



## 1. INTRODUCTION

### 1.1. History and Definition of Biotechnology

The first known use of the word "biotechnology" was in 1917 by a Hungarian agricultural engineer, Karl Ereky. He used it to describe a system for raising pigs on sugar beets as their primary food source. Biotechnology was defined by Ereky as: "all lines of work by which products are produced from raw materials with the aid of living things".

While most industries use mechanical devices (machines) to make things, biotechnology uses living organisms to make products of economic value. You can use this definition for most agricultural production, although "farming" is a more acceptable and widely understood term for this activity. Apart from the traditional cultivation of crops and raising of livestock, there are a number of other older technologies that can also be characterized as biotechnology:

**Fermentation:** brewing beer, winemaking, baking bread. All of these food-processing systems rely on yeast to alter the properties of a raw material. In the case of brewing and winemaking, yeasts convert the sugars in grains and grapes into alcohol. In bread, yeasts produce carbon dioxide to make the bread rise and soften the dough.

**Sewage treatment:** processes toxic domestic and industrial waste into less harmful materials. Organic wastes are degraded by the action of a complex community of microbes. Sewage treatment facilities provide an optimized environment for these organisms to process the waste, while at the same time allowing containment and monitoring of the process.

Some of the earliest industrial applications of biotechnology were involved in the production of ingredients to make explosives during the First World War. Supplies of these essential ingredients for production of munitions had been disrupted by naval blockades. Microbial fermentation tanks were used to convert readily available agricultural products into glycerol and acetone. Similar fermentation systems were developed to grow microbes for the production of antibiotics. Large-scale production of antibiotics was not possible until suitable fermentation systems were developed, and strains with higher yield of antibiotics were identified.

But the scope of biotechnology has been altered beyond this original vision by a series of developments in the life sciences. These developments focus on the ability to: isolate genes from any organism, modify and manipulate these genes, and put these altered genes back into various organisms.

A newer definition of biotechnology from the U.S. Office of Technology Assessment reads: "Any technique that uses living organisms to make or modify products, to improve plants or animals, or to develop microorganisms for specific purposes".

Some achievements through biotechnology:

- ✓ Tomatoes - with better flavor, longer shelf life, thicker paste



- ✓ Corn and cotton plants- with better insect resistance and less need for insecticides
- ✓ Canola- that produces better, healthier oils, and oils with specialized uses
- ✓ Corn and soybeans- that tolerate broad-spectrum herbicides such as Roundup

### 1.2.Objectives of Biotechnology

- creation of new biologically active substances and drugs for medicine and effective prevention, diagnosis and treatment of humans and animals;
- creation of methods of protection from diseases and pests, bacterial fertilizers, plant growth regulators, resistant to adverse environmental factors varieties and hybrids;
- creation of a valuable feed additives and biologically active substances for using in productivity of animals in order to increase it;
- creation of a new technologies for production of valuable products for using in food, microbiological and other industries;
- creation of a waste and environmentally safely recycling technologies and bioconversion of agricultural, industrial, municipal waste to produce energy, high-quality organic fertilizer, protein and vitamin feed additives;
- improvement and optimization of equipment for biotechnological processes in order to maximize the yield of products;

### 1.3.Biotechnology in Ethiopia

Conventional research approaches have tried to address the major research and development problems in Ethiopia. However, these approaches could not achieve the desired level of improvement unless they are supported by modern biotechnological tools. There is a **biosafety law** that countries are required to abide by and most countries including Ethiopia are signatories to the protocol. These countries believed that the utilization of transgenic plants on agriculture has its own demerit on the environment. But, so far, the utilization of modified plants did not bring any impact on the environment and on the biodiversity at large.

Biotechnological facilities exist scattered in different institutions in Ethiopia, a country with immense genetic biodiversity, but no precise information is available as to their capacities, capabilities and the associated technical and administrative gaps. Currently more than 15 institutions are engaged in biotechnology research and some outputs of the researches have been disseminated to pertinent bodies. In addition to these, research institutions in many universities have opened biotechnology departments and thousands of students graduate annually from these universities. Debrezeit, Jimma and Melkasa agricultural research institutions are involving in plant and animal research.

The government of Ethiopia is working on the expansion of textile industries, which need more cotton production. Currently, the nation could not satisfy its cotton demand from local market. Hence, it spends a significant amount of hard currency for cotton importation from abroad. Thus, unless the importation of cotton is substituted the outflow of hard currency further affects the currency reserve of the nation.



Therefore, producing better cotton which can be used as an input for the textile industries should be taken as option. Similar to the other countries, the traditional cotton, which is growing here, is highly vulnerable to African ball worm and the yield is insignificant to meet the demand.

Ethiopia was relaxed the previous biosafety law in 2015 and after that interested groups have signed agreement to import gene and to make research on plant genes. And currently, based on the agreement to improve the cotton production, the Ethiopian Agricultural Research Institute is experimenting BT cotton.

#### 1.4. Branches and Components of Biotechnology

Biotechnology has more than a few different branches which are referred to by dissimilar terms mainly noticeable with dissimilar colors to clarify the biotechnological field that it is used in. The majority extensively used ones are:

**Green Biotechnology:** is a biotechnology applied in agricultural processes. Examples are the production of larger number of plants from the existing stock of plants via micro propagation and the designing of transgenic plants to grow under specific environments in the presence (or absence) of chemicals through genetic engineering.

**Blue Biotechnology:** is a term used to describe the marine and aquatic applications of biotechnology, but its use is relatively rare.

**Bioinformatics:** also referred as *computational biology* and is an interdisciplinary field which addresses biological problems using computational techniques, and makes the rapid organization and analysis of biological data possible. It plays a key role in various areas, such as functional genomics, structural genomics, and proteomics.

**Red Biotechnology:** is also referred to as Medical Biotechnology. Some examples are the designing of organisms to produce antibiotics, and it helps to cure genetic issues in organisms through genetic manipulation.

**White Biotechnology:** is used and applied in industries and its processes. An example is the designing of an organism to produce a useful chemical.

#### 1.5. Applications of Biotechnology

Some of the applications of biotechnology and their potential benefits to society are described below:

##### a) Industry

Biotechnology can be used to develop alternative fuels; an example is the conversion of maize starch into ethanol by yeast, which is subsequently used to produce gasohol (a gasoline-ethanol mix). Bacteria are used to decompose sludge and landfill wastes. Through biotechnology, micro-organisms or their enzymes can be adapted to convert biomass into feed stocks, or they can be used for manufacturing biodegradable plastics (bioplastics). Other organisms (micro-organisms, plants and mammals) are used as bioreactors for producing chemical compounds that are extracted from them and processed as drugs



and other products. Plant and animal fibers are used for producing a variety of fabrics, threads and cordage. Biotechnology is applied to improve the quality and quantity of these products. Biopulping is a technique whereby a fungus is used to convert wood chips into pulp for papermaking.

#### **b) Agriculture**

Biotechnology is used for accelerating breeding programmes and the development of organisms with desirable characteristics. Also, it enables gene transfer across natural breeding boundaries, overcoming mating barriers. Likewise, it is possible to specifically introduce novel, desirable traits and characteristics into existing species. This biotechnological application is used to improve the yield of crop and animal species and their product quality such as nutritional value and shelf life. In addition to these benefits, this methodology reduces the need for agrochemicals by creating disease and pest-resistant species, thereby reducing environmental pollution from chemical runoff. Increased yields and higher food quality can contribute to reducing world hunger and malnutrition.

Technologies in the field of **agricultural biotechnology**:

- ✓ Molecular techniques are being used to monitor breeding populations and to diagnose animals and plants infected with diseases.
- ✓ Micropropagation techniques are being widely used to generate clonal plant materials, allowing rapid large-scale clonal propagation of many plant species including trees.
- ✓ Biofertilizers and biopesticides can be applied in place of conventional fertilizer and pesticides to promote plant growth and health in an environmentally sustainable way.

#### **c) Health and medicine**

Biotechnologies are used to develop diagnostic tools for identifying diseases. Biotechnology is also used to produce more effective and efficient vaccines, therapeutic antibodies, antibiotics, and other pharmaceuticals. For example, there are more than 370 drug products and vaccines obtained through biotechnology currently in clinical trials, targeting more than 200 diseases including various cancers, Alzheimer's disease, heart disease, diabetes, multiple sclerosis, AIDS and arthritis. Through the biotechnology of gene therapy, scientists are making efforts at curing genetic diseases by attempting to replace defective genes with the correct version.

#### **d) Environment**

Development and usage of alternative fuels that burn cleaner and improve air quality through reduced pollution of the environment is possible by biotechnological means. Micro-organisms are used to decompose wastes and clean up contaminated sites by the technology of bioremediation. The use of disease-resistant cultivars can make crop production less environmentally intrusive by reducing the use of agrochemicals.

#### **e) Forensics**

Since the DNA profile, i.e. the nucleotide sequence of the genome, is unique in every individual, it can be used as a powerful basis of identifying individuals in a population. DNA-based evidence is used in cases involving paternity disputes and family relationships. Furthermore, it is used in health care and judicial systems. In the judicial system, forensic experts use DNA profiling to identify suspects in criminal cases, especially when body fluids and other particles like hair and skin samples can be retrieved.



## 2. BASIC MOLECULAR TECHNIQUES

### 2.1. Isolation of nucleic acids

**DNA isolation** is a process of purification of DNA from sample using a combination of physical and chemical methods. The three basic steps of DNA extraction are 1) lysis, 2) precipitation, and 3) purification.

**Lysis:** In this step, the cell and the nucleus are broken open to release the DNA inside and there are two ways to do this. First, mechanical disruption breaks open the cells. This can be done with a tissue homogenizer (like a small blender), with a mortar and pestle, or by cutting the tissue into small pieces. Mechanical disruption is particularly important when using plant cells because they have a tough cell wall. Second, lysis uses detergents and enzymes such as Proteinase K to free the DNA and dissolve cellular proteins.

**Precipitation:** When you complete the lysis step, the DNA has been freed from the nucleus, but it is now mixed with mashed up cell parts. Precipitation separates DNA from this cellular debris. First, Na<sup>+</sup> ions (sodium) neutralize the negative charges on the DNA molecules, which makes them more stable and less water soluble. Next, alcohol (such as ethanol or isopropanol) is added and causes the DNA to precipitate out of the aqueous solution because it is not soluble in alcohol.

**Purification:** Now that DNA has been separated from the aqueous phase, it can be rinsed with alcohol to remove any remaining unwanted material and cellular debris. At this point the purified DNA is usually re-dissolved in water for easy handling and storage.

Some of the most common DNA extraction methods include **organic extraction**, **Chelex extraction**, and **solid phase extraction**. These methods consistently yield isolated DNA, but they differ in both the quality and the quantity of DNA yielded. When selecting a DNA extraction method, there are multiple factors to consider, including cost, time, safety, and risk of contamination. Organic extraction involves the addition of and incubation in multiple different chemical solutions, including a lysis step, a phenol chloroform extraction, an ethanol precipitation, and washing steps.

Organic extraction is often used in laboratories because it is cheap, and it yields large quantities of pure DNA. Though it is easy, there are many steps involved, and it takes longer than other methods. It also involves the unfavorable use of the toxic chemicals phenol and chloroform, and there is an increased risk of contamination due to transferring the DNA between multiple tubes.

Chelex extraction method involves adding the Chelex resin to the sample, boiling the solution, then vortexing and centrifuging it. The cellular materials bind to the Chelex beads, while the DNA is available in the supernatant. The Chelex method is much faster and simpler than organic extraction, and it only requires one tube, which decreases the risk of DNA contamination. Unfortunately, Chelex extraction does not yield as much quantity and the DNA yielded is single-stranded, which means it can only be used for PCR-based analyses.



Solid phase extraction such as using a spin-column based extraction method takes advantage of the fact that DNA binds to silica. The sample containing DNA is added to a column containing a silica gel or silica beads. The DNA binds to the silica, while the rest of the solution is washed out. The DNA can then be removed from the silica using a buffer. This method is very easy and uses only one tube; it also yields high quantities of pure, high-quality DNA, and the procedure can even be automated. Multiple solid phase extraction commercial kits are manufactured and marketed by different companies; the only problem is that they are more expensive than organic extraction or Chelex extraction.

After extraction, DNA digested with restriction enzymes. A restriction enzyme is a type of nuclease that cleaves a DNA duplex wherever the DNA molecule contains a particular short sequence of nucleotides matching the restriction site of the enzyme. A number of parameters need to be considered when choosing a particular restriction enzyme for DNA profiling experiments: length of the recognition sequence, frequency of cleavage, cost and sensitivity of the enzyme to cytosine methylation of the target sequence.

Most restriction sites consist of four or six nucleotides, within which the restriction enzyme makes a break in each DNA strand. Each break creates a free 3' hydroxyl (3'-OH) and a free 5' phosphate (5'-P) at the site of breakage.

## 2.2. Gel Electrophoresis

Gel electrophoresis is a commonly used technique for resolving proteins or nucleic acids. In general a sample of one particular molecule (protein, DNA, or RNA) is placed in a well at or near the end of the gel matrix (gel). The gel could be a polyacrylamide (preferred for proteins and for resolving DNA molecules, which differ by one nucleotide), agarose (is used routinely for medium sized nucleic acid molecules) or a starch. Charged molecules migrate according to their size when an electric field is applied across the gel. The end result of the gel electrophoresis is a gel with the molecules spread out from one end to the other. The progress of gel electrophoresis is monitored by observing the migration of a visible dye (tracking dye through the gel), if they have been colored.

## 2.3. Blotting

Blotting refers to the actual technique where molecules that have been separated on a gel are transferred or blotted onto a type of paper called nitrocellulose. After separation of fragments with gel electrophoresis, the separated fragments must be denatured and transferred to a thinner solid medium (such as nitrocellulose or nylon membrane) to prevent diffusion. For the blotting step, the gel is placed on a sponge which is sitting in a buffer solution. The nitrocellulose paper is placed on top of the gel and then covered with paper towels. **Southern blotting** (named after Edwin M. Southern) is a technique used to transfer the denatured, single-stranded fragments from a gel to a thin solid medium. The transfer of DNA from the gel to the paper happens by capillary action. After the single-stranded DNA fragments have been transferred, the membrane is placed in a hybridization solution of a radioactively or chemically labeled probe (a DNA or RNA molecule with a base sequence complementary to a sequence



in the gene of interest). The probe will bind to any DNA fragments on the membrane that bear complementary sequences. The binding of the probe to the target molecule is called hybridization. The membrane is then washed to remove any unbound probe; bound probe is detected by autoradiography or another method for chemically labeled probes.

RNA can be transferred from a gel to a solid support by a related procedure called **Northern blotting**. The hybridization of a probe can reveal the size of a particular mRNA molecule, its relative abundance, or the tissues in which the mRNA is transcribed. **Western blotting** is the transfer of protein from a gel to a membrane. Here, the probe is usually an antibody, used to determine the size of a particular protein and the pattern of the protein's expression.

## 2.4. Polymerase Chain Reaction (PCR)

A major problem in working at the molecular level is that each gene is a tiny fraction of the total cellular DNA. Because each gene is rare, it must be isolated and amplified before it can be studied. Before mid-1980, the only procedure available for amplifying DNA was **gene cloning** (placing the gene in a bacterial cell and multiplying the bacteria). Cloning is labor intensive and requires at least several days to grow the bacteria. In 1983, Kary Mullis of the Cetus Corporation conceptualized a new technique for amplifying DNA in a test tube. The PCR allows DNA fragments to be amplified a billionfold within just a few hours. It can be used with extremely small amounts of original DNA, even a single molecule. The PCR has revolutionized molecular biology and is now one of the most widely used of all molecular techniques. The basis of PCR is replication catalyzed by a **DNA polymerase** enzyme, which has two essential requirements: (1) a single-stranded DNA template from which a new DNA strand can be copied and (2) a primer with a 3'-OH group to which new nucleotides can be added.

Because a DNA molecule consists of two nucleotide strands, each of which can serve as a template to produce a new molecule of DNA, the amount of DNA doubles with each replication event. The starting point of DNA synthesis on the template is determined by the choice of primers. The primers used in PCR are short fragments of DNA, typically from 17 to 25 nucleotides long, that are complementary to known sequences on the template. A different primer is used for each strand.

To carry out PCR, one begins with a solution that includes the target DNA (the DNA to be amplified), DNA polymerase, all four deoxyribonucleoside triphosphates (dNTPs—the substrates for DNA polymerase), primers that are complementary to short sequences on each strand of the target DNA, and magnesium ions and other salts that are necessary for the reaction to proceed. A typical polymerase chain reaction includes three steps (Figure 2.1).

Implementation of PCR with conventional DNA polymerase is not practical, because at the high temperature necessary for denaturation, the polymerase is itself irreversibly unfolded and becomes inactive. However, DNA polymerase isolated from certain archaeobacteria is heat stable because the organisms normally live in hot springs at temperatures well above 90°C. Such organisms are said to be thermophiles. The most widely used heat-stable DNA polymerase is called *Taq* polymerase, because it



was originally isolated from the thermophilic archaebacterium *Thermus aquaticus*.

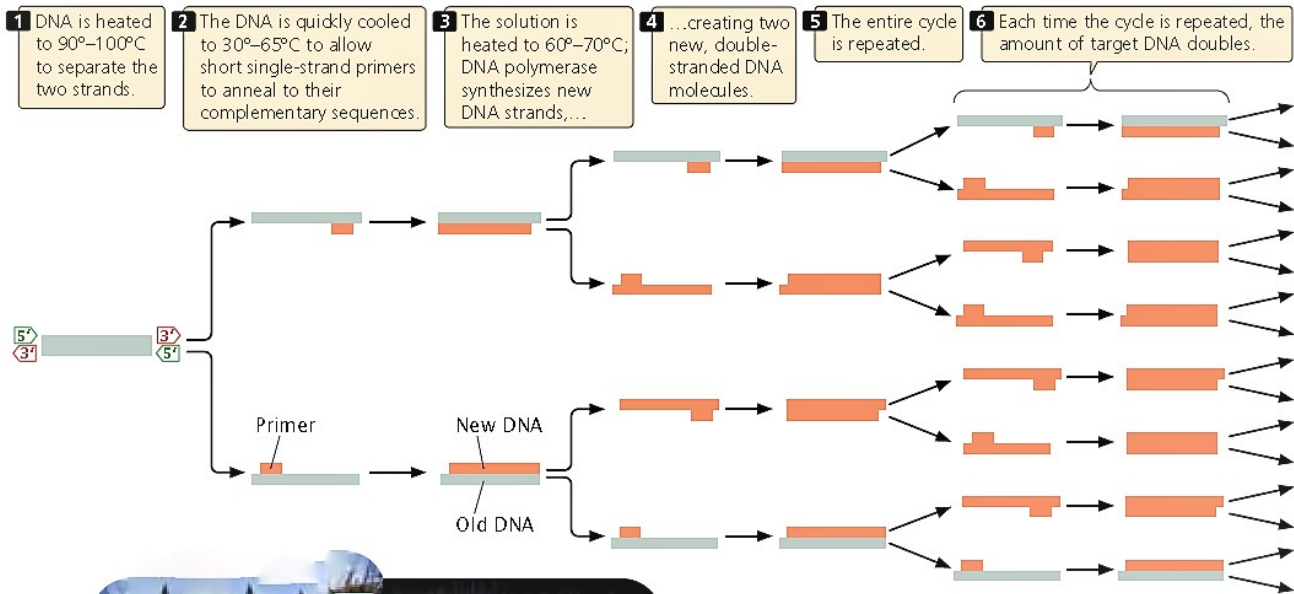


Figure 2.1. The polymerase chain reaction is used to amplify samples of DNA.

## 2.5.DNA Sequencing

DNA sequencing is the determination of the sequence of bases in DNA. Sequencing allows the genetic information in DNA to be read, providing an enormous amount of information about gene structure and function. The first methods for quickly sequencing DNA were developed between 1975 and 1977. Frederick Sanger and his colleagues created the dideoxy sequencing method based on the elongation of DNA; Allan Maxam and Walter Gilbert developed a method based on the chemical degradation of DNA. The Sanger method quickly became the standard procedure for sequencing any purified fragment of DNA.

**Maxam and Gilbert Method:** Maxam-Gilbert chemical degradation of DNA method involves chemical degradation (using dimethyl sulfate, piperidine, hydrazine, NaCl, NaOH, etc) of the original DNA. In this method, a fragment of DNA radiolabelled at one end is partially cleaved in five separate chemical reactions, each of which is specific for a particular base or type of base. This generates five populations of radiolabelled molecules that extend from a common point (the radiolabelled terminus) to the site of chemical cleavage. Each population consists of a mixture of molecules whose lengths are determined by the locations of a particular base along the length of the original DNA. These populations are then resolved by electrophoresis through polyacrylamide gels, and the end-labelled molecules are detected by autoradiography. This method has one clear advantage over the chain termination method: sequencing is obtained from the original DNA molecule and not from an enzymatic copy. However, because of its ease and rapidity, the Sanger technique is now the best choice for simple determination of DNA sequence.

**Sanger Method:** The Sanger, or dideoxy, method of DNA sequencing is based on the process of



replication. The fragment to be sequenced is used as a template to make a series of new DNA molecules. In the process, replication is sometimes (but not always) terminated when a specific base is encountered, producing DNA strands of different length, each of which ends in the same base.

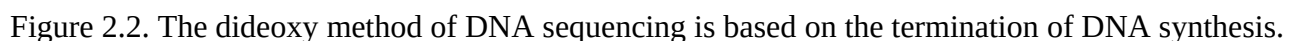
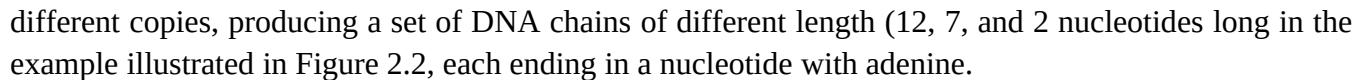
The method relies on the use of a special substrate for DNA synthesis. Normally, DNA is synthesized from **deoxyribonucleoside triphosphates** (dNTPs), which have an OH group on the 3'-carbon atom. In DNA synthesis, two phosphate groups on the 5'-carbon atom of a dNTP are removed, and a phosphodiester bond is formed between the remaining 5'-phosphate group of the dNTP and the 3'-OH group of the last nucleotide on the growing DNA chain. In the Sanger method, a special nucleotide, called a **dideoxyribonucleoside triphosphate** (ddNTP), is used as substrate. The ddNTPs are identical with dNTPs, except that they lack a hydroxyl residue at the 3' position of deoxyribose.

Like dNTPs, ddNTPs possess three phosphate groups on their 5' ends, and so they are incorporated into a growing DNA chain. When a ddNTP has been incorporated into a DNA chain, however, no more nucleotides can be added, because there is no 3'-OH group to form a phosphodiester bond with an incoming nucleotide. Thus, ddNTPs terminate DNA synthesis. A single DNA molecule cannot be sequenced; so any DNA fragment to be sequenced must first be amplified. Copies of the target DNA are isolated and split into four parts (Figure 2.2). Each part is placed in a different tube, to which the followings are added:

1. many copies of a primer that is complementary to one end of the target DNA strand;
2. all four deoxyribonucleoside triphosphates (dCTP, dATP, dGTP, and dTTP), the normal precursors of DNA synthesis;
3. a small amount of one of the four types of dideoxyribonucleoside triphosphates (ddCTP, ddATP, ddGTP, or ddTTP), which will terminate DNA synthesis as soon as it is incorporated into any growing chain (each of the four tubes received a different ddNTP); and
4. DNA polymerase.

Either the primer or one of the dNTPs is radioactively or chemically labeled so that newly produced DNA can be detected.

Within each of the four tubes, the DNA polymerase enzyme carries out DNA synthesis. Let's consider the reaction in one of the four tubes; the one that received ddATP. Within this tube, each of the single strands of target DNA serves as template for DNA synthesis. The primer pairs to its complementary sequence at one end of each template strand, providing a 3'-OH group for the initiation of DNA synthesis. DNA polymerase elongates a new strand of DNA from this primer, by using the target DNA strand as a template. Wherever DNA polymerase encounters a T on the template strand, it uses at random either a dATP or a ddATP to introduce an A in the newly synthesized strand. Because there is more dATP than ddATP in the reaction mixture, dATP is incorporated most often, allowing DNA synthesis to continue. Occasionally, however, ddATP is incorporated into the strand and synthesis terminates. The incorporation of ddA into the new strand occurs randomly at different positions in



Equivalent reactions take place in the other three tubes. In the tube that received ddCTP, all the chains terminate in a nucleotide with cytosine; in the tube that received ddGTP, all the chains terminate in a nucleotide with guanine; and, in the tube that received ddTTP, all the chains terminate in a nucleotide with thymine. After the completion of the polymerization reactions, all the DNA in the tubes is denatured, and the single-strand products of each reaction are separated by gel electrophoresis.

The contents of the four tubes are separated side by side on an acrylamide gel so that DNA strands differing in length by only a single nucleotide can be distinguished. After electrophoresis, the locations of the DNA strands in the gel are revealed by autoradiography. The shortest strands, which terminated at positions early in the DNA sequence, migrate quickly and end up near the bottom of the gel; longer



fragments, which terminated late in the sequence, migrate more slowly and end up near the top of the gel.

Reading the DNA sequence is simple and the shortest part of the procedure. In Figure 2.2, you can see that the band closest to the bottom of the gel is from the tube that contained the ddGTP reaction, which means that the first nucleotide synthesized had guanine (G). The next band up is from the tube that contained ddATP; so the next nucleotide in the sequence is adenine (A), and so forth. In this way, the sequence is read from the bottom to the top of the gel, with the nucleotides near the bottom corresponding to the 5' end of the newly synthesized DNA strand and those near the top corresponding to the 3' end. Keep in mind that the sequence obtained is not that of the target DNA but that of its complement.

### 3. CLONING GENES AND RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology is a set of molecular techniques for locating, isolating, altering, and studying DNA segments. The term *recombinant* is used because frequently the goal is to combine DNA from two distinct sources. Commonly called **genetic engineering**, recombinant DNA technology now encompasses an array of molecular techniques that can be used to analyze, alter, and recombine virtually any DNA sequences. Recombinant DNA Techniques are: Cutting and Joining DNA Fragments, Viewing DNA Fragments, Locating DNA Fragments with Southern Blotting and Probes, Cloning Genes, Finding Genes, Using the Polymerase Chain Reaction to Amplify DNA and Analyzing DNA Sequences.

#### 3.1. What is gene cloning?

Many recombinant DNA methods require numerous copies of a specific DNA fragment. One way to obtain these copies is to place the fragment in a bacterial cell and allow the cell to replicate the DNA. This procedure is termed gene cloning, because identical copies (clones) of the original piece of DNA are produced.

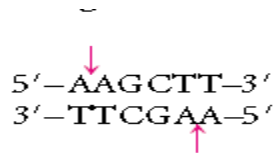
#### 3.2. Restriction enzymes and DNA ligases

The key development that made recombinant DNA technology possible was the discovery in the late 1960s of restriction enzymes (also called restriction endonucleases) that recognize and make double-stranded cuts in the sugar-phosphate backbone of DNA molecules at specific nucleotide sequences. These enzymes are produced naturally by bacteria, where they are used in defense against viruses. In bacteria, restriction enzymes recognize particular sequences in viral DNA and then cut it up. A bacterium protects its own DNA from a restriction enzyme by modifying the recognition sequence, usually by adding methyl groups to its DNA. Three types of restriction enzymes have been isolated from bacteria. Type I restriction enzymes recognize specific sequences in the DNA but cut the DNA at random sites that may be some distance (1000 bp or more) from the recognition sequence. Type III restriction enzymes recognize specific sequences and cut the DNA at nearby sites, usually about 25 bp away. Type II restriction enzymes recognize specific sequences and cut the DNA within the recognition

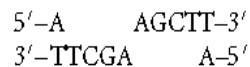


sequence. Virtually all work on recombinant DNA is done with type II restriction enzymes.

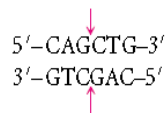
The sequences recognized by restriction enzymes are usually from 4 to 8 bp long; most enzymes recognize a sequence of 4 or 6 bp. Most recognition sequences are **palindromic** sequences that read the same forward and backward. All type II restriction enzymes recognize palindromic sequences. Some of the enzymes make staggered cuts in the DNA. For example, *Hind*III recognizes the following sequence:



*Hind*III cuts the sugar–phosphate backbone of each strand at the point indicated by the arrow, generating fragments with short, single-stranded overhanging ends:



Such ends are called **cohesive ends** or sticky ends, because they are complementary to each other and can spontaneously pair to connect the fragments. Thus DNA fragments can be “glued” together: any two fragments cleaved by the same enzyme will have complementary ends and will pair. When their cohesive ends have paired, two DNA fragments can be joined together permanently by the enzyme **DNA ligase**, which seals nicks between the sugar–phosphate groups of the fragments. Not all restriction enzymes produce staggered cuts and sticky ends. For instance, *Pvu*II cuts in the middle of its recognition site, producing blunt-ended fragments:



### 3.3. Vectors for cloning isolated DNA fragments

**Cloning vector** is a stable, replicating DNA molecule to which a foreign DNA fragment can be attached for introduction into a cell. An effective cloning vector has three important characteristics (Figure 3.1): (1) an origin of replication, which ensures that the vector is replicated within the cell; (2) selectable markers, which enable any cells containing the vector to be selected or identified; (3) one or more unique restriction sites into which a DNA fragment can be inserted. The restriction sites used for cloning must be unique; if a vector is cut at multiple recognition sites, generating several pieces of DNA, there will be no way to get the pieces back together in the correct order.

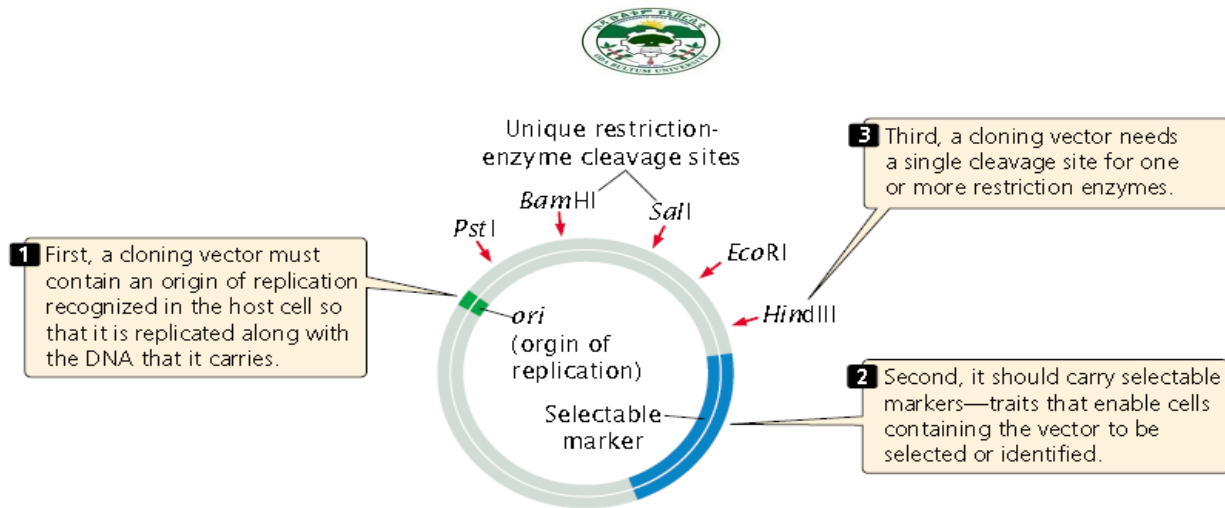


Figure 3.1. **Three characteristics of an idealized cloning vector.** An origin of replication, one or more selectable markers, and one or more unique restriction sites.

Three types of cloning vectors are commonly used for cloning genes in bacteria: plasmids, bacteriophages, and cosmids

**Plasmid vectors:** Plasmids are circular DNA molecules that exist naturally in bacteria. They contain origins of replication and are therefore able to replicate independently of the bacterial chromosome. The **pUC19** plasmid is a typical cloning vector which has an origin of replication and two selectable markers—an ampicillin-resistance gene and a *lacZ* gene. Ampicillin is an antibiotic that normally kills bacterial cells, but any bacterium that contains a pUC19 plasmid will be resistant to this antibiotic. The *lacZ* gene encodes the enzyme  $\beta$ -galactosidase, which normally cleaves lactose to produce glucose and galactose. The enzyme will also cleave a chemical called X-gal, producing a blue substance; when X-gal is placed in the medium, any bacterial colonies that contain intact pUC19 plasmids will turn blue and can be easily identified. The pUC19 plasmid also possesses a number of different unique restrictions sites grouped together (a polylinker) that allow DNA fragments to be inserted into the plasmid.

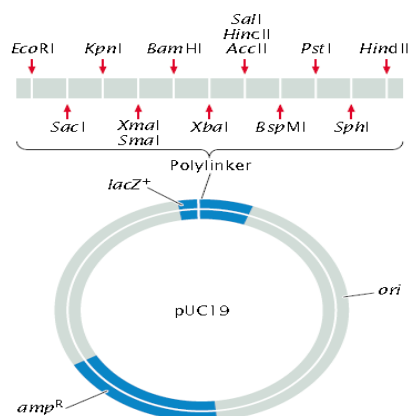


Figure 3.2. **The pUC19 plasmid is a typical cloning vector.** It contains a cluster of unique restriction sites, an origin of replication, and two selectable markers—an ampicillin-resistance gene and a *lacZ* gene.



**Bacteriophage vectors:** Bacteriophages offer a number of advantages as cloning vectors. The most widely used bacteriophage vector is bacteriophage  $\lambda$ , which infects *E. coli*. One of its chief advantages is the high efficiency with which it transfers DNA into bacteria cells. A second advantage is that about a third of the  $\lambda$  genome is not essential for infection and reproduction; without these genes, a  $\lambda$  particle will still faithfully inject its DNA into a bacterial cell and reproduce. These nonessential genes, which comprise about 15 kb, can be replaced by as much as about 23 kb of foreign DNA. A third advantage is that DNA will not be packaged into a  $\lambda$  coat unless it is 40 to 50 kb long; so fragments of foreign DNA are not likely to be transferred by the vector unless they are inserted into the  $\lambda$  genome, which ensures that the foreign DNA fragment will be replicated after it enters the cell.

**Cosmid vectors:** Although only about 23 kb of DNA can be cloned in  $\lambda$  vectors, DNA fragments as large as about 44 kb can be cloned in cosmids, which combine the properties of plasmids and phage vectors. Cosmids are small plasmids that carry phage  $\lambda$  *cos* sites; they can be packaged into viral coats and transferred to bacteria by viral infection. Because all viral genes except the *cos* sites are missing, a cosmid can carry more than twice as much foreign DNA as can a phage vector. Cosmid vectors have the following components: (1) a plasmid origin of replication (*ori*); (2) one or more unique restriction sites; (3) one or more selectable markers; and (4) *cos* sites to allow the packaging of DNA into phage heads. Foreign DNA is inserted into cosmids in the same way that DNA is introduced into plasmids: the cosmid and foreign DNA are both cut by a restriction enzyme that produces complementary (sticky) ends, and they are joined by DNA ligase. Recombinant cosmids are incorporated into the coats, and the phage particles are used to infect bacterial cells, where the cosmid replicates as a plasmid.

**Cloning vectors for eukaryotes:** The vectors discussed so far allow genes to be cloned in bacterial cells. Other cloning vectors have been developed for transferring genes into eukaryotic cells. Special plasmids, for example, have been developed for cloning in yeast, and retroviral vectors have been developed for cloning in mammals. **Shuttle vectors** are a vector (usually a plasmid) constructed so that it can propagate in two different host species (it used to shuttle genes back and forth between two hosts). Therefore, DNA inserted into a shuttle vector can be tested or manipulated in two different cell types. For example, plasmids have been engineered that allow gene sequences to be cloned and manipulated in bacteria and then transferred to yeast cells for study. For this reason, they must contain replication origins and selectable markers that work in both hosts. **Yeast artificial chromosomes (YACs)** are DNA molecules with a yeast origin of replication, a pair of telomeres, and a centromere. Mitotic spindle fibers attach to the centromere, and YACs segregate in the same way as yeast chromosomes; the telomeres ensure that YACs remain stable within the cell; and the origin of replication allows YACs to be replicated. YACs are particularly useful because they can carry DNA fragments as large as 600 kb, and some special YACs can carry inserts of more than 1000 kb. YACs have been modified so that they can be used in eukaryotic organisms other than yeast. **Bacterial artificial chromosomes (BACs)**, constructed from F factors, are used to clone large fragments ranging in length from 100 to 500 kb in bacteria.



### 3.4. Inserting DNA fragments into vectors

**Inserting DNA fragments into a plasmid:** The easiest method for inserting a foreign DNA fragment into a plasmid is to use **restriction cloning**, in which the foreign DNA and the plasmid are cut by the same restriction enzyme. Restriction cloning produces complementary sticky ends on the foreign DNA and the plasmid. The DNA and plasmid are then mixed together; some of the foreign DNA fragments will pair with the cut ends of the plasmid. DNA ligase seals the nicks in the sugar–phosphate backbone, creating a recombinant plasmid that contains the foreign DNA fragment. Although, restriction cloning has several disadvantages. First, restriction cloning requires that a single restriction site in the plasmid matches sites on both ends of the foreign sequence to be cloned. If this arrangement of restriction sites is not available, this relatively straightforward method cannot be used. Second, this technique often leads to undesirable products. The sticky ends of the plasmid are complementary to each other; so the two ends of the plasmid will often simply reanneal, reproducing the intact plasmid. Alternatively, the two complementary ends of the cleaved foreign DNA may anneal; or several pieces of foreign DNA or several plasmids may join. However, these undesirable products do not constitute a serious problem if an efficient method is used for screening bacterial cells for the presence of a recombinant plasmid.

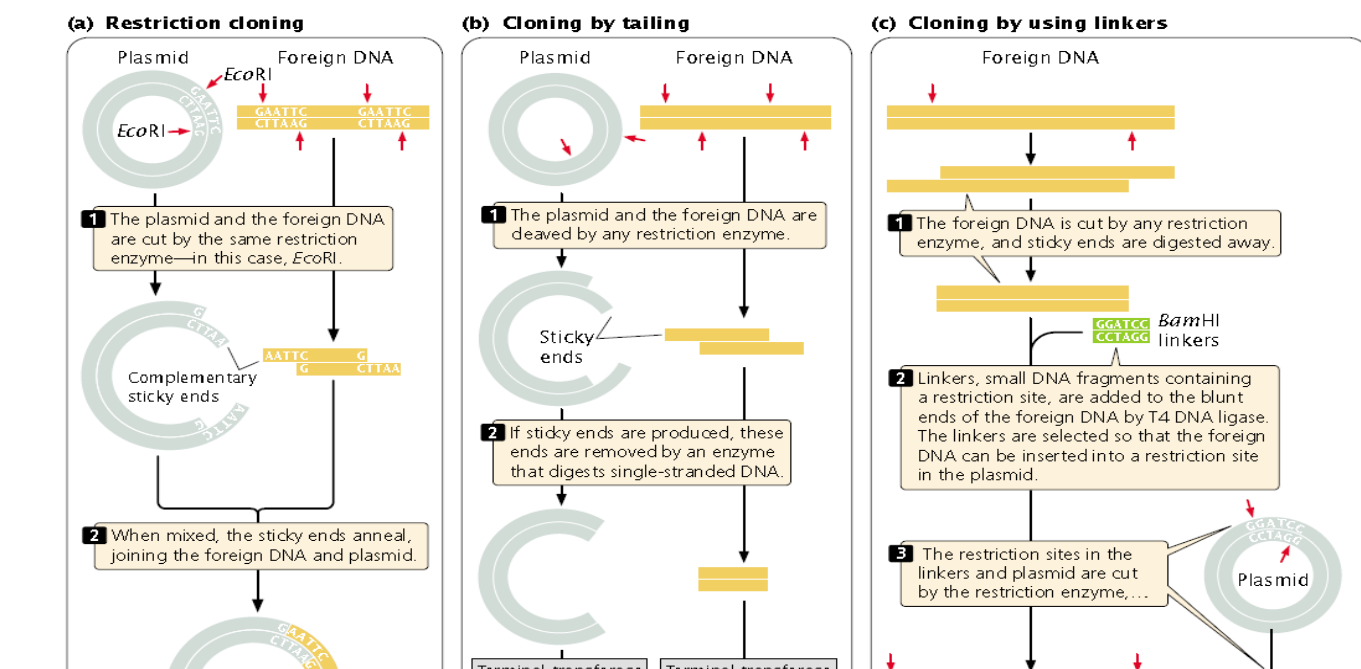
Another method for inserting DNA into a plasmid (a method that gets around the problem of undesired products) is **tailing**. In this procedure, complementary sticky ends are created on blunt-ended pieces of DNA. The plasmid and the foreign DNA are first cut by any restriction enzyme. If the restriction enzyme produces sticky ends, these ends are removed by an enzyme that digests single-stranded DNA. Alternatively, the plasmid and foreign DNA can be cut by a restriction enzyme that produces blunt ends. Once the plasmid and the foreign DNA have blunt ends, single-stranded sticky ends are added by an enzyme called terminal transferase, which adds any available nucleotides to the 3' end of DNA in a template-independent reaction. For example, terminal transferase and deoxyadenosine triphosphate (dATP) might be mixed with the plasmid DNA, creating poly (A) single-stranded tails on the 3' ends of the plasmid. Terminal transferase and deoxythymidine triphosphate (dTTP) could be mixed with the blunt-ended foreign DNA fragments, creating poly (T) single-stranded tails on their 3' ends. The poly(A) tail of the plasmid would be complementary to the poly(T) tail of the foreign DNA, allowing them to anneal and connecting the plasmid and foreign DNA together. DNA polymerase can be used to fill in any missing nucleotides, and DNA ligase can be used to seal the nicks in the sugar–phosphate backbone. One advantage of tailing is that it prevents the production of the undesired products created by restriction cloning: the single-stranded ends of the plasmid are complementary only to the single-stranded ends of the foreign DNA. Another advantage is that identical restriction sites are not required in plasmid and foreign DNA; any restriction site can be used for cleavage. First, it destroys the restriction site used to cut the original molecule, preventing later cleavage by the same restriction enzyme to retrieve the foreign DNA. Second, the new nucleotides (the complementary tails) introduced at the junctions between plasmid and foreign DNA sometimes interfere with the function of the cloned DNA.

A third method of inserting fragments into plasmids is to use the **enzyme T4 ligase**, which is capable of connecting any two pieces of blunt-ended DNA. Like tailing, this method requires no specific restriction sites and has great versatility; its chief drawback is that it creates a number of undesired products.



A fourth method, and one commonly used today, is the use of **linkers** to add complementary ends to DNA molecules. Linkers are small, synthetic DNA fragments that contain one or more restriction sites. The foreign DNA of interest is cut by any restriction enzyme; if sticky ends are created, they are digested to produce blunt ends. The linkers are then attached to the blunt ends by T4 ligase and are then cut by a restriction enzyme, generating sticky ends that are complementary to sticky ends on the plasmid, which have been generated by using the same restriction enzyme to cut the plasmid. Mixing the plasmid and foreign DNA leads to the formation of recombinant DNA that can be stabilized by ligase. The great advantage of using linkers is that a particular restriction site can be added at almost any desired location; so any two pieces of DNA can be cut and joined.

**Inserting DNA fragments into a bacteriophage:** The essential genes of the phage  $\lambda$  genome are located in a cluster. Strains of phage  $\lambda$ , called replacement vectors, have been engineered with unique *EcoRI* sites on either side of the nonessential genes so that, by using *EcoRI*, the nonessential genes can be removed. Foreign DNA cut with *EcoRI* will have sticky ends that are complementary to those on the ends of the essential  $\lambda$  genes, to which it can be connected by ligase. The  $\lambda$  chromosome possesses short, single-stranded ends called *cos* sites which are required for packaging  $\lambda$  DNA into a phage head. The recombinant phage chromosomes can then be packaged into protein coats and added to *E. coli*. The phages inject their recombinant DNA into the cell, where it will be replicated. Only DNA fragments of the proper size and containing essential genes will be packaged into the phage coats, providing an automatic selection system for recombinant vectors.



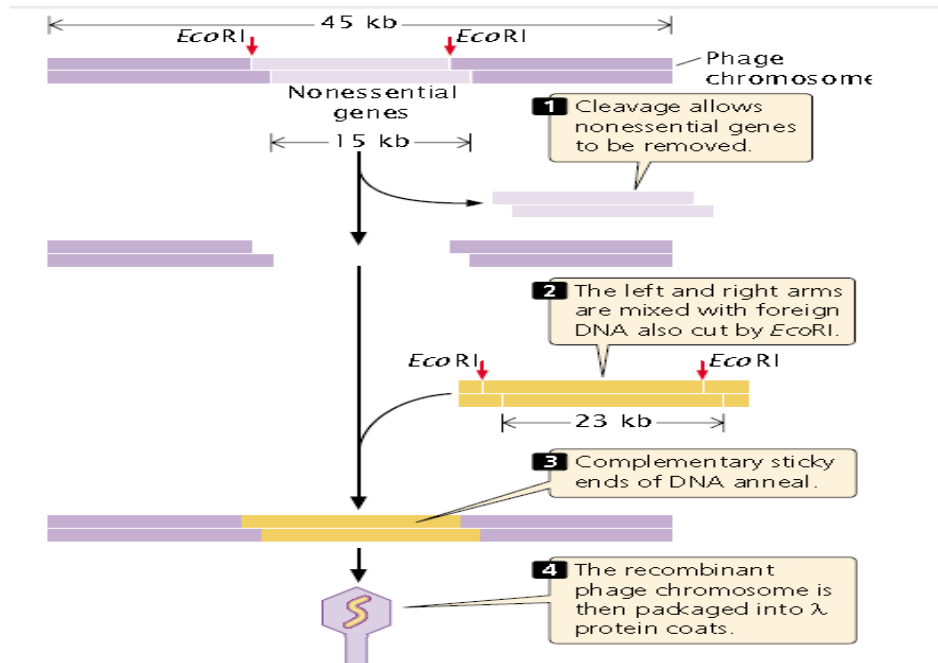
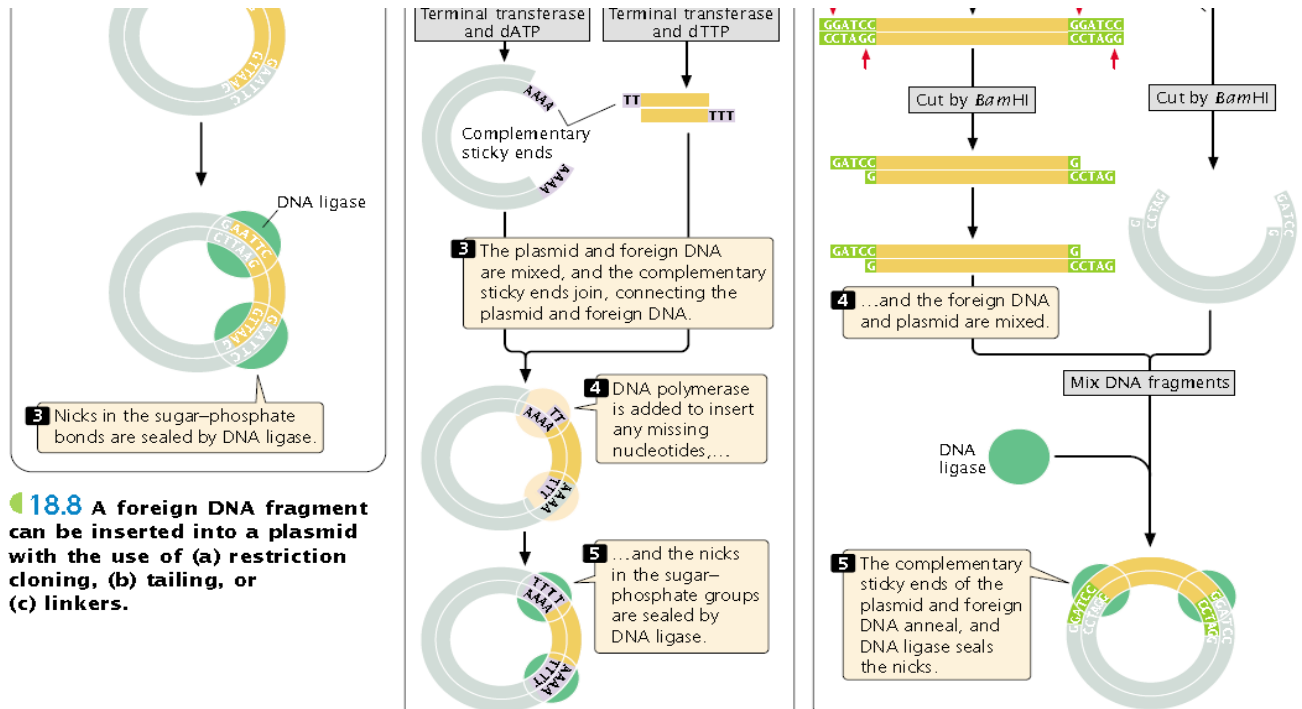


**Figure 3.4. Phage λ is an effective cloning vector.**

### 3.5. Transformation and selection of transformed colonies

Transformation is the capacity of cells to take up DNA from the medium. Some types of cells undergo



transformation naturally; others must be treated chemically or physically before they will undergo transformation. Inside the cell, cloning vector replicate and multiply.

Recombinant plasmid molecule is mixed with *E. coli* cells and most bacteria take up only a limited amount of DNA. Bacteriophage and cosmid vectors use infection method instead of transformation for their introduction into bacterial cells. Recombinant phages and cosmids are incorporated into the protein coats, and their particles are used to infect bacterial cells, where they will be replicated.

Methods for selecting transformants are **using a selectable markers** (for plasmids) and **selection on the basis of plaques** (for phages and cosmids). A selectable marker enables only cells that possess it to survive under selective conditions. pUC19 has an ampicillin-resistance gene and only transformants will grow on the media containing ampicillin. With phage vectors & cosmids, packaged vectors are used for infection of bacterial cells. The infected cells are **plated on a susceptible bacterial lawn** where they form plaques (clear zones) and plaques will form only due to the vector infected cells.

### 3.6. Finding Genes

How do we find the DNA sequence to be cloned? In fact, this problem is frequently the most significant one in cloning, because there are often millions or billions of base pairs of DNA in a cell. To solve this problem, one must often clone a gene to find it. This approach-**to clone first and search later**-is called “shotgun cloning” because it is like hunting with a shotgun. In shotgun cloning, one first clones a large number of DNA fragments, knowing that one or more contains the DNA of interest, and then searches for the fragment of interest among the clones. A collection of clones containing all the DNA fragments from one source is called a **DNA library**. For example, we might isolate genomic DNA from plant cells, break it into fragments, and clone all of them in bacterial cells or phages. The set of bacterial colonies or phages containing these fragments is a plant **genomic library**, containing all the DNA sequences found in the plant genome.

**Creating a genomic library:** to create a genomic library, DNA is isolated from the other cellular contents. When DNA has been extracted, it is cut into fragments by using a restriction enzyme. The fragments are then joined to plasmid, phage, or cosmid vectors, which can be transferred to bacteria. This technique produces a set of bacterial cells or phage particles containing the overlapping genomic fragments. A few of the clones contain the entire gene of interest, a few contain parts of the gene, but most contain fragments that have no part of the gene of interest. A genomic library must contain a large number of clones to ensure that all DNA sequences in the genome are represented in the library.



**Creating a cDNA library:** An alternative to creating a genomic library is, to create a library consisting only of those DNA sequences that are transcribed into mRNA (called a **cDNA library** because all the DNA in this library is *complementary* to mRNA). Much of eukaryotic DNA consists of repetitive (and other DNA) sequences that are not transcribed into mRNA and the sequences are not represented in a cDNA library. A cDNA library has two additional advantages. First, it is enriched with fragments from actively transcribed genes. Second, introns do not interrupt the cloned sequences.

To create a cDNA library, messenger RNA must first be separated from other types of cellular RNA (tRNA, rRNA, etc.). Most eukaryotic mRNAs possess a string of adenine nucleotides at the 3' end, and this poly(A) tail provides a convenient way for separating eukaryotic mRNA from the other types. Total cellular RNA is isolated from cells and poured through a column packed with short fragments of DNA consisting entirely of thymine nucleotides (oligo-dT chains). As the RNA moves through the column, the poly(A) tails of mRNA molecules pair with the oligo(dT) chains and are retained in the column, whereas the rest of the RNA passes through. The mRNA can then be washed from the column by adding a buffer that breaks the hydrogen bonds between poly(A) tails and oligo(dT) chains. The mRNA molecules are then copied into cDNA by reverse transcription. Short oligo(dT) primers are added to the mRNA. A primer pairs with the poly(A) tail at the 3' end of the mRNA, providing a 3'-OH group for the initiation of DNA synthesis. Reverse transcriptase, an enzyme isolated from retroviruses synthesizes single-stranded complementary DNA from the RNA template by adding DNA nucleotides to the 3'-OH group of the primer. The resulting RNA–DNA hybrid molecule is then treated with RNase to partly digest the RNA strand. Partial digestion leaves gaps in the RNA–DNA hybrid. DNA polymerase then synthesizes a second DNA strand by using the short undigested RNA pieces as primers and the first DNA strand as a template.

**Screening DNA libraries:** Creating a genomic or cDNA library is relatively easy compared with screening the library to find clones that contain the gene of interest. The screening procedure used depends on what is known about the gene. The first step in screening is to plate out the clones of the library. If a plasmid or cosmid vector was used to construct the library, the cells are diluted and plated so that each bacterium grows into a distinct colony. If a phage vector was used, the phages are allowed to infect a lawn of bacteria on a petri plate. Each plaque or bacterial colony contains a single, cloned DNA fragment that must be screened for the gene of interest.

One common way to screen libraries is **hybridization**, which uses probes. Probe labeled with radioactivity or fluorescent protein is used so that it gives fluorescent signals or colored precipitates. Then, labeled nylon membrane is pasted on culture plate, bacteria attach to a membrane and bacterial lyses occurred. Due to lyses bacterial genome is exposed, then double stranded DNA converted into single strands. Then, expose the probe to the membrane and provide the condition under which the probe can hybridize its complementary fragment.

Another method to screen libraries is **expression**. Vector for cloning is provided with expression system. The clones can be tested for the presence of the protein by using an antibody that recognizes the protein or by using a chemical test for the protein product. This method depends on the existence of a test for



the protein produced by the gene.

### 3.7. Plant Genetic Engineering and Gene Transfer Techniques

**Plant genetic engineering** is the deliberate, controlled manipulation of the genes in plants with the intent of making that plant better in some way. The result is a so-called genetically modified organism (GMO) or genetically modified crop. To date, most of the effort in genetic engineering has been focused on agriculture. Genetically engineered plants are generated in a laboratory by altering their genetic makeup. This is usually done by adding one or more genes to a plant's genome using genetic engineering techniques. Genes from sexually incompatible plants, or from animals, bacteria or insects can now be introduced into plants.

The uptake of foreign DNA by plant cell is called **transformation**. A variety of techniques have been used to introduce transgenes in to plant cells. These can be grouped in to the following two broad categories:

- i. Direct gene transfer method (chemical and physical)
- ii. Agrobacterium mediated (indirect method)

#### Direct gene transfer methods

In the direct gene transfer methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector. The methods used for direct gene transfer in plants are:

**Chemical mediated gene transfer** e.g. chemicals like polyethylene glycol (PEG) and dextran sulphate induce DNA uptake into plant protoplasts. Calcium phosphate is also used to transfer DNA into cultured cells.

#### Transformation by Physical Means

- a. **Microinjection:** where the DNA is directly injected into plant protoplasts or cells (specifically into the nucleus or cytoplasm) using fine tipped (0.5 - 1.0 micrometer diameter) glass needle or micropipette. This method of gene transfer is used to introduce DNA into large cells such as eggs, and the cells of early embryo.
- b. **Electroporation:** involves a pulse of high voltage applied to protoplasts/cells/ tissues to make transient (temporary) pores in the plasma membrane which facilitates the uptake of foreign DNA. The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.



- c. **Particle gun/Particle bombardment** - In this method, the foreign DNA containing the genes to be transferred is coated onto the surface of minute gold or tungsten particles (1-3 micrometers) and bombarded onto the target tissue or cells using a particle gun (also called as biolistics/gene gun/shot gun/microprojectile gun).

### **Agrobacterium mediated transformation (indirect method)**

*Agrobacterium tumefaciens* is a plant pathogen that causes a disease in dicotyledonous (broad-leafed) plants called crown gall. A crown gall is a tumor that usually forms on the stem of plants that consists of rapidly dividing cells. The crown gall is essentially a plant cancer. These galls do not kill the plant directly but they tap resources from the plant and can sometimes grow large enough to block the vascular system of the stem.

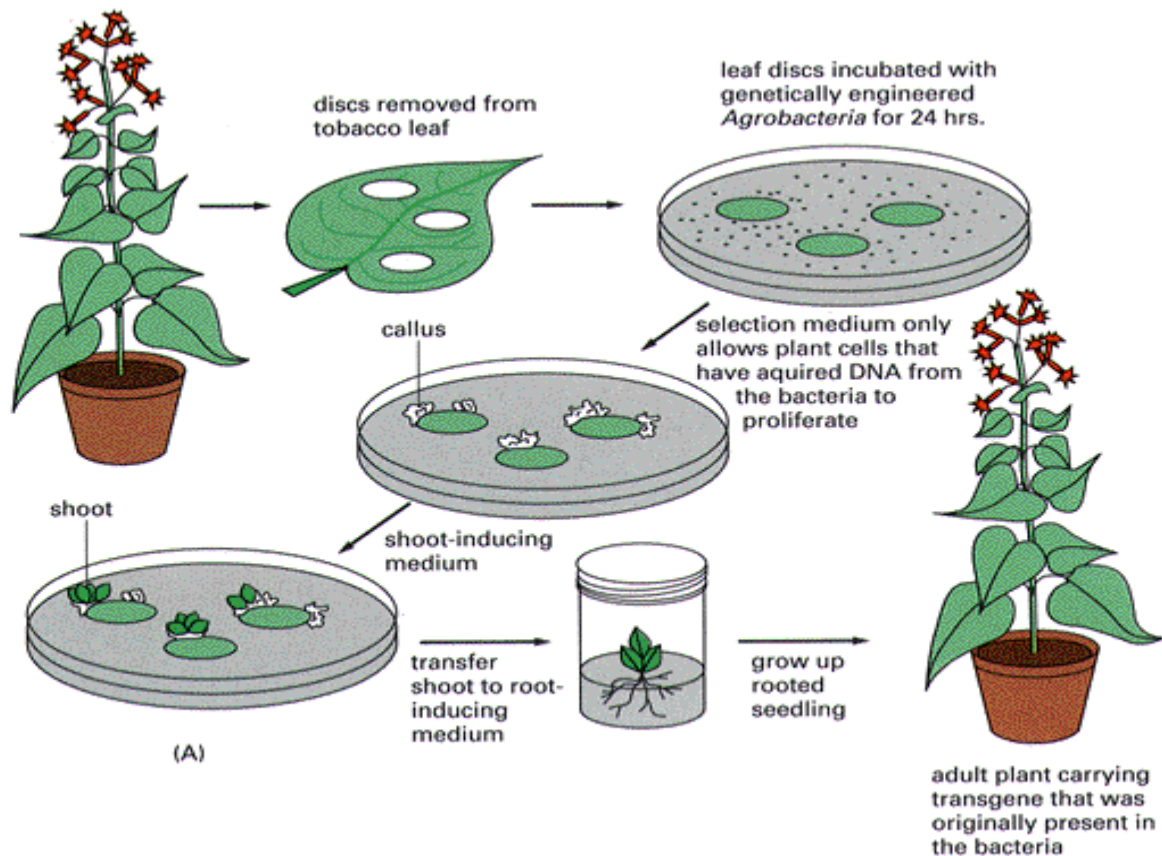


**Fig . A view of plant transformation using *Agrobacterium tumefaciens*.**

Virulent strains of *Agrobacterium* that are capable of causing tumors in plants carry large plasmids called Ti plasmids (Tumor inducing). These circular plasmids are typically several hundred nucleotides in size. When infection occurs, a portion of the Ti plasmid is transferred to the plant cell and is incorporated into the plant genome. The segment of the DNA that is transferred to the plant is called the



T-DNA. The T-DNA is flanked by direct repeats that are necessary for the transfer and includes several genes that are expressed in the plant cell host. Two of these genes encode enzymes that are involved in the production of the plant growth regulators auxin and cytokinin. These phytohormones, when present in abnormally high concentrations cause plant cells to grow into a rapidly proliferating undifferentiated tissue called callus. This mass of callus forms the crown gall. In addition to these tumor causing genes, the T-DNA also carry genes for enzymes that produces unusual amino acids, called an octopines, in the plant cell. Octopines, such as nopaline, cannot be used by the plant but the virulent *Agrobacterium* cells can use octopines as a food source. Therefore, when *Agrobacteria* infect a plant, they cause the growth of a tissue that will supply the surrounding *Agrobacteria* with food.

Therefore, *Agrobacterium* can carry out a natural form of genetic engineering; this system has been adapted by researchers to efficiently transfer other genes into plant tissues. To do this, it was necessary to remove the tumor-causing genes from the T-DNA of a Ti plasmid. Such a “disarmed” plasmid is still capable of transforming plant cells but does not cause tumors. Next, a synthetic gene that encodes an enzyme called neomycin phosphotransferase II (npt II) was introduced into the Ti plasmid. This enzyme breaks down the antibiotic kanamycin and expression of this enzyme in plant cells allows them to grow in the presence of this antibiotic. The synthetic npt II gene consisted of coding sequences derived from a transposon from *E. coli* under control of a promoter from a plant virus called the cauliflower mosaic virus (CaMV) 35S promoter. Therefore, infection of plant tissues with *Agrobacterium* that carry the modified Ti plasmid results in plant cells that express the npt II gene and are able to grow in tissue culture, on media that contains kanamycin. In this way, it was possible to transfer the T-DNA from the *Agrobacterium* to plant cells and select for those cells that were transformed by growing them in the presence of kanamycin.

Some plants, such as transformed tobacco, can be easily regenerated from tissue culture. So, it was a relatively simple matter to obtain transformed tobacco cells and cause these cells to regenerate into tobacco plants. These plants are perfectly normal, except that they carry a piece of T-DNA inserted in one of their chromosomes. When these plants flower and self pollinate, they produce seeds that inherit the T-DNA insert and the kanamycin resistance gene that it carries. In addition to the npt II gene, virtually any other gene can be introduced into the T-DNA and transferred to plant cells. In this way it is possible to genetically engineer plants to express genes that provide them with unique characteristics.

Since the Ti plasmid is large and difficult to manipulate in vitro, modified systems have been developed that use smaller, more easily manipulated T-DNA containing plasmids that can replicate in both *E. coli* and *Agrobacterium*. These are called binary vectors because the mobilization of the T-DNA to plant cells is catalyzed by the vir genes of a Ti plasmid from which the T-DNA has been deleted. The vir genes produce the enzymes that are responsible for cutting the T-DNA, copying it and transporting it to the plant cell. Since the vir genes work in trans, they can stimulate the transfer of T-DNA from separate plasmid.

Limitations of the *Agrobacterium*-based transformation system include:



1. Most monocot plants are resistant to *Agrobacterium* infection. Therefore, cereal crops such as wheat and maize cannot be transformed using this system.
2. Difficulty of regeneration of plants from transformed tissues.

For these reasons, other methods have been developed for the regeneration of recalcitrant species such as wheat and maize. One of the most interesting methods involves shooting DNA-coated gold particle into plant tissues. The original equipment used for this so-called particle bombardment technique was simply a modified 22 caliber nail gun. Currently used gene guns are powered by compressed helium and are more efficient and controllable. Using this method, tissue that is easily regenerated is shot with gold particles that are coated with plasmid that contain the gene of interest, along with a kanamycin resistance gene. Selection and regeneration of the transformed cells is then carried out to obtain a transgenic plant.

**Selection of transformed cells:** The selection of transformed plant cells from untransformed cells is an important step in the plant genetic engineering. For this, a marker gene (e.g. for antibiotic resistance) is introduced into the plant along with the transgene followed by the selection of an appropriate selection medium (containing the antibiotic).

### **3.8. Application of recombinant DNA technology**

In addition to providing valuable new information about the nature and function of genes, recombinant DNA technology has many practical applications. These applications include the production of pharmaceuticals and other chemicals, specialized bacteria, agriculturally important plants, and genetically engineered farm animals. The technology is also used extensively in medical testing and, in a few cases, is even being used to correct human genetic defects.

Production of transgenic organisms has created a great number of possibilities using plants. The ultimate goal of transgenic plants (introduction, integration and expression of foreign genes in plants) is to improve the crop with desired trait:

1. Resistant to biotic stresses ( insects, viruses, bacteria and fungi)
2. Resistant to abiotic stresses (herbicide, temperature, drought, salinity and etc.)
3. Improvement of crop quality, e.g. storage, longer shelf life of fruits and flowers, etc.
4. Transgenic plants with improved nutrition

#### **Production of crop varieties for biotic stress**

##### **a. Insect resistance**

Insect attack is a serious agricultural problem leading to yield losses and reduced product quality. Insects can cause damage both in the field as well as during storage. The damage to crop is mainly caused by insect larvae and to some extents adult insects. Even if multiple managements were undertaken, chemical pesticides were commonly used for control.



Limitation of pesticide uses:

- i. About more than three fourth of chemical applied is washed away from the plant surface and accumulated in the soil.
- ii. They are not degraded in the soil, causing environmental pollution.
- iii. Pesticides in general are toxic to humans, animals and non target crops.

Transgenic plants with insect resistant gene have been developed. Many genes obtained from microorganisms, higher plants and animals have been used to provide insect resistance in crop plants. One famous insect resistant gene is protoxin (insecticidal crystalline protein) from bacteria called *Bacillus thuringiensis* (Bt). This protein is sometimes called **Bt toxin**. Most of the Bt toxins are active against larvae.

By genetic transformation, it has been possible to genetically modify plants by inserting Bt genes and provide pest resistant to these transformed plants. Scientists isolated the gene (Bt gene) for protoxin from bacteria and inserted in to the plant genome. The gene is expressed in the plant. When the insects eat the plant, it affects the physiology of insects and kills them. E.g. Bt corn, Bt cotton

Advantages of transgenic plants with Bt genes:

- Bt genes can be expressed in all parts of the plant including roots, and internal regions of stems and fruits
- Toxin protein are produced within the plants (environmentally friendly).

#### **b. virus resistance**

Viruses cause significant damage to plants. The overall infections are growth retardation, lowering product yield, and sometimes complete plant failure. The chemicals used to control viruses are ineffective since the viruses are intracellular obligate parasites. In addition, the spread of most viruses is extremely difficult to control.

Certain safe agricultural practices used to control viral infection are:

- Use of seeds that are virus free
- Blocking of virus transmission (through controlling insects that spread plant viruses)
- Use varieties that are virus resistant

By genetic engineering, virus resistant plants can be produced. This is by immunization with viral coat protein gene. The virus coat protein gene can be inserted in to the plant genome by genetic engineering. The plants then express the gene which reduces the ability of plants to be infected by virus. All genetically modified virus resistant plants (e.g. papaya and squash) have coat protein mediated resistance. It may also be possible to confer resistance by taking a resistant gene naturally found in one plant and then transferring it to an important crop.

#### **c. bacteria and fungi resistance**



Bacteria and fungi cause extensive damages and losses of agricultural productivity. Currently, chemicals are being widely sprayed to control these pathogens which may have risks on ecology.

By using genetic engineering, bacteria and fungi resistant plants can be produced. When plants are attacked by particular pathogen, the plants produce pathogenesis related proteins (PR proteins) to defend themselves against pathogen. Plants can be engineered to express the pathogenesis related proteins. The transformed plants can produce pathogen related proteins and become resistant to specific bacteria or fungi.

One way of managing fungal and bacterial disease through genetic engineering is introducing genes from other plants or bacteria encoding enzymes. These enzymes breakdown chitin or glucan, which are essential components of fungal cell walls.

### **Production of crop varieties for abiotic stress**

Plants are subjected to environmental stresses such as herbicides, drought, salt concentration, etc. that may result in deterioration in crop plants and a very low or even no yield.

Some of the abiotic stresses and the recombinant technologies developed to overcome the problems are the following:

#### **A. Herbicide resistance**

Weeds are unwanted plants that grow along with crop plants. Weeds compete with crops for light, nutrients, water, air., etc, which causes yield reduction. Herbicides are chemicals that kill weeds. The majority of herbicides are broad spectrum, meaning no discrimination between weeds and crops. For this reason, crops can be affected by herbicides during application. Hence, it is needed to develop herbicide resistant crops.

Several crops have been genetically modified to be resistant to non selective herbicides. These transgenic crops contain gene that enable them to degrade the active ingredient in herbicide, rendering it harmless. Farmers, then can control weeds during the entire growing season and have more flexibility in choosing time for application.

Critics claim that in some cases, the use of herbicide resistant crops can lead to an increase in herbicide use, promote the development of herbicide resistant weeds and damage biodiversity on the farm.

Some of the methods of recombinant DNA technology to develop herbicide resistant crops are:

- i. Over expression of the target protein: this is the increasing of production of target protein being acted by the herbicide. So, the effect of herbicide on the target protein becomes insignificant.
- ii. Inhibiting the uptake of herbicide in crop plants
- iii. Detoxification of the herbicides by using foreign genes. By introducing foreign genes in to the crop plants, the herbicide can be effectively detoxified.



- iv. Making the protein to be not affected by the herbicide through mutation of the target protein: the target protein being affected by herbicide can be modified (reduce the binding to herbicide) and a protein can become resistant to inhibition by the herbicide. example: glyphosate resistant plants (GRP) (Roundup)

Herbicide tolerance is available for all of the major genetically modified crops, including soybean, maize, rapeseed and cotton.

## **B. Drought resistance**

Water is crucial for living things. Plants use water as a solvent, a transport medium, an evaporative coolant, physical support and as a major ingredient for photosynthesis. Without sufficient water, agriculture is impossible at all. Therefore, drought tolerance is an extremely important agricultural trait.

One way of engineering drought tolerance is by taking the genes from plants that are naturally drought tolerant and introducing them to crops. The plant which is native to dry regions possess a gene for a unique protein in its cell membrane. Hence plants given this gene are less prone to stress from drought. Some genes have been found that control the production of the thin, protective cuticle found on leaves.

## **C. Salt resistance**

Irrigation has enabled the transformation of arid regions into some of the world's most productive agricultural areas. Excess salinity, however, is becoming a major problem for agriculture in dry parts of the world. In several cases, scientists have used biotechnology to develop plants with enhanced tolerance to salty conditions.

Researchers have seen that plants with high tolerance to salt stress possess naturally high levels of a substance called glycinebetaine. Further, plants with intermediate levels of salinity tolerance have intermediate levels, and plants with poor tolerance to salinity have little or none at all. For example, GM tomatoes with enhanced glycinebetaine production have increased tolerance to salty conditions. These tomatoes have been genetically engineered enabling them to grow in salty water.

Another approach to engineering salt tolerance uses a protein that takes excess sodium and diverts it into a cellular compartment where it does not harm the cell. In the lab, this strategy was used to create test plants that were able to flower and produce seeds under extreme salt levels.

## **Production of crops with improved nutrition and quality**

Transgenic plants with altered nutritional composition offer diverse advantages to consumers:

- Modified oil content and composition: eg. for maize, soybeans and other oil crops. These modified crops can be important in fighting against cardiovascular diseases, obesity and certain forms of cancer.



- Golden rice: a rice cultivar with enhanced levels of vitamin A (enriched with carotenoids).
- Higher content of protein or amino acids: e.g. GM tomato
- Higher levels of beneficial antioxidant compound: to prevent cardiovascular diseases, E.g. in GM tomato
- Fruits with longer shelf life: e.g. Apples and melons with delayed ripening have also been developed.

#### 4. MOLECULAR MARKER TECHNOLOGY

A genetic marker is a measurable character that can detect variation in either a protein or DNA sequence. A difference, whether phenotypic or genotypic, may act as a genetic marker if it identifies characteristics of an individual's genotype and/or phenotype, and if its inheritance can be followed through different generations. It can be a gene or DNA sequence with a known location on a chromosome and associated with particular gene or trait. It can be described as a variation which may arise due to mutation or alteration in genomic loci, which can be observed. The technology has already delivered dramatic improvements for farmers using genes native to the crops.

##### Types of Genetic markers

A) **Morphological markers:** For many years, morphological markers/traditional genetic markers have been used which based on an easily observable characteristics such as seed shapes or flower characteristics.

##### Advantages:

- ✓ Readily available
- ✓ Usually require only simple equipment
- ✓ Form the most direct measure of phenotype

##### Disadvantages:

- ✓ Require expertise on crop and/or species
- ✓ Subject to environmental influences
- ✓ Limited in number

##### B) **Protein (biochemical) markers:**

Protein markers (seed storage proteins and isozymes) are generated through electrophoresis, taking advantage of the migrational properties of proteins and enzymes, and revealed by histochemical stains



specific to the enzymes being assayed.

**Advantage:**

- ✓ Require relatively simple equipment

**Disadvantages:**

- ✓ Subject to environmental influences
- ✓ Limited in number

**C) DNA (molecular) markers**

Molecular markers are DNA fragments associated with certain location in the genome. They are the most important and stable markers, which have been widely used in plant breeding and in other studies. The use of molecular markers to track loci and genome regions in crop plants is now routinely applied in many breeding programs. The location of major loci is now known for many disease resistance genes, tolerances to abiotic stresses and quality traits.

**Advantages:**

- ✓ Not subject to environmental influences
- ✓ Potentially unlimited in number
- ✓ Objective measure of variation

The major disadvantage of molecular marker is the need for technically more complex equipment.

**4.1. Types of Molecular Markers**

Types of molecular markers are the following:

**A. Restriction Fragment Length Polymorphism (RFLP)**

In molecular biology, the term RFLP refers to a difference between two or more samples of homologous DNA molecules arising from differing locations of restriction sites and to a related laboratory technique by which the segments can be distinguished. In RFLP analysis, the DNA sample is broken in to pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their length by gel electrophoresis. RFLP analysis was the first DNA profiling technique cheap enough to see wide spread application. In addition to genetic finger printing, it is used in genome mapping, to localize genes for genetic disorders, determination of the risk for diseases and paternity testing.

**B. Random Amplification of Polymorphic DNA (RAPD)**



It is the type of PCR reaction, but, the segments of DNA that are amplified are random. No knowledge of the DNA Sequence for the targeted gene is required as the primers will bind somewhere in the sequence. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community. Because, it relies on intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Currently, this technique has been used to characterize and trace the phylogeny of diverse plants animal species.

### **C. Microsatellite polymorphism or Simple Sequence Repeats (STR) or Short Tandem Repeats (STR)**

They are repeating sequences of 2-6 base pairs of DNA. They are used as molecular markers in genetics, population and other studies. They can also be used to study gene duplication or deletion. Example:

X can have sequence .....CACACACACACTGACCTTGAA...(CA)<sub>6</sub>

Y can have sequence.....CACACACACACACACACATGACG... (CA)<sub>10</sub>

These markers often present high levels of inter and intra-specific polymorphism, particularly when tandem repeats number ten or greater. The repeated sequence is often simple, consisting of two, three or four nucleotides and can be repeated 10 to 100 times.

### **D. Amplified Fragment Length Polymorphism ( AFLP)**

AFLP is a PCR based tool used in genetic research, DNA fingerprinting and in practice of genetic engineering. AFLP uses molecular scissors to digest genomic DNA, followed by ligation of adapters to sticky ends of the restriction fragments. A subset of restriction fragments is then selected to be amplified. This selection is achieved by using primers complementary to the adapter sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments the amplified fragments are visualized on denaturing agarose gels through fluorescence or other methodologies. In detail, the procedure of this technique is divided in to three steps:

- i. Digestion of total cellular DNA with one or more restriction enzymes and ligation of restriction half site specific adapters to all restriction fragments
- ii. Selective amplification of some of these fragments with two PCR primers that have corresponding adapter restriction site specific sequences
- iii. Electrophoretic separation of amplicon on gel matrix.

### **E. Single Nucleotide Polymorphism (SNP)**

It is a DNA sequence variation occurring when single nucleotide in the genome differs between members of the species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in single nucleotide. In this case, we say that there are two alleles. Almost all common SNPs have only two alleles.



Within the population, SNPs can be assigned a minor allele frequency at a locus that is observed in a particular population. There are variations between human populations, so, a SNP allele that is common in one geographical or ethnic group may be much rarer in another.

## 5. PLANT TISSUE CULTURE

Plant tissue culture is a practice used to propagate plant under sterile in vitro conditions, often to produce clones of a plant. It is the culture of plant seeds, organs, tissues, cells, or protoplasts on nutrient media under sterile conditions. It has the following advantages over traditional approach i.e.

- The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.
- To quickly produce mature plants.
- The production of multiples of plants in the absence of seeds.
- The regeneration of whole plants from genetically modified plant cells
- The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.
- The production of plants from seeds that have very low chances of germinating and growing, i.e orchids.
- To clean particular plant of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.

### 5.1. Requirement for a Tissue culture Laboratory

#### ***Laboratory space- divided in to 4 parts***

For standard tissue culture laboratory, the following minimum facilities are necessary.

1. A general washing area
2. A media preparation, sterilization, and storage area
3. An aseptic transfer area
4. Culture rooms or environmentally controlled incubators

Besides this, an observation /data collection area is also needed to record observations from time to time.

#### **1) Washing area**



An area with large sink (lead lined to resist acids and alkalis) and draining area is necessary with provision for running water, draining-boards or racks and ready access to a deionized, distilled and double-distilled apparatus.

Space should also be available to set up drying ovens, washing machines, plastic or steel buckets for soaking labware, acid or detergent baths, pipette washers, driers and cleaning brushes. For storage of washed and dried labware, the laboratory should be provided with dustproof cupboards or storage cabinets.

## **2) Media preparation Area**

This part is the central section of the laboratory where most of the activities are performed i.e., media preparation and sterilisation of media and glassware's needed for culture. There should be sufficient working bench as well as storage space. Laboratory should have ample storage space for the chemicals, culture vessels and closures.

It should have: different types of glassware Different kinds of balances, Required chemicals, Hot plates and Stirrer, Water bath, pH meter, Autoclave and Hot air oven, Microwave oven, Vortex, Shaker, Centrifuge, Storage cabinet (Dust-free), Refrigerator and Freezer.

**Refrigerators and freezers** – For storage of stocks solutions and chemicals

**Microwave or convection oven or hotplate-** for defrosting frozen stocks and melting agar

**Autoclave or domestic pressure cooker** – for sterilizing media glassware, and instruments. Some fungi and bacteria will only be killed at high temperature, 121<sup>0</sup>c, followed by 15 pound pressure per second.

**High quality water, i.e** high quality water is used for media preparation which is free from pathogenic substances produced by bacteria, gases and organic substances.

## **3) Aseptic transfer area**

Tissue culture techniques can only be successfully carried out in a very clean laboratory having dry atmosphere with protection against air-borne microorganisms. For this purpose a sterile dust-free room/cabinet is needed for routine transfer and manipulation work.

The laminar air flow cabinet is the most common accessory used for aseptic manipulations now adays. The cabinet may be designed with horizontal air flow or vertical air flow where the air is forced into the cabinet through a bacterial HEPA (High Efficiency Particulate Air) filter. The air flows over the working bench at a constant rate which prevents the particles (microorganisms) from settling on the bench.



Before operation in the laminar air flow cabinet, the interior of the cabinet is sterilised with the ultraviolet (UV) germicidal light and wiping the floor of cabinet with 70% alcohol. Inoculation chamber, a specially designed air tight glass chamber fitted with UV light, may also be used as transfer area.

All surfaces designed and constructed in such a manner that dust and microorganisms do not accumulate and the surfaces can be thoroughly cleaned and disinfected. Within the transfer area there should be a source of electricity, gas compressed air and vacuum.

#### **4) Culture Room-**

For keeping in vitro transferred material and for its growth; proper temperature, humidity, air circulation, light quality conditions are maintained.

Light duration – may influence the growth and differentiation process directly during culture or indirectly by affecting their response in subsequent generations. Temperature is maintained around  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , maintained using air conditioners, lighting is adjusted in terms of quantity and photoperiod duration by timer.

Relative Humidity – it is difficult to maintain inside growing vessel but room RH should not go below 50%, otherwise cultures will dry out and humidifiers can be used to correct this problem.

#### ***Observation / data collection room***

Monitoring – growth and development of tissues cultured in vitro is needed at regular intervals under controlled environment conditions. Data based on observations under aseptic conditions may be collected using laminar airflow cabinet or suitable area designed for the purpose.

### **5.2. Principles of aseptic culture**

All the material used in experimentation such as vessels, instruments, medium and plant material should be free of contamination, means free of microorganisms. This can be achieved by following approaches:

#### ***1) Heat sterilization***

***Dry heat sterilization*** – used for metal and glassware wrap in aluminum foil or keep in container and heat in hot air oven so heat uniformly reaches to every part of the glassware in initial 1 hr – it heats up at  $180^{\circ}\text{C}$  and keep it further two hours 2hrs- for sterilization at  $180^{\circ}\text{C}$

***Wet heat sterilization*** – For glassware, liquids or solids done in autoclave and pressure cooker water steam formed at high temperature  $121^{\circ}\text{C}$  and pressure (15 pas). Time of sterilization depend on volume cautions.

- Do not over autoclave, as it degrades media components
- Release pressure slowly

#### ***2) Flame sterilization***



Metal instrument like forceps, and scalper are sterilized by this way. Dip them in Absolute alcohol and heat on flame till turn red hot and use after cooling.

### **3) *Filter sterilization***

Growth factors, such as GA<sub>3</sub>, zeatin, ABA, etc (which are Thermolabile) should not be autoclaved, but sterilized separately by membrane filtration. 0.2  $\mu$ m filter use to sterilize chemicals (Millipore). Chemical added in liquid medium at room temperature for filtration and at 55<sup>0</sup>c in agar containing liquefied medium 0.45  $\mu$ m filters remove only particulates

### **4) *Chemical sterilization***

The plant material is sterilized by this method. Use 70% Ethyl alcohol for surface sterilization .

NaOCl (sodium hypochlorite) – use 10% for surface sterilization of explants.

**Time of treatment depends on type of tissue used**

Soft tissue – 3-5 min

Hard tissue -5-20 min

### **General safety rules**

- o Assume all cultures are hazardous since they may harbor latent viruses or other organisms that are un characterized
- o No eating, drinking, or smoking
- o Wash hands after handling cultures and before leaving the lab
- o Decontaminate work surfaces with disinfectant (before and after)
- o Properly dispose of biological and chemical waste
- o Do not expose bleach solution to uv light, it will produce cl gas
- o Do not use HgCl<sub>2</sub>, mercuric chloride as disinfectant it is highly toxic
- o Always wear lab coat

## **5.3. Tissue culture media preparation**

### ***Basic media component in tissue culture***

Culture media used for the in vitro cultivation of plant cells are composed of three basic

Components:



- (1) Essential elements of mineral ions, supplied as a compiled mixture of salts
- (2) An organic supplement supplying vitamins and/ or amino acids: and
- (3) A source of fixed carbon: usually supplied as the sugar sucrose.

### **(1). Essential elements**

For practical purposes, the essential elements are further divided into the following categories (1) macro elements (or macronutrients) (2) micro elements (of micronutrients); and (3) an iron source.

#### *Macroelements*

As is implied by the name the stock solution supplies those elements required in large amounts for plant growth and development. Nitrogen, phosphorus, potassium, magnesium, calcium and sulfur (and carbon which is added separately) are usually regarded as macroelements. These elements usually comprise at least 0.1% of the dry weight of plants. Nitrogen is most commonly supplied as a mixture of nitrate ions (from the  $\text{KNO}_3$ ) and ammonium ions (from the  $\text{NH}_4\text{NO}_3$ ).

Theoretically, there is an advantage in supplying nitrogen in the form of ammonium ions as  $\text{N}_2$  must be in the reduced form to be incorporated into macromolecules. Nitrate ions therefore, need to be reduced before incorporation. However, at high concentrations, ammonium ions can be toxic to plant cell cultures and uptake of ammonium ions from the medium causes acidification of the medium. In order to use ammonium ions as the sole nitrogen source, the medium needs to be buffered. High concentrations of ammonium ions can also cause culture problems by increasing the frequency of nitrification (the culture appears pale and 'glassy' and is usually unsuitable for further culture). Phosphorus is usually supplied as the phosphate ion of ammonium, sodium or potassium salts. High concentration of phosphate can lead to the precipitation of medium elements as insoluble phosphates.

#### *Microelements*

Elements that are required in trace amounts for plant growth and development and have many and diverse roles. Manganese, iodine, copper, cobalt, boron, molybdenum, iron and zinc usually comprise the microelements, although other elements such as nickel and aluminum are frequently found in some formulations.

#### *Iron source*

Iron is usually added as iron sulphate, although iron citrate can also be used.

Ethylene diamine tetra acetic acid (EDTA) is usually used in conjunction with the iron sulphate. The EDTA complexes with the iron allow the slow and continuous release of iron into the medium.

### **(2). Organic supplements**



### *Vitamins*

Plant cells and tissues in culture (like the natural plants) are capable of synthesizing vitamins but in suboptimal quantities, inadequate to support growth. Therefore the medium should be supplemented with vitamins to achieve good growth of cells. The vitamins added to the media include thiamine, riboflavin, niacin, pyridoxine, folic acid, pantothenic acid, biotin, ascorbic acid, myoinositol, Para amino benzoic acid and vitamin E.

### *Amino acids*

Although the cultured plant cells can synthesize amino acids to a certain extent, media supplemented with amino acids stimulate cell growth and help in establishment of cells lines. Further, organic nitrogen (in the form of amino acids such as L-glutamine, L-asparagine, L- arginine, L-cysteine) is more readily taken up than inorganic nitrogen by the plant cells.

### **(3).Carbon source**

As a carbon source, sucrose is cheap, easily available, readily assimilated and relatively stable and is therefore the most commonly used carbon source. Other carbohydrates such as glucose, Maltose and galactose can also be used in some circumstances.

### **Gelling agents**

Gelling agents media for plant cell culture in vitro can be used in either liquid or 'solid' forms, depending on the type of culture being grown. For any culture types that require the plant cells or tissues to be grown on the surface of the medium, it must be solidified (more correctly termed gelled). Agar , produced from seaweeds is the most common type of gelling agent, and is ideal for routine applications. However, because it is a natural product obtained from sea weed (red algae), the agar quality can vary from supplier to supplier and from batch to batch. In some cases the more purer form like phytogel can be used for more demanding applications.

**Table – some of the elements important for plant nutrition and their physiological function**

| Element                                                      | Function                                                                                                  |
|--------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"><li>• Nitrogen</li></ul>   | component of proteins, nucleic acids and some coenzymes required in greatest amount                       |
| <ul style="list-style-type: none"><li>• Potassium</li></ul>  | Regulates osmotic potential, principal inorganic cation                                                   |
| <ul style="list-style-type: none"><li>• Calcium</li></ul>    | cell wall synthesis, membrane function, cell signaling                                                    |
| <ul style="list-style-type: none"><li>• Magnesium</li></ul>  | component of chlorophyll                                                                                  |
| <ul style="list-style-type: none"><li>• Phosphorus</li></ul> | component of nucleic acids, energy transfer, component of Intermediates in respiration and photosynthesis |



- Sulphur component of some amino acids (methionine, cysteine ) and some cofactors
- Chlorine Required for photosynthesis
- Iron Electron transfer as a component of cytochromes
- Manganese Enzyme cofactor
- Cobalt Component of some vitamins
- Copper Enzyme cofactor , electron- transfer reactions
- Zinc Enzyme cofactor , chlorophyll biosynthesis
- Molybdenum Enzyme cofactor, component of nitrate reductase

However, other additions are made in order to manipulate the pattern of growth and development of the plant cell culture, those are -:

### **Plant growth regulators**

Specific media manipulations can be used to direct the development of plant cells in culture. Plant growth regulators are the critical media components in determining the developmental pathway of the plant cells. The plant growth regulators used most commonly are plant hormones or their synthetic analogues.

Classes of plant growth regulators

There are four main classes of plant growth regulators used in plant cell culture namely:

- (1) Auxins: (2) cytokinins: (3) gibberellins: (4) abscisic acid

#### ***Auxin***

- o Auxins promote both cell division and cell growth
- o The most important naturally occurring auxin is IAA ( indole-3 acetic acid) ,
- o The problems associated with the use of IAA that it is unstable to both heat and light.
- o 2,4-Dichlorophenoxy acetic acid (2,4-D) is the most commonly used auxin and is extremely effective in most circumstances .

Table ---- commonly used auxins, their abbreviation and chemical name

| Abbreviation /name | chemical name                       |
|--------------------|-------------------------------------|
| 2,4-D              | 2,4,-dichlorophenoxyacetic acid     |
| 2,4,5-T            | 2,4,5,- trichlorophenoxyacetic acid |
| IAA                | indole –e acetic acid               |
| IBA                | indole –e butyric acid              |

**Cytokinins**

- Cytokinins promote cell division.
- Naturally occurring cytokinins are purine derivatives
- These are zeatin and zip (2 isopentyl adenine) naturally occurring but zeatin unstable
- The synthetic analogues, kinetin and BAP (benzylaminopurine) are used frequently
- Non purine-based chemicals, such as substituted phenylureas, are also used as cytokinins

Table ----- commonly used cytokinins, their abbreviation and chemical name

| Abbreviation /name                                                                                            | Chemical name                                   |
|---------------------------------------------------------------------------------------------------------------|-------------------------------------------------|
| <i>BAP a</i>                                                                                                  | 6- benzylaminopurine                            |
| <i>Zip (PA) b</i>                                                                                             | (N6-(2-isopentyl) adenine)                      |
| <i>Kinetin a</i>                                                                                              | 6-furfurylaminopurine                           |
| <i>Zeatin b</i>                                                                                               | 4-hydroxy -e methyl -trans-2 butenylaminopurine |
| <ul style="list-style-type: none"> <li>• a: Synthetic analogues, b: Naturally occurring cytokinins</li> </ul> |                                                 |

**Gibberellins**

- Isolated from a fungus *Gibrella fujicurie*
- Involved in regulating cell elongation and are agronomically important in determining plant height and fruit set.
- Numerous, naturally occurring structurally related compounds termed gibberellins
- GA3 being in the most use

**Abscisic acid**

- Absciscic acid (ABA) inhibits cell division.
- It is mostly used in PTC to promote distinct developmental pathways such as somatic embryogenesis

**Ethylene**

Ethylene is a gaseous hormone produced during plant tissue culture. It is naturally occurring, plant growth regulator associated with controlling fruit ripening. Present a problem for plant by inhibiting the growth and development of the culture.



The type of culture vessel and its means of closure affect the gaseous exchange between the culture vessel and the outside atmosphere and thus the levels of ethylene present in the culture. Ratio of plant tissue culture affect the response of plant tissues or cells in vitro.

### **Cell, Tissue and organ culture**

Culture are generally initiated from sterile pieces of a whole plant these pieces are termed “explants’ and may consist of pieces of organs, such as leaves or roots, or may be specific cell types, such as pollen or endosperm. Many features of the explants are known to affect the efficiency of culture initiation. Generally, younger, more rapidly growing tissue (or tissue at an early stage of development) is most effective. Two hormones (auxin and cytokinin) mostly affect plant growth in tissue culture.

- ✓ Low auxin and high cytokinin ratio—induce shoot formation
- ✓ High auxin and low cytokinin ratio --- induce (root formation)
- ✓ Intermediate ratio of both hormones induce (callus formation)

### **5.4. Types of Tissue Culture**

#### **1) Seed Culture:**

Seeds may be cultured in vitro to generate seedlings or plants. It is the best method for raising the sterile seedling. The seed culture is done to get the different kinds of explants from aseptically grown plants which help in better maintenance of aseptic tissue.

#### **2) Embryo Culture:**

Embryo culture is the sterile isolation and growth of an immature or mature embryo in vitro with the goal of obtaining a viable plant. In some plants seed dormancy may be due to chemical inhibitors or mechanical resistance, structures covering the embryo. Excision of embryos and culturing them in nutrient media help in developing viable seedlings.

#### **3) Meristem Culture:**

The apical meristem of shoots of angiosperms and gymnosperms can be cultured to get the disease free plants. Meristem tips, between 0.2-0.5 mm, most frequently produce virus-free plants and this method is referred to as meristem-tip culture.

This method is more successful in case of herbaceous plants than woody plants. In case of woody plants, the success is obtained when the explant is taken after the dormancy period is over. After the shoot tip proliferation, the rooting is done and then the rooted plantlet is potted.

#### **4) Bud Culture:**

Buds contain quiescent or active meristems in the leaf axils, which are capable of growing into a shoot. Single node culture, where each node of the stem is cut and allowed to grow on a nutrient media to



develop the shoot tip from the axil which ultimately develops into new plantlet. In axillary bud method, where the axillary buds are isolated from the leaf axils and develop into shoot tip under little high cytokinin concentration.

### **5) Callus Culture:**

Callus is basically more or less un-organised dedifferentiated mass of cells arising from any kind of explant under in vitro cultural conditions. The cells in callus are parenchymatous in nature, but may or may not be homogenous mass of cells. They are meristematic tissue, under special circumstances they may be again organised into shoot primordia or may develop into somatic embryos.

The callus tissue from different plant species may be different in structure and growth habit. The callus growth is also dependent on factors like the type of explant and the growth conditions. After callus induction it can be sub-cultured regularly with appropriate new medium for growth and maintenance.

### **6) Cell Suspension Culture:**

The growing of individual cells that have been obtained from any kind of explant tissue or callus referred to as cell suspension culture. These are initiated by transferring pieces of tissue explant/callus into liquid medium (without agar) and then placed them on a gyratory shaker to provide both aeration and dispersion of cells. Like callus culture, the cells are also sub-cultured into new medium.

Cell suspension cultures may be done in batch culture or continuous culture system. In the later system, the culture is continuously supplied with nutrients by the inflow of fresh medium with subsequent draining out of used medium but the culture volume is constant. This culture method is mainly used for the synthesis of specific metabolite or for biomass production.

### **7) Anther Culture:**

An important aspect of plant tissue culture is the haploid production by anther culture or pollen culture. The anthers bearing the uni-nucleate microspores are selected and allowed to grow in medium to produce callus from the pollen mass. Then the triggering of these androgenic calli is directed to produce the embryos and haploid plants are developed from these androgenic embryos. The anther culture can be done with the isolated anthers on solid medium where anther wall will break open and the androgenic calli will be formed from the pollen.

In pollen culture, microspores of uni-nucleate stage are collected in liquid media and can be grown in suspension culture. In suspension, the uni-nucleate pollens may give rise to calli mass or the globular mass from which the plants can be raised either through embryogenic or organogenic pathway.

### **8) Protoplast Culture:**

It is the culture of plant protoplasts i.e., culture of cells devoid of cell wall. Isolated protoplasts are



usually cultured in either liquid or semisolid agar media plates. Protoplasts are isolated from soft parenchymatous tissue by enzymatic method and then viable protoplasts are purified and cultured.

The protoplast culture is aimed mainly to develop genetically transformed plant where the transgenic is put successfully within the plant protoplast and the transgenic plant is regenerated from that transformed protoplast. Another aspect of protoplast culture is somatic hybridization of two plant species through protoplast fusion.

### ***Plant regeneration***

All culture types lead to plant regeneration in the presence of suitable growth regulator.

Regeneration refers to the development of organized structures like roots, Shoots, flower buds, somatic Embryos (SEs), etc.

## **6. BIOTECHNOLOGY AND BIOSAFETY**

Safety of Recombinant DNA Technology:

- Long-term effects of transgenic manipulations are unknown
- Unforeseen problems arise from every new technology and procedure
- Natural genetic transfer could deliver genes from transgenic plants and animals into other organisms
- Transgenic organisms could trigger allergies or cause harmless organisms to become pathogenic
- In some areas, application of genetic engineering is encouraging stress.
- Many studies have not shown any risks to human health or environment
- But, there is potential effect of health from GM food crops.
- **Ethical Issues**
- Two issues that are opposing and supporting the GE:
  - Routine screenings?
  - Who should pay?
  - Genetic privacy rights?
  - Profits from genetically altered organisms?
  - Required genetic screening?
  - Forced correction of “genetic abnormalities”?
  - .....

