/l) and corre-

- 5 sponding selection agent: D-serine (3 mM, 5 mM, 10 mM), D-alanine (3 mM, 5 mM, 10 mM), bialaphos (3 mg/l).

PAW-4 (Regeneration Medium)

Ingredient	Concentration mg/l	Stock solutions	Supplier
MS macro, micro salts	4300		Duchefa M0221
Nicotinic acid	0.5	10ml	Sigma N4126
Pyridoxine HCl	0.5		Sigma P9755
Thiamine HCl	1.0		Sigma T4625
Myo-inositol	100		Duchefa I0609
CuSO ₄ ·5H ₂ O	0.5	1mg/ml	Duchefa C0508
MES	500		Duchefa M1503
Sucrose	20000		Duchefa S0809
Maltose	10000		Duchefa M0811
Gelrite	2500		Duchefa G1101
Zeatin	5.0	1mg/ml	Sigma Z0164

pH=5.65; Compounds added after autoclaving: Timentin (160mg/l) and corresponding selection agent: D-serine (3 mM, 5 mM, 10 mM); D-alanine (3 mM, 5 mM, 10 mM); bialaphos (3 mg/l).

5

PAW-5 (Hormone Free Medium for Shoots Elongation, Rooting and Embryos Germination)

Ingredient	Concentration mg/l	Stock solution	Supplier
MS macro, micro salts	2150g		Duchefa M0221
Nicotinic acid	0.5mg	10ml	Sigma N4126
Pyridoxine HCl	0.5mg		Sigma P9755
Thiamine HCl	1.0mg		Sigma T4625
Myo-inositol	100		Duchefa I0609
MES	500		Duchefa M1503
Sucrose	20000		Duchefa S0809
Gelrite	2500		Duchefa G1101

pH=5.65. Compounds added after autoclaving: Timentin (160 mg/l) and corresponding selection agent: D-serine (3 mM, 5 mM, 10 mM), D-alanine (3 mM, 5 mM, 10 mM), bialaphos (3 mg/l).

5 Supplier specification for supplements

Ingredient	Stock solutions	Supplier
Timentin	160 mg/ml	Duchefa T0190
Bialaphos	3 mg/ml	Duchefa B0178
Acetosyringone	100 mM (MW 196.0g)	Aldrich D13,440-6
D-serine	1M (MW 105.1g)	Sigma S4250
D-alanine	1 M (MW 89.1g)	Sigma A7377

TaqMan PCR primers/probes

GUSCommon-341 F 5' CCGGGTGAAGGTTATCTCTATGA 3' (SEQ ID NO: 17)

GUSCommon-414R 5' CGAAGCGGGTAGATATCACA CTCT 3' (SEQ ID NO: 18)

10 GUSCommon-366FAM 5' TGTGCGTCACAGCCAAAAGCCAGA 3' (SEQ ID NO: 19)

EcdsdA-860F 5' TCGCATTCGGGCTTAAACTG 3' (SEQ ID NO: 20)

EcdsdA-922R 5' GCGTTGGTTCGGCAAAAA 3' (SEQ ID NO: 21)

15 EcdsdA-883FAM 5' TTTGGCGATCATGTTCACTGC 3' (SEQ ID NO: 22)

TaGBSS: 1-F 5' TTCTGCATCCACAACATCTCGTA 3' (SEQ ID NO: 23)

TaGBSS: 1-R 5' TAGCCGTCGATGAAGTCGAA 3' (SEQ ID NO: 24)

TaGBSS: 1-TET 5' CGACGACTTCGCGCAGCTCAAC 3' (SEQ ID NO: 25)

20

dao1/pa-285F 5' GTT CGC GCA GAA CGA AGA C -3' (SEQ ID NO: 26)

dao1/pa-349R 5' GGC GGT AAT TTG GCG TGA -3' (SEQ ID NO: 27)

dao1/pa-308FAM 5TCC TTG TAC CAG TGC CCG AGC A -3' (SEQ ID NO: 28)

25 PCR primers/probes

For gusINT gene

Forward 5'-ACC GTT TGTGTGAACAACGA -3' (SEQ ID NO: 29)

Reverse 5'-GGCACAG CACATCAAAG AGA- 3' (SEQ ID NO: 30)

For dsdA gene

Forward 5'-GCTTTTTGTTTCGCTTGTTGTG -3' (SEQ ID NO: 31)

Reverse 5'-TCAATAATCCCCCAGTGGC- 3' (SEQ ID NO: 32)

5

For dao1 gene

Forward 5'-GACAAGCAAAATGGGAAGAATC -3' (SEQ ID NO: 33)

Reverse 5'-TCGGGGAATGATGTAGGC - 3' (SEQ ID NO: 34)

10 For dao1/ko gene

Forward 5'-AAGCAGGCCTTCTCACACTTGA -3' (SEQ ID NO: 35)

Reverse 5'-TTCCAACAAAGCCCGATGCG - 3' (SEQ ID NO: 36)

For PAT gene

15 Forward 5' - ATGTCTCCGGAGAGGAGACCAGTTGAGAT-3' (SEQ ID NO: 37)

Reverse 5' - GCCAAAAACCAACATCATGCCATCCA-3' (SEQ ID NO: 38)

For ahas gene:

Forward: 5' -TGACTTTGG CTCAR GGA ACG-3' (SEQ ID NO: 41)

20 Reverse: 5' -ATCTCACTTTCATTCTCTGGGTTT-3' (SEQ ID NO: 42)

Example 1: Wheat transformation protocol

25 1.1 Preparation of tissues for transformation

Plant material

Donor plants were produced from spring wheat *Triticum aestivum* variety Canon in an environmental controlled growth chambers with a 16/8-h photoperiod at 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ intensity and 70 % humidity. The day night temperature was 20/16
 30 °C. Two well developed seedlings per 4.2 l square pots (8:1 :1 Soil (K-jord): perlite: clay) (Weibulls, Sweden) were watered daily and fertilized 6 times during the vegetation including the basic fertilization with Superba vit (38 mg N per pot) (Weibulls, Sweden). Towards the end of the tillering before heading the side axes are removed so five strong uniform tillers per plant were selected for transformation.

When first anthers were extruded the individual spikes were marked with color tape and prepared for transformation by removing top and bottom flowers. Consequently the immature embryos from the middle part of the spikes were used for transformation. Immature caryopses were collected 13-14 days after anthesis.

5

Seed sterilization and immature embryos isolation

Immature seeds were sterilized by washing in 96% EtOH for 30 seconds followed by steering in 10% commercial bleach (Klorin®) + 0.1 % Tween-20 on the shaker for 10 min and five times rinsing in sterile distilled water. Immature embryos were dissected aseptically under the stereomicroscope and collected in 1ml PAW-infection medium with 300 µg/l acetosyringone added. Approximately 100 embryos with an optimal size 1.0 -1.2 mm in length were collected per micro tube, well developed milky scutellum and still translucent through the center.

15 1.2 Constructs

Super binary system was used in transformation experiments (WO 94/00977). Cloning vector pSB 11 was modified by replacing Sp gene with Km gene that is resulting in intermediate cloning vector pRLM175. Cloning expression cassettes with dsdA, dao1 and dao1 modified codons genes were cloned between RB and LB of T-DNA in intermediate cloning vector pRLM175. Constructs created and used for transformation are described in Table 2. Constructs maps are shown in Fig.1 - 3.

20

Table 2 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-ZmUbi = maize ubi promoter; t-OCS' = OCS' terminator; t-NOS = nos terminator; PsFedi = translational leader sequence)

Vector	LB-Selection marker	Reporter/Selection marker-RB	SEQ ID NO:
pRLM166	p-ZmUBI+l::c-dsdA::t-OCS	p-ScBV::c-gus ^{INT} ::t-NOS	10
pRLM167	p-ZmUBI+l::c-dsdA::t-OCS	p-ZmUBI+l::c-PAT::t-OCS	11
pRLM179	ZmAHASL2/Xi12	p-ZmUBI+l::c-dsdA::t-OCS	12
pRLM205	p-ZmUBI+l::c-dao1::t-OCS	p-ScBV::c-gus ^{INT} ::t-NOS	13
pRLM226	p-ZmUBI+l::l-PsFed1::c-dao1/ko	p-ScBV::c-gus ^{INT} ::t-NOS cas	14
pRLM151	p-ZmUBI+l::c-dsdA::t-OCS		39

Integration into *Agrobacterium* strain carrying super binary vector

The resulting intermediate plasmids were introduced by tri-parental mating cross (Ditta et al. 1980) Tri-parental mating is a term known in the art and involves a bacteria mating with 3 " sexes" .) in host bacteria LBA4404 (pSB1) that has a helper plasmid pAL4404 (having a complete *vir* region) and super virulence plasmid pSB1 obtained by inserting *virB*, *virC* and *virG* genes of a strongly virulent *Agrobacterium tumefaciens* strain A281 into pRK2 replicon. Both super virulence and intermediate plasmids share the regions of homology and recombine in *Agrobacterium*.

The presence of the transgenes in resulting recombined super binary vector system were confirmed in *Agrobacteria* by PCR using specific primers, e.g. as shown in SEQ ID No.: 29 to 41:

PCR reactions were performed using primers designed to amplify a 1000bp *gus*^{INT} fragment, a 500bp *ahas* fragment and a 442bp *pat* fragment, 485bp *dao1* fragment; 700bp *dsdA* fragment. Reaction conditions were as following for amplification of

gus^{INT} and dsdA fragments from pRLM166: " hot start" (95°C 5min) followed by 30 cycles of denaturation (94°C 30sec), annealing (62°C 30sec), extension (72°C 30 sec) followed by 1 cycle of 72 (5min) and then held at 4°C. Both fragments pat from pRLM167 and ahas from pRLM179 were amplified under similar conditions
5 except annealing temperature 63°C and 65°C respectively. Reaction conditions were as following for amplification of gus^{INT} and dao1 fragments from pRLM205: " hot start" (95°C 5min) followed by 35 cycles of denaturation (94°C 45sec), an-
nealing (66°C 30sec), extension (72°C 45 sec) followed by 1 cycle of 72 (5min) and then held at 4°C.

10

Preparation of Agrobacterium inoculum for transformation

Bacterial culture is initiated from the glycerol stock from the single colony growth on AB (Chilton et al. 1974) medium containing 50 mg/l spectinomycine or 50 mg/l kanamycin and 60 mg/l rifamycin respectively. Plates were incubated at 28°C in the
15 dark for 3 days or until single colonies are visible. For transformation fresh Agrobacterion culture is initiated from single colony on agar plate with YEP medium containing 10 g/l peptone, 5 g/l yeast extract, 5 g/l NaCl 15 g/l OXOID agar, 50 mg/l spectinomycin or 50 mg/l kanamycin respectively. Bacterial culture was grown for 2-3 days in dark at 26°C. Inoculum was initiated by dispersing Agrobacterium cells
20 (5 loops 2mm in 5ml medium) into PAWInf. medium Murashige & Skoog (1962) supplemented with 300 µM acetoseringone inverting and vortexing the tube for 5 min. Bacterial suspension was placed at 21° C for 3h on the shaker 200 rpm in dark. The density of cell population was adjusted to 1.0 -1.2 O.D. measured at λ 660 in spectrophotometer just before infection.

25

1.3 Transformation

Inoculation with Agrobacterium and co-cultivation

Explants were washed with PAW-Inf. medium and immersed in the above-described bacterial suspension for 2h at 26° C At the end of infection the explants
30 were placed with scutellum side up on PAW-1 medium. The residual bacterial sus-
pension is removed by pipeting out and air-drying of the infected embryos by open-
ing plates for 15 min on the sterile banch. Plates were sealed with Parafilm and placed in thermostat at 26°C in the dark for 5-6 days co-cultivation.

Selection of transgenic callus and tissues

After co-cultivation period the explants were washed with sterile water and 500 mg/L Cefotaxime and filter paper dried before being transferred to PAW-2 callus induction-recovery medium containing 160 mg/l Timentin for 14 days (7days dark/ 7 days semi light; $13.2\mu\text{mol m}^{-2}\text{s}^{-1}$). Explants with embryogenic callus were subculture to PAW-2 callus-proliferation medium containing 160 mg/l Timentin and corresponding selection 5 mM D-serine or 5 mM D-alanine or 3mg/l bialaphos. In some experiments the selection on D-amino acids was starting on PAW-2 callus induction medium 0, 7, 14 and 21 days after co cultivation. Embryogenic callus was subculture twice on fresh selective medium for callus maintaining and regeneration on PAW-4 medium with corresponding selection. Cultures were maintained at 23°C on light $60.2\mu\text{mol m}^{-2}\text{s}^{-1}$. Regenerated shoots were subculture to PAW-5 hormone free medium with corresponding selection (5 mM D-serine or 5 mM D-alanine or 3 mg/l bialaphos) for further growth and rooting. All media used in the transformation experiments were filter sterilized and are listed in above. After analyses transgenic plants were transferred to soil and placed for further growth in greenhouse.

1.4 Molecular and expression studies of the transgenic plants

20

TaqMan PCR

Leaf material was collected in 96 format plates, freeze dried and DNA was extracted using Wizard Magnetic 96 DNA plant system (Promega, Cat NoFF3760). Primarily transgenic plants were analyzed for gene integration using real-time PCR TaqMan chemistry (Ingham et al 2001) and specific primers and probes for the transgenes: SEQ ID NO: 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28.

25

Southern hybridization for copy number evaluation in wheat

Genomic DNA was extracted from silica gel dried leaf material following a method modified from Carlson et al. (1991). Twenty-five μg of gDNA was digested with BamHI, EcoRV or EcoRI and then separated by electrophoresis on a 0.8 % agarose gel. After depurination in 0.25N HCl for 25 min, DNA was transferred from the gel onto Hybond-N⁺ membrane by overnight capillary blotting using 0.4N NaOH as blotting solution.

30

PCR-amplified fragments recovered from gel with Zymoclean™ Gel DNA Recovery Kit (Zymo Research, CA USA) were used to generate ³²P-dCTP radioactively labelled probes using Rediprime™ Random Prime Labelling System (Amersham Biosciences). An 847 bp fragment of dsdA gene was used as a probe for plants transformed with pRLM151. An 884 bp fragment of gus^{INT} and an 1023 bp fragment of dsdA were used as probes for plants transformed with pRLM166. An 844 bp fragment of dsdA and an 828 bp of ahasL2 were used as probes for plants transformed with pRLM179. An 1156bp fragment of gus^{INT} was used as probe for transformants with pRLM205.

Prehybridization, hybridization and washing of membranes, and signal detection were performed as described in Sambrook et al. (1989).

Germination bioassay for resistance to D-serine

Well-developed intact T1 immature embryos (2.0-2.5 mm) were dissected aseptically from the young caryopses of T0 plants and cultured for germination on 1/2MS (PAW5 medium) medium supplemented with 1mM D-serine at 24°C. As a control non-transgenic Canon immature embryos were also included. Seedlings that grow and develop a strong rooting system on the selection medium scored 14 days after germination were considered to be transgenic.

Expression of pat and gus genes

GUS expression studies were done according to Jefferson et al. (1987) protocol and 20% methanol was added into the mixture. The expression of the pat gene was evaluated using the chlorphenolred test according to Kramer et al. (1993).

D-serine deaminase expression and activity in transgenic plants

Total of 19 events carrying dsdA gene representing T0 plants or T1 progenies were analyzed for D-serine deaminase by quantitative ELISA assay. Protein was extracted from 100 mg young leaf tissues according to Glick and Thompson (1993). About 20 µg of extracted protein per reaction was used in sandwich ELISA with goat IgG monoclonal antibodies (Harlow and Lane 1998). DSD activity was determined according to modified procedure (Szamosi et al. 1993).

D-Serine as a nitrogen source in hydroponics

T1 and T2 transgenic progenies were grown on hydroponics system where hydroponics solution (Gamborg and Wetter, 1975) was modified by replacing nitrogen with D-serine in five different concentrations (20, 30, 50 70 and 100 mM). Transgenic progenies and control seedlings were grown also on standard hydroponics solution as controls. The dry weigh of all 14 days old seedlings was measured.

Experiments were conducted using random block design with three replications. The obtained data were statistically analyses using the GLM ANOVA (Statgraphics Plus, Manugistics, Maryland, USA). Segregation of transgenes in sexual progenies was analyzed by χ^2 test for statistical deviation from Mendelian ratio for single-locus integration.

1.5 Killing curves

15 Immature embryos

In order to establish effective concentrations of D-serine and D-alanine on inhibiting growth of tissue cultured wheat 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 number of germinated embryos with well-developed shoots and brunched shoots were scored after 14 days.

Callus and regeneration

To define the sensitivity of wheat tissues during transformation when D-Serine and D-Alanine are present as selection agents regeneration experiments with immature embryos were designed. Immature embryos were immersed in PAWinf medium and subjected to all steps of callus induction, callus maintaining and regeneration in transformation protocol above.

Sensitivity of callus tissue under constant selection pressure were evaluated using range of concentrations: 0.5 mM, 1 mM, 3 mM, 5 mM, 10 mM, 25 mM, 50 mM. Experiments were performed in triplicates. Percentage of embryos forming embryogenic callus was scored on PAW-4 regeneration medium. At the end of shoots elongation regeneration capacity of the calluses was scored as calluses regenerating shoots and number of shoots per callus.

In vitro shoots

In order to define sensitivity of intact regenerants several concentrations of D-serine and D-alanine were evaluated: : 0.25 mM, 0.5 mM, 1 mM, 5 mM, 10 mM

In vitro shoots were regenerated and when roots were emerging shoots were transferred to selection. The effect of selection agents was observed two and tree weeks later. Number of plantlets with growing roots and green leaves were scored.

- 10 Example 2: Regeneration of transgenic plants using dual selection construct with dsdA and PAT genes and selection of transgenic plants on D-serine and bialaphos.

Freshly isolated immature embryos from Canon were inoculated with Agrobacterium. In all experiments Agrobacterium tumefaciens LBA4404 (pSB1/pRLM167) (SEQ ID NO: 11) was used. The pRLM167 is a super binary vector containing p-ZmUBI+l::c-dsdA::t-OCS and p-ZmUBI+l::c-PAT::t-OCS selectable marker genes in expression cassettes (Fig 1). Following co cultivation the explants were given a chance to recover for 14 days on callus induction selection free medium containing 160 mg/l Timentin to inhibit bacterial growth. Under these conditions 59% to 76% of the embryogenic callus developed over the all surface of immature embryos. Embryogenic callus was split in two and transferred to two the corresponding selection medium containing 5-mM D-serine or 3 mg/l bialaphos. During subsequent growth of callus, regeneration of plantlets, shoots elongation and roots formation the respective selection pressure was maintained in each of the corresponding medium.

25 After 7 weeks of selection 38% - 47% of the embryogenic calli regenerated with plants when selected on bialaphos or on D-serine respectively. Further selection of the regenerants was performed during the elongation and rooting of the regenerants for an additional 3 weeks. Intact in vitro transgenic shoots were selected within 10-12 weeks after co cultivation.

Table 3. Selection of transgenic plants containing dsdA/PAT selectable marker genes using Agrobacterium mediated transformation approach, construct pRLM167 and selection on D-serine or bialaphos

Experiments Repl. No	Construct	Genes of Interest	Explant No	Putative transgenic	Q-PSR (+)	TE/ER (%)*
2 (D-serine)	pRLM167	dsdA/pat	481	12	10	2.1/ 17
2 (bialaphos)	pRLM167	dsdA/pat	470	24	10	2.1/ 59

5 *TE-Transformation Efficiency calculated as% of transgenic plants out of the explants (freshly isolated immature embryos).

*ER - Escape Rate calculated as % of non-transgenic regenerants out of all selected plants.

10 More plants in bialaphos were able to root and grow in comparison to those selected on D-serine. Putative transgenic plants with branched root system and vigorous growth in presence of selection agents were acclimatized and analyzed by PCR or TaqMan PCR for their transgenic nature.

15 Using this protocol independent transformation events were obtained with a frequency in the average 2.1 % and the escape rate 17% when D-serine was used as selection (Table 3). The same transformation efficiency 2.1 % was achieved when bialaphos was used as selection agent however the escape rate was higher reaching 59%. This tendency was observed in our previous experiments using bialaphos
20 selection and the described transformation protocol (Data not shown).

All selected transgenic plants were tested by chlorophenol red test for PAT expression. Transgenic plants were changing the color of the medium to yellow that is the indication for the activity of PPT enzyme (Data not shown). DsdA gene expression
25 was detected by using bioassay for germinating T1 immature embryos on D-serine selective medium (Fig 4) (Discussed in Example 4). Furthermore germinated seedlings on selection were tested by TaqMan PCR for proving their transgenic nature. Grown to maturity transgenic T0 plants carrying dsdA/PAT genes show normal morphology, vigorous growth and full seed set (Fig 5).

The PAT gene from *Streptomyces viridochromogenes* conferring resistance to phosphinothricin (PPT)-based herbicides bialaphos and Basta®, has been used successfully as the selectable marker for wheat (Jones 2005). However it is recognized that pat gene as a selectable marker gene is resulting with selection of high number of untransformed plants (escapes) due to the presence of amino acids in tissue culture medium or "cross protection" that allows regeneration of untransformed cells (Christou et al.1991).

Our data suggest that the D-amino acid based selection system is comparable in resulting efficiency with phosphinothricin type herbicides based selection system in wheat. In respect of escape rate, the D-amino acid based selection system is resulting in limited number of escapes. Selected plants are developing strong phenotypic appearance of the toxicity so at the end of selection limited number of plants are entering greenhouse and are subjected for analyses, which is saving space and cost in production of transgenic plants. Both selectable marker genes dsdA and PAT integrated linked in T-DNA were shown to be active when introduced in wheat cells which is an advantage for implementation of gene stacking technologies and marker free transgenic plants.

20

Example 3 D-serine and D-alanine killing curves LD50 and LD100.

3.1 Dose curves of D-serine and D-alanine and constant selection pressure during callus initiation, callus maintaining and regeneration.

Range of the tested concentrations 0.5-50 mM D-ser and D-ala are evaluated in replicating regeneration experiments. The effect of selective compounds is estimated during callus growth and shoots formation following transformation procedure on medium with D-serine and D-alanine (Table 4).

30

Table 4. The effect of D-serine and D-alanine on wheat callus growth and regeneration

Concentrations (mM)	Canon -Regenerated Embryogenic Lines (%)		Bobwhite - Regenerated Embryogenic Lines (%)	
Compounds	D-serine	D-alanine	D-serine	D-alanine
0	93	88	94	100

0.5mM	84	63	83	85
3 mM	56	44	57	52
5 mM	23	19	32.5	14
10 mM	6	3	10	4
15 mM	3	0	6	0
25 mM	0	0	0	0
50 mM	0	0	0	0

The low concentrations of D-serine 0.5 mM shows clear increase in the regeneration capacity of the calli measured as the number of regenerants per callus line (Fig 6 A-B). The regenerating plants 6 weeks on selection were green and vigorous. On the medium with 3 mM D-serine the number of calli regenerating plants were reduced 50% but the capacity of the regenerating calli was profound and the shoots regenerated were green and normal. Regeneration capacity of the calli was reduced 70% already on 5 mM D-serine and D-alanine. Regenerating shoots after 7 weeks on selection medium were not vigorous, leaves were yellow with specific "a deer antler" phenotype. Despite of all this observation an individual plants were regenerated on the medium with 10 and 15 mM D-serine. Necrosis of callus was also observed on these and higher concentrations. The lethal doses (LD 100) for regeneration response of callus was 25 mM and 15 mM for D-serine and D-alanine respectively (tested in both varieties). None plants regenerated on medium with 25 mM D-serine and D-alanine and callus became necrotic. The lethal effect of D-amino acids on callus tissues is not completely understood yet. However D-serine and D-alanine applied as selective agents can influences callus proliferation and further regeneration in comparable manner as bialaphos.

3.2 Effect of D-serine and D-alanine on rooting of in vitro shoots.

In vitro regenerated shoots from media lacking the selection compounds were collected and transferred on medium for rooting (14 MS salts hormone free). Roots formation, growth of the shoots and leaves elongation was scored two and four weeks later after sub culturing of these shoots to the medium supplemented with D-amino acids (Table 5).

Table 5. The effect of D-serine and D-alanine on rooting stage of in vitro shoots.

Concentrations (mM)	Canon - Rooted Shoots (%)		Bobwhite - Rooted Shoots (%)	
Compounds	D-serine	D-alanine	D-serine	D-alanine
0	100	100	100	100
1mM	56	36	64	34
3 mM	16	11	24	13
5 mM	2	1	3	1
10mM	0	0	0	0
15mM	0	0	0	0
25 mM	0	0	0	0

A negative response in rooting and growth of the shoots on the medium with selection was clear after two weeks. On medium with 3 mM D-serine branching of the roots was affected but plants were still green and some of them continued to grow. In general non-transgenic shoots were not able to form roots on medium with 5 mM D-serine and D-alanine. Flag leaf of the plants showed specific "pin" like shape, became yellow, necrotic and died. All plant developed "a deer antler" phenotype and the leaves become yellowish. In individual cases plants were developing short thick green roots, thick stem and short broad flag leaf with hyper hydrated tissue of the lamina. The effect of D-amino acids on plant morphology, growth and rooting was profound (Fig 7).

3.3 The effect of D-serine and D-alanine on immature embryos germination and seedlings growth in in vitro conditions.

Immature embryos 2 mm in size were isolated from the sterilized caryopses. The embryos were cultured on PAW-5 hormone free medium for germination in dark. Seedlings growth on D-amino acid selection was observed after 2 weeks. Most of the embryos germinated but the seedlings growth was inhibited when roots emerge and the uptake of the selection compound was direct from the medium. The Lethal Dose (LD 100) for seedlings germination and growth was 1mM concentration of both D-amino acids tested (Table 6). Seedlings derived from embryos isolated from the immature caryopsis without endosperms were susceptible to the selection in concentration higher than 1mM (Fig 8).

Table 6. In vitro germination of immature embryos on medium containing D-serine and D-alanine.

Concentrations (mM)	Canon-Immature Embryos (%)		Bobwhite- Immature Embryos (%)	
Compounds	D-serine	D-alanine	D-serine	D-alanine
0	97	100	100	93
0.5mM	34	40	46	56
1 mM	0	0	0	0
5 mM	0	0	0	0
10 mM	0	0	0	0

- 5 The uptake of the selection compounds via scutellum and later on with roots enable fast accumulation of the selection agents in the tissues and cause the lethal effect on immature embryos denomination within one week. Both compounds prove to have lethal effect on the germination of the immature embryos when bioassay was carried out with 1mM selective compound.

10

Example 4: Regeneration of transgenic wheat plants with dsdA gene using selection on D-serine.

- Freshly isolated immature embryos from Canon were inoculated with Agrobacterium. In all experiments Agrobacterium tumefaciens super binary vector system based on LBA4404 (pSB1) was used. The constructs pRLM179, pRLM166, pRLM151 (Fig 2. I, II and III) containing dsdA selectable marker gene alone or with a second gene (ahas or gusINT) were used for transformation. The explants were cultured on selection medium right away after co-cultivation with Agrobacterium or were cultivated on callus induction - recovery medium for 7,14 and 21days containing only 160 mg/l Timentin for inhibiting bacterial growth. Furthermore calli were sub cultured to fresh selection PAW-2 medium for proliferation. Embryogenic calli were transferred on PAW-4 selection medium for regeneration 5-6 weeks after transformation. The emerging plantlets were further grown and rooted on PAW-5 hormone free selection medium. Well-developed and rooted on selection putative transgenic regenerants were selected in vitro within 9 better 10 -12 weeks.

Pilot transformation experiments were conducted with the selection pressure applied 7, 14 or 21 days after co cultivation. After transformation a total number of calli regenerating plants was not affected on medium supplemented with 3 mM D-serine. Individual callus lines show high regeneration potential. Roots growth and branching was faintly affected. All regenerants selected at this concentration proved to be escapes. Transgenic callus lines were not selected but regeneration capacity of the calli was inhibited on medium supplemented with 5 mM D-serine. Watery callus and necroses of the embryogenic calli with individual shoots regenerated were obtained when 10mM D-serine was incorporated in the medium.

Transgenic plants were obtained with comparable efficiency when 5 and 10 mM D-serine were included into the medium. Escapes rate was narrowed to 0 when shoots were selected on 10 mM D-serine (Table 7).

Table 7. Transformation of Canon with pRLM179 (dsdA/AHAS) with concentrations 3, 5 and 10 mM D-serine. Selection started 21 days after co-cultivation,

Experiment No.	D-Serine (mM)	Explants No	Putative Regenerant	TaqMan Positive	TE (%) [*]	ER(%) [*]
1	3	236	24	0	0	100
2	5	215	12	3	1.39	25
3	10	257	2	2	0.77	0

^{*}TE- Transformation Efficiency calculated as % of transgenic plants out of explants.

^{*}ER - Escapes Rate calculated as % of non transgenic regenerants out of all selected plants.

Large numbers of calluses were undergoing the selection of 3mM D-serine regenerating number of shoots. However no transgenic plants were identified using this particular concentration. Optimal for transformation was concentration 5mM D-serine as it was concluded from the killing curves regeneration experiments (Example 2).

The definition of the precise timing for application the selection pressure during transformation process required further experiments testing the defined in previous

experiments concentration 5 mM D-serine. Embryos with embryogenic callus were transferred on selection medium for callus proliferation immediately after co-cultivation or selection medium was introduced after 7, 14 or 21 days. When embryos were transferred immediately after co cultivation on selection medium number of embryos forming embryogenic callus was reduced. Postponed application of the selection pressure 7, 14 or 21 days was supporting recovery of the cells after co cultivation with Agrobacterium. Explants were entering the selection process when the embryogenic callus was proliferating. Callus selected on D-Serine for 5-6 weeks was friable type with green structures already appeared on the surface and comparable with the type of callus selected on bialaphos. Thus callus selected on D-Serine was not possessing specific morphology. Transgenic plants were obtained with different constructs when 5 mM D-serine selection was introduced 14 or 21 days after co cultivation (Table 8).

Table 8. Evaluation of different selection schema using D-Serine as selection compound, dsdA gene and wheat variety Canon

Transformation No	Constructs	Start sel. (days)	Selection	Explants No	Putative transgenics	Q-PCR (+)	TE (%)
WO04-46	pRLM179	21	D-ser	112	4	2	1.78
WF04-52	pRLM179	21	D-ser	286	2	1	0.35
WO04-63	pRLM166	14	D-ser	79	3	3	3.80
WO04-72	pRLM166	14	D-ser	90	1	1	1.10
WO04-69	pRLM166	21	D-ser	144	2	1	0.69
WO05-28	pRLM166	14	D-ser	280	3	2	0.71
WF04-71	pRLM166	14	D-ser	312	1	1	0.32
WF05-28	pRLM166	14	D-ser	199	1	1	0.50
WO05-28	pRLM166	14	D-ser	345	5	5	1.40
WO05-38	pRLM151	14	D-ser	373	6	6	1.60
WO05-36	PRLM151	14	D-ser	300	2	2	0.66
WF05-38	pRLM151	14	D-ser	176	9	2	1.10
WF05-39	PRLM151	14	D-ser	314	2	2	1.00

*TE- Transformation Efficiency calculated as% of transgenic plants out of explants.

*ER - Escapes Rate calculated as % of non transgenic regenerants out of all selected plants.

5 The transgenic plants were obtained using two different constructs with dsdA gene and second selectable marker gene ahas or marker gene gusINT. Transgenic plants were selected on D-serine with efficiency range of 0.35 to 3.8%. Average transformation efficiencies for both constructs were about 1%.

10 When the selection was applied immediately after co cultivation transgenic plants were not obtained. Immature embryos subjected to Agrobacterium transformation required period for recovery before going to selection. When selection was applied 7 days after co cultivation individual transgenic plant were recovered. Postponed selection for 14 and 21 days was resulting reproducibly with transgenic plants (Table 8).

15

Selection in respect of the escape rate on D-serine was better than one reported on bialaphos due to the clear phenotype performed at the end of selection. The escape rate shown in some experiments was strongly dependent by the duration on selection in rooting (2-4 weeks) in which plants were developing clear symptoms of D-amino acid toxicity. In vitro regenerants acclimatized before showing these symptoms were able to recover after transfer to soil. Applying strict selection criteria could lead to no escape rate as it was demonstrated in some of the experiments.

25 Moreover reducing 4 to 8 times nitrogen source in the rooting medium (PAW-5) was superior for vigorous growth of transgenic plants thus the escapes were eliminated completely. Non-transgenic plants were growing slower and the toxic effects were developing faster. Transgenic plants were strong and very well growing most probably using D-amino acid as a nutritional source (Data not shown).

30 The regenerants under the selection has distinguished phenotype. Transferred to the selection medium for rooting in vitro transgenic plants develop vigorous green leaves and strong-branched rooting system (Fig 9). Transgenic TO plants grown to maturity show normal morphology, vigorous growth and full seed set.

Expression of the reporter gene was measured by histochemical gus staining in different tissues. Expression in callus and in vitro leaf tissues were detected rarely. A total of 16 TO events grown in the greenhouse were analyzed at heading stage and mature seeds. Variation in tissue specificity and expression patterns were observed corresponding to the following distribution for plant/tissues: 88% in endosperm, 56% in embryos, 38 % in the roots, 25% in ovaries, 19 % in leaves and 13 % in anthers. In our constructs, the gus^{INT} gene was driven by SCBV promoter which resulted various intensity of the expression patterns.

10 Example 5: Regeneration of transgenic wheat plants with dao1 genes using selection on D-serine and D-alanine.

Freshly isolated immature embryos from Canon were inoculated with Agrobacterium. In all experiments Agrobacterium tumefactions super binary vector system based on LBA4404 (pSB1) was used. The constructs pRLM205 containing the original dao1 gene and pRLM226 carrying modified Dao1. Both constructs contain gus^{INT} reporter marker gene (Fig 3, I and II). Following co cultivation the explants were transferred on callus induction PAW-2 selection free medium containing 160mg/l Timentin to inhibit bacterial growth for 14 days. For selection embryogenic callus was transferred to PAW-2 selection medium containing 5 mM D-serine or 5 mM D-alanine respectively. Furthermore calli were sub cultured to fresh corresponding selection PAW-2 medium for maintaining. Embryogenic calli were transferred on PAW-4 selection medium for regeneration 5-6 weeks after transformation. The emerging plantlets were further grown and rooted on PAW-5 hormone free selection medium. Well-developed and rooted on selection putative transgenic regenerants were selected in vitro within. When D-alanine was used as selection compound similar effects in callus growth, shoots regeneration and rooting of transgenic plants or non transgenic escapes selected on D-serine was observed. Both selection agents resulted with 1.1% to 1.2 % transformation efficiency and no escape rate (Table 9). The transgenic plants showed normal vigorous growth under the selection pressure while non-transgenic plants were developing the phenotype described above. Transformation experiments with modified dao I gene (optimized codons) were resulting in transgenic plants in efficiency comparable with the experiments in which original gene was used (Table 9).

Table 9. Evaluation of D-serine and D-alanine selection compounds using with dao1 original and modified genes and wheat variety Canon.

Trafo No	Con-struct	Repli-cations	Selec-tion	Explants No	Putative transgenics	Transgen-ics Q-PCR(+)	TE (%)	ER (%)
WO04-78	pRLM205	2	D-ser	279	3	3	1.1	0
WF04-84	pRLM205	2	D-ala	298	2	2	1.27	0
WO05-03	pRLM226	2	D-ser	275	2	2	0.72	0

The D-amino acid based selection system using the dao1 gene and selection on D-serine and D-alanine were successfully utilized in wheat transformation. Both selective compounds were resulting with transgenic plants. Transformation efficiencies achieved with different constructs and the selectable marker genes dao1 and dao1/ko modified were comparable. All transgenic plants from this experiments were grown to maturity showing normal morphology, vigorous growth and full seed set. TO transgenic plants were evaluated for presence of the second gene using TaqMan assay and histochemical staining. Selectable marker gene was detected in all transgenic dao1 plants. Although gus^{INT} gene expression was found in individual plants measured as histochemical reaction in leaves, roots and anthers (Fig10).

T1 immature embryos were assayed with bioassay for germination of immature embryos on selection. All plants growing on selection were proved to be transgenic by TaqMan. Furthermore transgenic T1 seedlings were tested for negative selection using D-isoleucine and D-valine containing medium. Transgenic T1 dao1 seedlings were sensitive to the concentrations range from 10 to 30 mM D-isoleucine and 15 - 20 mM D-valine (Fig 11).

Example 6: Analyses of transgenes integration and inheritance

TO plants were grown to maturity in the greenhouse. Plants were self-pollinated secured by bagging all spikes individually. All TO plants had normal phenotype, vigorous growth and full seed set. T1 seedlings were produced in vitro and in the

greenhouse. Seedlings were tested by TaqMan PCR assay for genes integration and for expression measured as chlorophenol red test or herbicide tolerance for PAT and ahas transgenes, germination bioassay for dsdA and dao1 genes and histochemical staining for gus^{INT} reporter gene.

- 5 Total of 21 transgenic events (5 PAT/dsdA, 6 dsdA/GUS, 2 dsdA, 5 dao1/GUS, 3 dsdA/ ahas) were analyzed for copy number analyses by TaqMan[®] PCR and segregation in T1.

TO plants and their T1 progenies were assayed for copy number by Southern hybridization using dsdA probe for plants transformed with constructs pRLM 166, 179,
10 151, 167 and GUS probe for plants transformed with constructs pRLM205 and 226. Expression and enzyme activity for DSD (D-serine deaminase) enzyme was studied by enzymatic assay.

Transgenic plants were also evaluated for ability to grow in presence of D-serine as a solely nitrogen source using hydroponics culture.

15

6.1 . Inheritance of dsdA/PAT genes in T1 progenies selected on bialaphos

Expression of PAT gene in transgenic plants was evaluated and measured as change of pH and color (red to yellow) of the medium in chlorophenol red test and herbicide tolerance after spraying with Basta[®]. The same transgenic plants were
20 assayed for DsdA expression in T1 immature embryos measured with germination bioassay. Segregation analyses in T1 progenies were based on ability of transgenic plants to germinate, grow and root under selection conditions when 1mM D-serine is included in the medium. Thus the proposed in vitro bioassay based on germinating immature embryos detached from the endosperm on selective medium
25 is a handy tool for the screening of segregating transgenic populations carrying dsdA selectable marker gene as it was reported for bar selection on bialaphos (Stoger et al. 1998).

Progenies from three events show 3:1 segregation ratios suggesting single locus of
30 integration. Two other events confirm 15:1 segregation ratio that suggest integration in two loci (Table 10).

Table 10. Inheritance of the dsdA/PAT transgenes in T1 generation.

Plants No	Primary transformants (T0)		Inheritance T1 progeny (TaqMan PCR)			T1 Segregation	
	PAT expression	Min. No of copies dsdA	T1 No	Positive	Negative	χ^2	Segregation ratio
10	yes	1	2	10	2	0.49	3:1
11	yes	1	28	22	6	0.36	3:1
13	yes	1	30	26	5	0.46	3:1
14	yes	3	30	28	2	0.08	15:1
15	yes	3	25	25	2	0.44	15:1

χ^2 values are not significantly different from the ratio tested at a level of significance of 0.1

- 5 Unlike *Arabidopsis* (Ericson et al. 2004a), spraying of 5 days old control and transgenic seedlings with D-serine was not efficient in wheat. The concentrations 50 - 300mM D-serine were not sufficient to elicit toxicity symptoms in control seedlings (Data not shown). Watering of the plants was also not sufficient. Since the uptake, mobility in soil and detoxification by microorganisms are all potential barriers for D-serine in the soil, the expectation of in soil selection seems bleak. Therefore, that the transgenic plants will use the products from detoxification of D-serine as an additional nitrogen supply was not fulfilled in soil.

6.2. Inheritance of dsdA, dsdA/gus^{INT} and dsdA/ahas genes in T1 progenies

- 15 T1 progenies of the 2 dsdA, 6 dsdA/gas^{INT} and 3 dsdA/ahas events were grown in the greenhouse. Segregation ratios were calculated on the base of TaqMan analyses. All transgenic plants positive for dsdA were also positive for the second gene. Seven of the analyzed progenies confirmed both transgenic and show segregation ratio 3:1 that suggest single loci of integration. Four events show 15:1 ratio suggesting two loci of integration (Table 11). Copy number was ranging from 1 to 3 estimated by TaqMan (verified by Southern). GUS expression was detected histochemically in all T0 and T1 positive progenies confirming complete transfer, integration and inheritance of both transgenes.

6.3. Inheritance of dao1/GUSint genes in T1 progenies

T1 progenies of the 5-dao1/gusINT events were grown in the greenhouse. Segregation ratios were calculated on the base of TaqMan analyses. All transgenic plants positive for dao1 were positive for the second gene. All analyzed progenies confirmed both transgenes and show segregation ratio 3:1 that suggest single loci of integration in three events while two progenies show 15:1 ratio suggesting two loci of integration (Table 11). GUS expression was detected histochemically in all positive progenies.

10

Table 11. Inheritance of the dsdA and dao1 genes in T1 generation.

Plants No	Transgenes	Primary transformants (T0)		Integration (TaqMan PCR)			T1 Segregation	
		Min No of dsdA*/Dao1	GUS expression	T1 No	Positive	Negative	χ^2	Segregation ratio
1	dsdA/gusINT	2	yes	20	16	4	0.37	3:1
2	dsdA/gusINT	1	yes	24	18	6	0.03	3:1
3	dsdA/gusINT	2	yes	20	20	0	0.31	15:1
4	dsdA/gusINT	3	yes	20	17	3	0.36	3:1
5	dsdA/gusINT	1	yes	19	12	3	0.43	3:1
6	dsdA/gusINT	1	yes	20	16	2	0.41	3:1
423	dsdA/gusINT	3	yes	24	21	3	0.42	15:1
385	dsdA	1		33	24	9	0.31	3:1
451	dsdA	6		27	26	1	-	-
2256	dsdA/AHAS	2	nd	28	26	2	0,26	15:1
2258	dsdA/AHAS	3	nd	20	20	3	0,06	15:1
2263	dsdA/AHAS	1	nd	26	21	5	0,49	3:1
009	dao1/gusINT	nd	yes	20	20	0	0.07	15:1
026	dao1/gusINT	nd	yes	20	20	0	0.07	15:1
051	dao1/gusINT	nd	yes	19	16	3	0.42	3:1
052	dao1/gusINT	nd	yes	20	14	6	0.34	3:1

091	dao1/gusINT	Nd	yes	22	18	4	0.19	3:1
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χ^2 values are not significantly different from the ratio tested at a level of significance of 0.1

*Estimated copy number by TaqMan is not verified with true calibrator.

Nd: not detected

5

All analyzed transgenic progenies were expressing dsdA and dao 1 genes measured as bioassay germination of immature T2 embryos on 1mM D-Serine (Data not shown).

10 6.4. Molecular analyses of T0 and T1 plants

The dsdA gene copy number was analyzed in 33 samples representing 12 events. Genomic DNA from T0 primarily transformants and their T1 progenies were digested with BamHI or EcoRV correspondingly to the constructs. Restriction enzymes were selected to cut outside the dsdA gene coding regions within T-DNA generating different fragments due to the occurred cutting sides in the genomic DNA. The unique assembled hybridization patterns correspond to the independent events and also show copy number of the selectable marker gene (Fig 12 I, II, III). Plants with both single and multiple copy numbers were recovered with each of the tested constructs.

20 The inheritance of the dsdA gene in T1 progenies was revealed by identical T0 and T1 Southern hybridization profiles (Fig. 12 I, II). Segregation of bands was observed in lines 2258 and 2256 that have 2 and 3 copies, respectively, confirming integration at two independent loci (Fig. 12 III).

The dao1 gene copy number was analyzed in 14 samples representing 5 events T0 and T1 progenies. Genomic DNA from T0 primarily transformants and their T1 progenies were digested with BamHI or EcoRV correspondingly to detect gus or dao 1 gene. Restriction enzymes were selected to cut outside the probed genes coding regions within T-DNA generating different fragments due to the occurred cutting sides in the genomic DNA. The unique assembled hybridization patterns correspond to the independent events and also show copy number of the selectable marker gene (Fig 13). Plants with range of 1 to 4 copy numbers were recovered. The bands for gus in the T1 plants of event 51, 52 and 91 correspond to those in T0. T0 event 26 had three

copies, which are segregation in T1 progenies with one and two copies respectively suggesting two places of integration (Fig 13).

When all 53 transgenic events were analyzed for dsdA copy number by TaqMan
5 PCR the following distribution was detected: 60% single copy; 12% two copies 27%
more than two copies.

6.5. DsdA selectable marker gene expression

10 Leaf material from several T0 and T1 transgenic plants transformed with three different constructs and with different transgene copy number (CN) was evaluated by sandwich ELISA method for detecting D-serine deaminase expression (Table 12).

Transgenic plants with diverse levels of DSD expression were found irrespectively
15 from the construct used and copy number detected. Plants No 3, 4, 385 and 423
were showing high expression levels in a range of 30-45 ng/mg protein while DSD
was not detected in leaves of the plants No 10, 14, 413 and 463 (Table 12). DSD
was not present in controls: non-transgenic and transgenic plants with bar and
dao1 genes. Consistent data were obtained when enzymatic activity was measu-
20 red (Fig 14).

Table 12 T0 and T1 transgenic wheat plants characterized for copy number by Taq-Man PCR and D-serine deaminase activity by ELISA

Plant No	Progeny	Construct	Gene 1	Gene 2	CN dsdA	DSD (ng/mg)
1	T1	pRLM166	dsdA	gus	1	24,89
2	T1	pRLM166	dsdA	gus	1	24,35
3	T1	pRLM166	dsdA	gus	1	32,71
4	T1	pRLM166	dsdA	gus	2	40,70
5	T1	pRLM166	dsdA	gus	1	20,48
10	T1	pRLM167	dsdA	pat	1	0,00
11	T1	pRLM167	dsdA	pat	1	19,49
13	T1	pRLM167	dsdA	pat	1	8,78
14	T1	pRLM167	dsdA	pat	3	0,00
15	T1	pRLM167	dsdA	pat	3	28,50
385	T0	pRLM151	dsdA	-	1	47,57
413	T0	pRLM166	dsdA	gus	2	0,00
423	T0	pRLM166	dsdA	gus	3	33,74
429	T0	pRLM166	dsdA	gus	1	23,86
431	T0	pRLM151	dsdA	-	1	5,21
449	T0	pRLM151	dsdA	-	1	9,87
450	T0	pRLM151	dsdA	-	1	18,57
451	T0	pRLM151	dsdA	-	2	18,77
463	T0	pRLM151	dsdA	-	1	0,00
dao1 26	T1		dao1	gus	2	0,00
Control C	Canon		-	-		0,00
VC-05			bar		1	0,00

5 6.6. D-serine as a nitrogen source in hydroponic system

Catabolic products of D-serine deaminase are ammonium and pyruvate - key compounds in plant metabolism. Theoretically, transgenic plants equipped with a

functional *dsdA* gene are expected to utilize D-serine as a nitrogen source. We investigated this phenomenon in a hydroponic growth system by exchanging the nitrogen supply with D-serine. T2 progenies representing several independent transgenic events were subjected to two sets of hydroponic experiments.

5

The first hydroponics experiments were performed with plants from three independent transgenic lines 2256, 2258 and 2264 carrying the *dsdA* and *ahas* selectable marker genes and a range of D-serine concentrations (Fig. 15 I). Due to the accumulation of D-serine, the growth of non-transgenic plants was fully inhibited at all tested concentrations. Statistically significant differences between controls and transgenic plants on corresponding concentrations of D-serine were detected. Two of these transgenic lines were inhibited in all tested D-serine concentrations. Line No 2256 T2 seedlings grew in all tested D-serine concentrations with a performance comparable to seedlings grown in standard hydroponics solution demonstrating ability to use D-serine as a nitrogen source.

15

The second set of the experiments with four lines carrying the *dsdA* and *gus*^{INT} genes were grown in hydroponics solution supplemented with 30, 50 and 70 mM D-serine (Fig. 15 II). The results confirm that non-transgenic control plants were not able to grow at the tested concentrations. Transgenic events 3 and 15 were sensitive to all concentrations of D-serine while progenies from events 1 and 4 grew in presence of 30 mM D-serine. Transgenic progenies from event number 4 grew in hydroponics up to 70mM D-serine reaching the control's values.

20

25 Conclusions:

1. Transgenic wheat plants are selected with the D-amino acid based selection system with a frequency comparable to the *pat*/*bialaphos* selection. *PAT* and *dsdA* genes are not interfering when transferred in one T-DNA in wheat plants.
2. Selection system with *dsdA* and *dao1* provide a key advantage to minimize escape rate to zero and reduce cost of production of transgenic plants.
3. Selection effect is profound in rooting stage where non transgenic plants are developing abnormal leaf and root phenotypes leading to the selection of transgenic plants with very low or no escape rate.

30

4. Both selection agents D-serine and D-alanine are suitable for selecting wheat transgenic plants.
5. Selected transgenic plants with *dsdA* and *dao1* genes have normal phenotype, growth performance, seed set that are inherited in T1 progenies in Mendelian fesion.
6. The D-amino acid based selection system is a tool for gene stacking in wheat by re transformation or hybridization approaches.
7. The D-amino acid based selection system provides possibilities for producing " marker free" transgenic plants by applying negative selection.
- 10 8. D-serine as a substitute to the nitrogen in hydroponics system showed both inhibitory effect in plants growth and ability of individual lines to grow.

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Claims

1. A method for generating a transgenic wheat plant comprising the steps of
 - a. introducing into a wheat cell or tissue a DNA construct comprising at least one first expression construct comprising a promoter active in said wheat
5 plant and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine, and
 - b. incubating said wheat 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 3 mM to 100 mM for a time period of at least 5 days, and
10 c. transferring said wheat cell or tissue of step b) to a regeneration medium and regenerating and selecting wheat 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 wheat plant, and
15 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 at least one first expression construct comprising a
20 promoter active in said wheat plant and operably linked thereto a nucleic acid sequence encoding an enzyme capable to metabolize D-alanine and/or D-serine, and
 - c. transferring the co-cultivated immature embryos to a recovering medium,
25 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
30 ii. D-alanine and/or D-serine in a total concentration from 3 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 1 or 2, wherein the DNA construct of claim 1 or the T-DNA of claim 3 further comprises at least one second expression construct conferring to said wheat plant an agronomically valuable trait.
 - 5 4. The method of claim 2 or 3, wherein the effective amount of the auxin compound is equivalent to a concentration of 0.2 mg/l to 6 mg/l 2,4-D.
 5. The method of any of claim 1 to 4, 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).
 - 10 6. The method of any of claim 1 to 5, wherein the enzyme capable to metabolize D-serine is selected from the group consisting of
 - 15 i) the D-serine ammonia-lyase as shown in Table I,
 - 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 an amino acid sequence of a D-serine ammonia-lyase as shown in Table I;
 - 20 iii) enzymes having the same enzymatic activity and an identity of the encoding nucleic acid sequence 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 a nucleic acid sequence of a D-serine ammonia-lyase as shown in Table I, and
 - 25 iv) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence encoding the D-serine ammonia-lyase as shown in Table I,

and wherein selection is done on a medium comprising D-serine in a

 - 30 concentration from 3 mM to 100 mM;
- or wherein the enzyme capable to metabolize D-serine and D-alanine is selected from the group consisting of
- i) the D-amino acid oxidase as shown in Table I, 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 an amino acid sequence of a D-amino acid oxidase as shown in Table 1;
- 5 iii) enzymes having the same enzymatic activity and an identity of the encoding nucleic acid sequence 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 a nucleic acid sequence of a D-amino acid oxidase as shown in Table 1, and
- 10 iv) enzymes encoded by a nucleic acid sequence capable to hybridize to the complement of the sequence encoding the D-amino acid oxidase as shown in Table 1,
- and wherein selection is done on a medium comprising D-alanine and/or D-serine in a total concentration from 3 mM to 100 mM.
- 15
7. The method of any of claim 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
- 20 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 3 mM to 100 mM.
- 25
- or 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
- 30 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
- 35 D-serine in a total concentration from 3 mM to 100 mM.

8. The method of any of claim 1 to 7, wherein said promoter active in said wheat plant is an ubiquitin promoter, preferably the maize ubiquitin promoter.
- 5 9. The method of claim 8, wherein selection pressure is applied for 7 to 21 days after co-cultivation.
10. The method of claim 8 or 9, wherein the ubiquitin promoter is selected from the group consisting of
- 10 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 wheat,
- 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 wheat,
- 15 d) sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 5, and having promoter activity in wheat.
- 20 11. The method of any of claim 8 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 wheat,
- 25 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 wheat,
- d) sequences comprising a sequence hybridizing to the sequence as described by SEQ ID NO: 6, and having promoter activity in wheat.
- 30 12. The method of claim 1 or 2, wherein the selection of step b) of claim 1 or step d) of claim 2 is done using 5 to 10 mM D-alanine and/or D-serine.
13. The method of claim 1, 2, or 12, wherein the total selection time under differentiating conditions is from 3 to 4 weeks.
- 35

14. The method of claim 1, 2, or 13, wherein the selection of step b) of claim 1 or step d) of claim 2 is done in two steps, using a first selection step for 14 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 14 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 *Agrobacterium rhizogenes* bacterium.
17. The method of any of Claim 1 to 16, wherein said wheat plant is selected from the group of Triticum family.
18. The method of Claim 17, wherein said wheat cell or tissue or said immature embryo is isolated from a plant specie of the group consisting of Triticum spp.: common (T. aestivum), durum (T. durum), spelt (T. spelta), Triticum dicoccum (Emmer wheat), Triticum turgidum, and Triticum monococcum (Einkorn wheat).
19. The method of claim 1, wherein said method comprises the steps of:
- i) transforming a wheat plant cell with a first DNA construct comprising
 - a) at least one first expression construct comprising a promoter active in said wheat plant 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

- 5 ii) treating said transformed wheat 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
- 10 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
- 15 a) the promoter is defined as in any of claim 7 to 10, and/or
- b) D-amino acid oxidases is defined as in claim 5 or 7.
21. A wheat plant or cell comprising a promoter active in said wheat plants or cells 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.
- 20
22. The wheat plant of claim 21, wherein
- a) the promoter is defined as in any of claim 9 to 11, and/or
- 25 b) enzyme capable to metabolize D-alanine or D-serine is defined as in any of claim 6 to 8.
23. The wheat plant of claim 21 or 22, further comprising at least one second expression construct conferring to said wheat plant an agronomically valuable trait.
- 30
24. The wheat plant of any of claim 21 to 23, wherein said wheat plant is selected from the group of Triticum family.

25. The wheat plant of any of claim 21 to 24, wherein said plant is from the group consisting of *Triticum* spp.: common (*T. aestivum*), durum (*T. durum*), spelt (*T. spelta*), *Triticum dicoccum* (Emmer wheat), *Triticum turgidum*, and *Triticum monococcum* (Einkorn wheat).
- 5
26. A part of a wheat plant of any of claim 21 to 25.
27. A method for subsequent transformation of at least two DNA constructs into a wheat plant comprising the steps of:
- 10
- a) a transformation with a first construct said construct comprising at least one expression construct comprising a promoter active in said wheat plants 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-
- 15
- alanine or D-serine.
28. The method of claim 27, wherein said second marker gene is conferring resistance against at least one compound select from the group consisting of
- 20
- phosphinotricin, glyphosate, sulfonylurea- and imidazolinone-type herbicides.
29. A wheat plant comprising
- a) a first expression construct comprising a promoter active in said wheat plants and operably linked thereto a nucleic acid sequence encoding an
- 25
- 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.
30. A method for subsequent transformation of at least two DNA constructs into a wheat plant comprising the steps of:
- 30
- a) a transformation with a first construct said construct comprising an expression construct comprising a promoter active in said wheat plants 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 an expression construct comprising promoter active in said wheat plants and operably linked thereto a nucleic acid sequence encoding a dao enzyme and selecting with D-alanine.

5

31. A wheat plant comprising

- a) a first construct said construct comprising an expression construct comprising a promoter active in said wheat plants and operably linked thereto a nucleic acid sequence encoding an dsdA enzyme, and
- 10 b) a second construct said construct comprising an expression construct comprising promoter active in said wheat plants and operably linked thereto a nucleic acid sequence encoding a dao enzyme.

- 15 32 Composition for selection, regeneration, growing, cultivation or maintaining of a transgenic wheat plant cells, a transgenic wheat plant tissue, a transgenic wheat plant organs or a transgenic wheat plants or a part thereof comprising an effective amount of D-alanine, D-serine, or a derivative thereof allowing for selection of transgenic wheat plant cells, wheat plant tissue, wheat plant or-
- 20 gans or wheat plants or a part thereof and a transgenic wheat organism, a transgenic wheat cell, a transgenic cell culture, a transgenic wheat plant and/or a part thereof.

- 25 33. Cell culture comprising one or more embryogenic calli derived from immature wheat embryo(s), at least one auxin, wherein the effective amount of the auxin compound is equivalent to a concentration of 0.2 mg/l to 6 mg/l 2,4-D, and D-alanine and/or D-serine in a total concentration from 3 mM to 100 mM.

- 30 34. Recovery medium comprising an effective amount of at least one antibiotic that inhibits or suppresses the growth of the soil-borne bacteria, and/or L-proline in a concentration from 1 g/l to 10g/l, and/or silver nitrate in a concentration from 0 μ M to 50 μ M.

- 35 35. Selection medium comprising a wheat target tissue and D-alanine and/or D-serine or a derivative thereof in a phytotoxic concentration.

36. Regeneration medium comprising transformed wheat plant cells and one or more compounds selected from the group consisting of:
- i) cytokinins in a concentration from 0.5 to 10 mg/L,
 - ii) an effective amount of at least one antibiotic that inhibits or suppresses the growth of the soil-borne bacteria), and
 - iii) an effective amount of D-alanine, D-serine, or a derivative thereof allowing for selection of transgenic cells.
- 5

1/15

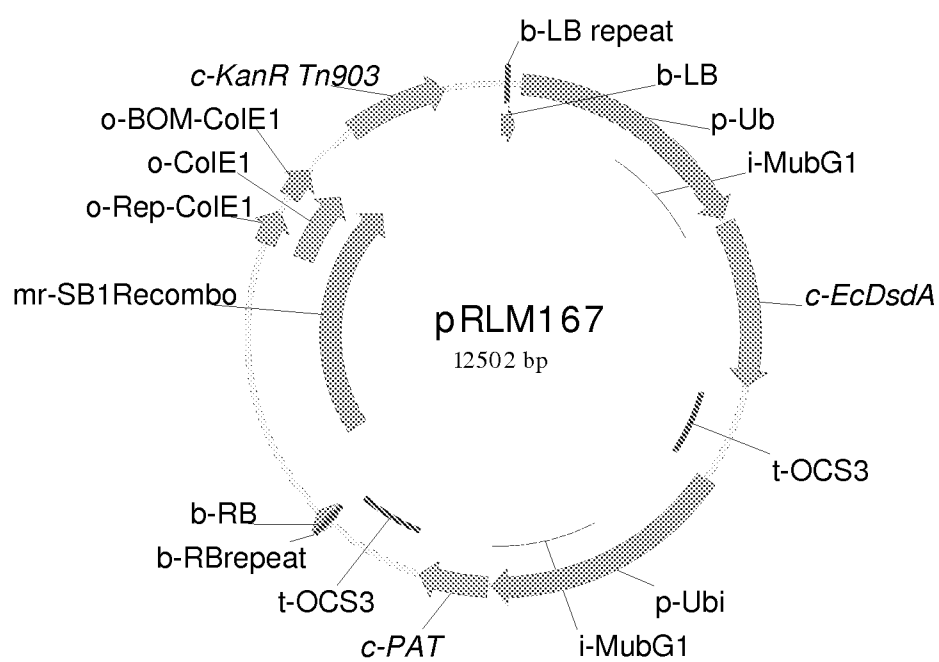


Fig. 1

2/15

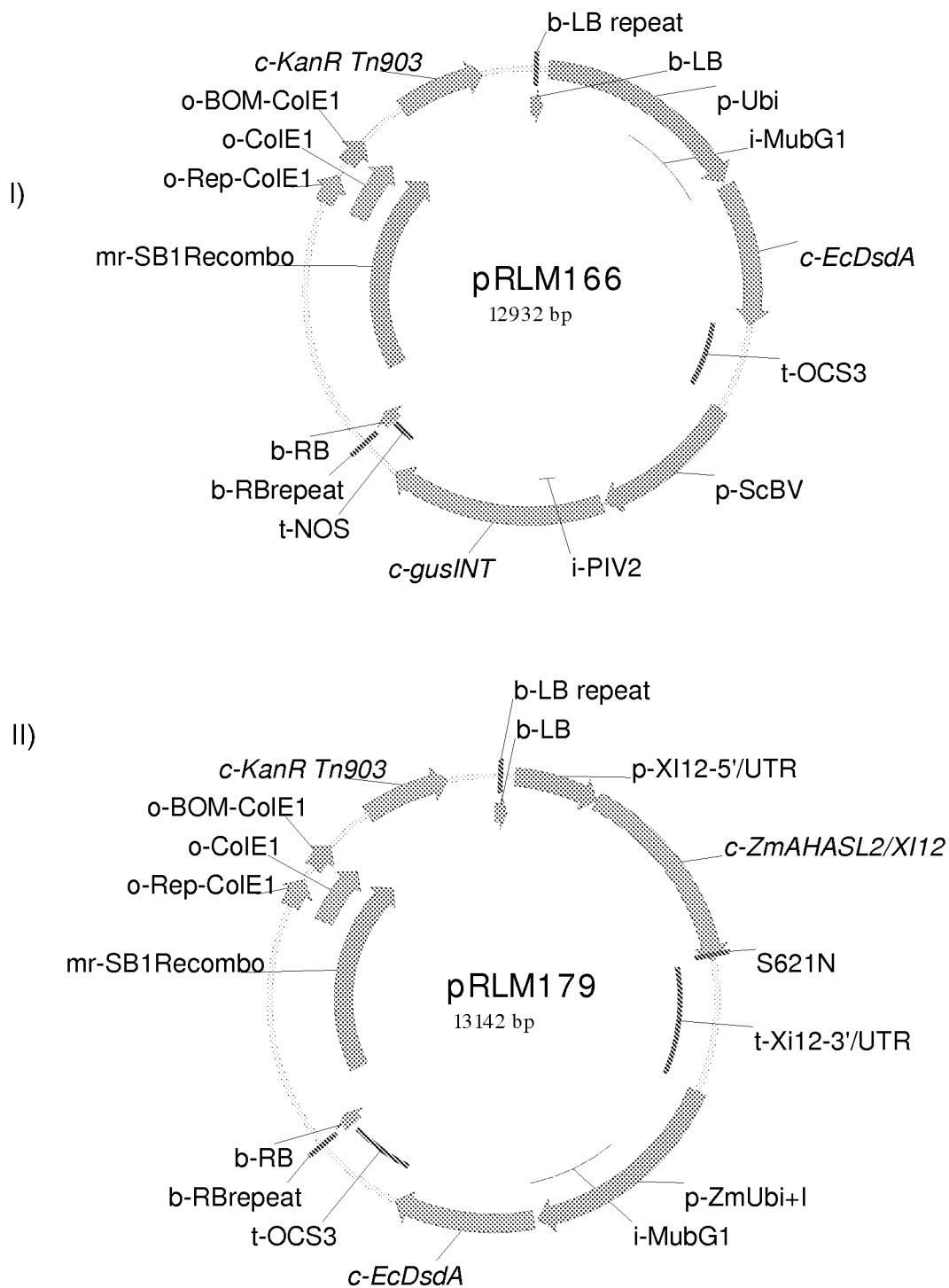


Fig. 2

3/15

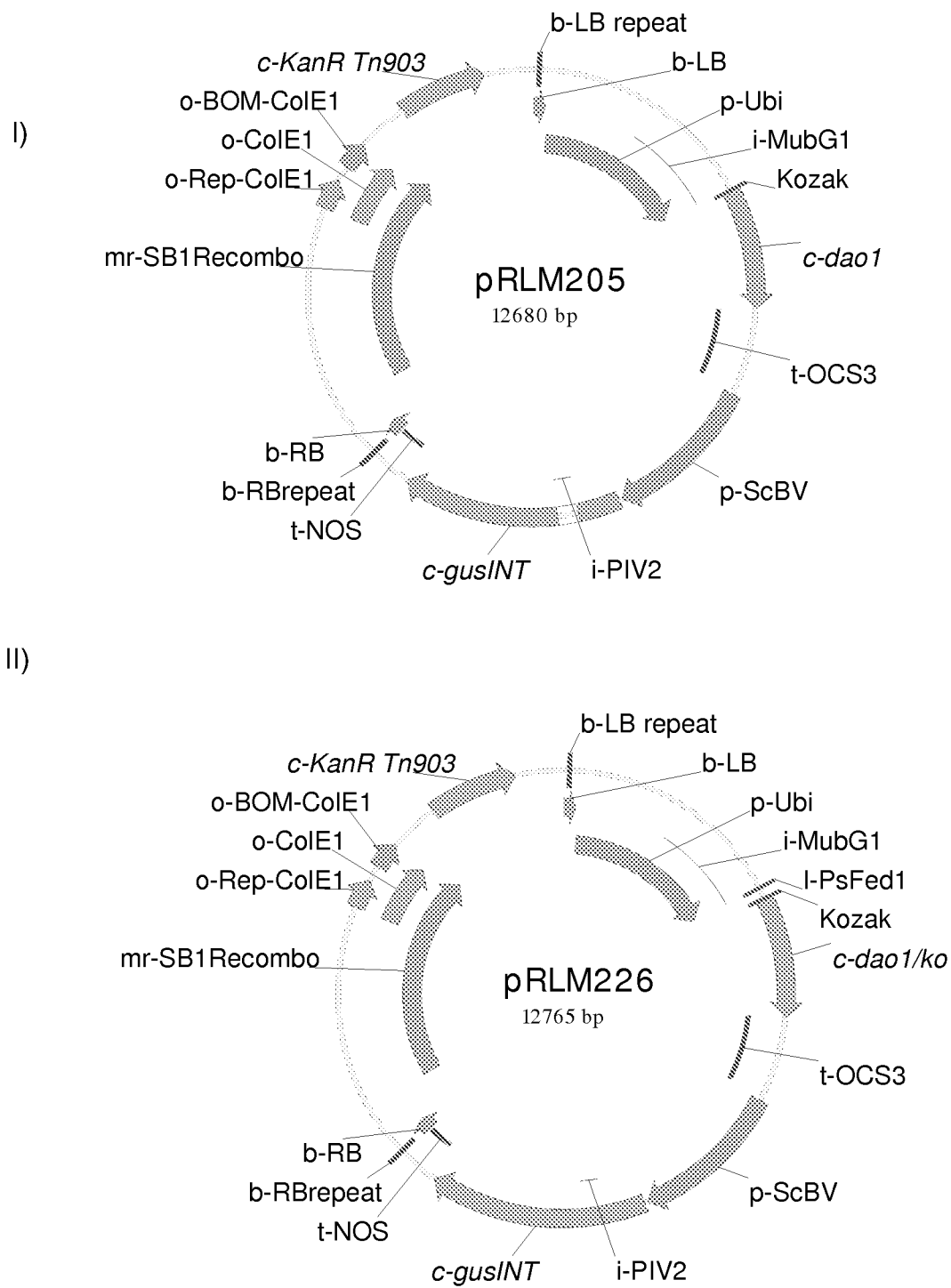


Fig. 3 (1 of 2)

3/15 cont.

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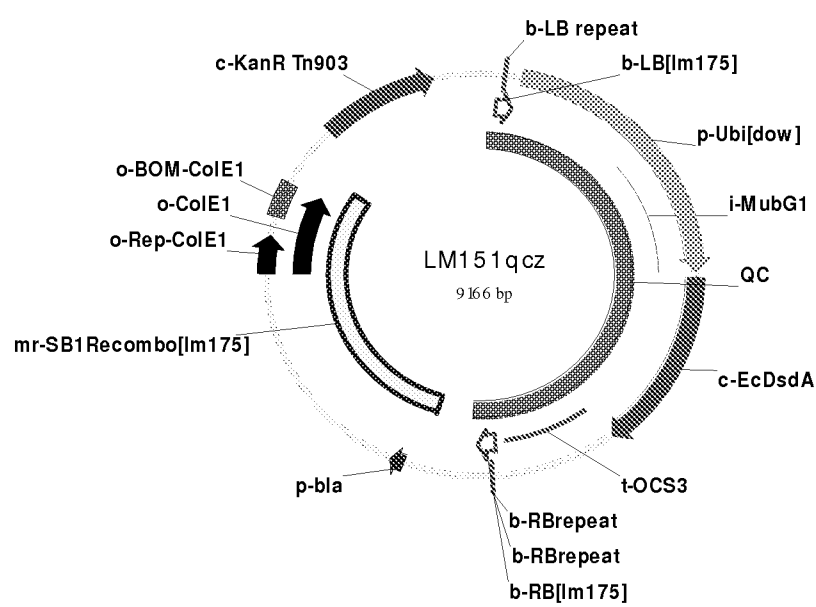
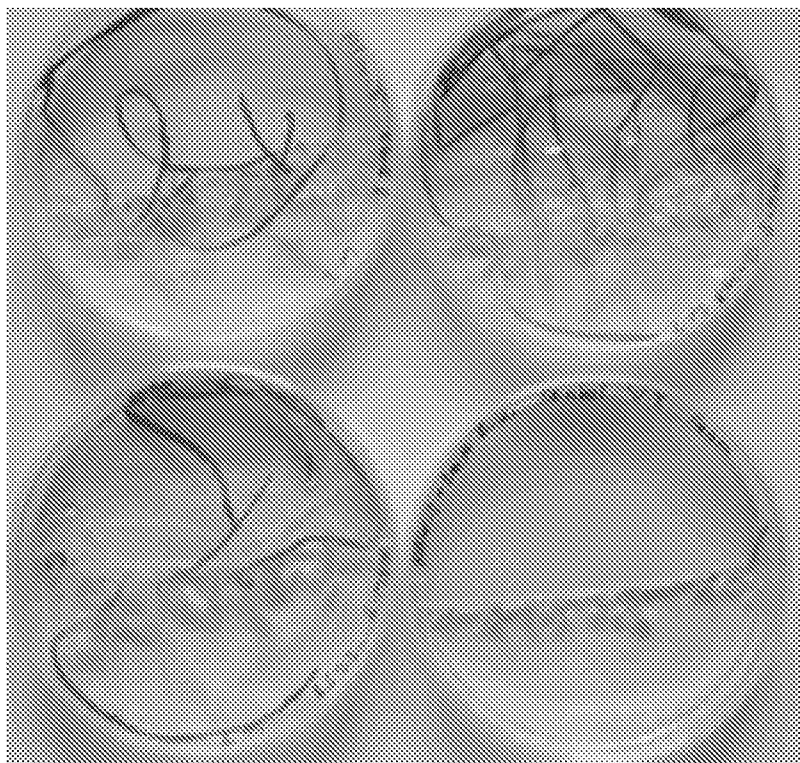


Fig. 3 (2 of 2)

4/15

I)



II)



Fig. 4

5/15

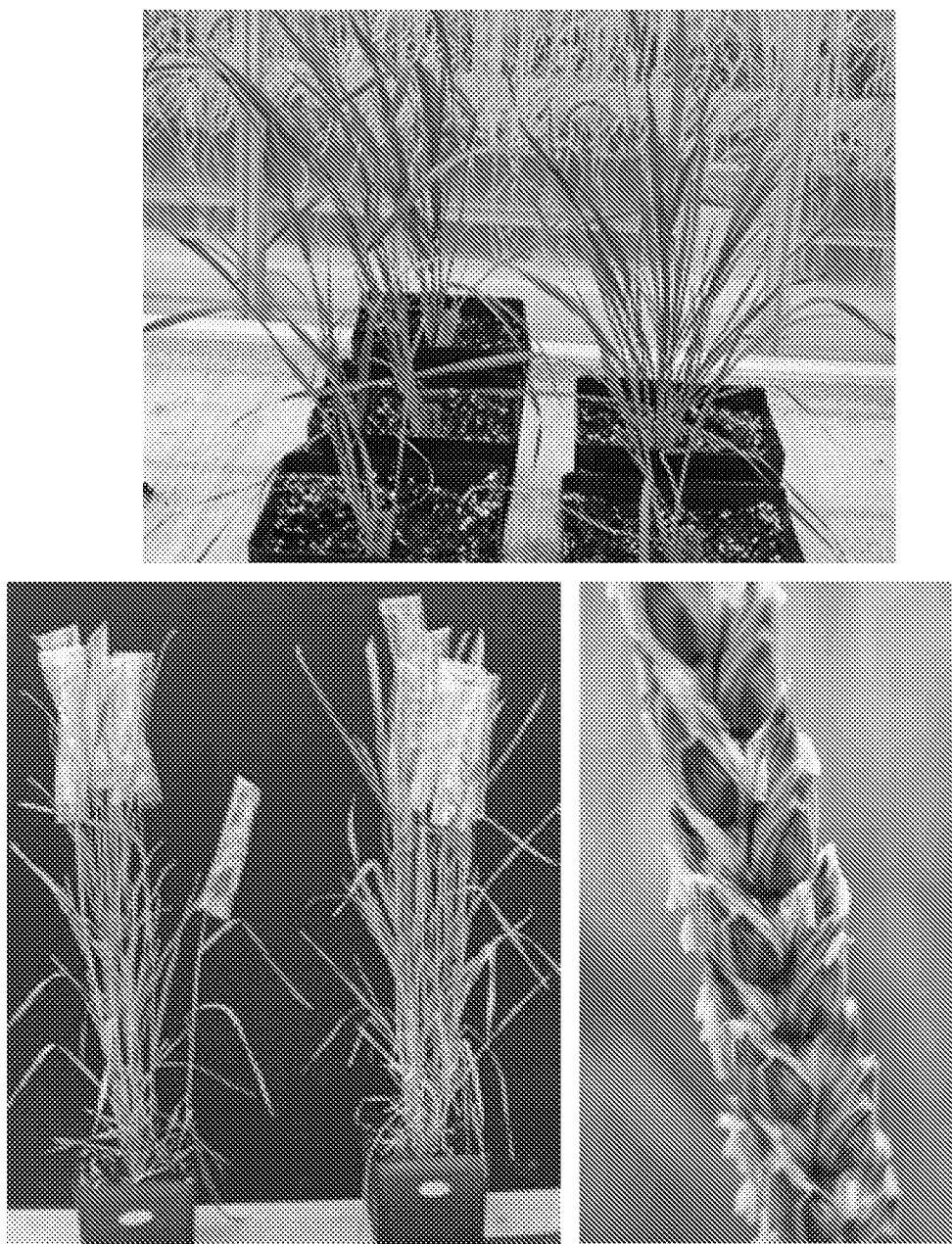
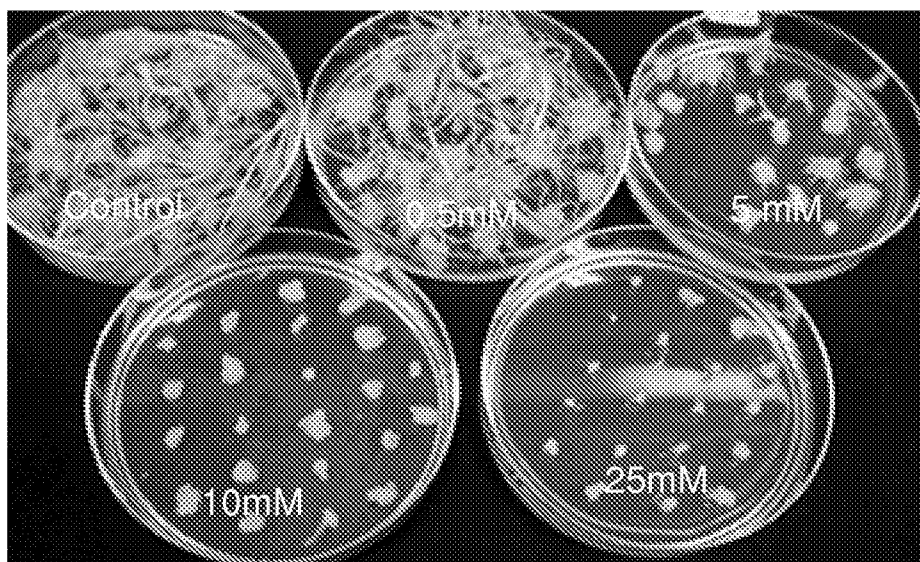


Fig. 5

6/15

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II)

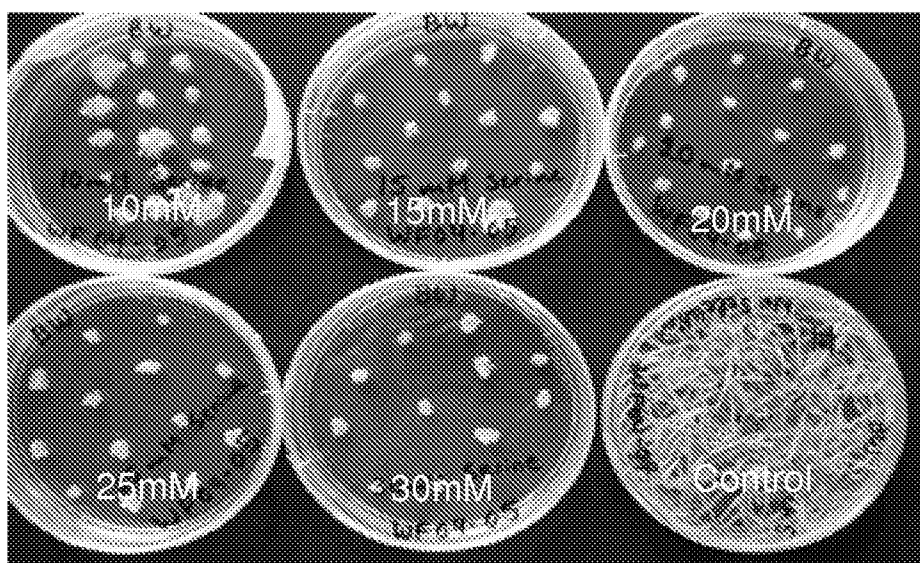


Fig. 6 (1 of 2)

6/15 cont.

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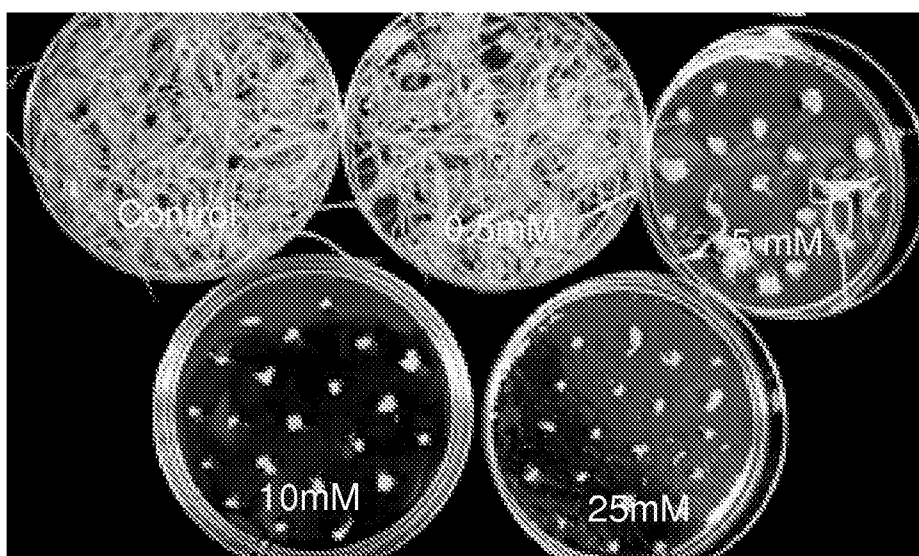


Fig. 6 (2 of 2).

7/15

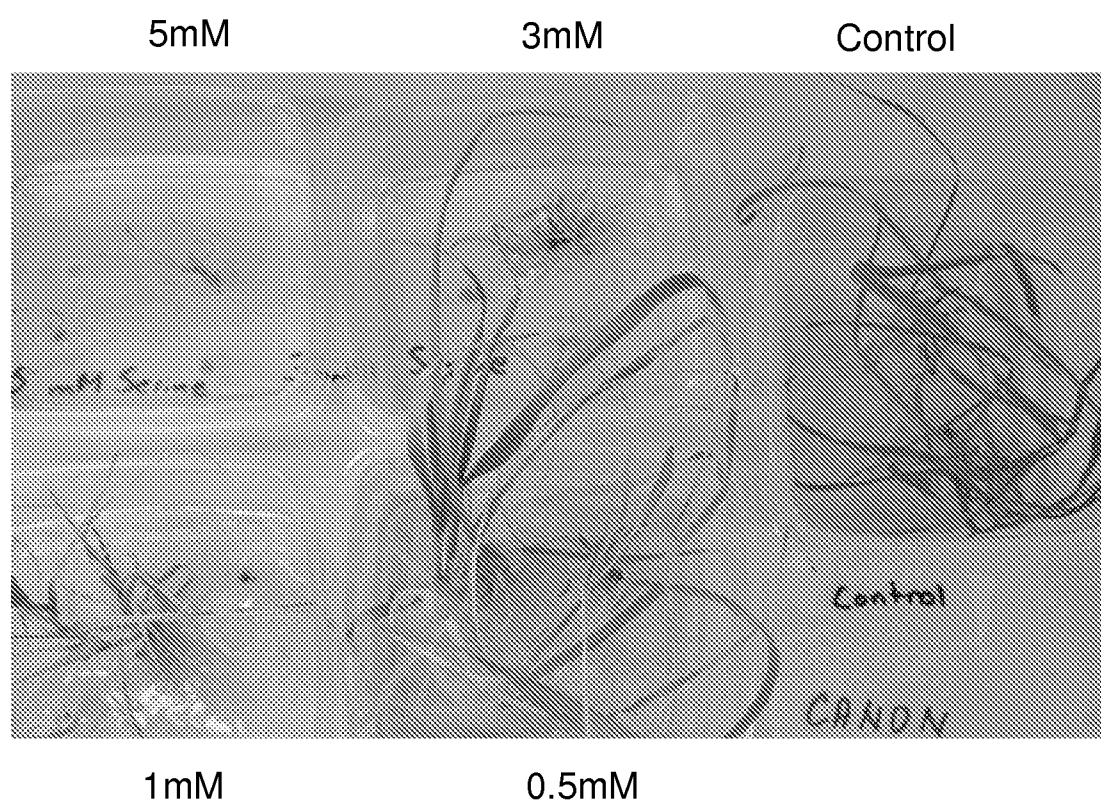


Fig. 7

8/15

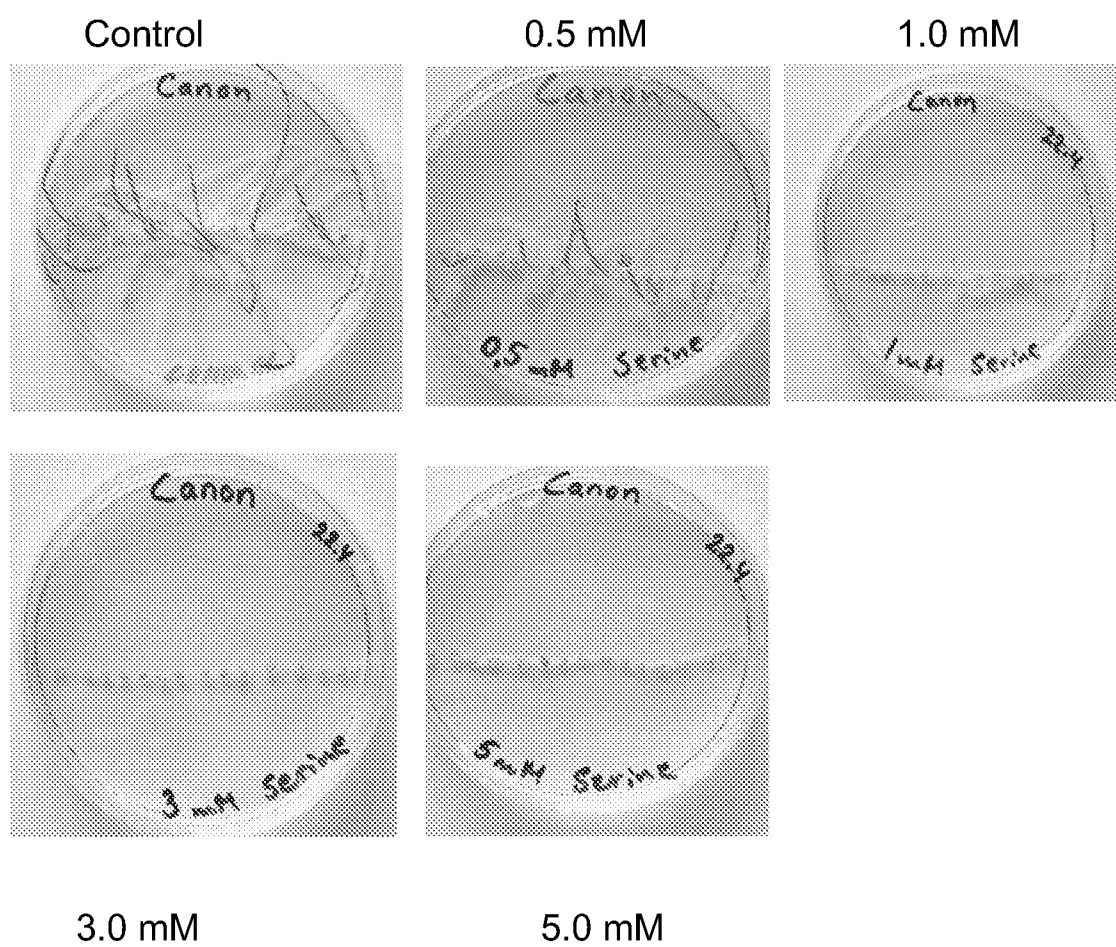


Fig. 8

9/15

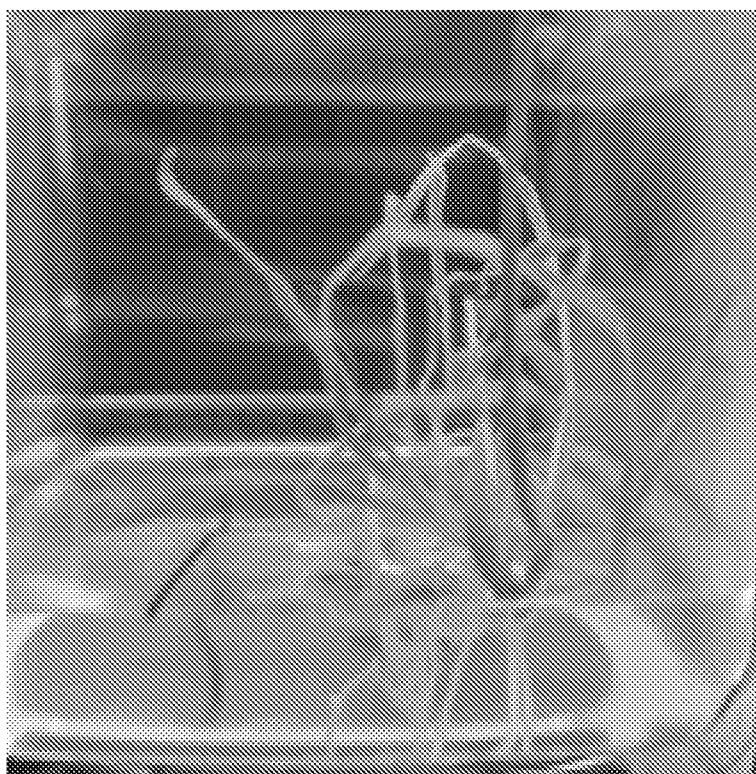


Fig. 9

10/15

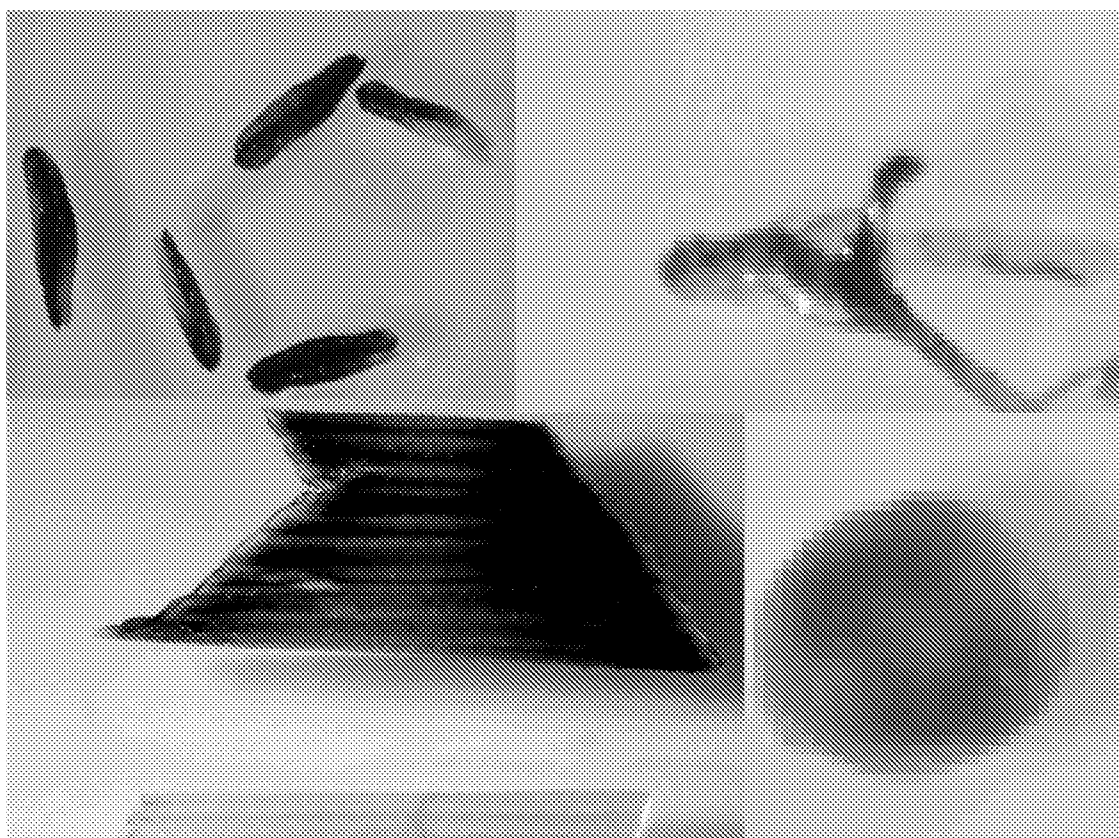


Fig. 10

11/15

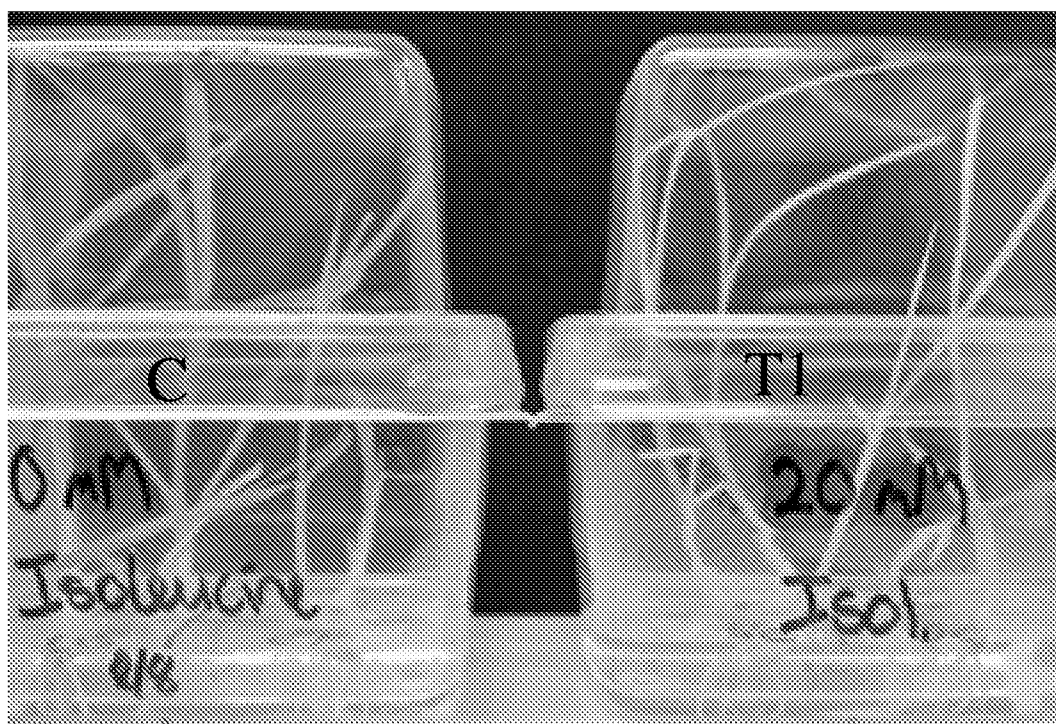
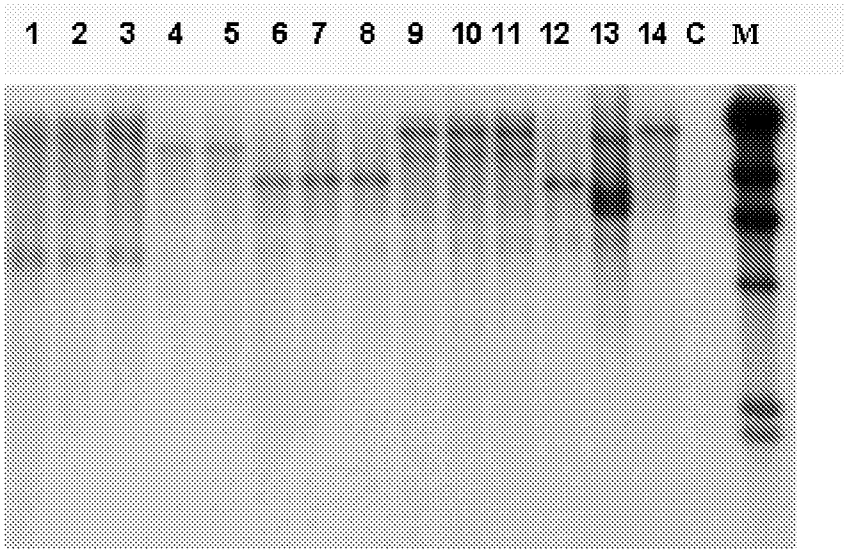


Fig. 11

12/15

I)



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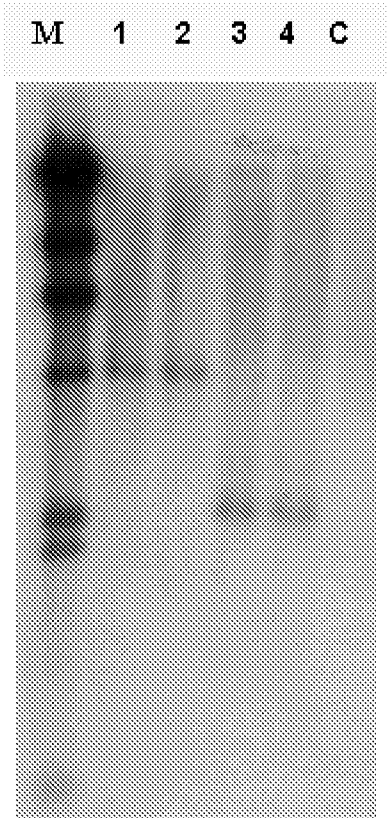


Fig. 12 (1 of 2)

12/15 cont.

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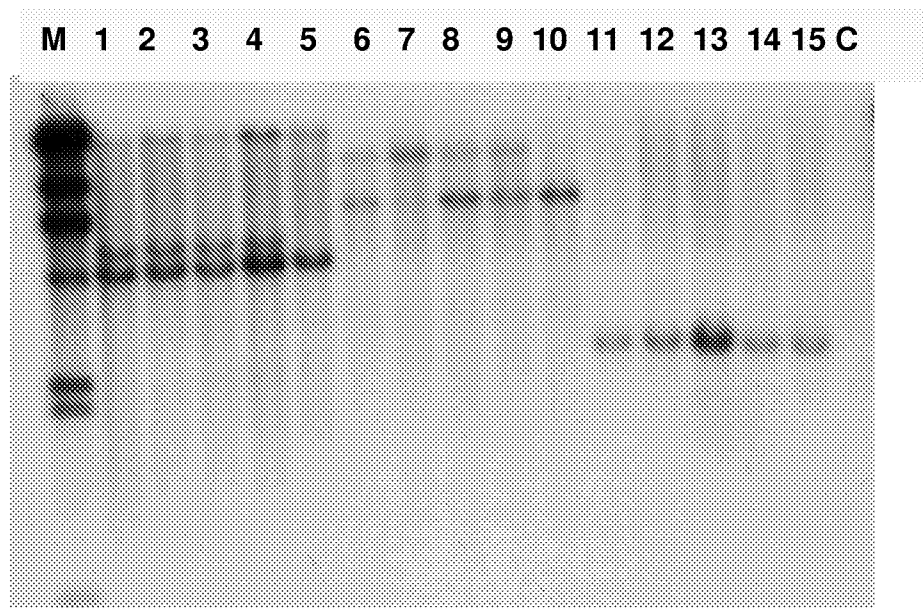


Fig. 12 (2 of 2)

13/15

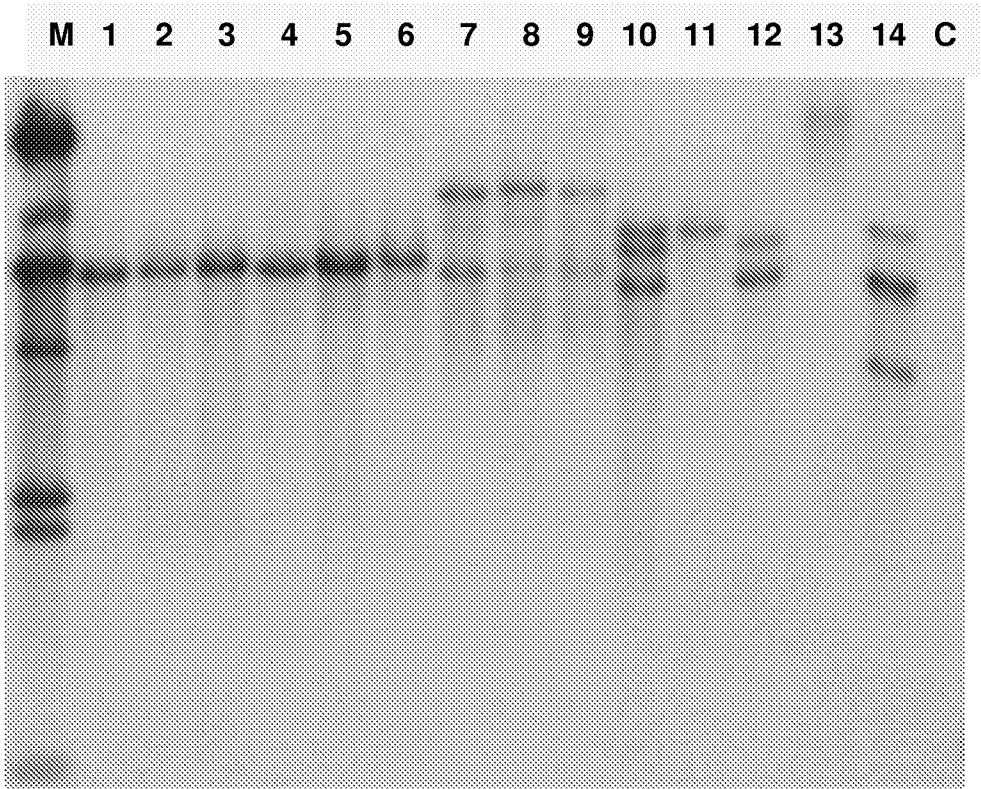


Fig. 13

14/15

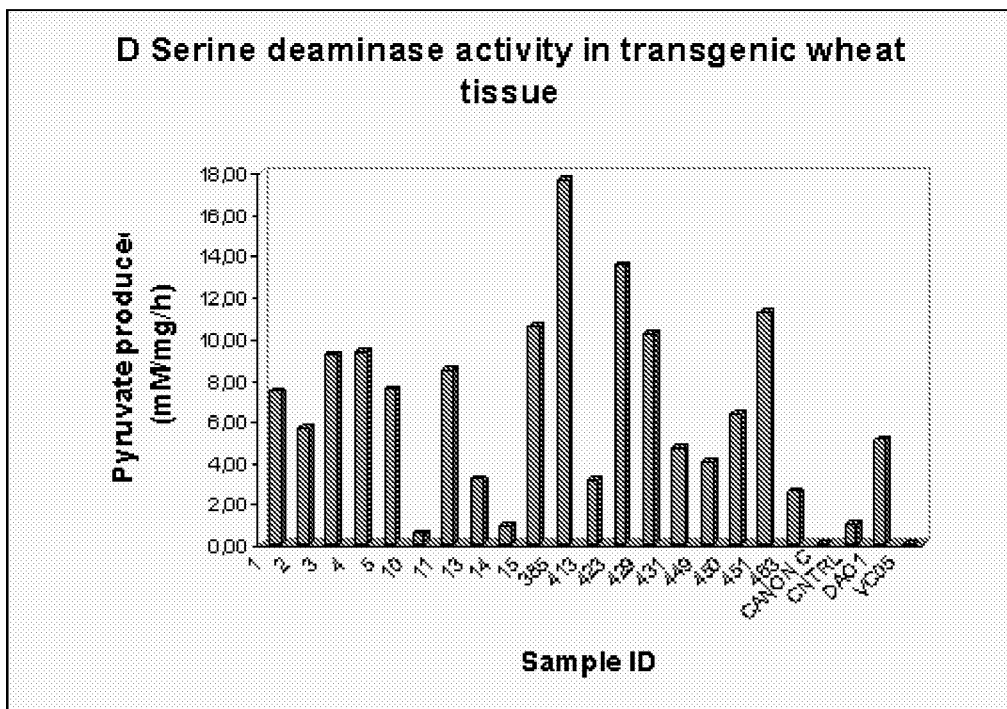
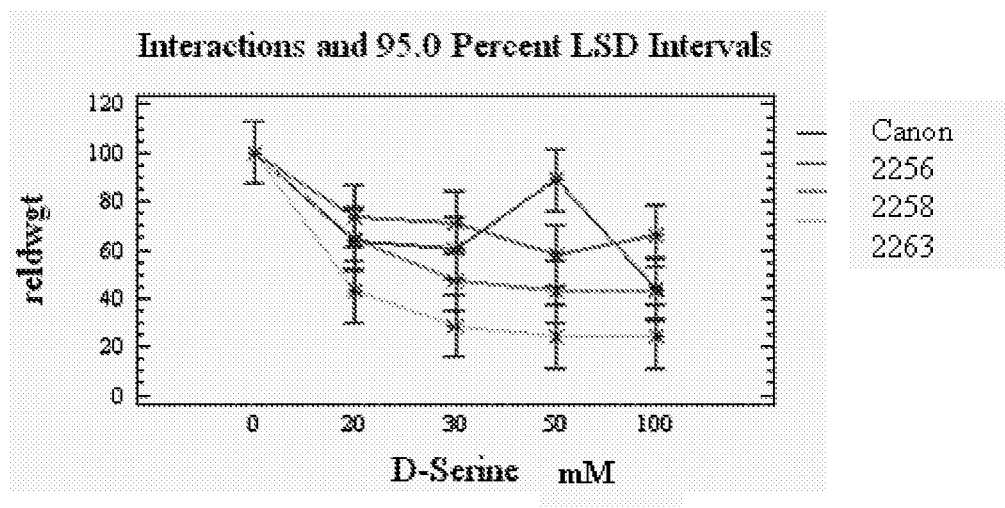


Fig. 14

15/15

I)



II)

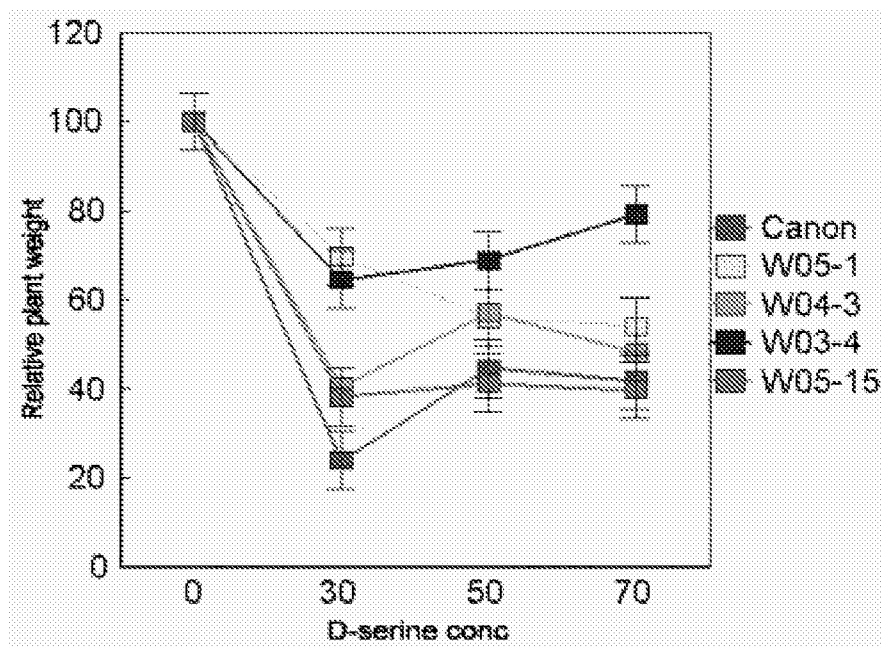


Fig. 15

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Val Val Gl u Tyr Gl u Gi n Asp Tyr Gly Val Al a Val Gl u Gl u Gly Arg
210 215 220

Lys Ala Ala Gi n Ser Asp Pro Asn Cys Phe Phe lie Asp Asp Gl u Asn
225 230 235 240

Ser Arg Thr Leu Phe Leu Gly Tyr ser val Al a Gly Gi n Arg Leu Lys
245 250 255

eol f - seq1 .txt

Ala Gln Phe Ala Gln Gln Gly Arg Ile Val Asp Ala Asp Asn Pro Leu
260 265 270

Phe Val Tyr Leu Pro Cys Gly Val Gly Gly Gly Pro Gly Gly Val Ala
275 280 285

Phe Gly Leu Lys Leu Ala Phe Gly Asp His Val His Cys Phe Phe Ala
290 295 300

Glu Pro Thr His Ser Pro Cys Met Leu Leu Gly Val His Thr Gly Leu
305 310 315 320

His Asp Gln Ile Ser Val Gln Asp Ile Gly Ile Asp Asn Leu Thr Ala
325 330 335

Al a Asp Gl y Leu Al a Val Gl y Arg Al a Ser Gl y Phe Val Gl y Arg Al a
340 345 350

Met Glu Arg Leu Leu Asp Gly Phe Tyr Thr Leu Ser Asp Gln Thr Met
355 360 365

Tyr Asp Met Leu Gly Trp Leu Ala Gln Glu Glu Gly Ile Arg Leu Glu
370 375 380

Pro Ser Ala Leu Ala Gly Met Ala Gly Pro Gln Arg Val Cys Ala Ser
385 390 395 400

Val Ser Tyr Gln Gln Met His Gly Phe Ser Ala Glu Gln Leu Arg Asn
405 410 415

Thr Thr H i s Leu val Trp A l a Thr G l y G l y G l y Met val Pro G u G u
420 425 430

Gl u Met Asn Gi n Tyr Leu Al a Lys Gl y Arg
435 440

<210>	3
<211>	1107
<212>	DNA
<213>	Rhodospirillum rubrum

```

<220>
<221> CDS
<222> (l) ..(1107)
<223> Rhodospirillum rubrum D-aminooxalate decarboxylase CDS

```

<400> 3
atg cac teg cag aag cgc gtc gtt gtc etc gga tea ggc gtt ate ggt 48
Met Hi s Ser Gi n Lys Arg Val Val Val Leu Gl y Ser Gl y Val I le Gl y
1 5 10 15

ctg age age gee etc ate etc get egg aag ggc tac age gtg cat att 96
Leu ser ser Ala Leu lie Leu Ala Arg Lys Gly Tyr ser val His lie
20 25 30

eolf-seql .txt																
etc Leu	gcg Ala	cgc Arg 35	gac Asp	ttg Leu	ccg Pro	gag Glu	gac Asp 40	gtc Val	teg Ser	age Ser	cag Gin	act Thr 45	ttc Phe	get Ala	tea Ser	144
cca Pro	tgg Trp 50	get Ala	ggc Gly	gcg Ala	aat Asn	tgg Trp 55	acg Thr	cct Pro	ttc Phe	atg Met	acg Thr 60	ctt Leu	aca Thr	gac Asp	ggt Gly	192
cct Pro 65	cga Arg	caa Gin	gca Ala	aaa Lys	tgg Trp 70	gaa Glu	gaa Glu	teg Ser	act Thr	ttc Phe 75	aag Lys	aag Lys	tgg Trp	gtc Val	gag Glu 80	240
ttg Leu	gtc Val	ccg Pro	acg Thr	ggc Gly 85	cat His	gcc Ala	atg Met	tgg Trp	etc Leu 90	aag Lys	ggg Gly	acg Thr	agg Arg	egg Arg 95	ttc Phe	288
gcg Ala	cag Gin	aac Asn	gaa Glu 100	gac Asp	ggc Gly	ttg Leu	etc Leu	ggg Gly 105	cac His	tgg Trp	tac Tyr	aag Lys	gac Asp 110	ate lie	acg Thr	336
cca Pro	aat Asn	tac Tyr 115	cgc Arg	ccc Pro	etc Leu	cca Pro	tct Ser 120	tec Ser	gaa Glu	tgt Cys	cca Pro	cct Pro 125	ggc Gly	get Ala	ate lie	384
ggc Gly	gta Val 130	ace Thr	tac Tyr	gac Asp	ace Thr	etc Leu 135	tec Ser	gtc Val	cac His	gca Ala	cca Pro 140	aag Lys	tac Tyr	tgc Cys	cag Gin	432
tac Tyr 145	ctt Leu	gca Ala	aga Arg	gag Glu	ctg Leu 150	cag Gin	aag Lys	etc Leu	ggc Gly 155	gcg Ala	acg Thr	ttt Phe	gag Glu	aga Arg	egg Arg 160	480
ace Thr	gtt Val	acg Thr	teg Ser	ctt Leu 165	gag Glu	cag Gin	gcg Ala	ttc Phe	gac Asp 170	ggt Gly	gcg Ala	gat Asp	ttg Leu	gtg Val 175	gtc Val	528
aac Asn	get Ala	acg Thr	gga Gly 180	ctt Leu	ggc Gly	gcc Ala	aag Lys	teg Ser 185	att lie	gcg Ala	ggc Gly	ate lie	gac Asp 190	gac Asp	caa Gin	576
gcc Ala	gcc Ala	gag Glu 195	cca Pro	ate lie	cgc Arg	ggg Gly	caa Gin 200	ace Thr	gtc val	etc Leu	gtc val	aag Lys 205	tec ser	cca Pro	tgc cys	624
aag Lys	cga Arg 210	tgc Cys	acg Thr	atg Met	gac Asp	teg Ser 215	tec Ser	gac Asp	ccc Pro	get Ala	tct Ser 220	ccc Pro	gcc Ala	tac Tyr	ate lie	672
att lie 225	ccc Pro	cga Arg	cca Pro	ggt Gly	ggc Gly 230	gaa Glu	gtc val	ate lie	tgc cys	ggc Gly 235	ggg Gly	acg Thr	tac Tyr	ggc Gly	gtg val 240	720
gga Gly	gac Asp	tgg Trp	gac Asp	ttg Leu 245	tct Ser	gtc Val	aac Asn	cca Pro	gag Glu 250	acg Thr	gtc Val	cag Gin	egg Arg	ate lie 255	etc Leu	768
aag Lys	cac His	tgc cys	ttg Leu 260	cgc Arg	etc Leu	gac Asp	ccg Pro	ace Thr 265	ate lie	teg ser	age ser	gac Asp	gga Gly 270	acg Thr	ate lie	816
gaa Glu	ggc Gly	ate lie 275	gag Glu	gtc Val	etc Leu	cgc Arg	cac His 280	aac Asn	gtc Val	ggc Gly	ttg Leu	cga Arg 285	cct Pro	gca Ala	cga Arg	864
cga Arg	ggc Gly 290	gga Gly	ccc Pro	cgc Arg	gtt Val	gag Glu 295	gca Ala	gaa Glu	egg Arg	ate lie	gtc Val 300	ctg Leu	cct Pro	etc Leu	gac Asp	912
egg	aca	aag	teg	ccc	etc	teg	etc	ggc	agg	ggc	age	gca	cga	gcg	gcg	960

eolf-seql .txt

Arg	Thr	Lys	Ser	Pro	Leu	Ser	Leu	Gly	Arg	Gly	Ser	Ala	Arg	Ala	Ala		
305					310					315					320		
aag	gag	aag	gag	gtc	acg	ctt	gtg	cat	gcg	tat	ggc	ttc	teg	agt	gcg	1008	
Lys	Glu	Lys	Glu	Val	Thr	Leu	Val	His	Ala	Tyr	Gly	Phe	Ser	Ser	Ala		
				325					330					335			
gga	tac	cag	cag	agt	tgg	ggc	gcg	gcg	gag	gat	gtc	gcg	cag	etc	gtc	1056	
Gly	Tyr	Gln	Gln	Ser	Trp	Gly	Ala	Ala	Glu	Asp	Val	Ala	Gln	Leu	Val		
				340					345				350				
gac	gag	gcg	ttc	cag	egg	tac	cac	ggc	gcg	gcg	egg	gag	teg	aag	ttg	1104	
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		355					360					365					
tag																1107	

<210> 4
 <211> 368
 <212> PRT
 <213> Rhodosporidium toruloides
 <400> 4

Met	His	Ser	Gln	Lys	Arg	Val	Val	Val	Leu	Gly	Ser	Gly	Val	His	Gly		
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Leu	Ser	Ser	Ala	Leu	Ile	Leu	Ala	Arg	Lys	Gly	Tyr	Ser	Val	His	Ile		
			20					25					30				
Leu	Ala	Arg	Asp	Leu	Pro	Glu	Asp	Val	Ser	Ser	Gln	Thr	Phe	Ala	Ser		
		35					40					45					
Pro	Trp	Ala	Gly	Ala	Asn	Trp	Thr	Pro	Phe	Met	Thr	Leu	Thr	Asp	Gly		
	50					55					60						
Pro	Arg	Gln	Ala	Lys	Trp	Glu	Glu	Ser	Thr	Phe	Lys	Lys	Trp	Val	Glu		
65					70					75					80		
Leu	Val	Pro	Thr	Gly	His	Ala	Met	Trp	Leu	Lys	Gly	Thr	Arg	Arg	Phe		
				85					90					95			
Ala	Gln	Asn	Glu	Asp	Gly	Leu	Leu	Gly	His	Trp	Tyr	Lys	Asp	Ile	Thr		
			100					105					110				
Pro	Asn	Tyr	Arg	Pro	Leu	Pro	Ser	Ser	Glu	Cys	Pro	Pro	Gly	Ala	Ile		
		115					120					125					
Gly	Val	Thr	Tyr	Asp	Thr	Leu	Ser	Val	His	Ala	Pro	Lys	Tyr	Cys	Gln		
	130					135					140						
Tyr	Leu	Ala	Arg	Glu	Leu	Gln	Lys	Leu	Gly	Ala	Thr	Phe	Glu	Arg	Arg		
145					150					155					160		
Thr	Val	Thr	Ser	Leu	Glu	Gln	Ala	Phe	Asp	Gly	Ala	Asp	Leu	Val	Val		
				165					170					175			

eolf-seql .txt

Asn Ala Thr Gly Leu Gly Ala Lys Ser lie Ala Gly lie Asp Asp Gi n
180 185 190

Al a Ala Gl u Pro lie Arg Gly Gi n Thr Val Leu Val Lys Ser Pro Cys
195 200 205

Lys Arg Cys Thr Met Asp Ser Ser Asp Pro Al a Ser Pro Al a Tyr lie
210 215 220

I i e Pro Arg Pro Gly Gly Gl u Val lie Cys Gly Gly Thr Tyr Gly Val
225 230 235 240

Gly Asp Trp Asp Leu ser val Asn Pro Gl u Thr val Gi n Arg lie Leu
245 250 255

Lys Hi s Cys Leu Arg Leu Asp Pro Thr lie Ser Ser Asp Gly Thr lie
260 265 270

Gl u Gly lie Gl u val Leu Arg Hi s Asn val Gly Leu Arg Pro Ala Arg
275 280 285

Arg Gly Gly Pro Arg Val Gl u Ala Gl u Arg I i e Val Leu Pro Leu Asp
290 295 300

Arg Thr Lys ser Pro Leu ser Leu Gly Arg Gly Ser Ala Arg Ala Ala
305 310 315 320

Lys Gl u Lys Gl u Val Thr Leu Val Hi s Ala Tyr Gly Phe Ser Ser Ala
325 330 335

Gly Tyr Gi n Gi n Ser Trp Gly Ala Ala Gl u Asp Val Ala Gi n Leu Val
340 345 350

Asp Gl u Ala Phe Gi n Arg Tyr Hi s Gly Ala Al a Arg Gl u Ser Lys Leu
355 360 365

<210> 5
<211> 275
<212> DNA
<213> Zea mays

<220>
<221> promoter
<222> (1)..(275)
<223> zea mays ubiquitin 275 bp core promoter

<220>
<221> mi sc_signal
<222> (61)..(74)
<223> Similar to consensus heat shock element

<220>
<221> mi sc_signal
<222> (71)..(84)
<223> Similar to consensus heat shock element

eolf-seql .txt

<220>
 <221> TATA_signal
 <222> (246)..(254)
 <223> TATA Box

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 ctggaccct ctcgagagtt ccgctccacc gttggacttg ctccgctgtc ggcatccaga 120
 aattgcgtgg cggagcggca gacgtgagcc ggcacggcag gcggcctcct cctcctctca 180
 cggcaccggc agctacgggg gattcctttc ccaccgctcc ttcgctttcc cttcctcgcc 240
 cgccgtaata aatagacacc ccctccacac cctct 275

<210> 6
 <211> 1988
 <212> DNA
 <213> Zea mays

<220>
 <221> promoter
 <222> (1)..(1988)
 <223> Zea mays ubiquitin promoter region with 5'UTR and intron

<220>
 <221> promoter
 <222> (1)..(899)
 <223> Zea mays ubiquitin promoter region

<220>
 <221> mi sc_signal
 <222> (685)..(698)
 <223> Similar to consensus heat shock element

<220>
 <221> mi sc_signal
 <222> (695)..(708)
 <223> similar to consensus heat shock element

<220>
 <221> TATA_signal
 <222> (870)..(878)
 <223> TATA Box

<220>
 <221> 5'UTR
 <222> (900)..(1988)

<220>
 <221> Intron
 <222> (983)..(1988)
 <223> Zea mays ubiquitin gene intron MubGl

<400> 6
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 gttataaaaa attaccacat attttttttg tcacacttgt ttgaagtga gtttatctat 120
 ctttatacat atatttaaac ttactctac gaataatata atctatagta ctacaataat 180
 atcagtgttt tagagaatca tataaatgaa cagttagaca tggctctaaag gacaattgag 240
 tattttgaca acaggactct acagttttat ctttttagtg tgcagtgtgt ctcctttttt 300
 tttgcaaata gcttcaccta tataatactt catccatttt attagtacat ccatttaggg 360

eolf-seql .txt

```

tttagggtta atgggttttta tagactaatt tttttagtac atctatttta ttctatttta 420
gcctctaaat taagaaaact aaaactctat ttttagtttt ttatttaata gtttagatat 480
aaaatagaat aaaataaagt gactaaaaat taaacaaata ccctttaaga aattaaaaaa 540
actaaggaaa catTTTTtctt gtttcgagta gataatgcca gcctgttaaa cgccgtcgac 600
gagtctaacg gacaccaacc agcgaaccag cagcgtcgcg tcgggccaag cgaagcagac 660
ggcacggcat ctctgtcgct gcctctggac ccctctcgag agttccgctc caccgttgga 720
cttgctccgc tgtcggcatc cagaaattgc gtggcggagc ggcagacgtg agccggcacg 780
gcaggcggcc tcctcctcct ctacacggac cggcagctac gggggattcc tttcccaccg 840
ctccttcgct ttcccttcct cgcccgccgt aataaataga caccctctcc acaccctctt 900
tccccaacct cgtgttggtc ggagcgcaca cacacacaac cagatctccc ccaaaccac 960
ccgtcggcac ctccgcttca aggtacgccg ctctctctcc ccccccccc ccctctctac 1020
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ctagatcgga gtagaattct gttcaaact acctggtgga ttattaatt ttggatctgt 1440
atgtgtgtgc catacatatt catagttacg aattgaagat gatggatgga aatatcgatc 1500
taggataggt atacatgttg atgcgggttt tactgatgca tatacagaga tgctttttgt 1560
tcgcttggtt gtgatgatgt ggtgtgggtt ggcggtcggt cattcgttct agatcggagt 1620
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ttgatatact tggatgatgg catatgcagc agctatatgt ggattttttt agccctgcct 1920
tcatacgcta tttatttgct tggtagtgtt tcttttgctg atgctcaccg tgttggttgg 1980
tgttactt 1988

```

<210> 7
 <211> 275
 <212> DNA
 <213> Sugarcane bacilli form virus

<220>
 <221> promoter
 <222> (1)..(275)
 <223> Sugarcane bacilli form virus 275 bp core promoter

eol f - seq1 .txt

<220>
 <221> TATA_signal
 <222> (224)..(243)
 <223> TATA Box

<400> 7
 gattgaggag gcattgacgt cagggatgac cgcagcggag agtactgggc ccattcagtg 60
 gatgctccac tgagttgtat tattgtgtgc ttttcggaca agtgtgtgtt ccactttctt 120
 ttggcacctg tgccacttta ttccttgtct gccacgatgc ctttgcttag cttgtaagca 180
 aggatcgagc tgcgtgtgtg acaccacccc ccttccgacg ctctgcctat ataaggcacc 240
 gtctgtaagc tcttacgac atcggtagtt caeca 275

<210> 8
 <211> 1398
 <212> DNA
 <213> Sugarcane bacilliform virus

<220>
 <221> promoter
 <222> (1)..(1398)
 <223> Sugarcane bacilliform virus [ScBV] promoter region

<220>
 <221> misc_signal
 <222> (98)..(118)
 <223> Heat shock factor

<220>
 <221> misc_signal
 <222> (365)..(385)
 <223> Heat shock factor

<220>
 <221> misc_signal
 <222> (606)..(626)
 <223> Heat shock factor

<220>
 <221> misc_signal
 <222> (705)..(719)
 <223> Heat shock element

<220>
 <221> TATA_signal
 <222> (1345)..(1364)
 <223> TATA BOX

<400> 8
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 ttggccatt atatccaaag acctcagaga aagggtgagc aaggctcaat tcagaagatt 120
 ggaagctgat caataggatc aagacaatgg tgagaacgct tccaaatctc actattccac 180
 cagaagatgc atacattatc attgaaacag atgcatgtgc aactggatgg ggagcagtat 240
 gcaagtggaa gaaaaacaag gcagacccaa gaaatacaga gcaaactctgt aggtatgcca 300
 gtggaaaatt tgataagcca aaaggaacct gtgatgcaga aatctatggg gttatgaatg 360
 gcttagaaaa gatgagattg ttctacttgg acaaaagaga gatcacagtc agaactgaca 420
 gtagtgcaat cgaaaggttc tacaacaaga gtgctgaaca caagccttct gagatcagat 480

eolf-seql .txt

```

ggatcagggtt catggactac atcactggtg caggaccaga gatagtcatt gaacacataa 540
aaggaagag caatggttta gctgacatct tgtccaggct caaagccaaa ttagctcaga 600
atgaaccaac ggaagagatg atcctgctta cacaagccat aaggaagta attccttattc 660
cagatcatcc atacactgag caactcagag aatggggaaa caaaattctg gatccattcc 720
ccacattcaa gaaggacatg ttcgaaagaa cagagcaagc ttttatgcta acagaggaac 780
cagttctact ctgtgcatgc aggaagcctg caattcagtt agtgtccaga acatctgccca 840
accaggaag gaaattcttc aagtgcgcaa tgaacaaatg ccattgctgg tactgggcag 900
atctcattga agaacacatt caagacagaa ttgatgaatt tctcaagaat cttgaagttc 960
tgaagaccgg tggcgtgcaa acaatggagg aggaacttat gaaggaagtc accaagctga 1020
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ccgcagaaga tgtgctagat ctccaagacg taagcaatga cgattgagga ggcattgacg 1140
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ttattgtgtg cttttcggac aagtgtgctg tccactttct tttggcacct gtgccacttt 1260
attccttgtc tgccacgatg cctttgctta gcttgtaagc aaggatcgca gtgctgtgtg 1320
gacaccaccc cccttccgac gctctgccta tataaggcac cgtctgtaag ctcttacgat 1380
catcggtagt tcaccaag 1398

```

```

<210> 9
<211> 5035
<212> DNA
<213> Artificial

```

```

<220>
<223> Nucleic acid sequence encoding pRLM175, a kanamycin resistant
pSB11-type binary vector.

```

```

<220>
<221> misc_structure
<222> (5)..(132)
<223> Left T-DNA border from pSB11

```

```

<220>
<221> miscjoinding
<222> (51)..(75)
<223> Left T-DNA border repeat region from pSB1

```

```

<220>
<221> misc_structure
<222> (232)..(376)
<223> Right T-DNA border from pSB11

```

```

<220>
<221> mi sc bi ndi ng
<222> (272)..(295)
<223> Right T-DNA border repeat region from pSB1

```

```

<220>
<221> misc_structure
<222> (777)..(3511)
<223> pSB1 recombination target

```

eolf-seql .txt

```

<220>
<221> rep_origi n
<222> (2654)..(2934)
<223> ColEI repl ication origi n from pSBll

<220>
<221> misc_feature
<222> (3074)..(3334)
<223> CoIEI BOM site from pSBll

<220>
<221> misc_feature
<222> (3793)..(4608)
<223> cds encoding Kanamycin Resistance selection gene from Tn903

```

```

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atgtactgaa ttggatccgc ccgggcggta ccgaattcgc ggccgcaagc ttgtacaacg      180
cgtaccgggtt aattaatcta gaggcgcgcc gggcccggcc ggccagatct tgattgtcgt      240
ttccccgctt cagtttaaac tatcagtgtt tgacaggata tattggcggg taaacctaaag      300
agaaaagagc gtttattaga ataatcggat atttaaaagg gcgtgaaaag gtttatccgt      360
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cttgaagacg aaagggcctc gtgatacgcc tatttttata ggtaaatgtc atgataataa      840
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ccttttttgc ggcattttgc cttcctgttt ttgtcaccac agaaacgctg gtgaaagtaa     1080
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cggatggcat gacagtaaga gaattatgca gtgctgccat aaccatgagt gataaacactg     1380
cggccaactt acttctgaca acgatcggag gaccgaagga gctaaccgct tttttgcaca     1440
acatggggga tcatgtaact cgccttgatc gttgggaacc ggagctgaat gaagccatac     1500
caaacgacga gcgtgacacc acgatgccgg gggggggggg ggggggacat aggttgcccc     1560
gtattcagtg tcgctgattt gtattgtctg aagttgtttt tacgttaagt tgatgcagat     1620

```

eolf-seql .txt

caattaatac	gataacctgcg	tcataattga	ttattttgacg	tggtttgatg	gcctccacgc	1680
acgtttgat	atgtagatga	taatcattat	cactttacgg	gtcctttccg	gtgatccgac	1740
aggttacggg	gcggcgacct	cgcggtttt	cgctatttat	gaaaattttc	cggtttaagg	1800
cgtttccgtt	cttcttcgtc	ataacttaat	gtttttat	aaaataccct	ctgaaaagaa	1860
aggaaacgac	agggtgctgaa	agcgagcttt	ttggcctctg	tcgtttcctt	tctctgtttt	1920
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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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 <212> DNA
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<220>
 <223> Nucleic acid sequence encoding T-DNA region of pRLM167, a pRLM175 derived binary vector containing p-ZmUBI+I: :c-dsdA: :t-OCS and p-ZmUBI+I: :c-PAT: :t-OCS cassettes.

<220>
 <221> misc_feature
 <222> (1)..(128)
 <223> Left T-DNA border from pSB11

<220>

eolf-seql .txt

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<221> misc_feature
<222> (47)..(71)
<223> Left T-DNA border repeat from pSBll

<220>
<221> promoter
<222> (162)..(2149)
<223> Zea mays ubiquitin promoter with first intron

<220>
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<222> (1144)..(2149)
<223> Zea mays ubiquitin gene first intron

<220>
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<222> (2192)..(3520)
<223> cds encoding E.coli D-serine dehydratase [dsdA]

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<222> (3558)..(4270)
<223> an octapine synthase terminator

<220>
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<223> Zea mays ubiquitin gene first intron

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<223> Phosphinothricin Acetyltransferase synthetic gene/CDS, which
confers BASTA/PPT resistance

<220>
<221> terminator
<222> (7009)..(7619)
<223> an octapine synthase terminator

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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 <223> Nucleic acid sequence encoding T-DNA region of pRLM179, a pRLM175 derived binary vector containing ZmAHASL2/Xi 12 and p-ZmUBI+I: :c-dsdA: :t-OCS cassettes.

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 <222> (1)..(128)
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eolf-seql .txt

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<223> S621N mutation in AHASL2 [XI12] for z.mays gene for
      acetohydroxyacid synthase (AHAS108), confers resistance to IMI
      herbicides.

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      synthase (AHAS108) .

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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<223> Left T-DNA border from pSBll

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(daol)

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<220>
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<220>
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used as terminator

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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<223> Left T-DNA border from pSBll

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<223> Left T-DNA border repeat from pSBll

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<223> leader fragment for pea Fedl gene

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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eolf-seql .txt

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gacgctcaca	ccgataccat	cagcgatctc	tttgatgtgc	tgtgcctgaa	ccgttattac	7200
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ctggcctggc	aggagaaact	gcatcagccg	attatcatca	ccgaatacgg	cgtggatacg	7320
ttagccgggc	tgcactcaat	gtacaccgac	atgtggagt	aagagtatca	gtgtgcatgg	7380
ctggatatgt	atcaccgcgt	ctttgatcgc	gtcagcgccg	tcgtcgggtga	acaggtatgg	7440
aatttcgcgc	attttgcgac	ctcgcgaaggc	atattgcgcg	ttggcggtaa	caagaaaggg	7500
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ggcatgaact	tcggtgaaaa	accgcagcag	ggaggcaaac	aatgaatcaa	caactctcct	7620
ggcgaccat	cgctcggtac	agcctcgga	attgctaccg	agctcgaatt	tccccgatcg	7680
ttcaaacatt	tggcaataaa	gtttcttaag	attgaatcct	gttgccggtc	ttgcgatgat	7740
tatcatataa	tttctgttga	attacgttaa	gcatgtaata	attaacatgt	aatgcatgac	7800
gttatttatg	agatgggttt	ttatgattag	agtcccgcga	ttatacat	aatacgcgat	7860
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actagatcgg	gaattcgggc	ccggccggcc	agatcttgat	tgtcgtttcc	cgccttcagt	7980
ttaaactatc	agtgtttgac	aggatatatt	ggcgggtaaa	cctaagagaa	aagagcgttt	8040
attagaataa	tcggatat	aaaagggcgt	gaaaagggtt	atccgttcgt	ccatttgat	8100
gt						8102

<210> 15
 <211> 1107
 <212> DNA

eol f - seq1 .txt

<213> Rhodospori di um torul oi des

<220>

<221> CDS

<222> (I) . . (1107)

<223> Zea mays codon opti mi ze Rhodospori di um torul oi des D-ami no aci d
oxi dase CDS

<400> 15

atg	cac	tct	caa	aaa	cgc	gtt	gta	gta	ttg	ggc	teg	gga	gtc	ata	ggc	48
Met	Hi s	Se r	Gi n	Lys	Arg	Val	Val	Val	Leu	Gly	Se r	Gly	Val	lie	Gly	
1			5						10					15		

ctt	tct	tct	get	ctt	ata	ctt	gca	cgc	aag	ggc	tat	tct	gtt	cat	att	96
Leu	Se r	Se r	Al a	Leu	lie	Leu	Al a	Arg	Lys	Gly	Tyr	Se r	Val	Hi s	lie	
			20					25					30			

ttg	gca	cgc	gat	etc	cca	gag	gac	gta	tct	age	caa	act	ttc	get	tec	144
Leu	Al a	Arg	Asp	Leu	Pro	Glu	Asp	Val	Se r	Se r	Gi n	Thr	Phe	Al a	Se r	
		35					40					45				

ccc	tgg	get	ggc	gca	aat	tgg	aca	cca	ttt	atg	aca	ttg	act	gac	ggc	192
Pro	Trp	Al a	Gly	Al a	Asn	Trp	Thr	Pro	Phe	Met	Thr	Leu	Thr	Asp	Gly	
	50					55					60					

cct	aga	cag	gca	aaa	tgg	gaa	gag	agt	ace	ttt	aaa	aaa	tgg	gtt	gag	240
Pro	Arg	Gi n	Al a	Lys	Trp	Glu	Glu	Se r	Thr	Phe	Lys	Lys	Trp	Val	Glu	
65					70					75					80	

ttg	gtc	cct	ace	gga	cat	get	atg	tgg	ctg	aag	gga	act	cgt	cgc	ttt	288
Leu	Val	Pro	Thr	Gly	Hi s	Al a	Met	Trp	Leu	Lys	Gly	Thr	Arg	Arg	Phe	
				85					90					95		

gca	cag	aat	gaa	gac	ggc	ttg	ctt	gga	cat	tgg	tac	aaa	gat	ata	ace	336
Al a	Gi n	Asn	Glu	Asp	Gly	Leu	Leu	Gly	Hi s	Trp	Tyr	Lys	Asp	lie	Thr	
			100					105					110			

ccg	aat	tac	cgc	ccc	ctt	ccc	teg	tct	gaa	tgt	cca	cca	ggc	gca	att	384
Pro	Asn	Tyr	Arg	Pro	Leu	Pro	Se r	Se r	Glu	Cys	Pro	Pro	Gly	Al a	lie	
		115					120					125				

ggc	gtg	act	tat	gac	aca	etc	tct	gta	cac	get	cct	aaa	tat	tgt	cag	432
Gly	Val	Thr	Tyr	Asp	Thr	Leu	Se r	Val	Hi s	Al a	Pro	Lys	Tyr	Cys	Gi n	
	130					135					140					

tat	ctt	gcc	aga	gaa	ctt	cag	aaa	etc	ggc	gca	act	ttt	gaa	aga	egg	480
Tyr	Leu	Al a	Arg	Glu	Leu	Gi n	Lys	Leu	Gly	Al a	Thr	Phe	Glu	Arg	Arg	
145					150					155					160	

acg	gtt	ace	tea	ctg	gaa	cag	gca	ttc	gac	ggc	gca	gac	etc	gtg	gtg	528
Thr	Val	Thr	Se r	Leu	Glu	Gi n	Al a	Phe	Asp	Gly	Al a	Asp	Leu	VaT	VaT	
				165					170					175		

aat	gca	aca	gga	ctg	gga	gca	aag	age	ata	gca	ggc	ata	gat	gat	caa	576
Asn	Al a	Thr	Gly	Leu	Gly	Al a	Lys	Se r	lie	Al a	Gly	lie	Asp	Asp	Gi n	
			180					185					190			

get	gcg	gag	cca	att	aga	ggc	cag	ace	gtg	ctg	gta	aaa	tea	cca	tgc	624
Al a	Al a	Glu	Pro	lie	Arg	Gly	Gi n	Thr	Val	Leu	Val	Lys	Se r	Pro	Cys	
		195					200					205				

aaa	aga	tgt	acg	atg	gat	agt	agt	gac	cca	gcg	agt	cca	get	tat	att	672
Lys	Arg	Cys	Thr	Met	Asp	Se r	Se r	Asp	Pro	Al a	Se r	Pro	Al a	Tyr	lie	
	210					215					220					

ata	ccg	aga	ccg	ggc	ggc	gaa	gtt	ata	tgc	gga	gga	act	tac	gga	gtg	720
lie	Pro	Arg	Pro	Gly	Gly	Glu	Val	He	Cys	Gly	Gly	Thr	Tyr	Gly	Val	
225				230						235					240	

eolf-seql .txt

ggt gat tgg gat ctt age gtt aac cec gaa aca gtc caa egg att ctg	768
Gly Asp Trp Asp Leu Ser Val Asn Pro Glu Thr Val Gin Arg lie Leu	
	245
	250
	255
aag cat tgc etc cgc ctt gac cca ace ata tea tet gac gga ace att	816
Lys His Cys Leu Arg Leu Asp Pro Thr lie Ser Ser Asp Gly Thr lie	
	260
	265
	270
gag gga ate gaa gta ctt agg cat aat gtt ggt ctt agg cec get cgt	864
Glu Gly lie Glu Val Leu Arg His Asn Val Gly Leu Arg Pro Ala Arg	
	275
	280
	285
aga gga gga cca cgt gta gaa get gag aga att gta ttg cec etc gat	912
Arg Gly Gly Pro Arg Val Glu Ala Glu Arg lie Val Leu Pro Leu Asp	
	290
	295
	300
aga ace aaa age cec ctt teg ctt gga aga gga age gca cgt gca get	960
Arg Thr Lys Ser Pro Leu Ser Leu Gly Arg Gly Ser Ala Arg Ala Ala	
	305
	310
	315
	320
aag gaa aaa gaa gtc aca ctg gta cat gca tat ggt ttt agt agt gcc	1008
Lys Glu Lys Glu Val Thr Leu Val His Ala Tyr Gly Phe Ser Ser Ala	
	325
	330
	335
gga tat cag caa tea tgg ggt get gca gaa gat gtt get cag ttg gtg	1056
Gly Tyr Gin Gin Ser Trp Gly Ala Ala Glu Asp Val Ala Gin Leu Val	
	340
	345
	350
gac gag gca ttc caa aga tac cac ggt gca gcg aga gag tea aaa ctt	1104
Asp Glu Ala Phe Gin Arg Tyr His Gly Ala Ala Arg Glu Ser Lys Leu	
	355
	360
	365
tag	1107

<210> 16
 <211> 368
 <212> PRT
 <213> Rhodosporidium toruloides

<400> 16

Met His Ser Gin Lys Arg Val Val Val Leu Gly Ser Gly Val lie Gly	
1 5 10 15	
Leu Ser Ser Ala Leu lie Leu Ala Arg Lys Gly Tyr Ser Val His lie	
20 25 30	
Leu Ala Arg Asp Leu Pro Glu Asp Val Ser Ser Gin Thr Phe Ala Ser	
35 40 45	
Pro Trp Ala Gly Ala Asn Trp Thr Pro Phe Met Thr Leu Thr Asp Gly	
50 55 60	
Pro Arg Gin Ala Lys Trp Glu Glu Ser Thr Phe Lys Lys Trp Val Glu	
65 70 75 80	
Leu Val Pro Thr Gly His Ala Met Trp Leu Lys Gly Thr Arg Arg Phe	
85 90 95	
Ala Gin Asn Glu Asp Gly Leu Leu Gly His Trp Tyr Lys Asp lie Thr	
100 105 110	

eolf - seq1 .txt

Pro Asn Tyr Arg Pro Leu Pro Ser Ser Glu Cys Pro Pro Gly Ala Ile
 115 120 125
 Gly Val Thr Tyr Asp Thr Leu Ser Val His Ala Pro Lys Tyr Cys Gin
 130 135 140
 Tyr Leu Ala Arg Glu Leu Gin Lys Leu Gly Ala Thr Phe Glu Arg Arg
 145 150 155 160
 Thr Val Thr Ser Leu Glu Gin Ala Phe Asp Gly Ala Asp Leu Val Val
 165 170 175
 Asn Ala Thr Gly Leu Gly Ala Lys Ser Ile Ala Gly Ile Asp Asp Gin
 180 185 190
 Ala Ala Glu Pro Ile Arg Gly Gin Thr Val Leu Val Lys Ser Pro Cys
 195 200 205
 Lys Arg Cys Thr Met Asp Ser Ser Asp Pro Ala Ser Pro Ala Tyr Ile
 210 215 220
 Ile Pro Arg Pro Gly Gly Glu Val Ile Cys Gly Gly Thr Tyr Gly Val
 225 230 235 240
 Gly Asp Trp Asp Leu Ser Val Asn Pro Glu Thr Val Gin Arg Ile Leu
 245 250 255
 Lys His Cys Leu Arg Leu Asp Pro Thr Ile Ser Ser Asp Gly Thr Ile
 260 265 270
 Glu Gly Ile Glu Val Leu Arg His Asn Val Gly Leu Arg Pro Ala Arg
 275 280 285
 Arg Gly Gly Pro Arg Val Glu Ala Glu Arg Ile Val Leu Pro Leu Asp
 290 295 300
 Arg Thr Lys Ser Pro Leu Ser Leu Gly Arg Gly Ser Ala Arg Ala Ala
 305 310 315 320
 Lys Glu Lys Glu Val Thr Leu Val His Ala Tyr Gly Phe Ser Ser Ala
 325 330 335
 Gly Tyr Gin Gin Ser Trp Gly Ala Ala Glu Asp Val Ala Gin Leu Val
 340 345 350
 Asp Glu Ala Phe Gin Arg Tyr His Gly Ala Ala Arg Glu Ser Lys Leu
 355 360 365

<210> 17
 <211> 23
 <212> DNA

eolf-seql .txt

<213> Artificial

<220>

<223> Nucleic acid sequence encoding qPCR primer GUSCommon-34IF

<400> 17

ccgggtgaag gttatctcta tga

23

<210> 18

<211> 24

<212> DNA

<213> Artificial

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<223> Nucleic acid sequence encoding qPCR primer GUSCommon-414R

<400> 18

cgaagcgggt agatatcaca ctct

24

<210> 19

<211> 24

<212> DNA

<213> Artificial

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<223> Nucleic acid sequence encoding qPCR probe GUSCommon-366FAM

<400> 19

tgtgcgtcac agccaaaagc caga

24

<210> 20

<211> 20

<212> DNA

<213> Artificial

<220>

<223> Nucleic acid sequence encoding qPCR primer EcdsdA-860F

<400> 20

tcgcatcgg gcttaaactg

20

<210> 21

<211> 18

<212> DNA

<213> Artificial

<220>

<223> Nucleic acid sequence encoding qPCR primer EcdsdA-922R

<400> 21

gcgttggttc ggcaaaaa

18

<210> 22

<211> 21

<212> DNA

<213> Artificial

<220>

<223> Nucleic acid sequence encoding qPCR probe EcdsdA-883FAM

<400> 22

tttggcgatc atgttcactg c

21

eolf-seql .txt

<210> 23
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 <212> DNA
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 <223> Nucleic acid sequence encoding qPCR primer TaGBSS:I-F
 <400> 23
 ttctgcatcc acaacatctc gta 23

<210> 24
 <211> 20
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 <223> Nucleic acid sequence encoding qPCR primer TaGBSS:I-R
 <400> 24
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<210> 25
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 <400> 25
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<210> 26
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 <223> Nucleic acid sequence encoding qPCR primer daol/pa-285F
 <400> 26
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<210> 27
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 <223> Nucleic acid sequence encoding qPCR primer daol/pa-349R
 <400> 27
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<210> 28
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 <400> 28

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<210> 29
 <211> 20
 <212> DNA
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<220>
 <223> Nucleic acid sequence encoding forward PCR primer for gusINT gene

<400> 29
 accgtttgtg tgaacaacga 20

<210> 30
 <211> 20
 <212> DNA
 <213> Artificial

<220>
 <223> Nucleic acid sequence encoding reverse PCR primer for gusINT gene

<400> 30
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<210> 31
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 <212> DNA
 <213> Artificial

<220>
 <223> Nucleic acid sequence encoding forward PCR primer for dsdA gene

<400> 31
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<210> 32
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 <212> DNA
 <213> Artificial

<220>
 <223> Nucleic acid sequence encoding reverse PCR primer for dsdA gene

<400> 32
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<210> 33
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 <212> DNA
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<220>
 <223> Nucleic acid sequence encoding forward PCR primer for daol gene

<400> 33
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<210> 34
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 <212> DNA
 <213> Artificial

<220>

eol f - seq1 .txt

<223> Nucleic acid sequence encoding reverse PCR primer for daol gene

<400> 34
tcggggaatg atgtaggc 18

<210> 35
<211> 22
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<220>
<223> Nucleic acid sequence encoding forward PCR primer for daol/ko gene

<400> 35
aagcaggcct tctcacactt ga 22

<210> 36
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<220>
<223> Nucleic acid sequence encoding reverse PCR primer for daol/ko gene

<400> 36
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<210> 37
<211> 29
<212> DNA
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<220>
<223> Nucleic acid sequence encoding forward PCR primer for PAT gene

<400> 37
atgtctccgg agaggagacc agttgagat 29

<210> 38
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<212> DNA
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<220>
<223> Nucleic acid sequence encoding reverse PCR primer for PAT gene

<400> 38
gccaaaaacc aacatcatgc catcca 26

<210> 39
<211> 4503
<212> DNA
<213> Artificial

<220>
<223> T-DNA region from RLM151, a RLM175-type binary vector containing a p-ZmUBI::c-dsdA::t-OCS selection cassette .

<220>
<221> misc_structure
<222> (D..C128)

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eolf-seql .txt
<223> Left T-DNA border from pSBll

<220>
<221> mi sc bi ndi ng
<222> (47) ..(71)
<223> Left T-DNA border repeat from pSBll

<220>
<221> promoter
<222> (162) ..(2149)
<223> Zea mays ubiquitin promoter region including intron

<220>
<221> Intron
<222> (1144) ..(2149)
<223> Zea mays ubiquitin intron MubGl

<220>
<221> CDS
<222> (2192) ..(3520)
<223> E. coli D-serine deaminase [dsdA] cds

<220>
<221> terminator
<222> (3562) ..(4270)
<223> Agrobacterium tumefaciens octapine synthase terminator region

<220>
<221> misc_structure
<222> (4359) ..(4503)
<223> Right T-DNA border from pSBll

<220>
<221> mi sc bi ndi ng
<222> (4399) ..(4422)
<223> Right T-DNA border repeat from pSBll

<400> 39
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actgaattgg atccgcccgg gcggtaccaa gcttccgcgg ctgcagtgca gcgtgacccg 180
gtcgtgcccc tctctagaga taatgagcat tgcattgtcta agttataaaa aattaccaca 240
tatttttttt gtcacacttg tttgaagtgc agtttatcta tctttataca tatatttaaa 300
ctttactcta cgaataatat aatctatagt actacaataa tatcagtggt ttagagaatc 360
atataaatga acagtttagac atggtctaaa ggacaattga gtattttgac aacaggactc 420
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taaaactcta ttttagtttt tttatttaaat agtttagata taaaatagaa taaaataaag 660
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cagcgaacca gcagcgtcgc gtcgggcca gcaagcaga cggcacggca tctctgtcgc 840
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ccagaaattg cgtggcggag cggcagacgt gagccggcac ggcaggcggc ctcctcctcc 960

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eolf-seql .txt

tctcacggca	ccggcagcta	cgggggattc	ctttcccacc	gctccttcgc	tttcccttcc	1020
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cggagcgcac	acacacacaa	ccagatctcc	cccaaatcca	cccgtcggca	cctccgcttc	1140
aaggtagccc	gctcgtcctc	ccccccccc	cccctctcta	ccttctctag	atcggcggtc	1200
cgggtccatgg	ttagggcccg	gtagttctac	ttctgttcat	gtttgtgtta	gatccgtggt	1260
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gcagacggga	tcgatttcat	gatttttttt	gtttcgttgc	atagggtttg	gtttgccctt	1440
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ggatcctcat	ctaagcgcaa	agagacgtac	t atg gaa aac get aaa atg aac			2212
			Met Glu Asn Ala Lys Met Asn			
			1	5		
teg etc ate gee cag tat ccg ttg gta aag gat ctg gtt get ctt aaa						2260
Ser Leu lie Ala Gin Tyr Pro Leu Val Lys Asp Leu Val Ala Leu Lys						
	10		15	20		
gaa ace ace tgg ttt aat cct ggc acg ace tea ttg get gaa ggt tta						2308
Glu Thr Thr Trp Phe Asn Pro Gly Thr Thr Ser Leu Ala Glu Gly Leu						
	25		30	35		
cct tat gtt ggc ctg ace gaa cag gat gtt cag gac gcc cat gcg cgc						2356
Pro Tyr Val Gly Leu Thr Glu Gin Asp Val Gin Asp Ala His Ala Arg						
	40		45	50		55
tta tec cgt ttt gca ccc tat ctg gca aaa gca ttt cct gaa act get						2404
Leu Ser Arg Phe Ala Pro Tyr Leu Ala Lys Ala Phe Pro Glu Thr Ala						
	60		65	70		
gcc act ggg ggg att att gaa tea gaa ctg gtt gcc att cca get atg						2452
Ala Thr Gly Gly lie lie Glu Ser Glu Leu Val Ala lie Pro Ala Met						
	75		80	85		
caa aaa egg ctg gaa aaa gaa tat cag caa ccg ate age ggg caa ctg						2500
Gin Lys Arg Leu Glu Lys Glu Tyr Gin Gin Pro lie ser Gly Gin Leu						
	90		95	100		

eolf-seql .txt																
tta Leu	ctg Leu 105	aaa Lys	aaa Lys	gat Asp	age Ser	cat His 110	ttg Leu	cec Pro	att lie	tec Ser	ggc Gly 115	tec Ser	ata lie	aaa Lys	gca Ala	2548
cgc Arg 120	ggc Gly	ggg Gly	att lie	tat Tyr	gaa Glu 125	gtc Val	ctg Leu	gca Ala	cac His	gca Ala 130	gaa Glu	aaa Lys	ctg Leu	get Ala	ctg Leu 135	2596
gaa Glu	gcg Ala	ggg Gly	ttg Leu	ctg Leu 140	acg Thr	ctt Leu	gat Asp	gat Asp	gac Asp 145	tac Tyr	age Ser	aaa Lys	ctg Leu	ctt Leu 150	tet Ser	2644
ccg Pro	gag Glu	ttt Phe	aaa Lys 155	cag Gin	ttc Phe	ttt Phe	age Ser	caa Gin 160	tac Tyr	age Ser	att lie	get Ala	gtg Val 165	ggc Gly	tea Ser	2692
ace Thr	gga Gly	aat Asn 170	ctg Leu	ggg Gly	tta Leu	tea Ser	ate lie 175	ggc Gly	att lie	atg Met	age Ser	gee Ala 180	cgc Arg	att lie	ggc Gly	2740
ttt Phe 185	aag Lys	gtg Val	aca Thr	gtt Val	cat His	atg Met 190	tct Ser	get Ala	gat Asp	gee Ala	egg Arg 195	gca Ala	tgg Trp	aaa Lys	aaa Lys	2788
gcg Ala 200	aaa Lys	ctg Leu	cgc Arg	age Ser	cat His 205	ggc Gly	gtt Val	acg Thr	gtc Val	gtg Val 210	gaa Glu	tat Tyr	gag Glu	caa Gin	gat Asp 215	2836
tat Tyr	ggt Gly	gtt Val	gcc Ala	gtc Val 220	gag Glu	gaa Glu	gga Gly	cgt Arg	aaa Lys 225	gca Ala	gcg Ala	cag Gin	tct Ser	gac Asp 230	ccg Pro	2884
aac Asn	tgt Cys	ttc Phe	ttt Phe 235	att lie	gat Asp	gac Asp	gaa Glu	aat Asn 240	tec Ser	cgc Arg	acg Thr	ttg Leu	ttc Phe 245	ctt Leu	ggg Gly	2932
tat Tyr	tec Ser	gtc Val 250	get Ala	ggc Gly	cag Gin	cgt Arg	ctt Leu 255	aaa Lys	gcg Ala	caa Gin	ttt Phe	gcc Ala 260	cag Gin	caa Gin	ggc Gly	2980
cgt Arg	ate lie 265	gtc val	gat Asp	get Ala	gat Asp	aac Asn 270	cct Pro	ctg Leu	ttt Phe	gtc val	tat Tyr 275	ctg Leu	ccg Pro	tgt cys	ggt Gly	3028
gtt Val 280	ggc Gly	ggt Gly	ggt Gly	cct Pro	ggt Gly 280 ⁵	ggc Gly	gtc Val	gca Ala	ttc Phe	ggg Gly 290	ctt Leu	aaa Lys	ctg Leu	gcg Ala	ttt Phe 295 ^r	3076
ggc Gly	gat Asp	cat His	gtt val	cac His 300	tgc cys	ttt Phe	ttt Phe	gcc Ala	gaa Glu 305	cca Pro	acg Thr	cac His	tec ser	cct Pro 310	tgt cys	3124
atg Met	ttg Leu	tta Leu	ggc Gly 315	gtc Val	cat His	aca Thr	gga Gly	tta Leu 320	cac His	gat Asp	cag Gin	att lie	tct Ser 325	gtt Val	cag Gin	3172
gat Asp	att lie	ggt Gly 330	ate lie	gac Asp	aac Asn	ctt Leu	ace Thr 335	gca Ala	gcg Ala	gat Asp	ggc Gly	ctt Leu 340	gca Ala	gtt val	ggt Gly	3220
cgc Arg	gca Ala 345	tea Ser	ggc Gly	ttt Phe	gtc Val	ggg Gly 350	egg Arg	gca Ala	atg Met	gag Glu	cgt Arg 355	ctg Leu	ctg Leu	gat Asp	ggc Gly	3268
ttc Phe 360	tat Tyr	ace Thr	ctt Leu	age Ser	gat Asp 365	caa Gin	ace Thr	atg Met	tat Tyr	gac Asp 370	atg Met	ctt Leu	ggc Gly	tgg Trp	ctg Leu 375	3316
gcg cag	cag	gaa	gaa	ggt	att	cgt	ctt	gaa	cct	teg	gca	ctg	gcg	ggt	atg	3364

eolf-seql.txt

Ala Gin Glu Gly Ile Arg Leu Glu Pro Ser Ala Leu Ala Gly Met
 380 385 390

gcc gga cct cag cgc gtg tgt gca tea gta agt tac caa cag atg cac 3412
 Ala Gly Pro Gin Arg Val Cys Ala Ser Val Ser Tyr Gin Gin Met HS
 395 400 405

ggt ttc age gca gaa caa ctg cgt aat ace act cat ctg gtg tgg gcg 3460
 Gly Phe Ser Ala Glu Gin Leu Arg Asn Thr Thr His Leu Val Trp Ala
 410 415 420

acg gga ggt gga atg gtg ccg gaa gaa gag atg aat caa tat ctg gca 3508
 Thr Gly Gly Gly Met Val Pro Glu Glu Glu Met Asn Gin Tyr Leu Ala
 425 430 435

aaa ggc cgt taa taacgtttca acgcagcatg gatcgtaccg agctcaatcg 3560
 Lys Gly Arg
 440

atcctgcttt aatgagatat gcgagacgcc tatgatcgca tgatatttgc tttcaattct 3620

gttgtgcacg ttgtaaaaaa cctgagcatg tgtagctcag atccttaccg ccggtttcgg 3680

ttcattctaa tgaatatatc acccgttact atcgtatttt tatgaataat attctccggt 3740

caatttactg attgtaccct actaettata tgtacaatat taaaatgaaa acaatatatt 3800

gtgctgaata ggtttatagc gacatctatg atagagcgcc acaataacaa acaattgcgt 3860

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cgttgggcgg gtccagggcg aattttgcga caacatgtcg aggtcagca ggatggggcc 4280

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cccgccgggc cagatcttga ttgtcgtttc ccgccttcag tttaaactat cagtgtttga 4400

caggatatat tggcgggtaa acctaaagaga aaagagcggt tattagaata atcggatatt 4460

taaaagggcg tgaaaagggt tatccgttcg tccatttgta tgt 4503

<210> 40
 <211> 442
 <212> PRT
 <213> Artificial

<220>
 <223> Synthetic Construct E. coli D-serine deaminase [dsdA] CDS

<400> 40

Met Glu Asn Ala Lys Met Asn Ser Leu lie Ala Gin Tyr Pro Leu Val
 1 5 10 15

Lys Asp Leu val Ala Leu Lys Glu Thr Thr Trp Phe Asn Pro Gly Thr
 20 25 30

eolf - seq1 .txt

Thr Ser Leu Ala Glu Gly Leu Pro Tyr Val Gly Leu Thr Glu Gln Asp
 35 40 45
 Val Gln Asp Ala His Ala Arg Leu Ser Arg Phe Ala Pro Tyr Leu Ala
 50 55 60
 Lys Ala Phe Pro Glu Thr Ala Ala Thr Gly Gly He He Glu Ser Glu
 65 70 75 80
 Leu Val Ala lie Pro Ala Met Gln Lys Arg Leu Glu Lys Glu Tyr Gln
 85 90 95
 Gln Pro lie Ser Gly Gln Leu Leu Leu Lys Lys Asp Ser His Leu Pro
 100 105 110
 lie Ser Gly Ser lie Lys Ala Arg Gly Gly lie Tyr Glu Val Leu Ala
 115 120 125
 His Ala Glu Lys Leu Ala Leu Glu Ala Gly Leu Leu Thr Leu Asp Asp
 130 135 140
 Asp Tyr Ser Lys Leu Leu Ser Pro Glu Phe Lys Gln Phe Phe Ser Gln
 145 150 155 160
 Tyr Ser lie Ala Val Gly Ser Thr Gly Asn Leu Gly Leu Ser lie Gly
 165 170 175
 lie Met Ser Ala Arg lie Gly Phe Lys Val Thr Val His Met Ser Ala
 180 185 190
 Asp Ala Arg Ala Trp Lys Lys Ala Lys Leu Arg ser His Gly val Thr
 195 200 205
 Val Val Glu Tyr Glu Gln Asp Tyr Gly Val Ala Val Glu Glu Gly Arg
 210 215 220
 Lys Ala Ala Gln ser Asp Pro Asn cys Phe Phe lie Asp Asp Glu Asn
 225 230 235 240
 Ser Arg Thr Leu Phe Leu Gly Tyr Ser Val Ala Gly Gln Arg Leu Lys
 245 250 255
 Ala Gln Phe Ala Gln Gln Gly Arg lie val Asp Ala Asp Asn Pro Leu
 260 265 270
 Phe Val Tyr Leu Pro Cys Gly Val Gly Gly Gly Pro Gly Gly Val Ala
 275 280 285
 Phe Gly Leu Lys Leu Ala Phe Gly Asp His Val His Cys Phe Phe Ala
 290 295 300

eolf-seql.txt

Glu Pro Thr His Ser Pro Cys Met Leu Leu Gly Val His Thr Gly Leu
 305 310 315 320

His Asp Gln Ile Ser Val Gln Asp Ile Gly Ile Asp Asn Leu Thr Ala
 325 330 335

Ala Asp Gly Leu Ala Val Gly Arg Ala Ser Gly Phe Val Gly Arg Ala
 340 345 350

Met Glu Arg Leu Leu Asp Gly Phe Tyr Thr Leu Ser Asp Gln Thr Met
 355 360 365

Tyr Asp Met Leu Gly Trp Leu Ala Gln Glu Glu Gly Ile Arg Leu Glu
 370 375 380

Pro Ser Ala Leu Ala Gly Met Ala Gly Pro Gln Arg Val Cys Ala Ser
 385 390 395 400

Val ser Tyr Gln Gln Met His Gly Phe ser Ala Glu Gln Leu Arg Asn
 405 410 415

Thr Thr His Leu Val Trp Ala Thr Gly Gly Gly Met Val Pro Glu Glu
 420 425 430

Glu Met Asn Gln Tyr Leu Ala Lys Gly Arg
 435 440

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 <211> 20
 <212> DNA
 <213> Artificial

<220>
 <223> Forward PCR primer for ahas gene

<400> 41
 tgactttggc tcarggaacg 20

<210> 42
 <211> 24
 <212> DNA
 <213> Artificial

<220>
 <223> Reverse PCR primer for ahas gene

<400> 42
 atctcacttt cattctctgg gttt 24