# Introduction to Gene Transfer

## (some practical applications of Molecular Biology)

# Genetic Engineering of Animals

- The term “transgenic animals” describes animals whose chromosomes contain stably integrated copies of genes or gene constructs derived from other species or not normally found in the host animal.

- The foreign or introduced genes are referred to as ***transgenes*** to distinguish it from the endogenous genes.

- The animals which receive the foreign or introduced genes are called ***transgenic animals***.

- Mice, rats or other small mammals are often used to introduce foreign DNA into oocytes or embryos (blastocysts).

- The application of genetic engineering technologies to higher animals has three major goals:

i. ***The development of gene-transfer techniques for future use in treating inherited diseases in humans by "gene therapy"***

- ***ii. The introduction or enhancement of desirable traits in domestic animals***

- ***iii. The production of valuable products by transgenic animals.***

- In all these, the mouse has been the organism of choice for developing gene-transfer methodologies in higher animals.

# Microinjection of DNA into Fertilized Eggs

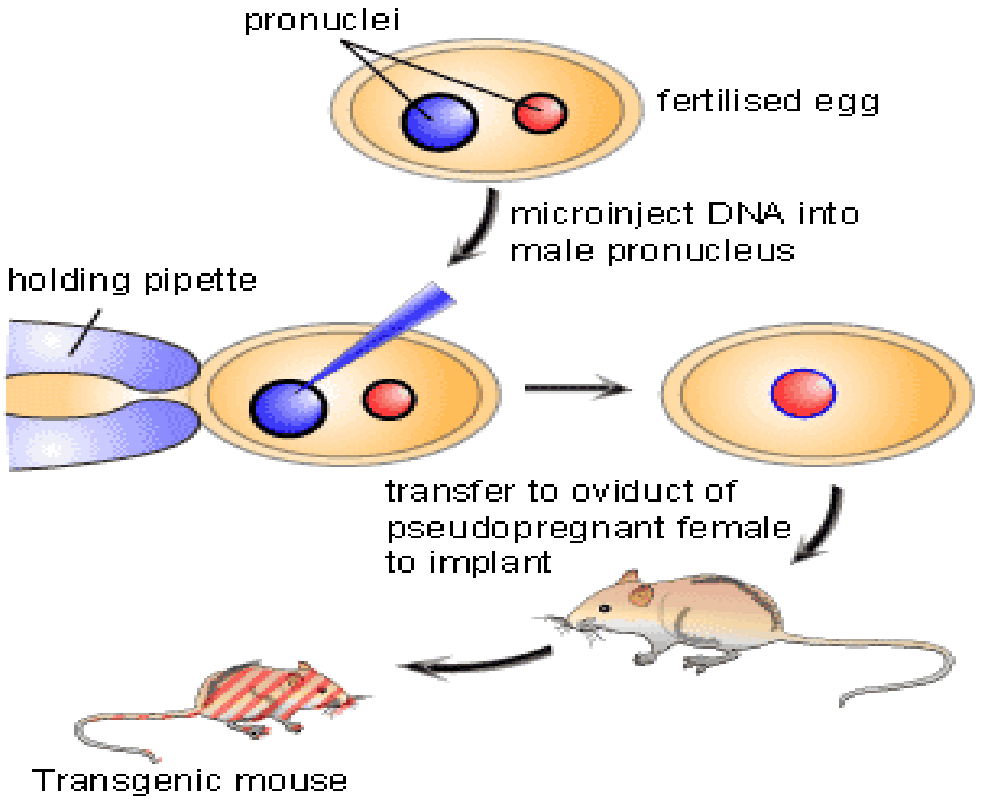
- Central to the process of making transgenic mice is the physical introduction of cloned DNA fragment into fertilized one-cell mouse eggs.

- Microinjection remains the most popular and successful of the methods currently available for generating transgenic animals.

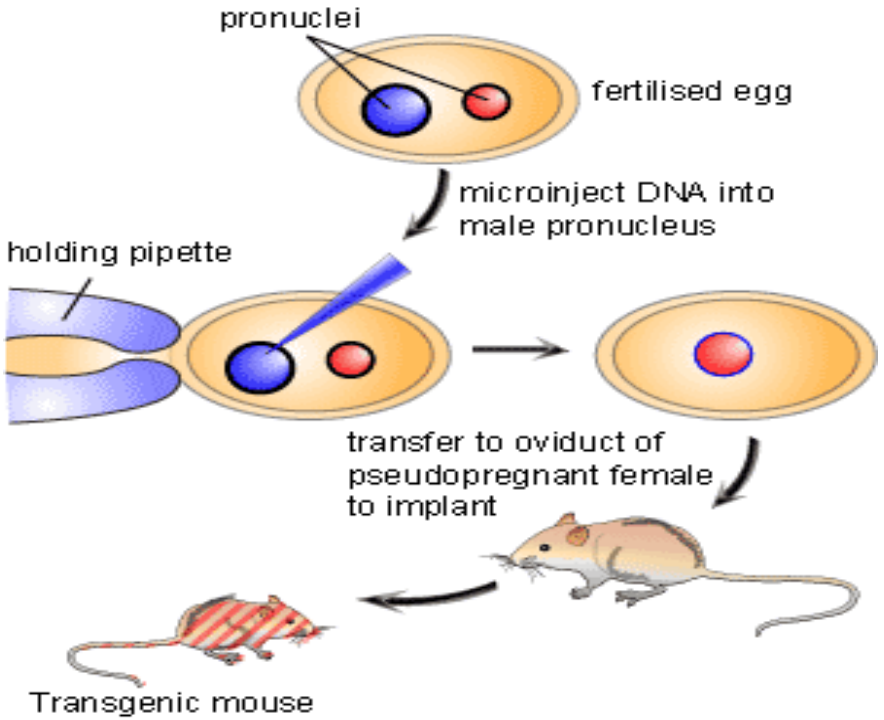
- Microinjection continues to be the method of choice, because the advantages of speed and reliability far outweigh the demands place on the investigator for precision technical skill and expensive equipment.

- The first successful production of transgenic mice using pronuclear microinjection was reported in 1980.

- A pronucleus is the nucleus of a sperm or an egg cell during the process of fertilization, after the sperm enters the ovum, but before they fuse.



- The pronuclear microinjection method of producing a transgenic animal begins with the introduction of linear DNA sequences into the chromosomes of fertilized eggs

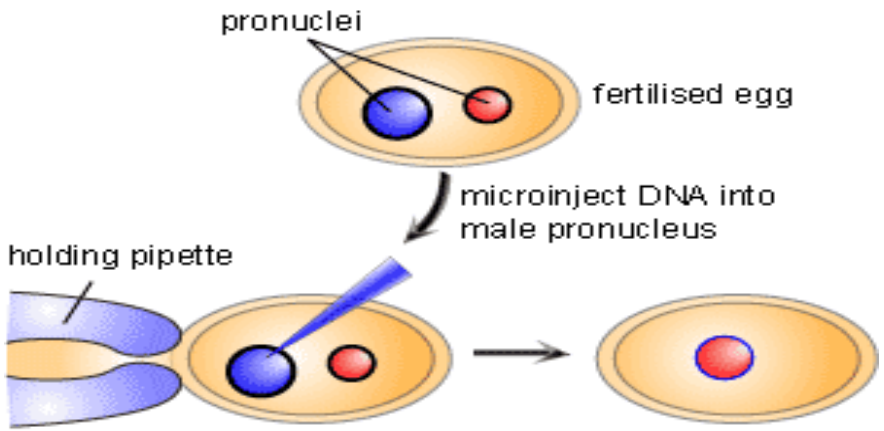


- If this transferred genetic material is integrated into one of the embryonic chromosomes, the animal will be born with a copy of this new information in every cell.

- The foreign DNA must be integrated into the genome prior to the doubling of the genetic material that precedes the first cleavage.

- If this does not occur, only a few cells will integrate the gene.

- For this reason, the DNA is introduced into the fertilized egg at the earliest stage, which is the pronuclear period immediately following fertilization.



- The DNA may be injected into either of these pronuclei with no difference in results.

- Usually the injection is into the male pronucleus because it is larger than the female nucleus and also closer to the oocyte surface.

- These oocytes are subsequently transferred into the uterus of the ***pseudopregnant*** recipient animals and then develop to term.

- Pseudopregnancy is the appearance of clinical signs and symptoms associated with pregnancy when the organism is not actually pregnant.

- It is generally estimated that false pregnancy is caused due to changes in the endocrine system of the body, leading to the secretion of hormones which translate into physical changes similar to those during pregnancy.

- Microinjection is performed when an embryo is brought into the injection position using the holding capillary.

- The tip of the injection capillary is aligned and inserted directly into the pronucleus.

- Approximately 1 pl DNA ($10^{-12}$ of a liter) solution is injected during every injection process.

- The injection is considered successful when the pronucleus increases in size.

- The percentage of eggs that survive the manipulation and develop to term varies, but it is usually between 10 and 30 percent.

- Of the survivors, the number that have the foreign DNA integration into their chromosome is between a few percentage and 40 percent.

- The DNA introduced will integrate randomly without preference for a particular chromosomal location.

# Transfer of embryonic stem cells

- Even though direct injection of DNA into the pronuclei of fertilized mouse eggs is an efficient way of producing transgenic mice, there is no opportunity to manipulate or otherwise control DNA integration.

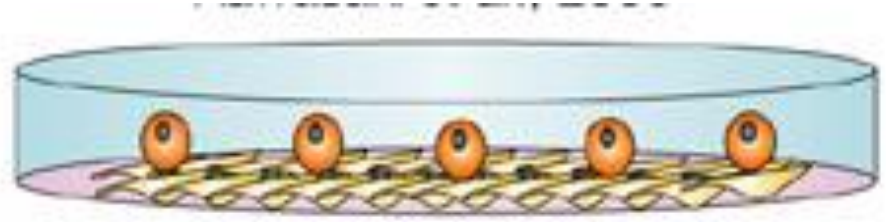
- However, this can be done by introducing the DNA into special cells called ***embryonic stem cells*** (**ES cell**) and then injecting the transfected cells into embryos, where they become incorporated into the developing embryo.

- Embryonic stem cells are derived from the inner cell masses of normal blastocysts (mostly early mouse embryos). OR

- They are obtained by culturing the inner cell mass of mouse blastocysts.

- They are grown in tissue culture just like other cells except that the ES cells must be prevented from differentiating by growing them on a feeder layer, or by adding ***leukemia inhibitory factor*** (**LIF**) to the culture medium.

A feeder layer

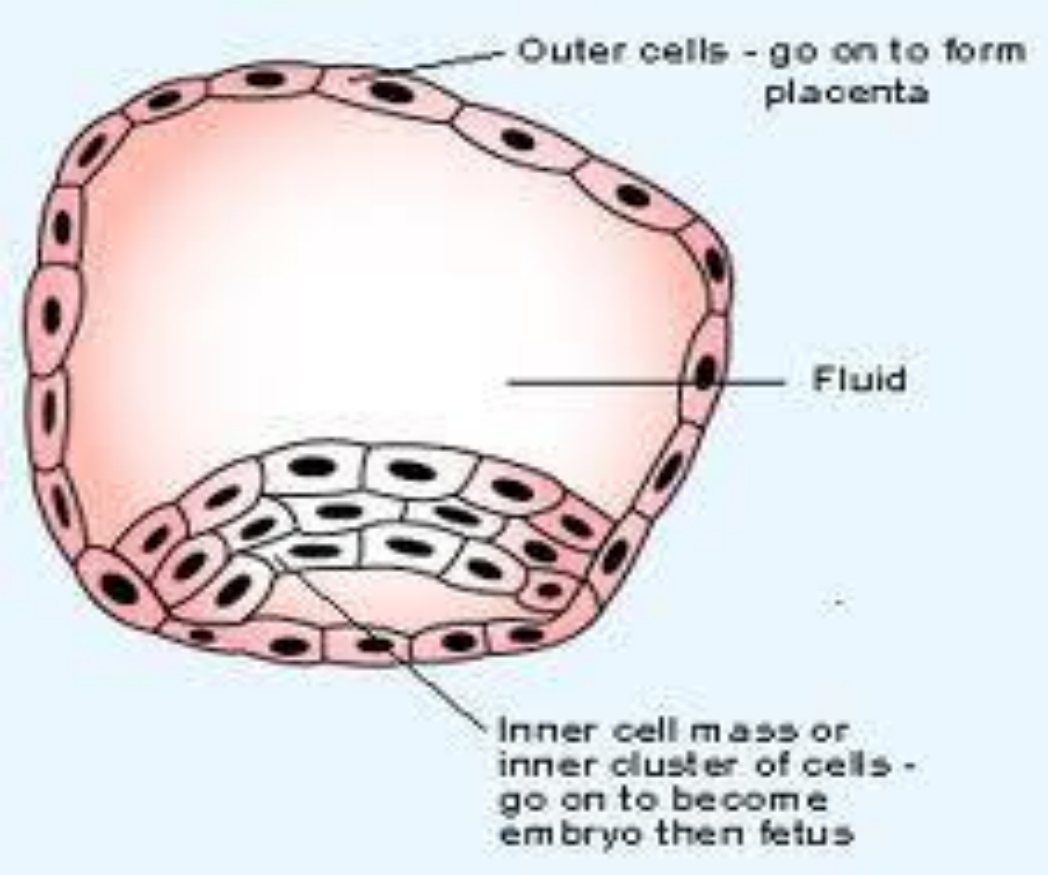


A monolayer of cells that has been treated so that the cells can no longer divide

- Under these conditions, ES cells can be grown for many weeks but will still not divide or differentiate.

- These extraordinary ES cells can be regarded as the equivalent of unicellular mice, and when they are injected into mouse **blastocysts**, they are able to participate in the formation of tissues or become part of the embryo.

A blastocyst is a structure formed in the early development of mammals.



- It possesses an inner cell mass (ICM) which subsequently forms the embryo.

- These cells are pluripotent; they can develop into any type of tissue.

- Transfection allows foreign genetic material to be inserted *in vitro* into these cells.

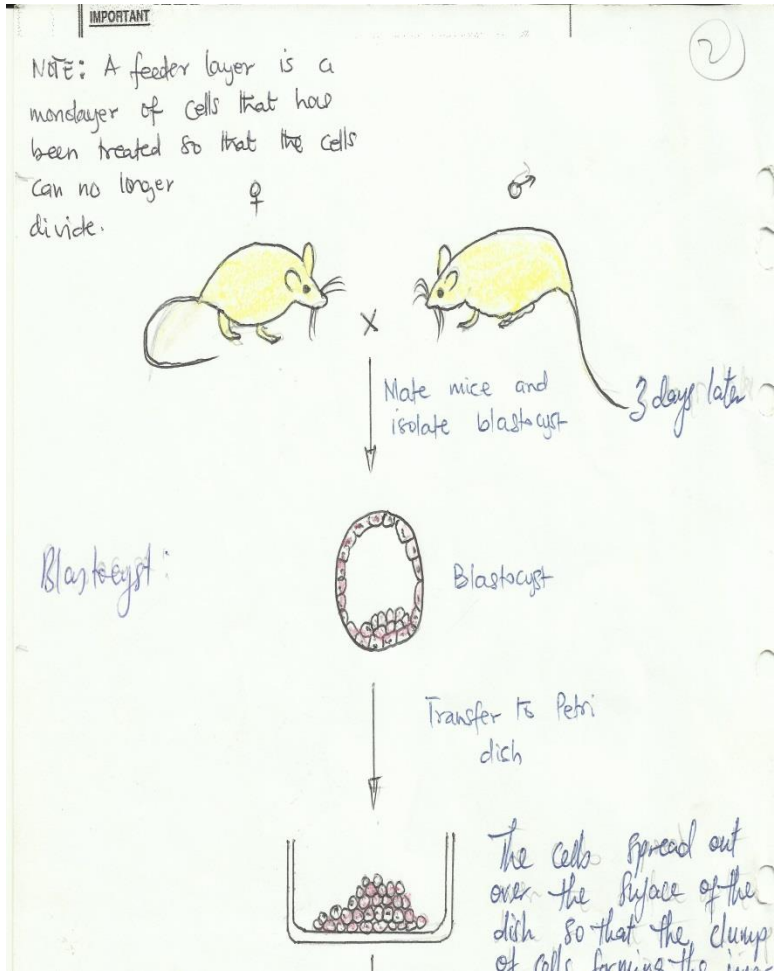
- The target of this process is homologous recombination with the chromosome of the cell, i.e., introduction or exchange of DNA at one location with homologous DNA sequences.

- The most important advantage for the gene transfer into mice through the ES cells is that, cells carrying the transgene can be selected for before being injected into a blastocyst.

# ES Cells produced from mouse blastocysts. summary

- Mice are mated, and 3 days later, blastocysts are isolated and cultured in petri dishes.

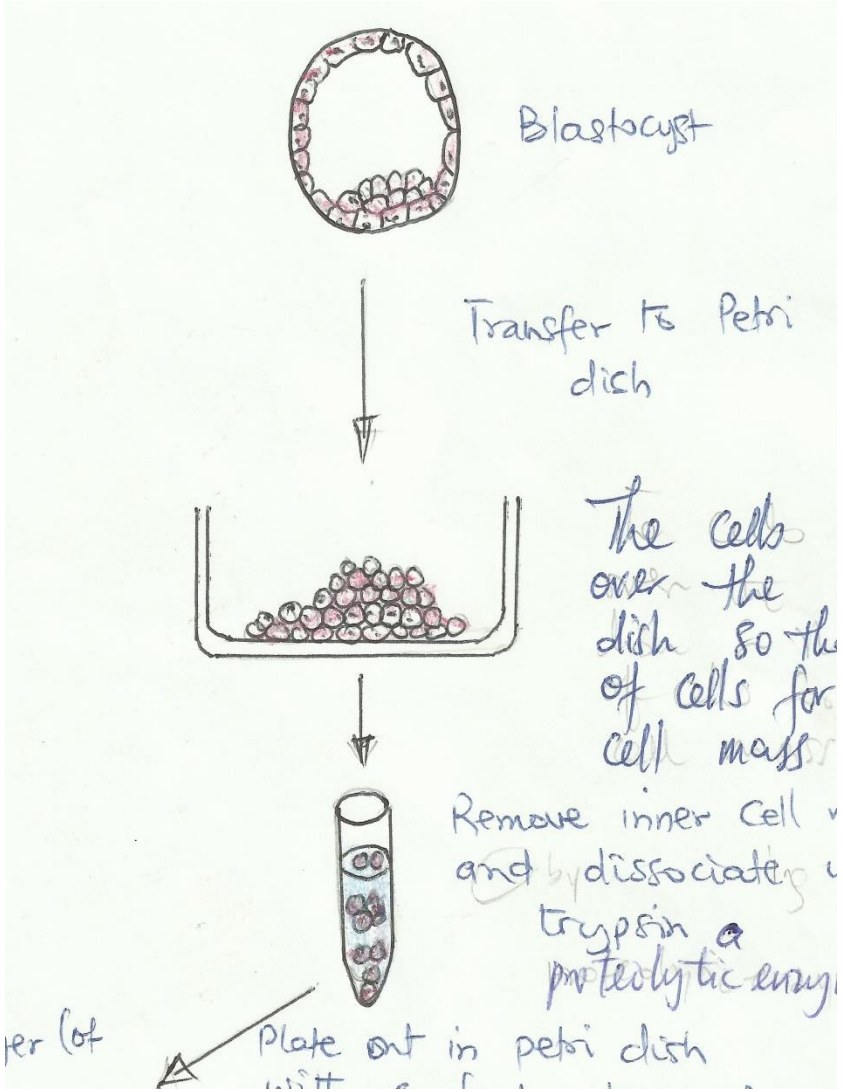
- The cells spread out over the surface of the dish so that the clump of cells forming the inner cell mass, and corresponding



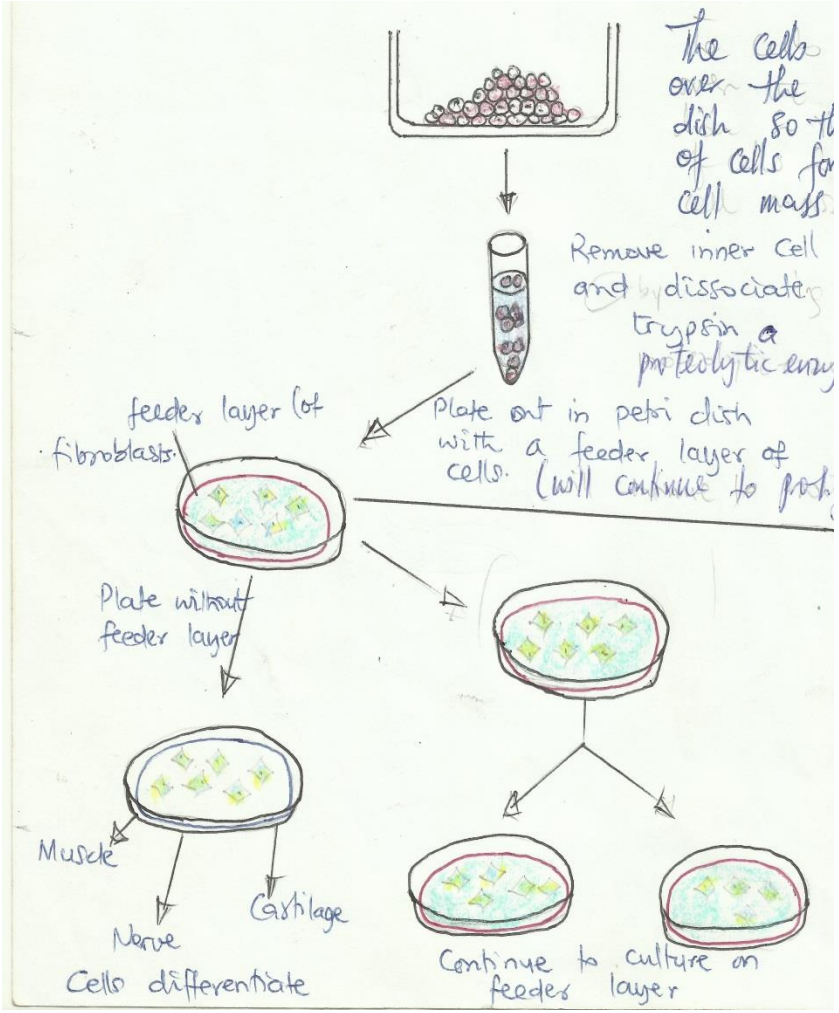
to the future embryo, can be removed.

- The clump of cells is dissociated into single cells using trypsin, which is a proteolytic enzyme.

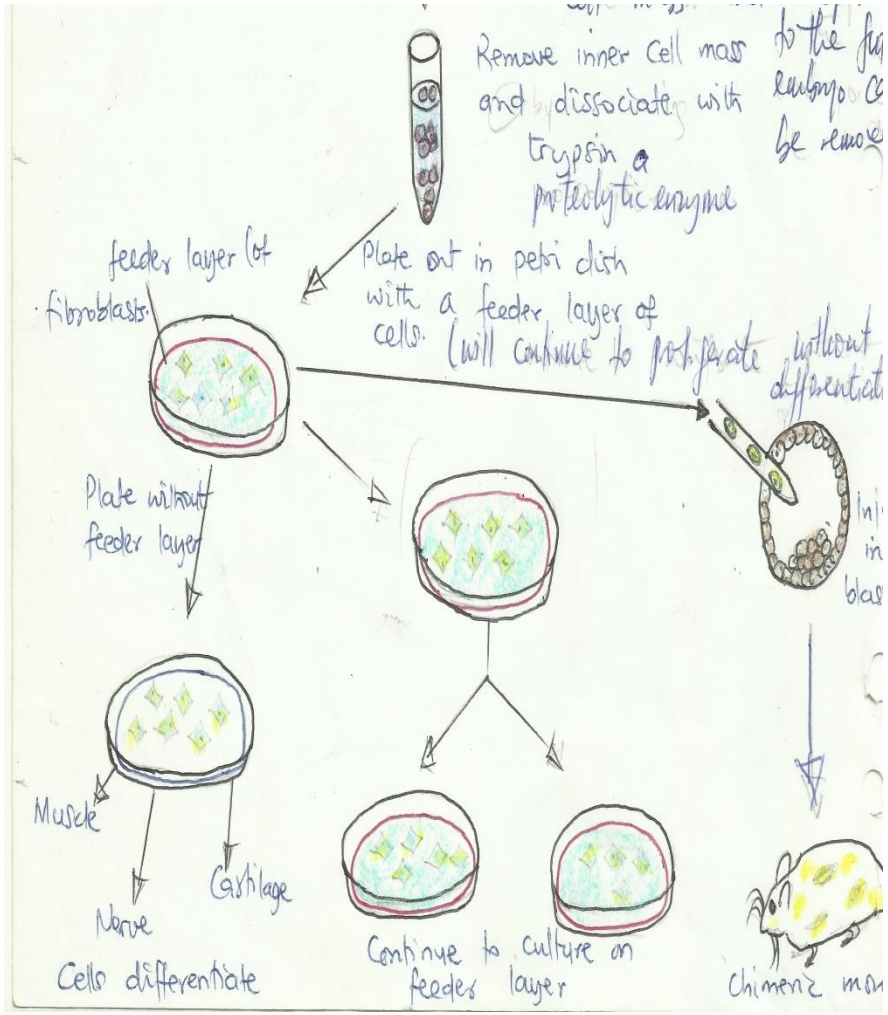
- If ES cells are plated



out on a plain culture dish surface, they will differentiate into a variety of tissues, but if they are grown on a feeder layer, they will continue to proliferate and can be subcultured repeatedly.



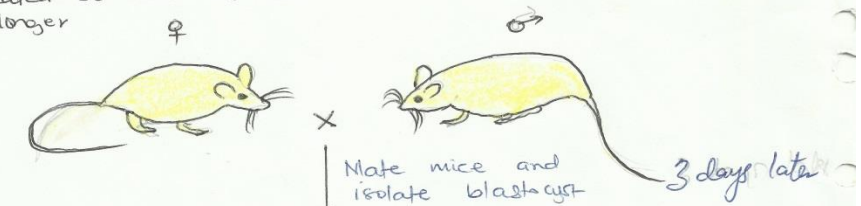
- The cells can be microinjected into a blastocyst where they will become integrated into the inner mass cell and take part in the formation of many tissues of the chimeric mouse.



- It is usual to use ES cells and recipient blastocysts derived from mice with different coat colours so that the contribution to ES cells to the chimeric offspring can be assessed by simply looking at their coat colour.

**IMPORTANT**

NOTE: A feeder layer is a monolayer of cells that have been treated so that the cells can no longer divide.



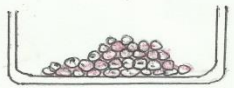

Mate mice and isolate blastocyst

3 days later

Blastocyst:

Blastocyst

Transfer to Petri dish



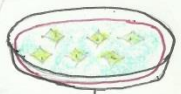
The cells spread out over the surface of the dish so that the clump of cells forming the inner cell mass and corresponds to the fu embryo ca be remove

Remove inner cell mass and dissociate with trypsin a proteolytic enzyme

Plate out in petri dish with a feeder layer of cells. (will continue to proliferate without differentiat

feeder layer (of fibroblasts)

Plate without feeder layer



Inj in blas

Muscle

Nerve

Cartilage

Cells differentiate

Continue to culture on feeder layer

Chimeric mo

- In early experiments, ES cells were infected with retroviral vectors, or transfected with plasmids carrying the ***neo*** gene.

- This gene confers resistance to the antibiotic G418.

- Only cells that have taken up the *neo* gene will grow in a medium containing G418.

- The selected G418-resistant cells were then introduced into mouse blastocyst.

- Not only will the resulting mice have the neo gene integrated into their genomes, but also the gene was transmitted to the offspring of the mice, and cell lines from the $F_2$ generation were also G418-resistant.

- Because ES cells can be manipulated in vitro before injection into the embryo, mouse geneticists can use homologous recombination to produce transgenic mice with mutations in specific genes, or to replace a mutant gene with a normal equivalent.

# Transgenes can be regulated in a tissue-specific pattern

- Although a transgene integrates in a chromosomal location different from that of its endogenous counterpart, it is often expressed in a manner that mimics the expression of the endogenous gene.

- To determine the pattern of expression, various tissues are analyzed for the presence of RNA or protein products encoded by the transgene.

- Species differences may be captalized on to distinguish the transgene product from the endogenous counterpart.

- For example, the RNA encoded by the human insulin gene can easily be differentiated from the RNA transcribed from the mouse insulin gene.

- *Insulin is a polypeptide hormone involved in the regulation of glycogen metabolism.*

- *This protein is normally produced in the β cells, which are found in the endocrine cells called, the islets of langerhans, in the pancreas.*

- When transgenic mice harboring the human insulin gene were analyzed, human insulin RNA was found in the pancreas but not in other tissues.

- Transcription of the human insulin transgene was induced by the same signals that induced the endogenous mouse insulin gene.

- Therefore, not only can a foreign transgene be expressed in the correct tissue, but it may be subjected to the same regulatory signals as the endogenous genes.

# Genetic Engineering of Plants

- Genetic manipulation of plants has been practiced for many hundreds of years with great success by plant breeders, and plant breeding has become a very sophisticated branch of applied genetics.

- Breeders have developed elegant schemes for crossing plants to introduce and maintain desirable traits, and the yields like maize and wheat have steadily increased over the past 70 years.

- Genetic engineering is an effective way to increase crop yield and ward off diseases or insects that kill crops.

- Genetically modified plants are already used by farmers in many places, and a reason for their widespread use may be the ease of creating genetically altered plants.

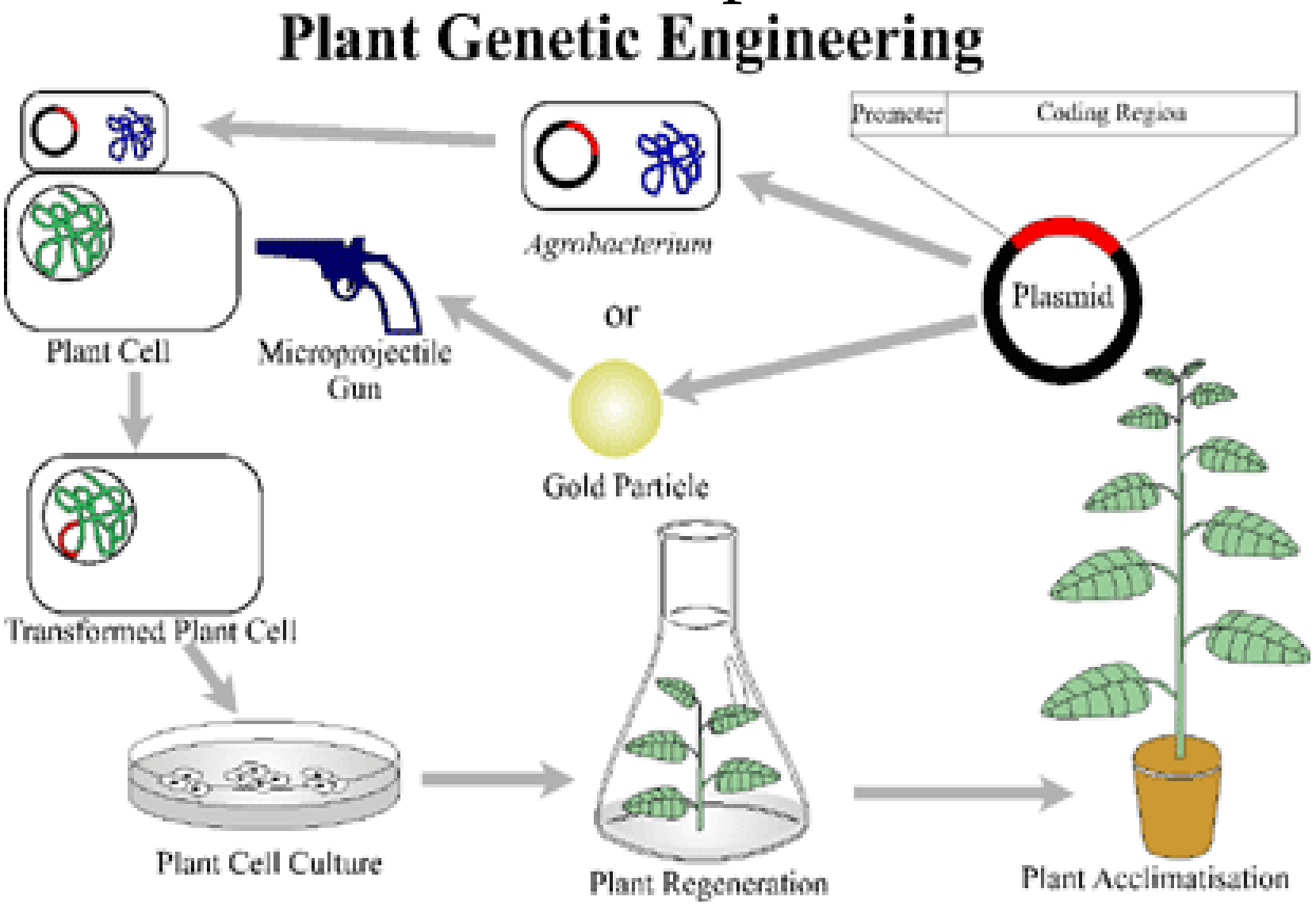
- However, the methods of classical plant breeding are slow and uncertain.

- To introduce a desire gene or set of genes by conventional methods requires a sexual cross between two lines, and then repeated back-crossing between the hybrid offspring and one of the parents until a plant with the desired characteristics is obtained.

- This process, however, is restricted to plants that can sexually hybridize.

- Recombinant DNA technology techniques promise to circumvent these limitations by enabling plant geneticists to identify and clone specific genes for desirable traits, such as resistance to insect pest, and to introduce these genes into already useful varieties of plants.

# Summarized process of



- In this case, sexual compatibility becomes irrelevant, and the process becomes faster because transgenic plants expressing the gene can be selected directly.

- Plants have a number of unique biological features that can be explored with recombinant DNA techniques.

- These features include their pattern of growth and photosynthesis.

- Most plants are totipotent, meaning an entire plant can grow from a single cell.

- Scientists can isolate a plant cell and introduce specific enzymes to the plant cell.

- These enzymes then digest the cell wall of the plant cell, creating a protoplast.

- Without a cell wall, manipulation of cellular DNA becomes much easier

- Scientists can, through various methods, introduce modified DNA into the plant genome.

- As the protoplast re-grows and divides, the modified DNA will be transferred to every cell in the new plant

# Whole Plants Can Be Regenerated from Single Cells

- An extraordinary phenomenon, and one that is very useful to the geneticists, is that whole plants can be regenerated from single cells.

- When a plant is wounded mechanically, a patch of soft cells called ***callus*** grows over the wound

- If a piece of young callus is removed and placed in a culture medium containing the appropriate nutrients and plant growth hormones, the cells

will continue to grow and divide as a suspension culture (*i.e. a culture of individual cells suspended in the liquid medium*).

- These cells can be plated out and will grow to form new calli.

- The callus will then differentiate into shoots and roots, and ultimately a whole flowering plants will be produced.

- Studies by Skoog and Miller have shown that the differentiation of the cells in a callus depends on the relative concentrations of the plant hormones (phytohormones) **auxins** and **cytokinins**.

- If the ratio of auxins to cytokinins is high, then roots will develop; shoots develop when the ratio is low.

- Like all plants, these cells are surrounded by cellulose wall and therefore DNA uptake is very difficult if not impossible.

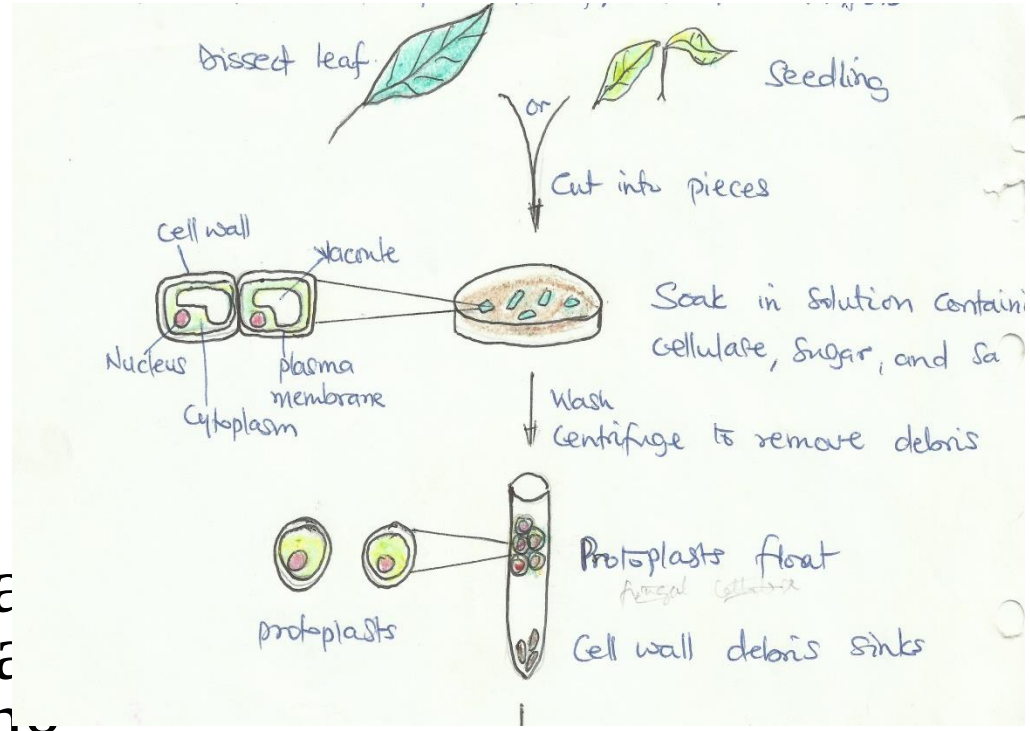
- However, the cellulose wall can be removed by treating the cells with fungal cellulase enzyme.

- The resulting ***protoplast*** is enclosed only by a plasma membrane which is more amenable to experimental manipulation.

- Protoplasts will take up macromolecules like DNA, and they are capable of regenerating whole plants through the formation of calli.

# Regeneration of Plants from Protoplasts

- The plasma membrane which is surrounded by a tough cellulose cell wall is removed by incubating pieces of plant tissues in a solution containing cellulase.




- Sugars and salts are a
  maintain osmotic bala
  protoplasts from lysing.

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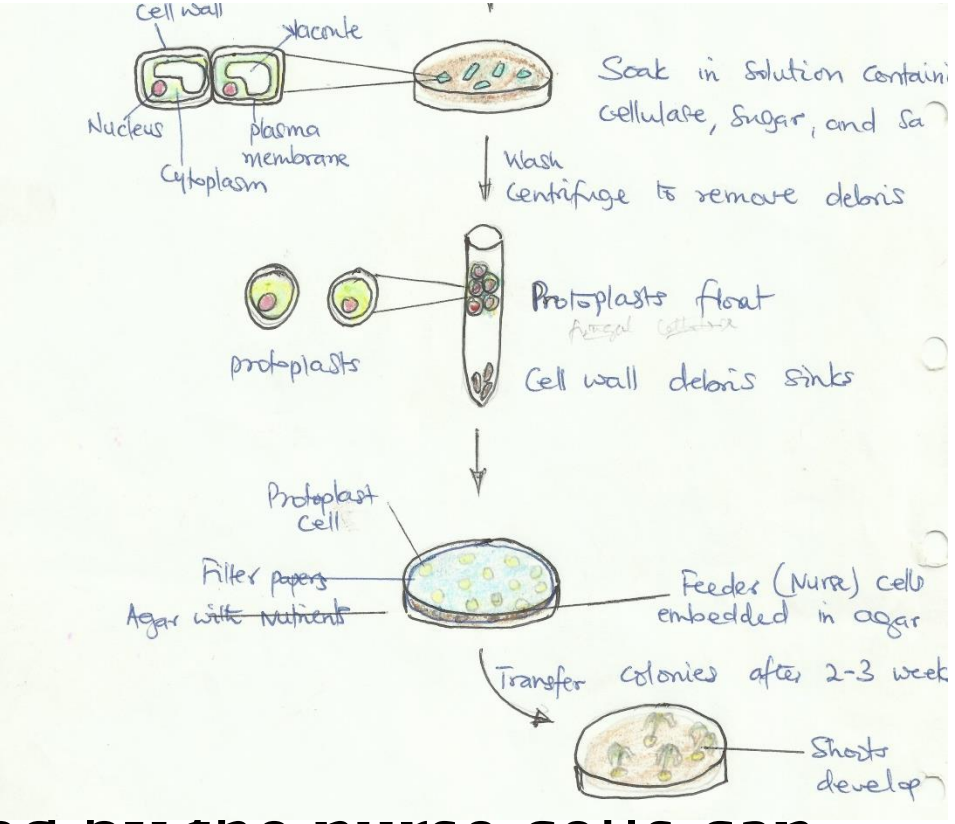
- Once the cell debris is removed, the protoplasts are placed on filter paper covering a layer of nurse cells. (*nurse cell will supply food material or nutrients to the protoplasts*).

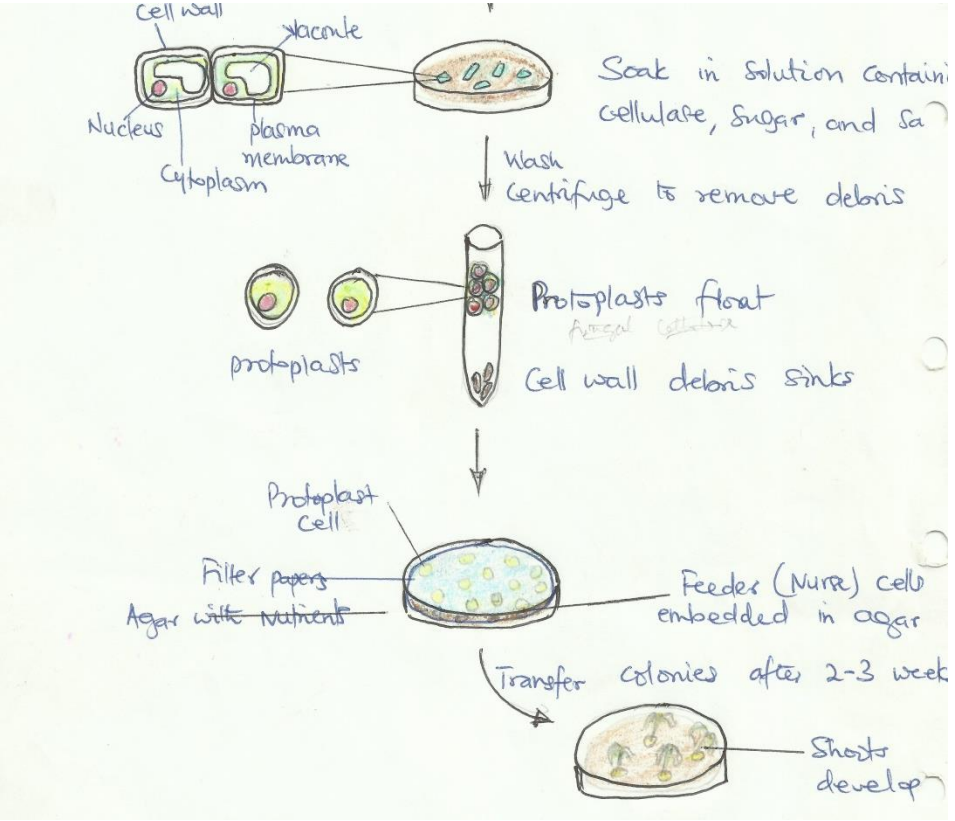



- The filter paper is impervious to the cells, but growth factors and other molecules produced by the nurse cells can

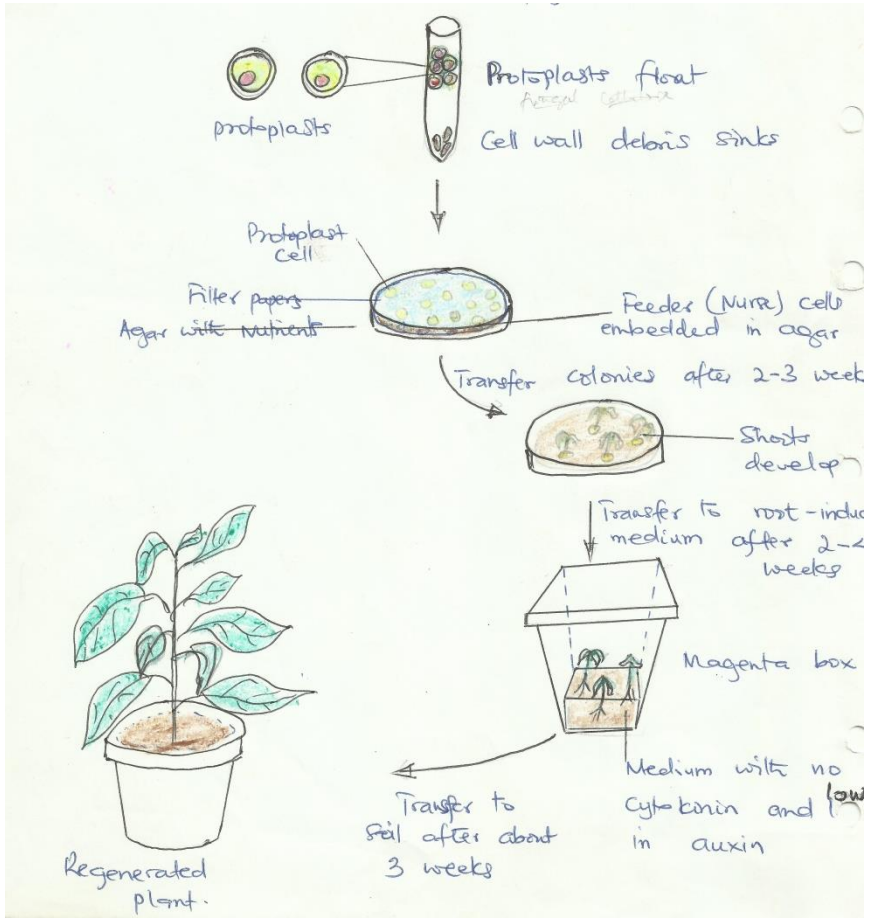
- diffuse into the protoplasts, which divide and grow to form macromolecules.

- The macromolecules are carefully transferred to a medium high in cytokinin and low in auxin.

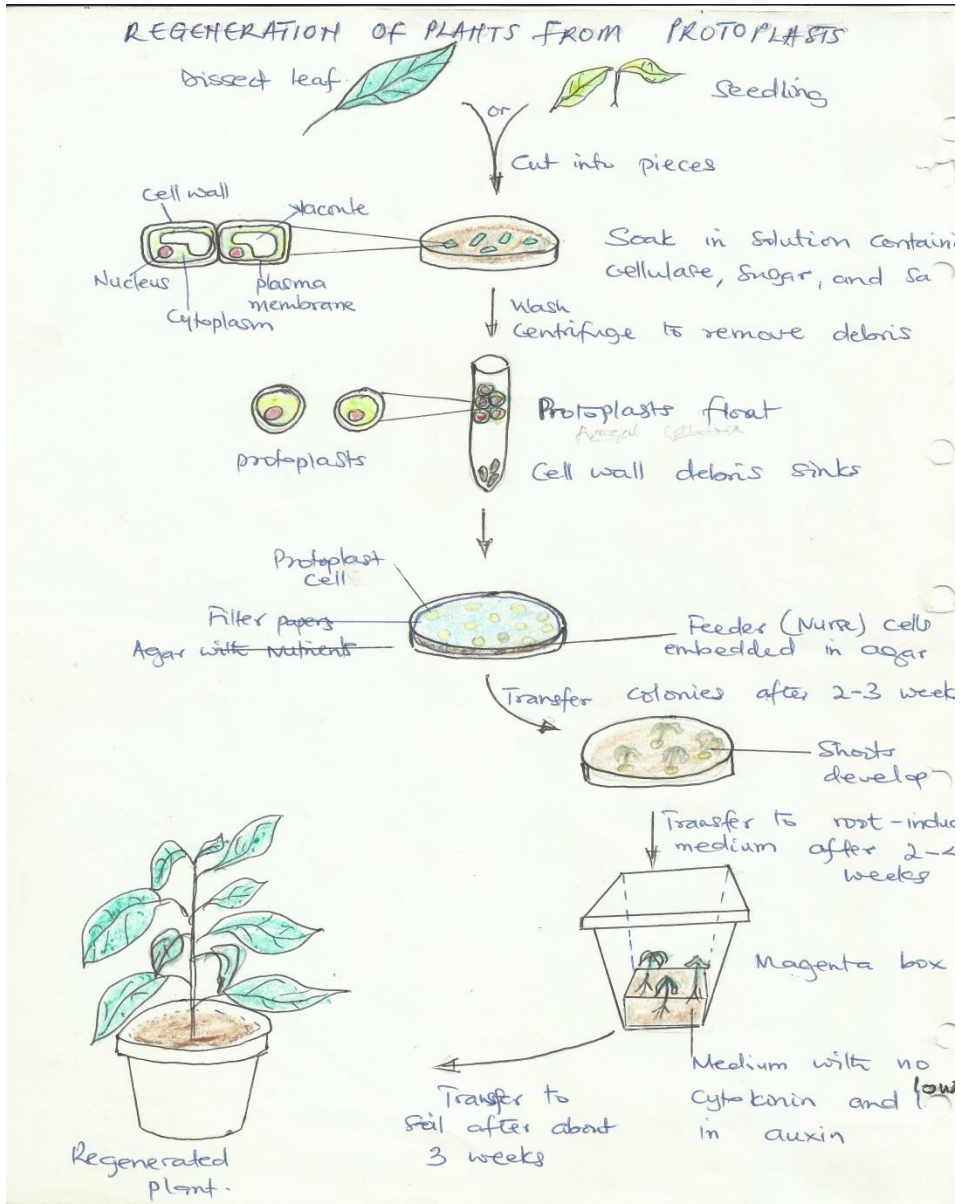
Shoots will appear in about 2 to 4 weeks.



- The cultured cells are then transferred to a container called a Magenta box, which contains root-inducing medium lacking cytokinin and low in auxin.

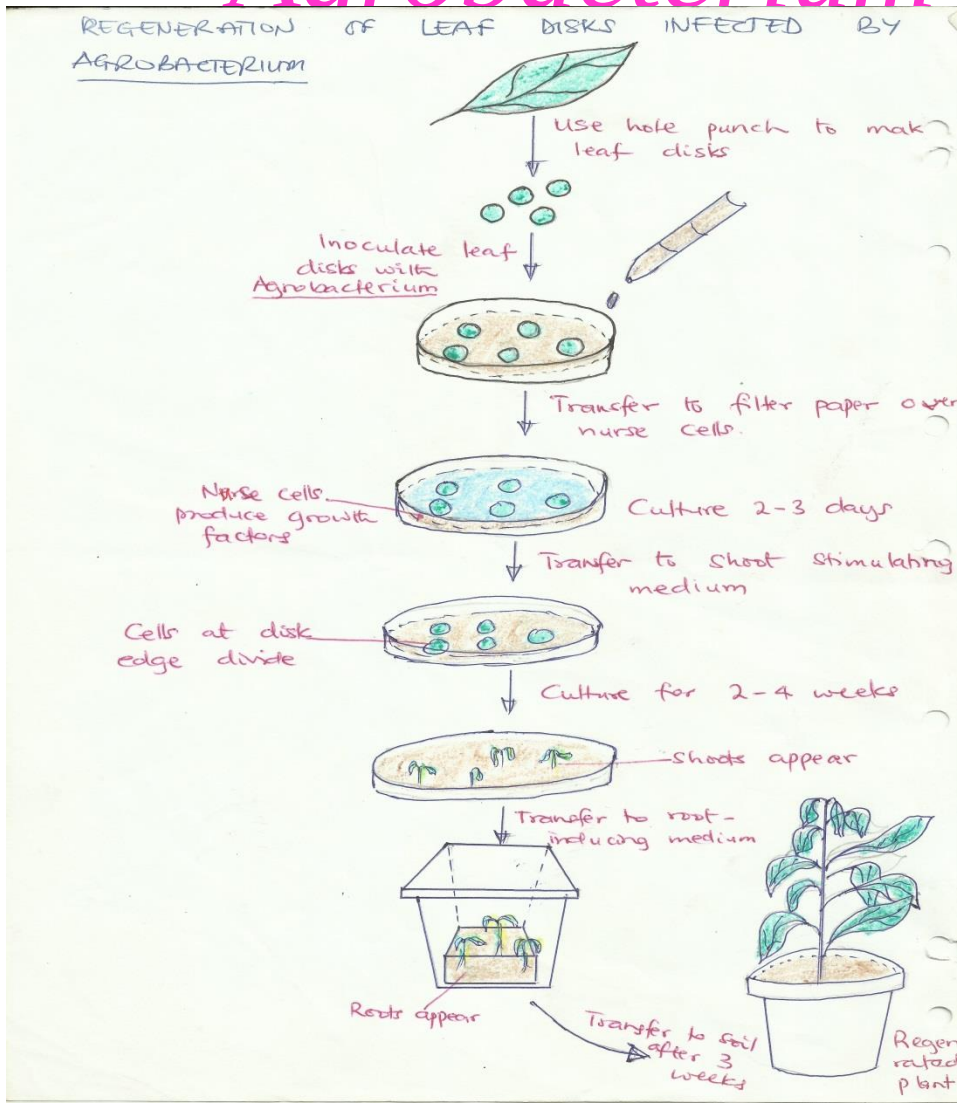



- Once the roots appear, the plantlets can be placed in soil, where they will develop into regenerated plants.


REGENERATION OF PLANTS FROM PROTOPLASTS
Dissect leaf
or
Seedling
Cut into pieces
Cell wall
Vacuole
Nucleus
Cytoplasm
plasma membrane
Soak in solution contain
cellulase, sugar, and sa
Wash
Centrifuge to remove debris
protoplasts
Protoplasts float
Cell wall debris sinks
Protoplast
Cell
Filter papers
Agar with nutrients
Feeder (NURSE) cells
embedded in agar
Transfer colonies after 2-3 week
Shoots
develop
Transfer to root-indu
medium after 2-
weeks
Magenta box
Medium with no
Cytokinin and low
in auxin
Transfer to
soil after about
3 weeks
Regenerated
plant.

- Even though protoplasts have been used successfully for many species, the most important group of agricultural plants and cereals, are very difficult to regenerate from protoplasts.

# Regeneration of Leaf disks infected by *Agrobacterium*



# Regeneration of Leaf disks infected by *Agrobacterium*

- Growing whole plants from protoplasts is not easy, even for the most amenable species of plants.

- A simple but very significant improvement came with the development of the ***leaf disc technique***.

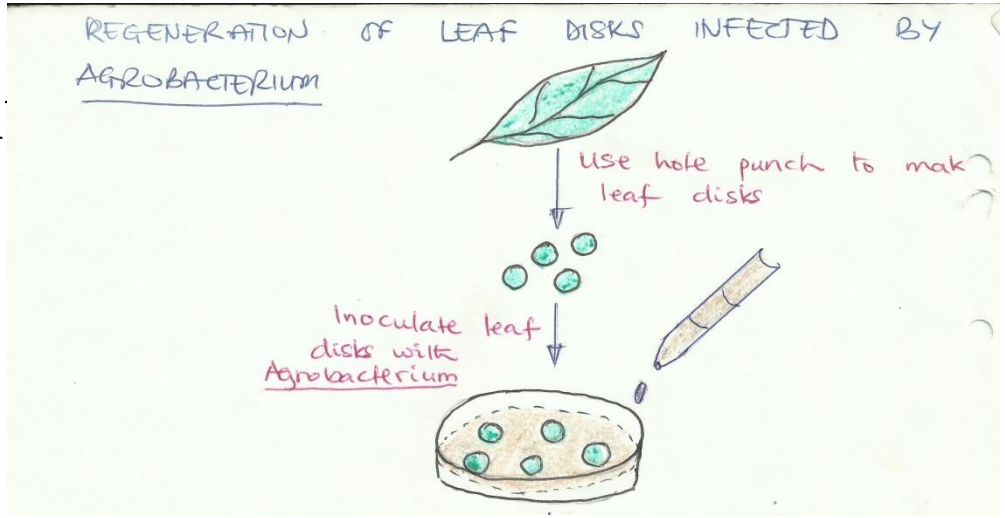
- The technique is so important because it can be used with the most effective system for

transferring genes into plants, a system that make use of the **Ti plasmid** which is carried by the bacterium ***Agrobacterium tumefaciens***.

- Leaf disks are cut ou

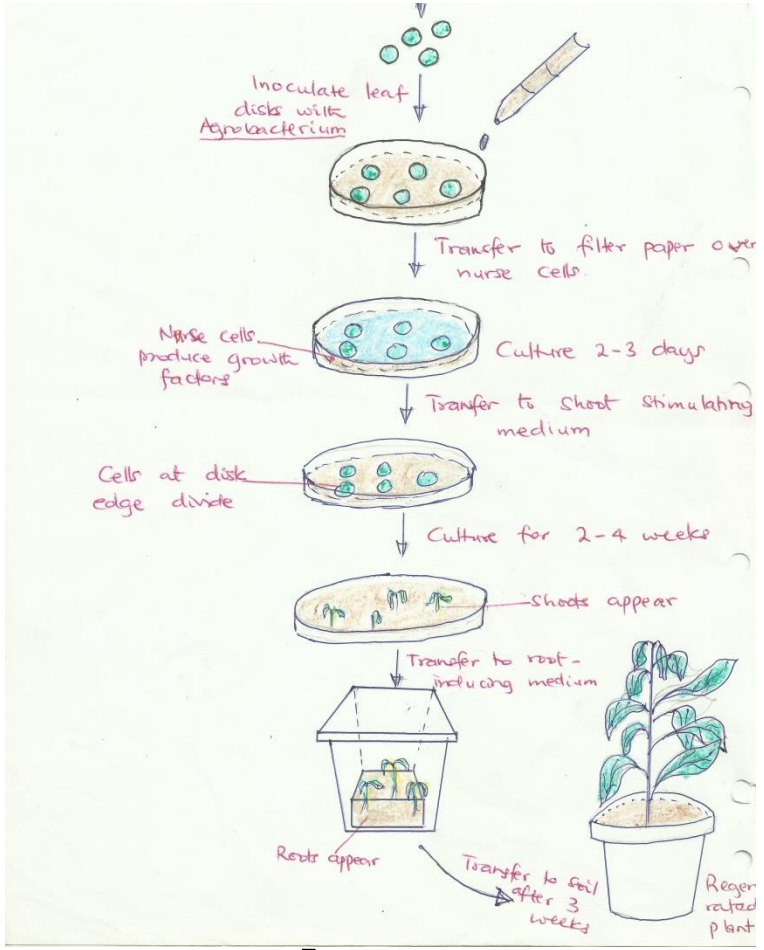
  And placed in a

  Shallow dish.




- A solution of Agrobacterium is added to efficiently expose the cells to the transferring agent.

- After a few minutes, the leaf disks are transferred onto nurse cultures containing medium that stimulates shoot development for several days.




- Wounded cells at the edge of the disk release factors that induce the Agrobacteria to infect the cells.
- The plant disks are then cultured just as described for protoplasts.

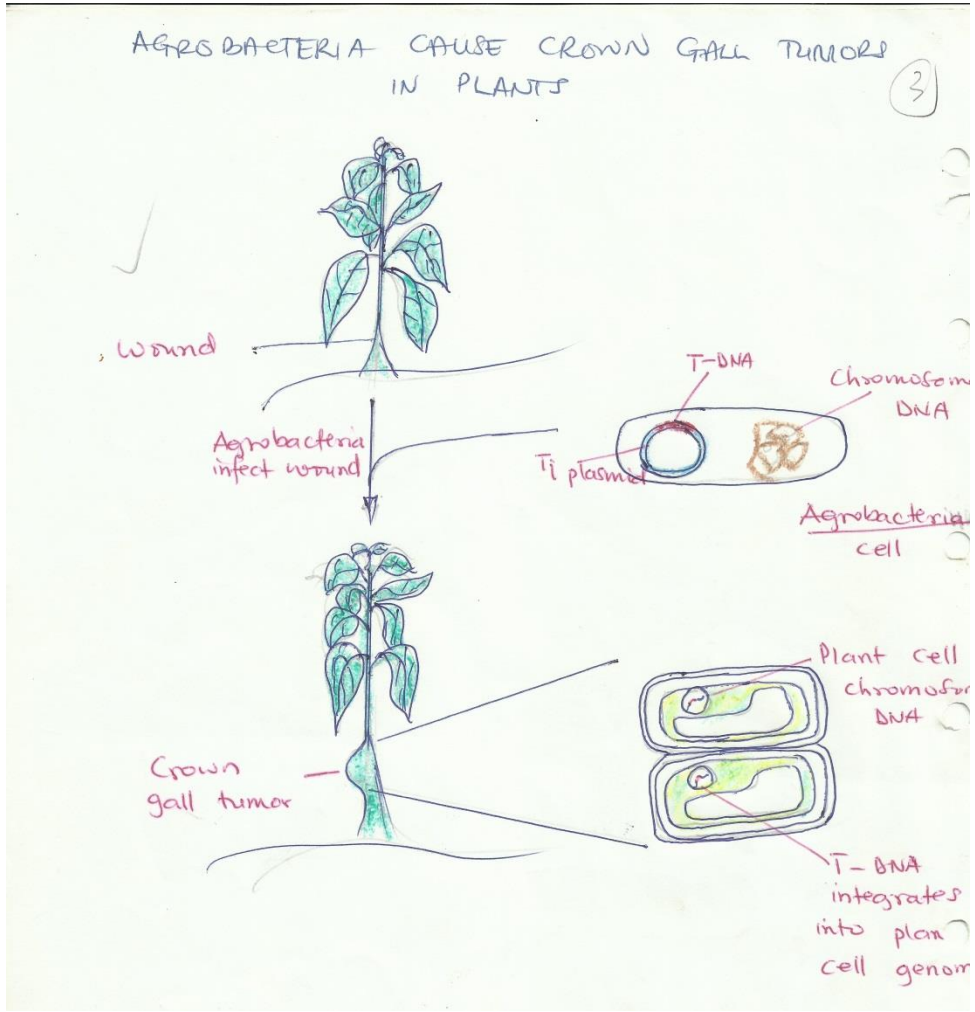
- Cells carrying the plasmids are selected by culturing in shoot-stimulating medium with appropriate antibiotic, such as kanamycin to kill the Agrobacterium but does not harm the plant cells to yield a regenerated plant.

- The whole process from, cutting out the leaf disk to having rooted plants, takes between 4 and 7 weeks.

- The process is very fast compared with protoplast cultures.

- Furthermore, this technique is applicable to a wide variety of dicotyledons and is now used routinely.

# Ti Plasmids of Agrobacterium Causes Crown Gall Tumors in Plants.

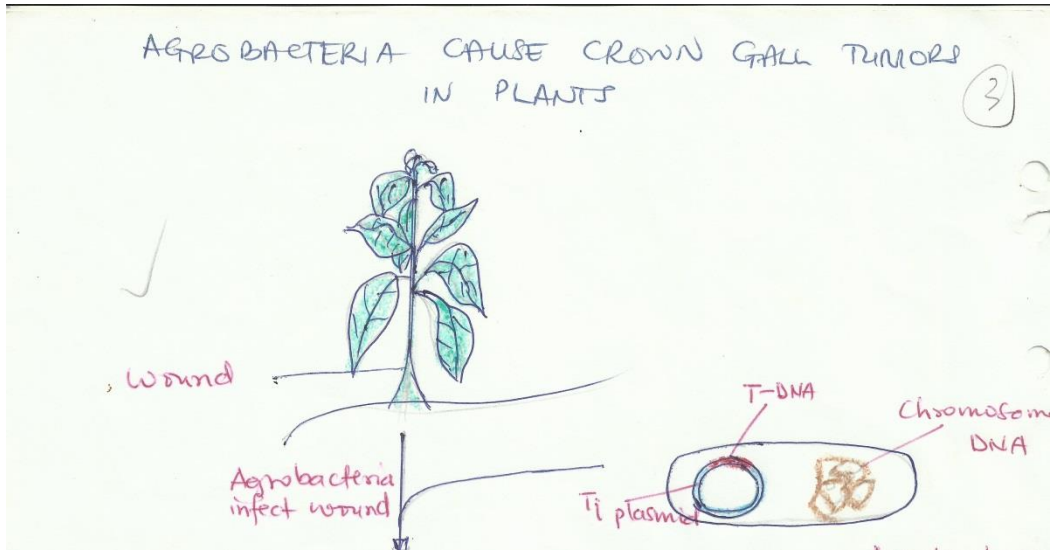


- Crown galls are tumors (i.e. cells that divide rapidly and uncontrollably) of plants that arise at the site of infection by some species of bacteriam called *Agrobacterium*.

- The cells of crown galls have the properties of independent and unregulated growth.

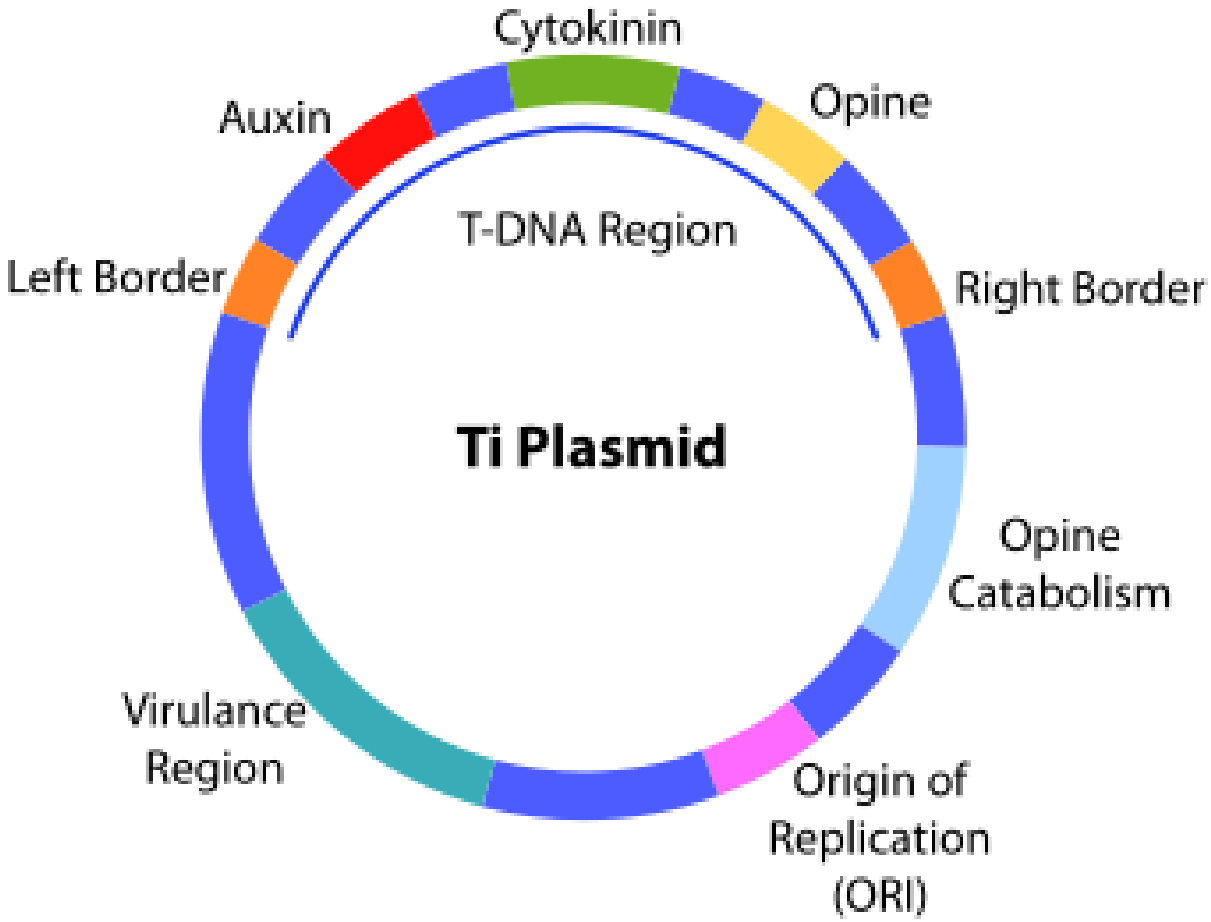
- In culture, these cells grow in the absence of the plant hormones that are necessary for the culture of normal plant cells.

- When a wounded plant is infected by *Agrobacterium*, the *Agrobacteria* cells do not enter the plant cell but transfer a DNA segment called the **T-DNA** from the circular extrachromosomal ***tumor-inducing*** (Ti) plasmid.



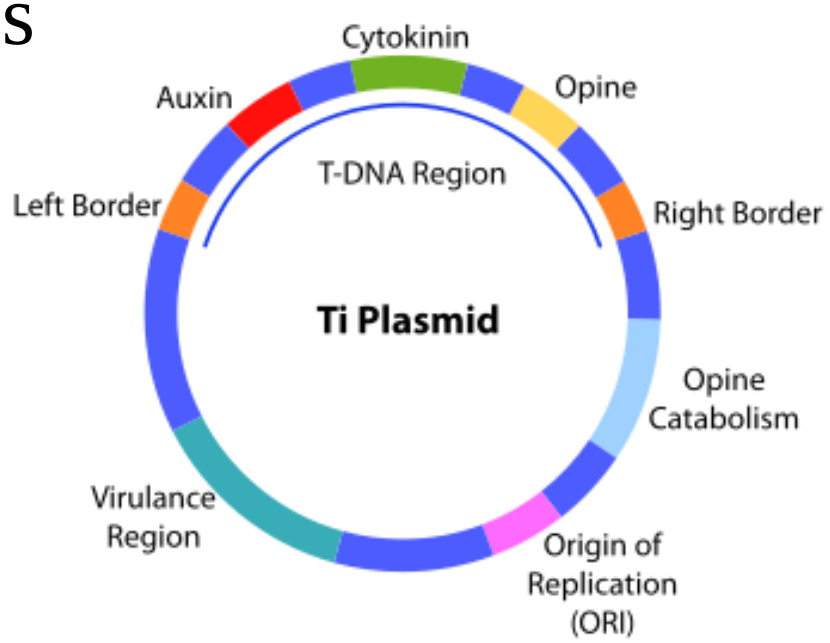
- Ti plasmids are large, circular, double stranded DNA molecules of about 200kb, and like other bacterial plasmids, they exist in *Agrobacterium* cells as independently replicating genetic units.

- Ti plasmids are maintained in *Agrobacterium* because a part of the plasmid DNA , called T-DNA, carries the genes coding for the synthesis of an unusual amino acids called opines.


Cytokinin
Auxin
Opine
T-DNA Region
Left Border
Right Border
Ti Plasmid
Opine
Catabolism
Virulance
Region
Origin of
Replication
(ORI)

- The infected plant cell is induced to synthesize these amino acids, although the plant cannot utilize them.

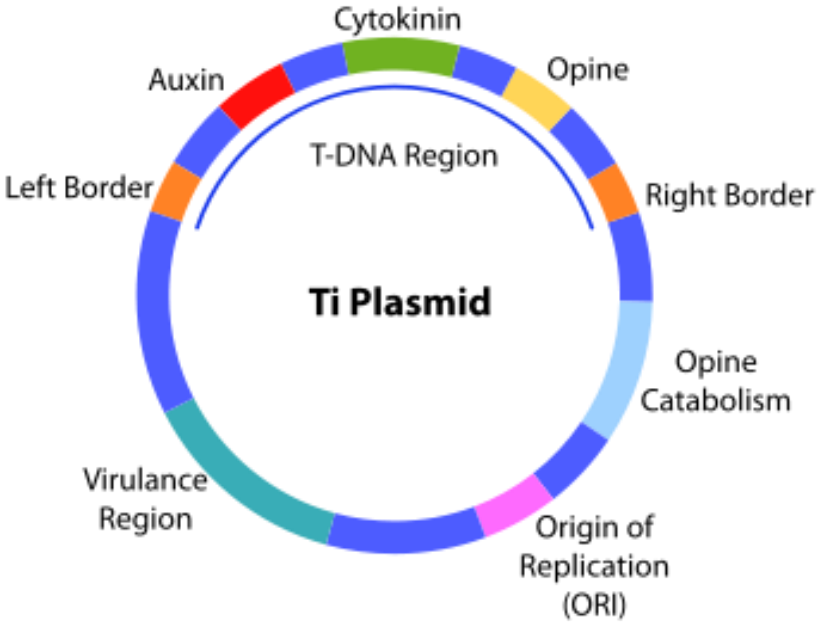
- Instead, the Ti plasmid is believed to carry genes coding for enzymes that can degrade opines to act as a nutrient for *Agrobacterium*.



- This subversion of the plant's metabolism could provide a selective for Agrobacterium.

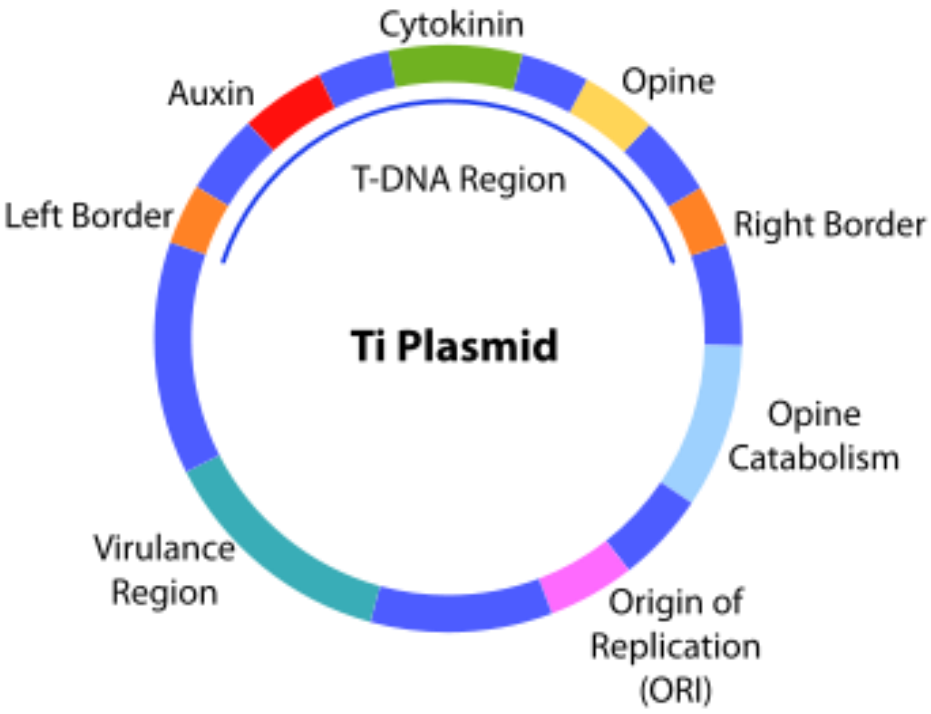
- A second set of genes in T-DNA causes the unregulated growth of the plant cell.

- Two of these genes, **iaaM** and **iaaH**, code for the enzymes that lead to the production of auxin.



- The third gene, **iptZ**, codes for an enzyme that causes the production of a second phytohormone.

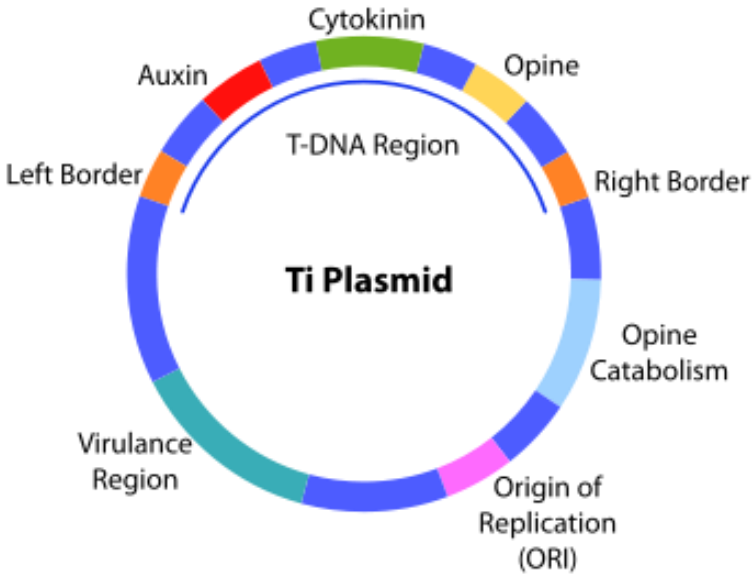
- These two hormones cause the infected plant cell to divide; and also affect the neighboring cells.



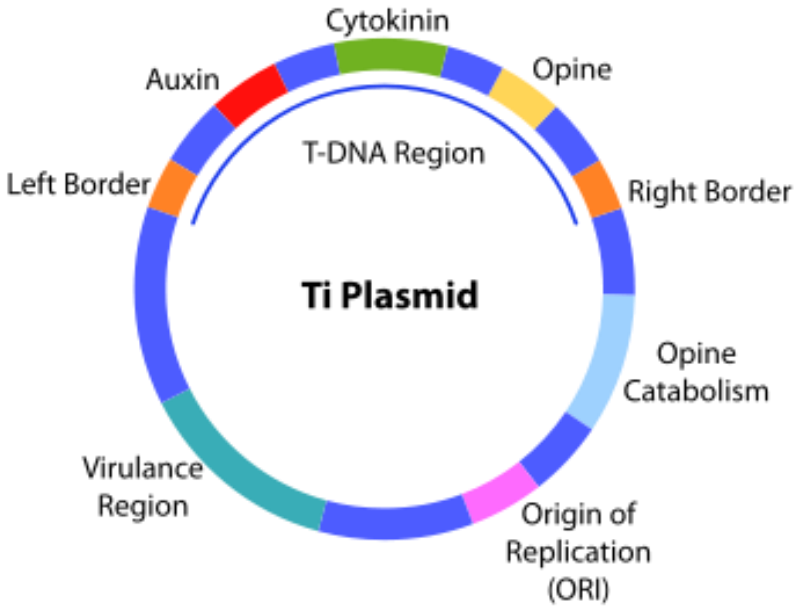
- The genes in the T-DNA are referred to as phyto-oncogenes because they induce tumor-producing, growth.

- To use the Ti plasmid as a vector for introducing new genes into plants, it is necessary to disarm the plasmid so that it

does not cause tumors.



- Researchers accomplished this task by deleting the genes in the T-DNA that encode the enzymes controlling auxin and cytokinin synthesis.




- In addition, it is necessary to introduce a gene into the T-DNA that will enable the investigator to select the transformed cells.

- Genes for antibiotic resistance are normally used for this purpose.

❖**READ ON THE MECHANISM OF TUMOR INDUCTION**

**OR**

❖**THE TRANSFER OF T-DNA FROM AGROBACTERIUM INTO A PLANT CELL**

# The Ti plasmid

- One metho d used to genetically engineer plants makes use of the Ti plasmid, found naturally in *Agrobacterium tumefaciens*.

- Naturally, *A. tumefaciens* infections are trig-gered by the wound response molecules, such as acetosynringone.

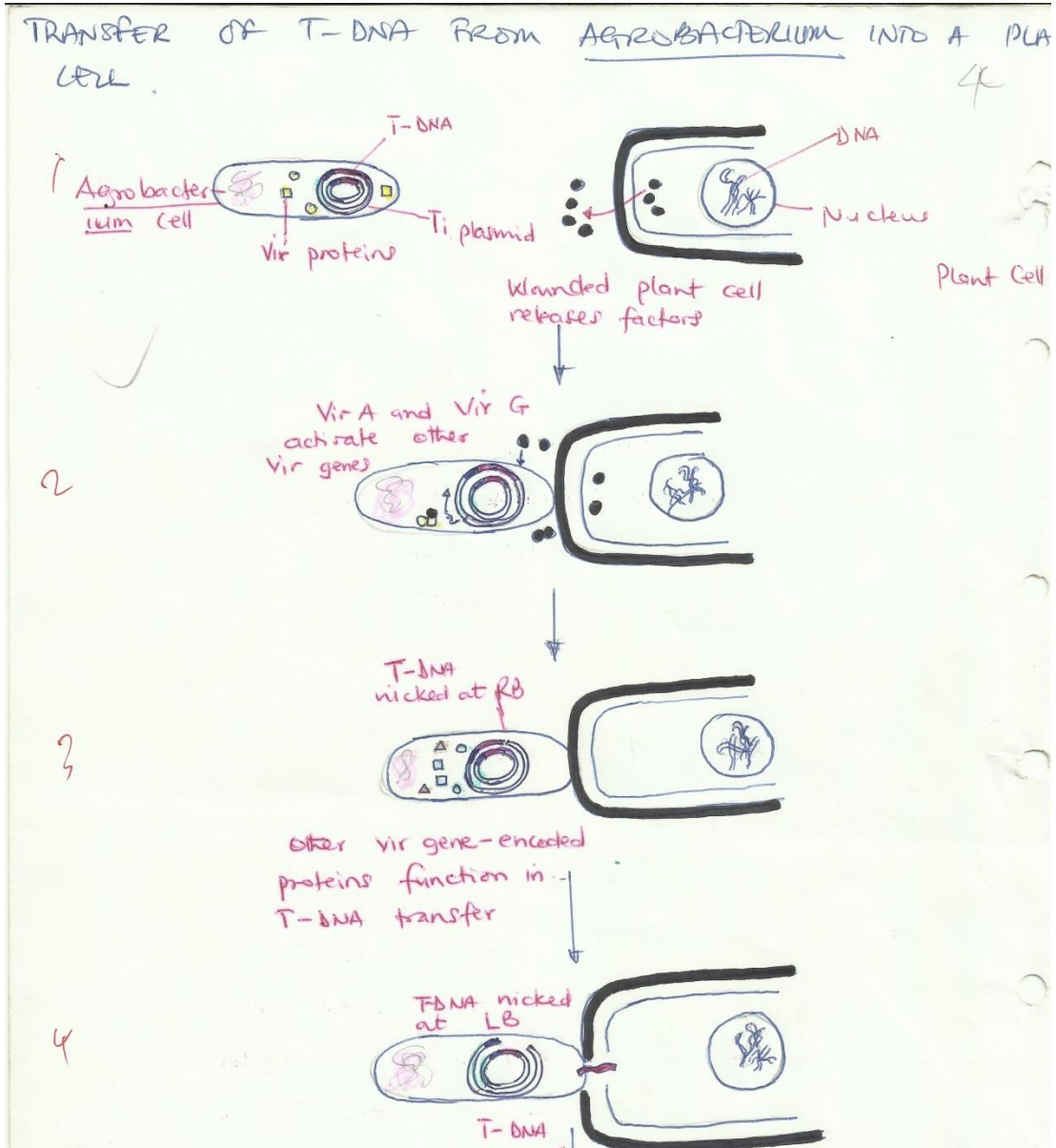
- The release of these molecules leads to the virulence of *A. tumefaciens*

- *A. tumefaciens* injects the Ti plasmid into plant cells' DNA, and then causes the growth of a crown gall (a tumor).

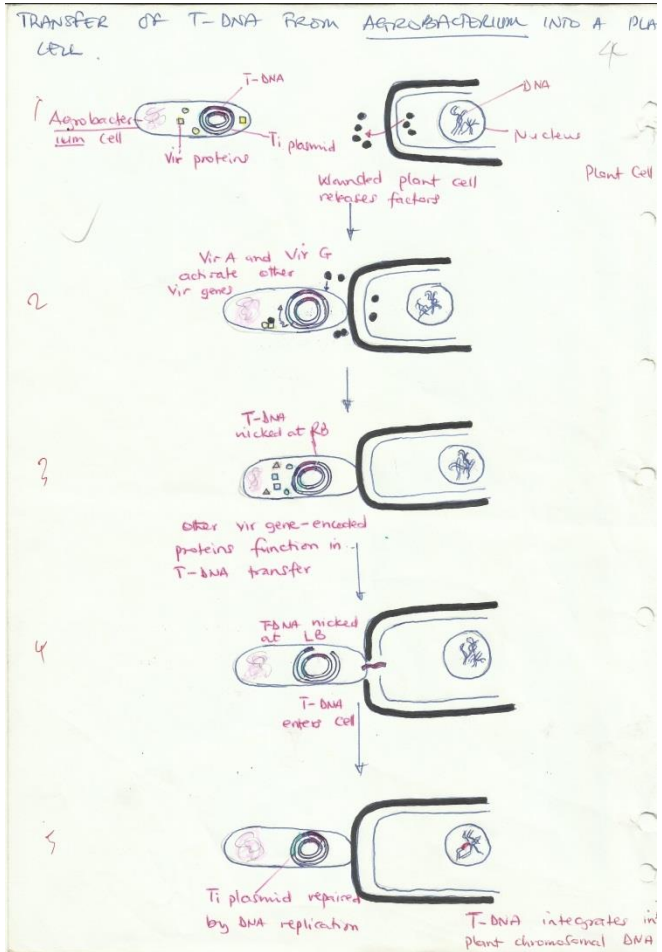
- The section of the plasmid that enters plant cells is called **T DNA**, and is about 30,000 base pairs long.

- The tumor-inducing region of the plasmid, called the *onc* gene, codes for plant growth hormones which cause plant cell

- proliferation and a resulting tumor.



TRANSFER OF T-DNA FROM AGROBACTERIUM INTO A PLA
CELL.



# Advantages and Disadvantages of Plants in Genetic Engineering

- Plants present advantages and disadvantages for the genetic engineer.

- The long history of plant breeding means that plant geneticists have a wealth of strains carrying genetically characterized mutations that can be exploited at the molecular level.

- Plants are particularly amenable or susceptible to genetic manipulation because many can be self-fertilized or *selfed*.

- When a plant heterozygous for a mutation is selfed, the progeny include wild-type plants, plants homozygous for the mutation, and also heterozygotes, in which the mutation is maintained.