Methods of Gene Transfer

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National Coordinator: Prof. S.C. Bhatla

Lesson:Methods of Gene Transfer

Lesson Developer: Dr Neetu Chaudhary[1], Dr. Parul Agarwal[2]

College: [1]Acharya Narender Dev College,[2]Department of Genetics, University of Delhi, South Campus

Lesson Reviewer: Prof. Suresh Chand, Professor and Head, School of Life Sciences, Devi Ahilya Vishwavidyalaya, Indore

Language Editor: Namrata Dhaka

Department/College: Department of Genetics, University of Delhi, South Campus

Lesson Editor: Dr Rama Sisodia, Fellow in Botany ILLL

Institute of Lifelong Learning, University of Delhi

Chapter: Methods of Gene Transfer

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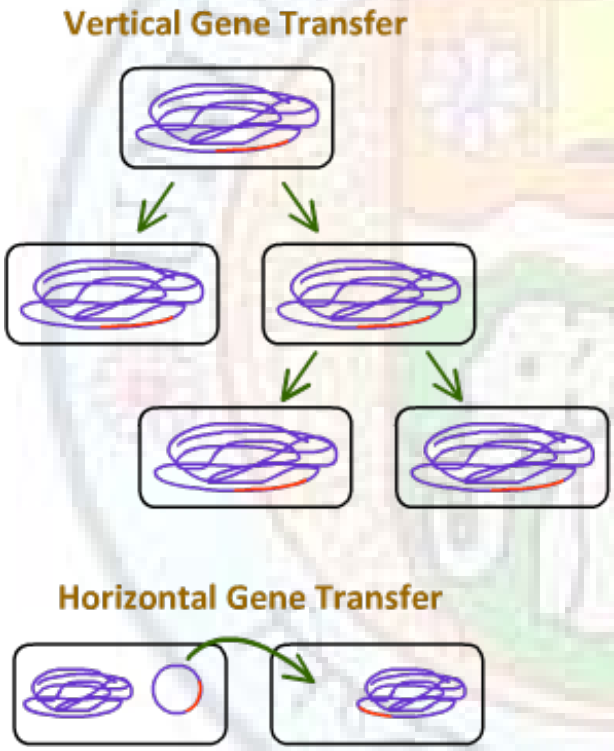
Introduction

Foreign genes are introducedartificially into crops by overcoming the fertility barriers.This process, also known asgenetic transformation,is a very important step in genetic engineering.

Horizontal vs vertical gene transfer

The natural transfer of genetic material from one organism to another is referred to as horizontal gene transfer or the lateral gene transfer. The foreign DNA is either randomly inserted into the host genome or recombines if there is sequence homology between the two genomes. This is different from the vertical gene transfer where the genetic material is transferred from the parents to the offsprings, through sexual reproduction.



Horizontal gene transfer is facilitated by various mechanisms. In prokaryotes mainly transformation (intake of genetic material from surrounding), conjugation (exchange of genetic material with the physical union of two cells) and transduction (transmission of DNA through bacteriophages from one cell to another) are responsible for the transfer of the gene within organisms. In eukaryotes, the presence of the outer cell membrane and the nuclear membrane makes transfer of DNA difficult between organisms. Horizontal gene transfer plays important role in evolution of both prokaryotes and eukaryotes.

Methods of Gene Transfer

Video: Horizontal vs Vertical gene transfer https://www.youtube.com/watch?v=EHTEFdSadXM

Plant transformation systems generally include following steps,

- Introduction of a DNA segment into totipotent cells.
- Its integration into host cells genome.
- Subsequent regeneration from transformed cellto produce whole plant.

Plant transformation methods therefore require an efficient wayto introduce DNA into cell and the regeneration of the transformed cells or tissues into whole plants.

TheDNA segment which is introduced in this process contains the gene of interestanda cassette containing additional genetic material. Additional genetic material includes

- A promoter which determines the site and timing of expression of the introduced gene
- A terminator to identify the end of transcription and
- Amarker gene which allows selection of plants having the introduced gene.

Various desirable traits have been efficiently introduced and stably expressed in almost 150 plant species.

Different methods are available to achieve genetic transformation of plants i.e. the delivery of the foreign DNA into the host plant. These are divided into two main groups

- Indirect methods: In this case vector is needed for insertion of the foreign DNA into the host genome.
- Direct methods: This method is vector independent. The DNA is directly inserted into the host genome.

Methods of Gene Transfer

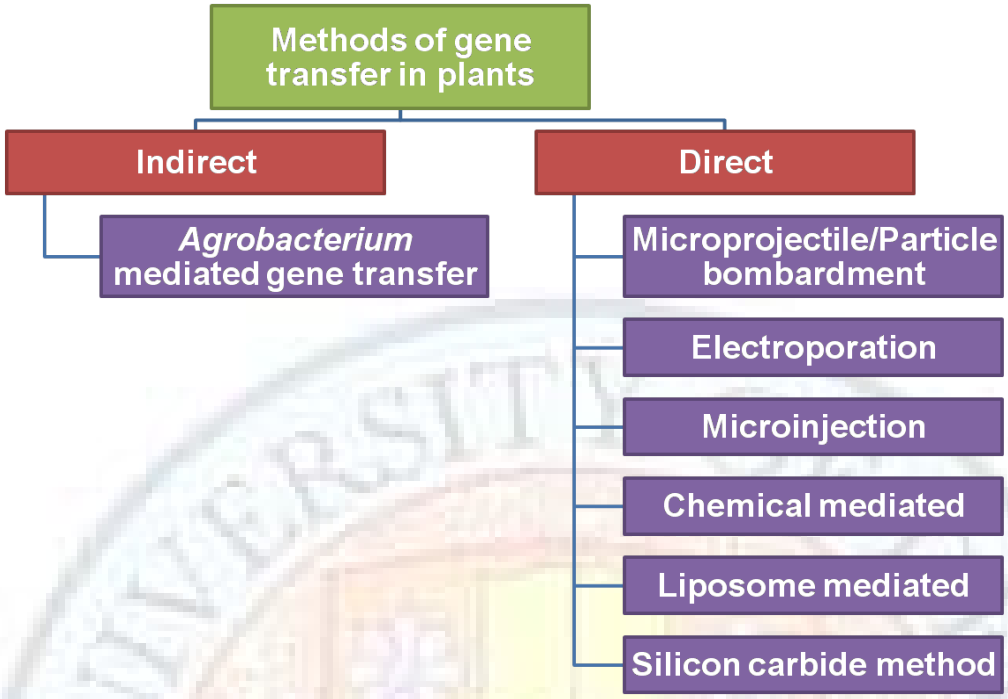


Figure: Types of methods for gene transfer in plants

Source: Namrata Dhaka, Research Scholar, Department of Genetics, University of Delhi, South Campus.

In-direct Methods

Agrobacterium-mediated genetic transformation

The method of genetictransformation, which employs bacteria as a vector to introduce the gene construct into the target cell,isknown as indirect method. Thismethod uses *Agrobacterium* (a gram-negative soil bacteria which causes crown gall disease in many plants) for the plant transformation experiments.

The most commonly studied species of this genus is *Agrobacterium tumefaciens,* which forms an efficient delivery system for genetic transformationin plants.These bacteria harbor a large plasmid called Ti plasmid (tumour inducing) having tumor-inducing genes (T-DNA) and other genes involved in integration of T-DNA into host genome.Wounded plants secrete a sap with high content of phenolic compounds which serve as chemical attractants for *Agrobacteria* and stimulateexpression of *vir*genes. It results ininfection of plantby *Agrobacterium*, insertion of T-DNA region at a random site inhost genome and proliferation of plant cells to form crown gall growth.

Another commonly used species is *Agrobacterium rhizogens*, which induces hairy roots in plants.It contains Ri plasmid (root inducing plasmid). The genus *Agrobacterium* has a wide host range and can infect a number of dicots and some monocots.

Figure: Crown gall disease caused by *Agrobacterium* on rose stem.

Source:http://www.bioimages.org.uk/html/p5/p56560.php (CC)

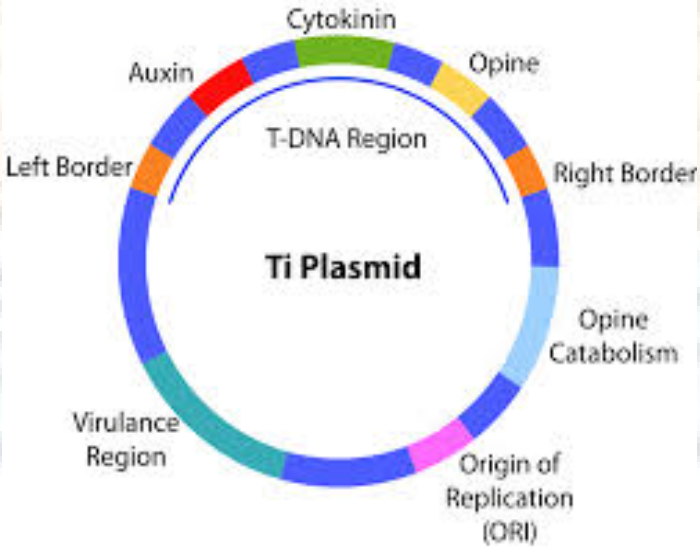


Figure: Ti plasmid with T-DNA region.

Source: http://en.wikipedia.org/wiki/Transfer_DNA (CC)

Methods of Gene Transfer

Structure of Ti Plasmid

In transformation experiments, vector is the genetic vehicle needed to transport the gene of interest, promoter, terminator and selectable marker genes to DNA of host plant. Virulence of *Agrobacterium* is conferred by Ti plasmid having genes for tumor-induction, T-DNA integration and synthesis of plant hormones and opines.

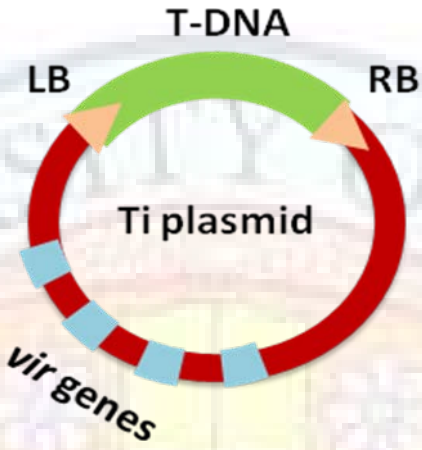


Figure: An illustration showing the important regions of a Ti plasmid.
Source: Namrata Dhaka, Department of Genetics, University of Delhi, South Campus.

Link for animation-
http://highered.mheducation.com/olcweb/cgi/pluginpop.cgi?it=swf::535::535::/sites/dl/free/0072437316/120078/bio40.swf::The+Ti+Plasmid

- **Origin or replication**

  This region is responsible for the replication of Ti plasmid independent of the bacterium cell.

- **Virulence region**

  This region contains genes called *vir* genes whose products enable processing and transfer of the T-DNA from bacterium to plant cells. Their expression is triggered by certain phenolic compounds like acetosyringone, which are secreted by plants in response to wounding.

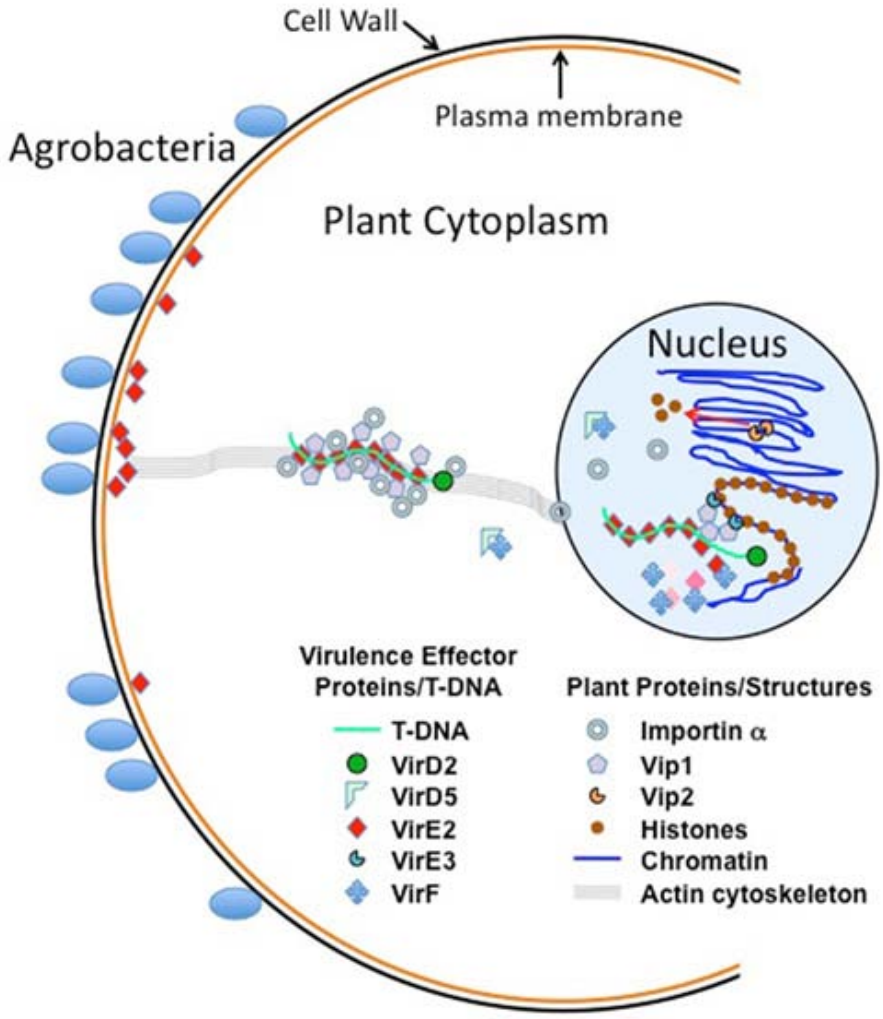


Figure: Figure showing a model of how different vir proteins interact with T-DNA to mediate its transfer from the bacterium to the host cell.

Source: Gelvin, Stanton B. "Traversing the cell: Agrobacterium T-DNA's journey to the host genome." *Frontiers in plant science* 3 (2012).

http://journal.frontiersin.org/Journal/10.3389/fpls.2012.00052/full

(cc)

- **T-DNA region**

It is a region of Ti plamid which contains genes from induction of tumour. It is flanked by 25 bp direct repeat sequences on both sides. These repeats are called as Left border (LB) and Right border (RB). The different genes present in this region are –

iaaM and iaaH genes - responsible for synthesis of indole acetic acid (an auxin),

ipt gene - responsible for synthesis of an enzyme isopentenyl adenine (a cytokinin)

tml gene – another gene involved in formation of tumours.

Opine biosynthesis genes–lead to synthesis of opines.

All these genes result in overgrowth of tissue in the plant cells leading to tumour formation.

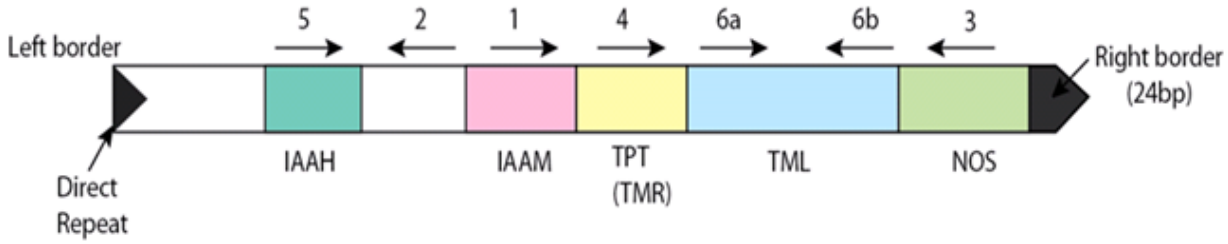


Figure: Arrangements of different genes in T-DNA region. Arrows represent the direction of transcription.

Source: http://nptel.ac.in/courses/102103016/25

(cc)

- **Region of opine catabolism**

It contains several other genes involved in metabolism of opines. This region of the plasmid is not transferred to the plant cells during infection.

For further information, read -
http://www.plantphysiol.org/content/107/4/1041.full.pdf

Use of Ti plasmid in genetic transformation

For its use in genetic transformation as a vector, most of the T-DNA region of bacterial plasmid is replaced with the gene of interest while leaving the left and right border sequences. The T-DNA region is defined not by its sequence but by its borderswhich enables itsinsertion into host plant genome.

Methods of Gene Transfer

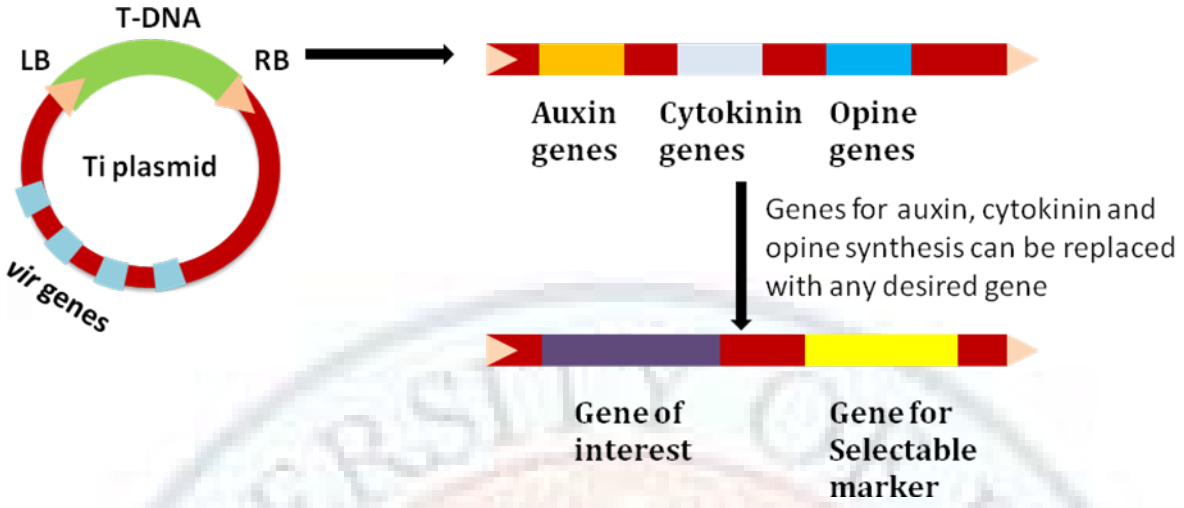


Figure: An illustration showing that the genes of Ti plasmid can be replaced with any gene of interest and selectable marker gene for selection of the transformed cells.

Source: Namrata Dhaka, Department of Genetics, University of Delhi, South Campus.

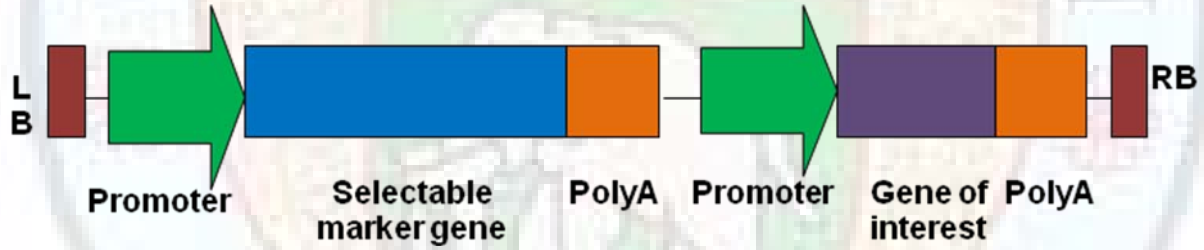


Figure: An illustration showing arrangement of different motifs in a cassette carrying gene of interest, used for transformation.LB: Left Border; RB: Right Border

Source: Namrata Dhaka, Department of Genetics, University of Delhi, South Campus.

Wild type Ti plasmid from *Agrobacterium* cannot be directly used as vector system due to their large size, presence of tumor causing genes and absence of marker genes and unique restriction sites within T-DNA region. Therefore they are genetically engineered to incorporate these sequences.There are two types of genetically engineered Ti plasmid based vectors.

1. **Binary vectors**

In this system, there is a pair of plasmids, which are independent of each other and present in the same *Agrobacterium* cell. This vector system is based on the fact that *vir* genes need not be present in the same plasmid with T-DNA for its transfer.

- **Helper Ti plasmid**

  The *vir* regionrequired for T-DNA integrationis present in this plasmid.

- **A disarmed Ti plasmid (Mini Ti plasmid)**

  T-DNA region having the gene of interest and plant selectable marker gene with promoter and terminator sequences + *ori* for both *Agrobacterium* and *E. coli* are present in a separate disarmed Ti plasmid.

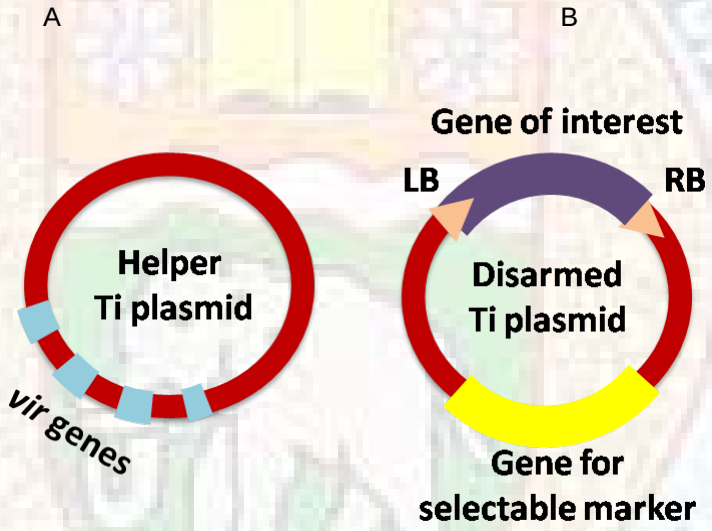


Figure:A binary vector system (A) A helper plasmid containing *vir* region but no T-DNA, (b) Another plasmid containinggene of interest within the border sequences of T-DNA and selectable marker gene but no *vir*region.
Source: Namrata Dhaka, Department of Genetics, University of Delhi, South Campus.

2. **Co-integrate vectors**

These vectors are also known as hybrid Ti plasmids. In these vectors both T-DNA region (having gene of interest) and *vir* genes are present in the same plasmid used for transformation.

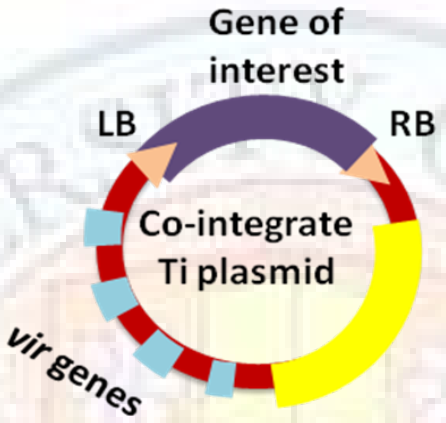


Figure: Co-integrate vectors contain vir genes as well as T-DNA region.

Source: Namrata Dhaka, Department of Genetics, University of Delhi, South Campus.

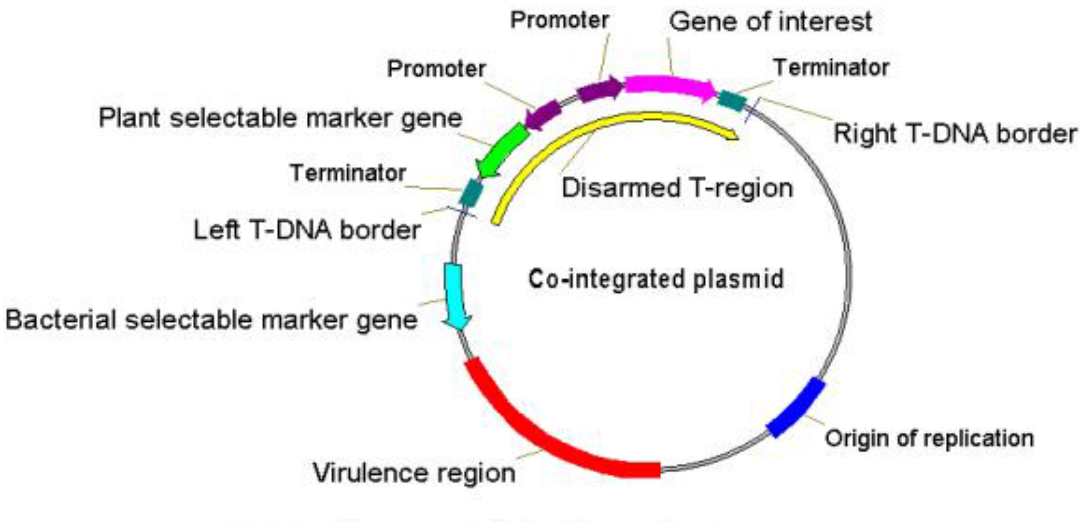


Figure: Vector map of co-integrated plasmid

Source: http://www.patentlens.net/daisy/AgroTran/g1/850.html (CC)

Methods of Gene Transfer

Steps involved in*Agrobacterium*-mediated genetic transformation of plants by 'Wounded explant' method

- Gene of interest isisolated/amplified from a source organism.
- An expression cassetteis developed including gene of interest flanked by promoter and terminator sequences to drive expression and marker genes to facilitate selection of transformed plants by tracking introduced genes in the host plant.
- Insertion of cassette into T-DNA region of the binary vector/co-integrate vector is carried out.
- Transformation of the above expression vector into *Agrobacterium*is done.
- Explants are obtained from plant that is to be transformed. They are wounded and then co-cultured for a brief period for *Agrobacterium* infection. This is done using tissue culture methods.
- The transformed explants are then grown in presence of a bacteriostatic agent to prevent the growth of Agrobacterium and in presence of a selective antibiotic which prevents survival of any untransformed explants.
- Positive transformants are tested by PCR analysis to check for the presence of desired gene.
- The transformed explants are maintained in vitro until they are ready for transplantation. After the transformed plants are obtained, DNA is isolated and Southern hybridization is carried out to confirm the site of integration and the copy number of the desired gene.

Methods of Gene Transfer

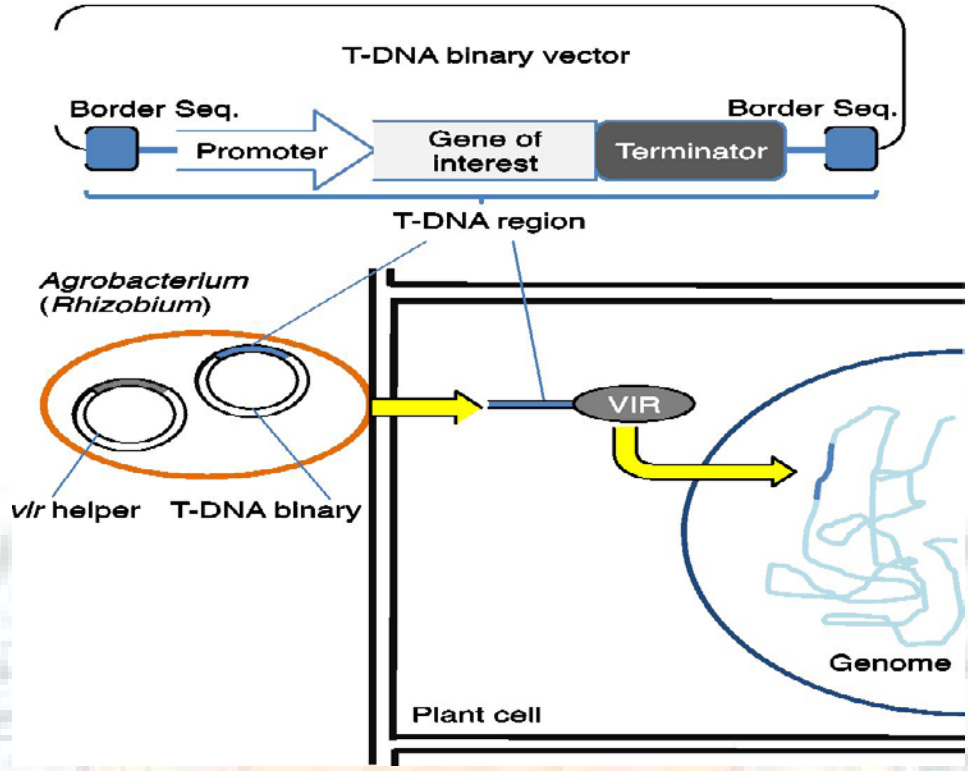


Figure: Figure showing how a desired gene is inserted into Ti plasmid, which is then used to transfer it to a plant.

Source:Narusaka, Yoshihiro, et al. "Methods to Transfer Foreign Genes to Plants." *IN: Transgenic Plants–Advances and Limitations, Yelda Ozden Çiftçi (Ed.), ISBN* (2012): 978-953.

http://www.intechopen.com/books/transgenic-plants-advances-and-limitations/methods-to-transfer-foreign-genes-to-plants (cc)

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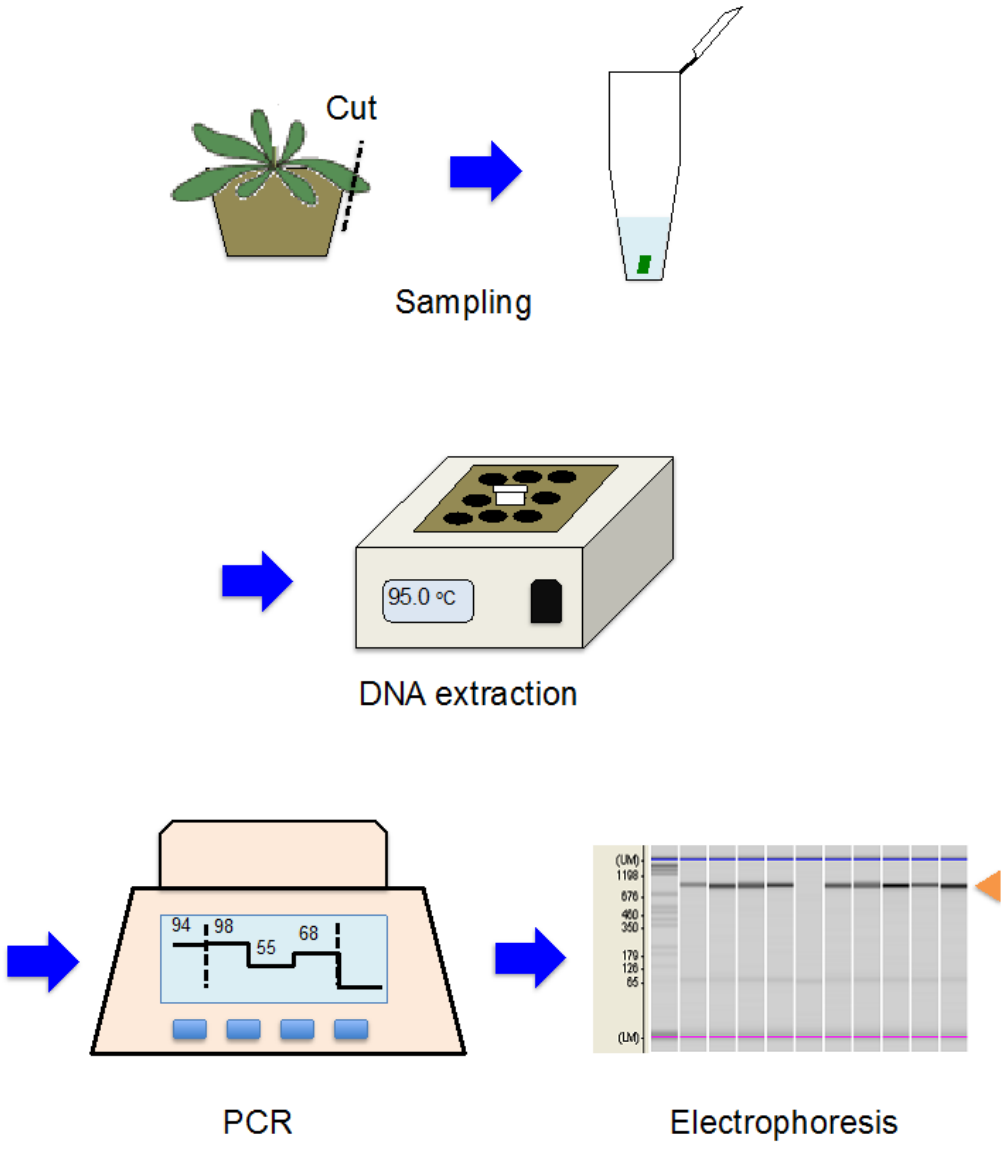

Figure: Steps in screening of tranformants by PCR analysis

Source:Narusaka, Yoshihiro, et al. "Methods to Transfer Foreign Genes to Plants." *IN: Transgenic Plants–Advances and Limitations, Yelda Ozden Çiftçi (Ed.), ISBN* (2012): 978-953.

http://www.intechopen.com/books/transgenic-plants-advances-and-limitations/methods-to-transfer-foreign-genes-to-plants (cc)

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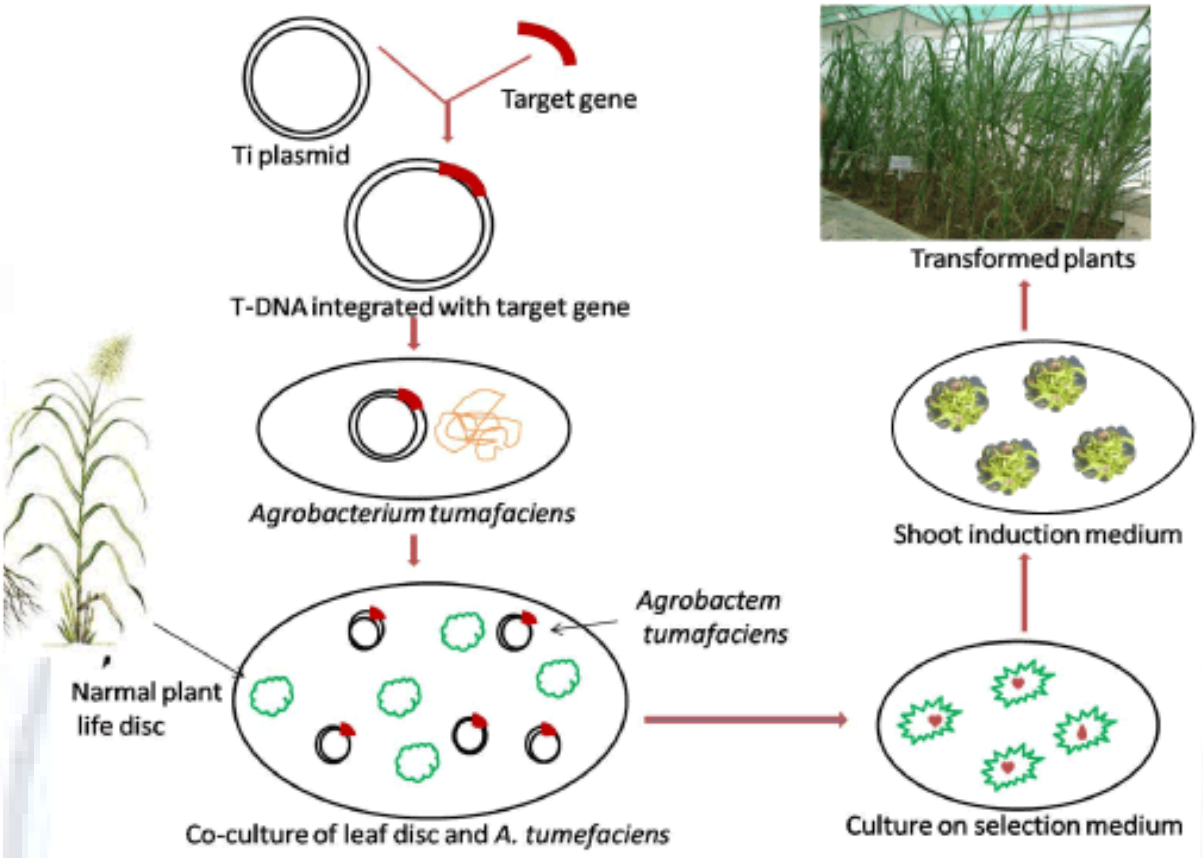

Figure: Diagram illustrating *Agrobacterium*-mediated transformation of sugarcane.

http://omicsgroup.org/journals/current-status-of-sugarcane-transgenic-an-overview-2169-0111.1000112.php?aid=18824 (CC)

For further details visit: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC150518/

Direct Methods

Direct methods are those methods which do not use bacteria as mediators for integration of DNA into host genome. These methods include microprojectile bombardment,electroporation and microinjection.

Microprojectile / particle Bombardment (biolistics)

Methods of Gene Transfer

Biolistics is a method where cells are physically impregnated with nucleic acids or other biological molecules.Abiolistic particle delivery system is a device for plant transformation where cells are bombarded with heavy metal particles coated with DNA/RNA. This technique was invented by John Stanford in 1984 for introduction of DNA into cells by physical means to avoid the host-range restrictions of *Agrobacterium.Agrobacterium*-mediated genetic transformation system works well for dicotyledonous plants but has low efficiency for monocots.Biolistic particle delivery system provides an effective and versatile way to transform almost all type of cells. It has been proven to be a successful alternative for creating transgenic organisms inprokaryotes, mammalian and plant species.

Figure: A biolistic microprojectile gun.

Source: http://en.wikipedia.org/wiki/Gene_gun (cc)

In this process, construct having gene of interest is coated on the surface oftiny particles of gold or tungsten (0.6 – 1 mm in size). Prior to coating, DNA is precipitated with calcium chloride, spermidine and polyethylene glycol.These coated microparticles are loaded on to the macrocarrier and accelerated to high speed by using pressurized helium gas.Plant cell suspensions, callus cultures, or tissues could be used as the target of thesemicroprojectiles. As the microprojectiles penetrate the plant cell walls and membranes to enter the cells, coated DNAis released from its surface and incorporated into the plant's genome.In biolistics, use of binary vectors with T-DNA border sequences is not required.

Methods of Gene Transfer

This method is especially importantformonocots, for which efficiency of othertransformation methods is not satisfactory. A wide range of tissues such as apical and floral meristems, embryos, seedlings, leaves, cultured cells and floral tissues could be used as target in this method.

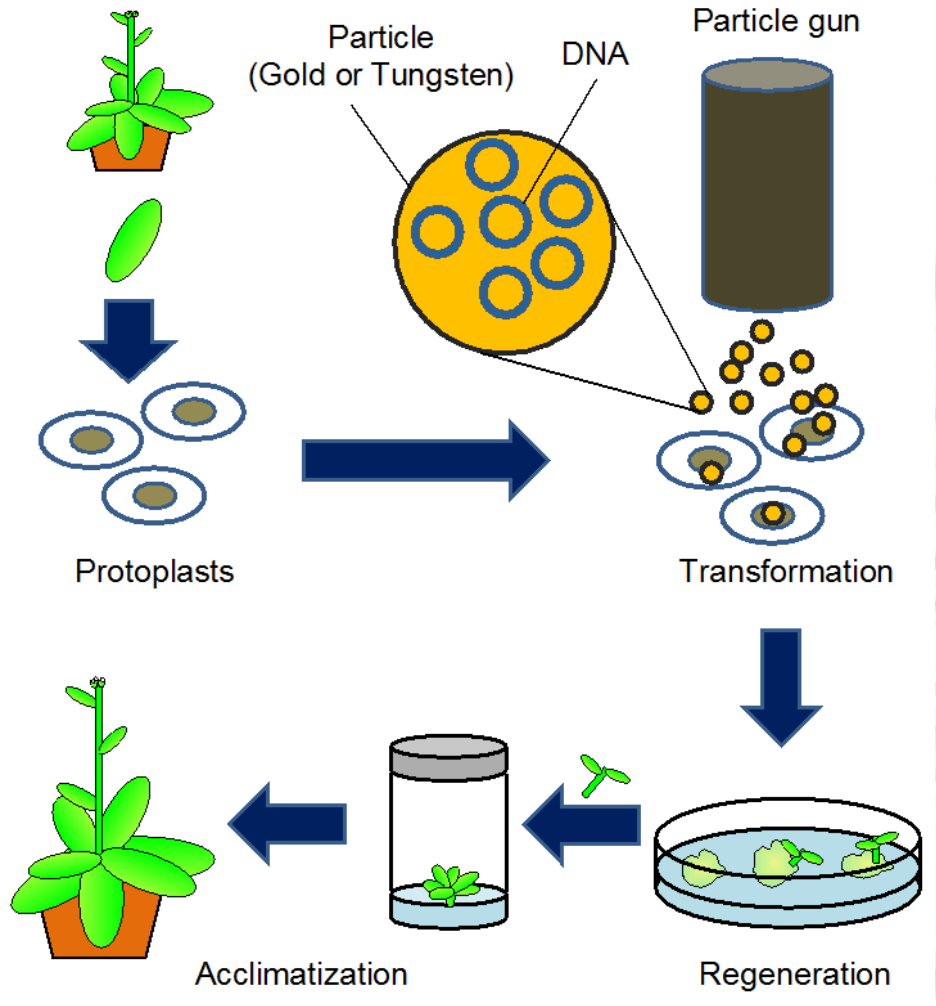

Figure: Particle bombardment method of Plant transformation (1) Isolation of protoplasts. (2)Injection of DNA-coated particles using particle gun. (3) Regeneration of transformed protoplasts into plantlets. (4) Acclimatizationof regenerated plantlets in a greenhouse.

Source: Narusaka, Yoshihiro, et al. "Methods to Transfer Foreign Genes to Plants." *IN: Transgenic Plants–Advances and Limitations, Yelda Ozden Çiftçi (Ed.), ISBN* (2012): 978-953.

http://www.intechopen.com/books/transgenic-plants-advances-and-limitations/methods-to-transfer-foreign-genes-to-plants (cc)

Methods of Gene Transfer

A number of parameters should be carefully considered before using particle bombardment. These can be classified under three categories:

- **Physical parameters**
  Nature, chemical and physical properties of the metal particles utilized to carry the foreign DNA. The nature and preparation of DNA,binding of DNA on the particles and target tissues.

- **Environmental parameters**
  Variables such as temperature, photoperiod and humidity of donor plants, explants, and bombarded tissues affect physiology of tissues and influence receptiveness of the target tissue.

- **Biological parameters**
  Choice and nature of explants, pre- and post bombardment culture conditions, osmotic pre- and post-treatment of explants.

Advantages of particle bombardment over Agrobacterium-mediated DNA transfer:

- This system is species independent and can been used successfully for a wide range of organisms.
- Many species which are recalcitrant to other direct transfer methods or are not readily amenable to Agrobacterium-mediated transformation have been transformed by this technique.
- Introduced DNA does not need sequences necessary for T-DNA replication and transfer as complex interaction between bacterium and plant tissue does not take place.
- Transformation of organelle DNA (mitochondria and chloroplasts) has also been achieved by this method.
- Multiple genes can be introduced in a single plant.
- Particles can be coated with DNA/RNA/siRNA/large fragments of nucleic acids.

Limitations of particle bombardment method :

- Limited regeneration capacity of tissue being bombarded
- Efficiency of stable integration of DNA.
- Insertion of multiple copies of the gene
- Integration of rearranged and/or truncated DNA sequences

- Damage to the cellular tissue.
- Specialized and expensive equipments are required

Electroporation

Electoporation is a method of transformation via direct gene transfer. In this technique mixture containing cells and DNA is exposed to very high voltage electrical pulses (4000 – 8000 V/cm) for very brief time periods (few milliseconds). It results in formation of transient pores in the plasma membrane, thorough which DNA seems to enter inside the cell and then nucleus.

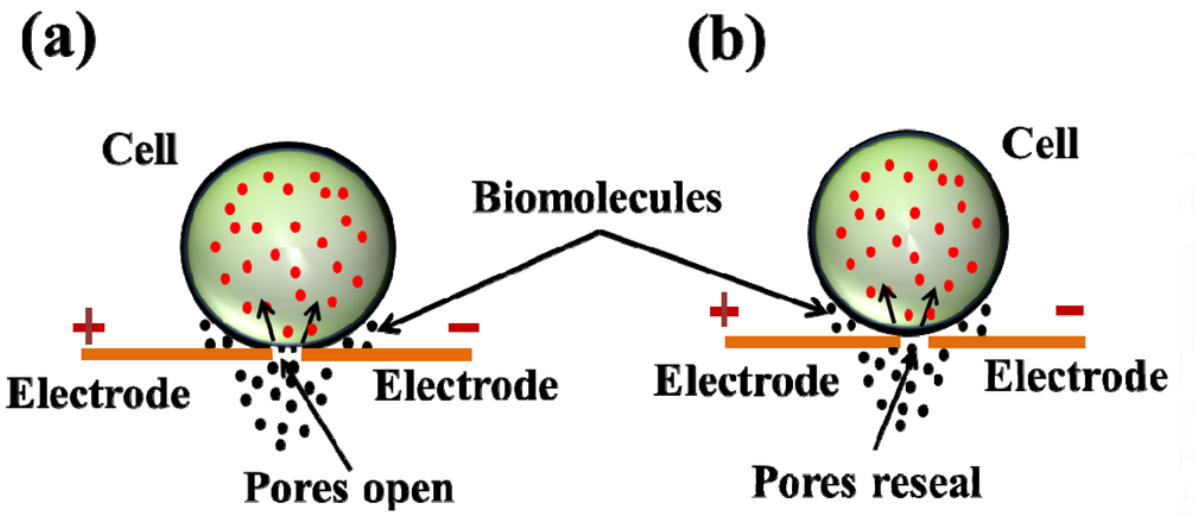

Figure: Electroporation (A) Diagram showing formation of transient pores in cell membrane on applying electrical pulse, entry of DNA inside thecell and sealing of pores afterwards.

Source: http://www.mdpi.com/2072-666X/4/3/333/htm
Santra, Tuhin Subhra, and Fang Gang Tseng. "Recent trends on micro/nanofluidic single cell electroporation." *Micromachines* 4.3 (2013): 333-356. (cc)

A suspension of cells with plasmid DNAis taken in an electroporation cuvette placed between electrodesand electrical pulses are applied.Temporary micropores are formed in cell membranes whichallow cells to take up plasmid DNA leading to stable or transient DNA expression.

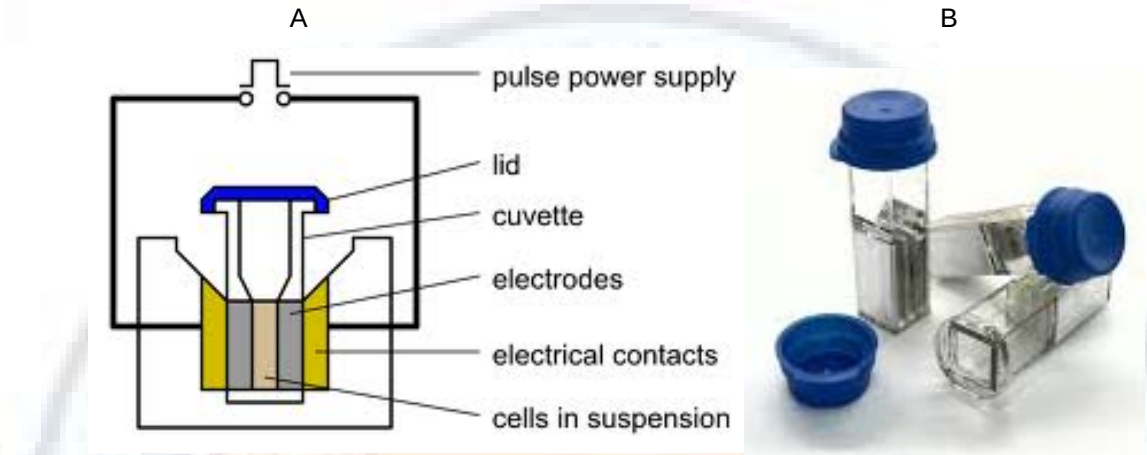

Figure:(A) Main components of an electroporator. (B) Cuvettes used for electroporation. These are plastic cuvettes with lid and aluminium electrodes, having a maximum capacity of 400 µl.

Source:

http://commons.wikimedia.org/wiki/File:Electroporation_Diagram.png (cc)

http://commons.wikimedia.org/wiki/File:Electroporation_Cuvettes.jpg (cc)

Cells which are arrested at metaphase stage of cell cycle are especially suitable for electroporation as these cells have absence of nuclear envelope and an unusual permeability of the plasma membrane. Protoplasts are used for electroporation of plant cells as thick plant cell walls restrict movement of DNA. The electroporation method was originally developed for protoplasts, but has given equally good results with cells and even tissues with easy recovery of regenerated plantlets. Immature zygotic embryos and embryogenic calli have also been used for electroporation to produce transgenic maize.

Transformation of protoplast is associated with low transient expression of transgenes as compared to organized tissues and low regeneration frequencyespecially in monocotyledonous plants. The electrical field and chemicalsubstancesapplied to disorganize cell walls strongly reducethe viability and capability of divisionof protoplasts.

Electoporation as a transformation method is fast, convenient, simple, and inexpensive and has low cell toxicity. The disadvantage associated with this technique is difficulty in regenerating plants from protoplasts, if protoplast is used for electroporation.

Microinjection

The process of using a fine glass micropipette to manually inject transgene at microscopic or borderline macroscopic level is known as microinjection.The transgene, in the form of plasmids, cosmids, phage, YACs, or PCR products, can be circular or linear and need not be physically linked for injection.

Microinjection involves direct mechanical introduction of DNA intothe nucleus or cytoplasm using a glass microcapillary injectionpipette. The protoplasts are immobilized in low melting agar, while working under a microscope, using a holding pipette and suction force. DNA is then directly injected into the cytoplasm or the nucleus. Theinjected cells are then cultured *invitro* and regenerated into plants. Successful examples of this process has been shown in rapeseed, tobacco and various other plants.

Stable transformants can be achieved through this method but it requires technical expertise and is atime consuming process. Also,microinjection has achieved only limited success in plant transformationdue to the thick cell walls of plants and a lack of availability of a single-cell-to-plant regenerationsystem in most plant species.

In this technique a traditional compound microscope (around 200X magnification) or aninverted microscope (around 200x magnification) or a dissecting stereomicroscope (around 40-50x)is used.Under the microscope target cell is positioned and cell membrane and nuclear envelope are penetrated with the help of two micromanipulators.One micromanipulator holds the pipette and another holds the microcapillary needle.

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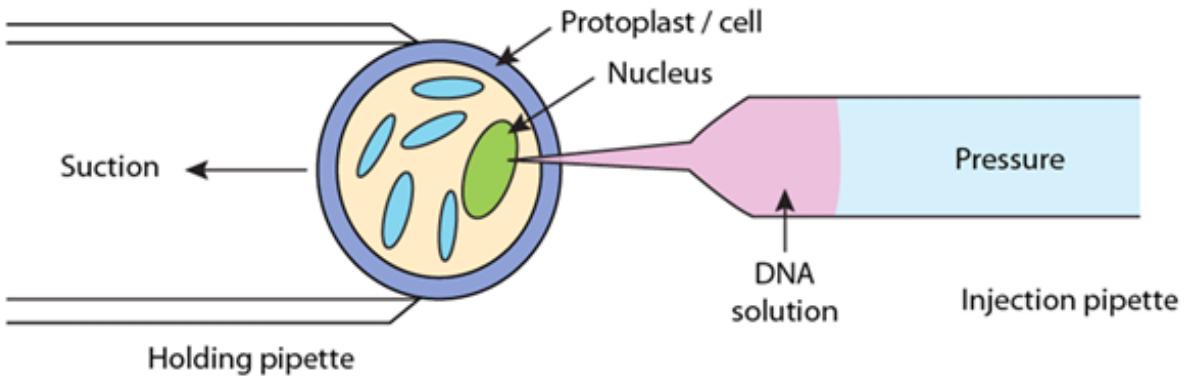


Figure: Illustration of microprojectile method

Source: http://nptel.ac.in/courses/102103016/module3/lec24/3.html (cc)

There are two types of microinjection systems; constant flow system and pulsed flow system.

- Inthe constant flow system the amount of sample injected is determined by the duration for which needle remains in the cell.The constant flow system is relatively simple and inexpensive but outdated.
- The pulsed flow systemhas greater control over thevolume of substance delivered, needle placement and movement and has better precision. This technique results in less damage to the receiving cell, however, the components of this system are quite expensive.

Chemical mediated gene transfer

Cells or protoplasts can be stimulated to take up foreign DNA using some chemicals. Polyethylene glycol (PEG) is the most commonly used chemical for this purpose. It helps in precipitation of DNA, which can then be taken up by the calls through the process of endocytosis.

Liposome mediated gene transfer

Plasmid containing foreign desired gene can be enclosed in small lipid bags called lipososmes, which can then be fused with protoplasts using chemicals like PEG.

Silicon carbide method

In this method, fibres of organic material like silicon carbide are used for gene transfer. These fibres, when mixed with plasmid DNA and plant tissue or cells, help in penetration of the foreign DNA into the plant tissue.

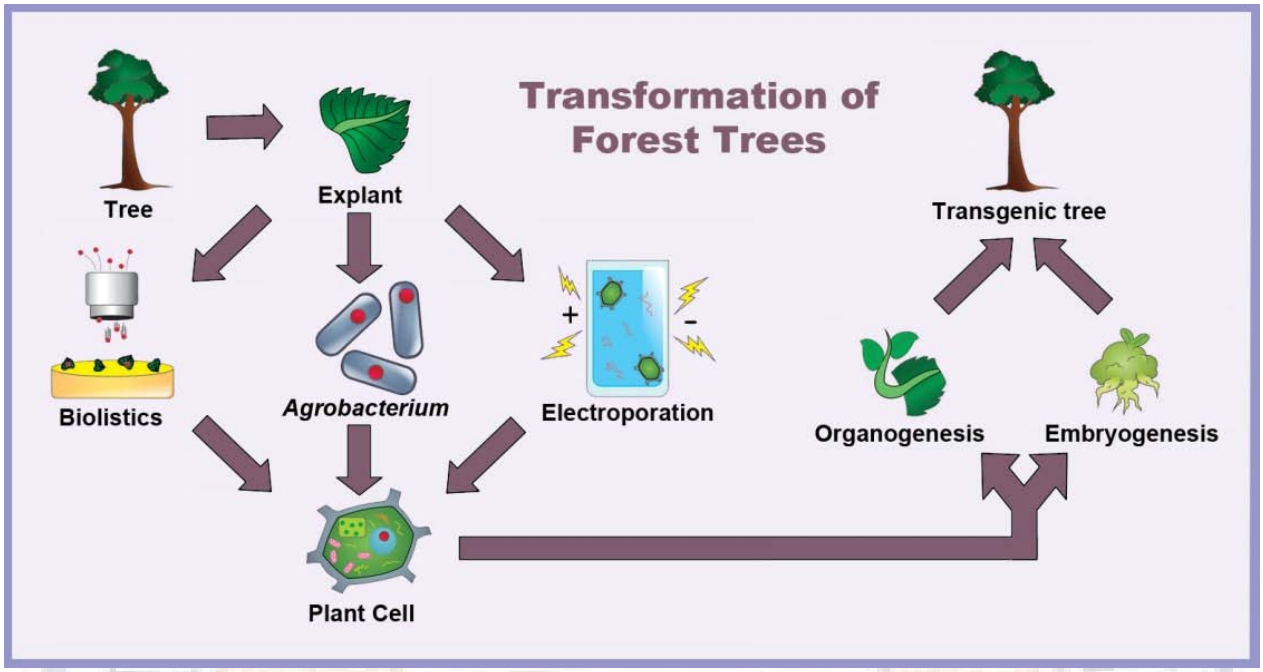


Figure: An illustration showing different techniques used for transformation of tree species.

Source: Castellanos-Hernández, Osvaldo A., et al. "Genetic Transformation of Forest Trees."

http://www.intechopen.com/books/genetic-transformation/genetic-transformation-of-forest-trees

(cc)

Selection of transformants

In a genetic transformation experiment, only one in a several million to billion cells may take up the transgene depending upon the efficiency of transformation. Rather than checking every single cell/organism, a selective agent that kills or gives a different phenotype to all the cells not carrying foreign DNA can be employed. These selective genes are called as marker genes. These genes also help in assessing the success rate of a genetic transformation study.

Marker gene is a gene introduced into cell along with the transgene. It is used to determine if the transgene has been successfully inserted into host organism's genome as marker gene's presence can be seen or detected.There are two types of marker genes:

Methods of Gene Transfer

- Selectable marker
- Screening marker.

Selectable marker

A selectable marker is a gene that confers a trait suitablefor artificial selection as it protects the organism from a selective agent that would normally kill it or prevent its growth.In most of the genetic transformation experiments only one in a several million or billion cells will take up the transgene. In order to find out transformed cell/organism a selective agent is utilized which kills all the cells withouttransgene, leaving only the transformed ones.

Selectable marker genes can be divided into several categories depending on whether they confer positive or negative selection and whether selection is conditional or non-conditional on the presence of external substrates.

Negative selectable marker genes result in death of the untransformed tissue whereas transformed cells are able to survive .e.g., antibiotics, herbicides,

Antibiotics or herbicides are the most common selective agents.When grown on medium containing antibiotic or herbicide the non-recombinants die due to lack of resistance. In bacteria, antibiotics are used almost exclusively. In plant system*nptII*, *hpt II*, and *bar* contribute to production of over 95% transgenic plants. In mammals, resistance to antibiotics that would kill the mitochondria is used as a selectable marker. All the regenerants obtained after a plant transformation experiment are grown on a medium containing an antibiotic, and those plants that can grow have successfully taken up and expressed the introduced gene. Efficiency, biosafety, scientific applications and commercialization issues for almost fifty marker genes used for transgenic and transplastomic plant research have been assessed.

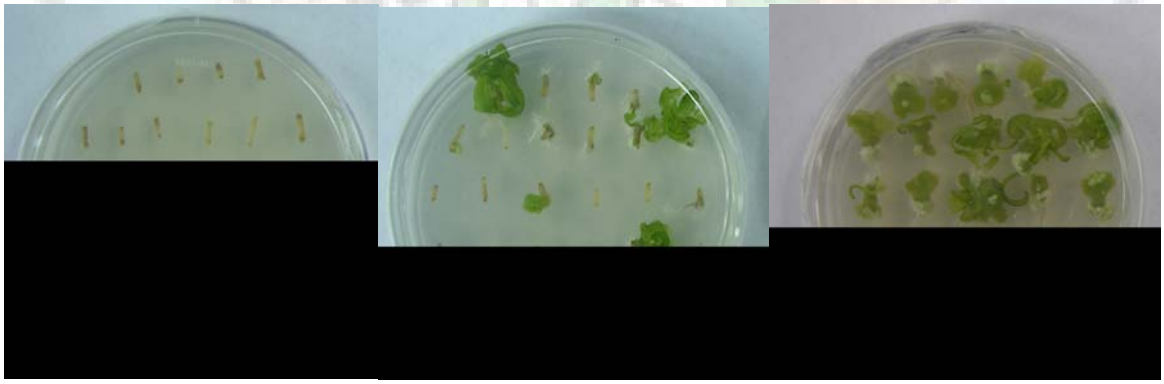
Examples of selectable markers

- *hpt*II gene codes for hygromycin phosphotransferase (HPT) enzyme, which detoxifies the aminocyclitol antibiotic hygromycin B. Hygromycin Bcauses mistranslocation of Mrna thereby inhibiting protein synthesis. *hpt*II gene has been used to transform a large numberof plants and including monocots. It has been found to be more effective than kanamycin in cereals. *hpt*II gene is also used for selecting transformed mammalian cells.

- *NptII* (neomycin phosphotransferase)gene from Tn5 confers resistance to kanamycin in bacteria and geneticin in eukaryotes.
- *bar* gene confers resistance to phosphinothricinin plants.
- *Beta-lactamase* gene confers resistance to ampicillin in bacterial system.
- Mutant *FabI* gene (*mFabI*) from *E. coli* which confers resistance to triclosan.

Figure:(A) Herbicide resistant transgenic coffee plant and (B) non-transformed coffee plant one week after spraying with herbicide (Ammonium glufosinate)

Source:http://www.scielo.br/scielo.php?pid=S1677-04202006000100007&script=sci_arttext (cc)



A B C

Figure: Petriplates showing result of a transformation experiment in *Brassica juncea*. A shows a plate used as negative control. In this no transformation has been carried out but the explants are grown in presence of selective antibiotic, therefore, none of them

survives. B shows a plate in which transformed explants are grown. Only the positive transformants are resistant to antibiotic and can grow, the untransformed explants die. C is a positive control used to ascertain that the explants are healthy. These are untransformed explants and the plate does not contain selective antibiotic, therefore all the explants show growth.

Source: Manisha Sharma, Department of Genetics, University of Delhi, South Campus.

Positive selectable marker genes are defined as those that promote the growth of transformed tissue however, untransformed tissue is not killed in the process.

Initially positive selectable marker genes conditional on the use of toxic agents such as antibiotics, herbicides or drugs were developed. Recently positive selectable marker genes which are conditional on non-toxic agents (compounds which induce growth and differentiationof transformed tissuesas theyare used as substrates)have been developed. The new generation of positive selectable marker genes is not conditional on external substrates but they modify the normal physiological processes involved in plant development.*Pmi* gene(derived from *E. coli*) coding for phosphomannose isomerase enzyme is used as a positive selection marker. Transformants carrying transgene and *pmi* gene are able to use mannose as a carbon source and grow in its presence. However, the untransformed tissue is unable to metabolize mannose and its growth is arrested.

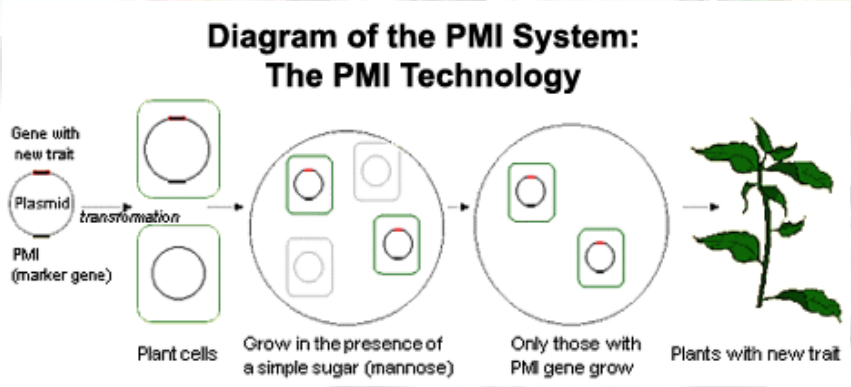


Figure: Mannose-based positive selection system in plants

Source: http://www.syngentabiotech.com/biotech_licensing/pmi_technology.aspx (cc)

Although a large number of selectable marker genes for plant system are known, only a few are used for crop development and plant research due to practical issues. Many of the marker

genes have not been thoroughly tested or displayspecific limitations. Biosafety issues are also considered while choosing markers for future crop developmentand widespread use.

Screening marker

A marker for screening is one which will make cells containing the gene look different and allows the researcher to distinguish between wanted and unwanted cells/organisms is known as screening marker. These markers do not provide a cell with a selective advantage, but are used to identify transgenic events by manually separating transgenic and non-transformed material.Reporter systems have been used to determinethe intracellular localization of a gene product, efficiency of gene delivery systems, detection of protein-protein or protein-DNA interactions and activity of promoter

A common example of reporter system is blue and white selection in bacteria. This assay is based upon insertional inactivation of *Lac-z*-gene which produces beta-galactosidase enzyme. This enzyme converts a colorless substrate into blue colored product which is responsible for blue color of colonies. Non recombinant colonies have intact *lac-z*gene thus they are blue colored, whereas recombinants have insertionally inactivated *lac-z*geneand colorless colonies.

There are three more types of screening markers commonly used:

GUS Assay

GUS assay is a simple method for detection oftransformed cell without needing any complicated equipment. β-glucuronidase, an enzyme from the bacterium *Escherichia coli* is utilized in this technique. This enzyme can transform colorless or non-fluorescent substrates into coloured or fluorescent products thereby giving the transformed cell a different phenotype.

Histochemical gusstaining is done using Xgluc i.e. 5-bromo-4-chloro-3-indolyl glucuronide, which produces blue color. Tissueexpressing the *gus* gene when incubated with Xgluc produces blue color. For fluorimetric estimation 4-methylumbelliferyl-beta-D-glucuronide (MUG) is used. In the reactionMUG acts as a substrate, which upon hydrolysis, produces glucuronic acid and the fluorescent 4-methylumbelliferone. For 4-MU the excitation is at 365 nm and emission at 455nm.

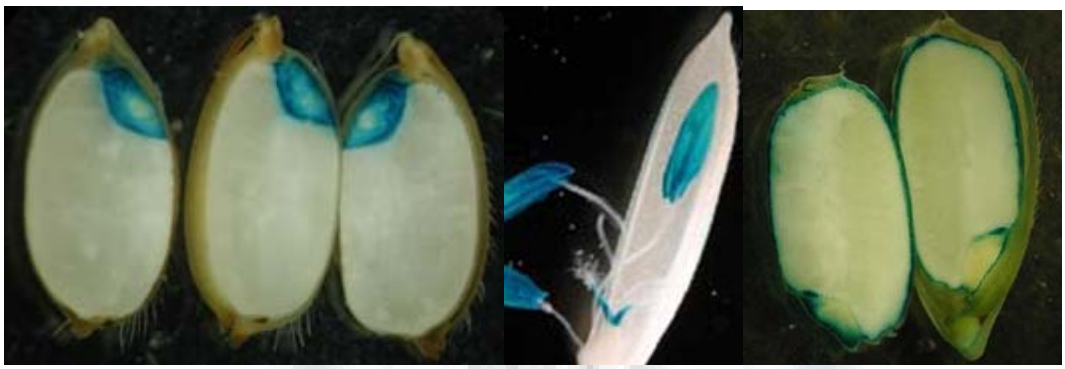


Figure: GUS histochemical assay in rice, (A) embryo, (B) anthers and style and (C) seed aleurone layer showing GUS expression.

Source: http://en.wikipedia.org/wiki/File:Rice_embryo.png

http://en.wikipedia.org/wiki/GUS_reporter_system#mediaviewer/File:RiceGUS.png

http://en.wikipedia.org/wiki/GUS_reporter_system#mediaviewer/File:Rice_aleurone.png (cc)

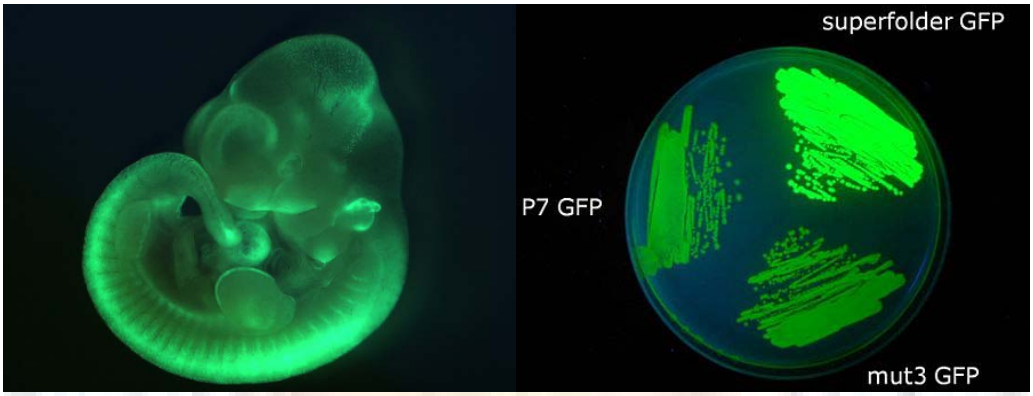
This technique is used to analyze the activity of regulatory elements by estimating expression of GUS gene either quantitativelyor bystudying activity in different tissues. It is very commonly used but the biggest drawback is that the cells are killed in the process.

GFP

The 2008 Nobel Prize in Chemistry was awarded to Martin Chalfie, Osamu Shimomura and Roger Y. Tsein for discovery and development of the green fluorescent protein (GFP). GFPis a protein which exhibits bright green fluorescence after exposure to light in the blue to ultraviolet range. The cells containing GFP glow green under UV light and can be seen with the help of a specialized microscope. GFP was first isolated from the jellyfish *Aequorea victoria* where it is expressed in small granules around the rim of the jellyfish bell.Itis made up of 238 amino acid residues and displays a major excitation peak at 395 nm and a minor one at 475 nm. It gives a green fluorescence as its emission peak (509 nm) falls in the lower green portion invisible region of spectrum.Other versions of this protein giving yellow and red

fluorescence are also available, so that multiple genes in one organismcan be followed at the same time.

A B



C

Figure:GFP expression in (A) mouse embryo (B) Enhanced fluorescence of super folder GFP mutant (C) Variants of GFP that emit different colors of light.

Source:

http://embryology.med.unsw.edu.au/embryology/index.php?title=File:Mouse_Wnt_signaling_01.jpg

http://openwetware.org/wiki/IGEM:Cambridge/2008/Improved_GFP

http://www.michaeleisen.org/blog/?p=59 (cc)

GFP gene is frequently used as a reporter which confirms expression of atransgene throughout the organism. It can be introduced into organisms through breeding, cell transformationor injection with a viral vector. This gene has been introduced and expressed in many bacteria, fungi, plant, fish, fly and mammalian cells.

Many different mutants of GFP have been engineered.

Methods of Gene Transfer

Examples of GFP
1. A single point mutation (S65T) in GFP dramatically improved the spectral characteristics of GFP, and resulted in increased fluorescence as well asphoto stability. The improved version is named as EGFP and is credited with use of GFPs in mammalian cells. 2. Super folder GFP, having a series of mutations that allow its rapid folding even when it is fused to poorly folding peptides, was reported in 2006. 3. Many other mutations resulting in color mutants have also been made e.g., derivatives of yellow fluorescent protein (YFP, Citrine, Venus, YPet), blue fluorescent protein (EBFP, EBFP2, Azurite, mKalama1) and cyan fluorescent protein (ECFP, Cerulean, CyPet).
Source: http://en.wikipedia.org/wiki/Green_fluorescent_protein (cc)

A 

B 

Figure: (A) GloFish, the first pet sold with artificially expressing fluorescent proteins, are available in bright red, green, orange-yellow, blue, and purple fluorescent colors. (B) Transgenic mice glowing green under UV light (left & right), compared to a normal mouse (center).

Source:http://en.wikipedia.org/wiki/File:GloFish.jpg

http://en.wikipedia.org/wiki/File:GFP_Mice_01.jpg (cc)

Luciferase

Production and emission of light by a living organism is known as Bioluminescence.It is a type of chemiluminescence where a chemical reaction results in release of light energy. Bioluminescence in nature is exhibited by both simple unicellular organisms (e.g., bacteria, dinoflagellates) and higher-order organisms (e.g., fish, insects). This phenomenon is more commonly seen in marine animals living in depths of the ocean, as compared to terrestrial organisms. It serves many natural purposes for these organisms. e.g., defense, camouflage, feeding, mating etc.

Luciferases are enzymes which emit light. Luciferase-mediated bioluminescence is used by many organisms to attract prey or mates or for protection from predators. Firefly luciferase from the firefly *Photinus pyralis*releases green light during the oxidation of its chemical substrate, luciferin. Other organisms which express the luciferase (*LUC*) gene will also glow faintly green when supplied with luciferin.

A B

Methods of Gene Transfer

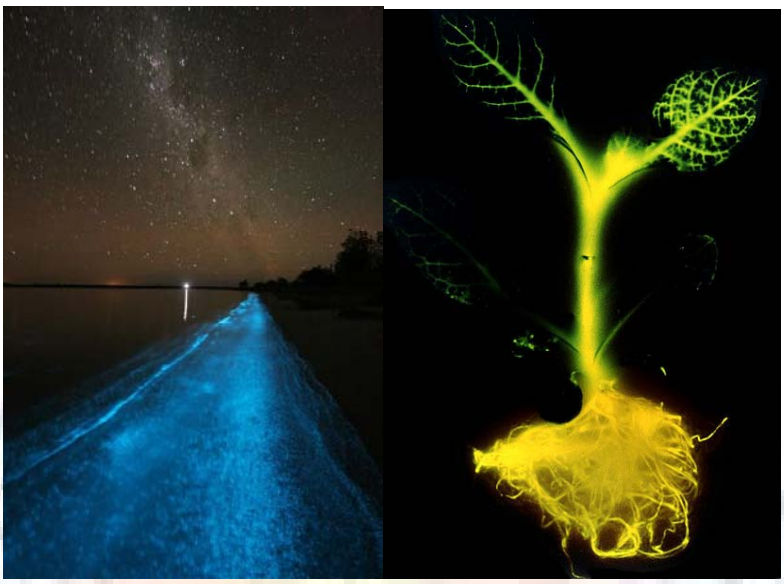


Figure: Luciferase-mediated bioluminescence. (A) marine dinoflagellate protist *N. scintillans* (known as sea sparkle) causing phosphorescent tide (B) glowing tobacco transformed with firefly luciferase gene.

Source: http://sydkab.wordpress.com/2012/02/21/bioluminescence/ (cc)

http://biobook.nerinxhs.org/bb/genetics/biotechnology.htm (CC)

Bioluminescence reaction takes place in the presence of two agents:the enzyme (firefly luciferase) which catalyzes the reaction, and thesubstrate(luciferase). Thisreaction takes place in two steps:

$$\text{Luciferin} + \text{ATP} \rightarrow \text{Luciferyl Adenylate} + O_2 \rightarrow \text{Oxyluciferin} + \text{light}$$

Oxyluciferin,formed in the end of this reaction in an electronically excited state and releases a photon of light while returning to the ground state.Light sensitive apparatus such as aluminometer or modified optical microscopes are used to detectthis photon emission.Luciferase bioluminescence does not require light excitation resulting in almost negligible autofluorescence which gives background-free glow.This glow is utilized as an assay for *LUC*(Luciferase gene) expression, which acts as a "reporter" to assessthe activity of regulatory elements.There is inherent variability in light emission form Luciferase enzymes isolated from different sources, therefore two or more luciferase enzymes can be used in combination to perform analysis of multiple genes.

Methods of Gene Transfer

Summary

1. The artificial or natural introduction of foreign genetic material into a cell is known as gene transfer.
2. Foreign genes can be introduced artificially into crops by a process known as genetic transformation.
3. Plant transformation systems generally include introduction of a DNA segment into plant cells or explants, its integration into host cells genome and subsequent regeneration from transformed cell to produce whole plant.
4. The DNA segment which is introduced in this process contains the gene of interest with flanking regions containing promoter, terminator and marker gene.
5. Methods available to achieve genetic transformation of plants can be divided into two main types; direct and indirect.
6. Methods of genetic transformation which employ bacteria as a vector to introduce the gene construct into the target cell are known as indirect methods. These methods use *Agrobacterium*, whichharbor Ti plasmid and causes crown gall disease in a number of plants. The genes to be integrated are present in T-DNA (transfer DNA), a specific region of the Ti plasmid which is transferred to the plant genome. This transfer is controlled by *vir* (virulence) genes present in some other region of Ti plasmid.
7. There are two types of genetically engineered Ti plasmid based vectors: Binary and co-integrate.
8. Direct methods are those methods which do not use bacteria as mediators for integration of DNA into host genome. These methods include microprojectile bombardment,electroporation and microinjection.
9. Biolistics is a method for plant transformation where cells are bombarded with heavy metal particles coated with DNA/RNA. As the microprojectiles penetrate the plant cell walls and membranes to enter the cells, coated DNA is released from its surface and incorporated into the plant's genome. It provides an effective and versatile way to transform almost all type of cells, including genetic material of the nucleus as well as organelles.
10. Electroporation is a method of transformation where transfection mixture containing cells and DNA is exposed to very high voltage electrical pulses (4000 – 8000 V/cm) for very brief time periods (few milliseconds). It results in formation of transient pores in

the plasma membrane, thorough which DNA seems to enter inside the cell and then nucleus.

11. Microinjection is the process of using a fine glass micropipette to manually inject transgene at microscopic or borderline macroscopic level. Selection of transgenics is needed in a transformation experiment as only one in many may take up the transgene depending upon the efficiency of transformation. Rather than checking every single cell/organism a marker gene is introduced into cell along with the transgene to determine if the transgene has been successfully inserted into host organism's genome, as marker gene's presence can be seen or detected. There are two types of marker genes: selectable and screening marker.
12. A selectable marker is a gene that protects the organism from a selective agent that would normally kill it or prevent its growth. In order to find out transformed cell/organism a selective agent is utilized which kills all the cells withouttransgene, leaving only the transformed ones. These genes can be divided into several categories depending on whether they confer positive or negative selection and whether selection is conditional or non-conditional on the presence of external substrates e.g., herbicides, antibiotics etc.
13. A marker for screening which will make cells containing the gene look different and allows the researcher to distinguish between wanted and unwanted cells/organisms is known as screening marker. These markers do not provide a cell with a selective advantage, but are used to identify transgenic events by manually separating transgenic and non-transformed material e.g., Gus, GFP, Luciferase.

Exercise

- How is *Agrobacterium*-mediatedtransformation is different from other genetic transformation systems?
- What is the role of Ti plasmid in manifestation of crown gall disease?
- How is Ti plasmid is modified to function as a vector for genetic transformation?
- Discuss the merits and demerits of particle bombardment methodof genetic transformation.
- What is Electroporation? How it is different from microinjection?
- Why do we need a marker gene in plant transformation experiment?

- Differentiate between selectable and screening marker? Give examples.
- What is the difference between positive and negative selection system?

Glossary

Crown gall disease: Adisease that affects many species of plants and is caused by bacteria (*Agrobacterium*species) which forms tumorous enlargements (several inches or more in diameter) usually at the junction of stem and root.

Fluorescence: Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation.

Homologous recombination: Homologous recombination is a type of genetic recombination in which nucleotide sequences are exchanged between homologous chromosomes.

Horizontal gene transfer: The acquisition by an organism of genetic information by transfer, for example via the agency of a virus, from an organism that is not its parent and is typically a member of a different species.

Provirus: A form of virus that is integrated into the genetic material of a host cell and by replicating with it can be transmitted from one cell generation to the next without causing lysis.

Reporter genes:A gene which is used to `tag' another gene or DNA sequence of interest, such as a promoter. Expression of the reporter is easily monitored, and permits the function or whereabouts of the `target' sequence to be tracked.

Retrotransposons: These aretransposable elements (transposons) that involve a retrovirus-like process of reverse transcription. The DNA element is transcribed into RNA, reverse-transcribed into DNA, and then inserted at a new site in the genome.

RNAi:A post-transcriptional genetic mechanism that suppresses gene expression and in which double-stranded RNA cleaved into small fragments initiates the degradation of a complementary messenger RNA.

Totipotent: Totipotency is the ability of a single cell to divide and produce all of the differentiated cells in an organism.

Methods of Gene Transfer

Transplastomic plant:A transplastomic plant is a genetically modified plant in which the new genes have not been inserted in the nuclear DNA but in the DNA of the chloroplasts.

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Methods of Gene Transfer

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