Abstract:
The present invention is concerned with a method for generating transgenic plants, said method comprising a) providing a population of transgenic and non-transgenic shoots to a hydroponic medium, and simultaneously allowing said shoots to root on said hydroponic medium and selecting for transgenic shoots.
Hydroponic systems for generating transgenic plants

The present invention is concerned with a method for generating transgenic plants, said method comprising a) providing a population of transgenic and non-transgenic rootless plant shoots grown under in vitro conditions, transferring said plant shoots to a hydroponic medium, and simultaneously allowing said shoots to root on said hydroponic medium and selecting for transgenic shoots.

Plant transformation, first described for tobacco in 1984, is now widely used for introduction of genes into plants for purposes of basic research as well as for generation of commercially used transgenic crops. Plants which can be successfully transformed include most major economic crops, vegetables, ornamental, medicinal, fruit, tree, and pasture plants.

Plant transformation is mostly done by Agrobacterium-mediated plant transformation. Agrobacterium is a naturally occurring pathogenic soil bacterium which is capable of transferring DNA into the genome of a plant cells. For Agrobacterium-mediated plant transformation, the gene of interest is placed between the left and right border repeats of Agrobacterium T-DNA (transfer DNA). Afterwards, the T-DNA region containing the gene of interest is stably integrated into the plant genome by using an appropriate plant transformation protocol (for a review see Gelvin, 2003 Microbiol Mol Biol Rev. 67(1):16-37).

Aside from Agrobacterium-mediated plant transformation, other plant transformation methods exist such as viral transformation, electroporation of plant protoplasts, and particle bombardment.

Generally, plant transformation techniques are based on the same principles. In a first step, the gene of interest is introduced in a suitable transformation vector. The transformation vector harbouring the gene of interest is then introduced into regenerable cells of a target plant. Since only a minor proportion of target cells receives the gene of interest, selection for transformed plant cells among a large excess of untransformed cells is carried out. Moreover, once the gene of interest has been stably introduced into the genome of a host cell, it is essential to establish regeneration conditions in order to regenerate whole plants from a single transformed plant cell (see, e.g., Birch, 1997, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 297-326).

One of the simplest available Agrobacterium-based plant transformation methods is "Floral Dip Transformation. Floral dip a germline transformation method by which the gene of interest is transformed into cells that give rise to the seeds. This method involves dipping
plants (in early flowering stage) in a suspension of Agrobacterium cells (Clough and Bent, 1998, Plant J 16:735-43). A few weeks after dipping, seeds of dipped plants are collected, and the seed population is selected for transformants. The advantage of Floral Dip transformation technique is that it avoids the use of tissue culture and plant regeneration which is cost-intensive and requires trained personnel. Unfortunately, small plant size, short generation time, and a large amount of seeds per plant are prerequisites for Floral Dip. Consequently, this transformation method has only been successfully applied to a few species, mainly for Arabidopsis thaliana (but also with Medicago truncatula).

Regeneration of whole transformed plants which are recalcitrant to germline transformation is considered as the bottleneck in plant transformation since regeneration is difficult to achieve, time-consuming, and requires specific equipment.

The first steps of plant regeneration are usually carried out under in vitro conditions, i.e. on a specific nutrient medium under sterile conditions. After transformation of target cells, cell division is induced by specific plant hormones in order to grow a callus from a transformed plant cell. After callus induction, the resulting callus is transferred to a medium allowing shoot induction. The callus is incubated (under in vitro condition) on said medium until shoots are formed. After shoot formation, the shoot is transferred to a medium that allows for root formation (under in vitro conditions). After root formation, regenerated plantlets (i.e. roots with shoots) are usually transferred from in vitro conditions to ex vitro conditions, mostly to soil under greenhouse conditions. Thus, callus induction, shoot induction and root induction are usually carried out under in vitro conditions.

The current methods for regenerating plants under in vitro conditions, however, have some disadvantages. Regenerating whole plants under in vitro conditions is expensive and requires specific nutrient media, specific equipment and trained personnel. There is, of course, always the risk of contamination (e.g. contamination with fungus). If a tissue culture gets contaminated, the work of weeks or even of months may become worthless.

Without tissue culture, however, plant transformation would be almost impossible since tissue culture a) allows for selection of transgenic plant cells (and plant shoots, respectively) and b) simultaneously suppresses growth of bacterial and fungal microorganisms. Without selection, it would be almost impossible to identify plants cells, plant shoots, plantletts (i.e. plants with shoots and roots) which carry the transgene. Moreover, suppressing the growth of bacterial and fungal microorganism is also necessary, since otherwise the regeneration of whole plants would be inhibited.
Transferring plants from in vitro conditions to ex vitro conditions (particularly, into the greenhouse) is another bottleneck of plant transformation and regeneration. After ex vitro transfer, the plantlets are frequently impaired by sudden changes of environmental conditions. During in vitro culture, the plantlets are grown in specific cultivation media under very specific conditions. Cultivation is carried out in relatively air-tight cultivation vessels resulting in increased air-humidity. Also, the cultivation media are often supplemented with saccharides as carbon source resulting in a decreased water potential. Furthermore, the cultivation media usually contain large amounts of growth regulators (see Pospisilova, 1999, Biologica Plantarum, 42(4):48 1-497).

Plantlets usually need a few weeks in order to acclimatize to ex vitro conditions and, thus, to start vegetative growth. E.g., under ex vitro conditions air humidity is much lower than under in vitro conditions. Therefore, a large proportion of plantlets transferred to ex vitro conditions may wilt or even die as a consequence of water loss, even if the plantlets are kept under humid conditions. Therefore, plantlets which are transferred from in vitro to ex vitro conditions must be carefully acclimatized to said ex vitro conditions.

Thus, regenerating transgenic plants (particularly, adapting transgenic shoots to ex vitro conditions) is generally a labor-intensive and very time-consuming task. For example, time needed from the isolation of putative transgenic in-vitro Brassica or Brachypodium shoots to ex-vitro adapted, greenhouse ready plants takes 12 weeks b 14 weeks (see e.g. for Brachypodium: Bablak et al (1995) Plant Cell, Tissue and Organ Culture 42: 97-107; or Christiansen et al. (2005) Plant Cell Report 23: 751-758; for Brassica: Cardoza and Stewart (2004), Transgenic crops of the World-Essential Protocols, 379-387; or Jonoubi et al. (2005).Biologia Plantarum 49 (2): 175-180).

Transformation procedures that avoid tissue culture or reduce tissue culture would be extremely valuable, especially for plants which are difficult to regenerate. Scientists have attempted to develop plant transformation procedures that do not require tissue culture, but these attempts have been met with limited success. For example, Graves and Goldman (1986 Plant Mol. Biol. 7: 43-50) reported that Agrobacterium could infect mesocotyl cells of germinating corn seeds, but the resulting transformed plants were chimeras and the transformation efficiency was extremely poor.

Hydroponics is a technology of growing plants in nutrient solutions without soil. The two main types of hydroponics are liquid hydroponic media and substrate hydroponic media. Substrate hydroponic systems include hydroponic, mostly inorganic substrates such as rockwool (see, e.g. WO01/87070), perlite, vermiculite in order to provide physical support
for the plant root system. These substrates are usually porous and absorb the nutrient solution. Liquid hydroponic systems (also known as solution hydroponic systems) have no other supporting medium for plant roots. All hydroponic mediums have in common that they provide a favourable buffer of nutrient solution around the root systems. Generally, the system contains the essential elements needed by the plant for growth and development.

Hydroponic substrates such as rock wool have been employed in the cultivation of a large number of plants such as tomatoes, cucumbers, peppers and even cut flowers such as roses and gerberas.

Schrammeijer et al. (Plant cell reports, 1990, 9:55-60) discloses a method for sunflower meristem transformation via Agrobacterium. Meristem explants were co-cultivated with an Agrobacterium strain containing a gene of interest. After co-cultivation, selection for transformants was done on a selection medium. However, only two plant shoots were able to grow on selective medium. Remarkably, the first shoot showed a normal growth pattern, whereas the second shoot showed an abnormal growth pattern (although both shoots were derived from transformation with the same construct). Both shoots were propagated and two propagated shoots were obtained from the normal first shoot and ten from the deviant second shoot. All twelve shoots were placed on rock wool for root production. However, only three of these rooted and only one set seeds. Two of the rooted plants were analyzed for transgene expression, i.e., for expression of the GUS reporter gene, and chimeric gene expression of the GUS-transgene was observed. Progeny of the only plant from which seeds could be selected, showed no GUS-activity.

Although many problems linked to the transformation and regeneration of plants have been overcome by the methods described in the art, there is still a significant need for improvement due to the use of tissue culture, low regeneration efficiencies, and time consuming regeneration of whole plants. Thus, although significant advances in the field of engineering transgenic plants were made, a need continues to exist for improved methods to facilitate the ease, speed and efficiency of generating transgenic plants.

The technical problem underlying the present invention can be seen as the provision of means and methods for complying with the aforementioned needs.

The technical problem is solved by the embodiments characterized in the claims and herein below.
Accordingly, the present invention relates to a method for generating transgenic plants comprising the steps,

a) providing a population of transgenic and non-transgenic rootless plant shoots grown under in vitro conditions,

b) transferring said plant shoots to a hydroponic medium, and

c) simultaneously allowing said shoots to root on said hydroponic medium and selecting for transgenic shoots.

The method of the present invention allows for the generation of transgenic plants. Said plants are, preferably, derived from single transgenic cells. The term "transgenic" as used herein (particularly, with regard to a plant cell, plant shoot, plantlet or plant), preferably, refers to plant cells, plant shoots, plantlets and/or plants (comprising plant cells) which contain at least one transgene, or whose genome has been altered by the introduction of at least one transgene. Accordingly, the term "transgene" as used herein, preferably, refers to any nucleic acid sequence, preferably a DNA construct, which is introduced into the genome of a cell by experimental manipulations. In the context of the present invention, the terms "transgene" and "gene of interest" may be used interchangeably.

A transgene may be an "endogenous DNA sequence," or a "heterologous DNA sequence" (i.e., "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence which is naturally found in the cell into which it is introduced so long as it does not contain some modification (e.g., a point mutation, the presence of a selectable marker gene, etc) relative to the naturally-occurring sequence. In the context of the present invention, the transgene can be virtually any gene. But, preferably, the transgene may encode for coding sequences of structural genes (e.g., reporter genes, selection marker genes, drug resistance genes, growth factors, etc), and non-coding sequences such as regulatory RNA. Of course, the transgene may further comprise nucleotide sequence allowing expression such as a promoter sequence, a polyadenylation sequence, a termination sequence, and/or an enhancer sequence, etc). The transgene in the context of the method of the present invention, preferably, encodes for an agronomically valuable trait. Preferably, the transgene is comprised by a suitable transformation vector or plasmid such as a T-DNA vector. Preferably, said transformation vector comprises also at least one expression cassette for at least one selectable gene, which allows for selection and/or identification of transformed plant cells, plant shoots, plantlets and plants. Preferred selectable marker genes allowing for said selection and/or said identification are described herein below.

Preferably, the transgene is stably integrated into the genome of the transgenic plant cell, or into plant cells comprised by said transgenic plant shoot, plantlet or plant. Preferably, the
transgene shall be stably comprised by the nuclear, i.e. chromosomal DNA of said plant cells resulting in stable heritability through meiosis. Thus, also progeny of transgenic plants shall comprise the transgene.

It is to be understood that transgenic plant cells, plant shoots, and plantlets, and plants shall comprise the transgene, whereas non-transgenic plant cells, plant shoots, and plantlets, and plants shall not comprise the transgene.

The method of the present invention, preferably, envisages the use plant shoots of all kind of plants. Preferably, the plant shoots (comprised by the populations of transgenic and non-transgenic plant shoots) are selected from shoots of monocotyledonous plants and dicotyle-
dedonous plants. More preferably, the plant shoots are selected from shoots of plants of the genus of Medicago, Lycopersicon, Brassica, Cucumis, Solanum (particularly, the species tuberosum), Juglans, Gossypium, Malus, Vitis, Antirrhinum, Eucalyptus, Populus, Fragaria, Arabidopsis (particularly the species thaliana), Picea, Capsicum, Chenopodium, Dendran-
themus, Pharbitis, Pinus, Pisum, Oryza, Zea, Triticum, Triticaceae, Secale, Lolium, Hordeum, Brachypodium, Glycine, Pseudotsuga, Kalanchoe, Beta, Helianthus and Nicotiana. Most preferably, said plant shoots are selected from shoots of plants of the genus Brassica, Arabidopsis (particularly, Arabidopsis thaliana), Oryza (particularly Oryza sativa), Nicotiana (particularly, Nicotiana tabacum), Zea (particularly Zea mays), Brachypodium, Triticum, and Glycine.

The most preferred species to be transformed are described in the Examples. Accordingly, the plant shoots are, preferably, from Brassica napus. Further preferred species are Glycine max and Brachypodium distachyon.

In step a) of the method of the present invention, a population of transgenic and non-
transgenic rootless shoots (grown under in vitro conditions) shall be provided. How to pro-
vide a population of transgenic and non-transgenic rootless shoots is well known in the art. Preferably, said population is derived from transforming a population of plant cells by any method deemed appropriate for the introduction of a gene of interest into a plant cell, and, thus by any method deemed appropriate for stable transformation of plant cells, see e.g. Transgenic Plants: Methods and Protocols Editor: Leandro Penal, Instituto Valenciano de InvestigacJones Agrarias, Valencia Spain Series: Methods in Molecular Biology (2004) Vol-
ume No.: 286.
Preferably, said population is derived from transforming a population of plant cells by Agrobacterium-mediated transformation, naked DNA transformation (particularly, electroporation and PEG-mediated transformation) or particle bombardment.

Agrobacterium is a soil born phytopathogen that integrates a piece of DNA (T-DNA) into the genome of a large number of dicotyledonous and few monocotyledonous plants (Chilton, et al., 1977 Cell 11: 263-271; Hoekema, et al., 1985 Nature 303: 179-180; Bevan, 1984 Nucl. Acids Res. 12: 871-8721; Sheng and Citovsky, 1996 The Plant Cell, Vol. 8.1699-1710). Preferred Agrobacterium strains are Agrobacterium tumefaciens which typically causes crown gall in infected plants, and Agrobacterium rhizogenes which typically causes hairy root disease in infected host plants. However, as set forth herein below, preferably, the Agrobacterium strains used for providing a population of transgenic and non-transgenic plant shoots shall lack the ability of causing crown gall disease and hairy root disease, respectively (which can be achieved by using disarmed Agrobacterium strains, see below).

The use of Agrobacterium, particularly, of Agrobacterium tumefaciens (but also of Agrobacterium rhizogenes) for plant transformation is known as such (for a review see Gelvin, 2003 Microbiol Mol Biol Rev. 67(1):16-37). For Agrobacterium-mediated plant transformation, the gene of interest is placed between the left and right border repeats of Agrobacterium T-DNA (transfer DNA). Afterwards, the T-DNA region containing the gene of interest is stably integrated into the plant genome by using an appropriate plant transformation protocol.

Various strains of Agrobacterium having different chromosomal backgrounds and Ti-plasmid content can be used for transformation. However, it is preferred that the Agrobacterium strain contains a disarmed Ti-plasmid or a disarmed Ri-plasmid. A disarmed Ti-plasmid is understood as a Ti-plasmid lacking its crown gall disease mediating properties but otherwise providing the functions for plant infection. A disarmed Ri-plasmid is understood as a Ri-plasmid lacking its hairy-root disease mediating properties but otherwise providing the functions for plant infection. Agrobacterium strains to be used for transforming plants cells are selected from LBA4404, GV2260, GV3600, EHA101, EHA105, AGL-1, LBA9402, GV3101, COR341, COR356, UIA143, pCH32, BIBAC2, C58C1, pMP90 and AGT121. In a preferred embodiment the Agrobacterium strain is selected from the group consisting of C58C1, EHA101, pMP90, and LBA4404. In another preferred embodiment the Agrobacterium strain is a disarmed variant of K599 (NCPPB 2659) which, preferably, carries a disarmed variant of pRi2659 as disclosed in WO03/017752.

For Agrobacterium-mediated plant transformation, plants cells, plant tissues or explants of plants to be transformed are co-cultivated with a suitable Agrobacterium strain which carries
(between the T-DNA left and right border) the gene of interest. The person skilled in the art
knows which plant tissues or plants cell should be selected for the co-cultivation step. Vari-
ous tissues are suitable as starting material for Agrobacterium-mediated transformation. The most commonly used starting materials are the meristematic ends of the plants like the
stem tip, auxiliary bud tip and root tip. Preferably, the starting material is selected from leafs
(e.g. for leaf disc transformation), immature embryos (EP-A1 672 752), pollen (U.S. Pat. No.
54,929,300), shoot apex (U.S. Pat. No. 5,164,310), mature seed-derived calli, immature
embryo-derived calli, cotyledonary nodes, hypocotyls, and inflorescences. Moreover, other
preferred starting materials are disclosed in WO03/017752. The optimal starting material
will depend on the plant species to be transformed. The person skilled in art, however, is
capable of selecting a suitable starting material (see also Examples).

The term “particle bombardment” as used herein, preferably, refers to the process of accel-
erating particles coated with the gene of interest towards a target biological sample (particu-
larly, cells, and plant tissue) in order to effectively wounding the cell membrane of a cell in
the target biological sample and/or entry of the particles into the target biological sample.
Methods for particle bombardment (frequently also referred to as "biolistic bombardment")
are known in the art, se, e.g., US 5,584,807), and are commercially available (e.g., the he-
lium gas-driven microprojectile accelerator (PDS-1000/He) (BioRad).

After transforming a population of plant cells, only a small proportion of said plant cells
comprises the gene of interest (and, thus, is transgenic), whereas a large proportion does
not comprise the gene of interest (and, thus, is not transgenic). Moreover, whole transgenic
plants are regenerated from single transgenic plant cells.

in accordance with the method of the present invention, the first steps of regenerating
whole plants from single transgenic plants cells (which are, e.g., comprised by an explant
that has been co-cultivated with Agrobacterium) are, preferably, carried out under in vitro
conditions, and, thus, on a nutrient medium under sterile conditions (plant tissue culture).

In order to provide a population of transgenic and non-transgenic plant shoots, the popula-
tion of transgenic and non-transgenic plant cells is incubated (in vitro) under conditions that
allow for shoot induction. Accordingly, the plant cells are, preferably, incubated on a regen-
eration medium comprising at least one plant growth factor. The use of plant growth factors
for plant regeneration is known as such (For a review see Gaspar et al. Plant hormones and
example, supplementing a nutrient medium with the plant growth factor cytokinin, prefera-
bly, will yield shoots, while supplementing the nutrient medium with the plant growth factor
auxin, preferably, will result in a proliferation of roots. A balance of both auxin and cytokinin will often result in unorganised growth of cells (thereby forming a callus). Plant growth regulators may act singly or in consort with one another or with other compounds (such as sugars, or amino acids). Auxins stimulate cellular elongation and division, differentiation of vascular tissue, fruit development, formation of adventitious roots, production of ethylene, and (in high concentrations) induce dedifferentiation (callus formation). The most common naturally occurring auxin is indoleacetic acid (IAA), which is transported polarly in roots and stems. Synthetic auxins are used extensively in modern agriculture. Auxin compounds comprise indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), and 2,4-dichlorphenoxyacetic acid (2,4-D). Cytokinins stimulate cellular division, expansion of cotyledons, and growth of lateral buds. Preferred cytokinins are 6-isopentenyladenine (IPA) and 6-benzyladenine/6-benzylaminopurine (BAP).

The employed nutrient media may further contain at least one compound, which in combination with the selectable marker gene allows for identification and/or selection of plant cells (e.g., a selective agent) may be applied. However, it is preferred that explants are incubated for a certain time, preferably 5 to 14 days, after transformation (e.g. by co-cultivation with Agrobacterium) on medium Sacking a selection compound. Establishment of a reliable resistance level against said selection compound needs some time to prevent unintended damage by the selection compound even to the transformed cells and tissue.

As set forth above, development of shoots from single transgenic or non-transgenic cells is, preferably, achieved under in vitro conditions. It is to be understood that shoot development can be achieved in a single cultivation step or in more than one cultivation step (preferably, incubating the transformed population of plant cells on a callus inducing medium, and then transferring the developed calli to a shoot inducing medium).

After shoots are developed under in vitro conditions, they are, preferably, sliced off. Thus, by slicing of the shoots, a population of rootless transgenic and non-transgenic plant shoots is provided (i.e. of transgenic or non-transgenic shoots not comprising root tissue). The rootless (transgenic and non-transgenic) shoots are then transferred to a hydroponic medium allowing for root induction.

The use of hydroponic mediums for the growth of plants is known as such (for a review see Hydroponics: A Practical Guide for the Soilless Grower, J. Benton Jones, published CRC Press, 2004). Hydroponics is a technology of growing plants in nutrient solutions without soil. The two main types of hydroponics are liquid hydroponic mediums and substrate hydroponic mediums. In the context of the method of the present invention the hydroponic
medium is a substrate hydroponic medium and, thus, a soil-less cultivation medium that comprises a hydroponic compound and a nutrient medium.

Preferably, the hydroponic compound comprised by the hydroponic medium is inorganic.

More preferably, the hydroponic compound is a mineral wool. Mineral wool hydroponic compounds for plant growth are known in the art and, preferably, comprised of coherent matrix of mineral fibers made from natural or synthetic minerals or metal oxides.

Preferably, the mineral wool is selected from the group consisting of glass wool, rock wool and slag wool. Also contemplated are mixtures of the aforementioned mineral wools. The most preferred mineral wool (and, thus, the most preferred hydroponic compound) in the context of the present invention is rock wool (see also WO01/87070).

Rock wool, frequently also referred to as stone wool, is a mineral wool manufactured from volcanic rock. It is comprised of pores (about 95%) and solids in the form of rock fibres (5%). Preferably, the rock wool is manufactured from basalt and limestone. For manufacturing rock-wool, these raw materials are, e.g., heated in an oven at about 1500°C, when they melt into lava. The lava may then poured onto a number of discs spinning at a high speed. The centrifugal force throws drops of lava from the discs, which are then transformed into threads. The threads are compressed to form a solid mass, which then may be sawn into slabs and blocks.

Alternatively, acidic phenol-formaldehyde-, urea formaldehyde- or cellulose-based foams can be applied as hydroponic compounds. Such foams, preferably, have an open cell structure mimicking the plant cellular structure. Preferred foams are available as Oasis® root medium from Smithers-Oasis Co., (Kent, OH, USA) or are available as cellular foam rooting sponges from Grow-Tech (Lisbon Falls, ME, USA). Moreover, particularly preferred acidic phenol formaldehyde based foams (also known as phenolic foams) are described in US 2,753,277.

Other hydroponic compounds that can be applied in the context of the method of the present invention are perlite, vermiculite, peat, sand, pumice, coconut husks (coir), styrofoam beads or other polymerized substrates or extruded foams.

It is to be understood that the hydroponic medium can be provided in many shapes and sizes, e.g., mini cubes, cubes, blocks, mats and slabs (see also Examples).
As set forth above, the hydroponic medium shall also comprise a nutrient medium. The nutrient medium, preferably, comprises all essential elements needed by the plant shoots for growth and development. Moreover, the nutrient medium may also comprise at least one plant growth regulator which induces root development, preferably, an auxin. Preferred auxins in the context of the present invention are mentioned above, particularly Indole butyric acid (IBA), Indole acetic acid (IAA), or Naphthalene acetic acid (NAA). Preferred concentrations in the nutrient medium are given in the Examples. Preferably, the concentrations are within a range between 0.1 to 50 µM (preferably, 10 to 15 µM) for IBA, between 0.1 to 50 µM (preferably, 10 to 15 µM) for IAA, and between 0.1 to 25 µM (preferably, 2 to 5 µM) for NAA. Moreover, preferred regulators as well as preferred concentrations for root-induction of shoots are described in the Examples. It is to be understood that the hydroponic medium shall not comprise soil.

In a preferred embodiment of the method of the present invention, the nutrient medium comprised by the hydroponic system does not comprise an auxin. Advantageously, it has been shown in the studies underlying the present invention that rooting in a hydroponic medium is stimulated even without the presence of auxin in the nutrient solution comprised by the hydroponic medium.

In the context of the method of the present invention, selection of transgenic plant shoots and root induction is carried out simultaneously (step c) of the method of the present invention. In order to achieve simultaneously selection and root induction, the plant shoots are contacted with a selecting agent (i.e. a compound that allows for selecting plants harbouring a selectable marker gene). Preferably, contacting the plant shoots is achieved by using a hydroponic medium comprising a nutrient medium supplemented with a selecting agent in an amount effective for allowing selection. Simultaneously, the plant shoots may be contacted with a plant growth regulator that induces root development (preferably auxin).

Preferably, the transgenic plant shoots comprised by the population of transgenic and non-transgenic shoots shall develop primary and lateral roots in said hydroponic medium. The terms "primary roots" and "lateral roots" are well known in the art. Lateral roots, preferably, extend horizontally from the primary roots (and serve to anchor the plant in the hydroponic medium).

Preferably, simultaneous selection and root development (step c) of the method of the present invention is carried out under ex vitro conditions, and, thus, under non-sterile conditions. More preferably, simultaneous selection and root development is carried out under greenhouse conditions.
Preferably, selection for transgenic shoots, plantlets and/or plants is done by using a plant selectable marker gene. Plant selectable marker genes are well known in the art.

In the context of the method of the present invention, plant selectable marker genes, preferably, allow for selecting and separating successfully transformed plant cells, shoots and/or plantlets. The markers, preferably, confer resistance or increase tolerance to specific selecting agents such as biocides, antibiotics, toxins, or heavy metals.

Selectable marker genes for plants in the context of the present invention, preferably, allow - in the presence of the corresponding selecting agent - for selection of transformed shoots which carry a marker gene.

Selectable marker genes for plants in the context of the present invention, preferably, confer resistance or increased tolerance to a biocidal compound (i.e. a selecting agent) such as a metabolic inhibitor (e.g., 2-deoxyglucose-6-phosphate, WO 98/45456), antibiotics (e.g., kanamycin, G 418, bleomycin or hygromycin) or herbicides (e.g., phosphinothricin or glyphosate). Particularly, selectable marker genes are those which confer resistance to herbicides.

Preferred selectable marker genes which confer resistance to a biocidal compound encode for polypeptides selected from the group consisting of phosphinothricin acetyltransferases (PAT; see US4,975,374, conferring resistance to phosphinothricin), glyphosate oxidoreductase gene (US 5,463,175) conferring resistance to Glyphosate (N-phosphonomethyl glycine); glyphosate degrading enzymes (Glyphosate oxidoreductase; gox); dalapon inactivating dehalogenases (deh); sulfonyleurea- and imidazolinone-inactivating acetolactate synthases, bromoxynil degrading nitrilases (bxn); kanamycin- or. G418- resistance genes (NPTII; NPTI), particularly coding for neomycin phosphotransferases which confer resistance to kanamycin and the related antibiotics neomycin, paromomycin, gentamicin, and G418; 2-deoxyglucose-6-phosphate phosphatase (D0GR1-Gene product; WO 98/45456; EP 0 807 836) conferring resistance against 2-desoxyglucose (Randez-Gil 1995), hygromycin phosphotransferase (HPT), which mediates resistance to hygromycin, and dihydrofolate reductase.

Other preferred selectable marker genes in the context of the present invention encode for a D-serine dehydratase (D-serine deaminase) or for a D-amino acid oxidase conferring increased tolerance to D-serine, or for mutated acetohydroxyacid synthase (AHAS), conferring increased tolerance to imidazolinone herbicides (see, e.g., WO/2004/005516).
It is to be understood that the population of transgenic and non-transgenic plants, plant shoots and/or plant cells has to be contacted with the respective selecting agent in order to allow for selecting. For hydroponic culture under ex vitro conditions the selecting agent is, preferably, added to the nutrient solution. If selection is also done during tissue culture (and, thus, under in vitro conditions), the selecting agent is, preferably, added to the medium used for tissue culture. Moreover, some selecting agents (such as phosphinotricin or imidazolinone, see Examples) may also be contacted with plants and/or plant shoots by spraying the plants and/or plant shoots with a solution containing the selecting agent under ex vitro conditions.

It is to be understood that the selecting agents are contacted with the non-transgenic and transgenic plants, plant shoots and/or plant cells in an effective amount, i.e. an amount allowing a) suppression of growth of non-transgenic and transgenic plants, plant shoots and/or plant cells and b) growth of transgenic plants, plant shoots and/or plant cells. Effective amount for the individual selecting agents are well known in the art and/or can be determined for individual plant species without further ado (see, e.g., Example 4 in which a D-serine kill curve is determined for soybean seedlings).

For example, with the AHAS gene (see above) as the selective marker, imidazolinone at a concentration of from about 0.1 to 10 \( \mu \text{M} \) may be included in the nutrient solution of the hydroponic system. Preferred concentrations for selection are within a range of 0.5 to 3uM. As mentioned above, plants can also be sprayed with imidazolinone, preferably with 0.3 - 20g/ha, more preferably, between 0.625 - 10 g/ha (see Examples).

For example, with the kanamycin resistance gene (neomycin phosphotransferase, NPTII) as the selective marker, kanamycin at a concentration of from about 3 to 200 mg/L may be included in the nutrient solution of the hydroponic system. Preferred concentrations for selection are 5 to 50 mg/L.

For example, with the phosphinotricin resistance gene (bar) as the selective marker, phosphinotricin at a concentration of from about 3 to 200 mg/L may be included in the nutrient solution of the hydroponic system. Preferred concentrations for selection are 5 to 50 mg/L. As mentioned above, plants can also be sprayed with phosphinotricin.

For example, with the D-serine dehydratase gene or the D-amino acid oxidase as the selective marker, D-serine at a concentration of from about 2 to 10 mM D-serine may be included
in the nutrient solution of the hydroponic system. Preferred concentrations for selection are 2 to 5 mM.

Once the transgenic plant shoots have developed roots (preferably, primary and lateral roots), the resulting plantlets (i.e. shoots with roots) are, preferably, transferred to soil and incubated under greenhouse conditions. Preferably, the plantlets develop transgenic seeds (and, thus, seeds stably comprising the gene of interest). Preferably, the seeds are collected.

Finally, the present invention relates to the use of a hydroponic medium for simultaneously allowing shoots to root and selecting for transgenic shoots in a population of transgenic and non-transgenic rootless plant shoots grown under in vitro conditions.

Advantageously, it was shown in the studies carried out in the context of the present invention that a) providing a population of transgenic and non-transgenic rootless plant shoots grown under in vitro conditions, b) transferring said plant shoots to a hydroponic medium, and c) simultaneously allowing said shoots to root on said hydroponic medium and selecting for transgenic shoots, allows for efficiently generating transgenic plants.

Specifically, populations of transgenic and non-transgenic plant shoots (of *Brassica napus* and soybean plants) were provided by *Agrobacterium-mediated* plant transformation. The shoots were grown under in vitro conditions (tissue culture) and then transferred to a hydroponic medium (rock wool, see Examples). On said medium, the shoots were allowed to root. Simultaneously, transgenic shoots were selected by using suitable selecting agents.

Surprisingly, the transferred transgenic shoots had a high rooting ability on rock wool. Moreover, selection on a hydroponic medium was very reliable, since the majority of the shoots that developed roots were transgenic. If at all, only a few "escape" plantlets were observed ("Escapes" are plants that survive on selection even though they lack the selection marker gene, but that do not give rise to transgenic seeds). Thus, the method of the present invention allows for significantly reducing the number of non-transgenic plants. Also, the vegetative growth started very early. Therefore, the time needed from the isolation of putative transgenic in vitro shoots to transgenic ex vitro adapted greenhouse plants could be significantly reduced (compared with plant that were rooted under in vitro conditions and, then, transferred to soil). Compared with methods for plant transformation and regeneration described in the prior art, the method of the present invention is advantageous, since it allows for a reliable, quick and cost-effective generation of transgenic plants. Further advantageous of the method of the present invention are High rooting ability (> 90%), vegetative
growth at the beginning of the cultivation of the crop, selection during the rooting and elongation phase proven to be reliable (< 5% escape rate), favourable cost efficiency, time needed from the isolation of putative transgenic in-vitro shoots to transgenic ex-vitro adapted, greenhouse ready plants takes 5 weeks only.

Particularly, the method of the present invention allows, if applied, for avoiding root induction under in vitro conditions, thereby reducing the amount of steps carried out under in vitro conditions. Moreover, the method allows for reducing the time needed for regenerating whole plants since the plantlets are already adjusted to greenhouse conditions before they are transferred to soil. Finally, almost 100% of plants generated by the method of the present invention are transgenic plants (and thus plants which give rise to transgenic seeds), rather than "escape" plants.

Moreover, it was shown that transgenic shoots transferred to the hydroponic medium also root even when the hydroponic medium does not comprise auxin, i.e. even when the hydroponic medium is not supplemented with auxin. Thus, by not adding auxin to the hydroponic medium, costs can be saved.

All references cited in this specification are herewith incorporated by reference with respect to their entire disclosure content and the disclosure content specifically mentioned in this specification.

The following Examples merely illustrate the invention and should not be construed, whatsoever, as limiting the scope of the invention.

Example 1: Root induction under in vitro conditions (on Agar-based media)

Etiolated, 5-day old hypocotyl explants of *B. napus* germplasm Kumily are co-cultivated with *Agrobacterium rhizogenes* (strain SHA001 which is a disarmed variant of K599 (NCPBP 2659) carrying a disarmed variant of pRI2659 including construct LLL957 (contains the Arabidopsis double mutated (A122T_S653N) AHAS gene as a selectable marker driven by the parsley ubiquitin promoter, and a reporter gene cassette). Primary selection of transformed explants is performed on imidazolinone containing Agar-based medium using optimized conditions for shoot regeneration.

Regenerated, putative transgenic, *in-vitro* shoots (2-3 cm) are isolated and transferred to Agar-based MS rooting medium with various concentrations of auxins or other compounds, which are known to promote root formation. Modified versions of the rooting media as de-

Shoots which developed roots are transferred into soil in small pots. Shoots are cultivated in small greenhouses in which an increased humidity (> 90%) and a decreased light intensity are applied. During the first 4 to 6 days, the small greenhouses are shaded. After 7 to 10 days, depending on shoot quality, these shoots are transferred to normal greenhouse conditions.

In general after 6 weeks cultivation on Agar-based rooting medium, less than 50% of all non-transgenic or transgenic shoots develop roots. An increased rooting percentage was often obtained after refreshing the cut area of the shoot as well as the Agar-based rooting medium leading however to higher production costs.

Moreover, none of the media tested resulted in an acceptable rooting capacity. Therefore, the application of selection pressure during the rooting and elongation phase, which enables us to distinguish between escape and real transgenic shoots, is not reliable.

**Summary of problems**

- Low rooting ability (< 50%) of transgenic and non-transgenic (!) shoots
- Low ability of shoots elongation
- Selection between escape and real transgenic shoots during the rooting and elongation phase is not reliable due to the low rooting ability in general
- Highly germplasm depending
- Laborious and cost intensive (sterile jars, sterile medium, prepare jars with medium, transfer plants in to the jar, wait until root formation occurs, transfer *in-vitro* shoots with roots to *ex-vitro* conditions in soil, adapt shoots to *ex-vitro* conditions)
- Time needed from the isolation of putative transgenic *in-vitro* shoots to *ex-vitro* adapted, greenhouse ready plants takes 8 (best case) to 14 weeks (re-rooting needed)

Example 2: Root induction in an hydroponic system
A hydroponic system is a growing system in which the root zone is physically supported by media. The plants are fed by applying nutrient solution to the media. The use of hydroponic substrate systems in general and Grodan rockwool in particular has been known and proven to be functional world-wide for several vine crops and some cut flower production.

Root induction on rockwool mats was tested. As described in Example 1, a population of transgenic and non-transgenic *Brassica napus*, Kumily shoots (2-3 cm) grown under in vitro conditions was provided. The shoots were transferred into drained Grodan rockwool mats. The medium, used for draining, included 1/10 MS and auxin to promote rooting. Grodan rockwool mats, including the small plantlets, were placed in small greenhouses ensuring a high humidity (>90%) for 2-3 weeks. The small greenhouses were placed in a plant growth chamber at 20°C in a 16h/day photoperiod of cool white illumination at 120µE/m²s⁻¹. During the first 24 hours, the small greenhouses were shaded. After three weeks the mats were uncovered and transferred to normal greenhouse conditions. Watering of the plants in the mats occurs with medium including 1/10 MS. The first shoots were already developing roots after 10 days cultivation on rockwool mats. Greenhouse adapted shoots were transferred to soil after 5 weeks cultivation on Grodan rockwool mats. Over 90% of the shoots developed roots.

Selection was applied by spraying of Imidazolinone. The most optimal time point for spraying is when shoots have developed roots and leaves under greenhouse conditions. The concentration of Imidazolinone used for spraying depends on the germplasm. Concentrations used were in a range of 0.3 up to 20 g/ha (preferably between 0.625 - 10 g/ha). Selection was clearly observed two weeks after spraying. The apical meristem of escape shoots was blocked in its further development and the leaves of escape shoots turns slowly red. The selection is extremely tight. Less than 5% of all escape shoots survived the spraying.

Table with Data included below:

<table>
<thead>
<tr>
<th>Conc. Pursuit (g/ha.) applied by spraying</th>
<th># of tested shoots</th>
<th># of vigorous looking shoots incl. roots (14 days after spraying)</th>
<th># of Q-PCR negative shoots</th>
<th>Escape rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>29</td>
<td>29</td>
<td>1</td>
<td>3.4</td>
</tr>
<tr>
<td>0.625</td>
<td>30</td>
<td>30</td>
<td>2</td>
<td>6.7</td>
</tr>
<tr>
<td>1.25</td>
<td>33</td>
<td>28</td>
<td>1</td>
<td>3.6</td>
</tr>
<tr>
<td>2.5</td>
<td>73</td>
<td>63</td>
<td>1</td>
<td>1.6</td>
</tr>
</tbody>
</table>
Benefits of using a hydroponic system

- High rooting ability (> 90%)
- Vegetative growth at the beginning of the cultivation of the crop
- Selection during the rooting and elongation phase proven to be reliable (< 5% escape rate)
- Favourable cost efficiency
- Time needed from the isolation of putative transgenic in-vitro shoots to transgenic ex-vitro adapted, greenhouse ready plants takes 5 weeks only

Example 3: Ex vitro rooting of soybean shoots in OASIS® Rootcubes®

Soybean seed germination, propagation, A. rhizogenes and axillary meristem explant preparation, and inoculations were done as previously described (WO2005/121345; Olhoft et al., 2007) with the exception that the constructs used for transformation were either RLM 545, RLM546, RLM701, or RLM702 and all contained the E.coli dsdA gene driven by the parsley ubiquitin promoter as a selectable marker. Selection during shoot initiation and shoot elongation was on D-serine in a range from 5-15mM. All experiments were conducted with soybean genotype Dairyland 93061. OASIS® Rootcubes® (Smithers-Oasis Co., Kent, OH) mats were pre-wetted with water (6 rinses) and then drenched with water containing 1 mg/L indole acetic acid (IAA). Elongated shoots (1-4 cm in length) were excised from shoot clumps and the stems were placed in the Rootcubes at a spacing of 1.0 to 2.0 cm apart. The rootcubes containing the shoots were transferred to a growth chamber set to 26°C and a16/8 hour photoperiod. Twenty-eight shoots were transferred and observed for root production. After 8 days, all 28 shoots had successfully rooted. Rooted shoots are transplanted in 4" pots containing Metromix 360 (or similar growing medium) when the shoots have demonstrated new apical growth. The entire Rootcube with Oasis medium and rooted shoot was transplanted. Transplant success was 100%.

A second experiment replicated the first, except that the constructs tested were RLM545, RLM546. Both constructs contained the E.coli dsdk gene driven by the parsley ubiquitin promoter as a selectable marker. Selection during shoot initiation and shoot elongation was on D-serine in a range from 5-15mM. Seventy-nine elongated shoots (1-4 cm in length) were excised from shoot clumps and the stems were placed into the Rootcubes. Condi-
tions for rooting were identical to those above. Rooting was assessed after 14 days. Seventy-three of the 79 (92%) had successfully rooted.

A third experiment tested rooting in OASiS® Rootcube® mats (entire, connected sheets of rootcube material) compared to OASiS® Rootcube® Wedges®, which are individual and contained in a plastic tray. OASiS® Rootcube® Wedges® are advantageous in that the roots do not intertwine with adjacent plantlets and transfer to soil is simplified. Shoots from experiments using construct RLM 702. Sixty-eight of 79 shoots (86%) placed in the Rootcube mats rooted successfully after 14 days and 61 of 73 (84%) had successfully rooted in the Rootcube wedges.

A second experiment with Rootcube wedges replicated the first, except that the shoots were from constructs RLM545 and RLM546. Two experimental conditions were tested: rootcubes were pre-wetted with water (6 rinses) before drenching with water containing 1mg/L IAA or rootcubes were not pre-wetted but instead were simply drenched with water containing 1mg/L IAA. In the first treatment, 84 of 95 (88%) shoots were successfully rooted after 14 days and 78 of 82 (95%) were rooted in the second treatment.

Ex vitro rooting for excised soybean shoots can be successfully accomplished in OASiS® Rootcube® Wedges®.

Example 4. D-serine kill curve in OASiS® Wedges® established with germinated soybean seedlings

Seeds of soybean genotype 93061 were germinated on Oasis Wedges moistened with tap water. Germination was sufficient after approximately one week and the epicotyls and germinated shoot was excised from the hypocotyl, root and cotyledons. The epicotyl end of the shoot was placed into the Oasis medium. Excised shoots were watered with tap water, or tap water containing either 0.5, 1, 1.5, 2 and 2.5mM D-serine. Nine individual Wedges with excised shoots were placed in a Plantcon container and the shoots were watered once after 3 days. The shoots were incubated in a growth chamber set to 26°C and a 16/8 hour photoperiod. Rooting was assessed 7 days after excision. Two mM D-serine was chosen as the minimal concentration for applying selection to transgenic shoots selected on D-serine.

<table>
<thead>
<tr>
<th>Concentration of D-serine (mM)</th>
<th># of shoots that rooted/Total # of shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>18/18</td>
</tr>
</tbody>
</table>
Example 5. Comparison of in vitro rooting and ex vitro rooting of soybean shoots in OASIS® Wedges® with selection on D-serine (5-15 mM range). Rooting was accomplished by transferring putatively transformed, elongating shoots to a semi-solid rooting medium in plantcons with or without 2 mM D-serine, or to OASIS® Rootcube® Wedges®, 9 per plantcon as described in the previous example and watered with tap water containing D-serine at a concentration of 4 mM. The shoots were incubated in a growth chamber set to 26°C and a 16/8 hour photoperiod. Total number of surviving shoots was determined 14 days after transfer to either rooting media.

<table>
<thead>
<tr>
<th></th>
<th>Total putative shoots</th>
<th>Number died in selection</th>
<th>Total Rooted shoots</th>
<th>% Shoots Eliminated</th>
</tr>
</thead>
<tbody>
<tr>
<td>R601 semi-solid rooting medium</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>0%</td>
</tr>
<tr>
<td>R601 medium + 2 mM D-ser</td>
<td>30</td>
<td>4</td>
<td>26</td>
<td>13%</td>
</tr>
<tr>
<td>Oasis® medium + 4 mM D-ser</td>
<td>85</td>
<td>69</td>
<td>16</td>
<td>81%</td>
</tr>
</tbody>
</table>

Example 6. Ex vitro rooting of soybean shoots in OASIS® Wedges® with selection on imazapyr.
Soybean seed germination, propagation, *A. rhizogenes* and axillary meristem expiant preparation, and inoculations were done as previously described (WO2005/121345; Olhoft et al., 2007) with the exception that the one of the following constructs, RJE396, RJE225, RJE289 and RJE400 each containing a mutated AHAS gene tolerant to imidazolinone herbicides and driven by the parsley ubiquitin promoter, was used for selection. Transplantation of shoots to OASIS® Wedges® was as described in the previous examples. Shoots in Oasis Wedges were watered with regular city water or water containing 3µM Imazapyr and were incubated in a growth chamber set to 26°C and a16/8 hour photoperiod. Elongated shoots that survived to transplantation were assayed for the presence of the transgene up to 3 months after the initiation of transformation experiments. The escape rate was calculated by determining the number of surviving shoots without the transgene as a percentage of the total number of elongated shoots that were analyzed. No escapes were identified from those shoots that were watered with 3 µM Imazapyr.

<table>
<thead>
<tr>
<th></th>
<th># Tested Shoots</th>
<th># Elongated Shoots Analyzed (Taqman)</th>
<th>#Taqman Positive Shoots</th>
<th>Escape Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>15</td>
<td>11</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Imazapyr, 3 µM</td>
<td>120</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

Example 7. Determining optimal level of imazapyr for ex vitro rooting and selection.

Soybean seed germination, propagation, *A. rhizogenes* and axillary meristem expiant preparation, and inoculations were done as previously described (WO2005/121345; Olhoft et al., 2007) with the exception that the following construct RJE396 was used for selection for selection. RJE396 contains a mutated AHAS gene tolerant to imidazolinone herbicides and is driven by the parsley ubiquitin promoter. Transplanting shoots to OASIS® Wedges® was as described in the previous examples. Shoots were watered with 1µM, 2µM or 3µM imazapyr for 2-3 weeks, and were incubated in a growth chamber set to 26°C and a16/8 hour photoperiod. The total number of surviving shoots was counted. Transgenic shoots survived and rooted on all concentrations of imazapyr. Surviving shoots were assayed for the presence of the transgene and the escape rate was calculated by determining the number of surviving shoots without the transgene as a percentage of the total number of surviv-
ing shoots. No escapes which were identified at any level of selective pressure. Overall
survival was best on 1µM imazapyr.

<table>
<thead>
<tr>
<th>Arsenal H2O (µM)</th>
<th>Total Tested Shoot Clusters (2-3wks on 521)</th>
<th>Survived Shoots</th>
<th>Survived Shoots Rate (%)</th>
<th>Escape Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>329</td>
<td>103</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>102</td>
<td>21</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>312</td>
<td>61</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

Example 8. Ex vitro rooting of Brachypodium regenerants in OASIS® Wedges®

*Brachypodium* regenerants derived from embryogenic calli were difficult to root in convention semi-solid agar rooting medium. Root production was slow (>30 days), roots were not well-formed and plantlets were weak. Instead of rooting in a agar medium, transplanting of either rooted or non-rooted seedlings to Oasis Wedges greatly improved the survival of in vitro cultured seedlings and shorten the overall time required for the seedling establishment from 60 days to 30 days.

Embryogenic *Brachypodium* cultures were induced to produce shoots by transferring callus to medium lacking 2,4-D and containing 0.5 mg/L 6-benzylamino purine (BAP) in the light at 27°C. Regenerated shoots were transferred to pre-moistened (with water) Oasis Wedges and maintained for two weeks in the light at 27°C. Rooted shoots were subsequently transferred to soil (Metromix 360) and grown to maturity in the greenhouse.

<table>
<thead>
<tr>
<th>Line name</th>
<th># Seedlings transferred to Oasis</th>
<th># Seedlings survived and set seeds</th>
<th>Survival rate of transplanting (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPTCK0021</td>
<td>30</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>DPTCK0202</td>
<td>30</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>DPTCK0208</td>
<td>30</td>
<td>30</td>
<td>100</td>
</tr>
</tbody>
</table>
Patent Claims

1. A method for generating transgenic plants comprising the steps,
   a) providing a population of transgenic and non-transgenic rootless
      plant shoots grown under in vitro conditions,
   b) transferring said plant shoots to a hydroponic medium, and
   c) simultaneously allowing said shoots to root on said hydroponic me-
      dium and selecting for transgenic shoots.

2. The method of claim 1, wherein step c) is carried out under ex vitro conditions.

3. The method of claim 2, wherein said ex vitro conditions are greenhouse conditions.

4. The method of any one claims 1 to 3, wherein said hydroponic medium comprises a
   hydroponic compound selected from the group consisting of rock-wool, glass-wool
   and slag wool.

5. The method of any one of claims 1 to 3, wherein in the step b) the plant shoots are
   transferred to a formaldehyde or cellulose-based foam.

6. The method of any one of claims 1 to 5, wherein said population of transgenic and
   non-transgenic rootless plant shoots provided in step a) is derived from transforming a
   population of plant cells by *Agrobacterium*-mediated transformation, electroporation or
   particle bombardment.

7. The method of any one of claims 1 to 6, wherein said plant shoots are selected from
   shoots of monocotyledonous and dicotyledonous plants.

8. The method of claim 7, wherein said plant shoots are selected from shoots of plants of
   the genus *Brassica, Arabidopsis, Oryza, Nicotiana, Zea, Glycine*.

9. The method of claim 7, wherein said plant shoots are from *Brassica napus*.

10. The method of claim 7, wherein said plant shoots are from *Glycine max*. 
11. The method of any one of claims 1 to 10, wherein said hydroponic medium is not supplemented with an auxin.

12. The method of any one of claims 1 to 11, wherein said transgenic plant shoots develop primary and lateral roots in said hydroponic medium.

13. The method of any one of claims 1 to 12, further comprising allowing the generated transgenic plants to develop seeds and collecting said seeds.

14. Use of a hydroponic medium for simultaneously allowing shoots to root and selecting for transgenic shoots in a population of transgenic and non-transgenic rootless plant shoots grown under in vitro conditions.
A. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
AOIH C12N AOIG

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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Date of the actual completion of the international search: 29 July 2010

Date of mailing of the international search report: 12/08/2010

Name and mailing address of the ISA/Authorized officer:
European Patent Office, P B 5818 Patentlaan 2
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Tel (+31-70) 340-2040
Fax (+31-70) 340-3016

Kania, Thomas
## INTERNATIONAL SEARCH REPORT

**International application No**  
PCT/EP2010/058377

### DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<td>A</td>
<td>WO 01/87070 A1 (ROCKWOOL INT [DK]; KUIPER DAAN [NL]; LUGTENBERG EGBERTUS JOHANNES J (N) 22 November 2001 (2001-11-22) cited in the application</td>
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</tr>
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