Genetic Engineering

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Genetic engineering, also known as recombinant DNA technology, means altering the genes in a living organism to produce a Genetically Modified Organism (GMO) with a new genotype.

or

The technology involving all processes of altering the genetic material of a cell/organism to make it capable of performing the desired functions, such as producing novel substances.

or

The branch of biology dealing with the splicing and recombining of specific genetic units from the DNA of living organisms in order to produce new genotype.

Importance

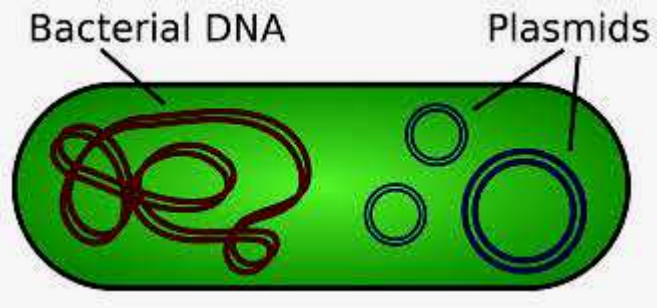
Genetic engineering is the transfer of DNA from one organism to another. By doing this organisms can be produced that have useful traits. For example, the human gene for insulin was put into bacteria, resulting in the production of a bacteria that produced insulin as a waste product. This break through allowed us to produce large quantities of human insulin for diabetics.

History

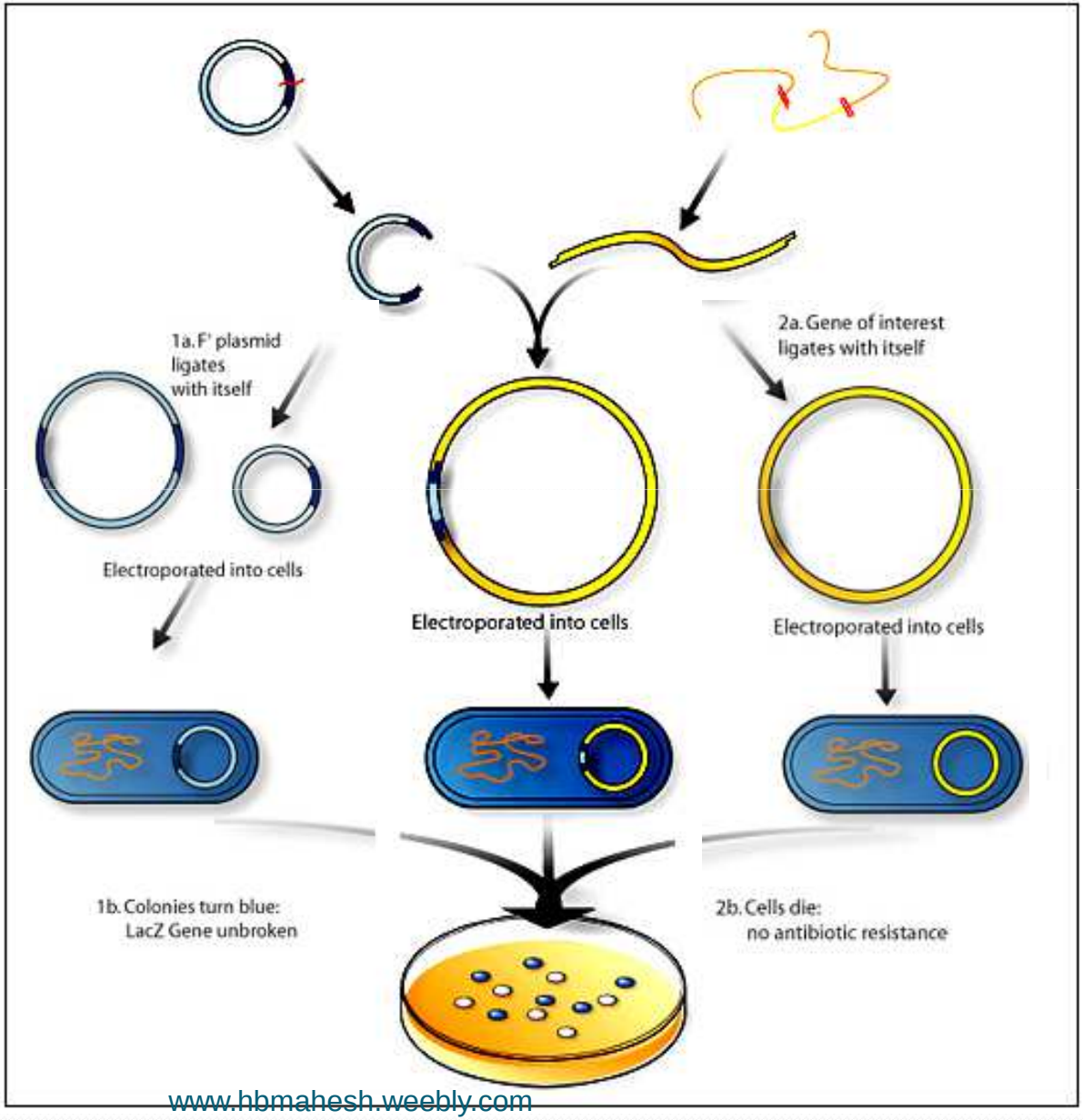
- Herbert Boyer and Stanley Norman Cohen 1973 successfully recombined two plasmids pSC 101 and pSC 102.

The "SC" stands for Stanley Cohen

Plasmid is a genetic structure in a cell that can replicate independently of the chromosomes, typically a small circular DNA strand in the cytoplasm of a bacterium or protozoan.



The Basic Principle of DNA Recombinant Technology



Developments

Some recent developments include:

- Creating mouse models of human cancers
- Creating porcine models to study the progression of heart diseases
- Knockout mice (mice with a certain gene missing from their genome) to study protein function and its consequences on metabolism and development.
- Anti-sense technology
- Inserting foreign genes into crops to increase yield (genetically modified foods)
- Inserting recombinant DNA into farm animals to create pharmaceutically relevant peptide therapeutics (like insulin and growth factor)

Restriction Enzymes

Definition: An enzyme produced chiefly by certain bacteria, that has the property of cleaving DNA molecules at or near a specific sequence of bases

OR

An enzyme that catalyzes the cleavage of DNA at restriction sites, producing small fragments used for gene splicing in recombinant DNA technology.

- **There are many different kinds of restriction endonucleases**

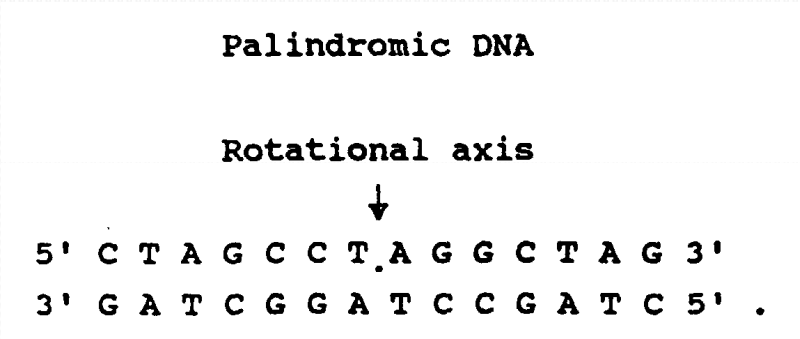
Restriction Enzymes continued.....

- **Restriction Enzymes are primarily found in bacteria and are given abbreviations based on genus and species of the bacteria.**
- **One of the first restriction enzymes to be isolated was from EcoRI.**
- **EcoRI is so named because it was isolated from *Escherichia* coli strain called RY13.**

TABLE 1: Characteristics of different types of restriction endonucleases

Type	*Salient features*
I	**A single enzyme with 3 subunits for recognition, cleavage and methylation. It can cleave up to 1000 bp from recognition site**
II	**Two different enzymes either to cleave or modify the recognition sequence. Cleavage site is the same or close to recognition site.**
III	**A single enzyme with 2 subunits for recognition and cleavage. Cleavage site is, 24-26 bp from recognition site.**
IIs	**Two different enzymes, cleavage site is up to 20 bp from recognition site**

In DNA a PALINDROME SITE is a SEQUENCE OF BASE PAIRS in double stranded DNA that reads the same backwards and forward.



Restriction Enzymes continued.....

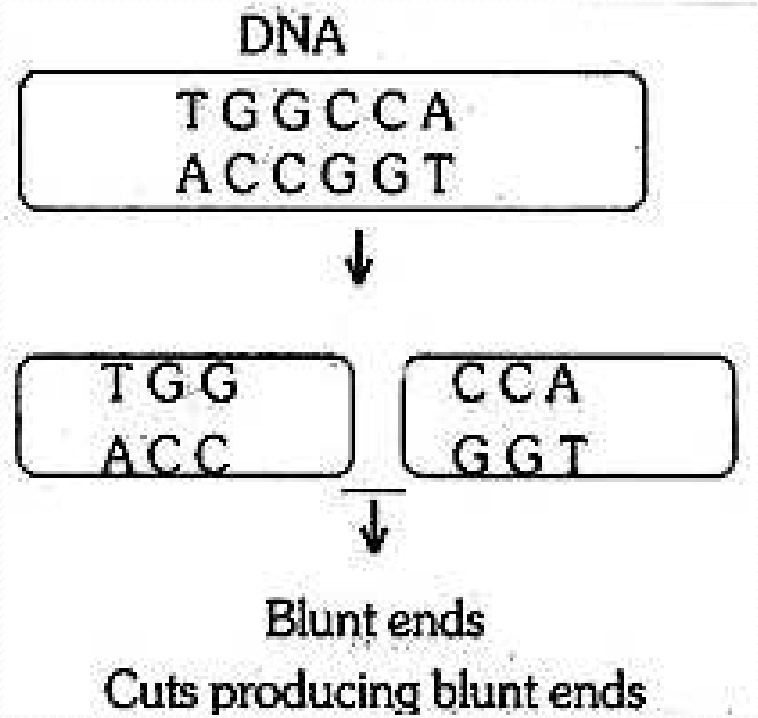
- A restriction enzyme cuts only double-helical segments that contain a particular sequence, and it makes its incisions only within that sequence known as a "recognition sequence".

Restriction Enzymes continued.....

- **Sticky end and blunt end are the two possible configurations resulting from the breaking of double-stranded DNA**

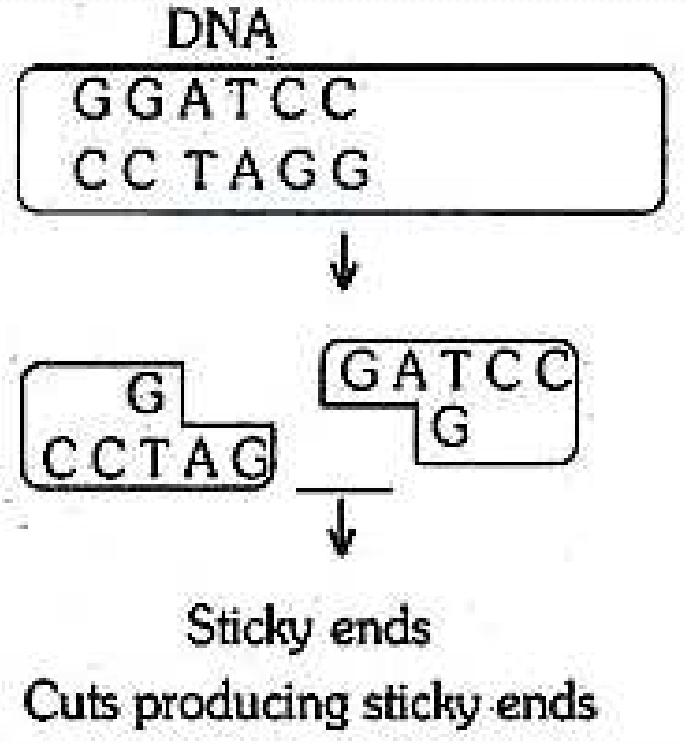
Restriction Enzymes continued.....

- If two complementary strands of DNA are of equal length, then they will terminate in a *blunt end*, as in the following example:



Restriction Enzymes continued.....

If another DNA fragment exists with a complementary overhang, then these two overhangs will tend to associate with each other and each strand is said to possess a *sticky end*:



Cuts producing sticky ends

Restriction Enzymes continued.....

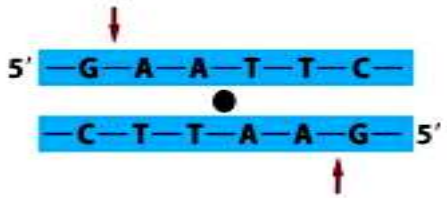
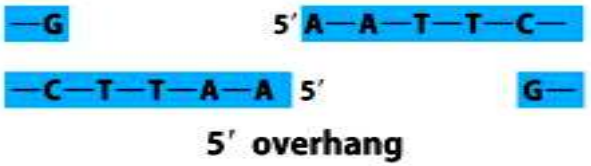
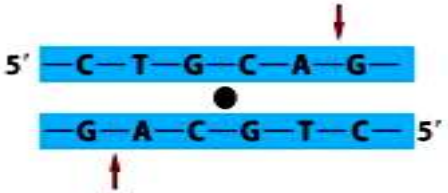
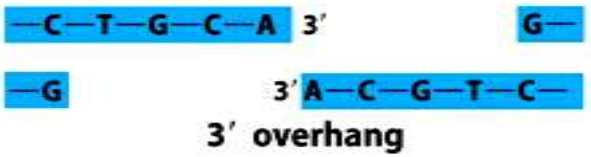
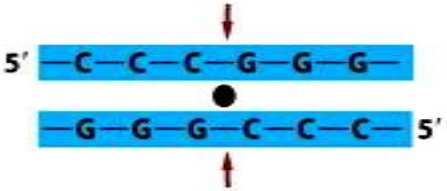
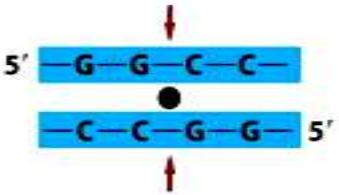
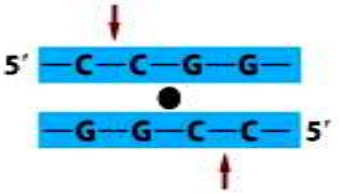
The cut ends join as

- 5'-ApTpCpTpGpApCpT pGpApTpGpCpGpTpApTpGpCpT-3'
- 3'-TpApGpApCpTpGpApCpTpApCpGp CpApTpApCpGpA-5'

Becomes

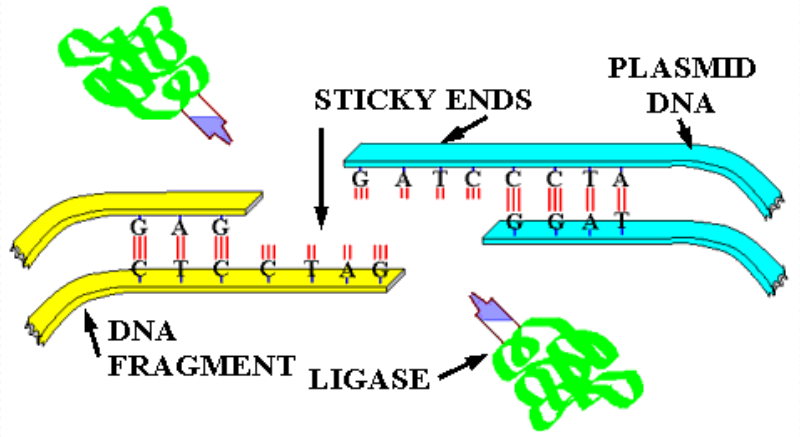
- 5'-ApTpCpTpGpApCpT pGpApTpGpCpGpTpApTpGpCpT-3'
- 3'-TpApGpApCpTpGpApCpTpApCpGp CpApTpApCpGpA-5'

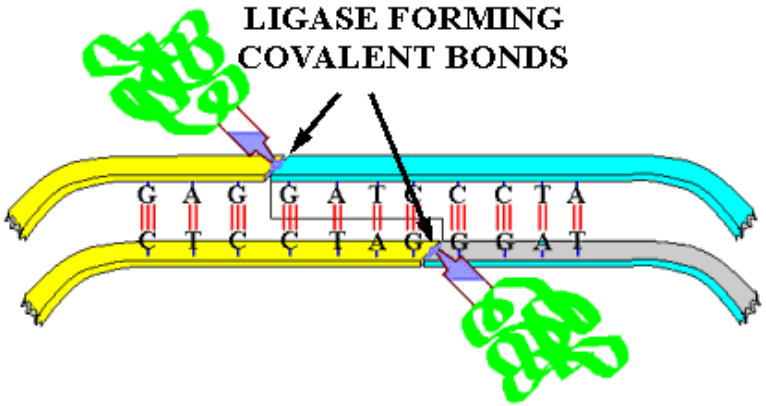
Some restriction enzymes

Enzyme	Source organism	Restriction recognition site in double-stranded DNA	Structure of the cleaved products
(a)			
EcoRI	*Escherichia coli*		
PstI	*Providencia stuartii*		
SmaI	*Serratia marcescens*		
(b)			
HaeIII	*Haemophilus aegyptius*		
HpaII	*Haemophilus parainfluenzae*		

Ligase

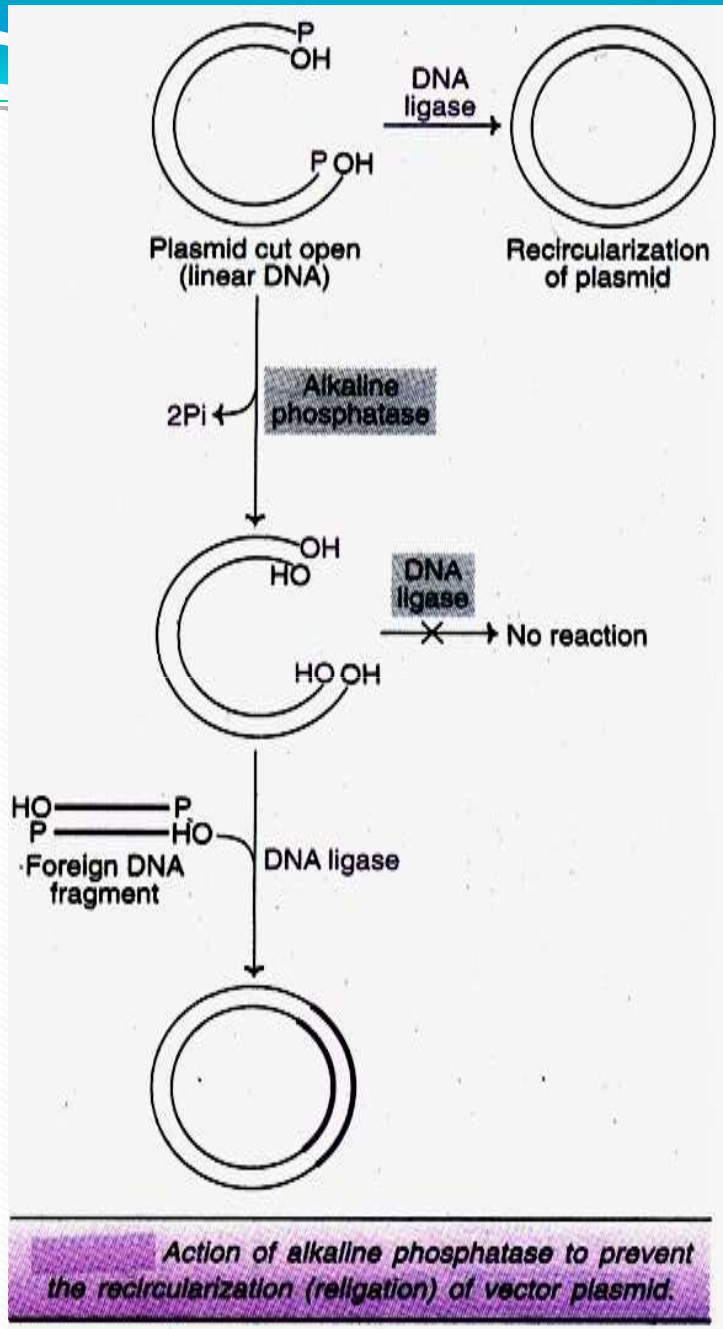
An enzyme that is able to join together two portions of DNA and therefore plays an important role in DNA repair. DNA ligase is also used in recombinant DNA technology as it ensures that the foreign DNA is bound to the plasmid into which it is incorporated.





Alkaline Phosphatase

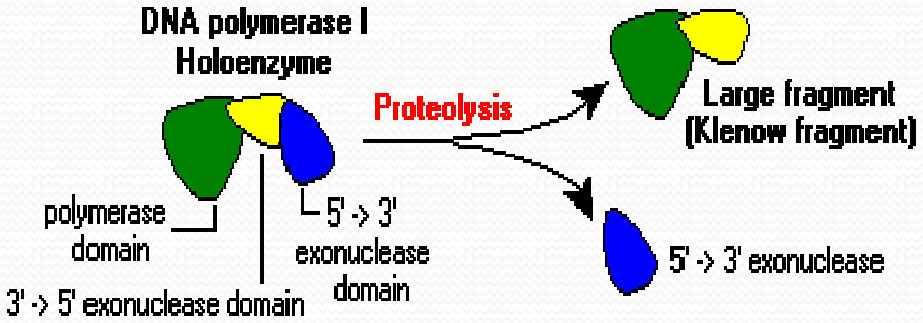
Alkaline phosphatase is an enzyme involved in the removal of phosphate groups. When the linear vector plasmid DNA is treated with alkaline phosphatase, the 5'-terminal phosphate is removed. This prevents both recircularization and plasmid DNA dimer formation. It is now possible to insert the foreign DNA through the participation of DNA ligase.



Action of alkaline phosphatase to prevent the recircularization (religation) of vector plasmid.

Klenow fragment

The 5'>3'exonuclease activity of *E. coli*'s *DNA Polymerase I* makes it unsuitable for many applications. However, this pesky enzymatic activity can readily be removed from the holoenzyme. Exposure of DNA polymerase I to the protease subtilisin cleaves the molecule into a small fragment, which retains the 5' > 3' exonuclease activity, and a large piece called Klenow fragment. **The large or Klenow fragment of DNA polymerase I has DNA polymerase and 3'>5' exonuclease activities, and is widely used in molecular biology.**



Taq DNA polymerase

***Taq* DNA polymerase is a heat stable enzyme used in the polymerase chain reaction (PCR) to amplify segments of DNA in the lab. It was discovered in the heat-loving bacterium *Thermus aquaticus*, and without it, we couldn't amplify DNA.**

Reverse transcriptase is a common name for an enzyme that functions as a RNA-dependent DNA polymerase.

In the retroviral life cycle, reverse transcriptase copies only RNA, but, as used in the laboratory, it will transcribe both single-stranded RNA and single-stranded DNA templates with essentially equivalent efficiency. In both cases, an RNA or DNA primer is required to initiate synthesis.

DNA can make DNA, DNA can make RNA, and RNA can make protein, but protein cannot make RNA, DNA, or another protein. Only under special circumstances can RNA make RNA or DNA.

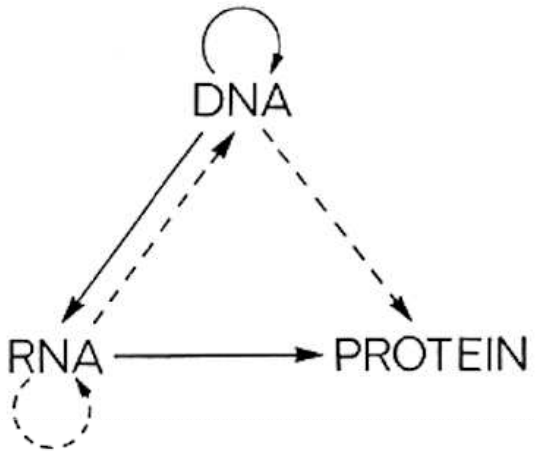


Fig. 3. A tentative classification for the present day. Solid arrows show general transfers; dotted arrows show special transfers. Again, the absent arrows are the undetected transfers specified by the central dogma.

S 1 nuclease

S1 nuclease selectively cuts and degrades single stranded portions of DNA. This enzyme breaks the phosphodiester bond between two nucleotides in single stranded portion of DNA and then degrades single stranded extensions. It does not degrade double-stranded portions of DNA and RNAs.

Uses:

1. S1 nuclease is used to degrade the hairpin loop formed while making a duplex DNA from complementary DNA strand (cDNA).
2. It is used to remove unwanted tail sequences from DNA fragments to make blunt ends.
3. It is used to remove the extra adenine base from DNAs prepared by polymerase chain reaction.
4. It can also be used to determine the degree of complementary base pairing between DNA strands during hybridization.

Ribonuclease

Enzymes that break down RNA.

RNase H activity: RNase H is a ribonuclease that degrades the RNA from RNA-DNA hybrids, such as are formed during reverse transcription of an RNA template. This enzyme functions as both an endonuclease and exonuclease in hydrolyzing its target.

Polynucleotide kinase

Polynucleotide kinase transfers a phosphate from ATP to *5'OH* group of dephosphorylated DNA or RNA.

Uses:

1. Polynucleotide kinase is used to rephosphorylate the 5' end of dephosphorylated vector DNA in rDNA. Then only DNA ligase can seal the nick between the vector DNA and target DNA.
2. It is used to transfer radioactive P32 from ATP to dephosphorylated *5'* end of DNA or RNA for labelling. The labelling technique is used.
    - i) To make hybridization probes.
    - ii) To make diagnostic kits.
    - iii) To analyse the base sequence of DNA.
    - iv) To construct restriction maps.

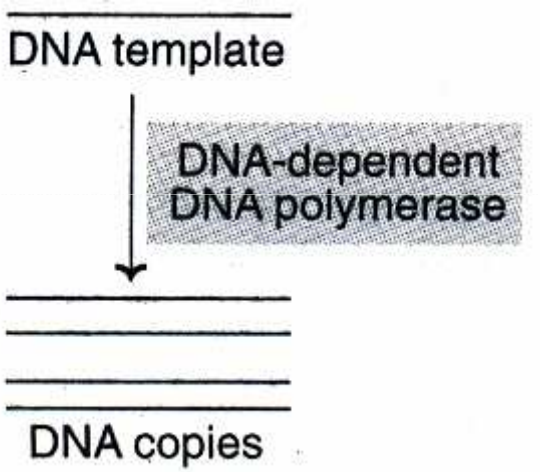
Terminal nucleotidyl transferase: *Terminal nucleotidyl transferase* adds mononucleotide triphosphates to 3' -OH group of DNA fragment without the aid of a template strand.

Uses:

1. Terminal transferase is used to make homopolymer cohesive tails at 3' end of DNA fragments. Thus it is of much use in joining blunt ended DNA fragments while constructing rDNA.
2. It is used to make radioactive DNA probes.

DNA polymerase

- Synthesizes DNA complementary to a DNA template.



Gene Cloning Vectors

The production of exact copies (clones) of a particular gene or DNA sequence using genetic engineering techniques.

Vectors are the DNA molecules, which can carry a foreign DNA fragment to be cloned

Examples:

Plasmids

Bacteriophages **is a virus that infects and replicates within a bacterium**

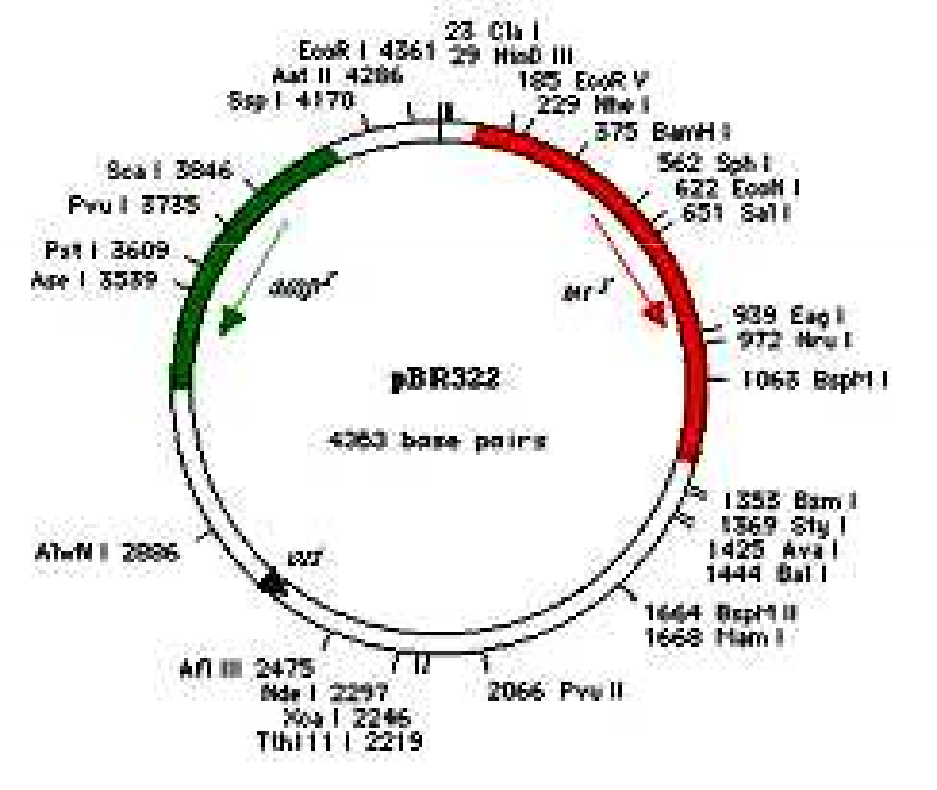
Cosmid: **is a hybrid plasmid that contains a Lambda phage cos sequence.**
Cosmids' (cos sites + plasmid = cosmids)

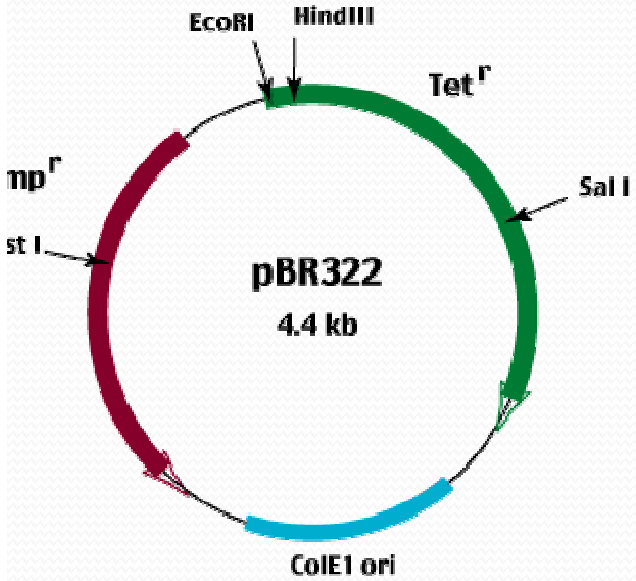
YACs

BACs

Gene CloningVectors

Importance of plasmids





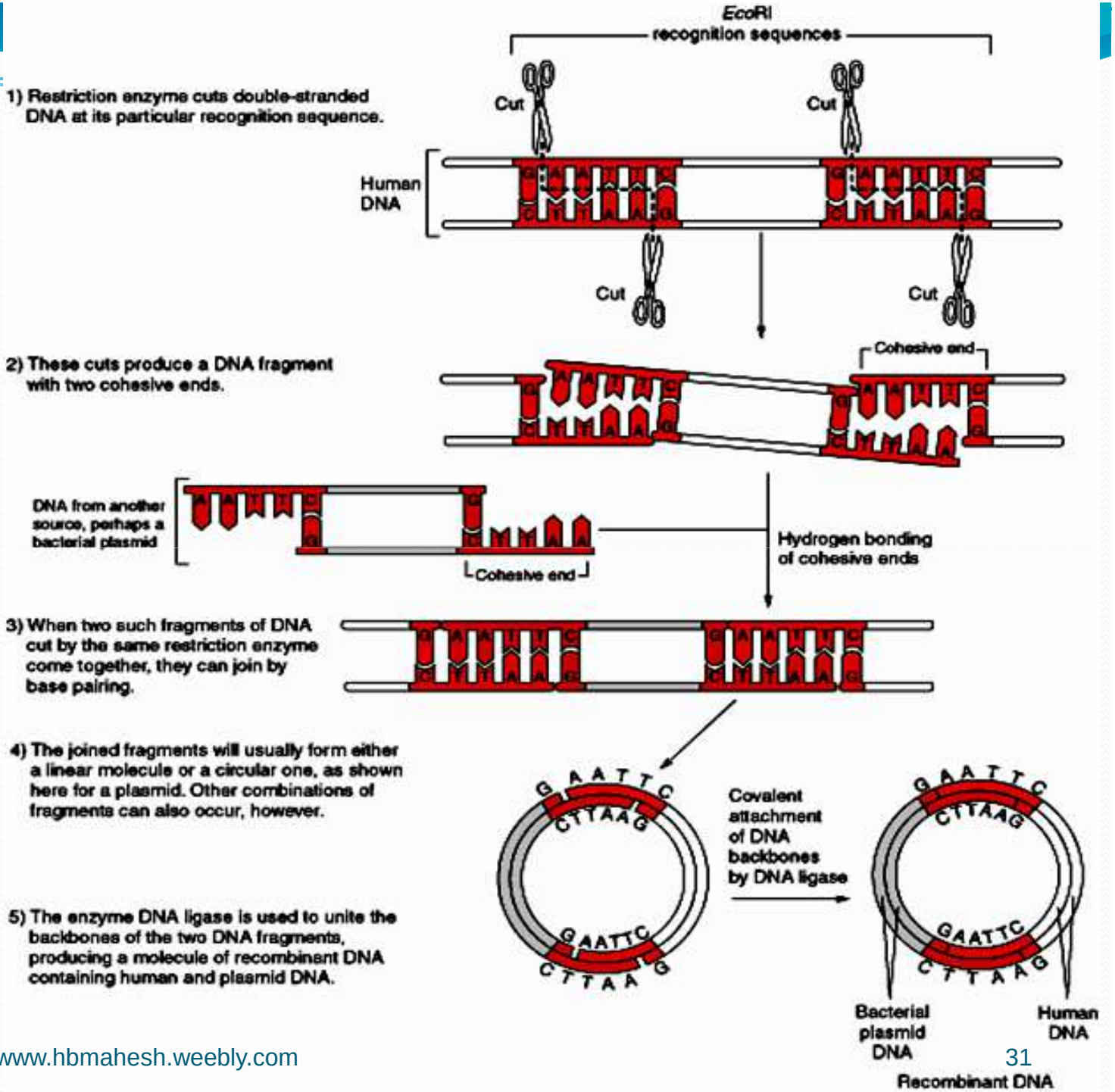
Methodology/steps in Genetic Engineering

1. **Preparation/Isolation of desired genes.**
   - a. Restriction digestion of genomic DNA & Separated by electrophoresis.
   - b. Reverse Transcription.
   - c. DNA synthesiser / gene machine.
1. **Isolation of DNA vector.**
2. **Construction of recombinant DNA.**
3. **Introduction of recombinant DNA into the host cell.**
4. **Screening & Selection of recombinants.**
5. **Expression of Cloned genes.**

Creating recombinant DNA

- **The first Recombinant DNA molecules were made by Paul Berg at Stanford University in 1972.**
- **In 1973 Herbert Boyer and Stanley Cohen created the first recombinant DNA organisms.**

Creating Recombinant DNA



GENE TRANSFER TECHNIQUES

Biological

***Agrobacterium* mediated gene transfer**

Non-Biological

Biolistic/particle gun delivery
Electroporation

Microinjection

Lipofection

UV Laser Microbeam

(Light amplification by stimulated emission of radiation)

Sonication/Ultrasonication

Silicon Carbide Fiber-Vortex

Chemical method of G T

Biolistic gene delivery

Particle Bombardment/microprojectile bombardment/etc.,

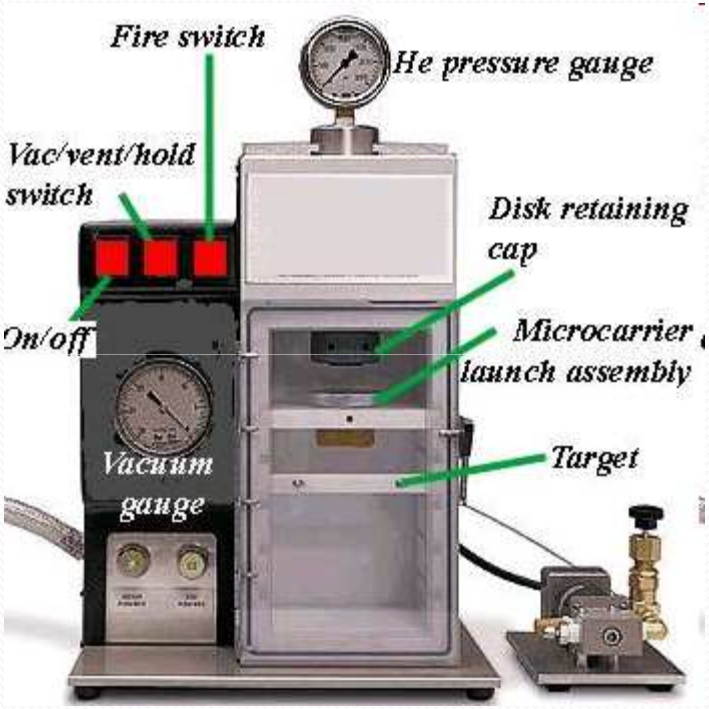
Originally developed for plant cells - animal "Human Gene Therapy"

"Technique uses high velocity microprojectile to incorporate the genes"

Gunpowder/nitrogen/compressed air/helium etc.,

Eg., Onion, Corn, immeture zygotic embryo of rice, wheat, suspension culture of cotton & soybean

GENE GUN


Fire switch
He pressure gauge
Vac/vent/hold switch
Disk retaining cap
On/off
Microcarrier launch assembly
Vacuum gauge
Target

MICROINJECTION

"Direct Physical approach overcomes many biological & other obstacles"

Technique uses fine capillary needle to deliver DNA into cells/nuclei.

Originally developed for animal cells – applied for plant cells

Advantages: 1. Amount of DNA delivery can be optimized

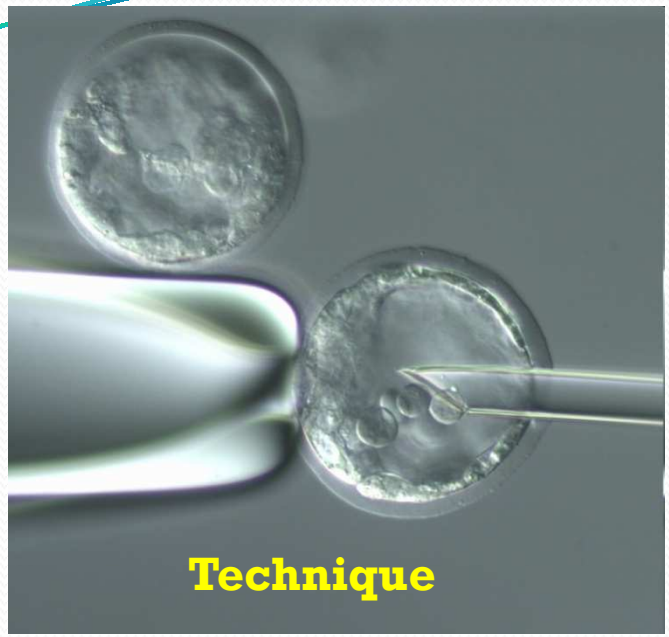
2. Delivery is precise

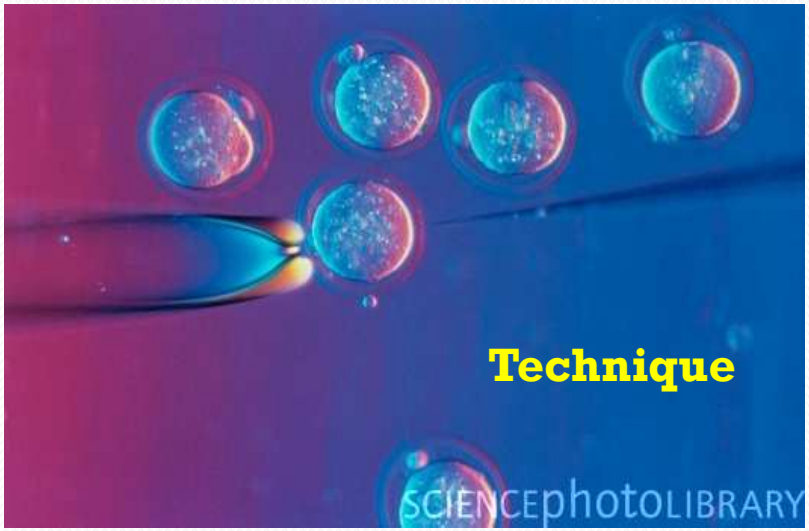
3. Small structure *i.e.*, organelles can be injected

Transgenic animal-Mouse

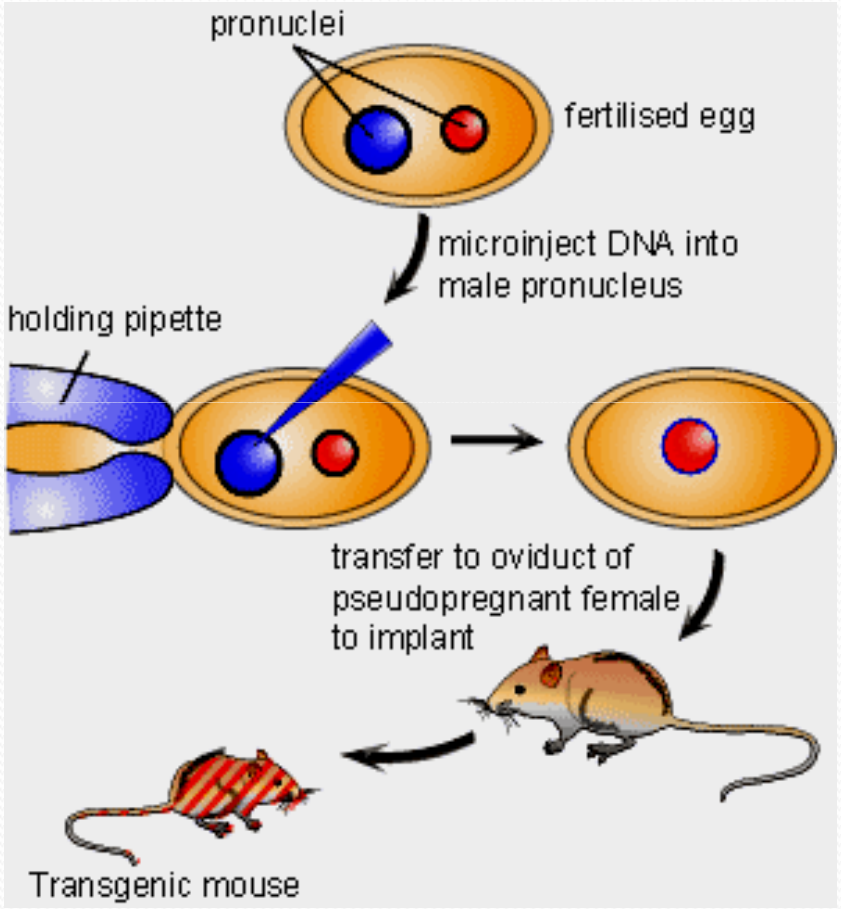
Fishes – Cat fish, Gold fish, Zebra fish, *etc.*,

MICROINJECTION continued





Technique



http://www.youtube.com/watch?v=h-Bfc1GPWpE

ELECTROPORATION

"Introduction of rDNA by electric shock"

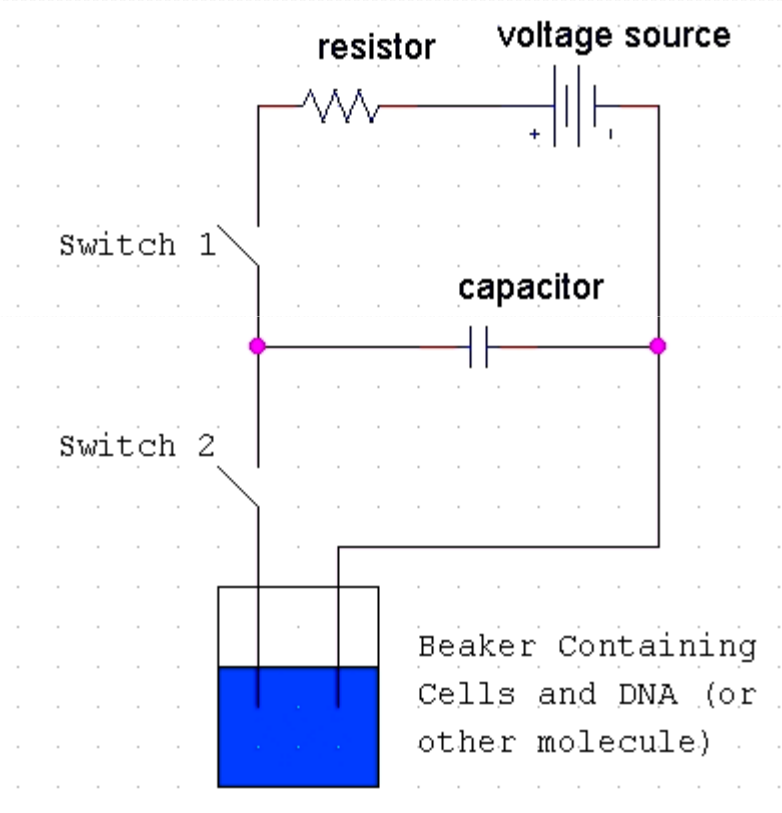
4-8kv/cm for 5 milliseconds *i.e.*, 0.005 seconds

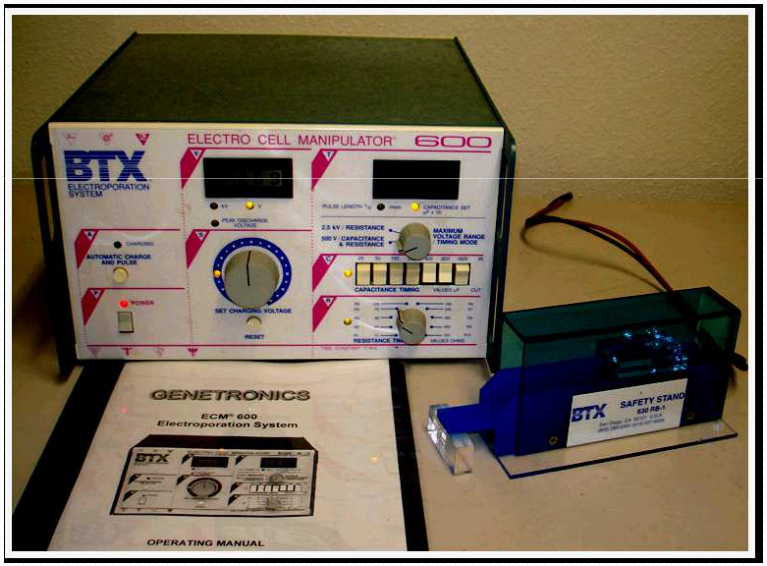
Limitations: Can not be adopted for intact plant cells

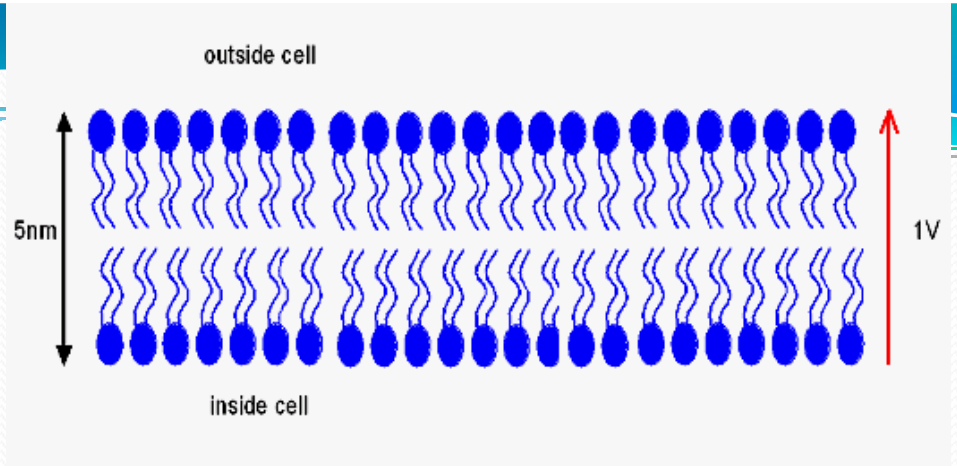
Animal cells are sensitive to electric treatment

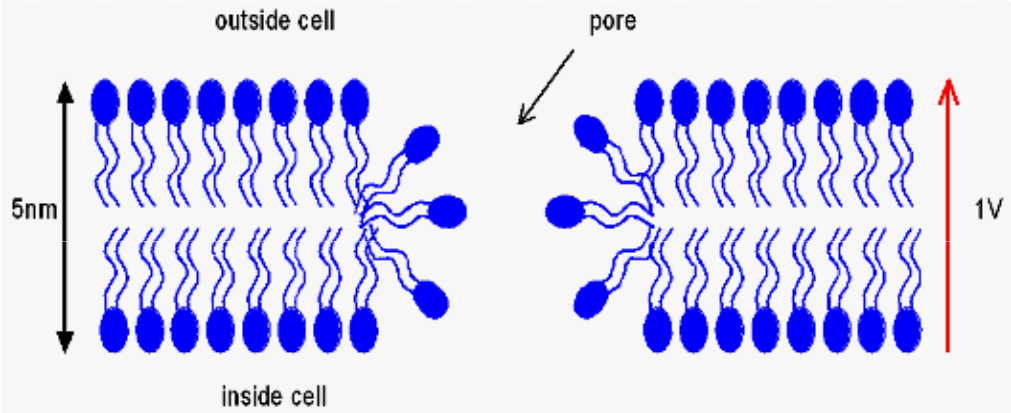
If the electric strength is too strong plant protoplasts may loose their viability

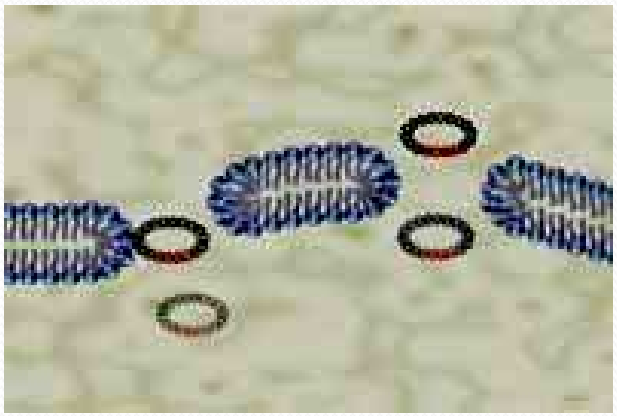
Mechanism


resistor
voltage source
Switch 1
capacitor
Switch 2
Beaker Containing Cells and DNA (or other molecule)


ELECTRO CELL MANIPULATOR 600
BTX
GENETRONICS
OPERATING MANUAL







http://www.youtube.com/watch?v=Hf0sen7bJ6A

LIPOFECTION

"Liposome mediated transformation"

An artificial microscopic vesicle consisting of an aqueous core enclosed in one or more phospholipid layers, used to convey DNA/RNA, vaccines, drugs, enzymes, or other substances to target cells or organs.

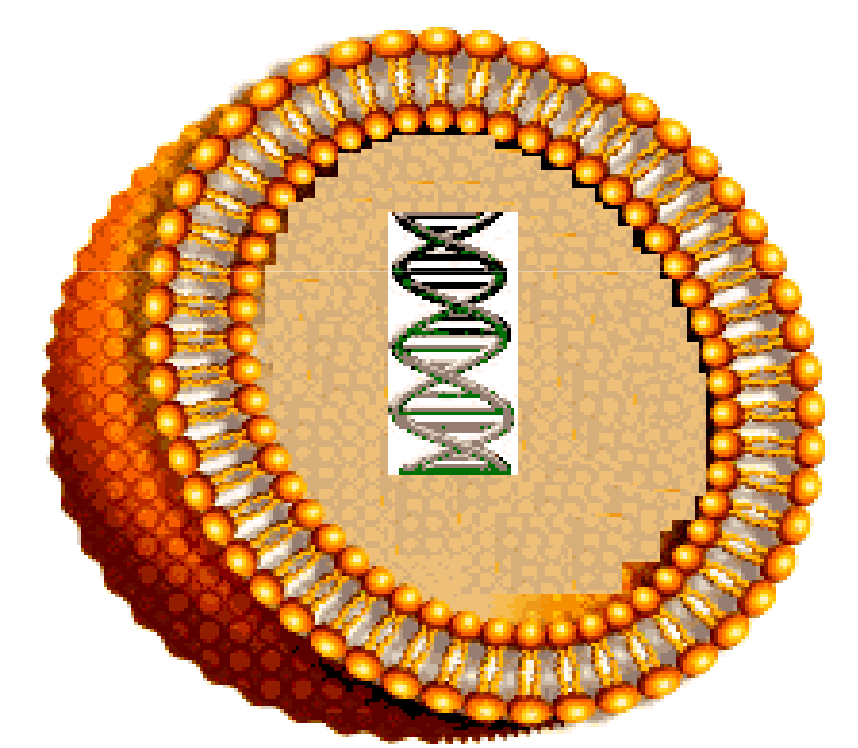
Or

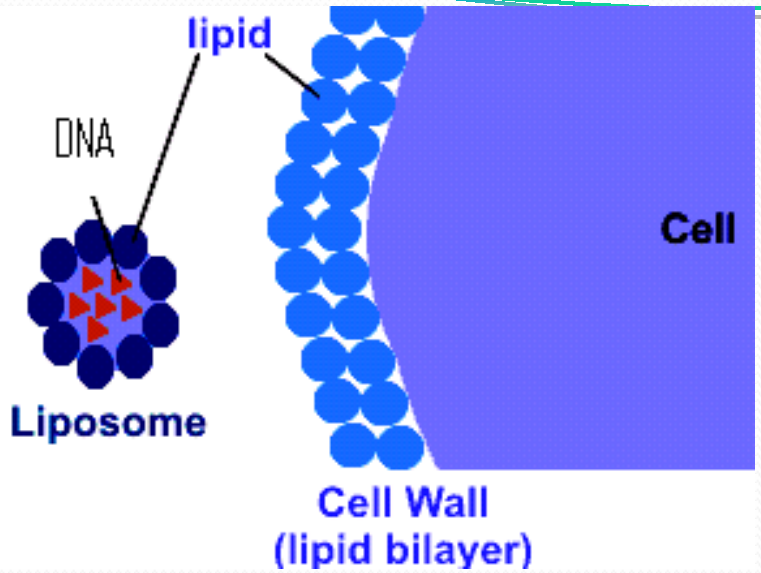
A **liposome** is a tiny bubble (vesicle), made out of the same material as a cell membrane. Liposomes can be filled with drugs, and used to deliver drugs for cancer and other diseases.

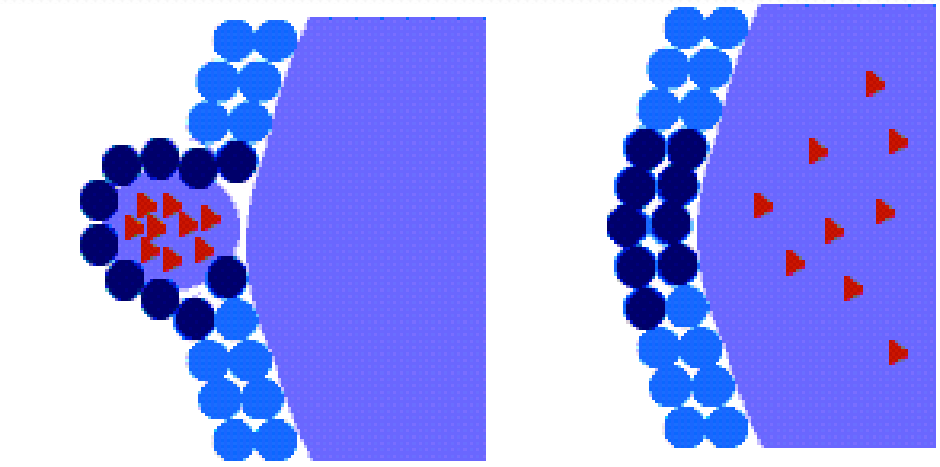
Adavntages

1. **Enhanced delivery**
2. **Protection from nucleases**
3. **Delivery into variety of cells**
4. **Delivery of intact small organelles**
5. **Protects from immunogenetic reaction**
6. **Composition can be manipulated for specific properties**

Liposome carries DNA/Drugs




lipid
DNA
Cell
Liposome
Cell Wall
(lipid bilayer)


Lipsome joins cell
DNA in cell

BIOLOGICAL- *Agrobacterium* mediated gene transfer

Inter-kingdom DNA transfer

***Agrobacterium tumefaciens* is a soil inhabitant, gram negative, rod shaped bacterium.**

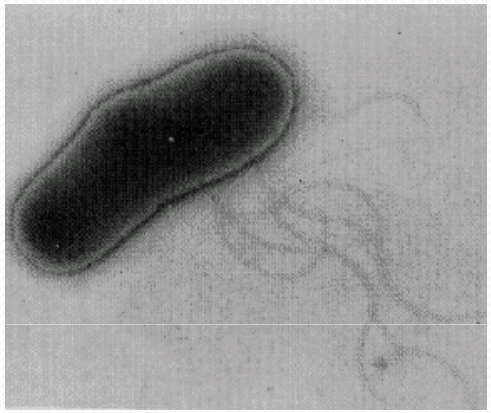
It infects crown, stem and roots of several dicotyledonous & gymnosperms through wounds. *Agrobacterium* Never infects monocotyledonous plants ?

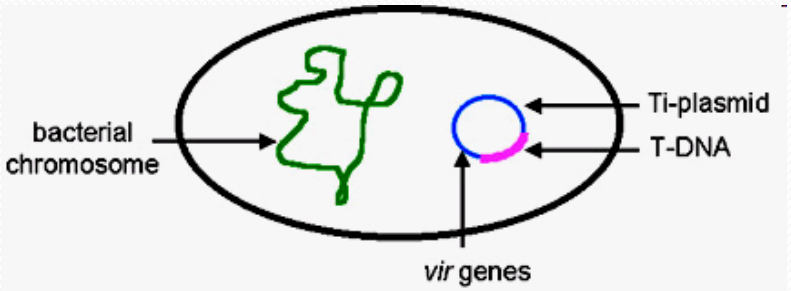
Positive Chemotaxi!

Agrobacterium tumefaciens

cells attached to a plant cell

Structure





Crown gall disease

Gall caused by *A. tumefaciens*

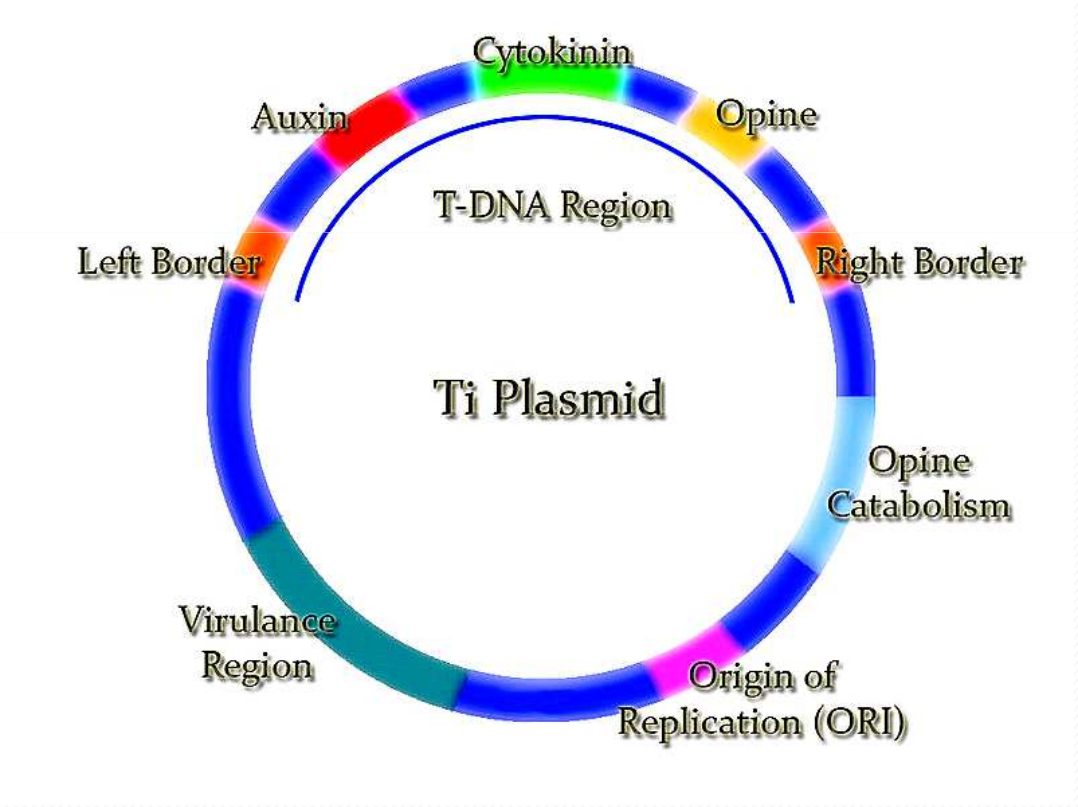
Ti-plasmid

Tumour inducing plasmids

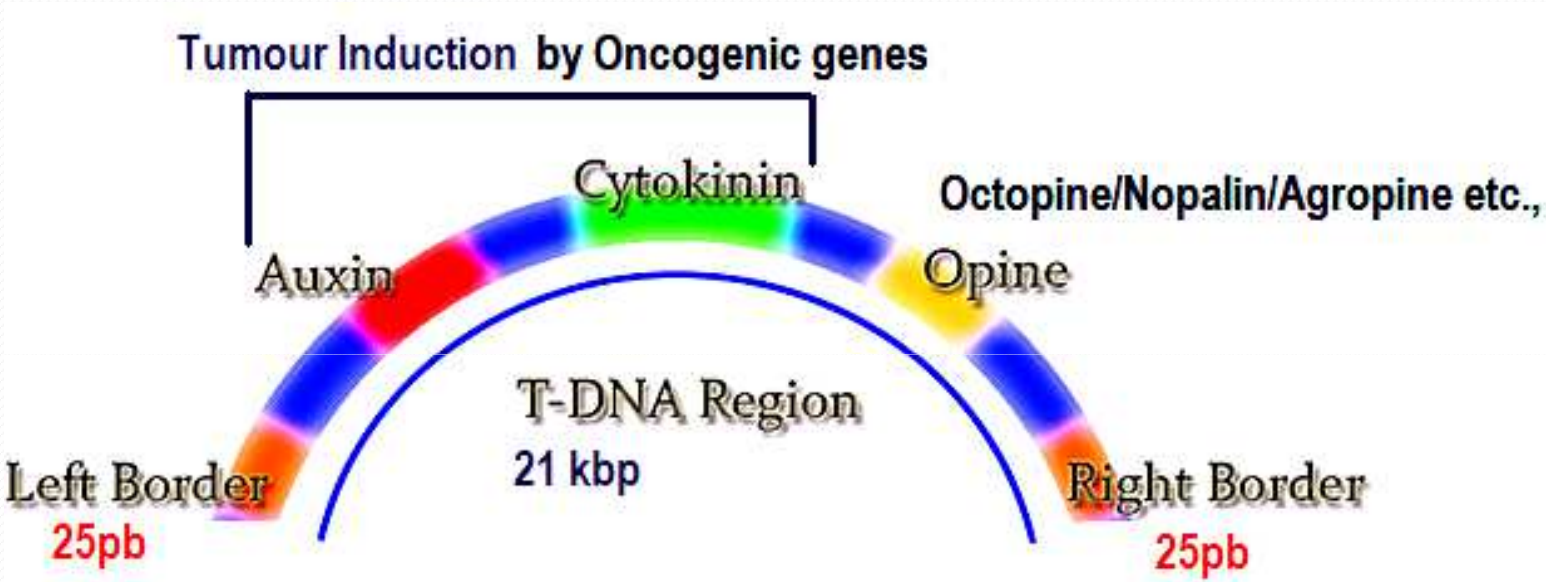
Large, circular, double stranded DNA molecule.

Size 150-230 kbp.

MW 120-160 mega daltons



T-DNA


Tumour Induction by Oncogenic genes
Cytokinin
Octopine/Nopalin/Agropine etc.,
Auxin
Opine
T-DNA Region
21 kbp
Left Border
25pb
Right Border
25pb

Ti-Plasmid: Ideal Cloning Vector

1. **Replicates autonomously**
2. **It carries foreign DNA into plant cell**
3. **It has T DNA which is integrated in chromosomal DNA of plant**
4. **It can be transferred from bacterium to bacterium by conjugation**

Ti-Plasmid Derived Vectors

The Wild Type Ti Plasmid is not suitable due to…

1. **It Induces tumourous growth in the recipient cell.**
2. **It is too large. Difficult to find out R S for inserting desired gene.**
3. **It has no selectable markers for identification of transformants.**
4. **It needs strong promoters for successful expression of cloned gene.**

In order to overcome DERIVATIVES of Ti plasmids are constructed & used

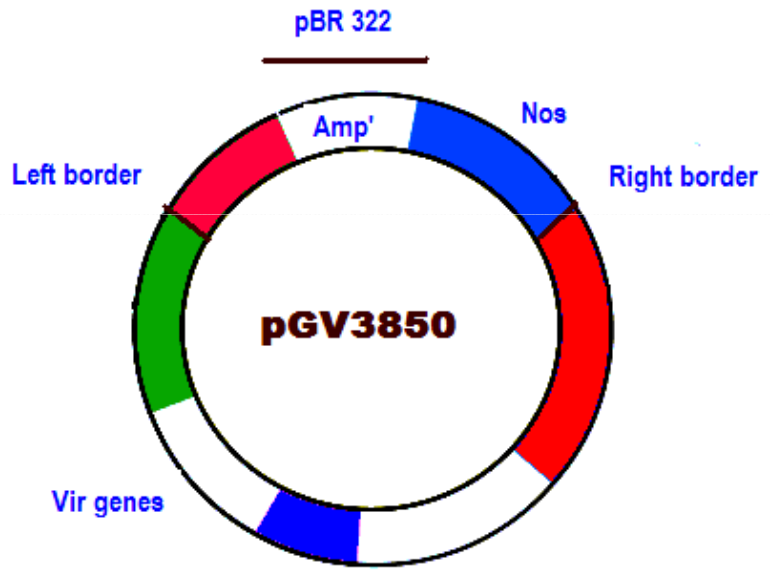
Two Types 1. Disarmed Ti Plasmids
2. Binary vectors

Binary vectors: Cloning vector that can propagate in both *Escherichia coli* and *Agrobacterium tumefaciens* for use in Biotechnology.

Disarmed Ti Plasmid

Non - oncogenic Ti plasmid

pGV3850 is a derivative of the nopaline Ti plasmid pTiC58



G E through disarmed Ti Plasmid

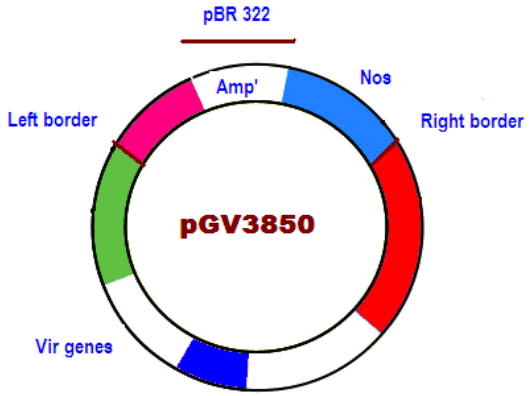
It is not so simple and it has three steps *i.e.*,

1. **Construction of *Agrobacterium* strain with rTi Plasmid.**
2. **Co cultivation of *Agrobacterium* with plant tissue.**
3. **Regeneration of plantlets.**

Construction of *Agrobacterium* Strain

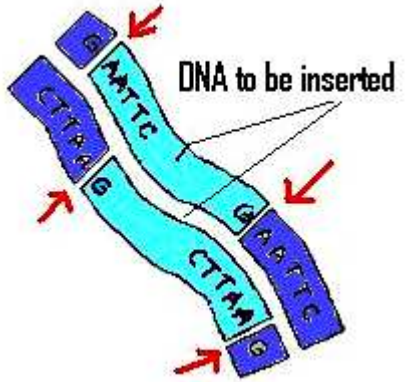
Agrobacterium strain with rDisarmed Ti plasmid

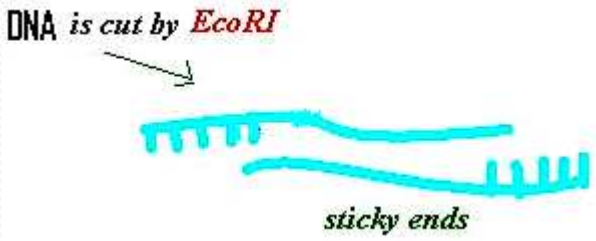
1. **The disarmed Ti plasmid pGV3850 is constructed from nopaline Ti plasmid pTiC58 & pBR322. It is introduced in to *E.coli* by transformation.**



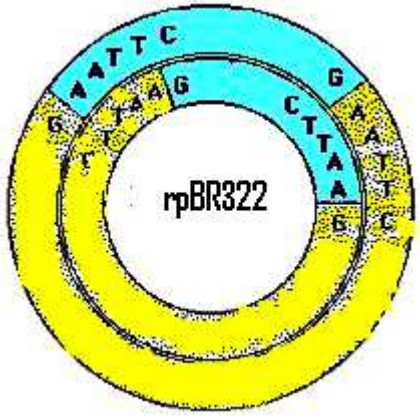
Introduction by electroporation in to *E. coli*

2. DNA of interest (foreign) inserted in to pBR322 using R E and formed rpBR322.

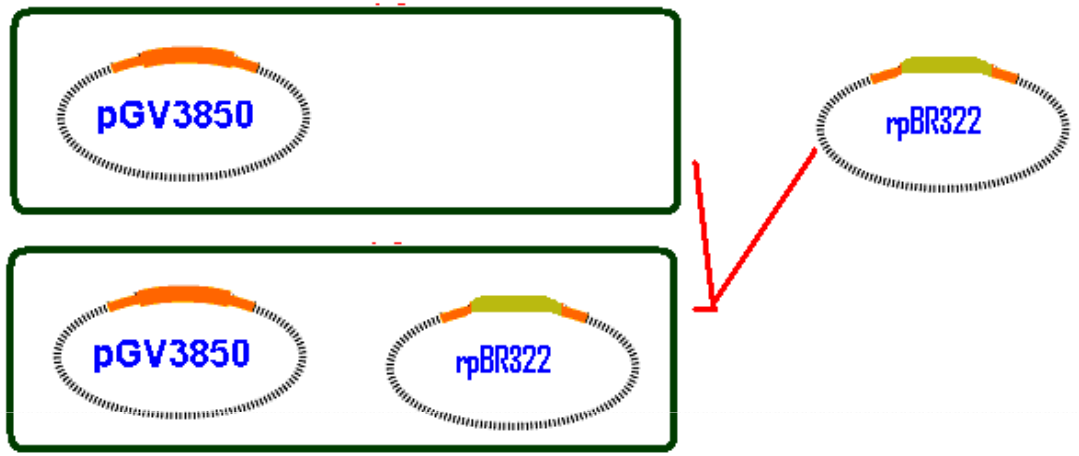


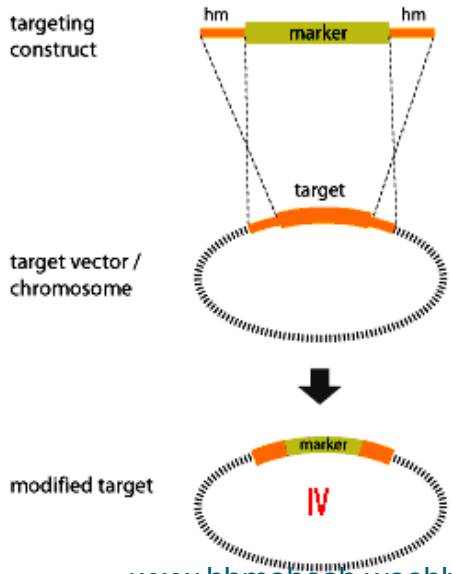


DNA LIGASE

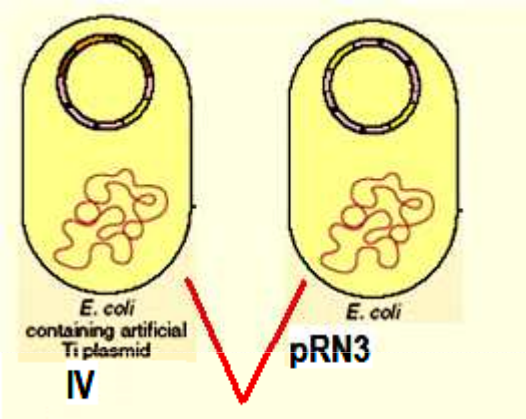


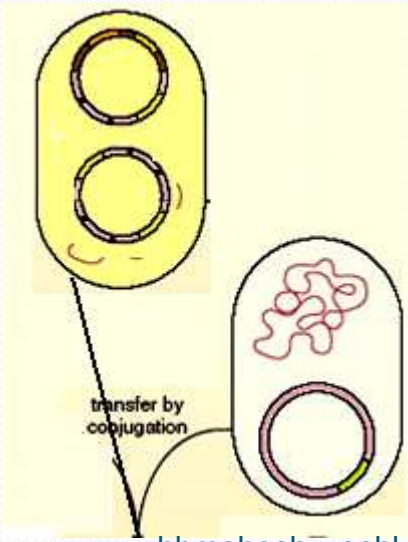
3. The rpBR322 is then introduced in *E.coli* that has pGV3850. These undergo homologous recombination so an INTERMEDIATE VECTOR (IV) is formed.

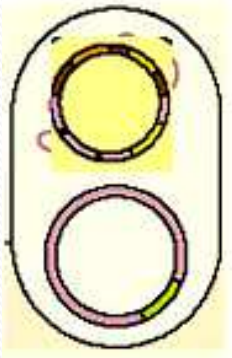




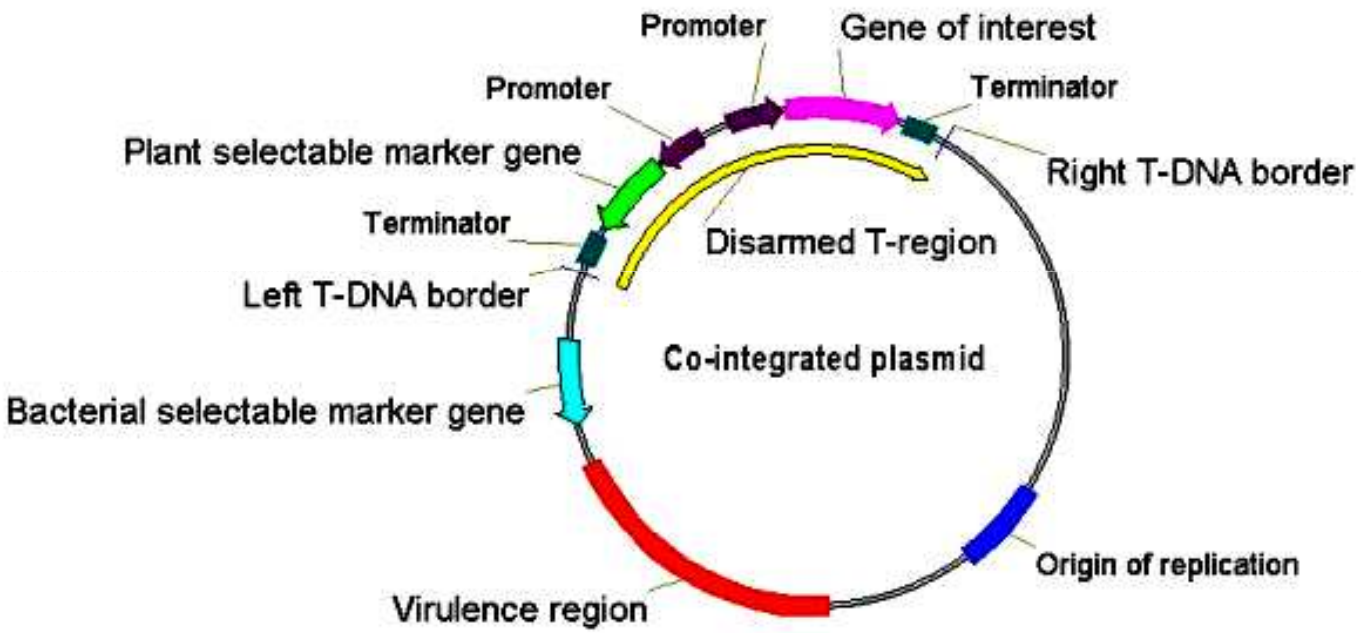
4. The IV present in *E.coli* is transferred to *A. tumefaciens* by conjugation using pRK2013 & pRN3 are helper plasmids.







Co-integrated plasmid

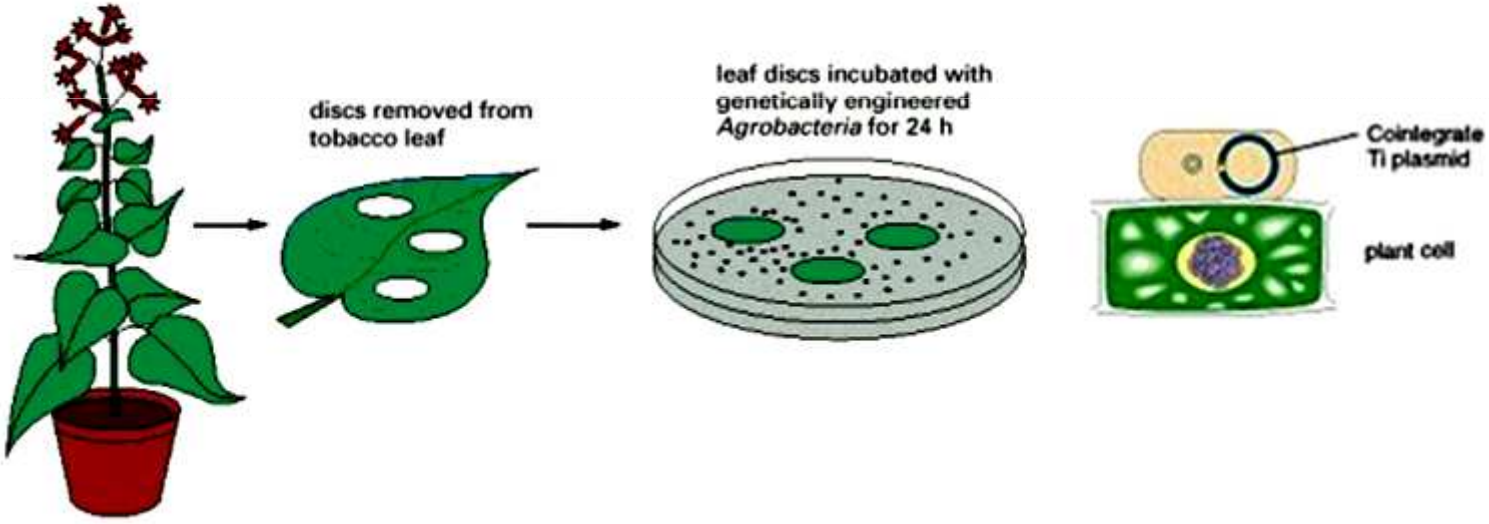


Co-Culturing

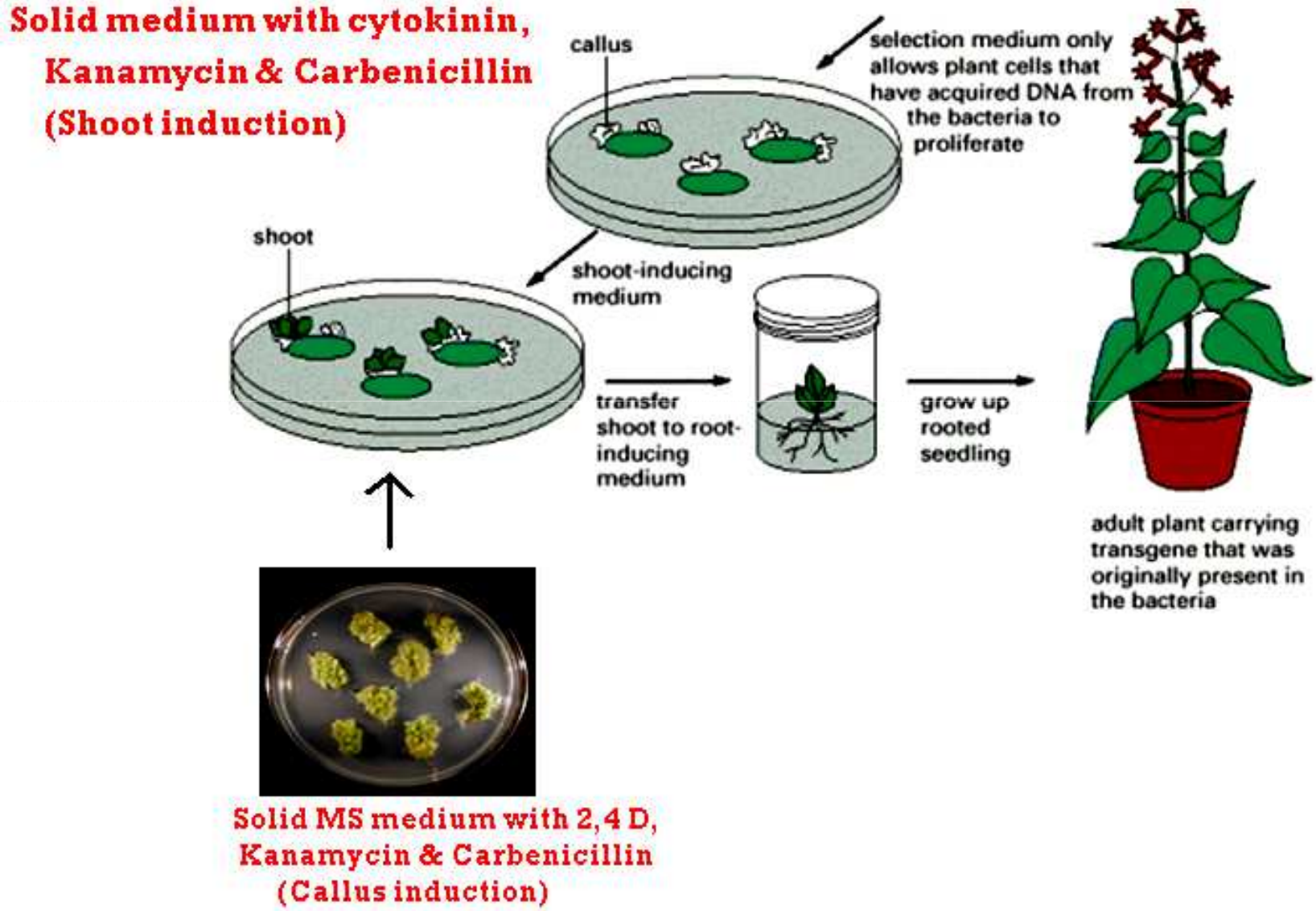
"Created *Agrobacterium* strain is allowed to infect either plant protoplast or small plant tissue"

De Block et al., 1984 – Protoplasts

Horsch et al., 1984 – Small leaf discs



Regeneration of Transformants



Applications in Sericulture

- **Pest Resistance Mulberry varieties (other than insect pests; as silkworm is also an insect).**
- **Disease Resistance Mulberry Varieties.**
- **Herbicide Resistance Mulberry Varieties.**
- **Nitrogen Fixing Mulberry varieties.**
- **High Leaf Yielding Mulberry Varieties.**
- **Improved Quality of Mulberry Leaf.**

Applications in Sericulture Con..

- **New strains of silkworm with improved yield and quality.**
- **New strains of silkworm with more resistance/tolerance against disease causing pathogens *i.e.*, Protozoan, Viral, Fungal & Bacterial. As well as Pests *i.e.*, Uzi fly.**
- **Production of beneficial proteins for human benefits *eg.*, Insulin, thrombolytic enzymes, growth hormone, *etc.*, instead of silk.**

Acknowledgements
to

INTERNET

FOR
PICTURES