Agrobacterium-mediated transformation of plants

This landscape was originally authored by Carolina Roa-Rodriguez with the assistance of Dr. Carol Nottenburg, and the web version was produced by Doug Ashton. It was updated in 2003 by Dr. Jorge Mayer, and the version you see here starts with a list of the updates done at that time. Technology landscapes, by their very nature, become outdated. While this landscape contains much useful information, some patents have lapsed and others have come into force. Accordingly, sections of this landscape are now gradually being updated again. Pages updated since 2003 show the dates of new searches. We have been grateful for input on some sections by Dr. Shoko Okada, Dr. Marie Connett Porceddu, Dr. Dianne Rees, and Dr. Yang Wei, and web production assistance by Dr. Nick dos Remedios, Steve Irwin, and Annet Maurer. We welcome updates and inputs by others through the comments interface available on every page of this version of the technology landscape.

Preface

Except where otherwise noted, patent information is current through to January 2003.

Summary

Both granted patents and pending patent applications are subject to **change**. A granted patent is typically in force for a 20 year term, calculated from the filing date, **as long as** the maintenance fees are paid, although some patents have been issued under rules that give them different terms (see tutorial).

The patent term is a period during which the patentee has the right to exclude others from using the technology. Technology described in a granted patent that lapses due to lack of payment or expiration of the term moves into the public domain, and unless the technology is covered by other patents still in force, people may work inventions in the public domain without infringement.

From the moment of filing, patent applications go through an interactive process between the applicant and the patent office, the so-called "prosecution", which eventually leads to the grant or rejection of a patent application. During this process, which may take several years, the claims, which define the scope of desired protection for the invention, are likely to be amended. Therefore, the claims of a published patent application **may differ** from those finally granted by a patent office. In addition, an application may be **abandoned** along the examination process if the applicant decides not to seek patent protection for the invention in a particular country.

This white paper on *Agrobacterium*-mediated transformation of plants was updated in March 2002 and June 2003, and is undergoing another revision now. The dynamic nature of intellectual property rights, especially in a rapidly evolving area such as biotechnology, makes regular updates necessary in order to keep abreast of new constraints to freedom to operate or of formerly patented technology that becomes freely accessible.

The main changes registered are:

- **Abandonment**, when the applicants have decided not to continue with the prosecution process or when maintenance fees have not been paid for granted patents;
- **Issuance of a patent**, when applications listed in previous versions of the paper have resulted in granted patents, which may have different claims;
- **Entering European (EP) phase**, or national phase in other national patent offices (applications filed under the Patent Cooperation Treaty may be converted to national patent applications after a maximum period of 30 months from the earliest priority date).

A summary table provides information on changes between 2002 and 2003. For convenience, the documents are presented according to the white paper sections to which they pertain and following the order set in the table of contents/index of the document. You will find out more detailed information by following the links provided for each patent application.

- New patents and patent applications

Many new patents and patent applications have emerged in the field of *Agrobacterium*-mediated transformation since 2002. Some of these patents are directed to new methods for transformation of plant tissues and crops, previously discussed in the white paper, and others are directed to new crops, such as coffee, onions, turfgrass and woody tree species.

The new patent documents are presented in a summary table. Documents are grouped according to the white paper sections set in the table of contents/index. You will find out more detailed information on each patent document by following the links provided in the table.

Changes in legal status of patents and patent applications since last update			
Document No.	Topic / Assignee		Change
AU 597 916 B	Transformation of poplar / Calgene	View Summary	Abandoned
AU 606 874 B	Transformation of Gramineae / Toledo Univ.	View Summary	Abandoned
AU 633 248 B	Transformation of Beans / Toledo Univ.	View Summary	Abandoned
AU 648 951 B	Transformation of Soybeans / Toledo Univ.	View Summary	Abandoned
US 5 376 543	Transformation of Soybeans / Toledo Univ.	View Summary	Abandoned
US 5 340 730	Transformation of *Gladiolus* / Toledo Univ.	View Summary	Abandoned

Patents and Patent Applications-Update July 2003

Note! Assignees listed in brackets are assumed (from related applications and patents), because the assignee is often not recorded on US applications.

Document No. and date of publication	Assignee	Title	More information
Methods			
US 2002/0088029 A1 (4 Jul 2002)	(Novartis Finance Corp (US))	Plant transformation methods.	See details
US 6,353,155 B1 (5 Mar 2002)	Paradigm Genetics, Inc. (US)	Methods for transforming plants.	See details
WO 02/066599 A2 (29 Aug 2002)	Scigen Harvest Co Ltd (KR)	Efficient method for the development of transgenic plants by gene manipulation.	See details
EP 1 236 801 A2 (4 Sep 2002)	The Agri-Biotechnology Research Center of Shanxi (CN)	Method of *Agrobacterium*-mediated plant transformation through treatment of germinating seeds.	See details
US2002/0184663 A1 (5 Dec 2002)	(The Agri-Biotechnology Research Center of Shanxi (CN))	Method of *Agrobacterium*-mediated plant transformation through treatment of germinating seeds.	See details
Monocots			
US 2002/0178463 A1 (28 Nov 2002)	(Japan Tobacco Inc (JP))	Method for transforming monocotyledons.	See details
US 2002/0112261 (15 Aug 2002)	(Univ. of Guelph (CA))	Transformation of monocotyledoneous plants using *Agrobacterium.*	See details
WO 00/58484 (15 Aug 2002)	(Univ. of Guelph (CA))	Transformation of monocotyledoneous plants using *Agrobacterium.*	See details
EP 1198985 A1 (14 Apr 2002)	Natl Inst of Agrobiological Resources (JP)	Method for superrapid transformation of monocotyledon.	See details
Gramineae			

US 2002/0002711 (3 Jan 2002)	(Univ. Toledo (US))	Process for transforming Gramineae and the products thereof.	See details
Onion (*Allium*)			
NZ 513184 (27 Sep 2002)	NZ Inst for Crop & Food Res (NZ)	Transformation and regeneration of *Allium* plants.	See details
WO 00/65903 (9 Nov 2000)	Seminis Vegetable Seeds, Inc. (US)	Transformation of *Allium* sp. with *Agrobacterium* using embryogenic callus cultures.	See details
Barley			
US 6,291,244 B1 (18 Sep 2001)	Sapporo Breweries Ltd (JP)	Method of producing transformed cells of barley.	See details
Maize			
US 2002/0104132 (1 Aug 2002)	Stine Biotechnology (US)	Methods for tissue culturing and transforming elite inbreds of *Zea mays* L.	See details
US 2002/0104131 (1 Aug 2002)	Stine Biotechnology (US)	Methods for tissue culturing and transforming elite inbreds of *Zea mays* L.	See details
US 6,420,630 B1 (16 Jul 2002)	Stine Biotechnology (US)	Methods for tissue culturing and transforming elite inbreds of *Zea mays* L.	See details
Rice			
US 6,329,571 B1 (11 Dec 2001)	Japan Tobacco, Inc. (JP)	Method for transforming indica rice.	See details
WO 02/057407 (25 Jul 2002)	Avestha Gengraine Technologies (IN)	Novel method for transgenic plants by transformation and regeneration of indica rice plant shoot tips.	See details
Sorghum			
US 2002/0138879 A1 (26 Sep 2002)	Pioneer Hi-Bred Intl.Inc. (US)	*Agrobacterium*-mediated transformed sorghum.	See details
US 6,369,298 B1 (9 Apr 2002)	Pioneer Hi-Bred Intl.Inc. (US)	*Agrobacterium*-mediated transformation of sorghum.	See details
Dicots			
US 6,323,396 B1 (27 Nov 2001)	Nunhems Zaden BV (NL)	*Agrobacterium*-mediated transformation of plants.	See details
Brassica			
US 6,316,694 B1 (13 Nov 2001)	AgrEvo Canada, Inc. (CA)	Transformed embryogenic microspores for the generation of fertile homozygous plants.	See details
US 6,455,761 B1 (24 Sep 2002)	Helsinki Univ.Licensing Ltd. (FI)	*Agrobacterium*-mediated transformation of turnip rape.	See details
Camelina sativa			
WO 02/38779 (16 May 2002)	Unicrop Ltd (FI)	A transformation system in *Camelina sativa.*	See details
Coffee			
US 6,392,125 B1 (21 May 2002)	Nara Inst.of Science and Technology (JP)	Method for producing the transformants of coffee plants and transgenic coffee plants.	See details
Cotton			
US 6,483,013 B1 (19 Nov 2002)	Bayer BioScience N.V. (BE)	Method for *Agrobacterium*-mediated transformation of cotton.	See details
Eucalyptus			

US 6,255,559 B1 (3 Jul 2001)	Genesis Research & Dev.Corp.NZ and Fletcher Challenge Forests Ltd. (NZ)	Methods for producing genetically modified plants, genetically modified plants, plant materials and plant products produced thereby.	See details
Guar			
US 2001/0034887 A1 (25 Oct 2001)	(Danisco A/S (DK))	Transformation of guar.	See details
US 6,307,127 B1 (23 Oct 2001)	Danisco A/S (DK)	Transformation of guar.	See details
Melon			
US 6,198,022 B1 (6 Mar 2001)	Groupe Limagrain Holding (FR)	Transgenic plants belonging to the species *Cucumis melo.*	See details
Soybeans			
US 2002/0157139 (24 Oct 2002)	Monsanto Co. (US)	Soybean transformation method.	See details
US 6,384,301 B1 (7 May 2002)	Monsanto Co. (US)	Soybean *Agrobacterium* transformation method.	See details
Strawberry			
US 6,274,791 B1 (14 Aug 2001)	(VPP Corp.) DNA Plant Technology Corp. (US)	Methods for strawberry transformation using *Agrobacterium tumefaciens.*	See details
Woody trees			
WO 02/14463 (21 Feb 2002)	Companhia Suzano de Papel e Celulose BR and Univ.de Sao Paulo (BR)	Method for genetic transformation of woody trees.	See details
Conifers (Pinus)			
US 6,255,559 B1 (3 Jul 2001)	Genesis Research & Dev. Corp.NZ and Fletcher Challenge Forests Ltd. (NZ)	Methods for producing genetically modified plants, genetically modified plants, plant materials and plant products produced thereby.	See details

Assignees in parentheses are assumed, based on related applications and patents, because they usually don't show on US applications

Introduction

Why a white paper on *Agrobacterium*-mediated transformation?

In our experience, the intellectual property landscape in biotechnology areas is often not very well understood by the research community, especially the public sector. All too often rumours and misstatements about patents are passed along from researcher to researcher. This is an unfortunate situation; however, it is understandable as scientists are not generally familiar with reading and understanding patents.

With the increasing importance and emphasis on patents, it is becoming necessary for scientists to be versed in the field of intellectual property. To assist researchers and others in gaining an overview and understanding of relevant intellectual property, we are preparing a series of white papers in chosen topic areas of agricultural biotechnology.

With this paper and others now present on or planned for the Patent Lens, we strive to provide a readable and understandable overview of patents in some key areas of biotechnology. In this way, we hope to contribute to the public awareness of intellectual property issues that surround these key biotechnological tools. The information in the white papers is not exhaustive, but consists of selected documents found to broadly encompass the area. To satisfy the myriad questions and issues raised by the research or the interests of each person who visits this site would require a host of attorneys and an enormous amount of time. Instead, this paper is provided in order to open the door into the patent world and furnish platform

knowledge from which additional self-directed investigation can be performed.

This first white paper is focused on the intellectual property concerning methods and materials used for *Agrobacterium*-mediated transformation of plants. This transformation method is currently one of the most widely used means of making transgenic plants. Although much of the basic research and findings that led to *Agrobacterium*-mediated transformation was done in public institutions, the private sector now holds many of the key patent positions. The patents were obtained by the private sector either from internal research and development or from public institutions in the form of a license or occasionally as the assignee. Thus, the science and the patent positions are of high interest to both public and commercial sectors.

We hope that you find this paper useful.

What is the present white paper about?

This white paper on *Agrobacterium* -mediated transformation of plants explains the basic scientific aspects of transformation as well as the key intellectual property aspects of methods and materials used in transformation.

This paper has been expanded to encompass transformation of organisms outside the plant realm. Patents directed to the transformation of fungi and algae are part of the new additions as well as patents related to improvements on plant transformation efficiency. The latest version of the paper is organized into the following 12 sections:

Introduction
The introduction first explains what the CAMBIA intellectual property resource intends to accomplish in this white paper and then provides brief summaries of each of the seven main sections of the paper. Importantly, the introduction informs you of some of the topics and subject matter areas you will *not* find analyzed within but that may still be important for obtaining freedom to practice some of the inventions described in this paper.

Because many web sites, workshops, and pamphlets that describe basic intellectual property principles (e.g., what is a patent; the requirements and standards for obtaining a patent) are widely available, we do not duplicate those efforts here. We do present, however, as a companion tutorial, guidelines on "How to read a patent". In addition, some key facts about patents that are often overlooked or forgotten by newcomers to patent literature are emphasized in the introduction. It is our belief that familiarity with these concepts will assist you in navigating the sometimes murky waters of patents.

Scientific aspects
This section provides some historical perspective and basic scientific information regarding *Agrobacterium*-mediated transformation of plant cells. The structure and use of two basic types of vectors, co-integrated vectors and binary vectors, are discussed.

The patent information in the following sections comprises an overview, a summary page presenting the key issues raised by the patents and patent applications (illustrated by comparing them and pointing out the most limiting aspects of the claimed inventions), and provides detailed information on each patent and patent application including bibliographic data, a summary of the claimed invention and independent claims.

Types of tissues to be transformed
Agrobacterium infects some tissues more efficiently than others. Reflecting this variability, specific protocols have been developed for different tissue types. Some of these methods have been patented, and it is these patents that are discussed in this section. The patents are generally directed to transformation of callus, immature embryo, pollen, shoot apex and live plants.

Binary vectors
Binary vectors are the major vector system used in *Agrobacterium*-mediated gene transfer. The binary vector system comprises two independent and complementing vectors: one vector having a T-region and the gene of interest and the other vector having a *vir* region. Two sets of patents and applications are presented and analyzed. The first set is directed to basic vector designs and methods of constructing them. The second set is directed to special applications using these vectors or improvements on the basic vector design.

Co-integrated vectors
Although historically the first vector system to be developed, co-integrated vectors are less widely used. In this system, a recombined vector is constructed from a Ti plasmid and a small plasmid containing a gene of interest between two T-DNA borders. The patents and applications in this section are directed to the basic

forms of the vectors, including the primary elements of the plasmids, and to basic methods for assembling the recombined, co-integrated vector. Additionally, a set of patents and applications is discussed that claim improved vector design and methods for their use.

Mobilisable vectors

This new system of vectors appears to be an alternate system to the binary and co-integrated vectors systems. The plasmids used in this system are derived from plasmids belonging to the family Enterobacteriaceae (e.g., *E. coli*). They are non-conjugative plasmids, thus, they are not able to transfer by themselves into a cell host as derived *Agrobacterium* Ti-plasmids are able to do. Mobilisable plasmids require the presence of a helper plasmid that supplies the transfer genes required for the transformation of the host cell. In addition, a gene of interest is not surrounded by T-DNA borders in a mobilisable plasmid. Although there is currently (September 2001) only a European application related to this vector system, we present it here as an alternative to the crowded patent landscape of the traditional vector systems.

Improvements on transformation efficiency

There are multiple protocols for *Agrobacterium*-mediated transformation that vary according to the tissue to be transformed, the plant and the purpose of transformation, among other reasons. Improvements of transformation efficiency can be gained by using compounds to control the growth of *Agrobacterium* and the undesired effects of tissue browning, as well as by using physical procedures to facilitate the inoculation of the bacterium into the host plant. The patents in this section are directed to methods for improving transformation efficiency and include methods of controlling *Agrobacterium* growth, inhibiting necrosis of the transformed plant tissue, reducing the weight of the explant to be transformed and applying physical treatments, such us sonication of the plant tissue and vacuum infiltration, to promote the intimate contact between the bacterium and the host plant cell.

Monocot transformation

The world of flowering plants with protected seeds (Angiosperms) is sometimes neatly divided into monocotyledonous (monocot) and dicotyledonous plants. Most of the important staple crops of the world, that is, cereals, are monocots. Initially it was difficult to transform monocots using *Agrobacterium*, but eventually this constraint was overcome. Several key patents were awarded to the entities able to accomplish this feat. The patents discussed in this section include those broad patents directed to transformation of any monocot as well as patents directed to transformation of any cereal plant (e.g., wheat, barley, rice, maize) and to transformation of a particular individual monocot plant (e.g., banana, pineapple, rice, sorghum).

Dicot transformation

The second major classification of flowering plants with protected seeds (Angiosperms) is dicotyledonous plants (dicots). Early on, dicots were readily transformed by *Agrobacterium* and so in general, there are fewer patents in this area. Following a presentation of the patents directed to general transformation methods, which generally are limited to the use of co-integrated vectors or binary vectors, patents and applications directed to particular dicot species are presented. Some of these particular dicots are beans, cacao, cotton, peas, roses, soybean, and tomato.

Conifer transformation

Non-flowering plants with naked seeds that appear in a cone are called Gymnosperms. Conifers are the largest group of plants within the Gymnosperms. Conifers such as Pines are very important as a source of timber for construction and for paper pulp. Several chemical compounds extracted from pines are used in the pharmaceutical, cosmetic and food industries. For many years, *Agrobacterium*-mediated transformation of conifers was deemed impossible but the barriers for their transformation have been overcome. Patents on this area describe several methods to attain transformation of pines.

Marine algae transformation

Algae are organisms found in virtually every ecosystem, in ecosystems as diverse as marine, freshwater and terrestrial habitats. Algae are commercially very valuable. For example, marine algae or seaweeds are used in many maritime countries as a source of food, for industrial applications and as a fertilizer. Marine algae's products such as gums are very important in the international market. Although *Agrobacterium*-mediated transformation of eukaryotic organisms was initially confined to plants for a while, nowadays, algae can also be transformed via this bacterium. Because transgenic marine algae with a large biomass are a potential source for valuable pharmaceutical and industrial products, patent activity in this area will possibly increase. Currently, there is a patent application directed to methods for transforming multicellular marine algae.

Fungus transformation

Fungi constitute a separate life kingdom from animals and plants. Most fungi are filamentous organisms that contain two nuclei per cell for most of their life cycle. Fungi are essential organisms required for the continuous cycle of nutrients through ecosystems. While they provide essential nutrients to vascular plants

through symbiosis, not all of their activity is beneficial. In this regard, many fungi are the cause of plant, animal and human diseases. The selected patents on *Agrobacterium*–mediated transformation of fungi are mainly directed to the transformation of filamentous fungi, commonly known as moulds. Transformation of yeasts, another group of fungi, is outside the scope of this paper.

What is the present white paper NOT about?

This white paper is not intended to make the reader an expert in patents nor will it serve as a legal opinion for the reader's particular issues. It should not be substituted for legal advice. More information

To learn more about patents and patentability, please visit our companion tutorial, "How to read a patent" and web sites such as the web site of the United States Patent Office and the web site of the World Intellectual Property Organization . Other resource sites may be found on the Links page.

The user should especially note that the materials provided in this site are not comprehensive. In particular, we do not analyze patents directed to methods of using or transforming eukaryotic cells or components of eukaryotic or bacterial vectors that are also used in agricultural R&D. Some of these patents may dominate the agricultural patents discussed on this site. As well, we present only a selected set of patents and applications. The set represents what we consider to be key in the field. It is inevitable that others would have a different opinion about what is key and, as a result, may well have chosen a different set of patents.

This white paper presents an overview of the field of *Agrobacterium*– mediated transformation with respect to intellectual property. The reader should gain an appreciation for the complexities of the field and insight into the types of intellectual property directed to this field.

What you NEED to know about patents

Claims define what is patented

The **claims** are the most important part of a patent. Not the title, not the text, not the examples, and not the figures.

It is the claims that define the boundaries of the patent owner's rights. Remember that the patent owner's rights are exclusionary: she may exclude others from making, using, selling, offering to sell, and importing the patented invention (e.g., a product or a process) and importing a product made by a process patented in the importing country. To determine if someone is infringing a patent, that is making, using, etc., without the patent owner's permission, the allegedly infringing product or process is compared only to the claims.

Don't fall into the trap of concluding that the title or the abstract or the general description found in the text of the patent indicates what is patented. For example, United States Patent No. 6 074 877 is titled "Process for transforming monocotyledonous plants". From the title, it sounds like these patent owners have protected a transformation process(es) for transforming all monocot plants. Examination of the claims shows, however, that only transformation of cereal plants is protected, and furthermore, that the method involves wounding an embryogenic callus or treating an embryogenic callus with an enzyme that degrades cell walls prior to transferring DNA into the cells with *Agrobacterium*. A bit different than what the title implied.

Yet, claims cannot to be interpreted in a vacuum. Although claims define the invention, the scope of the claimed invention is not always clear from reading the plain language of the claim. Claim interpretation can be difficult; a proper analysis is done by reading the claims in the context of the specification and in the context of the "prosecution history" (the back and forth negotiations between the patent applicant and the patent office regarding the claim language). In this case above, for example, several terms in the claims (e.g., "cereal plants", " embryogenic callus", and "enzyme that degrades cell walls") are unclear without additional insight hopefully provided by the specification and prosecution history.

Claims in this white paper and the claims written in "plain English" were analyzed from the plain language and the specification. The prosecution history was not examined. Thus, scope of the claimed inventions may not have always been precisely determined.

A patent application is not the same as a patent

A patent application is NOT the same as a patent. Claims in a published patent application have not been examined by a national patent office and may not be representative of a scope that will ultimately be granted.

During the application process, patent specifications are published 18 months after the earliest filing. The publications contain the claims as filed. Sometimes the claims are written much more broadly than is actually patentable. As the application is examined by a patent office and claim language negotiated, the claims may shrink in scope. In contrast, the specification of a granted patent will usually be the same as when filed; new matter is not allowed to be added to the text after it is filed.

Because the claims in an application are what the applicant hopes for and not what she will necessarily receive, it is important to know whether you are looking at a granted patent or a patent application.

How do you tell the difference between a granted patent and a patent application? Although every country uses its own system of identifying granted patents, some general guidelines will assist you for the major jurisdictions.

- **United States**:
  until 29 November 2000, all publications were issued patents. Currently, the United States identifies patents with a 7-digit number followed by a B1 (indicates a patent not previously published) or a B2 (indicates a patent previously published). E.g., US 6,174,724 B1, shown below.

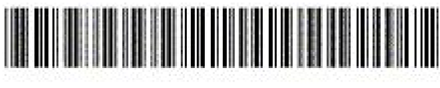
(12) **United States Patent** (10) **Patent No.: US 6,174,724 B1**
Rogers et al. (45) **Date of Patent: *Jan. 16, 2001**

Patent applications are indicated with the year as a 4-digit number and a publication number followed by an A1 (for the first publication), A2 (for republication) or A9 (corrected publication). E.g., US 2001/0002490 A1, shown below.

(19) **United States**
(12) **Patent Application Publication** (10) **Pub. No.: US 2001/0002490 A1**
Doerner et al. (43) **Pub. Date: May 31, 2001**

- **Europe**:
  patents are indicated with a 7-digit number followed by a B1, e.g., EP 0 458 846 B1 (shown below). A B2 number indicates that the claims have been modified after grant.

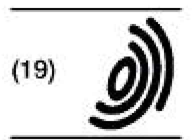
(19)
Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) **EP 0 458 846 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

Patent applications use the same numbering system but the number is followed by A1, A2, etc., e.g., EP 0 955 371 A1 (shown below).

(19) 
Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 0 955 371 A2**

(12) **EUROPEAN PATENT APPLICATION**

- **World Intellectual Property Organization (WIPO)**: often simply called PCT (Patent Cooperation Treaty) applications, publications from WIPO are only patent applications. The publication numbers have a WO, which stands for "world" followed by the year as 2 digits, followed by a publication number, and A1

(first publication), A2 (second publication), etc. e.g., WO 00/34491 A2 (shown below).

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :		(11) International Publication Number:	WO 00/34491
C12N 15/82, A01H 5/00	A2	(43) International Publication Date:	15 June 2000 (15.06.00)

The truth about international patents

There is no such thing as an international patent.

A patent is awarded by the government of a country and is valid only within its territorial boundaries. To obtain a patent that is valid in a particular country, a request must be made in that country's patent office.

The confusion and misunderstanding about "international patents" arises sometimes from the PCT process of pursuing patents. When looking at a PCT application, many people erroneously, but understandably, conclude that it is an application for a patent that will be valid in multiple countries. Indeed on the front page of a PCT application (presented below), in the upper right corner there is a heading titled "Designated states" followed by a list of two letter codes. Each of those codes stands for a country (e.g., AU, Australia; CA, Canada; CN, China, and so on). There can be as many as about 110 countries listed. However, this list does not mean that the application is a patent, or even will become a patent, in all of these countries.

The international (PCT) application is a "placeholder" application for national filings.

OK then, what does this list mean? Through an international treaty (Paris Convention Treaty), a group of countries agreed to not discriminate against each other by affording patent applicants in these countries a one-year period in which to file an application in one of the other countries without losing the benefit of their filing date. The advantage is that any "art" that became known after the original filing date in the home country but before the filing date in another country could not be cited against the application. Thus, for example, if you originally file an application for your invention in Canada, you could wait up to one year before filing the application in Mexico. This would give you time to see if the costs of filing in other countries is justified.

Later, a second treaty (Patent Cooperation Treaty (PCT)) established another route to delay the additional filings in other countries. In this method, an international office was set up (World Intellectual Property Office (WIPO)) to receive and process the applications. But now, the applicant has one year to file at the WIPO office and by designating member countries she preserves her rights and original filing date in those designated countries without having to go to the expense of actually filing in each country. This saves an enormous amount of money! Eventually to obtain a patent in these countries, the application does need to be filed in the national patent offices (the process is called "conversion"), pay fees, have translations done and comply with the regulations of each individual office. Depending on some procedural issues and fee payments, the applicant has either 20 months or 30 months from the original filing date (the date the application was filed in the home country) to file in each of these other countries. Given the costs, most applications are filed in a few other countries at most.

What is ownership of a patent

The legal owner of a patent is designated as the "Assignee" on United States patents and as the "Applicant" on patents in the rest of the world. However, the rights of a patent holder are like a bundle of sticks, and only one of the sticks is legal ownership.

Patent law gives the patent owner the right to exclude others from making, using, offering for sale, selling, and importing the patented product and from using the patented process, as well as using, offering for sale, selling, or importing a product obtained directly from a patented process. These rights are tradeable. The typical form of trade is a license, in which some or all of the rights may be transferred. For example, the patent owner may license only some of the claims in a patent, all of the claims but only in a particular field of research, all of the rights but only in certain countries, or the right to make and use but not the right to sell. Other types of licenses may also be granted.

Unlike the ownership of a patent, which is a matter of public record, licenses can be private. Unless the

parties to a license choose to reveal the relationship, it is impossible to know about.

In this paper, the legal owner is noted. The cautionary note is that the legal owner may not be the party that is in control of the rights you want access to.

Scientific aspects

Overview

Agrobacterium-mediated transformation of plants: from a naturally occurring nuisance to a major tool for plant transformation

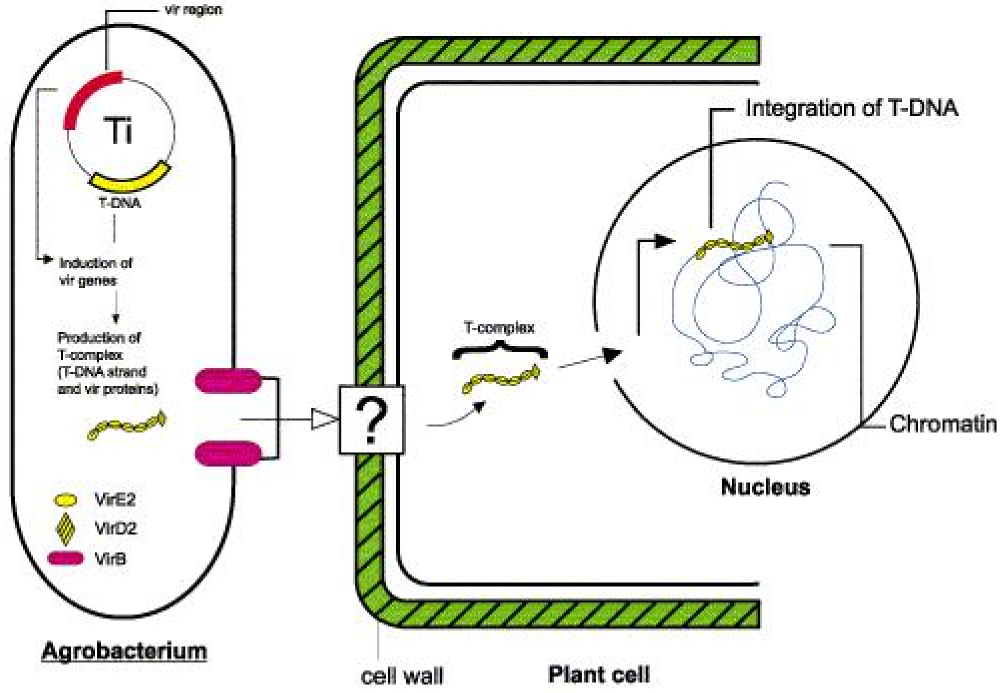
Agrobacterium tumefaciens is a common soil bacterium that naturally inserts its genes into plants and uses the machinery of plants to express those genes in the form of compounds that the bacterium uses as nutrients. In the process, *Agrobacterium* causes plant tumors commonly seen near the junction of the root and the stem, deriving from it the name of crown gall disease. The disease afflicts a great range of dicotyledonous plants, which constitute one of the major groups of flowering plants.

In 1907, the bacterium was identified by Smith and Townsend as the causative agent of the disease, but it was not until the end of the sixties that a correlation between the tumor and the presence of genetic material of the bacterium was established (Braun and Schilperoort).

During the 1970s, several laboratories investigated the biology, biochemistry, and molecular biology of *Agrobacterium*. The combined results of their investigations laid the foundation for generating transgenic plants.

Between the 1970s and 1980s, some striking aspects were discovered about the biology, biochemistry, and molecular biology of *Agrobacterium*. Tumorous plant cells were found to contain DNA of bacterial origin integrated in their genome. Furthermore, the **transferred DNA** (named **T-DNA**) was originally part of a small molecule of DNA located outside the chromosome of the bacterium. This DNA molecule was called **Ti** (**tumor-inducing**) **plasmid** (Zaenen *et al.*, Chilton *et al.*).

The Ti plasmid contains most of the genes required for tumor formation. Wounded plants exude phenolic compounds that stimulate the expression of the **virulence genes** (*vir* **-genes**), which are also located on the Ti plasmid (Wullems *et al.* , Hoekema *et al.*). The *vir* genes encode a set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant genome. The genes in the T-DNA region are responsible for the tumorigenic process. Some of them direct the production of plant growth hormones that cause proliferation of the transformed plant cells. The T-DNA region is flanked at both ends by 25 base pairs (bp) of nucleotides called **T-DNA borders** (Zambryski *et al.*). The T-DNA left border is not essential, but the right border is indispensable for T-DNA transfer.



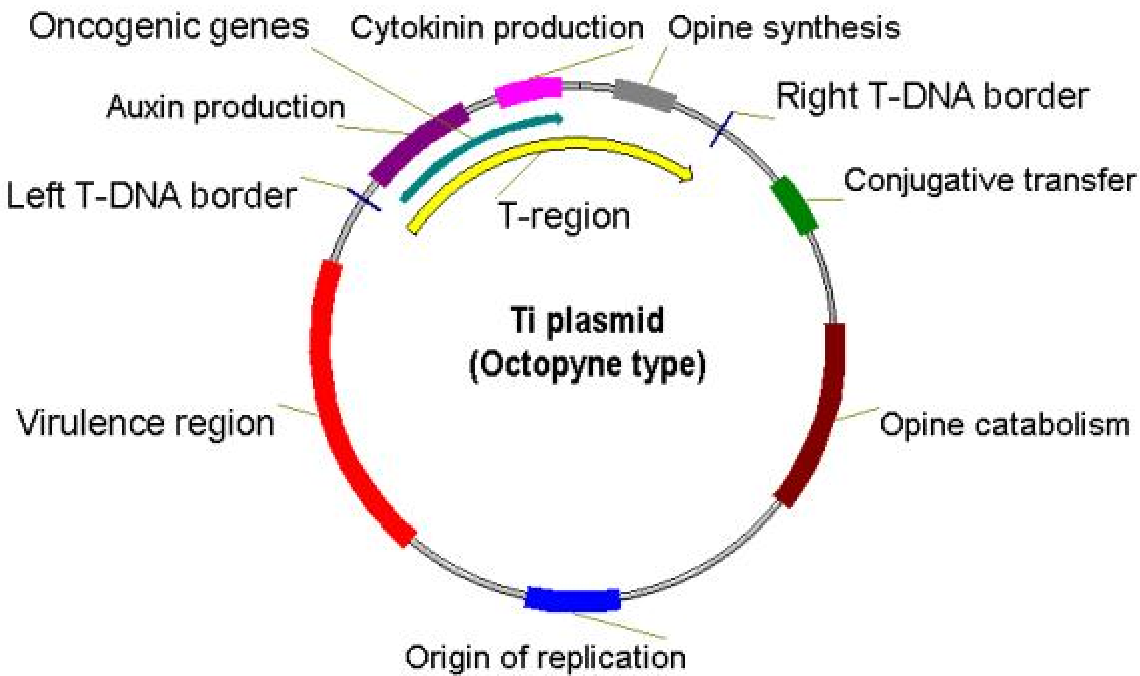
T-DNA transfer into the Plant's Genome
Adapted from Zupan et al 2000

Early 1980's - "the golden molecular age of *Agrobacterium*-mediated transformation." Major discoveries include finding that:

- a fragment of the Ti plasmid, the T-DNA, is responsible for plant tumor growth and is equipped with plant specific promoters and terminators, and
- continuous tumor growth is not dependent on the presence of agrobacteria in the tumor.

The study of *Agrobacterium* and its natural mechanism to alter the biology of infected plant cells sparked the design of molecules that would transfer genes of interest into plant cells. These engineered DNA molecules are commonly referred as **vectors**. The starting molecules can be native Ti-plasmids present in *Agrobacterium*
or native or modified plasmids from other bacteria known to deliver DNA into transformed cells.

The basic elements of the vectors designed for *Agrobacterium*-mediated transformation that were taken from the native Ti-plasmid:



- the **T-DNA border sequences**, at least the right border, which initiates the integration of the T-DNA region into the plant genome
- the ***vir* genes**, which are required for transfer of the T-DNA region to the plant, and
- a **modified T-DNA region** of the Ti plasmid, in which the genes responsible for tumor formation are removed by genetic engineering and replaced by foreign genes of diverse origin, e.g., from plants, bacteria, virus. When these genes are removed, transformed plant tissues or cells regenerate into normal-appearing plants and, in most cases, fertile plants.

Although other methodologies for plant transformation have been devised, *Agrobacterium* remains one of the preferred mechanisms to introduce exogenous genes into the plant cells. One of the reasons for this is the wide spectrum of plants that are susceptible to transformation by this bacterium. *Agrobacterium* was initially believed to be restricted to the transformation of certain dicotyledonous plants (flowering plants with two cotyledons in their seeds and broad leaves) such as potato and tomato, but nowadays, transformation of monocotyledonous plants (flowering plants with one cotyledon in their seeds and narrow leaves with parallel veins), such as maize and rice is routinely performed.

In summary, an *Agrobacterium* -mediated transformation system normally involves:

- an *Agrobacterium* strain carrying a *vir* region and a T-DNA with a gene of interest. The *vir* and T-DNA region are located either on the same or separate vectors;
- transfer of T-DNA region into a plant cell or tissue and its integration into the plant genome;
- expression of a gene of interest in the plant cell; and
- regeneration of the transformed plant cell or tissue into a complete plant.

Essential features

Several essential features are required for *Agrobacterium*-mediated transformation of plants:

- ***vir* genes**. Approximately 35 *vir* genes map outside the T-DNA (transferred DNA) region and encode products required for excision, transfer, and integration of T-DNA into a plant genome. *vir* genes act in *trans*, meaning they do not need to be physically attached to the T-DNA to cause integration into the plant genome.
- **T-DNA border sequences**. These are sequences of 25 bp imperfect repeats that flank the T-DNA and are required for its transfer. Border sequences encompass the recognition sites for a site-specific endonuclease, which is encoded by the *vir* D operon, part of the *vir* genes. The endonuclease cleaves the lower DNA strand of the T-DNA marking the starting point of the transfer.

- Apart from the T-DNA border sequences, most of the genes of the T-DNA can be replaced by genes of interest to be transferred into the plant. Such engineered T-DNA is generally referred as mutant T-DNA, engineered T-DNA or disarmed T-DNA.
  However, *the exact definition of the terms* in a patent application is often provided by the inventor.

- ***cis*-regulatory regions**. These include the right and left T-DNA borders, which are physically attached to the genes to be transferred into the plant genome. Other *cis* -regulatory regions include promoters and terminators that flank the transgenes and regulate their expression. Commonly used promoters and terminators are the nopaline synthesis gene (NOS) and Cauliflower Mosaic Virus (CaMV) 35S promoter.
- **Selectable marker genes.** In plants, they allow the identification and selection of cells with the gene of interest incorporated in their genome. Bacterial selectable markers permit the identification of bacteria transformed with a vector carrying the marker. Examples of plant and bacterial selectable marker are hygromycin phosphotransferase and kanamycin, respectively.

The above-mentioned elements are incorporated in two basic types of vectors used to transform a wide range of plants via *Agrobacterium*:

- **Binary vector.** In this system, the T-DNA and the *vir* region reside in **separate** plasmids within the same *Agrobacterium* strain. The *vir* genes are located in a disarmed (without tumor genes) Ti plasmid and the T-DNA with the gene of interest is located in a small vector molecule.
- **Co-integrated vector.** It results from the **recombination** of a small vector plasmid, for example an *E. coli* vector, and a Ti plasmid harbored in *A. tumefaciens*. The recombination takes place through a homologous region present in both of the plasmids. An engineered T-DNA containing the gene of interest can be in either one of the plasmids.

References

Binary vectors

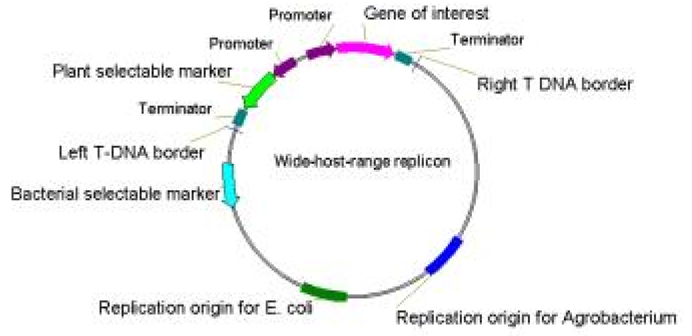
The discovery that the *vir* genes do not need to be in the same plasmid with a T-DNA region to lead its transfer and insertion into the plant genome led to the construction of a system for plant transformation where the T-DNA region and the *vir* region are on separate plasmids.

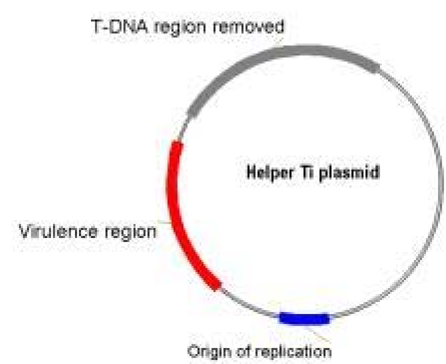
In the binary vector system, the two different plasmids employed are:

- a **wide-host-range small replicon**, which has an origin of replication (ori) that permits the maintenance of the plasmid in a wide range of bacteria including *E. coli* and *Agrobacterium*. This plasmid typically contains:
  1. foreign DNA in place of T-DNA,
  2. the left and right T-DNA borders (or at least the right T-border),
  3. markers for selection and maintenance in both *E. coli* and *A. tumefaciens*,
  4. a selectable marker for plants.
- The plasmid is said to be "disarmed", since its tumor-inducing genes located in the T-DNA have been removed.
- a **helper Ti plasmid**, harbored in *A. tumefaciens*, which lacks the entire T-DNA region but contains an intact *vir* region.

In general, the transformation procedure is as follows:

- the recombinant small replicon is transferred via bacterial conjugation or direct transfer to *A. tumefaciens* harboring a helper Ti plasmid,
- the plant cells are co-cultivated with the *Agrobacterium*, to allow transfer of recombinant T-DNA into the plant genome, and
- transformed plant cells are selected under appropriate conditions.





Possible pitfalls

A possible disadvantage may ensue from the fact that the stability of wide host range replicons in *E. coli* and *Agrobacterium*
varies considerably. Depending on the orientation, plasmids with two different origins of replication may be unstable in *E. coli* where both origins are active.

Advantages

Compared with co-integrated vectors, binary vectors present some advantages:

- No recombination process takes place between the molecules involved.
- Instead of a very large, recombinant, disarmed Ti plasmid, small vectors are used, which increases transfer efficiency from *E. coli* to *Agrobacterium*.

This vector system is most widely used nowadays. Different types of binary vectors have been devised to suit different needs in a plant transformation process.

Binary vector types

a. **pGA series vectors**, which contain:
    - an ori derived from RK2 for replication in *E. coli* and *Agrobacterium*,
    - a tetracycline resistance gene,
    - the *cis*-acting factor required for conjugal transfer,
    - the right (RB) and left (LB) T-DNA borders,
    - a neomycin phosphotransferase (*nptII*) gene, which confers resistance to kanamycin and G418 in transformed plants, and
    - a polylinker site (multicloning site).

    Specific vectors in this series are designed for cloning large fragments (colE1 origin of replication and phage l cos), analyzing promoters (multiple cloning site immediately upstream of a promoterless *cat* gene), and expressing a gene of interest (polylinker site between a plant promoter and a terminator).

b. **pCG series vectors**, which contain:
    - the origin of replication of the *Agrobacterium rhizogenes* root -inducing plasmid pRiHRI, which confers more stability in *Agrobacterium* than the ori derived from RK2, and a ColE1 origin of

replication from the vector pBR322 for maintenance in *E. coli*.

c. **pCIT series** which contain:
   - the hygromycin (*hph*) resistance gene for plants,
   - the lambda *cos* site for cloning long fragments.
d. **pGPTV** (glucuronidase plant transformation vector) **series** , which have:
   - different plant selectable marker genes close to the left T-DNA border. This design overcomes problems inherent with the preferential right to left border transfer of T-DNA and improves the chances of having the gene of interest transferred to the plant cell in cells expressing the selectable marker gene.
e. **pBECK2000 series**, which contain:
   - synthetic T-DNA borders and a *bar* gene, which confers the plants resistance to the herbicide phosphinothricin. Also, the vectors use the phage P1 Cre/*lox*P site-specific recombinase system, which permits the transfer and integration of a target and marker genes as a single T-DNA unit into the plant genome or as two independent T-DNAs within a single *Agrobacterium.* It also allows site-specific excision of marker genes from the plant genome after transformation.
f. **Binary-BAC (BiBAC) vector**
   - based on a bacterial artificial chromosome (BAC) vector and is suitable for *Agrobacterium*-mediated transformation of high-molecular-weight DNA
   - comprises low-copy number origins of replication for both *E. coli* and *Agrobacterium* to ensure replication of the plasmid as a single-copy in both bacteria; and
   - a helper plasmid carrying additional copies of *vir*-genes in order to clone very large T-DNAs (up to 150 kb) into the plant genome.
g. **pGreen series**, small plasmids of around 3.2 Kb containing:
   - a broad host range replication origin (ori pSa) and a ColE1 origin derived from pUC,
   - a pSa replicase gene (*rep* A) that provides replication functions in *trans* and is located in a compatible plasmid (pSoup) in *Agrobacterium*, and
   - multiple cloning sites based on the pBlueScript vector, which allow any arrangement of selectable marker and reporter genes.

References

Co-integrated vectors

Called **co-integrated vectors** or **hybrid Ti plasmids**, these vectors were among the first types of modified and engineered Ti plasmids devised for *Agrobacterium* -mediated transformation, but are not widely used today.

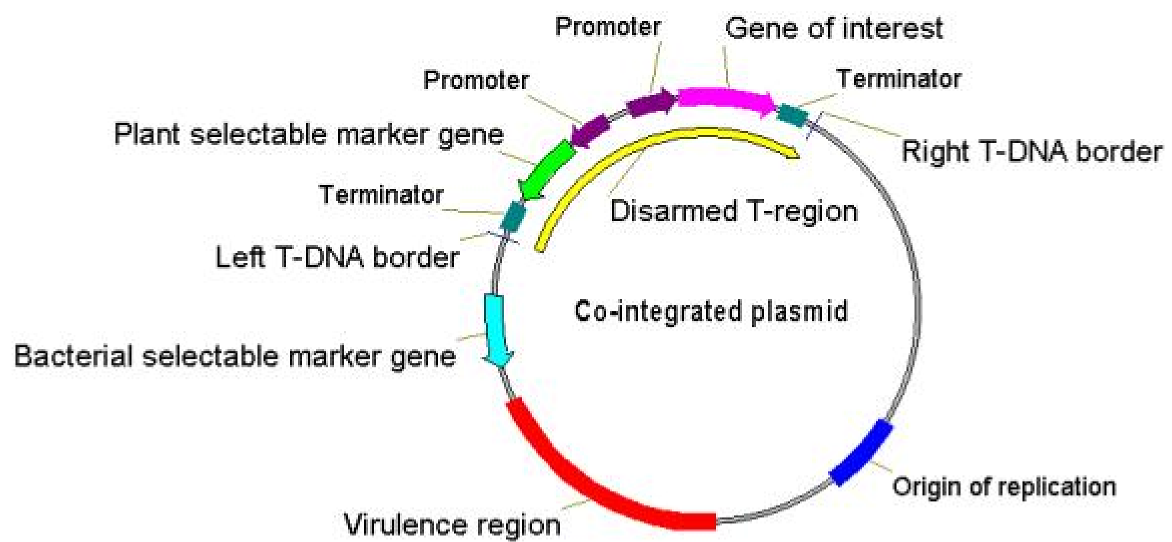
These vectors are constructed by homologous recombination of a bacterial plasmid with the T-DNA region of an endogenous Ti plasmid in *Agrobacterium*. Integration of the two plasmids requires a region of homology present in both.

Three vectors are necessary in this system:

- **Disarmed *Agrobacterium* Ti plasmids**
  In these Ti plasmids, the oncogenes located in the T-DNA region have been replaced by exogenous DNA.
  Examples of these vectors include:
  1. SEV series: the right border of the T-DNA together with the phytohormone genes coding for cytokinin and auxin are removed and replaced by a bacterial kanamycin resistance gene while the left border and a small part of the left segment (T_L) of the original T-DNA (referred to as Left Inside Homology (LIH)) are left intact.
  2. pGV series: the phytohormone genes are excised and substituted by part of pBR322 vector sequence. The left and right border sequences as well as the nopaline synthase gene of the Ti plasmid are conserved.
- **Intermediate vectors**
  These are small pBR322-based plasmids *(E. coli* vectors) containing a T-DNA region. They are used to overcome the problems derived from the large size of disarmed Ti plasmids and their lack of unique

restriction sites. Intermediate vectors are replicated in *E.coli* and are transferred into *Agrobacterium* by conjugation. They cannot replicate in *A. tumefaciens* and therefore, carry DNA segments homologous to the disarmed T-DNA to permit recombination to form a co-integrated T-DNA structure.

- **Helper vectors**
  These are small plasmids maintained in *E. coli* that contain transfer (*tra*) and mobilization (*mob*) genes, which allow the transfer of the conjugation-deficient intermediate vectors into *Agrobacterium*.



A resulting **co-integrated plasmid** assembled by *in vitro* manipulation normally contains:

1. the *vir* genes,
2. the left and right T-DNA borders,
3. an exogenous DNA sequence between the two T-DNA borders, and
4. plant and bacterial selectable markers.

Some drawbacks

Although co-integrated vectors have been designed to allow site-specific recombination based on the recombination system of the phage P1 (e.g., wP1 *lox*P-Cre series), co-integrated vectors in general are less popular due to:

- long homologies required between the Ti plasmid and the *E. coli* plasmids making them difficult to engineer and use, and
- relatively inefficient gene transfer compared to the binary vectors.

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Types of tissues to be transformed

Summary

The efficiency of T-DNA transfer via *Agrobacterium* to a plant varies considerably, not only among plant species and cultivars, but also among tissues. Various protocols for *Agrobacterium*-mediated transformation of plants use leaves, shoot apices, roots, hypocotyls, cotyledons, seeds and calli derived from various parts of a plant. In other methods, the transformed tissue is not removed from the plant but left in its natural environment, thus, the transformation takes place *in planta*.

Patents directed specifically to methods of transforming different tissues are relatively few, but the scope of their protection is rather broad. Some of the patents referred to in this section are considered key patents for widely used technologies by the research community.

The patents discussed in this section are directed to the transformation of **callus**, **immature embryo**, **pollen, seed, shoot apex parts** in culture as well as *in planta* . With the exception of **Japan Tobacco**'s patents directed to callus and immature embryo transformation of a monocotyledonous plant, claims in these patents are not restricted to the type or species of plant to be transformed. Therefore, any plant arguably falls within the scope of the claims of these patents. The bacterium used for transformation is *Agrobacterium* or specifically *A. tumefaciens.*

- Callus transformation. **Japan Tobacco** has two granted patents, one in Australia and the other in the United States. The Australian patent claims a method of transforming a monocot plant tissue with Agrobacterium. The plant tissue can be from any portion of any type of monocot plant and is either already dedifferentiated or is exposed to a dedifferentiation process. In the United States patent the tissue to be transformed must be not less than 7 days old.

- Immature embryo transformation. **Japan Tobacco** also has a patent in Australia claiming transformation of the scutellum of an immature embryo of a monocot plant with *Agrobacterium.* The transformation process takes place before the tissue has differentiated into a callus.

- Pollen transformation. A patent related to this topic was granted to the **United States Department of Agriculture (USDA)** in the US and a patent has also been **granted** in **Australia**. In these patents, transformed pollen fertilizes a second plant to obtain transgenic seed, which is germinated to obtain a transgenic plant.

- Shoot apex transformation. Transformation of an excised shoot apical tissue by inoculating the tissue with *A. tumefaciens* is disclosed by **Texas A & M University** in a granted United States patent. **Applications** filed in Europe and in Australia have been **abandoned**.

- *In planta* transformation. Three different entities have filed patent applications on *in planta* transformation. **Cotton Inc.**, **Rhobio** and **Performance Plants** ' patent applications refer to transformation of a plant tissue with *Agrobacterium* in its natural environment. **Performance Plants'** patent applications, however, were recently **abandoned**. In contrast to **Rhobio**, which does not claim a method of transforming a particular tissue of a plant, **Cotton Inc**. claims the injection of *Agrobacterium* into floral or meristematic tissue. Furthermore, **Cotton Inc.** claims appear to require the transformed plant cells or tissue to develop and regenerate within the plant, whereas **Rhobio**'s patent claims are directed to removing tissue from the plant and regenerating it *in vitro*.

- Floral transformation (Update July 2003) is basically an *in planta* method that has become very popular in the transformation of *Arabidopsis thaliana* (Brassicaceae), one of the best known model plants in genomic studies. It is also suitable for the transformation of monocotylenous plants. A US patent assigned to **Rhone-Poulenc Agro** and a PCT application assigned to **Paradigm Genetics Inc.** are described in the section " General Transformation Methods for Monocots." A US patent granted to **Cotton Inc.**
  which discloses transformation of floral or meristematic tissue (mentioned in the preceding paragraph) is discussed in the section "*In planta* transformation." A patent has been granted to **Paradigm Genetics Inc.**
  in which a new method to transform plants by direct treatment of flowers is described. The method is based on published literature and represents a simple modification to the adjustment of cell density of the *Agrobacterium* strain used in the transformation: cells are diluted rather than centrifuged.

- Seed transformation (Update July 2003). Two groups have filed patent applications on transformation of plants using seed as target tissue. **The Agri-Biotechnology Research Center of Shanxi** (China) has filed a US and an EP application based on a Chinese patent application. As filed, these two applications contain general claims to applying *Agrobacterium* to germinating seed, with no further treatment of the seed. A PCT application has also been filed by **Scigen Harvest Co Ltd** from Korea on a method using needle-wounded seed as target tissue in combination with *Agrobacterium tumefaciens*.

In conclusion

- Transformation of pollen with *Agrobacterium* is fairly broadly protected in the United States and in Australia. The situation is similar with shoot apex transformation in the United States, except that the bacterium used in this case is specifically *A. tumefaciens.* Thus, use of other species of *Agrobacterium* to transform apical shoots from any plant may fall outside the scope of the claimed invention.

- There appears to be more room to avoid infringing patents on *in planta* and callus transformation. Although other entities may have applications pending, only **Cotton Inc**. has been granted a United States patent, which particularly claims:

  1. transformation of floral or meristematic tissue, and
  2. the use of a needleless device to inject *Agrobacterium* into the tissue.

Thus, if one of these two elements is not part of an *in planta* transformation process, the process may be well outside the scope of the claims of Cotton Inc.'s patent.

With respect to callus transformation claimed by **Japan Tobacco**, at least in the United States, the tissue must be at least seven days old. Thus, if tissue can be used that is less than 7 days in culture, literal infringement of this patent may be avoided.

- Patent applications for the transformation of the tissues mentioned above are pending in Australia, Europe, United States and some other jurisdictions and may become an issue for freedom to operate if

they are granted as filed or may be prior art for other inventions related to the *Agrobacterium* transformation of these particular tissues.

- Transformation of germinating seed with *Agrobacterium* will be protected broadly if pending applications are granted, especially the one to **The Agri-Biotechnology Research Center of Shanxi .**

Callus transformation

Patents and application assigned to Japan Tobacco

In the disclosures, an explant of a monocot in the process of dedifferentiation or already dedifferentiated is used for transformation with *Agrobacterium*. A dedifferentiating tissue or a tissue in the process of dedifferentiation is described in the disclosures as an explant cultured on a dedifferentiation medium for not less than 7 days. Among the preferred tissues are a callus, an adventitious embryo-like tissue, and suspension cells.

Callus transformation
Patents granted to Japan Tobacco

Specific patent information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 5591616 A • Earliest priority - 7 July 1992 • Filed - 3 May 1994 • Granted - 7 January 1997 • Expected expiry - 6 July 2013	**Title** - Method of transforming monocotyledons **Claim 1** A method for transforming a monocotyledon callus, comprising contacting a cultured tissue of a monocotyledon during dedifferentiation wherein said dedifferentiation is obtained by culturing an explant on a dedifferentiation-inducing medium for not less than 7 days or a dedifferentiated cultured tissue of a monocotyledon, with a bacterium belonging to the genus *Agrobacterium* containing a desired gene. **Claim 17** A method for transforming a monocotyledon with a desired gene, comprising: contacting a cultured tissue of said monocotyledon during dedifferentiation thereof, or a dedifferentiated cultured tissue of said monocotyledon, with a suspension of *Agrobacterium tumefaciens* having a cell population of 10^6 to 10^{11} cells/ml for 3-10 minutes and then culturing said cultured tissue of said monocotyledon during dedifferentiation thereof, or said dedifferentiated cultured tissue of said monocotyledon, on a solid medium for several days together with said *Agrobacterium tumefaciens*, or adding said *Agrobacterium tumefaciens* to culture medium in which said cultured tissue of said monocotyledon during dedifferentiation thereof or said dedifferentiated cultured tissue of said monocotyledon is cultured, and continuously culturing said cultured tissue of said monocotyledon during dedifferentiation or said dedifferentiated cultured tissue of said monocotyledon together with said *Agrobacterium tumefaciens*, wherein said dedifferentiated cultured tissue of said monocotyledon is selected from the group consisting of a tissue cultured during the process of callus formation which is cultured for not less than 7 days after an explant is placed on a dedifferentiation-inducing medium and a callus, and wherein said *Agrobacterium tumefaciens* contains plasmid pTOK162, and said desired gene is present between border sequences of the T region of said plasmid pTOK162, or wherein said desired gene is present in another plasmid contained in said *Agrobacterium tumefaciens*. **US 5591616** claims: • a method for transforming monocotyledon callus by contacting	Japan Tobacco

	dedifferentiating tissue of **not less than 7 days** of culture or dedifferentiated tissue with *Agrobacterium* having a desired gene; and • a method of transforming such tissue or transforming a callus by contacting the tissue/callus with a suspension of *Agrobacterium* cells of 10^6 – 10^{11} cells/ml for 3-10 minutes. The bacterium contains the desired gene either between the T- borders of the plasmid pTOK162 or in another plasmid.
EP 604662 A1 • Earliest priority – 7 July 1992 • Filed – 6 July 1994 • Application pending	**Title** – Method of transforming monocotyledon **Claim 1** A method for transforming a monocotyledon comprising transforming a cultured tissue during dedifferentiation process or a dedifferentiated cultured tissue of said monocotyledon with a bacterium belonging to genus *Agrobacterium* containing a desired gene. The claims submitted in the European application **EP 604662 A1** are the same as the claims of the Australian patent, below.
AU 667939 B • Earliest priority – 7 July 1992 • Filed – 6 July 1993 • Granted – 18 April 1996 • Expected expiry– 6 July 2013	**Title** – Method of transforming monocotyledon **Claim 1** A method for transforming a monocotyledon comprising transforming a cultured tissue during dedifferentiation process or a dedifferentiated cultured tissue of said monocotyledon with a bacterium belonging to genus *Agrobacterium* containing a desired gene. The lead claim in the Australian patent AU 667 939 is broader than in the United States patent. In the Australian patent, a dedifferentiating or dedifferentiated tissue of a monocot is also used as the initial tissue for transformation, but there is **no restriction on the number of days of culture** in the medium to induce dedifferentiation.
US 2002/178463 A1 • Earliest priority – 7 July 1992 • Filed – 13 January 1999 • Application pending	**Title** – Method for transforming monocotyledons **Claim 1** A method for transforming a monocotyledon, comprising contacting a cultured tissue of said monocotyledon during dedifferentiation thereof obtained by culturing an explant on a dedifferentiation-inducing medium for less than 7 days with a bacterium belonging to the genus *Agrobacterium* containing a super binary vector having the virulence region of Ti plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281, left and right border sequences of T-DNA of a Ti plasmid or an Ri plasmid of a bacterium belonging to the genus *Agrobacterium*, and a desired gene located between said left and right border sequences. **Claim 13** A method for transforming a monocotyledon, comprising contacting a cultured tissue of said monocotyledon during dedifferentiation thereof obtained by culturing an explant derived from an immature tissue on a dedifferentiation-inducing medium for less than 7 days with a bacterium belonging to the genus *Agrobacterium* containing a desired gene and containing a vector having the virulence region of Ti plasmid contained in *Agrobacterium tumefaciens*. This application is a continuation of abandoned US 08/668464, which was a continuation of now granted US 5591616.

	Claims in this applicaiton recite *Agrobacterium*-mediated transformation of monocotyledon explants, where the explant is cultured on dedifferentiation medium for less than 7 days, then infected with *Agrobacterium* containing a vector that has the virulence region (in particular a vector that contains the virulence region of Ti plasmid pTiBo542 from *A. tumefaciens* A281 in the case of claim 1).	
Remarks	1. National phase entry of WO 1994/00977 in Canada (CA 2121545) is pending. 2. National phase entry of WO 1994/00977 in Japan (JP 2649287 B2) has been published as granted on 3 September 1997.	

Note: Patent information on this page was last updated on 21 February 2006.

Immature embryo transformation

Patents and application assigned to Japan Tobacco

This family of patents discloses use of an immature embryo of a monocot for *Agrobacterium* transformation. Within the embryo, the scutellum (name given to the single massive cotyledon (seed leaf) of monocot plants) is transformed. The scutellum is capable of producing dedifferentiated calli having the ability to regenerate normal plants after transformation.

The bacterium used for transformation contains either a Ti or Ri (root-inducing) plasmid with the desired gene and a plasmid having a virulence region derived from the *A. tumefaciens* Ti plasmid pTiBo542.

Immature embryo transformation
Patent and application assigned to Japan Tobacco

Specific patent information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
EP 672752 B1 • Earliest priority - 3 September 1993 • Filed - 1 September 1994 • Granted - 26 May 2004 • Expected expiry - 3 September 2013	**Title** - Method of transforming monocotyledon by using scutellum of immature embryo **Claim 1** A method for transforming monocotyledons comprising transforming scutellum of an immature embryo of a monocotyledon with a bacterium belonging to genus *Agrobacterium* containing a desired gene, which immature embryo has not been subjected to a dedifferentiation treatment, to obtain a transformant. EP B 672 752 contains the same independent claim as the Australian patent. Designated States at the time of grant are: Austria, Belgium, Switzerland, Germany, Denmark, Spain, France, United Kingdom, Greece (reported on INPADOC as lapsed), Ireland, Italy, Liechtenstein, Luxembourg, Monaco (reported on INPADOC as lapsed), Netherlands, Portugal, Sweden	Japan Tobacco
AU 687863 • Earliest priority - 3 September 1993 • Filed - 1	**Title** - Method of transforming monocotyledon by using scutellum of immature embryo **Claim 1** A method for transforming monocotyledons comprising transforming	

September 1994 • Granted – 5 March 1998 • Expected expiry – 3 September 2013	scutellum of an immature embryo of a monocotyledon with a bacterium belonging to genus *Agrobacterium* containing a desired gene, which immature embryo has not been subjected to a dedifferentiation treatment, to obtain a transformant. The claims of the Australian patent AU-B-687 863 are directed to: a method for transformation of a scutellum of an immature embryo of a monocotyledon with *Agrobacterium* having a desired gene. The embryo is not subjected to a dedifferentiation process prior the transformation with *Agrobacterium*.	
Remarks	1. National phase entry of WO 95/06722 in Canada (CA 2148499) is pending. 2. National phase entry of WO 95/06722 in Japan (JP 3329819 B2) has been published as granted on 30 September 2002.	

Note: Patent information on this page was last updated on 21 February 2006.

Pollen transformation

Patent granted to the United States Department of Agriculture (USDA)

The invention is a method for the genetic transformation of any plant by using pollen as starting material for transformation with *Agrobacterium*. A culture medium useful for pollen germination and pollen tube growth in presence of *Agrobacterium* is also claimed.

The previously analyzed Australian application **AU 84005/98 A1** was granted as the patent **AU-B-733 080** on **May 3, 2001**.

Bibliographic data

	US 5 929 300	AU-B-733 080
Title	Pollen-based transformation system using solid media	Pollen-based transformation system using solid media
Application No. & Filing Date	US 892735 July 15, 1997	AU 8400598 July 14, 1998
Issue Date	July 27, 1999	May 3, 2001
Language	English	
Remarks	Delta and Pine Land Co., acquired exclusive licensing rights to the pollen-transformation system developed by the USDA in the United States. (Source: Ag Biotech InfoNet, January 26, 2001).	

To view or download the patent as a PDF file, click on US 5 929 300 (677 kb).

Summary of the invention

The United States patent **5 929 300** claims

- a method for producing a transgenic plant by transforming pollen of a plant with *Agrobacterium* carrying a foreign gene and then fertilizing a second plant with the transgenic pollen to obtain transgenic seed. The transgenic plant is obtained by germination of the transgenic seeds.

The Australian patent **733 080** claims the same method for transforming pollen of a plant with *Agrobacterium*
as the United States patent. However, the Australian patent claims in addition a specific medium for pollen germination and pollen tube growth.

View Claims

Pollen transformation
Patents granted to the United States Department of Agriculture (USDA)

Claims in plain English

Disclaimer
THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

US 5 929 300 & AU-B-733 080*
Claim 1 A method for producing a transgenic plant by: A) applying Agrobacteria having a foreign gene to a pollen culture on solid medium; B) allowing germination and growth of the transgenic pollen on the medium; C) fertilizing a second plant with the transgenic pollen; D) obtaining transgenic seed and germinating to obtain a transgenic plant.

AU-B-733 080*
Claim 8 A medium for pollen germination and tube growth having agarose, sucrose, KNO_3, $MnSO_4$, H_3BO_3, $MgSO_4$, and gibberellic acid.

Actual granted Claims

US 5 929 300 & AU-B-733 080*
Claim 1 A method for producing a transgenic plant comprising: A) obtaining pollen from a first plant; B) applying a lawn of Agrobacteria to a solid pollen culture medium, the Agrobacteria comprising at least one heterologous gene sequence capable of being transferred to a plant cell; C) applying the pollen to the solid medium; D) allowing the pollen to germinate and grow on the medium, thereby producing transgenic pollen; E) applying the transgenic pollen to the stigma of a second plant capable of being fertilized by the pollen of the first plant, thereby fertilizing the second plant; F) obtaining transgenic seed from the second plant; G) germinating the transgenic seed to obtain a transgenic plant.

AU-B-733 080*
Claim 8 A medium for pollen germination and pollen tube growth comprising agarose, sucrose, KNO_3, $MnSO_4$, H_3BO_3, $MgSO_4$, and gibberellic acid.

*The Australian patent **AU-B-733 080** was granted on May 3, 2001.

Pollen Transformation
Patent application filed by the United States Department of Agriculture (USDA)

The invention disclosed in the European application is the same as in the United States patent and the recently granted Australian patent **AU-B-733 080** (former Australian application **AU 84005/98 A1**).

Bibliographic data

	EP 996 328 A1
Title	Pollen-based transformation system using solid media
Application No. & Filing Date	EP 934497 July 14, 1998
Publication Date	May 3, 2000
Language	English
Remarks	Applications also filed in Brazil (BR 9811791), China (CN 1263434), and South Africa (ZA 9806240).

To view or download the patent document as a PDF file, click on EP 996 328 (1,080 kb).

Summary of the invention

The independent claims as filed of the EP application **996 328 A1** are similar to the granted claims of its related United States and Australian patents. As in the Australian patent, the EP application also contains a filed claim referring to a medium for pollen germination and pollen tube growth.

View Claims

Pollen transformation
Patent application filed by the United States Department of Agriculture (USDA)

Claims in plain English

Disclaimer
THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

EP 996 328 A1
Claim 1 A method for producing a transgenic plant by: A) applying Agrobacteria having a foreign gene to a pollen culture on a solid medium; B) allowing germination and growth of the transgenic pollen on the medium; C) fertilizing a second plant with the transgenic pollen; D) obtaining seed; and E) germinating the seed to obtain a transgenic plant.
Claim 8 A medium for pollen germination and pollen tube growth having agarose, sucrose, KNO_3, $MnSO_4$, H_3BO_3, $MgSO_4$, and gibberellic acid.

Actual pending claims

EP 996 328 A1
Claim 1* A method for producing a transgenic plant comprising: A) obtaining pollen from a first plant; B) applying a lawn of Agrobacteria to a solid pollen culture medium, the Agrobacteria comprising at least one heterologous gene sequence capable of being transferred to a plant cell; C) applying the pollen to the solid medium; D) allowing the pollen to germinate and grow on the medium, thereby producing transgenic pollen; E) applying the transgenic pollen to the stigma of a second plant capable of being fertilized by the pollen of

the first plant, thereby fertilizing the second plant;
F) obtaining transgenic seed from the second plant;
G) germinating the transgenic seed to obtain a transgenic plant.

Claim 8**

A medium for pollen germination and pollen tube growth comprising agarose, sucrose, KNO_3, $MnSO_4$, H_3BO_3, $MgSO_4$, and gibberellic acid.

Note: The Australian application AU 84005/98 A1 was granted. See **Patents granted to the USDA** for more information on this new patent.

* Identical to claim 1 of the granted United States and Australian patents.

**Identical to claim 8 of the granted Australian patent.

Shoot apex transformation

Patent granted to The Texas A & M University System

In the invention disclosed in this United States patent, shoot apex tissue from any plant is subjected to gene transfer via *Agrobacterium*. According to the inventors, the use of such tissue permits rapid propagation of plant without encountering problems of somaclonal variation.

Bibliographic data

	US 5 164 310
Title	Method for transforming plants via the shoot apex
Application No. & Filing Date	US 650,685 February 5, 1991
Issued Date	November 17, 1992
Language	English
Remarks	Patents also granted in Ireland (IE 65516 B), Israel (IL 90440), Japan (JP 2996995 B2), Portugal (PT 9070 B), and Spain (ES 2017024 AF). Related patent applications: The previously analyzed Australian application **AU 37568/89** and the European application **EP 419 533 A1** were abandoned on April 26, 91 and on April 10, 1993, respectively. Applications appear to be pending in South Africa (ZA 8904379 A), New Zealand (NZ 229340 A), Denmark (DK 285590 A), and China (CN 1042638 A).

To view or download the patent as a PDF file, click on US 5 164 310 (764 kb).

Summary of the invention

The United States patent **5 164 310** claims a method to transform shoot apices, which contain the apical dome with meristematic tissue and some primordial leaves, with *A. tumefaciens*. According to the inventors, shoot cultures develop roots directly and rapidly, and plant regeneration is achieved within six weeks after transformation.

View Claims

Shoot apex transformation
Patent granted to the Texas A & M University System

Claims in plain English

Disclaimer

THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

US 5 164 310
Claim 1 A method for transforming shoot apical tissue by A) cutting out shoot apical tissue having the apical dome and two or more primordial leaves; B) placing the tissue in a growth medium and inoculating it with *Agrobacterium tumefaciens*.
Claim 8 A method for transforming a shoot apex by A) cutting out a shoot apex; B) placing the tissue in a growth medium and inoculating it with *Agrobacterium tumefaciens*

Actual granted claims

US 5 164 310
Claim 1 A method of transforming excised shoot apical tissue comprising: A) excising shoot apical tissue consisting essentially of apical dome and two or more primordial leaves; B) placing said excised tissue in a suitable growth medium; C) inoculating said apical tissue with *Agrobacterium tumefaciens* to transform said tissue.
Claim 8 A method for transforming an excised shoot apex comprising: A) excising a shoot apex; B) placing said apex in a suitable growth medium; C) inoculating said apical tissue with *Agrobacterium tumefaciens* to transform said tissue.

In planta transformation

Overview

The present inventions are directed to the transformation of a plant *in vivo* with *Agrobacterium*, in which the inoculation and co-cultivation process with *Agrobacterium* takes place as the plant develops normally.

According to the inventors, some advantages of this methodology derive from a close analogy to *Agrobacterium*'s natural environment for transformation and the production of a non-chimeric transgenic progeny from seeds of a treated plant when floral tissue is transformed.

Cotton Inc., **Paradigm Genetics Inc** , and **Rhobio** patents and applications are presented here.

Cotton Inc. claims

- the introduction of *Agrobacterium* into the floral tissue of the plant using a needleless-injection device. Development of transformed seed takes place in the plant. More information about this invention

Paradigm Genetics Inc claims (Update July 2003)

- a method for preparing a transformed plant or seed in which the *Agrobacterium* carrying the DNA to be transformed is grown to a certain density, diluted in an aqueous medium and then applied to floral tissues for transformation. More information about this invention

Rhobio claims

- the transformation of ***any*** tissue of a plant with *Agrobacterium* at the time when the tissue is still in its natural plant environment. Once transformed, the tissue is removed from the plant and regenerated *in vitro*. More information about this invention

The **Australian** and **PCT applications** filed by **Performance Plants Inc**. disclosing a method to transform plants *in vivo* with *A. tumefaciens* were **abandoned** on **June 1, 2000** and on **October 18, 2000**, respectively.

Patents granted to Cotton Inc.

Specific Patent Information

<table>
<tr><th>Patent Number</th><th>Title, Independent Claims and Summary of Claims</th><th>Assignee</th></tr>
<tr><td>US 5994624
Earliest priority - 20 October 1997
Filed - 20 October 1997
Granted - 30 November 1999
Expected expiry - 20 October 2017</td><td>Title - In planta method for the production of transgenic plants

Claim 1
A method for producing a transformed plant comprising, injecting Agrobacterium cells harboring a vector, comprising a nucleic acid molecule capable of conferring a desired phenotypic trait to a plant, into a plant floral or meristematic tissue using a needleless injection device, which can be adapted for the injection of small volumes of material in a precise manner without causing massive tissue damage.

Claim 16
A method for producing a transgenic seed comprising injecting a Agrobacterium cells harboring a vector, comprising a gene capable of conferring a desired phenotypic trait, into the floral tissues of a plant before the division of the egg cell using a needleless-hypodermic injection device.

Claim 17
A method of producing a transgenic seed comprising injecting a recombinant Agrobacterium into the foloral tissues of a plant using a needleless-hypodermic injection device.

Claim 18
A method for producing a transgenic seed comprising injecting a Agrobacterium cells harboring a vector, comprising a gene capable of conferring a desired phenotypic trait, into the floral tissues of a plant before the division of the egg cell using a needleless-hypodermic injection device.

The United States patent 5 994 624 discloses
a method to transform floral or meristematic tissue in vivo by injecting with Agrobacterium with a needleless hypodermic injection device; and
a method to transform floral tissue before the division of the egg cell using the same procedure as above.
The transformed floral tissue develops normally, forming seeds after pollination. Seeds are grown to generate a F1 transformed progeny.

Although claim 1 says that the injection device can be adapted for the injection of small volumes of material in a precise manner without causing massive tissue damage, it does not have to be. The specification, further, does not explicitly define what "massive tissue damage" is though it discloses that direct injection with a needle or particle bombardment can cause such damage.</td><td rowspan="2">Cotton Inc.</td></tr>
<tr><td>AU 752717
Earliest priority - 20 October 1997
Filed - 19 October 1998
Granted - 26 September</td><td>Title - In planta method for the production of transgenic plants

Claim 1
A method for producing a transformed plant including injecting a transforming agent comprising Agrobacterium cells harboring a vector having a nucleic acid molecule capable of conferring a desired phenotypic trait to a plant, into a plant floral or meristematic tissue using a needleless injection device which can be adapted for the injection of small volumes</td></tr>
</table>

2002 • Patent reported as ceased - 27 May 2004	of material in a precise manner without causing massive tissue damage. **Claim 12** A method for producing a transgenic seed comprising injecting a recombinant *Agrobacterium* into the floral tissues of a plant using a needleless injection device which can be adapted for the injection of small volumes of material in a precise manner without causing massive tissue damage. **Claim 13** A method for producing a transgenic seed comprising injecting *Agrobacterium* cells harboring a vector, comprising a gene capable of conferring a desired phenotypic trait, into the floral tissues of a plant before the division of the egg cell using a needleless injection device. **Claim 14** An *in planta* germline transformation method including needleless injection of a transforming agent comprising an *Agrobacterium* vector, said injection being directly into a tissue including the floral or meristematic germline cells of said plant, said injection being selected as to pressure, volume and trajectory so as to limit penetration of said transforming agent to a region of said tissue whereby said germline cells are transformed without causing excessive tissue damage. **Claim 15** A method for producing a transformed plant or a transgenic seed substantially as herein described in the detailed description of the invention with reference to the drawings. Unlike the US patent, the claims in the AU patent do not require that the needleless injection device be a hypodermic device. As in the US patent, although claim 1 and 12 say that the needleless injection device can be adapted for the injection of small volumes of material in a precise manner without causing massive tissue damage, the claims don't require the injection device to have these features/functions. The patent does not disclose what "excessive" tissue damage is other than to generally describe that it may occur through using a needle to inject a tissue. The patent does not explicitly state that "excessive" tissue damage and "massive" tissue damage are the same.
Remarks	Related patent application in South Africa (ZA 9809517).

Note: Patent information on this page was last updated on 22 February 2006.

Patent application filed by Cotton Inc.

Bibliographic data

	EP 1 025 247 A1
Title	In planta method for the production of transgenic plants
Application No. & Filing Date	EP 1 025 247 A1 October 19, 1998
Publication Date	August 9, 2000
Language	English
Remarks	Application also filed in Australia (AU 98019/98 A1).
	View Claims

To view or download the patent application as a PDF file, click on EP 1 025 247 A1 (1,710 kb).

Summary of the invention

The European application **EP 1 025 247 A1** also recites the transformation of floral tissues by injecting them with *Agrobacterium*. Additionally, it includes the injection of any transforming agent or DNA molecule having a gene of interest into the plant tissue.

In planta transformation
Patent application filed by Cotton Inc.

Claims in plain English

Disclaimer
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EP 1 025 247 A1

Claim 1

Transforming a plant by

- injecting, without a needle, a transforming agent into a plant tissue.

Claim 23

Producing a transgenic seed by

- injecting, without a needle, *Agrobacterium* having a vector with a gene of interest into plant floral tissues before egg cell division.

Claim 28

Producing a transgenic seed by

- injecting, without a needle, a DNA molecule with a gene of interest into plant floral tissues.

Actual pending claims

EP 1 025 247 A1
Claim 1 A method for producing a transformed plant comprising, injecting a transforming agent into a plant tissue using a needless injection device.
Claim 23 A method for producing a transgenic seed comprising injecting a recombinant *Agrobacterium* into the floral tissues of a plant using a needleless-hypodermic injection device.
Claim 28 A method for producing a transgenic seed comprising injecting a DNA molecule comprising a gene capable of conferring a desired phenotypic trait into the floral tissues of a plant using a needleless-hypodermic injection device.

Patent application filed by Rhobio

Bibliographic data

	WO 0063398
Title	Plant transformation method
Application No. & Filing Date	PCT/EP00/04177 April 19, 2000
Publication Date	October 26, 2000
Language	English
Remarks	The present PCT application was converted to the European application **EP 1 171 621 A1** published on **January 16, 2002**.
	View Claims

To view or download the patent application as a PDF file, click on WO 2000/63398 (EP 1 171 621 A1) (2,421 kb).

Summary of the invention

The PCT application **WO 0063398** (equivalent to the European application **EP 1 171 621 A1**), recites

- a method to transform a plant tissue *in vivo* by inoculating and co-cultivating *Agrobacterium* cells with the tissue. The transformed tissue is separated from the plant and submitted to a dedifferentiation and regeneration process.

In planta transformation
Patent application filed by Rhobio

Claims in plain English

Disclaimer
THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

WO 0063398 A1 (EP 1 171 621 A1*)
Claim 1 A transformation method comprising: A) inoculation and co-cultivation of a target tissue with *Agrobacterium* while the tissue is still in its natural plant environment; B) generation of a transgenic plant via dedifferentiation and regeneration of the target tissue.
Claim 3 A transformation method comprising: A) inoculation and co-cultivation of a target tissue with *Agrobacterium* as described in Claim 1; B) generation of a dedifferentiated transformed target tissue.
Claim 14 An *Agrobacterium* transformation method comprising: A) inoculation and co-cultivation of a target tissue with *Agrobacterium* as described in claim 1; B) generation of a transgenic plant via dedifferentiation of the target tissue.

Actual pending claims

WO 0063398 A1 (EP 1 171 621 A1*)
Claim 1 A transformation method comprising inoculation and co-cultivation of a target tissue, from a target plant, with *Agrobacterium*, at a time when the target tissue is in its natural plant environment, followed by generation of a transgenic plant via dedifferentiation and regeneration of the target tissue.

Claim 3
A transformation method comprising inoculation and co-cultivation of a target tissue, from a target plant, with *Agrobacterium*, at a time when the target tissue is in its natural plant environment, followed by generation of dedifferentiated tissue from the target tissue.
Claim 14
Use of *Agrobacterium* in a transformation method comprising inoculation and co-cultivation of a target tissue, from a target plant, with *Agrobacterium*, at a time when the target tissue is in its natural plant environment, followed by generation of transgenic plant material via dedifferentiation of the target tissue.

* The PCT application was converted to the EP application **EP 1 171 621 A1.**

Floral Transformation

Patent granted to Paradigm Genetics Inc. (Update July 2003)

Bibliographic data

The invention disclosed in this patent comprises an alternative protocol to adjust the cell density of the *Agrobacteria*
used in the transformation step. This method should be useful in high throughput transformation protocols.

	US 6 353 155 B1
Title	Method for transforming plants
Application No. & Filing Date	US 09/607,306 30 June 2000
Issue Date	5 March 2002
Language	English
	View Claims

To view or download the patent as a PDF file, click on US 6,353,155 (416 Kb).

Summary of the invention

United States patent **US 6 353 155** discloses

- a method to transform floral tissue *in vivo* by treatment with an *Agrobacterium* cell suspension adjusted to a certain density by dilution with an aqueous solution.

The invention comprises an alternative protocol to adjust the cell density of the Agrobacteria used in the transformation step. According to the inventors, centrifugation and resuspension are avoided by direct dilution of the bacteria in an aqueous solution, thereby allowing sequential treatment of many more plants. The method is well-suited for the transformation of *Arabidopsis thaliana* (Brassicaceae), a model plant where such a high throughput transformation protocol might be required. No other plants are used in the only example provided as part of the patent specification.

Patent granted to Paradigm Genetics Inc. (Update July 2003)

Granted independent claims

US 6 353 155 B1
Claim 1
A method for preparing a transgenic plant or seed comprising: a) growing a suspension of Agrobacterium cells until the optical density of the suspension is about 2 to about 2.4 at a wavelength of 600 nanometers, wherein said Agrobacterium cells contain at least one plasmid having a DNA sequence of interest flanked by T-DNA borders;

b) diluting said Agrobacterium cells of said suspension with an aqueous medium so that the optical density of the suspension is reduced to about 0.6 to about 1.5, wherein said diluting is not followed by pelleting via centrifugation with subsequent resuspension; and
c) treating the flower of said plant with said diluted suspension so that the Agrobacterium cells in said diluted suspension can transform said plant with the DNA-sequence of interest;
d) optionally, cultivating said treated plant to produce seed; and e) optionally, growing plants from said seed and selecting for transgenic plants having said DNA sequence of interest.

Claim 10

A method for preparing a transgenic plant or seed comprising:
a) growing a suspension of Agrobacterium cells until growth of Agrobacterium cells in the suspension is substantially completed, wherein said Agrobacterium cells contain at least one plasmid having a DNA sequence of interest flanked by T-DNA borders;
b) diluting said suspension with an aqueous medium to reduce the concentration of Agrobacterium cells and any other components in the growth medium and allow the Agrobacterium cells to infect the plant without harming it, wherein said diluting is not followed by pelleting via centrifugation with subsequent resuspension;
c) treating the flower of said plant with said diluted suspension so that the Agrobacterium cells in said diluted suspension can transform said plant with the DNA-sequence of interest;
d) optionally, cultivating said treated plant to produce seed; and e) optionally, growing plants from said seed and selecting for transgenic plants having said DNA sequence of interest.

Claim 17

A method for preparing a transgenic plant or seed comprising:
a) growing a suspension of Agrobacterium cells until growth of Agrobacterium cells in the suspension is substantially completed, wherein said Agrobacterium cells contains at least one plasmid having a DNA sequence of interest flanked by T-DNA borders;
b) diluting said Agrobacterium cells in said suspension with about 2 to about 10 volumes aqueous medium per volume of suspension, wherein said diluting is not followed by pelleting via centrifugation with subsequent resuspension;
c) treating the flower of said plant with said diluted suspension so that the Agrobacterium cells in said diluted suspension can transform said plant with the DNA-sequence of interest;
d) optionally, cultivating said treated plant to produce seed; and
e) optionally, growing plants from said seed and selecting for transgenic plants having said DNA sequence of interest.

Seed transformation

Patent application assigned to Scigen Harvest Co. Ltd. (Update July 2003)

The method disclosed in this patent application is based on wounding the cotyledonary node of a germinating seed. In the claims the node is simply described as the target plant tissue in the wounded seed. In the examples, only soybean is transformed, but the independent claim is unrestricted in terms of plant family or species. Dependent claims recite a long list of commercially important crop plants, monocots and dicots.

The numerous limitations in independent Claim 1 suggest various ways of inventing around this patent, e.g. using other than half a seed, pre-germinating more or less than a day, wounding with one or two needles instead of a bundle.

Bibliographic data

	WO 02/066599
Title	Efficient method for the development of transgenic plants by gene manipulation
Application No. & Filing Date	PCT/KR02/00232 14 February 2002
Publication Date	29 August 2002
Language	English

View Claims

To view or download the patent as a PDF file, click on WO 02/066599 (2.7 Mb).

Summary of the invention

PCT patent application **WO 02/066599** discloses

- a method to transform plants by wounding pre-germinated seeds with needles and treating the wounded areas with *Agrobacterium tumefaciens* containing a vector with the desired gene.

Seed transformation
Patent application assigned to Scigen Harvest Co. Ltd

Independent claims

WO 02/066599
Claim 1 A method for transforming plants, comprising the steps of: (a) selecting half a seed, which is germinated for one day as a target plant tissue; (b) wounding the target plant tissue using a bundle of needles; (c) inserting a target gene into the target plant tissue with *Agrobacterium tumefaciens* vector containing the target gene; and (d) regenerating a whole plant from the target plant tissue.

Seed transformation
Patent applications by The Agri-Biotechnology Research Center of Shanxi (Update July 2003)

The method disclosed in the following patent applications is based on wounding the apical meristem of the appearing bud in pre-germinated seeds. Although only enabled for one species, the independent claims are kept broad covering plants from any family or species.

Bibliographic data

	US 2002/0184663 A1	EP 1 236 801 A2
Title	Method of Agrobacterium mediated plant transformation through treatment of germinating seeds	Method of Agrobacterium mediated plant transformation through treatment of germinating seeds
Application No. & Filing Date	US 10/079,080 19 Feb 2002	EP 2 251 259 25 Feb 2002
Publication Date	5 Dec 2002	4 Sep 2002
Language	English	English

To view or download the patent as a PDF file, click on US 2002/0184663

Summary of the invention

United States application US 2002/0184663 and European application EP 1 236 801 disclose

- a method to transform plants by treating pre-germinated seed with *Agrobacterium tumefaciens* containing Ti plasmids with a desired gene.

As described in the specifications the method is based on wounding the apical meristem of the appearing bud. In independent Claim 1 this is described simply as germinating seed, without specifying a plant genus or species. Examples do not provide all the enablement. Dependent claims recite monocots and dicots.

The wording of independent Claim 1 is rather broad, requiring no more than the contacting of Agrobacterium containing a Ti plasmid with germinating seeds. The source of the seeds is unrestricted in terms of family or species of plants, whereas only maize seeds were exemplified in the patent text. As these

are patent applications, the final scope of any claims granted is unknown.

View Claims

Seed transformation
Patent applications by The Agri-Biotechnology Research Center of Shanxi (Update July 2003)

Independent claims

US 2002/0184663
1. A plant transformation method mediated by *Agrobacterium* comprising: providing germinating seeds; and co-culturing the germinating seeds with an Agrobacterium strain containing Ti plasmids with an inserted nucleic acid sequence so that the foreign nucleic acid sequence carried by the Agrobacterium strain is transferred and integrated into the genome of the germinating seeds through Ti plasmids.
11. A plant transformed by the method of claim 1.

EP 1 236 801 A2
Claim 1 A plant transformation method mediated by Agrobacterium, characterized in that germinating seeds were used as the receptors, an Agrobacterium strain containing Ti plasmids with an inserted nucleic acid sequence was used as the donor, the germinating seeds are co-cultured with the Agrobacterium strain so that the foreign nucleic acid sequence carried by the Agrobacterium strain can be transferred and integrated into the genome of the receptor through Ti plasmids.
Claim 11 A fertile transgenic plant produced by the method according to any of claims 1 to 10.
Claim 16 A progeny plant from a seed obtained from a transgenic plant according to any of claims 11 to 14.
Claim 17 A germinating seed transformed according to the method of any of claims 1 to 10.

Binary Vectors

Overview

Binary vector systems include the most commonly used vectors devised for *Agrobacterium* gene transfer to plants. In these systems, the T-DNA region containing a gene of interest is contained in one vector and the *vir* region is located in a separate disarmed (without tumor-genes) Ti plasmid. The plasmids co-reside in *Agrobacterium* and remain independent.

We will now introduce you to the entities that have applied for, and in some cases, been granted patents on **binary vectors** for plant transformation via *Agrobacterium*.

The patent applications are characterized according to the following criteria:

- Patents on basic binary vectors and methods. Directed to the basic binary vector molecules, including their primary elements and in some cases, the basic methods for their assembly and use; and
- Patents on modified binary vectors and methods. Directed to special applications or improved vectors and methods based on the basic binary vector molecules.

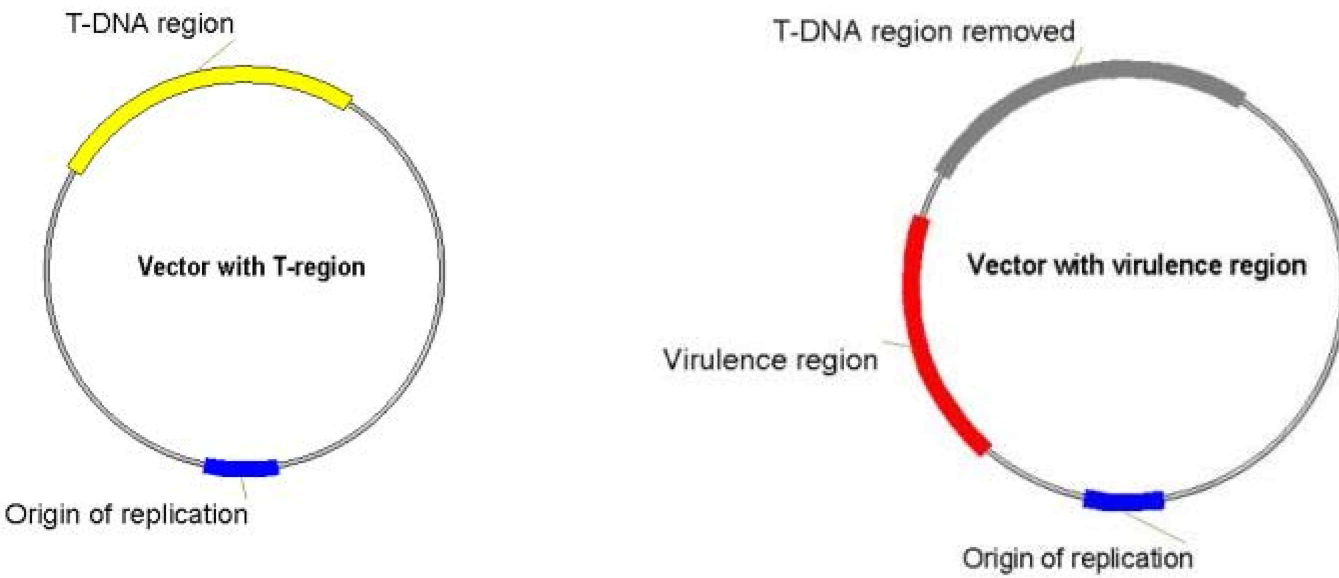
Summary of patents on basic binary vectors and methods

Important patents relating to basic binary vectors, which essentially claim the basics of binary vectors, were granted to **Mogen** in the United States (two patents) and in Europe (one patent). **Mogen** is now called **Syngenta Mogen B.V.** and belongs to **Syngenta Company**.

The claims are very broad and encompass basically **any** two-vector system located in the same *Agrobacterium* strain having

- a T-region in one vector, and
- a *vir* region in another vector,

as illustrated below.



A limitation of the claims in these patents is that the binary vectors are used for transformation of **dicotyledonous** (**dicots**) plants.

While it is difficult to form a conclusion that will apply to every reader, overall, these patents may encompass many transformation protocols in common use. In the United States and Europe, users of this binary vector system should keep these patents in mind when crafting a commercial research strategy.

Table of patents on basic binary vectors

The following table presents an overview of three patents that contain claims related to the components and methods of using **basic binary vectors** for gene transfer into plants via *Agrobacterium*. All of the patents are limited to using binary vectors for transformation of dicotyledonous plants (dicots).

This analysis is based only upon the published specification and claims and as such is preliminary and informal. A formal analysis would also entail examination of the patent prosecution (see Introduction) of each patent.

Assigned to Syngenta Mogen B.V.
Issued Patents Overview
US 4 940 838 & EP 120 516 B1 Process for the incorporation of foreign DNA into dicots using *Agrobacterium* containing at least two plasmids where one has a *vir* region but not a T-region, and the other one has a foreign gene inserted into the T-region but does not contain a *vir* region. Claims also recite elements of both plasmids and a method of producing an *Agrobacterium* harboring both of the plasmids. US 5 464 763 Process for the incorporation of foreign DNA into dicots using *Agrobacterium* containing at least two plasmids. The elements of the plasmids and methods to produce such *Agrobacterium* are as claimed in US patent **4 940 838** but this patent further claims the presence of 23 bp sequences at the ends of the T-DNA region and only foreign DNA between the 23 bp sequences and does not require homology between the *vir* and T-region plasmids. More detailed information on this patent.

Patents granted to Syngenta Mogen B.V.

The disclosed invention is directed to the transformation of dicots using an *Agrobacterium* carrying binary vectors where the T-DNA is located in one plasmid and the *vir* region is located in a different plasmid. The separation of the T-DNA and the *vir* region on two different plasmids is the basic principle of binary vector systems. The two United States patents are still in force, while the European and Japanese patents have reached their maximum 20 year protection term from filing and therefore have expired.

Specific Patent Information

<table>
<tr><th>Patent Number</th><th>Title, Independent Claims and Summary of Claims</th><th>Assignee</th></tr>
<tr><td>US 4940838
Earliest priority - 24 February 1983
Filed - 23 February 1984
Granted - 10 July 1990
Expected expiry - 10 July 2007</td><td>Title - Process for the incorporation of foreign DNA into the genome of dicotyledonous plants

Claim 1

A process for the incorporation of foreign DNA into chromosomes of dicotyledonous plants, comprising infecting the plants or incubating plant protoplasts with Agrobacterium bacteria, which contain plasmids, said Agrobacterium bacteria containing at least one plasmid having the vir-region of a Ti plasmid but no T-region, and at least one other plasmid having a T-region with incorporated therein foreign DNA but no vir-region.

Claim 13

A process for the production of Agrobacterium bacteria which contains at least one plasmid which has the vir-region of a Ti-plasmid but no T-region and at least one other plasmid which has a T-region with foreign DNA incorporated in it but no vir-region, comprising:

(a) using Escherichia coli as a host and incorporating foreign DNA into a plasmid therein which contains a T-region and a replicator having a broad bacterial host range
(b) introducing the resulting plasmid into Agrobacterium bacteria which contain at least one plasmid which has the vir-region of a Ti-plasmid but no T-region.

The United States patent 4 940 838 claims
an Agrobacterium having binary vectors where the T-DNA region and the vir region are located in two different plasmids. The plasmid that contains the T-region lacks the vir region, and vice versa. The vir region is fundamental for the transfer of the T-region into the plant genome.
the integration of foreign DNA into the genome of a dicot plant. The foreign DNA is located in the T-region.</td><td rowspan="2">Syngenta Mogen B.V.</td></tr>
<tr><td>US 5464763
Earliest priority - 24 February 1983
Filed - 23 December 1993
Granted - 7 November 1995
Expected expiry - 10 July 1997</td><td>Title - Process for the incorporation of foreign DNA into the genome of dicotyledonous plants

Claim 1

A process for incorporating into the genome of dicotyledonous plants foreign DNA, comprising infecting the plants or plant cells or incubating plant protoplasts with Agrobacterium strains, which contain plasmids, said Agrobacterium strains containing at least one plasmid having the vir-region of a Ti plasmid but no T-region, and at least one other plasmid having a T-region but no vir-region, said T-region being composed of naturally occurring border sequences consisting of about 23 base pairs at the extremities of said T-region and only foreign DNA between said border sequences, the vir-region plasmid and the T-region plasmid containing no homology which could lead to cointegrate formation.</td></tr>
</table>

Claim 2

Agrobacterium strains, comprising at least one plasmid having the vir-region of a Ti plasmid but no T-region, and at least one other plasmid having a T-region but no vir-region, said T-region being composed of naturally occurring border sequences consisting of about 23 base pairs at the extremities of said T-region and only foreign DNA between said sequences, the vir-region plasmid and the T-region plasmid containing no homology which could lead to cointegrate formation.

Claim 3

A process for the production of *Agrobacterium* strains comprising at least one plasmid having the vir-region of a Ti plasmid but no T-region, and at least one other plasmid having a T-region but no vir-region, said T-region being composed of naturally occurring border sequences consisting of about 23 base pairs at the extremities of said T-region and only foreign DNA between said border sequences, the vir-region plasmid and the T-region plasmid containing no homology which could lead to cointegrate formation, said process comprising:

(a) incorporating non-*Agrobacterium* foreign DNA into a plasmid having a T-region and a replicator having a broad bacterial host range, wherein said foreign DNA becomes part of the T-region,

(b) cloning the resulting plasmid in *Escherichia coli*; and

(c) introducing the resulting plasmid into *Agrobacterium* strains which contain at least one plasmid which has the vir-region of a Ti-plasmid but no T-region.

Claim 12

A process for incorporating in the genome of dicotyledonous plants foreign DNA, comprising infecting the plants or plant cells or incubating plant protoplasts with *Agrobacterium* strains, which contain plasmids, said *Agrobacterium* strains containing at least one plasmid having the vir-region of a Ti-plasmid but no T-region, and at least one other plasmid having a single T-region but no vir-region, said single T-region being composed of naturally occurring border sequences at the extremities of said single T-region, the vir-region plasmid and the T-region plasmid containing no homology which could lead to cointegrate formation.

Granted **US 5464763** is a continuation of US 07/550736 (now abandoned), which is a continuation of now granted **US 4940838** (see above).

The United States patent **US 5464763** includes the elements and methods claims of the United States patent **US 4940838** and additionally claims

- the presence of only foreign DNA between the 23 bp borders of the T-region, and
- the absence of a region of homology between the plasmid having the T-region and the plasmid bearing the *vir* region that might lead to cointegrate formation. This characteristic is essential to maintain the two plasmids as individual molecules.

EP 120516

- Earliest priority - 24 February 1983

Title - A process for the incorporation of foreign DNA into the genome of dicotyledonous plants; *Agrobacterium tumefaciens* bacteria and a process for the production thereof

Claim 1

• Filed - 21 February 1984 • Granted - 23 October 1991 • Expired - 21 February 2004	A process for the incorporation of foreign DNA into chromosomes of dicotyledonous plants comprising infecting the plants or incubating plant protoplasts with *Agrobacterium* bacteria, which contain plasmids, said *Agrobacterium* bacteria containing at least one plasmid having the vir-region of a Ti-plasmid but no T-region, and at least one other plasmid having an artificial T-region with only foreign DNA between the 23 base pairs at the extremeties of the wild type T-region, but no vir-region, the vir-region plasmid and the T-region plasmid containing no homology which could lead to cointegrate formation. Claim 2 *Agrobacterium* bacteria, comprising at least one plasmid having the vir-region of a Ti-plasmid but no T- region, and at least one other plasmid having an artificial T-region with only foreign DNA between the 23 base pairs at the extremeties of the wild type T-region, but no vir-region, the vir-region plasmid and the T-region plasmid containing no homology which could lead to cointegrate formation. Designated States at the time of grant are: Austria (cancelled as reported on INPADOC), Belgium (expired as reported on INPADOC), Switzerland (ceased as reported on INPADOC), Germany, France, United Kingdom (expired as reported on INPADOC), Italy, Liechtenstein, Luxembourg, Netherlands (lapsed as reported on INPADOC), Sweden The subject matter claimed in the European patent **120 516 B1** is a combination of the claims of the United States patents. In the European patent, the T-region of one of the binary vectors is claimed as an artificial T-region, which contains only foreign DNA between the 23 bp T-border sequences.
Remarks	Related patents granted in Japan (JP 7036751 and JP 7046993, both expired on 23 February 2004 as reported by the Japan Patent Office).

Note: Patent information on this page was last updated on 24 February 2006.

Patents on basic binary vectors and methods
Patents granted to Syngenta Mogen B.V.

Claims in plain English

Disclaimer
THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

US 4 940 838
Claim 1 Integration of foreign DNA into the chromosomes of dicots comprising: • infecting plants or incubating protoplasts with *Agrobacterium* having: (i) a plasmid with *a vir*-region and without a T-region, and (ii) a plasmid with foreign DNA in the T-region and without *a vir*-region.
Claim 2 An *Agrobacterium* comprising: *a vir*-region and without a T-region, and (ii) a plasmid with foreign DNA in the T-region and without *a vir*-region

Claim 13

Process to produce an *Agrobacterium* having the plasmids set out in Claim 2 comprising:

A) inserting foreign DNA into the T-region of a plasmid with a broad-host range replicator using *E.coli* as a host; and
B) introducing the resulting plasmid in an *Agrobacterium* that has a plasmid with a *vir* region and without a T-region.

US 5 464 763

Claim 1

Process for inserting foreign DNA into the genome of dicots by infecting plants or incubating protoplasts with *Agrobacterium* having: (i) a pl asmid with a *vir* region of Ti plasmid and without a T-region, and
(ii) a plasmid with a T-region and without a *vir* region.
The T-region has only foreign DNA between the 23 bp border sequences.
There is no homology region between the two plasmids.

Claim 2

Agrobacterium comprising:
(i) a plasmid with a *vir* region and without a T-region, and
(ii) a plasmid with a T-region and without a *vir* region.
The T-region has only foreign DNA between the 23 bp border sequences.
There is no homology region between the two plasmids.

Claim 3

Production of *Agrobacterium* as set out in Claim 2 comprising:

A) inserting foreign DNA within the T-region of a plasmid with a broad host range replicator;
B) cloning the resulting plasmid in *E. coli*; and
C) incorporating the resulting plasmid into *Agrobacterium* having a plasmid with a *vir* region and without a T-region.

EP 120 516 B1

Claim 1

Method of foreign DNA incorporation into dicot plants by:

- infecting plants or incubating protoplasts with *Agrobacterium* having:

(i) a plasmid with a *vir* region and without a T-region, and
(ii) a plasmid with an artificial T-region* and without a *vir* region
There is no homology region between the plasmids.

Claim 2

Agrobacterium containing:
(i) a plasmid with a *vir* region and without a T-region, and
(ii) a plasmid with an artificial T-region* and without a *vir* region
There is no homology region between the plasmids.

Claim 3

Production of *Agrobacterium* comprising:

A) incorporating in a plasmid with a broad-host-range replicator an artificial T-region* using *E.coli* as a host;
B) introducing the resulting plasmid in *Agrobacterium* having a plasmid with a *vir* region and without a T-region.

*An artificial T-region is defined by the inventors as one that contains only foreign DNA between the 23 base pairs at the extremes of such region.

Actual granted claims

US 4 940 838

Claim 1

A process for the incorporation of foreign DNA into chromosomes of dicotyledonous plants, comprising: infecting the plants or incubating plant protoplasts with *Agrobacterium* bacteria, which contain plasmids, said *Agrobacterium* bacteria containing:
(i) at least one plasmid having the *vir* region of a Ti plasmid but no T-region, and
(ii) at least one other plasmid having a T-region with incorporated therein foreign DNA but no *vir* region.

Claim 2

Agrobacterium bacteria, comprising:
(i) at least one plasmid which has the *vir* region of a Ti plasmid but no T-region, and
(ii) at least one other plasmid which has a T-region with foreign DNA incorporated in it but no *vir* region.

Claim 13

A process for the production of *Agrobacterium* bacteria which contains at least one plasmid which has the *vir* region of a Ti-plasmid but no T-region and at least one other plasmid which has a T-region with foreign DNA incorporated in it but no *vir* region, comprising:

A) using *Escherichia coli* as a host and incorporating foreign DNA into a plasmid therein which contains a T-region and a replicator having a broad bacterial host range;
B) introducing the resulting plasmid into *Agrobacterium* bacteria which contain at least one plasmid which has the *vir* region of a Ti-plasmid but no T-region.

US 5 464 763

Claim 1

A process for incorporating into the genome of dicotyledonous plants foreign DNA, comprising infecting the plants or plant cells or incubating plant protoplasts with *Agrobacterium* strains, which contain plasmids, said *Agrobacterium* strains containing:
(i) at least one plasmid having the *vir* region of a Ti plasmid but no T-region, and
(ii) at least one other plasmid having a T-region but no *vir* region,
said T-region being composed of naturally occurring border sequences consisting of about 23 base pairs at the extremities of said T-region and only foreign DNA between said border sequences,
the *vir* region plasmid and the T-region plasmid containing no homology which could lead to co-integrated formation.

Claim 2

Agrobacterium strains, comprising:
(i) at least one plasmid having the *vir* region of a Ti plasmid but no T-region, and
(ii) at least one other plasmid having a T-region but no *vir* region, said T-region being composed of naturally occurring border sequences consisting of about 23 base pairs at the extremities of said T-region and only foreign DNA between said sequences,
the *vir* region plasmid and the T-region plasmid containing no homology which could lead to co-integrated formation.

Claim 3

A process for the production of *Agrobacterium* strains comprising: (i) at least one plasmid having the *vir* region of a Ti plasmid but no T-region, and
(ii) at least one other plasmid having a T-region but no *vir* region,
said T-region being composed of naturally occurring border sequences consisting of about 23 base pairs at the extremities of said T-region and only foreign DNA between said border sequences,
the *vir* region plasmid and the T-region plasmid containing no homology which could lead to co-integrated formation, said process comprising:

A) incorporating non-*Agrobacterium* foreign DNA into a plasmid having a T-region and a replicator having a broad bacterial host range, wherein said foreign DNA becomes part of the T-region;
B) cloning the resulting plasmid in *Escherichia coli*; and
C) introducing the resulting plasmid into *Agrobacterium* strains which contain at least one plasmid which has the *vir* region of a Ti-plasmid but no T-region.

EP 120 516 B1
Claim 1 A process for the incorporation of foreign DNA into chromosomes of dicotyledonous plants comprising infecting the plants or incubating plant protoplasts with *Agrobacterium* bacteria, which contain plasmids, said *Agrobacterium* bacteria containing: (i) at least one plasmid having the *vir* region of a Ti-plasmid but no T-region, and (ii) at least one other plasmid having an artificial T-region with only foreign DNA between the 23 base pairs at the extremities of the wild type T-region, but no *vir* region, the *vir* region plasmid and the T-region plasmid containing no homology which could lead to co-integrated formation.
Claim 2 *Agrobacterium* bacteria containing: (i) at least one plasmid having the *vir* region of a Ti-plasmid but no T-region, and (ii) at least one other plasmid having an artificial T-region with only foreign DNA between the 23 base pairs at the extremities of the wild type T-region, but no *vir* region, the *vir* region plasmid and the T-region plasmid containing no homology which could lead to co-integrated formation.
Claim 3 A process for the production of *Agrobacterium* bacteria according to Claim 2 comprising: A) using *Escherichia coli* as a host and incorporating into a plasmid therein which contains said artificial T-region and a replicator having a broad host range; B) introducing the resulting plasmid into *Agrobacterium* bacteria which contain at least one plasmid which has the *vir* region of a Ti-plasmid, but no T-region.

Summary of patents on modified binary vectors and methods

Several patents claim inventions built on the basic components of the binary vector system and methods for their use. The patents referred under this section are:

- ***Agrobacterium* with more than one T-DNA or *vir* region. Leiden University and Schilperoort** have a granted United States patent and a granted European patent on this subject matter. The multiple T-DNA or *vir* regions are integrated into the chromosome of *Agrobacterium*. Thus, the T-DNA and *vir* regions are present not only in the binary vectors, but also in the bacterial chromosome. *Agrobacterium* with these features is used for transformation of dicots and monocots of the families Liliaceae and Amaryllidaceae.

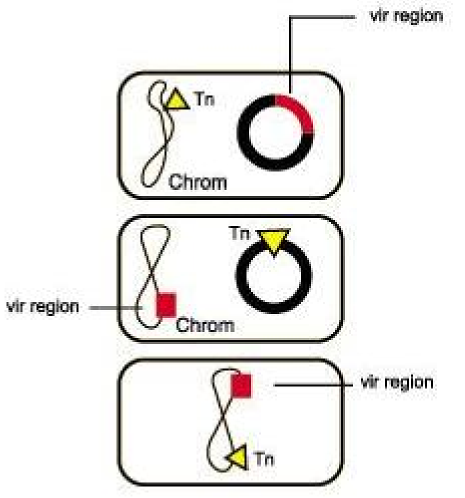


Diagram of Agrobacterium cells having either T-DNA or vir region and both integrated in the bacterial chromosome

Tn= Transponson containing T-DNA region
Chrom= Chromosome of the bacterium

Claimed by Leiden Uni & Schilperoort

The main limitations of this patent are the requirement of having at least one of either T-DNA or *vir*

region in the bacterial chromosome and the covering of monocots belonging to the families Liliaceae and Amaryllidaceae only.

- **Integration of foreign DNA in a plant target locus by homologous recombination** . The United States and European patents granted to **Mogen**, now **called Syngenta Mogen B.V.,** claim a vector with a region homologous to a part of the sequence of a target locus in the plant allowing homologous recombination of the target locus and the vector. This permits insertion of a gene of interest or a specific mutation in a particular locus of a plant genome.

  The most limiting factor of these patents is the use of sequences from a plant target locus as part of an *Agrobacterium* vector in order to allow homologous recombination between the vector and the plant locus.

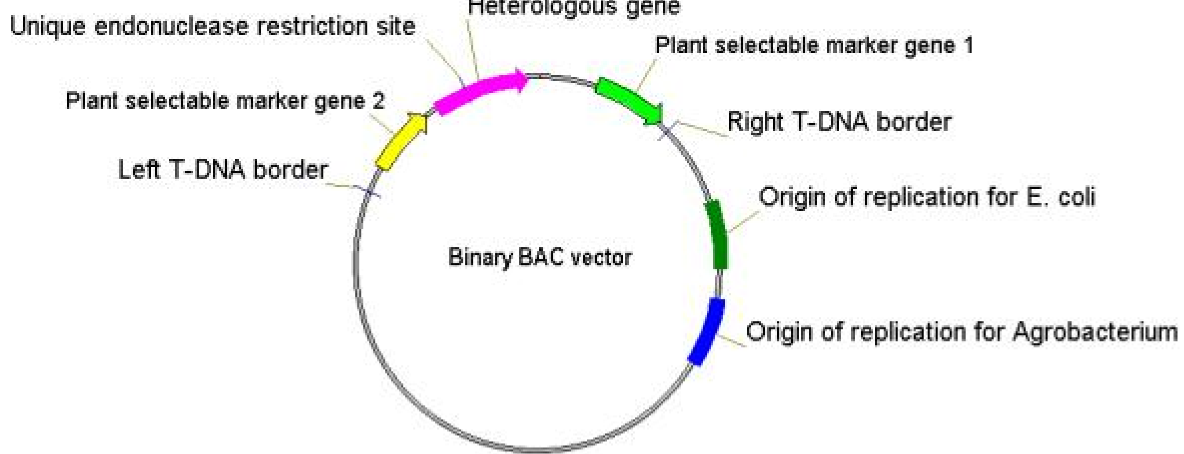
A series of binary vectors have been devised to suit different needs in a plant transformation. Different origins of replication for a plasmid, size of the insert a binary plasmid can carry and the size of the plasmid itself are among the features considered for the design of plasmids that are highly stable, easy to manipulate and transfer across diverse host bacteria. For example, origins of replication that allow low copy or high copy number of plasmids in a host cell are the subject of several granted patents and patent applications. Specifically the United States patent 6 165 780 (as PDF) and its related patent and applications filed by **The National Institute of Agrobiological Resources** (Japan) are directed to binary shuttle vectors containing two different origins of replication that confer stability and a low copy number of plasmids in both *E. coli* and *Agrobacterium*
host cells. One of the claimed binary vectors can be used to insert a clone of a genome library into a plant for complementation test.

More patents referring to this topic are likely to appear in the future as new combinations of origins of replication, number of restriction sites, capacity of the vector in terms of length of the carried insert, and number and nature of marker and reporter genes are envisioned and assemble in vector molecules according to the purpose or requirements of the research.

We analyze here one of those patents that refer to a binary vector maintained as a single copy in *E. coli* as well as in *Agrobacterium* and capable of carrying a large genome fragment.

- **Binary vectors based on the bacterial artificial chromosome (BAC)** . Two United States patents and a European application assigned to **Cornell** refer to this topic. The binary vector that contains the T-DNA region has
  - origin of replications for *E. coli* and *Agrobacterium*, wherein the plasmids are maintained as a single copy, and
  -
  - a unique restriction site for insertion of an exogenous sequence located between a right and a left T-DNA borders

  Although binary BAC vectors are devised for cloning long fragments of DNA (around 150 Kb), this feature is not part of the claim scope. As a result, the claimed vector type has a fairly broad scope except that the origins of replication are very specific, that is they must maintain plasmids as a single copy.



- **Co-transformation with two binary vectors** . The PCT and Australian applications recently filed by **Pioneer Hi-Bred** disclose the use of an *Agrobacterium* with two different binary vectors and a helper plasmid for co-transformation of plants. Because these are still applications, the scope of claims that may be granted is unknown.

  A related United States patent was granted to Pioneer Hi-Bred in July 2001. The granted claims are directed to the same subject matter as the filed claims of the Australian and the PCT application. Thus, the scope of the claims has already been defined in the United States.

Remember that any of the referred inventions are protected only in the countries or jurisdictions where the patent rights have been conferred.

Table of patents on variations based on binary vectors and methods

The following table presents an overview of nine patents and patent applications that contain claims related to **variations of basic components and methods** of **binary vectors** used for gene transfer into plants via *Agrobacterium*.

This analysis is based only upon the published specification and claims of each of the applications.

Assigned to Leiden University & Schilperoort
Issued Patents Overview
US 5 149 645 Process for transferring recombinant DNA into the plant cell genome by infecting or incubating plant cells with *Agrobacterium* having a binary vector and with one or more T-regions and/or the *vir* region integrated into the bacterial chromosome prior to the incubating or infecting step. At least one T-region contains recombinant DNA flanked by T-borders that is inserted into the plant protoplasts of dicot and monocot plants of the families Liliaceae and Amaryllidaceae. EP 176 112 B1 *patent abandoned in Austria, Switzerland, Liechtenstein, Luxemburg, and Sweden. *Agrobacterium* with one or more T-regions and a *vir* region. Either the T or *vir* region or both are introduced into the bacterial chromosome. At least one of the T-regions comprises an artificial T-region flanked by wild-type or analogous border sequences. More detailed information on these patents.

Assigned to Syngenta Mogen B.V.
Issued Patents Overview
US 5 501 967 & EP 436 007 B1 Process for the introduction of recombinant DNA into a plant genome by homologous recombination, where the recombinant DNA is a defined sequence and is contained in a plant transformation vector harbored in an *Agrobacterium* strain capable of T-region transfer. More detailed information on these patents.

Assigned to Cornell Research Foundation Inc.	
Issued Patents Overview	**Patent Applications Overview**
US 5 733 744 Vector and its components for transferring heterologous DNA into a plant cell. The vector contains an origin of replication for *E. coli* and an origin of replication for *Agrobacterium*, each of which maintains the DNA as a single copy. Method for introduction of heterologous DNA into plant and producing a gene product from transformed plant cell are further claimed. US 5 977 439 Methods of transforming a transgenic plant by introducing	EP 805 851 A1 Vector for transferring heterologous DNA into a plant cell comprising an origin of replication for *E. coli* and *Agrobacterium* for single copy maintenance of such DNA, a unique restriction site, and left and right T-DNA borders flanking unique restriction site. More detailed information on this patent application.

<table>
<tr><td>a vector having DNA heterologous to the plant cell. The vector contains: two separated origins of replication, one for Agrobacterium and one for E. coli, each of which maintains heterologous DNA as a single copy; a unique restriction site for insertion of heterologous DNA encoding a product; left and right Agrobacterium T-DNA border sequences flanking such unique restriction site.

More detailed information on these patents.</td><td></td></tr>
<tr><td>Assigned to Pioneer Hi-Bred Inc.</td><td></td></tr>
<tr><td>Issued Patent Overview</td><td>Patent Applications Overview</td></tr>
<tr><td>US 6 265 638

The recently granted United States patent is directed to the same subject matter as its related Australian and PCT applications. A method for co-transformation method of a plant with Agrobacterium having at least two binary vectors, each one with a heterologous gene of interest is the essence of the granted invention. Among the heterologous nucleotide sequences, they claim a plant scorable marker gene used for screening the transformed tissue.

More detailed information on this patent.</td><td>WO 0018939 A1 (EP 1 117 816 A1) & AU 61648/99

Agrobacterium strain having at least two binary vector plasmids and a helper plasmid. Each binary vector has one T-DNA region with a gene of interest. This Agrobacterium strain is used for co-transformation of a plant. The PCT application has entered the European phase (EP 1 117 816 A1 published July 2001).

More detailed information on these patent applications.</td></tr>
</table>

Patents granted to Leiden University & Schilperoort

Based on a binary vector system, the following patents use an *Agrobacterium* having either the T-region or the *vir*
region integrated in the chromosome of the bacterium. The target plants are dicots and monocot plants from the families *Amaryllidaceae* (e.g. onion, garlic) and *Liliaceae* (e.g. asparagus).

Bibliographic data

	US 5 149 645	EP 176 112 B1
Title	Process for introducing foreign DNA into the genome of plants	
Application No. Filing Date	US 449282 December 5, 1989	EP 85200871 June 3, 1985
Issue Date	September 22, 1992	May 16, 1990
Language	English	English (Claims in English, German and French)
Remarks		Patent lapsed in Austria, Switzerland, Liechtenstein, Luxemburg, and Sweden.
	View Claims in Plain English View Actual Granted Claims	View Claims in Plain English View Actual Granted Claims

To view or download the patents as a PDF file, click on US 5 149 645 (939 kb) and EP 176 112 B1 (588 kb).

Summary of the invention

The United States patent **5 149 645** claims:

- *Agrobacterium* having either a T-region or a *vir* region as part of the chromosome of the bacterium. When the T-region is integrated into the chromosome, the *vir* region is part of a Ti plasmid, and vice-versa.

- a process to transfer recombinant DNA that is part of a T-region in *Agrobacterium* into dicot cells and monocot cells of the families Amaryllidaceae and Liliaceae.

In the European patent **EP 176 112 B1**, claims recite:

- either a T-region or a *vir* region or both integrated into genome of *Agrobacterium*;
- a T-region that contains a desired DNA flanked by the wild T-border sequences;
- transformed plants.
- There is no mention of specific families of plants.

Patents on modified binary vectors and methods
Patents granted to Leiden University Schilperoort

Claims in plain English

Disclaimer THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

US 5 149 645
Claim 1 Agrobacteria with a T- and a *vir* region, having the T-region integrated into the bacterial chromosome.
Claim 2 Agrobacteria with a T- and a *vir* region, having the *vir* region integrated into the bacterial chromosome.
Claim 3 Process for transferring recombinant DNA into dicot cells and monocot cells from the Liliaceae and Amaryllidaceae families, comprising: • infecting plants or plant cells, or incubating plant protoplasts with *Agrobacterium* having: (i) a *vir* region from a Ti-plasmid integrated into the *Agrobacterium* chromosome, and (ii) a T-region with recombinant DNA flanked by wild type T-border sequences.
Claim 5 Process for transferring recombinant DNA into dicot cells and monocots from the Liliaceae and Amaryllidaceae families, comprising: • infecting plants or plant cells, or incubating plant protoplasts with *Agrobacterium* having: (i) a *vir* region from a Ti-plasmid, and (ii) a T-region with recombinant DNA flanked by wild type T-border sequences integrated into the *Agrobacterium* chromosome.

EP 176 112 B1
Claim 1 Process for introducing foreign DNA into a plant genome by: • infecting plants, explants or protoplasts, or incubating protoplasts with *Agrobacterium* having: (i) at least one artificial T-region*, and (ii) a *vir* region from a Ti-plasmid in the *Agrobacterium* genome.
Claim 3 Agrobacteria having at least one T- and *vir*-region. Either one or both of them are integrated into the

Agrobacterium chromosome.

*An artificial T-region, as defined by the inventors, contains desired DNA flanked by wild T-border sequences or functional analogous sequences.

Actual granted claims

US 5 149 645
Claim 1 Agrobacteria comprising a T-region and a *vir* region in their DNA, the T-region being stably integrated into the bacterial chromosome.
Claim 2 Agrobacteria comprising a T-region and a *vir* region in their DNA, the *vir* region being stably integrated into the bacterial chromosome.
Claim 3 A process for the transfer of recombinant DNA into the cells or protoplasts of plants selected from the group consisting of dicotyledonous plants and monocotyledonous plants of the families Liliaceae and Amaryllidaceae, said process comprising infecting the plants or the plant cells, or incubating protoplasts from the plants, with *Agrobacterium* bacteria which contain in their genetic material (i) a *vir* region from the Ti-plasmid of *Agrobacterium*, and (ii) at least one T-region, wherein said T-region comprises said recombinant DNA flanked on both sides by border sequences as present in wild type of *Agrobacterium*, and wherein said *vir* region is integrated into the chromosome of said *Agrobacterium* bacteria prior to said infecting or incubating step.
Claim 5 A process for the transfer of recombinant DNA into the cells or protoplasts of plants selected from the group consisting of dicotyledonous plants and monocotyledonous plants of the families Liliaceae and Amaryllidaceae, said process comprising infecting the plants or the plant cells, or incubating protoplasts from the plants, with *Agrobacterium* bacteria which contain in their genetic material (i) a *vir* region from the Ti-plasmid of *Agrobacterium*, and (ii) at least one T-region, wherein said T-region comprises said recombinant DNA flanked on both sides by border sequences as present in wild type of *Agrobacterium*, and wherein said T-region is integrated into the chromosome of said *Agrobacterium* bacteria prior to said infecting or incubating step.

EP 176 112 B1
Claim 1 Process for introducing foreign DNA into the genome of plants by infecting plants or explants thereof or plant protoplasts or by incubating plant protoplasts with *Agrobacterium*, characterized in that Agrobacteria are used, which contain one or more T-regions and a *vir* region from the Ti-plasmid of *Agrobacterium* in their genetic material and/or one or more T-regions comprising an artificial T-region which consists each of any DNA desired, flanked by border sequences as present in the wild type T-region of *Agrobacterium* or sequences being functionally analogous thereto.
Claim 3 Agrobacteria with one or more T-regions and a *vir* region in their DNA's characterized in that the T-region or the *vir* region or both have been introduced into the bacterial chromosome.

Patents granted to Syngenta Mogen B.V.

The following United States and EP patents assigned to **Syngenta Mogen B.V.** are directed to the insertion of specific sequences into a target locus of a plant genome through homologous recombination. The gene of interest and the homologous sequences are part of a recombinant DNA engineered in a T-region of a Ti plasmid. The *vir* region is located in a different plasmid within *Agrobacterium*.

Bibliographic data

	US 5 501 967	EP 436 007 B1
Title	Process for the site-directed integration of DNA into the genome of plants	
Application No. & Filing Date	US 87928 July 6, 1993	EP 90911630 July 26, 1990
Issue Date	March 26, 1996	March 26, 1997
Language	English	English (Claims in English, German and French)
Remarks		Patent lapsed in Austria, Switzerland, Liechtenstein, Luxemburg, and Sweden.
	View Claims in Plain English View Actual Granted Claims	View Claims in Plain English View Actual Granted Claims

To view or download the patents as a PDF file, click on US 5 501 967 (2,664 kb) and EP 436 007 B1 (2,418 kb).

Summary of the invention

The patents **US 5 501 967** and **EP 436 007 B1** claim

- the introduction of a defined sequence or a desired mutation into a target locus in a plant genome;
- a recombinant DNA having the defined mutation or sequence in a plant transformation vector within *Agrobacterium* . The recombinant DNA also contains:

  (i) at least one T-border, and
  (ii) a sequence homologous to a sequence in the plant target locus.
  The sequence to be inserted in the target locus is located between the homologous sequences in the vector. These sequences are in the same orientation but they are rearranged with respect to the orientation of the homologous sequence in the target locus.

Patents on modified basic binary vectors and methods
Patents granted to to Syngenta Mogen B.V.

Claims in plain English

Disclaimer
THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

US 5 501 967
Claim 1 Process for introducing a defined sequence, at least part of a recombinant DNA, into a target locus of a plant genome by homologous recombination. The process comprises the steps of: A) co-incubating plant cells with *Agrobacterium*, which is capable of T-region transfer and has a vector with a defined sequence, where: (i) one of T-borders may be absent; (ii) a DNA sequence homologous to sequence in target locus is long enough to promote recombination; and B) identifying plant cells with the defined sequence in target locus.

Claim 4

Process for the introducing a defined mutation into a target locus of plant genome. The recombinant DNA is basically the same as that described in Claim 1, but the region having a homologous sequence to the target locus also has a defined mutation. Identification of plant cells with the defined mutation in target locus is part of the process.

Claim 9

A recombinant vector capable of introducing a defined mutation in a target plant locus. The vector has:
(i) T-borders,
(ii) DNA sequences homologous to a target plant locus in the same orientation but rearranged with respect to homologous sequences in target locus, and
(iii) a defined mutation between homologous sequences.

EP 436 007 B1

Claim 1

Process for introducing a defined mutation into a target locus of a plant genome by homologous recombination.
The process comprises the steps of:

A) integrating at least part of the vector that has:
(i) one or two T-borders, and
(ii) a DNA sequence homologous to a sequence in target locus that is long enough to promote recombination B) identifying transformant plant cells with defined mutation in target locus.

Claim 8

A recombinant DNA capable of integrating part of itself (DNA *i*) in a target plant locus by homologous recombination. The recombinant DNA has:
(i) one or two T-borders,
(ii) DNA sequences homologous to target plant locus in the same orientation but rearranged with respect to homologous sequences in target locus, and
(iii) a DNA sequence *i* between homologous sequences that is inserted in the target locus.

Actual granted claims

US 5 501 967

Claim 1

A process for introducing a defined DNA sequence in a selected target locus of a nuclear plant cell genome by integrating at least a part of a recombinant DNA into said genome through homologous recombination at the target locus, comprising the steps of:

A) co-incubating plant protoplasts or plant cells under transforming conditions, with a strain of the genus *Agrobacterium* capable of T-region transfer and containing a plant transformation vector which comprises recombinant DNA of the formula [Figure]

- wherein in boxes 1 and 7 are T-DNA border sequences, and wherein one of box 1 or 7 may be absent; and
- wherein box 3 comprises a DNA sequence sufficiently homologous to a DNA sequence inside the target locus and sufficiently long to promote homologous recombination; and
- wherein box 4 comprises a DNA sequence not homologous to sequences occurring in the target locus; and
- wherein the lines connecting the boxes shown may comprise any number of nucleotides or base pairs, and

B) identifying protoplasts or cells that have obtained the said defined DNA sequence in their genomes at said target locus.

Claim 4

A process for introducing a defined mutation in a selected target locus of a nuclear plant cell genome by integrating at least a part of a recombinant DNA into said genome through homologous recombination at the target locus, comprising the steps of:

A) co-incubating plant protoplasts or plant cells under transforming conditions, with a strain of the genus *Agrobacterium* capable of T-region transfer and containing a plant transformation vector which comprises recombinant DNA of the formula [Figure]

- wherein in boxes 1 and 7 are T-DNA border sequences, and wherein one of box 1 or 7 may be absent; and
- wherein box 3 comprises a DNA sequence sufficiently homologous to a DNA sequence inside the target locus and sufficiently long to promote homologous recombination; and
- wherein box 4 comprises a DNA sequence sufficiently homologous to promote homologous recombination with a corresponding sequence in the target locus and wherein box (4) comprises the said defined mutation; and
- wherein the lines connecting the boxes shown may comprise any number of nucleotides or base pairs, and

B) identifying protoplasts or cells that have obtained the said defined mu tation in their genome at said target locus.

Claim 9

A recombinant DNA capable of introducing a defined mutation in a selected target locus of a nuclear plant cell genome through homologous recombination at the target locus, which recombinant DNA has the formula [Figure]

- wherein box I comprises the said defined mutation; and
- wherein boxes 1 and 7 are T-DNA border sequences, and wherein one of box 1 or 7 may be absent; and
- wherein boxes 3 and 4 individually is a DNA sequence sufficiently homologous to a DNA sequence inside the target locus and sufficiently long to promote homologous recombination; and
- wherein the DNA sequences of the boxes 3 and 4 have the same 5íto 3í orientation, but wherein said boxes 3 and 4 are rearranged with respect to the homologous sequences in the target locus.

EP 436 007 B1

Claim 1

A method for introducing a defined mutation in a selected target locus of a nuclear plant genome by:

A) integrating at least a part of a recombinant DNA into said genome through homologous recombination at the target locus, which the recombinant DNA has the following general structure, [Figure]

- in which the boxes 1 and 7 and the connecting lines represent DNA sequences that are capable of functioning as a T-DNA border in the DNA transfer process,
- in which box 1 or 7 may be absent but not both,
- in which box 3 comprises a DNA sequence which is sufficiently homologous to a DNA sequence inside the target locus and sufficiently long to promote homologous recombination,
- in which box 4 represents a DNA sequence that is not homologous to sequences occurring in the target locus, and
- in which the lines connecting the boxes may represent any number of base pairs, or no base pairs, and

B) identifying transformants having the defined mutation in said target locus using known methods.

Claim 8

A recombinant DNA capable of integrating a part of itself into the genome of a plant host via homologous recombination at the target locus, [Figure]

- in which the boxes 1 and 7 represent DNA sequences that are capable of functioning as a T-DNA border in the DNA transfer process,
- in which box 1 or 7 may be absent but not both,
- in which both box 3 and 4 represent DNA sequences which are sufficiently homologous and sufficiently long to promote homologous recombination with the target locus,
- in which the DNA sequences within the boxes have the same 5' to 3' orientation as in the target locus, but the order of the boxes themselves have been changed with respect to the situation in the target locus, resulting in insertion of box *i* in the target locus after homologous recombination,
- in which the lines connecting the boxes may represent any number of basepairs, or no basepairs.

Patents granted to Cornell

The inventions disclose a vector based on a bacterial artificial chromosome (BAC) designed for *Agrobacterium* -mediated plant transformation.

Some of the features of the invention are:

- the vector permits the maintenance of plasmids in *E. coli* as a single copy,
- the plasmids can be transferred from *E. coli* to *Agrobacterium* by electroporation, and
- *Agrobacterium* used for plant transformation contains the BiBAC vector having a heterologous gene and a helper plasmid to facilitate the transfer of the foreign gene into the plant (a binary vector system).

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 5733744 • Filed - 13 January 1995 • Granted - 31 March 1998 • Expected expiry - 30 March 2015	**Title** - Binary BAC vector Claim 1 A vector for transferring heterologous DNA into a plant cell, said vector comprising: • a backbone which includes a first origin of replication for maintaining heterologous DNA as a single copy in an Escherichia coli host cell, and which further includes a second origin of replication for maintaining heterologous DNA as a single copy in an *Agrobacterium tumefaciens* host cell; • a unique restriction endonuclease cleavage site for insertion of heterologous DNA; and • left and right *Agrobacterium* T-DNA border sequences flanking said unique restriction endonuclease cleavage site, said left and right T-DNA border sequences allowing introduction of heterologous DNA located between said left and right T-DNA border sequences into a plant cell. The United States patent 5733744 claims elements of a vector for transferring heterologous DNA into a plant. The elements are: • two different origins of replication, one for *Agrobacterium* and the other	Cornell

one for *E. coli*, each maintaining a plasmid as a single copy;

- a unique restriction site where the heterologous DNA is inserted; and
- right and left T-borders flanking the heterologous DNA.

US 5977439

- Filed - 22 December 1997
- Granted - 2 November 1999
- Expected expiry - 21 December 2017

Title - Binary BAC vector

Claim 1

A method of making a transgenic plant transformed with a vector comprising:

(1) providing a vector comprising: a backbone which includes a first origin of replication capable of maintaining heterologous DNA as a single copy in an *Escherichia coli* host cell, and which further includes a second origin of replication capable of maintaining heterologous DNA as a single copy in an *Agrobacterium tumefaciens* host cell, a unique restriction endonuclease cleavage site for insertion of heterologous DNA, a heterologous DNA encoding a gene product inserted into the unique restriction site, and left and right *Agrobacterium* T-DNA border sequences flanking said unique restriction endonuclease cleavage site, said left and right T-DNA border sequences allowing introduction of heterologous DNA located between said left and right T-DNA border sequences into a plant cell;
(2) introducing said vector into plant cells; and
(3) propagating plants from said plant cells.

Claim 17

A method of introducing heterologous DNA into a plant cell comprising:

(1) providing a vector comprising: a backbone which includes a first origin of replication capable of maintaining heterologous DNA as a single copy in an *Escherichia coli* host cell, and which further includes a second origin of replication capable of maintaining heterologous DNA as a single copy in an *Agrobacterium tumefaciens* host cell, a unique restriction endonuclease cleavage site for insertion of heterologous DNA, a heterologous DNA encoding a gene product inserted into the unique restriction endonuclease cleavage site, and left and right *Agrobacterium* T-DNA border sequences flanking said unique restriction endonuclease cleavage site, said left and right T-DNA border sequences allowing introduction of heterologous DNA located between said left and right T-DNA border sequences into a plant cell; and
(2) introducing said vector into plant cells.

Methods for transforming a plant with the vector and obtaining transformed plants are claimed in the United States patent 5 977 439.

EP 805851

- Filed - 11 January 1996
- Granted - 26 May 2004
- Expected expiry - 10 January 2016

Title - Binary BAC vector

Claim 1

A vector for transferring heterologous DNA into a plant cell, said vector comprising:

- a backbone which includes a first origin of replication capable of maintaining heterologous DNA as a single copy in an *Escherichia coli* host cell, and which further includes a second origin of replication capable of maintaining heterologous DNA as a single copy in an *Agrobacterium tumefaciens* host cell;
- a unique restriction endonuclease cleavage site for insertion of heterologous DNA; and

- left and right *Agrobacterium* T-DNA border sequences flanking said unique restriction endonuclease cleavage site, said left and right T-DNA border sequences allowing introduction of heterologous DNA located between said left and right T-DNA border sequences into a plant cell.

Designated contracting States at the time of grant are: Austria (patent ceased as reported by EPO), Belgium (patent lapsed as reported by EPO), Switzerland (patent ceased as reported by INPADOC), Germany, Denmark (patent lapsed as reported by EPO), Spain (patent lapsed as reported by EPO), France, United Kingdom, Greece (patent lapsed as reported by EPO), Ireland (patent lapsed as reported by INPADOC), Italy, Liechtenstein (patent lapsed as reported by INPADOC), Luxembourg, Monaco (patent lapsed as reported by EPO), Netherlands (patent lapsed as reported by INPADOC), Portugal, Sweden (patent lapsed as reported by INPADOC).

EP 805851 A1 • Filed - 11 January 1996 • Granted as EP 805851 (see above)	**Title** - Binary BAC vector **Claim 1** A vector for transferring heterologous DNA into a plant cell, said vector comprising: • a backbone which includes a first origin of replication capable of maintaining heterologous DNA as a single copy in an *Escherichia coli* host cell, and which further includes a second origin of replication capable of maintaining heterologous DNA as a single copy in an *Agrobacterium tumefaciens* host cell; • a unique restriction endonuclease cleavage site for insertion of heterologous DNA; and • left and right *Agrobacterium* T-DNA border sequences flanking said unique restriction endonuclease cleavage site, said left and right T-DNA border sequences allowing introduction of heterologous DNA located between said left and right T-DNA border sequences into a plant cell.
Remarks	National phase entry of WO 1996/21725 in Japan (JP H11/500306) is pending.

Note: Patent information on this page was last updated on 24 February 2006.

Granted patent and patent applications filed by Pioneer Hi-Bred

The newly granted United States patent discloses a co-transformation method in which an *Agrobacterium* strain has two binary vectors, each of them carrying a T-DNA region with a different heterologous sequence, and a helper plasmid. A plant scorable marker used for the selection of the transformed plant tissue is one of the heterologous sequences that can be present in the T-DNA region. The heterologous regions integrate into the genome of a plant in an unlinked manner facilitating the retention of the genes of interest and the elimination of undesirable genes in the progeny.

The PCT application, converted into a European application, and its related Australian application are also directed to the same topic.

Bibliographic data

	US 6 265 638	WO 0018939* †
Title	Method of plant transformation	

Application No. & Filing Date	US 407574 A September 28, 1999	WO99US22379 September 28, 1999
Issue Date	July 24, 2001	September 14, 2000 (Publication date)
Language	English	
Remarks	† The present PCT application was converted to the European application **EP 1 117 816 A1** published on **July 25, 2001**. The related Australian patent application **AU 61648/99 A1** * was published on **April 17, 2000**.	
	View Claims	

* The PCT application was filed under the Patent Cooperation Treaty (PCT) with the World Intellectual Property Organization (WIPO). It is important to remember that applications **are not issued patents** and the claims as presented have not been approved by any country. Thus, they are non-binding.

To view or download the patent and the patent application as a PDF file, click on US 6 265 638 (2,142 kb) and WO 0018939 (EP 1 117 816 A1) (2,430 kb).

Summary of the invention

The present United States patent claims:

- an *Agrobacterium* strain having at least two binary vectors with T-DNA regions containing a heterologous gene each, and a helper plasmid. One of the heterologous genes is a scorable marker; and
- a method to co-transform a plant tissue by co-cultivating the tissue with an *Agrobacterium* strain as described above, where the scorable marker is used to screen for the transformed tissue. A transformed plant is regenerated from the selected transformed tissue.

The PCT application, now EP application **EP 1 117 816 A1** and the Australian application recite the same invention in their claims as the granted United States patent.

Patents on modified binary vectors and methods
Granted patent and patent applications filed by Pioneer Hi-Bred Int.

Claims in plain English

Disclaimer
THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

US 6 265 638 & EP 1 117 816 A1*
Claim 1 An *Agrobacterium* strain having (i) a helper plasmid, and (ii) two binary vectors each one with a T-DNA region having a sequence of interest.
Claim 12 Co-transformation of a plant with at least two genes of interest by A) contacting plant tissue with *Agrobacterium* having (i) a helper plasmid, and (ii) two binary vectors each having a T-DNA region containing a gene of interest, with one of the genes being a plant scorable marker gene; B) co-cultivating seed with *Agrobacterium*; C) culturing the tissue in a medium with an antibiotic that works against *Agrobacterium*; D) screening the tissue for expression of the plant marker gene and regenerating the plants expressing that gene.

Actual granted (and pending) claims

US 6 265 638 & EP 1 117 816 A1*
Claim 1 An *Agrobacterium* strain comprising a helper plasmid and at least two binary vector plasmids that are stably inherited, wherein each of said binary vector plasmids comprises at least one T-DNA region comprising a heterologous nucleotide sequence of interest.
Claim 12 A method for co-transformation of a plant with at least two unique heterologous nucleotide sequences of interest, said method comprising the steps of: A) contacting a tissue from said plant with an *Agrobacterium* strain comprising a helper plasmid and said least two binary vector plasmids that are stably inherited, wherein each of said the binary vector plasmids comprises at least one T-DNA region, wherein each of said T-DNA regions comprises one of said unique nucleotide sequences, wherein at least one of said nucleotide sequences comprises at least an expression cassette comprising a plant scorable marker gene; B) co-cultivating seed tissue with said *Agrobacterium*; C) culturing the tissue in a culture medium comprising an antibiotic capable of inhibiting the growth of *Agrobacterium*; D) screening the tissue for expression of plant scorable marker gene; and E) regenerating the transformed plant from tissue that expresses said plant scorable marker gene.

* The PCT application **WO 0018939** was converted to the EP application **EP 1 117 816 A1**. The independent claims as filed of the Australian application **AU 61648/99 A1** are identical to the claims of related granted United States patent and European application.

Co-integrated vectors

Overview

In general, these vectors are constructed by recombining an *Agrobacterium* Ti plasmid lacking tumor-causing genes ("disarmed" Ti plasmid) and a small vector plasmid, which is engineered to carry a gene of interest between a right and a left T-DNA border of the T-DNA region (engineered or modified T-DNA region). Recombination takes place through a single crossover event in a homologous region present in both plasmids.

Although co-integrated vectors have become less popular in recent years due to some difficulties encountered in engineering them, they are still used to a certain extent when modified, for example, to allow site-specific recombination of the plasmids within *Agrobacterium* .

The granted patents and patent applications directed to methods and components for the assembling of co-integrated vectors presented under this section are divided as follows:

- Patents on basic vectors and methods . This group encompasses granted patents and patent applications directed to the basic forms of co-integrated vectors, including the primary elements of the plasmids that recombine and basic methods for their assembling.
- Patents on modified vectors and methods . This group includes granted patents and patent applications directed to improved co-integrated vectors and methods for their use.

Summary of patents on basic co-integrated vectors and methods

Max-Planck Society and **Monsanto Company** have both been granted basic patents on co-integrated vectors. The jurisdictions where these entities have filed applications and been awarded patents are:

Max-Planck	Monsanto
Granted patents	
Europe Australia Japan	Europe (2) Australia Russia

Pending applications	
Europe (2) Israel	Japan

A co-integrated plasmid is the product of homologous recombination through a single crossover between a small plasmid of bacterial origin and an *Agrobacterium* Ti plasmid. In the inventions filed by both entities, the resulting co-integrated plasmid or hybrid Ti plasmid contains at least:

- a gene of interest between the left and right T-DNA border sequences, and
- a *vir*
  region, which allows the transfer of the gene of interest located between the two border sequences into the plant genome.

Both entities claim two plasmids involved in the assembling of a co-integrated plasmid:

- a vector molecule containing a gene of interest to be transferred into a plant, called an intermediate cloning vector by **Max-Plank** and a first plasmid by **Monsanto**, and
- a Ti plasmid containing the *vir* region and free of tumor-inducing genes, which is called an acceptor Ti plasmid by **Max-Plank** and a Ti plasmid by **Monsanto**.

In addition, they both claim a homologous region present in both plasmid types through which the recombination of the plasmids takes place to form a co-integrated vector. The main difference lies in the source of the homologous DNA sequence:

- **Max-Planck**
  claims a homologous region derived from a small vector molecule of bacterial origin different from *Agrobacterium* .
- **Monsanto**
  claims a homologous region derived from the external and attached left side to the left T-DNA border of *Agrobacterium* Ti plasmid, called left inside homology (LIH) region.

In conclusion

- the most limiting factor under these patents is the use of a co-integrated plasmid with a homologous region derived either

  1. from a vector molecule, i.e. *E. coli*-derived vector, or
  2. from a LIH region of a Ti-plasmid.

Table of patents assigned to Max-Planck and Monsanto

The following table presents an overview of nine patent and patent applications that claim components and methods of using basic co-integrated vectors for gene transfer into plants via *Agrobacterium*. This analysis is based only upon the granted patents and published patent applications.

Notably, neither **Max-Planck** nor **Monsanto** have a patent in the United States Until very recently the United States did not publish patent applications and the application process is secret. Many rumors abound about possible interferences in the United States between **Max-Planck** and **Monsanto**. An interference is a procedure in which the inventor who is earliest-in-time is determined. That inventor is then awarded the patent. The interference process can be extremely lengthy. Thus, there may yet be a patent issuing in the United States

Assigned to Max-Planck Society	
Issued Patents * Overview	**Patent Applications Overview**
EP 116 718 B2 Combinations of acceptor Ti plasmids and intermediate cloning vectors for the introduction	**EP 290 799 A2** Oncogenic-free recombinant plant DNA genome containing an integrated foreign gene obtained by

of gene(s) of interest into the plant genome, and hybrid plasmids resulting from such combinations. **AU-B-546 542** Different acceptor Ti plasmids and intermediate vectors as well as elements of a hybrid Ti-plasmid. More detailed information on these patents.	infecting a plant cell with an *Agrobacterium* having a hybrid Ti-plasmid. **EP 320 500 A2** Elements of a non-oncogenic Ti plasmid and an intermediate cloning vector having as a distinctive element at least a right border sequence of a wild-type T-region of a Ti plasmid associated with a gene of interest. More detailed information on these patent applications.
Assigned to Monsanto Company	

Issued Patents Overview
EP 131 620 B1 Method for transforming plant cells by contacting the cells with *A. tumefaciens* having a co-integrated plasmid with a disarmed T-DNA. **AU-B-559 562** Method of creating transformed plant cells by insertion of a co-integrated plasmid formed by a single crossover event between a first plasmid and a Ti plasmid. Elements of a region of the co-integrated plasmid. **EP 131 624 B1** Elements of a co-integrated plasmid for transforming plant cells produced by a single crossover recombination event between a chimeric plasmid and a Ti plasmid. More detailed information on these patents.

* Japanese patent not analyzed.

Patents granted to Max-Planck

Two patents granted to the **Max-Planck Society** (Germany) related to basic forms of co-integrated vectors have issued in Europe (EP) and in Australia (AU).

In general, the claims of both patents are directed to the same invention:

- hybrid plasmids that are the product of co-integration, through a single crossover event, of acceptor Ti plasmids and intermediate cloning vectors.

The difference between the two patents lies in the way the claims are written. In the European patent, the plasmids are claimed as vector combinations. In the Australian patent, each vector that is part of the co-integration process is independently claimed. Yet, both of the patents have similar claim scope.

Bibliographic data

	EP 116 718 B2	AU-B-546 542
Title	Process for the production of expressible genes into plant cell genomes and *Agrobacterium* strains carrying hybrid Ti plasmid vectors useful for this process.	Introduction of expressible genes into plant genomes and *Agrobacterium* strains carrying hybrid Ti plasmid.
Application No. & Filing Date	EP 83112985 December 22, 1983	AU 84232/74 January 13, 1984
Issue Date	August 5, 1996	September 5, 1985
Language	English (Claims in English, German and French)	English
	View Claims in Plain English View Actual Granted Claims	View Claims in Plain English View Actual Granted Claims

To view or download the patent as a PDF file, click on EP 116 718 B2 (2,223 kb).

Summary of the invention

The invention claimed in patent **EP 116 718 B2** relates to vector combinations consisting of acceptor Ti plasmids and intermediate cloning vectors that result in the formation of a hybrid Ti plasmid. The co-integration of both plasmid types is achieved through a single crossover event. The homologous region derives from a cloning vector and is present in both the acceptor plasmid and the intermediate cloning vector. The resultant hybrid plasmid contains at least:

- a gene of interest under the control of a promoter, located between two T-DNA borders; and
- a DNA sequence from Ti plasmid that is essential for T-DNA transfer into the plant genome.

The patent **AU-B-546 542** claims acceptor Ti plasmids and intermediate cloning vector molecules that through a single crossover event form a co-integrated or hybrid Ti plasmid. The hybrid plasmid contains a gene of interest located between two border sequences.

The resulting hybrid plasmid claimed in both of the patents is incapable of inducing tumors in plants transformed via *Agrobacterium*.

Patents on basic co-integrated vectors and methods
Patents granted to Max-Planck

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
EP 116718 B2 • Earliest priority - 13 January 1983 • Filed - 22 December 1983 • Granted - 8 May 1996 • Expected expiry - 21 December 2003	**Title** - Process for the introduction of expressible genes into plant cell genomes and agrobacterium strains carrying hybrid Ti plasmid vectors useful for this process **Claim 1** A vector combination **consisting** of: A) an acceptor Ti plasmid which is substantially free of internal T-DNA sequences of wild-type Ti plasmid and incapable of inducing tumors in plants comprising: (i) the two border sequences of the T-region of the wild-type Ti plasmid; (ii) a DNA sequence derived from a cloning vehicle, located between the two border sequences; and (iii) a DNA segment of a wild type Ti plasmid containing DNA sequences essential for the transfer by *Agrobacterium* of the T-region of wild-type Ti plasmids into plant cell genomes, and B) an intermediate cloning vector, said cloning vector comprising: (i) at least one gene of interest under the control of a promoter capable of directing gene expression in plants; and (ii) a cloning vehicle segment containing a DNA sequence which is homologous with the DNA sequence (ii) in said acceptor Ti plasmid permitting a single crossover event. **Claim 5** A vector combination **consisting** of: A) an acceptor Ti plasmid which is incapable of inducing tumors in plants being free of border sequences and	Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.

internal T-DNA sequences of a wild-type Ti plasmid, comprising:
(i) a DNA segment of a wild-type Ti plasmid without the T-region and without the two border sequences of the T-region; and
(ii) a DNA sequence derived from a cloning vehicle, and

B) an intermediate cloning vector comprising:
(i) a cloning vehicle segment containing the two border sequences of the T-region of a wild-type Ti plasmid; and
(ii) a DNA sequence located between said two border sequences which is homologous with the DNA sequence (ii) in said acceptor Ti plasmid permitting a single crossover event, wherein the region between said border sequences is substantially free of internal T-DNA sequences of a wild-type Ti plasmid; and
(iii) at least one gene of interest under the control of a promoter capable of directing gene expression in plants located between the two border sequences in a manner allowing its integration into the plant genome.

Claim 15

A hybrid Ti plasmid vector obtained by cointegration *either* between

A) an acceptor Ti plasmid which is incapable of inducing tumors in plants comprising:
(i) the two border sequences of the T-region of the wild-type Ti plasmid;
(ii) a DNA sequence devoid of oncogenic internal T-DNA regions of the wild-type T-DNA, derived from a cloning vehicle, located between the two border sequences, and containing a DNA sequence which is homologous with at least a part of a DNA sequence in an intermediate cloning vector permitting a single crossover event; and
(iii) a DNA segment of the wild-type Ti plasmid containing DNA sequences essential for the transfer by *Agrobacterium* of the T-region of wild-type Ti plasmids into plant cell genomes; and

B) an intermediate cloning vector comprising
(i) at least one gene of interest under the control of a promoter capable of directing gene expression in plants; and
(ii) a cloning vehicle segment containing a DNA sequence which is homologous with the above DNA sequence (ii) in the acceptor Ti plasmid; *or* between
I) an acceptor Ti plasmid comprising:
(a) a DNA segment of the wild-type Ti plasmid without the T-region and without the two border sequences of the T-region; and
(b) a DNA sequence derived from a cloning vehicle; and
II) an intermediate cloning vector comprising:
(a) a cloning vehicle segment containing the two border sequences of the T-region of a wild-type Ti plasmid and a DNA sequence located between said two border sequences which is homologous with the above DNA sequence (b) in the acceptor Ti plasmid permitting a single crossover event, wherein the region between said border sequences is substantially free of internal T-DNA sequences of a wild-type Ti plasmid; and

(b) at least one gene of interest under the control of a promoter capable of directing gene expression in plants located between the two border sequences in a manner allowing its integration into the plant genome; III) said hybrid Ti plasmid vector comprising at least:
(1) the two border sequences in the T-region of a wild-type Ti plasmid;
(2) non-oncogenic DNA sequences derived from a cloning vehicle;
(3) a DNA segment of the wild-type Ti plasmid containing DNA sequences essential for the transfer of the T-region of wild type Ti plasmid by *Agrobacterium* into plant cell genomes; and
(4) at least one gene of interest under the control of a promoter capable of directing gene expression in plants which is located between the two border sequences.

Designated contracting States at the time of grant are: Austria, Belgium (patent lapsed as reported on INPADOC), Switzerland (patent lapsed as reported on INPADOC), Germany, France, United Kingdom (patent lapsed as reported on INPADOC), Italy, Liechtenstein, Luxembourg, Netherlands (patent lapsed as reported on INPADOC), Sweden (patent lapsed as reported on INPADOC).

The invention claimed in patent EP 116 718 B2 relates to vector combinations consisting of acceptor Ti plasmids and intermediate cloning vectors that result in the formation of a hybrid Ti plasmid. The hybrid plasmid is also claimed.

In certain claims, the region between border sequences is said to be substantially free of internal T-DNA sequences of a wild type Ti plasmid. How free "substantially free" is, is not defined in the disclosure. Further, the claims do not exclude the presence of sequences from a mutated Ti plasmid.

The co-integration of both plasmid types is achieved through a single crossover event. The homologous region derives from a cloning vector and is present in both the acceptor plasmid and the intermediate cloning vector.

The resultant hybrid plasmid contains at least:

- a gene of interest under the control of a promoter, located between two T-DNA borders which must be from a wild-type Ti plasmid; and
- a DNA sequence from Ti plasmid that is essential for T-DNA transfer into the plant genome.

AU 546542 B

- Earliest priority - 13 January 1983
- Filed - 13 January 1984

Title - Introduction of exressible genes into plant genomes and Agrobacterium strains carrying hybrid Ti plasmid

Claim 1

Acceptor Ti plasmid comprising: (i) two border sequences of the T-region of the wild-type Ti plasmid; and
(ii) a non-oncogenic DNA segment derived from a

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- Granted - 5 September 1985
- Patent expired - 12 August 2004

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cloning vehicle located between the two border sequences containing a DNA sequence which is homologous with at least a part of a DNA in an intermediate cloning vector permitting a single crossover event; and
(iii) a segment of the wild type Ti plasmid containing DNA sequences essential for the transfer by *Agrobacterium* of the T-region of wild-type Ti plasmids into plant cell genomes.

Claim 2

Acceptor Ti plasmid comprising: (i) a DNA segment of a wild-type Ti plasmid without the T-region and without the two border sequences of the T-region; and
(ii) a DNA sequence derived from a cloning vehicle which is homologous with a DNA sequence of an intermediate cloning vector which contains the two border sequences of the T-region of the wild-type Ti plasmid.

Claim 9

Hybrid Ti plasmid vector comprising: (i) the two border sequences of the T-region of the wild-type Ti plasmid;
(ii) non-oncogenic DNA segments derived from a cloning vehicle;
(iii) a segment of the wild-type Ti plasmid containing DNA sequences essential for the transfer by *Agrobacterium* of the T-region of wild-type Ti plasmids into plant cell genomes; and
(iv) at least one gene of interest which is located between the two border sequences.

The patent **AU-B-546 542** claims acceptor Ti plasmids and intermediate cloning vector molecules that through a single crossover event form a co-integrated or hybrid Ti plasmid. The hybrid plasmid contains a gene of interest located between two border sequences.

The resulting hybrid plasmid disclosed in both of the patents is incapable of inducing tumors in plants transformed via *Agrobacterium;* however, the "comprising" language used in the claims doesn't exclude the addition of oncogenic sequences.

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EP 290799 B1

- Earliest priority - 13 January 1983
- Filed - 22 December 1983
- Granted - 26 November 2003
- Reprinted (B9) - 1 September 2004

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Title - Transgenic dicotyledonous plant cells and plants

Claim 1

A cell of a dicotyledonous plant, obtainable by *Agrobacterium* transformation, which contains stably integrated into its genome a foreign DNA which is characterised in that:

(a) it does not contain T-DNA genes that control neoplastic growth and it is substantially free of internal T-DNA sequences of a wild-type Ti-plasmid; and
(b) it comprises at least one gene of interest containing:
(i) a coding sequence; and
(ii) a promoter region that contains a promoter sequence other than the natural promoter sequence of said coding sequence, and wherein said promoter sequence regulates transcription of downstream sequences containing said

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coding sequence to produce an RNA in said cell.

This granted patent is a divisional of now granted **EP 116718** (see above).

Designated contracting States at the time of grant are: Austria, Belgium (patent lapsed as reported on INPADOC), Switzerland (patent lapsed as reported on INPADOC), Germany, France, United Kingdom, Italy, Liechtenstein, Luxembourg, Netherlands (patent lapsed as reported on INPADOC), Sweden (patent lapsed as reported on INPADOC).

- Expected expiry - 21 December 2003

Independent claim 1 of granted **EP 290799 B2** does not claim a co-integrated vector system (see application **EP 290799 A2** below), but a transformed cell of a dicotoledonous plant that:

1. is substantially free of T-DNA from a wild-type Ti plasmid (as above, the definition of term "substantially free" is unclear, and the construct could include T-DNA from a non-wild-type Ti plasmid),
2. contains a gene of interest that has a coding sequence (so might not cover a siRNA-producing sequence), and
3. a promoter that regulates expression of the gene of interest.

EP 320500 B1

- Earliest priority - 13 January 1983
- Filed - 22 December 1983
- Granted - 17 November 2004
- Expected expiry - 21 December 2003

Title - Non-oncogenic Ti plasmid vector system and recombinant DNA molecules for the introduction of expressible genes into plant cell genomes

Claim 1

A cloning vector which comprises:

(a) a cloning vehicle segment (3') containing a left border sequence (1) and a right border sequence (2) of a T-region of a wild-type Ti-plasmid, and
(b) a DNA segment which is located between said border sequences in a manner allowing its integration into a plant genome, wherein said DNA segment does not contain T-DNA genes that control neoplastic growth and wherein said DNA segment is substantially free of internal T-DNA sequences of a wild-type Ti-plasmid, except for promoter sequences, and which contains at least one gene of interest which comprises:
(i) a coding sequence, and
(ii) a promoter region that contains a promoter sequence other than the natural promoter sequence of said coding sequence, and wherein said promoter sequence regulates transcription of downstream sequences containing said coding sequence to produce an RNA in a cell of a plant.

Claim 9

A vector combination **consisting** of
(i) an acceptor Ti plasmid, which is incapable of inducing tumors in plants being free of border sequences and intemal T-DNA sequences of a wild-type Ti plasmid,

comprising:
(a) a DNA segment (4) of a wild-type Ti plasmid without the T-region and without the two border sequences of the T-region; and
(b) a DNA sequence (3) derived from a cloning vehicle, and
(ii) an intermediate cloning vector comprising:
(c) a cloning vehicle segment (3') containing the two border sequences of the T-region of a wild-type Ti plasmid (1; 2) and a DNA sequence located outside of said two border sequences which is homologous with the DNA sequence (b) in said acceptor Ti plasmid permitting a single cross-over event wherein the region between said border sequences is substantially free of internal T-DNA sequences of a wild-type Ti plasmid; and
(d) at least one gene of interest (5) under the control of a promoter capable of directing gene expression in plants located between the two border sequences in a manner allowing its integration into the plant genome.

Claim 16

A hybrid Ti plasmid vector obtained by co-integration between an acceptor Ti plasmid comprising:
(a) a DNA segment (4) of a wild-type Ti plasmid without the T-region and without the two border sequences of the T-region; and
(b) a DNA sequence (3) derived from a cloning vehicle; and an intermediate cloning vector comprising:
(a') a cloning vehicle segment (3') containing the two border sequences of the T-region of a wild-type Ti plasmid (1; 2) and a DNA sequence located outside of said two border sequences which is homologous with the above DNA sequence (b) in the acceptor Ti plasmid permitting a single crossover event, wherein the region between said border sequences is substantially free of internal T-DNA sequences of a wild-type Ti plasmid; and

(b') at least one gene of interest (5) under the control of a promoter capable of directing gene expression in plants located between the two border sequences in a manner allowing its integration into the plant genome;

said hybrid Ti plasmid vector comprising at least:

(å) the two border sequences (1; 2) of the T-region of a wild-type Ti plasmid;

(ß) non-oncogenic DNA sequences (3; 3') derived from a cloning vehicle;

(y) a DNA segment (4) of the wild-type Ti plasmid containing DNA sequences essential for the transfer of the T-region of wild-type Ti plasmids by *Agrobacterium* into plant cell genomes; and

(∂) at least one gene of interest (5) under the control of a promoter capable of directing gene expression in plants which is located between the two border sequences (1; 2).

This granted patent is a divisional of now granted **EP 116718** (see above).

Designated contracting States at the time of grant are: Austria (patent lapsed as reported on INPADOC), Belgium (patent lapsed as reported on INPADOC), Switzerland (patent lapsed as reported on INPADOC), Germany, France, United Kingdom, Italy, Liechtenstein, Luxembourg, Netherlands (patent lapsed as reported on INPADOC), Sweden.

EP 290799 A2

- Earliest priority - 13 January 1983
- Filed - 22 December 1983
- Granted as EP 290799 B1 (see above)

Title - Process for the production of expressible genes into plant cell genomes and *Agrobacterium* strains carrying hybrid Ti plasmid vectors useful for this process

Claim 1

Recombinant plant DNA genome being free of oncogenic internal T-DNA regions of the wild-type Ti plasmid containing an integrated gene of interest foreign to said plant DNA obtainable by infecting a plant cell with an Agrobacterium harboring a hybrid Ti plasmid vector created by homologous recombination between:

A) an acceptor Ti plasmid which is incapable of inducing tumors in plants and comprises:
(i) the two border sequences of the T-region of the wild-type Ti plasmid;
(ii) a DNA segment devoid of oncogenic internal T-DNA regions of the wild-type T-DNA, derived from a cloning vehicle, located between the two border sequences, and containing a DNA sequence "A" which is homologous with at least a part of a DNA sequence in an intermediate cloning vector permitting a single crossover event; and
(iii) a segment of the wild-type Ti plasmid containing DNA sequences essential for the transfer by *Agrobacterium* of the T-region of wild-type Ti plasmid into plant cell genomes, and

B) an intermediate cloning vector which comprises:
(i) at least one gene of interest; and
(ii) a cloning vehicle segment containing a DNA sequence which is homologous to the DNA sequence "A" in said acceptor Ti plasmid,
or a hybrid Ti plasmid vector created by homologous recombination between:
I) an acceptor Ti plasmid which is incapable of inducing tumors in plants and which comprises:
(a) a DNA segment of a wild-type Ti plasmid without the T-region and without the two border sequences of the T-region; and
(b) a DNA sequence derived from a cloning vehicle which is homologous with at least a part of a DNA sequence of an intermediate cloning vector which contains the two border sequences of the T-region of the wild-type Ti plasmid, and II) an intermediate cloning vector which comprises:
(a) a cloning vehicle segment containing the two border sequences of the T-region of the wild-type Ti plasmid and a DNA sequence which is homologous with the DNA sequence (b) in said acceptor Ti plasmid; and
(b) at least one gene of interest located between the two border sequences in a manner allowing its integration into the plant genome; said hybrid Ti plasmid comprising

at least:
1) the two border sequences of the T-region of the wild-type Ti plasmid;
2) non-oncogenic DNA segments derived from a cloning vehicle;
3) a segment of the wild-type Ti plasmid containing DNA sequences essential for the transfer by Agrobacterium of the T-region of wild-type Ti plasmid into plant cell genomes, and
4) at least one gene of interest which is located between the two border sequences.

The European application **EP 290 799 A2** claims a recombinant plant genome obtained after infection of plant cells with an *Agrobacterium* strain having a hybrid Ti plasmid. The recombinant genome contains an exogenous gene of interest and is free of oncogenic sequences. The hybrid plasmid is the product of homologous recombination between an acceptor plasmid and an intermediate cloning vector. The elements of the combined acceptor plasmids and intermediate cloning vectors are set out in Claim 1.

EP 320500 A2

- Earliest priority - 13 January 1983
- Filed - 22 December 1983
- Granted as EP 320500 B1 (see above)

Title - Non-oncogenic Ti plasmid vector system and recombinant DNA molecules for the introduction of expressible genes into plant cell genomes

Claim 1

A non-oncogenic Ti plasmid vector system free of T-DNA genes controlling neoplastic growth of transformed plants which comprises:

(i) DNA sequences coding for functions which are essential for the transfer of the T-region of a wild-type Ti plasmid by Agrobacterium into a plant cell genome; and
(ii) at least one gene of interest which has been inserted into said vector system and which is capable of being expressed in plants which are susceptible to infection by *Agrobacterium*,
said gene being under the control of at least one promoter capable of directing the expression of said gene in the plant and associated with at least the right border sequence of the T-region of a wild-type Ti plasmid, said border sequence allowing the integration of said gene into the plant genome.

Claim 4

An intermediate cloning vector comprising:

(i) a cloning vehicle segment containing the right border sequence of the T-region of a wild-type Ti plasmid and a DNA sequence which is homologous with a DNA sequence in an acceptor Ti plasmid; and
(ii) at least one gene of interest under the control of a promoter capable of directing gene expression in plants which is associated with said border sequence in a manner allowing its integration into the plant genome.

The European application **EP 320 500 A2** claims a

	non-oncogenic Ti plasmid vector containing a gene of interest and its controlling promoter associated with at least the right border of the wild type T-region. The border allows the integration of the gene into the plant genome. Also, the Ti plasmid contains the sequences needed for transfer of T-region into plant genome (claim 1). In another independent claim, a gene of interest and its promoter that is associated with a right T-border are contained in an intermediate cloning vector (claim 4).	
Remarks	1. Related patent of EP 116718 B2 in Canada (CA 1341419) has been granted. 2. Related patent of EP 116718 B2 in Japan and their status are: • JP 1633546 C - granted and expired • JP 2726267 B2 - granted and expired • JP 2769539 B2 (divisional of now granted JP 1633546) - granted and expired • JP (H)03/108478 A (divisional of now granted JP 2769539) - application rejected • JP (H)06/105629 A (divisional of now granted JP 2769539) - application rejected • JP 2001/029092 A (divisional of JP (H)06/105629 A) - application rejected	

Note: Patent information on this page was last updated on 28 February 2006.

Patents granted to Monsanto

Monsanto Company
has been granted three patents related to co-integrated vectors in Europe (EP) and in Australia (AU). These patents are:

- directed to elements of a co-integrated plasmid and methods for transforming plants using co-integrated plasmids (EP 131 620 B1 and AU-B-559 562) and
- directed to the elements of a co-integrated plasmid and the plasmids used to generate the co-integrated (EP 131 624 B1).

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
EP 131620 B1 • Earliest priority - 17 January 1983 • Filed - 16 January 1984 • Granted - 21 August 1991	Title - Genetically transformed plants Claim 1 A method for transforming plant cell which comprises • contacting plant cells, which are susceptible to genetic transformation by *Agrobacterium* cells, with *Agrobacterium tumefaciens* cells containing a co-integrated Ti plasmid comprising a disarmed T-DNA region which comprises in sequence: (i) a left *Agrobacterium* T-DNA border sequence, (ii) a chimeric selectable marker gene which functions in plant cells	Monsanto

comprising: (a) a promoter which functions in plant cells;
(b) a structural coding sequence encoding a neomycin phosphotransferase; and
(c) a 3í non-translated region encoding a polyadenylation signal, and (iii) a right *Agrobacterium* T-DNA border sequence.

- Expected expiry - 16 January 2004

Designated contracting States at the time of grant are: Austria (patent lapsed as reported by INPADOC), Belgium, Switzerland (patent lapsed as reported by INPADOC), Germany, France (patent lapsed as reported by INPADOC), United Kingdom (patent lapsed as reported by INPADOC), Liechtenstein, Luxembourg, Netherlands (patent lapsed as reported by INPADOC), Sweden (patent lapsed as reported by INPADOC)

The patents **EP 131 620 B1** and **AU-B-559 562** are both directed to methods for transforming plant cells. The patent **EP 131 620 B1** claims transformation methods by contacting plant cells with *Agrobacterium* harboring a co-integrated plasmid. The **AU-B-559 562** patent further specifies a series of steps to carry out such transformation.
In addition, both of the patents claim elements of the co-integrated plasmid used in the transformation process. One of the differences between them is that the **EP 131 620 B1** limits the gene to be expressed in plants to a chimeric selectable marker gene that includes:

- a promoter,
- a sequence coding for neomycin phosphotransferase; and
- a polyadenylation signal coding sequence.

AU-B 559562

- Earliest priority - 17 January 1983
- Filed - 16 January 1984
- Granted - 12 March 1987
- Patent expired - 14 August 2003

Title - Genetically transformed plants

Claim 1

A method of creating transformed plant cells, comprising the following steps:

(i) culturing a microorganism containing

- a first plasmid containing:

(a) at least one first gene that is expressed in plant cells and
(b) at least one second gene which serves as a marker in a selected microorganism, and

- a Ti second plasmid, under conditions which allow the first plasmid to recombine with the Ti second plasmid in the microorganism, thereby creating a third plasmid having at least one T-DNA border on each side of the first gene, said gene being located in said first plasmid between a region of homology from the T-region of the Ti plasmid and a T-DNA border;

(ii) selecting those microorganisms containing the third plasmid;
(iii) inserting the third plasmid, or a portion thereof, into plant cells; and
(iv) culturing the plant cells under conditions which allow a segment of DNA from the third plasmid to be inserted into the genome of the plant cells.

Claim 13

A method of transforming plant cells, comprising

- inserting into the plant cells a co-integrated plasmid, or a portion thereof, which was formed by a single crossover event between a first

plasmid and a Ti second plasmid, wherein the co-integrated plasmid contains a region comprising:

(i) a first T-DNA border;
(ii) a gene which expressed in plant cells; and
(iii) a second T-DNA border which is complementary to the first T-DNA border,
wherein the region does not contain any sequences which would render the plant cells incapable of being regenerated into morphologically normal plants.

Remarks on related patents of WO 1984/02920:

- WO 1984/02920 entered national phase in Japan (JP (S)60/500795) and Union of Soviet Socialist Republics (SU 1582990).

EP 131624 B1

- Earliest priority - 17 January 1983
- Filed - 16 January 1984
- Granted - 16 September 1992
- Expected expiry - 15 January 2004

Title - Plasmids for transforming plant cells

Claim1

A co-integrated plasmid for use in transforming plant cells, and produced by recombination by a single crossover event of:

A) a chimeric plasmid comprising a gene which functions in plants to express an encoded polypeptide, said plasmid comprising in sequence:

(i) a region of DNA which is homologous to T-DNA located near the left T-DNA border of a tumor-inducing plasmid of *Agrobacterium* and which is capable of causing in vivo recombination of the chimeric plasmid with said tumor-inducing plasmid of Agrobacterium by a single crossover event;
(ii) a gene comprising a promoter which functions in plant cells, a structural coding sequence and a 3í non-translated region encoding a polyadenylation signal, said promoter and polyadenylation signal being operably linked to said structural coding sequence; and
(iii) an *Agrobacterium* plasmid T-DNA right border sequence which enables the transfer and incorporation of T-DNA into the genome of a plant cell;
said chimeric plasmid containing no plant tumorigenic genes between and including the region (i), the gene (ii) and the border sequence (iii), and

B) a Ti plasmid capable of transferring the T-DNA region into the genome of a plant cell,

such that the co-integrated plasmid comprises a T-DNA region having the following elements in sequence:
1) a left T-DNA border sequence;
2) the gene (ii) of the chimeric plasmid;
3) at least one right T-DNA border sequence;
wherein said T-DNA border sequences enable the transfer of the T-DNA region into the genome of a plant cell, and wherein, between and including the left T-DNA border sequence, the gene (ii) and the right T-DNA border sequence or (if there is more than one right T-DNA border sequence) between the gene (ii) and the right T-DNA border sequence which is nearest to the left T-DNA border sequence, there are no genes which would render a transformed plant cell tumorous or incapable of regeneration into a morphologically normal plant, and there is no duplication of DNA sequences, which duplication could, through homologous recombination, result in loss of the gene (ii).

Designated contracting States at the time of grant are: Austria (patent lapsed as reported by INPADOC), Belgium, Switzerland (patent lapsed as reported by INPADOC), Germany, France (patent lapsed as reported by INPADOC), United

Kingdom (patent lapsed as reported by INPADOC), Liechtenstein, Luxembourg, Netherlands (patent lapsed as reported by INPADOC), Sweden (patent lapsed as reported by INPADOC)

The patent **EP 131 624 B1** claims elements of a chimeric plasmid that produce a co-integrated plasmid by recombination with a Ti plasmid. The chimeric plasmid comprises in order:

1. a left-inside homology (LIH) region, which is naturally located near the left T-DNA border of a Ti plasmid
2. a gene of interest with a promoter and a polyadenylation signal; and
3. a right T-DNA border

The elements present in the T-region of the co-integrated plasmid are also part of the claims.

Remarks on related patents of WO1984/02919:

- WO 1984/02919 entered national phase in Japan (JP (S)60/500438)

Note: Patent information on this page was last updated on 1 March 2006.

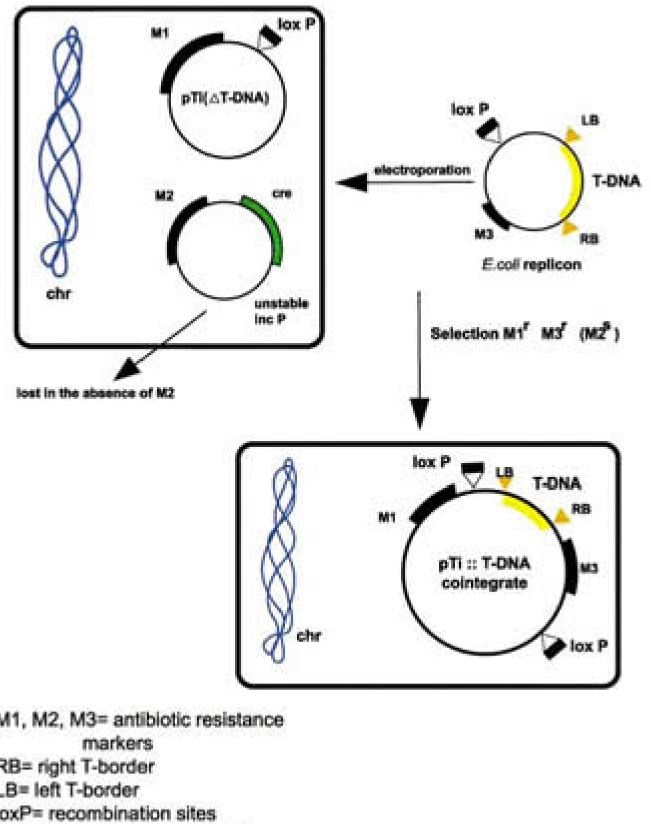
Summary of patents on modified co-integrated vectors and methods

Several entities own inventions built on the basic components of co-integrated vectors and the basic methods to assemble them. Three different inventions are referred to in this section:

- **Stable co-integrated vector**. The invention of **Schilperoort and Hille** in the United States and in Europe discloses a way to combine in *E. coli* a vector molecule (pR772) having a modified T-region with a Ti plasmid (pTiB6). After the recombination of the molecules, the co-integrated plasmid pAL969 is transferred to an *Agrobacterium* strain lacking a native Ti-plasmid. The co-integrated vector and its derivatives are stable in both *E.coli* and *Agrobacterium* and are used for transformation of dicot plants.

  The very narrow scope of this patent will not present undue difficulties for most researchers. Quite simply, by using different plasmids than those claimed, infringement of this patent can likely be avoided.

- **Site-specific co-integrated vector**. The granted United States patent and the European application assigned to **Mogen**, now called **Syngenta Mogen B.V.**, discloses an alternate way to attain homologous recombination in order to form a co-integrated vector. The recombination process is triggered by the action of Cre (*causing recombination*) recombinase on recombination sites (i.e. lox P) present in both a small vector and a disarmed (tumor-genes free) Ti plasmid. The following diagram illustrates this method.



Construction of a site specific co- integrated vector
(Taken from Mozo and Hooykaas 1992)

In conclusion, the most limiting factor in this patent is the presence and use of the Cre recombinase system to form a stable co-integrated plasmid within an *Agrobacterium* strain.

- **Co-transformation with two different T-DNAs** . **Japan Tobacco** has one United States granted patent and a European application directed to this subject matter. This invention discloses an *Agrobacterium* strain containing a first T-DNA, which has a selectable marker, e.g. a drug resistance gene, and a second T-DNA, which has a desired gene or a cloning site and is part of a co-integrated vector. The first T-DNA may or may not be part of the same vector as the second T-DNA. Although when both are in the same co-integrated vector, they are distant enough to be inherited independently.

  Thus, an *Agrobacterium* strain having two different T-DNAs as described above is very likely to fall within the scope of the claimed invention in the United States patent. Note, however, that the second vector used in this invention must be a co-integrated vector.

Table of patents on modified co-integrated vectors

The following table presents an overview of six patents and patent applications that claim **co-integrated vectors** having **variations on their basic elements** .

This analysis is limited by being based only upon the published specification and claims of the issued patents and patent applications.

Assigned to Schilperoort & Hille
Issued Patents Overview
US 4 693 976 Method for incorporating foreign DNA into a dicot genome via *A. tumefaciens* using a stable co-integrated plasmid. The co-integrated plasmid, composed of a Ti-plasmid (pTiB6) and a broad host range plasmid (R772), contains foreign DNA in the T-region of the Ti component of the co-integrated plasmid. The insertion of the new genes into the T-region of the R::Ti co-integrate is achieved by homologous recombination of an engineered *E.coli* vector with a modified T-region and the R::Ti co-integrate.

EP 120 515 B1
Process for incorporating foreign DNA into a dicot genome by infecting plant protoplasts with *A. tumefaciens* containing a plasmid derived from the co-integrated plasmid pAL969. Such plasmid contains only foreign DNA between the 23 base pairs of the wild-type T-region from the Ti plasmid pTiB6.

More detailed information on these patents.

Assigned to Syngenta Mogen B.V.	
Issued Patents Overview	
US 5 635 381 *Agrobacterium* strains having a site-specific recombinase (Cre) capable of causing recombination between two different recombination sites (i.e. loxP site) present in the same strains. The site-specific recombination event between two separated DNA molecules each carrying a recombination site results in the formation of a site-specific co-integrated plasmid. EP 628 082 B1 This recently granted patent (former application **EP 628 082 A1**) is directed to an *Agrobacterium* strain having a gene encoding a recombinase and a sequence capable of controlling its expression. The recombinase causes site-specific recombination between two recombination sites. The scope of the claims as granted is the same as the claims filed in the EP application. More detailed information on these patents.	

Assigned to Japan Tobacco Inc.	
Issued Patents Overview	Patent Applications Overview
US 5 731 179 Method for co-transforming plant cells with two T-DNAs via *Agrobacterium* where the first T-DNA contains a selectable marker, and the second T-DNA has a gene of interest. Either both T-DNAs are located in the same hybrid vector or at least the second T-DNA is in a hybrid vector, which is the product of homologous recombination between an acceptor vector and an intermediate vector. More detailed information on this patent.	EP 687 730 A1 Method for co-transforming plant cells with a first T-DNA containing a drug resistance gene and a second T-DNA containing a desired gene and contained in a hybrid vector prepared by homologous recombination between an acceptor vector and an intermediate vector. A hybrid vector with a first T-DNA as described and a second T-DNA with a cloning site is further claimed. More detailed information on this patent.

Patents granted to Schilperoort & Hille

Two patents granted to **Schilperoort & Hille** are related to transformation of dicotyledonous (dicot) plants using stable co-integrated plasmids. The granted patents were obtained in Europe (EP) and in the United States of America (US). The European patent was assigned to the **University of Leiden** and **Prof. Dr. R. Schilperoort**.

In general, both patents disclose transformation of dicot plant protoplasts by infecting them with an *A. tumefaciens*
strain containing a stable co-integrated plasmid. The co-integrated plasmid results from the combination of the Ti plasmid pTiB6 and the broad host range plasmid R772. In the European patent, the *A. tumefaciens* strain used for plant transformation contains a plasmid derived from the co-integrated plasmid pAL969, which has a T-region from pTiB6 and contains only foreign DNA between the border sequences.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 4693976 • Earliest priority - 24 February	**Title** - Process for the incorporation of foreign DNA into the genome of dicotyledonous plants using stable cointegrate plasmids Claim 1	Schilperoort & Hille

1983 • Filed - 23 February 1984 • Granted - 15 September 1987 • Expected expiry - 14 September 2007	A process for the incorporation of foreign DNA into the genome of dicotyledonous plants comprising • infecting the plants or incubating plant protoplasts with *A. tumefaciens* bacteria, which contain one or more Ti (tumor inducing) plasmids, characterized in that as Ti plasmid a stable co-integrated plasmid composed of the plasmid R772 and the plasmid pTiB6 with foreign DNA incorporated in the T-region of the Ti component of the co-integrated plasmid is applied. Claim 3 A process for the production of *Agrobacterium tumefaciens* bacteria, which contain one or more Ti plasmids, comprising • combining in *Escherichia coli* a vector known per se for use in *E. coli*, provided with T-DNA region in which foreign DNA has been incorporated, with co-integrated plasmid pAL969 and the co-integrated plasmid with foreign DNA incorporated by double crossing-over in the T-region of the Ti component of transferring the co-integrated plasmid to *A. tumefaciens*. Claims of the US patent **4 693 976** recite • the incorporation of foreign DNA into the dicot genome by infecting or incubating plant protoplasts with *A. tumefaciens* having a stable co-integrated plasmid (R772::pTiB6). Such plasmid bears a modified T-region with foreign DNA. The co-integrated is formed within *E. coli* by homologous recombination (double crossing) of R772, a broad host range plasmid, and pTiB6, a Ti plasmid with an engineered T-region. • the process for the production of *A. tumefaciens* bacteria comprising: • 1. combining in *E. coli* a vector having a T-region with foreign DNA and the co-integrated plasmid pAL969; and 2. transferring the recombinant to *A. tumefaciens*.	
EP 120515 B1 • Earliest priority - 24 February 1983 • Filed - 23 February 1984 • Granted - 22 November 1990 • Expected expiry - 22 February 2004	**Title** - A process for the incorporation of foreign DNA into the genome of dicotyledonous plants; a process for the production of *Agrobacterium tumefaciens* bacteria Claim 1 A process for the incorporation of foreign DNA into the genome of dicotyledonous plants comprising • infecting the plants or incubating plant protoplasts with *Agrobacterium tumefaciens* bacteria containing at least one plasmid which is derived from the co-integrated plasmid pAL969 by the incorporation of foreign DNA in the T-region of the component pTiB6 characterized in that the co-integrated plasmid with foreign DNA contains only foreign DNA between the 23 base pairs of the wild-type T-region. Designated contracting States at the time of grant are: Austria, Belgium (patent lapsed as reported by INPADOC), Switzerland (patent lapsed as reported by INPADOC), Germany, France, United Kingdom	University of Leiden and Schilperoort

	(patent lapsed as reported by INPADOC), Italy, Liechtenstein, Luxembourg, Netherlands (patent lapsed as reported by INPADOC), Sweden (patent lapsed as reported by INPADOC) The claims of the patent **EP 120 515** recite: • a process for foreign DNA incorporation into a dicot genome by infecting plant protoplasts with *A. tumefaciens* containing a plasmid derived from the co-integrated plasmid pAL969. The transferred plasmid contains only foreign DNA between the 23 bp ends of the wild-type T-region of the Ti plasmid pTiB6.	
Remarks	Related patents include: • JP 2523468 B2 - granted and expired • JP 2528270 B2 (divisional of now granted JP 2523468 B2) - granted and expired • NL 8300699 A - application lapsed as reported by INPADOC	

Note: Patent information on this page was last updated on 1 March 2006.

Patent granted to Mogen Int. (now Syngenta Mogen B.V.)

The United States patent granted to **Syngenta Mogen B.V.**, is directed to the use of a Cre (**c** ausing **re**combination)-recombinase capable of causing site-specific recombination of two separated DNA molecules present in the same *Agrobacterium* strain. The resulting plasmid is a co-integrated plasmid having a gene of interest and is useful for plant transformation.

The related European patent is similar to the United States patent as both disclose a site-specific recombination system for the generation of a site-specific co-integrated plasmid for plant transformation. In the European patent, however, the recombinase is **not** limited to Cre recombinase.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 5635381 • Earliest priority - 26 February 1992 • Filed - 20 January 1995 • Granted - 3 June 1997 • Expected expiry - 19 January 2015	**Title** - *Agrobacterium* bacteria capable of site-specific recombination **Claim 1** An *Agrobacterium* strain comprising: (i) a structural DNA sequence encoding a site-specific recombinase that is Cre recombinase; and (ii) a DNA sequence linked thereto which operationally controls expression of said Cre recombinase, said strain further comprising (iii) a first recombination site. The patent discloses that a first plasmid bearing a recombination site combines, in a site-specific fashion, with a second plasmid also bearing a recombination site to generate a site-specific co-integrated plasmid. The second plasmid contains a gene of interest linked to a right T-border. The production of the co-integrated plasmid **does not** require homologous recombination. The *Agrobacterium* strains used for plant transformation contain the site-specific co-integrated plasmid with the exogenous DNA to plants. The patent claims an *Agrobacterium* strain with • a DNA sequence encoding Cre recombinase linked to a sequence that controls its expression and	Syngenta Mogen B.V.

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- a first recombination site (which can be on a plasmid or in the genome).

The claim is a bit ambigious in that it doesn't state that the recombination site is necessarily recognized by the Cre recombinase, though the specification discloses that an exemplary site is a loxP seqence.

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<tr><td>

EP 628082 B1

- Earliest priority - 26 February 1992
- Filed - 25 February 1993
- Granted - 16 May 2001
- Expected expiry - 24 February 2013

</td><td>

Title - *Agrobacterium* strains capable of site-specific recombination

Claim 1

An *Agrobacterium* strain capable of producing a site-specific recombinase capable of effecting site-specific recombination of a first and second recombination site in said *Agrobacterium* strain, when present therein, comprising a structural DNA sequence encoding said recombinase and a DNA sequence capable of controlling expression in said *Agrobacterium* strain.

Designated contracting States at the time of grant are: Austria (patent lapsed as reported by INPADOC), Belgium, Switzerland, Germany, Denmark (patent lapsed as reported by INPADOC), Spain, France, United Kingdom, Greece, Ireland (patent lapsed as reported by INPADOC), Italy, Liechtenstein, Luxembourg, Monaco, Netherlands, Portugal, Sweden (patent lapsed as reported by INPADOC).

Both of **Syngenta Mogen's** patents in the United States and in Europe encompass *Agrobacterium* strains having a plasmid with a gene encoding a recombinase, a sequence to control its expression, *vir* functions, and a first recombination site.

Unlike the United States patent, the claims of the European patent are **broader** as the recombinase is not limited to a Cre recombinase. Other site-specific recombinases may be encompassed by the European patent claims. The European patent does explicitly claim that the recombinase can mediate recombination between the first recombination site and a second recombination site but claim 1 does not require that the first or second site be on a plasmid.

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Note: Patent information on this page was last updated on 1 March 2006.

Patent granted to Japan Tobacco Inc.

The United States patent granted to **Japan Tobacco** discloses a method commonly called co-transformation. In this method, two T-DNAs containing genes that encode different products (e.g. gene of interest and selectable marker) are inserted into the plant genome via *Agrobacterium*. The method results in transformed plants having the gene of interest and lacking the selectable marker gene.

Bibliographic data

	US 5 731 179
Title	Method for introducing two T-DNAs into plants and vectors therefor
Application No. & Filing Date	US 500952 August 8, 1995
Issue Date	March 24, 1998
Language	English
	View Claims

To view or download the patent as a PDF file, click on US 5 731 179 (1,889 kb).

Summary of the invention

The patent US 5 731 179 claims

- a method for transforming plants via *Agrobacterium* by inserting two different T-DNAs. The first T-DNA contains a plant selectable marker gene and the second T-DNA contains a gene of interest (claim 1) and a restriction site (claim 16). The second T-DNA is contained in a hybrid vector formed by homologous recombination between an acceptor vector and an intermediate vector;
- a transformed plant with both of the T-DNAs and the subsequent selection of transformed plants in the next generation that contain the desired gene but not contain the selectable marker gene;
- the elements of the hybrid vector, the acceptor vector, and the intermediate vector.

Patents on modified co-integrated vectors and methods
Patent granted to Japan Tobacco

Claims in plain English

Disclaimer
THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

US 5 731 179
Claim 1 Method for transforming a plant with *Agrobacterium* comprising: A) co-transforming a plant with 2 different T-DNAs: • a first T-DNA with a plant selectable gene • a second T-DNA with a desired gene in a hybrid vector, B) obtaining a transformed plant with both the selectable gene and the desired gene C) cultivating the transformed plant and D) selecting a plant in next generation with the desired gene but without the selectable gene. The hybrid plasmid is prepared in A*grobacterium* by homologous recombination between: I) an acceptor plasmid containing: (i) an *ori* region* for *E. coli* and for *Agrobacterium;* (ii) *vir*B and *vir*G genes from plasmid pTiBo542 of *A. tumefaciens*; and (iii) a region "A" homologous to intermediate vector, and II) an intermediate vector containing (i) an *ori* region* only for *E. coli*; (ii) a DNA with at least part of second T-DNA; and (iii) a region homologous to "A".
Claim 16 A hybrid vector comprising: 1) a first T-DNA with a plant selectable marker and 2) a second T-DNA with a restriction site, where both T-DNAs are distant enough from each other to be independently inherited. The hybrid vector is formed in *Agrobacterium* by homologous recombination between an acceptor plasmid and an intermediate vector, both as described in Claim 1.

* an *ori*
region corresponds to a replication origin region that is recognized by either both or only one of the bacteria involved in the process of formation of a hybrid vector.

Actual granted claims

US 5 731 179

Claim 1

A method for transforming and cultivating a plant using a bacterium belonging to the genus *Agrobacterium*, comprising:

A) co-transforming plant cells with a first T-DNA (1) and a second T-DNA (2); and selecting cells based on a selection marker gene;
said first T-DNA (1) containing a selection marker gene which functions in said plant; said second T-DNA (2) containing a desired DNA fragment to be introduced into said plant, the second T-DNA (2) being contained in a hybrid vector;

said hybrid vector being prepared by homologous recombination between an acceptor vector and an intermediate vector in said bacterium belonging to the genus *Agrobacterium*;

- said acceptor vector containing at least:
- (i) a DNA region having a replication origin allowing replication of a plasmid in both a bacterium belonging to the genus *Agrobacterium* and in *Escherichia coli*,
  (ii) a DNA region containing *vir*B gene and *vir*G gene in virulence region of Ti plasmid pTiBo542 of *Agrobacterium tumefaciens*, and
  (iii) a DNA region which is homologous with a part of said intermediate vector, which is subjected to homologous recombination in said bacterium belonging to the genus *Agrobacterium*;
- said intermediate vector containing at least:

(i) a DNA region having a replication origin allowing replication of a plasmid in *Escherichia coli*, which does not function in said bacterium belonging to the genus *Agrobacterium*,
(ii) a DNA region which is homologous with a part of said acceptor vector, which is subjected to homologous recombination in said bacterium belonging to the genus *Agrobacterium*, and
(iii) a DNA region which constitutes at least a part of said second T-DNA; B) obtaining a plant transformed with said selection marker gene and said desired DNA fragment; and
C) cultivating said plant and selecting a plant in the next generation, which contains said desired DNA fragment but does not contain said selection marker gene.

Claim 16

A hybrid vector comprising: 1) a first T-DNA containing a selection marker that functions in a plant, and 2) a second T-DNA having a restriction site; wherein there is sufficient distant in said hybrid vector between said first T-DNA and said second T-DNA to be independently inherited, said hybrid vector being prepared by homologous recombination between an acceptor vector and an intermediate vector in a bacterium belonging to the genus *Agrobacterium*;

- said acceptor vector containing at least:
- (i) a DNA region having a replication origin allowing replication of a plasmid in both a bacterium belonging to the genus *Agrobacterium* and in *Escherichia coli*,
  (ii) a DNA region containing *vir*B gene and *vir*G gene in virulence region of Ti plasmid pTiBo542 of *Agrobacterium tumefaciens*, and
  (iii) a DNA region which is homologous with a part of said intermediate vector, which is subjected to homologous recombination in said bacterium belonging to the genus *Agrobacterium*;
- said intermediate vector containing at least:
- (i) a DNA region having a replication origin allowing replication of a plasmid in *Escherichia coli*, which does not function in said bacterium belonging to the genus *Agrobacterium*,
  (ii) a DNA region which is homologous with a part of said acceptor vector, which is subjected to homologous recombination in said bacterium belonging to the genus *Agrobacterium*, and
  (iii) a DNA region which constitutes at least a part of said second T-DNA.

Patent application filed by Japan Tobacco Inc.

The European application filed by **Japan Tobacco** discloses methods for co-transforming plants with two T-DNAs. One of the T-DNAs has a gene conferring drug-resistance and the other has a gene of interest and is part of a hybrid plasmid. The hybrid plasmid is constructed by homologous recombination of an acceptor and an intermediate plasmid.

Bibliographic data

	EP 687 730 A1
Title	Method of transforming plants and vector therefor
Application No. & Filing Date	EP 95902308 December 6, 1994
Publication Date	December 20, 1995
Language	English (claims in English, French and German)
Remarks	The European application is still pending (European Patent Register Information March 28, 2001).
	View Claims

To view or download the patent application as a PDF file, click on EP 687 730 A1 (2,154 kb).

Summary of the invention

The claims of the application **EP 687 730 A1** recite the transformation of a plant with two different T-DNAs via *Agrobacterium*. The first T-DNA contains a gene conferring drug resistance to the plant and the second one contains a gene of interest. In the method claim (claim 1), the second T-DNA is part of a hybrid vector formed by homologous recombination between an acceptor vector and an intermediate vector.

A hybrid vector (claim 16) harboring the first T-DNA as described and a second T-DNA with a cloning site is also formed by homologous recombination of the vectors mentioned above.

Elements of the acceptor vector and the intermediate vector are also recited in the claims.

Patents on modified co-integrated vectors and methods
Patent application filed by Japan Tobacco

Claims in plain English

Disclaimer
THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

EP 687 730 A1
Claim 1 Method for transforming plant with *Agrobacterium* comprising: A) co-transforming plant cells with two different T-DNAs: (i) a first T-DNA with a drug-resistance gene (ii) a second T-DNA with a desired gene, which is part of a hybrid vector, and B) selecting the drug resistant cells. The hybrid plasmid is prepared in *Agrobacterium* by homologous recombination between: I) an acceptor plasmid having: (i) an *ori* region* for *E. coli* and *Agrobacterium* (ii) *vir*B and *vir*G genes from plasmid pTiBo542 of *A. tumefaciens* (iii) a region of homology "A", and II) an intermediate vector having: (i) an *ori* region* only for *E. coli*; (ii) DNA with at least part of second T-DNA; and (iii) a region homologous to region "A" of the acceptor plasmid.
Claim 16 A hybrid vector comprising: 1) a first T-DNA with a plant drug-resistance marker, 2) a second T-DNA with a restriction site. The hybrid vector is formed in *Agrobacterium* by homologous recombination between an acceptor plasmid and an intermediate vector, both as described in Claim 1.

* an *ori*

region corresponds to a replication origin region that is recognize by either both or only one of the bacteria involved in the process of formation of a hybrid vector.

Actual pending claims

EP 687 730 A1
Claim 1 A method for transforming a plant through a bacterium belonging to the genus *Agrobacterium*, comprising: A) co-transforming plant cells with a first T-DNA (1) and a second T-DNA (2); and B) selecting the cells which acquired drug resistance; said first T-DNA (1) containing a gene giving said drug resistance, which function in said plant; said second T-DNA (2) containing a desire DNA fragment to be introduced into said plant, the second T-DNA (2) being contained in a hybrid vector; said hybrid vector being prepared by homologous recombination between an acceptor vector and an intermediate vector in said bacterium belonging to genus *Agrobacterium*; • said acceptor vector containing at least: • (i) a DNA region having a function to replicate a plasmid in said bacterium belonging to the genus *Agrobacterium* and *Escherichia coli*, (ii) a DNA region containing *vir* B gene and *vir* G gene in virulence region of Ti plasmid pTiBo542 of *Agrobacterium tumefaciens*, and (iii) a DNA region which is homologous with a part of said intermediate vector, which is subjected to homologous recombination in said bacterium belonging to genus *Agrobacterium*; • said intermediate vector containing at least: • (i) a DNA region having a function to replicate a plasmid in *Escherichia coli*, which does not function in said bacterium belonging to genus *Agrobacterium*, (ii) a DNA region which is homologous with a part of said acceptor vector, which is subjected to homologous recombination in said bacterium belonging to genus *Agrobacterium*, and (iii) a DNA region which constitutes at least a part of said second T-DNA.
Claim 16 A hybrid vector comprising a first T-DNA containing: 1) a gene giving a drug resistance, which functions in plant, and 2) a second T-DNA giving a restriction site; said hybrid vector being prepared by homologous recombination between an acceptor vector and an intermediate vector in a bacterium belonging to genus *Agrobacterium*; • said acceptor vector containing at least: • (i) a DNA region having a function to replicate a plasmid in said bacterium belonging to the genus *Agrobacterium* and *Escherichia coli*, (ii) a DNA region containing *vir* B gene and *vir* G gene in virulence region of Ti plasmid pTiBo542 of *Agrobacterium tumefaciens*, and (iii) a DNA region which is homologous with a part of said intermediate vector, which is subjected to homologous recombination in said bacterium belonging to genus *Agrobacterium*; • said intermediate vector containing at least: • (i) a DNA region having a function to replicate a plasmid in *Escherichia coli*, which does not function in said bacterium belonging to genus *Agrobacterium*, (ii) a DNA region which is homologous with a part of said acceptor vector, which is subjected to homologous recombination in said bacterium belonging to genus *Agrobacterium*, and (iii) a DNA region which constitutes at least a part of said second T-DNA.

Mobilisable vectors

Summary

This section introduces a new type of vectors for *Agrobacterium*-mediated transformation. Some scientific information and patenting aspects are discussed.

Background

Conjugation is a bacterial mechanism through which a plasmid genome or a host chromosome is transferred from one bacteria cell to another. Conjugation requires a whole complex of sequences and gene products. Some bacterial plasmids are **conjugative plasmids** that have the ability of **transfer themselves** into another host.

Mobilisable plasmids are **not** able to promote their **own transfer** unless an appropriate conjugation system is provided by a helper plasmid. Mobilisable vectors contain a site for transfer initiation called **origin of transfer**, *ori*T, and have sequences encoding proteins involved in the mobilization of the DNA during the conjugative process. The mobilization proteins (**Mob**) alone are not sufficient to achieve the transfer of the genome. Additional proteins for transfer (**Tra**) are involved in the formation of a pore or pilus through which the genome passes to the recipient. Mobilisable plasmids **lack Tra proteins** and for this reason they require a helper plasmid providing the *tra* genes. In general, the process involves the following steps:

- double-strand plasmid DNA is nicked at a specific site in *ori*T
- a single-strand DNA is released to the recipient through a pore or pilus structure

The enzyme that cleaves the double-strand DNA at *ori*T and binds to a release 5' end is called **relaxase,** and the intermediate structure formed is called **relaxosome**. A complex of auxiliary proteins assemble at *ori*T and assist in the nick process to form this intermediate in the DNA transfer.

Agrobacterium T-DNA transfer

The transfer of the *Agrobacterium* T-DNA to a host cell is comparable to a conjugation process. The virulence (*vir*) genes are involved in the mobilization and transfer of the T-DNA to the host plant cell. The ***vir*D operon** contains genes that encode:

- proteins for DNA cleavage at T-DNA borders: *vir*D1 and *vir*D2;
- a protein that remains covalently bound to the 5' end of the T-strand and contains a nuclear localization site: *vir*D2; and
- a coupling protein, which binds to the T-DNA complex (T-strand plus *vir* proteins attached to it) and mediates its transfer through the mating bridge: *vir*D4.

The T-DNA transfer apparatus is encoded by the ***vir*B operon.** The proteins of the *vir*B genes are located in the inner and outer membrane of the bacterium and are involved in the production of the pilus/pore structure. They also play an essential role in tumorigenesis.

IP aspects

Leiden University, in The Netherlands, has a European patent application related to the use of **mobilisable plasmids** for genetic transformation of **eukaryotic cells**. The invention disclosed by the applicants combines mobilisable plasmids with ***Agrobacterium*-mediated transformation**. A **mobilisable plasmid contains** at least an *ori*T and some **mob genes**, and ***Agrobacterium* provides** the transfer genes, basically a ***vir*B operon**.

Unlike the binary and co-integrate vector systems, there is **no *Agrobacterium* T-DNA or nucleotide sequences surrounded by T-borders** transferred in the mobilisable vector system. The mobilisable plasmid is engineered into an *Agrobacterium* and the genetic material contained in the mobilisable plasmid is transferred to the eukaryotic host using the transfer machinery of *Agrobacterium*. VirD4, the coupling factor, and mobilization functions can either be part of the mobilisable plasmid or can be provided by *Agrobacterium*.

A mobilisable plasmid is defined by the inventors as "a plasmid that has the capability of forming a relaxosome in a suitable surrounding such as *Agrobacterium* and being capable of being transferred by an *Agrobacterium vir*-like system into eukaryotic cells."

According to the applicants, the advantages of mobilisable plasmids such as the plasmid CloDF13 include:

- small size;
- easy to manipulate;

- can be maintained at high copy number in enterobacteria (e.g. *E. coli*);
- can be transferred using the *Agrobacterium* virulence system;
- their transfer to fungi and plants is very efficient compared to other plasmids;
- can be used for nuclear and organelle transformation as well as homologous and site-specific recombination.

Note that this is still an application and the scope of the claims that may be granted is unknown.

The following table contains some bibliographic information of the European patent application.

Bibliography

	EP 1 130 105 A1
Title	Transformation of eukaryotic cells by mobilisable plasmids
Application No. and Filing date	EP 2000200726 A March 1, 2000
Publication No.	September 5, 2001
Language	English
	View Claims

To view or download the patent application as a PDF file, click on EP 1 130 105 A1 (WO 0164925 A1) (1,353 kb).

Summary of the invention

The independent claims of the present patent application recite:

- a method for transferring material to a eukaryotic cell by:
  - using a mobilisable plasmid capable of forming a relaxosome;
  - placing the plasmid into *Agrobacterium* , which provides a functional virB operon; and
  - co-cultivating the *Agrobacterium* with the eukaryotic host.
- a mobilisable plasmid for *Agrobacterium* -mediated transfer to an eukaryotic host comprising:
  - a functional *ori*T;
  - *vir*D-like mobilization products;
  - *vir*D4-like coupling factor; and
  - *vir*B-like activities.

If the claims granted as submitted, the resulting patent would provide protection for a broad spectrum of plasmids used for transformation via *Agrobacterium*. Binary and co-integrated vectors are based on the Ti-plasmid of *Agrobacterium* and the DNA transferred is always surrounded by T-DNA borders, while the plasmids of the present invention are derived from other bacterial sources, mainly bacteria from the family Enterobacteriaceae and do not use T-DNA borders for DNA transfer. Notice that the invention as filed is not limited to plants; all eukaryotic organisms may be encompassed by the claims in case of being granted as filed.

Mobilisable vectors
Patent application filed by University of Leiden

Claims in plain English

Disclaimer

THE FOLLOWING CLAIMS ARE MEANT ONLY TO ASSIST READING OF THE CLAIMS AND ARE NOT MEANT AS A LEGAL INTERPRETATION OF THE CLAIM SCOPE.

EP 1 130 105 A1

Claim 1

A method for transferring genetic material, not typically surrounded byT-DNA borders, to a eukaryotic host cell, comprising: A) having the genetic material on a mobilisable plasmid, which is capable of forming a relaxosome;
B) placing the mobilisable plasmid in an *Agrobacterium* having at least transfer genes that provide the same or similar activity as a functional *vir*B operon; and
C) co-cultivating the *Agrobacterium* with the eukaryotic host cell.

Claim 10

A mobilisable plasmid with genetic material to be transferred into a eukaryotic cell by *Agrobacterium* transfer, comprising: 1) a functional *ori*T,
2) sequences encoding *vir*D-like mobilization products,
3) a *Vir*D4-like coupling factor, and
4) sequences encoding virB-like activity.

Actual pending claims

EP 1 130 105 A1

Claim 1

A method for transferring genetic material which is not a typical T-DNA surrounded by *Agrobacterium* T-borders from an *Agrobacterium* virulence system to a eukaryotic host cell, comprising: A) providing said genet ic material on a mobilisable plasmid, capable of forming a relaxosome;
B) bringing said mobilisable plasmid in an *Agrobacterium* having at least the activity of the transfer genes of *Agrobacterium* not present on said mobilisable plasmid, whereby the necessary gene products providing the same or similar activity as a functional *Vir* B operon are also present and
C) co-cultivating said *Agrobacterium* with said eukaryotic host cell.

Claim 10

A mobilisable plasmid comprising genetic material to be transferred into a eukaryotic cell by *Agrobacterium* transfer, said mobilisable plasmid further comprising: 1) a functional *ori*T,
2) sequences encoding functional *vir*D-like mobilization products,
3) a *Vir*D4-like coupling factor, and
4) sequences encoding functional virB-like activity.

Improvements on transformation efficiency

Overview

New update July 2003

As in any technology for plant transformation, there are multiple factors involved in *Agrobacterium*-mediated transformation that influence the success or failure of the transfer of gene of interest into plants and their subsequent stable integration and expression. The different factors can affect transformation differently, depending in part on the plant species.

Aspects of transformation that affect success include:

- Maturity of the plant - as a general rule young plants are easier to transform than old ones;
- Selected tissue to be transformed;
- *Agrobacterium* strain selected for transformation;
- Extent of time and conditions for inoculation of the tissue with *Agrobacterium*;
- Growth of *Agrobacterium* with respect to the transformed plant cells. If there is overgrowth of

Agrobacterium, the chances of regenerating complete plants from the transformed tissue dwindle;

- Plant tissue necrosis caused by *Agrobacterium*.

In this section we present patents and patent applications disclosing improvements related to one or more of the factors mentioned above. The inventors were motivated by a need to enhance the efficiency of *Agrobacterium*-mediated transformation of plants. The selected inventions refer to:

- Inhibition of plant necrosis caused by *Agrobacterium*. **Novartis** (now **Syngenta**) and the **University of Minnesota**
  each have several granted patents and patent applications filed in the United States, Europe and Australia that teach methods for inhibiting *Agrobacterium*-induced necrosis. The methods entail heat shock treatment of the plant tissue, chemical inhibitors, and gene products expressed in the transformed plants that inhibit necrosis and enzyme inhibitors.
- Inhibition of *Agrobacterium* growth. Overgrowth of *Agrobacterium* has a negative effect on the survival rate of the transformed plant cells and increases the number of copies of T-DNA inserts in the transformed plant. **Nunhems Zaden** has granted patents describing the use of auxotrophic *Agrobacterium* mutants that allow control of their growth by omission of defined vital nutrients. **Monsanto**
  has a PCT application describing the use of compounds containing heavy metals and antibiotics to control the growth rate of *Agrobacterium*.
- Reduction of the weight of the transformed explant . Weight reduction of the explant to be transformed facilitates the DNA transfer and favors embryogenic callus formation. **Monsanto**'s applications filed in United States and Europe discuss methods for reducing the weight of the explant during the co-cultivation period by extracting moisture from the explant.
- Sonication of the plant tissue . In this improvement, the target tissue is subjected to ultrasound before, during or after immersion in an *Agrobacterium* suspension. **The Ohio State Research Foundation** has a United States patent and a European patent application on this subject matter.
- Vacuum infiltration of *Agrobacterium* into the plant. *Agrobacterium* establishes a more intimate contact with the cells of a plant when subjected to a vacuum environment. The method is applied *in planta* thus avoiding both *in vitro* culture and regeneration steps. **The Samuel Robert Noble Foundation** and **Paradigm Genetics Inc.** have patent applications on the use of vacuum to assist the transformation of any plant, monocots and some particular crops with *Agrobacterium*.

In conclusion,
The granted patents and patent applications discussed in this section are directed to fairly specific methods applied in *Agrobacterium*-mediated transformation protocols. With regard to these patents and any patents yet to issue freedom to operate will become an issue only if any one of the particular procedures mentioned above is part of a transformation protocol carried out in the countries where the patents have been granted. Remember that claims as filed in patent applications do not have a defined scope and may vary if the applications become granted patents.

Inhibition of *Agrobacterium*-induced necrosis

Summary

In plant biology, necrosis means death of a plant tissue; the tissue first turns brown and subsequently dies. Disrupted plant cells of a tissue at the cut edge release colorless phenolic compounds that come into contact with each other and in the presence of oxygen suffer brown discoloration accumulating in brown spots in the cells. Oxidation extends throughout the tissue and the culture media, and, if not controlled, the tissue finally dies. Enzyme activity and polymerization of phenolic compounds are some of the causes of the oxidation-browning process.

Inoculation of a plant tissue with *Agrobacterium* is in itself a disruptive process and triggers a hypersensitive response in the tissue. As a result, there is a poor survival rate of the target tissue. Therefore, the design of an adequate artificial environment to minimize damage due to the interaction of *Agrobacterium* with the plant tissue is critical for the success of genetic transformation experiments. Anti-necrotic/anti-browning treatments, applied during the transformation process, include addition of reducing agents, heat inactivation of enzymes participating in the oxidative process, lowering pH and the addition of enzyme inhibitors.

IP aspects

There are two entities with patents directed to reducing browning/necrosis induced by *Agrobacterium*:

- **Syngenta (formerly Novartis)** has patents in the United States, Australia and Europe and patent applications in the United States and Europe on different **methods for overcoming** the **necrosis induced by *Agrobacterium***, especially in Gramineae plants. According to the applicants, *Agrobacterium* induces in some plants a necrotic process analogous to apoptosis in animal cells, where cell death is characterized by DNA fragmentation and defined morphological changes. The claimed methods for controlling plant cell death include:
    - heat shock treatment of the plant tissue;
    - chemical inhibitors, such as ethylene inhibitors; and
    - foreign gene products expressed in the transformed plants.
- The **University of Minnesota** has PCT, United States, and Australian patent applications disclosing the use of **agents to inhibit enzymatic browning of the plant tissue** during *Agrobacterium*-mediated transformation. The agents include:
    - sulfhydryl-containing agents (e.g. L-cysteine); and
    - iron and copper chelators.

Granted patents and applications filed by Syngenta (formerly Novartis)

Updated July 2003
According to the applicants the necrosis seen in some plants, i.e. Gramineae, upon *Agrobacterium* exposure is a programmed cell death that is different from the passive death experienced during oxidative browning and exposure to toxins. It is an active process in which the cells undergo morphological changes in part as a result of *de novo* gene expression and DNA cleavage.

The patents and patent applications disclose the **use of physical and chemical methods for inhibiting *Agrobacterium*-induced necrosis (AIN)**. **Heat shock treatment** is one of the physical methods and among the chemical methods;**chemical compounds** are used as inhibiting agents of AIN. **Nucleotide sequences such as p35 and iap**
(see below) stably or transiently expressed in the cell to be transformed also inhibit AIN. In addition, the applicants teach the use of ***Agrobacterium* strains** that are less likely to induce necrosis in the transformed tissue.

Bibliography

	US 6,162,965	US 2002/0088029	AU 735 472 B	EP 986 299 A2
Title	Plant transformation methods			
Application No. & Filing Date	US 09/089,111 2 June 1998	US 09/741,297 19 Dec 2000	AU 85355/98 29 May 1998	EP 98/936297 29 May 1998
Issue date	19 December 2000	4 Jul 2002 (Publ. date)	12 July 2001	22 March 2000 (Publ. date)
	View Claims	View Claims	View Claims	View Claims
Language	English			
Remarks	The Australian and the United States patents **do not** belong to the same patent family. The Australian patent is related to European application **EP 986 299 A2** and to the following patent applications: Brazil (BR 98/09899 A), Canada (CA 2290863), China (CN 1258316 T), Hungary (HU 2000/02903 AB), Israel (IL 132768 A0), Poland (PL 336979 A1), Turkey (TR 9902941) and South Africa (ZA 9804681 A).			

To view or download the patent and patent application as a PDF file, click on US 6,162,965 (1,193 KB) & EP 986 299 A2 (WO 98/54961 A2) (2,153 KB).

Summary of the invention

Granted patents

United States patent US 6,162,965 claims several methods to inhibit AIN in plants:

- **heat shock** to treat plant cells or tissues before co-cultivating with *Agrobacterium*
- transformation of a plant cell via *Agrobacterium* with **sequences** such as:
  - **p35** and **iap**, which are apoptosis-inhibiting genes from baculovirus; and
  - **dad-1**, a gene capable of suppressing disease response in plants.

The patent also claims methods for inhibiting AIN in **Gramineae** in general and in **maize** in particular. Besides the methods mentioned above, gramineaceous plants and maize may be cultured in a necrosis inhibiting medium containing an inhibitor of ethylene or ethylene biosynthesis.

The methods for inhibiting AIN claimed in the granted **Australian patent** are **similar to** the ones claimed in the **United States patent**. However, the group of chemical inhibitors additionally includes **gibberellin antagonists** and **phosphatase inhibitors**. Also, the nucleotide sequences whose products inhibit AIN are not limited to specific genes, but encode **any mRNA** or **protein inhibiting AIN**. Furthermore, unlike the United States patent, the **Australian patent does not** claim transformation of Gramineae or maize in particular.

Patent applications

United States application US 2002/0088029 (Update July 2003) is a continuation of application No. 09/089,111, which is now patent US 6,162,965 described above. The new elements in this application include a method to select an *Agrobacterium* strain with reduced necrosis-inducing capacity from a population of regenerable plant cells from the family Gramineae. Further, the application also claims the *Agrobacterium*
strain with reduced or eliminated production of the necrosis factor, obtained through the selection process or through genetic manipulation. Finally, a method is claimed to transform regenerable cells from the family Gramineae using such strains.

The claims as filed in the **European application** are **broader** in scope than the granted claims of both the Australian and the US patents. For example, one claim broadly recites "conditions which inhibit AIN" without specifying details of suitable conditions. In another claim the culture medium for the plant cell or tissue contains a chemical inhibitor without defining what sort of chemical. A couple of claims of the European application make reference to the use of an ***Agrobacterium* strain** that **does not induce** significant levels of **necrosis**. The *Agrobacterium* strain has been modified to reduce or eliminate expression of a necrosis factor. This strain of *Agrobacterium* may be used for transforming a totipotent Gramineae cell.

Remember that as a patent application, the actual scope of the claims of the present European and United States applications are yet to be determined.

Inhibition of *Agrobacterium*-induced necrosis
Patents granted to Novartis

Actual granted claims

US 6 162 965
Claim 1 A method for transforming a plant cell or tissue with a gene construct, comprising: • heat shocking said plant cell or tissue before co-cultivating with *Agrobacterium*, wherein said heat shock treatment inhibits *Agrobacterium*-induced necrosis in said plant cell or tissue, and said *Agrobacterium* comprises a vector comprising said gene construct.
Claim 2 A method for producing a fertile transgenic plant comprising a gene construct, which method comprises: A) transforming a plant cell or tissue comprising heat shocking said plant cell or tissue before co-cultivating with *Agrobacterium* , wherein

- said heat shock treatment inhibits *Agrobacterium*-induced necrosis in said plant cell or tissue and
- said *Agrobacterium* comprises a vector comprising said gene construct; and

B) regenerating the transformed plant cell or tissue to produce said fertile transgenic plant.

Claim 14

A method for transforming a plant cell or tissue with a gene construct, comprising:

- exposing said plant cell or tissue to *Agrobacterium* under conditions which inhibit *Agrobacterium*-induced necrosis,
  wherein said conditions comprise
-
  - delivering to or expressing in said plant cell or tissue a nucleotide sequence comprising a coding sequence of a p35, iap or dad-1 gene,
  - said delivery or expression of said nucleotide sequence inhibits *Agrobacterium*-induced necrosis in said plant cell or tissue, and
  - said *Agrobacterium* comprises a vector comprising said gene construct.

Claim 15

A method for producing a fertile transgenic plant comprising a gene construct, which method comprises:
A) transforming a plant cell or tissue comprising exposing said plant cell or tissue to *Agrobacterium* under conditions which inhibit *Agrobacterium*-induced necrosis, wherein said conditions comprise

- delivering to or expressing in said plant cell or tissue a nucleotide sequence comprising a coding sequence of a p35, iap or dad-1 gene,
- said delivery or expression of said nucleotide sequence inhibits *Agrobacterium* induced necrosis in said plant cell or tissue, and
- said *Agrobacterium* comprises a vector comprising said gene construct; and

B) regenerating the transformed plant cell or tissue to produce said fertile transgenic plant.

Claim 35

A transgenic plant, plant tissue or plant cell comprising a nucleotide sequence of heterologous origin which comprises a coding sequence of a p35, iap or dad-1 gene.

Claim 36

A transgenic plant, plant tissue or plant cell comprising a genome having a stably integrated nucleotide sequence of heterologous origin which comprises a coding sequence of a p35, iap or dad-1 gene.

AU-B-735 472

Claim 1

A method of transforming a plant cell or tissue with a gene of interest, comprising:

- exposing said plant cell or tissue to *Agrobacterium* under conditions which inhibit *Agrobacterium*-induced necrosis (AIN), wherein
  said *Agrobacterium* comprises a vector comprising said gene of interest, wherein
  said conditions which inhibit AIN comprise:
  - exposing said plant cell or tissue to *Agrobacterium* after heat shock treatment; or
  - exposing said plant cell or tissue to *Agrobacterium* in the presence of an agent inhibiting AIN, wherein said agent comprises:
    - a chemical inhibitor, wherein said chemical inhibitor is a compound selected from the group consisting of ethylene inhibitors other than silver nitrate, ethylene synthesis inhibitors, gibberellin antagonists, and phosphatase inhibitors; or
    - a nucleotide sequence encoding mRNA or protein inhibiting AIN.

Claim 18

A method of making a fertile, transgenic plant comprising: A) transforming plant tissue by exposing the tissue to *Agrobacterium* under conditions which inhibit *Agrobacterium* -induced necrosis (AIN); and B) regenerating tissue thus transformed, wherein
said *Agrobacterium* comprises a vector comprising said gene of interest, wherein
said conditions which inhibit AIN comprise:

- exposing said plant cell or tissue to *Agrobacterium* after heat shock treatment; or
- exposing said plant cell or tissue to *Agrobacterium* in the presence of an agent inhibiting AIN, wherein said agent comprises:
  - a chemical inhibitor, wherein said chemical inhibitor is a compound selected from the group consisting of ethylene inhibitors other than silver nitrate, ethylene synthesis inhibitors, gibberellin antagonists, and phosphatase inhibitors; or
  - a nucleotide sequence encoding mRNA or protein inhibiting AIN.

Claim 39

A plant, plant tissue or plant cell comprising a nucleotide sequence of heterologous origin which inhibits AIN.

Claim 50

A plant cell or tissue culture medium, comprising:

1. a chemical inhibitor, wherein said chemical inhibitor is a compound selected from the group consisting of ethylene inhibitors other than silver nitrate, ethylene synthesis inhibitors, gibberellin antagonists, and phosphatase inhibitors;
2. an *Agrobacterium* comprising a plasmid comprising a gene of interest; and
3. water and essentials salts.

Inhibition of *Agrobacterium*-induced necrosis
Patent application filed by Novartis

Actual pending claims

EP 986 299 A2

Claim 1

A method of transforming a plant cell with a gene of interest, comprising

- exposing said plant cell to *Agrobacterium* under conditions which inhibit *Agrobacterium*-induced necrosis (AIN), wherein
  said *Agrobacterium* comprises a vector comprising said gene of interest.

Claim 8

A method of making a fertile, transgenic plant comprising: A) transforming plant tissue by exposing the tissue to *Agrobacterium* under conditions which inhibit *Agrobacterium*- induced necrosis (AIN); and B) regenerating tissue thus transformed, wherein said *Agrobacterium* comprises a vector comprising a gene of interest.

Claim 9

A plant, plant tissue or plant cell comprising a nucleotide sequence of heterologous origin which inhibits AIN.

Claim 10

A plant cell or tissue culture medium, comprising:

A) a chemical inhibitor of AIN;
B) an *Agrobacterium* comprising a plasmid comprising a gene of interest; and
C) water and essential salts.

Claim 11

A method of transforming a totipotent cell of a plant of the family Gramineae, comprising

- exposing a population of said totipotent cells to *Agrobacterium* comprising a plasmid comprising a gene of interest, wherein
  the *Agrobacterium* is of a strain which does not induce significant levels of necrosis in said population at an exposure duration and concentration sufficient to achieve transformation of said cell.

Claim 12

A method for determining the suitability of an *Agrobacterium* strain for use in the transformation of a regenerable cell of a plant of the family Gramineae comprising: A) exposing a population of said regenerable cells of the plant to the *Agrobacterium* strain; and
B) observing the necrosis in said cell population.

Claim 13

An *Agrobacterium* strain which has been genetically modified to reduce or eliminate expression of the *Agrobacterium* necrosis factor or a derivative of such a modified strain.

Inhibition of Agrobacterium-induced necrosis

Independent Claims of US application

US 2002/0088029
1. A method of transforming a plant cell with a gene of interest, comprising exposing said plant cell to *Agrobacterium* under conditions which inhibit *Agrobacterium* induced necrosis (AIN), wherein said *Agrobacterium* comprises a vector comprising said gene of interest.
8. A method of making a fertile, transgenic plant comprising transforming plant tissue by exposing the tissue to *Agrobacterium* under conditions which inhibit *Agrobacterium* induced necrosis (AIN) and regenerating tissue thus transformed, wherein said *Agrobacterium* comprises a vector comprising a gene of interest.
9. A plant, plant tissue or plant cell comprising a nucleotide sequence of heterologous origin which inhibits AIN.
10. A plant cell or tissue culture medium, comprising a) a chemical inhibitor of AIN, b) an *Agrobacterium* comprising a plasmid comprising a gene of interest, and c) water and essential salts.
11. A method of transforming a totipotent cell of a plant of the family Gramineae, comprising exposing a population of said totipotent cells to *Agrobacterium* comprising a plasmid comprising a gene of interest, wherein the *Agrobacterium* is of a strain which does not induce significant levels of necrosis in said population at an exposure duration and concentration sufficient to achieve transformation of said cell.
12. A method for determining the suitability of an Agrobacterium strain for use in the transformation of a regenerable cell of a plant of the family Gramineae comprising exposing a population of said regenerable cells of the plant to the *Agrobacterium* strain and observing the necrosis in said cell population.
13. An *Agrobacterium* strain which has been genetically modified to reduce or eliminate expression of the *Agrobacterium* necrosis factor or a derivative of such a modified strain.

Patent applications filed by the University of Minnesota

The present applications describe **methods for inhibiting enzymatic browning of plant tissue** , cells or parts of a plant in response to wounding. The disclosure describes agents that inhibit the activity or production of enzymes associated with browning such as polyphenol oxidase (PPO) and peroxidase (POD), chelators of metals required for enzymatic activity, and sulphydryl-containing agents that also inhibit PPO activity.

Bibliography

	US 20010034888 A1	WO 0144459 A2
Title	Method to enhance *Agrobacterium* -mediated transformation of plants	
Application No. & Filing date	US 738398 December 15, 2000	PCT/US00/34081 December 15, 2000
Publication date	October 25, 2001	June 21, 2001
Language	English	
Remarks	Related application filed in Australia (AU 200122672).	

To view or download the patent applications as a PDF file, click on US 20010034888 A1 (5,000 kb) and WO 0144459 A2 (4,492 kb).

Summary of the invention

The claims as filed of the present applications recite:

- a method for inhibiting enzymatic browning in cotyledon explant to be infected with *Agrobacterium* by introducing an agent that inhibits browning
- a method to identify an agent that enhances *Agrobacterium*-mediated transformation of a plant cell, tissue or plant part
- methods where agents that inhibit browning are either put into contact with the explant to be transformed or present in the plant medium. The agents are metal chelators, sulphydryl-containing agents or other enzyme inhibitors.

View Claims

Inhibition of enzymatic browning of *Agrobacterium*-transformed tissue
Patent applications filed by the University of Minnesota

Actual pending claims

US 20010034888 A1 & WO 0144459 A2
Claim 1 A method for transforming plant explant tissue, comprising: A) contacting a cotyledon explant from a plant seedling infected with an *Agrobacterium* containing DNA to be introduced into the explant with an agent that inhibits enzymatic browning of a wounded plant, plant tissue or plant cell so as to yield transformed explant tissue; and B) identifying transformed explant tissue.
Claim 22 A method to identify an agent that enhances *Agrobacterium* -mediated transformation of a plant cell, plant tissue or plant part, comprising: A) contacting the plant cell, plant tissue or plant part with *Agrobacterium* containing DNA to be introduced into the plant cell, plant tissue or plant part and the agent so as to yield a transformed plant cell, plant tissue or plant, wherein the agent is not a phenolic compound; and B) detecting or determining whether the agent enhances *Agrobacterium*-mediated transformation of the plant cell, plant tissue or plant part relative to *Agrobacterium*-mediated transformation of a plant cell, plant tissue or plant part in the absence of the agent.
Claim 37 A method for the stable transformation of plant tissue or cells, comprising:

A) contacting plant tissue or cells with an *Agrobacterium* containing DNA and an agent selected from the group consisting of a sulthydryl-containing agent, an iron chelator, a copper chelator, 10 an inhibitor of plant polyphenol oxidase and an inhibitor of plant peroxidase; and
B) identifying stably transformed plant tissue or cells.

Claim 44

A plant medium comprising:
an amount of an agent effective to inhibit the enzymatic browning of a plant organ, tissue or cell, wherein the agent is selected from the group consisting of a sulfhydryl-containing agent, an iron chelator, a copper chelator, an inhibitor of polyphenol oxidase and an inhibitor of peroxidase.

Inhibition of *Agrobacterium* growth
Patent application filed by Monsanto

Overgrowth of *Agrobacterium* jeopardizes the survival of the transformed plant cells and also has an effect on the T-DNA transfer process. Insertion of multiple copies of the gene of interest into the plant cell is influenced by the frequency of T-DNA transfer. *Agrobacterium*-mediated transformation protocols strive to attain transformation events with a limited number of copies of the introduced DNA. The presence of multiple inserts can lead to gene silencing or reduce expression levels of the transformed genes, which is caused by several mechanisms including recombination between the multiple copies. Inhibiting agents of *Agrobacterium*
growth should be effective against the bacterium but remain neutral with respect to plant cell growth.

The present invention disclosed by **Monsanto** relates to the **control of *Agrobacterium* growth** during the transformation process in order to improve transformation efficiency. The use of inhibiting agents during inoculation and co-culture of *Agrobacterium* with a transformable plant cell results, according to the inventors, in increased transformation efficiencies and a low copy number of the introduced genetic component in several plant systems. Preferred growth inhibiting agents are compounds containing heavy metals such as silver nitrate or silver thiosulfate, antibiotics such as carbenicillin, and a combination of antibiotics and a clavulanic acid such as augmentin or timentin.

Bibliography

	WO 01/09302 A2
Title	A novel *Agrobacterium*-mediated plant transformation method
Application No. & Filing Date	WO 00US20634 29 July 1999
Publication date	8 Feb 2001
Language	English
Remarks	Priority US 09/364,254. Related application filed in Australia (AU 63892/00 A5) and Europe (EP 1 200 613 A2)

To view or download the patent application as a PDF file, click on WO 01/09302 A2 (2.79 Mb).

Summary of the invention

The claims as filed of the PCT application recite:

- methods of transforming plant cells or tissues with *Agrobacterium* , where the growth of *Agrobacterium* cells is inhibited during:
  - the inoculation phase, where *Agrobacterium* and plant cells are first brought into contact with each other;
  - the co-cultivation phase, where *Agrobacterium* and plant cells are grown together for a period of several hours to days; or
  - both the inoculation and co-cultivation phases.

View Claims

Inhibition of *Agrobacterium* growth
Patent application filed by Monsanto

Actual pending claims

WO 0109302 A2
Claim 1 A method of transforming a plant cell or plant tissue using an *Agrobacterium* mediated process comprising the steps of: A) inoculating a transformable plant cell or tissue with *Agrobacterium* containing at least one genetic component capable of being transferred to the plant cell or tissue in the presence of at least one growth inhibiting agent; B) co-culturing the transformable plant cell or tissue after inoculation in a media capable of supporting growth of plant cells or tissue expressing the genetic component, said media not containing a growth inhibiting agent; C) selecting transformed plant cells or tissue; and D) regenerating a transformed plant expressing the genetic component from the selected transformed plant cells or tissue.
Claim 23 A method of transforming a plant cell or plant tissue using an *Agrobacterium* mediated process comprising the steps of: A) inoculating a transformable plant cell or tissue with *Agrobacterium* containing at least one genetic component capable of being transferred to the plant cell or tissue; B) co-culturing the transformable plant cell or tissue after inoculation in a media capable of supporting growth of plant cells or tissue expressing the genetic component, said media further containing a growth inhibiting agent; C) selecting transformed plant cells or tissue; and D) regenerating a transformed plant expressing the genetic component from the selected transformed cells or tissue.
Claim 45 A method of transforming a plant cell or plant tissue using an *Agrobacterium* mediated process comprising the steps of: A) inoculating a transformable plant cell or tissue with *Agrobacterium* containing at least one genetic component capable of being transferred to the plant cell or tissue in the presence of at least one growth inhibiting agent; B) co-culturing the transformable plant cell or tissue after inoculation in a media capable of supporting growth of the plant cells or tissue expressing the genetic component, said media further containing a growth inhibiting agent; C) selecting transformed plant cells or tissue; and D) regenerating a transformed plant expressing the genetic component from the selected transformed cells or tissue.

Patent granted to Nunhems Zaden BV

The present United States patent granted to **Nunhems Zaden** from Holland discloses a process to obtain transgenic plants by using *Agrobacterium* mutants deficient in the biosynthesis of specified vital biomolecules. This allows a controlled systemic infection of the tissues to be transformed to be maintained for longer periods, thereby increasing the probability of successful infection. The *Agrobacterium* can then be eliminated by omission of those nutrients from the incubation medium.

A corresponding patent has been granted in Australia.

	US 6,323,396 B1
Title	*Agrobacterium*-mediated transformation of plants
Application No. & Filing Date	US 09/512,650 24 Feb 2000
Publication date	27 Nov 2001

Remarks	Priority: EP 1 009 844 (equivalent to WO 99/10512). Related patent in Australia (AU 736 349 B2). Applications are pending in Canada (CA 2301707, China (CN 1268184), in Japan (JP 2001/514009 T2) and in Europe (EP 1 009 844 A1).

To view or download the patent as a PDF file, click on US 6 323 396 (1.01 Mb); AU 736 349 (1.5 Mb).

Summary of the invention

The United States patent **US 6,323,396 B1** claims

- A process for the production of transgenic plants (Claims 1 and 13) utilizi ng an *Agrobacterium* strain deficient in the biosynthesis of one of the following:
  - methionine
  - cysteine
  - adenosine and histidine
- Co-cultivating a plant or plant tissue with one of the auxotrophic *Agrobacterium* mutants described above carrying a desired foreign DNA and regenerating a transgenic plant from such a treated tissue (Claim 13).
- Systemically infecting a plant with one of the auxotrophic *Agrobacterium* mutants described above carrying a desired foreign DNA and regenerating a transgenic plant from such a treated tissue (Claim 1).
- Two auxotrophic *Agrobacterium* strains as described (Claims 11 and 12).

View Claims

Inhibition Agrobacterium Growth
Patents granted Nunhems Zaden BV (NL)

Independent claims

Patent granted to Nunhems Zaden BV (NL)
US 6 323 396 B1
Claim 1 1. A process to produce a dicotyledonous transgenic plant, said plant comprising a foreign DNA fragment integrated into the genome of at least some of its cells, said process comprising the following steps: 1) providing a plant which is systemically infected with an *Agrobacterium* strain auxotrophic for methionine or cysteine, or adenine and histidine, harboring a DNA of interest which is operably linked to at least one T-DNA border sequence; and 2) generating a transgenic plant from a single cell or a group of cells isolated from said systemically infected plant.
Claim11 Bacterial strain LBA4404metHV, deposited as LMG P-18486.
Claim12 Bacterial strain ATHV ade,his, deposited as LMG P-18485.
Claim13 A method for producing a transgenic plant, said plant comprising a foreign DNA fragment integrated into the genome of at least some of its cells, said process comprising: a. cocultivating a plant cell, plant tissue, explant or plant with an *Agrobacterium* strain auxotrophic for methionine or cysteine, or for adenine and histidine, to generate a transgenic cell; and b. regenerating a transgenic plant from said transgenic cell.

Weight reduction of the transformed plant tissue

Patent applications filed by Monsanto

During co-cultivation of plant tissue with *Agrobacterium* it is desirable to reduce the weight of the explant in order to facilitate the DNA transfer process and the formation of embryogenic callus.

Monsanto's disclosure teaches methods for **reducing** the **weight of the explant during the co-cultivation period**. Preferred methods of the inventors include reduction of moisture conditions, applying vacuum, increasing the osmotic potential of the media by use of mannitol, sorbitol or polyethylene glycol, air drying the explant by evaporation or applied air, or applying chemicals (desiccants) such as calcium oxide to extract moisture from the explant.

Bibliography

	US 20010054186 A1	EP 1 137 790 A2
Title	An improved efficiency *Agrobacterium*-mediated plant transformation method	
Application No. & Filing Date	US 609794 March 1, 1996	EP 97906796 February 28, 1997
Publication date	December 2, 1997	March 31, 1999
Language	English	
Remarks	Related patent applications also filed in Australia (AU 20492/00 A5), and Brazil (BR 9916103 A). The claims as filed of the Australian application are worded exactly the same as the United States and Australian applications.	
	View Claims	View Claims

To view or download the patent applications as a PDF file, click on US 20010054186 A1 (1,192 kb) and EP 1 137 790 A2 (WO 0034491 A2) (1,519 kb).

Summary of the invention

The claims as filed of both the **United States and the European applications** recite a method for producing a fertile transgenic plant by co-culturing a plant cell or tissue with *Agrobacterium* having the genes of interest under conditions that decrease the weight of the explant. The transformed cell lines are selected and regenerated into a fertile transgenic plant.

Weight reduction of the transformed plant tissue
Patent applications filed by Monsanto

Actual pending claims

US 20010054186 A1 & EP 1 137 790 A2
Claim 1 A method for producing a fertile transgenic plant, comprising the steps of: A) introducing one or more genetic component(s) one desires to introduce into the genome of a plant by co-culturing a regenerable plant cell or tissue with *Agrobacterium* containing said genetic component(s); B) co-culturing said *Agrobacterium* and regenerable plant cells or tissues of step (A) under conditions that decrease the weight of said *Agrobacterium*-inoculated explant; C) identifying or selecting a transformed cell line; and D) regenerating a fertile transgenic plant therefrom.

Sonication of plant tissue
Granted patent and patent application filed by The Ohio State Research Foundation

The inventors describe a method called sonication-assisted *Agrobacterium*-mediated transformation. It consists of subjecting the target tissue to ultrasound while immersed in an *Agrobacterium* suspension. The enhanced transformation rates probably result from micro-wounding both on the surface of and deep within the target tissue caused by the energy released in the process. High intensity ultrasound results in cell lysis, but sublethal doses cause temporary suppression of mRNA and protein synthesis as well as moderate

rupture of the cell wall. The wounding caused by lower energy ultrasonic frequency may aid in the production of signal phenolics and enhance the accessibility of putative cell-wall binding factors to the bacterium.

The disclosure describes a method for transforming a plant sample with *Agrobacterium* by sonicating the plant in the presence of *Agrobacterium*. Preferably, the process does not take longer than 60 seconds and not less than 0.1 seconds.

Bibliography

	US 5 693 512	EP 904 362 A1
Title	Method for transforming plant tissue by sonication	Method for transforming plant tissue
Application No. & Filing Date	US 609794 March 1, 1996	EP 97906796 February 28, 1997
Issue date	December 2, 1997	March 31, 1999
Language	English	

To view or download the patent and patent application as a PDF file, click on US 5 693 512 (2,541 kb) & EP 904 362 A1 (WO 9732016 A1) (1,858 kb).

Summary of the invention

The **United States patent** claims

- a method for transforming a plant where the plant sample is combined with a non-oncogenic *Agrobacterium*
  and sonicated. The sonication is applied before, during or after the plant sample is combined with *Agrobacterium*.

The claims as filed of the **European application** recites a method fairly **similar** to the one claimed in the United States patent. The **difference** lies in the phase where sonication takes place: **only** during **combination** of the plant sample with *Agrobacterium*.

View Claims

Sonication of plant tissue
Granted patent and patent application filed by the Ohio State University

Actual granted claims

US 5 693 512
Claim 1 A method for transforming a plant sample comprising the following steps: A) providing a non-tumor inducing vector containing nucleic acid to be transferred to the plant sample, wherein the vector is a non-tumor inducing *Agrobacterium*; B) combining the plant sample with said vector; C) sonicating the plant sample; wherein the vector is combined with the plant sample before, during, or after sonication; D) lastly growing the plant sample and selecting for the transformed plant sample.

Actual pending claims

EP 904 362 A1
Claim 1 A method for transforming a plant sample comprising the following steps: A) providing a non-tumor inducing vector containing nucleic acid to be transferred to the plant sample; B) combining the plant sample with said vector;

C) sonicating the plant sample;
D) lastly growing the plant sample.

Agrobacterium-mediated transformation assisted by vacuum infiltration

Summary

Vacuum infiltration had already been used by plant physiologists before researchers interested in improving transformation efficiency started using it. Plant physiologists use the method to allow the penetration of pathogenic bacteria into the inter cell spaces and in that way study the interaction between plants and pathogenic bacteria.

Physically, vacuum generates a negative atmospheric pressure that causes the air spaces between the cells in the plant tissue to decrease. The longer the duration and the lower the pressure of the vacuum, the less air space within the plant tissue. The increase in the pressure allows the infiltration medium, including the infective transformation vector to relocate into the plant tissue. For plant transformation, vacuum is applied to a plant part in the presence of *Agrobacterium* for a certain time period. The length of time that a plant part or tissue is exposed to vacuum is critical as prolonged exposure causes hyperhydricity.

Vacuum infiltration-facilitated transformation can be performed *in planta*, in which the plant part to be transformed, e.g. flower, is not excised from the plant, thus eliminating *in vitro* regeneration of plants. It also offers other several advantages such as the generation of many independently transformed plants from a single plant, a reduction in somaclonal variation by avoiding tissue culture steps, the possibility of testing many constructs in a short time frame as the process is in itself fast, and is potentially useful for transformation of plants recalcitrant to plant tissue culture and regeneration.

The use of *Agrobacterium*-mediated transformation assisted by vacuum infiltration was first reported in 1993 for transforming *Arabidopsis* and since then many improvements have been made. Others plants such as soybeans, duckweed, wheat, petunia, and rice have also been transformed by this method.

IP aspects

Several organizations have patents and patent applications directed to *Agrobacterium*-mediated transformation assisted by vacuum infiltration. The selected disclosures describe either transformation of **any plant** or transformation of **monocotyledonous**. They also discriminate between **any plant part** or a **selected plant part** or tissue, e.g. flower.

Transformation of any plant

- The **Samuel Roberts Foundation Noble Foundation** has patent applications filed in Australia and Europe on methods for direct plant transformation of any plant with *Agrobacterium* using vacuum infiltration. The limitations of the filed claims lie in the plant part or plant phase to be transformed: **seedlings** and plants in **flowering stage.** The method entails suspending *Agrobacterium* cells containing a vector with a gene of interest in vacuum infiltration medium, and the plant portions to be transformed are immersed in the suspension and subjected to vacuum infiltration. Infiltrated plants subsequently produce transformed seeds from which transformed plants are obtained. More information about these applications.

Transformation of monocot plants

- **Paradigm Genetics** has a PCT application disclosing the use of **vacuum infiltration** to transform **monocots** with *Agrobacterium*. The claims as filed are limited to the use of a monocot flower as tissue to be transformed with *Agrobacterium* and subjected to vacuum. The **apparatus** to perform the vacuum infiltration is also part of the filed claims. In addition, the disclosure describes the transformation of **rice** by applying vacuum to a **rice panicle** immersed in an *Agrobacterium* suspension.

  This disclosure is discussed in detail under the section Monocots - General transformation methods .

Patent applications filed by The Samuel Roberts Noble Foundation

The present European applications describe methods for direct plant transformation via *Agrobacterium* **using vacuum**. In one of the disclosures, *Agrobacterium* containing a vector with a gene of interest contacts the aerial portions of a plant at **flowering stage** under vacuum conditions. The vacuum applied is of sufficient strength to force the *Agrobacterium* cells into intimate contact with the plant such that the T-DNA transfer to the plant takes place.

A plant at flowering stage is defined by the inventors as a plant form about the beginning of the first flower bud formation to about the time of the last flower set. The flowering plants are grown from vernalised seeds. These are seeds subjected to a period of chilling before germination. The seeds are incubated at 4°C for a period of time, preferably in the dark, and kept moist. Vernalisation is used to speed up the formation of flowers.

In the other disclosure, the material selected for transformation is a **seedling**.

As part of the both disclosures, the plant material is transformed with a mix of *Agrobacterium* cells containing different T-DNA to be inserted into the plants. The transformed plant is allowed to grow into maturity and produce seeds. Progeny from the seed is selected by the use of selectable markers and the presence of an additional transferred gene.

Bibliography

	EP 1 141 356 A2	EP 1 171 618 A2
Title	Plant transformation process	
Application No. and Date	EP 99968550 December 23, 1999	EP2000923550 April 20, 2000
Publication Date	October 10, 2001	January 16, 2002
Language	English	
Related applications	Australia (AU 200025943 A5)	Australia (AU 200043652 A5)
Remarks	The present PCT applications are not related to one another as they have different priority patent documents.	
	View Claims	View Claims

To view or download the patent applications as a PDF file, click on EP 1 141 356 A2 (WO 0037663 A2) (1,850 kb) and EP 1 171 618 A2 (WO 0063400 A2) (1,684 kb).

Summary of the invention

The claims as filed of the European application **EP 1 141 356 A2** recite:

- a method for direct transformation of seedlings with *Agrobacterium* by placing the plant material with *Agrobacterium* having a vector with a gene of interest in its T-DNA and applying vacuum to them so *Agrobacterium* enters into contact with the seedling and transfers the T-DNA to the plant cells;
- a method as the one described above but the material is in contact with a mixture of *Agrobacterium* cells harboring different genes in their T-DNAs; and
- a method as the one first mentioned where after the transformation plants s et seed and the progeny from these seeds is selected for the presence of a gene of interest and a selectable marker.

The claims of the European application **EP 1 171 618 A2** recite similar methods to the ones described above. However, the material subjected to vacuum infiltration are the aerial parts of a flowering plant.

Vacuum infiltration of seedling plants
Patent application filed by The Samuel Roberts Noble Foundation

Actual pending claims

EP 1 141 356 A2
Claim 1 A method for direct plant transformation using seedlings and *Agrobacterium* comprising: A) contacting at least one seedling with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to said seedling; B) applying a vacuum to said seedling in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said seedling such that

said *Agrobacterium* cells transfer said T-DNA to cells of said seedling at a second time, wherein said first and second time are the same or different.

Claim 9

A method for direct plant transformation using seedlings and *Agrobacterium* comprising: A) contacting at least one seedling with a mixture of *Agrobacterium* cells, said mixture comprising cells from a *Agrobacterium* strain harboring a vector with a DNA fragment and cells from said *Agrobacterium* strain harboring said vector a second DNA fragment, said vector enabling said *Agrobacterium* cells to transfer said T-DNA to said seedling;
B) applying a vacuum to said seedling in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said seedling such that said *Agrobacterium* cells transfer T-DNA to cells of said seedling at a second time, wherein said first and second time are the same or different.

Claim 18

A method for direct plant transformation using seedlings and *Agrobacterium* comprising: A) contacting at least one seedling with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment and a selectable marker gene to said seedling;
B) applying a vacuum to said seedling in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said seedling such that said *Agrobacterium* cells transfer said T-DNA to cells of said seedling at a second time, wherein said first and second time are the same or different;
C) allowing said transformed seedling to grow to maturity and set seed;
D) germinating said seed to form progeny;
E) exposing said progeny to an agent enabling detection of selectable marker gene expression;
F) selecting for progeny expressing said selectable marker gene and at least one gene, said expression of said selectable marker gene and at least one gene indicating gene transfer.

Vacuum infiltration of flowering plants
Patent application filed by The Samuel Roberts Noble Foundation

Actual pending claims

EP 1 171 618 A2

Claim 1

A method for direct plant transformation using plants and *Agrobacterium* comprising: A) contacting the aerial portions of at least one plant at the time of flowering with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to said plant; and
B) applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer said T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different.

Claim 9

A method for direct transformation of a plant comprising: A) vernalizing and germinating initial seed to form said plant contacting the aerial portions of said plant at the time of flowering with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment to said plant; and
B) applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer said T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different.

Claim 17

A method for direct plant transformation using plants at the time of flowering and *Agrobacterium* comprising:
A) contacting aerial portions of at least one plant at the time of flowering with a mixture of *Agrobacterium*

cells, said mixture comprising cells from a *Agrobacterium* strain harboring a vector with a DNA fragment and cells from said *Agrobacterium* strain harboring said vector with a second DNA fragment, said vector enabling said *Agrobacterium* cells to transfer said T-DNA to said plant; and
B) applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different.

Claim 25

A method for direct transformation of a plant at the time of flowering comprising: A) vernalizing and germinating initial seed to form said plant contacting aerial portions of said plant at the time of flowering with a mixture of *Agrobacterium* cells, said mixture comprising cells from a *Agrobacterium* strain harboring a vector with a DNA fragment and cells from said *Agrobacterium* strain harboring said vector with a second DNA fragment, said vector enabling said *Agrobacterium* cells to transfer said T-DNA to said plant; and
B) applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different.

Claim 33

A method for direct plant transformation using plants at the time of flowering and *Agrobacterium* comprising: A) contacting aerial portions of at least one plant at the time of flowering with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment and a selectable marker gene to said plant;
B) applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer said T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different;
C) allowing said transformed plant to grow to maturity and set seed;
D) germinating said seed to form progeny;
E) exposing said progeny to an agent enabling detection of selectable marker gene expression; and
F) selecting for progeny expressing said selectable marker gene and at least one gene, said expression of said selectable marker gene and at least one gene indicating gene transfer.

Claim 36

A method for direct transformation of a plant at the time of flowering comprising: A) vernalizing and germinating initial seed to form said plant;
B) contacting aerial portions of said plant at the time of flowering with *Agrobacterium* cells, said *Agrobacterium* cells harboring a vector, said vector enabling said *Agrobacterium* cells to transfer T-DNA containing at least one gene or gene fragment and a selectable marker gene to said plant;
C) applying a vacuum to said plant portions in contact with said *Agrobacterium* cells at a first time, said vacuum of sufficient strength to force said *Agrobacterium* cells into intimate contact with said plant such that said *Agrobacterium* cells transfer said T-DNA to cells of said plant at a second time to form a transformed plant, wherein said first time and said second time are the same or different;
D) allowing said transformed plant to grow to maturity and set seed; germinating said seed to form progeny;
E) exposing said progeny to an agent enabling detection of selectable marker gene expression; and
F) selecting for progeny expressing said selectable marker gene and at least one gene, said expression of said selectable marker gene and at least one gene indicating gene transfer.

Monocots

Overview

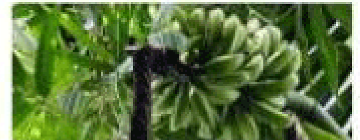
Monocots (Monocotyledonous) comprise one of the large divisions of Angiosperm plants (flowering plants with seeds protected within a vessel). They are herbaceous plants with parallel veined leaves and have an embryo with a single cotyledon, as opposed to dicot plants (dicotyledonous), which have an embryo with two cotyledons.

Most of the important staple crops of the world, the so-called cereals, such as wheat, barley, rice, maize, sorghum, oats, rye and millet, are monocots. Other food crops such as onion, garlic, ginger, banana, plantain, yam and asparagus are also classified as monocots.

Agrobacterium-mediated transformation of commercially important monocots was first attained in rice and maize in the mid 90's. Following these achievements, other monocot crops were successfully transformed and refinements of techniques led to improved regeneration of transformed monocot tissue.

In this section of the document, the selected patents directed to *Agrobacterium* transformation of monocots are categorized as:

- General transformation methods, regardless the vector type used for transformation.
- **Monocot plants**, which are divided into:
  - Gramineae and Cereals, which are large monocot groups.
  - Particular plants, including grains, tropical fruits and flowers. In alphabetical order, patents on *Agrobacterium*-mediated transformation of the following monocots are analyzed individually: banana, barley, duckweed, gladiolus, maize, onions, pineapple, rice, sorghum, turfgrass and wheat. The inventions cover aspects such as the initial tissue used for transformation, transformation protocols, media composition, and in some cases, the insertion of particular genes.



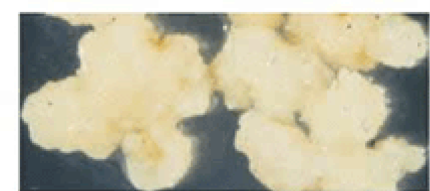
General Monocot Transformation Methods

Summary

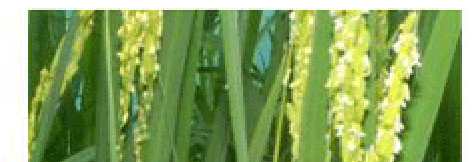
Japan Tobacco (in Japan), **Rhône-Poulenc Agro** (in France), **University of Guelph** (in Canada) and recently, **Paradigm Genetics** (in the US), **the Department of Primary Industries of Queensland** (AU) and the **National Institute of Agrobiological Resources** (in Japan) have granted patents or patent applications directed to methods for *Agrobacterium*-mediated transformation of any monocot with a gene of interest. The main difference among them lies in:

- the initial plant tissue or explant used for the transformation process, and
- the application of additional treatments, such as vacuum infiltration or the addition of phenolic compounds to facilitate the transformation process.

Japan Tobacco claims the transformation of a **monocot callus** during a dedifferentiation process and the transformation of the **scutellum of an immature embryo** prior to dedifferentiation. Thus, these patents granted in the United States and Australia cover transformation of monocot tissues that are widely and commonly used. In the United States, the breadth of the claimed monocot callus transformation is limited only by the minimum number of days in culture before the transformation process takes place. This time limit is not claimed in either the Australian patent or European patent application.



Rhône-Poulenc Agro (now Bayer Crop Science) claims the transformation of a **monocot inflorescence** via *Agrobacterium*. The inflorescence can be dissected and then transformed. Alternatively, callus formation is induced from an inflorescence in culture, and the derived callus is transformed with *Agrobacterium*. A transgenic monocot plant is then



regenerated from the transformed inflorescence-derived callus. The invention is thus limited to transformation of a monocot inflorescence. Other tissues are not part of the scope of the claims.

In contrast to the previous two inventions that disclose particular tissue types for transformation, the **Paradigm Genetics** application discloses the use of **vacuum infiltration in the presence of a phenolic compound** for monocot transformation with *Agrobacterium*. The major limitation of the claims as filed in the patent application is the use of a **monocot flower**. An **apparatus to perform vacuum** infiltration of the monocot plant is also part of the disclosed invention. It remains to be seen what claim scope is ultimately granted.

The Department of Primary Industries of Queensland does not disclose a particular tissue to be transformed in its PCT patent application. Neither are particular conditions stated for the transformation process. One limitation of the invention consists of formation of an **organogenic callus** by the transformed plant cells.

The **University of Guelph** has a US application derived from a PCT application. An Australian application lapsed in 2002. The applications describe the use of vacuum infiltration in combination with a phenolic compound for the transformation of a monocot with *Agrobacterium*.

The **National Institute of Agrobiological Resources** (Japan) have a PCT and a European application that disclose a method for transforming a monocot by treatment of intact seed with *Agrobacterium* containing a recombinant gene of interest.

Patents and applications assigned to Japan Tobacco

The following patents appear to be some of the broadest patents granted on *Agrobacterium*-transformation of monocots.

In this invention an explant of a monocot in the process of dedifferentiation or already dedifferentiated is used for transformation with *Agrobacterium*. A dedifferentiating tissue or a tissue in the process of dedifferentiation is described by the inventors as an explant cultured on a dedifferentiation medium for not less than 7 days. The cultured tissue can be a callus, an embryo-like tissue derived from any explant, or suspension cells.

According to the scientific literature, differentiation of a cell is the process through which a cell becomes specialized to perform a particular function. A dedifferentiation process is thus the opposite of specialization. During cell dedifferentiation, more random planes of cell division increase progressively and there is a loss of organized structures.

Patents granted to Japan Tobacco

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 5591616 A • Earliest priority - 7 July 1992 • Filed - 3 May 1994 • Granted - 7 Jan 1997 • Expected expiry - 2 May 2014	**Title - Method of transforming monocotyledon** **Claim 1** A method for transforming a monocotyledon callus, comprising contacting a cultured tissue of a monocotyledon during dedifferentiation wherein said dedifferentiation is obtained by culturing an explant on a dedifferentiation-inducing medium **for not less than 7 days** or a dedifferentiated cultured tissue of a monocotyledon, with a bacterium belonging to the genus *Agrobacterium* containing a desired gene. **Claim 17** A method for transforming a monocotyledon with a desired gene, comprising: A) contacting a cultured tissue of said monocotyledon during dedifferentiation thereof, or a dedifferentiated cultured tissue of said	Japan Tobacco

monocotyledon, with a suspension of *Agrobacterium tumefaciens* having a cell population of 10^6 to 10^{11} cells/ml for 3-10 minutes, and then
B) culturing said cultured tissue of said monocotyledon during dedifferentiation thereof, or said dedifferentiated cultured tissue of said monocotyledon, on a solid medium for several days together with said *Agrobacterium tumefaciens*, or
C) adding said *Agrobacterium tumefaciens* to culture medium in which said cultured tissue of said monocotyledon during dedifferentiation thereof or said dedifferentiated cultured tissue of said monocotyledon is cultured, and continuously culturing said cultured tissue of said monocotyledon during dedifferentiation or said dedifferentiated cultured tissue of said monocotyledon together with said *Agrobacterium tumefaciens*,

wherein said dedifferentiated cultured tissue of said monocotyledon is selected from the group consisting of a tissue cultured during the process of callus formation which is cultured for not less than 7 days after an explant is placed on a dedifferentiation-inducing medium and a callus, and wherein said *Agrobacterium tumefaciens* contains plasmid pTOK162, and said desired gene is present between border sequences of the T region of said plasmid pTOK162, or wherein said desired gene is present in another plasmid contained in said *Agrobacterium tumefaciens*.

The patent discloses that "the term 'dedifferentiated tissue' ...means a callus or an adventitious embryo-like tissue obtained by culturing an explant in a medium containing a plant growth regulator such as an auxin or a cytokinin."

US 7060876

- Earliest priority - 7 July 1992
- Filed - 13 Jan 1999
- Granted - 13 Jun 2006
- Expected expiry - 2 May 2014

Title - Method for transforming monocotyledons

Claim 1

A method for transforming a monocot plant comprising:

i) culturing an explant of said monocot plant, or a tissue isolated from said explant, for one to six days in a medium comprising at least one auxin to obtain a cultured tissue;
ii) co-culturing the cultured tissue from step i) with an *Agrobacterium* bacterium comprising a polynucleotide of interest;
iii) selecting cultured tissue into which the polynucleotide of interest has been introduced; and
iv) culturing the selected tissue on a regeneration medium to obtain a transformed monocot plant.

Claim 16

A method for transforming a tissue of a monocot plant comprising:

i) culturing an explant of an immature tissue of a monocot plant for one to six days on a medium comprising at least one auxin and that induces dedifferentiation of the cells of the explanted tissue to obtain a dedifferentiating or dedifferentiated cultured immature tissue;
ii) contacting the dedifferentiating or dedifferentiated cultured immature tissue with cells of *Agrobacterium* bacteria that comprise a vector comprising at least one virulence gene of a Ti plasmid, a left T-DNA border, a right T-DNA border and a polynucleotide of interest located between the left T-DNA border and the right T-DNA border;
thereby obtaining a transformed plant tissue.

Claim 20

A method for obtaining a transformed monocot plant comprising

i) culturing an explant of an immature tissue of a monocot plant for one to

six days on a medium comprising at least one auxin and that induces dedifferentiation of the cells of the explanted tissue to obtain a dedifferentiating or dedifferentiated cultured immature tissue;
ii) contacting the dedifferentiating or dedifferentiated cultured immature tissue with cells of *Agrobacterium* bacteria that comprise a vector comprising at least one virulence gene of a Ti plasmid, a left T-DNA border, a right T-DNA border and a polynucleotide of interest located between the left T-DNA border and the right T-DNA border; thereby obtaining a transformed plant tissue; and
iii) culturing the transformed plant tissue on at least one regeneration medium, thereby obtaining a transformed monocot plant.

Granted US 7060876 is a continuation of US 08/668464 (now abandoned), which is a continuation-in-part of now granted US 5591616.

The claims are generally drawn towards:

- a method for transforming a monocot plant comprising culturing an explant or a tissue from an explant for **one to six days** in a medium comprising at least one auxin (claim 1)
- a method for transforming a tissue of a monocot plant comprising culturing an explant of an immature tissue for **one to six days** on a medium comprising at least one auxin (claim 16)
- a method for obtaining a transformed monocot plant comprising culturing an explant of an immature tissue for **one to six days** on a medium comprising at least one auxin (claim 20)

Granted US 7060876 and US 5591616 together cover the whole duration of the dedifferentiation period of monocot plant tissue that can be used as transformation material by *Agrobacterium* in the United States.

The term "less than seven days" in the application US 2002/0178463 has been changed to "one to six days", and there is no limit on the type of plasmid that is contained in the *Agrobacterium* that is used to transform the monocot plant in granted US 7060876.

US 2002/0178463

- Earliest priority - 7 July 1992
- Filed - 13 Jan 1999
- Pubished - 28 November 2002
- Granted as US 7060876 (see above)
- Expected expiry - N/A

Title - Method of transforming monocotyledons

Claim 1

A method for transforming a monocotyledon, comprising contacting a cultured tissue of said monocotyledon during dedifferentiation thereof obtained by culturing an explant on a dedifferentiation-inducing medium for less than 7 days with a bacterium belonging to the genus *Agrobacterium* containing a super binary vector having the virulence region of Ti plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281, left and right border sequences of T-DNA of a Ti plasmid or an Ri plasmid of a bacterium belonging to the genus *Agrobacterium*, and a desired gene located between said left and right border sequences.

Claim 13

A method for transforming a monocotyledon, comprising contacting a cultured tissue of said monocotyledon during dedifferentiation thereof obtained by culturing an explant derived from an immature tissue on a dedifferentiation-inducing medium for less than 7 days with a bacterium belonging to the genus *Agrobacterium* containing a desired gene and containing a vector having the virulence region of Ti plasmid contained in *Agrobacterium tumefaciens*.

United States patent application **US 2002/0178463** is a

	continuation-in-part of application US 08/193,058 (now patent US 5,591,616, see above). The content of this application is very similar to the parent application. The main difference in the claims is that independent Claim 1 recites the use of a superbinary vector having the virulence region of a defined Ti plasmid and contained in a specific strain of *Agrobacterium tumefaciens* and tissue is cultured in dedifferentiating medium for **less than** 7 days. In claim 13, the explant is derived from an immature tissue.The application discloses that the "term 'immature' means that the tissue has not reached the matured state of the tissue and will mature under conditions which allow the maturation." The description doesn't clarify what physiological or phenotypic properties an immature tissue has but merely basically restates that "immature" means not mature.	
AU 667939 B • Earliest priority - 7 July 1992 • Filed - 6 July 1993 • Granted - 18 April 1996 • Expected expiry - 5 July 2013	**Title - Method of transforming monocotyledon** The lead claim in the Australian patent **AU 667 939 B** is broader than in the United States patent. In the Australian patent, a dedifferentiating or dedifferentiated tissue of a monocot is also used as the initial tissue for transformation, but there is no restriction with respect to a minimum number of days of culture in the medium to induce dedifferentiation. Claim 1 A method for transforming a monocotyledon comprising transforming a cultured tissue during dedifferentiation process or a dedifferentiated cultured tissue of said monocotyledon with a bacteria belonging to genus *Agrobacterium* containing a desired gene.	
EP 604662 A1 • Earliest priority - 7 July 1992 • Filed - 6 July 1993 • Application pending	**Title - Method of transforming monocotyledon** The claims submitted in the European application **EP 604 662 A1** are the same as the claims of the Australian patent.	
Remarks	National phase entry of PCT application WO 94/0977 in Canada (CA 2121545) is still pending.	

Note: Patent information on this page was last updated on 3 February 2006.

Patents and application assigned to Japan Tobacco

This family of patents discloses the use of an immature embryo of a monocot for *Agrobacterium*-mediated transformation. Within the embryo, the tissue to be transformed is the scutellum, which is the name given to the cotyledon of monocot plants. The transformed scutellum can be induced to become dedifferentiated calli that have the ability to regenerate normal plants after transformation.

In addition, they disclose an *Agrobacterium* used for transformation that contains a Ti or Ri (root-inducing) plasmid with a desired gene and a plasmid having a virulence region derived from the *A. tumefaciens* Ti plasmid pTiBo542.

Specific Patent Information

<table>
<tr><th>Patent Number</th><th>Title, Independent Claims and Summary of Claims</th><th>Assignee</th></tr>
<tr><td>AU 687863 B
Earliest priority – 3 September 1993
Filed – 1 Sept 1994
Granted – 5 March 1998
Expected expiry – 31 Aug 2014</td><td>Title – Method of transforming monocotyledon by using scutellum of immature embryo

The claims of the Australian patent AU 687863 are directed to
a method for transforming a scutellum of an immature embryo of a monocotyledon with Agrobacterium having a desired gene. The embryo is not submitted to a dedifferentiation process prior to transformation with Agrobacterium.
Claim 1

A method for transforming monocotyledons comprising transforming scutellum of an immature embryo of a monocotyledon with a bacterium belonging to genus Agrobacterium containing a desired gene, which immature embryo has not been subjected to a dedifferentiation treatment, to obtain a transformant.</td><td rowspan="4">Japan Tobacco</td></tr>
<tr><td>EP 672752 B1
Earliest priority – 3 September 1993
Filed – 1 Sept 1994
Granted – 26 May 2004
Expected expiry – 31 Aug 2014</td><td>Title – Method of transforming monocotyledon by using scutellum of immature embryo

Claim 1

A method for transforming monocotyledons comprising transforming scutellum of an immature embryo of a monocotyledon with a bacterium belonging to genus Agrobacterium containing a desired gene, which immature embryo has not been subjected to a dedifferentiation treatment, to obtain a transformant.

The independent claim is the same as the Australian patent.

Designated contracting States at the time of grant are: Austria, Belgium, Switzerland, Germany, Denmark, Spain, France, United Kingdom, Greece (reported on INPADOC as lapsed), Ireland, Italy, Liechtenstein, Luxembourg, Monaco (reported on INPADOC as lapsed), Netherlands, Portugal, Sweden.</td></tr>
<tr><td>EP 672752 A1
Earliest priority – 3 September 1993
Filed – 1 September 1994
OPI – 20 September 1995
Granted as EP 672752 B1 (see above)</td><td>Title – Method of transforming menocotyledon by using scutellum of immature embryo

This application has been granted as EP 672752 B1 (see above).

The independent claim in this application has been granted without amendments.</td></tr>
<tr><td>Remarks</td><td>The United States application No. 428238, corresponding to a PCT application, was filed on 3 May 1995. Specification and claims of the PCT application WO 956722 are in Japanese.
National phase entry of the PCT application WO 956722 in Japan (JP 3329819) has been granted on Sept 30 2002.
National phase entry of the PCT application WO 956722 in Canada</td></tr>
</table>

Note: Patent information on this page was last updated on 2 February 2006.

Patent assigned to Rhône-Poulenc Agro

The invention disclosed in the following patent is directed to the use of an inflorescence of a monocot as a target tissue to be transformed with *Agrobacterium*. In the method, a dissected inflorescence or a callus derived from the dissected inflorescence is co-cultivated with *Agrobacterium*.

Specific Patent Information

<table>
<tr><th>Patent Number</th><th>Title, Independent Claims and Summary of Claims</th><th>Assignee</th></tr>
<tr><td>US 6037522 A
Earliest priority - 23 June 1998
Filed - 23 June 1998
Granted - 14 March 2000
Expected expiry - 22 June 2018</td><td>Title- Agrobacterium-mediated transformation of monocots

Claim 1

A method of transforming a monocot comprising co-cultivating a monocot inflorescence with Agrobacterium containing a plasmid comprising a heterologous nucleic acid.

Claim 5

A method of transforming a monocot comprising:

A) dissecting an inflorescence from a monocot;
B) initiating a callus from the inflorescence to generate an inflorescence-derived callus; and
C) co-cultivating the inflorescence-derived callus with Agrobacterium containing a plasmid comprising a heterologous nucleic acid.

Claim 13

A method of making a transgenic monocot comprising:

A) dissecting an inflorescence from a monocot;
B) initiating a callus from the inflorescence to generate an inflorescence-derived callus;
C) co-cultivating the inflorescence-derived callus with Agrobacterium containing a plasmid comprising a heterologous nucleic acid; and
D) regenerating a transgenic monocot from the callus.

Claim 16

A method of making a transgenic monocot comprising:

A) dissecting an inflorescence from a monocot;
B) co-cultivating the inflorescence with Agrobacterium containing a plasmid comprising a heterologous nucleic acid;
C) initiating a callus from the inflorescence; and
D) regenerating a transgenic monocot from the callus.

The United States patent US 6037522 claims
an inflorescence of a monocot transformed with Agrobacterium having foreign DNA;
a method where the inflorescence to be transformed is first dissected and then co-cultivated with Agrobacterium; and
a method where the inflorescence to be transformed is first cultured in a medium to induce callus formation and then the callus is transformed with Agrobacterium.</td><td>Rhône-Poulenc Agro

(now Aventis CropScience, now owned by Bayer)</td></tr>
</table>

Remarks	The related Australian application **AU 46163/99 A1** was **abandoned** on **March 15, 2001.**	

Note: Patent information on this page was last updated on 2 February 2006.

Patent application filed by Paradigm Genetics Inc.

The present disclosure refers to transformation of monocot plants with *Agrobacterium* using **vacuum infiltration**. The explant to be transformed is a **flower** . The monocot flower is in contact with a solution containing *Agrobacterium* while the vacuum is applied. An **apparatus** to carry out the vacuum infiltration is also described.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
WO 0112828 A1 • Earliest priority - 18 August 1999 • Filed - 17 August 2000 • OPI - 22 February 2001	**Title** - Methods and apparatus for transformation of monocotyledonous plants using *Agrobacterium* in combination with vacuum filtration **Claim 1** An *in planta* method of transforming a monocotyledonous plant comprising: A) contacting at least one flower of the monocotyledonous plant with a solution or suspension comprising an *Agrobacterium* clone; and B) subjecting said plant to a vacuum effective to cause entry of the *Agrobacterium* clone into at least one flower of the plant. **Claim 40** An apparatus for the transformation of a monocotyledonous plant, comprising: 1. a vacuum chamber of sufficient size to contain at least one monocotyledonous plant; 2. means for generating a vacuum; 3. a connector that connects the means for generating a vacuum with the vacuum chamber; and 4. means for affixing the monocotyledonous plant inside the vacuum chamber. The present PCT application recites: • an *in planta* method of transforming a **monocot flower** by contacting the plant part with *Agrobacterium* in suspension or in solution and applying **vacuum** to cause the entry of *Agrobacterium* into the plant flower. • an **apparatus** for transforming a monocot plant comprising a vacuum chamber of sufficient size to contain the plant, means to generate the vacuum and affix the plant inside the chamber. The present application also contains independent claims reciting methods for transforming **rice** plants. They are discussed under the section Particular monocots - Rice.	Paradigm Genetics Inc
Remarks	A related patent application filed in Australia (**AU 67807/00**) has **lapsed** on 2 May 2002.	

Note: Patent information on this page was last updated on 2 February 2006.

Patent application filed by The Department of Primary Industries of Queensland

The present disclosure refers to transformation of monocot cells through *Agrobacterium*. The explant to be transformed can be any. An advantage of the disclosed method according to the applicants is that the transformed monocot cells form an **organogenic callus** instead of an embryogenic callus. A modified monocot plant is regenerated from the organogenic callus formed by the selected transformed plant cells.

The applicants do not provide a definition for either organogenic or embryogenic callus. According to commonly accepted definitions in the scientific literature (which may or may not be valid for construing the patent claims), in organogenesis the shoot or root organ, usually a shoot, is induced to form first, followed by root or shoot formation from that shoot or root. In the latter callus type, embryo-like structures develop, called somatic embryos, that then simultaneously develop shoots and roots. Use of embryogenic callus, according to the applicants, is time consuming, labor intensive and not always successful.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
WO 01/33943 A1 • Earliest priority - 5 November 1999 • Filed - 3 November 2000 • OPI - 17 May 2001	Title - A method of plant transformation **Claim 1** A method of transforming cells of a monocotyledonous plant with genetic material, said method comprising: A) obtaining an explant from said plant; B) co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without said *Agrobacterium* overgrowing the plant cells; and C) selecting for the transformed plant cells and permitting the cells to form organogenic callus. **Claim 21** A method for producing a genetically modified monocotyledonous plant, said method comprising: A) obtaining explant from a plant to be genetically modified; B) co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising genetic material to be transformed into said plant cells for a time and under conditions sufficient for the genetic material to transfer to plant cells without said *Agrobacterium* overgrowing the plant cells; C) selecting transformed plant cells and permitting the cells to form organogenic callus; and then D) regenerating a plant from selected transformed plant cells. **Claim 41** A method for producing a genetically modified monocotyledonous plant, said method comprising: A) obtaining an explant from said plant to be genetically modified; B) co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without the *Agrobacterium* overgrowing the plant cells; C) selecting for the transformed plant cells and permitting the cells to form organogenic callus; and D) regenerating a plant from said transformed organogenic	The Department of Primary Industries of Queensland

callus.

The present PCT application recites:

- method of transforming cells of a monocot plant by co-cultivating an explant with *Agrobacterium* having a T-DNA with genetic material to be transferred into the plant cells. The bacteria **does not** overgrow the plant cells and the selected transformed cells form an **organogenic callus**.
- a method for producing modified monocot plants by regenerating a plant from the organogenic callus containing the transformed cells.

The present application also contains independent claims directed to methods for transforming of pineapple plants (Claims 60 and 77). They are discussed under the section **Particular monocots** - Pineapple.

AU 779510 B2

- Earliest priority - 5 November 1999
- Filed - 3 Nov 2000
- Granted - 27 Jan 2005
- Expected expiry - 2 Nov 2020

Title - A method of plant transformation

Claim 1

A method of transforming cells of a **pineapple** plant with genetic material, said method comprising:

A) obtaining an explant from said plant;
B) co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without said *Agrobacterium* overgrowing the plant cells; and
C) selecting for the transformed plant cells and permitting the cells to form organogenic callus.

Claim 19

A method for producing a genetically modified **pineapple** plant, said method comprising:

A) obtaining explant from a plant to be genetically modified;
B) co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising genetic material to be transformed into said plant cells for a time and under conditions sufficient for the genetic material to transfer to plant cells without said *Agrobacterium* overgrowing the plant cells;
C) selecting transformed plant cells and permitting the cells to form organogenic callus; and then
D) regenerating a plant from selected transformed plant cells.

Claim 37

A method for producing a genetically modified **pineapple** plant, said method comprising:

A) obtaining an explant from said plant to be genetically modified;
B) co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without the *Agrobacterium* overgrowing the plant cells;
C) selecting for the transformed plant cells and permitting the cells to form organogenic callus; and

	D) regenerating a plant from said transformed organogenic callus. This granted patent is a national phase entry of WO 01/33943 (see above). All three independent claims in the granted patent are limited to a "pineapple plant" instead of a "monocotyledonous plant".	

Note: Patent information on this page was last updated on 5 February 2006.

Patent application filed by the University of Guelph (Canada)

The present disclosure is directed to transformation of any explant from monocot plants using *Agrobacterium*. According to the applicants, an advantage of the disclosed method is that vacuum infiltration of the tissue in the presence of *Agrobacterium* and a phenolic compound such as acetosyringone yields better transformation efficiency than by wounding. According to the inventors, the method is also simpler than previously described methods. Because the known patent applications have lapsed, the disclosed methods may be in the public domain. If you'd like to use it and are uncertain that it is in the public domain in your jurisdiction, it may be best to contact the assignee, for which a recent contact address disclosed on the website is plorenz@uoguelph.ca.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
WO 0058484 A2 • Earliest priority - 26 March 1999 • Filed - 26 September 2001 • OPI - 5 October 2000	**Title** - Transformation of monocotyledoneous plants using *Agrobacterium* **Claim 1** A method for the transformation of a monocot plant comprising, i) exposing explant tissue of said monocot plant to an *Agrobacterium* strain under vacuum in the presence of a phenolic compound, said *Agrobacterium* strain comprising a heterologous gene of interest within a vector; ii) removing said *Agrobacterium* from said explant tissue; iii) adding an antibiotic against said *Agrobacterium*; and iv) selecting explant tissue for occurrence of said heterologous gene of interest. **Claim 10** A method for the transformation of a monocot plant comprising, i) placing explant tissue of said monocot plant into media comprising a suspension of *Agrobacterium* to obtain a mixture, said *Agrobacterium* strain comprising a heterologous gene of interest within a vector; ii) maintaining said mixture under vacuum in the presence of acetosyringone; iii) releasing said vacuum and further incubating said explant tissue in the presence of said *Agrobacterium*; iv) transferring said explant tissue to fresh media comprising acetosyringone and incubating said explant tissue in the dark v) washing said explant tissue with an antibiotic against said *Agrobacterium*, vi) transferring said explant tissue to fresh media and allowing said explant tissue to differentiate, thereby producing differentiated calli; vii) placing said differentiated calli onto media containing a selection agent, and maintaining said differentiated calli in the light; and viii) obtaining calli that grow in the presence of the selection agent.	University of Guelph (Canada)

	Claim 17 A method for the transformation of a monocot plant comprising, i) placing explant tissue of said monocot plant into media comprising a phenolic compound, and a suspension of *Agrobacterium* to obtain a mixture, said *Agrobacterium* strain comprising a heterologous gene of interest within a vector; ii) washing said explant tissue with an antibiotic against said *Agrobacterium* and transferring said explant tissue to fresh media comprising acetosyringone and incubating said explant tissue in the dark; iii) transferring said explant tissue to fresh media and allowing said explant tissue to differentiate, thereby producing differentiated calli; iv) placing said differentiated calli to media containing a selection agent, and maintaining said differentiated calli in the light; and v) obtaining calli that grow in the presence of the selection agent. The present PCT application recites: • method of transforming monocot plant • by vacuum infiltration of a plant tissue • with *Agrobacterium* having a heterologous gene of interest on a vector • in the presence of a phenoloic compound, e.g. acetosyringone.	
Remarks	Related applications in Australia (AU 112261/00), Canada (CA 2368841), and US (US 2002/112261) have lapsed or been withdrawn.	

Note: Patent information on this page was last updated on 5 February 2006.

Patent application filed by the National Institute of Agrobiological Resources (JP)

The present disclosure is directed to transformation of monocot plants by infecting intact seed with *Agrobacterium*. Dependent claims recite that the seed is pre-germinated. Gramineae and more specifically rice are preferred embodiments mentioned in dependent claims.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Asignee
EP 1 198 985 A1 • Earliest priority - 22 July 1999 • Filed - 22 July 1999 • Application pending	**Title** - Method for super-rapid transformation of monocotyledon **Claim 1** A method for transforming a monocotyledon, comprising a step of infecting an intact seed with an *Agrobacterium* which contains a desired recombinant gene. The present EP application recites: • method of transforming monocot plant by infecting intact seed of a monocot with *Agrobacterium*. Note: because intact seed are mentioned in the broadest claim, any damage to the seed could be outside the scope of the patent claims, thus creating a possible way to circumvent the claims without infringement.	National Institute of

AU775233 B2 • Earliest priority - 22 July 1999 • Filed - 22 July 1999 • Granted - 22 July 2004 • Expected expiry - 21 July 2019	**Title** - Method for super-rapid transformation of monocotyledon **Claim 1** A method for transforming a monocotyledon, comprising a step of infecting an intact seed with an *Agrobacterium* which contains a desired recombinant gene wherein the seed is a germinated seed which is germinated by preculturing four to five days after sowing on a medium comprising 2,4-D. The granted independent claim of this patent is limited to intact seed that has been precultured for four to five days with 2,4-D.	Agrobiological Resources (JP)
Remarks	National phase entry of PCT application WO 01/06844 in Canada (CA 2366104) and China (CN 1352522) are still pending.	

Note: Patent information on this page was last updated on 5 February 2006.

Gramineae and Cereals

Summary

Gramineae is one of the largest families of monocot plants. Mostly herbaceous, grass-like plants, this family includes several important staple crops (cereals) such as wheat, rice, maize, sorghum, barley, oats, and millet. It also encompasses plants such as bamboos, palms, and foraging grasses (*e.g.* turfgrass, king grass (*Pennisetum purpureum*), *Brachiaria*).

Remember that,

patents addressing the Gramineae family embrace cereals,
but patents directed to cereals *do not embrace* all Gramineae.

Gramineae transformation. The United States and Australian patents granted to the **University of Toledo** and the United States patent granted to **Goldman and Graves** belong to the same patent family. They all claim a method for transforming seedlings of a Gramineae with a *vir*+ *Agrobacterium*. Furthermore, in this invention transformed pollen grains are obtained from a transformed seedling. Claims of both United States patents limit the inoculation of the bacterium to a particular area in the seedling.

Remarkably, the United States patent granted to **Goldman and Graves** also contains broad claims to the transformation of Gramineae with *Agrobacterium*. This particular claim encompasses **any Gramineae**, constituting one of the broadest claims recently issued in the area of plant transformation technologies. This could mean that any United States patent claiming *Agrobacterium* transformation of any tissue of a Gramineae may be dominated by this patent. The grant of this patent has wreaked havoc in the scientific community and multiple parties with interest in *Agrobacterium*- mediated transformation of Gramineae.

In 1998 the same inventors filed a related United States patent application **US 2002/0002711 A1** directed to transformation of Gramineae and corn in particular. One of the independent claims recites: "**A transformed Gramineae**". This could be the broadest claim one could think of, aiming to cover genetic transformation with **any technique** of an **entire botanical family** of enormous economic interest. If the above United States patent granted to **Goldman and Graves** caused outrage in the scientific community, the eventual granting of a claim as broad as the one filed in the present patent application could have had a disturbing impact on Gramineae transformation, for already existing transformants as well as for future transgenic Gramineae. Luckily,

according to the USPTO, the patent application has been abandoned.

Cereal transformation. **Plant Genetic Systems** (now part of **Bayer Crop Science**) has a granted United States patent and a European application disclosing the transformation of any cereal with *Agrobacterium*. The most limiting elements in the claims are the wounding of a cereal tissue and the enzymatic disruption of a tissue cell wall before transformation. The European application additionally recites different transformation methods besides *Agrobacterium*. It remains to be seen what claim scope will be granted in Europe.

Patents granted to The University of Toledo

The invention disclosed in the following patents provides a method for transforming of Gramineae with a *vir*+ *A. tumefaciens*. A seedling is inoculated with the bacterium in an area of rapid cell division, which gives rise to germ cell lines. By inoculating this area, transformation of pollen is attained.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims
US 5187073 A • Earliest priority – 30 June 1986 • Filed – 13 November 1989 • Granted – 16 February 1993 • Expected expiry – 15 February 2010	**Title** – Process for transforming Gramineae and the products thereof **Claim 1** A method of producing transformed Gramineae, said method comprising: A) making a wound in a graminaceous seedling with newly emerging radicle and stem, said being made in an area of the seedling containing rapidly dividing cells, wherein said area e the base of the scutellar node to slightly beyond the coleoptile node; and B) inoculating the wound with *vir*+ *A. tumefaciens*. The United States patent **5 187 073** claims • the transformation of a seedling of a Gramineae plant with *vir*+ *A. tumefaciens* in a wo between the scutellar node and the coleoptile node
US 6020539 • Earliest priority – 30 June 1986 • Filed – 27 June 1994 • Granted – February 1 2000 • Expected expiry – 15 February 2010	**Title** – Process for transforming Gramineae and the products thereof **Claim 1** A transformed pollen grain of a Gramineae produced by a plant grown from a seedling infe *vir+ Agrobacterium tumefaciens* containing a vector comprising genetically-engineered T- **Claim 3** A transformed Gramineae plant derived from a seedling infected with *vir+ Agrobacterium t* which contains a vector comprising genetically-engineered T-DNA. **Claim 6** A transformed Gramineae produced by making a wound in a graminaceous seedling with n emerged radicle and stem, the wound being made in an area of the seedling containing rap cells, wherein said area extends from the base of the scutellar node to slightly beyond the node; and inoculating the wound with *vir+ Agrobacterium tumefaciens*. **Claim 17** A transformed Gramineae produced by making a wound in a graminaceous seedling with n emerged radicle and stem, the wound being made in an area of the seedling containing rap cells, wherein said area extends from the base of the scutellar node to slightly beyond the node; and inoculating the wound with *vir+ Agrobacterium tumefaciens*; the transformed Gr

containing a foreign gene which is an opine synthesis gene that is a nopaline synthase gen octopine synthase gene.

Claim 22

An Agrobacterium--mediated transformed Gramineae.

Claim 25

A transformed Gramineae plant comprising a genetically-engineered T-DNA further compr heterologous gene and a transcription unit in operable order.

This patent is a continuation of now abandoned US 08/016600, which is a continuation of r US 5187073.

Although assignment of this patent is not stated in the records provided by the Internationa Documentation Center (INPADOC) and USPTO PAIR, the patent can be licensed through the U **Toledo** according to the information provided by the Office of Technology Licensing of the u

The claims of the United States patent US 6,020,539 embrace the subject matter claimed in States patent US 5,187,073 and the Australian patent AU 606 874 B2 granted to the Univers That is the transformation of a Gramineae seedling in an area of high cellular division with *tumefaciens*. Transformed pollen and plants derived from the seedling inoculated with *vir+ tumefaciens* are also claimed.

United States patent US 6,020,539 further claims

- the transformation of a Gramineae plant with a T-DNA having a foreign gene and a tra unit;
- either a nopaline synthase gene or an octopine synthase gene as a foreign gene;
- the transformation of a Gramineae with *Agrobacterium* (claim 22).

This last, fairly broad claim does not restrict

- the initial tissue to be transformed; any part, form or plant of a Gramineae can be use process;
- the mechanism or procedure to achieve transformation with *Agrobacterium*; or
- the species of *Agrobacterium* used to transform the Gramineae.

AU 606874 B2

- Earliest priority – 30 June 1986
- Filed – 30 June 1987
- Granted – 21 February 1991
- Expected expiry – 29 June 2007

Title – Transformation of Gramineae and products thereof

Claim 1

A method of producing transformed Gramineae, as hereinbefore defined, comprising:
A) making a wound in a seedling in an area of the seedling containing rapidly dividing cells rise to germ line cells; and
B) inoculating the wound with *vir*$^{+}$ *A. tumefaciens*.

The Australian patent **606 874** claims the transformation of a seedling with *vir*$^{+}$ *A. tumefac* but it does not specify the area where the inoculation occurs.

It claims

- wounding of the seedling in an area that gives rise to germ cell lines;
- transformed pollen derived from the transformed seedling having inserted foreign DN
- transformed Gramineae plant derived from the seedling transformed with *A. tumefaci* vector with engineered DNA

<table>
<tr><td>CA 1341455 A1
Earliest priority - 30 June 1986
Filed - 29 June 1987
Granted - 27 April 2004
Expected expiry - 26 April 2021</td><td>Title - Process for transforming Gramineae and the products thereof

Claim 1
A method of producing transformed Gramineae comprising:
A) making a wound in a seedling in an area of the seedling containing rapidly dividing cells
B) inoculating the wound with vir+ A. tumefaciens.

Claim 10
A transformed pollen grain of a Gramineae.

Claim 11
A transformed pollen grain of a Gramineae produced by a plant grown from a seedling infe
Agrobacterium tumefaciens.

Claim 12
A transformed pollen grain of a Gramineae produced by a plant grown from a seedling infe
Agrobacterium tumefaciens which contains a vector comprising genetically-engineered T-[

Claim 13
A transformed pollen grain of a Gramineae produced by a plant grown from a seedling infe
Agrobacterium tumefaciens which contains a vector comprising genetically-engineered T-[

This patent claims
a method of Gramineae transformation by inoculating vir+ Agrobacterium tumefacier seedling that is wounded where the cells are rapidly dividing
a transformed Gramineae pollen grain (this claim is broad due to the fact that there is the method of transformation)</td></tr>
<tr><td>Remarks</td><td>A corresponding patent and its divisional patent has been granted in Japan (JP 26934 3234534 respectively).
A continuation of now granted US 6020539 (US 2002-0002711 A1) has been abandor</td></tr>
</table>

Note: Patent information on this page was last updated on 14 February 2006.

Patent and application assigned to Plant Genetic Systems (now Bayer Crop Science)

The invention disclosed in the United States patent assigned to **Plant Genetic Systems** uses an embryogenic callus of a cereal, **any cereal**, as starting material for transformation with *Agrobacterium*. The tissue is either wounded or treated with an enzyme prior to the transformation process.

Plant Genetic Systems is now part of **Bayer Crop Science**, for which a recent contact address from their website on licensing is annette.josten@bayercropscience.com.

Specific Patent Data

<table>
<tr><th>Patent Number</th><th>Title, Independent Claims and Summary of Claims</th><th>Assignee</th></tr>
<tr><td>US 6074877
Earliest priority - 23 November 1990
Filed - 28</td><td>Title - Process for transforming monocotyledonous plants

Claim 1
A process for the stable integration of a DNA, comprising a gene that is functional in a cell of a cereal plant, wherein said DNA is integrated into the nuclear genome of said cereal plant, said process comprising the</td><td></td></tr>
</table>

<table>
<tr>
<td>May 1998
• Granted - 13 June 2000
• Expected expiry - 27 May 2018</td>
<td>steps of:

A) providing a compact embryogenic callus of said cereal plant;
B) wounding said compact embryogenic callus or treating said compact embryogenic callus with a cell wall degrading enzyme for a period of time so as not to cause a complete disruption of tissues, and transferring said DNA into the nuclear genome of a cell in said compact embryogenic callus by means of Agrobacterium-mediated transformation to generate a transformed cell; and
C) regenerating a transformed cereal plant from said transformed cell.

The United States patent 6,074,877 claims

• Agrobacterium-mediated transformation of an embryogenic callus of a cereal, which is either wounded or treated with an enzyme that degrades cell walls;
• introduction and stable integration of a gene into the nuclear genome of the callus cell;
• regeneration of a transformed plant.</td>
<td>Science)</td>
</tr>
<tr>
<td>EP 955 371 A2

• Earliest priority - 23 November 1990
• Filed - 21 November 1991
• Granted as EP 955371 (see below)</td>
<td>Title - Process for transforming monocotyledonous plants

Claim 1

A method for the stable integration of a DNA comprising a gene that is functional in a cell of a cereal plant, into the nuclear genome of a cereal plant, said method comprising:

A) providing a compact embryogenic callus of a corn plant;
B) wounding and/or degrading said compact embryogenic callus and transferring said DNA in the nuclear genome of a cell in said compact embryogenic callus by means of electroporation, bombardment with DNA-coated microprojectiles or Agrobacterium-mediated transformation to generate a transformed cell; and optionally
C) regenerating a transformed cereal plant from said transformed cell.

Claim 18

The use of compact embryogenic callus of a cereal plant as starting material for transferring a DNA comprising a gene that is functional in a cell of a cereal plant, by means of electroporation, bombardment with DNA-coated microprojectiles or Agrobacterium-mediated transformation, into the nuclear genome of said cereal plant.

The patent application EP 955 371 A2 additionally recites the use of electroporation and microbombardment for the transformation of an embryogenic callus of a cereal or corn. The claims submitted in the EP application include:

• the use of an embryogenic callus of a cereal as starting tissue for transformation;
• transformation of the tissue by either electroporation, microbombardment or Agrobacterium infection;
• the insertion of a functional gene into the genome of the transformed cereal; and
• the transformation of an embryogenic callus of corn.</td>
<td></td>
</tr>
</table>

<table>
<tr><td>EP 955371 B1
Earliest priority - 23 November 1990
Filed - 21 November 1991
Granted - February 22 2006
Expected expiry - 20 November 2011</td><td>Title - Process for transforming monocotyledonous plants

Claim 1

A method for the stable integration of a DNA comprising a gene that is functional in a cell of a cereal plant, into the nuclear genome of a cereal plant, said method comprising:

a) providing a compact embryogenic callus of a cereal plant;
b) wounding and/or degrading said compact embryogenic callus and transferring said DNA in the nuclear genome of a cell in said compact embryogenic callus by means of electroporation, bombardment with DNA-coated microprojectiles or Agrobacterium-mediated transformation to generate a transformed cell; and optionally
c) regenerating a transformed cereal plant from said transformed cell.

Claim 18

The use of compact embryogenic callus of a cereal plant as starting material for transferring a DNA comprising a gene that is functional in a cell of a cereal plant, by means of electroporation, bombardment with DNA-coated microprojectiles or Agrobacterium-mediated transformation, into the nuclear genome of said cereal plant.

Granted EP 955371 recites a method of cereal transformation that is not limited to Agrobacterium-mediated DNA transfer, but also includes electroporation and bombardment with DNA-coated microprojectiles.</td></tr>
<tr><td>Remarks</td><td>The European application was assigned to Aventis CropScience N.V. on 7 June 2000. This may now be assigned to Bayer Crop Science.
A corresponding patent application in Canada (CA 2096843) is still pending.
A corresponding patent in Japan (JP 3234598) has been granted with cereals limited to those of corn, wheat and rice.</td></tr>
</table>

Note: Patent information on this page was last updated on 8 March 2006.

Particular monocot plants

Find out more information about patents on particular monocot plants by following the links shown below.

- Banana (*Musa spp.*)
- Barley (*Hordeum vulgare*)
- Duckweed (*Lemna* spp.)
- *Gladiolus* spp.
- Maize (*Zea mays*)
- Onions *(Allium spp.)*
- Pineapple (*Ananas spp.*)
- Rice (*Oryza sativa*)
- *Sorghum* spp.
- Turfgrass
- Wheat (*Triticum*)

Banana

Summary

Banana is the first of the particular monocot plants presented in this section. Some of the patents discussed here refer to the genus *Musa*, to which banana and plantain belong.

The inventions assigned to **Texas A&M University** disclose transformation of a wounded meristematic tissue from a *Musa* plant with *A. tumefaciens* carrying an engineered T-DNA plasmid. Embryogenic material of banana transformed with *Agrobacterium* containing a gene of interest is disclosed by a United States patent and a European application assigned to **Zeneca & DNA Plant Technology Corp.**

The most limiting factors in the claims of these inventions are:

- the use of a wounded meristematic tissue of a *Musa* plant (**Texas A & M University**),
- the additional application of microbombardment to the already wounded meristematic *Musa* tissue (claimed in the Australian and European patents granted to **Texas A & M University**) and
- the use of embryogenic material of banana (Australian patent granted to **Syngenta and DNA Plant Technology Corporation**) and embryogenic material from banana inflorescences (United States patent granted to **Zeneca & DNA Plant Technology Corp.**).

Banana (*Musa)* - Specific Patent Information - part 1

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 5792935 A • Earliest priority - 9 December 1993 • Filed - 9 December 1994 • Granted - 11 August 1998 • Expected expiry - 10 August 2015	**Title** - *Agrobacterium tumefaciens* transformation of *Musa* species **Claim 1** A method for transforming a *Musa* plant, said method comprising: A) wounding meristematic tissue from a *Musa* plant to generate a wounded *Musa* plant tissue and to facilitate access of *Agrobacterium tumefaciens* to *Musa* plant cells competent for transformation and regeneration; and B) applying to said wounded *Musa* plant tissue at least one transformation competent *Agrobacterium tumefaciens* to transform said *Musa* plant, wherein said at least one transformation competent *Agrobacterium tumefaciens* harbors at least one Ti plasmid and at least one virulence gene, wherein said at least one Ti plasmid comprises at least one genetically engineered T-DNA to effect transformation of said *Musa* plant. Transformation of an apical or adventitious meristem of a *Musa* plant by wounding the tissue and inserting *A. tumefaciens* carrying a T-DNA with foreign DNA. The plant can be transformed for the production of pharmaceutical products or the alteration of phenotypic traits of the fruit.	Texas A & M University
AU 693506 B2 • Earliest priority - 9 December 1993	**Title** - *Agrobacterium tumefaciens* transformation of *Musa* species **Claim 1** A method for transforming a *Musa* plant, said method including:	

• Filed - 9 December 1994 • Granted - 2 July 1998 • Expected expiry - 8 December 2014	A) wounding meristematic tissue from a *Musa* plant, followed by bombarding the said wounded tissue with microparticles, to generate a wounded *Musa* plant tissue and to facilitate access of *Agrobacterium tumefaciens* to *Musa* plant cells competent for transformation and regeneration; and B) applying to said wounded *Musa* plant tissue at least one transformation competent *Agrobacterium tumefaciens* to transform said *Musa* plant, wherein said at least one transformation competent *Agr obacterium tumefaciens* harbors at least one Ti plasmid and at least one virulence gene, wherein said at least one Ti plasmid includes at least one genetically engineered T-DNA to effect transformation of said *Musa* plant. Microbombardment of wounded meristematic tissue of a *Musa* plant to facilitate *A. tumefaciens* infection. The transformation method is used to obtain *Musa* plants producing pharmaceutical products and fruits with improved phenotypic traits.
EP 731632 B1 • Earliest priority - 9 December 1993 • Filed - 9 December 1994 • Granted - 7 November 2001 • Expected expiry - 8 December 2014	**Title** - *Agrobacterium tumefaciens* transformation of *Musa* species **Claim 1** A method for transforming a *Musa* plant, said method comprising: A) pre-wounding the meristematic tissue from a *Musa* plant prior to bombarding said plant with microparticles; B) wounding the pre-wounded meristematic tissue by microparticle bombardment to generate a wounded *Musa* plant tissue and to facilitate access of *Agrobacterium tumefaciens* to *Musa* plant cells competent for transformation and regeneration; and B) applying to said wounded *Musa* plant tissue at least one transformation competent *Agrobacterium tumefaciens* to transform said *Musa* plant, wherein said at least one transformation competent *Agrobacterium tumefaciens* harbors at least one Ti plasmid and at least one virulence gene, wherein said at least one Ti plasmid comprises at least one genetically engineered T-DNA to effect transformation of said *Musa* plant. Designated contracting States at the time of grant are: Austria, Belgium, Germany, Spain, France, United Kingdom, Greece (reported on INPADOC as lapsed), Ireland, Italy, Netherlands, Portugal, Sweden. A method for transforming a *Musa* meristematic tissue similar to the method disclosed in the related Australian patent. The tissue is wounded prior to the wounding by microbombardment. Double wounding of the tissue facilitates access of *A. tumefaciens* to *Musa* plant cells. The claims as filed of the EP application had disclosed a single wounding step by microbombardment. The granted claims are more limiting as a prior wounding step is part of the method.
CA 2177267 A • Earliest priority - 9 December 1993 • Filed - 9 December 1994	**Title** - *Agrobacterium tumefaciens* transformation of *Musa* species **Claim 1** A method for transforming a *Musa* plant, said method comprising: A) wounding meristematic tissue from a *Musa* plant by microparticle bombardment to generate a wounded *Musa* plant tissue and to facilitate access of *Agrobacterium tumefaciens* to *Musa* plant cells

• Granted – 28 September 2004 • Expected expiry – 8 December 2014	competent for transformation and regeneration; and B) applying to said wounded *Musa* plant tissue at least one transformation competent *Agrobacterium tumefaciens* to transform said *Musa* plant, wherein said at least one transformation competent *Agrobacterium tumefaciens* harbors at least one Ti plasmid and at least one virulence gene, wherein said at least one Ti plasmid comprises at least one genetically engineered T-DNA to effect transformation of said *Musa* plant. Claims as filed are similar to the Australian patent but a single step of wounding of the *Musa* tissue by microbombardment prior to the transformation with *A. tumefaciens* is disclosed.	
EP 1087016 A2 • Earliest priority – 9 December 1993 • Filed – 9 December 1994 • Deemed to be withdrawn – 10 August 2005	**Title** – *Agrobacterium tumefaciens* transformation of Musa species **Claim 1** A method for transforming a *Musa* plant, said method comprising: A) wounding meristematic tissue from a Musa plant by microparticle bombardment to generate a wounded *Musa* plant tissue and to facilitate access of *Agrobacterium tumefaciens* to *Musa* plant cells competent for transformation and regeneration; and B) applying to said wounded *Musa* plant tissue at least one transformation competent *Agrobacterium tumefaciens* to transform said *Musa* plant, wherein said at least one transformation competent *Agrobacterium tumefaciens* harbors at least one Ti plasmid and at least one virulence gene, wherein said at least one Ti plasmid comprises at least one genetically engineered T-DNA to effect transformation of said *Musa* plant; C) growing said transformed *Musa* plant for a sufficient time to identify the presence of chimeric features; D) producing non-chimeric tissue by dividing said transformed *Musa* plant into segments which have at least one meristem which can regenerate into an intact plant and which have cells that are uniformily transformed to produce non-chimeric tissue; and E) growing said non-chimeric tissue into a non-chimeric plant. This application was a divisional application to the application EP 731 632 A1. Microbombardment is also used to wound a meristematic tissue of a Musa plant prior to the transformation with *A. tumefaciens*. In addition, the transformed Musa tissue is grown to identify chimeras and regenerate an intact plant from non-chimeric tissue.	
Remarks	Application filed in Japan (JP 9508786 T2) is deemed to be withdrawn.	

Note: Patent information on this page was last updated on 17 February 2006.

Banana (Musa) - Specific Patent Information - part 2

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 6133035 A • Earliest priority – 16 July 1997 • Filed – 16 July 1997 • Granted – 17 October 2000	**Title** – Method of Genetically Transforming Banana Plants **Claim 1** A method of producing a transformed banana plant comprising transforming banana embryogenic material from inflorescences with *Agrobacterium* containing a gene of interest and regenerating a transformed banana plant from the transformed embryogenic material.	Zeneca & DNA Plant Technology Corp. (Zeneca is now Syngenta)

<table>
<tr><td>

- Expected expiry - 15 July 2017

</td><td>

Claim 7

A method of producing a transformed banana plant comprising transforming a banana somatic embryo from inflorescences with *Agrobacterium* containing a gene of interest and regenerating a transformed banana plant from the transformed somatic embryo.

Claim 11

A method of genetically transforming banana, the method comprising: A) culturing somatic banana plant tissue from inflorescences in a medium to obtain at least one somatic embryo structure or pro-embryo structure;
B) culturing the somatic embryo or pro-embryo structure in a medium to obtain embryogenic material;
C) transforming the embryogenic material with *Agrobacterium* cells having at least one exogenous DNA sequence to produce transformed embryogenic material;
D) culturing the transformed embryogenic material in a medium to produce at least one transformed somatic embryo; and
E) germinating the transformed somatic embryo in a medium to produce a mature plantlet capable of being transferred to soil conditions.

Claim 19

A method of genetically transforming banana, the method comprising:
A) culturing somatic banana plant tissue from inflorescences in a medium to obtain at least one somatic embryo structure or pro-embryo structure;
B) culturing the somatic embryo or pro-embryo structure in a medium to obtain embryogenic material;
C) culturing the embryogenic material in a medium to produce at least one somatic embryo;
D) transforming the somatic embryo produced in step (c) with *Agrobacterium* cells having at least one exogenous DNA sequence to produce transformed somatic embryos;
E) multiplying the transformed somatic embryo to produce additional transformed somatic embryos; and
F) germinating the transformed somatic embryo to produce a mature plantlet capable of being transferred to soil conditions.

Methods for transformation of embryogenic material from banana inflorescences with *Agrobacterium* having a gene of interest. Production of plantlets capable of being transferred to soil conditions.

</td><td></td></tr>
<tr><td>

AU 744496 B2

- Earliest priority - 16 July 1997
- Filed - 13 July 1998
- Granted - 28 February 2002
- Expected expiry - 12 July 2018

</td><td>

Title - Method of Genetically Transforming Banana Plants

Claim 1

A method of producing a transformed banana plant comprising transforming banana embryogenic material with *Agrobacterium* containing a gene of interest and regenerating a transformed banana plant from the transformed embryogenic material.

Claim 7

A method of producing a transformed banana plant comprising transforming a banana somatic embryo with *Agrobacterium* containing a gene of interest and regenerating a transformed

</td><td>Syngenta Ltd. & DNA Plant Technology Corp.</td></tr>
</table>

banana plant from the transformed somatic embryo.

Claim 11

A method of genetically transforming banana, the method comprising:
A) culturing somatic banana plant tissue in a medium to obtain at least one somatic embryo structure or pro-embryo structure;
B) culturing the somatic embryo or pro-embryo structure in a medium to obtain embryogenic material;
C) transforming the embryogenic material with *Agrobacterium* cells having at least one exogenous DNA sequence to produce transformed embryogenic material;
D) culturing the transformed embryogenic material in a medium to produce at least one transformed somatic embryo; and
E) germinating the transformed somatic embryo in a medium to produce a mature plantlet capable of being transferred to soil conditions.

Claim 19

A method of genetically transforming banana, the method comprising:
A) culturing somatic banana plant tissue in a medium to obtain at least one somatic embryo structure or pro-embryo structure;
B) culturing the somatic embryo or pro-embryo structure in a medium to obtain embryogenic material;
C) culturing the embryogenic material in a medium to produce at least one somatic embryo;
D) transforming the somatic embryo produced in step (c) with *Agrobacterium* cells having at least one exogenous DNA sequence to produce transformed somatic embryos;
E) multiplying the transformed somatic embryo to produce additional transformed somatic embryos; and
F) germinating the transformed somatic embryo to produce a mature plantlet capable of being transferred to soil conditions.

The invention claimed in Australia is similar to the related United States patent but the embryogenic material is not from a specific part of a banana plant.

EP 996329 A1

- Earliest priority - 16 July 1997
- Filed - 13 July 1998
- Application pending

Title - Method of Genetically Transforming Banana Plants

Claim 1

A method of producing a transformed banana plant comprising transforming banana embryogenic material with *Agrobacterium* containing a gene of interest and regenerating a transformed banana plant from the transformed embryogenic material.

Claim 7

A method of producing a transformed banana plant comprising transforming a banana somatic embryo with *Agrobacterium* containing a gene of interest and regenerating a transformed banana plant from the transformed somatic embryo.

Claim 11

A method of genetically transforming banana, the method comprising:

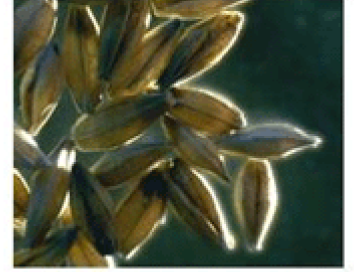
A) culturing somatic banana plant tissue in a medium to obtain at least one somatic embryo structure or pro-embryo structure;

	B) culturing the somatic embryo or pro-embryo structure in a medium to obtain embryogenic material; C) transforming the embryogenic material with *Agrobacterium* cells having at least one exogenous DNA sequence to produced transformed embryogenic material; D) culturing the transformed embryogenic material in a medium to produce at least one transformed somatic embryo; and E) germinating the transformed somatic embryo in a medium to produce a mature plantlet capable of being transferred to soil conditions. Claim 19 A method of genetically transforming banana, the method comprising: A) culturing somatic banana plant tissue in a medium to obtain at least one somatic embryo structure or pro-embryo structure; B) culturing the somatic embryo or pro-embryo structure in a medium to obtain embryogenic material; C) culturing the embryogenic material in a medium to produce at least one somatic embryo; D) transforming the somatic embryo produced in step (C) with *Agrobacterium* cells having at least one exogenous DNA sequence to produce transformed somatic embryos; E) multiplying the transformed somatic embryo to produce additional transformed somatic embryos; and F) germinating the transformed somatic embryo to produce a mature plantlet capable of being transferred to soil conditions. The claims as filed in the European application recite the same as the claims granted in the United States patent **US 6133035** with the exception of **claim 7**, where a banana somatic embryo is **not limited** to an embryo derived from an inflorescence. Transformation of somatic embryos of banana with *Agrobacterium* having a gene of interest. Multiplication of transformed somatic embryos and production of plantlets capable of being transferred to soil conditions.	
Remarks	National phase entry of WO 1999/03327 in Japan (JP 2001510021 T2) is still pending.	

Note: Patent information on this page was last updated on 14 February 2006.

Barley

Barley is one of the major cereal crops worldwide and as such biotechnological



genetic improvement technologies are a desirable avenue for the introduction of novel traits, like disease resistance or modified starch production.

This patent discloses a method for transformation of barley using *Agrobacterium* in combination with acetosyringone (a phenolic compound that induces DNA transfer by *Agrobacterium*) and suspending this mixture together with callus cells as target tissue for transformation.

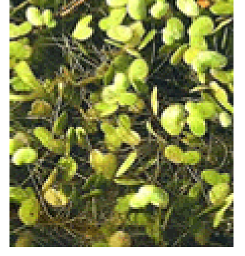
Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 6291244 B1 • Earliest priority - 25 July 1997 • Filed - 24 Jan 2000 • Granted - 18 Sep 2001 • Expected expiry - 23 Jan 2020	**Title** - Method of producing transformed cells of barley **Claim 1** 1. A method of producing transformed cells of barley, comprising: (a) suspending, in a suspension medium containing 200 to 1000 mg/l acetosyringone, a microorganism belonging to the genus *Agrobacterium* comprising a foreign gene; (b) culturing, in a co-culture medium containing about 1000 mg/l acetosyringone, the microorganism belonging to the genus *Agrobacterium* and barley callus cells; (c) separating the cultured barley callus cells from the co-culture medium; and (d) placing the separated barley callus cells on a selective medium to select the transformed cells into which the foreign gene has been introduced. The present invention provides variations on existing protocols for the transformation of cereals and other monocots. In the case of barley the concentration of the widely used phenolic compound acetosyringone has been adjusted to the special requirements given for the interaction between *Agrobacterium* and the plant cells to achieve optimal transformation efficiency. If sub-optimal concentrations are used or a different or no phenolic compound is utilized, thereby possibly sacrificing transformation efficiency, infringement can likely be avoided.	Sapporo Breweries Ltd (JP)
Remarks	related patents and applications in Japan JP 2002509440 T2 (deemed withdrawn), Australia AU 79377/98 (lapsed), WO 99/04618	

Note: Patent information on this page was last updated on 6 February 2006.

Duckweed

Summary



Duckweeds are small, fresh-water plants with a world-wide distribution. They are exploited for protein production due to two unusual aspects of their growth: the plants reproduce vegetatively by budding and under intensive culture they accumulate a very high rate of biomass. The level of protein production can achieve that obtained with yeast gene expression systems.

Two entities have patents and patent applications directed to *Agrobacterium*-mediated transformation of duckweed:

- **North Carolina State University** has been granted a United States patent directed to transformation of duckweed tissue with *Agrobacterium* having a gene of interest. The invention further comprises a method for mass production of recombinant proteins or peptides from duckweed cultures.
- **Yeda Research & Development Co**. has filed patent applications in Europe, Australia and Canada directed to a transformed Lemnaceae plant with *Agrobacterium.* Lemnaceae is the name of the botanical family to which duckweed belongs. Duckweeds from the genera *Spirodela*, *Lemna* and *Wolffia* are used for the production of various chemical and biological products. The claims as filed recite different methods for the transformation of Lemnaceae such as *in planta* transformation, microinjection of *Agrobacterium* cells into meristematic plant cells and incubation of meristematic

plant cells with *Agrobacterium.*

Although the inventions disclosed by both institutes may overlap in terms of the subject matter, they have been filed in different countries. The claims as filed by **Yeda Research & Development Co.** in the European patent application are broad and may have a different scope if granted.

Duckweed - Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 6 040 498 • Filed - 11 August 1998 • Granted - 21 March 2000	**Title** - Genetically Engineered Duckweed **Claim 1** A method for stably transforming duckweed tissue with a nucleotide sequence of interest, the method comprising the steps of: A) inoculating a duckweed plant tissue with *Agrobacterium* comprising a vector which comprises a nucleotide sequence of interest; and B) co-cultivating the tissue with the *Agrobacterium* to produce stably transformed tissue. **Claim 20** A stably transformed duckweed plant comprising a heterologous nucleic acid of interest incorporated in its genome wherein said plant is produced via an *Agrobacterium*-mediated method. **Claim 30** A method of producing recombinant proteins or peptides, comprising the steps of: A) culturing a stably transformed duckweed plant that expresses at least one heterologous protein or peptide; and B) collecting the at least one heterologous protein or peptide from the duckweed cultures. **Claim 48** A method for stably transforming duckweed tissue from the genus *Lemna* with a nucleotide sequence of interest, the method comprising the steps of: A) inoculating a duckweed plant tissue with *Agrobacterium* comprising a vector which comprises a nucleotide sequence of interest, wherein the duckweed plant tissue is from the genus *Lemna*; and B) co-cultivating the tissue with the *Agrobacterium* to produce stably transformed tissue. **Claim 59** A stably transformed duckweed plant from the genus *Lemna* comprising a heterologous nucleic acid sequence incorporated in its genome wherein said plant is produced via an *Agrobacterium* -mediated method. **Claim 62** A method of producing recombinant proteins or peptides, comprising: A) culturing a stably transformed duckweed plant from the genus *Lemna* that expresses at least one heterologous protein or peptide; and B) collecting the at least one protein or peptide from the duckweed cultures.	North Carolina State University

<table>
<tr><td></td><td>A method for transforming a duckweed tissue by co-cultivating the tissue with Agrobacterium having a sequence of interest. A method for producing recombinant proteins or peptides by culturing transformed duckweed plants expressing the proteins or peptides and collecting them form the cultured plants.
The same methods are used to transform a duckweed from the genus Lemna.</td><td></td></tr>
<tr><td>EP 1 021 552 A1
• Filed - 8 October 1998
• Granted -</td><td>Title - Transgenic Lemnaceae

Claim 1
A genetically stable, transformed Lemnaceae plant and progeny thereof.

Claim 12*
A method for the stable genetic transformation of Lemnaceae plants which comprises:
incubating Lemnaceae plants and/or tissue with Agrobacterium cells containing a transforming DNA molecule, whereby cells in said plant tissue become stably transformed with said DNA.

Claim 19*
A method for the genetic transformation of a plant comprising: A) cutting the plant into particles of a size such that they still contain undamaged meristematic tissue capable of developing into full plants;
B) incubating said particles with Agrobacterium cells containing transforming DNA molecules, whereby said transforming DNA is introduced into meristematic cells in said particles; and
C) producing transformed plants from the transformed meristematic tissue.

Claim 23*
A method for the stable genetic transformation of a Lemnaceae plant comprising
microinjecting Agrobacterium cells containing a transforming DNA into the meristematic zone of the plant, whereby the meristemic tissue becomes stably transformed with said DNA.

Claim 25
A) method for the in planta transformation of Lemnaceae plants comprising: A) exposing the plant's meristematic zone by removal of the daughter fronds;
B) incubating the plant with Agrobacterium cells capable of targeting to the meristemic tissue.

Claim 37*
A booster medium for enhancing Agrobacterium cell's virulence comprising plant tissue culture at a pH below about 5.2.

Claim 43*
A booster medium for enhancing Agrobacterium cell's virulence comprising an extract from Lemnaceae plants.

Claim 45
A method for maintaining morphogenetic Lemnaceae calli for long-periods of time comprising culturing the calli in a medium having a low level of sucrose.</td><td>Yeda Research & Development Co.</td></tr>
</table>

	Claim 47 A method for the regeneration of plants from calli wherein the plant's growth medium has sucrose levels below 1.5% and comprises: B5, minerals and organic compounds. **Claim 48** A method for the production of highly regenerative calli, wherein the calli's growth medium has sucrose levels below 1.5% and comprises B5, minerals and organic compounds. **Claim 50** A method for the production of highly regenerative calli, wherein the calli's growth medium has sucrose levels below 1.5% and comprises B5, minerals, organic compounds and selection agents. **Claim 52** A method for the production of stable transformed plants, wherein the growth media has sucrose levels below 1.5% and comprises B5, minerals and organic compounds. * Claims directly related to *Agrobacterium* -mediated transformation of a Lemnaceae plant. Different methods for stable genetic transformation of a Lemnaceae plant with *Agrobacterium* containing a gene of interest. The methods include incubating the plants with *Agrobacterium*, incubating meristematic tissue with the bacterium, injecting the bacterium into the meristematic tissue and *in planta* transformation. The invention also includes methods for enhancing the virulence of *Agrobacterium* and for regenerating calli and transformed plants.
Remarks	Related applications to **EP 1 021 552 A1** also filed in Australia (AU 94572/98 A1) and Canada (CA 2312008).

Gladiolus

The present patent claims a method to transform a corm tissue from a *Gladiolus* plant with a vir+ *A. tumefaciens*.

The patent is thus limited to a gladiolus corm, an underground stem modified into a mass storage tissue as tissue to be transformed.
Other types of gladiolus tissues are not encompassed by the claims.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 5340730 A • Earliest priority - 31 March 1988 • Filed - 17 June 1992 • Granted - 23 August 1994 • Expired - 25 September	**Title** - Process for Transforming *Gladiolus* **Claim 1** A method of producing a transformed *Gladiolus* plant comprising: A) removing a piece of tissue from a corm; B) inoculating the tissue with *vir+* *Agrobacterium*	University of Toledo

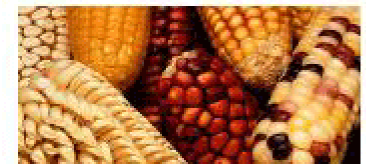
2002 (due to non-payment of maintenance fees)	*tumefaciens* strain; C) incubating the inoculated tissue until a tumor forms; D) culturing at least a portion of the tumor in hormone-free medium until a cormel forms; and E) growing the cormel to produce the transformed plant.	
Remarks	This patent has been abandoned according to the USPTO database. While there don't seem to be any patents with specific claims to *Gladiolus* transformation using *Agrobacterium*, other patents related to general methods or transformation of monocots still may apply.	

Note: Patent information on this page was last updated on 7 February 2006.

Maize

Summary

The **University of Toledo** (US), **Pioneer Hi-Bred** (US), and **Stine Biotechnology** (US) have been granted United States patents directed to *Agrobacterium*-mediated transformation of maize. The main difference among them is the type of maize tissue used as target for transformation:



- The **University of Toledo** claims a maize seedling wounded in a specific area for inoculation of the bacterium.
- **Pioneer Hi Bred**
  claims an immature embryo of maize and a medium formula for cultivation of a transformed maize embryo
- **Stine Biotechnology** also employs immature embryos as target tissue for transformation with *Agrobacterium* in their patent. Claims in their applications disclose a method to improve the production of so-called Type II callus (a preferred target tissue for the transformation of corn) by including a monosaccharide like glucose in addition to the usual sucrose in the medium.

Maize - Specific Patent Information - part 1

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 5177010 A • Earliest priority - 30 June 1986 • Filed - 5 September 1990 • Granted - 5 Jan 1993 • Expected expiry - 4 September 2010	**Title** - Process for Transforming Corn and the Products Thereof **Claim 1** A method of producing transformed corn, said method comprising: A) making a wound in a corn seedling with newly emerging radicle and stem, said wound being made in an area of the seedling containing rapidly dividing cells, wherein said area extends from the base of the scutellar node to slightly beyond the coleoptile node; and B) inoculating the wound with *vir*+ *A. tumefaciens*. Transformation of a corn seedling by inoculating *vir*+ *A. tumefaciens* in a wounded area located between the scutellar node to slightly beyond the coleoptile node. This area presents a high rate of cell division.	University of Toledo
US 2002/0002711 A1 • Earliest priority - 27 June 1994 • Filed - 10 June	Title - Process for Transforming Germinae and the Products Thereof **Claim 1** 1. A transformed Gramineae.	Goldman and Graves

<table>
<tr><td>

1998

- Abandoned - 28 May 2002

</td><td>

<table>
<tr><td>Claim 7

A fertile transgenic Zea mays plant comprising stably incorporated exogenous DNA.</td></tr>
<tr><td>Claim 10

A transformed corn plant having a transformed gene, the plant produced by an Agrobacterium-mediated transformation or direct gene transfer transformation.</td></tr>
<tr><td>Claim 11

A transformed corn plant produced by an Agrobacterium or direct cell transfer whereby the plant or other differentiated organs or tissues provide an expression of exogenous DNA in the corn product.</td></tr>
<tr><td>Claim 12

A transformed corn plant produced by a regenerated protoplast or single cell cultures.</td></tr>
<tr><td>Claim 13

A transformed corn plant produced by a method equivalent to an Agrobacterium tumefaciens-mediated transformation.</td></tr>
</table>

Transformed corn plant having a transformed gene produced by: Agrobacterium -mediated transformation or an equivalent method, direct cell transfer, regenerated protoplasts and single cell cultures. Fertile transgenic Zea mays having an exogenous gene and transformed Gramineae are also part of the filed claims.

This patent application has been abandoned (failure to respond to a USPTO action) according to the USPTO.

</td><td></td></tr>
<tr><td>Remarks</td><td>A related patent has been granted in Japan (JP3234534, which is a divisional of now granted JP 2693443 claiming Agrobacterium-mediated transformation of Gramineae).</td><td></td></tr>
<tr><td>

US 5981840 A

- Earliest priority - 24 January 1997
- Filed - 24 January 1997
- Granted - 9 Nov 1999
- Expected expiry - 23 January 2017

</td><td>

Title - Method for Agrobacterium-Mediated Transformation

<table>
<tr><td>Claim 1

A method for transforming maize using Agrobacterium comprising the steps of:

A) contacting at least one immature embryo from a maize plant with Agrobacterium capable of transferring at least one gene to the embryo;
B) co-cultivating the embryo with Agrobacterium;
C) culturing the embryo in a medium comprising N6 salts, an antibiotic at concentrations capable of inhibiting the growth of Agrobacterium, and a selective agent to select for embryos expressing the gene; and
D) regenerating plants expressing the gene.</td></tr>
<tr><td>Claim 4

A method for transforming maize using Agrobacterium comprising the steps of:

A) contacting at least one immature embryo from a maize plant with Agrobacterium capable of transferring at least one gene to the embryo in a medium comprising N6 salts;
B) co-cultivating the embryo with Agrobacterium in a medium</td></tr>
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</td><td>Pioneer Hi-Bred</td></tr>
</table>

comprising N6 salts;
C) culturing the embryo in a medium comprising N6 salts, an antibiotic at concentrations capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos expressing the gene; and
D) regenerating plants expressing the gene in a medium comprising MS salts.

Claim 7

A method for transforming maize using *Agrobacterium* comprising the steps of:

A) contacting at least one immature embryo from a maize plant with *Agrobacterium* capable of transferring at least one gene to the embryo in a medium comprising N6 or MS salts;
B) co-cultivating the embryo with *Agrobacterium* in a medium comprising MS salts;
C) culturing the embryo in a medium comprising N6 salts, an antibiotic at concentrations capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos expressing the gene; and
D) regenerating plants expressing the gene in a medium comprising MS salts.

Claim 10

A method for optimizing the production of transgenic maize plants of a first genotype using *Agrobacterium*-mediated transformation comprising the steps of:

A) isolating immature embryos from maize;
B) separating the embryos into treatment groups;
C) incubating each treatment group separately in a medium comprising N6 or MS salts and in a suspension of *Agrobacterium* at concentrations ranging from about 1×10^8 cfu/ml to about 1×10^{10} cfu/ml;
D) co-cultivating the embryos with *Agrobacterium* on a solid medium;
E) culturing the embryos in a medium comprising N6 salts, an antibiotic at concentrations capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos transformed by *Agrobacterium*;
F) identifying the treatment group with the highest transformation frequency; and
G) using the concentration of *Agrobacterium* generating the highest transformation frequency to transform other embryos from the first genotype.

Claim 13

A method for transforming maize using *Agrobacterium* comprising the steps of:

A) contacting at least one immature embryo from a maize plant with *Agrobacterium* capable of transferring at least one gene to the embryo;
B) co-cultivating the embryo with *Agrobacterium*;
C) culturing the embryo in a medium containing salts other than MS salts, an antibiotic at concentrations capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos expressing the gene; and
D) regenerating plants expressing the gene.

	Transformation of an immature embryo of maize by contact and co-cultivation with *Agrobacterium* having a gene of interest. The media used for culturing the embryo contains N6 or MS salts, an antibiotic against *Agrobacterium,* and a selective agent for embryos expressing the gene. Regeneration of plants expressing the gene is also recited in the claims.
AU 727849 B2 • Earliest priority – 24 January 1997 • Filed – 23 January 1998 • Granted – 4 January 2001 • Expected expiry – 22 January 2018	**Title** – Methods for *Agrobacterium*-mediated transformation **Claim 1** A method for transforming maize using *Agrobacterium* comprising the steps of: A) contacting at least one immature embryo from a maize plant with *Agrobacterium* capable of transferring at least one gene to the embryo; B) co-cultivating the embryo with *Agrobacterium*; C) culturing the embryo in a medium comprising N6 salts, an antibiotic at concentrations capable of inhibiting the growth of *Agrobacterium*, and a selective agent to select for embryos expressing the gene; and D) regenerating plants expressing the gene. Claim 1 states the same method of maize transformation as that of US 5981840.
EP 971578 A1 • Earliest priority – 24 January 1997 • Filed – 23 January 1998 • Application deemed to be withdrawn – 25 June 2003	**Title** – Methods for *Agrobacterium*-mediated Transformation **Claim 1** A method for transforming maize using *Agrobacterium* comprising the steps of: A) contacting tissue from a maize plant with *Agrobacterium* capable of transferring at least one gene to the tissue in a non-LS salt medium; B) co-cultivating the tissue with *Agrobacterium* in a non-LS salt medium; C) culturing the tissue in a non-LS salt medium and a selective agent to select for tissue expressing the gene; and D) regenerating, in a non-LS salt medium, plants expressing the gene. The transformation method disclosed in these applications is very similar to the method claimed in the United States patent **US 5981840**. The difference consists in the use of a co-cultivation medium free of LS salts.
Remarks	1. A divisional patent of now granted US 5981840 has been granted in the US (US 6822144), which claims a stably transformed maize plant (limited to PHN46 or PHJ90 inbred plants) using *Agrobacterium* to transform an immature embryo. 2. National phase entry of WO 1998/32326 in Canada (CA 2278618 AA) is pending.

Note: Patent information on this page was last updated on 7 February 2006.

Maize - Specific Patent Information - part 2

<table>
<tr><th>Patent Number</th><th>Title, Independent Claims and Summary of Claims</th><th>Assignee</th></tr>
<tr><td>US 6420630 B1
Earliest priority - 1 December 1998
Filed - 1 December 1998
Granted - 16 July 2002
Expected expiry - 30 November 2018</td><td>Title - Methods for tissue culturing and transforming elite inbreds of Zea mays L.

Claim 1

A method for transforming a line of corn using Agrobacterium comprising the steps of:
(a) initiating co-cultivation of an immature embryo from said line with Agrobacterium capable of transferring at least one gene to tissue of said line to produce an infected embryo;
(b) applying heat shock treatment during said co-cultivation;
(c) culturing the infected embryo to initiate callus on a medium comprising an antibiotic;
(d) culturing the resulting callus tissue on a medium comprising a selective agent;
(e) selecting transformed callus tissue comprising growing Type II callus; and
(f) regenerating transgenic plants from said Type II callus.

A method of transforming maize by co-cultivating Agrobacterium carrying a gene of interest with immature embryos. Heat shock is applied to enhance DNA integration.</td><td rowspan="2">Stine Biotechnology</td></tr>
<tr><td>US 6919494 B2
Earliest priority - 1 December 1998
Filed - 30 July 2001
Granted - 19 July 2005
Expected expiry - 29 July 2021</td><td>Title - Methods for tissue culturing and transforming elite inbreds of Zea mays L.

Claim 1

A method for producing a corn plant comprising the steps of:

(a) co-cultivating an immature embryo from said tissue at a temperature of about 18°C. to 20°C. with Agrobacterium capable of transferring at least one DNA sequence of interest to said tissue to produce an infected embryo;

(b) culturing the infected embryo on a medium comprising an antibiotic to produce a resulting tissue;

(c) culturing said resulting tissue on a medium comprising a selective agent and an antibiotic;

(d) selecting transformed tissue having Type II callus; and

(e) regenerating transgenic plants from said Type II callus.

Claim 3

A method for transforming a line of corn comprising the steps of:

(a) co-cultivating an immature embryo from said line with Agrobacterium capable of transferring at least one DNA sequence of interest to said tissue of said line to produce an infected embryo;

(b) culturing the infected embryo to initiate callus on a medium comprising an antibiotic and a compound selected from the group consisting of glucose, maltose, lactose, sorbitol and mannitol, wherein the concentration of said compound is from 5 g/L to 30 g/L;

(c) culturing the resulting callus tissue on a medium comprising a selective agent and an antibiotic;</td></tr>
</table>

(d) selecting transformed callus tissue comprising growing Type II callus; and

(e) regenerating transgenic plants from said growing Type II callus.

Claim 4

A method for producing a transformed corn plant using *Agrobacterium* comprising the steps of:

(a) initiating co-cultivation of an immature embryo from said tissue with *Agrobacterium* capable of transferring at least one DNA sequence of interest to said tissue to produce an infected embryo;

(b) applying heat shock treatment during said co-cultivation;

(c) culturing the infected embryo to initiate callus on a medium comprising an antibiotic and glucose;

(d) culturing the resulting callus tissue on a medium comprising a selective agent and an antibiotic;

(e) selecting transformed callus tissue having Type II callus; and

(f) regenerating transgenic plants from said Type II callus.

Granted patent of application US 2002/0104131 (see below).

Continuation of US Patent Application 09/203,679 (now US Patent 6,420,630 B1)

US 2002/0104131 A1

- Earliest priority - 1 December 1998
- Filed - 30 July 2001
- Granted as US 6919494 (see above)

Title - Methods for tissue culturing and transforming elite inbreds of *Zea mays* L.

Claim 1 (not granted in US 6919494)

A method for stimulating a high frequency production of Type II callus from immature embryos of elite corn inbreds which comprises culturing said embryos on a solid medium comprising sucrose and a monosaccharide sugar, wherein the concentration of said monosaccharide sugar is between about 5 g/L and about 30 g/L.

Claim 4

A method for transforming elite lines of corn using *Agrobacterium* comprising the steps of:

(a) co-cultivating an immature embryo from said elite line with *Agrobacterium* capable of transferring at least one gene to tissue of said elite line on a solid medium to produce an infected embryo;

(b) culturing the infected embryo on a solid medium comprising an antibiotic;

(c) culturing the resulting tissue on a solid medium comprising a selective agent to select for transformed tissue;

(d) selecting transformed tissue with growing Type II callus capable of forming water tower embryo structures; and

(e) regenerating plants from said embryo structures.

Claim 22

A method for transforming elite lines of corn using *Agrobacterium* comprising the steps of:

(a) co-cultivating an immature embryo from said elite line with *Agrobacterium* capable of transferring at least one gene to tissue of said elite line on a solid medium to produce an infected embryo;

(b) culturing the infected embryo on a solid medium comprising an antibiotic and a monosaccharide sugar in an amount of from 5 g/L to 30g/L;

(c) culturing the resulting tissue on a solid medium comprising an antibiotic and a selective agent;

(d) culturing the resulting tissue on a solid medium comprising a selective agent to select for transformed tissue;

(e) selecting transformed tissue with growing Type II callus capable of forming water tower embryo structures; and

(f) regenerating plants from said embryo structures.

Claim 32

A method for transforming elite lines of corn using *Agrobacterium* comprising the steps of:

(a) co-cultivating at a temperature of 19[deg.] C. an immature embryo from said elite line with *Agrobacterium* capable of transferring at least one gene to tissue of said elite line on a solid medium to produce an infected embryo, said *Agrobacterium* is selected from *Agrobacterium* one to two days after rescue from frozen glycerol stocks;

(b) culturing the infected embryo on a solid medium comprising an antibiotic at a concentration of 15 mg/L to 75 mg/L and a monosaccharide sugar selected from the group consisting of glucose, maltose, lactose, sorbitol and mannitol in an amount of from 5 g/L to 30 g/L;

(c) culturing the resulting tissue on a solid medium comprising an antibiotic and a selective agent;

(d) culturing the resulting tissue on a solid medium comprising a selective agent to select for transformed tissue;

(e) selecting transformed tissue with growing Type II callus capable of forming water tower embryo structures; and

(f) regenerating plants from said embryo structures.

A method in which formation of Type II callus (a preferred form of target tissue for the transformation with *Agrobacterium*) from immature embryos is enhanced by adding sucrose and a monosaccharide to the medium.

Continuation of US Patent Application 09/203,679 (now US Patent US 6420630 B1)

US 2002/0104132 A1	**Title** - Methods for tissue culturing and transforming elite inbreds of *Zea mays* L.

- Earliest priority - 1 December 1998
- Filed - 30 July 2001
- Abandoned - 19 September 2005

Claim 1

A method for stimulating a high frequency production of Type II callus from immature embryos of elite corn inbreds which comprises

- culturing said embryos on a solid medium comprising sucrose and a monosaccharide sugar, wherein the concentration of said monosaccharide sugar is between about 5 g/L and about 30 g/L.

Claim 4

A method for transforming elite lines of corn using *Agrobacterium* comprising the steps of:

(a) co-cultivating an immature embryo from said elite line with *Agrobacterium* capable of transferring at least one gene to tissue of said elite line on a solid medium to produce an infected embryo;

(b) culturing the infected embryo on a solid medium comprising an antibiotic;

(c) culturing the resulting tissue on a solid medium comprising a selective agent to select for transformed tissue;

(d) selecting transformed tissue with growing Type II callus capable of forming water tower embryo structures; and

(e) regenerating plants from said embryo structures.

Claim 22

A method for transforming elite lines of corn using *Agrobacterium* comprising the steps of:

(a) co-cultivating an immature embryo from said elite line with Agrobacterium capable of transferring at least one gene to tissue of said elite line on a solid medium to produce an infected embryo;

(b) culturing the infected embryo on a solid medium comprising an antibiotic and a monosaccharide sugar in an amount of from 5 g/L to 30g/L;

(c) culturing the resulting tissue on a solid medium comprising an antibiotic and a selective agent;

(d) culturing the resul ting tissue on a solid medium comprising a selective agent to select for transformed tissue;

(e) selecting transformed tissue with growing Type II callus capable of forming water tower embryo structures; and

(f) regenerating plants from said embryo structures.

Claim 32

A method for transforming elite lines of corn using *Agrobacterium* comprising the steps of:

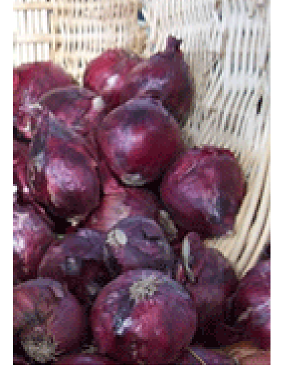
(a) co-cultivating at a temperature of 19[deg.] C. an immature embryo from said elite line with *Agrobacterium* capable of transferring at least one gene to tissue of said elite line on a solid medium to produce an infected embryo, said *Agrobacterium* is selected from *Agrobacterium* one to two days after rescue from

	frozen glycerol stocks.; (b) culturing the infected embryo on a solid medium comprising an antibiotic at a concentration of 15 mg/L to 75 mg/L and a monosaccharide sugar selected from the group consisting of glucose, maltose, lactose, sorbitol and mannitol in an amount of from 5 g/L to 30 g/L; (c) culturing the resulting tissue on a solid medium comprising an antibiotic and a selective agent; (d) culturing the resulting tissue on a solid medium comprising a selective agent to select for transformed tissue; (e) selecting transformed tissue with growing Type II callus capable of forming water tower embryo structures; and (f) regenerating plants from said embryo structures. A method as in the application above but specifically designed for elite corn inbred lines. Divisional application from US Patent Application 09/203,679 (now US Patent **US 6420630 B1**), this application has been abandoned due to failure to respond to a USPTO action according to the USPTO.	
US 2005/0278802 A1 • Earliest priority - 1 December 1998 • Filed - 18 July 2005 • Application pending	Title - Methods for tissue culturing and transforming elite inbreds of *Zea mays* L. **Claim 35** A method for producing a corn plant comprising the steps of: (a) culturing a corn embryo on a medium comprising a compound selected from the group consisting of glucose, maltose, lactose, sorbitol, and mannitol, wherein said compound is in an amount of from about 5 g/L to 30 g/L, to produce a type II callus; and (b) regenerating a plant. This is a divisional application of now granted **US 6420630**. All of the independent claims preceeding claim 35 has been cancelled.	

Note: Patent information on this page was last updated on 7 February 2006.

Onions (*Allium* spp)

Summary



The **New Zealand Institute for Crop & Food Research** and **Seminis Vegetable Seeds** have filed patent applications directed to the transformation of *Allium* spp. (onions) using *Agrobacterium*.

- **The New Zealand Institute for Crop & Food Research** : the PCT application contains a very broad independent claim reciting a method of transforming plants of the *Allium* genus but lacks recitation of any steps. As such, it is highly unlikely to be granted as filed. If allowed, such a claim would cover any method used to this end. It is possible that converted patents will not contain such a claim. New Zealand application NZ 513184, for example, specifies the target tissue for transformation (embryos or embryo-derived cell cultures), and that transformation is carried out without passage through a callus stage. A preferred explant used in the transformation procedure with *Agrobacterium* are wounded immature embryos.

- **Seminis Vegetable Seeds** discloses a method that uses embryogenic callus, embryos or flower buds as a target explant for transformation with *Agrobacterium*.

Because of their limited subject matter, these applications may be affected by other patents with granted claims to general transformation methods for monocots.

Onions - Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
WO 2000/44919 A1 • Earliest priority - 29 January 1999 • Filed - 10 December 1999 • OPI - 3 August 2000	**Title** - Transformation and Regeneration of *Allium* plants A preferred embodiment utilizes the alliinase gene, which encodes an enzyme involved in sulfur metabolism. **Claim 1** A method of transforming plants of the *Allium* genus. **Claim 3** A method of transforming plants of the *Allium* genus comprising inoculating an embryo culture of an *Allium* species with an *Agrobacterium tumefaciens* strain containing a suitable vector or plasmid. **Claim 9** A method of transforming *Allium* using immature embryos as an explant source, including: (a) isolating immature embryos of the *Allium* plant to be transformed; (b) innoculating cultures of the immature embryos with an *Agrobacterium tumefaciens* strain containing a binary vector; (c) wounding embryos and infiltrating embryos with agrobacteria; (d) transferring embryos to a selective medium; (e) culturing embryo pieces; (f) selecting putative transgenic cultures; and (g) regenerating plants.	New Zealand Institute for Crop & Food Research Ltd.
AU 763531 C • Earliest priority - 29 January 1999 • Filed - 10 December 1999 • Granted - 24 July 2003 • Amended - 26 February 2004 • Expected expiry - 9 December 2019	**Title** - Transformation and regeneration of *Allium* plants **Claim 1** A method of transforming plants of the *Allium* genus comprising the following steps: (a) delivering previously manipulated DNA into embryo, or embryo derived culture cell types of the *Allium* genus via vector or direct gene transfer; (b) selecting transformed plant material; (c) culturing and regenerating the transformed plants; wherein the transformation is carried out without passage through a callus phase. **Claim 7** A method of transforming *Allium* using immature embryos as an explant source, including: (a) isolating immature embryos of the *Allium* plant to be transformed; (b) innoculating cultures of the immature embryos with an *Agrobacterium tumefaciens* strain containing a binary vector; (c) wounding embryos and infiltrating embryos with agrobacteria; (d) transferring embryos to a selective medium; (e) culturing embryo pieces;	

	(f) selecting putative transgenic cultures; and (g) regenerating plants.	
Remarks	1. Related patent granted in New Zealand (NZ 513184). 2. National phase entries of WO 2000/44919 in Canada (CA 2361143 A1) and Europe (EP 1144664 A1) are still pending.	
EP 1180927 B1 • Earliest priority – 5 May 1999 • Filed – 5 May 2000 • Granted – 21 December 2005 • Expected expiry – 4 May 2020	**Title** – Transformation of *Allium sp* with *Agrobacterium* using embrogenic callus cultures A method of transforming *Allium* species (onions) with *Agrobacterium* carrying a heterologous gene. A preferred embodiment utilizes immature embryos and flower buds as target tissue for transformation. **Claim 1** A method for transforming an *Allium* species with a heterologous gene, the method comprising the step of: contacting embryogenic callus material from an *Allium* species with a bacterium belonging to the genus *Agrobacterium* which contains a heterologous gene. **Claim 8** A method for transforming an *Allium* species with a heterologous gene, the method comprising the steps of: a. culturing immature embryos or flower buds from an *Allium* species on an initiation medium for a period of from about 2 to about 6 months until embryogenic callus material forms on the embryos or flower buds; b. transferring the embryogenic callus material to a coculture medium and contacting the embryogenic callus material with a suspension of *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* containing a heterologous gene; c. incubating the embryogenic callus material with the *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* for a period of from about 2 to about 4 days; and d. removing the *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* from the transformed embryogenic callus material. Designated contracting State at the time of grant is Spain.	Seminis Vegetable Seeds Inc. (US)
EP 1180927 A1 • Earliest priority – 5 May 1999 • Filed – 5 May 2000 • Granted as **EP 1180927 B1** (see above)	**Title** – Transformation of *Allium* sp with *Agrobacterium* using embrogenic callus cultures A method of transforming *Allium* species (onions) with *Agrobacterium* carrying a heterologous gene. A preferred embodiment utilizes immature embryos and flower buds as target tissue for transformation. **Claim 1 (granted)** A method for transforming an *Allium* species with a heterologous gene, the method comprising the step of: contacting embryogenic callus material from an *Allium* species with a bacterium belonging to the genus *Agrobacterium* which contains a heterologous gene. **Claim 9 (granted)** A method for transforming an *Allium* species with a heterologous gene, the method comprising the steps of:	

a. culturing immature embryos or flower buds from an *Allium* species on an initiation medium for a period of from about 2 to about 6 months until embryogenic callus material forms on the embryos or flower buds;
b. transferring the embryogenic callus material to a coculture medium and contacting the embryogenic callus material with a suspension of Agrobacterium rhizogenes or *Agrobacterium tumefaciens* containing a heterologous gene;
c. incubating the embryogenic callus material with the *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* for a period of from about 2 to about 4 days; and
d. removing the *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* from the transformed embryogenic callus material.

AU 780954 B2

- Earliest priority - 5 May 1999
- Filed - 5 May 2000
- Granted - 28 April 2005
- Expected expiry - 4 May 2020

Title - Transformation of *Allium sp.* with *Agrobacterium* using embryogenic callus cultures

Claims of this patent are limited to transforming *Allium cepa* and *A. fistulosum* using *Agrobacterium*.

Claim 1

A method for transforming an *Allium cepa* or *Allium fistulosum* with a DNA of interest from a heterologous gene, the method comprising the steps of: contacting embryogenic callus material from an *Allium cepa* or *Allium fistulosum* with a bacterium belonging to the genus *Agrobacterium* which contains a DNA of interest from a heterologous gene and obtaining a transformed *Allium cepa* or *Allium fistulosum* embryogenic callus under selective conditions.

Claim 8

A method for transforming a plant or plant tissue of an *Allium cepa* or *Allium fistulosum* with a DNA of interest from a heterologous gene, the method comprising the steps of:
a. culturing immature embryos or flower buds from an *Allium cepa* or *Allium fistulosum* on an initiation medium for a period of from about 2 to about 6 months until an embryogenic callus forms on the embryos or flower buds;
b. transferring the embryogenic callus to a coculture medium and contacting the embryogenic callus with a suspension of *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* containing a DNA of interest from a heterologous gene; and
c. obtaining a transformed *Allium cepa* or *Allium fistulosum* embryogenic callus under selective conditions.

Note: Patent information on this page was last updated on 7 February 2006.

Pineapple

Summary

The invention disclosed by **DNA Plant Technology Corp**. in a granted United States patent is directed to the transformation of embryogenic pineapple cells or pineapple callus with *Agrobacterium* having a T-DNA with a heterologous gene.

The recently granted Australian patent also uses an **embryogenic cell or an embryogenic callus cell** as a starting material for transformation.

In a recently filed PCT application, the **Department of Primary Industries of Queensland**

(Australia) discloses methods for transforming pineapple cells and genetically modified pineapple by co-cultivating a pineapple explant with *Agrobacterium* having a gene of interest. Unlike the method used by **DNA Plant Technology Corp**., the transformed pineapple cells form an **organogenic callus**, which according to the applicants is easier and faster to obtain than an embryogenic callus.

Pineapple - Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 5952543 A • Earliest priority - 25 February 1997 • Filed - 24 February 1998 • Granted - 14 September 1999 • Expected expiry - 23 February 2018	**Title** - Genetically transformed pineapple plants and methods for their production **Claim 1** A method for modifying the genotype of a pineapple cell, said method comprising: A) contacting said pineapple cell with *Agrobacterium* comprising a T-DNA containing a DNA segment, such that said DNA segment is integrated into the genome of said pineapple cell; and B) selecting a pineapple cell comprising said integrated DNA segment wherein said pineapple cell is an embryogenic cell or an embryogenic callus cell. **Claim 11** A method for modifying the genotype of a pineapple cell, said method comprising: A) culturing pineapple tissue to produce pineapple embryogenic cells; B) contacting said pineapple embryogenic cells with *Agrobacterium* comprising a T-DNA containing a DNA segment, such that said DNA segment is integrated into the genome of said pineapple cells; and C) selecting a pineapple cell comprising said integrated DNA segment.	DNA Plant Technology Corp.
AU 740294 B2 • Earliest priority - 25 February 1997 • Filed - 25 February 1998 • Granted - 1 November 2001 • Expected expiry - 24 February 2018	**Title** - Genetically transformed pineapple plants and methods for their production **Claim 1** A method for modifying the genotype of a pineapple cell, said method comprising: A) contacting said pineapple cell with *Agrobacterium* comprising a T-DNA containing a DNA segment, such that said DNA segment is integrated into the genome of said pineapple cell; and B) selecting a pineapple cell comprising said integrated DNA segment wherein said pineapple cell is an embryogenic cell or an embryogenic callus cell. **Claim 11** A method for modifying the genotype of a pineapple cell, said method comprising: A) culturing pineapple tissue to produce pineapple embryogenic cells; B) contacting said pineapple embryogenic cells with	

<table>
<tr><td></td><td>

Agrobacterium comprising a T-DNA containing a DNA segment, such that said DNA segment is integrated into the genome of said pineapple cells; and
C) selecting a pineapple cell comprising said integrated DNA segment.

Claim 39

A method for modifying the genotype of a pineapple cell, said method being substantially as hereinbefore described with reference to any one of the examples.

Claim 41

A pineapple plant cell comprising an integrated *Agrobacterium* T-DNA sequence comprising a heterologous gene, substantially as hereinbefore described with reference to any one of the examples.

Claim 51

A pineapple plant comprising an integrated *Agrobacterium* T-DNA sequence comprising a heterologous gene, substantially as hereinbefore described with reference to any one of the examples.

* Claims 1 and 11 of the Australian granted patent are the same as those of the United States patent. The three additional independent claims in this patent (claims 39, 41, 51) recite methods and transgenic pineapple plants stated in the examples of the specification.

</td><td></td></tr>
<tr><td>

WO 2001/33943 A1

- Earliest priority - 5 November 1999
- Filed - 3 November 2000
- OPI - 17 May 2001

</td><td>

Title - A method of plant transformation

Claim 1 - See below *

Claim 21 - See below *

Claim 41 - See below *

Claim 60

A method of transforming cells of a pineapple plant or a related plant with genetic material, said method comprising: A) obtaining explant from said pineapple plant or a related species; B) co-cultivating same with *Agrobacterium* species having T-DNA or T-DNA region comprising genetic material to be transformed into said pineapple plant cells for a time and under conditions sufficient for transfer of the genetic material to occur; C) selecting for transformed pineapple or related cells and permitting the cells to form organogenic callus.

Claim 77

A method of genetically modifying a pineapple or related plant, said method comprising: A) obtaining an explant from a pineapple or related plant to be genetically modified;
B) co-cultivating the explant with *Agrobacterium* species having a T-DNA comprising genetic material to be transferred into the pineapple or related cells for a time and under conditions sufficient for the genetic material to transfer to said cells;
C) selecting for transformed pineapple or related cells and permitting the cells to form organogenic callus; and
D) regenerating a pineapple or related plant from said selected

</td><td>Department of Primary Industries of Queensland</td></tr>
</table>

transformed cells.

Method for transforming pineapple cells by co-cultivating an explant with *Agrobacterium* having T-DNA with genetic material to be transformed into the plant cells.

The selected transformed pineapple cells form an organogenic callus. A genetically modified pineapple plant is regenerated from the organogenic callus.

* The present application also contains claims (1, 21, 41) directed to methods for transforming monocot plants in general, which are discussed under the section General transformation methods.

AU 779510 B

- Earliest priority - 5 November 1999
- Filed - 3 November 2000
- Granted - 27 January 2005
- Expected expiry - 2 November 2020

Title - A method of plant transformation

Claim 1

A method of transforming cells of a pineapple plant with genetic material, said method comprising:-

(a) obtaining an explant from said plant;

(b) co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without said Agrobacterium overgrowing the plant cells; and

(c) selecting for the tranformed plant cells and permitting the cells to form organogenic callus.

Claim 19

A method for producing a genetically modified pineapple plant, said method comprising:-

(a) obtaining explant from a plant to be genetically modified;

(b) co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising genetic material to be transformed into said plant cells for a time and under conditions sufficient for the genetic material to transfer to plant cells without said Agrobacterium overgrowing the plant cells;

(c) selecting transformed plant cells and permitting the cells to form organogenic callus; and

(d) then regenerating a plant from selected transformed plant cells.

Claim 37

A method for producing a genetically modified pineapple plant, said method comprising:

(a) obtaining an explant from said plant to be genetically modified;

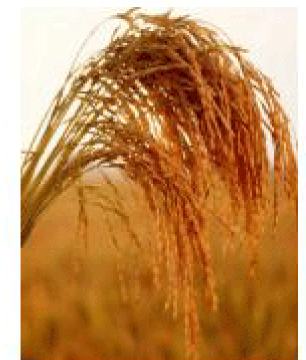
(b) co-cultivating the explant with *Agrobacterium* species having a T-DNA or T-DNA region comprising the genetic material to be transformed into the plant cells for a time and under conditions sufficient for the genetic material to transfer into the plant cells without the Agrobacterium overgrowing the plant cells;

	(c) selecting for the transformed plant cells and permitting the cells to form organogenic callus; and (d) regenerating a plant from said transformed organogenic callus. Granted independent claims in this patent recite the same method of pineapple transformation as claims 60 and 77 in **WO 2001/33943**, except that the plant is limited to pineapple only and not those of "related plants".	

Note: Patent information on this page was last updated on 7 February 2006.

Rice

Summary



- United States and Australian patents were granted to **Japan Tobacco** on methods for the transformation of Indica rice. Patents are pending in other jurisdictions. The patent specification discloses the use of **immature embryo cells** of **Indica rice** for transformation with *Agrobacterium* spp. The claims granted recite wide ranges for typical components of media used in the selection medium. Note that other varieties of rice, such as **Japonica rice,** are **not covered** by the claims as granted (Update July 2003).
- In 2001, the **National Science Council of R.O.C.** in Taiwan was granted a United States patent directed to a method for the transformation of **immature rice embryos** with *Agrobacterium*. The invention is **not limited** to any particular rice variety. A particular feature of the transformation method is that the rice embryos and *Agrobacterium* are co-cultivated with a **dicot suspension culture**. In one of the preferred embodiments, the dicot suspension culture is made of potato cells. According to the inventors, such culture is rich in phenolic compounds, which induce the *vir* genes of the Ti plasmid. Thus, the phenolic compounds of the culture assist the transformation process.
- **Paradigm Genetics** filed a PCT application disclosing the transformation of a **rice panicle** with *Agrobacterium* using **vacuum infiltration**. This method is carried out *in planta* (the transformed plant part is still attached to the whole plant) and avoids *in vitro* regeneration steps.
- The Indian company **Avestha Gengraine Technologies** has filed a PCT application disclosing a method of transformation of Indica rice using excised shoot tip tissue as a target for *Agrobacterium*.

Rice - Specific Patent Information - part 1

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 6329571 B1 • Earliest priority - 22 October 1996 • Filed - 22 October 1997 • Granted - 11 December 2001 • Expected expiry - 21 October 2017	**Title** - Method for Transforming Indica Rice **Claim 1** A method for transforming rice comprising transforming immature embryo cells of Indica rice by *Agrobacterium* method and selecting transformed cells, characterized in that a medium containing • 2000 to 4000 mg/l of KNO_3, • 60 to 200 mg/l of $MgSO_4$, • 200 to 600 mg/l of KH_2PO_4, • 100 to 450 mg/l of $CaCl_2$,	Japan Tobacco

- 200 to 600 mg/l of $(NH_4)_2SO_4$,
- 1 to 7 mg/l of H_3BO_3,
- 2 to 20 mg/l of $MnSO_4$,
- 20 to 50 mg/l of EDTA or a salt thereof,
- 3 to 8 mg/l of Fe,
- 50 to 200 mg/l of myoinositol,
- 0.5 to 10 mg/l of 2,4-dichlorophenoxyacetic acid,
- 0.01 to 5 mg/l of a cytokinin,
- 5000 to 80,000 mg/l of a sugar, and
- a gelling agent,which medium has a pH of 4.5 to 6.5,

is used as a medium for selecting said transformed cells.

A method for transformation of Indica rice using immature embryo cells as target tissue for *Agrobacterium*. Ranges for the media components are disclosed in the independent claim.

AU 736027 B2

- Earliest priority - 22 October 1996
- Filed - 22 October 1997
- Granted - 26 July 2001
- Expected expiry - 21 October 2017

Title - Method for Transforming Indica Rice

Claim 1

A method for transforming rice comprising transforming immature embryo cells of Indica rice by *Agrobacterium* method and selecting transformed cells, characterized in that a medium containing:

- 2000 to 4000 mg/l of KNO_3,
- 60 to 200 mg/l of $MgSO_4$,
- 200 to 600 mg/l of KH_2PO_4,
- 100 to 450 mg/l of $CaCl_2$,
- 200 to 600 mg/l of $(NH_4)_2SO_4$,
- 1 to 7 mg/l of H_3BO_3,
- 2 to 20 mg/l of $MnSO_4$,
- 20 to 50 mg/l of EDTA or a salt thereof,
- 3 to 8 mg/l of Fe,
- 50 to 200 mg/l of myoinositol,
- 0.5 to 10 mg/l of 2,4-dichlorophenoxyacetic acid,
- 0.01 to 5 mg/1 of a cytokinin,
- 5000 to 80,000 mg/l of a sugar, and
- a gelling agent, which medium has a pH of 4.5 to 6.5,

is used as a medium for selecting said transformed cells.

Transformation of immature embryo cells of **Indica rice** by *Agrobacterium*. The composition of the medium used to select the transformed cells is disclosed.

EP 897013 A1

- Earliest priority - 22

Title - Method for Transforming Indica Rice

October 1996 • Filed – 27 October 1997 • Application deemed withdrawn – 18 December 2003	**Claim 1** A method for transforming rice comprising transforming immature embryo cells of Indica rice by *Agrobacterium* method and selecting transformed cells, characterized in that a medium containing: • 2000 to 4000 mg/l of KNO_3, • 60 to 200 mg/l of $MgSO_4$, • 200 to 600 mg/l of KH_2PO_4, • 100 to 450 mg/l of $CaCl_2$, • 200 to 600 mg/l of $(NH_4)_2SO_4$, • 1 to 7 mg/l of H_3BO_3, • 2 to 20 mg/l of $MnSO_4$, • 20 to 50 mg/l of EDTA or a salt thereof, • 3 to 8 mg/l of Fe, • 50 to 200 mg/l of myoinositol, • 0.5 to 10 mg/l of 2,4-dichlorophenoxyacetic acid, • 0.01 to 5 mg/1 of a cytokinin, • 5000 to 80,000 mg/l of a sugar, and • a gelling agent, which medium has a pH of 4.5 to 6.5, is used as a medium for selecting said transformed cells.	
Remarks	1. National phase entry of WO 1998/17813 in Canada (CA 2240454) has lapsed according to CIPO. 2. National phase entry of WO 1998/17813 in China (CN 1206435) is deemed withdrawn according to CNPO. 3. National phase entry of WO 1998/17813 in Japan (JP H10-117776) has been rejected by the JPO.	

Note: Patent information on this page was last updated on 8 February 2006.

Rice - Specific Patent Information - part 2

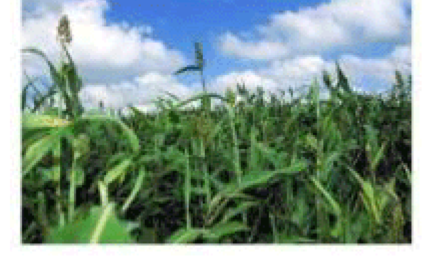
Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 6215051 A • Earliest priority – 4 November 1992 • Filed – 4 May 1998 • Granted – 10 April 2001 • Expected expiry – 3 May 2018	Title – *Aarobacterium*-mediated method for transforming rice (note that *Agrobacterium* has the incorrect spelling, as shown above, on the patent) **Claim 1** A method for the production of a transgenic plant of rice crop comprising the steps: A) infecting an immature embryo of rice crop with the genus *Agrobacterium* for transformation; B) co-culturing the infected embryo with a dicot suspension culture during the step of transformation; C) allowing the transformed embryo in step (B) to grow into a callus in a selective medium comprising a sufficient amount of a	National Science Council of R.O.C.

	plant growth hormone for the growth of rice crop; and D) allowing the cultured callus to regenerate root and shoot in a regeneration medium comprising a pre-determined amount of nutrients for the growth of rice crop. **Claim 8** A method for the production of a transgenic rice plant comprising the steps of: A) transforming an immature rice embryo with a gene encoding a desired gene product by culturing the embryo in a dicot suspension culture with bacteria from the genus *Agrobacterium*, said bacteria comprising said gene; B) growing the transformed embryo from step (A) into a callus in a selective medium comprising a rice plant growth hormone; and C) regenerating root and shoot from said cultured callus in a regeneration medium comprising nutrients for the growth of rice crop. Method for the production of transgenic rice plants by co-culturing immature rice embryo and *Agrobacterium* with a **dicot suspension culture**. The transformed embryo grows into a callus that in turn regenerates roots and shoots.	
Remarks	Related Japanese applications and United States patents are not directed to transformation of rice with *Agrobacterium*. They refer to a gene expression system with a promoter region from the alpha amylase genes.	
WO 2001/12828 A1 • Earliest priority – 18 August 1999 • Filed – 17 August 2000 • OPI – 22 February 2001	**Title** – Methods and Apparatus for transformation of Monocotyledenous plants using *Agrobacterium* in combination with vacuum filtration Claim 1 – See below * **Claim 20** An *in planta* method of transforming a rice plant comprising: A) contacting at least one panicle of the rice plant with a solution or suspension comprising at least one *Agrobacterium* clone; and B) subjecting the rice plant to a vacuum effective to cause entry of the *Agrobacterium* clone into at least one flower of the panicle. **Claim 38** An *in planta* method of producing a transgenic rice plant, comprising: A) contacting at least one panicle of a first rice plant with a solution comprising at least one *Agrobacterium* clone wherein the *Agrobacterium* clone comprises at least one heterologous gene; B) subjecting the first rice plant to a vacuum effective to cause entry of the *Agrobacterium* clone into at least one flower of the panicle; C) cultivating the first rice plant to maturity; and D) collecting seeds of the first rice plant expressing the heterologous gene. Claim 40 – See below * An *in planta* method for transforming rice panicles by contacting the panicle with *Agrobacterium* in suspension containing a heterologous gene and subjecting the plant to vacuum so *Agrobacterium* enters the plant part. After the transformation the plant is cultivated into maturity and seeds express the heterologous	Paradigm Genetics

	gene. * Independent claims 1 and 40 recite use of vacuum infiltration to transform monocotyledonous plants, and are introduced in the General Monocot Transformation Methods section.	
Remarks	National phase entry of WO 01/12828 in Australia (AU 67807/00 A) has lapsed.	
WO 02/057407 A2 • Earliest priority - 17 January 2001 • Filed - 14 January 2002 • OPI - 25 July 2002	**Title** - Novel Method for Transgenic Plants by Transformation and Regeneration of Indica Rice Plant Shoot Tips **Claim 1** A novel method of transforming excised shoot tip tissue of the Indica rice cultivars by using the *Agrobacterium* method. A method of transformation of Indica rice using excised shoot tip tissue as a target for *Agrobacterium*.	Avestha Gengraine Technologies Pty. Ltd.
Remarks	1. related application in India IN 2001CH00047 2. National phase entry of WO 02/57407 in Europe (EP 1444339) is still pending.	

Note: Patent information on this page was last updated on 8 February 2006.

Sorghum
Summary



An Australian patent, AU 743 706 B2, was granted to **Pioneer Hi-Bred Inc**. in January 2002 and a United States Patent, US 6 369 298 B1, was granted in April. The subject matter is the transformation of sorghum via *Agrobacterium*. A related application was filed in South Africa.

The granted **Australian** claims are **more limited** than the initially filed claims. The sorghum tissue to be transformed is now restricted to a certain group of tissues and the concentration of *Agrobacterium* cells in suspension is fixed. Another limiting factor of the granted invention is the number of copies of the sequence of interest inserted into the genome of a sorghum cell, tissue or plant, offering another avenue for designing around.

The method disclosed in the **US patent** utilizes an immature embryo as target for *Agrobacterium*. The gene cassette must contain a gene which confers resistance to a selection agent. Furthermore, during culture the medium must contain an antibiotic to eliminate the bacteria as well as contain the selection agent.

The broadest claim in the pending **US application** recites a sorghum plant transformed with *Agrobacterium*. The main restriction is that fewer than 5 copies of the introduced construct are present in the genome (multiple copies of integrated foreign DNA are generally undesirable).

Sorghum - Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
AU 743706 B2 • Earliest priority - 30 April 1997 • Filed - 14	**Title** - *Agrobacterium* Mediated Transformation of Sorghum **Claim 1** A method for transforming sorghum with a nucleotide sequence of interest, said method comprising the steps of:	Pioneer Hi-Bred Inc.

April 1998

- Granted - 31 January 2002
- Expected expiry - 13 April 2018

A) contacting tissue selected from the group consisting of: an immature embryo or cells derived from an immature embryo, immature inflorescence, the basal portion of young leaves and tissue capable of forming callus and/or secondary embryos from a sorghum plant with an *Agrobacterium* comprising a vector which comprises said nucleotide sequence, wherein said nucleotide sequence comprises at least an expression cassette comprising a gene which confers resistance to a selection agent;
B) co-cultivating the tissue with said *Agrobacterium* in a concentration from about 1×10^3 cfu/ml to about 1.5×10^{10} cfu/ml;
C) culturing the tissue in a medium comprising an antibiotic capable of inhibiting the growth of *Agrobacterium* and said selection agent;
D) regenerating transformed sorghum plants.

Claim 16

A method for transforming sorghum with a nucleotide sequence, said method comprising the steps of:

A) contacting a tissue selected from the group consisting of: an immature embryo or cells derived from an immature embryo, immature inflorescence, the basal portion of young leaves and tissue capable of forming callus and/or secondary embryos from a sorghum plant with an *Agrobacterium* comprising a super-binary vector which comprises said nucleotide sequence, wherein said nucleotide sequence comprises at least an expression cassette co mprising a gene which confers resistance to a selection agent;
B) co-cultivating the tissue with said *Agrobacterium* in a concentration from about 1×10^3 cfu/ml to about 1.5×10^{10} cfu/ml;
C) culturing the tissue in a medium comprising an antibiotic capable of inhibiting the growth of *Agrobacterium* and said selection agent;
D) regenerating transformed sorghum plants.

Claim 31

A *Agrobacterium*-mediated transformed sorghum plant, said plant comprising fewer than 5 copies of a nucleic acid of interest flanked by at least one T-DNA border sequence incorporated in its genome, wherein said plant is derived from tissue selected from the group consisting of: an immature embryo or cells derived from an immature embryo, immature inflorescence, the basal portion of young leaves and tissue capable of forming callus and/or secondary embryos.

Claim 41

A *Agrobacterium*-mediated transformed plant cell, said cell comprising fewer than 5 copies of a nucleic acid of interest flanked by at least one T-DNA border sequence incorporated in its genome, wherein said plant cell is sorghum and wherein said plant cell is derived from tissue selected from the group consisting of: an immature embryo or cells derived from an immature embryo, immature inflorescence, the basal portion of young leaves and tissue capable of forming callus and/or secondary embryos.

Claim 44

A *Agrobacterium*-mediated transformed plant tissue, said tissue comprising fewer than 5 copies of a nucleic acid of interest flanked by at least one T-DNA border sequence incorporated in its genome of cells of said tissue, wherein said plant is sorghum and wherein said plant tissue is derived from tissue selected from the group consisting of: an immature embryo or cells derived from an immature embryo, immature inflorescence, the basal portion of young leaves and tissue capable of forming callus and/or secondary embryos.

	Method for sorghum transformation with *Agrobacterium* containing a gene that confers resistance to a selective agent. The tissue to be transformed is selected from: immature embryo, immature inflorescence, basal portion of young leaves and tissue capable of forming callus or secondary embryos. *Agrobacterium* contains a vector or a super binary vector having the mentioned gene. The transformed sorghum tissue or cell has less than 5 copies of the gene of interest flanked by at least one T-border.
US 6369298 B1 • Earliest priority - 30 April 1997 • Filed - 7 April 1998 • Granted - 9 April 2002 • Expected expiry - 6 April 2018	**Title** - Agrobacterium Mediated Transformation of Sorghum **Claim 1** A method for transforming sorghum with a nucleotide sequence of interest, said method comprising the steps of: contacting an immature embryo from a sorghum plant with an Agrobacterium comprising a vector which comprises said nucleotide sequence, wherein said nucleotide sequence comprises at least an expression cassette comprising a gene which confers resistance to a selection agent; co-cultivating said immature embryo with said Agrobacterium; culturing said immature embryo in a medium comprising an antibiotic capable of inhibiting the growth of said Agrobacterium and said selection agent; regenerating transformed sorghum plants. **Claim 15** A method for transforming sorghum with a nucleotide sequence, said method comprising the steps of: contacting an immature embryo from a sorghum plant with an Agrobacterium comprising a super-binary vector which comprises said nucleotide sequence, wherein said nucleotide sequence comprises at least an expression cassette comprising a gene which confers resistance to a selection agent; co-cultivating said immature embryo with said Agrobacterium; culturing said immature embryo in a medium comprising an antibiotic capable of inhibiting the growth of said Agrobacterium and said selection agent; regenerating transformed sorghum plants. A method for transformation of sorghum in which an immature embryo is the target tissue for *Agrobacterium*. The gene cassette must contain a gene which confers resistance to a selection agent. During further culture the medium must contain an antibiotic to eliminate the bacteria as well as contain the selection agent.
US 2002/0138879 A1 • Earliest priority - 30 April 1997 • Filed - 19 March 2002 • Application pending	**Title** - Agrobacterium Mediated Transformation of Sorghum **Claim 1** An Agrobacterium-transformed sorghum plant wherein said plant comprises fewer than 5 copies of a nucleic acid of interest flanked by at least one T-DNA border sequence incorporated in its genome. **Claim 12** A sorghum plant transformed by contacting an immature embryo from a sorghum plant with an Agrobacterium comprising a nucleic acid of interest; co-cultivating said immature embryo with said Agrobacterium; culturing said immature embryo in a medium comprising an antibiotic capable of inhibiting the growth of said Agrobacterium; and regenerating a transformed sorghum plant, said plant comprising fewer than 5 copies of said nucleic acid flanked by at least one T-DNA border sequence incorporated in its genome.

	Claim 19 A sorghum plant transformed by contacting an immature embryo from a sorghum plant with an Agrobacterium comprising a vector which comprises; a nucleic acid comprising at least one expression cassette comprising a gene of interest; and a nucleic acid comprising at least one expression cassette comprising a gene which confers resistance to a selection agent; co-cultivating said immature embryo with said Agrobacterium; culturing said immature embryo in a medium comprising an antibiotic capable of inhibiting the growth of said Agrobacterium and a selection agent; and regenerating a transformed sorghum plant, said plant comprising fewer than 5 copies of said vector flanked by at least one T-DNA border sequence incorporated in its genome. This application is a division of now granted US 6369298. A sorghum plant transformed with *Agrobacterium* with fewer than 5 copies of the introduced construct integrated in the genome.	
Remarks	National phase entry of WO 98/49332 has been accepted in South Africa (ZA 98/03603).	

Note: Patent information on this page was last updated on 8 February 2006.

Turfgrass

Summary

Rutgers University has filed a PCT application directed to transformation of turfgrass callus with *Agrobacterium*. The bacterium contains a vector with a selectable marker gene and an insertion site for any coding sequence, both of them controlled by promoters isolated from monocot plants. The promoters, according to the applicants, function efficiently in turfgrass cells.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
EP 1100876 A1 • Earliest priority - 17 July 1998 • Filed - 13 July 1999 • Application pending	**Title** - *Agrobacterium*-mediated transformation of turfgrass **Claim 1** A method of producing a transgenic turfgrass plant, comprising the steps of: A) providing regenerable callus tissue from the turfgrass plant; B) inoculating the tissue with *Agrobacterium* carrying at least one vector for transformation, the vector comprising virulence genes that confer strong infectivity to *Agrobacterium*, in which vector is inserted a heterologous DNA construct operably linked to a promoter from a monocotyledonous species, and a selectable marker gene conferring antibiotic resistance to transformed cells operably linked to a promoter from a monocotyledonous species; C) culturing the inoculated tissue under conditions that enable the *Agrobacterium* vector to transform cells of the tissue; D) selectively culturing the inoculated tissue on a selection medium comprising the antibiotic; and E) regenerating a transformed turfgrass plant from the selectively cultured tissue. **Claim 11** A superbinary vector system for *Agrobacterium*-mediated transformation of turfgrass, which comprises: A) a virulence region from a Ti plasmid of an *A. tumefaciens* strain that	Rutgers University

	confers to the strain as strong a virulence as that displayed by *A. tumefaciens* strain 281; B) a selectable marker gene operably linked to a promoter obtained from a gene of a monocotyledonous plant; and C) a site for insertion of at least one additional coding sequence, operably linked to a promoter obtained from a gene of a monocotyledonous plant, the promoter being the same as or different from the promoter operably linked to the selectable marker gene. A method of producing a transgenic turfgrass plant by inoculating a regenerable turfgrass callus with *Agrobacterium* containing an antibiotic resistance linked to a monocot promoter. The antibiotic resistance is used as a selectable marker for the transformed tissue. A transformed turfgrass plant is regenerated from the selected transformed tissue. A super-binary vector used for the *Agrobacterium*-mediated transformation of turfgrass comprising: a virulence region, a selectable marker linked to a monocot promoter, and an insertion site for a coding sequence. This site is linked to the same or a different monocot promoter.	
Remarks	National phase entry of WO 2000/04133 in Australia (AU 52136/99 A) has lapsed.	

Note: Patent information on this page was last updated on 9 February 2006.

Wheat
Summary

In 2001 **Monsanto** was granted an Australian patent directed to transformation of wheat with *Agrobacterium*. Related applications are still pending in Europe and Canada.

The granted claims of the Australian patent are narrower in their scope than the claims as filed in the European and Canadian applications. In the Australian patent the wheat tissues to be transformed are **restricted** to certain types of tissues while the applications disclose the transformation of wheat cells derived from **any** tissue.

Both the Australian patent and the applications disclose the insertion into the cells of genes that confer resistance to selective agents such as gentamycin, kanamycin, and hygromycin. Production of fertile and transgenic wheat plants is also disclosed.

Note that the claim language may be modified during the prosecution process and when granted might not encompass the same scope as the filed claims.

Wheat - Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
AU 738153 C • Earliest priority - 21 June 1996 • Filed - 20 June 1997 • Granted - 13 September 2001 • Amended -	**Title** - Methods for the production of stably-transformed, fertile wheat employing *agrobacterium*-mediated transformation and compositions derived therefrom **Claim 1** A fertile, transgenic wheat plant, the genome of which has been altered through the genomic introduction of a pre-selected genetic component, said component comprising an exogenous gene positioned under the control of one or more pre-selected genetic control elements, the plant prepared by a process comprising:	Monsanto

1 July 2004

- Expected expiry - 19 June 2017

A) preparing a DNA composition *in vitro*, which composition includes the genetic component one desires to introduce into the genome of a wheat plant;
B) introducing said DNA composition into recipient wheat cells by *Agrobacterium* transformation;
C) regenerating wheat plants from said cells which have received said genetic component; and
D) identifying a fertile, transgenic wheat plant whose genome has been altered through the stable introduction of said genetic component, and wherein said recipient cells comprise an immature embryo, a callus tissue, or suspension cells.

Claim 7

A fertile, transgenic wheat plant, the genetic complement of which has been altered through the addition of a DNA composition comprising a pre-selected functional genetic element that includes a transgene selected from the group consisting of an *npt*II gene, a *bla* gene, a *npt*I gene, a *dhfr* gene, a *aph*IV gene, a *aac*C3 gene, a *aac*C4 gene and a GUS gene, wherein said functional genetic element confers on said wheat plant a phenotypic trait that is not found in the parentage of said plant, wherein said DNA composition was added using *Agrobacterium* transformation of recipient cells comprising an immature embryo, a callus tissue, or suspension cells.

Claim 10

A fertile, transgenic wheat plant, the genome of which has been altered through the genomic introduction of a pre-selected genetic component, said component comprising an exogenous gene positioned under the control of one or more pre-selected genetic control elements, substantially as hereinbefore described with reference to any one of the examples.

Claim 11

A fertile, transgenic wheat plant, the genetic complement of which has been altered through the addition of a DNA composition comprising a pre-selected functional genetic element, substantially as hereinbefore described with reference to any one of the examples.

Claim 14

A method for producing a fertile transgenic wheat plant, comprising the steps of:

A) establishing a regenerable culture from a wheat plant to be transformed, wherein said culture comprises an immature embryo, a callus tissue, or suspension cells;
B) introducing a DNA composition comprising a genetic component one desires to introduce into the genome of said wheat plant, by *Agrobacterium* transformation;
C) identifying or selecting a transformed cell line; and
D) regenerating a fertile transgenic wheat plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny, wherein said progeny comprises a selectable or screenable marker gene, and wherein said marker gene is chromosomally integrated.

Claim 18

A method for producing a fertile transgenic wheat plant, substantially as hereinbefore described with reference to any one of the examples.

Claim 19

A method for producing a transgenic wheat plant, comprising the steps of:

A) establishing a culture from a wheat plant to be transformed, wherein said culture comprises an immature embryo, a callus tissue, or suspension cells;
B) transforming said culture with an *Agrobacterium* comprising a DNA composition comprising a genetic component one desires to introduce into the genome of said wheat plant;
C) identifying or selecting a transformed cell line; and
D) regenerating a transgenic wheat plant therefrom.

Claim 21

A method for producing a transgenic wheat plant, substantially as hereinbefore described with reference to any one of the examples.

Process for the production of a fertile, transgenic wheat plant by transforming an immature embryo, a callus tissue or suspension cells of wheat with *Agrobacterium* having an exogenous gene.
The transgene can be selected from: an nptII gene, bla gene, nptI gene, dhfr gene, aphIV gene, aacC3, aacC4 and a GUS gene.
The fertile transgenic wheat plant produces progeny comprising a selectable or screenable marker integrated into the chromosomes.

EP 856060 A1

- Earliest priority - 21 June 1996
- Filed - 20 June 1997
- Application pending

Title - Methods for the Production of Stably-transformed, Fertile Wheat employing *Agrobacterium*-mediated transformation and compositions derived therefrom

Claim 1

A fertile, transgenic wheat plant, the genome of which has been altered through the genomic introduction of a pre-selected genetic component, said component comprising an exogenous gene positioned under the control of one or more pre-selected genetic control elements, the plant prepared by a process comprising:

A) preparing a DNA composition *in vitro*, which composition includes the genetic component one desires to introduce into the genome of a wheat plant;
B) introducing said DNA composition into recipient wheat cells by *Agrobacterium* transformation;
C) regenerating wheat plants from said cells which have received said genetic component; and
D) identifying a fertile, transgenic wheat plant whose genome has been altered through the stable introduction of said genetic component.

Claim 8

A fertile, transgenic wheat plant, the genetic complement of which has been altered through the addition of a DNA composition comprising a pre-selected functional genetic element that includes a transgene selected from the group consisting of an nptII gene, a bla gene, a nptI gene, a dhfr gene, a aphIV gene, a aacC3 gene, a aacC4 gene and a GUS gene, wherein said functional genetic element confers on said wheat plant a phenotypic trait that is not found in the parentage of said plant.

Claim 14

A method for producing a fertile transgenic wheat plant, comprising the steps of:

A) establishing a regenerable culture from a wheat plant to be transformed;
B) introducing a DNA composition comprising a genetic component one desires to introduce into the genome of said wheat plant, by

Agrobacterium transformation;
C) identifying or selecting a transformed cell line; and
D) regenerating a fertile transgenic wheat plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny, wherein said progeny comprises a selectable or screenable marker gene, and wherein said marker gene is chromosomally integrated.

Claim 18

A method for producing a transgenic wheat plant, comprising the steps of:

A) establishing a culture from a wheat plant to be transformed;
B) transforming said culture with an *Agrobacterium* comprising a DNA composition comprising a genetic component one desires to introduce into the genome of said wheat plant;
C) identifying or selecting a transformed cell line; and
D) regenerating a transgenic wheat plant therefrom.

National phase entry of WO 97/48814, this application is still pending.

Insertion of a foreign gene into a wheat plant via *Agrobacterium* . The foreign gene is selected from a group of genes conferring resistance to different antibiotics, i.e. kanamycin, hygromycin, and also the GUS gene. Regenerating and obtaining fertile transformed wheat plants is also covered by the claims.

Remarks

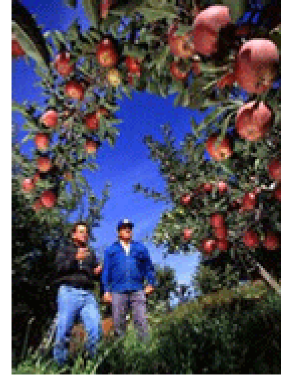
1. National phase entry of WO 97/48814 in Canada (CA 2230216) is still pending.
2. National phase entry of WO 97/48814 in China (CN 1208437) is deemed to be withdrawn on 26 January 2005.
3. WO 97/48814 has also entered national phase in Czech Republic (CZ 9800867), Hungary (HU 9902123), and Turkey (TR 9800294).
4. A related patent application in the US (US 20030024014 A1) is still pending.

Note: Patent information on this page was last updated on 9 February 2006.

Dicots

Overview

Dicotyledonous plants (dicots) are the second major group of plants within the *Angiospermae* division (flowering plants with seeds protected in vessels). The other major group is the monocots.



In contrast to monocots, dicots have an embryo with two cotyledons, which give rise to two seed leaves. The mature leaves have veins in a net-like pattern, and the flowers have four or five parts.

Apart from cereals and grasses that belong to the monocot group, most of the fruits, vegetables, spices, roots and tubers, which constitute a very important part of our daily diet, are classified as dicots. In addition, all legumes, beverages such as coffee and cocoa, and a great variety of flowers, oil seeds, fibers, and woody plants belong to the dicot group.

General transformation methods. Several patents encompass transformation of dicots, although they are mainly directed to the use of co-integrated vectors and binary vectors for the incorporation of foreign DNA into plants. A broad patent directed to transformation of dicots using an *Agrobacterium* strain lacking functional tumor genes was granted to **Washington University**. The invention teaches transformed dicot cells, regenerated plants and their progeny.

Agrobacterium transformation of particular dicots. Only patents with broad claims reciting methods for *Agrobacterium*-mediated transformation of dicot plants are presented here. Inventions directed to insertion of specific genes and generation of transformed plants exhibiting determined traits are beyond the scope and goal of this white paper.

The cited patents disclose inventions directed to general methods to obtain transgenic plants through *Agrobacterium*-mediated transformation from plant groups as diverse as pulses, vegetables, fiber crops, oil-producing crops and ornamental trees. The inventions cover aspects such as the initial tissue used for transformation, transformation protocols, media composition, and in some cases the insertion of particular genes into plants.

General transformation methods

Summary

A patent with broad claims to transformation of dicots in general with a non-oncogenic *Agrobacterium* was issued to **Washington University**.

Although issued in 2000 in the United States, this patent has an initial priority date of 1983. Thus, the prosecution process took approximately 17 years until the patent was finally granted by the United States Patent and Trademark Office (USPTO). The patent could be considered one of the broadest in scope granted in the area of *Agrobacterium* transformation. The patent rights under this patent may overlap with the rights already granted in previous patents related to transformation of dicots with *Agrobacterium*.

One of the distinctive factors of that patent is the knocking out of the cytokinin function in the Ti plasmid in order to get a non-tumorigenic *Agrobacterium* strain. As a general practice in *Agrobacterium*-mediated transformation, "disarmed" strains lacking functional tumorigenic genes are used. The present patent thus may constitute a blow for a widely used and standard procedure carried out to regenerate complete transformed dicot plants.

With respect to enablement of the invention, the examples referred to transformation of tobacco only, a model plant at the time the invention was initially filed and one of the easiest dicot plants to be transformed with *Agrobacterium*. No other plant examples are provided in the disclosure.

Most other patents analyzed here claim transformation of dicots in conjunction with the use of co-integrated or binary vectors, the vectors being the main subject matter of the claimed inventions. This group of patents are reviewed under the section of "Binary vectors and co-integrated vectors". In the following table, you will find a reference to those patents and links to more information on them.

Assigned to	Patent No.	Title	
Washington University	US 6,051,757	Regeneration of plants containing genetically engineered T-DNA	More information on this patent
Schilperoort &	US 4,693,976	Process for the incorporation of foreign DNA into the genome of dicotyledonous plants using stable co-integrated plasmids.	More information

Hille	EP 120 515 B1	A process for the incorporation of foreign DNA into the genome of dicotyledonous plants; a process for the production of *Agrobacterium tumefaciens* bacteria.	on these patents
Syngenta Mogen B.V.	US 4,940,838	Process for the incorporation of foreign DNA into the genome of dicotyledonous plants.	More information on these patents
	US 5,464,763		
	EP 120 516 B1	A process for the incorporation of foreign DNA into the genome of dicotyledonous plants; *Agrobacterium tumefaciens* bacteria and a process for the production thereof.	
Leiden University & Schilperoort	US 5,149,645	A process for introducing foreign DNA into the genome of plants	More information on this patent

Patent granted to Washington University

The present United States patent granted to **Washington University** discloses the transformation of dicot plants with an *Agrobacterium* vector having the cytokinin gene of the T-DNA region inactivated. According to the USPTO assignments database, this patent was exclusively licensed to Syngenta.

In a wildtype T-DNA of a Ti plasmid, the genes encoding phytohormones are responsible for the tumorous state of a transformed tissue. Cytokinin, one of those phytohormones, induces the formation of shoots in a tumor.

In the disclosed invention, regeneration of a transformed dicot plant is achieved by inactivating the cytokinin gene. Additionally, the cytokinin gene is replaced by foreign DNA. The plasmid containing the mutant T-DNA, with foreign DNA replacing the cytokinin gene, is accomplished by homologous recombination within *Agrobacterium*.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 6051757 A • Earliest priority - 4 November 1983 • Filed - 5 June 1995 • Granted - 18 April 2000 • Expected expiry - 17 April 2017	**Title** - Regeneration of plants containing genetically engineered T-DNA **Claim 1** A method of transforming a dicotyledonous plant susceptible to transformation by *Agrobacterium*, comprising: contacting the plant with an *Agrobacterium tumefaciens* bacterium comprising a gene vector, the vector comprising (i) DNA foreign to the *Agrobacterium*, and (ii) the vector not comprising a functional cytokinin autonomy gene. **Claim 2** A method for producing a morphologically and developmentally normal dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated in the plant's genome, said method comprising the following steps: A) transforming a dicotyledonous plant cell susceptible to transformation by *Agrobacterium* with an *Agrobacterium* -derived gene vector, said vector comprising (i) non-*Agrobacterium* foreign DNA and (ii) the vector not comprising a functional cytokinin autonomy gene; and	Washington University, exclusively licensed to Syngenta

B) regenerating said transformed plant cell to produce a morphologically and developmentally normal transformed plant with said foreign DNA stably integrated in the plant's genome.

Claim 5

A method for producing a transgenic dicotyledonous plant comprising a stably integrated non-*Agrobacterium* foreign DNA, the method comprising:

A) sexually propagating a dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated into its genome, said foreign DNA having been introduced into the genome by an *Agrobacterium*-derived gene vector not comprising a functional cytokinin-autonomy gene; and
B) selecting for progeny plants which comprise the non-*Agrobacterium* foreign DNA stably integrated into the genome of said progeny plants.

Claim 6

A method for producing a transgenic dicotyledonous plant comprising stably integrated non-*Agrobacterium* foreign DNA, the method comprising:

A) sexually propagating a dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated into its genome, said plant derived from a dicotyledonous plant which was transformed by *Agrobacterium*-mediated transformation with a gene vector comprising
(i) said non-*Agrobacterium* foreign DNA and
(ii) not comprising a functional cytokinin autonomy gene; and

B) obtaining a progeny plant which comprises the non-*Agrobacterium* foreign DNA stably integrated into its genome.

Claim 7

A method for producing a transgenic dicotyledonous plant comprising stably integrated non-*Agrobacterium* foreign DNA, the method comprising:

A) propagating a dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated into its genome, said plant derived from a dicotyledonous plant which was transformed by *Agrobacterium*-mediated transformation with a gene vector comprising
(i) said non-*Agrobacterium* foreign DNA and
(ii) not comprising a functional cytokinin autonomy gene; and

B) obtaining a plant which comprises the non-*Agrobacterium* foreign DNA stably integrated into its genome.

Claim 8

A method for producing a transgenic dicotyledonous plant comprising stably integrated non-*Agrobacterium* foreign DNA, the method comprising:

growing a seed of a dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated into its genome, said plant derived from a dicotyledonous plant which was transformed by *Agrobacterium*-mediated transformation with a gene vector comprising

(i) said non-*Agrobacterium* foreign DNA and
(ii) not comprising a functional cytokinin autonomy gene.

Claim 9

A method for producing seed of a transgenic dicotyledonous plant comprising stably integrated non-*Agrobacterium* foreign DNA, the method comprising:

A) propagating a dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated into its genome, said plant derived from a dicotyledonous plant which was transformed by *Agrobacterium*-mediated transformation with a gene vector comprising
(i) said non-*Agrobacterium* foreign DNA and
(ii) not comprising a functional cytokinin autonomy gene; and

B) harvesting seed from said propagated plant.

Claim 10

A method of transforming a dicotyledonous plant of a species that is a naturally susceptible host for *Agrobacterium*, comprising:

contacting the plant with an *Agrobacterium* bacterium comprising a gene vector, the vector comprising
(i) DNA foreign to the *Agrobacterium* and
(ii) the vector not comprising a functional cytokinin autonomy gene.

Claim 11

A method for producing a transgenic dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated in the plant's genome, said method comprising the following steps:

A) transforming a cell of a dicotyledonous plant species that is a naturally susceptible host for *Agrobacterium* by *Agrobacterium* -mediated transformation with a gene vector comprising
(i) non-*Agrobacterium* foreign DNA and
(ii) not comprising a functional cytokinin autonomy gene; and

B) regenerating said transformed plant cell to produce a normal transformed dicotyledonous plant with said foreign DNA stably integrated in the plant's genome.

Claim 14

A method for producing a transgenic dicotyledonous plant comprising a stably integrated non-*Agrobacterium* foreign DNA, the method comprising:

A) sexually propagating a dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated into its genome, said plant derived from a dicotyledonous plant which is of a species that is a naturally susceptible host for *Agrobacterium* and which was transformed by *Agrobacterium*-mediated transformation with a gene vector comprising
(i) said non-*Agrobacterium* foreign DNA and
(ii) not comprising a functional cytokinin autonomy gene; and

B) obtaining a progeny plant which comprises the non-*Agrobacterium* foreign DNA stably integrated into its genome.

Claim 15

A method for producing a transgenic dicotyledonous plant comprising a stably integrated non-*Agrobacterium* foreign DNA, the method comprising:

A) propagating a dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated into its genome, said plant derived from a dicotyledonous plant which is of a species that is a naturally susceptible host for *Agrobacterium* and which was transformed by *Agrobacterium*-mediated transformation with a gene vector comprising
(i) said non-*Agrobacterium* foreign DNA and
(ii) not comprising a functional cytokinin autonomy gene; and

B) obtaining a plant which comprises the non-*Agrobacterium* foreign DNA stably integrated into its genome.

Claim 16

A method for producing a transgenic dicotyledonous plant comprising stably integrated non-*Agrobacterium* foreign DNA, the method comprising:

growing a seed of a dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated into its genome, said plant derived from a dicotyledonous plant which is of a species that is a naturally susceptible host for *Agrobacterium* and which was transformed by *Agrobacterium*-mediated transformation with a gene vector comprising
(i) said non-*Agrobacterium* foreign DNA and
(ii) not comprising a functional cytokinin autonomy gene.

Claim 17

A method for producing seed of a transgenic dicotyledonous plant comprising stably integrated non-*Agrobacterium* foreign DNA, the method comprising:

A) propagating a dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated into its genome, said plant derived from a dicotyledonous plant which is of a species that is a naturally susceptible host for *Agrobacterium* and which was transformed by *Agrobacterium*-mediated transformation with a gene vector comprising
(i) said non-*Agrobacterium* foreign DNA and
(ii) not comprising a functional cytokinin autonomy gene; and

B) harvesting seed from said propagated plant.

Claim 18

An *Agrobacterium*-mediated method for genetically engineering a dicotyledonous plant comprising:

A) producing a transgenic plant cell by transforming a cell of dicotyledonous plant species that is a naturally susceptible host for *Agrobacterium* with a gene vector comprising non-*Agrobacterium* foreign DNA and not comprising a functional cytokinin autonomy gene by *Agrobacterium tumefaciens*-mediated transformation; and
B) regenerating a whole normal plant from the transgenic plant cell which contains said foreign DNA stably integrated into its genome.

	Claim 20 A method for producing a transgenic dicotyledonous plant comprising intact T-DNA comprising non-*Agrobacterium* foreign DNA stably integrated into the genome of said plant, the method comprising: A) propagating a dicotyledonous plant comprising non-*Agrobacterium* foreign DNA stably integrated into its genome, said plant derived from a dicotyledonous plant which is of a species that is a naturally susceptible host for *Agrobacterium* and which was transformed by *Agrobacterium*-mediated transformation with a disarmed T-DNA gene vector comprising (i) said non-*Agrobacterium* foreign DNA and (ii) not comprising a functional cytokinin autonomy gene; and B) obtaining a plant which comprises the intact T-DNA stably integrated into its genome. The United States patent US 6051757 claims • transformation of a dicot plant, which is either susceptible to transformation (claim 1) or a naturally susceptible host (claim 10) with *A. tumefaciens* (claim 1) or an *Agrobacterium* (claim 10) having a vector that lacks the cytokinin function and contains foreign DNA instead; • regeneration of a transformed dicot cell and production of a morphologically normal plant; • sexual or non-sexual propagation of the transformed dicot plant and generation of a progeny bearing the foreign gene; • seed production and harvesting from a propagated transformed dicot plant.	
Remarks	A related United States application (US 07/155092) was in interference, the process by which the United States Patent Office determines who was the earliest inventor when there are competing claims (in this case, from Monsanto). According to the USPTO status database PAIR, Syngenta lost the interference case in 2004.	

Note: Patent information on this page was last updated on 10 March 2006.

Particular dicot plants

Find out more information about patents on particular dicot plants by following the links shown below.

- *Acacia* spp
- Beans (*Phaseolus*)
- *Brassica* spp
- Cacao
- *Camelina sativa*
- Carnations
- Chrysanthemums
- *Citrus*
- Coffee (*Coffea* spp)

- Cotton (*Gossypium* spp)
- *Eucalyptus*
- Guar (*Cyamopsis tetragonolobus*)
- *Impatiens*
- Melon (*Cucumis spp*)
- Peas (*Pisum* spp)
- *Pelargonium*
- Pepper (*Piper* spp)
- Poplar (*Populus* spp)
- Roses (*Rosa* spp)
- Soybean (*Glycine* spp)
- Squash (*Cucurbita* spp)
- Strawberry (*Saxifraga* spp)
- Sugar beet (*Beta* spp)
- Tomato (*Lycopersicum* spp)
- Woody tree species

Acacia
Summary

The genus *Acacia*
is composed of as many as 8000 species native to the tropics and subtropics. The commercially valuable species *Acacia mangium* is variously known as "Mangium", "Black Wattle", "Hickory Wattle", and is traded as brown salwood. Its distribution covers the northern part of Queensland in Australia, Papua New Guinea and the Molucca Islands of Indonesia. Mangium is a fast-growing tree, relatively short-lived (30-50 years) and adapted to a wide range of acidic soils in most tropical lowlands. It is a pioneer species that colonize disturbed sites. Major plantations of *A. mangium* are grown in Southeast Asia for paper pulp. Wood from this species is used in furniture, handles of sporting goods, door frames and light construction. Plantations of *A. mangium*
are also used for erosion control, shade and shelter. This species is playing an important role in efforts to sustain commercial supply of tree products while reducing pressure on natural forest ecosystems.

The **Institute of Molecular Agrobiology** from Singapore has filed applications related to the genetic transformation of *A. mangium* with *Agrobacterium* and to the regeneration of the species. The PCT applications disclose methods for transforming *A. mangium* with a gene of interest using *A. tumefaciens*. One of the recited methods uses stems as initial tissue to be transformed with the bacterium.

Acacia (*Acacia mangium*)		
Assigned to The Institute of Molecular Agrobiology		
Application No.	Publication Date	Summary of the claims
WO 0153452 A2 Full PCT application text (1,865 kb)	July 26, 2001	Methods for transforming explants of *A. mangium* with a gene of interest by co-cultivating *A. tumefaciens* having the gene of interest with pre-cultured explants of the tree species. In one of the methods the tissue selected for transformation is stems. Transgenic cells and plants of *A. mangium* are also part of the recited invention. Additional claims recite methods for the regeneration of non-transformed *A. mangium* from different tissues. View Claims

Remarks: The present PCT application entered the European phase on October 17, 2001. Applications also filed in Australia (AU 41625/00) and Norway (NO 200104331).

Acacia mangium
Patent application filed by the Institute of Molecular Agrobiology

Actual filed claims*

WO 0153452 A2
Claim 1 A method for regenerating *Acacia mangium* comprising: A) inducing callus formation from an explant; B) culturing said callus to produce adventitious buds; C) culturing said adventitious buds to elongate and produce pinnate leaves; and D) culturing elongated buds of step (c) such that they produce roots and become plantlets.
Claim 11 A method for regenerating *Acacia mangium* comprising: A) culturing auxiliary buds from an *Acacia mangium* tree to produce adventitious buds comprising phyllodes; B) subculturing said adventitious buds comprising phyllodes to produce adventitious shoots; and C) culturing said adventitious shoots.
Claim 14* A method of transforming *Acacia mangium* with a gene of interest comprising the steps of: A) activating *Agrobacterium tumefaciens* comprising said gene of interest to form activated *Agrobacterium tumefaciens*; B) preculturing an explant of *Acacia mangium* to yield a precultured explant; C) co-cultivating said activated *Agrobacterium tumefaciens* and said precultured explant to produce infected explants; D) culturing said infected explants to induce callus and adventitious buds; and E) culturing said callus or adventitious buds on a selective medium.
Claim 25 A method for promoting root formation from transformed adventitious buds comprising culturing transformed adventitious buds on a medium comprising 1/2 MS basic medium supplemented with: A) cx-naphthaleneacetic acid, B) kinetin, C) casein enzymatic hydrolysate, D) L-ascorbic acid, E) L-glutamine, F) L-asparagine, G) L-proline, H) sucrose and I) phytagel.
Claim 28* A method of preparing transgenic *Acacia mangium* cells comprising the steps of: a) preculturing stem pieces of *Acacia mangium* in a culture medium; and b) co-cultivating said stem pieces of step (a) with activated *Agrobacterium tumefaciens*.
Claim 41 A transgenic *Acacia mangium* cell.

Claim 42
A transgenic *Acacia mangium* plant.

* Independent **claims 1** and **11** recite methods for regenerating *A. mangium,* but they do not recite transformed plants. The rest of the independent claims as filed are directed to transformed or transgenic *A. mangium*, with **claims 14** and **28** being directly relevant to transformation with *A. tumefaciens*.

Beans (*Phaseolus vulgaris*)
Summary

The **University of Toledo** has been granted three patents in the United States, Australia and Europe directed to the transformation of *Phaseolus vulgaris* (common beans) with *Agrobacterium.*

One of the distinctive features of the claimed intentions is a lack of regeneration of transformed bean plants in a tissue culture media. Mesocotyl cells of beans seedlings are inoculated with *Agrobacterium* and then the plant is allowed to grow normally. Note that the European patent claims transformation of *Phaseolus vulgaris* (common beans) as well as *Glycine max* (soybeans).

The table shown below presents a summary of the scope of the claims of the mentioned patents. Full text of patents can be accessed as PDF.

Beans (*Phaseolus vulgaris*)		
Assigned to The University of Toledo		
Patent No.	Issue Date	Summary of the claims
US 5 169 770 & AU 633 248 B Full United States patent text (923 kb)	December 8, 1992 & January 28, 1993	Production of a transgenic bean plant through a non-tissue culture process. In it, the meristematic or mesocotyl cells are inoculated with an armed or disarmed *Agrobacterium* vector having a gene of interest. The cells differentiate into a mature plant. View Claims
EP 397 687 B1 Full patent text (830 kb)	December 5, 1994	The method for producing a transgenic bean plant is the same as the one disclosed in the related United States andAustraliapatents (above mentioned), but transformation of soybean (*Glycine max*) is also claimed. View Claims
AU 633 248 B deemed to be abandoned according to IP Australia database (Update July 2003).		

Beans (*Phaseolus vulgaris*)
Patent granted to The University of Toledo

Actual granted claims

US 5 169 770 & AU-B- 633 248
Claim 1 A non-tissue culture process for producing a transgenic plant, which process comprises: A) germinating a seed of a *Phaseolus vulgaris* plant for about 24 to 48 hours; B) inoculating the meristematic or mesocotyl cells produced by the germinating seed of step (A), prior to differentiation of said cells, with an armed or disarmed *Agrobacterium* strain containing an *Agrobacterium*-derived vector, said vector containing a transferable gene; and

C) allowing the cells to differentiate into a mature plant.

EP 397 687 B1

Claim 1

A non-tissue culture process for producing a transgenic plant, which comprises:

A) germinating a seed of a *Phaseolus vulgaris* or a *Glycine max* plant for 24 to 48 hours;
B) inoculating the meristematic or mesocotyl cells produced during germination, prior to differentiation of the seed, with a virulent or non-virulent *Agrobacterium* strain containing a transferable gene in an *Agrobacterium* -derived vector; and
C) allowing the cells to differentiate into a mature plant.

Brassica

Summary

The genus *Brassica* encompasses crops such as broccoli, brussel sprouts, cabbage and cauliflower. In addition turnips and choy sum, an Asian vegetable, belong to the *Brassica* genus. Finally mustards, canola or rapeseed and rutabaga are species of *Brassica* as well.

The patents granted to **Calgene** in the United States and in Europe are directed to transgenic *Brassica* cells containing expression cassettes inserted in their genome through co-cultivation with a disarmed *Agrobacterium tumefaciens*. *Brassica* explants, such as leaf and hypocotyl tissue, and protocols to achieve transformation are claimed as well as the component elements of the expression cassettes.

One the most limiting aspects of the claimed inventions is the sequences of the expression cassette or construct inserted into the genome of *Brassica* plants. In this regard the expression cassette must contain the following elements in 5' to 3' order:

- a transcription initiation region;
- an open reading frame (ORF) or a sequence complementary to an endogenous sequence of a plant;
- a right T-DNA border; and
- a structural gene.

AgrEvo (now Bayer Crop Science) has a granted United States patent that is directed to a method to produce a transgenic *Brassica* microspore using *Agrobacterium* . The microspore is treated first with a mucolytic enzyme to kill the bacteria and then develops into a haploid or doubled haploid embryo and ultimately a homozygous transgenic plant (Update July 2003).

The **University of Helsinki** has granted Australian and United States patents and a European application directed to the transformation of **turnip rape** (*Brassica rapa*) with *A. tumefaciens*. The claims as granted in Australia and in the US and claims as filed in Europe are fairly narrow in scope; the methods comprise very detailed steps, e.g. type of tissue to be transformed and specific, pre-cultivation and co-cultivation conditions (Update July 2003).

The following table presents basic bibliographic data and a summary of the scope of the claimed inventions. Full text of patents can be accessed as PDF files.

Brassica spp.		
Assigned to Calgene		
Patent No.	Issue Date	Summary of the claims
US 5 188 958 Full patent text (1,643 kb)	23 Feb 1993	Transformation of *Brassica* with a disarmed *A. tumefaciens* having a cassette with a right T-DNA border from a Ti or a Ri-plasmid that is free of oncogenic genes. Induction of callus and shoot formation are key steps in the method to regenerate plants having an altered phenotype. The elements of the expression cassette are also recited in the claims.

		View Claims
EP 270 615 B1 Full patent text (1.6 kb)	4 Aug 1993	Transgenic *Brassica* cells and progeny having an expression cassette that confers an altered phenotype. A method to transform those cells with a disarmed *A. tumefaciens* is also claimed. The method is the same as the one claimed in US 5 188 958. View Claims
US 5 463 174 Full patent text (1,877 kb)	31 Oct 1995	Method to transform *Brassica* leaf and hypocotyl explants with a disarmed *A. tumefaciens* having a cassette with a right T-DNA border. Media composition for callus and shoot regeneration are claimed. A claimed expression cassette contains *npt II* gene for conferring resistance to kanamycin; other elements of the cassette are also recited in the claims. View Claims
US 5 750 871 Full patent text (1,746 kb)	12 May 1998	The inventors claim elements of an expression cassette that is integrated into the genome of *Brassica* cells. Hypocotyl and leaf explants are used for transformation. Medium components for callus and shoot regeneration are also claimed. View Claims
Remarks: Applications were also filed in Finland (FI 880383 A), Japan (JP 1500718 T2) and Sweden (SE 8800157 A).		
Assigned to AgrEvo Canada Inc. (now Bayer Crop Science) (Update July 2003)		
Patent No.	Issue Date	Summary of the claims
US 6 316 694 Full patent text (738 Kb)	13 Nov 2001	A method to produce a transgenic *Brassica* microspore using an *Agrobacterium* carrying a gene of interest. After treatment with a mucolytic enzyme to kill the bacteria the microspore develops into haploid or doubled haploid embryo which can further develop into a homozygous transgenic plant. View Claims
Remarks: related patent in Australia (AU 710 201 B2), applications in Canada (CA 2 215 763) and Europe (EP 832 259)		
Turnip rape (*Brassica rapa*) (Update July 2003)		
Assigned to Helsinki University		
Issued patent		
Patent No.	Issue Date	Summary of the claims
AU 732 372 B2	26 April 2001	A method of transforming mature turnip rape plants by immersing an internode section of the inflorescence-carrying stem in MS medium with *A. tumefaciens* carrying at least one heterologous gene. Conditions for pre-cultivating the tissue to be transformed, co-cultivating the tissue with the bacteria, and regenerating transgenic shoots are also recited in the claims.
US 6,455,761	24 Sep 2002	see Australian equivalent. View Claims
Application		
Application No.	Publication Date	Summary of the claims
EP 1 009 845 A1* Full patent application	21 June 2000	The independent claims as filed are worded the same as the granted Australian claims. View Claims

text (WO 99/14349 A1) (1,231 kb)		

Remarks: A related patent has been granted in Finland (**FI 104907 B1**). The document is in Finnish and Swedish.
There is also a related application filed in Canada (CA 2302835).

*It is important to remember that applications are **not issued patents** and the claims as filed have not been approved by any country. Thus, they are non-binding. To view or to download patents as a PDF file click on AU 732 372 (1.226 Kb) or US 6 455 761 (720 Kb)

Brassica
Patents granted to Calgene

Actual granted claims

US 5 188 958
Claim 1 Transgenic *Brassica* species cells and progeny thereof comprising an expression cassette, wherein said cells are characterized as oncogene-free and capable of regeneration to morphologically normal whole plants, and wherein said expression cassette comprises, in the 5' to 3' direction of transcription: (i) a transcription initiation region functional in *Brassica* species cells; (ii) a DNA sequence comprising an open reading frame having an initiation codon at its 5' terminus or a nucleic acid sequence complementary to an endogenous transcription product which when expressed will alter the phenotype of said transgenic cells; (iii) a transcription termination region functional in *Brassica* species cells; (iv) a right border of T-DNA; and (v) a structural gene capable of expression in said Brassica providing for selection of transgenic *Brassica* species cells; wherein said expression cassette is capable of altering the phenotype of said *Brassica* species cells when said cells are grown under conditions whereby said DNA sequence or said nucleic acid sequence is expressed.
Claim 8 A transformed *Brassica* plant produced according to the method comprising: A) co-cultivating *Brassica* cells with disarmed *A. tumefaciens* comprising a plasmid containing an insertion sequence resulting from joining in vitro of a transcription cassette to at least the right T-DNA border of a Ti or Ri plasmid whereby said *Brassica* cells are transformed with said insertion sequence which becomes integrated into the plant cell genome to provide transformed oncogene-free cells; B) transferring said transformed oncogene-free cells to callus inducing medium, wherein said callus inducing medium contains at least one auxin and a means for selecting for transformed cells as a result of a marker carried on said plasmid whereby callus comprising transformed cells is produced; C) transferring said callus to regeneration medium containing less than about 2% sucrose or an organic caloric equivalent thereto to produce shoots; and D) transferring said shoots to a growing medium to produce plants capable of having an altered phenotype when grown under condition whereby a DNA sequence in said insertion sequence is expressed.

EP 270 615 B1
Claim 1 Transgenic *Brassica* species cells having a DNA construct resulting from *in vitro* joining of at least two fragments, wherein said fragments comprise: (i) a transcription initiation region functional in said *Brassica*; (ii) a DNA sequence comprising an open reading frame having an initiation codon at its 5' terminus or a sequence complementary to an endogenous transcription product; (iii) a transcription termination region functional in said *Brassica*; (iv) a right border of T-DNA; and (v) a structural gene capable of expression in said *Brassica* providing for selection of transformed *Brassica*

cells;
wherein said fragments provide an expression cassette capable of expression in said *Brassica* cells.

Claim 7

A method for transforming *Brassica* cells to produce *Brassica* plants said method comprising:

A) co-cultivating *Brassica* cells with *A. tumefaciens* comprising a plasmid comprising an insertion sequence resulting from the *in vitro* joining of a transcription cassette to at least the right T-DNA border and a marker which provides for selection of cells containing said marker, whereby said *Brassica* cells are transformed with said insertion sequence which becomes integrated into the plant cell genome;
B) transferring said transformed *Brassica* cells to callus inducing media containing at least one auxin and selecting for cells comprising said marker to produce callus from said transformed cells;
C) transferring said callus to regeneration media containing less than about 2% sucrose or an organic caloric equivalent to produce shoots; and
D) transferring said shoots to a growing medium to produce plants.

US 5 463 174

Claim 1

Transgenic *Brassica* species cells and progeny thereof comprising an expression cassette, wherein said cells are characterized as oncogene-free and capable of regeneration to morphologically normal whole plants, and wherein said expression cassette comprises, in the 5' to 3' direction of transcription:
(i) a transcription initiation region functional in *Brassica* species cells;
(ii) a DNA sequence comprising an open reading frame having an initiation codon at its 5' terminus or a nucleic acid sequence complementary to an endogenous transcription product; and
(iii) a transcription termination region functional in *Brassica* species cells;
wherein at least one of said transcription initiation region and transcription termination region is not naturally associated with said DNA sequence or said nucleic acid sequence; and
wherein said expression cassette imparts a detectable trait when said *Brassica* species cells are grown under conditions whereby said DNA sequence or said nucleic acid sequence is expressed.

Claim 8

A method for transforming *Brassica* species cells to produce morphologically normal whole *Brassica* plants capable of having an altered phenotype as a result of said transformation, said method comprising:

A) co-cultivating a *Brassica* leaf explant with disarmed *A. tumefaciens* comprising a plasmid containing an expression cassette joined to at least a right T-DNA border, whereby said expression cassette becomes integrated into the genome of cells in said *Brassica* explant to provide transformed oncogene-free cells;
B) transferring said explant to callus inducing medium comprising approximately 1 mg/l of one or more growth regulators selected from the group consisting of 2,4-D, kinetin and zeatin to allow callus to form on said explant;
C) transferring said callus to regeneration medium containing less than about 2% sucrose, or an organic caloric equivalent thereto, and comprising at least one milligram per liter of a cytokinin, and a means for selecting for transformed cells as a result of a marker carried on said plasmid, whereby shoots comprising transformed cells are produced from said callus; and
D) transferring said shoots to a growing medium to produce plants capable of having an altered phenotype when grown under conditions whereby a DNA sequence in said expression cassette is expressed.

Claim 11

A transformed *Brassica* plant produced according to the method comprising:

A) co-cultivating a *Brassica* leaf or hypocotyl explant with disarmed *A. tumefaciens* comprising a plasmid containing an expression cassette joined to at least a right T-DNA border, whereby said expression cassette becomes integrated into the genome of cells in said *Brassica* explant to provide transformed oncogene-free cells, and wherein said expression cassette comprises a neomycin phosphotransferase II gene conferring kanamycin resistance;
B) transferring said explant to callus inducing medium comprising at least one auxin to allow callus to form on said explant;
C) transferring said callus to regeneration medium containing less than about 2% sucrose, or an organic caloric equivalent thereto, and comprising at least one milligram per liter of a cytokinin, and a means for selecting for transformed cells as a result of expression of said neomycin phosphotransferase II gene,

whereby shoots comprising transformed cells are produced from said callus; and
D) transferring said shoots to a growing medium to produce plants.

Claim 19

A cell culture of stably transformed *Brassica* species cells, wherein said cells are capable of regeneration into morphologically normal whole *Brassica* plants capable of having an altered phenotype as a result of said transformation and regeneration, and wherein said cells are produced according to a method comprising:

A) co-cultivating a *Brassica* leaf or hypocotyl explant with disarmed *A. tumefaciens* comprising a plasmid containing an expression cassette joined to at least a right T-DNA border, whereby said expression cassette becomes integrated into the genome of cells in said *Brassica* tissue explant to provide transformed oncogene-free cells;
B) transferring said tissue explant to callus inducing medium to produce callus comprising stably transformed cells, wherein said callus inducing medium contains at least one auxin and a means for selecting for transformed cells as a result of a marker carried on said plasmid.

Claim 20

Transgenic *Brassica* species cells and progeny thereof comprising an expression cassette, wherein said cells are characterized as oncogene-free and capable of regeneration to morphologically normal whole plants, and wherein said expression cassette comprises, in the 5' to 3' direction of transcription:
(i) a transcription initiation region functional in *Brassica* species cells;
(ii) a DNA sequence comprising a gene of interest encoding a protein product or a nucleic acid sequence complementary to an endogenous transcription product which when expressed will alter the phenotype of said transgenic cells; and
(iii) a transcription termination region functional in *Brassica* species cells;
wherein said expression cassette further comprises a structural gene capable of expression in said *Brassica* species cells and providing for selection of *Brassica* species cells comprising said structural gene, and a right border region of T-DNA capable of providing for integration of said expression cassette into the genome of said *Brassica* species cells; and
wherein said gene of interest or said nucleic acid sequence is expressed and imparts a detectable trait to said *Brassica* species cells.

US 5 750 871

Claim 1

Transgenic *Brassica* species cells and progeny thereof comprising an expression cassette, wherein said cells are characterized as oncogene-free and capable of regeneration to morphologically normal whole plants, and wherein said expression cassette comprises, in the 5' to 3' direction of transcription:
(i) a transcription initiation region functional in *Brassica* species cells;
(ii) a DNA sequence comprising an open reading frame having an initiation codon at its 5' terminus or a nucleic acid sequence complementary to an endogenous transcription product; and
(iii) a transcription termination region functional in *Brassica* species cells;
wherein at least one of said transcription initiation region and transcription termination region is not naturally associated with said DNA sequence or said nucleic acid sequence; and
wherein said expression cassette is integrated into the genome of said *Brassica* species cells and imparts a detectable trait when said *Brassica* species cells are grown under conditions whereby said DNA sequence or said nucleic acid sequence is expressed.

Claim 15

A method for transforming *Brassica* species cells to produce morphologically normal whole *Brassica* plants having an altered phenotype as a result of said transformation, said method comprising:

A) co-cultivating a *Brassica* hypocotyl or leaf explant with disarmed *A. tumefaciens* comprising a plasmid containing an expression cassette joined to at least a right T-DNA border, whereby said expression cassette becomes integrated into the genome of cells in said *Brassica* explant to provide transformed oncogene-free cells;
B) transferring said explant to callus inducing medium to allow callus to form on said explant, wherein said callus inducing medium comprises about 1 mg/l of an auxin and from about 0 to 1 mg/l of a cytokinin;
C) transferring said callus to regeneration medium containing less than about 2% sucrose, or an organic caloric equivalent thereto, and comprising at least one milligram per liter of a cytokinin, and a means for

selecting for transformed cells as a result of a marker carried on said plasmid, whereby shoots comprising transformed cells are produced from said callus; and
D) transferring said shoots to a growing medium to produce plants having an altered phenotype when grown under conditions whereby a DNA sequence in said expression cassette is expressed.

Brassica
Independent claims

Patent US 6,316,694 B1 Assigned to AgrEvo Canada Inc. (now Bayer Crop Science)

Summary of the invention

This patent discloses a method to generate transgenic plants from the Brassica family by using microspores as a target tissue for Agrobacterium carrying a gene of interest. After infection bacteria are eliminated by treatment with mucolytic enzymes. Microspores are further cultivated to produce haploid or doubled haploid embryos from which fully regenerated transgenic plants are obtained.

Claims in the corresponding European patent application are not limited to plants from the Brassica family.

Patent assigned to AgrEvo Canada Inc. (now Bayer Crop Science)
US 6,316,694 B1
Claim 1 A method for producing a stably transformed Brassica embryogenic microspore, capable of leading to a non-chimeric transformed haploid or doubled haploid embryo which develops into a fertile homozygous Brassica plant within one generation, said process comprising the following steps: a. infecting an embryogenic microspore with Agrobacteria, which contain a plasmid carrying a gene of interest under regulatory control of initiation and termination signals bordered by at least one T-DNA border, and b. washing out and killing the Agrobacteria after co-cultivation using mucolytic enzymes, thereby producing a stably transformed Brassica embryogenic microspore.
Claim 2 A method for producing a non-chimeric Brassica plant, containing a foreign DNA stably incorporated into its genome, said method comprising: a. co-cultivating a Brassica embryogenic microspore with Agrobacteria which contains a plasmid carrying a gene of interest under regulatory control of initiation and termination signals; b. washing out and killing the Agrobacteria after co-cultivation using mucolytic enzymes; and c. regenerating a non-chimeric haploid or doubled haploid Brassica embryo from said microspore, wherein the embryo contains said gene of interest stably integrated into its genome, thereby producing a non-chimeric Brassica plant.

Patent US 6,455,761 B1 assigned to Helsinki University

Summary of the invention

The independent claim of United Stated patent US 6,455,761 discloses a quite detailed protocol of how to obtain transgenic turnip rape (*Brassica rapa*). This offers a number of opportunities to develop similar methods and avoid infringement with respect to this specific patent at the same time. Other patents have more general claims that cover the use of Agrobacterium to produce transgenic dicots for example.

Independent claims

Patent assigned to Helsinki University
US 6,455,761 B1
Claim 1 1. A method for transforming mature plants of turnip rape, comprising (i) excising an internode section of the inflorescence-carrying stem of a mature turnip rape plant, (ii) sterilizing said internode section, and cutting it in 4-8 mm segments to obtain an internode segment,

(iii) placing an internode segment in a horizontal position on an agar pre-cultivation medium supplemented with 15-90 μM of silver nitrate and 2,4-dichlorophenoxyacetic acid (2,4-D) hormone,
(iv) pre-cultivating the internode segment on said medium for 1 day,
(v) immersing the internode segment in a MS solution inoculated with *Agrobacterium* tumefaciens bacteria carrying at least one gene heterologous to said turnip rape,
(vi) placing the immersed internode segment in a horizontal position on an agar MS co-cultivation medium,
(vii) co-cultivating the internode segment with Agrobacteria for 2 days,
(viii) washing the internode segment to remove the Agrobacteria,
(ix) placing the internode segment in a vertical position with the basal side down on MS agar medium for selection with an antibiotic, the medium being supplemented with cytokinine hormones and silver nitrate, to obtain an internode segment with regenerated primordia or embryonic green nodules,
(x) placing the internode segment with regenerated primordia or embryogenic green nodules on a hormone-free regeneration medium, and
(xi) recovering the transgenic shoots regenerated.

Turnip rape (*Brassica rapa*)
Granted patent and patent application assigned to the University of Helsinki

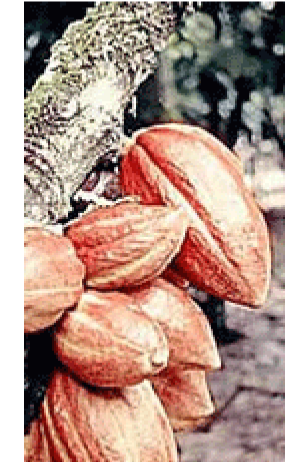
Actual granted (and pending*) claims

AU-B-732 372 & EP 1 009 845*
Claim 1 A method for transformation of mature plants of turnip rape, comprising: A) excising an internode section of the inflorescence-carrying stem of a mature turnip rape plant, B) sterilizing said internode section, and cutting it in 4-8 mm segments, C) placing a segment in a horizontal position on an agar pre-cultivation medium supplemented with silver nitrate and 2,4-dichlorophenoxyacetic acid (2,4-D) hormone, D) pre-cultivating the segment on said medium for 1 day, E) immersing the segment in a MS solution inoculated with *Agrobacterium tumefaciens* bacteria carrying at least one gene heterologous to said turnip rape, F) placing the immersed segment in a horizontal position on an agar MS co-cultivation medium, G) co-cultivating the segment with Agrobacteria for 2 days, H) washing the segment from the Agrobacteria, I) placing the segment in a vertical position with the basal side down on MS agar medium for selection with an anti biotic, the medium being supplemented with cytokinine hor mones and silver nitrate, and J) placing the segment with regenerated primordia or embryogenic green nodules on a hormone free regeneration medium, and K) recovering the transgenic shoots regenerated.

*
The independent claim as filed in the present European application is worded the same as the granted Australian independent claim.

Cacao
Summary

The **Penn State Research Foundation** has filed patent applications in Europe and



Australia (now abandoned) and has been granted a United States patent related to transformation of cacao floral tissue or a cacao cell with *Agrobacterium*.

The Australian application was abandoned in March 2000.

The invention sets out different steps for a method to transform cacao tissue. The steps include

- induction of primary callus growth in a specified culture medium;
- induction of secondary callus growth in a medium with specific requirements; and
- embryo development in a specific hormone-free medium.

In contrast to the claims of the European application, the claims of the United States patent do not require regeneration of transformed cacao plantlets; they just encompass embryo development.

The following table presents basic bibliographic data and a summary of the invention. Full text of the patent and patent application can be accessed as PDF.

Cacao		
Assigned to The Penn State Research Foundation		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 6 150 587 Full patent text (2,703 kb)	November 21, 2000	Transformation of cacao floral tissue or cacao cells with a non-tumorigenic *Agrobacterium*. Media for cultivation of embryogenic callus and i nduction of embryo differentiation are also claimed. View Claims
Application		
Application No.	Publication date	Summary of the claims
EP 999 737 A1 * Full EP application text (3,133 kb)	May 17, 2000	In contrast to the related United States patent **6 150 587**, the submitted claims recite transformation of **any** cacao tissue with a non-tumorigenic *Agrobacterium*. Additionally, they recite a method for regeneration of cacao plantlets starting from transformed cacao somatic embryo. View Claims
Remarks: the Australian application **AU-81743/98 A1** was **abandoned** on **March 9, 2000.**		

*It is important to remember that applications **are not issued patents** and the claims as filed have not been approved by any country. Thus, they are non-binding.

Cacao
Patent granted to The Penn State Research Foundation

Actual granted claims

US 6 150 587
Claim 1 A method of inducing *Agrobacterium* -mediated transformation of cacao, which method comprises: A) co-culturing a cacao floral tissue explant or cell with a non-tumorigenic *Agrobacterium* to produce a transformed embryogenic callus, wherein the cacao tissue explant or cell is cultured on a primary callus

growth medium, said primary callus growth medium comprising DKW basal salts, a carbon source, and at least two growth regulators;
B) culturing the embryogenic callus produced in step (A) on a secondary callus growth medium having the property of inducing homeostatic growth and bipolar callus development, wherein the secondary callus growth medium is comprised of a low salt WPM basal medium, at least one growth regulator and a carbon source; and
C) culturing the callus produced in step (B) on a hormone-free embryo development medium having the property of inducing embryo differentiation, wherein the embryo development medium is comprised of a DKW basal medium and a carbon source.

Patent application filed by The Penn State Research Foundation
Actual pending claims

EP 999 737 A1
Claim 1 A method of inducing *Agrobacterium* -mediated transformation of cacao comprising the steps of: A) co-culturing a cacao tissue explant or cell with a non-tumorigenic *Agrobacterium* to produce a transformed embryogenic callus; B) culturing the embryogenic callus produced in step (A) on an embryo development medium, said medium having the property of inducing embryo differentiation to obtain mature transformed somatic embryos.
Claim 16 A method of regenerating transformed cacao plantlets comprising the steps of: A) providing a transformed cacao somatic embryo; B) germinating said somatic embryo on a primary embryo conversion medium; and C) regenerating a transformed cacao plantlet on a secondary embryo conversion medium from a germinated embryo produced in step (B).
Claim 24 A method of regenerating transformed cacao plantlets comprising the steps of: A) providing a transformed cacao somatic embryo; B) culturing said somatic embryo on a plant regeneration medium.

The pending Australian application **AU 81743/98 A1** was **abandoned**.

Camelina sativa (Gold of pleasure)

(Update July 2003)

Camelina sativa
belongs to the family Brassicaceae. This plant is native to Eastern Europe and Southwest Asia, where there are also wild weedy forms. The developed crop form was widely grown across Europe until the 1950s. The seeds yield an oil of excellent nutritional quality which in the past was used as an illuminant and for cosmetic purposes, while the stems were utilized for making brushes, packaging, and thatching temporary buildings. There were additional uses of the green crop as fodder and of the seed for fattening poultry, while the protein-rich press cake was a valued livestock food. Small areas have been grown in recent years for use in the soap and cosmetic industries and as a constituent of birdseed. Experiments are being conducted to assess its future potential.

Summary of the invention

The present invention discloses a general method for transformation of *Camelina sativa* using *Agrobacterium*. Independent Claim 1 is not limited in the sort of explant used for transformation. The only limitation seems to be that an explant is used as starting material, which could mean that full plants are excluded from protection.

Independent Claims 27-30 recite broad claims on various industrial application of the genetically modified

plant, which is seen as having potential for the production of a number of metabolites and products.

Naturally, this patent application must be viewed in the light of the many other patents on Agrobacterium-mediated transformation, including general methods, methods for dicots and vectors.

Camelina sativa		
Assigned to Unicrop Ltd (Finland)		
Application No.	Publication date	Summary of the claims
WO 02/38779 A1 * Full PCT application text (322 KB)	16 May 2002	*Agrobacterium* mediated transformation of *Camelina sativa* explants and the subsequent regeneration of the transformed cells into whole Camelina sativa plants. The use of transgenic *Camelina sativa* for the production of homologous or heterologous recombinant products are also claimed. View Claims
Remarks: Priority application in Finland (FI 2000/2478); related Australian application (AU 14078/02).		

*It is important to remember that applications **are not issued patents** and the claims as filed have not been approved by any country. Thus, they are non-binding.

WO 02/38779 A1	
Title	A transformation in *Camelina sativa*
Appl. No. & Filing date	PCT/FI01/00978 12 Nov 2001
Publ. date	16 May 2002

Camelina sativa (Gold of pleasure) (Update July 2003)

Independent claims

Assigned to Unicrop Ltd
WO 02/38779 A1
Claim 1 1. A method for *Agrobacterium*-mediated genetic transformation, characterized in that the method is *Agrobacterium* -mediated genetic transformation of *Camelina sativa* comprising the steps of: (a) providing explants from *Camelina sativa* ; (b) contacting the explants of *Camelina sativa* with *Agrobacterium* containing at least one recombinant DNA construct; (c) allowing the transformation to take place on culture medium optionally supplemented with at least one hormone; (d) inducing formation (regeneration) of one or more shoots and roots from the transformed explants on a cell culture medium optionally containing at least one hormone; and (e) growing the shoots into a whole *Camelina sativa* plant.
Claim 27 The use of *Camelina sativa* as an alternative model plant in *Agrobacterium*-mediated transformation.
Claim 28 The use of transgenic *Camelina sativa* for producing heterologous or homologous products.
Claim 29 The use of transgenic *Camelina sativa* for producing proteins.

Claim 30
The use of transgenic *Camelina sativa* for producing metabolites.

Carnations
Summary

In a European application and a United States granted patent, the invention claimed by **Florigene Europe** is directed to

- a method for transforming carnation plant material with *A. tumefaciens* or *A. rhizogenes.* The material to be transformed encompasses any explant of a carnation plant and in certain methods specifically leaves from shoots grown in culture.
- methods for producing genetically altered carnation plants. A method for micropropagating shoots from initially propagated vitrified shoots is also covered.
- methods to alter the normal phenotype of carnation plants. The characteristics included are prolonged vase life, resistance to a herbicide and modification of color.

The table shown below presents basic bibliographic data and a summary of the invention. Full text of patent and patent application can be accessed as PDF.

Carnations		
Assigned to Florigene Europe		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 5 589 613 Full patent text (1,776 kb)	December 31, 1996	Transformation of carnation plant material with *A. tumefaciens* or *A. rhizogenes* carrying a gene of interest. Carnation leaves are transformed to alter the phenotype of the plants. A controlled senescence, resistance to a herbicide, resistance to diseases, and alteration of color are part of the desirable characters introduced into the plants via *Agrobacterium* transformation. View Claims
Application		
Application No.	Publication date	Summary of the claims
EP 582 603 A1* Full patent application text (3,205 kb)	February 16, 1994	The claims of the European application recite transformation of carnation plant material with *Agrobacterium*. There is no mention of the plant material used for transformation. Methods for shoot formation and rooting as well as a method for whole plant regeneration are recited in the claims. View Claims

*It is important to remember that applications are **not issued patents** and the claims as filed have not been approved by any country.

Carnations
Patent application filed by Florigene Europe B.V.

Actual pending claims

EP 582 603 A1

Claim 1

A method for genetically transforming carnation plant material, said method comprising: A) co-cultivating carnation plant material with *Agrobacterium* cells carrying an exogenous DNA sequence;
B) initiating callus formation in the plant material; and
C) selecting transformed plant cells.

Claim 5

A method for producing genetically altered carnation plants, said method comprising:

A) co-cultivation of carnation plant material with *Agrobacterium* cells carrying an exogenous DNA sequence including a selectable marker gene in a co-cultivation medium containing nutrients, an energy source, and an induction compound under conditions which allow the *Agrobacterium* cells to infect the plant material and transfer the exogenous DNA to the carnation chromosomes;
B) culturing plant material from step (A) in a callus initiation medium containing nutrients, an energy source, an auxin, a cytokinin, an anti-*Agrobacterium* antibiotic, and a plant selection agent which inhibits callus and shoot formation from plant material which does not express the selectable marker gene to produce transformed callus material; and
C) culturing transformed callus material in a regeneration medium containing nutrients, an energy source, an auxin, a cytokinin, an anti-*Agrobacterium* antibiotic, and the plant selection agent, present in amounts effective to produce transformed shoots.

Claim 23

A method for micropropagating shoots from carnation plant material, said method comprising:

A) culturing carnation plant material to produce a plurality of shoots; and
B) placing vitrified shoots from step (A) in a normalizing medium containing nutrients and an energy source but being substantially free from growth regulators, whereby new shoots are produced which are free from vitrification.

Claim 26

A method for micropropagating shoots from previously established carnation shoots, said method comprising:

A) separating individual shoots; and
B) culturing individual shoots in a multiplication medium comprising nutrients, an energy source, growth regulators and a solidifying agent for a time sufficient to produce at least about 50 shoots for each individual shoot cultured.

Claim 27

A method for regenerating carnation plants, said method comprising: culturing carnation plant material on a regeneration medium containing nutrients, an energy source, a solidifying agent, indole butyric acid at a concentration in the range from about 1 to 5 M, and thidiazuron at a concentration in the range from about 0.5 to 2 M, whereby shoots are produced at a regeneration frequency above about 20 percent.

Claim 28

Carnation callus material which expresses an exogenous DNA sequence.

Claim 30

A carnation plant having cells which express an exogenous DNA sequence.

Carnations
Patent granted to Florigene Europe B.V.

Actual granted claims

US 5 589 613

Claim 1

A method for genetically transforming carnation plant material, said method comprising: A) co-cultivating

carnation plant material with *Agrobacterium tumefaciens* or *rhizogenes* cells carrying an exogenous DNA sequence;
B) initiating callus formation in the plant material; and
C) selecting transformed plant cells.

Claim 5

A method for producing genetically altered carnation plants, said method comprising:

A) co-cultivation of carnation plant material with *Agrobacterium tumefaciens* or *rhizogenes* cells carrying an exogenous DNA sequence including a selectable marker gene in a co-cultivation medium containing nutrients, an energy source, and an induction compound under conditions which allow the *Agrobacterium* cells to infect the plant material and transfer the exogenous DNA to the carnation chromosomes, wherein the carnation plant material is leaf obtained from shoots grown in culture;
B) culturing plant material from step (A) in a callus initiation medium containing nutrients, an energy source, an auxin, a cytokinin, an anti-*Agrobacterium* antibiotic, and a plant selection agent which inhibits callus and shoot formation from plant material which does not express the selectable marker gene to produce transformed callus material; and
C) culturing transformed callus material in a regeneration medium containing nutrients, an energy source, an auxin, a cytokinin, an anti-*Agrobacterium* antibiotic, and the plant selection agent, present in amounts effective to produce transformed shoots.

Claim 22

A method for micropropagating shoots from transformed carnation plant material, said method comprising:
A) culturing transformed carnation plant material obtained from callus to produce a plurality of vitrified shoots; and
B) placing vitrified shoots from step (A) in a medium containing nutrients and an energy source but being substantially free from growth regulators for a period of at least about one month, whereby new shoots are produced which are free from vitrification.

Claim 24

Carnation callus material derived from an explant material which has been transformed with an exogenous DNA sequence, wherein said DNA sequence comprises a functional gene capable of imparting a phenotype not possessed by the explant material and wherein said DNA sequence has been integrated into the carnation genome.

Claim 26

A carnation plant having cells derived from an explant material which have been transformed with an exogenous DNA sequence, wherein said DNA sequence comprises a functional gene capable of imparting a phenotype not possessed by the explant material and wherein said DNA sequence has been integrated into the carnation genome.

Claim 28

A carnation plant having cells derived from an explant material which have been transformed with an exogenous DNA sequence so that flowers of the plant display a phenotype characterized by controlled senescence resulting in prolonged vase life relative to the vase life of flowers from plants propagated from non-transformed cells of the explant material.

Claim 29

A transgenic carnation plant derived from an explant material comprising an exogenous DNA sequence so that flowers of the plant display a phenotype charac terized by controlled senescence resulting in prolonged vase life relative to the vase life of flowers from plants propagated from non-transformed cells of the explant material.

Claim 31

A carnation plant having cells derived from an explant material which have been transformed with an exogenous DNA sequence to display a phenotype characterized by resistance to a herbicide.

Claim 32

A transgenic carnation plant derived from an explant material comprising an exogenous DNA sequence to display a phenotype characterized by resistance to a herbicide.

Claim 34
A carnation plant having cells derived from an explant material which have been transformed with an exogenous DNA sequence so that flowers of the plant display a phenotype characterized by a color conferred by said exogenous DNA sequence which color is modified relative to a flower color of the explant material.
Claim 35 A transgenic carnation plant derived from an explant material comprising an exogenous DNA sequence so that flowers of the plant display a phenotype characterized by a modified color conferred by said exogenous DNA sequence which color is modified relative to a flower color of the explant material.
Claim 37 A carnation plant derived from an explant material having cells which have been transformed with an exogenous DNA sequence to display a phenotype characterized by enhanced resistance to disease relative to the disease resistance of plants propagated from non-transformed cells of the explant material.
Claim 38 A transgenic carnation plant derived from an explant material comprising an exogenous DNA sequence to display a phenotype characterized by enhanced resistance to disease relative to the disease resistance of plants propagated from non-transformed cells of the explant material.
Claim 40 A carnation plant having cells which have been transformed with the ACC synthase gene.
Claim 41 A carnation plant having cells which have been transformed with a chlorsulfuron resistance gene.

Chrysanthemums
Summary

A transformed chrysanthemum plant capable of expressing a gene of interest is the subject of the invention granted to **Florigene Europe** in the United States. In this patent, the explants or plant material transformed with *Agrobacterium* are limited to stem, leaf, peduncle, petiole, meristem and shoot apex. The invention discloses a method to regenerate a chrysanthemum shoot and a chrysanthemum plant expressing an exogenous gene.

A related pending Australian application was abandoned.

The table shown below presents basic bibliographic data and a summary of the invention. Full text of United States patent can be accessed as PDF.

Chrysanthemums		
Assigned to Florigene Europe		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 5 567 599 Full patent text (1,422 kb)	October 22, 1996	Production of a transformed chrysanthemum plant expressing a gene of interest by transforming different explants with *Agrobacterium* carrying the exogenous gene. The group of explants transformed include leaf, stem, peduncle, petiole, meristem and shoot apex. To obtain a chrysanthemum plant, shoot formation and rooting are induced. View Claims

Remarks: the related Australian application AU 84330/91 was abandoned on May 13, 1993.

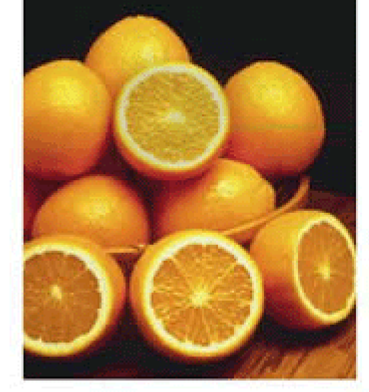
Chrysanthemum
Patent granted to Florigene Europe B.V.

Actual granted claims

US 5 567 599
Claim 1 A method for producing a chrysanthemum plant comprising an exogenous DNA fragment, which chrysanthemum plant is capable of expressing a gene within the exogenous DNA fragment, comprising the steps of: A) isolating an explant from source material from a chrysanthemum plant, wherein said source material is selected from the group consisting of leaf, stem, peduncle, petiole, meristem, and shoot apex; B) inoculating the explant from source material from the chrysanthemum plant with a culture of *Agrobacterium*, which *Agrobacterium* comprises an exogenous DNA fragment comprising a gene under the control of a promoter capable of promoting the transcription of the gene within the exogenous fragment, to obtain an inoculated explant; C) incubating the inoculated explant of step (B) on an incubation medium; D) culturing the incubated explant of step (C) on a regeneration medium to obtain regenerated shoots of the chrysanthemum plant; and E) culturing the regenerated shoots of step (D) on a rooting medium to obtain a chrysanthemum plant comprising the exogenous DNA fragment, in which the chrysanthemum plant is capable of expressing the gene within the exogenous DNA fragment.
Claim 60 A method for producing a chrysanthemum plant comprising an exogenous DNA fragment using *Agrobacterium* as a vector, comprising the steps of: A) incubating chrysanthemum plant source material on a pretreatment medium; B) isolating a leaf explant from source material from the chrysanthemum plant; C) inoculating the explant from source material from the chrysanthemum plant with a culture of *Agrobacterium*, which *Agrobacterium* comprises an exogenous DNA fragment comprising a gene under the control of a promoter capable of promoting the transcription of the gene within the exogenous fragment, to obtain an inoculated explant; D) incubating the inoculated explant of step (C) on an incubation medium; E) culturing the incubated mixture of step (D) on a regeneration medium to obtain regenerated shoots of the chrysanthemum plant; and F) culturing the regenerated shoots of step (E) on a rooting medium to obtain a chrysanthemum plant comprising the exogenous DNA fragment.

Citrus
Summary

The INIA[1] and the IVIA[2] from Spain have a granted United States patent directed to a method for transforming adult citrus plants with *A. tumefaciens* having a gene of interest.



The invention discloses *in vitro* micrografting of transformed shoots onto stocks, at least twice, in order to generate complete adult plants. The second *in vitro* micrografting can be skipped by planting the first micrografted plants directly into soil.

The related European application EP 870 838 A3, which is not presented here, does not refer to a transformation method in particular, and instead inoculation of a vector carrying a gene of interest into a citrus plant can be performed by any method, including Agrobacterium as disclosed in dependent Claim 2. Furthermore, independent Claim 1 covers any woody species, therefore this application is mentioned in the section 'Woody Tree Species'.

The following table presents basic bibliographic data and a summary of the invention. Full text of United

States patent can be accessed as PDF.

Citrus		
Assigned to INIA[1] & IVIA[2]		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 6,103,955 Full patent text (595 kb)	15 Aug 2000	Method for transforming explants of adult citrus plants with a non-oncogenic *A. tumefaciens* having a gene of interest. The transformed shoots are micrografted onto other stocks to allow regeneration of complete adult plants. View Claims

[1] Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria (INIA) , Spain

[2] Instituto Valenciano de Investigaciones Agrarias (IVIA) , Spain

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Citrus
Patent granted to Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria (IN IA) and Instituto Valenciano de Investigaciones Agrarias (IVIA)

Actual granted claims

US 6 103 955
Claim 1 A procedure for the genetic transformation of citrus adult plants consisting of: A) co-culturing explants of adult tissue from citrus plant species, from the first flushes of the grafts of buds of citrus adult plants onto stocks, with a non-oncogenic strain of *Agrobacterium tumefaciens*, optionally modified to further contain genes which encode the characteristics of interest to be inserted into the citrus plant, in feeder plates, and subsequently, in a culture medium which favors the induction of transgenic shoots and permits the selection thereof; B) *in vitro* micrografting said transgenic shoots, their buds or apices, onto stocks cultivated *in vitro*; and C) grafting the resulting *in vitro* micrografted plants, their buds and apices, onto other stocks which give vigor and allowing the successful grafts to grow to generate complete adult plants, or directly transplanting the *in vitro* micrografted plants into the soil to generate complete adult plants.

Coffee
Summary

Coffee belongs to the botanical family Rubiaceae. There are at least 25 species of the genus *Coffea*, all indigenous to Africa, and some islands in the Indian Ocean. Today coffee is cultivated in some eighty countries in South and Central America, the

Caribbean, Africa and Asia, generally in areas lying between the Tropics of Cancer and Capricorn. The two biggest producers by far are Brazil and Colombia, followed by Indonesia, Vietnam and Mexico.

The coffee tree is a tropical evergreen shrub with two beans per fruit, which when ripe resemble a red cherry.

The two most commercially important species grown are *Coffea canephora* (robustas) and *Coffea arabica* (arabicas). The latter, which accounts for 70% of the world production, grows at higher altitudes, requires less rain, and its beans have a lower caffeine content than that of robustas. Arabica coffee is highly susceptible to pests and diseases; therefore resistance is a major goal of plant breeding programs. It is grown throughout Latin America, in Central and East Africa, in India and to some extent in Indonesia. Robusta coffee is grown in West and Central Africa, throughout Southeast Asia and in Brazil. Two other species, which are grown on a much smaller scale are *Coffea liberica* (Liberica coffee), grown in Malaysia and in West Africa, and *Coffea dewevrei* (Excelsa coffee).

After oil, coffee is the most important traded commodity in the world, and is the primary export of many developing countries. More than two thirds of current world coffee production is exported from Latin America and the Caribbean, with much of the rest coming from African and Asian producers. However, most coffee is consumed in the developed world; the United States and the European Community together import two out of every three bags of coffee produced in the world.

IP aspects

The **Nara Institute of Science and Technology** has granted patents in **Australia** and in the **United States**, and a pending patent in **Japan** related to genetic transformation of coffee with *A. tumefaciens*. In the Australian granted patent there is **no** limitation on the species of *Coffea* to be transformed or on the gene to be introduced into the plants. The major limitation of the granted invention lies in the strain of *A. tumefaciens* used for the transformation process.

Furthermore, the patent application has only been filed in countries where coffee is not a major agricultural product, at least not at the same scale as the well-known coffee producing countries. For instance, in Australia there are only two places with commercial production. One is located in north of Queensland and the other one is in the Northern Rivers region of New South Wales. The production of coffee in Australia was around US$800,000 dollars worth in 1998/1999, while the world coffee trade is estimated at around US$13 billion dollars per year. Thus, the patents on the present invention will likely have very little impact for the most important coffee producers' countries.

Actual granted claims

Coffee		
Assigned to Nara Institute of Science and Technology		
Patent No.	Issue Date	Summary of the claims
AU 729 635 B2	8 Feb 2001	Methods for producing transformed coffee plants by infecting embryogenic coffee callus with *A. tumefaciens* EHA101 strain having a gene of interest. Somatic embryos are induced from the transformed coffee calli and transformed coffee plants are regenerated from the somatic embryos.
US 6,392,125 B1 (Update July 2003)	21 May 2002	Same as Australian patent. One limitation is that iso-pentenyl adenosine must be included in the culture medium. View Claims
Remarks: Related application also filed in Japan (JP 2000245485 A2).		

Coffee
Independent claim

Patent assigned to Nara Institute of Science and Technology (JP) (Update July 2003)
US 6,392,125
Claim 1 A method for producing a transformant of Coffea arabica, the method comprising the steps of: infecting an embryogenic callus of Coffea arabica with *Agrobacterium tumefaciens* EHA101 strain that comprises a

vector containing an exogenous gene and a gene available for the selection of transformed embryonic callus to produce a transformed embryogenic callus in a medium containing N6 -[2-isopentenyl]-adenosine, selecting said transformed embryonic callus, forming a somatic embryo from said transformed embryogenic callus and regenerating a transformed Coffea arabica from said somatic embryo.

Cotton

Summary

Five different entities have patents and patent applications related to methods for transforming cotton with *Agrobacterium*.

Agracetus (now owned by **Monsanto**) has been granted two patents in the United States and one in Europe, directed to transformation of immature cotton plants with *A. tumefaciens*. The Agracetus patents have the earliest priority date in the group of cotton transformation patents, dating back to 1986. The major aspects of the inventions are:

- **hypocotyl cotton tissue** is selected for transformation;
- at least two sequences including a foreign chimeric gene and a resistance gene are introduced into plants. The product of the foreign gene is, in one of the United States patents, either a foreign protein or a negative RNA stand; and
- completely transformed plants are regenerated.

Calgene (also owned by **Monsanto**) has one United States patent related to this topic and recently a European and an Australian patent have also been granted. In both **Agracetus' and Calgene's** inventions hypocotyl cotton tissue is transformed with *Agrobacterium.* However, in contrast to **Agracetus'** inventions, **Calgene** claims **any** exogenous gene and *Agrobacterium* species. Other distinctive features of the invention by **Calgene** include the use of a **cotton seedling grown in the dark** as source material for the tissue to be transformed and the induction of embryogenic callus formation in a hormone-free medium.

Differing from those discussed above, **Cotton Inc**. and **The Institute of Molecular Agrobiology** (SG) disclose in their patent applications the use of **meristematic cells of apical shoot tips** of cotton, and **cotton petiole** and **root callus**, respectively, as tissues to be transformed with *Agrobacterium*.

Aventis CropScience (now Bayer Crop Science) and Bayer BioScience patents and applications disclose the use of **cotton embryogenic callus** as target tissue for transformation with *Agrobacterium*. The addition of **a plant phenolic compound** prior or during the transformation of the cotton tissue for *vir* gene induction constitutes a disclosed improvement of cotton transformation methodology. This group has the most recent priority date (19 May 1999) among the cotton transformation patents (Update July 2003).

The following table presents basic bibliographic data and a summary of the scope of the claimed inventions. Full text of patents and patent applications can be accessed as PDF files.

Cotton		
Assigned to Agracetus (Monsanto)		
Issued patents		
Patent No.	Issue date	Summary of the claims
US 5,004,863 Full patent text (1,020 kb)	2 Apr 1991	Transformation of hypocotyl tissue of immature cotton plants with *A. tumefaciens* having a T-DNA with a chimeric gene and a resistance gene. Cotton plants are regenerated from somatic embryos induced from the transformed tissue. A protocol for the introduction of foreign T-DNA into cotton plants is also claimed. View Claims
EP 270 355 B1	16 Mar 1994	As in **US 5,004,863**, hypocotyl tissue of immature cotton plants is transformed with *A. tumefaciens* having a T-DNA containing a chimeric gene and a resistance gene. Cotton seeds that give rise to transformed cotton

Full patent text (1,203 kb)		plants expressing the product of foreign gene are also claimed. View Claims
US 5,159,135* Full patent text (915 kb)	27 Oct 1992	Transformation of cotton plants with two different foreign gene constructions via *Agrobacterium*. The first one contains either a foreign protein or a negative strand of RNA, and the second construction contains a resistance gene that acts as a selectable marker. Cotton plants containing both constructions are claimed. View Claims

Remarks: Applications also filed in Brazil (BR 8706530), China (CN 87107233), and India (IN 168950). European patent converted in Spain (ES 2052582 T3).
* This patent was re-examined and the claims were again found to be patentable (Official Gazette 24 October 2000).

Assigned to Calgene (Monsanto)

Issued patents

Patent No.	Issue date	Summary of the claims
US 5,846,797 Full patent text (980 kb)	8 Dec 1998	Transformation of hypocotyl cotton tissue grown in the dark with *Agrobacterium* having a gene of interest. Embryogenic callus induced from the transformed tissue in a hormone-free medium regenerates into transformed cotton plants. View Claims
EP 910 239 B1 Full patent text (130 kb)	5 Dec 2001	Unlike the related United States patent, the cotton explant to be transformed with *Agrobacterium* is not defined. Any transformed cotton tissue is induced to produce embryogenic callus on a free-hormone media. View Claims
AU 727 910 B2	4 Jan 2001	The claims of the Australian patent are substantially the same as the claims of the United States patent. In addition, the Australian patent refers to methods for regenerating cotton plants. View Claims

Remarks:
Application also filed in China (CN 1198665).

Assigned to Cotton Inc.

Applications

Application No.	Publication date	Summary of the claims
AU 26865/99** & EP 1 056 334 A1** † Full EP application text (WO 99/41975 A1) (8,566 kb)	26 Aug 1999 & 27 July 2000	Method of transforming apical shoot tips of cotton by exposing the meristematic cells to a recombinant *Agrobacterium* having a gene of interest. Regeneration of transgenic cotton plants from the transformed meristematic shoot tips cells. View Claims

† Remarks: The PCT application **WO 99/41975** was converted into the European application **EP 1 056 334 A1** and published on **July 27, 2000**. The Australian application and European application have the same filed claims

Assigned to the Institute of Molecular Agrobiology

(SG)

Applications

Application No.	Publication date	Summary of the claims
EP 1 194 579 A1**‡ Full patent application text (WO 0077230 A1) (1,184 kb)	23 Jan 2002	Method for producing a transgenic cotton plant by exposing petiole explants to *A. tumefaciens* carrying a vector having a gene of interest. This is followed by induction of callus and embryoid formation and regeneration of a whole plant. View Claims
‡ Remarks: The PCT application **WO 00/77230 A1** was converted into the European application **EP 1 194 579 A1** on **23 January 2002**. A related application was filed in Australia (AU 48170/99).		
EP 1 159 436 A1 Full patent application text (WO 00/53783 A1) (1,293 kb)	5 Dec 2001	In this invention the cotton explant selected for transformation with *A. tumefaciens* is callus originated from fibrous roots. Whole plants are regenerated from somatic embryos induced from the transformed callus. This European application and its related Australian application are not related to the above mentioned applications filed by the same institute. View Claims
Remarks: A related application was filed in Australia (AU 29693/99)		

Assigned to Aventis CropScience (Bayer Crop Science)		
Applications		
Application No.	Publication date	Summary of the claims
EP 1 183 377 A1 Full patent application text (WO 0071733 A1) (1,160 kb)	9 Dec 2001	Methods for transformation of cotton embryogenic callus with *Agrobacterium* by incubating *Agrobacterium* cells with a plant phenolic compound. The incubation with the phenolic compound can be prior or during co-cultivation of the bacterium cells with the cotton tissue. View Claims
Remarks: A related application was filed in Australia (AU 52148/00)		

Assigned to Bayer BioScience (BE)		
Patent No.	Issue date	Summary of the claims
US 6,483,013 B1 Full patent application text (804 kb)	10 Nov 2002	A method and a process for transformation of cotton which includes the addition of a phenolic compound for *Agrobacterium vir* gene induction during co-cultivation of embryogenic callus with *Agrobacterium*. View Claims

** It is important to remember that applications **are not issued patents** and the claims as filed have not been approved by any country.

Cotton
Patents granted to Agracetus

Actual granted claims

US 5 004 863

Claim 1

A method of introducing genes into cotton plants and plant lines comprising the steps of:

A) exposing hypocotyl tissue of immature cotton plants to a culture of transformation competent non-oncogenic *Agrobacterium tumefaciens* harboring a Ti plasmid having a T-DNA region including both a foreign chimeric gene and a selection agent resistance gene, both genes including appropriate regulatory sequences so as to be expressed in the cells of cotton plants;
B) culturing the exposed tissue in the presence of a selection agent for which the resistance gene encodes for resistance so as to select for plant cells transformed with the T-DNA region;
C) inducing somatic embryo formation in the exposed tissue in culture; and
D) regenerating the somatic embryos into whole cotton plants.

Claim 16

A method for introducing genes into cotton plants and plant lines, comprising the following steps in sequence:

A) surface sterilizing cotton seeds;
B) allowing said cotton seeds to germinate thus forming immature cotton plants, said immature cotton plants including hypocotyl tissue;
C) exposing said hypocotyl tissue to a culture of transformation competent non-oncogenic *Agrobacterium tumefaciens* harboring a Ti plasmid having a T-DNA region including both a foreign chimeric gene and a selection agent resistance gene;
D) culturing said hypocotyl tissue on a medium containing at least one antibiotic toxic to said *Agrobacterium tumefaciens* but not toxic to cotton cells;
E) culturing said tissue of step (D) in the presence of a selection agent for which the resistance gene encodes for resistance so as to select for plant cells transformed with the T-DNA region;
F) inducing somatic embryo formation in the exposed tissue in culture; and
G) regenerating the somatic embryos into whole cotton plants.

EP 270 355 B1

Claim 1

A method of introducing genes into cotton plants and plant lines comprising the steps of:

- exposing hypocotyl tissue of immature cotton plants to a culture of transformation competent non-oncogenic *Agrobacterium tumefaciens* harboring a Ti plasmid having a T-DNA region including both

(i) a foreign chimeric gene, and
(ii) a selection agent resistance gene.

Claim 16

Cotton seeds capable of germination into cotton plants comprising in their genome:

- a chimeric gene construction including:
  (i) a foreign gene, and
  (ii) promoter and control sequences operable in plant cells,
  the chimeric gene construction being effective in the cells of the cotton plant to express a cellular product coded by the foreign gene.

US 5 159 135

Claim 1

Cotton seed capable of germination into a cotton plant comprising in its genome a chimeric recombinant gene construction including: (i) a foreign gene, and
(ii) promoter and control sequences operable in cotton cells,

- the chimeric gene construction being effective in the cells of the cotton plant to express a cellular product coded by the foreign gene;
- the cellular product imbuing the plant with a detectable trait;

- the cellular product selected from the group consisting of a foreign protein and a negative strand RNA.

Claim 5

A cotton plant comprising in the genome of at least some of its cells a foreign gene construction including promoter and control sequences effective in cotton cells,

- said gene construction further including a heterologous coding sequence;
- the foreign gene construction effective to cause expression of a detectable cellular product coded by the heterologous coding sequence in the plant cells;
- the cellular product selected from the group consisting of a foreign protein and a negative strand RNA.

Claim 6

A cotton plant comprising in its genome at least two foreign gene constructions each including promoter and control sequences effective in cotton cells,

- both gene constructions further including heterologous coding sequences;
- both foreign gene constructions effective to cause the expression of a detectable cellular product coded by the heterologous coding sequence in the plant cells;
- the cellular product of one of the foreign gene constructions selected from the group consisting of a foreign protein and a negative strand RNA;
- the other foreign gene construction being a selectable marker gene which imbues the cotton cells with the trait of resistance to a selection agent.

Claim 7

A cotton plant comprising in its genome at least two foreign gene constructions each including promoter and control sequences effective in cotton cells,

- both gene constructions further including heterologous coding sequences;
- both foreign gene constructions effective to cause the expression of a detectable cellular product coded by the heterologous coding sequence in the plant cells;
- the cellular product of one of the foreign gene constructions selected from the group consisting of a foreign protein and a negative strand RNA;
- the other foreign gene construction being a selectable marker gene which imbues the cotton cells with the trait of resistance to a selection agent;
- the foreign gene constructions having been transformed into the cotton plant or the progenitors of the cotton plant by *Agrobacterium*-mediated plant transformation.

Cotton
Patents granted to Calgene

Actual granted claims

US 5 846 797

Claim 1

In a method for regenerating transformed cotton plants from explant tissue, the improvement whereby embryogenic callus is generated from a transformed cotton tissue explant which is cultivated on cotton callus initiation media which is not supplied with exogenous plant hormones, wherein said explant tissue is hypocotyl tissue cut from a seedling which has been grown in the dark.

Claim 8

A method for the transformation of cotton plants, said method comprising the steps of:

A) cutting cotton hypocotyl tissue to form an explant, wherein said hypocotyl tissue is cut from seedling which has been grown in the dark;
B) co-cultivating said cotton explant tissue with *Agrobacterium* comprising a DNA sequence of interest; and
C) culturing said co-cultivated explant on cotton callus initiation media comprising a selective agent and no exogenous plant hormones,
whereby transformed cells are induced to produce embryogenic callus on said hormone-free selective media.

EP 910 239 B1*

Claim 1

A method of regenerating cotton plants from explant tissue comprising generating embryogenic callus from a cotton tissue explant cultivated on cotton initiation media not supplied with exogenous plant hormones.

Claim 12

A method for the transformation of cotton plants, said method comprising:

A) cutting cotton tissue to form an explant;
B) co-cultivating said cotton explant tissue with *Agrobacterium* comprising a DNA sequence of interst; and
C) culturing said co-cultivated explant on cotton initiation media comprising a selective agent and no exogenous plant hormones,
whereby transformed cells are induced to produce embryogenic callus on said hormone-free selective media.

AU-B-727 910*

Claim 1

A method for regenerating transformed cotton plants from explant tissue, characterized by an improvement whereby embryogenic callus is generated from a transformed cotton tissue explant which is cultivated on cotton callus initiation media not supplied which is not supplied with exogenous plant hormones, wherein said explant tissue is hypocotyl tissue cut from a seedling which has been grown in the dark.

Claim 8

A method for regenerating cotton plants from explant tissue, the method being substantially as hereinbefore described with reference to any one of the examples.

Claim 9

A method for regenerating cotton plants from explant tissue, the method being substantially as hereinbefore described with reference to the 'New' Regime in Figure 1.

Claim 11

A method for the transformation of cotton plants, the method comprising the steps of:

A) cutting cotton hypocotyl tissue to form an explant, wherein said hypocotyl tissue cut from a seedling which has been grown in the dark;
B) co-cultivating said cotton explant tissue with *Agrobacterium* comprising a DNA sequence of interest; and
C) culturing said co-cultivated explant on cotton callus initiation media comprising a selective agent and no exogenous plant hormones,
whereby transformed cells are induced to produce embryogenic callus on said hormone-free selective media.

Claim 14

A method for the transformation of cotton plants, the method being substantially as hereinbefore described with reference to any one of the examples.

* The European and Australian patents were issued in 2001.

Cotton
Patent applications filed by Cotton Inc.

Actual pending claims

EP 1 056 334 A1* & AU 26865/99
Claim 1 A method for producing a transformed plant comprising: A) isolating apical shoot tips from three day old seedlings; B) chilling the isolated apical shoot tips; C) dissecting apical shoot tips to expose meristematic cells; D) introducing a transforming agent into the dissected apical shoot tips; and E) regenerating a plant from shoots which form on the meristematic cells.
Claim 23 A method for producing a transformed cotton plant comprising: A) isolating apical shoot tips from three day old seedlings; B) chilling the isolated apical shoot tips; C) dissecting apical shoot tips to expose meristematic cells; D) introducing a transforming agent into the dissected apical shoot tips; and E) regenerating a plant from shoots which form on the meristematic cells.
Claim 27 A transformed cotton plant produced by: A) isolating apical shoot tips from three day old seedlings; B) chilling the isolated apical shoot tips; C) dissecting apical shoot tips to expose meristematic cells; D) exposing the dissected meristematic cells to the recombinant *Agrobacterium* comprising a gene conferring the desired phenotypic trait to a plant; and E) regenerating a transgenic cotton plant from the shoots which form from the meristematic cells.

* The former PCT application WO 9941975 was converted into the present European patent application.

Cotton
Patent applications filed by The Institute of Molecular Agrobiology

Actual pending claims

EP 1 194 579 A1*
Claim 1 A method for producing a transgenic cotton plant comprising the steps of: A) obtaining cotton petiole explants; B) exposing the petiole explants to a culture of *Agrobacterium tumefaciens* that harbors a vector comprising an exogenous gene and a selectable marker, the *Agrobacterium* being capable of effecting the stable transfer of the exogenous gene and selection agent resistance gene to the genome of the cells of the petiole explant; C) culturing the petiole explants to induce callus formation; D) selecting transformed callus that expresses the exogenous gene; E) culturing the selected callus in suspension culture to induce formation of embryoids; F) regenerating the embryoids into whole transgenic cotton plants.

* The present European application was the result of a conversion of the PCT application WO 0077230 A1.

EP 1 159 436 A1**

Claim 1

A method for producing a transgenic cotton plant comprising the steps of: A) obtaining cotton fibrous root explants;
B) culturing the fibrous root explants to induce callus formation;
C) exposing root callus to a culture of *Agrobacterium tumefaciens* that harbors a vector comprising an exogenous gene and a selectable marker, the *Agrobacterium* being capable of effecting the stable transfer of the exogenous gene and selection agent resistance gene to the genome of the cells of the callus;
D) culturing the callus in the presence of the selection agent to which the selection agent resistance gene confers resistance so as to select for transformed cells;
E) inducing somatic embryo formation in the selected callus culture; and
F) regenerating the induced somatic embryos into whole transgenic cotton plants.

** The present European application is equivalent to its related PCT application WO 0053783 A1. The entire patent application found in the patent databases corresponds to the PCT application.

Cotton
Patent application filed by Aventis CropScience

Actual pending claims

EP 1 183 377 A1*
Claim 1 A method for producing a transgenic cotton plant, comprising the step of: • incubating *Agrobacterium* cells comprising a DNA fragment of interest operably linked to at least one T-DNA border with a plant phenolic compound prior to or during the cocultivation of cotton embryogenic callus with said *Agrobacterium* cells.
Claim 2 A method for producing a transgenic cotton plant, said method comprising: • cocultivating cotton embryogenic callus with *Agrobacterium* cells, said *Agrobacterium* cells comprising a DNA fragment of interest operably linked to at least one T-DNA border, in the presence of a plant phenolic compound, for a time sufficient to generate embryogenic callus comprising a transformed cotton cell; and • regenerating a transgenic cotton plant from said transformed cell.
Claim 10 A method for producing a transgenic cotton plant by *Agrobacterium*-mediated transformation comprising • co-cultivating *Agrobacterium* cells comprising a DNA fragment of interest operably linked to at least one T-DNA border with cotton embryogenic callus, characterized in that said cocultivating occurs in the presence of a plant phenolic compound.
Claim 11 Use of a plant phenolic compound for *Agrobacterium*-mediated transformation of cotton embryogenic callus.
Claim 13 A method for producing a transgenic cotton plant by *Agrobacterium*-mediated transformation characterized in that *Agrobacterium* cells comprising a DNA fragment of interest operably linked to at least one T-DNA border are cocultivated with cotton embryogenic callus in the presence of a plant phenolic compound.

* The present European application is equivalent to its related PCT application WO 0071733 A1. The entire

patent application found in the patent databases corresponds to the PCT application.

Cotton
Patent filed by Bayer BioScience N.V.

Actual pending claims

US 6 483 013
Claim 1 A method for producing a transgenic cotton plant comprising the steps of: • incubating *Agrobacterium* cells comprising a DNA fragment of interest operably linked to at least one T-DNA border, with a plant phenolic compound capable of inducing increased *vir* gene expression in said *Agrobacterium* cells; • co-cultivating solid cotton embryogenic callus cultivated on solid media with said *Agrobacterium* cells to generate embryogenic callus comprising a transformed cotton cell; and • regenerating a transgenic cotton plant from said transformed cell; wherein said incubating step occurs prior to or during said co-cultivation step.
Claim 2 A method for producing a transgenic cotton plant, comprising the steps of: • co-cultivating solid cotton embryogenic callus cultivated on solid media with *Agrobacterium* cells, said *Agrobacterium* cells comprising a DNA fragment of interest operably linked to at least one T-DNA border, in the presence of a plant phenolic compound capable of inducing increased *vir* gene expression in said *Agrobacterium* cells, for a time sufficient to generate embryogenic callus comprising a transformed cotton cell: and • regenerating a transgenic cotton plant from said transformed cell.
Claim 10 A process for producing a transgenic cotton plant comprising: • co-cultivating solid cotton embryogenic callus cultivated on solid media, wherein said cotton embryogenic callus has not been generated from a cotton explant comprising transformed cells, with *Agrobacterium* cells in the presence of a plant phenolic compound capable of inducing increased *vir* gene expression in said *Agrobacterium* cells; said *Agrobacterium* cells comprising a DNA fragment of interestoperably linked to at least one T-DNA border; wherein said *Agrobacterium* cells are co-cultivated with said cotton embryogenic callus for a time sufficient to generate embryogenic callus comprising a transformed cotton cell; and • regenerating a transgenic cotton plant from said transformed cell.

Eucalyptus

Because of the long generation time of woody species and the presence of lignified tissues, a main problem in transformation of these species is the rapid generation of tissues amenable to DNA introduction by various methods. This is reflected in the methods described in the patents and applications described below.

Summary

Patent applications on *Agrobacterium*-mediate transformation of *Eucalyptus* have been filed mainly by four private companies:

- **Shell International Research Maatschappij** has a granted Australian patent related to the production of genetically

modified *Eucalyptus* by transforming *Eucalyptus* tissue or cells with *Agrobacterium* having gene(s) of interest. As part of the invention, phenylurea is used to induce shoot formation from the transformed *Eucalyptus* cells. This patent has expired, so the technology described in it is now in the public domain.

- **Oji Paper Co.**, from Japan, has granted patents in Australia and the United States related to the transformation of adventitious shoots from a mature *Eucalyptus* tree, using *A. tumefaciens*; and
- **Genesis Research & Development Corp. and Fletcher Challenge Forests Ltd** (both based in NZ), had a granted patent in the United States on a method used to produce material amenable to *Agrobacterium*-mediated transformation. The method is described for eucalypt and pine trees. However this patent and other patent applications in this patent family have been abandoned. Genesis filed an unrelated patent application published in 2005.
- **ArborGen**, now the assignee for related patent applications by **Westvaco**, has patent applications pending in the USA and Brazil on methods used for *Agrobacterium*-mediated transformation.

Notice that the claims in pending applications may vary in scope if granted.

Eucalyptus

This page provides specific information on patents that were granted: Shell, Oji Paper, Genesis/Fletcher (several pending applications recently published will be shown on a new page). Note that the patents of Shell and Genesis/Fletcher shown below are no longer in force. This means that the technology described in them is now in the public domain.

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
AU 706650 B2 • Earliest priority - 17 February 1995 • Filed - 16 February 1996 • Granted - 17 June 1999 • Patent ceased - 20 September 2001	**Title** - Genetic modification of plants **Claim 1** A process for producing genetically modified *Eucalyptus* plant material comprising one or more stably incorporated DNA sequences of interest, which process comprises (a) subjecting *Eucalyptus* cells or tissue to *Agrobacterium* mediated transfer of the DNA sequence(s) of interest, inducing shoot formation in transformed cells or tissue, **and** (b) selecting transformed material, the induction of shoot formation being carried out in the presence of N-(2-chloro-4-pyridyl)-N'-phenylurea or another phenylurea. Process for the production of genetically modified *Eucalyptus* with stable DNA incorporated in its genome by transforming *Eucalyptus* tissue or cells with *Agrobacterium* having gene(s) of interest. The induction of shoot formation must have been carried out in presence of phenylurea for the process to be covered by this claim.	Shell International Research Maatschappij
WO 1996/25504 A1 • Earliest priority - 17 February 1995 • Filed - 16 February 1996 • OPI - 22 August 1996	**Title** - Genetic modification of plants Claims identical to the granted Australian patent AU 706650 B2 Remarks: 1. National phase entries of WO 1996/25504 in Europe (EP 808372) deemed to be withdrawn on 14 February 2001. 2. National phase entry of WO 1996/25504 in New Zealand (NZ 303170) was granted on 16 February 1996 and lapsed on 16 September 2003 as reported by IPONZ. 3. Parent application of WO 1996/25504 in the United Kingdom (GB 2298205 A1) deemed to be withdrawn as reported by INPADOC on 11 June 1997.	

<table>
<tr><td></td><td>4. Other national phase entries of WO 1996/25504 include Brazil (BR 9607723; application refused as reported by INPADOC on 18 November 2003), Israel (IL 117023), African Intellectual Property Organization (OA 10503), South Africa (ZA 9601200).</td><td></td></tr>
<tr><td>AU 772053 B2
Earliest priority – 7 May 1999
Filed – 2 May 2000
Granted – 8 April 2004
Expected expiry – 2 May 2020</td><td>Title – Process for transformation of mature trees of Eucalyptus plants

Claim 1

A process for production of transgenic Eucalyptus plants from a mature tree of Eucalyptus plant, comprising the steps of:
(1) preparing a shoot tip explant from a mature tree of Eucalyptus plant;
(2) inducing the shoot tip explant to form adventitious shoots;
(3) pre-culturing the adventitious shoots to prepare infection in an infection induction medium;
(4) infecting segments of explant cut out from the pre-cultured adventitious shoots, with Agrobacterium tumefaciens in an infection medium;
(5) rotary-culturing the segments of the explant from the step (4) in a medium for sterilization containing antibiotics so as to sterilize the Agrobacterium tumefaciens attached to the segments of the explant, to form calli and to select transformed calli;
(6) forming shoot primordia from the transformed calli;
(7) regenerating transgenic plants from the transformed shoot primordia.

Granted AU 772053 recites a process to produce transgenic Eucalyptus plants using A. tumefaciens to transform "adventitious shoots" induced to form from "shoot tips" of a "mature" Eucalyptus tree. Note that all the steps above must be used in order for the claim to cover the process; additional steps may be added and the claim would still cover the process if all the steps above are used.</td><td rowspan="4">Oji Paper Co.</td></tr>
<tr><td>US 6563024 B1
Earliest priority – 7 May 1999
Filed – 5 May 2000
Granted – 13 May 2003
Expected expiry – 5 May 2020</td><td>Title – Process for transformation of mature trees of Eucalyptus plants

Independent claim 1 of granted US 6563024 recites the same process of producing transgenic Eucalyptus plants as that of AU 772053.</td></tr>
<tr><td>EP 1050209 A2
Earliest priority – 7 May 1999
Filed – 4 May 2000
Application pending</td><td>Title – Process for transformation of mature trees of Eucalyptus plants

Independent claim 1 of this patent application recites the same process of producing transgenic Eucalyptus plants as that of AU 772053. Note that any granted claims may be different in scope.</td></tr>
<tr><td>Remarks</td><td>Parent application in Japan (JP 2000/316403) is pending.</td></tr>
</table>

US 6255559 B1 - Earliest priority – 15 September 1998 - Filed – 15 September 1998 - Granted – 3 July 2001 - Patent expired – 3 August 2005	**Title** – Methods for producing genetically modified plants, genetically modified plants, plant materials and plant products produced thereby Claim 1 A method for producing genetically modified plant material comprising: (1) preparing an in vitro shoot culture of a target plant, the target plant being of the *Eucalyptus* or *Pinus* species; (2) maintaining and growing the shoot culture until it has produced multiple modes and stem segments; (3) selecting and excising the stem segments from one or more nodes of a shoot; (4) transforming a stem segment by stably incorporating a genetic contruct comprising a selection marker and a polynucleotide of interest into the stem segment to form a putatively transformed stem segment; (5) exposing a putatively transformed stem segment to a selection medium comprising a selection agent that permits survival of transformed stem segments and is lethal to stem bud on the that were not successfully transformed; (6) selectively inducing the formation of an adventitious bud on the putatively transformed stem segment to form a putatively transformed adventitious bud; (7) selectively regenerating the putatively transformed adventitious bud by excising the putatively transformed adventitious bud and exposing the putatively transformed adventitious bud to a selection medium comprising a selection agent that permits survival of transformed adventitious buds and is lethal to adventitious buds that were not successfully transformed to identify a transformed adventitious bud; and (8) elongating the transformed adventitious bud to form a transformed shoot. This patent expired due to non-payment of maintenance fees according to USPTO. Granted **US 6255559** recites a method for transformation of *Eucalyptus* or *Pinus* species based on introducing a desired gene into an in vitro shoot culture and using excised stems with multiple nodes grown from that culture as targets for transformation. Transgenic adventitious buds are induced in a medium containing a selection agent and regenerated into plants. The mode of transformation is not limited to that using *A. tumefaciens*.	Genesis Research & Development Corp.; Fletcher Challenge Forest Ltd
US 2002/016981 A1 - Earliest priority – 15 September 1998 - Filed – 20 March 2001 - Application abandoned – 6 October 2003	**Title** – Methods for producing genetically modified plants, genetically modified plants, plant materials and plant products produced thereby Claim 1 A method for producing genetically modified plant material of the *Eucalyptus* or *Pinus* species, comprising: (1) culturing nodal stem segments of a target plant selected from the *Eucalyptus* and *Pinus* species; (2) transforming the stem segments with a genetic construct by incubating the nodal stem segments with an *Agrobacterium* culture transformed with the genetic construct;	

	(3) promoting regeneration of adventitious shoot buds from the transformed stem segments; (4) selecting transformed adventitious shoot buds; and (5) regenerating transformed plant material from the transformed adventitious shoot buds. Patent application US 2002/016981 has been abandoned due to failure to respond to an office action, according to the USPTO.	
Remarks	Related PCT application WO 2000/15813 recites a method to produce genetically modified plant material that is not limited to *Eucalyptus* species, nor via *Agrobacterium*-mediated transformation.	

Note: Patent information on this page was last updated on 17 March 2006.

Eucalyptus
Independent claim

Patent assigned to Genesis Research & Development Corp. (NZ) and Fletcher Challenge Forest Ltd (NZ)
US 6,255,559
Claim 1 A method for producing genetically modified plant material comprising: preparing an in vitro shoot culture of a target plant, the target plant being of the Eucalyptus or Pinus species; maintaining and growing the shoot culture until it has produced multiple nodes and stem segments; selecting and excising the stem segments from one or more nodes of a shoot; transforming a stem segment by stably incorporating a genetic construct comprising a selection marker and a polynucleotide of interest into the stem segment to form a putatively transformed stem segment; exposing a putatively transformed stem segment to a selection medium comprising a selection agent that permits survival of transformed stem segments and is lethal to stem bud on the that were not successfully transformed; selectively inducing the formation of an adventitious bud on the putatively transformed stem segment to form a putatively transformed adventitious bud; selectively regenerating the putatively transformed adventitious bud by excising the putatively transformed adventitious bud and exposing the putatively transformed adventitious bud to a selection medium comprising a selection agent that permits survival of transformed adventitious buds and is lethal to adventitious buds that were not successfully transformed to identify a transformed adventitious bud; and elongating the transformed adventitious bud to form a transformed shoot.

Guar (*Cyamopsis tetragonolobus,* Fabaceae)

(Update July 2003)

Guar, also called "cluster bean", and formerly referred to as *Cyamopsis psoralioides*, is a native plant of India where it is grown principally for its green fodder and for the pods that are used for food and feed. As a legume it is capable of fixing nitrogen through symbiotic rhizobia. Its seed gum (galactomannan gum) has a wide variety of food and other commercial uses.

Patent application assigned to Danisco A/S (DK)

	US 6,307,127 B1	US 2001/0034887 A1
Title	Transformation of guar	
Appl. No. & Filing date	US 08/750,267 6 June 1995	US 09/861,575 22 May 2001
Publ. date	23 Oct 2001 (issued)	25 Oct 2001 (publ)
Remarks: Related patent in Australia (AU 609 999 B2) and patent applications in Brazil (BR 95/07970), Europe (EP 766 743 A2), Japan (JP 10501139 T2), New Zealand (NZ 287848), Poland (PL 317578 A1),		

Brief description of the invention

The granted United States patent discloses a method to transform the leguminous plant guar using *Agrobacterium* .

- An auxin or ethylene inhibitor is added during selection to inhibit callus formation and induce shoot formation.
- A beta-lactamase inhibitor is added to inhibit the formation of auxin-like substances produced by degradation of carbenicillin added to kill the bacteria after co-cultivation. This is an additional means of preventing callus proliferation.

The related application claims transformed guar plants without mention of *Agrobacterium*.

View Claims

Guar (*Cyamopsis tetragonolobus*, Fabaceae) Update July 2003

Independent claims

US 6,307,127
Claim 1 A method for producing a genetically modified plant or part thereof of the genus *Cyamopsis*, comprising the steps of introducing a recombinant DNA sequence into at least one cell or protoplast by means of a beta-lactamase producing *Agrobacterium* and generating genetically modified explants using at least one selection or shoot growth medium comprising (a) at least one beta-lactamase inhibitor and (b) at least one auxin inhibitor or ethylene inhibitor, so as to obtain a genetically modified plant or part thereof containing in its genome at least one recombinant DNA sequence.
Claim 22 A method for producing a genetically modified plant or part thereof of the genus *Cyamopsis*, comprising the steps of introducing a recombinant DNA sequence into at least one cell or protoplast by means of a beta-lactamase producing *Agrobacterium* and generating genetically modified explants using at least one selection or shoot growth medium comprising a nickel salt, so as to obtain a genetically modified plant or part thereof containing in its genome at least one recombinant DNA sequence.
Claim 28 A method for producing a genetically modified plant or part thereof of the genus *Cyamopsis*, comprising the steps of introducing a recombinant DNA sequence into at least one cell or protoplast by means of a beta-lactamase producing *Agrobacterium* and generating genetically modified explants using at least one selection or shoot growth medium comprising at least one beta-lactamase inhibitor.

Impatiens

Summary

Impatiens is the genus of popular ornamental bedding plants. **Ball Horticultural Co.** has a granted United States patent related to a method for the production of transgenic *Impatiens* plants by transforming an *Impatiens* tissue with *Agrobacterium* having either a vector with a selectable marker gene and a foreign gene or two expression vectors where one of them has a selectable marker gene and the other one a foreign gene.

The table shown below presents basic bibliographic data and a summary of the invention. Full text of United States patent can be accessed as PDF.

Impatiens
Assigned to Ball Horticultural Co.

Issued patent

Patent No.	Issue date	Summary of the claims
US 6 121 511 Full patent text (1,414 kb)	September 19, 2000	A method to produce transgenic *Impatiens* plants by introducing into an *Impatiens* tissue either one expression vector having a selectable marker and a second foreign gene or two expression vectors where one of them contains a selectable marker and the other one contains a second foreign gene. The introduction of one or two vectors into the tissue is via *Agrobacterium*. The transformed tissue is regenerated into fertile transgenic plants with the foreign gene stably integrated in the genome. The progeny of these plants also contain the foreign gene. View Claims

Impatiens
Patent granted to Ball Horticultural Co.

Actual granted claims

US 6 121 511
Claim 1 A method for producing transgenic *Impatiens* plants, comprising the steps of: A) introducing an expression vector into a plant tissue explant via *Agrobacterium* to produce a transformed explant, wherein said expression vector comprises a selectable marker gene and a second foreign gene, or A') introducing two expression vectors into said plant tissue explant via *Agrobacterium* to produce a transformed explant, wherein one of said expression vectors comprises a selectable marker gene, and wherein the second of said expression vectors comprises a second foreign gene; B) culturing said transformed explant on a selection medium; C) culturing said transformed explant on regeneration medium; and D) recovering fertile transgenic plants from said transgenic explants capable of transmitting said foreign gene to progeny.
Claim 36 A fertile transgenic *Impatiens* plant having stably integrated in the plant genome a foreign gene, wherein said transgenic *Impatiens* plant is capable of transmitting said foreign gene to progeny.

Melon (*Cucumis melo*)
Summary

The patents granted to **Biosem** in the United States and in Europe claim

- the production of transgenic diploid melon (*C. melo*) plants having a DNA molecule introduced by *A. tumefaciens;*
- a method to transform cotyledons of *C. melo* by contacting with *A. tumefaciens*;
- the insertion of a sequence conferring resistance to cucumber mosaic virus; and
- media for inducing shoot formation from transformed cotyledons and for regeneration of transformed plantlets.

The European patent was assigned to **Groupe Limagrain Holding** on March 1, 2001.

The following table presents basic bibliographic data and a summary of the invention. Full text of patents can be accessed as PDF.

Melon (*Cucumis melo*)

Assigned to Biosem (Groupe Limagrain Holding)		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 5,422,259 Full patent text (1,825 kb)	6 June 1995	Process for the production of transgenic diploid melon plantlets by transforming cotyledons of *C. melo* having a gene of interest introduced via *A. tumefaciens*. The process includes media components and protocols for inducing formation of transformed shoot buds and formation of plantlets. View Claims
US 5,789,656 Full patent text (1,896 KB)	4 Aug 1998	Process for the production of transgenic diploid melon plantlets by transforming cotyledons of *C. melo* having a gene of interest introduced via *A. tumefaciens*. Transgenic diploid melon having the gene of interest, i.e. gene for cucumber mosaic virus, are also claimed. View Claims
US 6,198,022 (Update July 2003) Full patent text (1.6 KB)	6 Mar 2001	The process described above applied to the production of cucumber mosaic virus-resistant plants by expression of the viral capsid protein (this is a divisional application of the patent listed immediately above)
EP 412 912 B1 Full patent text (2,055 KB)	16 Mar 1994	The process for the production of transgenic diploid melon plantlets is very similar to the process claimed in the related United States patents. Media components for shoot buds induction and plantlet development are also part of the claims View Claims
Remarks: Patent also granted in Portugal (PT 94967 B). Applications filed in Israel (IL 95334 A0), and Japan (JP 3103127 A2).		

Melon (*Cucumis melo*)
Patents granted to Biosem

Actual granted claims

US 5 422 259
Claim 1 Process for the production of transgenic plantlets having diploid phenotype from genetically transformed explants, said plantlets belonging to the species *Cucumis melo* and containing at least one gene introduced by the intermediary of *Agrobacterium tumefaciens* , comprising the following steps: A) inducing genetically transformed shoot buds from cotyledons of *Cucumis melo* in a shoot bud induction medium without forming calli, wherein the cotyledons are obtained from embryos which have germinated from 0 to 4 days before being contacted with *A. tumefaciens*, wherein the induction medium comprises about 440 to about 2,200 mg/L of calcium chloride calculated as $CaCl_2R_2H_2$ O, and about 0.3 to about 1.13 mg/L 6-benzyl aminopurine (BAP); and B) forming genetically transformed plantlets from genetically transformed shoot buds, wherein the step of forming comprises: (i) culturing the genetically transformed shoot buds in a medium having 6-benzyl aminopurine (BAP) until the shoot buds have reached a height of at least 3 mm; and

(ii) transferring and incubating the shoot buds in a suitable macro-element plant cell culture medium sufficiently to form the genetically transformed plantlets.

Claim 2

Process for production of transgenic plantlets with a diploid phenotype, from genetically transformed explants, said plantlets belonging to the species *Cucumis melo* and containing at least one gene introduced by the intermediary of *A. tumefaciens* comprising the following steps: A) inducing genetically transformed shoot buds from cotyledons of *Cucumis melo* in a shoot bud induction medium without forming calli, wherein the cotyledons are obtained from embryos which have germinated from 0 to 4 days before being contacted with *A. tumefaciens*, and wherein the induction medium comprises about 440 to about 2,200 mg/L of calcium chloride calculated as $CaCl_2R_2H_2$ O, about 0.3 to about 1.13 mg/L of 6-benzyl aminopurine (BAP), and about 0 to about 1.3 mg/L indole-3-acetic acid (IAA); and
B) forming genetically transformed plantlets from the genetically transformed shoot buds, wherein the step of forming comprises: (i) culturing the shoot buds in a medium having 6-benzyl aminopurine (BAP) until the shoot buds have reached a height of at least 3 mm; and
(ii) transferring and incubating the shoot buds in a suitable macro-elements plant cell culture medium comprising:

- KH_2PO_4 from about 50 to about 100 mg/L;
- $MgSO_4$from about 75 to about 300 mg/L;
- $CaCl_2R_2H_2O$ from about 500 to about 2500 mg/L;
- KNO_3 from about 750 to about 1200 mg/L; and
- NH_4NO_3 from about 150 to about 200 mg/L sufficiently to form the genetically transformed plantlets.

US 5 789 656

Claim 1

Transgenic plants having a diploid phenotype belonging to the species *Cucumis melo* comprising at least one DNA sequence introduced by the intermediary of *Agrobacterium tumefaciens*.

Claim 2

Transgenic plant tissue having a diploid phenotype belonging to the species *Cucumis melo* comprising at least one DNA sequence that confers resistance to cucumber mosaic virus.

Claim 9

Transgenic plants having a diploid phenotype and belonging to the species *Cucumis melo* comprising at least one DNA sequence that confers resistance to Cucumber mosaic virus.

Claim 15

Transgenic plants having diploid phenotype, belonging to the species *Cucumis melo*, and containing at least one gene introduced by the intermediary of *Agrobacterium tumefaciens*, wherein the plant tissue is produced from genetically transformed explants by a process comprising the following step: A) inducing genetically transformed shoot buds from cotyledons of *Cucumis melo* in a shoot bud induction medium without forming calli, wherein the cotyledons are obtained from embryos which have germinated from 0 to 4 days before being contacted with *A. tumefaciens*, wherein the induction medium comprises about 440 to about 2,200 mg/L of calcium chloride calculated as $CaCl_2R_2H_2$ O, and about 0.3 to about 1.3 mg/L 6-benzyl aminopurine (BAP); and
B) forming genetically transformed plantlets from genetically transformed shoot buds, wherein the step of forming comprises: (i) culturing the genetically transformed shoot buds in a medium having 6-benzyl aminopurine (BAP) until the shoot buds have reached a height of at least 3 mm; and
(ii) transferring and incubating the shoot buds in a suitable macro-element plant cell culture medium sufficiently to form the genetically transformed plantlets.

EP 412 912 B1

Claim 1

Process for production of transgenic, phenotypically normal plantlets from genetically transformed

explants, said plantlets belonging to the species *Cucumis melo* and containing at least one gene, which has been introduced through *Agrobacterium tumefaciens*, characterized by the following steps: A) induction of genetically transformed shoot buds from cotyledons of *Cucumis melo* which have germinated for 0 to 4 days and, after this period, have been brought into contact with *A. tumefaciens*, the induction being carried out on an induction medium for genetically transformed shoot buds which comprises all of the minerals, salts and vitamins normally required for the induction of shoot buds from genetically non-transformed explants and containing, amongst the mineral salts, approximately 440 to approximately 2,200 mg/L of calcium chloride calculated as $CaCl_2R_2H_2O$, and approximately 0.8 to approximately 1.2% of bacto-agar or agar-agar, said induction medium being supplemented with approximately 0.3 to about 1.13 mg/L of 6-benzyl aminopurine (BAP); and approximately 0 to approximately 1.3 mg/L indole-3-acetic acid (IAA); B) culturing the resulting genetically transformed shoot buds in two successive stages, the first of these culture stages taking place on a plant cell culture medium containing a cytokinin and, more particularly, 6-benzyl aminopurine (BAP), and the second stage, which is carried out when the shoot buds have reached a length of at least 3 mm, taking place on a plant cell culture medium containing, as macroelements:

- KH_2 PO_4 approximately 50 to approximately 100 mg/L
- $MgSO_4$ approximately 75 to approximately 300 mg/L
- $CaCl_2R_2H_2O$ approximately 500 to approximately 2500 mg/L
- KNO_3 approximately 750 to approximately 1200 mg/L
- NH_4NO_3 approximately 150 to approximately 200 mg/L.

Claim 15

Cell culture medium suitable for the development of shoot buds into plantlets in the course of the regeneration of a plant, characterized in that it contains, as macro-elements:

- KH_2 PO_4 approximately 50 to approximately 100 mg/L
- $MgSO_4R2H_2O$ approximately 75 to approximately 300 mg/L
- $CaCl_2R_2H_2O$ approximately 1000 to approximately 2500 mg/L
- KNO_3 approximately 750 to approximately 1200 mg/L
- NH_4NO_3 approximately 150 to approximately 200 mg/L.

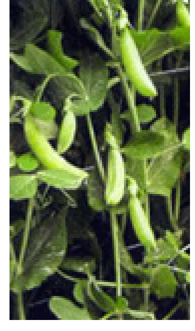
Claim 18

Shoot bud induction medium composed of a plant cell culture medium, which comprises all the minerals, salts and vitamins normally required for inducing shoot buds from non-genetically transformed explants and containing, amongst its mineral salts, calcium chloride, and bacto-agar or agar-agar, characterized in that the $CaCl_2$ content of this medium is 1000 to 2200 mg/L calculated as $CaCl_2$ R_2H_2O, and the bacto-agar or agar-agar content is 0.8 to 1.2%, said medium being supplemented with 0.3 to about 2.0 mg/L of BAP and 0 to 1.3 mg/L of IAA.

Peas

Summary

FreshWorld



has a granted patent in the United States related to a method for transforming plumule material or cotyledons of a pea seed, either from a garden pea plant or an edible pod, with *A. tumefaciens* or *A. rhizogenes* carrying a gene of interest. A related Australian application was abandoned.

The following table presents basic bibliographic data and a summary of the invention. Full text of United States patent can be accessed as PDF.

Peas		
Assigned to FreshWorld		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 5 286 635 Full patent text (1,421 kb)	February 15, 1994	Method for transforming the plumule of a pea seed and cotyledons with *A. tumefaciens* or *A. rhizogenes* having an exogenous gene. Shoot regeneration and production of viable pea plants expressing the gene of interest are also claimed View Claims
Remarks: The Australian patent application **AU 26441/92 A1** was abandoned on June 16, 94.		

Peas
Patent granted to FreshWorld

Actual granted claims

US 5 286 635
Claim 1 A method for genetically transforming a pea plant, said method comprising: A) culturing explant material from the seed plumule of the pea plant with *Agrobacterium tumefaciens* or *rhizogenes* cells carrying an exogenous DNA sequence, wherein the strain of *Agrobacterium* is selected to be capable of infecting and transferring DNA to the explant material; B) regenerating shoots from the explant material from step (A), wherein said shoots are obtained from non-callus material; C) selecting regenerated shoots from step (B) which express the exogenous DNA sequence, and D) rooting said regenerated shoots to produce viable pea plants expressing the exogenous DNA sequence.
Claim 7 A method for genetically transforming a pea plant, said method comprising: A) obtaining plumule material from a pea plant seed; B) culturing the plumule material with *Agrobacterium tumefaciens* or *rhizogenes* cells carrying an exogenous DNA sequence in a co-cultivation medium for a time and under conditions selected to effect transfer of the exogenous DNA sequence to cells of the plumule material, wherein the strain of *Agrobacterium* is selected to be capable of infecting and transferring DNA to the explant material; C) separating the *Agrobacterium* cells form the plumule material; D) regenerating shoots from the plumule material from step (C) in a regeneration medium for a time and under conditions selected to produce shoots from non-callus regions of the plumule; (E) selecting regenerated shoots from step (D) which express the exogenous DNA sequence; and (F) rooting the selected regenerated shoots from step (E) in a rooting medium to produce plants which express the exogenous DNA sequence.
Claim 19 A method for genetically transforming a pea plant, said method comprising: A) preparing an explant by removing the seed coat from a sterilized seed of the pea plant and cutting off the cotyledons where they are attached to the embryo; B) culturing explant material from sterilized seed of the pea plant with *Agrobacterium tumefaciens* or *rhizogenes* cells carrying an exogenous DNA sequence, wherein the strain of *Agrobacterium* is selected to be capable of infecting and transferring DNA to the explant material; C) regenerating shoots from the explant material from step (A), wherein said shoots are obtained from non-callus material;

D) selecting regenerating shoots from step (B) which express the exogenous DNA sequence, and
E) rooting said regenerated shoots to produce viable pea plants expressing the exogenous DNA sequence.

Note: the pending Australian patent application **AU 26441/92 A1** was abandoned.

Pelargonium (Geranium)
Summary

The present United States patent granted to **Institut National de la Recherche Agronomique** discloses a method for modifying a *Pelargonium spp*. via transformation with *A. rhizogenes*. A pelargonium plant or tissue is transformed *in vivo* . Flower scent is one of the specific characteristics to be modified by the transformation process.

The table shown below presents basic bibliographic data and a summary of the invention. Full text of United States patent can be accessed as PDF.

Pelargonium		
Assigned to Institut National de la Recherche Agronomique		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 5 648 598 Full patent text (840 kb)	July 15, 1997	Method for the transformation of a live *Pelargonium* plant or live tissue with T-DNA of *A. rhizogenes*. The transformation is aimed at modifying an ornamental characteristic or the scent of a geranium plant. View Claims

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Pelargonium
Patent granted to Institut National de la Recherche Agronomique

Actual granted claims

US 5 648 598
Claim 1 A method of genetically modifying a plant of the genus *Pelargonium* to modify at least one ornamental characteristic of said plant, the method comprising the steps of, transforming a live *Pelargonium* plant or live tissue thereof with the T-DNA of the Ri plasmid of *Agrobacterium rhizogenes*, and propagating the resulting transformant to obtain a plant for ornamental use.
Claim 5 A method of genetically modifying a *Pelargonium* species or variety having a unique scent comprising transforming live plants or live tissues of said species or variety with the T-DNA of the Ri plasmid of *Agrobacterium rhizogenes*, and propagating the resultant transformants to obtain plants characterized by improved ornamental characteristics.

Pepper
Summary

DNA Plant Technology has been granted a United States patent directed to the transformation of a pepper explant (young cotyledon) with *A. tumefaciens* or *A. rhizogenes*. Shoot formation is then induced from the transformed cells. Gibberellin and an inhibitor of ethylene are used as components of an elongation/selection culture medium for shoots.

Related Australian and PCT patent applications were abandoned.

The following table presents basic bibliographic data and a summary of the invention. Full text of United States patent can be accessed as PDF.

Pepper		
Assigned to DNA Plant Technology		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 5 262 316 Full patent text (2,104 kb)	November 16, 1993	Method for transforming young embryonic and expanded cotyledons of pepper with *A. tumefaciens* or *A. rhizogenes* having a gene of interest. Media for shoot induction, elongation and rooting are part of the claimed invention. View Claims
Remarks: The Australian patent application **AU 30743/92 A1** and the PCT application **WO 9309665 A1** were abandoned on August 11, 94 and November 11, 1994.		

Pepper
Patent granted to DNA Plant Technology Corporation

Actual granted claims

US 5 262 316
Claim 1 A method for genetically transforming a pepper plant, said method comprising: A) co-cultivating explant material from the pepper plant with *Agrobacterium tumefaciens* or *rhizogenes* cells carrying an exogenous DNA sequence wherein the explant material is selected from the group consisting of young embryonic cotyledons and young expanded cotyledons; B) selecting and inducing shoots from the explant material from step (A), wherein said shoots are obtained from non-callus material and express the exogenous DNA sequence; and C) elongating and further selecting the shoots from step (B)
Claim 11 A method for genetically transforming a pepper plant, said method comprising: A) obtaining the explant material from the pepper plant seed; B) co-cultivating the explant material obtained in step (A) with *Agrobacterium tumefaciens* or *rhizogenes* cells carrying an exogenous DNA sequence in a co-cultivation medium for a time and under conditions selected to effect transfer of the exogenous DNA sequence to cells of the explant material obtained in step (A), wherein the explant material is selected from the group consisting of young embryonic cotyledons and young expanded cotyledons; C) removing the *Agrobacterium* cells from the explant material obtained in step (A); D) selecting and inducing shoots from the explant material from step (C) in a selection/induction medium for a time and under conditions chosen to produce shoots from non-callus regions of the explant material and select shoots which express the exogenous DNA sequence, wherein the elongation/selection medium contains a gibberellin in the amount of about 0.1 to 50 mg/l and an inhibitor of ethylene action; E) elongating and further selecting the shoots from step (D) in an elongation/selection medium; and F) rooting the selecting regenerated shoots from step (E) in a rooting medium.
Claim 25 A method for generating a pepper plant, said method comprising: A) obtaining young explant material from the pepper plant, wherein the young explant material is selected from the group consisting of embryonic cotyledons and young expanded cotyledons; B) inducing shoots from the explant material from step (A) in an induction medium containing BA for a time and under conditions chosen to induce about regeneration from non-callus regions of the explant material; and

C) elongating the shoots from step (B) in an elongation medium containing a gibberellin and an inhibitor of ethylene action.
Claim 31 A method for regenerating a pepper plant, said method comprising: A) obtaining young explant material from a pepper plant seed; B) inducing shoots from the young explant material from step (A) in an induction medium containing BA for a time and under conditions chosen to induce shoot regeneration from non-callus regions of the explant material, wherein the young explant material is selected from the group consisting of embryonic cotyledons and young expanded cotyledons; C) elongating the shoots from step (B) in an elongation medium containing a gibberellin and an inhibitor of ethylene action; and rooting the shoots from step (C) in a rooting medium.

Note: The Australian patent application **AU 30743/92 A1** was abandoned.

Poplar
Summary

The invention disclosed by **Fillatti & Comai** in patents granted in the United States and Australia is directed to the transformation of poplar leaves with an armed (tumor-causing genes) *A. tumefaciens* having a gene of interest.

The construct inserted into a transformed poplar comprises a transcription initiation region, an open reading frame (ORF) expressing a peptide and a transcription termination region.

The table shown below presents basic bibliographic data and a summary of the inventions. Full text of United States patent can be accessed as PDF.

Poplar		
Assigned to Fillatti & Comai		
Issued patents		
Patent No.	Issue date	Summary of the claims
US 4 795 855 Full patent text (1,071 kb)	January 3, 1989	Method to transform poplar leaves explants with *A. tumefaciens* having a construct with a gene of interest flanked by a right T-DNA border. Regeneration of transformed poplar plants from the explants, and elements of the construct are also claimed. View Claims
AU 597 916 B	June 14, 1990	The claims of the Australian patent are exactly the same as the United States patent. View Claims
Remarks: The Australian patent was assigned to **Calgene**. Deemed to be abandoned according to the IP Australia database (Update July 2003).		

Poplar
Patents granted to Fillatti & Comai

Actual granted claims

US 4 795 855 & AU-B-597 916
Claim 1 * A transformed poplar plant comprising transformed cells, said cells comprising a DNA construct as a result of transforming of poplar cells with said DNA construct, which construct comprises in the 5'-3' direction: (i)

a transcription-initiation region;
(ii) an open reading frame other than T-DNA expressing a peptide downstream from said transcription-initiation region, and under the transcriptional regulation of said transcription-initiation region; and
(iii) a transcription-termination region to provide an expression cassette capable of expression in said cells,

- wherein at least one of said open reading frame, transcription initiation region, and transcription termination region is from a plant other than poplar, and
- wherein said expression cassette is bordered by at least the right T-DNA border, and
- wherein said construct is a result of joining *in vitro* at least two of (i), (ii), and (iii) and said right T-DNA border.

Claim 7

*
Stably transformed poplar seedlings comprising cells comprising a DNA construct as a result of transformation of poplar cells with said DNA construct, which construct comprises in the 5'-3' direction: (i) a transcription initiation region,
(ii) an open reading frame, other than T-DNA, encoding a peptide, said open reading frame downstream from said transcription initiation region and under the transcriptional regulation of said transcription initiation region, and
(iii) a transcription termination region, to provide an expression cassette capable of expression in said poplar cells,

- wherein at least one of said open reading frame, transcription initiation region, and transcription termination region is from a plant other than poplar, and
- wherein said construct is as a result of joining *in vitro* at least two of (i),(ii), and (iii).

Claim 9

*
A method for transforming a poplar plant which comprises:

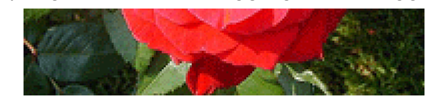
A) pre-incubating poplar leaf explants from shoot cultures with a medium conditioned with plant cells;
B) co-cultivating said leaf explants with *Agrobacterium tumefaciens* comprising an armed Ti plasmid containing:
(i) *vir* genes,
(ii) an expression construct comprising transcriptional initiation and termination regulatory regions functional in said poplar, and
(iii) a gene other than the wild-type gene of one or both of the initiation and termination regions and under their regulatory control, bordered by at least the right T-DNA border,
whereby said expression construct becomes integrated into the genome of cells of said leaf explant; C) transferring said leaf explants after co-cultivation to a regeneration medium comprising plant hormones and phytohormones produced by a Ti-plasmid comprising *A. tumefaciens* strain, whereby callus is formed and shoots develop; and
D) transferring shoots to growing medium to produce a poplar plant.

* Correspondence of the United States claims to the Australian claims: United States claim 1 corresponds to the Australia claim 1, United States claim 7 corresponds to Australia claim 8 and United States claim 9 corresponds to claim 10 of the Australian issued patent.

Roses
Summary

Florigene's inventions disclosed in two granted United States patents and in a European application are directed to:

- a method for producing a transformed somatic rose embryo, which expresses an exogenous gene,
- a method for transforming embryogenic, friable, granular rose callus



cells with *Agrobacterium*,

- a method for transforming a rose plant and producing transformed rose plantlets, and
- culture media for callus cultivation and somatic embryo maintenance.

An additional protocol is disclosed to obtain somatic embryo out of a mature somatic tissue, a stamen filament and a leaf explant. These protocols are not limited to transformed tissues.

A new United States patent application has been filed by **Florigene**. Unlike the granted United States patents, the application describes *Agrobacterium*-mediated transformation of rose callus cells, **without** specifying the origin of the callus.

The table below presents basic bibliographic data and a summary of the invention. Full text of patents and patent application can be accessed as PDF.

Roses		
Assigned to Florigene Europe		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 5 480 789 Full patent text (1,504 kb)	January 2, 1996	Method for the production of a transformed somatic rose embryo expressing a gene of interest by transforming an embryogenic callus with *Agrobacterium* carrying the exogenous gene. View Claims
US 5 792 927 Full patent text (1,431 kb)	August 11, 1998	Method for transforming rose callus cells with *Agrobacterium* carrying an exogenous gene and selecting callus cells containing that exogenous gene. Production of transformed somatic rose embryo and a method for transforming a rose plant starting from a transformed granular callus are part of the claimed invention. View Claims
Applications		
Application No.	Publication date	Summary of the claims
EP 536 327 A1* Full patent application text (3,745 kb)	April 14, 1993	The claims of the European application recite methods to regenerate a rose plantlet from a somatic embryo and to obtain somatic embryo from somatic rose tissue, i.e. stamen filament, and leaf. Similar to its related United States patent, methods are also claimed to transform a rose callus with *Agrobacterium* having an exogenous gene, to produce a transformed somatic embryo and transformed rose plantlets. View Claims
US 20010007157 A1* Full patent application text (1,155 kb)	July 5, 2001	Methods for transforming a rose callus with *Agrobacterium* cells carrying an exogenous DNA. Unlike the granted patent **US 5 792 927** , the type of callus to be transformed is not specified. A method for producing a somatic rose embryo expressing an exogenous sequence is also described. Rose callus, somatic embryo and plant expressing an exogenous gene are recited in the filed claims. View Claims

*It is important to remember that applications are **not issued patents** and the claims as filed have not been approved by any country. Thus, they are non-binding.

Roses
Patents granted to Florigene Europe B.V.

Actual granted claims

US 5 480 789
Claim 1 A method for producing a somatic rose embryo, which expresses an exogenous DNA sequence including a selectable marker gene, said method comprising: A) culturing tissue from a rose plant on a callus induction medium containing nutrients, an energy source, an auxin, and a cytokinin in amounts effective to induce formation of embryogenic callus, wherein the tissue is cultured until a friable, granular embryogenic callus is produced; B) combining cells from the embryogenic callus of step (A) with *Agrobacterium* cells carrying the exogenous DNA sequence in a co-cultivation medium containing nutrients, an energy source, and an induction compound under conditions which allow the *Agrobacterium* cells to infect the embryogenic callus cells and transfer the exogenous DNA sequence to the embryogenic callus cell chromosomes; C) culturing embryogenic callus cells from step (B) in a selection medium containing nutrients, an energy source, an auxin, a cytokinin, and an agent which inhibits the growth of embryogenic callus cells which do not express the selectable marker gene; and D) culturing the cells selected in step (C) in a maintenance medium containing nutrients, an energy source, an antibacterial agent, and a growth regulator, other than an auxin or a cytokinin, present in amounts effective to produce viable somatic embryos capable of being regenerated into transformed plantlets.

US 5 792 927
Claim 1 A method for genetically transforming callus cells from a rose plant, said method comprising: A) incubating friable, granular callus cells with *Agrobacterium* cells carrying an exogenous DNA sequence; and B) selecting callus cells which express at least a portion of the exogenous DNA sequence.
Claim 5 A method for genetically transforming a rose plant, said method comprising: A) culturing tissue from the rose plant under conditions selected to produce a friable, granular callus; B) incubating cells from the callus of step (A) with *Agrobacterium* cells carrying an exogenous DNA sequence; C) selecting callus cells from step (B) which express at least a portion of the DNA sequence; and D) producing transformed plantlets from the selected callus cells of step (C).
Claim 15 A somatic rose embryo produced by the method comprising: A) culturing tissue from a rose plant on a callus induction medium containing nutrients, an energy source, an auxin, and a cytokinin in amounts effective to induce callus formation; B) combining cells from the callus of step (A) with *Agrobacterium* cells carrying the exogenous DNA sequence in a co-cultivation medium containing nutrients, an energy source, and an induction compound under conditions which allow the *Agrobacterium* cells to infect the callus cells and transfer the exogenous DNA sequence to the callus cell chromosomes; C) culturing callus cells from step (B) in a selection medium containing nutrients, an energy source, an auxin, a cytokinin, and an agent which inhibits the growth of callus cells which do not express the selectable marker gene; and D) culturing the cells selected in step (C) in a maintenance medium containing nutrients, an energy source, an antibacterial agent, and a growth regulator, other than an auxin or a cytokinin, present in amounts effective to produce somatic embryos.

Roses
Patent application filed by DNA Plant Technology Co. & Florigene B.V.*

Actual pending claims

EP 536 327 A1
Claim 1 A method for controlled regeneration of a rose plantlet from a somatic embryo which comprises: A) providing a somatic embryo; B) culturing the somatic embryo on a maturation medium capable of inducing differentiation of the embryo

to yield a differentiated embryo;
C) germinating the differentiated embryo on germination medium to yield a germinated embryo; and
D) propagating the germinated embryo on propagation medium to produce a mature plantlet capable of being transferred to soil conditions.

Claim 2

A method for obtaining at least one somatic embryo from mature somatic tissue of rose plant, which comprises: A) culturing mature somatic tissue on callus induction medium comprising effective amounts of a nutrient medium, an energy source, an auxin and a cytokinin to obtain at least one induced callus; and
B) culturing the induced callus in a regeneration media capable of inducing completion of the development of somatic embryos comprising effective amounts of a nutrient medium, an energy source, an auxin and a cytokinin in which the source of the auxin and cytokinin in the regeneration media differs from the source of the auxin and cytokinin in the callus induction medium to obtain at least one somatic embryo.

Claim 3

A method for obtaining a somatic embryo from a stamen filament of rose plant which comprises: A) culturing the stamen filament on callus induction medium comprising effective amounts of a nutrient medium, an energy source, an auxin and a cytokinin to obtain at least one induced callus; and
B) culturing the induced callus in a regeneration media capable of inducing completion of the development of somatic embryos comprising effective amounts of a nutrient medium, an energy source, an auxin and a cytokinin in which the ratio of auxin to cytokinin is decreased by a factor of two to about 15 relative to the ratio of auxin to cytokinin in the callus induction medium to obtain a somatic embryo.

Claim 4

A method for obtaining a somatic embryo from a leaf explant of rose plant, which comprises: A) culturing the leaf explant on a callus induction medium comprising effective amounts of a nutrient medium, an energy source, an auxin and a cytokinin to obtain at least one induced callus; and
B) culturing the induced callus in a regeneration media capable of inducing completion of the development of somatic embryos comprising effective amounts of a nutrient medium, an energy source, an auxin and a cytokinin to which the source of the auxin and cytokinin in the regeneration media differs from the source of the auxin and cytokinin in the callus induction medium to obtain a somatic embryo.

Claim 5

A method for genetically transforming callus cells from a rose plant, said method comprising: A) incubating the callus cells with *Agrobacterium* cells carrying an exogenous DNA sequence; and
B) selecting callus cells which express at least a portion of the exogenous DNA sequence.

Claim 6

A method for genetically transforming a rose plant, said method comprising: A) culturing tissue from the rose plant under conditions selected to produce a callus;
B) incubating cells from the callus of step (A) with *Agrobacterium* c ells carrying an exogenous DNA sequence;
C) selecting callus cells from step (B) which express at least a portion of the DNA sequence; and
D) producing transformed plantlets from the selected callus cells of step (C).

Claim 7

A method for producing a somatic rose embryo which expresses an exogenous DNA sequence, said method comprising: A) culturing tissue from a rose plant on a callus induction medium containing nutrients, an energy source, an auxin, and growth regulator, a cytokinin in amounts effective to induce callus formation wherein the tissue is derived from a plant part selected from the group consisting of stamen filaments, leaf explants, stem sections, shoot tips, petal, sepal, petiole, and peduncle;
B) combining cells from the callus of step (A) with *Agrobacterium* cells carrying the exogenous DNA sequence in a co-cultivation medium containing nutrients, an energy source, and an induction compound under conditions which allow the *Agrobacterium* cells to infect the callus cells and transfer the exogenous DNA sequence to the callus cell chromosomes;
C) culturing callus cells from step (B) in a selection medium containing nutrients, an energy source, an auxin, a cytokinin, and an agent which inhibits the growth of callus cells which do not express the selectable marker gene; and
D) culturing the cells selected in step (c) in a regeneration medium containing nutrients, an energy source, an antibacterial agent, and a growth regulator selected from abscisic acid and giberellic acid, other than an

auxin or a cytokinin, present in amounts effective to produce somatic embryos.

* The present European application was filed by both companies, but the only assignee for Europe is **Florigene Europe B.V.**

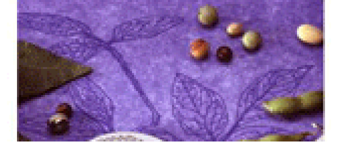
Roses
Patent application filed by Florigene B.V.

Actual pending claims

US 20010007157 A1
Claim 1 A method for genetically transforming callus cells from a rose plant, said method comprising: A) incubating the callus cells with *Agrobacterium* cells carrying an exogenous DNA sequence; and B) selecting callus cells which express at least a portion of the exogenous DNA sequence.
Claim 6 A method for genetically transforming a rose plant, said method comprising: A) culturing tissue from the rose plant under conditions selected to produce a callus; B) incubating cells from the callus of step (a) with *Agrobacterium* cells carrying an exogenous DNA sequence; C) selecting callus cells from step (b) which express at least a portion of the DNA sequence; and D) producing transformed plantlets from the selected callus cells of step (c).
Claim 13 A method for producing a somatic rose embryo which expresses an exogenous DNA sequence including a selectable marker gene, said method comprising: A) culturing tissue from a rose plant on a callus induction medium containing nutrients, an energy source, an auxin, and a cytokinin in amounts effective to induce callus formation; B) combining cells from the callus of step (a) with *Agrobacterium* cells carrying the exogenous DNA sequence in a co-cultivation medium containing nutrients, an energy source, and an induction compound under conditions which allow the *Agrobacterium* cells to infect the callus cells and transfer the exogenous DNA sequence to the callus cell chromosomes; C) culturing callus cells from step (b) in a selection medium containing nutrients, an energy source, an auxin, a cytokinin, and an agent which inhibits the growth of callus cells which do not express the selectable marker gene; and D) culturing the cells selected in step (c) in a maintenance medium containing nutrients, an energy source, an antibacterial agent, and a growth regulator, other than an auxin or a cytokinin, present in amounts effective to produce somatic embryos.
Claim 38 A rose callus cell which expresses an exogenous DNA sequence.
Claim 39 A rose plant having cells which express an exogenous DNA sequence.
Claim 40 A somatic rose embryo which expresses an exogenous DNA sequence.

Soybean
Summary

The **University of Toledo**, **Monsanto** and **Pioneer Hi-Bred** collectively have several patents granted in the United States, Canada, Australia and Europe which are directed to *Agrobacterium* transformation of soybean (*Glycine max*).



The **University of Toledo** has both an Australian and a United States patent which

claim a non-tissue culture process to transform meristematic or mesocotyl cells of a soybean seed. An *Agrobacterium* strain of any species is used for the transformation and can contain either an armed (with tumor-causing genes) or a disarmed vector. A related European patent additionally claims a method for transforming beans (*Phaseolus vulgaris*).

In the United States patents granted to **Monsanto** a cotyledon from a soybean seedling or an embryonic axis are used for transformation with *Agrobacterium* containing a disarmed vector. Additionally they claim the insertion of a gene encoding for neomycin phosphotransferase II (*npt*II) and a gene encoding EPSPS. The first gene confers resistance to kanamycin and neomycin among other antibiotics, and the later confers resistance to the herbicide glyphosate.

A recent United States patent **(US 6,384,301 B1**) and published application **(US 2002/0157139 A1**) disclose a method for soybean transformation using a wounded embryonic axis isolated from germinating seed as target for *Agrobacterium* -mediated transformation. The explant is cultured in a medium containing a selection agent. The difference between the granted patent and the application is that in the patent application the wounding step is omitted, thereby providing an alternative protocol to obtain transgenic soybean plants. **(Update July 2003)**

In the European and Australian applications, **Monsanto** discloses a method to prepare a transformed germline of soybean starting with an embryonic axis of a soybean seed for transformation process.

The inventions disclosed by **Pioneer Hi-Bred** in patents granted in the United States (2), Australia (1) and Canada (1) use a hypocotyl or a cotyledonary node of a soybean seed transformed with any species of *Agrobacterium*. Detailed protocols for co-cultivation of a soybean explant with the bacterium are also claimed. One of the United States patents also encompasses soybean transformation by microbombardment.

The table below presents a summary of the claims of the different patents and patent applications. Full text of patents and patent applications can be accessed as PDF.

Soybean		
Assigned to The University of Toledo		
Patent No.	Issue Date	Summary of the claims
US 5,376,543 & AU 648 951 B Full United States patent text (897 KB)	27 Dec 1994 & 5 May 1994	Production of a transgenic soybean plant through a non-tissue culture process. Meristematic or mesocotyl cells are inoculated with an armed or disarmed *Agrobacterium* vector having a gene of interest. The cells differentiate into a mature plant. View Claims
EP 397 687 B1 Full patent text (852 KB)	December 5, 1994	The method for producing a transgenic soybean plant is the same as the one disclosed in the related United States and Australian patents (above mentioned), but transformation of beans (*Phaseolus vulgaris*) is also included in the claims. View Claims
Remark: US 5,376,543 is deemed to have been abandoned according to the USPTO database, and Australian patent AU 648 951 B is deemed to have been abandoned according to IP Australia database (Update July 2003).		
Assigned to Monsanto		
US 5,416,011 Full patent text (1,037 KB)	16 May 1995	Transformation of a cotyledon of a soybean seedling by inoculation with a disarmed *A. tumefaciens* vector having a chimeric gene. As part of the invention, the chimeric gene encodes for *npt* II. View Claims

US 5,569,834 Full patent text (865 KB)	29 Oct 1996	Method to obtain a soybean plant resistant to kanamycin via *A. tumefaciens* transformation. The explant for transformation is a cotyledon isolated from a soybean seedling. View Claims
US 5,824,877 Full patent text (927 KB)	20 Oct 1998	As in the previous United States patents, a cotyledon explant is transformed by inoculation and co-cultivation with *A. tumefaciens* containing a gene encoding glyphosate resistance. View Claims
US 5,959,179 Full patent text (8,192 KB)	28 Sep 1999	A method for transforming a soybean cotyledon by inoculating into the axillary bud region a disarmed *A. tumefaciens* vector containing DNA of interest. View Claims
US 6,384,301 B1 (**Update July 2003**) Full patent text (581 KB)	7 May 2002	A method for soybean transformation using a wounded embryonic axis isolated from germinating seed as a target for *Agrobacterium* -mediated transformation. The explant is cultured in a medium containing a selection agent. View Claims
Remark: related patent appl. in the United States (US 2002/0157139 A1)		
Applications		
Application No.	**Publication Date**	**Summary of the claims**
EP 1 141 346 A1 and **AU 28488/00*** Full PCT patent application text (840 KB)	24 July 2001 & 1 Aug 2000	Method of preparing a germline-transformed soybean plant by exposing the embryonic axis of a freshly germinated soybean seed to a disarmed *Agrobacterium* vector having a gene of interest. View Claims
Remarks: The PCT application **WO 00/42207 A2** was converted into the European application **EP 1 141 346 A1** published 24 July 2001.		
US 2002/0157139 A1 **(Update July 2003)** Full PCT patent application text (567 KB)	24 Oct 2002	A method for soybean transformation using an embryonic axis isolated from germinating seed as a target for Agrobacterium-mediated transformation. The explant is cultured in a medium containing a selection agent. View Claims
Remarks: related patent in the United States (US 6,384,301 B1)		
Assigned to Pioneer Hi-Bred		
Issued patents		
Patent No.	**Issue Date**	**Summary of the claims**
US 5,563,055 Full patent text (791 KB)	8 Oct 1996	Transformation of cotyledonary nodes of soybean seeds in a medium containing *A. tumefaciens*, an auxin, and a signal molecule that induces virulence. Transformed tissue is subsequently selected. View Claims
AU 670 316 B	11 July 1996	Transformation of a hypocotyl or a cotyledonary node of a soybean seed by co-cultivation with *Agrobacterium* in a medium having specific signal compounds that induce *Agrobacterium* virulence, in order to regenerate transgenic soybean plants. View Claims

CA 2140910	23 Mar 1999	Claims are the same as for the Australian patent. View Claims
US 5,968,830 Full patent text (1,140 KB)	19 Oct 1999	Transformation of a hypocotyl soybean expant by cultivation with *Agrobacterium* in a medium having a virulence-inducing signal and cytokinin. A method for regeneration of soybeans (*G. max*) via organogenesis is also disclosed. Additionally, the explant can be transformed by microbombardment. View Claims
Remarks: Granted Japanese patent **JP 2952041 B2** is not analyzed. The Australian patent **AU 691 423 B** is also part of this patent family, but its claim scope is not directed to *Agrobacterium* -mediated transformation.		
Applications		
Application No.	**Publication Date**	**Summary of the claims**
EP 652 965 A1 * Full patent application text (1,352 KB)	17 May 1995	The applications disclose a transformation method using a hypocotyl or a cotyledonary node of a soybean seed. The explant is co-cultivated with *Agrobacterium* in the same conditions as specified in the Australian patent. Also, a method to regenerate soybean plants from cotyledonary nodes is disclosed. View Claims
Remarks: Applications also filed in Brazil (BR 9306802 A) and Hungary (HU 70467 A2).		

* It is important to remember that applications **are not issued patents** and the claims as filed have not been approved by any country. Thus, they are non-binding.

Soybean
Patents granted to The University of Toledo

Actual granted claims

US 5 376 543 & AU-B-648 951
Claim 1 A non-tissue culture process for producing a transgenic soybean plant, which process comprises: A) germinating a seed of a *Glycine max* plant for about 24 to 48 hours; B) inoculating the meristematic or mesocotyl cells produced by the germinating seed of step (A), prior to differentiation of said cells, with an armed or disarmed *Agrobacterium* strain containing an *Agrobacterium*-derived vector, said vector containing a transferable gene; and C) allowing the cells to differentiate into a mature plant.

EP 397 687 B1
Claim 1 A non-tissue culture process for producing a transgenic plant, which comprises: A) germinating a seed of a *Phaseolus vulgaris* or a *Glycine max* plant for 24 to 48 hours; B) inoculating the meristematic or mesocotyl cells produced during germination, prior to differentiation of the seed, with a virulent or non-virulent *Agrobacterium* strain containing a transferable gene in an *Agrobacterium* -derived vector; and C) allowing the cells to differentiate into a mature plant.

Soybean
Patents granted to Monsanto

Actual granted claims

US 5 416 011

Claim 1

A method for transforming soybeans which comprises:

A) preparing a cotyledon explant from a soybean seedling by:
(i) removing the hypocotyl region by cutting just below the cotyledonary node,
(ii) separating the two cotyledons at the cotyledonary node by tearing the cotyledons apart, and
(iii) removing the epicotyl from the cotyledon to which it remains attached, B) inserting a chimeric gene into the explant of part (A) by inoculation of at least the region adjacent to the axillary bud of the explant with a disarmed *Agrobacterium tumefaciens* vector containing said chimeric gene;
C) selecting transformed explant tissue, and
D) regenerating a differentiated transformed plant from the transformed explant tissue of part (C).

Claim 10

A method for transforming soybeans which comprises:

A) preparing a cotyledon explant from a soybean seedling by:
(i) removing the hypocotyl region by cutting just below the cotyledonary node,
(ii) separating the two cotyledons at the cotyledonary node by tearing the cotyledons apart, and
(iii) removing the epicotyl from the cotyledon to which it is attached, and
(iv) wounding the explant by making at least one cut in the axillary bud region of the explant, B) inserting a chimeric gene into the explant of part (A) which gene encodes for neomycin phosphotransferase II by inoculation and co-cultivation of the explant with a disarmed *Agrobacterium tumefaciens* vector containing said chimeric gene;
C) selecting transformed explant tissue by growing the explant in the presence of kanamycin, and
D) regenerating a differentiated transformed plant from the transformed explant tissue of part (C).

Claim 12

A method for transforming soybeans which comprises:

A) preparing a cotyledon explant from a soybean seedling by:
(i) removing the hypocotyl region by cutting just below the cotyledonary node,
(ii) separating the two cotyledons at the cotyledonary node, and
(iii) removing the epicotyl from the cotyledon to which it remains attached, B) inserting a chimeric gene into the explant of part (A) by inoculation of at least the region adjacent to the axillary bud of the explant with a disarmed *Agrobacterium tumefaciens* vector containing said chimeric gene;
C) selecting transformed explant tissue, and
D) regenerating a differentiated transformed plant from the transformed explant tissue of part (C).

US 5 569 834

Claim 1

A soybean plant comprising a chimeric gene and associated DNA resulting from an *Agrobacterium tumefaciens* -mediated transformation, said chimeric gene capable of conferring kanamycin resistance to said soybean plant, produced by the method which comprises:

A) preparing a cotyledon explant from a soybean seedling by:
(i) removing the hypocotyl region by cutting just below the cotyledonary node,
(ii) separating the two cotyledons at the cotyledonary node by tearing the cotyledons apart, and
(iii) removing the epicotyl from the cotyledon to which it remains attached, B) inserting a chimeric gene into the explant of part (A) by inoculation and co-cultivation of the explant with a disarmed *Agrobacterium tumefaciens* vector containing said chimeric gene;
C) selecting transformed explant tissue, and
D) regenerating a differentiated transformed plant from the transformed explant tissue of part (C).

US 5 824 877

Claim 1

A method for transforming soybean which comprises:

A) preparing a cotyledon explant from a soybean seedling by:
(i) removing the hypocotyl region by cutting just below the cotyledonary node,
(ii) separating the two cotyledons at the cotyledonary node by tearing the cotyledons apart, and
(iii) removing the epicotyl from the cotyledon to which it remains attached, B) inserting a chimeric gene into the explant of part (A) by inoculation and co-cultivation of the explant with a disarmed *Agrobacterium tumefaciens* vector containing said chimeric gene;
C) selecting transformed explant tissue, and
D) regenerating a differentiated transformed plant from the transformed explant tissue of part (C).

Claim 10

A method for transforming soybean which comprises:

A) preparing a cotyledon explant from a soybean seedling by:
(i) removing the hypocotyl region by cutting just below the cotyledonary node,
(ii) separating the two cotyledons at the cotyledonary node by tearing the cotyledons apart, and
(iii) removing the epicotyl from the cotyledon to which it is attached, and
(iv) wounding the explant by making at least one cut in the axillary bud region of the explant, B) inserting a chimeric gene into the explant of part (A) which gene encodes for 5-enolpyruvylshikimatephosphate synthase by inoculation and co-cultivation of the explant with a disarmed *Agrobacterium tumefaciens* vector containing said chimeric gene;
C) selecting transformed explant tissue by growing the explant in the presence of glyphosate, and
D) regenerating a differentiated transformed plant from the transformed explant tissue of part (C).

US 5 959 179

Claim 1

A method for transforming soybean, comprising:

A) preparing a cotyledon explant from a soybean seedling by:
(i) incubating said seedling at about 0°C to about 10°C for at least 24 hours;
(ii) removing the hypocotyl region by cutting in the region of from about 0.2 to about 1.5 cm below the cotyledonary node;
(iii) splitting and completely separating the remaining attached hypocotyl segment, also thereby separating the two cotyledons;
(iv) removing the epicotyl from the cotyledon to which it remains attached; and
(v) wounding the cotyledon in the region of the axillary bud; B) inserting DNA to be introduced into said explant of step (a) by inoculating at least the region adjacent to the axillary bud of the explant with a disarmed *Agrobacterium tumefaciens* vector containing said DNA;
C) selecting transformed explant tissue; and
D) regenerating a differentiated transformed plant from said transformed explant tissue of step (c).

Soybeans
Independent claims

Assigned to Monsanto (Update July 2003)

US 6,384,301 B1

Claim 1

1. A method of making germline-transformed soybean plants using Agrobacterium mediation, the method comprising:
(a) initiating the germination of a soybean seed;
(b) isolating the embryonic axis including the embryonic meristem from the soybean seed to prepare an explant;
(c) wounding the explant;
(d) exposing the explant to a disarmed Agrobacterium vector comprising a heterologous genetic construct comprising a selectable marker gene wherein the heterologous genetic construct is transferred into at least

one cell in the explant;
(e) culturing the explant in the presence of a selection agent in a manner allowing identification of soybean cells of the explant to which the heterologous genetic construct has been transferred;
(f) inducing formation of one or more shoots from the explant, the shoot comprising germline transformed cells;
(g) cultivating the shoot into a whole fertile mature soybean plant.

Assigned to Monsanto (Update July 2003)
US 2002/0157139 A1
Claim 1 1. A method of making germline-transformed soybean plants using Agrobacterium mediation, the method comprising: (a) initiating the germination of a soybean seed; (b) isolating the embryonic axis from the soybean seed to prepare an explant; (c) exposing the explant to a disarmed Agrobacterium vector containing a heterologous genetic construct including a selectable marker gene under conditions in which the heterologous genetic construct is transferred into at least one cell in the explant; (d) culturing the explant in the presence of a selection agent in a manner capable of identifying soybean cells of the explant to which the heterologous genetic construct has been transferred; (e) inducing formation of one or more shoots from the explant, the shoot comprising germline transformed cells; (f) cultivating the shoot into a whole fertile mature soybean plant.

Soybean
Patent applications filed by Monsanto

Actual pending claims

EP 1 141 346 A1* & AU 28488/00
Claim 1 A method of preparing a germline-transformed soybean plant using *Agrobacterium* mediation, the method comprising: A) initiating the germination of a soybean seed; B) isolating the embryonic axis from the soybean seed to prepare an explant; C) wounding the explant; D) exposing the explant to a disarmed *Agrobacterium* vector comprising a heterologous genetic construct including a selectable marker gene under conditions in which the heterologous genetic construct is transferred into at least one cell in the explant; E) culturing the explant in the presence of a selection agent in a manner capable of identifying soybean cells of the explant to which the heterologous genetic construct has been transferred; F) inducing formation of one or more shoots from the explant, the shoot comprising germline transformed cells; G) cultivating the shoot into a whole fertile mature soybean plant.

* The PCT application WO 0042207 A2 was converted into the present European application.

Soybean
Patents granted to Pioneer Hi-Bred

Actual granted claims

US 5 563 055
Claim 1 A method for transforming soybean cells, comprising the steps of: A) providing a complete plant medium that supports rapid division of plant cells, said medium comprising (i) a virulence-inducing amount of a signal molecule,

(ii) a growth promoting amount of an auxin, and
(iii) *Agrobacterium tumefaciens* bacteria in log growth phase, such that said bacteria are present in said medium in a concentration of about 10^8 viable cells per ml,
wherein said bacteria contain a chimeric gene and said medium is buffered at a pH below 6.0;
B) introducing into a first portion of said medium a plurality of germinated soybean seeds, from each of which seeds seed coat and radicle have been removed, and separating the cotyledons of each seed so as to expose the cotyledonary node of each seed, whereby a plurality of explants is produced; then
C) macerating said cotyledonary node, without cutting entirely through each of said explants to the abaxial side thereof, and thereafter maintaining said explants in said first portion, at room temperature, for at least about 30 minutes; then
D) transferring said explants to a second portion of said medium in solidified form, such that said explants are embedded in said medium, adaxial side up and level with the surface of said medium, and culturing said explants for about 3 days at about 22° C.;
E) treating said explants in counterselection medium;
F) cultivating said explants in agarose-solidified selection medium, wherein said explants are embedded adaxial side down in said selection medium, whereby transformed cells in said explants are favored; and then
G) selecting transformed cells from said explants.

AU-B-670 316

Claim 1

A genotype-independent method for producing a transgenic soybean plant which comprises:

A) co-cultivating an explant derived from a hypocotyl or cultured cotyledonary nodes of a germinated soybean seed with cells of an *Agrobacterium* species containing a chimeric gene at a concentration of 10^8 to 3×10^8 cells/ml and in the presence of a signal compound selected from the group consisting of acetosyringone, alfa-hydroxyacetosyringone, acetovanillone, syringaldehyde, syringic acid, sinapinic acid and mixtures thereof;
B) maintaining a temperature for co-cultivation of from 18 to 28°C; and
C) inducing virulence of the *Agrobacterium* by decreasing the pH of the plant culture media below pH 6.0.

CA 2140910

Claim 1

A genotype-independent method for producing a transgenic soybean plant which comprises:

A) co-cultivating an explant derived from a hypocotyl or cultured cotyledonary node of a germinated soybean seed with cells of an *Agrobacterium* species containing a chimeric gene at a concentration of 10^8 to 3×10^8 cells/ml and in the presence of a signal compound selected from the group consisting of acetosyringone, alpha-hydroxyacetosyringone, acetovanillone, syringaldehyde, syringic acid, sinapinic acid and mixtures thereof;
B) maintaining a temperature for co-cultivation of from about 22 to about 25°C;
C) inducing virulence of the *Agrobacterium* by decreasing the pH of the plant culture media below pH 6.0; and
D) regenerating a transgenic soybean plant.

US 5 968 830

Claim 1

A method of regenerating soybeans (*G. max*) via organogenesis, comprising:

A) obtaining hypocotyl explants from germinated seedlings of soybean plants whose regeneration is desired,
B) maintaining said hypocotyl explants on a shoot induction medium comprising a cytokinin until shoots form at the acropetal end of said hypocotyl explant,
C) excising shoots from said hypocotyl explant and maintaining said shoots on a shoot elongation medium until said shoots are competent on a rooting medium, and maintaining said shoots on a rooting medium until rooted plantlets are formed, and
D) transplanting said plantlets to soil.

Claim 7

A method for transforming soybean plants to express exogenous DNA, comprising:

A) obtaining a hypocotyl explant from germinated seedlings of a soybean plant,
B) maintaining said explant on a shoot induction medium comprising a cytokinin for 16-32 hours,
C) bombarding said explant, with the acropetal end facing up, with microparticles of an inert metal coated with exogenous DNA comprising a plasmid which comprises an expression gene, said expression gene encoding the expression of a protein exogenous to said soybean plant,
D) maintaining said bombarded hypocotyl explant on a medium selective for growth of transformed tissues, followed by maintenance on a shoot elongation medium and preparing plantlets from shoots so obtained.

Claim 10

A method of transforming soybeans, comprising:

A) obtaining a hypocotyl soybean explant from germinated seedlings, and
B) maintaining on its side or upright on a shoot induction medium comprising a cytokinin with a basipetal end thereof submerged in said medium,
C) pre-culturing and/or co-culturing an *Agrobacterium* strain with a virulence enhancing substance, and
D) adding said pre- or co-cultured bacteria to the acropetal end of said upright hypocotyl explant or by complete submersion,
E) co-incubating said hypocotyl explant for a time sufficient to permit transfection of said hypocotyl explant by said bacteria,
F) disinfecting said hypocotyl explant, maintaining said hypocotyl explant on a shoot induction medium and assaying said explant for expression of introduced foreign DNA, and regenerating hypocotyl explants positive for expression of said DNA.

Soybean
Patent application filed by Pioneer Hi-Bred

Actual pending claims

EP 652 965 A1

Claim 1

A genotype-independent method for producing a transgenic soybean plant which comprises:

A) co-cultivating an explant derived from a hypocotyl or cultured cotyledonary nodes of a germinated soybean seed with *Agrobacterium* species containing a chimeric gene in the presence of a signal compound selected from the group consisting of acetosyringone, alpha-hydroxyacetosyringone, acetovanillone, syringaldehyde, syringic acid, and sinapinic acid and mixtures thereof;
B) maintaining a temperature for co-cultivation of from 18 to 28°C; and
C) inducing virulence of the *Agrobacterium* by decreasing the pH of the plant culture media below pH 6.0.

Claim 6

A method of regenerating soybean plants from cotyledonary nodes which comprises:

A) dividing the node;
B) culturing the divided node on a nutrient medium until callus tissue develops which contains shoots; and
C) removing the shoots from said callus and rooting the shoots on a nutrient medium containing hormone and pyroglutamic acid to form a plantlet.

Squash (*Cucurbita pepo*)
Summary

The United States patent granted to Asgrow Seed refers to

- a method for transforming squash embryogenic calli with *Agrobacterium*,
- regeneration of transformed squash plants, and

- components of an induction medium for regeneration of the transformed calli.

The following table presents basic bibliographic data and a summary of the invention. Full text of United States patent can be accessed as PDF.

Squash (*Cucurbita pepo*)		
Assigned to Asgrow Seed Co.		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 5 677 157 Full patent text (1,029 kb)	October 14, 1997	Methods to transform embryogenic calli derived from squash shoot tips and squash seeds with *Agrobacterium* having a gene of interest. Media for culturing the transformed explant and embryogenic regeneration procedure are claimed. Additionally, transformation of the same explants by microprojectile bombardment is also claimed. The claims referring to this transformation method are not shown here. View Claims
Remarks: The related European patent **EP 491 733 B1** claims a general method for regenerating transformed squash calli, and *Agrobacterium* transformation of squash is recited in **dependent** claims. Applications filed in Australia (AU 62840/90 A1), and Japan (JP 5500308 T2).		

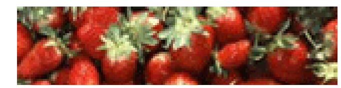
Squash (*Cucurbita pepo*)
Patent granted to Asgrow Seed Co.

Actual granted claims

US 5 677 157
Claim 1 A method of transforming and regenerating squash plants, which comprises: A) excising shoot tips from germinating squash; B) transforming embryogenic calli by inoculating the excised squash tissue with *Agrobacterium* comprising a DNA construct having a beneficial gene and a plant expressible selection marker gene and culturing the resulting explant on an induction media comprising MS media, 2,4,5-T, BAP, and Kn; C) selectively growing the transformed embryogenic calli on media containing a selection agent for the plant expressible selection marker gene; and D) subjecting the transformed embryogenic calli to an embryogenic regeneration procedure from which whole transformed squash plants can be obtained.
Claim 4 A method of transforming and regenerating squash plants, which comprises: A) excising tissue from mature squash seeds; B) transforming embryogenic calli by inoculating said excised squash tissue with *Agrobacterium* comprising a DNA construct having a beneficial gene and a plant expressible selection marker gene and culturing on an induction media comprising MS media, 2,4-D or 2,4,5-T, BAP, and Kn; C) selectively growing the transformed embryogenic calli on media containing a selection agent for the plant expressible selection marker gene; and D) subjecting the transformed embryogenic calli to an embryogenic regeneration procedure from which whole transformed squash plants can be obtained.

Strawberry
Summary

Update July 2003



A United States patent was issued to **(VPP Corp) DNA Plant Technology Corp.** with granted claims disclosing a method to produce transgenic strawberry plants by

co-cultivating *Agrobacterium* carrying a gene of interest with explants in the presence of glucose or fructose.

The related PCT application **WO 99/35903 A1** and the Australian application **AU 23231/99** filed by **VPP Corp.** were both abandoned in the years 2001 and in 2000, respectively.

The US patent is broad with respect to the target tissue used in transformation. The main limitation of the patent is with additives to the culture medium. If the procedure is carried out in medium containing a carbohydrate that is not glucose or fructose or no carbohydrate, it is unlikely there would be infringement.

Bibliography

Strawberry		
Assigned to (VPP Corp) DNA Plant Technology Corp.		
Issued Patent		
Patent No.	Issue Date	Summary of the Claims
US 6,274,791 B1 (1.010 Kb)	14 Aug 2001	Methods for strawberry transformation using *Agrobacterium tumefaciens*
Appl. No. & Filing date	US 09/232,085 15 Jan 1999	

Sugar beet
Summary

The invention claimed by **Biosem** in a granted European patent is directed to the transformation of **callus** of sugar beet by contacting the calli with *Agrobacterium* having a vector with a gene of interest. The transformation process takes place in a liquid culture medium.

A transgenic sugar beet plant resistant to the sugar beet necrotic yellow vein virus is also part of the disclosed invention. A cDNA or genomic fragment conferring resistance to this virus is specifically limited to a certain nucleotide sequence that encodes at least part of the protein responsible for the resistance.

Monsanto
has recently filed applications in Australia, United States and a PCT application related to a method for transforming a sugar beet **leaf** with *Agrobacterium*. The transformed leaf is initially derived from a selected region of a sugar beet seedling.

The following table presents basic bibliographic data and a summary of the inventions. Full text of the granted European patent and the PCT application can be accessed as PDF.

Sugar beet		
Assigned to Biosem		
Issued patents		
Patent No.	Issue date	Summary of the claims

EP 517 833 B1 Full patent text	November 2, 1995	Method for transforming calli of sugar beet by contacting a suspension of them with *Agrobacterium* having a vector with a gene to be introduced into the plant cells. The gene of interest confers resistance to the infection caused by the sugar beet necrotic yellow vein virus. View Claims
Remarks: Granted French patent **FR 2658987 B1** is not analyzed.		
Assigned to Monsanto		
Applications		
Application No.	**Publication date**	**Summary of the claims**
WO 0142480 A2* Full patent application text (1,181 kb)	June 14, 2001	A method for preparing transgenic sugar beet cells by selecting part of the cotyledon and hypocotyl region of a sugar beet seedling and micropropagating this part to form a shoot, from which a leaf is selected and put in contact with *Agrobacterium* cells. The *Agrobacterium* cells contain a vector with an exogenous gene. Transgenic sugar beet plants capable of expressing the exogenous gene are also recited in the claims. View Claims
US 20010042257 A1* Full patent application text (880 kb)	November 15, 2001	The filed claims are worded the same as the claims of the related PCT patent application. View Claims
Remarks: A related application was also filed in Australia (AU 200125757 A5).		

*It is important to remember that applications are **not issued patents** and the claims as filed have not been approved by any country. Thus, they are non-binding.

Sugar beet
Patent granted to Biosem

Actual granted claims

EP 517 833 B1
Claim 1 Method for transforming plant cells belonging to species *Beta vulgaris*, characterized in that it comprises the bringing into contact of the dispersion of friable white calluses in a liquid plant cell culture medium containing 0 to about 3.0 mg/liter of the cytokinin, or of a suspension of friable white calluses in a liquid plant cell culture medium containing about 0.1 to about 3.0 mg/liter of the cytokinin, with *Agrobacterium* containing a vector carrying a gene intending to be introduced into the plant cells, following by co-culturing the plant cells and the bacteria in order to give rise to transformed friable calluses.
Claim 21 Transgenic plant belonging to the *Beta vulgaris* species and resistance to infection by the sugar beet necrotic yellow vein virus (BNYVV), the said plant being transformed in a stable manner by a nucleic acid fragment whose expression product is capable of conferring the said resistance, the said fragment being derived from the 5' end of genomic or subgenomic RNA2 of BNYVV, or from the corresponding cDNA, this fragment encoding at least a portion of the proteins encoded by nucleotides 145 to 3285 of the wild type sequence of RNA2, and being under the control of a promoter allowing the expression of the fragment in the plant cells and being in this sense or antisense orientation.

Sugar beet
Patent applications filed by Monsanto

Actual pending claims

WO 0142480 A2 & US 20010042257 A1
Claim 1 A method for the preparation of transgenic sugarbeet cells, the method comprising: A) selecting a sugarbeet seedling, the seedling comprising a cotyledon region and a hypocotyl region; B) removing the cotyledon region and upper half of the hypocotyl region from the seedling; C) contacting the cotyledon region and upper half of the hypocotyl region with micropropagation media to form a micropropagated shoot, the micropropagated shoot comprising at least one leaf or portion thereof comprising a leaf base; D) removing a leaf from the micropropagated shoot at the leaf base; and E) contacting the leaf at the leaf base with Agrobacteria in a manner forming a tissue comprising a transgenic sugarbeet cell, capable of expressing an exogenous structural nucleic acid sequence wherein: the Agrobacteria comprises a vector; and the vector comprises operatively linked in the 5' to 3' orientation: 1. a promoter that directs transcription of an exogenous structural nucleic acid sequence; 2. an exogenous structural nucleic acid sequence; and 3. a 3' transcription terminator.
Claim 7 A method for the preparation of a transgenic sugarbeet plant, the method comprising: A) selecting a sugarbeet seedling, the seedling comprising a cotyledon region and a hypocotyl region; B) removing the cotyledon region and upper half of the hypocotyl region from the seedling; C) contacting the cotyledon region and upper half of the hypocotyl region with micropropagation media to form a micropropagated shoot, the micropropagated shoot comprising at least one leaf or portion thereof comprising a leaf base; D) removing a leaf from the micropropagated shoot at the leaf base; E) contacting the leaf base with Agrobacteria in a manner forming a tissue comprising a transgenic sugarbeet cell; F) culturing said tissue to form a transgenic shoot; G) culturing the transgenic shoot to form a transgenic rooted shoot; and H) growing the transgenic rooted shoot to form a transgenic sugarbeet plant capable of expressing an exogenous structural nucleic acid sequence, wherein: > the Agrobacteria comprise a vector; and the vector comprises operatively linked in the 5' to 3' orientation: 1. a promoter that directs transcription of an exogenous structural nucleic acid sequence; 2. an exogenous structural nucleic acid sequence; and 3. a 3' transcription terminator.

Tomato

Summary

In a United States granted patent **Calgene** discloses methods to transform tomato cotyledons with *A. tumefaciens* having a gene of interest. The construct to be integrated into the tomato cells contains transcription initiation and termination regulatory regions, a gene of interest and at least a right T-DNA border. In one of the claimed methods, the bacterium carries binary vectors. Tomato cells resistant to the herbicide glyphosate are also part of the invention.

The following table presents basic bibliographic data and a summary of the inventions. Full text of United States patent can be accessed as PDF.

Tomato

Assigned to Calgene		
Issued patent		
Patent No.	Issue date	Summary of the claims
US 5 565 347 Full patent text (1,480 kb)	October 15, 1996	Transformation of tomato cotyledon sections with *A. tumefaciens* carrying a construct with a foreign gene. Transformed tissues are regenerated into tomato plants. Disarmed *A. tumefaciens* having a binary vector with a foreign gene is used to transform tomato cells. View Claims
Remarks: Applications also filed in Australia (AU 73351/87 A1), China (CN 87104202 A), Israel (IL 82704 A0), Japan (JP 63068088 A2) and New Zealand (NZ 220642 A).		

Tomato
Patent granted to Calgene

Actual granted claims

US 5 565 347
Claim 1 A transformed *Lycopersicon esculentum* cotyledon cell, wherein said cell is present in an *in vitro* cell culture.
Claim 2 A method for transforming tomato species cells, said method comprising: A) pre-incubating tomato cotyledon sections with medium conditioned by a plant cell feeder culture; B) co-cultivating said cotyledon sections with *Agrobacterium tumefaciens* cells comprising *vir* genes, wherein said *Agrobacterium* cells further comprise DNA construct comprising: (i) transcriptional initiation and termination regulatory regions functional in tomato plant cells and (ii) a gene other than the wild-type gene associated with at least one of said transcriptional initiation and termination regions, and (iii) at least a right T-DNA border, whereby said construct becomes integrated into the genome of cells in said cotyledon section to provide transformed tomato plant cells; C) incubating said transformed tomato plant cells in a regeneration medium comprising a bacteriocide and a means for selection of said transformed tomato plant cells as the result of a marker on said DNA construct, whereby transformed tomato shoots develop; and D) transferring said transformed shoots to a rooting medium to produce transformed tomato plants.
Claim 9 A method for modifying the genotype of tomato plant cells, said method comprising: A) pre-incubating tomato cotyledon sections with medium conditioned by a plant cell feeder culture, B) contacting said cotyledon sections with a culture of a disarmed *Agrobacterium tumefaciens* strain comprising *vir* genes and a binary vector plasmid comprising at least the right T-DNA border and a gene of interest, wherein said gene of interest is under regulatory control of transcriptional initiation and termination regions functional in tomato plant cells, and wherein said gene of interest is integrated into the genome of cells in said tomato cotyledon sections, and C) isolating said tomato cells comprising said integrated gene of interest.
Claim 16 A method for transforming tomato species cells, said method comprising: A) co-cultivating tomato cotyledon sections with *Agrobacterium tumefaciens* cells comprising *vir* genes, wherein said *Agrobacterium* cells further comprise a DNA construct comprising transcriptional initiation and termination regulatory regions functional in tomato plant cells and a gene other than the wild-type gene

associated with at least one of said transcriptional initiation and termination regions, and at least a right T-DNA border, whereby said construct becomes integrated into the genome of cells in said cotyledon section to provide transformed tomato plant cells;
B) incubating said transformed tomato plant cells in a regeneration medium comprising a bacteriocide and a means for selection of said transformed tomato plant cells as the result of a marker on said DNA construct, whereby transformed tomato shoots develop; and
C) transferring said transformed shoots to a rooting medium to produce transformed tomato plants.

Claim 19

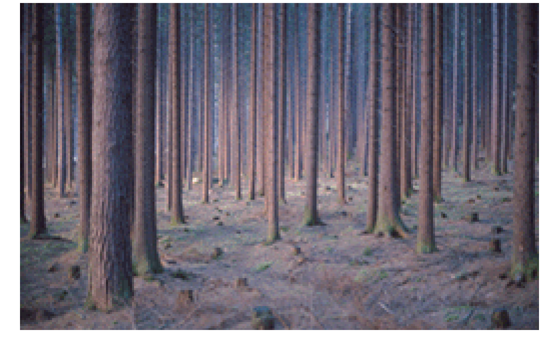
A method for modifying the genotype of tomato plant cells, said method comprising:

A) contacting tomato cotyledon sections with a culture of a disarmed *Agrobacterium tumefaciens* strain comprising *vir* genes and a binary vector plasmid comprising at least the right T-DNA border and a gene of interest, wherein said gene of interest is under regulatory control of transcriptional initiation and termination regions functional in tomato plant cells, and wherein said gene of interest is integrated into the genome of cells in said tomato cotyledon sections, and
B) isolating said tomato cells comprising said integrated gene of interest.

Woody tree species

(Update July 2003)

Forestry industry --which relies on woody tree species-- is a major economical factor for several countries. Tree species like *Eucalyptus* and *Pinus* are very important in the paper and cellulose industry. Because of their long generation times, novel breeding methodologies are sought to introduce disease resistance genes, influence lignin production or modulate flowering time for example. Patents covering transformation technologies could be of commercially strategic relevance.



Summary

- The European application assigned to the Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria (INIA) and the Instituto Valenciano de Investigaciones Agrarias (IVIA) , both in Spain, is related to a United States patent analyzed in the 'Citrus' section. The present application discloses a procedure for the transformation of any woody species using adult plant material as primary explant. The explant is grafted on a stock to generate shoots which are then used as target tissue for *Agrobacterium*-mediated transformation. Treated shoots are then re-grafted in vitro to select for transformed shoots, which are then regenerated into full transgenic plants. This method addresses the problem of how to obtain improved clones from elite woody trees from which it is usually hard to obtain totipotent, regenerable material.
- The PCT application assigned to the Companhia Suzano de Papel e Celulose and the University of Sao Paulo
in Brazil discloses a method to transform woody tree species starting from pre-germinated seed material. The method disclosed was developed for transformation of *Eucalyptus* spp but the claims have been worded to encompass any woody tree species, which could include conifers. The method disclosed in the independent claims is rather detailed in terms of timing of the multiple steps. This timing could be very different for other tree species than *Eucalyptus*, which would allow ways to design around the claim in this patent (if granted) and thus avoid infringement.

Woody tree species

Assigned to the Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria (INIA)

and the Instituto Valenciano de Investigaciones Agrarias (IVIA) , Spain

Patent

Applications

Patent Appl. No.	Publ. date	Summary of the claims

EP 870 838 A2 Full patent text (569 Kb)	14 Oct 1998	A procedure for transformation of adult woody species with appropriate vectors, including Agrobacterium. Adult tissue is grafted on stocks to generate new buds or apices that are used as target tissue for transformation followed by in vitro micrografting of transgenic shoots. View Claims
Remarks: Priority application in Spain (ES 2151338). Related United States patent (US 6,103,955) restricted to citrus spp.		
Assigned to Companhia Suzano de Papel e Celulose and Univ. de Sao Paulo (BR)		
WO 02/14463 A2 Full patent text (1.21 Kb)	21 Feb 2002	A method for transformation of woody trees using pre-germinated seed as target tissue for Agrobacterium-mediated transformation. View Claims
Remarks: Priority application in Brazil (BR 00/03908); application also filed in Australia (AU 79510/01).		

Woody Trees
Independent claims

Assigned to Companhia Suzano de Papel e Celulose and Univ. de Sao Paulo (BR)
WO 02/14463 A2
Claim 1 METHOD FOR GENETIC TRANSFORMATION OF WOODY TREES, which comprises the following steps: -sterilization and washing of seeds of woody trees; -transference of said seeds to an appropriate medium of culture; -germination for a period between about 2 and 17 days; -collection of germinated material; -inoculation with *Agrobacterium*, containing one or more genes of interest and optionally one or more marker genes, under concentration between about 10E+7 and 10E+9 cells per ml; -await inoculation between about 20 and 30 hours in a liquid medium; -transference of the material to a solid medium for a period between about 38 and 50 hours in the dark, under temperature between about 25 and 31 C and ambient humidity; -transference of the material to start plantlet growth in the light; -withdrawal of a plantlet leaf obtained between about 5 and 17 days during growth in the light; -transference of the plantlet leaf to the MS medium containing auxins derived from urea; -await germination of the plant tissue for approximately 20 days; and -identification and selection of the transformed plantlets.
Claim 2 METHOD FOR GENETIC TRANSFORMATION OF WOODY TREES, which comprises the following steps: -sterilization and washing of seeds of woody trees; -transference of said seeds to an appropriate medium of culture; -germination for a period between about 2 and 17 days; -collection of germinated material; -sonication of the material as collected ; inoculation with *Agrobacterium*, containing one or more genes of interest and optionally one or various marker genes, under concentration between about 107 and 109 cells per ml; -await inoculation between about 20 and 30 hours in a liquid medium; -transference of the material to a solid medium for a period between about 38 and 50 hours in the dark, under temperature between about 25 and 31 C and ambient humidity; -transference of the material to start the plantlet growth in the light; -withdrawal of a plantlet leaf obtained between about 5 and 17 days during growth in the light; -transference of the plantlet leaf to the MS medium containing auxins derived from urea; -await germination of the plant tissue for approximately 20 days and -identification and selection of transformed plantlets.

Claim 9

METHOD TO OBTAIN TRANSGENIC WOODY TREE PLANTS, which comprises the following steps:
-sterilization and washing of seeds of woody trees;
-transference of said seeds to an appropriate medium of culture;
-germination for a period between about 2 and 17 days;
-collection of germinated material;
-inoculation with *Agrobacterium*, containing one or more genes of interest and optionally one or more marker genes, under concentration between about 107 and 109 cells per ml;
-await inoculation between about 20 and 30 hours in a liquid medium ;
-transference of the material to a solid medium for a period between about 38 and 50 hours in the dark, under temperature between about 25 and 31 C and ambient humidity;
-transference of the material to start the plantlet growth step in the light;
-withdrawal of a plantlet leaf obtained between about 5 and 17 days during growth in the light;
-transference of plantlet leaf to the MS medium containing auxins derived from urea;
-await germination of the plant tissue for approximately 20 days;
-identification and selection of transformed plantlets;
-maintaining transforming plantlets in a multiplication and elongation medium for about 20 days;
-regeneration of the final transformed plant from one of the following regions hypocotyl; cotyledon; primary leaves and col.

Assigned to the Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria (INIA)

and the Instituto Valenciano de Investigaciones Agrarias (IVIA)

EP 870 838 A2

Claim 1

A genetic transformation procedure for adult plants of woody species which consists of:
inoculating explants of adult tissue of woody species, from the first shoots of the grafts of buds of adult plants of woody species on stocks, with appropriate vectors which carry the genes which encode the characteristics of interest, under conditions which permit the development of transgenic shoots; and micrografting in vitro said transgenic shoots, their buds or apices, on stock cultivated in vitro and, subsequently, grafting the resulting micrografted plants onto other stocks which give vigor and let the taken shoots grow to generate complete adult transgenic plants, or directly transplanting the in vitro micrografted plants into the soil, to give complete adult transgenic plants.

Conifers

Summary

Botanical aspects

Conifers is a major group within the Gymnosperms plants (plants with naked seeds that appear in a cone). They usually have needle-shaped or scale-like leaves, and nearly all are evergreen. The trees have a conical shape and have cones for pollen and seed production.

Within Conifers, *Pinus*
is the largest genus with about 120 species. It is also the most widespread genus of trees in the Northern Hemisphere. The natural distribution of pines ranges from arctic and subarctic regions of Eurasia and North America to subtropical and tropical (usually montane) regions of Central America and Asia. Pines are also extensively planted in temperate regions of the Southern Hemisphere. Many pines are fast growing species tolerant of poor soils and relatively arid conditions, making them popular in reforestation. Due to their occurrence in some very cold and dry environments where disease and stand-destroying disturbance are rare, pines are collectively the most long-lived of conifers. Ages of over 1000 years are common for some pine species.

Important pine products include wood, turpentine, and edible seeds. The wood of several *Pinus* species (e.g. *P. elliottii*) is widely used for construction. Synthetic products derived from turpentine (e.g. terpin, anethole, camphor, and dl-menthol) are used in pharmaceutical preparations (e.g. expectorant in humans), in perfumery, in the elaboration of cigarettes, cosmetic, toilet products, and to impart flavor in different products.

IP aspects

There are a couple of patents directed to transformation of conifers by microprojectile bombardment, but very few related to transformation through *Agrobacterium*.

A United States patent on transformation of **pine tissue** with *Agrobacterium* was granted to **North Carolina State University**
in 1989. The patent claims a method for the transformation of a differentiated tissue of pine with *A. tumefaciens*. The strain of *A. tumefaciens* used for transformation can be selected from: a strain capable of causing crown gall, a strain with a co-integrated Ti-plasmid and a strain with a binary vector system.

Recently, **Genesis Research and Development Corporation** has filed a United States patent application directed to transformation of nodal stem segments of a plant from the genus *Pinus* with *Agrobacterium*.

Conifers - Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 4 886 937 • Filed - 22 June 1998 • Granted - 12 December 1989	**Title** - Method for Transforming Pine A method for transforming a differentiated pine tissue with a strain of *A. tumefaciens* selected from: a strain capable of causing crown gall in pines, a strain with a Ti-plasmid having both the *vir* genes and the T-borders and a strain having two plasmids, one with the *vir* genes and the other with the T-borders. A transformed pine attained with the mentioned method is also claimed.	North Carolina State University
US 2002/0016981 A1 • Filed - 20 March 2001	**Title** - Methods for Producing Genetically Modified Plants, Plant Materials and Plant Products Produced Thereby A method for producing genetically modified material of *Pinus* by incubating nodal stem segments with an *Agrobacterium* culture transformed with a genetic construct. Transformed plant material is regenerated from transformed adventitious shoot buds. The invention also includes transformation of *Eucalyptus* species. This part is discussed under Particular dicots - Eucalyptus.	Genesis Research and Development Corporation
Remarks	Related applications also filed in Australia (AU 61270/99 A1), Brazil (BR 9913740 A) and China (CN 1326510 T). The European application EP 1 114 169 A1 and the granted United States patent US 6 255 559 are also related to this application, but *Agrobacterium* -mediated transformation of *Pinus* is not the subject matter of the invention.	

Marine algae

Summary

Algal aspects

Algae are organisms relatively undifferentiated, which unlike plants, have no true roots, leaves, flowers or seeds. They are found in marine, freshwater and terrestrial habitats. Their size varies from tiny microscopic unicellular forms of 3-10 μm (microns) to large macroscopic multicellular forms up to 70 meters long and growing at up to 50 cm per day. Algae do not have water-conducting tissues, as they are, at some stage, surrounded by water, which is also important for reproduction by spores. The spores may be motile or non-motile. Most of the algae are photosynthetic organisms that have chlorophyll. Apart from it, they contain additional pigments, which are the basis of classification.

Phytoplankton, seaweeds and symbiotic dinoflagellates (unicellular, biflagellate organisms) in corals and sea anemones are marine algae. Seaweeds are classified as Green algae (Chlorophyta), Brown algae (Phaeophyta), Red algae (Rhodophyta) and some filamentous Blue-green algae (Cyanobacteria). Most of the seaweeds are red (6000 species) and the rest known are brown (2000 species) or green (1200 species). Seaweeds are used in many maritime countries as a source of food, for industrial applications and as a fertilizer. Nori (*Porphyra spp.*), a Japanese red seaweed, is very popular in the Japanese diet, has a high protein content (25-35% of dry

weight), vitamins (e.g. vitamin C) and mineral salts, especially iodine. Industrial utilization is at present largely confined to extraction for phycocolloids, industrial gums classified as agars, carrageenans and alginates. Agars, extracted from red seaweeds such as *Gracilaria* , are used in the food industry and in laboratory media culture. Carrageenans, extracted from red seaweeds such as *Chondrus*, *Gymnogongrus*, and *Eucheuma*
among others, are used to provide gel particular qualities. Alginates are derivatives of alginic acid extracted from large brown algae such as *Laminaria*. They are used in printers' inks, paints, cosmetics, insecticides, and pharmaceutical preparations. In the USA, alginates are used as stabilizers in ice cream and also as a suspending agent in milk shakes. In 1995, the estimated value of international seaweed gums market was $560 million dollars.

IP aspects

Northeastern University, in the United States, has filed a PCT application related to transformation of multicellular marine algae via *Agrobacterium*. Marine algae are defined by the applicants as non-angiosperm photosynthetic eukaryotic organisms that live in ocean or saline water. According to the disclosure, marine algae could serve as a source of valuable pharmaceutical compounds through genetic engineering. They naturally have a very high protein content and are easy to grow for biomass.

With respect to genetic transformation, the applicants claim to provide a stable method for genetic transformation of multicellular marine algae. The method comprises wounding the outer cell wall layer of an alga in order to facilitate the access of *Agrobacterium* T-DNA with a gene of interest. The transformation of algae cells takes place in an environment containing seawater to ensure the survival of the transformed algae. To exemplify their invention, the applicants describe the transformation of the red algae *Porphyra*, known as nori, for which worldwide production is estimated at $1.5 billion dollars annually.

Algae transformation - Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
WO 0062601 A1 • Filed - 14 April 2000	**Title** - *Agrobacterium*-mediated genetic transformation of multicellular marine algae, resultant strains and their products **Claim 1** A method for causing genetic transformation of multicellular marine algae, said method comprising: A) culturing cells of a transformation-competent *Agrobacterium* species, said cells containing a Ti plasmid that contains a gene of interest; B) wounding a multicellular marine algae to be transformed in a manner that is sufficient to penetrate at least the cuticle, or outer cell wall layer of said alga; C) applying cells of said transformation-competent *Agrobacterium* species to wounded cells of said alga; and D) co-culturing said applied cells of said *Agrobacterium* species with said wounded algal cells for a time sufficient to effect transformation of some of said algal cells. **Claim 20** A stable transgenic multicellular marine algae, said alga comprising a DNA sequence coding for a gene foreign to said alga, wherein said alga is further capable of expressing said DNA sequence and of transferring said expressible DNA sequence to progeny of said alga. **Claim 22** A transgenic strain of marine algae comprising and capable of expressing a DNA sequence coding for an antigen of a pathogenic microorganism or an antigenic determinant thereof, wherein said antigen or antigenic determinant thereof is capable of eliciting a secretory immune response in	Northeastern University

a human or other animal upon oral administration of cellular material from said algae.

Claim 23

A transgenic strain of marine algae with enhanced disease resistance to marine fungi compared to a non-transformed said strain.

Claim 24

A transgenic strain of marine algae capable of producing a compound having an enhanced health benefit compared to a non-transformed said strain.

Claim 26

A method for eliciting a secretory immune response in a human or other animal, said method comprising:

- orally administering an effective amount of a composition comprising transgenic marine algae, or tissue thereof,
  wherein said transgenic algae, or algal tissue, comprise and are capable of expressing a DNA sequence coding for an antigen of a pathogenic microorganism or an antigenic determinant thereof,
  wherein said antigen or antigenic determinant thereof is capable of eliciting a secretory immune response in a human or other animal upon oral administration of cellular material from said algae.

The claims as filed of the present PCT application recite:

- a method for the transformation of multicellular marine algae with *Agrobacterium* by
-
  - wounding the cuticle of the algae
  - applying competent *Agrobacterium* containing a gene of interest to the wounded alga; and
  - co-culturing both *Agrobacterium* and algae cells allowing enough time for the transformation process
- a stable transgenic marine algae capable of expressing a foreign sequence and transferring such sequence to its progeny;
- transgenic strains of marine algae comprising:
-
  - antigens as elicitors for human or animal immune response
  - resistance to marine fungi
  - a compound for health benefit
- a method to elicit human or animal immune response by orally administering a transgenic algae or algal tissue containing an antigen that acts as an elicitor of an immune response.

The present PCT application needs to be converted into a national application in order to be granted. The subject matter of the claims as filed may change during the granting process.

Fungi

Summary

"Fungal" aspects

Fungi constitute one of the life kingdoms. Fungi are eukaryotic (eu=true; karyon=nucleus) organisms with a cell wall like plants, but they do not have chlorophyll. Fungi are not able to ingest their food like animals do,

nor can they manufacture their own food the way plants do. Instead, fungi feed by absorption of nutrients from the surrounding environment. They accomplish this by growing through and within the substrate on which they are feeding.

Fungi are divided into two big groups: **yeasts** and **moulds**. Yeasts are solitary rounded forms that reproduce by making more rounded forms through mechanisms such as budding or fission. Moulds, on the other hand, have bodies composed of thread-like long cells called **hyphae**. Thus, moulds are also known as **filamentous fungi**. The filamentous cells are connected end-to-end and grow in a branching fashion forming a network called **mycelium**. The mycelium that grows over and within a substrate that is used as a source of nourishment is called **vegetative mycelium** . In the life cycle, the vegetative mycelium may give rise to a large organized reproductive structure called **fruit body**, which bears the reproductive cells or **spores** and is produced solely for the release of spores.

In taxonomic terms, moulds are present in all five divisions of Eumycota (Eu=true; mycota=fungus): Mastigomycotina (e.g. *Phytophtora* , *Achlya*), Zygomycotina (e.g. *Rhizopus*, *Mucur*), Ascomycotina (e.g. some species of *Aspergillus*, *Neurospora*), Basidiomycotina (e.g. *Agaricus*, *Pleurotus*) and Deuteromycotina (e.g. *Fusarium*, *Trichoderma*).

Filamentous fungi or moulds are vital for the maintenance of ecosystems. By breaking down dead organic material, they continue the cycle of nutrients through ecosystems. Some of them act as plant pathogens causing severe crop losses from disease and post-harvest food spoilage. In the reagent industry and medicine areas, filamentous fungi are the source of commercial enzymes, organic acids, and numerous drugs such as antibiotics (e.g. penicillin, cefalosporin). Among filamentous fungi are highly appreciated edible fungi such as *Agaricus bisporus*, the popular cultivated mushroom; *Pleurotus spp.*, the "oyster mushroom", *Tuber spp.*, "truffles", and *Morchella spp.*, "Morels", among others. Thus, in many areas, the industrial production of genetically engineered fungi has tremendous potential.

IP aspects

The selected patent and patent applications presented in this section are directed to transformation of moulds or filamentous fungi with *Agrobacterium*.

There are two institutions that have filed patent applications related to *Agrobacterium*-mediated transformation of filamentous fungi: **Unilever N.V.**, in The Netherlands, has a granted United States patent and several applications around the world, and **The Penn State Research Foundation** , in the United States, has a PCT and a United States patent application.

Unilever's invention is directed to a transformed mould with *A. tumefaciens* having a vector containing an expressible gene between T-DNA borders. Although the **Penn State** 's invention also refers to the transformation of a mould, which the inventors called filamentous fungi, they limit the invention to a particular tissue to be transformed: the **fruit body tissue** of a filamentous fungi. **Unilever** does **not** claim the transformation of a tissue in particular, and their claims are therefore broader in that respect. In addition, some of the claims filed in the **Penn State's** applications are also directed to the transformation of a particular filamentous fungus: *Agaricus bisporus* , the cultivated mushroom.

In conclusion

- There is an overlap between the inventions as both refer to transformation of **any filamentous fungi or mould** with *Agrobacterium*. However, the invention disclosed by **Penn State Research Foundation** is more defined as encompasses a particular fungi tissue to be transformed.
- It remains to be seen whether the claims as filed in the applications by **Penn State Research Foundation** are granted as filed. **Unilever**'s United States patent is fairly broad, however, and may pose freedom to operate problems if the species *A. tumefaciens* is used for transforming **any** mould.

Granted patent and patent application filed by Unilever Patent Holdings B.V.

Unilever discloses in its applications a process for producing a transformed mould with *A. tumefaciens*. In the method, an *A. tumefaciens* vector containing at least one expressible gene is introduced into the mould.

Moulds are defined by the inventors to include fungi from all five subdivisions of the division Eumycota. The examples in the disclosures include fungi from the genera *Aspergillus*, *Fusarium*, *Trichoderma*, *Neurospora* and *Colletotrichum*. The first three genera contain species important in large scale fermentation and production of homologous and heterologous proteins. Other species within these genera are fungal

pathogens andfungi that serve as important model organisms for basic research.

Specific Patent Information

Patent Number	Title, Independent Claims and Summary of Claims	Assignee
US 6 255 115 • Filed - 7 October 1999 • Granted - 3 July 2001	**Title** - *Agrobacterium*-mediated transformation of moulds, in particular those belonging to the genus *Aspergillus* **Claim 1** A process for producing a transformed mould, comprising: A) inserting a DNA fragment containing at least one expressible gene to be introduced into a mould into a vector of *Agrobacterium tumefaciens* between the T-DNA borders present in that vector; B) introducing the vector containing the DNA fragment between the T-DNA borders into an *Agrobacterium tumefaciens* strain containing a *vir* region in its DNA; C) inducing *vir* genes to release T-DNA containing said DNA fragment from said *Agrobacterium tumefaciens*, and incubating the *Agrobacterium tumefaciens* strain with the mould to be transformed; and D) selecting the transformed mould from the untransformed mould depending on the characteristics of the introduced DNA or its expression product, and optionally culturing the transformed mould. The United States patent claims • a method for producing a transgenic mould by inserting into it at least an expressible gene carried in an *A. tumefaciens* vector. *A. tumefaciens* containing the vector and mould are co-cultivated and the transformed mould is selected. Note that although the title of the patent refers to the genus *Aspergillus*, the independent claims are **not** limited to this genus or any other genera of filamentous fungi.	Unilever
EP 973 917 A1 • Filed - 24 March 1998	**Title** - *Agrobacterium*-mediated transformation of moulds, in particular those belonging to the genus *Aspergillus* **Claim 1** A process for producing a transformed mould, characterized in that: A) a DNA fragment containing at least one expressible gene to be introduced into a mould is first cloned into a vector of *Agrobacterium tumefaciens* between the T-DNA borders present in that vector; B) the vector containing the DNA fragment between the T-DNA borders is introduced into an *Agrobacterium tumefaciens* strain containing a *vir* region in its DNA; C) release of T-DNA containing said DNA fragment from said *Agrobacterium tumefaciens* by addition of a *vir*-inducing compound, and the *Agrobacterium tumefaciens* strain is incubated with the mould to be transformed; and D) the transformed mould is selected from the untransformed mould depending on the characteristics of the introduced DNA or its expression product, and optionally the transformed mould is cultured. The claims as filed in the European application are directed to the same subject matter as the granted claims of the United States patent. * As the independent claims of the United States patent and the European patent application are worded slightly different, the claims are presented independently. However, the claims have practically the same scope.	

		Patent Holdings B.V.
Remarks	Related applications also filed in Brazil (BR 9807941 A), Canada (CA 2286307), China (CN 1259167 T), Japan (JP 2001518786 T2) and South Africa (ZA 9802905 A).	

Patent applications filed by The Penn State Research Foundation

The present PCT and United States patent applications relate to the transformation of the **fruit body tissue** of a filamentous fungi with *Agrobacterium*. The genetically modified fungi can serve as biofermentators for the mass production of commercial products. As examples of filamentous fungi, the applicant mentions fungi belonging to phyla Ascomycota and Basidiomycota such as *Coprinus*, *Agaricus*, *Morchella* , and *Coriolus*, among others. In particular, the invention contemplates the transformation of cultivated mushroom *Agaricus bisporus,* which accounts for 38% of the world production of cultivated mushrooms.

Specific Patent Information

Patent Number	Specific Patent Information	Assignee
WO 0200896 A2 • Filed – 28 June 2001	**Title** – Methods and compositions for highly effective transformation of filamentous fungi **Claim 1** A method of transforming filamentous fungi comprising: introducing to a fruit body tissue cell of said fungi a polynucleotide construct said construct comprising sequences for the expression of a structural gene or for the inhibition of an endogenous gene, the presence of which is desired in said fungi cell. **Claim 16** A filamentous fungi transformation method comprising: A) obtaining an *Agrobacterium* vector, said vector comprising a polynucleotide sequence the presence of which is desired in a recipient filamentous fungi cell; and B) introducing said vector to fruit body tissue cells of said fungi in the presence of an active *vir Agrobacterium* region and a *vir* inducing compound. **Claim 26** A method for transforming a fungi cell comprising: A) obtaining an *Agrobacterium*-derived Ti plasmid, said plasmid comprising a polynucleotide sequence the expression of which is desired in a host fungal cell; B) introducing said plasmid to said cell in the presence of *vir* active genes and a *vir* inducing agent. **Claim 30** A method for transformation of *Agaricus bisporus* cells comprising: A) obtaining an *Agrobacterium* vector, said vector comprising a polynucleotide sequence the presence of which is desired in a recipient filamentous fungi cell; and B) introducing said vector to fruit body tissue cells of said *Agaricus bisporus* cells in the presence of an active *vir Agrobacterium* region and a *vir* inducing compound.	Penn State Research Foundation

	The claims as filed in the PCT and United States application recite: • a method for transforming **any** filamentous fungi by introducing into the **fruit body tissue** a construct for the expression of a structural gene or for the inhibition of an endogenous gene; and • a method for transforming the **fruit body tissue** of **any** filamentous fungi and *Agaricus bisporus* in particular with an *Agrobacterium* vector in the presence of a *vir* acting region and a *vir* inducing compound. Although these claims are fairly broad as they refer to the transformation of **any** filamentous fungi **without** specifying a method in one case and specifying *Agrobacterium* in another claim, an apparent limitation lies in the type of fungal tissue to be transformed. The invention recites **only** transformation of **fruit body tissue**. It remains to be seen whether granted claims will have the same breadth as the filed claims.	
Remarks	US 20020016982 A1 is the United States application, with the same claims as the PCT application above.	

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