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(54) Title: SOMATIC EMBRYOGENESIS OF SQUASH (57) Abstract A method involving somatic embryogenesis of various squash (<i>Cucurbita pepo</i> L) tissues is described which can be used for the regeneration of normal squash plants. This regeneration method is also used for the transfer and integration of genetic materials into the genome of squash plants, which belong to the family Cucurbitaceae, followed by regeneration of such transformed plants. Thus regenerated transformed whole squash plants are produced by this method.		

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SOMATIC EMBRYOGENESIS OF SQUASH

FIELD OF INVENTION

The present invention relates to a regeneration method for squash (Cucurbita pepo
5 L.) and the use of the sources of regenerable tissue for the transfer of genetic material.
More specifically, the invention relates to a process for preparing squash tissues,
obtaining somatic embryos, and regenerating these embryos into normal squash plants.
This invention also provides for the transfer of genetic material into squash embryos for
the purpose of producing transformed plant cells and transformed squash plants which
10 are resistant to plant viruses, insects, weeds, pathogenic microbes and to obtain other
beneficial traits as a result of the transfer of genetic information.

BACKGROUND OF THE INVENTION

Squash (Cucurbita pepo L.) is an important crop in many regions of the temperate
zones of both hemispheres, and improvement in the ability of this crop to resist viruses,
15 insects, weeds, and to improve its growth vigor would be of considerable commercial
value. Present plant breeding programs have made considerable improvements to this
species with respect to these characteristics; however, it is believed that these
characteristics could be made more rapidly with the development of cellular events such
as tissue culture regeneration and molecular events such as the transfer of genetic
20 material.

The regeneration of cucurbit species from tissue cultures has been reported for
cucumber (Cucumis sativas L.) (Nadolska-Orczyk and Malepszy, 1984) and melon
(Cucumis melo L.) Moreno et al, (1985). Schroder (1968) reported the production of
embryogenic tissue from pericarp tissues of squash. Jelaska (1972, 1973) reported
25 somatic embryogenesis in hypocotyl and cotyledon-derived callus of pumpkins and
demonstrated that embryos could develop into normal plants. Pink and Walkey (1984)
reported a rapid propagation method for pumpkin through apical meristem culture.
However, the literature contains no reports on whole plant regeneration of squash, while
we describe here two methods for the efficient regeneration of squash. These
30 regeneration methods also provide the material which can be utilized for the transfer of
genetic material into the squash genome.

One method for the transfer of genetic information into the genomes of

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dicotyledonous plants involves the use of two pathogenic species of Gram-negative soil bacteria, Agrobacterium tumefaciens and Agrobacterium rhizogenes (see review by Bevan and Chilton, 1982) which results in the diseases known as Crown Gall and Hairy Root, respectively. In each of these diseases the causative agent has been identified as
5 resulting from the transfer and integration of a segment of the large pTi or pRi DNA plasmid, known as the T-DNA regions (Chilton et al., 1977; Chilton et al., 1982). The identification of several common steps in the pTi and pRi T-DNA transfer mechanism and the genes contained within these T-DNA regions which are responsible for the disease has resulted in the engineering of avirulent Agrobacterium strains (Hepburn et
10 al., 1985; Hood et al., 1986; Vilaine and Casse-Delbart, 1987). This information has also led to the construction of smaller, wide-host-range, plasmids which are capable of replication in both E.coli and Agrobacterium strains. Such plasmids contain the Agrobacterium-derived pTi or pRi DNA signals necessary for the transfer of an engineered T-DNA region from the bacteria into plant cells. These plasmids are referred to as
15 binary plasmids (Bevan, 1984; An et al., 1985).

Many of the species belonging to the family Cucurbitaceae are known to be susceptible to infection by these Agrobacterium pathogens (see review by Anderson and Moore, 1979; Smarrelli et al., 1986). Regeneration of squash (Cucurbita pepo L.) is described below. The combination of these two facts suggests that Agrobacterium-
20 mediated transfer of genetic information into the genome of cucurbit species (such as Cucurbita pepo L.) should be readily achievable.

In addition to the use of Agrobacterium-mediated transformation of regenerable plant tissues a second method for gene transfer has recently been developed: the use of microprojectiles to transfer DNA molecules into plant cells (Klein et al., 1987). The
25 method (described below) for the regeneration of squash provides tissues which can be readily transformed by the microprojectile procedure. The development of regeneration and transformation systems for the transfer and stable integration of genetic material into the genome of cucurbit species (cucumber, melon, and squash) will be useful for the transfer of genetic traits which are difficult or impossible to transfer via conventional
30 plant breeding techniques.

The development of gene transfer technology for cucurbit species (such as squash) will be used to transfer engineered genes which will: confer resistance to viral infection (Powell-Able et al., 1986; Cuozzo et al., 1988), herbicide resistance (Comai et al.,

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1985; della-Cioppa et al., 1987; Lee et al., 1988; Stalker et al., 1985), or any other useful genetic traits such as genes for resistance to insect pests, microbes and other pests, see examples listed below.

INFORMATION DISCLOSURE

5 The transformation and regeneration of transformed cucumber plants which express the NPT II gene has been previously reported by Trulson et al. (1986). This transformation was achieved using Agrobacterium rhizogenes. European patent application EP O 223 452 describes plants that are resistant to viral diseases and methods for producing them. PCT patent application PCT/US86/00614 refers generally to a
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SUMMARY OF THE INVENTION

This invention provides:

A method of regenerating and transforming Cucurbita pepo L. (squash) plants, which belong to the family Cucurbitaceae, which comprises (1) excising squash tissue selected from the group consisting of shoot tips from germinating squash seeds and squash tissue from mature seeds, (3) producing embryogenic calli from said tissues, being either non-transformed or transformed, (4) selectively growing the transformed embryogenic calli on media containing kanamycin, and (5) subjecting the transformed embryogenic calli to an embryogenic regeneration procedure from which whole transformed squash plants can be obtained.

When the excised tissue is shoot tips from germinating squash seed, the transformed embryogenic calli is produced by (a) inoculating said excised squash tissue with virulent or avirulent strains of Agrobacterium and (b) culturing the resulting explants on an induction media consisting of MS media, 2,4,5-T, BA, Kn.

When the excised tissue is tissue from mature squash seed, the transformed embryogenic calli is produced by (a) inoculating said excised squash tissue with virulent or avirulent strains of Agrobacterium and (b) culturing the resulting explants on an induction media consisting of MS media, 2,4-D or 2,4,5-T, BA, Kn.

DETAILED DESCRIPTION OF THE INVENTION

In general the process of this invention involves the steps schematically shown in Chart 1.

Formation and Regeneration of Embryonic Squash Calli

Method 1 Somatic embryos derived from shoot tip explants

Plant Material

Seeds of summer squash (Cucurbita pepo L.) cv. YC 60 were obtained from the Asgrow Seed Co., Kalamazoo, MI and they were soaked in tap water for approximately 15 minutes and then their seed coats were removed by hand. The de-coated seeds were treated with 95% alcohol for two minutes followed by immersion in a 25% (v/v) solution

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of commercial hypochlorite (Clorox, which contains 5.25% sodium hypochlorite) for 25 minutes and then rinsed four times with sterile distilled water. Sterilized seeds were placed in Magenta boxes with 50 ml of Murashige and Skoog (1962) (MS) basal medium contains 30 g/l sucrose, 8 g/l Difco agar (Difco Laboratories). The seeds were germinated in a Percival incubator at 28°C in darkness.

Induction of somatic embryogenesis and plant regeneration:

Shoot apices consisting of the apical domes and some supporting tissue were excised from seven-day old *in vitro* grown seedlings. The apices were cut into longitudinal halves and cultured horizontally on MS plus 1.2 mg/l of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) plus 0.8 mg/l 6-benzylaminopurine (BAP) and 0.1 mg/l β -naphthyleneacetic acid (NAA). All tissue culture media were supplemented with 3% sucrose and solidified with 0.8% Phytagar (Gibco). The pH of the media was adjusted to 5.8 prior to being autoclaved at 121°C for 20 minutes. All subsequent tissue cultures were maintained in darkness at 28°C. After two weeks of culturing on this medium, the shoot tips explants became swollen. The tissue lost its green color and became amber. Embryogenic callus tissues were propagated by successive subculturing of the slimy, translucent callus tissue onto plates containing fresh MS medium plus 1.2 mg/l 2,4,5-T, 0.8 mg/l BAP, and 0.1 mg/l NAA, every week. After six weeks, the callus tissue propagated appeared shiny, translucent and slimy. Following eight weeks of incubation using the above conditions, putative somatic embryos were observed to organize from sectors on the surface of the slimy callus. To promote further development of the putative somatic embryos, they were transferred to conversion medium, which was composed of MS medium plus 0.05 mg/l NAA and 0.05 mg/l kinetin. These structures were maintained on this medium at 28°C under diffuse cool-white fluorescent lamps (4 klx) with a 16-hour photoperiod. Within three weeks, shoot-tips of the embryos became green and plantlets were obtained three weeks later. About 75% of somatic embryos developed into whole plants. A superficial evaluation revealed that regenerated plants displayed no gross phenotypic abnormalities. Regenerated plants flowered and set seed.

Method 2 Somatic embryos derived from mature seeds.

Plant material

Mature seeds of summer squash, *Cucurbita pepo* L. cv. YC 60, were obtained from the Asgrow Seed Company, Kalamazoo, MI. The seeds were surfaced sterilized for 10 minutes with a 15% (v/v) solution of Clorox (commercial bleach containing

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5.25% sodium hypochlorite) and rinsed three times with sterile water. The seed coats were removed manually and the seeds sterilized again with 25% Clorox for 25 minutes, rinsed three times with sterile water, following by 70% alcohol for one minute and rinsed three more times with sterile water.

5 Induction of somatic embryogenesis and plant regeneration:

(A) Sterilized seeds were cut transversely into two unequal sections: one section containing the embryonic axis and one-third of the cotyledon and the other section containing two-thirds of the cotyledon. The explants containing the embryonic axis were cultured at 26°C., in darkness, with the cut surface facing up, and the explants containing only the cotyledons were cultured horizontally on MS basal medium containing a factorial combination of eight concentrations of 2,4-D (0.5, 1, 2, 3, 5, 10, 25, 50 mg/l) and five concentrations of kn (0, 0.5, 1.2, 3 mg/l). After nine weeks with one subculture, the embryogenic callus tissue containing putative somatic embryos were formed on the surface of explants. These somatic embryos were transferred to conversion medium composed of MS basal medium containing 0.05 mg/l NAA and 0.05 mg/l kinetin. These structures were maintained on this medium at 28°C under diffuse cool-white fluorescent lamps (4 klx) with a 16-hour photoperiod. As soon as the apical regions displayed development, the plantlets were transferred to Magenta boxes containing one-half strength MS basal medium. After plantlets developed a root system, they were transferred to potting mix and covered with a Ziploc\ storage bag for hardening-off. Subsequently, the hardened plants were transplanted to soil and grown to maturity in a greenhouse. Regenerated plants displayed no gross phenotypic abnormalities, flowered and set seeds which were viable and produced subsequent generations.

25 B. Explants were cultured on MS basal medium containing a factorial combination of three concentrations of IAA (0, 1.5 and 3 mg/l) and five concentrations of kn (0, 1.5, 3.0, 4.5 and 6.0 mg/l). After nine weeks with one subculture, the tissues were scored for embryogenic callus formation.

C. The explants were cultured on MS basal medium containing 2,4,5-T (1.2 mg/l), BA (0.8 mg/l) and kinetin (0.1 mg/l) at 26°C. in darkness. After nine weeks with one subculture, the embryogenic callus tissues containing putative somatic embryos that formed on the surface of the explants were transferred to conversion medium and incubated under diffuse cool white fluorescent lamps (4 klx) with a sixteen-hour

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photoperiod. Subsequent procedures were followed as described in experiment 1.

Abbreviations:

BA = 6-benzylaminopurine,

2,4-D = 2,4-dichlorophenoxyacetic acid,

5 IAA = indoylbutyric acid,

NAA = naphthylacetic acid,

2,4,5-T = 2,4,5-trichlorophenoxyacetic acid,

Kn = kinetin

MS = Murashige and Skoog (1962).

10 Transformation procedures

Example 1 The use of *Agrobacterium* for transformation

Transformation of squash tissues, derived either from the shoot tips or freshly cut mature seeds was done using methods similar to those described by Horsch et al. (1985). Shoot apices consisting of the apical domes and some supporting tissues were removed
15 aseptically from young squash seedlings and they were submerged in an overnight culture of either the avirulent *Agrobacterium* strain C58Z707 (Hepburn et al., 1985), virulent strain C58 (Depicker et al., 1980) or any other strains of *Agrobacterium tumefaciens* or *rhizogenes*. In addition, squash tissues derived from freshly cut mature seeds and are subjected to transformation by *Agrobacterium* in a similar manner. *Agrobacterium*
20 strains should contain a binary plasmid, such as, pGA482 (An et al., 1985; An, 1986) which has been modified (see examples) to contain a beneficial gene(s) for transfer and integration into the squash genome. After gentle shaking to ensure that all edges were infected, the plant tissues were blotted dry and cultured abaxial side down in a sterile petri dish containing 10 ml of initiation medium (MS basal medium supplemented with
25 1.2 mg/l of 2,4,5-T; 0.8 mg/l of BAP; 0.1 mg/l of kinetin) as described above, followed by growth in the dark at 26°C. Tissues derived from mature seeds would be treated using MS medium containing 5 mg/l 2,4-D or 1.2 mg/l 2,4,5-T and 0.8 mg/l BA and 0.1 mg/l kinetin. After four days, the *Agrobacterium*-infected tissues are transferred to petri plates containing the same medium supplemented with 500 µg/ml carbenicillin
30 and between 100 to 200 µg/ml kanamycin and cultured for six additional weeks in the dark at 28°C. In cases where *Agrobacterium* overgrowth was a problem, the tissues were rinsed with MS salt containing 500 µg/ml carbenicillin prior to transferring to subsequent petri plates.

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Regeneration of potentially transformed squash callus tissues was done according to the respective procedures described above in the embodiments of the invention. After incubation for about nine weeks (two to three subcultures) characteristic gel like callus formed at the surface of the squash tissues. Embryogenic calli containing putative
5 somatic embryos were excised and placed on MS basal medium containing 0.05 mg/l of NAA; 0.05 mg/l of kinetin (this medium is identical for both squash tissue sources); 100 to 200 mg/l of kanamycin sulfate; 500 mg/l of carbenicillin and grown under a 16-hour photoperiod using 2 klx of cool white light. As soon as the apical regions displayed development, the plantlets were transferred to magenta boxes containing one-half strength
10 MS basal medium. After plantlets developed a root system, they were transferred to potting mix. Plants regenerated in this manner displayed no gross phenotypic abnormalities. They flowered and set seeds which produced subsequent generations.

Example 2 The use of microprojectiles for transformation

Squash somatic embryos derived from squash shoot tips are obtained as described
15 in the embodiment of the invention (MS medium containing 1.2 mg/l of 2,4,5-T; 0.8 mg/l BAP; 0.1 mg/l of kinetin). In addition, squash somatic embryos can be obtained from mature squash seeds as described in the invention (MS medium containing 5 mg/l 2,4-D or 1.2 mg/l 2,4,5-T and 0.8 mg/l BA and 0.1 mg/l kinetin). These embryo tissues were bombarded with microprojectiles which have been coated with DNA
20 containing a plasmid encoding a beneficial gene(s), as described by the manufacturer. After bombardment these tissues were allowed to grow for two days on these plates followed by transfer to plates containing the respective fresh media. If the DNA construction used contained the plant expressible NPT II gene, the selection drug, kanamycin 100 to 200 mg/ml was added to the fresh plates. Squash tissues bombarded
25 with microprojectiles were regenerated using the procedures described above (depending on the original tissue source) in the embodiment of the invention.

The transfer of useful genetic material

Example 3 The transfer of virus coat protein genes

The purpose of this example is to generate a construction for the expression of
30 a plant virus coat protein gene which, when expressed in a plant, results in reduced symptoms or resistance to later infections by that virus. In general, after the identification of a coat protein gene by nucleotide sequencing, its sequences can be modified by using specific oligomers and the technique referred to as polymerase chain reaction

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(PCR), to attach specific restriction enzyme sites to any coat protein gene. These restriction enzyme sites can be used to clone the coat protein gene into a plant expression vector which contains the necessary gene regulatory elements needed for controlling expression of the gene after transfer into the genome of various plants. A scheme
5 describing the use of the plant expression vector p18UCcpexp for the cloning of various coat protein genes is shown in Chart 2. To facilitate Agrobacterium-mediated gene transfer the plant expressible coat protein is cloned into a binary vector and to facilitate microprojectile-mediated gene transfers a marker gene such as the β -glucuronidase gene (Jefferson, et al. 1987) is attached to the plasmid. (see Chart 2).

10 Plants that are resistant to virus diseases and methods for producing them are described in EP 223,452, and in Patent Application PCT/US89/03288, published March 8, 1990, entitled "Cucumber Mosaic Virus Coat Protein Gene", and Patent Application PCT/US89/03094 published March 8, 1990, entitled "Potyvirus Coat Protein Genes and Plants Transformed Therewith". The use of viral coat protein gene to obtained
15 resistance in transgenic plants has been described by Powell-Abel et al (1986). Viral coat protein genes are isolated from any number of plant virus classes (tobamo, cucumo, poty, tobra, AMV, etc.) and expressed constitutively in plants after the attachment of the CaMV promoter and downstream 3' plant polyadenylation signals.

The construction of plant expressible coat protein genes is described in Examples
20 3 and 4 and accompanying charts of Application PCT/US89/03288 and in Examples 8, 11 and 14 and accompanying charts of Application PCT/US89/03094. These Examples and accompanying charts are herein incorporated by reference.

Example 11 Transfer of Herbicide Resistance Gene

This example illustrates how to generate plant expressible genes which allow a
25 plant to be resistant to specific classes of herbicides. Such plants are useful for several reasons: (1) herbicides normally lethal can be used, and (2) different crops can be used in close rotations on soil which may contain residual amounts of a previously used herbicide that is normally lethal to the second crop. Two genes of interest are mutant derivatives (derived from plant or bacterial sources) of the acetolactate synthase (ALS)
30 gene which are not sensitive to chlorsulfuron and sulfometuron methyl herbicides (Falco et al., 1985) and mutants of the gene encoding enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Stalker et al., 1985) which are not sensitive to the herbicide glyphosate.

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A gene which encodes an important enzyme which is either resistant to or detoxifies a specific herbicide is cloned downstream from a plant promoter, such as: CaMV 35S (Pietrzak et al, 1986), ribulose-1,5-biphosphate carboxylase small subunit gene (Mazur and Chui, 1985) or other strong plant gene promoter and upstream from
5 a plant gene poly (A) signal sequence (see Chart 3).

This gene is then cloned into an Agrobacterium-derived vector (either binary or cis) and using the above-described plant transformation methods this genetic material can be transferred and integrated into the squash genome (Chart 3). This gene can also be cloned into a vector containing a plant expressible marker gene (Chart 3) and transferred
10 into squash plants using the microprojectile-mediated gene transfer method (see Example 2).

Example 5 Transfer of Insect-Resistance Genes

In nature, numerous polypeptides exist which are toxic to insects and nematodes. The best known protein toxins are those associated with different strains Bacillus
15 thuringiensis; for example, B. israelensis is active against Diptera (mosquitoes and blackflies), B. Thuringiensis is active against Lepidoptera, and B. san diego is active against Colioptera. The toxin protein found in each of these bacteria is highly specific to insect pests; they are not toxic to other organisms. Thus, the transfer and expression of genes encoding such toxic proteins in plants are beneficial in reducing insect damage
20 without using chemical insecticides thereby avoiding risk to other organisms. The genes encoding many of these toxic proteins have been isolated and sequenced (Schmepf et al., 1985; Waalwijk et al., 1985; Sekar et al., 1987). The transfer of the B. thuringiensis toxin gene into tobacco and its usefulness in protecting the plant from insect damage has been reported by Vaeck et al. (1987). Thus, the combination of using the regeneration
25 and transformation systems described here and plant expressible Bacillus toxin genes (Chart 4) allows for the transfer of a useful genetic trait to squash (see Examples 1 and 2 for gene transfer methods).

Example 6 Transfer of Antimicrobial Gene

This example is to illustrate how to generate plant expressible genes which allow
30 a plant to be resistant to infections by bacterial and fungi. In nature, several classes of polypeptides have been isolated and found to convey broad-spectrum antimicrobial activity; for example, magainins (Zasloff, 1987), defensins (Daher et al., 1988), lysozymes (Boman et al., 1985), Cericorpins (Boman et al., 1989), attacins (Haltmark

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et al., 1983), thionins (Bohlmann et al., 1988), and the like. Genes encoding these antimicrobial peptides or their more active modified forms can be synthesized and engineered for expression in plants. Promoters for the expression of these antimicrobial polypeptide genes can include the constitutive type or others which have tissue
5 specificity. The use of tissue specific promoters, or wound inducible promoters (Sanchez-Serrano et al., 1987) would be useful so as to produce the antimicrobial peptide only when it is needed or in tissues which are more vulnerable to attack by bacterial or fungi, or both pests. Thus the engineering of genes encoding antimicrobial polypeptide genes combined with the plant transformation and regeneration schemes described in the
10 embodiment and Examples 1 and 2 would allow for the transfer of a useful trait to squash plants. Chart 5 summarizes the construction of plasmids which could be used with the Agrobacterium-mediated and microprojectile-mediated gene transfer systems.

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CHART 1

Remove Seed Coats, Treat with Clorox, Then Alcohol

5

Germinate Seed For Three to Five days

or

Obtain Mature Squash Seeds and Treat with Clorox

10

Excise Shoot Apices From Germinating Seedlings

Use Prescribed Method to Produce Somatic Embryos

or

Cut Seeds and Use Prescribed Method to

Produce Somatic Embryos

15

Dip Plant Tissues Into Agrobacterium

or

Bombard Tissues With DNA Coated Microprojectiles

20

Transfer to Culture Plates and Cultivate For Four Days

Transfer To Fresh Medium

25

Culture Nine Weeks In Darkness

(Can See Callus Growing On Side)

Excise Putative Somatic Embryos And

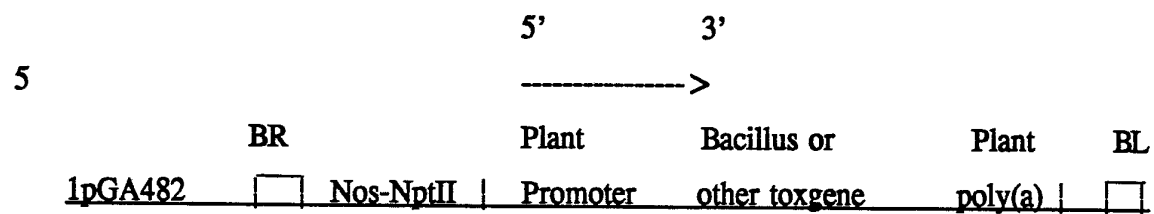
Put In Regeneration Media In Regeneration to Produce Plant

30

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Chart 2

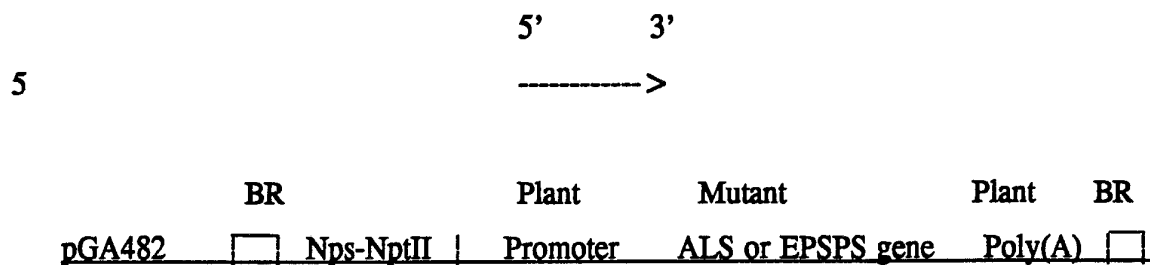
Coat protein gene vectors



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Chart 3

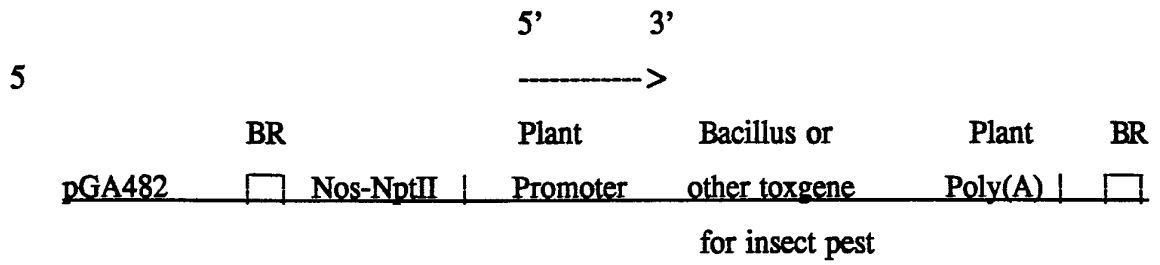
Herbicide gene vectors



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Chart 4

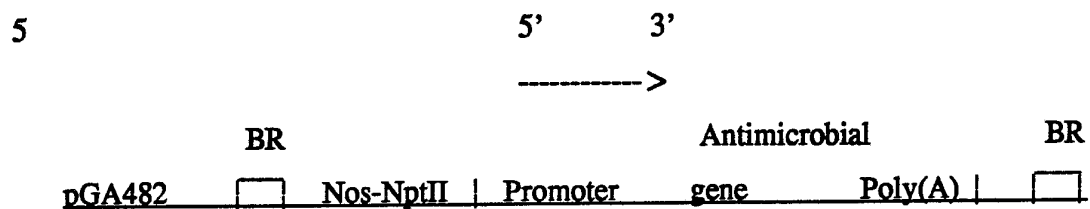
Insect resistance gene vectors



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Chart 5

Antimicrobial gene vectors



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CLAIMS

1. A method of regenerating and transforming Cucurbita pepo L. (squash) plants, which belong to the family Cucurbitaceae, which comprises (1) excising squash tissue
5 selected from the group consisting of shoot tips from germinating squash seeds and squash tissue from mature seeds, (3) producing embryogenic calli from said tissues, being either non-transformed or transformed, (4) selectively growing the transformed embryogenic calli on media containing kanamycin, and (5) subjecting the transformed embryogenic calli to an embryogenic regeneration procedure from which whole
10 transformed squash plants can be obtained.
2. A method according to claim 1, wherein the excised tissue used a shoot tip from young squash plants.
- 15 3. A method according to claim 2 wherein the transformed embryogenic calli is produced by (a) inoculating said excised squash tissue with virulent or avirulent strains of Agrobacterium and (b) culturing the resulting explant on an induction media consisting of MS media, 2,4,5-T, BA, Kn.
- 20 4. A method according to claim 2 wherein the transformed embryogenic calli is produced by culturing the excised squash tissue on an induction media consisting of MS media, 2,4,5-T, BA, Kn, and introducing foreign DNA into the resulting embryoid tissues by bombardment with microprojectiles.
- 25 5. A method according to claim 1 wherein transformed embryoids are identified by their expression of antibiotic or herbicide resistance.
6. A method according to claim 1 wherein the excised tissue is tissue from mature seeds.
- 30 7. A method according to claim 6 wherein the transformed embryogenic calli is produced by (a) inoculating said excised squash tissue with virulent or avirulent strains of Agrobacterium and (b) culturing on an induction media consisting of MS media, 2,4-

-21-

D or 2,4,5-T, BA, Kn.

8. A method according to claim 6 wherein the transformed embryogenic calli is produced by (1) culturing the excised squash tissue on an induction media consisting of
- 5 MS media, 2,4-D or 2,4,5-T, BA, Kn and (2) introducing foreign DNA into the resulting embryoid by microprojectile bombardment.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/04692

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : C 12 N 15/82, A 01 H 4/00											
II. FIELDS SEARCHED Minimum Documentation Searched ⁷ <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;">Classification System </td> <td style="width: 50%; border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; padding: 10px;">IPC⁵</td> <td style="border: none; padding: 10px;">C 12 N, A 01 H</td> </tr> </table> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸			Classification System	Classification Symbols	IPC ⁵	C 12 N, A 01 H					
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category ⁹</th> <th style="width: 70%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;">Commonwealth Agricultural Bureau, 871657363 CAB, Quest Acc. no. 87245434, S. Jelaska: "Cucurbits" & Biotechnology in agriculture and forestry 2. Crops I (edited by Bajaj, Y. P. S.), Berlin; Springer-Verlag, 1986, P. 371-386, 485-519, Many ref. in English, (JA: 8703) see the abstract --</td> <td style="text-align: center; vertical-align: top;">1</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;">Biological Abstracts, volume 80, 1985, Jelasca, Sibila: "Induction of embryogenic callus in Cucurbita pepo hypocotyl explants by indole-3- ethanol and its sugar conjugates", see page AB-819, abstract 89878, & Physiol. Plant 64(2): 237-242. 1985 -- ./.</td> <td style="text-align: center; vertical-align: top;">1</td> </tr> </tbody> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	A	Commonwealth Agricultural Bureau, 871657363 CAB, Quest Acc. no. 87245434, S. Jelaska: "Cucurbits" & Biotechnology in agriculture and forestry 2. Crops I (edited by Bajaj, Y. P. S.), Berlin; Springer-Verlag, 1986, P. 371-386, 485-519, Many ref. in English, (JA: 8703) see the abstract --	1	A	Biological Abstracts, volume 80, 1985, Jelasca, Sibila: "Induction of embryogenic callus in Cucurbita pepo hypocotyl explants by indole-3- ethanol and its sugar conjugates", see page AB-819, abstract 89878, & Physiol. Plant 64(2): 237-242. 1985 -- ./.	1
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IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; padding: 5px;"> Date of the Actual Completion of the International Search 5th December 1990 </td> <td style="width: 50%; border: none; padding: 5px;"> Date of Mailing of this International Search Report 22.12.1990 </td> </tr> <tr> <td style="width: 50%; border: none; padding: 5px;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="width: 50%; border: none; padding: 5px;"> Signature of Authorized Officer </td> </tr> </table>			Date of the Actual Completion of the International Search 5th December 1990	Date of Mailing of this International Search Report 22.12.1990	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer 					
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Biological Abstracts, volume 59, no. 2, 1985, Jelaska, Sibila: "Embryogenesis and organogenesis in pumpkin explants", see page 1120, abstract 10484 & Physiol. Plant 31(4): 257-261. illus. 1974. --	1
A	WO, A, 89/05859 (UPJOHN) 29 June 1989 see page 17, lines 10-15; page 8, lines 19-22 --	3-8
A	EP, A, 0262972 (PLANT CELL RESEARCH INSTITUTE) 6 April 1988 see the whole document --	3,7
A	Commonwealth Agricultural Bureau, 861655509 CAB, Quest Acc. No. 86482970 J. Smarrelli Jr. et al.: "Response of various cucurbits to infection by plasmid harboring strains of agrobacterium", & Dep. Biol., Loyola Univ. Chicago, Chicago, Illinois 60626, USA, Plant Physiology, Vol. 82, No. 2, 1986, P. 622-624, 17 Ref. in English (JA: 8701) see the abstract --	3,7
A	Commonwealth Agricultural Bureau, 871658967 CAB, Quest Acc. No. 87251406 R.D. Vaultx et al.: "Obtention of embryos and plants from in vitro culture of unfertilized ovules of cucurbita pepo", & Genetic manipulation in plant breeding. Proceedings international symposium organized by Eucarpia, September 8-13, 1985, Berlin (West), Germany, Walter de Gruyter, 1986, P. 295-297. 8 ref. in English, (JA:05) -----	1

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9004692

SA 39639

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 14/01/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8905859	29-06-89	AU-A- 2818789 EP-A- 0397687	19-07-89 22-11-90
EP-A- 0262972	06-04-88	AU-A- 8151887 WO-A- 8802405	21-04-88 07-04-88