ARULMIGU PALANI ANDAVAR ARTS COLLEGE FOR WOMEN, PALANI

PG DEPARTMENT OF BOTANY

LEARNING RESOURCES

PLANT BIOTECHNOLOGY

Plant Biotechnology

Biotechnology is a multidisciplinary field that involves the integration of natural sciences and engineering sciences in order to achieve the application of organisms, cells, parts thereof and molecular analogues for products and services.

The term *biotechnology* was first used by Károly Ereky in 1919, to refer to the production of products from raw materials with the aid of living organisms. The core principle of biotechnology involves harnessing biological systems and organisms, such as bacteria, yeast, and plants, to perform specific tasks or produce valuable substances.

Biotechnology had a significant impact on many areas of society, from medicine to agriculture to environmental science. One of the key techniques used in biotechnology is genetic engineering, which allows scientists to modify the genetic makeup of organisms to achieve desired outcomes. This can involve inserting genes from one organism into another, creating new traits or modifying existing ones.

Other important techniques used in biotechnology include tissue culture, which allows researchers to grow cells and tissues in the lab for research and medical purposes, and fermentation, which is used to produce a wide range of products such as beer, wine, and cheese.

The applications of biotechnology are diverse and have led to the development of essential products like life-saving drugs, biofuels, genetically modified crops, and innovative materials. It has also been used to address environmental challenges, such as developing biodegradable plastics and using microorganisms to clean up contaminated sites.

Biotechnology is a rapidly evolving field with significant potential to address pressing global challenges and improve the quality of life for people around the world; however, despite its numerous benefits, it also poses ethical and societal challenges, such as questions around genetic modification and intellectual property rights. As a result, there is ongoing debate and regulation surrounding the use and application of biotechnology in various industries and fields.

The concept of biotechnology encompasses a wide range of procedures for modifying living organisms for human purposes, going back to domestication of animals, cultivation of the plants, and "improvements" to these through breeding programs that employ artificial selection and hybridization. Modern usage also includes genetic engineering, as well as cell and tissue culture technologies. The American Chemical Society defines *biotechnology* as the application of biological organisms, systems, or processes by various industries to learning about the science of life and the improvement of the value of materials and organisms, such as pharmaceuticals, crops, and livestock. As per the European Federation of Biotechnology, biotechnology is the integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services. Biotechnology is based on the basic biological sciences (e.g., molecular biology, biochemistry, cell biology, embryology, genetics, microbiology) and conversely provides methods to support and perform basic research in biology.

Biotechnology is the research and development in the laboratory using bioinformatics for exploration, extraction, exploitation, and production from any living organisms and any source of biomass by means of biochemical engineering where high value-added products could be planned (reproduced by biosynthesis, for example), forecasted, formulated, developed, manufactured, and marketed for the purpose of sustainable operations (for the return from bottomless initial investment on R & D) and gaining durable patents rights (for exclusives rights for sales, and prior to this to receive national and international approval from the results on animal experiment and human experiment, especially on the pharmaceutical branch of biotechnology to prevent any undetected side-effects or safety

concerns by using the products). The utilization of biological processes, organisms or systems to produce products that are anticipated to improve human lives is termed biotechnology.

Although not normally what first comes to mind, many forms of human-derived agriculture clearly fit the broad definition of "utilizing a biotechnological system to make products". Indeed, the cultivation of plants may be viewed as the earliest biotechnological enterprise.

Agriculture has been theorized to have become the dominant way of producing food since the Neolithic Revolution. Through early biotechnology, the earliest farmers selected and bred the best-suited crops (e.g., those with the highest yields) to produce enough food to support a growing population. As crops and fields became increasingly large and difficult to maintain, it was discovered that specific organisms and their by-products could effectively fertilize, restore nitrogen, and control pests. Throughout the history of agriculture, farmers have inadvertently altered the genetics of their crops through introducing them to new environments and breeding them with other plants — one of the first forms of biotechnology.

These processes also were included in early fermentation of beer. These processes were introduced in early Mesopotamia, Egypt, China and India, and still use the same basic biological methods. In brewing, malted grains (containing enzymes) convert starch from grains into sugar and then adding specific yeasts to produce beer. In this process, carbohydrates in the grains broke down into alcohols, such as ethanol. Later, other cultures produced the process of lactic acid fermentation, which produced other preserved foods, such as soy sauce. Fermentation was also used in this time period to produce leavened bread. Although the process of fermentation was not fully understood until Louis Pasteur's work in 1857, it is still the first use of biotechnology to convert a food source into another form.

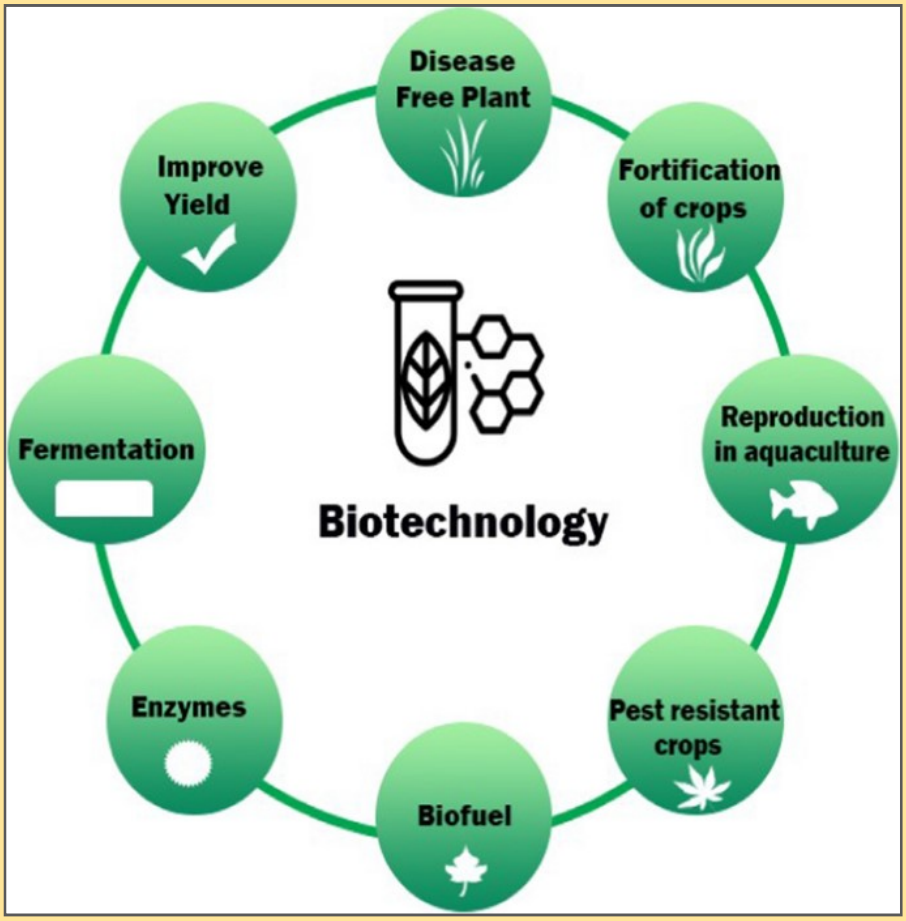
Before the time of Charles Darwin's work and life, animal and plant scientists had already used selective breeding. Darwin added to that body of work with his scientific observations about the ability of science to change species. These accounts contributed to Darwin's theory of natural selection.

For thousands of years, humans have used selective breeding to improve the production of crops and livestock to use them for food. In selective breeding, organisms with desirable

characteristics are mated to produce offspring with the same characteristics. For example, this technique was used with corn to produce the largest and sweetest crops.

In the early twentieth century scientists gained a greater understanding of microbiology and explored ways of manufacturing specific products. In 1917, Chaim Weizmann first used a pure microbiological culture in an industrial process, that of manufacturing corn starch using *Clostridium acetobutylicum,* to produce acetone, which the United Kingdom desperately needed to manufacture explosives during World War I.

Biotechnology has also led to the development of antibiotics. In 1928, Alexander Fleming discovered the mold *Penicillium*. His work led to the purification of the antibiotic compound formed by the mold by Howard Florey, Ernst Boris Chain and Norman Heatley – to form what we today know as penicillin. In 1940, penicillin became available for medicinal use to treat bacterial infections in humans.



The field of modern biotechnology is generally thought of as having been born in 1971 when Paul Berg's (Stanford) experiments in gene splicing had early success. Herbert W. Boyer (Univ. Calif. at San Francisco) and Stanley N. Cohen (Stanford) significantly advanced the new technology in 1972 by transferring genetic material into a bacterium, such

that the imported material would be reproduced. The commercial viability of a biotechnology industry was significantly expanded on June 16, 1980, when the United States Supreme Court ruled that a genetically modified microorganism could be patented in the case of *Diamond v. Chakrabarty*. Indian-born Ananda Chakrabarty, working for General Electric, had modified a bacterium (of the genus *Pseudomonas*) capable of breaking down crude oil, which he proposed to use in treating oil spills. (Chakrabarty's work did not involve gene manipulation but rather the transfer of entire organelles between strains of the *Pseudomonas* bacterium).

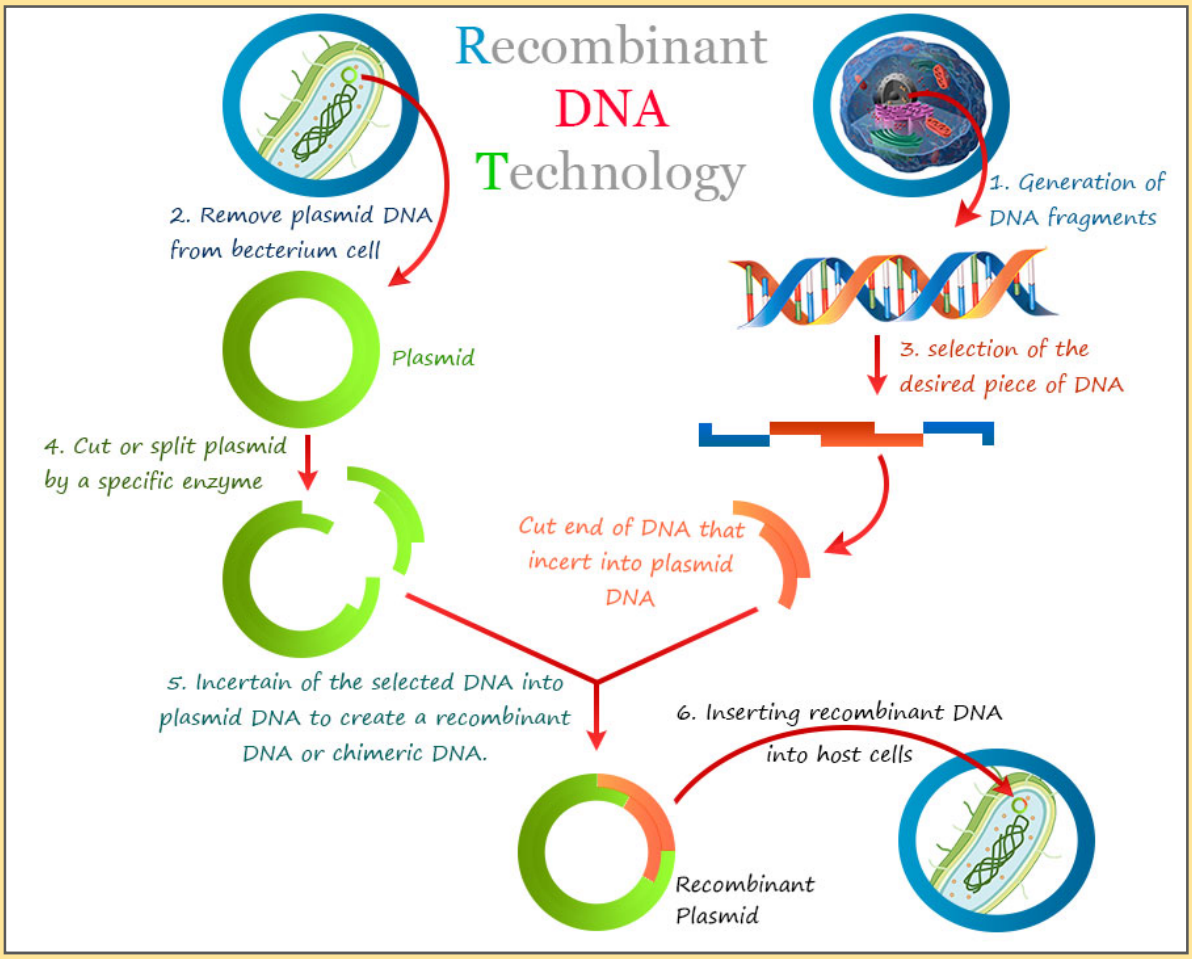
A factor influencing the biotechnology sector's success is improved intellectual property rights legislation—and enforcement—worldwide, as well as strengthened demand for medical and pharmaceutical products to cope with an ageing, and ailing, U.S. population.

Rising demand for biofuels is expected to be good news for the biotechnology sector, with the Department of Energy estimating ethanol usage could reduce U.S. petroleum-derived fuel consumption by up to 30% by 2030. The biotechnology sector has allowed the U.S. farming industry to rapidly increase its supply of corn and soybeans—the main inputs into biofuels—by developing genetically modified seeds that resist pests and drought. By increasing farm productivity, biotechnology boosts biofuel production.

Recombinant DNA

Recombinant DNA (**rDNA**) molecules are DNA molecules formed by laboratory methods of genetic recombination (such as molecular cloning) that bring together genetic material from multiple sources, creating sequences that would not otherwise be found in the genome.

Recombinant DNA is the general name for a piece of DNA that has been created by combining two or more fragments from different sources. Recombinant DNA is possible because DNA molecules from all organisms share the same chemical structure, differing only in the nucleotide sequence. Recombinant DNA molecules are sometimes called **chimeric DNA** because they can be made of material from two different species like the mythical chimera. rDNA technology uses palindromic sequences and leads to the production of sticky and blunt ends.



The DNA sequences used in the construction of recombinant DNA molecules can originate from any species. For example, plant DNA can be joined to bacterial DNA, or human DNA can be joined with fungal DNA. In addition, DNA sequences that do not occur anywhere in nature can be created by the chemical synthesis of DNA and incorporated into recombinant

DNA molecules. Using recombinant DNA technology and synthetic DNA, any DNA sequence can be created and introduced into living organisms.

Proteins that can result from the expression of recombinant DNA within living cells are termed *recombinant proteins*. When recombinant DNA encoding a protein is introduced into a host organism, the recombinant protein is not necessarily produced. Expression of foreign proteins requires the use of specialized expression vectors and often necessitates significant restructuring by foreign coding sequences.

Recombinant DNA differs from genetic recombination in that the former results from artificial methods while the latter is a normal biological process that results in the remixing of existing DNA sequences in essentially all organisms.

DNA Creation

Molecular cloning is the laboratory process used to create recombinant DNA. It is one of two most widely used methods, along with polymerase chain reaction (PCR), used to direct the replication of any specific DNA sequence chosen by the experimentalist. There are two fundamental differences between the methods. One is that molecular cloning involves replication of the DNA within a living cell, while PCR replicates DNA in the test tube, free of living cells. The other difference is that cloning involves cutting and pasting DNA sequences, while PCR amplifies by copying an existing sequence.

Formation of recombinant DNA requires a cloning vector, a DNA molecule that replicates within a living cell. Vectors are generally derived from plasmids or viruses, and represent relatively small segments of DNA that contain necessary genetic signals for replication, as well as additional elements for convenience in inserting foreign DNA, identifying cells that contain recombinant DNA, and, where appropriate, expressing the foreign DNA. The choice of vector for molecular cloning depends on the choice of host organism, the size of the DNA to be cloned, and whether and how the foreign DNA is to be expressed. The DNA segments can be combined by using a variety of methods, such as restriction enzyme/ligase cloning or Gibson assembly.

In standard cloning protocols, the cloning of any DNA fragment essentially involves seven steps: (1) Choice of host organism and cloning vector, (2) Preparation of vector DNA, (3) Preparation of DNA to be cloned, (4) Creation of recombinant DNA, (5) Introduction of recombinant DNA into the host organism, (6) Selection of organisms containing recombinant DNA, and (7) Screening for clones with desired DNA inserts and biological properties

DNA expression requires the transfection of suitable host cells. Typically, either bacterial, yeast, insect, or mammalian cells (such as Human Embryonic Kidney cells or CHO cells) are used as host cells.

Following transplantation into the host organism, the foreign DNA contained within the recombinant DNA construct may or may not be expressed. That is, the DNA may simply be replicated without expression, or it may be transcribed and translated and a recombinant protein is produced. Generally speaking, expression of a foreign gene requires restructuring the gene to include sequences that are required for producing an mRNA molecule that can be used by the host's translational apparatus (e.g. promoter, translational initiation signal, and transcriptional terminator).

Specific changes to the host organism may be made to improve expression of the ectopic gene. In addition, changes may be needed to the coding sequences as well, to optimize translation, make the protein soluble, direct the recombinant protein to the proper cellular or extracellular location, and stabilize the protein from degradation.

Properties of Organisms containing Recombinant DNA

In most cases, organisms containing recombinant DNA have apparently normal phenotypes. That is, their appearance, behavior and metabolism are usually unchanged, and the only way to demonstrate the presence of recombinant sequences is to examine the DNA itself, typically using a polymerase chain reaction (PCR) test. Significant exceptions exist, and are discussed below.

If the rDNA sequences encode a gene that is expressed, then the presence of RNA and/or protein products of the recombinant gene can be detected, typically using RT-PCR or western hybridization methods. Gross phenotypic changes are not the norm, unless the recombinant gene has been chosen and modified so as to generate biological activity in the host organism. Additional phenotypes that are encountered include toxicity to the host organism induced by the recombinant gene product, especially if it is over-expressed or expressed within inappropriate cells or tissues.

In some cases, recombinant DNA can have deleterious effects even if it is not expressed. One mechanism by which this happens is insertional inactivation, in which the rDNA becomes inserted into a host cell's gene. In some cases, researchers use this phenomenon to "knock out" genes to determine their biological function and importance. Another mechanism by which rDNA insertion into chromosomal DNA can affect gene expression is by inappropriate

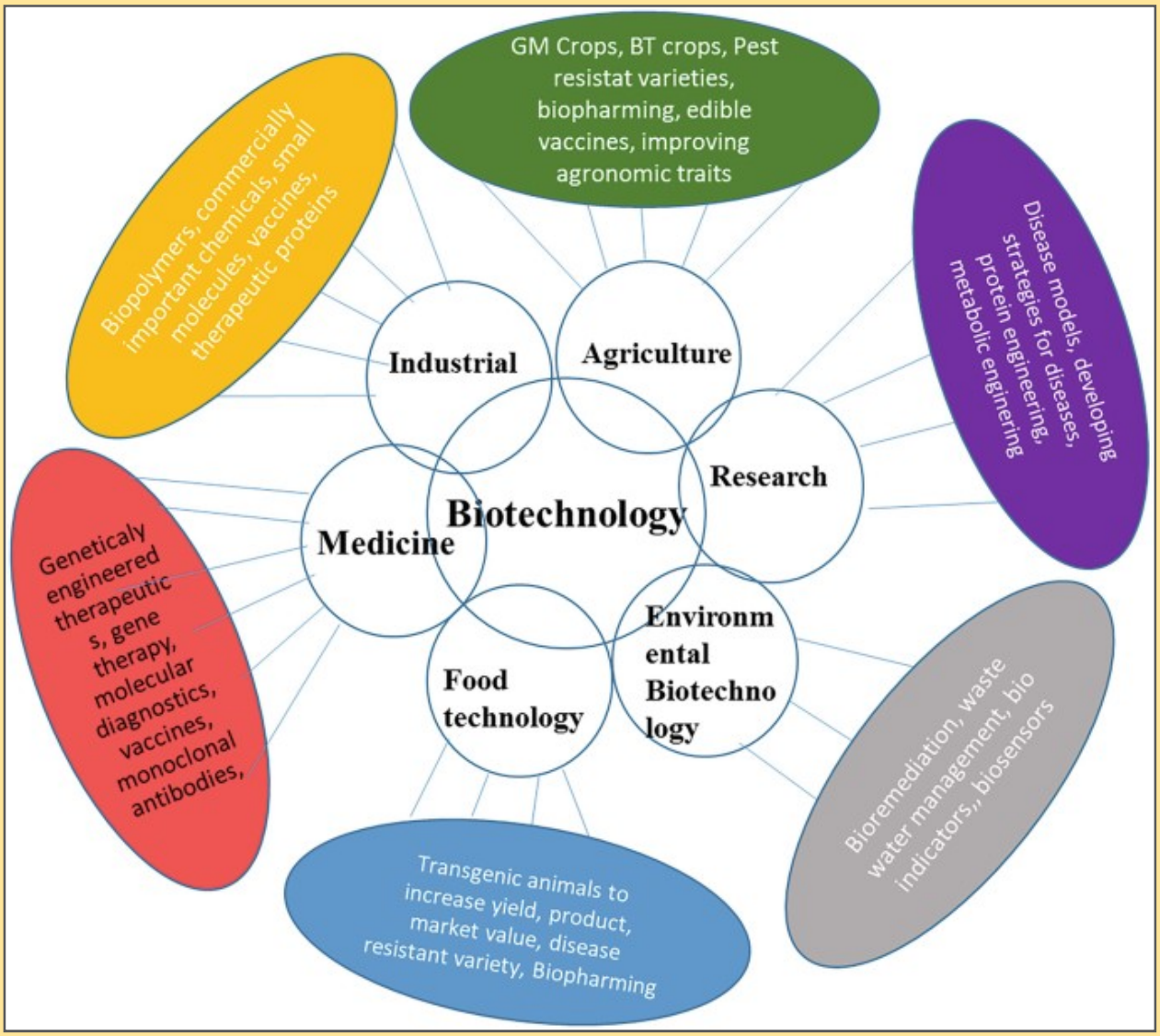
activation of previously unexpressed host cell genes. This can happen, for example, when a recombinant DNA fragment containing an active promoter becomes located next to a previously silent host cell gene, or when a host cell gene that functions to restrain gene expression undergoes insertional inactivation by recombinant DNA.

Applications of Recombinant DNA

Recombinant DNA is widely used in biotechnology, medicine and research. Today, recombinant proteins and other products that result from the use of DNA technology are found in essentially every western pharmacy, physician or veterinarian office, medical testing laboratory, and biological research laboratory. In addition, organisms that have been manipulated using recombinant DNA technology, as well as products derived from those organisms, have found their way into many farms, supermarkets, home medicine cabinets, and even pet shops, such as those that sell GloFish and other genetically modified animals.

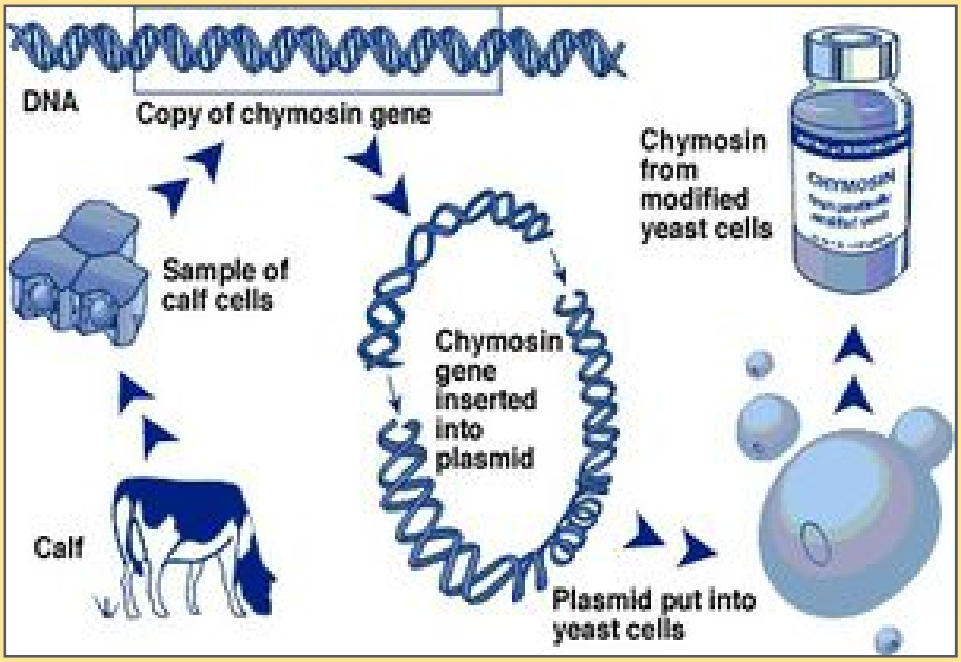
The most common application of recombinant DNA is in basic research, in which the technology is important to most current work in the biological and biomedical sciences. Recombinant DNA is used to identify, map and sequence genes, and to determine their function. rDNA probes are employed in analyzing gene expression within individual cells, and throughout the tissues of whole organisms. Recombinant proteins are widely used

as reagents in laboratory experiments and to generate antibody probes for examining protein synthesis within cells and organisms.



Many additional practical applications of recombinant DNA are found in industry, food production, human and veterinary medicine, agriculture, and bioengineering. Some specific examples are identified below.

Recombinant chymosin

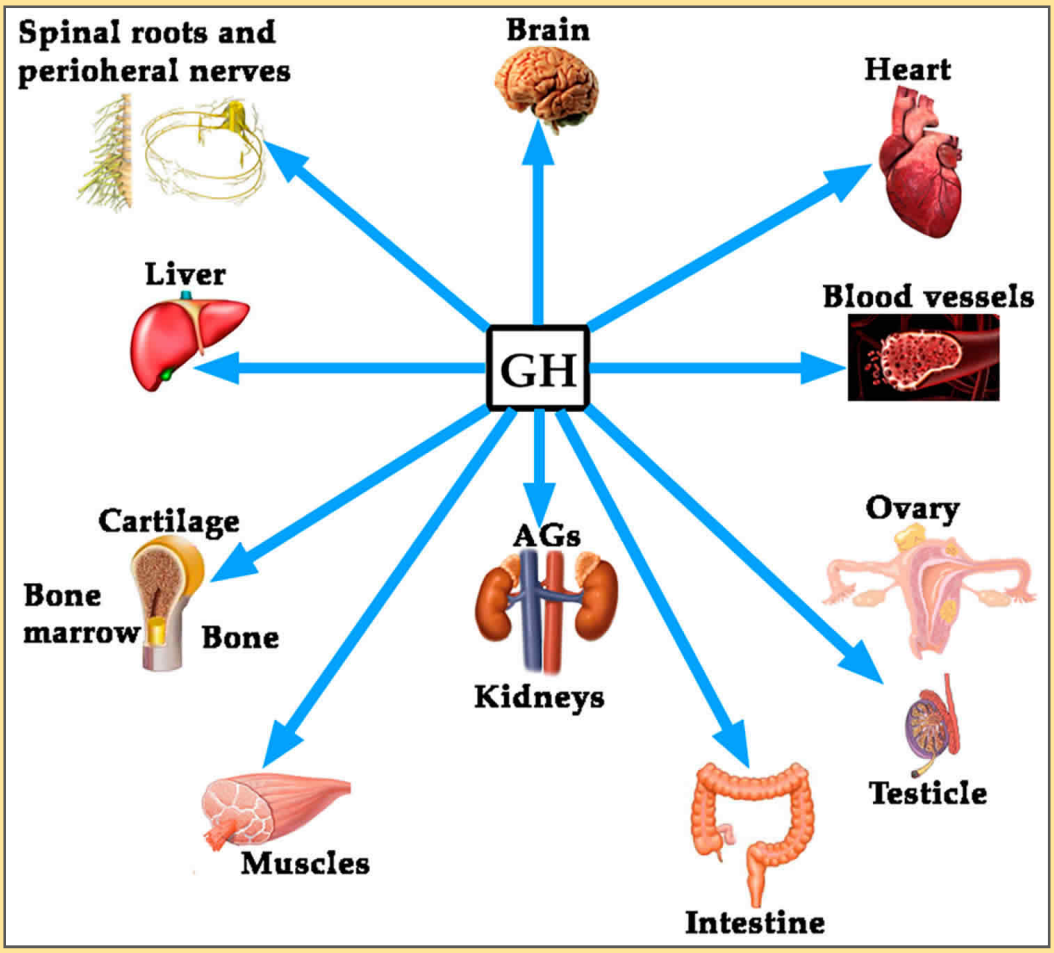


Found in rennet, chymosin is an enzyme required to manufacture cheese. It was the first genetically engineered food additive used commercially. Traditionally, processors obtained chymosin from rennet, a preparation derived from the fourth stomach of milk-fed calves. Scientists engineered a non-pathogenic strain (K-12) of *E. coli* bacteria for large-scale laboratory production of the enzyme. This microbiologically produced recombinant enzyme, identical structurally to the calf derived enzyme, costs less and is produced in abundant quantities. Today about 60% of U.S. hard cheese is made with genetically engineered chymosin. In 1990, FDA granted chymosin "generally recognized as safe" (GRAS) status based on data showing that the enzyme was safe.

Recombinant human insulin

Almost completely replaced insulin obtained from animal sources (e.g. pigs and cattle) for the treatment of insulin-dependent diabetes. A variety of different recombinant insulin preparations are in widespread use. Recombinant insulin is synthesized by inserting the human insulin gene into *E. coli*, or yeast (Saccharomyces cerevisiae) which then produces insulin for human use. Insulin produced by E. coli requires further post translational modifications (glycosylation) whereas yeast are able to perform these themselves by virtue of being a more complex host organism. The advantage of recombinant human insulin is after chronic use patients don't develop an immune defence against it the way animal sourced insulin stimulates the human immune system.

Recombinant human growth hormone (HGH, somatotropin)

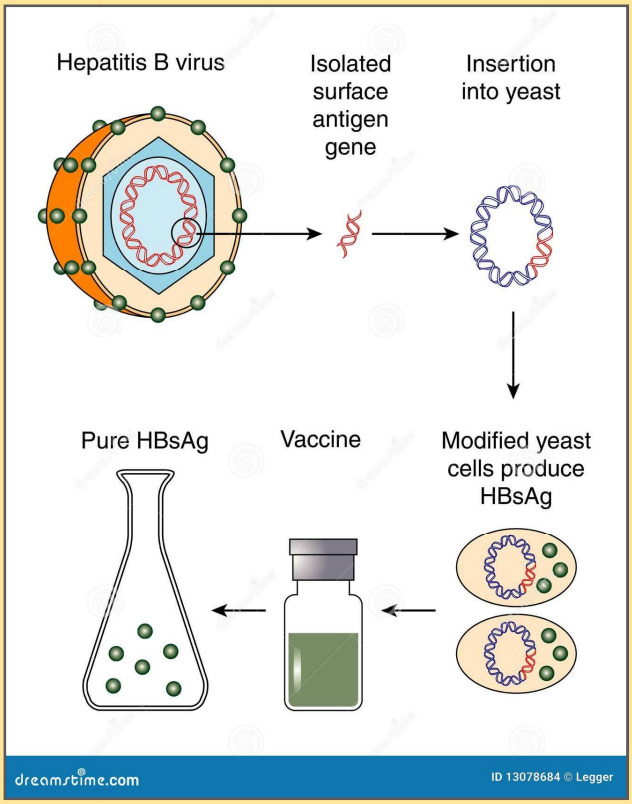


Administered to patients whose pituitary glands generate insufficient quantities to support normal growth and development. Before recombinant HGH became available, HGH for therapeutic use was obtained from pituitary glands of cadavers. This unsafe practice led to some patients developing Creutzfeldt–Jakob disease. Recombinant HGH eliminated this problem, and is now used therapeutically. It has also been misused as a performance-enhancing drug by athletes and others. DrugBank entry

Recombinant blood clotting factor VIII

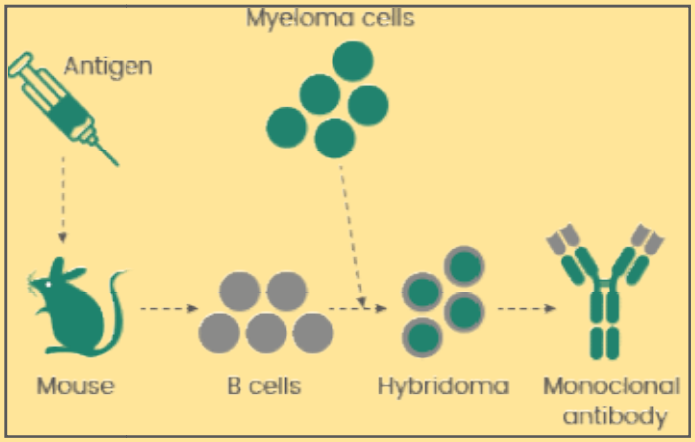
A blood-clotting protein that is administered to patients with forms of the bleeding disorder hemophilia, who are unable to produce factor VIII in quantities sufficient to support normal blood coagulation. Before the development of recombinant factor VIII, the protein was obtained by processing large quantities of human blood from multiple donors, which carried a very high risk of transmission of blood borne infectious diseases, for example HIV and hepatitis B. Drug Bank entry

Recombinant hepatitis B vaccine



Hepatitis B infection is controlled through the use of a recombinant hepatitis B vaccine, which contains a form of the hepatitis B virus surface antigen that is produced in yeast cells. The development of the recombinant subunit vaccine was an important and necessary development because hepatitis B virus, unlike other common viruses such as polio virus, cannot be grown in vitro. Vaccine information from Hepatitis B Foundation

Recombinant antibodies



Recombinant antibodies (rAbs) are produced in vitro by the means of expression systems based on mammalian cells. Their monospecific binding to a specific epitope makes rAbs eligible not only for research purposes, but also as therapy options against certain cancer types, infections and autoimmune diseases.

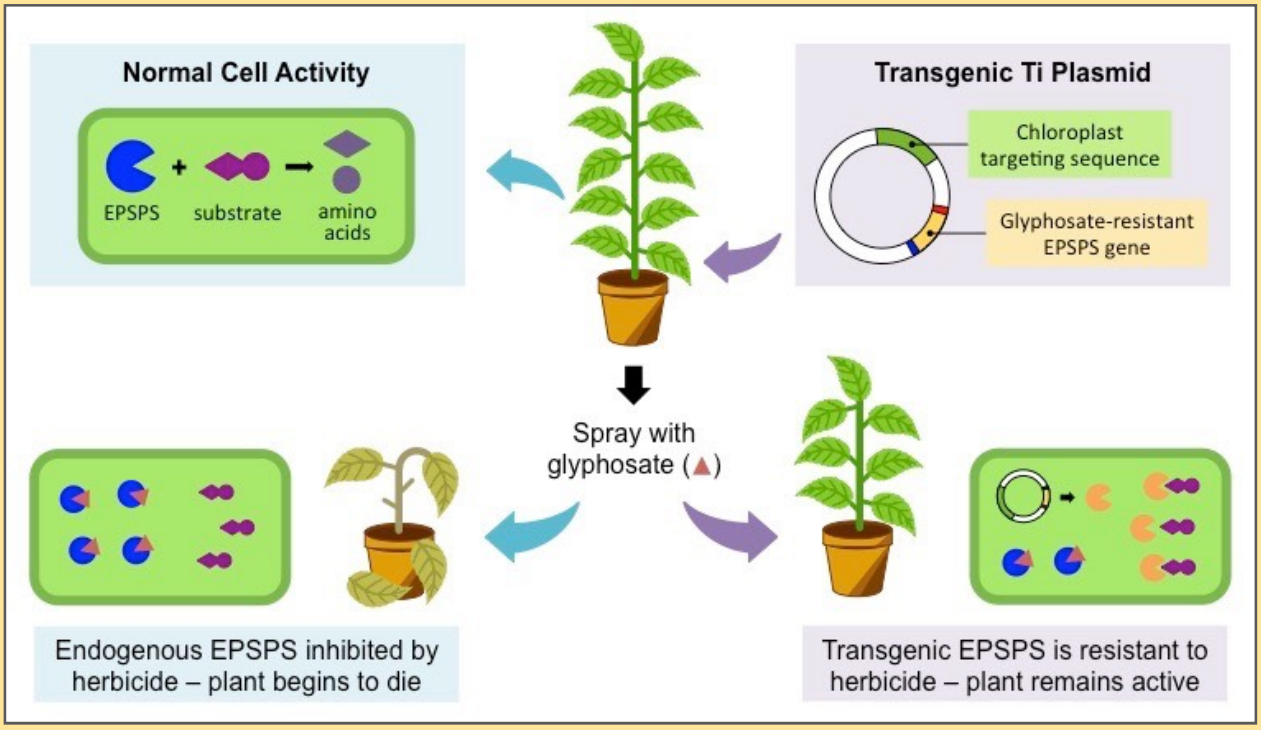
Diagnosis of infection with HIV

Each of the three widely used methods for diagnosing HIV infection has been developed using recombinant DNA. The antibody test (ELISA or western blot) uses a recombinant HIV protein to test for the presence of antibodies that the body has produced in response to an HIV infection. The DNA test looks for the presence of HIV genetic material using reverse transcription polymerase chain reaction (RT-PCR). Development of the RT-PCR test was made possible by the molecular cloning and sequence analysis of HIV genomes. HIV testing page from US Centers for Disease Control (CDC)

Golden rice

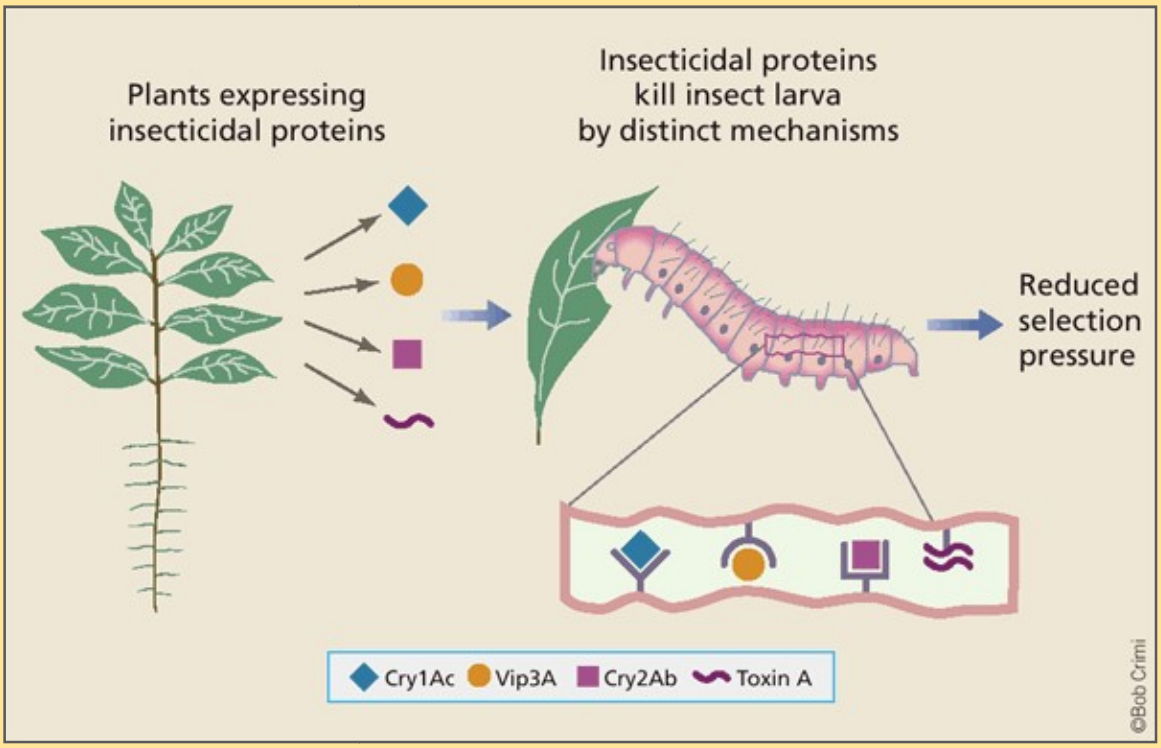
A recombinant variety of rice that has been engineered to express the enzymes responsible for β-carotene biosynthesis. This variety of rice holds substantial promise for reducing the incidence of vitamin A deficiency in the world's population. Golden rice is not currently in use, pending the resolution of regulatory and intellectual property issues.

Herbicide-resistant crops

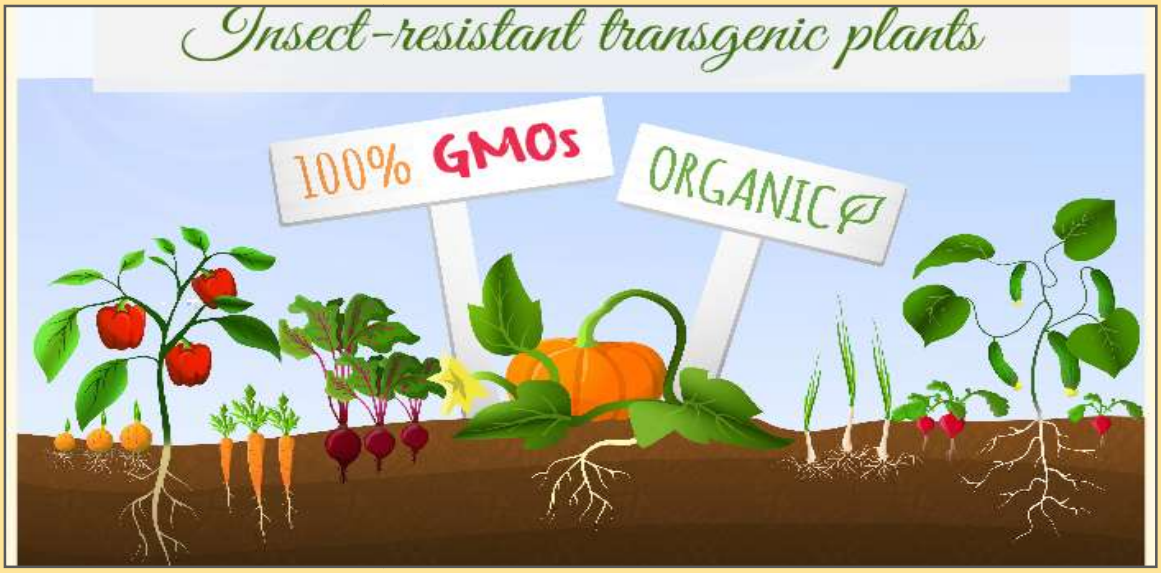


Commercial varieties of important agricultural crops (including soy, maize/corn, sorghum, canola, alfalfa and cotton) have been developed that incorporate a recombinant gene that results in resistance to the herbicide glyphosate (trade name *Roundup*), and simplifies weed control by glyphosate application. These crops are in common commercial use in several countries.

Insect-resistant crops



Bacillus thuringeiensis is a bacterium that naturally produces a protein (Bt toxin) with insecticidal properties. The bacterium has been applied to crops as an insect-control strategy for many years, and this practice has been widely adopted in agriculture and gardening. Recently, plants have been developed that express a recombinant form of the bacterial protein, which may effectively control some insect predators. Environmental issues associated with the use of these transgenic crops have not been fully resolved.



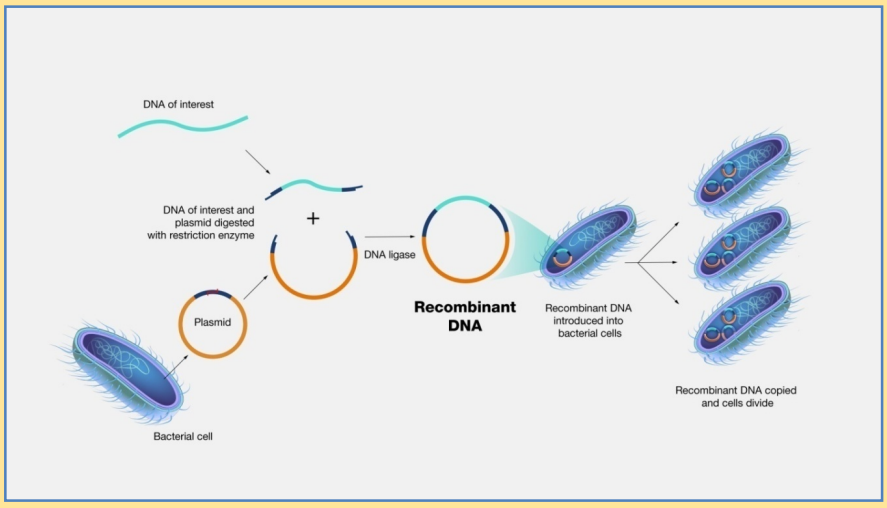
History

The idea of recombinant DNA was first proposed by Peter Lobban, a graduate student of Prof. Dale Kaiser in the Biochemistry Department at Stanford University Medical School. The first publications describing the successful production and intracellular replication of recombinant DNA appeared in 1972 and 1973, from Stanford and UCSF. In 1980 Paul Berg, a professor in the Biochemistry Department at Stanford and an author on one of the first papers was awarded the Nobel Prize in Chemistry for his work on nucleic acids "with particular regard to recombinant DNA". Werner Arber, Hamilton Smith, and Daniel Nathans shared the 1978 Nobel Prize in Physiology or Medicine for the discovery of restriction endonucleases which enhanced the techniques of rDNA technology.

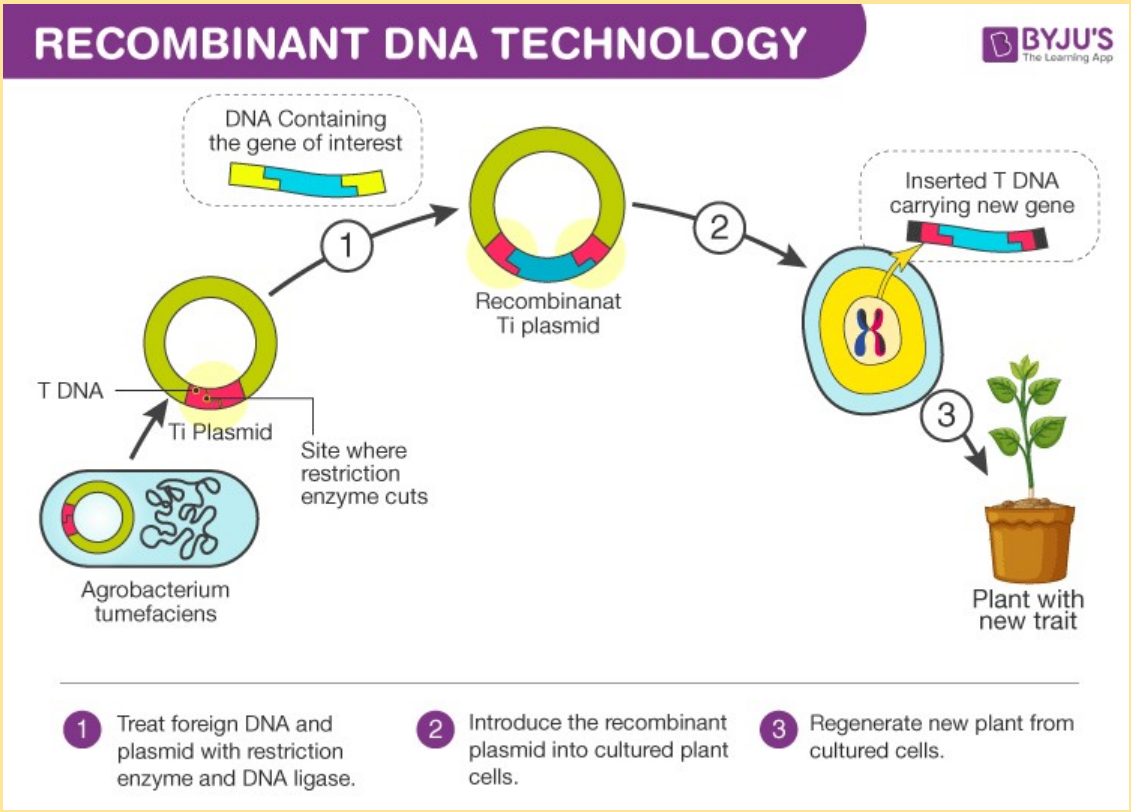
Stanford University applied for a US patent on recombinant DNA in 1974, listing the inventors as Herbert W. Boyer (professor at the University of California, San Francisco) and Stanley N. Cohen (professor at Stanford University); this patent was awarded in 1980. The first licensed drug generated using recombinant DNA technology was human insulin, developed by Genentech and licensed by Eli Lilly and Company.

Recombinant DNA technology

Recombinant DNA technology involves using enzymes and various laboratory techniques to manipulate and isolate DNA segments of interest. This method can be used to combine (or splice) DNA from different species or to create genes with new functions. The resulting copies are often referred to as recombinant DNA. Such work typically involves propagating the recombinant DNA in a bacterial or yeast cell, whose cellular machinery copies the engineered DNA along with its own.



Recombinant DNA Technology. Recombinant DNA technology is an extremely important research tool in biology. It allows scientists to manipulate DNA fragments in order to study them in the lab. It involves using a variety of laboratory methods to put a piece of DNA into a bacterial or yeast cell. Once in, the bacteria or yeast will copy the DNA along with its own. Recombinant DNA technology has been successfully applied to make important proteins used in the treatment of human diseases, such as insulin and growth hormone.



Human insulin production by genetic engineering

- **Insulin** is a hormone produced by β-cells of islets of Langerhans of pancreas. It was discovered by sir Edward Sharpey Schafer (1916) while studying Islets of Langerhans.
- Pancreas is a mixed gland situated transversely across the upper abdomen behind stomach and spleen.
- Insulin is a peptide hormone produced by pancreas and is a central regulator of carbohydrates and fat metabolism in the body.

Structure of Human Insulin:

- Chemically Human insulin is small, simple protein composed of 51 amino acids sequences and has a molecular weight of 5808 Da.
- Insulin hormone is a dimer of a A- chain and a B-chain which are linked together by a disulphide bond.
- Fredrick Sanger et al (1954) gave the first complete description of insulin. Insulin consists of two polypeptide chain,
  - o Chain A- 21 amino acids long
  - o Chain B-30 amino acids long
  - o Both chains are joined together by disulphide bond between two cysteine residue

Insulin produced inside pancreas:

- At first Pancreatic β-cells synthesize pre-pro-insulin, which is a 109 amino acids long polypeptide
- Among 109 amino acids, 23 amino acids are signal molecules which allows the pre-pro-insulin to pass through cell membrane.
- Entering inside cell, it become 86 amino acids long pro-insulin. It is still inactive.
- Some Proteolytic enzymes cut and expose the active site of pro insulin converting it into active form of insuin of 51 amino acids long.

Insulin produced by recombinant DNA technology.

- The basic step in recombinant DNA technology is similar for insulin production also.
  - At first suitable vector (plasmid) is isolated from E. coli and then it is cut open by restriction endonuclease enzyme.
  - The gene of interest (ie. Insulin coding gene) is isolated from β-cell and inserted in opened plasmid.
  - Plasmid and gene of interest are recombined together by DNA ligase enzyme
  - This recombined plasmid is inserted into suitable host cell (ie E. coli) and now this recombined host cell starts producing insulin hormone.
- Hakura et al (1977) chemically synthesize DNA sequence of insulin for two chains A and B and separately inserted into two PBR322 plasmid vector.
  - These gene are inserted by the side of β-galactosidase gene of the plasmid.
  - The recombinant plasmid were then separately transformed into E. coli host.

- The recombinant host produced pro-insulin chains ie. fused β-galactosidase-A chain and β-galactosidase-B-chain separately.
- These pro-insulin chains A and B were separated from β-galactosidase by treatment with cyanogen bromide. The detachment of pro-insulin chains from β-galactosidase is possible because an extra codon form methionine was added at N-terminal of each gene for A and B-chain.
- After detachment, A and B chains are joined invitro to reconstitute the naïve insulin by sulphonating the peptide chains with sodium disulphonate and sodium sulphite.

- Another method of insulin production by recombinant DNA technology is designed by Gilbert and Villokomaroff.
  - In this method, m RNA for pre-pro-insulin is isolated from islets of Langerhans cell
  - mRNA is reverse transcribed to form DNA and then it is inserted into PBR 322 plasmid in the middle of the gene for penicillinase.
  - Then the recombinant plasmid is transformed into suitable host ie E. coli cell
  - The host produced penicillinase + pre-pro insulin
  - Insulin is later separated by trypsin treatment

Roles of insulin in body:

1. Insulin causes cells in liver, skeletal muscles and fat tissue to take up glucose from the blood. In liver and skeletal muscle, glucose is stored as glycogen and in adipose tissue, it is stored as triglyceride.

2. Insulin stops the use of fat as energy source by inhibiting the release of glucagon hormone.

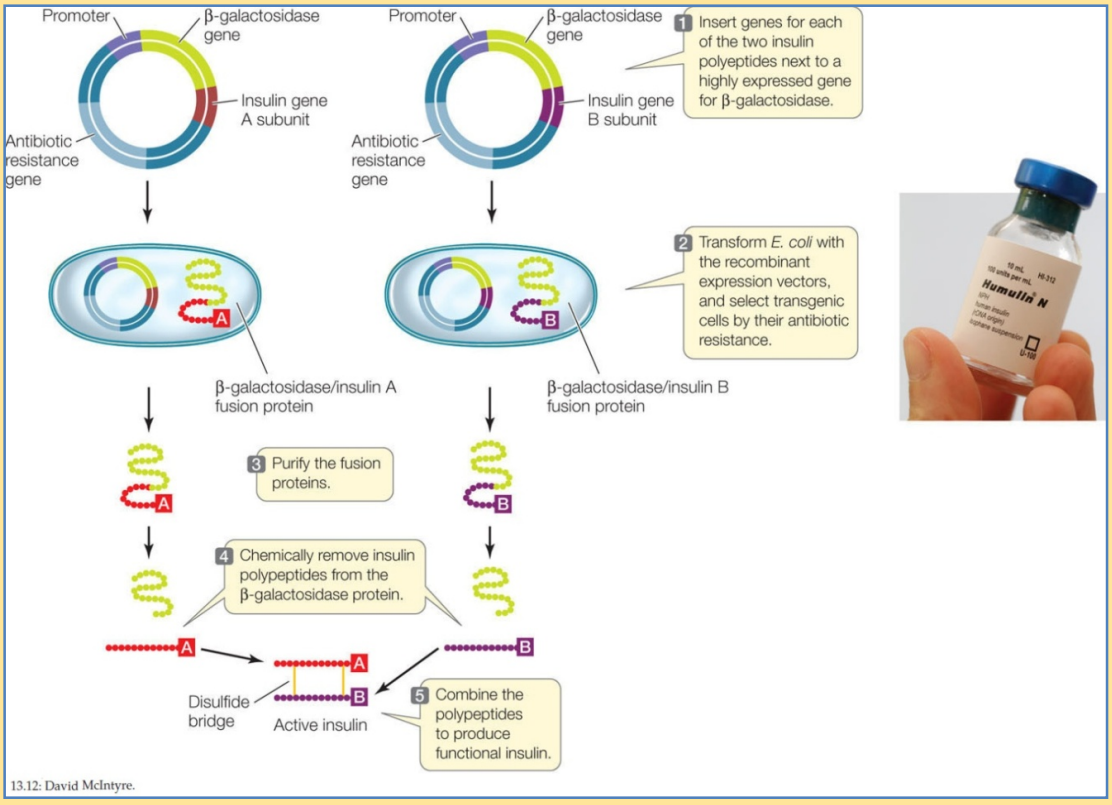
3. With the exception of the metabolic disorder such as Diabetes mellitus and metabolic syndrome, insulin maintain constant proportion of glucose in blood by removing excess glucose from the blood which otherwise would be toxic.

4. When blood glucose levels fall below a certain level, body begins to use stored glycogen as energy source through glycogenolysis; which breaks down glycogen stored in liver and muscles into glucose, which is then utilized as energy source.

5. Failing to control the level of insulin in body results in a disorder called diabetes mellitus. As a consequences Insulin is used medically to treat some forms of diabetes mellitus.

6. Patients with type I diabetes depends on insulin shots. Most commonly insulin is injected subcutaneously for the patients because the hormone is no longer produced in their body. Type I diabetes is also known as Insulin dependent diabetes mellitus.

7. Patients with type II diabetes are often resistant to insulin and because of such resistance many suffer from relative insulin deficiency. This is also known as Insulin independent diabetes. Some patients with type II diabetes may eventually require insulin shots if other medication fails to control blood glucose level. Over 40% of type II diabetes patients require insulin shots as part of their diabetes management plan.



Plasmid

Plasmids are extrachromosomal DNA molecules. They are small, circular and have the ability to replicate autonomously. Replication of plasmid is not under the control of chromosomal DNA. They are mostly found in bacteria. Some of the eukaryotes like yeast and plants also contain plasmids.Their ability to replicate independently makes plasmid a cloning vector in the recombinant DNA technology for transferring and manipulating genes.

- Many antibiotic-resistant genes in bacteria are present in plasmids.
- The size of plasmid varies from a few base pairs to thousands of bp.
- Plasmids also get transferred from one bacterial cell to another by the process of conjugation.
- Plasmids carrying a specific gene are introduced into bacterial cells, which multiply rapidly and the required DNA fragment is produced in larger quantities.
- Plasmids are used to prepare recombinant DNA with the desired gene to transfer genes from one organism to another. This is known as genetic engineering.
- Joshua Lederberg coined the term plasmid.

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Plasmid Structure

- Plasmids are extrachromosomal and not essential. They are useful but not necessarily present in every organism of the species
- Plasmids are not a part of the genome and the same plasmid can exist in different species and gets transferred from one another
- Plasmids have their own origin of replication (ORI) and they replicate along with the cell so that each daughter cell possesses a copy of the plasmid also

- Apart from the origin of replication, often it contains genes for antibiotic resistance, for the production of toxins and other useful genes, that may be required for the survival of cells

Plasmid Vector

Plasmids and bacteriophages are frequently used as cloning vectors in DNA recombinant technology.

- The ease with which plasmids can be modified and replicated makes it a great tool in genetic engineering and biotechnology
- For genetic engineering purposes, plasmids are artificially prepared in the lab
- The lab-grown plasmids, which are used as a vector contain an origin of replication, cloning site and selection marker

Vector Element	**Description**
Origin of Replication (ORI)	DNA sequence where initiation of replication starts
Selectable Marker	For selecting bacteria containing desired plasmid, e.g. **antibiotic resistance genes** and other specific genes
Multiple Cloning Sites (MCS)	Recognition sites to insert foreign DNA fragment by using restriction enzymes, a few or single recognition site is preferred to avoid getting several fragments
Promoter Region	Promotes transcription of the target gene to get the desired protein
Primer Binding site	The sequence of DNA used as a start point for PCR amplification and sequence verification

- DNA is cut at the specific points by using restriction enzymes (molecular scissors), which make sticky ends of the DNA

Herbert Boyer and Stanley Norman Cohen together discovered recombinant DNA technology by recombining DNA segments as desired and inserting them into the bacteria cell to get the desired protein

- The desired genes are then inserted by using DNA Ligase
- The recombinant DNA molecule is then introduced to the host bacteria cell by the process of **transformation**
- The recombinant plasmid then multiplies using host DNA polymerase
- The first plasmid used as a cloning vector was **pSC101** of ***Salmonella typhimurium***. They showed that a gene from a frog can be expressed in the bacterial cell
- *E.coli.* plasmid is frequently used as a cloning vector

pBR322 Plasmid

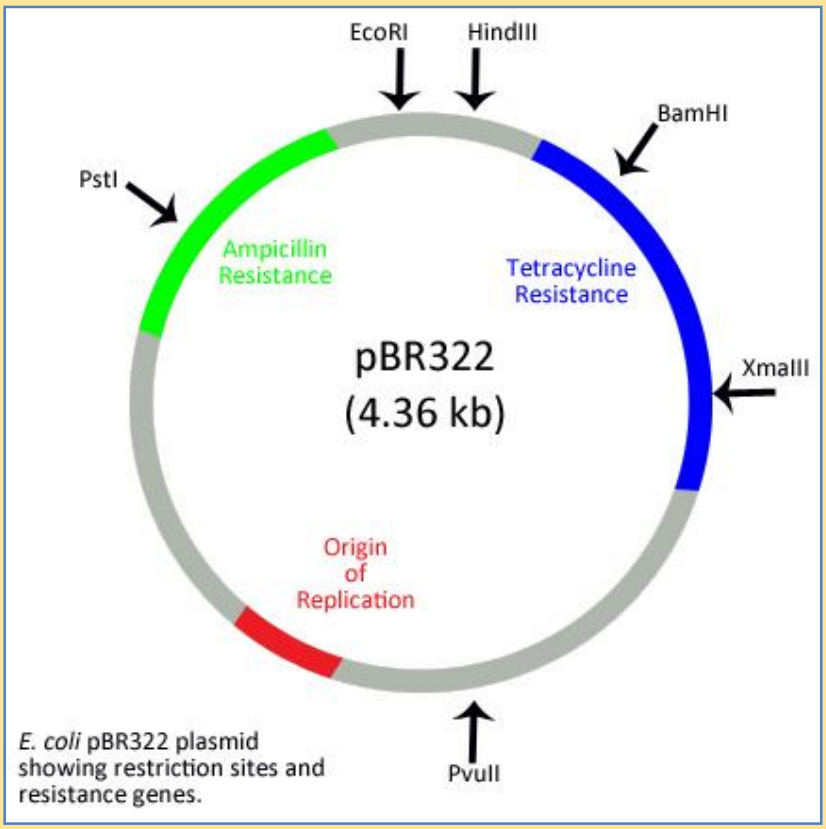
The main characteristics of pBR322 are:

- **Restriction sites:** BamH I, Hind III, Sal I, Pvu I, Pvu II, Pst I, EcoR I, Cla I
- **Selectable marker:** antibiotic resistance genes for ampicillin (amp^R) and tetracycline (tet^R)
- **ORI:** the origin of replication
- **ROP:** It codes for proteins, which are involved in the process of replication of plasmid

Different antibiotic resistance genes act as a restriction site and to ligate foreign DNA and for the selection of transformants. The gene, where the foreign DNA is inserted becomes inactive.

Alternative selectable marker: Mostly these have the ability to produce some colour after reacting with a chromogenic substance. The alternative markers are used for the ease of differentiating recombinants from non-recombinants, e.g. gene coding for β-galactosidase.

When a foreign gene is inserted between the gene coding for β-galactosidase**,** the recombinant cell does not produce the enzyme β-galactosidase due to inactivation of the gene. In the presence of a chromogenic substrate, non-recombinants form blue colour colonies and recombinants form colourless colonies.



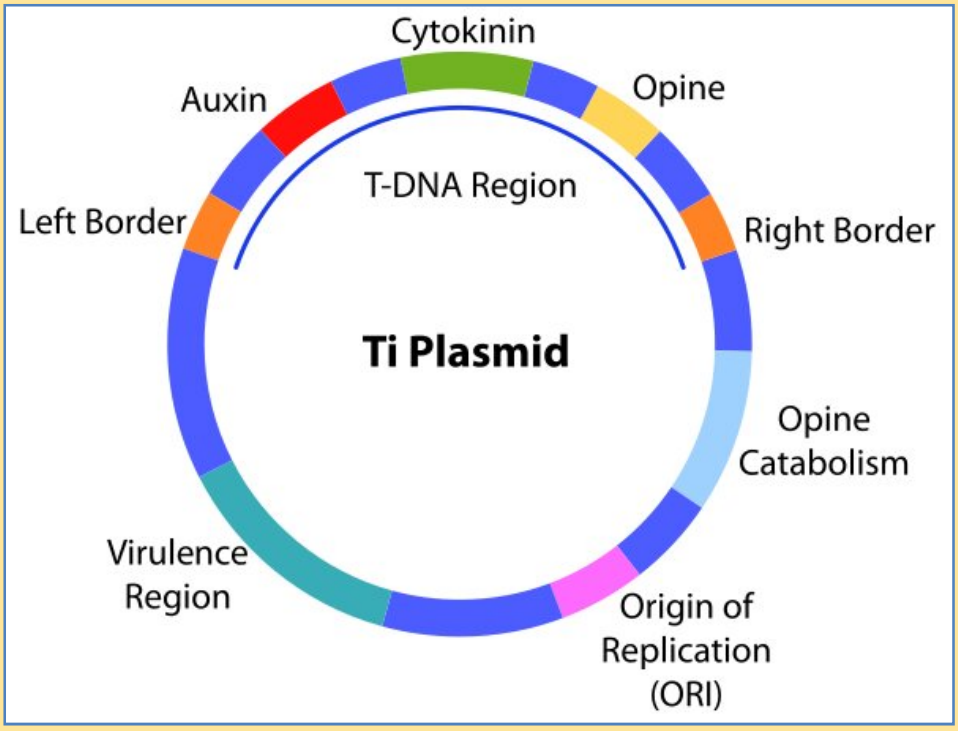
Ti Plasmid

The Tumour inducing or Ti plasmid is present in the bacterium *Agrobacterium tumifaciens.*

It is widely used now as a cloning vector to deliver desirable genes to the host plant to get **transgenic plants.** The main characteristics of Ti plasmid are:

- Size of the plasmid is ~ 250kbp
- There are different kinds of Ti plasmids based on the different genes they possess, which code for different opines, e.g. leucinopine, nopaline, octopine, etc.
- It is a pathogenic species to many dicotyledonous plants. It causes crown gall disease in plants.
- It contains one or more T-DNA region
- *Agrobacterium tumifaciens* has the ability to transform the normal cells into tumour cells by inserting a DNA piece known as T DNA and it starts producing chemicals, that are required by the bacterium
- After inserting the desired gene into Ti plasmid, it loses its pathogenic ability but is still able to insert the desired gene into the plant cell

- It contains *vir* or virulence genes**,** which transfer T-DNA region to plant cells and gets integrated into the plant genome
- Ti plasmid can be modified as per the requirement to insert the desired genes
- *Agrobacterium tumifaciens* is called “nature’s genetic engineer”



Recombinant Plasmid

Recombinant plasmids are the plasmids into which a foreign DNA fragment of gene is inserted. These recombinant plasmids independently replicate from the chromosomal DNA of the host.

Cells that contain recombinant plasmids usually are selected by taking to advantage the antibiotic resistance marker found on the vector plasmid. The cells containing recombinant plasmids can be recognized as containing recombinant plasmids by screening for insertional inactivation of a second genetic marker on the plasmid.

Advantages of Plasmids

- Plasmids are used as tools to transfer, manipulate and clone genes
- Since bacteria can rapidly divide, it can be used as factories to copy DNA fragments in large quantities

- Using plasmids as vectors is advantageous as it is easy to isolate and manipulate because of small size
- Due to its circular configuration, it is more stable
- Replicate independant of the host

Agrobacterium tumefaciens

Agrobacterium radiobacter (more commonly known as ***Agrobacterium tumefaciens***) is the causal agent of **crown gall disease** (the formation of tumours) in over 140 species of eudicots. It is a rod-shaped, Gram-negative soil bacterium. Symptoms are caused by the insertion of a small segment of DNA (known as the T-DNA, for 'transfer DNA', not to be confused with tRNA that transfers amino acids during protein synthesis), from a plasmid into the plant cell, which is incorporated at a semi-random location into the plant genome. Plant genomes can be engineered by use of *Agrobacterium* for the delivery of sequences hosted in T-DNA binary vectors.

Agrobacterium tumefaciens is an Alphaproteobacterium of the family Rhizobiaceae, which includes the nitrogen-fixing legume symbionts. Unlike the nitrogen-fixing symbionts, tumor-producing *Agrobacterium* species are pathogenic and do not benefit the plant. The wide variety of plants affected by *Agrobacterium* makes it of great concern to the agriculture industry.

Economically, *A. tumefaciens* is a serious pathogen of walnuts, grape vines, stone fruits, nut trees, sugar beets, horse radish, and rhubarb, and the persistent nature of the tumors or galls caused by the disease make it particularly harmful for perennial crops.

Agrobacterium tumefaciens grows optimally at 28 °C (82 °F). The doubling time can range from 2.5–4h depending on the media, culture format, and level of aeration. At temperatures above 30 °C (86 °F), *A. tumefaciens* begins to experience heat shock which is likely to result in errors in cell division.

Conjugation

To be virulent, the bacterium contains a tumour-inducing plasmid (Ti plasmid or pTi) 200 kbp long, which contains the T-DNA and all the genes necessary to transfer it to the plant cell. Many strains of *A. tumefaciens* do not contain a pTi.

Since the Ti plasmid is essential to cause disease, prepenetration events in the rhizosphere occur to promote bacterial conjugation - exchange of plasmids amongst bacteria. In the presence of opines, *A. tumefaciens* produces a diffusible conjugation signal called 30C8HSL or the *Agrobacterium* autoinducer .This activates the transcription factor TraR, positively regulating the transcription of genes required for conjugation

Infection methods

Agrobacterium tumefaciens infects the plant through its Ti plasmid. The Ti plasmid integrates a segment of its DNA, known as T-DNA, into the chromosomal DNA of its host plant cells. *A. tumefaciens* has flagella that allow it to swim through the soil towards photoassimilates that accumulate in the rhizosphere around roots. Some strains may chemotactically move towards chemical exudates from plants, such as acetosyringone and sugars, which indicate the presence of a wound in the plant through which the bacteria may enter. Phenolic compounds are recognised by the VirA protein, a transmembrane protein encoded in the virA gene on the Ti plasmid. Sugars are recognised by the chvE protein, a chromosomal gene-encoded protein located in the periplasmic space.

At least 25 *vir* genes on the Ti plasmid are necessary for tumor induction. In addition to their perception role, *virA* and *chvE* induce other *vir* genes. The VirA protein has autokinase activity: it phosphorylates itself on a histidine residue. Then the VirA protein phosphorylates the VirG protein on its aspartate residue. The virG protein is a cytoplasmic protein produced from the *virG* Ti plasmid gene. It is a transcription factor, inducing the transcription of the *vir* operons. The ChvE protein regulates the second mechanism of the *vir* genes' activation. It increases VirA protein sensitivity to phenolic compounds.

Attachment is a two-step process. Following an initial weak and reversible attachment, the bacteria synthesize cellulose fibrils that anchor them to the wounded plant cell to which they were attracted. Four main genes are involved in this process: *chvA*, *chvB*, *pscA*, and *att*. The products of the first three genes apparently are involved in the actual synthesis of the cellulose fibrils. These fibrils also anchor the bacteria to each other, helping to form a microcolony. VirC, the most important virulent protein, is a necessary step in the recombination of illegitimate recolonization. It selects the section of the DNA in the host plant that will be replaced and it cuts into this strand of DNA.

After production of cellulose fibrils, a calcium-dependent outer membrane protein called rhicadhesin is produced, which also aids in sticking the bacteria to the cell

wall. Homologues of this protein can be found in other rhizobia. Currently, there are several reports on standardisation of protocol for the *Agrobacterium*-mediated transformation. The effect of different parameters such as infection time, acetosyringone, DTT, and cysteine have been studied in soybean (*Glycine max*).

Possible plant compounds that initiate *Agrobacterium* to infect plant cells:

- Acetosyringone and other phenolic compounds
- alpha-Hydroxyacetosyringone
- Catechol
- Ferulic acid
- Gallic acid
- p-Hydroxybenzoic acid
- Protocatechuic acid
- Pyrogallic acid
- Resorcylic acid
- Sinapinic acid
- Syringic acid
- Vanillin

Formation of the T-pilus

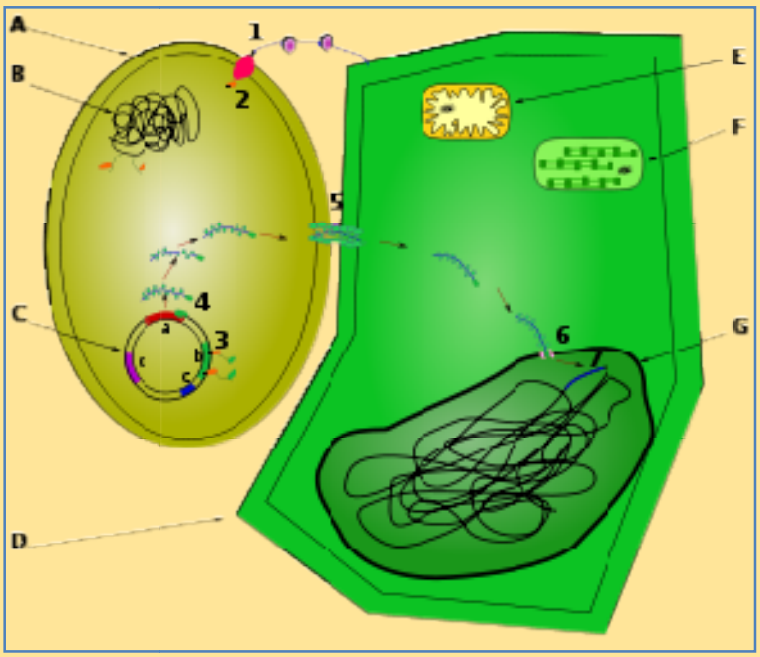
To transfer the T-DNA into the plant cell, *A. tumefaciens* uses a type IV secretion mechanism, involving the production of a T-pilus. When acetosyringone and other substances are detected, a signal transduction event activates the expression of 11 genes within the VirB operon which are responsible for the formation of the T-pilus.

The pro-pilin is formed first. This is a polypeptide of 121 amino acids which requires processing by the removal of 47 residues to form a T-pilus subunit. The subunit was though to be circularized by the formation of a peptide bond between the two ends of the polypeptide. However, high-resolution structure of the T-pilus revealed no cyclization of the pilin, with the overall organization of the pilin subunits being highly similar to those of other conjugative pili, such as F-pilus.

Products of the other VirB genes are used to transfer the subunits across the plasma membrane. Yeast two-hybrid studies provide evidence that VirB6, VirB7, VirB8, VirB9 and

VirB10 may all encode components of the transporter. An ATPase for the active transport of the subunits would also be required.

Transfer of T-DNA into the plant cell



A: *Agrobacterium tumefaciens*

B: Agrobacterium genome

C: Ti Plasmid: a: T-DNA, b: Vir genes, c: Replication origin, d: Opines catabolism genes

D: Plant cell

E: Mitochondria

F: Chloroplast

G: Nucleus

The T-DNA must be cut out of the circular plasmid. This is typically done by the Vir genes within the helper plasmid. A VirD1/D2 complex nicks the DNA at the left and right border sequences. The VirD2 protein is covalently attached to the 5' end. VirD2 contains a motif that leads to the nucleoprotein complex being targeted to the type IV secretion system (T4SS). The structure of the T-pilus showed that the central channel of the pilus is too narrow to allow the transfer of the folded VirD2, suggesting that VirD2 must be partially unfolded during the conjugation process.

In the cytoplasm of the recipient cell, the T-DNA complex becomes coated with VirE2 proteins, which are exported through the T4SS independently from the T-DNA

complex. Nuclear localization signals, or NLSs, located on the VirE2 and VirD2, are recognised by the importin alpha protein, which then associates with importin beta and the nuclear pore complex to transfer the T-DNA into the nucleus. VIP1 also appears to be an important protein in the process, possibly acting as an adapter to bring the VirE2 to the importin. Once inside the nucleus, VIP2 may target the T-DNA to areas of chromatin that are being actively transcribed, so that the T-DNA can integrate into the host genome.

Hormones

To cause gall formation, the T-DNA encodes genes for the production of auxin or indole-3-acetic acid via the IAM pathway. This biosynthetic pathway is not used in many plants for the production of auxin, so it means the plant has no molecular means of regulating it and auxin will be produced constitutively. Genes for the production of cytokinins are also expressed. This stimulates cell proliferation and gall formation.

Opines

The T-DNA contains genes for encoding enzymes that cause the plant to create specialized amino acid derivatives which the bacteria can metabolize, called opines. Opines are a class of chemicals that serve as a source of nitrogen for *A. tumefaciens*, but not for most other organisms. The specific type of opine produced by *A. tumefaciens* C58 infected plants is nopaline (Escobar *et al.*, 2003).

Two nopaline type Ti plasmids, pTi-SAKURA and pTiC58, were fully sequenced. "*A. fabrum*" C58, the first fully sequenced pathovar, was first isolated from a cherry tree crown gall. The genome was simultaneously sequenced by Goodner *et al.* and Wood *et al.* in 2001. The genome of *A. tumefaciens* C58 consists of a circular chromosome, two plasmids, and a linear chromosome. The presence of a covalently bonded circular chromosome is common to Bacteria, with few exceptions. However, the presence of both a single circular chromosome and single linear chromosome is unique to a group in this genus. The two plasmids are pTiC58, responsible for the processes involved in virulence, and pAtC58, once dubbed the "cryptic" plasmid.

The pAtC58 plasmid has been shown to be involved in the metabolism of opines and to conjugate with other bacteria in the absence of the pTiC58 plasmid. If the Ti plasmid is removed, the tumor growth that is the means of classifying this species of bacteria does not occur.

Disease Cycle

Agrobacterium tumefaciens overwinters in infested soils. Agrobacterium species live predominantly saprophytic lifestyles, so its common even for plant-parasitic species of this genus to survive in the soil for lengthy periods of time, even without host plant presence. When there is a host plant present, however, the bacteria enter the plant tissue via recent wounds or natural openings of roots or stems near the ground. These wounds may be caused by cultural practices, grafting, insects, etc. Once the bacteria have entered the plant, they occur intercellularly and stimulate surrounding tissue to proliferate due to cell transformation. Agrobacterium performs this control by inserting the plasmid T-DNA into the plant's genome. See above for more details about the process of plasmid DNA insertion into the host genome. Excess growth of the plant tissue leads to gall formation on the stem and roots. These tumors exert significant pressure on the surrounding plant tissue, which causes this tissue to become crushed and/or distorted. The crushed vessels lead to reduced water flow in the xylem. Young tumors are soft and therefore vulnerable to secondary invasion by insects and saprophytic microorganisms. This secondary invasion causes the breakdown of the peripheral cell layers as well as tumor discoloration due to decay. Breakdown of the soft tissue leads to release of the Agrobacterium tumefaciens into the soil allowing it to restart the disease process with a new host plant

Disease Management

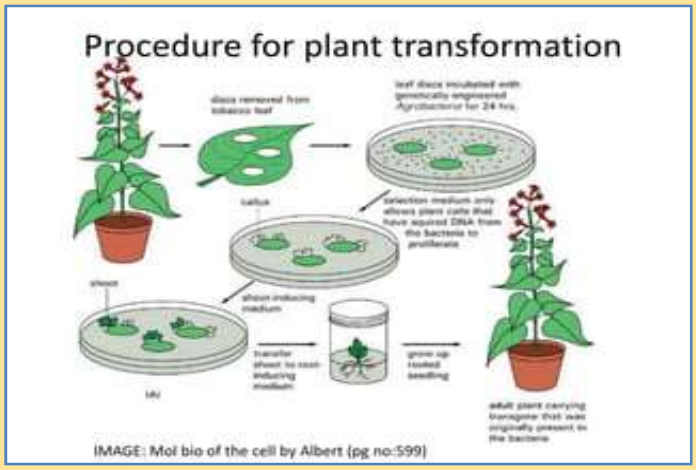
Crown gall disease caused by *Agrobacterium tumefaciens* can be controlled by using various methods. The best way to control this disease is to take preventative measures, such as sterilizing pruning tools so as to avoid infecting new plants. Performing mandatory inspections of nursery stock and rejecting infected plants as well as not planting susceptible plants in infected fields are also valuable practices. Avoiding wounding the crowns/roots of the plants during cultivation is important for preventing disease. In horticultural techniques in which multiple plants are joined to grow as one, such as budding and grafting these techniques lead to plant wounds. Wounds are the primary location of bacterial entry into the host plant. Therefore, it is advisable to perform these techniques during times of the year when *Agrobacteria* are not active. Control of root-chewing insects is also helpful to reduce levels of infection, since these insects cause wounds (aka bacterial entryways) in the plant

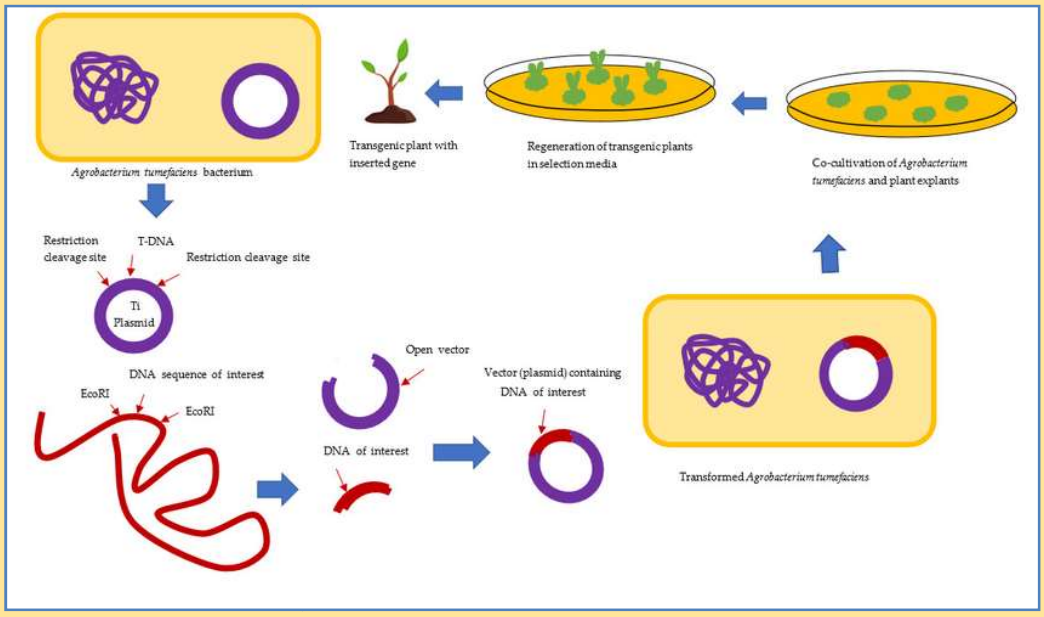
roots. It is recommended that infected plant material be burned rather than placed in a compost pile due to the bacteria's ability to live in the soil for many years.

Biological control methods are also utilized in managing this disease. During the 1970s and 1980s, a common practice for treating germinated seeds, seedlings, and rootstock was to soak them in a suspension of K84. K84 is composed of *A. radiobacter,* which is a species related to *A. tumefaciens* but is not pathogenic. K84 produces a bacteriocin (agrocin 84) which is an antibiotic specific against related bacteria, including *A. tumefaciens*. This method, which was successful at controlling the disease on a commercial scale, had the risk of K84 transferring its resistance gene to the pathogenic *Agrobacteria.* Thus, in the 1990s, the use of a genetically engineering strain of K84, known as K-1026, was created. This strain is just as successful in controlling crown gall as K84 without the caveat of resistance gene transfer.

Environment

Host, environment, and pathogen are extremely important concepts in regards to plant pathology. Agrobacteria have the widest host range of any plant pathogen, so the main factor to take into consideration in the case of crown gall is environment. There are various conditions and factors that make for a conducive environment for A. tumefaciens when infecting its various hosts. The bacterium can't penetrate the host plant without an entry point such as a wound. Factors leading to wounds in plants include cultural practices, grafting, freezing injury, growth cracks, soil insects, and other animals in the environment causing damage to the plant. Consequently, in exceptionally harsh winters, it is common to have an increased incidence of crown gall due to the weather-related damage. Along with this, there are methods of mediating infection of the host plant. For example, nematodes can act as a vector to introduce Agrobacterium into plant roots. More specifically, the root parasitic nematodes damage the plant cell, creating a wound for the bacteria to enter through. Finally, temperature is a factor when considering A. tumefaciens infection. The optimal temperature for crown gall formation due to this bacterium is 22 °C (72 °F) because of the thermosensitivity of T-DNA transfer. Tumor formation is significantly reduced at higher temperature conditions.





Gene Transfer Methods Applicable to Agricultural Organisms

The transfer of genes from one organism to another is a natural process that creates variation in biological traits. This fact underlies all attempts to improve agriculturally important species, whether through traditional agricultural breeding or through the techniques of molecular biology. In both cases, human beings manipulate a naturally occurring process to produce varieties of organisms that display desired traits, for example, food animals with a higher proportion of muscle to fat, or disease-resistant corn.

The major differences between traditional agricultural breeding and molecular biological methods of gene transfer lie neither in aims nor in processes, but rather in speed, precision,

reliability, and scope. When traditional, or classical, breeders cross two sexually reproducing plants or animals, they mix tens of thousands of genes in the hope of obtaining progeny with the desired trait or traits. Through the fusion of sperm and egg, each parent contributes half of its genome (an organism's entire repertoire of genes) to its offspring, but the composition of that half varies in each parental sex cell and hence in each cross. In addition, because the traits desired usually come from only one parent and may be controlled by one or a few genes, many crosses are necessary before the "right" chance recombination of genes results in expression of the trait in the offspring. Even then, the progeny usually have to be crossed back to the parental variety to ensure stable adoption of the new trait. Sometimes undesired traits derived from one parent of a new, improved variety persist whereas the desired traits are lost.

Such are the difficulties and limitations of classical breeding. Molecular biological methods of gene transfer alleviate some of these problems by allowing the process to be manipulated at a more fundamental level. Instead of gambling on recombination of large numbers of genes, scientists can insert individual genes for specific traits directly into an established genome. They can also control the way in which these genes express themselves in the new variety of plant or animal. In short, by homing in on desired traits, molecular gene transfer can shorten the breeding time for new varieties and, in addition, lead to improvements not possible by traditional breeding.

Laboratory methods to move individual genes between organisms capitalize on naturally occurring mechanisms of gene transfer other than sexual reproduction. These include uptake of DNA by cells and cell-to-cell transfer of packaged genetic material such as viruses. Scientists began by studying these mechanisms in simple systems—bacteria and the viruses that infect them. Research has progressed at a remarkable rate. Now scientists can transfer genes into organisms as diverse as soybeans and sheep. Much work remains, however, to perfect gene transfer and its attendant technologies of embryo culture and plant regeneration.

Scientists have relied heavily on favorite model organisms such as the bacterium *Echerischia coli* and the fruit fly *Drosophila melanogaster* , because of their ease of manipulation and the large body of scientific knowledge accumulated about them. Model systems are critical to the progress of research. Nevertheless, molecular biologists must extend their techniques to commercially important agricultural organisms. Movement in this direction will not replace

all traditional agricultural breeding with molecular gene transfer. It will, however, expand the array of methods available to improve agriculturally important species.

Direct DNA Uptake

The earliest and still most widely used method for introducing DNA into animal cells grown in culture in the laboratory is direct uptake of DNA from the surrounding culture medium. The conditions are in principle the same as those used for bacterial cells: DNA must enter the cell and become stably maintained and inherited in the cell line in such a way that its new genetic information is expressed to confer a new trait on the cell.

The mechanics differ because animal cells differ structurally from bacterial cells. On the one hand, animal cells have only a membrane surrounding their contents, whereas bacterial cells (and plant, fungal, and yeast cells) have both a membrane and a wall. The rigid cell wall of the latter organisms often must be removed to allow DNA to enter the cell. On the other hand, most of the genetic information in animal, plant, fungal, and yeast cells is sequestered in the nucleus, an organelle surrounded by its own membrane. (Organisms that have cell nuclei are known as eucaryotes.) New genetic material usually must pass through this second membrane in order to be permanently added to a eucaryotic cell. Bacteria (known as procaryotes) lack an organized nucleus and usually accept new DNA more easily.

The major advantages of direct DNA uptake (facilitated by chemical or electrical treatments, as will be described) are its simplicity and applicability to many organisms and cell types. Hundreds of thousands of cells may be simultaneously treated, in contrast to microinjection of DNA into individual cells (described later), which is laborious and time consuming. Because it is so simple and rapid, direct uptake is extremely useful for basic studies of gene expression in cell culture. These studies are important for characterizing a gene's function, before researchers attempt elaborate and time-consuming gene transfer experiments in whole animals or plants.

Foreign genes introduced by direct uptake are expressed in their new host cells after a short period, usually 1 or 2 days. Direct DNA uptake thus quickly reveals the function of newly isolated or engineered genes during this period of "transient expression." For long-term studies the genes must integrate into the cell's own chromosomes, or be carried in by the uptake of new chromosomes, to ensure that they are stably inherited. Integration occurs at a high frequency after direct DNA uptake into animal cells because so many copies of the

foreign genes have been introduced. (Maintenance on new chromosomes is discussed in the sections on Cell Fusion and Vector-Mediated Gene Transfer.)

In addition, gene transfer into cultured cells by direct DNA uptake is used for the commercial production of genetically engineered proteins. Drugs, hormones, food additives, and other valuable substances can be manufactured by cells into which the appropriate genes have been transferred. Human insulin for treatment of diabetics is now manufactured in bacteria in this way.

The limitations of direct uptake, particularly for animals, center on the fact that intact organisms usually are not suitable recipients. Thus, gene transfer into an animal embryo usually must be accomplished by other means. For plants this is not a strict limitation, as many species can be regenerated into whole plants from a single cultured cell.

Chemical Treatments

Chemical treatments can induce animal cells to take up DNA from their medium; most frequently these cells are in culture rather than in living animals. In the simplest and most popular method, cells are mixed with DNA that has been precipitated with calcium phosphate (Graham and van der Eb, 1973). This treatment compacts the DNA, so cells take up many copies of the foreign genes. Alternatively, the chemical DEAE-dextran may be used to facilitate DNA uptake (McCutchan and Pagano, 1968).

Cells in culture are relatively unspecialized and often do not correctly regulate genes as would the specialized organs of an intact animal. Researchers have therefore developed a technique to introduce DNA directly into intact organs, such as the liver or spleen, of living animals. Calcium phosphate-precipitated DNA is injected directly into the organs, in combination with low concentrations of enzymes that allow the DNA to enter (Dubensky et al., 1984). This technique enables researchers to quickly study an isolated gene's function in the differentiated, specialized cells of an intact organ, which more accurately reflect the gene's proper function in an animal. A variation of the organ transformation technique involves injecting the calcium phosphate-precipitated DNA intraperitoneally, where it is taken up and expressed by animal tissues, such as those of the liver and spleen (Benvenisty and Reshef, 1986).

Plant cells have been difficult to transform by chemical methods, but recently breakthroughs have been made. Polyethylene glycol has been used to obtain direct uptake and stable

maintenance of DNA by protoplasts from a species of wheat, *Triticum monococcum* (Lörz et al., 1985), another monocot grass, *Lolium multiforum* (Potrykus et al., 1985a), and the dicots oilseed rape, tobacco, and petunias (Potrykus et al., 1985b). The frequency of integration of DNA after direct uptake is sometimes lower than for vector-mediated gene transfer into plants (discussed later), but there are no species restrictions on the type of host cell. However, protoplasts are used as recipients, so they must be capable of regenerating into plants for direct uptake to yield genetically altered species for agriculture.

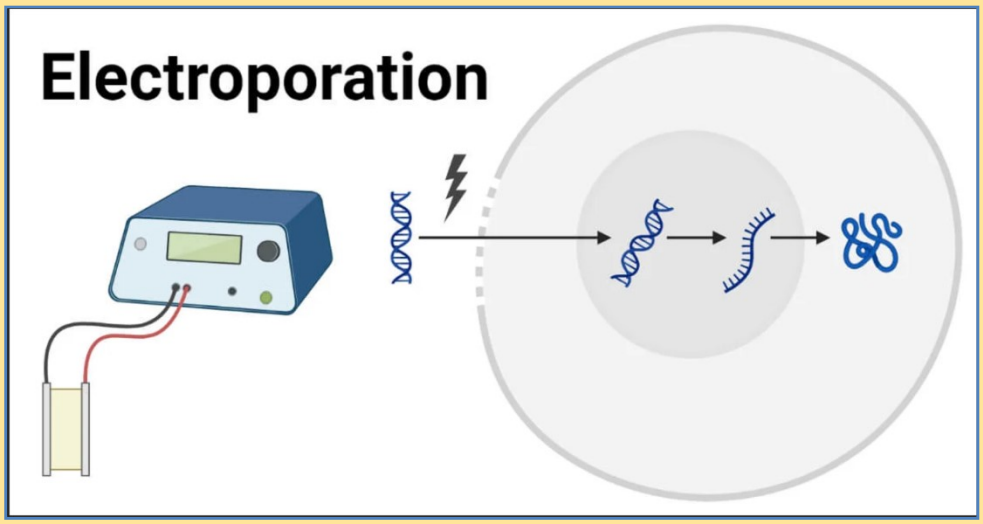
Insect, fungal, yeast, and bacterial cells are all amenable to variations of calcium phosphate or other chemical treatments for direct DNA uptake. Often, direct uptake is used to introduce vector DNA molecules containing engineered genes. Direct uptake procedures simply place foreign genes inside the cell; vectors can help to integrate the genes into the cell's chromosomes or stably maintain the genes within the cell on the vector's minichromosome (see the section on Vector-Mediated Gene Transfer).

Electroporation

A newer method that is being widely adopted is electroporation (Neumann et al., 1982; Potter et al., 1984). Cells are mixed with DNA in solution and subjected to a brief pulse of electrical current. It is thought that the current pulse creates transient pores in the cell's membrane that allow DNA to enter efficiently.

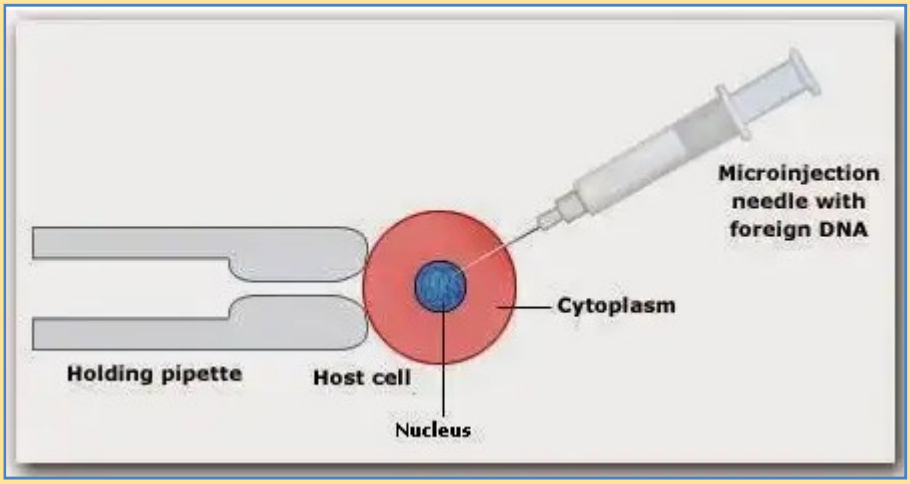
Electroporation may work for any type of cell, even those that have resisted DNA uptake by chemical treatments, for example, cells of the immune system.

Electroporation can introduce DNA into protoplasts of both major categories of plants—dicots (e.g., carrots and tobacco) and monocots (e.g., corn; Fromm et al., 1985). Electroporation provides a transient gene expression system for plants. As discussed previously, transient expression systems are very useful for preliminary characterization of new genes. The lack of such a system for plants had previously held up progress in characterizing plant genes. Electroporation also permits stable integration of genes into plant chromosomes. It has been used successfully to stably transform corn and tobacco cells



DNA Microinjection

NA can be injected directly into single living cells using very fine glass pipettes (hollow needles). Experimenters use an elaborate apparatus consisting of a microscope and delicate micromanipulators to view the cell, hold it steady, and inject a solution containing DNA. As with chemical or electrical uptake methods, foreign genes can be in the form of isolated molecules or attached to vectors. A disadvantage compared to direct uptake is that relatively few individual cells can be injected; however, the frequency of successful incorporation of DNA per injected cell is higher.



Animals

Microinjection has been very successful for delivering foreign genes into mouse embryos at an early stage of development. Usually DNA is injected directly into a particular structure, the male pronucleus, of a fertilized mouse egg. This is the most receptive structure to the

incorporation of foreign DNA. The embryos are subsequently reimplanted into foster mothers for development to term. Foreign genes are incorporated into the developing cells' chromosomes and are often present in every cell of the mature animal. Animals given new genes by this procedure are called "transgenic." Their new genes are usually passed on normally to their progeny. These foreign genes can be expressed, that is, make their protein products, which can confer new characteristics on the animal. The now classic example is transgenic mice containing foreign genes for growth hormone. Expression of these genes caused the mice to grow to up to twice their normal size (Hammer et al., 1984; Palmiter et al., 1983).

Many other animal genes have now been transferred into fertilized mouse eggs by microinjection and correctly expressed in the resulting mature mice. These include the chicken transferrin gene expressed in the liver (McKnight et al., 1983); a mouse immunoglobulin gene expressed in the spleen (Brinster et al., 1983); the rat elastase gene expressed in the pancreas (Swift et al., 1984); the rat skeletal muscle myosin gene expressed in skeletal muscle (Shani, 1985); a chimaeric mouse/human β-globin gene in blood, bone marrow, and spleen (Chada et al., 1985); and a swine histocompatibility gene (Frels et al., 1985).

Traits of potential economic value to the farmer that might be transferred by microinjection include increased levels of certain circulating hormones, antibiotic resistance, and immunoglobulins (antibodies) for "genetic vaccination" against pathogens. As noted previously, the introduction and expression of such genes has been successful in mice.

A necessary supporting technology for in vitro microinjection of mammalian embryos is embryo transfer into surrogate mother animals, for in vivo development of the embryos to term. Embryos of each livestock species must be handled in a slightly different manner, which must be experimentally determined.

Hammer and his collegues (1985) reported the successful production of transgenic farm animals (rabbits, sheep, and pigs) by microinjection. The same foreign gene for growth hormone used to produce transgenic mice was used for these other species. New techniques were needed to visualize pronuclei for microinjection, because of differences in the fertilized eggs of each species. The microinjected gene was integrated into the chromosomes of all three species, and was expressed in some of the transgenic rabbits and pigs.

Scientists have been very successful in microinjecting genes into embryos of the laboratory fruit fly *Drosophila melanogaster* for studies on the molecular biology of this insect. Rubin and Spradling (1982) pioneered this approach with their transposable P-element vector (discussed in the section on Vector-Mediated Gene Transfer). This vector or others similar to it might be

adapted for both beneficial and harmful insects of agronomic importance.

Researchers routinely microinject genes into frog eggs, which are very large and metabolically active cells, for basic studies on gene expression in animals. More recently, microinjection was used to transfer DNA into the chromosomes of developing fish eggs (Chourrout et al., 1986; Zhu et al., 1985). Projects are aimed at basic studies of fish molecular biology and questions of how fish respond to their environment at the molecular level, as well as at aquacultural applications.

Both bovine and fish growth hormone genes have been injected into fish eggs. It has already been shown that injection of the purified protein hormones augments fish growth (Gill et al., 1985; Sekine et al., 1985). Transferred genes should be even more effective than purified hormones in promoting fish growth. Researchers have injected metallothionein genes from both mammals and fish into fish eggs, with the goal of engineering fish resistant to toxic metals. They have injected "antifreeze" genes obtained from winter flounder (also found in all antarctic fishes) to increase the cold tolerance of commercially valuable fish.

Plants

Microinjection can be used to deliver genetic material into plant cells. Segments of DNA, whole chromosomes, and even cellular organelles such as chloroplasts, which contain their own DNA molecules, can be microinjected by methods used for animal cells, although certain physical properties of plant cells complicate the technique.

Key elements for protoplast microinjection include microscopic resolution of the cell nucleus, which is enhanced by staining with dyes; immobilization of the cell by a holding pipette, embedding within agarose, or adhesion to glass surfaces; and efficient cell culture techniques. Researchers can successfully transform up to 14 percent of the cells they microinject with DNA (Crossway et al., 1986). This high frequency might be increased further by using microinjection in conjunction with specially developed vectors, derived from the Ti plasmid or plant transposable elements (see sections on these vectors). Because of the high

transformation frequency possible with microinjection, a direct selection scheme (e.g., drug resistance) is unnecessary. Furthermore, specific hostrange requirements associated with the Ti plasmid or viral vectors are obviated.

Although at present the recipient plant species must be amenable to cell culture and regeneration from protoplasts, suspension cultures or pollen grains may be used in the future, which would bypass the problem of regeneration. Alternatively, DNA may be injected into the developing floral side-shoots of plants, where it can pass into germ cells. Researchers have reported that the cereal rye (a monocot) can be transformed in this way (de la Peña et al., 1987).

Microinjection of individual chromosomes or cellular organelles (e.g., chloroplasts, mitochondria, and nuclei) could potentially produce improved cultivars with new traits such as herbicide resistance or cytoplasmic male sterility. Transfer of traits by microinjection would be more direct, precise, and faster than by breeding or cell fusion (described in the next section), because microinjection transfers a specific, limited amount of genetic information. There would be less need for selection or backcrossing, which are often time-consuming, difficult processes.

Most agronomic traits are polygenic, that is, they are caused by the interplay of several different genes in the plant. Genetic studies often reveal that these genes are linked in blocks on specific segments of chromosomes. Classical plant breeding can sometimes transfer such traits between species via interspecific crosses, but these crosses are not always successful. Transfer of individual chromosomes would permit researchers to introduce traits that result from the interaction of several genes linked on that chromosome.

Chromosome microinjection would also enable the transfer of traits that are encoded by single genes that have not yet been identified and isolated. Much of the sophisticated biochemistry and genetics of single-gene traits known for animals and used to isolate important genes is lacking for plants. Consequently, few plant genes of agronomic importance have been isolated. Whole chromosome transfer may allow scientists to genetically engineer plants that would not be tractable at this time by more sophisticated gene-splicing (recombinant DNA) techniques. Attempts are being made to transform plant cells by microinjection of isolated chromosomes (Greisbach, 1983, 1987).

Cell Fusion

Cell fusion combines the entire genetic contents of two cells, producing hybrid cells that often express certain traits from both parents. The parent cells can be from different species or from different types of the same species. Fusion is usually mediated by chemicals such as polyethylene glycol or dimethylsulfoxide, although newer techniques use electrofusion.

Animal Cells

Cell fusion is the basis for the manufacture of monoclonal antibodies. Monoclonal antibody-producing cell lines (hybridomas) are created by fusing antibody-producing B-cells from animals with myeloma cells, which grow indefinitely in culture. The pure, highly specific antibodies thus obtained are important reagents for research, medicine, and agriculture. Diagnostic kits and vaccines for animal health based on monoclonal antibodies are already on the market (Gamble, 1986). Diagnosis of plant pathogens such as viruses, bacteria, fungi, and nematodes can also be facilitated by tests based on monoclonal antibodies; commercial products should be available in the near future (Gamble, 1986).

Certain agricultural applications have been held back by lack of suitable myeloma lines for fusion with B-cells from farm animals, as opposed to standard laboratory animals such as the mouse. However, this problem can be surmounted by creating hybridomas by direct DNA uptake. DNA from B-cells and myeloma cells is simultaneously introduced into recipient cells by calcium phosphate coprecipitation or by electroporation (Gamble, 1986). This approach obviates the need to fuse interspecific cell lines, and thus solves the problem of finding suitable myeloma lines for different livestock species.

Fusion of animal cell lines in culture is also exploited to map genes to specific chromosomes, an important step in locating genes to use in transfer experiments and in breeding strategies. Gene maps for mice and men are quite advanced. Those for livestock lag behind, but efforts are starting, notably for swine (Fries and Ruddle, 1986). To map these genes, swine cells are fused to mouse cells in culture. The interspecies cell hybrids reject most of the swine chromosomes. Ideally, a set of cell lines, each harboring a single different swine chromosome, is made. Known DNA sequences are used as probes for particular genes with those sequences. These probes bind to defined lengths of DNA from the fused cells. Because swine and mouse chromosomes can be distinguished by small differences in DNA sequences (known as restriction fragment length polymorphisms), differences in the lengths of DNA

containing the gene detected by the probe indicate whether that gene is on a swine or a mouse chromosome of the hybrid cell. Location on a swine chromosome pinpoints the gene to that single particular swine chromosome, which is the only swine chromosome in the hybrid cell. Gene mapping is expected to play an important role in finding genes for transfer of complex traits in livestock, such as lactation, fertility, growth, and disease resistance.

Plant Cells

In eucaryotic cells the cytoplasm—that part of the cell surrounding the nucleus—contains organelles that have their own separate DNA. In plants, protoplast fusion is used to transfer genes from both the nucleus and the cytoplasm. Fusion combines the genomes of two parents, as in traditional breeding, but results can sometimes be obtained faster, even though the fusion product must be backcrossed to the recipient line for several generations to create a new, stable line possessing the one trait desired from the donor. Protoplast fusion can be used for transferring genes that are hard to identify, isolate, and clone or for polygenic traits. Furthermore, protoplast fusion can be used for plants that cannot be crossed sexually (although plants regenerated from such fused hybrids may sometimes be sterile)

Vector –mediated gene transfer

A vector is a molecule of DNA that is attached to a foreign gene to facilitate its transfer, maintenance, and expression within the target cell. Vectors offer many advantages: high frequency of gene transfer, transfer into specific cell types, more control over the final copy number of a transferred gene, and certain properties that make them easy to track, permit them to be stably maintained in the target cell, and enable them to express foreign genes. Vectors can, therefore, greatly improve gene transfer. However, different species and cell types may require different types of vectors, and often much work must go into creating an appropriate vector system before genes can be transferred into a specific organism.

Plant Viruses

Cauliflower Mosaic Virus

Only small steps have been taken with viral vectors for plants, in contrast to the great strides in virally mediated gene transfer into animals. There are no known plant retroviruses and only a few, small DNA viruses. The best-studied virus is cauliflower mosaic virus (CaMV), a

small double-stranded DNA virus that infects cruciferous plants, such as cabbage and mustard. CaMV is transmitted in nature by aphids, but its DNA can infect plants if simply rubbed onto their leaves. CaMV causes systemic infection and replicates abundantly throughout the plant. It thus should transfer many copies of a gene per cell into all tissues of a mature plant. Furthermore, powerful CaMV gene regulation sequences can promote high-level expression of foreign genes. In fact, CaMV promoters are being used to augment the expression of plant genes transferred via other systems, as most plants recognize these promoters even when they are detached from the rest of CaMV.

The biggest obstacles to the development of a CaMV vector have been the severe limitation on the virus's size and thus on the quantity of DNA that can be inserted, and the instability of the genetically engineered virus. This instability may be caused both by the packaging limitation on extra DNA and by the way the virus replicates. Furthermore, CaMV does not integrate into plant genomes under normal conditions of infection. Some success in introducing foreign genes into plants using CaMV has been reported, however. Bacterial drug resistance genes were expressed and stably propagated in CaMV-infected turnip plants (Brisson et al., 1984).

Geminiviruses

Geminiviruses are single-stranded DNA viruses of plants that are transmitted by insects, such as leafhoppers. Viruses in this group infect many crops, including the monocots wheat and corn and the dicots beans, tobacco, and tomatoes. Work on developing a vector system based on these viruses is in progress (Kridl and Goodman, 1985; Lazarowitz, 1987).

Recently published experiments indicate that geminiviruses can be combined with the *Agrobacterium* Ti plasmid delivery system (described in a subsequent section) to obtain "agroinfection" of corn plants with the geminivirus maize streak virus (Grimsley et al., 1987). This dual system may prove useful in introducing engineered geminivirus vectors into plants, because often their DNA is not infectious unless transmitted as as intact virus by the natural insect mechanism. These experiments also demonstrated that *Agrobacterium* can transfer DNA to corn, a monocot, which was thought not to be amenable to the Ti plasmid gene transfer system.

RNA Viruses

Although there are many known plant RNA viruses, progress has been limited by the fact that manipulations developed to recombine DNA cannot be done on RNA directly. However, scientists can construct complementary DNA copies of RNA virus genomes. These copies can be used to construct a vector that will carry a foreign gene. The DNA can then be transcribed back into RNA, enabling the engineered virus to infect cells.

Transposable Elements

Transposable elements (also called "transposons") can move from place to place within an organism's genome and take extra pieces of DNA along for the ride. These elements have some physical and functional properties in common with retroviruses, but they do not spread from cell to cell by infection and therefore are not considered to be viruses. Barbara McClintock first recognized transposable elements in corn 40 years ago, for which she won a Nobel prize in 1983.

Transposable elements have since been found to be widespread in nature: examples have been described in bacteria, yeast, nematodes, fruit flies, mice, corn, soybeans, and snapdragons. It is likely that they will be found to exist for all species. Their apparent ubiquity in nature may make transposons especially useful for genetic modification of agronomically important insects and plants. Already, transposons have been used to modify *Pseudomonas fluorescens* bacteria that live on corn roots by insertion of an insecticidal gene from *Bacillus thuringiensis* (Obukowicz et al., 1986). In addition, studies of gene function aided by the use of transposable elements are very important for understanding basic aspects of gene expression in insects and plants. This knowledge is essential to the application of genetic engineering.

The jumping abilities of transposable elements have been used to isolate important genes from corn (Fedoroff et al., 1984). This is done by inducing the transposable element to jump into the corn gene of interest, thereby inactivating the gene and producing a mutant plant. When DNA from the mutant plant is compared to DNA from a normal plant, the characteristic sequence of the transposable element identifies its location, thus acting as a tag for the mutant gene. The DNA surrounding the transposable element is then cloned, yielding copies of the gene of interest. Although these copies are inactive because of the insertion of the transposable element, their sequences can be used as probes to find the active gene copy

from a normal plant. This gene *isolation* strategy contrasts with gene *transfer* via transposable elements in that for isolation the transposable element is inserted into the gene, whereas for transfer the gene is inserted into the transposable element.

The Ti Plasmid

The most successful gene transfer vector developed thus far for plant cells is the Ti plasmid found in the soil bacterium *Agrobacterium tumefaciens*. Plasmids are circular DNA molecules that exist independently of the cell's main chromosomes; the Ti plasmid is a naturally occurring variety that is quite large. *Agrobacterium* infects most species of dicots and causes a tumorous disease called crown gall. The disease is instigated by natural gene transfer of part of the bacterium's Ti plasmid, called T-DNA, into the plant's chromosomes. Plant cells acquire new properties as a consequence of the transferred genes. Besides metabolic changes that incite their uncontrolled growth into a tumor, the cells are programmed to manufacture certain chemical compounds called opines, which are used by the parasitic *Agrobacterium* as food. Thus *Agrobacterium tumefaciens* is a natural genetic engineer that forces a plant to do its bidding! It inserts its bacterial genes to create tumors composed of altered plant cells that provide it with specialized food.

Researchers have adapted the Ti plasmid to transfer foreign genes into plants and to obtain stable and heritable expression of the genes in normal, nontumorous plants. In order to be able to regenerate plants from cells transformed with T-DNA in culture, they modified the Ti plasmid to eliminate its tumor-promoting properties. Transferred genes can be expressed under the control of their own normal regulatory signals, or T-DNA signals can be used to turn on the foreign

Plants currently amenable to Ti plasmid vectors include petunias, tobacco, soybeans, carrots, tomatoes, alfalfa, and oilseed rape. Genes transferred include the small subunit of the plant photosynthetic enzyme ribulose 1,5-bisphosphate carboxylase (Broglie et al., 1984; Herrera-Estrella et al., 1984), the bean storage protein phaseolin (Murai et al., 1983; Sengupta-Gopalan et al., 1985), the corn storage protein zein (Matzke et al., 1984), the wheat photosynthetic chlorophyll a/b binding protein (Lamppa et al., 1985), and a bacterial enzyme for resistance to the herbicide glyphosate (Comai et al., 1985).

Although many experiments focus on a basic understanding of plant gene regulatory mechanisms, experiments with herbicide and pest resistance genes are already introducing

agronomic modifications into dicotyledonous crops such as soybeans, tomatoes, turnips, tobacco, and oilseed rape. Likewise, the nutritional improvement of seed crops is an important goal. One

Fungal and Bacterial Plasmids

Plasmids occur naturally in yeast, fungi, and bacteria. Scientists have used plasmid vectors extensively for basic research on the molecular biology of strains of these organisms commonly studied in the laboratory. With recombinant DNA techniques, researchers can cut and splice genes into small plasmids quite easily. Likewise, they can combine useful parts from different plasmids to create new plasmid vectors better suited to a particular gene transfer operation. Small plasmids can be introduced into cells by direct DNA uptake. Once inside the cell they replicate and stably maintain themselves and can express foreign genes that have been engineered into them. Furthermore, under certain conditions plasmids can transfer the foreign genes they carry into the host cells' chromosomes, where the genes can also be maintained and expressed. Thus plasmids are versatile vectors for gene transfer into procaryotes (bacteria) and simple eucaryotes (yeast and fungi).

Until recently, transformation systems were lacking for fungi of agricultural and industrial importance. For instance, the fungal corn pathogen *Cochliobolus heterostrophus* contains a toxin gene that might be manipulated to create a weed control agent or to develop resistant strains of corn. Progress was stymied, however, until the development of a plasmid-based gene transfer system for *C. heterostrophus* (Turgeon et al., 1985). Work on other pathogenic fungi is also progressing. The systems for pathogenic fungi rely on elements of a plasmid vector developed for the laboratory model fungus *Aspergillus nidulans* (Yelton et al., 1984).

Pathogenic and beneficial fungi and bacteria are important candidates for agronomically valuable gene transfer strategies. Pathogenic fungi and bacteria can be used as biological control agents for pest insects or weeds. Isolation and transfer of pathogenicity genes has a twofold purpose: construction of improved agents for biological control, and discovery of resistance genes in the plant or insect that counteract the pathogenicity. The use of transposons and plasmids to isolate and study pathogenic genes from fungi and bacteria that attack crop plants will lead to an understanding of the molecular bases of many agronomically critical diseases and suggest ways to combat them.

Beneficial fungi and bacteria may be improved and their host range extended to help other plants and animals. In addition, transformation of beneficial fungi and bacteria should prove

advantageous for introducing improved traits for commercial production of special metabolites such as antibiotics and pigments and for food processing and waste disposal. For example, studies on bacteria with a natural capacity to degrade toxic herbicide and pesticide residues should yield improved strains that may prove useful in detoxifying the environment (Ghosal et al., 1985; Serdar and Gibson, 1985).

Another important aspect of bacterial gene transfer is basic and applied research on strains of *Rhizobium* that fix nitrogen for legumes. These studies have the following goals: improved strains of *Rhizobium*, engineered strains of other bacteria that can fix nitrogen for other crops such as cereals, and perhaps even crops that can fix nitrogen themselves. *Rhizobium* might also be used for the commercial production of ammonia.

Bacteria, as described at the outset, are generally easy targets for gene transfer. However, details must often be worked out for species that differ significantly from the laboratory model *Escherichia coli*.

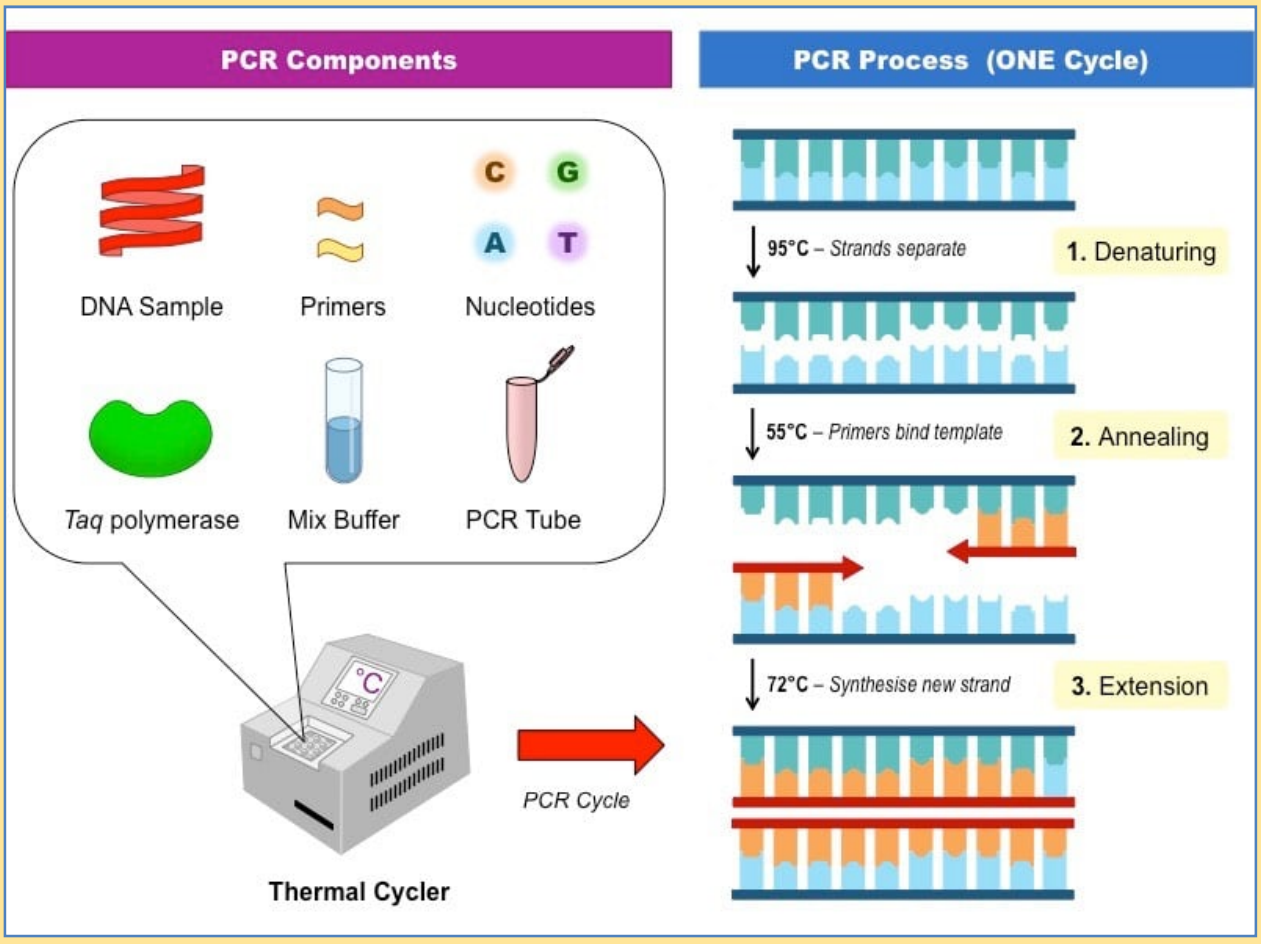
An example of bacterial gene transfer for agricultural purposes is the transfer of an insecticidal toxin gene from *Bacillus thuringiensis* to a *Pseudomonas fluorescens* strain that colonizes corn roots, to extend the number of plant hosts that can be protected against pest insects by the bacterial toxin. *B. thuringiensis* itself has been marketed as an insecticide for many years. After ingestion, its toxin is activated in the insect's gut. There are different strains of *B. thuringiensis* that make toxins capable of killing over 100 different lepidopteran and dipteran pests. These toxins are harmless except to targeted insects, and delivery via bacteria with a specific range of plant hosts ensures a high level of specificity for the pesticide.

Scientists at Monsanto Company have transferred the *B. thuringiensis* toxin gene into *P. fluorescens* via a plasmid and also into the *P. fluorescens* chromosome via a transposon (Obukowicz et al., 1986; Watrud et al., 1985). The new biological insecticide is intended to protect corn against the black cutworm. *P. fluorescens* does not persist in the field, so the genetically engineered bacteria should kill off insects after application early in the growing season and then die.

A second strategy is to transfer the toxin gene into crops, to make them self-protecting. Scientists at the Belgian company Plant Genetic Systems engineered the *B. thuringiensis* toxin gene into plants, which then expressed the toxin and resisted insect predators (Vaeck et al., 1987).

Polymerase chain reaction (PCR)

The **polymerase chain reaction** (**PCR**) is a method widely used to make millions to billions of copies of a specific DNA sample rapidly, allowing scientists to amplify a very small sample of DNA (or a part of it) sufficiently to enable detailed study. PCR was invented in 1983 by American biochemist Kary Mullis at Cetus Corporation. Mullis and biochemist Michael Smith, who had developed other essential ways of manipulating DNA, were jointly awarded the Nobel Prize in Chemistry in 1993.



PCR is fundamental to many of the procedures used in genetic testing and research, including analysis of ancient samples of DNA and identification of infectious agents. Using PCR, copies of very small amounts of DNA sequences are exponentially amplified in a series of cycles of temperature changes. PCR is now a common and often indispensable technique used in medical laboratory research for a broad variety of applications including biomedical research and criminal forensics.

The majority of PCR methods rely on thermal cycling. Thermal cycling exposes reactants to repeated cycles of heating and cooling to permit different temperature-dependent reactions—specifically, DNA melting and enzyme-driven DNA replication. PCR employs two main reagents—primers (which are short single strand DNA fragments known as oligonucleotides that are a complementary sequence to the target DNA region) and a DNA polymerase. In the first step of PCR, the two strands of the DNA double helix are physically separated at a high temperature in a process called nucleic acid denaturation. In the second step, the temperature is lowered and the primers bind to the complementary sequences of DNA. The two DNA strands then become templates for DNA polymerase to enzymatically assemble a new DNA strand from free nucleotides, the building blocks of DNA. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the original DNA template is exponentially amplified.

Almost all PCR applications employ a heat-stable DNA polymerase, such as *Taq* polymerase, an enzyme originally isolated from the thermophilic bacterium *Thermus aquaticus*. If the polymerase used was heat-susceptible, it would denature under the high temperatures of the denaturation step. Before the use of *Taq* polymerase, DNA polymerase had to be manually added every cycle, which was a tedious and costly process.

Applications of the technique include DNA cloning for sequencing, gene cloning and manipulation, gene mutagenesis; construction of DNA-based phylogenies, or functional analysis of genes; diagnosis and monitoring of genetic disorders; amplification of ancient DNA; analysis of genetic fingerprints for DNA profiling (for example, in forensic science and parentage testing); and detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases.

Principles

PCR amplifies a specific region of a DNA strand (the DNA target). Most PCR methods amplify DNA fragments of between 0.1 and 10 kilo base pairs (kbp) in length, although some techniques allow for amplification of fragments up to 40 kbp. The amount of amplified product is determined by the available substrates in the reaction, which becomes limiting as the reaction progresses.

A basic PCR set-up requires several components and reagents, including:

- a *DNA template* that contains the DNA target region to amplify
- a *DNA polymerase*; an enzyme that polymerizes new DNA strands; heat-resistant *Taq* polymerase is especially common, as it is more likely to remain intact during the high-temperature DNA denaturation process
- two DNA *primers* that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strands of the DNA target (DNA polymerase can only bind to and elongate from a double-stranded region of DNA; without primers, there is no double-stranded initiation site at which the polymerase can bind); specific primers that are complementary to the DNA target region are selected beforehand, and are often custom-made in a laboratory or purchased from commercial biochemical suppliers
- *deoxynucleoside triphosphates*, or dNTPs (sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), the building blocks from which the DNA polymerase synthesizes a new DNA strand
- a *buffer solution* providing a suitable chemical environment for optimum activity and stability of the DNA polymerase
- *bivalent cations*, typically magnesium (Mg) or manganese (Mn) ions; Mg^{2+} is the most common, but Mn^{2+} can be used for PCR-mediated DNA mutagenesis, as a higher Mn^{2+} concentration increases the error rate during DNA synthesis; and *monovalent cations*, typically potassium (K) ions

The reaction is commonly carried out in a volume of 10–200 μL in small reaction tubes (0.2–0.5 mL volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction (see below). Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibrium. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermal cyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

Procedure

Typically, PCR consists of a series of 20–40 repeated temperature changes, called thermal cycles, with each cycle commonly consisting of two or three discrete temperature steps (see figure below). The cycling is often preceded by a single temperature step at a very high

temperature (>90 °C (194 °F)), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters, including the enzyme used for DNA synthesis, the concentration of bivalent ions and dNTPs in the reaction, and the melting temperature (T_m) of the primers. The individual steps common to most PCR methods are as follows:

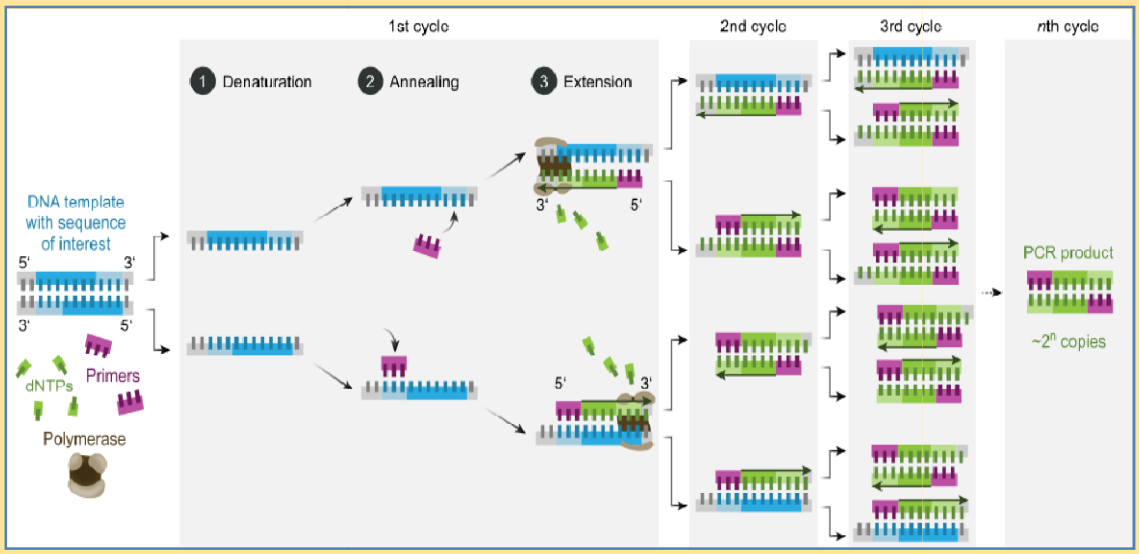
- *Initialization*: This step is only required for DNA polymerases that require heat activation by hot-start PCR. It consists of heating the reaction chamber to a temperature of 94–96 °C (201–205 °F), or 98 °C (208 °F) if extremely thermostable polymerases are used, which is then held for 1–10 minutes.
- *Denaturation*: This step is the first regular cycling event and consists of heating the reaction chamber to 94–98 °C (201–208 °F) for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.
- *Annealing*: In the next step, the reaction temperature is lowered to 50–65 °C (122–149 °F) for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand.

  It is critical to determine a proper temperature for the annealing step because efficiency and specificity are strongly affected by the annealing temperature. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should bind *only* to a perfectly complementary part of the strand, and nowhere else. If the temperature is too low, the primer may bind imperfectly. If it is too high, the primer may not bind at all. A typical annealing temperature is about 3–5 °C below the T_m of the primers used. Stable hydrogen bonds between complementary bases are formed only when the primer sequence very closely matches the template sequence. During this step, the polymerase binds to the primer-template hybrid and begins DNA formation.

- *Extension/elongation*: The temperature at this step depends on the DNA polymerase used; the optimum activity temperature for the thermostable DNA polymerase of *Taq* polymerase is approximately 75–80 °C (167–176 °F), though a temperature of 72 °C (162 °F) is commonly used with this enzyme. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that is complementary to the template in the 5'-to-3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand. The precise time required for elongation depends both on the DNA polymerase used and on the length of the DNA target region to amplify. As a rule of thumb, at their optimal temperature, most DNA polymerases polymerize a thousand bases per minute. Under optimal conditions (i.e., if there are no limitations due to limiting substrates or reagents), at each extension/elongation step, the number of DNA target sequences is doubled. With each successive cycle, the original template strands plus all newly generated strands become template strands for the next round of elongation, leading to exponential (geometric) amplification of the specific DNA target region.

  The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is 2^n, where n is the number of cycles. Thus, a reaction set for 30 cycles results in 2^{30}, or 1,073,741,824, copies of the original double-stranded DNA target region.

  - *Final elongation*: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.
  - *Final hold*: The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and may be employed for short-term storage of the PCR products.

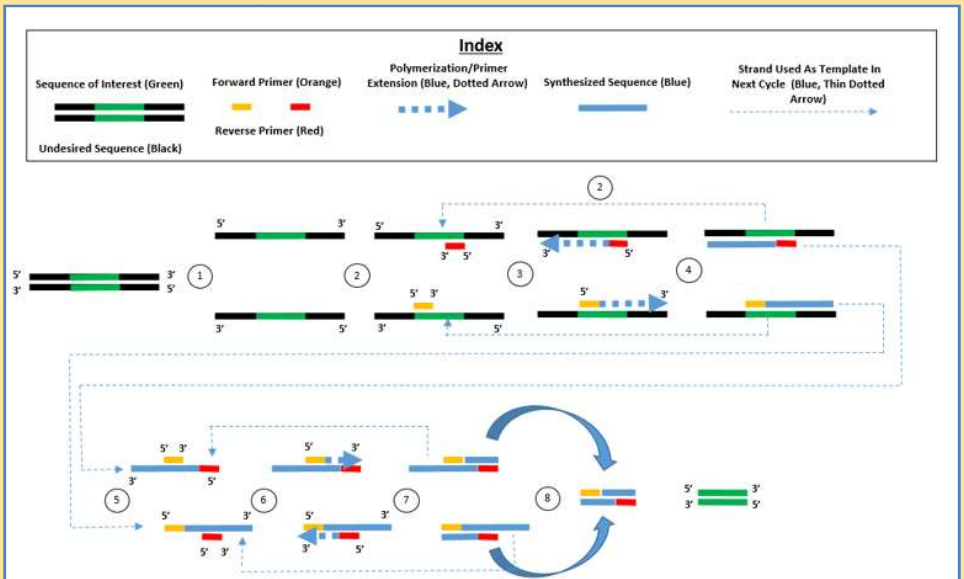


To check whether the PCR successfully generated the anticipated DNA target region (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis may be employed for size separation of the PCR products. The size of the PCR products is determined by comparison with a DNA ladder, a molecular weight marker which contains DNA fragments of known sizes, which runs on the gel alongside the PCR products.

Stages

As with other chemical reactions, the reaction rate and efficiency of PCR are affected by limiting factors. Thus, the entire PCR process can further be divided into three stages based on reaction progress:

- *Exponential amplification*: At every cycle, the amount of product is doubled (assuming 100% reaction efficiency). After 30 cycles, a single copy of DNA can be increased up to 1,000,000,000 (one billion) copies. In a sense, then, the replication of a discrete strand of DNA is being manipulated in a tube under controlled conditions. The reaction is very sensitive: only minute quantities of DNA must be present.
- *Leveling off stage*: The reaction slows as the DNA polymerase loses activity and as consumption of reagents, such as dNTPs and primers, causes them to become more limited.
- *Plateau*: No more product accumulates due to exhaustion of reagents and enzyme.



1. The DNA double helix is melted apart at T> 90°C and its strands separate.
2. The temperature is decreased to slightly below the T_m of both the primers being used. Both primers bind to the available strands. These primers are supplied in excess to insure that the strands do not only come back and reanneal to one another.
3. Polymerization (extension) occurs via DNA Polymerase in the 5' to 3' direction on each strand.
4. Incorporated additional nucleotides give rise to new strands that extend past the sequence of interest.
5. The previously polymerized strands act as template for the other primer (if forward primer bound first, reverse primer now binds and vice versa).
6. Polymerization occurs via DNA Polymerase in the 5' to 3' direction on each strand, this time ending at the end of the sequence of interest.
7. Incorporated additional nucleotides give rise to new strands that only encode the sequence of interest.
8. The synthesized strands encoding the sequence of interest anneal to one another to form the end product.

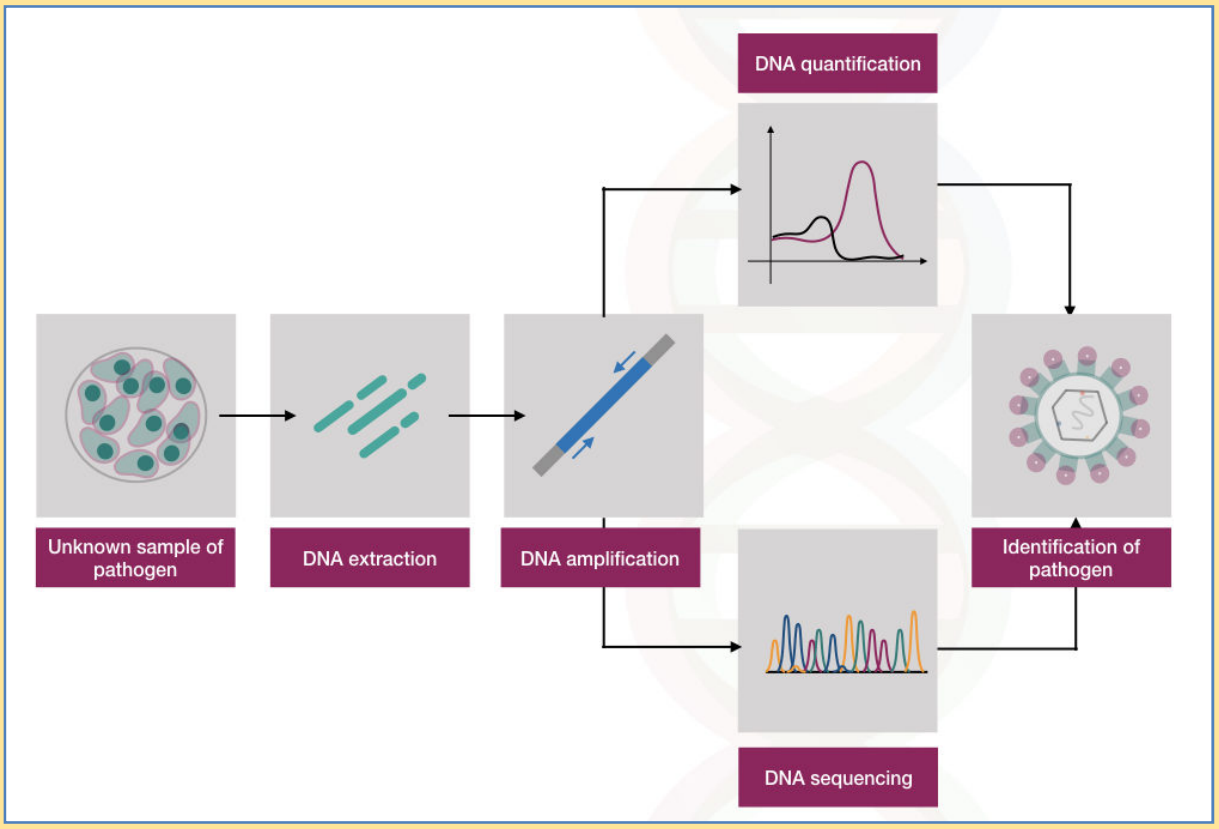
Optimization

In practice, PCR can fail for various reasons, such as sensitivity or contamination. **Contamination** with extraneous DNA can lead to spurious products and is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants. For instance, if DNA from a crime scene is analyzed, a single DNA molecule from lab personnel could be amplified and misguide the investigation. Hence the PCR-setup areas is separated from the analysis or purification of other PCR products, disposable plasticware used, and the work surface between reaction setups needs to be thoroughly cleaned.

Specificity can be adjusted by experimental conditions so that no spurious products are generated. Primer-design techniques are important in improving PCR product yield and in avoiding the formation of unspecific products. The usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA. For instance, Q5 polymerase is said to be ~280 times less error-prone than Taq polymerase. Both the running parameters (e.g. temperature and duration of cycles), or the addition of reagents, such as formamide, may increase the specificity and yield of

PCR. Computer simulations of theoretical PCR results (Electronic PCR) may be performed to assist in primer design.

Applications

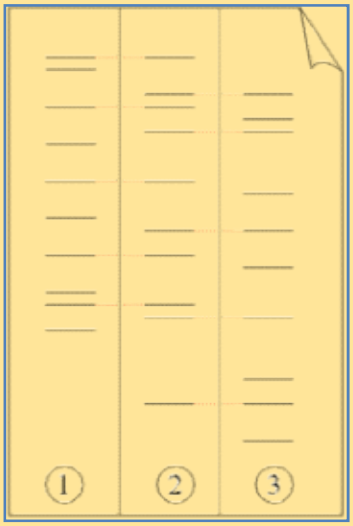


Selective DNA isolation

PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many ways, such as generating hybridization probes for Southern or northern hybridization and DNA cloning, which require larger amounts of DNA, representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material.

Other applications of PCR include DNA sequencing to determine unknown PCR-amplified sequences in which one of the amplification primers may be used in Sanger sequencing, isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid, phage, or cosmid (depending on size) or the genetic material of another organism. Bacterial colonies *(such as E. coli)* can be rapidly screened by PCR for correct DNA vector constructs. PCR may also be used for genetic

fingerprinting; a forensic technique used to identify a person or organism by comparing experimental DNAs through different PCR-based methods.



Electrophoresis of PCR-amplified DNA fragments:

1. Father
2. Child
3. Mother

The child has inherited some, but not all, of the fingerprints of each of its parents, giving it a new, unique fingerprint.

Some PCR fingerprint methods have high discriminative power and can be used to identify genetic relationships between individuals, such as parent-child or between siblings, and are used in paternity testing (Fig. 4). This technique may also be used to determine evolutionary relationships among organisms when certain molecular clocks are used (i.e. the 16S rRNA and recA genes of microorganisms).

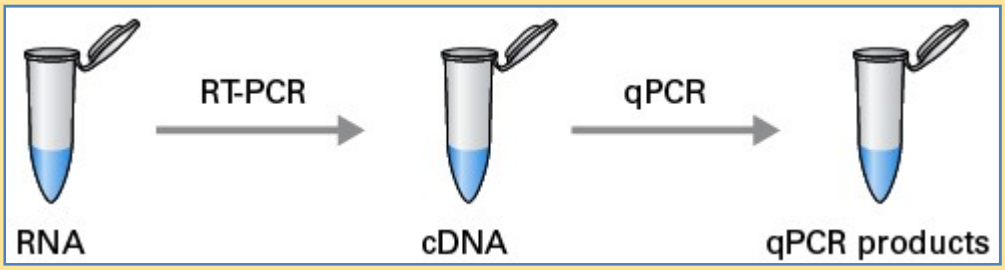
Amplification and quantification of DNA

Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on

human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian tsar and the body of English king Richard III.

Quantitative PCR or Real Time PCR (qPCR, not to be confused with RT-PCR) methods allow the estimation of the amount of a given sequence present in a sample—a technique often applied to quantitatively determine levels of gene expression. Quantitative PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

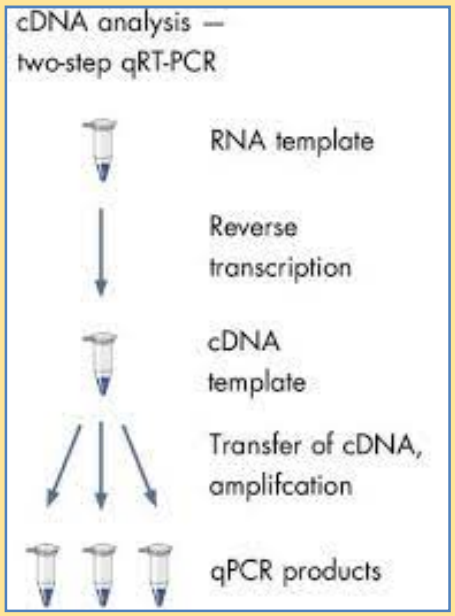
qPCR allows the quantification and detection of a specific DNA sequence in real time since it measures concentration while the synthesis process is taking place. There are two methods for simultaneous detection and quantification. The first method consists of using fluorescent dyes that are retained nonspecifically in between the double strands. The second method involves probes that code for specific sequences and are fluorescently labeled. Detection of DNA using these methods can only be seen after the hybridization of probes with its complementary DNA (cDNA) takes place. An interesting technique combination is real-time PCR and reverse transcription. This sophisticated technique, called RT-qPCR, allows for the quantification of a small quantity of RNA. Through this combined technique, mRNA is converted to cDNA, which is further quantified using qPCR. This technique lowers the possibility of error at the end point of PCR, increasing chances for detection of genes associated with genetic diseases such as cancer. Laboratories use RT-qPCR for the purpose of sensitively measuring gene regulation. The mathematical foundations for the reliable quantification of the PCR and RT-qPCR facilitate the implementation of accurate fitting procedures of experimental data in research, medical, diagnostic and infectious disease applications.



Medical and diagnostic applications

Prospective parents can be tested for being genetic carriers, or their children might be tested for actually being affected by a disease. DNA samples for prenatal testing can be obtained by amniocentesis, chorionic villus sampling, or even by the analysis of rare fetal cells circulating in the mother's bloodstream. PCR analysis is also essential to preimplantation genetic diagnosis, where individual cells of a developing embryo are tested for mutations.

- PCR can also be used as part of a sensitive test for *tissue typing*, vital to organ transplantation. As of 2008, there is even a proposal to replace the traditional antibody-based tests for blood type with PCR-based tests.
- Many forms of cancer involve alterations to *oncogenes*. By using PCR-based tests to study these mutations, therapy regimens can sometimes be individually customized to a patient. PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000 fold higher than that of other methods. PCR is very useful in the medical field since it allows for the isolation and amplification of tumor suppressors. Quantitative PCR for example, can be used to quantify and analyze single cells, as well as recognize DNA, mRNA and protein confirmations and combinations.



Infectious disease applications

PCR allows for rapid and highly specific diagnosis of infectious diseases, including those caused by bacteria or viruses. PCR also permits identification of non-cultivatable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.

Characterization and detection of infectious disease organisms have been revolutionized by PCR in the following ways:

- The *human immunodeficiency virus* (or *HIV*), is a difficult target to find and eradicate. The earliest tests for infection relied on the presence of antibodies to the virus circulating in the bloodstream. However, antibodies don't appear until many weeks after infection, maternal antibodies mask the infection of a newborn, and therapeutic agents to fight the infection don't affect the antibodies. PCR tests have been developed that can detect as little as one viral genome among the DNA of over 50,000 host cells. Infections can be detected earlier, donated blood can be screened directly for the virus, newborns can be immediately tested for infection, and the effects of antiviral treatments can be quantified.
- Some disease organisms, such as that for *tuberculosis*, are difficult to sample from patients and slow to be grown in the laboratory. PCR-based tests have allowed detection of small numbers of disease organisms (both live or dead), in convenient samples. Detailed genetic analysis can also be used to detect antibiotic resistance, allowing immediate and effective therapy. The effects of therapy can also be immediately evaluated.
- The spread of a disease organism through populations of domestic or wild animals can be monitored by PCR testing. In many cases, the appearance of new virulent sub-types can be detected and monitored. The sub-types of an organism that were responsible for earlier epidemics can also be determined by PCR analysis.
- Viral DNA can be detected by PCR. The primers used must be specific to the targeted sequences in the DNA of a virus, and PCR can be used for diagnostic analyses or DNA sequencing of the viral genome. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease. Such early detection may give physicians a significant lead time in treatment. The amount of virus ("viral load") in a

patient can also be quantified by PCR-based DNA quantitation techniques (see below). A variant of PCR (RT-PCR) is used for detecting viral RNA rather than DNA: in this test the enzyme reverse transcriptase is used to generate a DNA sequence which matches the viral RNA; this DNA is then amplified as per the usual PCR method. RT-PCR is widely used to detect the SARS-CoV-2 viral genome.

- Diseases such as pertussis (or whooping cough) are caused by the bacteria *Bordetella pertussis*. This bacteria is marked by a serious acute respiratory infection that affects various animals and humans and has led to the deaths of many young children. The pertussis toxin is a protein exotoxin that binds to cell receptors by two dimers and reacts with different cell types such as T lymphocytes which play a role in cell immunity. PCR is an important testing tool that can detect sequences within the gene for the pertussis toxin. Because PCR has a high sensitivity for the toxin and a rapid turnaround time, it is very efficient for diagnosing pertussis when compared to culture.

Forensic applications

The development of PCR-based genetic (or DNA) fingerprinting protocols has seen widespread application in forensics:

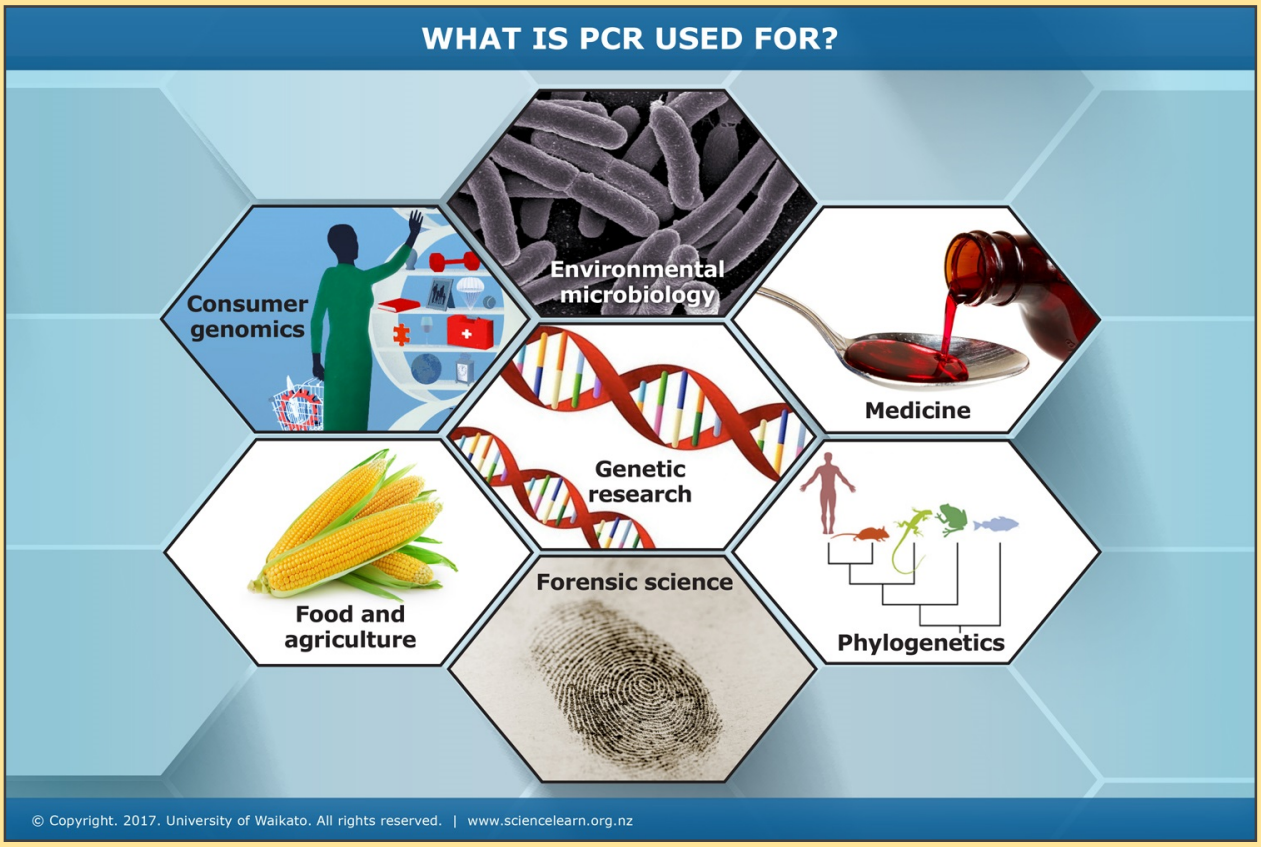
- DNA samples are often taken at crime scenes and analyzed by PCR.In its most discriminating form, *genetic fingerprinting* can uniquely discriminate any one person

from the entire population of the world. Minute samples of DNA can be isolated from a crime scene, and compared to that from suspects, or from a DNA database of earlier evidence or convicts. Simpler versions of these tests are often used to rapidly rule out suspects during a criminal investigation. Evidence from decades-old crimes can be tested, confirming or exonerating the people originally convicted.

- Forensic DNA typing has been an effective way of identifying or exonerating criminal suspects due to analysis of evidence discovered at a crime scene. The human genome has many repetitive regions that can be found within gene sequences or in non-coding regions of the genome. Specifically, up to 40% of human DNA is repetitive. There are two distinct categories for these repetitive, non-coding regions in the genome. The first category is called variable number tandem repeats (VNTR), which are 10–100 base pairs long and the second category is called short tandem repeats (STR) and these consist of repeated 2–10 base pair sections. PCR is used to amplify several well-known VNTRs and STRs using primers that flank each of the repetitive regions. The sizes of the fragments obtained from any individual for each of the STRs will indicate which alleles are present. By analyzing several STRs for an individual, a set of alleles for each person will be found that statistically is likely to be unique. Researchers have identified the complete sequence of the human genome. This sequence can be easily accessed through the NCBI website and is used in many real-life applications. For example, the FBI has compiled a set of DNA marker sites used for identification, and these are called the Combined DNA Index System (CODIS) DNA database. Using this database enables statistical analysis to be used to determine the probability that a DNA sample will match. PCR is a very powerful and significant analytical tool to use for forensic DNA typing because researchers only need a very small amount of the target DNA to be used for analysis. For example, a single human hair with attached hair follicle has enough DNA to conduct the analysis. Similarly, a few sperm, skin samples from under the fingernails, or a small amount of blood can provide enough DNA for conclusive analysis.
- Less discriminating forms of DNA fingerprinting can help in *DNA paternity testing*, where an individual is matched with their close relatives. DNA from unidentified human remains can be tested, and compared with that from possible parents, siblings, or children. Similar testing can be used to confirm the biological parents of an adopted (or kidnapped) child. The actual biological father of a newborn can also be confirmed (or ruled out).

- The PCR AMGX/AMGY design has been shown to not only facilitate in amplifying DNA sequences from a very minuscule amount of genome. However it can also be used for real-time sex determination from forensic bone samples. This provides a powerful and effective way to determine gender in forensic cases and ancient specimens.

Research applications



PCR has been applied to many areas of research in molecular genetics:

- PCR allows rapid production of short pieces of DNA, even when not more than the sequence of the two primers is known. This ability of PCR augments many methods, such as generating *hybridization probes* for Southern or northern blot hybridization. PCR supplies these techniques with large amounts of pure DNA, sometimes as a single strand, enabling analysis even from very small amounts of starting material.
- The task of *DNA sequencing* can also be assisted by PCR. Known segments of DNA can easily be produced from a patient with a genetic disease mutation. Modifications to the

amplification technique can extract segments from a completely unknown genome, or can generate just a single strand of an area of interest.

- PCR has numerous applications to the more traditional process of *DNA cloning*. It can extract segments for insertion into a vector from a larger genome, which may be only available in small quantities. Using a single set of 'vector primers', it can also analyze or extract fragments that have already been inserted into vectors. Some alterations to the PCR protocol can *generate mutations* (general or site-directed) of an inserted fragment.
- *Sequence-tagged sites* is a process where PCR is used as an indicator that a particular segment of a genome is present in a particular clone. The Human Genome Project found this application vital to mapping the cosmid clones they were sequencing, and to coordinating the results from different laboratories.
- An application of PCR is the phylogenic analysis of DNA from *ancient sources*, such as that found in the recovered bones of Neanderthals, from frozen tissues of mammoths, or from the brain of Egyptian mummies. In some cases the highly degraded DNA from these sources might be reassembled during the early stages of amplification.
- A common application of PCR is the study of patterns of *gene expression*. Tissues (or even individual cells) can be analyzed at different stages to see which genes have become active, or which have been switched off. This application can also use quantitative PCR to quantitate the actual levels of expression
- The ability of PCR to simultaneously amplify several loci from individual sperm has greatly enhanced the more traditional task of *genetic mapping* by studying chromosomal crossovers after meiosis. Rare crossover events between very close loci have been directly observed by analyzing thousands of individual sperms. Similarly, unusual deletions, insertions, translocations, or inversions can be analyzed, all without having to wait (or pay) for the long and laborious processes of fertilization, embryogenesis, etc.
- Site-directed mutagenesis: PCR can be used to create mutant genes with mutations chosen by scientists at will. These mutations can be chosen in order to understand how proteins accomplish their functions, and to change or improve protein function.

DNA fingerprinting

DNA fingerprinting, also called **DNA typing, DNA profiling, genetic fingerprinting, genotyping,** or **identity testing**, in genetics, method of isolating and identifying variable elements within the base-pair sequence of DNA (deoxyribonucleic acid). The technique was developed in 1984 by British geneticist Alec Jeffreys, after he noticed that certain sequences of highly variable DNA (known as minisatellites), which do not contribute to the functions of genes, are repeated within genes. Jeffreys recognized that each individual has a unique pattern of minisatellites (the only exceptions being multiple individuals from a single zygote, such as identical twins).

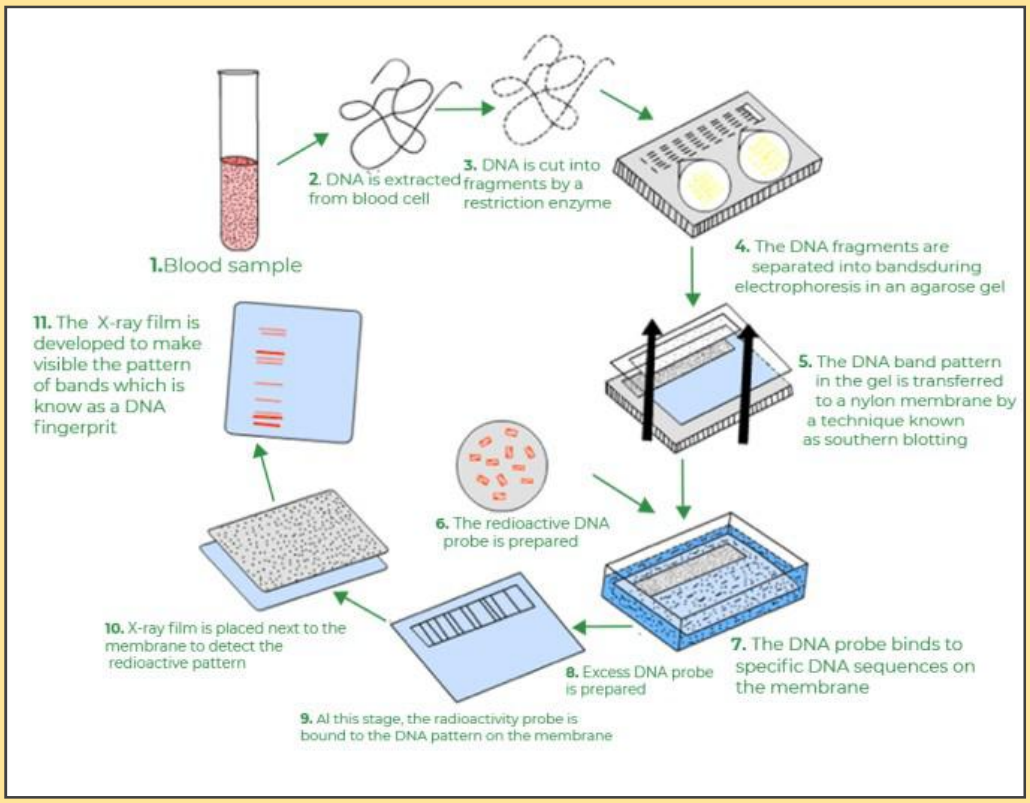
Learn how a genetic fingerprint is made using agarose gel, Southern blotting, and a radioactive DNA probe

The procedure for creating a DNA fingerprint consists of first obtaining a sample of cells, such as skin, hair, or blood cells, which contain DNA. The DNA is extracted from the cells and purified. In Jeffreys's original approach, which was based on restriction fragment length polymorphism (RFLP) technology, the DNA was then cut at specific points along the strand with proteins known as restriction enzymes. The enzymes produced fragments of varying lengths that were sorted by placing them on a gel and then subjecting the gel to an electric current (electrophoresis): the shorter the fragment, the more quickly it moved toward the positive pole (anode). The sorted double-stranded DNA fragments were then subjected to a blotting technique in which they were split into single strands and transferred to a nylon sheet. The fragments underwent autoradiography in which they were exposed to DNA probes—pieces of synthetic DNA that were made radioactive and that bound to the minisatellites. A piece of X-ray film was then exposed to the fragments, and a dark mark was

produced at any point where a radioactive probe had become attached. The resultant pattern of marks could then be analyzed.

The assay developed by Jeffreys has been supplanted by approaches that are based on the use of the polymerase chain reaction (PCR) and so-called microsatellites (or short tandem repeats, STRs), which have shorter repeat units (typically 2 to 4 base pairs in length) than minisatellites (10 to more than 100 base pairs in length). PCR amplifies the desired fragment of DNA (e.g., a specific STR) many times over, creating thousands of copies of the fragment. It is an automated procedure that requires only small amounts of DNA as starting material and works even with partially degraded DNA. Once an adequate amount of DNA has been produced with PCR, the exact sequence of nucleotide pairs in a segment of DNA can be determined by using one of several biomolecular sequencing methods. Automated equipment has greatly increased the speed of DNA sequencing and has made available many new practical applications, including pinpointing segments of genes that cause genetic diseases, mapping the human genome, engineering drought-resistant plants, and producing biological drugs from genetically altered bacteria.

An early use of DNA fingerprinting was in legal disputes, notably to help solve crimes and to determine paternity. Since its development, DNA fingerprinting has led to the conviction of numerous criminals and to the freeing from prison of many individuals who were wrongly convicted. However, making scientific identification coincide exactly with legal proof is often problematic. Even a single suggestion of the possibility of error is sometimes enough to persuade a jury not to convict a suspect. Sample contamination, faulty preparation procedures, and mistakes in interpretation of results are major sources of error. In addition, RFLP requires large amounts of high-quality DNA, which limits its application in forensics. Forensic DNA samples frequently are degraded or are collected postmortem, which means that they are lower-quality and subject to producing less-reliable results than samples that are obtained from a living individual. Some of the concerns with DNA fingerprinting, and specifically the use of RFLP, subsided with the development of PCR- and STR-based approaches.



DNA Finger Printing Techniques

Plant tissue culture

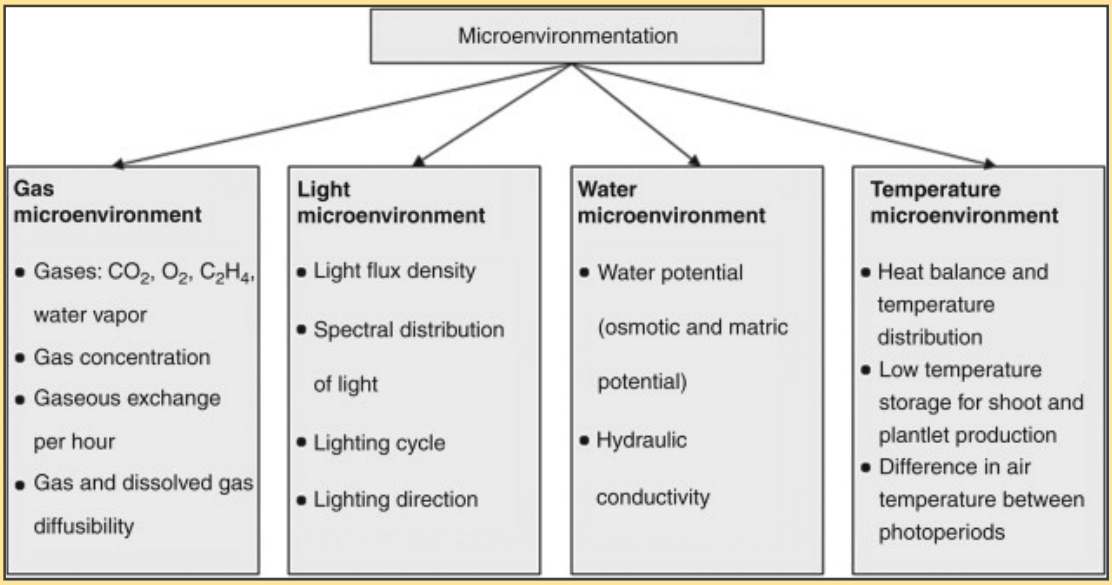
Plant tissue culture broadly refers to the *in vitro* cultivation of all plant parts under aseptic conditions. Any laboratory in which tissue culture techniques are performed must contain a number of basic facilities. These include areas for general washing and media preparation, sterilization, storage, aseptic transfer, observation/data collection, and environmentally controlled incubators or culture rooms. The most important work area is the culture transfer room where the core activity takes place. Plant tissue cultures should be incubated under well-controlled temperature, humidity, air circulation, light quality, and for specific duration. The plant culture technique requires various organic and inorganic chemicals for the preparation of culture media. Growth room is an equally important area where plant cultures are maintained under controlled environmental conditions to achieve optimal growth. Plants regenerated from *in vitro* tissue cultures are transplanted to vermiculite pots. The potted plants are ultimately transferred to greenhouses or growth cabinets and maintained for further observations under controlled conditions of light, temperature, and humidity.

Plant tissue culture is a widely known technique for the production of large numbers of genetically identical plantlets. This technology exhibits several advantages over conventional propagation techniques. Propagules derived from plant tissue culture exhibit several applications in horticulture, crops, and forestry. Genetic expression of such propagules governs their growth and development; however, the environmental conditions have a huge effect on genotype and expression of *in vitro* propagated plant cell/tissue. Utilization of traditional methods to control the physical and chemical environment is time consuming and limited for the large-scale production of propagules. With modernization in technology currently several engineering techniques (robust, automated, and computerized) have been applied to micropropagation with the objective of providing optimum environmental conditions to *in vitro* plant stock at a larger level. Usually *in vitro* propagation is practiced by using three general steps: (i) preparation and sterilization of plant material, (ii) culture medium composition, and (iii) physical environmental conditions in the culture room and culture vessel. Microenvironmentation is essential for the last two steps but especially so for the last step for providing the optimum physical environment to the culture.

Unlike the greenhouse effect where plants have to compromise with environmental conditions, microenvironmentation is investigated in closed plant tissue culture vessels, with their caps or closures, which creates the boundaries between the internal microenvironment

and the external environment. This will be helpful in exploring benefits of micropropagation over conventional propagation techniques, such as: rapid clonal propagation, decreasing diseases of plantlets and the period of acclimatization *ex vitro*, cutting down motherstock requirements, improving the survival of micropropagation plantlets after transfer to *ex vitro* conditions, etc., and then reducing the cost of micropropagation plantlets. Physical environmental factors such as temperature, light, air movement, physical boundaries of the culture vessel, and physical characteristics of the culture medium are predetermined and can be maintained constant or varied during the growth cycle. Similarly the chemical environment of the tissue culture is predetermined and variables such as pH and the composition of the medium are maintained in such a way that the optimum conditions are always provided for the nourishment of young propagules. Physical parameters or culture room conditions can be optimized by changing the growth room temperature and humidity, or physically moving the culture to alter growth conditions. High relative humidity, stable temperature, low CO_2 concentration in light and high CO_2 concentration in dark, and high C_2H_4 concentrations are favorable for the proper growth of plant tissue culture . High CO_2 concentrations in the light period inhibit growth of plantlets and induce senescence . High relative humidity reduces the transpiration and induces stomatal malfunction .

In addition, the physical properties of the vessels and caps or closures affect the growth microenvironment of plantlets by virtue of the interface between inside and outside environments. The most important specifications for vessels are to provide uniform and adequate light quality, to isolate contamination of microorganisms, and to allow gas exchange. Therefore, growth conditions of plantlets are significantly affected by the inside microenvironment of culture vessels. However, the microenvironment of culture vessels is not easily adjusted. Various essential parameters of micro environmentation during plant tissue culture are mentioned.



Procedure

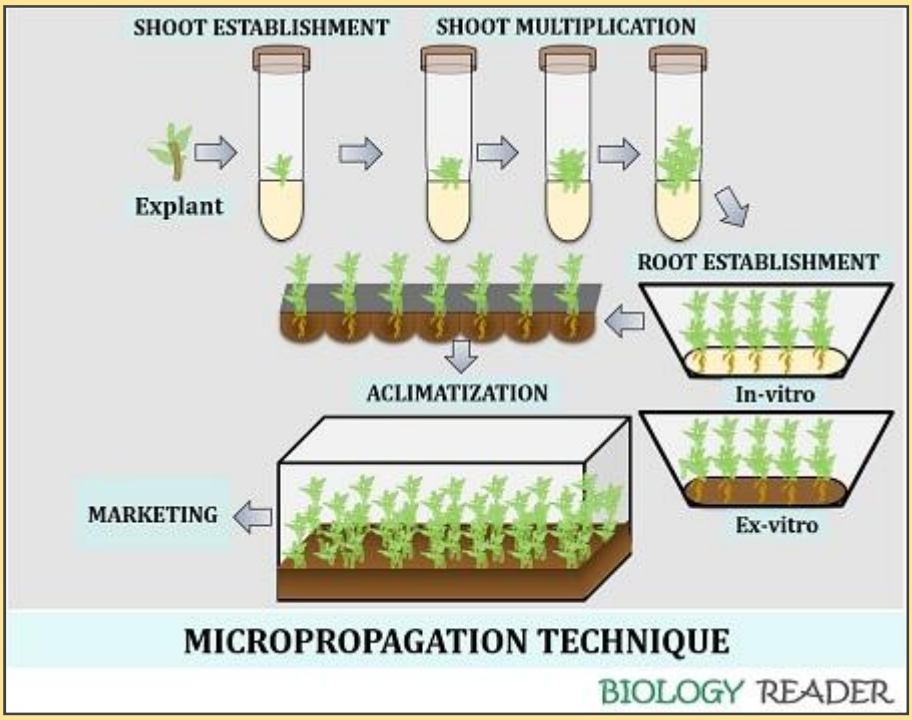
Preparation and sterilization of growing medium

These steps will make 1 L of growth medium which is enough to prepare about 65 growing tubes. 1. Dissolve the MS mixture in about 800 ml of distilled water. Stir the water continuously while adding the salt mixture. Add 30 g sugar and stir to dissolve. Adjust pH to 5.8 using 1M NaOH or 1M HCl as necessary while gently stirring. Add distilled water to make the total volume up to 1 L. 2. Weigh out 10 grams of agar and add it to the MS solution. Heat the solution gently while stirring until all the agar has dissolved. 3. Pour the still warm medium into the polycarbonate tubes to a depth of about 4 cm which will use about 15ml of media per tube. 4. Place the tubes (with lids sitting on the tubes but not tightened) in a pressure cooker and sterilize for 20 minutes. Cool the pressure cooker, then remove the tubes and tighten the lids. Alternatively the tubes can be placed in boiling water for 30 minutes, but make sure that none of the water is able to enter the tubes.

Preparation of a sterile transfer chamber and equipment

A classroom transfer chamber can be made from a clean glass aquarium turned on its side. Scrub the aquarium thoroughly with a 30% bleach solution, making sure that you wear gloves and do not inhale the fumes. Rinse with sterile distilled water, turn upside down on a clean counter or paper towels and allow to dry. Cut holes in a clean plastic sheet to allow arms to reach into the chamber and reinforce the cut edges with tape if necessary. Tape the clean plastic sheet over the open side of the aquarium making sure that the arm holes are located at

a convenient height. Plastic sleeves could also be fitted to these holes if you wish to make it easier to prevent the entry of airborne spores into the chamber. The finished aquarium chamber can be sterilized by spraying with 10% chlorox bleach just prior to each use and drying with sterile paper towel. Wrap the forceps, scalpels, razor blades, paper towel and gloves (rubber or surgical) in aluminum foil, seal with tape and sterilize by processing them in a pressure cooker for twenty minutes. These items can also be sterilized by placing in an oven at 350o F for 15 minutes. You can wrap each item separately or put together a "kit" so that each student will have their own sterile equipment to use.



Alternatively the forceps and blades can be sterilized by dipping in 10% bleach and then rinsing in sterile water, or dipping in alcohol and then placing in a flame, although this is not recommended for use in crowded classrooms. If you choose to dip in bleach and rinse in sterile water, it is best if fresh solutions are available for each 3-4 students since the water can easily be contaminated if care is not used. These liquid containers should only be opened once they are inside of the sterile chamber.

Plant preparation

Your plant material must first be surface sterilized to remove any bacteria or fungal spores that are present. We aim to kill all microorganisms, but at the same time not cause any adverse damage to the plant material. 1. Cauliflower should be cut into small sections of

florets about 1 cm across. If using a rose or other cuttings, cut the shoots into about 5 to 7 cm lengths. Whole African violet leaves can also be used. 2. Wash the prepared plant material in a detergent-water mixture for about 20 minutes. If trying hairy plant material scrub with a soft brush (toothbrush). This will help remove fungi etc., and the detergent will help wet the material and remove air bubbles that may be trapped between tiny hairs on a plant. 3. Transfer the washed plant material to the sterilizing chlorox solution. Shake the mixture for 1 minute and then leave to soak for 10- 20 minutes. Carefully pour off the bleach solution using the lid to keep the plant tissue from coming out and then carefully cap the container.

Transfer of plant material to tissue culture medium

Use the sterile gloves and equipment for all of these steps.

1. Place the plant material still in the chlorox bleach sterilizing container, the containers of sterile water, the sterilized forceps and blades, some sterile paper towel to use as a cutting surface and enough tubes containing sterile medium into the sterile aquarium. The outside surfaces of the containers, the capped tubes and the aluminum wrapped supplies should be briefly sprayed with 70% alcohol before moving them into the chamber.

2. The gloves can be sprayed with a 70% alcohol solution and hands rubbed together to spread the alcohol just prior to placing hands into the chamber. Once students have gloves on and sprayed they must not touch anything that is outside of the sterile chamber.

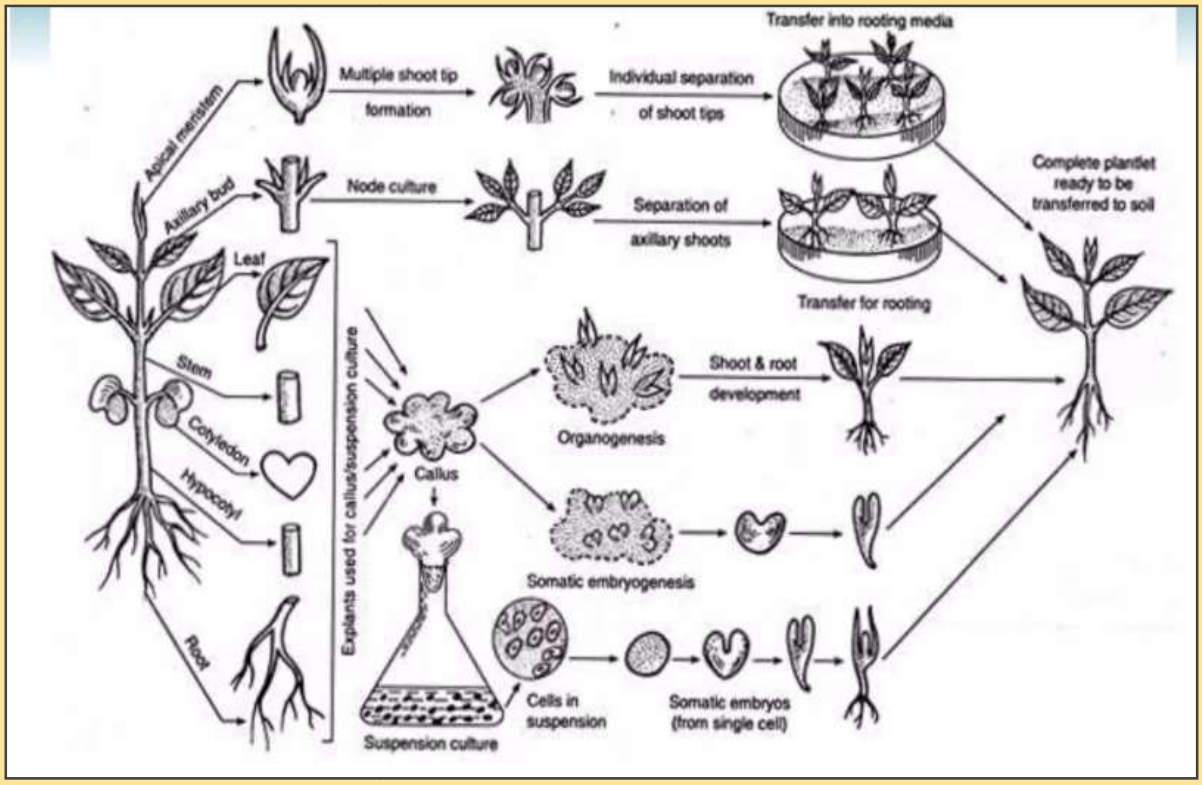
3. Carefully open the container with the plant material and pour in enough sterile water to half fill the container. Replace the lid and gently shake the container to wash tissue pieces (explants) thoroughly for 2-3 minutes to remove the bleach. Pour off the water and repeat the washing process 3 more times.

4. Remove the sterilized plant material from the sterile water, place on the paper towel or sterile petri dish. Cut the cauliflower into smaller pieces about 2 to 3 mm across. If using rose cut a piece of stem about 10 mm in length with an attached bud. The African violet leaf can be cut into small squares about 1-1.5 cm across. Be sure to avoid any tissue that has been damaged by the bleach, which is apparent by its' pale color.

5. Take a prepared section of plant material in sterile forceps and place into the medium in the polycarbonate tube. Cauliflower pieces should be partly submerged in the medium, flower bud facing up. Rose or other cuttings should be placed so that the shoots are level with the

medium surface. The African violet leaf pieces should be laid directly onto the medium surface. 6. Replace the cap tightly on the tube.

6. Replace the cap tightly on the tube.



Growing the plants

1. The tubes containing plant sections may be placed in a well-lit area of the classroom although not in direct sunlight. The shoots will probably grow more quickly if the explants are placed under fluorescent or growlights to provide at least 12 hours of light per day. The aquarium can be used as a growth chamber with the lighting about 8-10" overhead. This will also help maintain a more regular and warm temperature. Ensure that the temperature does not go over 28o C. New shoots should develop within 2 weeks, and should be well advanced in 3 to 4 weeks. Check the tubes daily and discard any that show signs of infection (before discarding first sterilize in the pressure cooker or add bleach into the tube).

2. Roots can appear within 6 weeks on cauliflowers. The roses, African violet and other cuttings will need to be moved into rooting media for roots to properly develop. This transfer to the second, rooting media must be conducted under the same sterile conditions as at the

initiation of the culture. All necessary equipment and the aquarium should be set up as before and properly sterilized.

3. Working inside the sterile aquarium chamber, remove the cap from the culture tube. There will usually be several shoots that have arisen from each explant. These shoots should be carefully separated by gently removing the whole explant from the media with sterile forceps and then separating the shoots by gently pulling them apart using two pairs of forceps. Each shoot should then be placed into a tube of rooting media and the bottom of the shoot pushed into the media so that good contact is made. The cap is replaced and the shoots are then allowed to grow as in step 1 until roots are formed, usually within 2-3 weeks.

Potting the clones

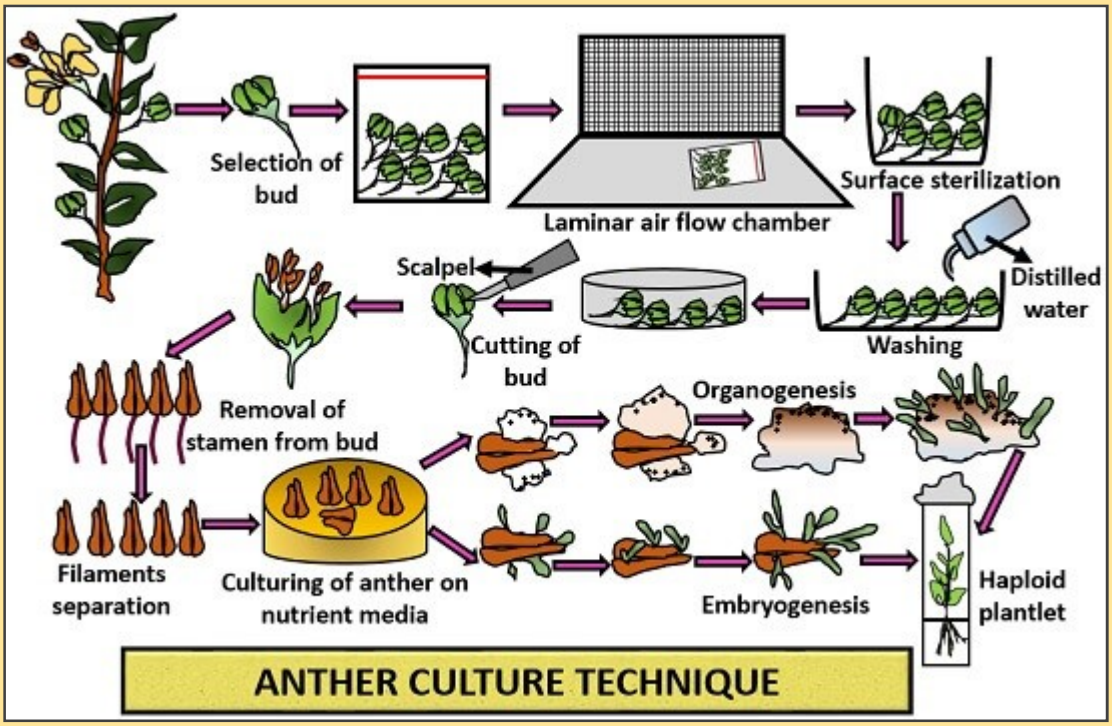
Once roots are well formed the plants are ready to be transferred into soil. Figure 2: Roots are fully developed prior to moving plants to pots of soil

1. Each plant should be carefully removed from its tube of media and planted into a small pot containing a clean light potting mix. Gently wash off all the agar medium prior to planting. The plants will still need to be protected at this stage since they are not accustomed to the drier air of the classroom when compared to the moist environment of the tube of media.

2. Place all of the pots onto a tray and cover lightly with a plastic dome or tent. Place the plants in an area with 12-16 hours of light (either natural or artificial) but not direct sunlight.

3. After a week the cover can be gradually removed and the plants acclimated to stronger light and drier atmospheric conditions.

4. You now have a collection of plants in your classroom that are genetically exactly the same. You could use these plants to carry out other experimental tests knowing that one of the main variables in the experiment has been eliminated. Some of these tests could include looking at plant responses to low light levels, to drought or to saline soil conditions.



Advantages of Tissue Culture

There are several advantages to using the tissue culture process. We already mentioned its effectiveness in helping developing countries to increase food production, but what are some other advantages that may be relevant to you?

- The new plantlets can be grown in a short amount of time.
- Only a small amount of initial plant tissue is required.
- The new plantlets and plants are more likely to be free of viruses and diseases.
- The process is not dependent on the seasons and can be done throughout the year.
- You need only a relatively small space to perform the process (ten times the plants in one-tenth of the space).
- On a larger scale, the tissue culture process helps to supply the consumer market with new subspecies and variety.
- People looking to cultivate challenging plants such as specific breeds of orchid find more success with the tissue culture process than traditional soil.

Disadvantages of Plant Tissue Culture

- Tissue culture can require more labor and cost more money in building the facility and equipping the lab with all the instruments and chemicals.
- There is a chance that the propagated plants will be less resilient to diseases when grown in outside conditions due to the type of environment they are grown in.
- It is imperative that, before being cultured, the material is screened; failure to pick up any abnormalities could lead to the new plants being infected.
- While the success rate is high if the correct procedures are followed, success with the tissue culture is not a guarantee. That's why accurate protocols are necessary to grow plants in tissue culture setting, which can be laborious when you try to create one working protocol by yourself.
- Contamination is the major issue in tissue culture setting. Plants can get infected by bacteria, fungi, and viruses. That's why all measures should be taken and PPE kit should be used while performing tissue culture in your lab.
- Tissue culture is an advanced technique and require some advanced knowledge and practice for anyone to get started in the field.

As you can see, the advantages do seem to outweigh the disadvantages. Sure, you may have to spend a bit more money to get your DIY tissue culture going, but the rewards certainly outweigh the initial cost. So, let's take a look at the Tissue Culture Process and see if we can break down the complicated terms into something a little more digestible.

Types of Tissue Culture Technique

Tissue culture is a technique in which healthy tissues are extracted from living matter or organisms. In plant tissue culture, this could be either the leaves or other parts of the plant- depending on the protocol.

Based on the explant (starting material/plant tissue used to grow plants), tissue culture is classified into the following types:

- **Callus Culture:** A callus refers to a cluster of undifferentiated cells with the remarkable ability to give rise to various plant parts. When the plant tissues derived from any plant organ are artificially induced in laboratory settings, they form callus, which further give rise to different plant organs, roots and shoots.
- **Protoplast Culture:** A protoplast is a plant cell lacking a cell wall. In this technique, the cell wall of plant cells is eliminated through mechanical or enzymatic means. The resulting protoplasts are purified and subsequently, under controlled conditions, the cell wall is regenerated before transferring them to appropriate media for further growth into a complete plant.
- **Meristem Culture:** Meristem culture involves the isolation of the meristematic region, such as shoot tips, from plants and its transfer to a growth medium containing nutrients, vitamins, and plant hormones. This technique promotes cell division and tissue differentiation in the cultured cells. Meristem culture finds diverse applications, including the production of disease-free plants, regeneration of complete plants, generation of transgenic and haploid plants, crop enhancement, and preservation of germplasm.
- **Embryo Culture:** Embryo culture involves the isolation and cultivation of either immature or mature plant embryos to support their development into complete plants. Instead of individually sterilizing the embryos, this method involves sterilizing the organ (such as ovule, seed, or fruit) from which the embryos are derived and utilizing it in the culture process.
- **Ovary Culture:** The technique involves culturing fertilized or unfertilized ovaries of plant species in a suitable environment to facilitate their development into complete

plants. This method, also known as gynogenesis, is primarily employed to overcome barriers before and after fertilization. Additionally, it has been utilized to achieve interspecific hybrids.

- **Anther/Pollen culture:** Pollen/anther culture is a technique in plant biotechnology where pollen grains or anthers (the male reproductive parts of flowers) are isolated and cultured in a nutrient-rich medium. This method allows for the development and regeneration of haploid plants or callus tissues from the cultured pollen or anther cells. It is commonly used in plant breeding and genetic studies to produce new plant varieties or to study the behavior of plant cells in a controlled environment.

By using the tissue culture process, a plant's yield can be increased dramatically, and in a short amount of time. The plant can also be genetically altered so that it becomes immune to certain diseases and viruses. The genetic modification enables growers to ensure that plants carry very specific characteristics. In many cases, businesses and individuals will propagate the plants to carry specific traits that are more profitable for their business, or more desirable for personal use.

On another note, the tissue culture process can be used to promote the survival of a rare plant or endangered species.

Lastly, the tissue culture technique relies on the plant's innate ability to rejuvenate cells quickly, and these rejuvenated cells are copies most often referred to as clones. This

technique can be used in a lab with expensive and complicated equipment, or it can be simply adapted for a home DIY.