

SELECTION MARKER

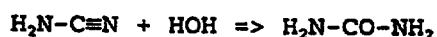
FIELD OF THE INVENTION

This application is concerned with a novel selectable marker,
5 especially with the use of cyanamide hydratase as a selection marker
in transformation experiments, more specifically in plant
transformation experiments.

BACKGROUND ART

10 Cyanamide ($\text{H}_2\text{N}-\text{C}\equiv\text{N}$) is a nitrile derivate which, like other nitrile
derivates, is used in agriculture for stimulation of growth and for
plant protection. Cyanamide in aqueous solution or in the form of its
calcium salt is used as a fertilizer by providing ammonia to the soil
by its metabolic conversion. It has, however, the additional advantage
15 of acting as a herbicide. To use it as fertilizer it has to be applied
before sowing.

Chemically, cyanamide belongs to the class of nitriles. In spite of
the relatively rare occurrence in nature of compounds containing the
nitrile group, enzymes that hydrate this group have been found in
20 bacteria and plants (e.g. Nagasawa T., et al. (1988) Biochem. Biophys.
Res. Commun. 155 1008-1016; Endo T. and Watanabe I. (1989) FEBS Lett.
243 61-64). Also in the fungus *Myrothecium verrucaria* a nitrile
hydrating enzyme was found (Stransky H. and Amberger A. (1973) Z.
Pflanzenphysiol. 70 74-87), which hydrates the nitrile group of
25 cyanamide with formation of urea:



Maier-Greiner et al. have isolated the enzyme and cloned the gene
30 coding for it (Proc. Natl. Acad. Sci. USA 88, 4260-4264, 1991). They
have demonstrated that this enzyme shows an extremely narrow substrate
specificity, where compounds chemically related to cyanamide are not
recognized as substrates.

35 Selectable markers have to confer a dominant phenotype on transformed
cells which is able of being used as a selection criteria. These fall
into two classes: one class of genes which confers either cell
viability or lethality in the presence of a selective agent and a
class of genes which has negligible effects on cell survival but which

confers transformed cells with some distinguishing physical characteristic.

In plant transformation the fraction of cells incorporating the novel DNA is generally low, so most stable transformation schemes use markers which ensure the survival of transformed cells in the presence of a selective agent.

A number of selection markers of this first group has been known and used for plant transformation experiments for several years. Included are the enzyme neomycin phosphotransferase (npt) which confers resistance to a group of antibiotics including kanamycin, paromomycin, geneticin and neomycin, mutant forms of the enzyme acetolactate synthase (als) which confer resistance to imidazolinones, sulfonylureas, triazolopyrimidines and pyrimidyloxybenzoates and the enzyme hygromycin 3'-O-phosphotransferase (hpt) which confers resistance to hygromycin. Also available are chloramphenicol transferase (cat) which detoxifies chloramphenicol and dihydrofolate reductase (dhfr) which neutralizes the toxic effects of methotrexate. Another possibility is to use the bar gene for resistance to the herbicide bialaphos (WO 97/05829).

Although there already are a number of selectable markers available, there is still need for another marker. This is due to several reasons:

- when transgenic plants are being transformed for a second time with a new construct it is necessary to select for the newly formed transformants with the help of a second selectable marker.
- the above mentioned selection markers are not applicable on in all plant species.
- some of the compounds which have to be added to enable selection are antibiotics. Spreading of genes which give resistance to antibiotics or herbicides should be minimized as much as possible to avoid the risk of conferring resistance to pathogens.
- some of the compounds which have to be added to enable selection are relatively expensive. There is a need for cheaper selection agents.

SUMMARY OF THE INVENTION

The invention now provides the use of a gene coding for cyanamide hydratase (CAH) as a new selection marker. Preferably this can be used for the transformation of plants. The gene comprises the nucleotide sequence of SEQIDNO: 1 or muteins thereof having cyanamide hydratase function.

The invention further comprises a method for the selection of transformed plants which comprises constructing a vector carrying a coding sequence for CAH and a gene of interest, transforming the vector to plants or plant parts or plant cells or callus and growing the resulting transformants in a medium which comprises cyanamide.

The invention is also directed to the use of cyanamide for the selection of plants transformed with a gene coding for CAH.

Further part of the invention are expression cassettes comprising a nucleotide sequence coding for cyanamidehydratase and a gene of interest. Also part of the invention are vectors with this expression cassette and hosts, including Agrobacterium, harboring such a vector. Further, plants transformed with such a vector and/or such an Agrobacterium form part of the invention.

DESCRIPTION OF THE FIGURES

- Fig. 1. Outline of the T-DNA in pMOG874
Fig. 2. Outline of the T-DNA in pMOG1156
Fig. 3 Outline of the T-DNA in pMOG22
Fig. 4 Outline of the T-DNA in pMOG1005
Fig. 5 Outline of the T-DNA in pMOG1278
Fig. 6 Outline of the T-DNA in pMOG1295
Fig. 7 Outline of the T-DNA in pMOG1253
Fig. 8 Outline of the expression cassette in pMOG873
Fig. 9 Outline of the expression cassette in pMOG617
Fig. 10. Arabidopsis explants transformed with a) pMOG1156 or b) pMOG410 selected on 50 mg/l cyanamide both.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to the use of a gene coding for cyanamide hydratase as a selectable marker.

5 The enzyme cyanamide hydratase (CAH) confers resistance to cyanamide which is a compound that has herbicidal activity. It has now been found that this property of the gene can be used in transformation technology to help in discerning transformed plants from non-transformed plants. However, the herbicidal activity alone is not sufficient to make a gene useful as a selectable marker. For that it is also needed that the gene is expressed in those cells which are submitted to selective conditions. This can be either by constitutive expression or expression in specific tissues like callus, seed, embryogenic tissues and meristematic tissues. Furthermore, it is needed that the gene converts susceptibility of a plant to a toxic compound into tolerance without any residual toxic activity. Also the presence of a large enough 'window' between the concentration of toxic compound needed for selection and the concentration which in the presence of the selection gene at which still growth can be seen is of importance for the use of a selection marker gene. In addition, the system should preferably function sufficiently cell autonomously, such that in a chimaeric tissue (i.e. a tissue with a mosaic of transformed and untransformed cells) untransformed cells are not protected by neighboring transformed cells and therefore survive selection. Surprisingly, the combination of the gene coding for CAH and the toxic properties of cyanamide qualify for their use as selection marker system.

This invention shows that it is possible to select transformants on basis of their tolerance to cyanamide.

30 An additional advantage is that the cyanamide is converted into urea which is converted in various plants in NH_3 and CO_2 . The NH_3 can be used by the plant as source of nitrogen. This is an additional selection possibility to increase the "window" between tolerance and selection. Normally, the culture media contain ammonia and nitrate (contained in the Murashige and Skoog media, see Table 2 and 4). If these are left out or their concentration is decreased the transformed plants containing the CAH gene will convert the cyanamide present in the medium as selection agent into urea and further into ammonia which can be used as nitrogen source. The non-transformed plants are unable to do so, thus in addition to the herbicidal effect of cyanamide they

will also suffer from a competitive disadvantage in the area of nitrogen uptake.

5 The nucleotide sequence coding for CAH is preferably the sequence as depicted in SEQIDNO:1. Also muteins of this sequence may be considered as being part of the invention. Muteins are nucleotide sequences which alter in their nucleotide sequence but still have similar functional and immunological characteristics as the sequence presented in SEQIDNO:1. These muteins are also called functional variants. In
10 addition, the polynucleotides of the invention specifically include those sequences substantially identical (determined as described below) with the gene sequences of the invention and that encode proteins that retain the functional activity of the proteins of the invention. Thus, in the case of the CAH gene disclosed here, the above
15 term includes variant polynucleotide sequences which have substantial identity with the sequences disclosed here and which encode proteins which still have cyanamide degrading activity.

"Percentage of sequence identity" for polynucleotides and polypeptides is determined by comparing two optimally aligned
20 sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e. gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is
25 calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of match positions, dividing the number of match positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage
30 of sequence identity. Optimal alignment of sequences for comparison may be conducted by computerized implementations of known algorithms (e.g., GAP, BESTFIT, FASTA and TFAST in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI, or BlastN and BlastX available from the National Center
35 for Biotechnology Information), or by inspection.

The term "substantial identity" or "substantial similarity" means that a polypeptide comprises a sequence that is able to hybridize with the target polypeptide under stringent conditions. With

stringent conditions a solution of 2 * SSC and a temperature of 65°C is meant.

Polypeptides which are "substantially similar" share sequences as noted above except that residue positions which are not identical may differ by conservative amino acid changes. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine and isoleucine: a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine: a group of amino acids having amide-containing side chains is asparagine and glutamine: a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan: a group of amino acids having basic side chains is lysine, arginine and histidine: and a group of amino acids having sulfur-containing side chains is cysteine and methionine.

Substantial identity of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90% and most preferably at least 95%. Another indication that nucleotide sequences are substantially identical is if two molecules specifically hybridize to each other under stringent conditions. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. The T_m of a hybrid, which is a function of both the length and the base composition of the probe, can be calculated using information in Sambrook, T. et al., (1989) Molecular Cloning -- A Laboratory Manual (second edition), Volume 1-3, Cold Spring Harbor Laboratory, Cold Spring. Typically, stringent conditions for a Southern blot protocol involve washing at 65°C with 0.2 X SSC. For preferred oligonucleotide probes, washing conditions are typically about 42°C in 6X SSC.

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The present invention provides a chimeric DNA sequence which comprises an open reading frame capable of encoding a protein having cyanamide hydratase activity. The term chimeric DNA sequence shall mean to comprise any DNA sequence which comprises DNA sequences not

naturally found in nature. For instance, chimeric DNA shall mean to comprise DNA comprising the said open reading frame in a non-natural location of the plant genome, even if said plant genome would normally contain a copy of the said open reading frame in its natural chromosomal location. Similarly, the said open reading frame may be incorporated in the plant genome wherein it is not naturally found, or in a replicon or vector where it is not naturally found, such as a bacterial plasmid or a viral vector. Chimeric DNA shall not be limited to DNA molecules which are replicable in a host, but shall also mean to comprise DNA capable of being ligated into a replicon, for instance by virtue of specific adaptor sequences, physically linked to the open reading frame according to the invention. The open reading frame may or may not be linked to its natural upstream and downstream regulatory elements.

The open reading frame may be derived from a genomic library. In this latter it may contain one or more introns separating the exons making up the open reading frame that encodes a protein according to the invention. The open reading frame may also be encoded by one uninterrupted exon, or by a cDNA to the mRNA encoding a protein according to the invention. Open reading frames according to the invention also comprise those in which one or more introns have been artificially removed or added. Each of these variants is embraced by the present invention.

Preferably the open reading frame is derived from the soil fungus *Myrothecium verrucaria* (as described in Maier-Greiner, U.H. et al., Proc. Natl. Acad. Sci. USA 88, 4260-4264, 1991).

In order to be capable of being expressed in a host cell in a way that the expressed protein can confer resistance to the toxic selection agent, a chimeric DNA according to the invention will usually be provided in an expression cassette with regulatory elements enabling it to be recognized by the biochemical machinery of the host and allowing for the open reading frame to be transcribed and translated in the host. It will usually comprise a transcriptional initiation region which may be suitably derived from any gene capable of being expressed in the host cell of choice, as well as a translational initiation region for ribosome recognition and attachment. In eukaryotic plant cells, an expression cassette usually comprises in addition a transcriptional termination region located

downstream of said open reading frame, allowing transcription to terminate and polyadenylation of the primary transcript to occur. In addition, the codon usage may be adapted to accepted codon usage of the host of choice. The principles governing the expression of a chimeric DNA construct in a chosen host cell are commonly understood by those of ordinary skill in the art and the construction of expressible chimeric DNA constructs is now routine for any sort of host cell, be it prokaryotic or eukaryotic.

In order for the open reading frame to be maintained in a host cell it will usually be provided in the form of a replicon comprising said open reading frame according to the invention linked to DNA which is recognized and replicated by the chosen host cell. Accordingly the selection of the replicon is determined largely by the host cell of choice. Such principles as govern the selection of suitable replicons for a particular chosen host are well within the realm of the ordinary skilled person in the art.

A special type of replicon is one capable of transferring itself, or a part thereof, to another host cell, such as a plant cell, thereby co-transferring the open reading frame according to the invention to said plant cell. Replicons with such capability are herein referred to as vectors. An example of such vector is a Ti-plasmid vector which, when present in a suitable host, such as *Agrobacterium tumefaciens*, is capable of transferring part of itself, the so-called T-region, to a plant cell. Different types of Ti-plasmid vectors (vide: EP 0 116 718 B1) are now routinely being used to transfer chimeric DNA sequences into plant cells, or protoplasts, from which new plants may be generated which stably incorporate said chimeric DNA in their genomes. A particularly preferred form of Ti-plasmid vectors are the so-called binary vectors as claimed in (EP 0 120 516 B1 and US 4,940,838). Other suitable vectors, which may be used to introduce DNA according to the invention into a plant host, may be selected from the viral vectors, e.g. non-integrative plant viral vectors, such as derivable from the double stranded plant viruses (e.g. CaMV) and single stranded viruses, gemini viruses and the like. The use of such vectors may be advantageous, particularly when it is difficult to stably transform the plant host. Such may be the case with woody species, especially trees and vines.

The expression "host cells incorporating a chimeric DNA sequence according to the invention in their genome" shall mean to comprise

cells, as well as multicellular organisms comprising such cells, or essentially consisting of such cells, which stably incorporate said chimeric DNA into their genome thereby maintaining the chimeric DNA, and preferably transmitting a copy of such chimeric DNA to progeny cells, be it through mitosis or meiosis. Such host cells can be prokaryotic organisms such as bacteria, but also eukaryotic organisms such as yeast. Also cells from eukaryotes in tissue culture, such as cell cultures of plants or animals like mammals can be envisaged to stably incorporate the chimeric DNA. According to a preferred embodiment of the invention plants are provided, which essentially consist of cells which incorporate one or more copies of said chimeric DNA into their genome, and which are capable of transmitting a copy or copies to their progeny, preferably in a Mendelian fashion. By virtue of the transcription and translation of the chimeric DNA according to the invention those cells that produce the CAH will show enhanced resistance to cyanamide. Although the principles which govern transcription of DNA in plant cells are not always understood, the creation of chimeric DNA capable of being expressed in tissue which is subject to selection by cyanamide, such as callus, seed, embryogenetic tissues or meristematic tissues, or constitutive expression, is now routine. Transcription initiation regions routinely in use for expression of the transformed polynucleotide in a constitutive way are promoters obtainable from the cauliflower mosaic virus, notably the 35S RNA and 19S RNA transcript promoters and the so-called T-DNA promoters of *Agrobacterium tumefaciens*. In particular to be mentioned are the nopaline synthase promoter, octopine synthase promoter (as disclosed in EP 0 122 791 B1) and the mannopine synthase promoter. In addition plant promoters may be used, which may be substantially constitutive, such as the rice actin gene promoter. The choice of the promoter is not essential, although it must be clear that constitutive high-level promoters should show expression in tissue on which the selection takes place. It is further known that duplication of certain elements, so-called enhancers, may considerably enhance the expression level of the DNA under its regime (vide for instance: Kay R. et al. (1987), Science 236, 1299-1302: the duplication of the sequence between -343 and -90 of the CaMV 35S promoter increases the activity of that promoter). In addition to the 35S promoter, singly or doubly enhanced, examples of high-level promoters are the light-inducible ribulose biphosphate carboxylase small subunit (*rbcSSU*) promoter and

the chlorophyll a/b binding protein (Cab) promoter. Also envisaged by the present invention are hybrid promoters, which comprise elements of different promoter regions physically linked. A well known example thereof is the so-called CaMV enhanced mannopine synthase promoter (US Patent 5,106,739), which comprises elements of the mannopine synthase promoter linked to the CaMV enhancer.

Specifically with monocot transformation the use of introns between promoter and selectable marker gene enhances expression.

The term "promoter" thus refers to a region of DNA upstream from the structural gene and involved in recognition and binding RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells. A "constitutive promoter" is a promoter which is active under most environmental conditions and states of development or cell differentiation.

A constitutive promoter is preferable for this invention because selection for transformants may be made at various stages and with various tissues. Thus a constitutive promoter does not limit the selection possibilities.

Choice of an appropriate constitutive promoter in this respect is of importance for the use of other promoters in the same transformation process. It is known that duplication of promoters is influential to the expression of the genes under control of said promoters. Since it is the goal of the expression of a selection marker only to be used for selection of plants which are simultaneously transformed with a gene of interest one should keep in mind that using the same promoter for the selectable marker gene and the gene of interest can cause problems.

As regards the necessity of a transcriptional terminator region, it is generally believed that such a region enhances the reliability as well as the efficiency of transcription in plant cells. Use thereof is therefore strongly preferred in the context of the present invention.

As regards the applicability of the invention in different plant species, it has to be mentioned that one particular embodiment of the invention is merely illustrated with transgenic tomato, potato, rice and Arabidopsis plants as an example, the actual applicability being in fact not limited to these plant species.

Although some of the embodiments of the invention may not be practicable at present, e.g. because some plant species are as yet recalcitrant to genetic transformation, the practicing of the invention in such plant species is merely a matter of time and not a matter of principle, because the amenability to genetic transformation as such is of no relevance to the underlying embodiment of the invention.

"Transformation of plants" is meant to be any method in which DNA is introduced into a plant. Such a transformation process should not necessarily contain a regeneration and/or tissue culture period.

Transformation of plant species is now routine for an impressive number of plant species, including both the *Dicotyledoneae* as well as the *Monocotyledoneae*. In principle any transformation method may be used to introduce chimeric DNA according to the invention into a suitable ancestor cell. Methods may suitably be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. et al., 1982, *Nature* 296, 72-74; Negrutiu I. et al, June 1987, *Plant Mol. Biol.* 8, 363-373), electroporation of protoplasts (Shillito R.D. et al., 1985 *Bio/Technol.* 3, 1099-1102), microinjection into plant material (Crossway A. et al., 1986, *Mol. Gen. Genet.* 202, 179-185), (DNA or RNA-coated) particle bombardment of various plant material (Klein T.M. et al., 1987, *Nature* 327, 70), infection with (non-integrative) viruses, in planta *Agrobacterium tumefaciens* mediated gene transfer by infiltration of plants or transformation of mature pollen or microspores (EP 0 301 316) and the like. A preferred method according to the invention comprises *Agrobacterium*-mediated DNA transfer. Especially preferred is the use of the so-called binary vector technology as disclosed in EP A 120 516 and U.S. Patent 4,940,838).

Tomato transformation is preferably done essentially as described by Van Roekel et al. (Van Roekel, J.S.C., et al. *Plant Cell Rep.* 12, 644-647). Potato transformation is preferably done essentially as described by Hoekema et al. (Hoekema, A., et al. 7, 273-278 1989).

Although considered somewhat more recalcitrant towards genetic transformation, monocotyledonous plants are amenable to transformation and fertile transgenic plants can be regenerated from transformed cells or embryos, or other plant material. Presently, preferred methods for transformation of monocots are microprojectile bombardment

of embryos, explants or suspension cells, and direct DNA uptake or (tissue) electroporation (Shimamoto, et al., Nature 338, 274-276, 1989). Transgenic maize plants have been obtained by introducing the *Streptomyces hygrosopicus* bar-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microprojectile bombardment (Gordon-Kamm, Plant Cell, 2, 603-618, 1990). Wheat plants have been regenerated from embryogenic suspension culture by selecting embryogenic callus for the establishment of the embryogenic suspension cultures (Vasil Bio/Technol. 8, 429-434, 1990). The combination with transformation systems for these crops enables the application of the present invention to monocots.

Monocotyledonous plants, including commercially important crops such as rice and corn are also amenable to DNA transfer by *Agrobacterium* strains (vide WO 94/00977; EP 0 159 418 B1; Gould J, Michael D, Hasegawa O, Ulian EC, Peterson G, Smith RH, Plant. Physiol. 95, 426-434, 1991).

To obtain transgenic plants capable of expressing more than one chimeric gene, a number of alternatives are available including the following:

A. The use of DNA, e.g. a T-DNA on a binary plasmid, with a number of modified genes physically coupled to a second selectable marker gene. The advantage of this method is that the chimeric genes are physically coupled and therefore migrate as a single Mendelian locus. The invention is especially useful in this respect, since it enables for a second selectable marker which can be introduced next to an already existing selectable marker-gene of interest combination. Thus selection for retransformants can be performed irrespective of the nature of the first selectable marker.

B. Cross-pollination of transgenic plants each already capable of expressing one or more chimeric genes, preferably coupled to a selectable marker gene, with pollen from a transgenic plant which contains one or more chimeric genes coupled to another selectable marker. Afterwards the seed, which is obtained by this crossing, maybe selected on the basis of the presence of the two selectable markers, or on the basis of the presence of the chimeric genes themselves. The plants obtained from the selected seeds can afterwards be used for further crossing. In principle the chimeric genes are not on a single

locus and the genes may therefore segregate as independent loci. Also here the option to select for both selectable markers is one of the advantages of the present invention.

C. The use of a number of a plurality of chimeric DNA molecules, e.g. plasmids, each having one or more chimeric genes and a selectable marker. If the frequency of co-transformation is high, then selection on the basis of only one marker is sufficient. In other cases, the selection on the basis of more than one marker is preferred.

D. Consecutive transformation of transgenic plants already containing a first, second, (etc), chimeric gene with new chimeric DNA, optionally comprising a selectable marker gene. As in method B, the chimeric genes are in principle not on a single locus and the chimeric genes may therefore segregate as independent loci.

E. Combinations of the above mentioned strategies.

The actual strategy may depend on several considerations as maybe easily determined such as the purpose of the parental lines (direct growing, use in a breeding program, use to produce hybrids) but is not critical with respect to the described invention.

Although not necessary for this invention, it is known that practically all plants can be regenerated from cultured cells or tissues. The means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Shoots may be induced directly, or indirectly (from callus) via organogenesis or embryogenesis and subsequently rooted. Next to the selective compound, the culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. Efficient regeneration will depend on the medium, on the genotype and on the history of the culture. If these three variables are controlled regeneration is usually reproducible and repeatable.

After stable incorporation of the transformed gene sequences into the transgenic plants, the traits conferred by them can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Example 1Cloning the fungal gene encoding cyanamide hydratase (CAH)
in a heterologous expression cassette5 **a. Constructs for transformation to dicots**

Construct pMOG874 contains the coding region from the cyanamide hydratase gene from the soil fungus *Myrothecium verrucaria* which is operably linked to the CaMV 35S promoter and the CaMV 35S terminator. This chimeric gene is cloned in the binary vector pBI101 (Jefferson et al. EMBO J. 6, 3901, 1987) replacing the β -glucuronidase coding region and the nopaline synthase terminator.

The construct is obtained by adding an XhoI site at the 5' end and a SstI site at the 3' end of a 899 bp cDNA fragment of CAH (position 235-1197 of sequence published by Maier-Greiner et al. (1991) Proc. Natl. Acad. Sci. USA 88:4260-4264) by PCR using the primers p1:

5'ACCGAGCTCGAATTCGGCACGAGGTTGACATGATACCTTCCTG 3' and

p2: 5'GACCTCGAGAATTCGGCACGAGGTACGATCCTACTTCCTCGC 3' between the sites XhoI and SstI of the plant expression vector pRT101, both sites belonging to the polylinker which is inserted between the 35S promoter and the 35S termination signal of pRT101 (Töpfer et al. 1987, Nucl. Acids Res. 15: 5890).

The chimeric gene is then cleaved with PstI, overhanging ends are polished with T4 DNA polymerase and the fragment is cloned blunt in the SmaI site of pBIN19 (Bevan, M. Nucl. Acids Res. 12:8711-8721, 1984).

In construct pMOG1156 an additional β -glucuronidase gene operably linked to the 35S promoter and the 35S terminator is inserted as XhoI/SalI fragment in the SalI site of pMOG874.

Both constructs contain in addition to the novel CAH selection marker the conventional NPTII selection marker linked to the nopaline synthase promoter and the nopaline synthase terminator as it is in pBIN19.

35 **b. Constructs for transformation to monocots**

In the same way as pMOG874 was made the expression cassette was cloned into a high copy vector (pRT101, Töpfer, R. et al., Nucl. Acids Res. 15, 5890, 1987) resulting in pMOG873 (fig. 8)

A derivative of pMOG22 (fig. 3, deposited at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands on January 29, 1990, under

no. CBS 101.90) was made by introducing a *Kpn* I restriction site in the polylinker of pMOG22 between the *Eco*R I and *Sma* I site. The orientation of the polylinker was also reversed. This plasmid, denominated pMOG1005, contains a hygromycin resistance gene between the left and right T-DNA borders (fig. 4). The 1.7 kb expression cassette comprising the *cah* gene under control of the 35S promoter and the 35S terminator was cloned between the *Hind* III and *Bam*H I restriction sites. This plasmid was denominated pMOG1278 (fig. 5). Binary vector pMOG1295 (fig. 6) is a derivative of pMOG1278 and contains in the *Sal* I restriction site a GUS-expression cassette as described in Vancanneyt, G. et al. (Mol. Gen. Genet., 220, 245-250, 1990).

pMOG1253 was made departing from pMOG18 (Sijmons, P.C. et al., Bio/Technol. 8, 217-221, 1990) which contains the double enhanced 35S promoter, the AlMV RNA4 leader sequence, the GUS-gene and the nos-terminator in an expression cassette as an *Eco*R I - *Hind* III fragment. The plasmid p35S GUS INT (Vancanneyt, 1990) was digested with *Sna*B I and *Msc* I; the resulting 426 bp fragment, containing part of the coding region for the GUS gene and ST-LS1 intron, was isolated and cloned into pMOG18 linearized with *Sna*B I and *Msc* I. From the resulting plasmid a 3189 bp *Eco*R I - *Hind* III fragment was isolated and cloned into pMOG22; resulting in pMOG1253 (fig. 7).

pMOG617 (fig. 9) was made by cloning the hygromycin expression cassette from pMOG22 in the *Hind* III site of high copy vector pMOG18.

Example 2

Potato transformation

Described below is the method used for transformation of stem segments of *Solanum tuberosum* cv. Kardal using *Agrobacterium tumefaciens*. Nodal explants from in vitro grown potato plants were used 3 to 8 weeks after transfer. The plants were grown on Multiplication Medium (MUM) under a 16 h light period (1700 lux) at 24°C and a 8 h dark period at 21°C (The various media can be found in Table 2). Stem segments of approximately 5 mm were cut on sterile filter paper soaked with Washing Medium (WAM) and collected in a flask containing Washing Medium. For approximately 300 explants the Washing Medium was replaced

by Pre cultivation Medium (PRM). The flasks were cultured at 80 rpm at the same culture conditions as described above for approximately 24 h. All binary vectors used in this study contained the nptII gene as a plant selectable marker and the nptIII as bacterial selectable marker. Plasmid pMOG410 additionally harbored a chimeric gus gene containing an intron (Vancanneyt et al. Mol. Gen. Genet., 220, 245-250, 1990). Plasmid pMOG1156 additionally harbored the gus gene and the chimeric cah gene encoding cyanamide hydratase. Plasmid pMOG874 additionally harbored the cah gene. Plasmids were maintained in *E.coli* and *A. tumefaciens* under kanamycin selection.

The *Agrobacterium* strain used in this study harbored a rifampicin selection marker in a C58 chromosomal background. The construction of the helper strain EHA105 is described by Hood et al. (1993), Transg. Res. 2, 208-218.

Agrobacteria were grown overnight in LB medium with antibiotics (rifampicin 20 mg/l, kanamycin 100 mg/l). The overnight culture was diluted to $OD_{600} = 0.1$ and grown to $OD_{600} = 0.3$ in LB without antibiotics in approximately 2 h time. Bacterial suspensions were centrifuged at 1600xg for 15 minutes at room temperature. Bacteria were resuspended in Washing Medium and used for cocultivation experiments. The Pre cultivation Medium was removed from the flasks and replaced by the *Agrobacterium* suspension. The flasks were incubated for 20 minutes after which the explants were rinsed twice with Washing Medium. The explants were dried on sterile filter paper and incubated for 48 h on plates containing Cocultivation Medium (COM). Then, the explants were transferred to Post cultivation Medium (POM) and incubated for 72 h. The explants were then transferred to Shoot inducing Medium (SIM) containing several concentrations cyanamide or kanamycin. After two weeks the explants were subcultured on the same medium and approximately three weeks later the explants were placed on Shoot elongation Medium (SEM) containing cyanamide or kanamycin as mentioned above. When shoots were large enough to cut they were transferred to Root inducing Medium (RIM). Shoots that were able to root were then transferred to Root inducing Medium containing 50 mg/l cyanamide or 30 mg/l kanamycin. Simultaneously the transgenic nature of the shoots was determined by testing leaflets of the rooted shoots for expression of the gus gene using a histochemical GUS assay. It appeared that for pMOG1156 rooting of transgenic shoots on medium containing cyanamide was completely correlated with expression of the gus gene.

Table 1. Transformation frequencies of potato stem segments

pMOG1156 (cah-gus-nptII)

Selection during regeneration (mg/l)	Nr. of inoculated explants	No. of excised shoots	No. of rooting shoots	No. of gus + shoots
Cyanamide 40	48	15	2	2
Cyanamide 30	48	50	12	12
Cyanamide 20	48	56	6	6
Kanamycin 50	48	26	16	16

pMOG410 (gus-nptII)

Selection during regeneration (mg/l)	Nr. of inoculated explants	No. of excised shoots	No. of rooting shoots	No. of gus + shoots
Cyanamide 40	62	0	0	0
Cyanamide 30	62	10	0	0
Cyanamide 20	62	48	0	0
Kanamycin 50	58	24	13	12

pMOG874 (cah-nptII)

Selection during regeneration (mg/l)	Nr. of inoculated explants	No. of excised shoots	No. of rooting shoots	No. of gus + shoots
Cyanamide 40	58	10	1	n.d.
Cyanamide 30	58	40	8	n.d.
Cyanamide 20	58	81	2	n.d.
Kanamycin 50	58	34	26	n.d.

n.d.:not determined.

5

Table 2. Composition of the various media.

Medium:	WAM	PRM	COM	POM	SIM	SEM	RIM	MUM
Macro salts	1x MS	1x MS	1x MS	1x MS	1x MS	1x MS	1/2x MS	1/2x MS
Vitamins	B5	B5	B5	B5	B5	B5	1/2R3	1/2R3
Sucrose	3%	3%	3%	3%	3%	3%	1%	1%
Agar	-	0.8%	0.8%	0.8%	0.8%	0.8%	0.8%	0.8%
MES (gr/l)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Zeatin Riboside (mg/l)	-	0.5	0.5	3.0	3.0	0.3	-	-
2,4-D	-	1.0	1.0	-	-	-	-	-
IBA	-	-	-	-	-	-	0.1	-
Cefotaxim	-	-	-	200	200	200	100	-
Vancomycin	-	-	-	100	100	100	50	-

MS: Murashige and Skoog, *Physiol.* **15**, 473-479, 1962B5: Gamborg B5 (Gamborg, Orl et al., *Exp. Cell Res.* **50**, 151-158, 1986)

Example 3Tomato transformation

Described below is the method used for transformation of cotyledons of *Lycopersicon esculentum* cv. Money Maker using *Agrobacterium*

5 *tumefaciens*. The binary vectors and *Agrobacteria* strains for this transformation method are identical to those described above.

Tomato seedlings were germinated on Germination Medium (GEM) under a 16 h light period (1700 lux) at 24°C and a 8 h dark period at 21°C (The contents of the various media can be found in Table 4). Cotyledon
10 explants of 5 to 7 day old seedlings were cut on sterile filter paper soaked with Washing Medium (WAM) and placed on plates containing Cocultivation Medium (COM). The plates, each containing approximately 50 explants, were incubated overnight under the same conditions as described above.

15 The pre incubated explants were carefully submerged in the *Agrobacterium* inoculum for 20 minutes.

The explants were then blotted dry on sterile filter paper and incubated for 48 h on the second set of Cocultivation plates. In procession the explants were incubated for 72 h on plates containing
20 Postcultivation Medium (POM) after which the explants were transferred to Shoot inducing Medium (SIM) containing several concentrations of cyanamide or kanamycin. Every three weeks the explants were subcultured on the same medium. After approximately 8-12 weeks shoots were excised and placed on Root inducing Medium (RIM). Shoots that
25 were able to root were then transferred to Root inducing Medium containing 50 mg/l cyanamide or 30 mg/l kanamycin. Simultaneously leaflets of the rooted shoots were tested for expression of the *gus* gene in a histochemical GUS assay.

Table 3. Transformation results of tomato cotyledon explants

pMOG1156 (gus-nptII-cah)

Selection during regeneration (mg/l)	Nr. of inoculated explants	No. of excised shoots	No. of rooting shoots	No. of gus + shoots
Cyanamide 40	30	5	3	3
Cyanamide 30	30	5	0	0
Cyanamide 20	30	0	0	0
Kanamycin 100	30	5	3	4

pMOG410 (gus-nptII)

Selection during regeneration (mg/l)	Nr. of inoculated explants	No. of excised shoots	No. of rooting shoots	No. of gus + shoots
Cyanamide 40	30	0	0	0
Cyanamide 30	30	3	0	0
Cyanamide 20	30	0	0	0
Kanamycin 100	30	4	3	3

pMOG874 (cah-nptII)

Selection during regeneration (mg/l)	Nr. of inoculated explants	No. of excised shoots	No. of rooting shoots	No. of gus + shoots
Cyanamide 40	35	1	1	n.d.
Cyanamide 30	35	0	0	n.d.
Cyanamide 20	35	2	0	n.d.
Kanamycin 100	35	4	1	n.d.

n.d.: not determined

Table 4. Composition of the various media

Medium:	WAM	COM	POM	SIM	RIM	GEM
MS Macro salts	1x	1x	1x	1x	1x	1/2x
Vitamins	B5	B5	B5	B5	B5	B5
Sucrose	3%	3%	3%	-	1%	1%
Glucose	-	-	-	1%	-	-
Agar	-	0.8%	0.8%	0.8%	0.8%	0.8%
MES (gr/l)	0.5	0.5	0.5	0.5	0.5	0.5
pH	5.8	5.8	5.8	5.8	5.8	5.8
Zeatin Riboside (mg/l)	-	2.0	2.0	2.0	-	-
IAA	-	0.1	0.1	0.1	-	-
2,4-D	-	0.05	-	-	-	-
IBA	-	-	-	-	0.25	-
Carbenicillin	-	-	-	500	-	-
Cefotaxim	-	-	200	-	200	-
Vancomycin	-	-	50	-	-	-
Acetosyringone (mM)	-	0.2	-	-	-	-

5

Example 4
Arabidopsis transformation

Described below is the method used for transformation of root segments of *Arabidopsis thaliana* cv. C24 using *Agrobacterium tumefaciens*. The binary vectors for this transformation method are identical to those described above.

Six mg of *Arabidopsis* seeds were germinated in a flask containing liquid Germination Medium (GM) under 16 h light period (1700 lux) at 24°C and a 8 h dark period at 21°C at 80 rpm. (The contents of various media can be found in Table 4). Roots of 9 days old seedlings were isolated in a sterile petridish and collected in a drop of Germination Medium (GM). Roots were cut in segments of approximately 3-5 mm and approximately 100 explants were spread evenly on a nylon membrane (0.8 cm) which was placed on plates containing Callus

20

Inducing Medium (CIM). The plates were incubated 3 days under the same conditions as described above.

The *Agrobacterium* strain used in this study harbored a rifampicin selection marker in a C 58 chromosomal background. The construction of the helper strain MOG101 is described by Hood et al. (1993).

5 *Agrobacteria* were grown overnight in LB medium with antibiotics (rifampicin 20 mg/l, kanamycin 100 mg/l). The overnight culture was diluted 1:10 in LB without antibiotics and grown for approximately 3 hours. Bacterial suspensions were centrifuged at 1600xg for 15 minutes at room temperature. Bacteria were resuspended in GM and adjusted to OD600=0.1 and used for cocultivation.

10 The membrane containing approximately 100 explants was incubated for 2 minutes with the *Agrobacterium* suspension and dried on sterile filter paper to remove excess of bacteria. The membrane with explants are cultured for 48 h on CIM plates. After rinsing the membrane and
15 explants with liquid GM these were incubated on Shoot Induction Medium (SIM) plates containing several concentrations of cyanamide or kanamycin. After 5 days the membrane with the explants was transferred to the same medium (SIM) for subculture. The second
20 subculture was after 2 weeks. Approximately four weeks after cocultivation 60 shoots per cyanamide concentration were excised and placed on plates with Shoot Elongation Medium (SEM) containing 30 mg/l cyanamide. Shoots which were able to root are tested on their transgenic character by testing leaflets and flowers for expression of the gus gene using a histochemical GUS assay.

25 Three experiments were performed. Shoots obtained from Exp. 98-8 and 98-11 were transferred to rooting medium (SEM) containing 30 mg/litre cyanamide. Shoots obtained from Exp. 98-13 were transferred to rooting medium containing the same concentration as the selection medium (SIM), for results see Table 4a. The shoots obtained from the
30 kanamycin selection (50 mg/litre) were transferred to rooting medium containing 25 mg/litre Kanamycin.

Table 4. Media required for *Arabidopsis thaliana* C24 root transformation

Media components	Media	GM	CIM	SIM	SEM
Ingredients	macro elements	B5	B5	B5	MS
	micro elements	B5	B5	B5	MS
	vitamins	B5	B5	B5	B5
	sucrose (g/l)				10
	glucose (g/l)	20	20	20	
	Daichin agar (g/l)		10	10	10
Hormones	2,4-D		0.5		
	kinetin		0.05		
	2-ip			5	
	IAA			0.15	
Antibiotics	vancomycin			100	50
	carbenicillin			500	
	cefotaxime				100

Root explants transformed with pMOG 410 were not able to regenerate on cyanamide containing medium. Even 20 mg/litre cyanamide was already enough to prevent regeneration of explants transformed with a construct without the cah gene. At 20 till 40 mg/litre cyanamide some callus development was observed, but at 50 mg/litre and higher explants were not viable and turned completely brown.

On the other hand explants transformed with the cah gene (pMOG 1156) were able to regenerate at all cyanamide concentrations, even at 80 mg/litre. At lower concentrations the regeneration of shoots was faster than with kanamycin.

Although more shoots were available 60-65 shoots were harvested per treatment and placed on rooting medium. At the lower cyanamide concentrations the same amount of shoots developed as with kanamycin selection (approx. 70-100 per petridish).

There is a clear correlation between callus development and GUS expression on cyanamide selection with root explants transformed with pMOG 1156 (fig. 4b). GUS analysis of shoots obtained on cyanamide 0 mg/litre (NS) showed no staining, indicating that cyanamide is needed to obtain transgenic shoots.

Table 4a. Results Arabidopsis transformation with pMOG 1156

Experiment 98-8			Experiment 98-11		Experiment 98-13	
Conc. of Cyanamide (mg/litre)	Nr. of plants ¹	% Shoots ² rooting	Nr. of plants	% Shoots ² rooting	Nr. of plants	% Shoots ² rooting
C 20	0	0	1	1.7	3	4.8
C 30	4	6.3	2	3.2	9	14.5
C 40	5	8.2	3	4.5	1	1.6
C 50	nd.	nd.	5	8.1	0	0
C 60	5	8.2	nd.	nd.	4	6.0
C 80	9	13.8	10	15.6	8	11.4
K 50	29	46.8	19	47.5	14	20.6

(1) : The total number of plants consists of those shoots developed into plants and able to root on cyanamide containing medium.

(2) : % Shoots rooting = Nr. of plants / Total nr. of shoots * 100%

(3) : % of blue stained plants compared to nr. of plants

Table 4b. Percentage of GUS expressing Arabidopsis plants obtained via cyanamide or kanamycin selection.

Treatment	pMOG 410	pMOG 1156	
	C ³ 30 ³	C30 ³	Same conc.as treatment ⁴
C20	nd	0 ¹	0
C30	nd	75-100	78
C40	nd	40-100	100
C50	nd	80	nd.
C60	nd	80	100
C80	nd	67-90	100
K ⁴ 50	100 ²	82-92	100

(1) : % of plants staining blue

(2) : All pMOG 410 shoots are rooted on Kanamycin 25 mg/litre

(3) : C = Cyanamide (mg/litre)

(4) : K = Kanamycin (mg/litre)

(5) : concentration in rooting medium

Example 5

Rice transformation

Described below is the method used for transformation of callus derived from scutellum of mature embryos of *Oryza sativa* cv. Taipei 309 using *Agrobacterium tumefaciens* strain LBA1119-pMOG1295

(harboring the *cah*-gene) and strain LBA1119-pMOG1253 (control).

Sterile dehusked rice seeds were germinated on plates containing Callus Induction Medium (CIM) in the dark at 28°C. (The contents of various media can be found in Table 5). After 3 weeks embryogenic callus derived from the scutellum is isolated and subcultured on the

same medium under the same conditions. After 2-3 weeks embryogenic calli were cut in segments of approximately 2-3 mm and cultured plates containing CIM for 4 days. The *Agrobacterium* strains used in this study harbored a rifampicin selection marker in a C 58 chromosomal background. The construction of the helper strain EHA105 is described by Hood et al. (1993). *Agrobacteria* were grown for 4 days on plates containing AB medium with antibiotics (rifampicin 20 mg/l, kanamycin 100 mg/l). *Agrobacteria* were collected in LIM and the OD600 was adjusted till 1.0-1.5. This suspension was used for cocultivation. Calli were incubated for 10 minutes with the *Agrobacterium* suspension and dried on sterile filter paper to remove excess of bacteria. Calli were cultured for 48 h on Coculture Medium (COM) plates at 25°C in the dark. 50 pMOG1295 calli and 20 pMOG1253 calli were cultured per concentration of cyanamid. The following concentrations of cyanamide were used: 0, 15, 30, 60, 100, 150, 200, 300 and 500 mg/l. Hygromycin was applied in a concentration of 50 mg/l. Calli were incubated on First Selection Medium (FSM) plates containing several concentrations of cyanamide or hygromycin at 28°C in the dark. After 3 weeks the calli were transferred to Embryo Induction Medium I (EIM I) containing the same concentration of cyanamide or hygromycin. After another 3 weeks the calli were subcultured on Embryo Induction Medium II (EIM II) containing the same concentration of cyanamide or an increased concentration of hygromycin (75 mg/l). Calli were transferred to Shoot Induction Medium (SIM) containing the same concentration of cyanamide as during FSM, EIM I, EIM II and were cultured under 12 hours light period (2600 LUX) and 12 hours dark at 28 °C. Approximately 3 weeks after transferring calli to SIM, shoots were regenerating and excised and placed in jars containing Pre-Greenhouse Medium (PGM). No calli were formed at concentrations of 100 mg/l or higher of cyanamid. At 15 mg/l cyanamide the regeneration frequency of callus from both constructs was the same (pMOG1253 7 out of 16 calli were able to regenerate, pMOG1295 17 out of 44). At 30 mg/l cyanamide only 11 calli of pMOG1295 showed green callus development and 6 were able to be regenerated.

Table 5. Media required for *Oryza sativa* Taipei 309 transformation

Media components	Media	CIM	COM	LIM	FSM	EIM I	EIM II	SIM	PGM
Ingredients (g/l)	Macro elements	N6	R2	R2	R2	LS	LS	LS	1/2 MS
	Micro elements	B5	R2	R2	R2	LS	LS	LS	1/2 MS
	Vitamins	B5	R2	R2	R2	LS	LS	LS	1/2 B5
	Sucrose	30			30	30	30	40	10
	Glucose		10	10					
Hormones (mg/l)	Agarose Type I		7		7	7	7	7	
	Phytigel	2.5							2.5
	pH	5.8	5.2	5.2	6.0	5.8	5.8	5.8	5.8
	2,4-D	2.5	2.5	2.5	2.5	2.5	2.5		
	IAA								0.5
Additives	BAP								0.3
	NAA								0.05
	Proline (mg/l)	500							
	Glutamine (mg/l)	500							
	Casein Enzymatic Hydrolysate (mg/l)	300							
Antibiotics (mg/l)	Acetosyringone (μ M)		100	100					
	Cocosnutwater (ml)					100	100		
	Vancomycin				100	100	100	100	
	Cefotaxime				400	100	100	100	

Example 6Rice transformation by particle gun

Described below is the method used for transformation of non-morphogenic cell suspensions of *Oryza sativa* cv. IR 52 using a particle inflow gun (PIG) according to Finer et al. (Plant Cell Rep. 11, 323-328, 1992).

A long-term, non-morphogenic suspension culture of *Oryza sativa* cv. IR 52 was subcultured in weekly intervals in liquid LS-4 (Linsmaier and Skoog, Physiol. Plant. 18, 100-127, 1962) medium and maintained on a rotary shaker (110 rpm) at 28°C in the dark. (The contents of the LS-4 medium can be found in Table Z). 3 - 4 days after the last subculture 1.5 ml of this cell suspension (approx. 1.5×10^6 cells) were evenly spread on a filter paper (Whatman no 4) which was subsequently placed on solidified LS-4 medium and cultivated in the dark at 28°C for 24 h and directly used for bombardment thereafter. For microprojectile bombardment a home-made particle inflow gun (PIG) according to Finer et al. (1992) was used. 300 μ g tungsten particles

coated with either pMOG 617 (35S-gus and 35S-hyg) or pMOG 873 (35S-cah) were loaded on a particle support. The particles were accelerated by a 2.5 bar helium pulse and had to pass a 500 µm metal stop screen, placed 2 cm under the particle support. The suspension cells were placed 15 cm under the particle support. The FIG was evacuated to 30 mbar before bombardment. After bombardment the cells were cultured at 28°C in the dark for 3 days. Then the filters with the cells were transferred to solid LS-4 medium containing various concentrations of cyanamide or 50 mg/l hygromycinB (see table 6). The subculture was repeated every 9 days. Resistant microcalli that were visible after 4-6 weeks were transferred to fresh LS-4 medium containing the respective selective agent. From two experiments 7 + 41 calli transformed with pMOG617 were found resistant to hygromycin, while for the transformation with pMOG873 7 calli survived cyanamide 20 mg/l in the first experiment (results from the second experiment not yet available) and 0 + 4 calli remained viable on 40 mg/l cyanamide. No calli were formed at concentrations of 50 mg/l cyanamide or higher. The transgenic nature was confirmed by testing parts of the developing callus for the presence of the DNA in the callitransformed with pMOG873. One of the 4 surviving calli on 40 mg/l cyanamide showed positive in a PCR experiment on the cah-gene.

Table 6. Media required for *Oryza sativa* cv. IR 52 transformation

	LS-4 liquid	LS-4 solid
Macro elements	LS	LS
Micro elements	LS	LS
Vitamins	LS	LS
Sucrose (g/l)	30	30
Agarose Type I (g/l)	-	7
pH	5.8	5.8
2,4-D (mg/l)	4	4

Table 7. pMOG617

Selective agent (mg/l)	Number of plates	resistant clones	gus positive
Cyanamide 20	4		
Cyanamide 30	2		
Cyanamide 40	4		
Cyanamide 50	2		
Cyanamide 60	2		
Cyanamide 70	2		
Cyanamide 80	2		
Hygromycin 50	8		

pMOG 873

Selective agent (mg/l)	Number of plates	resistant clones	PCR positive
Cyanamide 0	4		
Cyanamide 20	6		
Cyanamide 30	8		
Cyanamide 40	8		
Cyanamide 50	7		
Cyanamide 60	4		
Cyanamide 70	4		
Cyanamide 80	2		
Hygromycin 50	4		

5

Example 8Maize killing curve

Stock solutions of cyanamide were prepared in water at 10 and 100 mg/ml and filter sterilised. Aliquots were stored at -20°C.

10

Media were prepared by adding MS medium (4.4g), sucrose (20g), 2,4-D (2.0mg) and agar (8g) to 1 litre of water. After autoclaving the appropriate amount of cyanamide (0, 10, 30, 50, 100, 150 mg/L cyanamide) was added and the media was poured into 9cm petri dishes. BMS liquid was prepared as above minus agar.

BMS cells were added to media containing cyanamide in three ways:

a. BMS cell suspension added to falcon tube and liquid removed. Then the BMS cells were arranged on the surface of the agar in clumps of approximately 5mm in diameter, 5 clumps per plate, 3 plates per concentration, while on the base of each petri dish the outline of each clump was marked;

b. Approximately 0.5ml pack cell volume plus 1.5ml BMS liquid added to surface of agar and the cells were spread finely over surface of agar. Three plates per treatment were set up.

c. Approximately 0.5ml pack cell volume plus 1.5ml BMS liquid were added to filter paper overlying agar. Cells were spread evenly over surface of filter. One plate per treatment was set up.

The plates were sealed with micropore tape and incubated at 25°C in the dark. The growth of the cells was observed after 7 and 14 days.

RESULTS

Day 8

The growth of BMS cells on cyanamide was assessed after 8 days.

The clumps of BMS cells arranged on the surface of the control media had increased in size and outgrown their original outline. Cells on 10 mg/l had not outgrown their outline but the height of the clumps had increased forming an uneven surface. A slight reduction of growth with increased cyanamide concentration was apparent with the maximum effect on growth observed at 50 mg/l cyanamid.

The cells which were spread over the surface of the control media had grown well and densely covered the surface of the media. A

significant reduction in growth was observed on the lowest level of cyanamide (10 mg/l), however, an increased cell density was clearly visible. A slight increase in cell density was evident on 30 mg/l cyanamid, but it was difficult to distinguish different growth rates on higher concentrations.

5

Cells on all levels of cyanamide remained a milky white colour, no browning of cells was observed.

Day 15 (Table 9)

10

The reduction in growth of BMS cells on 10mg/l was still very clear after 15 days on cyanamide, however, the cells arranged in clumps had outgrown their original outline. Cells spread directly over the surface of the agar showed a similar response to those arranged in clumps with a notable reduction in growth observed on 30mg/l plus.

15

Cells on 50mg/l plus showed no signs of growth and the surface of the clumps remained very flat but the cells were still milky white in colour. A similar response was observed with cell spread over a filter. However, small raised lumps were observed on the surface on all filters but these did not develop further into colonies and were evidently comprised of the larger cell aggregates from a mixed population of sizes typical in BMS suspensions.

20

Samples were taken from the clumps of cells on all levels of cyanamide for observation under a light microscope. With increasing levels of cyanamide there were increasing numbers of dead cells where the cell contents had shrunk away from the cell wall, and an increase in the number of dark bodied starch grains. Cells were observed resuspended in water and with FDA stain under UV light.

25

Table 9.

Cyanamide Level (mg/l)	Observations
0	Normal healthy cells with occasional dead cells.
10	Signs of stress. Odd clump of dead cells amongst healthy cells. Approx 5% dead.
30	Striking differences from control cells. Accumulation of starch grains. Dead cells with cell contents shrunk away from cell wall. Approx 15-20% dead.
50	Quite different to 30. Increasing number of dead cells and accumulation of starch grains. Approx 50% dead.
100	Majority of cell dead. Small clear spherical objects possibly oil droplets accumulating in cells. Cells decreasing in size. Approx 90% dead.
150	Majority of cells dead. Cells appear darker in colour due to accumulation of starch grains and phenols. Odd living cell visible in clump of dead cells. Approx 95-98% dead.

5 The experiment was repeated with cyanamide concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/l cyanamid. The results were similar to those described above, i.e. for the cells aggregated in clumps a slight reduction in growth was seen at 10 mg/l. From a concentration of 20 mg/l cyanamide on the cell clumps showed no
10 outgrowing from their original outline, but at the lower concentrations (<50 mg/l) the cell clumps showed an increase in height (decreasing with higher concentrations). Above 50 mg/l the clumps showed a slight orange tinge.

The results with cells spread over the surface of the agar or on the
15 filters were similar in that at a concentration of 10 mg/l showed a slight growth (approximately doubling of the number of original cells), while at concentrations of 20 mg/l and higher exhibited limited signs of growth.

Example 8Kill curves in banana (Musa)

To test the potential of cyanamide as a selection agent for transformation in banana two kill curves were set up with a regenerable embryogenic suspension of Grand nain 6 day old embryogenic suspension (Ed6b) cultures, subbed routinely in M2 2,4D liquid, containing 4.32 g/l MS salts, 45g/l sucrose, standard 1x concn. MS vitamins, 100mg/l glutamine, 100 mg/l myo-inositol, 100 mg/l biotin, 100mg/l malt extract at pH 5.3 and added after autoclaving 1.2mg/l 2,4-D and 0.8 mg/l picloram.

Cultures were sieved ($>250\mu$, $<710\mu$) and aliquots of approx 50 μ l sieved culture in 300 μ l volume of liquid were pipetted onto two kill curve media as detailed below (3 reps per plate). Culture growth and survival were monitored over the following 3 weeks and cell survival assessed after 21 days via FDA staining.

Kill Curve Medium A : M2/MS/1.0 2,4-D (as M2/MS/2,4-D except only 1.0 mg/l 2,4-D, no picloram and +2.25 g/l gelrite): this medium promotes the rapid division and growth of embryogenic callus, but not embryos.

Kill Curve Medium B : M2/SH/0.5Pic, 0.5 2,4-D (as M2/MS/2,4-D except only 0.5 mg/l 2,4-D and 0.5 mg/l picloram, SH salts (4.32g/l) instead of MS, + 2.25 g/l gelrite): This medium promotes the early development of embryos which can be matured and germinated by transfer to alternative media.

Cyanamide was added to both media types, after autoclaving, to concentrations of 0,20,30,50,75,100,150 mg/l.

The results are depicted in Table 10, where the figures on cell growth are approximate visual estimates, not precise measurements of callus volume. There is no significant visual browning of cultures and release of phenolics until concentrations of >75 mg/l. Generally cultures just stop growing, with cell division being widely inhibited. Cyanamide inhibits the growth of embryogenic callus by 40-

50% at even low concentrations of 20mg/l, without causing significant visual damage. Embryogenesis was totally inhibited at the lowest concentration tested here.

5 Table 10. Results of cyanamide concentrations on banana cell culture.

Medium	Cyanamide Concentra tion	% Live cells as assessed by FDA	Embryos Formed (average)	% Callus Growth
M2/MS/2,4-D	0	95%	N/A	+100%
M2/MS/2,4-D	20	60%	N/A	+30%
M2/MS/2,4-D	30	50%	N/A	+20%
M2/MS/2,4-D	50	20%	N/A	0
M2/MS/2,4-D	75	10%	N/A	0
M2/MS/2,4-D	100	10%	N/A	0
M2/MS/2,4-D	150	0	N/A	0
M2/SH/0.5Pic +0.5 2,4-D	0	95%	51	N/A
M2/SH/0.5Pic +0.5 2,4-D	20	50%	0	N/A
M2/SH/0.5Pic +0.5 2,4-D	30	40%	0	N/A
M2/SH/0.5Pic +0.5 2,4-D	50	30%	0	N/A
M2/SH/0.5Pic +0.5 2,4-D	75	20%	0	N/A
M2/SH/0.5Pic +0.5 2,4-D	100	10%	0	N/A
M2/SH/0.5Pic +0.5 2,4-D	150	0	0	N/A

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT:
 (A) NAME: MOGEN International nv
 (B) STREET: Einsteinweg 97
 (C) CITY: Leiden
 (E) COUNTRY: The Netherlands
 10 (F) POSTAL CODE (ZIP): 2333 CB
 (G) TELEPHONE: 31-(0)71-5258282
 (H) TELEFAX: 31-(0)71-5221471
- (ii) TITLE OF INVENTION: New selection marker
 15 (iii) NUMBER OF SEQUENCES: 4
- (iv) COMPUTER READABLE FORM:
 (A) MEDIUM TYPE: Floppy disk
 20 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

25 (2) INFORMATION FOR SEQIDNO: 1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 900 base pairs
 (B) TYPE: nucleic acid
 30 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- 35 (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 40 (A) ORGANISM: Myrothecium verrucaria
- (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 47..782
- 45 (xi) SEQUENCE DESCRIPTION: SEQIDNO: 1:

GTACGATCCT ACTTCCTCGC TTATCTGCTC TAAACGATTC AACAAG ATG TCT TCT
 55

50 Met Ser Ser
 1

TCA GAA GTC AAA GCC AAC GGA TGG ACT GCC GTT CCA GTC AGC GCA AAG
 103

55 Ser Glu Val Lys Ala Asn Gly Trp Thr Ala Val Pro Val Ser Ala Lys
 5 10 15

GCC ATT GTT GAC TCC CTG GGA AAG CTT GGT GAT GTC TCC TCA TAT TCT
 151

Ala Ile Val Asp Ser Leu Gly Lys Leu Gly Asp Val Ser Ser Tyr Ser
 20 25 30 35
 5 GTG GAA GAT ATC GCG TTC CCT GCG GCA GAC AAA CTT GTT GCC GAG GCA
 199
 Val Glu Asp Ile Ala Phe Pro Ala Ala Asp Lys Leu Val Ala Glu Ala
 40 45 50
 10 CAG GCC TTT GTG AAG GCC CGA TTG AGT CCC GAA ACC TAC AAT CAC TCC
 247
 Gln Ala Phe Val Lys Ala Arg Leu Ser Pro Glu Thr Tyr Asn His Ser
 55 60 65
 15 ATG CGC GTT TTC TAC TGG GGA ACC GTC ATC GCG AGA CGT TTA CTT CCC
 295
 Met Arg Val Phe Tyr Trp Gly Thr Val Ile Ala Arg Arg Leu Leu Pro
 70 75 80
 20 GAG CAA GCT AAA GAC TTG TCT CCA AGT ACA TGG GCA CTG ACA TGT CTT
 343
 Glu Gln Ala Lys Asp Leu Ser Pro Ser Thr Trp Ala Leu Thr Cys Leu
 85 90 95
 25 CTG CAT GAC GTT GGT ACT GCG GAG GCA TAC TTT ACA TCT ACA CGA ATG
 391
 Leu His Asp Val Gly Thr Ala Glu Ala Tyr Phe Thr Ser Thr Arg Met
 100 105 110 115
 30 TCC TTC GAT ATT TAC GGT GGC ATT AAG GCT ATG GAG GTG CTC AAG GTC
 439
 Ser Phe Asp Ile Tyr Gly Gly Ile Lys Ala Met Glu Val Leu Lys Val
 120 125 130
 35 CTT GGG AGT AGC ACC GAC CAG GCT GAG GCT GTT GCC GAG GCC ATC ATT
 487
 Leu Gly Ser Ser Thr Asp Gln Ala Glu Ala Val Ala Glu Ala Ile Ile
 135 140 145
 40 CGT CAT GAG GAT GTG GGG GTA GAT GGC AAC ATC ACA TTC CTC GGT CAG
 535
 Arg His Glu Asp Val Gly Val Asp Gly Asn Ile Thr Phe Leu Gly Gln
 150 155 160
 45 TTG ATC CAG CTG GCT ACG CTT TAT GAC AAT GTC GGG GCC TAC GAT GGG
 583
 Leu Ile Gln Leu Ala Thr Leu Tyr Asp Asn Val Gly Ala Tyr Asp Gly
 165 170 175
 50 ATT GAT GAT TTT GGT AGC TGG GTT GAT GAC ACC ACA CGC AAC AGT ATC
 631
 Ile Asp Asp Phe Gly Ser Trp Val Asp Asp Thr Thr Arg Asn Ser Ile
 180 185 190 195
 55 AAC ACG GCA TTC CCA CGA CAT GGT TGG TGT TCT TGG TTT GCC TGC ACG
 679
 Asn Thr Ala Phe Pro Arg His Gly Trp Cys Ser Trp Phe Ala Cys Thr
 200 205 210

GTT CGT AAG GAA GAA AGT AAC AAG CCT TGG TGC CAC ACA ACG CAT ATC
 727
 Val Arg Lys Glu Glu Ser Asn Lys Pro Trp Cys His Thr Thr His Ile
 215 220 225
 5
 CCT CAG TTC GAT AAA CAG ATG GAA GCG AAC ACT TTG ATG AAG CCT TGG
 775
 Pro Gln Phe Asp Lys Gln Met Glu Ala Asn Thr Leu Met Lys Pro Trp
 230 235 240
 10
 GAG TAA C TCTGAGTAAG CAGAGAATAT TTAGCCGGGT AGCTATAGAT GAATCTGGAC
 832
 Glu *
 245
 15
 AAATTCAGGC ACATTGGTT TCACGATACA GGTATTGGAA ATAGCTTGCA GGAAGGTATC
 892
 ATGTCAAC
 20 900

(2) INFORMATION FOR SEQIDNO: 2:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 245 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQIDNO: 2:

35 Met Ser Ser Ser Glu Val Lys Ala Asn Gly Trp Thr Ala Val Pro Val
 1 5 10 15
 Ser Ala Lys Ala Ile Val Asp Ser Leu Gly Lys Leu Gly Asp Val Ser
 20 25 30
 40 Ser Tyr Ser Val Glu Asp Ile Ala Phe Pro Ala Ala Asp Lys Leu Val
 35 40 45
 Ala Glu Ala Gln Ala Phe Val Lys Ala Arg Leu Ser Pro Glu Thr Tyr
 50 55 60
 45 Asn His Ser Met Arg Val Phe Tyr Trp Gly Thr Val Ile Ala Arg Arg
 65 70 75 80
 50 Leu Leu Pro Glu Gln Ala Lys Asp Leu Ser Pro Ser Thr Trp Ala Leu
 85 90 95
 Thr Cys Leu Leu His Asp Val Gly Thr Ala Glu Ala Tyr Phe Thr Ser
 100 105 110
 55 Thr Arg Met Ser Phe Asp Ile Tyr Gly Gly Ile Lys Ala Met Glu Val
 115 120 125
 Leu Lys Val Leu Gly Ser Ser Thr Asp Gln Ala Glu Ala Val Ala Glu
 130 135 140

Ala Ile Ile Arg His Glu Asp Val Gly Val Asp Gly Asn Ile Thr Phe
 145 150 155 160
 5 Leu Gly Gln Leu Ile Gln Leu Ala Thr Leu Tyr Asp Asn Val Gly Ala
 165 170 175
 Tyr Asp Gly Ile Asp Asp Phe Gly Ser Trp Val Asp Asp Thr Thr Arg
 180 185 190
 10 Asn Ser Ile Asn Thr Ala Phe Pro Arg His Gly Trp Cys Ser Trp Phe
 195 200 205
 Ala Cys Thr Val Arg Lys Glu Glu Ser Asn Lys Pro Trp Cys His Thr
 210 215 220
 15 Thr His Ile Pro Gln Phe Asp Lys Gln Met Glu Ala Asn Thr Leu Met
 225 230 235 240
 Lys Pro Trp Glu *
 20 245

(2) INFORMATION FOR SEQIDNO: 3:

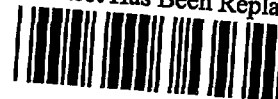
25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 43 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 30 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (xi) SEQUENCE DESCRIPTION: SEQIDNO: 3:
 35 ACCGAGCTCG AATTCGGCAC GAGGTTGACA TGATACCTTC CTG
 43

(2) INFORMATION FOR SEQIDNO: 4:

40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 42 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 45 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 50 (xi) SEQUENCE DESCRIPTION: SEQIDNO: 4:
 GACCTCGAGA ATTCGGCACG AGGTACGATC CTA CTTCTCCTC GC
 42

CLAIMS

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1. Use of cyanamide hydratase as a selection marker.
2. Use of cyanamide hydratase as a selection marker in plant transformation experiments.
3. Use of claim 2, characterised in that the plants are transformed with a nucleotide sequence coding for cyanamide hydratase.
4. Use of claim 3, characterised in that the nucleotide sequence as depicted in SEQIDNO:1 is used.
5. Method for the selection of transformed plants comprising:
 - a. constructing a vector comprising a coding sequence for cyanamide hydratase and a gene of interest
 - b. transforming said vector to plants or plant parts or plant cells, and
 - c. growing said transformants in a medium comprising cyanamide.
6. Method according to claim 5, characterized in that the nucleotide sequence of SEQIDNO:1 is used.
7. Use of cyanamide for the selection of plants transformed with a vector comprising a nucleotide sequence coding for cyanamide hydratase.
8. Expression cassette comprising a nucleotide sequence coding for cyanamide hydratase and a gene of interest.
9. Vector comprising an expression cassette according to claim 8.
10. Host cell comprising a vector according to claim 9.



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11. Host cell according to claim 10, according that it is *Agrobacterium*.

12. Plant transformed with a vector according to claim 9.

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13. Plant transformed by using a host cell wall according to claim 11.



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