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(54) PROCESS FOR SELECTION OF TRANSGENIC PLANT CELLS
VERFAHREN ZUR SELEKTION VON TRANSGENEN PFLANZENZELLEN
PROCEDE DE SELECTION DE CELLULES VEGETALES TRANSGENIQUES

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The present invention relates to genetically transformed plants, and more particularly to a process for the
selection of transformed plant cells cultured under heterotrophic conditions, a process for selectively increasing the
number of transformed plants regenerated from a mixture of transformed and non-transformed plant cells cultured
under heterotrophic culture conditions, as well as to transformed rooted plants and a kit for forming transformed plants.

Techniques for selection of transgenic plant cells that are used currently involve the utilization of prokaryotic
genes conferring resistance to toxic substances that are added to the regeneration medium, but that themselves are
not essential for cell growth. These toxic additives are either antibiotics, herbicides, amino acids or amino acid analogs added in toxic concentrations. Although generally effective in selecting genetically modified plant cells, these systems have limitations. For example, they may not be effective in some types of plants, or they may result in abnormal phenotypes of the genetically engineered plants. Additionally, the use of these selective agents can have undesirable side effects and thus have raised public concern about their environmental safety.


Additionally, there are negative aspects of antibiotic use, primarily, but not exclusively, due to their high toxicity against eukaryotic cells. For example, hygromycin has a broad spectrum of toxicity against prokaryotic and eukaryotic cells due to interference with protein synthesis [Waldron et al., Plant Mol. Biol., 5:103-108 (1985)]. Bleomycin is a cytotoxic drug used in human cancer therapy and has known pulmonary toxicity [Gatignol et al., FEBS Lett., 230: 171-175 (1988); Hille et al., Plant Mol. Biol., 7:171-176 (1986); Mulsant et al., Som. Cell Mol. Gen., 14:243-252 (1988)]. Many antibiotics are also potent allergens. Thus, extensive use of antibiotics in routine selections of transgenic plants can pose a health hazard to the research staff. It is also possible that upon lysis of transgenic plant cells, the DNA encoding for the antibiotic resistance may be taken up by bacteria, rendering them antibiotic-resistant and thereby a threat to the public health.

Herbicides such as chlorsulfuron, 2,4-D, glyphosate, phosphinotricin, and others, have been proposed as selective agents [De Block et al., EMBO J., 6:2513-2518 (1987); Dekeyser et al., Plant Phys., 90:217-223 (1989); Li et al., Plant Phys., 100:662-668 (1992); Sireber et al., Bio/Technology, 7:811-816 (1989); Shah et al., Science, 233: 478-481 (1986); White et al., Nucl. Acid Res., 18:1062 (1990)]. The use of herbicide resistance in identification of transgenic plants can result in increased weediness of transgenic plants because they can become herbicide-resistant weeds in the alternate years of crop rotation. Out-crossing to wild relatives is also a concern. If the mechanism of resistance to the herbicide is based on detoxification, there is a potential for one or more of its metabolites to be toxic (Stalker, et al., Science, 242:419-423 (1988)). Further, the use of herbicide-resistant crops probably will increase the herbicide load in the environment.

Certain amino acids such as lysine and threonine, or the lysine derivative amino ethyl cysteine (AEC), can also be used as selective agents due to their ability to inhibit cell growth when applied at high concentrations [Shafl et al., The Plant J., 2:203-209 (1992); Perl et al., Bio/Technology, 11:715-718 (1993)]. In this selection system, expression of the selectable marker gene, which permits the transgenic cells to grow under selection, results in overproduction of specific amino acids by transgenic cells, which counteracts the selective pressure. In some cases, this results in abnormal plant development [Shafl et al., The Plant J., 2:203-209 (1992)]. Moreover, transgenic plants that are selected in such a system have permanently altered amino acid composition, which may affect the nutritional value of the transgenic plants [Perl et al., Bio/Technology, 11:715-718 (1993)].

To be generally useful, a selectable marker must meet certain criteria. Selection must be stringent with a minimum of non-transformed plant tissue escaping the selection process. The selection should result in a large number of independent transformation events and not significantly interfere with regeneration. In addition, the marker should work well in a large number of plant species, and there should be an assay to score transformed tissue for confirmation that the marker gene is being expressed.

Another phenomenon associated with selectable markers is that once transformed plant cells are selected
by means of the marker and rooted plants are regenerated, the marker gene continues to be expressed in the mature, autotrophically-grown plants. Thus, as was noted before, a transgenic plant can permanently express an altered amino acid composition or a gene for antibiotic resistance. It would be beneficial if once selected and regenerated, the autotrophic rooted plants expressed the marker to a lessened extent or not at all.

The present invention provides a process for selecting transformed plant cells that meets the above criteria, while avoiding toxic or environmentally unsafe substances. The present invention also provides a process for selectively increasing the number of transformed plant cells regenerated from a mixture of transformed and non-transformed plant cells cultured under heterotrophic culture conditions. The present invention further provides a means by which expression of the selectable marker can be lessened or shut off after the selection process.

The present invention contemplates a process for selectively growing transformed plant cells cultured under heterotrophic conditions. Also contemplated is a process for selectively increasing the number of transformed plant cells regenerated from a mixture of transformed and non-transformed plant cells cultured under heterotrophic culture conditions. Still further contemplated is a method for increasing the number of transformed plant cells regenerated from a mixture of transformed and non-transformed plant cells under delayed heterotrophic culture conditions. Still even further contemplated are transformed plants whose genome contains an identifiable heterologous, exogenously supplied DNA segment that contains at least one expression cassette. A kit useful for transforming plant cells is also contemplated.

Thus, in one embodiment, a selection process for transformed plant cells is contemplated. In accordance with this process,

(a) a mixture of transformed and non-transformed plant cells is cultured under heterotrophic culture conditions in a culture medium that contains minimal nutrients required for growth and proliferation by those plant cells except for a source of carbon that is utilized to support that growth and proliferation. The source of carbon utilized is replaced by an encrypted or latent (growth-limiting) carbon source that does not support growth and proliferation by the non-transformed cells. The transformed cells of the mixture contain a genomic heterologous DNA segment that contains at least two expression cassettes.

The first expression cassette contains a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that on expression converts the encrypted carbon source into a carbon source that supports growth and proliferation by the transformed plant cells under heterotrophic culture conditions. The first gene is operatively linked to (ii) a first promoter DNA segment that controls expression of the heterologous gene, and (iii) a termination DNA segment. The second expression cassette contains (i) a second gene that is expressed in a transformed plant, and that gene is operatively linked to (ii) a second promoter DNA segment that controls expression of that second gene and (iii) a termination DNA segment.

(b) The heterotrophic culture conditions are maintained for a time period sufficient for the transformed plant cells to express the heterologous enzyme, proliferate and grow. Inasmuch as the non-transformed plant cells cannot utilize the encrypted or latent carbon source, those cells do not grow and proliferate. The transformed cells that do grow and proliferate can thereby be selected from the non-transformed cells.

A particularly preferred first gene encodes the enzyme phosphomannose isomerase (pmi; EC 5.3.1.8) that converts non-utilizable mannose-6-phosphate into fructose-6-phosphate that can be used by plant cells as a carbon source to support cell growth and proliferation. The pmi gene is also known as manA, and this gene is often referred to herein as pmi/manA. The encrypted (growth-limiting) carbon source useful with this first gene is mannose. Another preferred useful gene encodes mannitol-1-oxidoreductase that converts mannitol into mannose, and here, mannitol is the encrypted (growth-limiting) carbon source. This second gene and its encrypted carbon source are used in plant cells that have previously been transformed with a pmi/manA gene. Another preferred first gene encodes human L-iditol dehydrogenase (EC 1.1.1.14) that converts sorbitol into fructose, so that sorbitol is used as the encrypted (growth-limiting) carbon source. Similar aldehyde reductase enzyme genes can also be used.

The proliferating cells so produced and selected can thereafter be harvested or regenerated by culture in appropriate media into mature plants via meristematic tissue or embryos, or via callus tissue conversion into meristematic tissue or embryos. Thus, the selected proliferating cells are preferably collected and thereafter regenerated into mature plants that grow autotrophically. The above process therefore more preferably utilizes the added steps of:

(c) recovering the selected proliferating cells; and/or
(d) regenerating plants from those proliferating cells.

The promoter of the first expression cassette is repressed by a product of the normal autotrophic metabolism
of the transgenic plant, which product is also present in a non-transgenic plant. Exemplary preferred promoters include the cucumber isocitrate lyase promoter and the rice α-amylase Amy3A promoter.

[0013] The second gene and the genes in the subsequent cassettes can be any gene desired to be expressed in a plant, and its promoter and termination DNA segments can be any desired promoter and terminator that operate in plants.

[0014] In a second embodiment, a selection process for increasing the number of transformed plant cells from a mixture of transformed and non-transformed plant cells cultured under heterotrophic culture conditions is contemplated. In accordance with this process,

(a) a mixture of transformed and non-transformed plant cells is cultured under heterotrophic culture conditions in a culture medium that contains minimal nutrients required for proliferation and growth by non-transformed plant cells except for a source of carbon that supports growth and proliferation and about 1.5 to 3 times the standard amount of phosphorus. The source of carbon utilized is replaced by an encrypted carbon source that does not support growth and proliferation of said non-transformed cells. The transformed cells of the mixture have a heterologous DNA segment inserted into their genome that contains at least one expression cassette.

At least one expression cassette contains a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that on expression converts the encrypted carbon source into a carbon source that supports growth and proliferation by the transformed plant cells under heterotrophic culture conditions. The first gene is operatively linked to (ii) a first promoter DNA segment that controls expression of the heterologous gene, and (iii) a termination DNA segment.

(b) The heterotrophic culture conditions are maintained for a time period sufficient for the transformed plant cells to express the heterologous enzyme, proliferate and grow.

[0015] In a third embodiment, a selection process for increasing the number of transformed plant cells from a mixture of transformed and non-transformed plant cells cultured under delayed selective culture conditions is contemplated. In accordance with this process,

(a) a mixture of transformed and non-transformed plant cells is cultured for up to two weeks in a first culture medium that contains the minimal nutrients required for proliferation and growth by both, transformed and non-transformed plant cells including a source of carbon that supports growth and proliferation of both the transformed and non-transformed plant cells. The transformed plant cells have a heterologous genomic DNA segment that contains at least one expression cassette.

At least one expression cassette contains a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that on expression converts an encrypted carbon source into a carbon source that supports growth and proliferation of said transformed plant cells under heterotrophic culture conditions, said first gene being operatively linked to (ii) a first promoter DNA segment that controls expression of said heterologous gene, and (iii) a termination DNA segment.

(b) After sufficient time in the first medium, the transformed and non-transformed plant cells are removed from the first culture medium.

(c) The transformed and non-transformed plant cells are then placed under heterotrophic culture conditions in a second culture medium that contains the minimal nutrients required for proliferation and growth of the non-transformed plant cells except for an encrypted carbon source that does not support growth and proliferation of said non-transformed plant cells and 1.5 to 3 times the standard amount of phosphorous.

(d) The heterotrophic culture conditions are maintained for a time period sufficient for said transformed plant cells to express said heterologous enzyme, proliferate and grow.

[0016] A transgenic plant whose genome comprises a heterologous DNA segment that contains at least two expression cassettes is further contemplated.

[0017] The first expression cassette contains a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that on expression during heterotrophic culture of cells from the transformed plant converts an encrypted carbon source that does not support growth and proliferation of non-transformed plant cells of the same type into a carbon source that supports growth and proliferation of those transformed cells. That first gene is operatively linked to (ii) a promoter DNA segment that controls expression of the heterologous gene and (iii) a termination DNA segment. The second expression cassette contains (i) a second gene that is expressed in the transformed plant that is operatively linked to (ii) a second promoter DNA segment that controls expression of the second gene and (iii) a termination DNA segment.

[0018] The before-noted preferences are also followed for the first gene and its promoter in the transgenic plant. The second gene and its promoter are also as discussed before.
A kit for forming transformed plant cells is also contemplated. That kit comprises:

(a) a first package containing a DNA segment for transforming plant cells that contains an expression cassette operatively linked to a linker segment containing at least one restriction endonuclease site. The expression cassette contains a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that on expression during heterotrophic culture of transformed plant cells converts an encrypted carbon source that does not support growth and proliferation of non-transformed plant cells into a carbon source that supports growth and proliferation the transformed cells. The first heterologous gene is operatively linked to (ii) a promoter DNA segment that controls expression of the heterologous gene and (iii) a termination DNA segment.

(b) a second package is also present that contains minimal nutrients required for growth and proliferation of plant cells during heterotrophic culture except for a source of carbon and about 1.5 to 3 times the standard amount of phosphorous. That source of carbon is replaced by an encrypted carbon source that does not support growth and proliferation of non-transformed plant cells but does support growth and proliferation of a transformed plant cell whose genome contains the DNA segment of the first package. Instructions for use of the kit components are also preferably provided.

The present invention has several benefits and advantages.

One benefit of the invention is that its selective growth process does not rely upon potentially harmful antibiotics, herbicides or other possibly toxic materials.

One advantage of the invention is that its encrypted (growth-limiting) carbon source can be and preferably is a carbohydrate.

Another benefit of the invention is that expression of the selectable marker can be repressed in the regenerated plant under autotrophic growth conditions.

Another advantage of the invention is that the selectable marker gene can be used with any second expressed gene.

Still another benefit of the invention is that the kit provides a ready means for inserting a second expression cassette into a plant transforming vector and an appropriate selection medium for the enhanced transformation and selected growth of transformed plant cells.

Still another advantage is that successive transformations can be made in which one encrypted (growth-limiting) carbohydrate can be converted by a second selectable marker gene into another encrypted (growth-limiting) carbohydrate that is converted into a useful carbon source by a first selectable marker gene also present in the transformed cells.

Still further benefits and advantages of the present invention will be apparent to the skilled worker from the disclosure that follows.

Brief Description of the Sequence Listing

SEQ ID NO:1 is a sequence listing for the 1650 base pair ClaI fragment that contains the Salmonella typhimurium phosphomannose isomerase (pmi) gene.

SEQ ID NO:2 is the amino acid sequence for pmi.

SEQ ID NO:3 is the sequence of one of the primers used to facilitate the cloning of the pmi gene into a plant transformation vector. The primer corresponds to bases 391 to 410 of SEQ ID NO:1 with a substitution of an adenine for a thymine at base 403.

SEQ ID NO:4 is the sequence of the other primer used to facilitate the cloning of the pmi gene into a plant transformation vector. The primer corresponds to a complementary sequence to bases 1617 to 1641 of SEQ ID NO:1, with the substitution of a cytosine for a guanine at base 1628 and a cytosine for thymine at base 1630.

Brief Description of the Drawings

Fig. 1 shows the relationship between mannitol and sucrose biosynthesis in plants. Circled numbers identify enzymes that catalyze the reaction shown as follows: (1) mannitol-1-phosphate phosphatase, (2) mannose kinase, (3) mannose-6-phosphate reductase, (4) mannose-6-phosphate isomerase, and (5) sucrose phosphate synthase. Fig. 2 illustrates the construction of plasmid vector pETO148. The recombinant DNAs manipulated and produced
by the construction process are indicated in the figure by the circles. The construction proceeds by a series of steps as indicated by the arrows connecting the circles in the figure and as described in detail herein. Landmark and utilized restriction enzyme recognition sites are indicated on the circles by labeled lines intersecting the circles. The relative location of individual genetic elements and their orientation are indicated by the labeled arrows inside the circles.

Fig. 3 shows the relative NPTII expression in populations of plants selected on kanamycin and mannose with plants regenerated with no selection. NPTII expression was determined by an ELISA, and the units of expression are absorbance values at 405 nM. Data for non-transformed plants (negative control) are shown as closed circles. Data for use of kanamycin (positive control) for selection of transformed plants are shown as closed triangles. Data for the transformed plants selected on mannose are shown as closed squares.

Detailed Description of the Invention

A. Definition of Terms

Amino Acid: All amino acid residues identified herein are in the natural L-configuration.

Autologous: A DNA segment or protein normally present in a non-transformed cell.

Explant: A piece of plant tissue.

Expression: The combination of intracellular processes, including transcription and translation undergone by a protein-encoding gene to produce a polypeptide.

Expression cassette: A DNA segment construct comprising a gene to be expressed operatively linked to a promoter DNA and to a DNA termination segment and sequences sufficient for translation, as well as any other regulatory signals needed to effect proper processing of the expression product.

Expression vector: A DNA sequence that forms control elements that regulate expression of a desired gene when operatively linked to that gene within a vector. An expression vector of particular interest also contains DNA segments that permit integration into the plant genome.

Gene: A sequence of nucleotides in the genome of an organism to which a specific function can be ascribed.

Heterologous: A DNA segment or protein not present in a non-transformed cell.

Integrated: A heterologous DNA sequence incorporated into a host chromosome is integrated.

Operatively linked or inserted: A first DNA sequence such as a promoter DNA sequence is operatively linked to a second DNA sequence such as a heterologous gene DNA sequence if the two are situated so that the promoter DNA sequence influences the transcription or translation of the heterologous gene DNA sequence.

Promoter: A recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

Protein-encoding gene: A DNA sequence that through an RNA intermediate encodes for a polypeptide; i.e., an amino acid residue sequence.

Recombinant DNA molecule: A hybrid DNA sequence comprising at least two nucleotide sequences not that are created in vitro.

Same type or same strain plant: A plant of the same cross as or a clone of the untransformed plant.

Transgenic plant: A plant that contains chromosomally integrated heterologous or foreign DNA whose expression may be constitutive or regulatable.

Vector: A DNA molecule capable of replication in a cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A plasmid is an exemplary vector.

B. Introduction

Higher plants (plants) in their adult (mature) forms are autotrophic organisms. Thus, mature plants use carbon dioxide for most of their carbon requirements. When grown from seed, the germinating seedling initially uses part of the nutrient rich seed to supply its carbon requirements until it is capable of carbon fixation. Similarly, when plant cells are grown in a culture medium, as cell suspensions, microspores, protoplasts or as explants (collectively referred to as plant cells in that each is from a plant and has a nucleus and cytoplasm surrounded by a membrane), the cultured plant cells are heterotrophic and require the provision of an external source of carbon as well as other nutrients. That carbon source is typically sucrose, glucose or another carbohydrate that can be readily metabolized by the growing cells so that they can grow, proliferate and differentiate, and then be regenerated into mature plants.

However, not all sources of carbon, including some carbohydrates, can be metabolized by cultured plant cells.
Cultured plants do not possess all of the necessary enzymes needed to convert many simple organic molecules into sources of carbon that can support growth and proliferation under tissue culture conditions.

In some cases, a gene that is functionally similar to the first gene of the first expression cassette is present in the genome of the non-transformed plant. That endogenous gene is, however, functionally not present in the heterotrophically cultured plant cells inasmuch as the non-transformed plant cells do not grow and proliferate using the same encrypted carbon source that the heterologous gene product of that first gene converts into a useful source of carbon. This carbon source is encrypted (in code) because the plant cells cannot use it to grow and proliferate, and it is only after the selectable marker gene product that is present in the transformed callus decodes (decrypts) or acts upon that carbon source does it become useful to support growth and proliferation in the transformed plant cells.

The present invention utilizes the fact that plant cells cannot grow and proliferate using many small, carbon-containing compounds as a source of carbon during heterotrophic culture as a means of selectively growing transformed (genetically engineered) plant cells. This is accomplished, inter alia, by using a marker gene for cell transformation that converts a source of carbon that does not support cell growth and proliferation (a non-useful carbon source) into a carbon source that supports cell growth and proliferation (a useful carbon source).

In one embodiment, a selection process for transformed plant cells is contemplated. In accordance with this process,

(a) a mixture of transformed and non-transformed plant cells is cultured under heterotrophic culture conditions in a culture medium that contains minimal nutrients required for proliferation and differentiation by those plant cells except for a useful source of carbon. The normally present useful source of carbon is replaced by an encrypted, latent or growth-limiting carbon source, only those plant cells grow and proliferate that are transformed with a selectable marker gene whose expressed product converts the encrypted carbon source into a useful source of carbon. This carbon source is encrypted (in code) because the plant cells cannot use it to grow and proliferate, and it is only after the selectable marker gene product that is present in the transformed callus decodes (decrypts) or acts upon that carbon source does it become useful to support growth and proliferation in the transformed plant cells.

(b) The heterotrophic culture conditions are maintained for a time period sufficient for the transformed plant cells to express the heterologous enzyme, grow, and proliferate. The non-transformed plant cells do not utilize the encrypted or latent carbon source and do not grow and proliferate as well as the transformed cells. The transformed cells that do grow and proliferate are thereby selected away from the non-transformed cells, and are preferably collected for regeneration into plants.

The transformed cells of the mixture contain a genomic (chromosomally integrated) heterologous DNA segment that contains two expression cassettes.

The first expression cassette contains a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that on expression converts the encrypted carbon source into a useful carbon source that supports growth and proliferation of the transformed plant cells under heterotrophic culture conditions. That first heterologous gene is operatively linked to (ii) a first promoter DNA segment that controls expression of the heterologous gene, and (iii) a termination DNA segment. The second expression cassette contains (i) a second heterologous gene that is expressed in a transformed plant, and that second heterologous gene is operatively linked to (ii) a second promoter DNA segment that controls expression of the second heterologous gene and (iii) a termination DNA segment.

The first expression cassette DNA thus contains three operatively linked elements: (1) a first heterologous gene that encodes a heterologous enzyme that converts an encrypted carbon source into a useful carbon source, (2) a first promoter DNA segment that controls the expression of the heterologous gene, and (3) a first termination DNA segment that causes termination of transcription and polyadenylation of the 3'-end of the translated RNA.

In some cases, a gene that is functionally similar to the first gene of the first expression cassette is present in a mixture of transformed and non-transformed plant cells. This concept is exemplified by the fact that tomato plants do not grow and proliferate when heterotrophically cultured using either lactose or salicin as carbon sources. Lactose is susceptible to cleavage by a β-galactosidase into glucose and galactose, whereas salicin can be cleaved by a β-glucosidase to form glucose. Nonetheless, one commercially available β-galactosidase is recovered from jack beans, whereas a commercially available β-galactosidase is recovered from sweet almonds (Sigma Chemical Co.), indicating that the plant genomes contain genes for each
enzyme that are not sufficiently expressed at all times.

[0039] The choice of the first gene depends upon its encoded enzyme and the function of that enzyme, which, in this invention, is to convert an encrypted, non-useful carbon source into a useful carbon source that supports the growth and proliferation of heterotropically-cultured transgenic plant cells. Continuing this choice of the first gene backwards, one assays carbon sources that the plant cells to be transformed cannot utilize to grow and proliferate.

[0040] Such an assay is readily carried out by culturing the desired non-transformed plant cells in a medium that contains minimal nutrients required for cell growth and proliferation except for a source of carbon. Such media are well known in the art and are exemplified by the well known medium of Murashigi and Skoog, Physiol. Plant. 15:437-498 (1962); MS salts. The glucose or sucrose usually present in that medium is replaced with the carbon source to be assayed that is present at about the same concentration as was the glucose or sucrose.

[0041] The plant cells are then cultured in the resulting medium following usual procedures. An assayed carbon source that does not support growth and proliferation of the heterotropically cultured cells is determined. Exemplary carbon sources that do not support the growth and proliferation of the illustrative tomato cells used herein include mannose, mannotol, sorbitol, lactose, trehalose and salicin. In addition, oat, maize, melon and squash cells are shown not to grow and proliferate using mannose as the sole carbon source.

[0042] An enzyme is then selected that converts the non-utilizable carbon source into a carbon source that can be utilized by the plants. Exemplary sources of carbon that can be used by plant cells to support growth and proliferation are the compounds of the Calvin cycle and include glucose, fructose, ribulose and glyceral acid, as well as the compounds of the Hatch-Slack pathway that include oxaloacetate, malate and pyruvate. Thus, supplying the heterotropically cultured plant cells with one of the before-mentioned carbon sources and the other required minimal nutrients of the medium results in growth and proliferation of the plant cells in that the plant cells contain endogenous enzymes that utilize such compounds for growth and proliferation.

[0043] Inasmuch as living organisms all utilize carbon sources for growth and proliferation, many such enzymes are available for use. An enzyme that converts a non-useful carbon source to a useful 6-carbon source such as glucose or fructose is preferred, and such enzymes are utilized illustratively herein.

[0044] One preferred enzyme used illustratively herein is phosphomannose isomerase (pmi/manA; E.C. 5.3.1.8) that converts mannose-6-phosphate into fructose-6-phosphate; the latter compound that supports plant cell growth and proliferation. When mannose is used as the encrypted carbon source, mannose-6-phosphate is formed in the cell by the enzymatic action of an endogenous kinase. The phosphomannose isomerase gene is designated as the pmi gene in Rhizobium mellioti (Gene, 122: 35-43), Pseudomonas aeruginosa (Gene, 42:293-302), Saccharomyces cerevisiae (Molecular and Cellular Biology, 12:2924-2930) and Salmonella typhimurium (Gene, 103:135-136). In Escherichia coli, the phosphomannose isomerase gene is called manA (Gene, 32:41-48).

[0045] Another useful enzyme is L-iditol dehydrogenase (EC 1.1.1.14) that converts sorbitol into fructose. Several aldehydoreductase enzymes are known that can be used to convert sorbitol as encrypted carbon source into the useful carbon source glucose. One exemplary enzyme is D-sorbitol 1-oxidoreductase (EC 1.1.0.24). Lactase (EC 3.2.1.108) that converts the encrypted carbon source lactose into glucose and galactose, as do several other β-galactosidases, and α,α-trehalase (EC 3.2.1.28) that converts the encrypted carbon source α,α-trehalose into glucose are yet other useful enzymes.

[0046] Upon determination of the heterologous marker enzyme for use, the gene that encodes that enzyme is obtained. The genes that encode many heterologous marker enzymes have been reported in the literature and can be obtained from the authors, as was the case of the pmi gene used here. Many useful gene sequences are also reported in the GenBank database such as the human L-iditol dehydrogenase gene (GenBank accession number L29008) or the rat aldehyde dehydrogenase genes (GenBank accession numbers X74673 and D10484) or the human aldehyde reductase gene (GenBank accession number J04794). Sequences for several useful β-galactosidases are also available in the GenBank data base. For example, the enzyme from Brassica oleracea has accession Number X84684, that of the Granny Smith apple (Malus domestica) has accession Number L29451, and three genes from E. coli have the accession Numbers X03228, M13700 and M13797. The sequence of the S. typhimurium pmi gene used illustratively here has GenBank accession Number X57117.

[0047] Where the desired gene is unavailable, but its sequence is known, PCR technology can be used with a DNA or RNA prepared from a source reported or otherwise known to contain the gene to obtain a useful copy of that gene.

[0048] Where a sequence of a desired gene is not available, the enzyme itself is obtained following usual procedures, and its termini are sequenced sufficiently so that sets of redundant DNA probes can be prepared and used with PCR technology to obtain a DNA copy of the gene from a DNA or RNA library of the organism from which the enzyme was isolated.

[0049] The preparation and use of DNA and RNA libraries for obtaining desired genes from host organisms is well known in this art and will not be dealt with further herein.

[0050] The DNA segment encoding the heterologous selectable marker enzyme (first gene) is typically isolated and purified containing appropriate endonuclease restriction sites at the 5' - and 3'-termini so that that gene can be oper-
An above-described first gene is operatively linked to a promoter DNA segment. That promoter is a segment that is operative in plant cells. Exemplary useful promoters include the constitutive CaMV 35S promoter of the cauliflower mosaic virus, the octopine synthase promoter (P-Ocs) and the nopaline synthase promoter (P-Nos) that are constitutive promoters.

In one embodiment, the promoter utilized is repressed by a product of the normal metabolism of the transgenic (or non-transgenic) plant cultured under autotrophic growth conditions. Thus, once a mature plant has been regenerated, and selection is no longer necessary, the promoter controlling expression of the marker enzyme is shut off or repressed so that none or a reduced amount of the selecting enzyme is expressed.

An exemplary promoter whose activity is repressed by a normal product of plant metabolism is the rice α-amylase Amy3A promoter [Thomas et al., Plant Physiol., 106:1235-1239(1994)]. Expression from this α-amylase promoter in rice is repressed by the presence of sucrose, a product of the normal, autotrophic, metabolism of both transformed and non-transformed rice cells.

Two additional promoters that are repressible by normal products of plant metabolism are the cucumber malate synthase and isocitrate lyase promoters reported in Graham et al., Plant Cell, 6: 761-772 (1994). Glucose, fructose and raffinose in culture media repressed both promoters. Inasmuch as the malate synthase promoter is repressed when plant cells are in the presence of mannose, that promoter is not utilized in conjunction with the pmu/manA gene and mannose as the first encrypted carbon source.

The final element of the first cassette is a first termination DNA segment that is operatively linked to the 3'-end of the first gene. Several termination segments useful in plants are well known and can be used herein. One exemplary segment is the 3'-non-translated region of the nopaline synthase gene [Nos-T; Fraley et al., Proc. Natl. Acad. Sci. USA, 80:4803-4807(1983)] used herein. Another is the 3'-non-translated region of the pea rbcS-E9 gene [E9; Coruzzi et al., Embo J., 3:1671-1679 (1984)].

The promoter and termination DNA segments are also preferably terminated by appropriate endonuclease restriction sites for ligation and operable linkage to the termini of the first gene and ligation to the second expression cassette as part of the genomic heterologous DNA segment, and also into an appropriate vector. Blunt end ligation can also be used for operative linking to the first gene and/or a vector.

The second expression cassette contains a second gene that is expressed in a transformed plant. That second or target gene encodes for the expression of a protein in a transformed plant.

The second gene can be any gene that is desired. Exemplary genes include those listed in Table I, below, whose transformations into plants have been disclosed in the patent citations shown in that Table.

<table>
<thead>
<tr>
<th>Second Gene Product</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>U.S. 5,202,422</td>
</tr>
<tr>
<td>Mammalian Peptide</td>
<td>WO 87/00865</td>
</tr>
<tr>
<td>HMG-CoA Reductase</td>
<td>U.S. 5,306,862</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>U.S. 5,387,756</td>
</tr>
<tr>
<td>Waxy Locus of Wheat (Antisense)</td>
<td>U.S. 5,365,016</td>
</tr>
<tr>
<td>ADP-Glucose pyrophosphorylase, antisense</td>
<td>EP 0 368 506 A2</td>
</tr>
<tr>
<td></td>
<td>EP 0 455 316 A2</td>
</tr>
<tr>
<td>Potato L-amylase</td>
<td>WO 92/11382</td>
</tr>
<tr>
<td>Sucrose phosphate Synthase</td>
<td>EP 0 470 145 B1</td>
</tr>
<tr>
<td></td>
<td>EP 0 466 995 A2</td>
</tr>
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<td></td>
<td>EP 0 530 978</td>
</tr>
<tr>
<td>E. coli inorganic pyrophosphorylase</td>
<td>EP 0 485 044 A2</td>
</tr>
<tr>
<td>Maize 1,4-α-glucan branching enzyme, antisense</td>
<td>WO 92/11375</td>
</tr>
<tr>
<td>Granule-bound starch synthase, antisense</td>
<td>WO 92/11376</td>
</tr>
<tr>
<td>Tomato vacuolar invertase, antisense</td>
<td>WO 92/14831</td>
</tr>
<tr>
<td>E. Herbicola genes in the carotenoid synthetic pathway</td>
<td>WO 91/13078</td>
</tr>
</tbody>
</table>

The second gene is operatively linked to a second promoter DNA segment that controls expression of the
second gene. This promoter is not repressed by a product of normal plant metabolism, and can be a constitutive promoter such as the CaMV 35S, P-Ocs and P-Nos promoters discussed before, or an organ-enhanced promoter that causes expression in one or more organs of the transformed plant.

[0060] Expression in one or more preselected organs with little or no expression in other organs such as roots versus leaves or stems is referred to herein as enhanced or preferential expression.

[0061] The final element in the second expression cassette is a termination DNA segment. Any termination segment can be used, as was discussed before in regard to the first cassette.

[0062] The elements of the second expression cassette are operatively linked together, usually via ligatedendonuclease sites, although blunt end ligation can also be utilized, as before.

[0063] It is also noted that either or both of the heterologous enzyme encoded by the first gene and the product of the second gene can be portions of a polyprotein that actually contains two or more proteins, e.g. enzymes, linked together by a peptide bond as has been found for several viral genes. See, for example, Vardi et al., Proc. Natl. Acad. Sci. USA, 90:7413-7417 (1993) and Maiti et al., Proc. Natl. Acad. USA, 90:6110-6114 (1993).

[0064] The first and second expression cassettes are linked together in the genomic heterologous DNA segment so that the two expression cassettes constitute very tightly linked loci that are transformed into the plant's chromosomal DNA. Thus, as is the case with other selectable marker systems, transformation of plant cells with one of the cassettes is very strongly correlated with transformation with the other. In this instance, growth and proliferation of cells that were putatively transformed indicates that the transformation with the second cassette was successful.

[0065] Use of the DNA segment containing the two linked expression cassettes to transform plant cells is discussed in detail hereinafter.

[0066] In some instances, it is desirable to transform plant cells on two separate occasions. Such double transformations require the sequential use of two selectable markers and two target genes. In the terminology used herein, such a double transformation utilizes four expression cassettes and two encrypted (growth-limiting) sources of carbon for heterotrophic growth. Two heterologous genomic DNA segments are present in the twice-transformed plants.

[0067] For ease of discussion as to this embodiment, the first and second expression cassettes are those already discussed, and those expression cassettes are present in the first-named genomic heterologous DNA segments. A second genomic heterologous DNA segment is also present in a twice-transformed plant and contains a third and a fourth expression cassettes.

[0068] The third expression cassette contains a second heterologous DNA selectable marker segment that includes (i) a second heterologous gene that encodes a second heterologous enzyme that is operatively linked to (ii) a third promoter DNA sequence that controls the expression of the second heterologous gene and (iii) a termination DNA segment.

[0069] The third promoter DNA can be the same promoter used with the first gene that encodes a heterologous enzyme. The termination DNA segment is also any termination segment useful in plants as is also discussed before. The third promoter and this termination DNA segment are linked as discussed previously.

[0070] The second gene that encodes a second heterologous enzyme is different from those genes discussed before. The second heterologous enzyme converts a second encrypted carbon source into the first-named encrypted carbon source that supports growth and proliferation of the transformed plant cells. That second encrypted carbon source also does not support growth and proliferation of non-transformed plant cells of the same type.

[0071] Thus, the second encrypted carbon source can be viewed as a precursor to the first-named encrypted carbon source that is converted into a useful source of carbon to support cell growth and proliferation by the first heterologous gene. More specifically, where mannose is illustratively the first encrypted carbon source and phosphomannose isomerase is the first heterologous gene, mannitol can be used as the second encrypted carbon source along with mannitol 1-oxidoreductase as the second heterologous gene.

[0072] Thus, plant cells transformed with a first and second expression cassette containing the pmi/manA gene as a first gene selectable marker are transformed again with third and fourth expression cassettes whose third expression cassette contains a gene that encodes the mannitol 1-oxidoreductase gene and whose fourth cassette contains another desired gene. The resulting mixture of twice-transformed and once-transformed plant cells is heterotrophically cultured in a minimal nutrient medium that contains mannitol as the carbon source. Inasmuch as only the twice transformed cells contain a gene encoding mannitol 1-oxidoreductase and phosphomannose isomerase, only those cells grow and proliferate because they convert mannitol to mannose and mannose into fructose-6-phosphate.

[0073] Those proliferating twice-transformed cells are preferably recovered and regenerated into mature plants as discussed elsewhere.

[0074] It is noted that reference herein to "first", "second", "third" and "fourth" is for convenience only. Thus, the second cassette can be upstream of the first cassette, as can the fourth cassette be upstream of the third cassette.

[0075] In a second embodiment, a process for selectively increasing the number of transformed plant cells regenerated from a mixture of transformed and non-transformed plant cells cultured under heterotrophic culture conditions is contemplated. In accordance with this process,
(a) a mixture of transformed and non-transformed plant cells is cultured under heterotrophic culture conditions in a culture medium that contains standard nutrients required for proliferation and differentiation by those plant cells except for a useful source of carbon and 1.5 to 3 times the standard amount of phosphorus. The normally present useful source of carbon is replaced by an encrypted, latent or growth-limiting carbon source that does not support growth and proliferation of the non-transformed cells. The transformed cells of the mixture contain a genomic (chromosomally integrated) heterologous DNA segment that contains at least one expression cassette.

At least one expression cassette contains a heterologous DNA selectable marker segment that includes (i) a heterologous gene that encodes a heterologous enzyme that on expression converts the encrypted carbon source into a useful carbon source that supports growth and proliferation of the transformed plant cells under heterotrophic culture conditions. The gene is operatively linked to (ii) a first promoter DNA segment that controls expression of the heterologous gene, and (iii) a termination DNA segment. This expression cassette may be identical to the first expression cassette described earlier as the first embodiment of this invention. Therefore, the previous descriptions relating to the first expression cassette may be applicable to the expression cassette used in the second embodiment of this invention.

The heterologous DNA segment must contain at least the expression cassette described above. Optionally, the heterologous DNA segment may contain additional expression cassettes which contain any gene(s) that are expressed in transformed plants. These expression cassettes may be identical to the second through fourth expression cassettes described above with respect to the first embodiment of this invention. Therefore, the previous descriptions relating to these expression cassettes may be applicable in the second embodiment of this invention.

(b) The heterotrophic culture conditions are maintained for a time period sufficient for the transformed plant cells to express the heterologous enzyme, proliferate and grow.

[0076] The second embodiment of the invention utilizes the discovery that when additional phosphorous is added to the culture medium containing the minimal nutrients required for growth and proliferation of the non-transformed plant cells except for an encrypted or latent carbon source that does not support growth and proliferation of the non-transformed plant cells, that there is an increase in the quantity and quality of transformed plant cells regenerated.

[0077] "The standard amount of phosphorous" as used herein refers to the amount or level of phosphorous contained in the commonly used Murashige and Skoog culture medium which is frequently used in this art. The Murashige and Skoog culture medium typically contains 170.00 mg/L of phosphorous such as potassium phosphate monobasic. Nonetheless, it is well known in the art that the Murashige and Skoog culture medium can be modified to meet certain requirements. Generally, when the Murashige and Skoog medium is modified, these modifications are in the composition of microelements, vitamins or inclusion of other additives. An example of a modified Murashige and Skoog medium in which only the vitamins are altered is the Linsmaier and Skoog medium (described in Physiologia Plantarum 18: 100-127 (1965). This medium contains 170.00 mg/L of phosphorous.

[0078] In this invention, the inventors have discovered that when the culture medium that contains the encrypted or latent carbon source also contains 1.5 to about 3 times the standard amount of phosphorous normally contained in said medium that an enhanced number of regenerated transformed plant cells can be obtained. Additionally, among those regenerates, a higher percentage of normal shoots can be recovered as well.

[0079] While not wishing to be bound by any theory, the inventors believe that the reason why transformation experiments on the growth medium containing the encrypted or latent carbon source with supplemented levels of phosphates are more successful than experiments on the growth medium containing only the encrypted carbon source is because that when the growth medium contains the additional levels of phosphate the transformed plant cells have at least two growth advantages over the non-transformed plant cells. The first advantage is that the transformed plant cells can access additional carbon from the medium. The second advantage is that the phosphate confers an additional growth advantage to transformed cells capable of growing on the limited source of carbon over the non-transformed plant cells which are not capable of growing on such a limited carbon source.

[0080] The phosphorous added to the culture medium can be any water soluble phosphate salt. For example, potassium phosphate (monobasic, dibasic and tribasic), sodium phosphate (monobasic, and dibasic) calcium phosphate (monobasic, dibasic and tribasic), sodium hexameta-phosphate, ammonium phosphate (monobasic and dibasic), orthophosphoric acid, ferric phosphate, sodium glycerophosphate, ferric glycerophosphate and organic phosphorous (such as ATP, ADP, etc) can be used.

[0081] In a third embodiment, a process for selectively increasing the number of transformed plant cells regenerated from a mixture of transformed and non-transformed plant cells cultured under delayed heterotrophic culture conditions is contemplated. In accordance with this process,

(a) a mixture of transformed and non-transformed plant cells is cultured for up to two weeks in a first culture medium that contains the minimal nutrients required for proliferation and growth by non-transformed plant cells including a source of carbon that supports growth and proliferation. The transformed plants cells containing a genomic
heterologous DNA segment that contains at least one expression cassette.

At least one expression cassette contains a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that on expression converts an encrypted carbon source into a carbon source that supports growth and proliferation of said transformed plant cells under heterotrophic culture conditions, said first gene being operatively linked to (ii) a first promoter DNA segment that controls expression of said heterologous gene, and (iii) a termination DNA segment. This expression cassette may be identical to the first expression cassette described earlier as the first embodiment of this invention. Therefore, the previous descriptions relating to the first expression cassette in the first embodiment of this invention may be applicable to the expression cassette used in the third embodiment of this invention.

The heterologous DNA segment must contain at least the expression cassette described above. Optionally, the heterologous DNA segment may contain additional expression cassettes which contain any gene(s) that are expressed in transformed plants. Therefore, the previous description in the first embodiment relating to such expression cassettes may be applicable to the third embodiment of this invention.

(b) After sufficient time in the first medium, transformed and non-transformed plant cells are removed from the first culture medium.

(c) After removal from the first culture medium, the transformed and non-transformed plant cells are placed under heterotrophic culture conditions in a second culture medium that contains the minimal nutrients required for proliferation and growth of the non-transformed plant cells except for an encrypted carbon source that does not support growth and proliferation of said non-transformed plant cells and 1.5 to 3 times the standard amount of phosphorous.

(d) The heterotrophic culture conditions are maintained for a time period sufficient for said transformed plant cells to express said heterologous enzyme, proliferate and grow.

As discussed earlier with respect to the second embodiment, the addition of 1.5 to 3 times the standard amount of phosphorous increases the quantity and quality of transformed plant cells regenerated. Also as in the second embodiment, the phosphorous added to the culture medium can be any water soluble phosphate salt.

Recombinant DNA Molecules

A recombinant DNA molecule useful herein can be produced by operatively linking a vector to an isolated heterologous DNA segment that contains two expression cassettes to form a plasmid. Particularly preferred recombinant DNA molecules are discussed in detail in the examples, hereafter. Vectors capable of directing the expression of the gene are referred to herein as "expression vectors".

In one preferred embodiment, a vector includes a prokaryotic replicon; i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell transformed therewith. Such replicons are well known in the art.

Those vectors that include a prokaryotic replicon can also include a prokaryotic promoter region capable of directing the expression of gene in a host cell, such as E. coli, transformed therewith. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing one or more convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC18, pUC19, and pBR322 available from Gibco BRL, Gaithersburg, MD. These vectors are utilized in the synthesis of the DNA segments present in the integrating expression vectors.

Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors that contain portions of the tumor-inducing (Ti) plasmid of Agrobacterium tumefaciens described by Rogers et al., Meth. Enzymol., 153:253-277 (1987). These vectors are plant integrating vectors in that on transformation, a portion of the vector DNA is integrated into the genome of the host plant. For integrating vectors containing portions of the Ti plasmid, the region integrated into the host plant chromosomes is that between the right and left borders of the Ti plasmid, or TDNA.


Another plant transformation system is based on Agrobacterium rhizogenes that induces hairy roots rather than a tumor on transformation. Application PCT/US87/02512 (WO 88/02405 published April 7, 1988) describes the use of A. rhizogenes strain A4 and its Ri plasmid along with A. tumefaciens vectors pARC8 or pARC16 to transform the cucumber Cucumis sativus, cv. Straight Eight, and form regenerated cucumber plants.

An Agrobacterium-based transformation system for melon (Cucumis melo), which is another member of the cucumber family of Cucurbitaceae, was reported by Dong et al., Bio/Technology, 9:858-863 (1991). Those workers used a binary vector that utilized the constitutive CaMV 35S promoter, and found evidence of transformation via a
Introducing genes into higher plants

Methods for transforming higher, multicellular flowering plants include Agrobacterium-mediated plant cell transformation, protoplast transformation, gene transfer into pollen, injection into reproductive organs, transformation of microspores and injection into immature embryos. Each of these methods has distinct advantages and disadvantages. Thus, one particular method of introducing genes into a particular plant species may not necessarily be the most effective for another plant species, but it is well known which methods are useful for a particular plant species.

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells. The use of Agrobacterium-mediated expression vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described by Fraley et al., Biotechnology, 3:629 (1985) and Rogers et al., Methods in Enzymology, 153:253-277 (1987). Further, the integration of the T-DNA is a relatively precise process resulting in few rearrangements. The region of DNA to be transferred is within the border sequences, and intervening DNA is usually inserted into the plant genome as described by Spielmann et al., Mol. Gen. Genet., 205:34 (1986) and Jorgensen et al., Mol. Gen. Genet., 207:471 (1987).

Agrobacterium transformation vectors such as those discussed before are capable of replication in E. coli as well as Agrobacterium, allowing for convenient manipulations as described by Klee et al., in Plant DNA Infectious Agents, T. Hohn and J. Schell, eds., Springer-Verlag, New York (1985) pp. 179-203.

Moreover, technological advances in vectors for Agrobacterium-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate construction of vectors capable of expressing various polypeptide coding genes. The vectors described by Rogers et al., Methods in Enzymology, 153:253 (1987), have convenient multilinker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes.

In those plant species where Agrobacterium-mediated transformation is efficient, i.e., in dicotyledonous plants, it is a method of choice because of the facile and defined nature of the gene transfer. Particularly preferred dicotyledonous plants include those of Brassicaceae (cabbage and broccoli), Compositae (lettuce), Umbelliterae (carrots), Solanaceae (pepper and tomato) and Cucurbitaceae (melon and squash) families of higher plants.

Monocots such as cereals and grasses appear not to be natural hosts for Agrobacterium, although transgenic plants have been produced in asparagus using Agrobacterium vectors as described by Bytebier et al., Proc. Natl. Acad. Sci. U.S.A., 84:5345 (1987). Commercially important cereal grains such as rice, corn, oats and wheat are usually transformed using alternative methods.

To transform plant species that cannot be successfully transformed using Agrobacterium, a "particle gun" or high-velocity microprojectile technology can be utilized. Using such technology, DNA is carried through the cell wall and into the cytoplasm on the surface of small metal particles as described in Klein et al., Nature, 327:16 (1986); Yamada et al., Biotechnology, 5:923 (1988); Uchimiya et al., Mol. Gen. Genet., 205:34 (1986); Wang et al., Mol. Gen. Genet., 1:923 (1988); and Vasil et al., Mol. Gen. Genet., 207:471 (1987). Metal particles penetrate through several layers of cells and thus allow the transformation of cells within tissue explants.

The microprojectile mediated transformation is a preferred source of transgenic cells from monocotyledonous plants. Particularly preferred monocotyledonous plants include maize, wheat, oats, barley, rice, sorghum and forage grasses. Metal particles coated with DNA have been used to successfully transform maize and wheat cells. Fertile, stably transformed tobacco and soybean plants have also been transformed by this method. Regeneration of cereals from immature embryos or explants can be effected as described by Vasil, Biotechnology, 6:397 (1988).


Application of these systems to different plant species depends upon the ability to regenerate that particular plant species from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described in Fujimura et al., Plant Tissue Culture Letters, 2:74 (1985); Toriyma et al., Theor Appl. Genet., 73:16 (1986); Yamada...


[0101] The regeneration of plants from transformed plant cells such as either single plant protoplasts or various explants is well known in the art. See, for example, *Methods for Plant Molecular Biology*, A. Weissbach and H. Weissbach, eds., Academic Press, Inc., San Diego, CA (1988). The regeneration and growth process includes the steps of selection of transformant cells and shoots, rooting the transformant shoots and growth of the plantlets in soil.

[0102] Growth media for plant cells contemplated herein are those familiar to workers of ordinary skill in these arts except that an encrypted carbon source replaces the usually used carbon source such as glucose or sucrose. Exemplary media contain Murashige and Skoog (MS) salts and RO vitamins. Regeneration media are similarly well known and also contain plant hormones such as benzyladenine, gibberellic acid, indoleacetic acid, naphthylacetic acid, zeatin, thiamine hydrochloride and the like, and utilize an encrypted carbon source.

[0103] As discussed earlier, one special feature of a preferred nutrient medium for regeneration is that at approximately 1.5 to about 3 times the standard amount of phosphorous is present as a water-soluble phosphate salt. The basis for this preference is the finding that an enhanced number of healthy regenerated shoots were obtained using the enhanced amount of phosphate salt as well as a greater percentage of normal shoots among those regenerates.

[0104] The regeneration of plants containing the foreign gene introduced by *Agrobacterium* from leaf explants can be achieved as described by Horsch et al., *Science*, 227:1229-1231 (1985). In this procedure, transformants are grown in the presence of the appropriate encrypted carbon source in a medium that induces the regeneration of shoots in the plant species being transformed as described by Fraley et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:4803 (1983). This procedure typically produces shoots within two to four months and these transformant shoots are then transferred to an appropriate root-inducing medium and an antibiotic to prevent bacterial growth.

[0105] Transformant shoots that begin to root in the presence of an encrypted carbon source and form plantlets are then transplanted to soil or other media to allow the production of roots. These procedures vary depending upon the particular plant species employed, such variations being well known in the art.

**Genetics**

[0106] A transgenic plant formed using *Agrobacterium* transformation methods can contain a single transgene on one chromosome. Such transgenic plants can be referred to as being heterozygous for the added gene. However, inasmuch as use of the word "heterozygous" usually implies the presence of a complementary gene at the same locus of the second chromosome of a pair of chromosomes, and there is no such gene in a plant containing one added gene as here, a more accurate name for such a plant is a hemizygote. The original transgenic plant formed using *Agrobacterium* is referred to as a hemizygote.

[0107] More preferred is a transgenic plant that is homozygous for the added expression cassettes, e.g., a transgenic plant that contains two sets of added expression cassettes, one set at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can exhibit at least enhanced expression of the selectable marker as compared to both a native, non-transgenic plant and an independent segregant transgenic plant. A homozygous transgenic plant can also be prepared from transformed microspores, as noted before. Even more preferred is a plant created by a cross of different inbred lines. Plant seed produced by such a cross are referred to as hybrid seed. There are many benefits to growing hybrid seed that include, a more uniform product, higher yields, and healthier plants. Collectively, these advantages are well known in agriculture and are often referred to as hybrid vigor. If one of the inbred parents carry the transgenic cassettes in a homozygous condition, then the hybrid progeny would all contain a single copy of the transgenic cassettes (i.e., they would be hemizygotic for the transgenes). In such a crossing scheme, the hybrid progeny will have both the advantages of hybrid vigor and the advantage of the effects conferred by the transgenes.

[0108] It is to be understood that two different transgenic plants can be mated to produce offspring that contain two sets of independently added segregating cassette sets. Selfing of appropriate progeny can produce plants that are homozygous for both added gene sets. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.
A transgenic plant of this invention thus has heterologous DNA containing two expression cassettes (a set). A preferred transgenic plant is an independent segregant for the added set of cassettes and can transmit those cassettes and their activity to its progeny. A more preferred transgenic plant is homozygous for those heterologous cassette sets, and transmits those genes to all of its offspring on sexual mating.

Plant Transformation Kit

Another aspect of this invention is a kit for transforming plant cells. This kit, which itself is usually contained in a package, contains two packages and also preferably contains a set of directions for use of the kit.

A first package contains a DNA segment heterologous to the plant to be transformed. That DNA segment contains an expression cassette operatively linked to a linker segment that includes at least one restriction endonuclease site, and preferably includes a plurality of such sites. The expression cassette is a first (or third) expression cassette discussed before that contains a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that converts an encrypted carbon source that does not support growth and proliferation of non-transformed plant cells into a carbon source that supports growth and proliferation of the transformed cells upon expression during heterotrophic culture of the transformed plant cells. That gene is operatively linked to (ii) a promoter DNA segment that controls the expression of that heterologous gene and (iii) a termination DNA segment.

The first gene can encode any of the before-discussed heterologous genes, with the pmi gene being one preferred gene. Another preferred gene is the mannitol 1-oxidoreductase gene that converts mannitol into mannose and is useful for situations where double transformation is contemplated.

The promoter and termination DNA segments are those discussed before in connection with the first expression cassette. A repressible promoter DNA segment is particularly preferred.

The linker segment of this DNA preferably contains a plurality of endonuclease restriction sites as are commonly found in vectors. This linker and its one or more restriction sites facilitates incorporation of a second expression cassette of the user's choice into the heterologous DNA segment. The linker segment can be located upstream or downstream of the first expression cassette.

The DNA of the first package is itself preferably within the T-DNA borders of the Ti plasmid of Agrobacterium tumefaciens in a vector adapted for Agrobacterium-mediated plant transformation. Thus, an expression vector is contemplated for Agrobacterium-mediated plant transformation that contains all of the elements needed for that transformation except for the second expression cassette containing the second, target, gene and its promoter and termination segments that are supplied by the user.

Another embodiment of this aspect of the invention includes one or the other or both of 5'-promoter and 3'-termination DNA segments with an operatively-linked linker containing at least one restriction site in between so that all that the user need do is insert (ligate) a desired second, target, gene. These segments can be located together upstream or downstream of the recited expression cassette.

The second package of the kit contains minimal nutrients required for growth and proliferation of non-transformed plant cells during heterotrophic culture, except for a source of carbon. The source of carbon usually present in such nutrient media is replaced by an encrypted carbon source that does not support growth and proliferation of non-transformed plant cells but supports growth and proliferation of transformed plant cells whose genome contains a before-described DNA segment containing a first expression cassette.

A nutrient medium contemplated here as present in the second package can be any useful medium such as any of the media discussed herein like MS medium containing MS salts and RO vitamins that is free of a usual carbon source. The encrypted carbon source is also a previously discussed encrypted carbon source such as mannose, lactose, sorbitol or mannitol.

A third package can also be included that contains components of a regeneration medium and the same encrypted carbon source. Thus, nutrient materials such as MS salts and RO vitamins are included along with plant hormones such as benzyladenine and gibberellic acid, and the like as are well known. Antibiotics such as carbenicillin and timentin or ticarcillin that eliminate Agrobacteria can also be included. Each of the ingredients is present in its usually-used amount except for phosphorous that is preferably present at about 1.5 to about 3 times its usual amount in such a medium as a water-soluble phosphate salt.

Results

The first step in the heterotrophic complementation selection system is to identify a carbon source such as a carbohydrate that is not metabolized by plant cells to support growth and proliferation. Examples of such carbohydrates are mannose, mannitol, sorbitol, lactose, trehalose and salicin.

The second step in the carbon source-based selection system is to select a gene that encodes an enzyme
that permits the transformed tissue to utilize that encrypted carbon source, whereas that carbon source is not normally metabolized by non-transformed plant tissue. One particular exemplary gene encodes the enzyme phosphomannose isomerase (*pmi*; EC 5.3.1.8). This enzyme reversibly catalyzes the conversion of fructose 6-phosphate to mannose 6-phosphate. This gene is designated as the *pmi* gene in *Rhizobium meliloti* (Gene, 122: 35-43), *Pseudomonas aeruginosa* (Gene, 42:293-302), *Saccharomyces cerevisiae* (Molecular and Cellular Biology, 12:2924-2930) and *Salmonella typhimurium* (Gene, 103:135-136). In *Escherichia coli*, the phosphomannose isomerase gene is called *manA* (Gene, 32:41-48).

**Selection of a Growth-limiting Carbohydrate**

All media used herein was that of Murashige and Skoog, Physiol. Plant, 15:437-498 (1962) (Medium MS) salts and RO vitamins (composition listed below) that were adjusted to pH = 5.7 and solidified with 9 g/L of Noble Agar (Gibco). Medium R1F was MS supplemented with 1 mg/L indoleacetic acid (IAA), 0.65 mg/L zeatin and 16 g/L glucose. Medium MR1 was MS supplemented with 1 mg/L IAA, 0.65 mg/L zeatin and 16 g/L mannose. Medium MR1/2 was MS supplemented with 0.5 mg/L IAA, 0.325 mg/L of zeatin and 16 g/L mannose. Medium RO was MS supplemented with 16 g/L glucose. Medium MRO was MS supplemented with 16 g/L mannose.

**Table 1.**

<table>
<thead>
<tr>
<th>Medium MS Salts</th>
<th>mg/L</th>
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</thead>
<tbody>
<tr>
<td>Ammonium nitrate</td>
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<tr>
<td>Boric acid</td>
<td>6.200</td>
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<tr>
<td>Calcium chloride</td>
<td>440.000</td>
</tr>
<tr>
<td>Cobaltous chloride</td>
<td>0.025</td>
</tr>
<tr>
<td>Cupric sulfate pentahydrate</td>
<td>0.025</td>
</tr>
<tr>
<td>Ferrous sulfate sephatydrate</td>
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<td>Magnesium sulfate sephatydrate</td>
<td>370.000</td>
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<td>Manganese sulfate monohydrate</td>
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</tr>
<tr>
<td>Myo-inositol</td>
<td>100.000</td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
</tr>
</tbody>
</table>

Tomato seeds were sterilized in a solution containing 20 percent of a commercial household bleach containing 5.25 percent sodium hypochlorite for 20 minutes, rinsed 3 times in sterile distilled water and planted on RO medium solidified with 10 g/L of Noble agar (Gibco) in 135 mm Phytacon™ tissue culture vessels (Sigma, St. Louis, MO). Seeds were germinated for 72 hours at 25°C in the dark, then moved to a lighted shelf under approximately 80 µmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD), at 24-26°C. Plant tissue used for regeneration was prepared by removing cotyledons from 7-day-old seedlings and cutting them into three parts (proximal, middle and distal to the growing point). The middle and proximal parts were used for the evaluation of growth on potential non-useful carbohydrates.

The following carbohydrates were considered for use as a non-useful encrypted, replacement of a carbon source in plant regeneration medium: mannose, mannitol, sorbitol, lactose, α,α-trehalose and salicin. Different R1F media were prepared, each containing one of those carbohydrates added at 0.09 M concentration as a sole carbon source. Control R1F medium contained 0.09M (16 g/L) glucose as a carbon source. Tomato tissue was placed abaxial side down on the media and incubated in sealed petri plates at 24-26 °C, under 30 µmolm⁻²s⁻¹ PPFD, 16 hours photo-
to period. Tomato tissue was observed for signs of regeneration weekly.

[0125] Throughout the carbohydrate evaluation, which lasted four weeks, none of the altered media containing the above-listed carbohydrates supported regeneration of tomato shoots, whereas tomato tissue placed on the control glucose-supplemented R1F medium produced abundant shoots.

Selection of a Gene to Convert an Encrypted Carbohydrate

[0126] It is known that plants can take mannose up from media, and subsequently phosphorylate mannose into mannose-6-phosphate. Sheu-Hwa et al. New Phyto., 74:383-392 (1975). It is concluded that mannose-6-phosphate does not support growth and proliferation of plant cells because plant cells cultured using mannose as the sole carbon source do not grow and proliferate. A gene was therefore chosen that encodes an enzyme that converts mannose-6-phosphate into fructose-6-phosphate. Fructose-6-phosphate is a compound known to be metabolized by plants. Thus, plant cells that express this gene are able to utilize mannose to grow and proliferate, and have a selective growth advantage when placed on media containing mannose as the sole carbon source.

Construction of the Transfer Vector

[0127] A pmi gene from a prokaryotic species was selected and used to create a plant transformation vector that included a selectable marker of the present invention. Molecular biology techniques, that are well known to those skilled in the art, were used to create the plant transformation vector containing the selectable marker gene. The steps used to move the pmi gene into a plant transformation vector are shown in Fig. 2. Enzymes used to manipulate DNA can be purchased either from New England Biolabs or Boehringer Mannheim.

[0128] Briefly, plasmid pADE253, containing the S. typhimurium pmi gene (GenBank accession number X57117), was a gift from Dr. James Hackett, University of Adelaide. Plasmid pADE253 is a derivative of the commonly used E. coli cloning vector pBR322 that contains a 1650 base pair (bp) Clal fragment (Fig. 2) that contains the pmi gene. SEQ ID NO:1 shows the sequence of the 1650 bp Clal fragment, which contains 432 bases 5’ to the beginning of the pmi coding region, 391 codons that contain the coding capacity for the pmi gene product (a protein of predicted Mr of 42.6 kDa), and 43 bases 3’ to the TAG stop codon.

[0129] SEQ ID Nos:3 and 4 are two oligonucleotides that were prepared to facilitate cloning the pmi gene from pADE253 into a plant transformation vector. The oligonucleotide of SEQ ID NO:3 is 20 bases in length and is identical to bases 391-410 of the Clal fragment (coding strand), except for position 403 where there is single mismatch. The oligonucleotide of SEQ ID NO:4 is 25 bases in length and is of complimentary sequence to bases 1641-1617 of the Clal fragment, except for mismatches at positions 1628 and 1630.

[0130] The two oligonucleotides and DNA from plasmid pADE253 were used in a polymerase chain reaction (PCR) to amplify a population of approximately 1250 bp DNA fragments containing the pmi coding region and small portions of the upstream and downstream regions. As a result of the mismatched bases between the oligonucleotides and the Clal fragment and because most of the DNA synthesized in the PCR reaction used newly synthesized DNA as a template rather than the pADE253 DNA as a template, the majority of the approximately 1250 bp fragments synthesized contained two new restriction endonuclease recognition sites that facilitated the cloning of the pmi gene into a plant transformation vector.

[0131] The new restriction endonuclease recognition sites added were a BglII recognition site (AGATCT), located between 24 and 29 bases 5’ to the initiator methionine codon of the pmi gene, and a SalI recognition site (GAGCTC), located between 21 and 26 bases 3’ to the TAG stop codon of the pmi gene. The DNA from the PCR reaction was digested with BglII and SalI for several hours at 37°C, and then electrophoresed on a 1 percent low melting point agarose gel. An approximately 1225 bp band was excised from the gel, and the DNA was isolated and purified.

[0132] This fragment, containing the pmi gene, was cloned into the plasmid pGEM3Zf(−) (Fig. 2). Plasmid pGEM3Zf(-) is a commercially available vector (Promega Corporation), and is 3199 bps in length. More specifically, pGEM3Zf(-) DNA was digested with BamHI and SalI, and the vector fragment was gel-purified. Digestion with SalI leaves a four base, 3’ overhang of the sequence AGCT. Although the recognition sites for BamHI and BglII are different, both enzymes produce an identical four base, 5’ overhang of the sequence GATC. These overhanging regions greatly facilitate directional cloning.

[0133] The approximately 1.2 kilobase pair kbp BglII-SalI fragment containing the pmi gene and the BamHI and SalI digested pGEM3Zf(−) DNA were ligated together overnight (about 18 hours) at 16°C. The following day, the ligation reaction product was used to transform E. coli strain MV1190. Various aliquots from the transformation were spread onto LB agar plates supplemented with carbenicillin (100 mg/L), isopropyl-1-thio-β-D-galactoside (IPTG) (0.1 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (1 ml/L of a 20 mg/mL stock dissolved in N,N dimethylformamide).

[0134] Plasmid pGEM3Zf(−), like many commonly used plasmids in molecular biology, has a color marker selection
system to indicate when DNA has been inserted into the vector's polylinker. When _E. coli_ containing the omega fragment of β-galactosidase, which is usually contained on the F’ plasmid, and harboring the pGem3zf(·) plasmid are plated onto medium supplemented with IPTG and X-gal, the α-fragment of β-galactosidase, which is encoded by the plasmid, can complement the omega fragment and produce a functional β-galactosidase enzyme. β-galactosidase can cleave the X-gal in the medium, yielding a blue color. Normally, when DNA is inserted into the polylinker region of pGem3zf(·), α-complementation is destroyed, and the colonies appear white instead of blue. The day following transformation, plasmid DNA was purified from several of the white colonies that grew on the LB-carbenicillin-IPTG-X-gal plates. To confirm the presence of the _pmi_ gene, these plasmid DNAs were examined by their gel electrophoresis profiles after digestion with various restriction endonuclease enzymes. A clone that contained the _pmi_ gene was selected from this population and the plasmid was designated as pET0147 (Fig. 2).

**[0135]** The _pmi_ gene was excised from plasmid pET0147 and inserted into a binary vector that facilitated transformation. pET0107 is approximately 13 kbp in length, and is a derivative of the commonly used binary plant transformation vector BIN19 [Bevan, Nucleic Acids Res., 12: 8711-8721(1984)]. Like BIN19, plasmid pET0107 has both a right and left TDNA border region. These 25 base pair TDNA regions specify the sites that are recognized by _Agrobacterium tumefaciens_, which mediates the transfer of DNA from the plasmid to the plant chromosome; DNA between these borders is inserted into the plant chromosome by _A. tumefaciens_. Infecting a number of different plant tissue types, such as leaf discs or cotyledons with _A. tumefaciens_ that harbor a binary vector is a preferred method of transforming many plant species. Outside the TDNA borders, plasmid pET0107 contains the bacterial nptII gene, which can confer kanamycin resistance to _E. coli_ that harbor this plasmid.

**[0136]** Plasmid pET0107 has two expression cassettes of interest between the TDNA borders. Starting from the right TDNA border and proceeding clockwise for convenience (Fig. 2), the first cassette is composed of three elements that are operationally linked. The first element is the nopaline synthase promoter (P-Nos), which when inserted into the plant chromosome directs the constitutive expression in most plant cells of genes that are positioned downstream of this DNA (clockwise in Fig. 2). The next element is the coding region for the neomycin phosphotransferase type II gene (NPTII). If NPTII is expressed in most plant cells in sufficient quantities, it confers resistance to those cells to the antibiotic kanamycin. The third and final element of the first cassette is the 3' termination end of the nopaline synthase coding region (Nos-T), which contains the polyadenylation recognition site. The first cassette is commonly used in plant transformation vectors as a selectable marker to identify transformed tissue; the kanamycin resistance conferred to transformed cells results in a selective growth advantage on medium supplemented with kanamycin. The nptII expression cassette is part of the original plasmid, and is not essential for the current invention, but is rather an example of a before-discussed second expression cassette.

**[0137]** Proceeding clockwise in Fig. 2, the second expression cassette in plasmid pET0107 also contains three elements. The first element is the 35S promoter (35S) from cauliflower mosaic virus (CaMV 35S). Like the P-Nos promoter, CaMV 35S directs constitutive expression in plant cells. Generally, the level of expression in plant cells is greater with CaMV 35S than P-Nos. The next element is a polylinker and includes the recognition sites for the following restriction endonuclease enzymes: XbaI, BamHI, SmaI, KpnI and SacI. The third and final element of this cassette is the 3' termination end of the nopaline synthase coding region (Nos-T). The polylinker region of this second element was used to insert the XbaI-SacI fragment from pET0147, which contains the _pmi_ gene, into the XbaI and SacI sites that are located between the CaMV 35S and Nos-T elements.

**[0138]** DNA from plasmids pET0147 and pET0107 were digested with XbaI and SacI. The approximately 1.2 kbp XbaI-SacI fragment from plasmid pET0147, and the 13 kbp XbaI-SacI fragment from plasmid pET0107 were gel-purified. In a ligation reaction containing the 1.2 kbp XbaI-SacI fragment from plasmid pET0147 containing the _pmi_ gene, and the 13 kbp XbaI and SacI plasmid pET0107 fragment, the 4-base overhangs facilitated the directional insertion of the _pmi_ gene between the CaMV 35S promoter and Nos-T. The ligation reaction was performed overnight (about 18 hours) at 16°C.

**[0139]** The following day, the ligation reaction product was used to transform the _E. coli_ strain XL1-Blue MRF. Various aliquots from the transformation were spread onto LB agar plates supplemented with kanamycin (50 mg/L). The following day, plasmid DNA was purified from several colonies, and gel electrophoresis profiles from restriction endonuclease digestions were used to identify a plasmid containing a _pmi_ gene that was inserted between the CaMV 35S and Nos-T elements. One such plasmid was designated pET0148 (Fig. 2).

**[0140]** Plasmid pET0148 was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD on April 6, 1995 and was given ATCC accession number 97107. The present deposit was made in compliance with the Budapest Treaty requirements that the duration of the deposit should be for 30 years from the date of deposit or for five years after the last request for the deposit at the depository or for the enforceable life of a U.S. patent that matures from this application, whichever is longer. The vector will be replenished should it become non-viable at the depository.

**[0141]** _E. coli_ harboring plasmid pET0148 were mobilized into the disarmed _A. tumefaciens_ strain LBA4404 using the triparental mating system to form _A. tumefaciens_ pET0148::LHA4404. Transconjugants were used to transform
tomato (Lycopersicon esculentum).

Example 1: Transformation of Tomato Plants

Tomato seeds were sterilized in a solution containing 20 percent of a commercial household bleach containing 5.25 percent sodium hypochlorite, rinsed three times in sterile distilled water and planted on Murashige and Skoog (MS) medium ( Gibco) solidified with 10 grams of Noble agar ( Gibco) in 135 mm Phytacon™ tissue culture vessels (Sigma, St. Louis, MO). Seeds were germinated for 72 hours at 25°C in the dark, then moved to a lighted shelf under approximately 80 μmol m⁻² s⁻¹ PPFD, at 24-26°C. Plant tissue used for transformation was prepared by removing cotyledons from 7-day-old seedlings and cutting them into three parts (proximal, middle and distal to the growing point).

The middle and proximal parts were used for co-cultivation with Agrobacterium. Those parts were placed abaxial side down on a sterile filter paper overlaying co-cultivation medium R1F supplemented with 0.09 M (16 g/L) glucose, and incubated in the dark for 24 hours. Next, they were incubated for 20 minutes with bacterial inoculum containing 5x10⁸ CFU/mL of Agrobacterium tumefaciens, pPET0148::LBA4404, blotted dry, and cultured for 48 hours, at 24°C, in the dark.

Bacterial inoculum was prepared by growing A. tumefaciens, pPET0148::LBA4404, in 25 ml of AB medium [Chilton et al., 1974, Proc. Natl. Acad. Sci. USA, 71:3672-3676 (1974)] supplemented with 50 mg/L kanamycin (K) and 25 mg/L streptomyces (St) (ABK50St25) on a shaker at 28°C, 180 rpm, for 24 hours. One mL of this culture was transferred to 25 mL of fresh ABK50St25 medium and grown on a shaker at 28°C, 180 rpm for 24 hours. The bacteria were then pelleted by centrifuging at 8000 rpm for 10 minutes in a Beckman J2-21 centrifuge using a JA-20 rotor. The bacterial pellet was resuspended in sterile MS medium and their concentration was adjusted to 5x10⁸ CFU/mL using spectrophotometric optical density reading at 550 nm (0.1 OD₅₅₀ 2x10⁸ CFU/mL). Prior to co-cultivation, the inoculum was supplemented with acetosyringone (3',5'-dimethoxy-4' hydroxy-acetophenone, Sigma, St. Louis, MO) to a final concentration of 600 micromol.

Selection of Transformed Tomato Plants

After the 48 hour co-cultivation period with the Agrobacterium (pPET0148::LBA4404) on the R1F medium, tomato tissue was moved to MR1 regeneration medium containing 0.09 M mannose as the sole carbon source. The MR1 medium was supplemented with 100 mg/L of the antibiotic ticarcillin (Duchefa Co.) to eliminate Agrobacterium.

After two weeks of culture, tomato tissue was moved to fresh MR1 medium. At this time small calli were visible on the cut ends of co-cultivated tissue, whereas control tissue that was not co-cultivated with the bacteria but was also cultured on the MR1 medium, showed yellowing and did not produce any callus. After two more weeks of culture all tissues were moved to MR1/2 regeneration medium also containing 0.09 M mannose as a sole carbon source. At this time the co-cultivated tissue produced a thick ridge of callus with small shoot primordia, whereas the control tissue was yellow in color, did not enlarge, nor produce callus. After two weeks on the MR1/2 medium the calli and shoot primordia that formed on the co-cultivated tissue were detached from the original tissue and moved to MRO medium supplemented with 0.9M mannose. On this medium the shoots elongated and developed roots. Rooted plants were potted and moved to the greenhouse.

Biochemical and Molecular Analysis of Regenerated Plants

Plants regenerated on mannose were assayed biochemically and molecularly to determine whether they were transformed. These plants were selected by their ability to grow on a medium containing mannose as an encrypted carbon source, but because the pETO148 transformation vector contained both the pmi and the nptII genes, plants were assayed for transformation indirectly by scoring for the expression of the second gene (nptII) using a commercially available (5 Prime → 3 Prime, Inc.) NPTII enzyme linked immunosorbant assay (ELISA).

Fig. 3 shows the relative NPTII expression from three rank-ordered populations. One population included five tomato plants that were non-transformed (negative control). The second population of five plants was from a transformation study that used kanamycin as a selective agent. In these positive control plants, the NPTII ELISA assays directly for the expression of the selectable marker gene which detoxifies the selective agent. The expression of NPTII in these plants covered a wide range. The third population were 16 plants selected on mannose. The NPTII expression in the plants selected on mannose also covered a wide range. The expression of NPTII in the plants selected on mannose indicated that the nptII expression cassette that is incorporated with the pmi expression cassette was functioning in...
plants, and supplied indirect evidence that these plants were transformed. Further, these data indicate that the expression from the \textit{pmi} gene, which confers the ability to grow on mannose, did not affect expression of an adjacent gene in the plant's chromosome.

To assay directly for transformation, a Southern blot was performed on several putative transformants. A Southern blot is a commonly performed assay in molecular biology that can show how many copies of the transgene of interest are incorporated into the genome. Five grams of young leaf tissue were harvested and used to isolate genomic DNA. DNA yields were quantitated using a Hoefer Model TKO100 Fluorometer.

For each sample, 10 micrograms of DNA was digested with \textit{HindIII}, electrophoresed on a 1 percent agarose gel and transferred to a nylon membrane. DNAs were fixed to the membrane using a Stratagene CL-100 ultraviolet crosslinker. The Genius\textsuperscript{T,M} non-radioactive detection system (Boehringer Mannheim) was used to probe the membrane and the blot results were recorded by fluorography. Using primers specific for the \textit{pmi} gene, a 788 bp fragment was amplified using PCR. During amplification, digoxigenin-11-dUTP was incorporated into the PCR fragments. The \textit{pmi}-specific, digoxigen-labeled fragments were used to probe the genomic blot. Protocols for the hybridization, blot washing and visualization were supplied by the manufacturer.

A blot of 11 putative transgenic samples was prepared using molecular mass markers from \textit{HindIII}-digested bacteriophage lambda DNA and DNA from a non-transformed tomato plant as a negative control. The fluorogram showed that the \textit{pmi} probe did not hybridize to the DNA from the non-transformed parental control, but did decorate specific bands in nine of the eleven putative transformants. The number of copies inserted into the tomato genome varied (data not shown).

**Example 2: Monocot Cell Growth on Mannose**

To assay whether mannose could be used for selection of transgenic cells in monocot plants the growth of cell suspensions of both oats and corn (maize) was evaluated in sucrose-supplemented media (standard protocol) and in media in which sucrose was replaced with mannose at the same molar concentration.

**Oats**

Approximately 500 mg of drained oat suspension cells were inoculated to 40 ml of liquid MS2-D medium (4.4 g/L MS salts, Sigma #M5524; 0.5 mg/L thiamine HCl; 2 mg/L 2,4-D; 150 mg/L L-asparagine; pH=5.8) containing either 0.06 M sucrose (20 g/L) or 0.06 M mannose (10.6 g/L). Each treatment was replicated three times. The flasks were placed on a shaker at 160 rpm, 24-26°C, using 16 hours of photoperiod. After 25 days, the suspension cells were allowed to settle at the bottom of a test tube, the liquid medium was aspirated and the cells were weighed. The net weights of the cells are listed in Table 2, below.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Sucrose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.03</td>
<td>0.79</td>
</tr>
<tr>
<td>2</td>
<td>3.33</td>
<td>0.66</td>
</tr>
<tr>
<td>3</td>
<td>3.41</td>
<td>0.92</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>3.26 ± 0.12</td>
<td>0.79 ± 0.08</td>
</tr>
</tbody>
</table>

**Corn (Maize)**

A rapidly growing suspension culture of black Mexican sweet corn (BMS) was washed several times in mannose-supplemented (10.6 g/L) MS-F medium (4.4 g/L MS salts, Sigma #M5524; 1.3 mg/L nicotinic acid; 0.25 mg/L pyridoxine HCl; 0.25 mg/L thiamine HCl; 0.25 mg/L calcium pantothenate; 2 mg/L 2,4-D; 0.1 g/L inositol; 150 mg/L L-asparagine; pH=5.8). A five mL aliquots of a thick (1:1 cells to liquid ratio) cell slurry made from the washed cells were inoculated to flasks containing 35 mL of either mannose-supplemented (0.06 M) or sucrose-supplemented (0.06 M) medium. Each treatment was replicated four times. The flasks were placed on a shaker at 160 rpm, 24-26°C, in the dark. The growth of the suspension cells was assessed after 32 days of culture by measuring the packed cell volume. The results are presented in Table 3, below.
Both oat and corn cell suspensions showed severe inhibition of growth when cultured in mannose-supplemented medium as compared to the standard sucrose-containing medium. This indicates that a replacement of sucrose with mannose provides an effective selection for genetically engineered cells from monocot plants that can utilize mannose as a carbon source under heterotrophic growth conditions.

Example 3: Regeneration of transgenic tomatoes

Tomato tissue was prepared and cocultivated with Agrobacterium containing the pmi gene as described in Example 1. After cocultivation tomato tissue was cultured either on the standard MR1 medium or on the MR1 medium supplemented with 170.00 mg/L of monobasic potassium phosphate (MR1/P medium). The supplementation of the mannose-containing medium with additional phosphorus resulted in a significant increase in the number of transformed plants that were regenerated. The comparison of regeneration on MR1 medium and MR1/P medium is shown in Table 4.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Frequency of regeneration (Percent)</th>
<th>Number of regenerated shoots</th>
<th>Percent normal shoots</th>
<th>Percent abnormal shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR1</td>
<td>66.4</td>
<td>83</td>
<td>48.2</td>
<td>51.8</td>
</tr>
<tr>
<td>MR1/P</td>
<td>82.4</td>
<td>103</td>
<td>80.6</td>
<td>19.4</td>
</tr>
</tbody>
</table>

These observations indicate that an additional modification of the basic medium composition results in increased regeneration of transformed plants. Doubling of the concentration of phosphorus, which is an essential component of the regeneration medium, gave further growth advantage to tomato cells containing the pmi gene, resulting in a more efficient regeneration of genetically engineered plants using mannose for selection.

Example 4: Transgenic Melon

Decoated melon seeds were surface sterilized in a solution containing 10 percent of a commercial household bleach containing 5.25 percent sodium hypochlorite for ten minutes, rinsed six times in sterile, distilled water and germinated on a blotter paper soaked with sterile water for 24 hours, in the dark. After germination, cotyledons were detached from the seed axis and cultured on B0 medium for two days under 20 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) PPFD. Tissue for transformation was cut out using a No. 1 corkborer and inoculated with Agrobacterium tumefaciens pETO156::EHA105 by first briefly vortexing and then by soaking the tissue in the bacterial inoculum for 20 minutes. Inoculated tissue was cultured for three days on a fresh B0 medium overlayed with a sterile filter paper. Cocultivated explants were washed three times in a liquid B0 medium containing 1000 mg/L of the antibiotic carbenicillin and 200 mg/L of the antibiotic ticarcillin (Duchefa Co.) to eliminate the Agrobacterium, followed by two weeks of culture on shoot induction medium B300 supplemented with 16 gm/L of mannose, at 24-26°C, under 60-80 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) PPFD of light, at 16 hours of photoperiod.

After two weeks on the B300 medium melon tissue was transferred to shoot elongation medium E500 also containing 16 gm/L mannose, and cultured for another two weeks. At the time of transfer to the E500 medium the cocultivated tissue showed rapid cell proliferation, whereas non-transformed control tissue did not enlarge and did not produce any callus. Shoots that developed on the cocultivated tissue were rooted on medium N500 containing 16 gm/L mannose. Noncocultivated control tissue never produced any shoots. Rooted plants were potted. Effective selection of transformed melon plants was confirmed by staining plant tissue in the chemical reagent X-Gluc, which causes the

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Volume (cc) of Corn Suspension Cells After Culture in Media Supplemented Either with Sucrose or Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>Sucrose</td>
</tr>
<tr>
<td>1</td>
<td>8.4</td>
</tr>
<tr>
<td>2</td>
<td>7.6</td>
</tr>
<tr>
<td>3</td>
<td>8.0</td>
</tr>
<tr>
<td>4</td>
<td>10.4</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>8.60 ± 0.62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Comparison of Regeneration of Transformed Plants on MR1 and MR1/P Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Frequency of regeneration (Percent)</td>
</tr>
<tr>
<td>MR1</td>
<td>66.4</td>
</tr>
<tr>
<td>MR1/P</td>
<td>82.4</td>
</tr>
</tbody>
</table>
transgenic tissue to turn blue due to the production of indigo dye by transgenic cells.

**[0160]** All media used in melon regeneration contained 4.3 gm/L of MS salts (Gibco #11117-074) and 1 ml/L of MS vitamins (Sigma #7150), were solidified with 8.0 gm/L of Noble agar and had pH adjusted to 5.8. Media B300, E500 and N500 contained 1000 mg/L carbenicillin and 200 mg/L ticarcillin (Duchefa Co.) to eliminate the Agrobacterium. The B0 medium used for preculture and cocultivation was supplemented with 30 gm/L of sucrose, whereas media B300, E500 and N500 contained 16 gm/L mannose as a sole carbon source. Medium B300 contained 1.0 mg/l benzyladenine; medium E500 contained 0.15 mg/L benzyladenine and 3 mg/L gibberellic acid; medium N500 contained 50 mg/L 1-naphthylacetic acid and 16 mg/L thiamine HCl.

**[0161]** A preferred way to regenerate transgenic melon plants using mannose as a selective agent is to use B300, E500 and N500 media supplemented with additional 170 mg/L or 340 mg/L monobasic potassium phosphate.

**Example 5: Transgenic Squash**

**[0162]** Decoated squash seeds were surface sterilized in a solution containing ten percent of a commercial household bleach containing 5.25 percent sodium hypochlorite for ten minutes, rinsed five times in sterile, distilled water and germinated for 24 hours in the dark at 27°C on filter paper soaked with sterile water. Plant tissue used for transformation was prepared by removing the shoot axis cotyledonary node tissue and the distal half of the cotyledonary tissue. The remaining proximal half was cut into 4 explant pieces.

**[0163]** All explants were inoculated for 10 minutes in 2.5x10⁸ CFU/mL of Agrobacterium tumefaciens, pETO 156: EHA 105 in liquid MS medium supplemented with 0.2 mM acetosyringone. Explants were blotted on sterile filter paper and cocultivated adaxial side down on MS medium supplemented with 2 mg/L 2,4,5-T and 0.5 mg/L kinetin. Explants were cocultivated for 3 days in the dark in unsealed Petri dishes.

**[0164]** After the 3 day cocultivation period, explants were washed for 3 hours in MS liquid medium supplemented 10 mg/L 2,4-D, 0.5 mg/L kinetin, 400 mg/L cefotaxime and 750 mg/L carbenicillin. The rinse medium was changed 3 times during the procedure. Explants were blotted on sterile filter paper and cultured abaxial side down on selection medium consisting of MS salts and vitamins, 10 mg/L 2,4-D, 0.5 mg/L kinetin, 16.2 g/L mannose, 1000 mg/L carbenicillin and 170 mg/L monobasic potassium phosphate. Explants were cultured in unsealed Petri dishes and kept in diffused light. Explants were subcultured to fresh medium every two weeks.

**[0165]** After 8 weeks of selection, clusters of embryos formed on the edges of the explants cultured on mannose, whereas control explants did not produce any embryos. The transgenic character of the regenerating embryos was confirmed by staining in the chemical reagent X-Gluc, which causes transgenic tissue to turn blue. Regeneration of transgenic plants from embryos was achieved by subculture to hormone-free medium for rooting and further development. The transgenic character of the plants regenerated on mannose-containing medium was confirmed by staining in Xgluc.

**Example 6: Transgenic Squash**

**[0166]** Two experiments were performed in squash using the mannose-based selection. In both experiments the preparation of the explants and their inoculation was as follows:

**[0167]** Decoated squash seeds were surface sterilized in a solution containing twenty percent of a commercial household bleach containing 5.25 percent sodium hypochlorite (Clorox™) for ten minutes, rinsed briefly five times in sterile, distilled water and germinated for 24 hours in the dark at 27°C on filter paper soaked with sterile water. Plant tissue used for transformation was prepared by removing the shoot axis cotyledonary node tissue and the distal half of the cotyledonary tissue. The remaining proximal half was cut into 4 explant pieces, which are referred to as explants.

**[0168]** The explants were inoculated for 10 minutes in 10 ml per 32 explants of MS medium supplemented with 0.2 mM acetosyringone, and 2.5 x 10⁸ CFU/ml of the disarmed Agrobacterium tumefaciens, strain EHA 105, that was harboring the binary plasmid pETO156. After incubation with the Agrobacterium, the explants were blotted on sterile filter paper and placed adaxial side down on MS medium supplemented with 2 mg/l 2,4,5-T and 0.5 mg/l kinetin. Explants were cultured for 3 days in unsealed Petri dishes, in the dark.

**[0169]** After the 3 days of culture, explants were washed for 3 hours in 20-30 ml of MS liquid medium supplemented with 10 mg/l 2,4-D, 0.5 mg/l kinetin, 400 mg/l cefotaxime and 750 mg/l carbenicillin. The rinse medium was changed every hour for a total of 3 washes. After washing, explants were blotted on sterile filter paper and placed abaxial side down in Petri plates containing selection medium consisting of MS salts and vitamins, 10 mg/l 2,4-D, 0.5 mg/l kinetin, 1000 mg/l carbenicillin.

**[0170]** In the first experiment the medium contained 16.2 g/l mannose and various concentrations of KH₂PO₄ (170 mg/l, 340 mg/l or 510 mg/l). Explants were cultured in unsealed Petri dishes and kept in diffused light of approximately 10 µmol·m⁻²·s⁻¹ Photosynthetic Photon Flux Density (PPFD). Explants were subcultured to fresh medium every two weeks.
[0171] In the second experiment explants were cultured on the MS medium supplemented with the following:

16.2 g/l mannose,
16.2 g/l mannose plus 170 mg/l KH$_2$PO$_4$,
30 g/l sucrose for one week, then selection on
16.2 g/l mannose plus 170 mg/l KH$_2$PO$_4$,
30 g/l sucrose for two weeks, then selection on
16.2 g/l mannose plus 170 mg/l KH$_2$PO$_4$.

[0172] In both experiments clusters of embryos formed on the edges of the cocultivated with Agrobacterium explants after 8 weeks of culture, whereas non-cocultivated control explants did not produce embryos. Transformation of the regenerating embryos was confirmed by assaying for the expression of the GUS gene, which causes transgenic tissue to turn blue in the presence of the chemical 5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronic Acid Cyclohexylammonium Salt (X-Gluc). Because the GUS gene contains an intron, expression of this gene can occur in the eukaryotic cells, but not in the cells of the prokaryotic Agrobacterium. Explants were scored as transgenic if a blue signal was observed in the embryos. The percentage of explants producing transgenic embryos in the first experiment is shown in Table 5 and the percentage of explants producing transgenic embryos in the second experiment is shown in Table 6.

<table>
<thead>
<tr>
<th>Table 5. Effect of Phosphate Concentration on the Selection of Squash Embryos Transformed with the pmi Gene.</th>
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<tr>
<td>Treatment</td>
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<tr>
<td>MS medium + 16.2 g/l mannose</td>
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<tr>
<td>MS medium + 16.2 g/l mannose + 170 mg/l KH$_2$PO$_4$</td>
</tr>
<tr>
<td>MS medium + 16.2 g/l mannose + 340 mg/l KH$_2$PO$_4$</td>
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<td>MS medium + 16.2 g/l mannose + 510 mg/l KH$_2$PO$_4$</td>
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<th>Table 6. Effect of Carbon and Phosphate on the Selection of Squash Embryos Transformed with the pmi Gene.</th>
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<td>Treatment</td>
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<td>MS medium + 16.2 g/l mannose</td>
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<td>MS medium + 16.2 g/l mannose + 170 mg/l KH$_2$PO$_4$</td>
</tr>
<tr>
<td>MS medium + 30 g/l sucrose for 1 week, then 16.2 g/l mannose + 170 mg/l KH$_2$PO$_4$</td>
</tr>
<tr>
<td>MS medium + 30 g/l sucrose for 2 weeks, then 16.2 g/l mannose + 170 mg/l KH$_2$PO$_4$</td>
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[0173] In the first experiment the frequency of transgenic embryo production using mannose selection was increased by 5-fold if the concentration of phosphate in the selection medium was doubled and 6-fold if the concentration was tripled when compared to the control (MS medium) concentration of phosphate. The MS medium is a widely used formulation in the regeneration of many plants from tissue culture.

[0174] In the second experiment the use of mannose plus phosphate for selection resulted in a nearly two-fold increase in transformation frequency. When the explants were first grown on an MS medium containing 30 g/l sucrose and transferred a week later to an MS medium containing 16.2 g/l mannose and 170.00 mg/l KH$_2$PO$_4$, no significant increase in the transformation frequency was observed over the explants kept on MS + mannose + KH$_2$PO$_4$ medium. In contrast, a two week growth on MS medium supplemented with sucrose followed by growth on MS + mannose + KH$_2$PO$_4$ medium resulted in a nearly two-fold increase in transformation frequency over explants grown on MS + mannose + KH$_2$PO$_4$ medium. Further, the two week sucrose treatment followed by the mannose + KH$_2$PO$_4$ medium displayed a 3.5-fold increase in transformation efficiency over the mannose only treatment.

[0175] Both experiments showed that the addition of phosphate to the MS-mannose medium increased the frequency of regeneration of transgenic embryos from 3.5-fold up to 6-fold. The second experiment also showed that delay in the selection by up to two weeks resulted in an increased recovery of transgenic embryos. The differences in the frequen-
cies of transformation in these two experiments were characteristic of a high experiment to experiment variation frequently observed in tissue culture.

Example 7. Transgenic Tomato.

[0176] Seeds from two transgenic tomato lines, X11-28 and X11-40, transformed with the pmi gene were sterilized in 20% Clorox™ for 20 minutes, rinsed 3 times in sterile distilled water and planted on Murashigi and Skoog medium (Gibco) solidified with 7 g/L of commercial grade Phytagar in 135 mm Phytacon™ tissue culture vessels (Sigma, St. Louis, MO). Seeds were germinated for 72 hours at 25°C in the dark, then moved to a lighted shelf under approximately 80 µmol·m⁻²·s⁻¹ PPDF, at 24-26°C. Plant tissue used in the experiment was prepared by removing cotyledons from 7-day-old seedlings and cutting them into three sections, proximal, middle and distal to the growing point. The middle and proximal parts were placed either on the control R1 medium supplemented with 16g/L glucose or on the MR1 regeneration medium containing 16 g/L mannose. Both the R1 and MR1 media were subdivided into three treatments, which differed in the amount of monobasic potassium phosphate: 1x MS (standard concentration of 170 mg/L KH₂PO₄), 2x MS (340 mg/L total phosphate concentration) and 3x MS (510 mg/L total phosphate concentration). Each treatment was replicated three times, each replicate consisted of 9 explants. Explants were cultured for three weeks under approximately 30 µmol m⁻² s⁻¹ PPDF, at 24-26°C, 16 hr photoperiod, then moved to a lighted shelf under approximately 80 µmol m⁻² s⁻¹ PPDF. The effect of the phosphate concentration and carbon source on the growth of the explants was assessed after 6 weeks of culture by weighing each explant. The results are presented in Tables 7 and 8.

Table 7.

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<th>KH₂PO₄ level</th>
<th>Carbon source</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Replicate 3</th>
<th>Mean ± Standard Error</th>
<th>Percent Difference</th>
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<tbody>
<tr>
<td>1x (standard)</td>
<td>Glucose</td>
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<tr>
<td></td>
<td>Mannose</td>
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<td>Mannose</td>
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<td>3x</td>
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<td>Mannose</td>
<td>370.4</td>
<td>340.3</td>
<td>420.0</td>
<td>376.9 ±</td>
<td>+37.4</td>
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Table 8.

<table>
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<tr>
<th>KH₂PO₄ level</th>
<th>Carbon source</th>
<th>Replicate 1</th>
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<th>Replicate 3</th>
<th>Mean ± Standard Error</th>
<th>Percent Difference</th>
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<td>Mannose</td>
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<td>550.1</td>
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<td>2x</td>
<td>Glucose</td>
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<td></td>
<td>Mannose</td>
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<td>3x</td>
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<td>Mannose</td>
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<td>404.9</td>
<td>nt</td>
<td>431.3 ±</td>
<td>+30.3</td>
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</table>

[0177] Tomato explants transformed with the pmi gene had on average 30% higher fresh weight when cultured on the mannose medium supplemented with two- or three-fold higher levels of phosphate as compared to medium containing glucose as a carbon source. This result indicates that elevated phosphate provides a growth advantage for transgenic cells transformed with the mannose isomerase gene on mannose medium.
In a transformation experiment, there are many nontransgenic cells and a few transgenic cells. The purpose of selection is to identify and provide a growth advantage to the transgenic cells with totipotency to regenerate plants. Cells derived either from photosynthetically active tissues, such as leaves, or storage organs, such as cotyledons of germinating seeds, are typically rich in metabolizable carbon. In a transformation experiment with the mannose isomerase gene and the use of mannose medium, both the nontransgenic cells, which are present in great excess, and the transgenic cells, which are relatively scarce, have the potential to grow by using the residual intracellular supply of carbon. The transgenic cells have an additional source of carbon available to them because they can access the carbon that is present in the medium in the form of mannose. If the amount of growth due to the residual intracellular supply of carbon is significant, it will be more difficult to identify the few cells that are transgenic.

Data shown in Tables 7 and 8 demonstrate that the addition of phosphate confers a growth advantage to transgenic cells grown on mannose over transgenic cells grown on glucose. Further, these experiments suggest why transformation experiments on mannose medium supplemented with elevated phosphate levels are far more successful than experiments with mannose only. In the mannose plus phosphate environment, the transgenic cells have two growth advantages over the glucose-utilizing (i.e. nontransgenic) cells: (1) they can access additional carbon from the medium, and (2) the phosphate confers an additional growth advantage to cells capable of growing on mannose over cells utilizing glucose. This double growth advantage results in an efficient selection of transgenic cells that have the totipotency to regenerate healthy transgenic plants.
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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1650 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

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(B) LOCATION: 432..1607

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(A) LENGTH: 391 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
Claims

1. A process for selectively increasing the number of transformed plant cells regenerated from a mixture of transformed and non-transformed plant cells cultured under heterotrophic culture conditions, the method comprising the steps of:

   (a) culturing a mixture of transformed and non-transformed plant cells under heterotrophic culture conditions in a culture medium that contains minimal nutrients required for proliferation and growth by non-transformed plant cells except for a source of carbon that supports growth and proliferation and about 1.5 to 3 times the standard amount of phosphorus, said source of carbon being replaced by an encrypted carbon source that does not support growth and proliferation of said non-transformed cells and that is selected from the group of: mannose, mannitol, sorbitol, lactose, trehalose and salicin, said transformed cells having a heterologous genomic DNA segment that contains at least one expression cassette,

   the one expression cassette containing a heterologous DNA selectable marker segment that includes

   (i) a heterologous gene that encodes a heterologous enzyme that is selected from the group of: phosphomannose isomerase, mannitol-1-oxidoreductase, L-iditol dehydrogenase, D-sorbitol-1-oxidoreductase, lactase, β-galactosidase and α,α-trehalase and that on expression converts said encrypted carbon source into a carbon source that supports growth and proliferation of said transformed plant cells under heterotrophic culture conditions, said first gene being operatively linked to

   (ii) a promoter DNA segment that controls expression of said heterologous gene, and

   (iii) a termination DNA segment;

   (b) maintaining said heterotrophic culture conditions for a time period sufficient for said transformed plant cells to express said heterologous enzyme, grow and proliferate; and

   (c) recovering said transgenic proliferating cells.

2. The process according to claim 1 wherein said heterologous gene is phosphomannose isomerase and said encrypted carbon source is mannose.

3. The process according to claim 1 wherein said phosphorous is a water-soluble phosphate salt.
4. The process of claim 3 wherein the phosphate salt is selected from the group consisting of: potassium phosphate, sodium phosphate, calcium phosphate, sodium hexametaphosphate, ammonium phosphate, orthophosphoric acid, ferric phosphate, sodium glycerophosphate, ferric glycerophosphate and organic phosphate.

5. The process according to claim 1 wherein expression from said promoter DNA sequence is repressed by a product of the normal metabolism of said transgenic plant cells under autotrophic growth.

6. The process according to claim 5 wherein the repressible promoter DNA sequence is that of the cucumber isocitrate lyase promoter.

7. The process according to claim 5 wherein the repressible promoter DNA sequence is that of the rice α-amylase Amy3A promoter.

8. The process according to claim 1 wherein the promoter is a constitutive promoter.

9. The process according to claim 8 wherein the constitutive promoter is selected from the group consisting of: the 35S promoter of the cauliflower mosaic virus, the octopine synthase promoter and the nopaline synthase promoter.

10. A process for selectively increasing the number of transformed plants regenerated from a mixture of transformed and non-transformed plant cells cultured under heterotrophic culture conditions, the method comprising the steps of

(a) culturing a mixture of transformed and non-transformed plant cells under heterotrophic culture conditions in a culture medium that contains minimal nutrients required for proliferation and growth by non-transformed plant cells except for a source of carbon that supports growth and proliferation and about 1.5 to 3 times the standard amount of phosphorus; said source of carbon being replaced by an encrypted carbon source that does not support growth and proliferation of said non-transformed cells and that is selected from the group of: mannose, mannitol, sorbitol, lactose, trehalose and salicin, said transformed cells containing a heterologous genomic DNA segment that contains at least one expression cassette, the one expression cassette containing a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that is selected from the group of: phosphomannose isomerase, mannitol-1-oxidoreductase, L-iditol dehydrogenase, D-sorbitol-1-oxidoreductase, lactase, β-galactosidase and α,α-trehalase and that on expression converts said encrypted carbon source into a carbon source that supports growth and proliferation of said transformed plant cells under heterotrophic culture conditions, said first gene being operatively linked to (ii) a first promoter DNA segment that controls expression of said heterologous gene, and (iii) a termination DNA segment;
(b) maintaining said heterotrophic culture conditions for a time period sufficient for said transformed plant cells to express said heterologous enzyme, grow and proliferate;
(c) recovering said proliferating cells; and
(d) regenerating plant meristematic tissues or plant embryos from said proliferating cells.

11. The process according to claim 10 wherein said heterologous gene is phosphomannose isomerase and said encrypted carbon source is mannose.

12. The process according to claim 10 wherein said phosphorous is a water-soluble phosphate salt.

13. The process of claim 12 wherein the phosphate salt is selected from the group consisting of: potassium phosphate, sodium phosphate, calcium phosphate, sodium hexametaphosphate, ammonium phosphate, orthophosphoric acid, ferric phosphate, sodium glycerophosphate, ferric glycerophosphate and organic phosphate.

14. The process according to claim 10 wherein expression from said promoter DNA sequence is repressed by a product of the normal metabolism of said transgenic plants under autotrophic growth.

15. The process according to claim 14 wherein the repressible promoter DNA sequence is that of the cucumber isocitrate lyase promoter.

16. The process according to claim 14 wherein the repressible promoter DNA sequence is that of the rice α-amylase Amy3A promoter.
17. The process according to claim 10 wherein the promoter is a constitutive promoter.

18. The process according to claim 17 wherein the constitutive promoter is selected from the group consisting of: the 35S promoter of the cauliflower mosaic virus, the octopine synthase promoter and the nopaline synthase promoter.

19. A process for selectively increasing the number of transformed plant cells regenerated from a mixture of transformed and non-transformed plant cells placed under selective heterotrophic culture conditions, the method comprising the steps of:

(a) culturing a mixture of transformed and non-transformed plant cells for up to two weeks in a first culture medium that contains the minimal nutrients required for proliferation and growth by non-transformed plant cells including a source of carbon that supports growth and proliferation, said transformed plants cells containing a genomic heterologous DNA segment that contains at least one expression cassette, the one expression cassette containing a heterologous DNA selectable marker segment that includes (i) a heterologous gene that encodes a heterologous enzyme that is selected from the group of: phosphomannose isomerase, mannitol-1-oxidoreductase, L-iditol dehydrogenase, D-sorbitol-1-oxidoreductase, lactase, β-galactosidase and α,α-trehalase and that on expression converts an encrypted carbon source into a carbon source that supports growth and proliferation of said transformed plant cells under heterotrophic culture conditions, said first gene being operatively linked to (ii) a promoter DNA segment that controls expression of said heterologous gene, and (iii) a termination DNA segment;

(b) removing the mixture of transformed and non-transformed plant cells from the first culture medium;

(c) placing the transformed and non-transformed plant cells under heterotrophic culture conditions in a second culture medium that contains the minimal nutrients required for proliferation and growth of the non-transformed plant cells except for an encrypted carbon source that does not support growth and proliferation of said non-transformed plant cells and that is selected from the group of: mannose, mannitol, sorbitol, lactose, trehalose and salicin, and 1.5 to 3 times the standard amount of phosphorous;

(d) maintaining said heterotrophic culture conditions for a time period sufficient for said transformed plant cells to express said heterologous gene, grow and proliferate; and

(e) recovering said proliferating cells.

20. The process according to claim 19 wherein said heterologous gene is phosphomannose isomerase and said encrypted carbon source is mannose.

21. The process according to claim 19 wherein said phosphorous is a water-soluble phosphate salt.

22. The process according to claim 21 wherein the phosphate salt is selected from the group consisting of: potassium phosphate, sodium phosphate, calcium phosphate, sodium hexametaphosphate, ammonium phosphate, orthophosphoric acid, ferric phosphate, sodium glycerophosphate, ferric glycerophosphate and organic phosphate.

23. The process according to claim 19 wherein expression from said promoter DNA sequence is repressed by a product of the normal metabolism of said transgenic plants under autotrophic growth.

24. The process according to claim 23 wherein the repressible promoter DNA sequence is that of the cucumber iso-citrate lyase promoter.

25. The process according to claim 23 wherein the repressible promoter DNA sequence is that of the rice α-amylase Amy3A promoter.

26. The process according to claim 23 wherein the promoter is a constitutive promoter.

27. The process according to claim 26 wherein the constitutive promoter is selected from the group consisting of: the 35S promoter of the cauliflower mosaic virus, the octopine synthase promoter and the nopaline synthase promoter.

28. A process for selectively growing twice-transformed plant cells from a mixture of twice- and once-transformed plant cells comprising the steps of:

(a) culturing a mixture of twice- and once-transformed plant cells under heterotrophic culture conditions in a culture medium that contains minimal nutrients required for proliferation and growth by said once-transformed....
plant cells except for a source of carbon that supports growth and proliferation of said once-transformed cells and about 1.5 to 3 times the standard amount of phosphorus, said source of carbon being replaced by a second encrypted carbon source that does not support growth and proliferation of said once-transformed plant cells and that is selected from the group of: mannose and mannitol; said twice-transformed cells containing first and a second heterologous DNA segments that contains at least two expression cassettes, wherein at least one expression cassette is in the first heterologous DNA segment and at least one expression cassette is in the second heterologous DNA segment;

the expression cassette in the first heterologous DNA segment containing a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that is selected from the group of: phosphomannose isomerase and mannitol-1-oxidoreductase and that on expression converts a first encrypted carbon source into a carbon source that supports growth and proliferation of said once- and twice-transformed plant cells under heterotrophic culture conditions but does not support growth and proliferation of non-transformed plant cells, said first gene being operatively linked to (ii) a first promoter DNA segment that controls expression of said heterologous gene, and (iii) a termination DNA segment;

the expression cassette in the second heterologous DNA segment containing a second heterologous DNA selectable marker segment that includes (i) a second heterologous gene that encodes a second heterologous enzyme that is selected from the group of: phosphomannose isomerase, and mannitol-1-oxidoreductase and that on expression during heterotrophic culture of said twice-transformed cells converts said second encrypted carbon source that does not support growth and proliferation of once-transformed and non-transformed plant cells of the same type into said first encrypted carbon source that supports growth and proliferation of said twice- and once-transformed cells, said second gene being operatively linked to (ii) a second promoter DNA segment that controls expression of said second heterologous gene and (iii) a termination DNA segment; (b) maintaining said heterotrophic culture conditions for a time period sufficient for said twice-transformed plant cells to express said first and second heterologous enzymes, grow and proliferate; and

(c) recovering said proliferating cells.

29. The process according to claim 28 wherein said second heterologous DNA selectable marker segment encodes the enzyme mannitol-1-oxo-reductase, said second encrypted carbon source is mannitol, said first gene that encodes a heterologous gene encodes phosphomannose isomerase and said first encrypted carbon source is mannose.

30. A process for selectively growing twice-transformed plants from a mixture of twice- and once-transformed plant cells comprising the steps of:

(a) culturing a mixture of twice- and once-transformed plant cells under heterotrophic culture conditions in a culture medium that contains minimal nutrients required for proliferation and growth by said once-transformed plant cells except for a source of carbon that supports growth and proliferation of said once-transformed cells and about 1.5 to 3 times the standard amount of phosphorus, said source of carbon being replaced by a second encrypted carbon source that does not support growth and proliferation of said once-transformed plant cells and that is selected from the group of: mannose and mannitol; said twice-transformed cells containing first and a second heterologous DNA segments that contains at least two expression cassettes, wherein at least one expression cassette is in the first heterologous DNA segment and at least one expression cassette is in the second heterologous DNA segment;

the expression cassette in the first heterologous DNA segment containing a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that is selected from the group of: phosphomannose isomerase and mannitol-1-oxidoreductase and that on expression converts a first encrypted carbon source into a carbon source that supports growth and proliferation of said once- and twice-transformed plant cells under heterotrophic culture conditions but does not support growth and proliferation of non-transformed plant cells, said first heterologous gene being operatively linked to (ii) a first promoter DNA segment that controls expression of said first heterologous gene, and (iii) a termination DNA segment;

the expression cassette in the second heterologous DNA segment containing a second heterologous DNA selectable marker segment that includes (i) a second heterologous gene that encodes a second heterologous enzyme that is selected from the group of: phosphomannose isomerase and mannitol-1-oxidoreductase and that on expression during heterotrophic culture of said twice-transformed cells converts said second encrypted carbon source that does not support growth and proliferation of once-transformed and non-transformed plant cells of the same type into said first encrypted carbon source that supports growth and proliferation of said twice- and once-transformed cells, said second heterologous gene being operatively linked to (ii) a
second promoter DNA segment that controls expression of said second heterologous gene and (iii) a termination DNA segment;
(b) maintaining said heterotrophic culture conditions for a time period sufficient for said twice-transformed plant cells to express said first and second heterologous enzymes, grow and proliferate;
(c) recovering said proliferating cells; and
(d) regenerating plant meristematic tissues or plant embryos from said proliferating cells.

31. The process according to claim 30 wherein said second heterologous DNA selectable marker segment encodes the enzyme mannitol-1-oxo reductase, said second encrypted carbon source is mannitol, said first gene that encodes for the expression of phosphomannose isomerase and said first encrypted carbon source is mannose.

32. A kit for forming transformed plant cells comprising:
(a) a first package containing a DNA segment for transforming plant cells that contains an expression cassette operatively linked to a linker segment containing at least one restriction endonuclease site, said expression cassette containing a heterologous DNA selectable marker segment that includes (i) a first heterologous gene that encodes a heterologous enzyme that is selected from the group of: phosphomannose isomerase, mannitol-1-oxidoreductase, L-iditol dehydrogenase, D-sorbitol-1-oxidoreductase, lactase, β-galactosidase and α,α-trehalase and on expression during heterotrophic culture of transformed plant cells converts an encrypted carbon source that does not support growth and proliferation of non-transformed plant cells into a carbon source that supports growth and proliferation of said transformed cells and that is selected from the group of: mannose, mannitol, sorbitol, lactose, trehalose and salicin, said first gene being operatively linked to (ii) a promoter DNA segment that controls expression of said first heterologous gene and (iii) a termination DNA segment; and
(b) a second package that contains minimal nutrients required for proliferation and growth of non-transformed plant cells during heterotrophic culture except for a source of carbon and about 1.5 to 3 times the standard amount of phosphorus, said source of carbon being replaced by an encrypted carbon source that does not support growth and proliferation of non-transformed plant cells but supports growth and proliferation of transformed plant cells whose genome contains the DNA segment of said first package and that is selected from the group of: mannose, mannitol, sorbitol, lactose, trehalose and salicin.

33. The kit according to claim 32 wherein said heterologous gene is phosphomannose isomerase and said encrypted carbon source is mannose.

34. The kit according to claim 32 wherein said first promoter DNA segment that controls expression of said heterologous gene is repressed by a product of the normal metabolism of said transgenic plants under autotrophic growth.

35. The kit according to claim 32 wherein said linker segment contains a plurality of endonuclease restriction sites.

36. The kit according to claim 32 wherein said DNA segment of (a) is located within the TDNA borders of the Ti plasmid of Agrobacterium tumefaciens.

37. The kit according to claim 32 wherein said phosphorous is a water-soluble phosphate salt.

38. The kit according to claim 37 wherein the phosphate salt is selected from the group consisting of: potassium phosphate, sodium phosphate, calcium phosphate, sodium hexametaphosphate, ammonium phosphate, orthophosphoric acid, ferric phosphate, sodium glycerophosphate, ferric glycerophosphate and organic phosphate.

Patentansprüche

1. Verfahren zur selektiven Erhöhung der Anzahl von transformierten Pflanzenzellen, welche aus einer Mischung von transformierten und nicht-transformierten Pflanzenzellen, die unter heterotrophen Kulturbedingungen gezüchtet werden, regeneriert werden, wobei das Verfahren die Schritte umfaßt:

(a) Züchten einer Mischung von transformierten und nicht-transformierten Pflanzenzellen unter heterotrophen Kulturbedingungen in einem Kulturmedium, das die minimalen Nährstoffe, welche für die Proliferation und das Wachstum der nicht-transformierten Pflanzenzellen erforderlich sind, außer einer Kohlenstoffquelle, welche
das Wachstum und die Proliferation fördert, und etwa das 1,5- bis 3-fache der üblichen Menge an Phosphor enthält, wobei die Kohlenstoffquelle durch eine maskierte Kohlenstoffquelle ersetzt ist, welche nicht das Wachstum und die Proliferation der nicht-transformierten Zellen fördert, und welche gewählt ist aus der Gruppe, bestehend aus Mannose, Mannit, Sorbit, Lactose, Trehalose und Salicin, wobei die transformierten Zellen ein heterologes genomisches DNA-Segment besitzen, das mindestens eine Expressionskassette enthält, wobei die eine Expressionskassette einen selektierbaren Marker in Form eines heterologen DNA-Segments enthält, umfassend (i) ein heterologes Gen, welches ein heterologes Enzym codiert, ausgewählt aus der Gruppe, bestehend aus Phosphomannose-Isomerase, Mannit-1-Oxidoreductase, L-Iditol-Dehydrogenase, D-Sorbit-1-Oxidoreductase, Lactase, β-Galactosidase und α,α-Trehalase, das bei der Expression die maskierte Kohlenstoffquelle in eine Kohlenstoffquelle umwandelt, welche das Wachstum und die Proliferation der transformierten Pflanzenzellen unter heterotrophen Kulturbedingungen fördert, wobei das erste Gen mit (ii) einem Promotor-DNA-Segment, welches die Expression des heterologen Gens reguliert, und (iii) einem TerminatordNA-Segment funktionell verbunden ist; (b) Aufrechterhalten der heterotrophen Kulturbedingungen während eines ausreichenden Zeitraums, um zu ermöglichen, daß die transformierten Pflanzenzellen das heterologe Enzym exprimieren, wachsen und sich vermehren; und (c) Gewinnen der transgenen proliferierenden Zellen.

2. Verfahren nach Anspruch 1, wobei das heterologe Gen eine Phosphomannose-Isomerase ist, und worin die maskierte Kohlenstoffquelle Mannose ist.

3. Verfahren nach Anspruch 1, wobei der Phosphor in Form eines wasserlöslichen Phosphatsalzes vorliegt.


5. Verfahren nach Anspruch 1, wobei die Expression ausgehend von der Promotor-DNA-Sequenz durch ein Produkt des normalen Stoffwechsels der transgenen Pflanzenzellen unter autotrophen Wachstumsbedingungen reprimiert wird.

6. Verfahren nach Anspruch 5, wobei die reprimierbare Promotor-DNA-Sequenz aus dem Isocitrat-Lyase-Promotor der Gurke abgeleitet ist.

7. Verfahren nach Anspruch 5, wobei die reprimierbare Promotor-DNA-Sequenz aus dem α-Amylase-Amy3A-Promotor von Reis abgeleitet ist.

8. Verfahren nach Anspruch 8, wobei der Promotor ein konstitutiver Promotor ist.


10. Verfahren zur selektiven Erhöhung der Anzahl von transformierten Pflanzenzellen, welche aus einer Mischung von transformierten und nicht-transformierten Pflanzenzellen, die unter heterotrophen Kulturbedingungen gezüchtet werden, regeneriert werden, wobei das Verfahren die Schritte umfaßt:

(a) Züchten einer Mischung von transformierten und nicht-transformierten Pflanzenzellen unter heterotrophen Kulturbedingungen in einem Kulturmedium, das die minimalen Nährstoffe, welche für die Proliferation und das Wachstum der nicht-transformierten Pflanzenzellen erforderlich sind, außer einer Kohlenstoffquelle, welche das Wachstum und die Proliferation fördert, und etwa das 1,5- bis 3-fache der üblichen Menge an Phosphor enthält, wobei die Kohlenstoffquelle durch eine maskierte Kohlenstoffquelle ersetzt ist, welche nicht das Wachstum und die Proliferation der nicht-transformierten Zellen fördert, und welche gewählt ist aus der Gruppe, bestehend aus Mannose, Mannit, Sorbit, Lactose, Trehalose und Salicin, wobei die transformierten Zellen ein heterologes genomisches DNA-Segment enthalten, welches mindestens eine Expressionskassette enthält, wobei die eine Expressionskassette einen selektierbaren Marker in Form eines heterologen DNA-Segments enthält, umfassend (i) ein erstes heterologes Gen, welches ein heterologes Enzym codiert, ausgewählt aus der Gruppe, bestehend aus Phosphomannose-Isomerase, Mannit-1-Oxidoreductase, L-Iditol-Dehydrogena-
5 se, D-Sorbit-1-Oxidoreductase, Lactase, β-Galactosidase und α-α-Trehalase, welches bei der Expression die maskierte Kohlenstoffquelle in eine Kohlenstoffquelle umwandelt, welche das Wachstum und die Proliferation der transformierten Pflanzenzellen unter heterotrophen Kulturbedingungen fördert, wobei das erste Gen mit (ii) einem ersten Promotor-DNA-Segment, welches die Expression des heterologen Gens reguliert, und (iii) einem Terminator-DNA-Segment funktionell verbunden ist; 
(b) Aufrechterhalten der heterotrophen Kulturbedingungen während eines ausreichenden Zeitraums, um zu ermöglichen, daß die transformierten Pflanzenzellen das heterologe Enzym exprimieren, wachsen und sich vermehren;
(c) Gewinnen der proliferierenden Zellen; und 
(d) Regenerieren von pflanzlichen Meristemgeweben oder Pflanzenembryonen aus den proliferierenden Zellen.

11. Verfahren nach Anspruch 10, wobei das heterologe Gen eine Phosphomannose-Isomerase ist, und worin die maskierte Kohlenstoffquelle Mannose ist.

12. Verfahren nach Anspruch 10, wobei der Phosphor in Form eines wasserlöslichen Phosphatsalzes vorliegt.


15. Verfahren nach Anspruch 14, wobei die reprimierbare Promotor-DNA-Sequenz aus dem Isocitrat-Lyase-Promotor der Gurke abgeleitet ist.


17. Verfahren nach Anspruch 10, wobei der Promotor ein konstitutiver Promotor ist.


19. Verfahren zur selektiven Erhöhung der Anzahl von transformierten Pflanzenzellen, welche aus einer Mischung von transformierten und nicht-transformierten Pflanzenzellen unter selektiven heterotrophen Kulturbedingungen regeneriert werden, wobei das Verfahren die Schritte umfaßt:

(a) Züchten einer Mischung von transformierten und nicht-transformierten Pflanzenzellen während bis zu zwei Wochen in einem ersten Kulturmedium, enthaltend die minimalen Nährstoffe, welche für die Proliferation und das Wachstum der nicht-transformierten Pflanzenzellen erforderlich sind, einschließlich einer Kohlenstoffquelle, welche das Wachstum und die Proliferation fördert, wobei die transformierten Pflanzenzellen ein heterologes genomisches DNA-Segment enthalten, welches mindestens eine Expressionskassette enthält, wobei die eine Expressionskassette einen selektierbaren Marker in Form eines heterologen DNA-Segments enthält, umfassend (i) ein heterologes Gen, welches ein heterologes Enzym codiert, ausgewählt aus der Gruppe, bestehend aus Phosphomannose-Isomerase, Mannit-1-Oxidoreductase, L-Iditol-Dehydrogenase, D-Sorbit-1-Oxidoreductase, Lactase, β-Galactosidase und α-α-Trehalase, welches bei der Expression eine maskierte Kohlenstoffquelle in eine Kohlenstoffquelle umwandelt, welche das Wachstum und die Proliferation der transformierten Pflanzenzellen unter heterotrophen Kulturbedingungen fördert, wobei das erste Gen mit (ii) einem Promotor-DNA-Segment, welches die Expression des heterologen Gens reguliert, und (iii) einem Terminator-DNA-Segment funktionell verbunden ist; 
(b) Entfernen der Mischung von transformierten und nicht-transformierten Pflanzenzellen aus dem ersten Kulturmedium; 
(c) Überführen der transformierten und nicht-transformierten Pflanzenzellen unter heterotrophen Kulturbedingungen in ein zweites Kulturmedium, welches die minimalen Nährstoffe, welche für die Proliferation und das Wachstum der nicht-transformierten Pflanzenzellen erforderlich sind, außer einer maskierten Kohlenstoffquelle, welche nicht das Wachstum und die Proliferation der nicht-transformierten Pflanzenzellen fördert, und wel-

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che gewählt ist aus der Gruppe, bestehend aus Mannose, Mannit, Sorbit, Lactose, Trehalose und Salicin, und etwa das 1,5- bis 3-fache der üblichen Menge an Phosphor enthält;
(d) Aufrechterhalten der heterotrophen Kulturbedingungen während eines ausreichenden Zeitraums, um zu ermöglichen, daß die transformierten Pflanzenzellen das heterologe Enzym exprimieren, wachsen und sich vermehren; und
(e) Gewinnen der proliferierenden Zellen.

20. Verfahren nach Anspruch 19, wobei das heterologe Gen eine Phosphomannose-Isomerase ist, und worin die maskierte Kohlenstoffquelle Mannose ist.

21. Verfahren nach Anspruch 19, wobei der Phosphor in Form eines wasserlöslichen Phosphatsalzes vorliegt.


27. Verfahren nach Anspruch 26, wobei der konstitutive Promotor gewählt ist aus der Gruppe, bestehend aus dem 35S-Promotor des Blumenkohlmosaikvirus, dem Octopinsynthase-Promotor und dem Nopalinsynthase-Promotor.

28. Verfahren zur selektiven Vermehrung von zweimal transformierten Pflanzenzellen aus einer Mischung von zweimal und einmal transformierten Pflanzenzellen, umfassend die Schritte:

(a) Züchten einer Mischung von zweimal und einmal transformierten Pflanzenzellen unter heterotrophen Kulturbedingungen in einem Kulturmedium, das die minimalen Nährstoffe, welche für die Proliferation und das Wachstum der einmal transformierten Pflanzenzellen erforderlich sind, außer einer Kohlenstoffquelle, welche das Wachstum und die Proliferation der einmal transformierten Zellen fördert, und etwa das 1,5- bis 3-fache der üblichen Menge an Phosphor enthält, wobei die Kohlenstoffquelle durch eine zweite maskierte Kohlenstoffquelle ersetzt ist, welche nicht das Wachstum und die Proliferation der einmal transformierten Pflanzenzellen fördert, und welche gewählt ist aus der Gruppe, bestehend aus Mannose und Mannit, wobei die zweimal transformierten Zellen ein erstes und ein zweites heterologes DNA-Segment enthalten, worin mindestens zwei Expressionskassetten enthalten sind, wobei mindestens eine Expressionskassette in dem ersten heterologen DNA-Segment enthalten ist und mindestens eine Expressionskassette in dem zweiten heterologen DNA-Segment enthalten ist,

wobei die Expressionskassette in dem ersten heterologen DNA-Segment einen selektierbaren Marker in Form eines heterologen DNA-Segments enthält, umfassend (i) ein erstes heterologes Gen, welches ein heterologes Enzym codiert, ausgewählt aus der Gruppe, bestehend aus Phosphomannose-Isomerase und Mannit-1-Oxidoreductase, welches bei der Expression eine erste maskierte Kohlenstoffquelle in eine Kohlenstoffquelle umwandelt, welche das Wachstum und die Proliferation der einmal und zweimal transformierten Pflanzenzellen unter heterotrophen Kulturbedingungen fördert, aber nicht das Wachstum und die Proliferation von nicht-transformierten Pflanzenzellen begünstigt, wobei das erste Gen mit (ii) einem ersten Promotor-DNA-Segment, welches die Expression des heterologen Gens reguliert, und (iii) einem Terminator-DNA-Segment funktionell verbunden ist;

wobei die Expressionskassette in dem zweiten heterologen DNA-Segment einen zweiten selektierbaren Marker in Form eines heterologen DNA-Segments enthält, umfassend (i) ein zweites heterologes Gen, welches ein zweites heterologes Enzym codiert, ausgewählt aus der Gruppe, bestehend aus Phosphomannose-Isomerase und Mannit-1-Oxidoreductase, welches bei der Expression während der heterotrophen Kultur der zweimal transformierten Zellen die zweite maskierte Kohlenstoffquelle, welche nicht das Wachstum und die
Proliferation der einmal und nicht-transformierten Pflanzenzellen desselben Typs fördert, in die erste maskierte Kohlenstoffquelle umwandelt, welche das Wachstum und die Proliferation der zweimal und einmal transformierten Zellen begünstigt, wobei das zweite Gen mit (ii) einem zweiten Promotor-DNA-Segment, welches die Expression des zweiten heterologen Gens reguliert, und (iii) einem Terminator-DNA-Segment funktionell verbunden ist;

(b) Aufrechterhalten der heterotrophen Kulturbedingungen während eines ausreichenden Zeitraums, um zu ermöglichen, daß die zweimal transformierten Pflanzenzellen das erste und das zweite heterologe Enzym exprimieren, wachsen und sich vermehren; und

(c) Gewinnen der proliferierenden Zellen.

29. Verfahren nach Anspruch 28, wobei der zweite selektierbare Marker in Form eines heterologen DNA-Segments das Enzym Mannit-1-Oxidoreductase codiert; die zweite maskierte Kohlenstoffquelle Mannit ist; das erste Gen, welches ein heterologes Enzym codiert, eine Phosphomannose-Isomerase codiert; und die erste maskierte Kohlenstoffquelle Mannose ist.

30. Verfahren zur selektiven Züchtung von zweimal transformierten Pflanzen aus einer Mischung von zweimal und einmal transformierten Pflanzenzellen, umfassend die Schritte:

(a) Züchten einer Mischung von zweimal und einmal transformierten Pflanzenzellen unter heterotrophen Kulturbedingungen in einem Kulturmedium, das die minimalen Nährstoffe, welche für die Proliferation und das Wachstum der einmal transformierten Pflanzenzellen erforderlich sind, außer einer Kohlenstoffquelle, welche das Wachstum und die Proliferation der einmal transformierten Zellen fördert, und etwa das 1,5- bis 3-fache der üblichen Menge an Phosphor enthält, wobei die Kohlenstoffquelle durch eine zweite maskierte Kohlenstoffquelle ersetzte ist, welche nicht das Wachstum und die Proliferation der einmal transformierten Pflanzenzellen fördert, und welche gewählt ist aus der Gruppe, bestehend aus Mannose und Mannit; wobei die zweimal transformierten Zellen ein erstes und ein zweites heterologes DNA-Segment enthalten, worin mindestens zwei Expressionsskassetten enthalten sind. wobei mindestens eine Expressionsskasse in dem ersten heterologen DNA-Segment enthalten ist und mindestens eine Expressionsskasse in dem zweiten heterologen DNA-Segment enthält, wobei die Expressionen des ersten heterologen Gens reguliert, und (iii) einem Terminator-DNA-Segment funktionell verbunden ist;

wobei die Expressionsskasse in dem ersten heterologen DNA-Segment einen selektierbaren Marker in Form eines heterologen DNA-Segments enthält, umfassend (i) ein erstes heterologes Gen, welches ein heterologes Enzym codiert, ausgewählt aus der Gruppe, bestehend aus Phosphomannose-Isomerase und Mannit-1-Oxidoreductase, welches bei der Expression eine erste maskierte Kohlenstoffquelle in eine Kohlenstoffquelle umwandelt, welche das Wachstum und die Proliferation der einmal und zweimal transformierten Pflanzenzellen unter heterotrophen Kulturbedingungen fördert, aber nicht das Wachstum und die Proliferation von nicht-transformierten Zellen begünstigt, wobei das zweite heterologe Gen mit (ii) einem ersten Promotor-DNA-Segment, welches die Expression des ersten heterologen Gens reguliert, und (iii) einem Terminator-DNA-Segment funktionell verbunden ist;

wobei die Expressionsskasse in dem zweiten heterologen DNA-Segment einen zweiten selektierbaren Marker in Form eines heterologen DNA-Segments enthält, umfassend (i) ein zweites heterologes Gen, welches ein zweites heterologes Enzym codiert, ausgewählt aus der Gruppe, bestehend aus Mannit-1-Oxidoreductase, welche bei der Expression während der heterotrophen Kultur der zweimal transformierten Zellen die zweite maskierte Kohlenstoffquelle, welche nicht das Wachstum und die Proliferation von einmal und nicht-transformierten Pflanzenzellen desselben Typs fördert, in die erste maskierte Kohlenstoffquelle umwandelt, welche das Wachstum und die Proliferation der zweimal und einmal transformierten Zellen fördert, wobei das zweite heterologe Gen mit (ii) einem zweiten Promotor-DNA-Segment, welches die Expression des zweiten heterologen Gens reguliert, und (iii) einem Terminator-DNA-Segment funktionell verbunden ist;

(b) Aufrechterhalten der heterotrophen Kulturbedingungen während eines ausreichenden Zeitraums, um zu ermöglichen, daß die zweimal transformierten Pflanzenzellen das erste und das zweite heterologe Enzym exprimieren, wachsen und sich vermehren; und

(c) Gewinnen der proliferierenden Zellen; und

(d) Regenerieren von pflanzlichen Meristemgeweben oder Pflanzenembryonen aus den proliferierenden Zellen.

31. Verfahren nach Anspruch 30, wobei der zweite selektierbare Marker in Form eines heterologen DNA-Segments das Enzym Mannit-1-Oxidoreductase codiert; die zweite maskierte Kohlenstoffquelle Mannit ist; das erste Gen die Expression einer Phosphomannose-Isomerase codiert; und die erste maskierte Kohlenstoffquelle Mannose ist.
32. Kit zur Erzeugung von transformierten Pflanzenzellen, umfassend:

(a) einen ersten Behälter, enthaltend ein DNA-Segment zur Transformation von Pflanzenzellen, das eine Expressionskassette enthält, welche funktional verbunden ist mit einem Linker-Segment, das mindestens eine Endonuclease-Restriktionsstelle enthält, wobei die Expressionskassette einen selektierbaren Marker in Form eines heterologen DNA-Segments enthält, umfassend (i) ein erstes heterologes Gen, welches ein heterologes Enzym codiert, ausgewählt aus der Gruppe, bestehend aus Phosphomannose-Isomerase, Mannit-1-Oxidoreductase, L-Iditol-Dehydrogenase, D-Sorbit-1-Oxidoreductase, Lactase, β-Galactosidase und α,α-Trehalsa-

β

se, welches bei der Expression während der heterotrophen Kultur von transformierten Pflanzenzellen eine maskierte Kohlenstoffquelle, welche nicht das Wachstum und die Proliferation von nicht-transformierten Pflanzenzellen fördert, in eine Kohlenstoffquelle umwandelt, welche das Wachstum und die Proliferation der transformierten Zellen fördert, ausgewählt aus der Gruppe, bestehend aus Mannose, Mannit, Sorbit, Lactose, Trehalose und Salicin, wobei das erste Gen mit (ii) einem Promotor-DNA-Segment, welches die Expression des ersten heterologen Gens reguliert, und (iii) einem Terminator-DNA-Segment funktionell verbunden ist; und (b) einen zweiten Behälter, der die minimalen Nährstoffe, welche für die Proliferation und das Wachstum von nicht-transformierten Pflanzenzellen während der heterotrophen Kultur erforderlich sind, außer einer Kohlenstoffquelle, und etwa das 1,5- bis 3-fache der üblichen Menge an Phosphor enthält, wobei die Kohlenstoffquelle durch eine maskierte Kohlenstoffquelle ersetzt ist, welche nicht das Wachstum und die Proliferation von nicht-transformierten Pflanzenzellen fördert, aber das Wachstum und die Proliferation von transformierten Pflanzenzellen begünstigt, deren Genom das DNA-Segment aus dem ersten Behälter enthält, und welche aus der Gruppe von Mannose, Mannit, Sorbit, Lactose, Trehalose und Salicin gewählt ist.

33. Kit nach Anspruch 32, wobei das heterologe Gen eine Phosphomannose-Isomerase ist, und worin die maskierte Kohlenstoffquelle Mannose ist.

34. Kit nach Anspruch 32, wobei das erste Promotor-DNA-Segment, welches die Expression des heterologen Gens reguliert, durch ein Produkt des normalen Stoffwechsels der transgenen Pflanzen unter autotrophen Wachstumsbedingungen reprimiert wird.


36. Kit nach Anspruch 32, wobei das DNA-Segment aus (a) innerhalb der T-DNA-Grenzen des Ti-Plasmids von Agro-
bacterium tumefaciens angeordnet ist.

37. Kit nach Anspruch 32, wobei der Phosphor in Form eines wasserlöslichen Phosphatsalzes vorliegt.


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Revendications

1. Procédé destiné à augmenter de manière sélective le nombre de cellules végétales transformées régénérées à partir d'un mélange de cellules végétales transformées et non transformées cultivées dans des conditions de culture hétérotrophes, le procédé comprenant les étapes de :

(a) culture d'un mélange de cellules végétales transformées et non transformées dans des conditions de culture hétérotrophes dans un milieu de culture qui contient les nutriments minimaux nécessaires à la prolifération et la croissance des cellules végétales non transformées à l'exception d'une source de carbone qui promeut la croissance et la prolifération et environ 1,5 à 3 fois la quantité standard de phosphore, ladite source de carbone étant remplacée par une source de carbone cryptée qui ne promeut pas la croissance et la prolifération des cellules non transformées et qui est choisie parmi le groupe composé : du mannose, du mannitol, du sorbitol, du lactose, du tréhalose et de la salicine, lesdites cellules transformées possédant un segment d'ADN génomique héterologue qui contient au moins une cassette d'expression, la cassette d'expression contenant un segment marqueur sélectionnable d'ADN héterologue qui comprend (i) un gène héterologue qui code pour une enzyme héterologue qui est choisie parmi le groupe composé : de la phosphomannose isomérase, de la mannitol-1-oxydoréductase, de la L-iditol déshydrogénase, de la D-
sorbitol-1-oxydoréductase, de la lactase, de la β-galactosidase et de l’α,α-tréhalase et qui lors de l’expression convertit ladite source de carbone cryptée en une source de carbone qui promeut la croissance et la prolifération desdites cellules végétales transformées dans des conditions de culture hétérotrophes, ledit premier gène étant lié de manière opérante (ii) à un segment d’ADN promoteur qui contrôle l’expression dudit gène hétérologue, et (iii) à un segment d’ADN de terminaison ;
(b) maintien desdites conditions de culture hétérotrophes pendant un temps suffisant pour que lesdites cellules végétales transformées expriment ladite enzyme hétérologue, croissent et prolifèrent ; et
(c) récupération desdites cellules proliférantes transgéniques.

2. Procédé selon la revendication 1 dans lequel ledit gène hétérologue est celui de la phosphomannose isomérase et ladite source de carbone cryptée est le mannose.

3. Procédé selon la revendication 1, dans lequel ledit phosphore est un sel de phosphate soluble dans l’eau.


5. Procédé selon la revendication 1, dans lequel l’expression de ladite séquence d’ADN promoteur est réprimée par un produit du métabolisme normal desdites cellules végétales transgéniques en croissance autotrophe.

6. Procédé selon la revendication 5 dans lequel la séquence d’ADN promoteur répressible est celle du promoteur de l’isocitrates lyase du concombre.


8. Procédé selon la revendication 1 dans lequel le promoteur est un promoteur constitutif.


10. Procédé destiné à augmenter de manière sélective le nombre de végétaux transformés régénérés à partir d’un mélange de cellules végétales transformées et non transformées cultivées dans des conditions de culture hétérotrophes, le procédé comprenant les étapes de :

(a) culture d’un mélange de cellules végétales transformées et non transformées dans des conditions de culture hétérotrophes dans un milieu de culture qui contient les nutriments minimaux nécessaires à la prolifération et la croissance des cellules végétales transformées à l’exception d’une source de carbone qui promeut la croissance et la prolifération et environ 1,5 à 3 fois la quantité standard de phosphore, ladite source de carbone étant remplacée par une source de carbone cryptée qui ne promeut pas la croissance et la prolifération desdites cellules non transformées et qui est choisie parmi le groupe composé : du mannose, du mannitol, du sorbitol, du lactose, du tréhalose et de la salicine, lesdites cellules transformées contenant un segment d’ADN génomique hétérologue qui contient au moins une cassette d’expression,

la cassette d’expression contenant un segment marqueur sélectionnable d’ADN hétérologue qui comprend (i) un premier gène hétérologue qui code pour une enzyme hétérologue qui est choisie parmi le groupe composé : de la phosphomannose isomérase, de la mannitol-1-oxydoréductase, de la L-iditol déshydrogénase, de la D-sorbitol-1-oxydoréductase, de la lactase, de la β-galactosidase et de l’α,α-tréhalase et qui lors de l’expression convertit ladite source de carbone cryptée en une source de carbone qui promeut la croissance et la prolifération desdites cellules végétales transformées dans des conditions de culture hétérotrophes, ledit premier gène étant lié de manière opérante (ii) à un premier segment d’ADN promoteur qui contrôle l’expression dudit gène hétérologue, et (iii) à un segment d’ADN de terminaison ;
(b) maintien desdites conditions de culture hétérotrophes pendant un temps suffisant pour que lesdites cellules végétales transformées expriment ladite enzyme hétérologue, croissent et prolifèrent ; et
(c) récupération desdites cellules proliférantes ; et
(d) régénération de tissus méristématiques végétaux ou d’embryons végétaux à partir desdites cellules pro-
11. Procédé selon la revendication 10 dans lequel ledit gène hétérologue est celui de la phosphomannose isomérase et ladite source de carbone cryptée est le mannose.

12. Procédé selon la revendication 10 dans lequel ledit phosphore est un sel de phosphate soluble dans l'eau.


14. Procédé selon la revendication 10 dans lequel l'expression de ladite séquence d'ADN promoteur est réprimée par un produit du métabolisme normal desdits végétaux transgéniques en croissance autotrophe.

15. Procédé selon la revendication 14 dans lequel la séquence d'ADN promoteur répressible est celle du promoteur de l'isocitrate lyase du concombre.

16. Procédé selon la revendication 14 dans lequel la séquence d'ADN promoteur répressible est celle du promoteur de l'α-amylose Amy3A du riz.

17. Procédé selon la revendication 10 dans lequel le promoteur est un promoteur constitutif.

18. Procédé selon la revendication 17 dans lequel le promoteur constitutif est choisi parmi le groupe composé : du promoteur 3SS du virus de la mosaïque du chou-fleur, du promoteur de l'octopine synthase et du promoteur de la nopaline synthase.

19. Procédé destiné à augmenter de manière sélective le nombre de cellules végétales transformées régénérées à partir d'un mélange de cellules végétales transformées et non transformées placées dans des conditions de culture héterotrophes sélectives, le procédé comprenant les étapes de :

   (a) culture d'un mélange de cellules végétales transformées et non transformées pendant jusqu'à deux semaines dans un premier milieu de culture qui contient les nutriments minimaux nécessaires à la prolifération et la croissance des cellules végétales non transformées comprenant une source de carbone qui promeut la croissance et la prolifération, lesdites cellules végétales transformées contenant un segment d'ADN hétérologue génomique qui contient au moins une cassette d'expression, la cassette d'expression contenant un segment marqueur sélectionnable d'ADN hétérologue qui comprend (i) un gène hétérologue qui code pour une enzyme hétérologue qui est choisie parmi le groupe composé : de la phosphomannose isomérase, de la mannitol-1-oxydoréductase, de la L-iditol déshydrogénase, de la D-sorbitol-1-oxydoréductase, de la lactase, de la β-galactosidase et de l'α,α-tréhalase et qui lors de l'expression convertit une source de carbone cryptée en une source de carbone qui promeut la croissance et la prolifération desdites cellules végétales transformées contenant un segment d'ADN hétérologue génomique qui contient au moins une cassette d'expression, et (iii) à un segment d'ADN de terminaison ;

   (b) retrait du mélange de cellules végétales transformées et non transformées du premier milieu de culture ;

   (c) placement des cellules végétales transformées et non transformées dans des conditions de culture héterotrophes dans un second milieu de culture qui contient les nutriments minimaux nécessaires à la prolifération et la croissance des cellules végétales non transformées à l'exception d'une source de carbone cryptée qui ne promeut pas la croissance et la prolifération desdites cellules végétales non transformées et qui est choisie parmi le groupe composé : du mannose, du mannitol, du sorbitol, du lactose, du tréhalose et de la salicine, et 1,5 à 3 fois la quantité standard de phosphore ;

   (d) maintien desdites conditions de culture héterotrophes pendant un temps suffisant pour que lesdites cellules végétales transformées expriment ladite enzyme hétérologue, croissent et prolifèrent ; et

   (e) récupération desdites cellules proliférantes.

20. Procédé selon la revendication 19 dans lequel ledit gène hétérologue est celui de la phosphomannose isomérase et ladite source de carbone cryptée est le mannose.
21. Procédé selon la revendication 19 dans lequel ledit phosphore est un sel de phosphate soluble dans l'eau.


23. Procédé selon la revendication 19 dans lequel l'expression de ladite séquence d'ADN promoteur est réprimée par un produit du métabolisme normal desdits végétaux transgéniques en croissance autotrophe.

24. Procédé selon la revendication 23 dans lequel la séquence d'ADN promoteur pouvant être réprimée est celle du promoteur de l'isocitrate lyase du concombre.

25. Procédé selon la revendication 23 dans lequel la séquence d'ADN promoteur pouvant être réprimée est celle du promoteur de l'α-amylase Amy3A du riz.

26. Procédé selon la revendication 19 dans lequel le promoteur est un promoteur constitutif.


28. Procédé destiné à cultiver de manière sélective des cellules végétales transformées deux fois à partir d'un mélange de cellules végétales transformées deux fois et transformées une fois comprenant les étapes de :

(a) culture d'un mélange de cellules végétales transformées deux fois et transformées une fois dans des conditions de culture hétérotrophes dans un milieu de culture qui contient les nutriments minimaux nécessaires à la prolifération et à la croissance desdites cellules végétales transformées une fois à l'exception d'une source de carbon hydrate qui promeut la croissance et la prolifération desdites cellules transformées une fois et environ 1,5 à 3 fois la quantité standard de phosphore, ladite source de carbon hydrate étant remplacée par une seconde source de carbon hydrate cryptée qui ne promeut pas la croissance et la prolifération desdites cellules végétales transformées une fois et qui est choisie parmi le groupe composé : du mannose et du mannitol ; lesdites cellules transformées deux fois contenant un premier et un second segment d'ADN hétérologue qui contient au moins deux cassettes d'expression, au moins une cassette d'expression étant dans le premier segment d'ADN hétérologue et au moins une cassette d'expression étant dans le second segment d'ADN hétérologue ;

la cassette d'expression dans le premier segment d'ADN hétérologue contenant un segment marqueur sélectionnable d'ADN hétérologue qui comprend (i) un premier gène hétérologue qui code pour une enzyme hétérologue qui est choisie parmi le groupe composé : de la phosphomannose isomérase et de la mannitol-1-oxydoréductase et qui lors de l'expression convertit une première source de carbon hydrate cryptée en une source de carbon hydrate qui promeut la croissance et la prolifération desdites cellules végétales transformées une fois et transformées deux fois dans des conditions de culture hétérotrophes mais qui ne promeut pas la croissance et la prolifération de cellules végétales transformées deux fois contenante un premier et un second segment d'ADN hétérologue qui contient au moins deux cassettes d'expression, ledit premier gène étant lié de manière opérante (ii) à un segment d'ADN promoteur qui contrôle l'expression dudit gène hétérologue, et (iii) à un segment d'ADN de terminaison ;

la cassette d'expression dans le second segment d'ADN hétérologue contenant un second segment marqueur sélectionnable d'ADN hétérologue qui comprend (i) un second gène hétérologue qui code pour une enzyme hétérologue qui est choisie parmi le groupe composé : de la phosphomannose isomérase et de la mannitol-1-oxydoréductase et qui lors de l'expression convertit la première source de carbon hydrate transformées deux fois contenant la deuxième source de carbon hydrate cryptée qui ne promeut pas la croissance et la prolifération des cellules végétales transformées deux fois et transformées une fois, ledit second gène étant lié de manière opérante (ii) à un second segment d'ADN promoteur qui contrôle l'expression dudit second gène hétérologue, et (iii) à un segment d'ADN de terminaison ;

(b) maintien desdites conditions de culture hétérotrophes pendant un temps suffisant pour que lesdites cellules végétales transformées deux fois expriment lesdites premières enzyme hétéroologue et seconde enzyme hétérologue, croissent et prolifèrent ;

(c) récupération desdites cellules proliférantes.
29. Procédé selon la revendication 28, dans lequel ledit second segment marqueur sélectionnable d’ADN hétérologue code pour l’enzyme mannitol-1-oxo-réductase, ladite seconde source de carbone cryptée est le mannitol, ledit premier gène qui code pour un gène hétérologue code pour la phosphomannose isomérase et ladite première source de carbone cryptée est le mannose.

30. Procédé destiné à cultiver de manière sélective des végétaux transformés deux fois à partir d’un mélange de cellules végétales transformées deux fois et transformées une fois comprenant les étapes de :

(a) culture d’un mélange de cellules végétales transformées deux fois et transformées une fois dans des conditions de culture hétérotrophes dans un milieu de culture qui contient les nutriments minimaux nécessaires à la prolifération et à la croissance desdites cellules végétales transformées une fois à l’exception d’une source de carbone qui promeut la croissance et la prolifération desdites cellules transformées une fois et environ 1,5 à 3 fois la quantité standard de phosphore, ladite source de carbone étant remplacée par une seconde source de carbone cryptée qui ne promeut pas la croissance et la prolifération desdites cellules végétales transformées une fois et qui est choisie parmi le groupe composé : du mannose et du mannoïde ; lesdites cellules transformées deux fois contenant un premier et un second segment d’ADN hétérologue qui contient au moins deux cassettes d’expression, au moins une cassette d’expression étant dans le premier segment d’ADN hétérologue et au moins une cassette d’expression étant dans le second segment d’ADN hétérologue ;

(b) maintien desdites conditions de culture hétérotrophes pendant un temps suffisant pour que lesdites cellules végétales transformées deux fois expriment lesdites première enzyme et seconde enzyme, croissent et prolifèrent ;

(c) récupération desdites cellules proliférantes ; et

(d) régénération de tissus mésistomatiques végétaux ou d’embryons végétaux à partir desdites cellules proliférantes.

31. Procédé selon la revendication 30 dans lequel ledit second segment marqueur sélectionnable d’ADN hétérologue code pour l’enzyme mannitol-1-oxo-réductase, ladite seconde source de carbone cryptée est le mannitol, ledit premier gène qui code pour l’expression de la phosphomannose isomérase et ladite première source de carbone cryptée est le mannose.

32. Kit pour former des cellules végétales transformées comprenant :

(a) un premier ensemble contenant un segment d’ADN pour transformer des cellules végétales qui contient une cassette d’expression liée de manière opérante à un segment espaceur contenant au moins un site d’endonucléase de restriction, ladite cassette d’expression contenant un segment marqueur sélectionnable d’ADN hétérologue qui comprend (i) un premier gène hétérologue qui code pour une enzyme hétérologue qui est choisie parmi le groupe composé : de la phosphomannose isomérase, de la mannitol-1-oxoréductase, de la L-iditol déshydrogénase, de la D-sorbitol-1-oxoréductase, de la lactase, de la β-galactosidase et de l’α, α-tréhalase et lors de l’expression en culture hétérotrophe des cellules végétales transformées convertit une
source de carbone cryptée qui ne promeut pas la croissance et la prolifération de cellules végétales non transformées en une source de carbone qui promeut la croissance et la prolifération desdites cellules transformées et qui est choisie parmi le groupe composé : du mannose, du mannotol, du sorbitol, du lactose, du tréhalose et de la salicine, ledit premier gène étant lié de manière opérante (ii) à un segment d’ADN promoteur qui contrôle l’expression dudit premier gène hétérologue et (iii) à un segment d’ADN de terminaison ; et (b) un second ensemble qui contient les nutriments minimaux nécessaires à la prolifération et à la croissance de cellules végétales non transformées lors d’une culture hétérotrophe à l’exception d’une source de carbone et environ 1,5 à 3 fois la quantité standard de phosphore, ladite source de carbone étant remplacée par une source de carbone cryptée qui ne promeut pas la croissance et la prolifération de cellules végétales non transformées mais qui promeut la croissance et la prolifération de cellules végétales transformées dont le génome contient le segment d’ADN dudit premier ensemble et qui est choisie parmi le groupe composé : du mannose, du mannotol, du sorbitol, du lactose, du tréhalose et de la salicine.

33. Kit selon la revendication 32 dans lequel ledit gène hétérologue est celui de la phosphomannose isomérase et ladite source de carbone cryptée est le mannose.

34. Kit selon la revendication 32 dans lequel ledit premier segment d’ADN promoteur qui contrôle l’expression dudit gène hétérologue est réprimé par un produit du métabolisme normal desdits végétaux transgéniques en croissance autotrophe.

35. Kit selon la revendication 32 dans lequel ledit segment espaceur contient une pluralité de sites de restriction d’endonucléases.

36. Kit selon la revendication 32 dans lequel ledit segment d’ADN de (a) est situé à l’intérieur des limites de l’ADNT du plasmide Ti de Agrobacterium tumefaciens.

37. Kit selon la revendication 32 dans lequel ledit phosphore est un sel de phosphate soluble dans l’eau.

FIG. 2

PCR, Digest with BglII & SacI
Digest pGEM3Zf(-) with BamHI & SacI

Ligate

Digest with XbaI & SacI

RB

P-NOS

NPTII

NOS-T

35S

pmi

SacI

pET0148
14 Kb

pET0147
4.2 Kb

pET0107
12.8 Kb

LB

SacI

XbaI

NOS-T