

Block

4

BIOTECHNOLOGY - TECHNIQUES AND APPLICATIONS

UNIT 13

Molecular Techniques	87
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UNIT 14

Recombinant DNA Technology	107
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UNIT 15

Application of Plant Biotechnology	147
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BLOCK 4 :BIOTECHNOLOGY - TECHNIQUES AND APPLICATIONS

Block 4 focuses on biotechnology techniques and applications. It is made up of three units: 13, 14, and 15. Biotechnology is a broad discipline that employs a variety of techniques and technologies to produce medicines, diagnostics, and therapeutics such as monoclonal antibodies, stem cells, and gene therapy, as well as for bioremediation and agricultural biotechnology. It is a biological and engineering combination that uses living organisms, cells, or components to create useful products for human welfare. As a result, you will comprehend the potential applications of biotechnology in this section.

Blotting (Northern, Southern, and Western), DNA fingerprinting, molecular markers, DNA sequencing, PCR, and ELISA are all discussed in Unit 13.

Recombinant DNA technology is discussed in Unit 14. (RDT). It describes the fundamental tools of RDT, such as restriction enzymes, cloning vectors, and gene cloning. The concept of building genomic and c DNA libraries, as well as colony hybridization, is also discussed. Plant genetic engineering methods are also described. Agrobacterium-mediated genetic transformation, direct gene transfer methods (electroporation, microinjection, microprojectile bombardment), and crisper technology are among them.

Unit 15, the final unit in this block, provides an overview of the major applications of Plant Biotechnology. A detailed account of the role of plant biotechnology in agriculture, horticulture, and the development of herbicide and pest-resistant crops is provided. This unit also includes a brief description of genetically modified crops such as golden rice, Bt cotton, and Flavr Savr tomato.

Objectives

After studying this block, you will be able to :

- ❖ describe the molecular techniques such as Blotting (Northern, Southern and Western), DNA fingerprinting, molecular markers, DNA sequencing, PCR and ELISA,
- ❖ explain the Recombinant DNA technology;
- ❖ describe the concept of gene cloning and its various steps;
- ❖ discuss genomic and c DNA libraries, colony hybridization;
- ❖ enumerate the methods and application of plant genetic engineering;
- ❖ enlist the applications of biotechnology and;
- ❖ explain the role of plant biotechnology in development of crops with high nutritional value, better tolerance capacity and long shelf life.



UNIT 13

MOLECULAR TECHNIQUES |

Structure

13.1	Introduction Objectives	13.6	Hybridoma and Monoclonal Antibodies
13.2	Northern, Southern, and Western Blotting	13.7	ELISA and Immunodetection
13.3	DNA Fingerprinting	13.8	Summary
13.4	Molecular DNA Markers RAPD, RFLP, SNPs	13.9	Terminal Questions
13.5	DNA sequencing, PCR, and Reverse Transcriptase-PCR	13.10	Answers

13.1 INTRODUCTION

Earlier units have introduced you to the basics of biotechnology, emphasizing various plant tissue culture methods. You have also read about applications of plant tissue culture, such as micropropagation, androgenesis, gynogenesis, embryo culture and germplasm conservation. The present unit provides information about molecular biology and plant biotechnology techniques such as Southern, Northern, and Western blotting used to identify DNA, RNA and proteins. In addition, DNA amplification techniques, DNA markers, and hybridoma technology have been dealt with in detail.

Objectives

After studying this unit, you would be able to:

- ❖ describe the techniques of Southern, Northern, and Western blotting;
- ❖ know about the ELISA technique;
- ❖ describe different types of DNA markers;
- ❖ appreciate the details of the DNA fingerprinting technique; and
- ❖ describe hybridoma technology.

13.2 NORTHERN, SOUTHERN, AND WESTERN BLOTTING

The mixture of DNA, RNA and protein fragments can be separated by gel electrophoresis. Further, the separated gel bands can be stained with specific dyes and studied. The identity of these bands can be confirmed by using a known molecular probe that illustrates/indicates the similarity to one or more bands. The separated bands can be hybridized using probes within the gel. Hybridization using probes is carried out after transferring the bands to the nitrocellulose membrane. Blotting refers to transferring bands onto a membrane and then hybridizing them with a specific probe. In a sample, Southern blotting detects specific DNA sequences, Northern blotting detects specific RNA sequences, and Western blotting detects specific protein sequences. As a result, the main distinction between the three blotting techniques is the macromolecule type that they detect.

Southern Blotting

The technique was named after a biologist **Edward M. Southern** who developed this technique in 1975. Southern blot hybridization refers to detecting specific DNA fragments that have been separated by gel electrophoresis (Fig. 1). The DNA segments are first digested with a restriction enzyme, and the digested sample is subjected to electrophoresis. After that, the separated DNA fragments in the gel are denatured in the buffer with the help of an alkali solution. The single-stranded fragments from the gel get transferred to the surface of the nitrocellulose membrane. The bands firmly adhere to the paper based on the affinity of DNA for nitrocellulose paper.

The gel is laid on a buffer-saturated filter paper placed on a solid support. The two edges of the gel are immersed in the buffer. A nitrocellulose membrane is placed on top of the gel, which is then topped with a stack of papers. A weight of about 1 kg is placed on the top of the stack. The buffer solution is drawn by the filter paper wick, then through the gel to the nitrocellulose membrane, and finally through the layer of paper. As the buffer passes through the gel, it carries single-stranded DNA, which binds to the nitrocellulose membrane. The sheet is exposed to the probe under conditions favoring hybridization. The set is left up for several hours or overnight (Fig. 13.1). The paper towels are discarded, and the nitrocellulose membrane has single-stranded DNA bands that get blotted onto it. The membrane is baked at 80°C for 2 to 3 h to fix the DNA permanently on the membrane. The membrane with DNA bands can hybridize with labelled DNA or RNA probe (^{32}P) (Fig. 13.1). A probe is a labelled DNA sequence that hybridizes with one or more fragments of the digested DNA sample after they were separated by gel electrophoresis, thus revealing a unique blotting pattern characteristic of a specific genotype at a specific locus. The nitrocellulose sheet containing the bound single-stranded DNA fragments is placed in a sealed box along with the buffer containing labelled DNA probe specific for the target DNA sequence. After the hybridization, the sheet is washed thoroughly to remove unhybridized probes (unbound DNA). The sheet is viewed using autoradiography or ultraviolet light depending on the labels used (radioactive or fluorescent). Autoradiographs are obtained by exposing the X-ray film to the hybridized membrane.

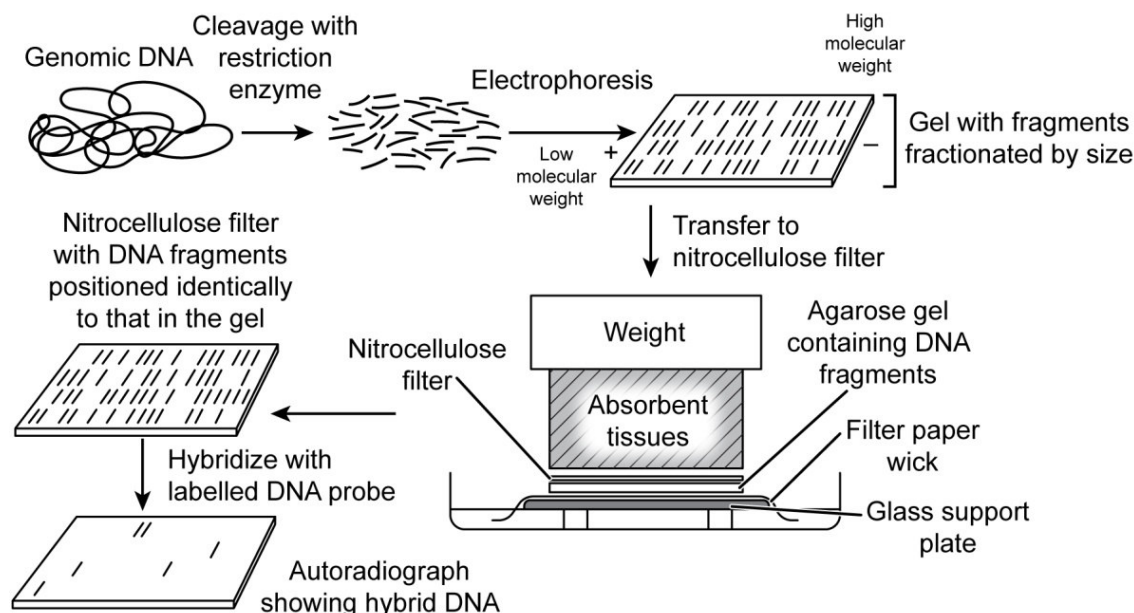


Fig. 13.1 Diagram showing set up in the Southern blotting.

Southern blotting is used to detect specific DNA fragments of varying sizes. The technique is used in DNA profiling or DNA fingerprinting. The techniques are helpful in the discovery of genes located on a particular DNA sequence or gene within the entire genome. It is used as a confirmatory test to ensure that a particular DNA segment has been successfully incorporated into the genome of another organism and also helps in assessing the evolutionary relationship among organisms. The Southern blotting can also be used to determine the genetic alterations causing a mutation due to structural change in the gene controlling the trait of interest.

Northern blotting

Northern blotting is a method to detect specific RNA molecules from a mixture of RNA. The technique is used to analyze RNA samples from a particular tissue or cell type to measure the RNA expression of a particular gene. The technique was developed by **James Alwine, David Kemp, and George Stark** at Stanford University in 1977. The mRNA bands are transferred from the gel to the nitrocellulose membrane or diazotized amino benzyloxy methyl paper. The mRNA bands blotted on the nitrocellulose membrane can be hybridized with labelled DNA or RNA probes. The RNA samples are then loaded onto an agarose gel and an electric current is passed through the gel, causing the RNA in each sample to reach the bottom. The distance moved depends on the size of the RNA fragment. The RNA molecules are separated according to their sizes using gel electrophoresis. Small RNAs move faster while larger RNAs migrate slowly. The RNA within the sample is denatured to get single strands, formaldehyde is used as a denaturant.

After separation, the RNA is transferred from the gel onto a blotting membrane. Each RNA molecule retains its position relative to all the other molecules, a probe, which is a small piece of DNA or RNA with a complementary sequence to a specific RNA sequence in the sample, is used to treat the nitrocellulose membrane. It allows the probe to hybridize, or bind, to a specific RNA fragment on the membrane (Fig.13.2). Next, the probe is radioactively labelled or using a fluorescent dye (Fig. 13.2). The filter is then

placed on a film for autoradiography, and finally, the film will be developed. The probes can be seen on the autoradiograph if paired with a specific region of RNA on the filter. The gene gets expressed shows a band that will be visible in the column containing the tissue or cells only.

The technique of Northern blotting is helpful for the study of gene expression. The position of bands on the blot provides size RNA. The Northern blot analysis of RNA samples from different cells or tissues enables researchers to determine the expression of the gene along with the relative levels of expression in cells. Hence the technique helps to identify where and when a specific section of DNA of a known sequence gets transcribed. This technique also detects gene variants and identifies closely related species.

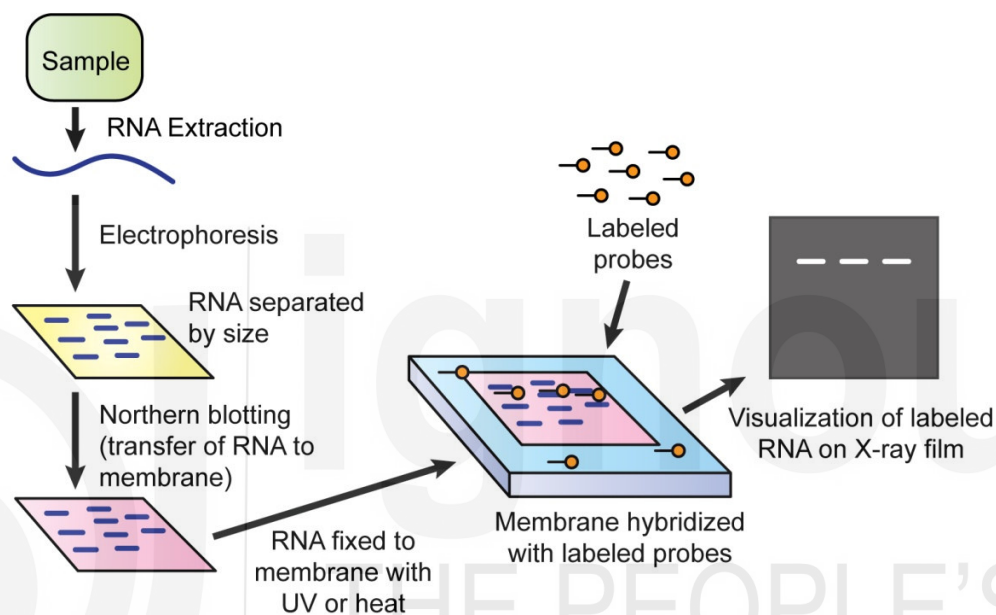


Fig. 13.2: Diagram showing set up in the Northern blotting.

Western blotting

Towbin and Julian Gordon (Friedrich Miescher Institute) and **Theophil Staehelin** (at Hoffmann-La Roche) introduced western blotting in 1979. It is a rapid and sensitive assay for detecting and characterizing proteins. It is based on the ability of a specific antibody to bind a given protein or peptide in a mixture of is used to detect its antigen-specific. It is based on the immunochromatography principle, in which proteins are separated into polyacrylamide gel based on their molecular weight. The proteins are separated using polyacrylamide gel electrophoresis and then transferred to the nitrocellulose membrane to which they bind. The proteins are detected using a specific primary antibody, and secondary enzyme labelled antibody and substrate. The membrane is used for probing with a specific labelled antibody. The nitrocellulose membrane is first incubated with a primary antibody to the protein of interest. The antibody binds to the protein, and an antibody-protein complex is formed. Next, the nitrocellulose paper is incubated with a secondary antibody, and it specifically binds to the primary antibody, finally, a secondary antibody tagged with an enzyme helps detect and visualize the bound antibody. For enzymatic reaction, the substrate is added, and the reaction mixture is allowed to incubate. The colored bands can be noted at sites where the primary antibody-secondary antibody-enzyme complex is formed (Fig.13.3).

If the radiolabelled antibody is used, the signal is detected with the help of autoradiography. The target protein is marked using a primary and secondary antibody for visualization. The unbound antibody is washed off, leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present. The technique detects proteins of a particular specificity and determines the size and amount of protein in a given sample. For example, the western blotting technique is the confirmatory test for HIV that detects anti-HIV antibodies in the patient's serum and detects defective proteins.

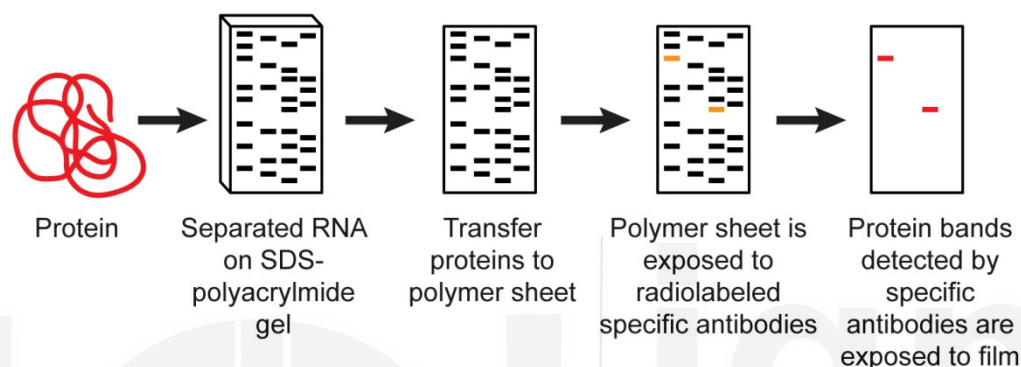


Fig.13.3: Various steps involved in Western blot.

SAQ 1

Match the items in column A with those in Column B.

Column A	Column B
a) Detection of DNA fragments	1. Western blots
b) Detection of RNA molecules	2. Southern blots
c) Towbin & Julian Gordon	3. Northern blots
d) Helps in the identification of specific bands	4. Molecular probe

13.3 DNA FINGERPRINTING

The technique of DNA fingerprinting was developed in 1984 by British geneticist **Alec Jeffreys**, who discovered that certain sequences of highly variable DNA (known as minisatellites), do not contribute to the functions of genes, are repeated within genes. In most cases, minisatellites are defined as the repeated in the tandem repetition of a short (6 to 100 bp) motif that spans 0.5 kb to several kilobases in length. They show polymorphism, which results from variations in the number of repeats. They can cross-hybridize with tens of other similar loci throughout the genome. Hence, they are used in DNA fingerprinting to identify an individual. DNA fingerprinting is used to isolate and identify variable elements within DNA sequences.

DNA is extracted from various places, including skin, hair, or blood cells (WBCs). The DNA is purified and cut at specific points along the strand with restriction enzymes. Specific restriction endonucleases digest the DNA. The fragments produced of varying lengths are placed on the gel and then subjected to electrophoresis. The DNA fragments are separated based on length (Fig.13.4). The shorter the fragment, the more quickly it moves toward the positive pole (anode). It is based on restriction fragment length polymorphism (RFLP). The shorted double-stranded DNA fragments are then subjected to a blotting technique in which they were split into single strands and transferred to a nylon membrane. The fragments are exposed to DNA probes. Fragments of DNA are made radioactive and made to bind to minisatellites. The DNA strands are subjected to autoradiography. A piece of X-ray film was then exposed to the fragments, and a dark mark was produced at any point where a radioactive probe had become attached.

The location of the DNA fragments containing specific DNA sequences in the gel is determined using a labelled probe having complementary sequences. The DNA fragments that bind these probes show variable lengths from one person to the other because of a variable number of short tandem repeats (STR) in the genome. Generally, 13 STR, which is supposed to be polymorphic, are analyzed. The amount of polymorphism generated is used to identify the individual or specimens.

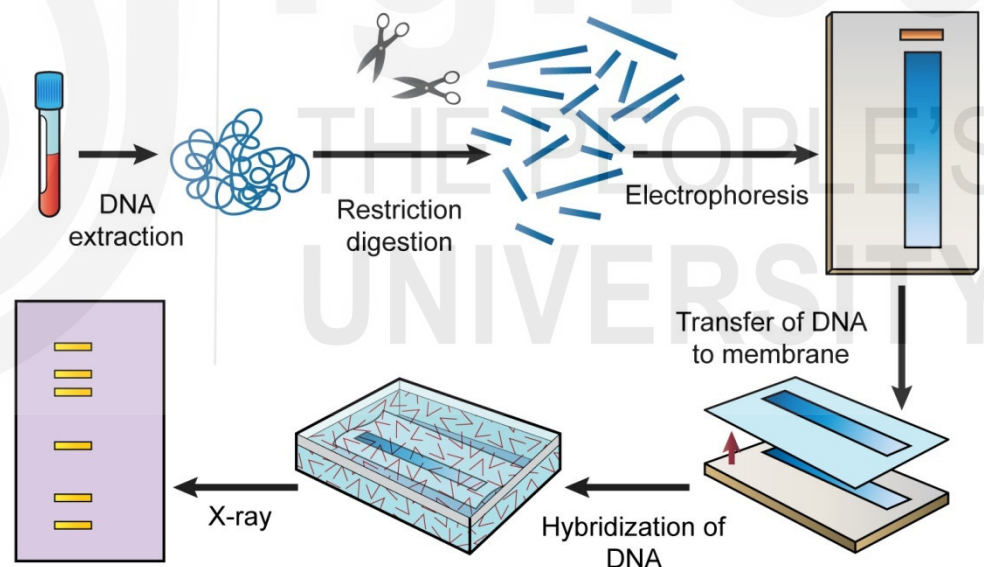


Fig. 13.4: Various steps involved in DNA fingerprinting technique.

13.4 MOLECULAR DNA MARKERS (RAPD, RFLP, SNPs)

Molecular markers are DNA sequences that are used in molecular biology and biotechnology to distinguish one particular sequence of DNA from another in a pool of unidentified DNA. The DNA sequences provide many visual markers that can be used to map their locations in the chromosome of various species. The molecular markers that are extensively used in the preparation of genome maps include RFLP, RAPD, and SNPs.

Restriction Fragment Length Polymorphism (RFLPs)

The technique detects alterations in specific regions in the chromosome of different strains/species. The technique was developed by **Botstein and coworkers in 1980**. It is noted whether or not a recognition site for the same restriction endonuclease exists in the same region of a chromosome from a different individual of a species. The variation appears in the form of bands. An RFLP map depicts the location of different DNA sequences used as probes (Fig.13.5). The enzyme treatment produces fragments of different lengths for the same chromosome region of different individuals. Gel electrophoresis detects the differences, generally followed by hybridization using a labelled probe specific for the chromosome region. The DNA sequences used as a probe have features like a strong hybridization signal, produce small bands, and show polymorphism in different species strains.

DNA is isolated separately from the parent strains, the progeny, i.e. F_1 and F_2 plants, are used to determine RFLPs with various probes showing hybridization. DNA is digested with a restriction enzyme, and the product obtained is subjected to electrophoresis and Southern hybridization using different probes. Short, single- or low-copy genomic DNA or cDNA clones are typically used as RFLP probes.

The probes are used to screen different or related species, few species show polymorphism for several probes. Instead, probes reveal a single band that shows the difference in mobility in the two parental strains. One probe can detect a slow-moving band, while the other probe can detect a fast-moving band. The distance between the two markers can be measured to prepare a map. This map depicts the relative positions of the chromosome.

We can detect the loci affecting the trait by mapping using RFLP markers. These markers are specific to a single clone/restriction enzyme combination. Most RFLP markers are co-dominant (both alleles in the heterozygous sample will be detected) and highly locus-specific. The RFLP probes are frequently used in genome mapping and in variation analysis (genotyping, forensics, paternity tests, genetic disease diagnostics, and many more).

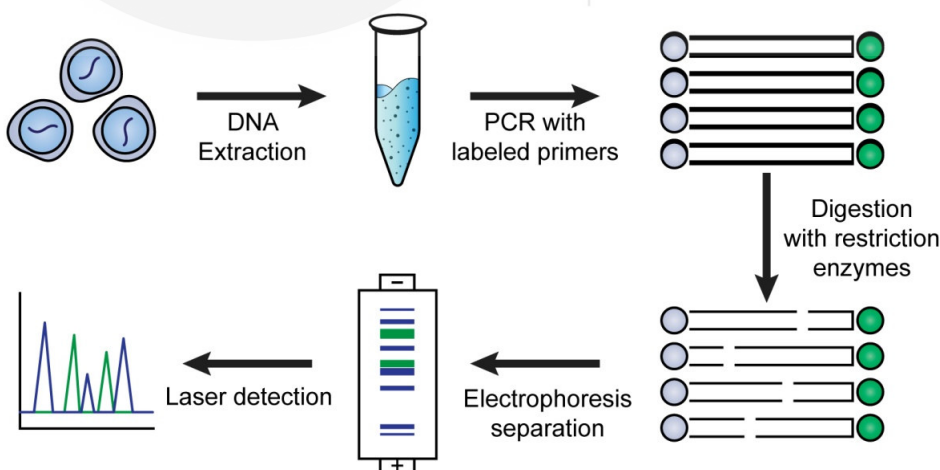


Fig. 13.5: Steps involved in RFLP.

Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) is a PCR based technique for identifying genetic variation. RAPDs are generated using random sequences

generally 10 oligonucleotides long. These markers are DNA fragments generated from PCR amplification of random segments of genomic DNA with a single primer of the arbitrary nucleotide sequence. RAPD markers are used as primers for PCR amplification of genomic DNAs. They act as dominant markers and are recognized by the primer used for amplification. The RAPD technology was developed by **Williams and his coworkers** in 1990. They used short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR.

RAPD involves using a single arbitrary primer in a PCR reaction, resulting in the amplification of many discrete DNA products. The target DNA sequence is amplified with the help of primers, DNA polymerase, di-deoxynucleotide tri-phosphates, magnesium and reaction buffer. The DNA is made single-stranded by raising the temperature to 94°C (denaturation) followed by annealing primer to their target sequences on the template DNA at a lower temperature (about 40 to 65°C). The temperature at which the thermostable Taq DNA polymerase activity is optimal (i.e., usually 72°C) is chosen. The polymerase extends the DNA-primer hybrids' 3' ends toward the other primer binding site. On both DNA strands, this occurs at both primer-annealing sites. The fragment that is being replicated is the target fragment. Repetition of PCR cycles (in three to four steps about 40 to 50 times) results in the target amplification between the 5' ends of the two primer binding sites.

A single primer binds to the genomic DNA at two different sites on opposite strands of the DNA template (Fig.13.6). If these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through amplification. The polymorphisms between individuals result from sequence differences in one or both of the primer binding sites. The technique detects nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single primer of the nucleotide sequence. Amplification products are separated by gel electrophoresis and visualized by ethidium bromide staining under gel documentation system.

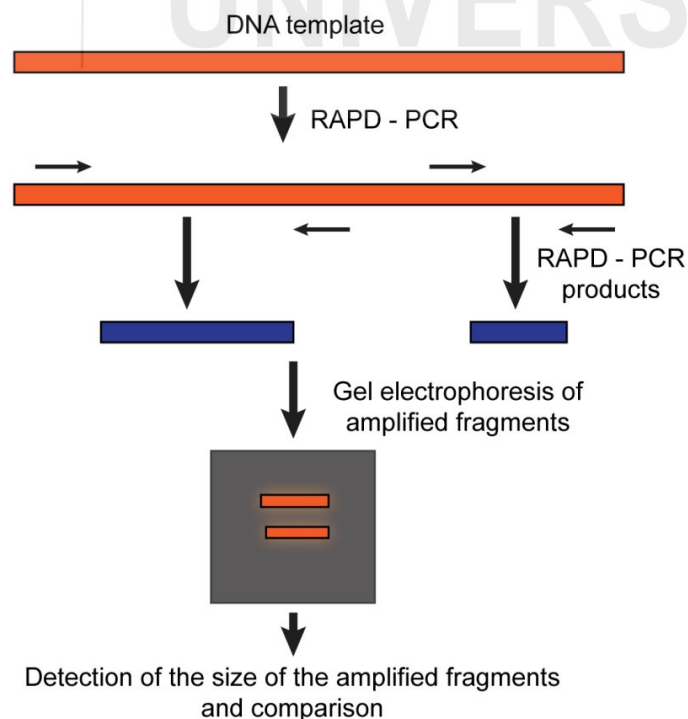


Fig. 13.6: Various steps in the RAPD technique.

Welsh and McClelland, 1990 developed a method that known as random amplified polymorphic DNA (RAPD) (RAPD). It is a PCR-based DNA fingerprinting technique using primers whose nucleotide sequence is arbitrarily selected primer at low stringency for first and second strand cDNA synthesis. Finally, cDNA sequences amplify and electrophoresis are carried out under different conditions than RAPD.

Single Nucleotide Polymorphisms (SNPs)

SNPs are the genetic markers that represent sites where DNA sequences differ by a single base. They represent a DNA sequence (a single nucleotide), abundant in populations, but having a low mutation rate. SNPs give a measure of the frequency of occurrence of polymorphism at a particular locus, i.e., provide an idea about true polymorphism. RFLP or AFLP can detect the presence of SNPs conducted on PCR products. Methods for SNP detection require gel-based assays. If SNPs is present at the 3' end of the PCR primer binding site, it can be detected by the failure of amplification due to a mismatch between the primer sequence and binding site on the template. The detection of SNP at the internal position of an amplicon need PCR amplification followed by a non-gel-based assay (Fig. 13.7). The non-gel-based SNPs detection assays are based on detecting a mismatch between the PCR product and oligonucleotides at the internal site as a probe. It helps in distinguishing the wild and mutant alleles.

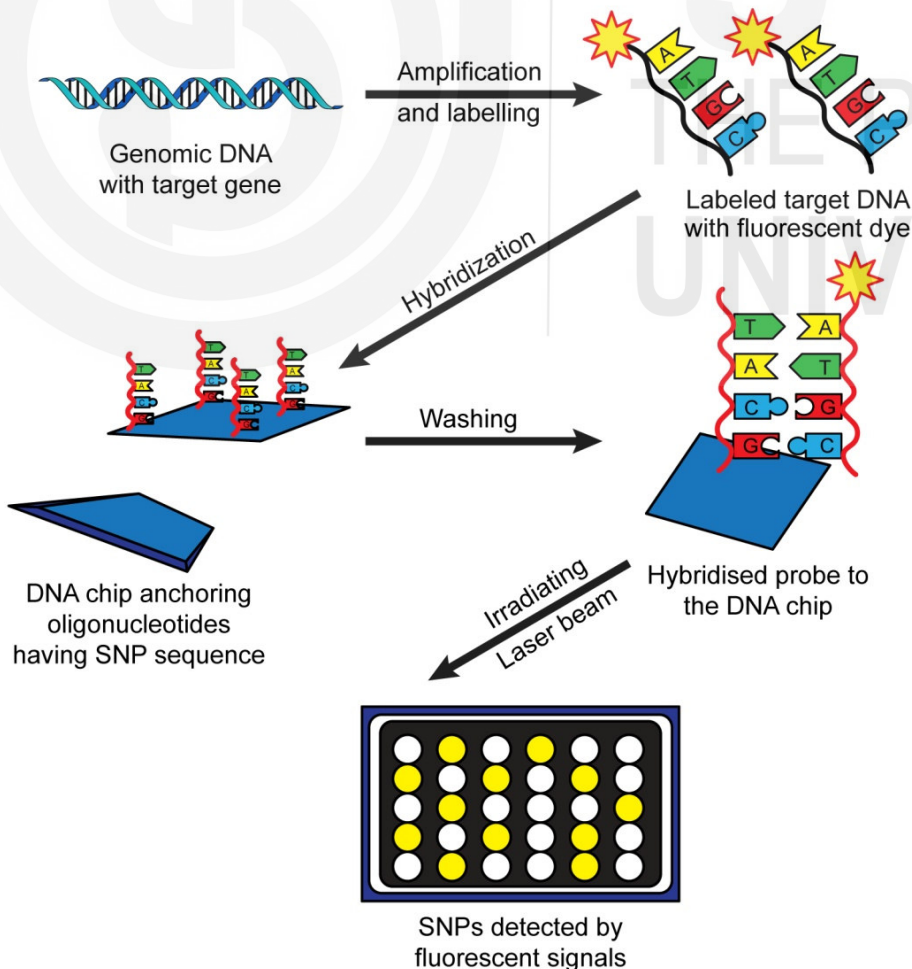


Fig. 13.7: Various steps involved in the formation of SNPs.

The polymorphism has been found in the human genome with an estimated average frequency of one SNP per kilobase pair. Their inheritance's stability and relative fidelity are higher than the other markers. Therefore, methods for SNP detection and the construction of the SNP genetic map have been employed. SNPs have been detected in many crops like barley, wheat, and maize. Some assays like TaqMan and Molecular beacon used to detect the SNP in various crops.

- a) **TaqMan assay-** An oligonucleotides probe is labelled with a fluorescent reporter molecule at the 5' end and a quencher at the 3' end. The probe is degraded after hybridization due to the *exonuclease* activity of the Taq polymerase enzyme during the extension phase of PCR. A high fluorescence signal is generated. Due to the SNP, the probe mismatches the template leading to a failure in duplex formation, no such degradation at the 5' end of the probe is possible, and no rise in the fluorescence signal will be detected.
- b) **Use of Molecular beacon-** Molecular beacon is a short oligonucleotide (25 - 40 nucleotides) that forms a hairpin structure with a loop and stem. The loop is designed to hybridize specifically to a 15 - 25 nucleotide portion of the target DNA sequence, while 5 - 6 nucleotide long stem sequences are complementary to each other. A fluorescent reporter molecule is attached at the 5'- end of the molecular beacon while a non-fluorescent quencher is attached at 3'- end. In real-time PCR, during annealing, binding of molecular beacon to its target leads to increased fluorescence signal, due to separation of reporter and quencher. If SNP is available, the probe fails to form a duplex with the template DNA, and generates a hairpin-like structure due to the self-annealing of its two ends, thereby quenching the reporter. A sensing device can detect the very less or no signals fluorescence signal.
- b) **Oligonucleotide Ligation Assay (OLA) -** Two independent probes are used for hybridization with PCR product. When probes match the product, the two probes anneal with the PCR product and undergo ligation resulting in oligonucleotides which is biotinylated at 5' end, and labelled at 3' end. The ligation product is captured on the streptavidin-coated matrix, and a signal is detected by autoradiography. When SNP is present, there is a mismatch. The labelled and biotinylated oligonucleotides are unable to ligate. When captured by streptavidin, they carry no signal.
- c) **DNA microarray or chip-** An array of oligonucleotides of known overlapping sequence differing at solitary specific nucleotides can be used to detect SNPs. Four oligonucleotides that differ at SNP position are hybridized with biotinylated PCR products. The perfect match will allow binding while the mismatched products will be washed away.
- d) **Dynamic allele-specific hybridization –** The technique is based on differences in temperature between duplexes due to perfectly match and mismatch between PCR product and oligonucleotide. The PCR product is immobilized on a solid support and denatured to give a single-stranded DNA which is hybridized to an oligonucleotide probe containing

an SNP site. The duplex formed is detected using a fluorescent dye specific for double-stranded DNA. An increase in temperature denatures the duplex leading to a reduction in fluorescence. A sudden decrease in fluorescence indicates a mismatch, thus identifying the SNP.

- e) **Minisequencing** - SNPs can also be detected using the Sanger dideoxynucleotide method. The oligonucleotide primer has a sequence of one or more base pairs upstream of the SNP site.

SAQ 2

- a) State whether these statements are 'True' or 'False'.
- i) Minisatellites are non-variable sequence elements present in the genome.
 - ii) DNA fingerprinting technique helps in the identification of variable elements within DNA sequences.
 - iii) RFLP technique depicts the location of different DNA sequences.
 - iv) RAPD involves using many primers in a PCR reaction resulting in the amplification of many discrete DNA products.
 - v) Single Nucleotide Polymorphisms (SNPs) measure the frequency of occurrence of polymorphism at a particular locus.
- b) Define SNPs. Mention their significance.

13.5 DNA SEQUENCING, PCR AND REVERSE TRANSCRIPTASE-PCR (RT-PCR)

DNA sequencing

Frederick Sanger & Colleagues developed DNA sequencing technique in 1980. First, DNA template molecules obtained from PCR or cloned DNA fragments are taken. Then, the template DNA is mixed with the primer that is complementary to the 3' end of one strand of the region to be sequenced. Taq polymerase, all four deoxyribonucleoside triphosphate precursors (dNTPs), and a low concentration of modified precursors called dideoxyribonucleoside triphosphates (ddNTPs) are all present in the reaction mixture. Each ddNTP is modified by adding colored fluorescent dye to its 3' end.

The reaction begins by heating the mixture to a temperature so that two strands denature. The reaction is cooled, and the primer can hybridize with the template DNA. One primer is present so that only one DNA strand can hybridize with the primer. The Taq polymerase adds dNTPs to the end of the primer that is complementary to the template molecule, synthesizing a new complementary strand of DNA. The polymerase inserts a ddNTP instead of dNTP. Dideoxyribonucleotides lack a hydroxyl group at both 2' and 3' positions. When the nucleotide gets incorporated into the end of the growing chain, the lack of 3' OH makes it impossible for the polymerase to add another nucleotide leading to chain termination. The incorporation of ddNTP ceases the growth of the chain.

After the extension of the chain is complete, the temperature is raised to denature the new double-stranded DNA molecules. The cycle of hybridization, synthesis and denaturation is repeated many times, generating a large population of daughter DNA strands with ddNTP molecules incorporated at every position. When the reaction is complete, the products are separated by gel electrophoresis. High-resolution gel electrophoresis separates fragments that differ by one nucleotide in length. The bands in the gel contain one nucleotide longer than those in the previous band. Each ddNTP is labelled with the fluorescent dye, and the color of the band is read by an automated laser detector. It reveals the identity of the terminal nucleotide on each daughter molecule. The order of the colors in the gel corresponds to the base sequence of the template molecule.

Massive parallel sequencing (2D generation sequencing) has been developed in the last few years. The fragments are prepared directly from the genome, and each genome fragment is amplified to form a considerable population. This technique is based on polymerase dependent DNA synthesis. In this method, premature termination of strands or separation of strands by electrophoresis is not required. The nucleotides are identified as they get incorporated by polymerase in real-time. Automated sequencers use giant DNA molecules immobilized on the surface and incubated with DNA polymerase in the presence of dNTPs (Fig.13.8). The nucleotides that get incorporated in the complementary strands are identified using various procedures, including DNA sequencing technologies to study individual genomes from a species. Recently the third-generation sequencers have also been used. These sequencers act on single DNA molecules (eliminates the requirement of DNA amplification), generate long reads (about 1000 nucleotides), operate at low cost and a much faster rate. Nucleotide identification in these sequencers occurs based on ionic properties or sequencing by the synthesis in which a laser-based detection system identifies fluorescently labelled nucleotides. Various software systems are used to analyze the nucleotide sequence of the DNA fragment.

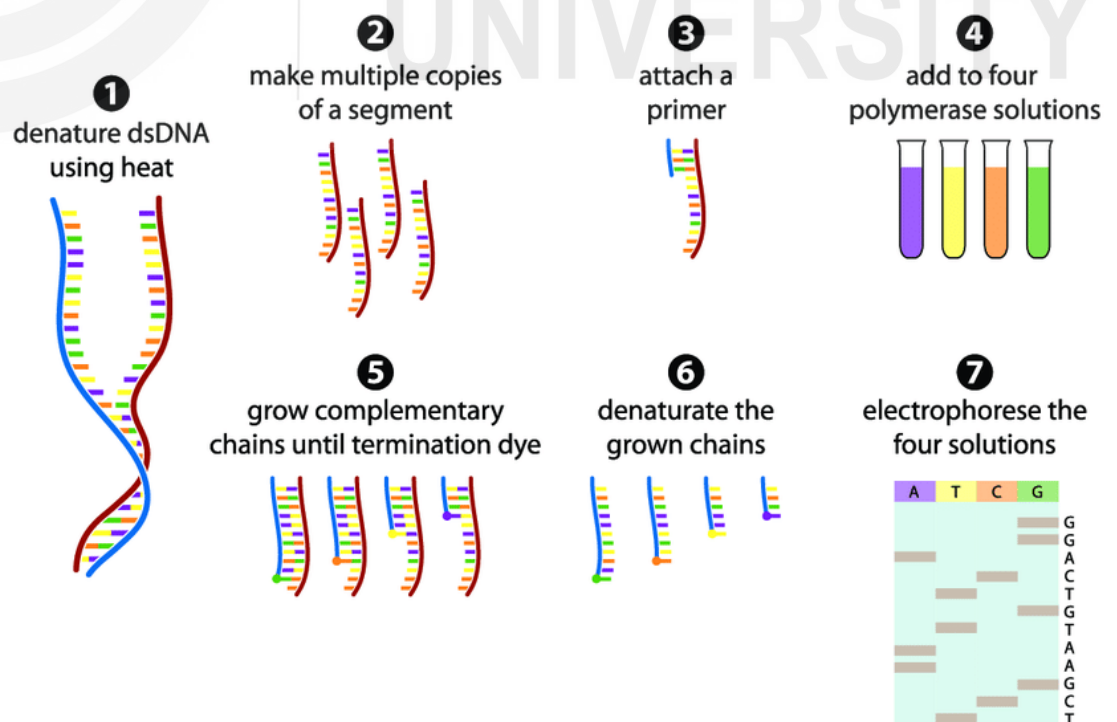


Fig.13.8: Diagrammatic representation of various steps involved in DNA sequencing via Sanger method.

PCR and Reverse Transcriptase-PCR

In 1983, **Kary Mullis** developed a new technique of amplifying DNA fragments that is known as Polymerase Chain Reaction (PCR). In this reaction unlimited copies of a DNA segment can be quickly produced using this technique. DNA fragments having a single DNA molecule can be amplified many times. This technique proves beneficial if the DNA sample is small. The technique is based on the principle that two strands of DNA comprise the double helix complement each other can be separated at high temperatures and reannealed at low temperatures, and single-stranded fragments are referred to as templates. RNA templates can be converted to complementary DNA using reverse transcriptase.

The Taq polymerase enzyme, four building blocks of DNA- the nucleotides (ATP, CTP, GTP, TTP), forward and reverse primer sequence, and DNA template are required for carrying out the PCR (Fig. 13.9). A heat-stable Taq polymerase isolated from bacterium *Thermus aquaticus* that lives in hot springs at a temperature above 90°C. The PCR technique involves repeated cycles of DNA synthesis or replication. Each cycle comprises three basic steps- separating the strands, annealing the primer to the template, and synthesizing new strands. The product of the first cycle becomes the template for the next cycle.

The oligonucleotides act as primers to which the nucleotides are added during the replication steps. The mixture is heated to 90°C, which causes the DNA strands to separate into two strands. The mixture is then cooled to about 60°C. It allows the primers to hybridize to the strands of the target DNA. The temperature is then increased to 72°C to allow the polymerase to add nucleotides at the 3' end of the primers. The polymerase extends the primer. The DNA gets copied, forming new complementary strands. The temperature is raised once more to separate the newly formed complementary strands from the original DNA strands; the sample is then cooled to allow the synthetic primers in the mixture to bind to the target DNA once more. The cycle is repeated repeatedly, each time doubling the specific region of DNA flanked by the bound primers. Hundreds of copies of a specific region of DNA can be generated in a few hours using a thermal cycler that automatically changes the temperature of the reaction mixture to allow each step in the cycle to take place.

PCR technique can generate many copies of the specific DNA fragment before cloning the fragment. Therefore, DNA can be amplified without cloning; hence the procedure is less time-consuming. In about 25 rounds of PCR, more than one million copies of DNA are produced. If the target sequence is known, then the nucleotide sequence of the complementary primers can be specified. PCR technique has been used in criminal investigations to generate the quantities of DNA from the dried blood sample from the cloth of the suspect or even a hair follicle. A highly polymorphic genomic region is selected for amplification because every individual possesses no similar-sized DNA fragments. PCR can also be used to study the DNA fragments from preserved fossil remains. The PCR technique finds its application in forensic science, medical diagnostics, food analysis, and research studies.

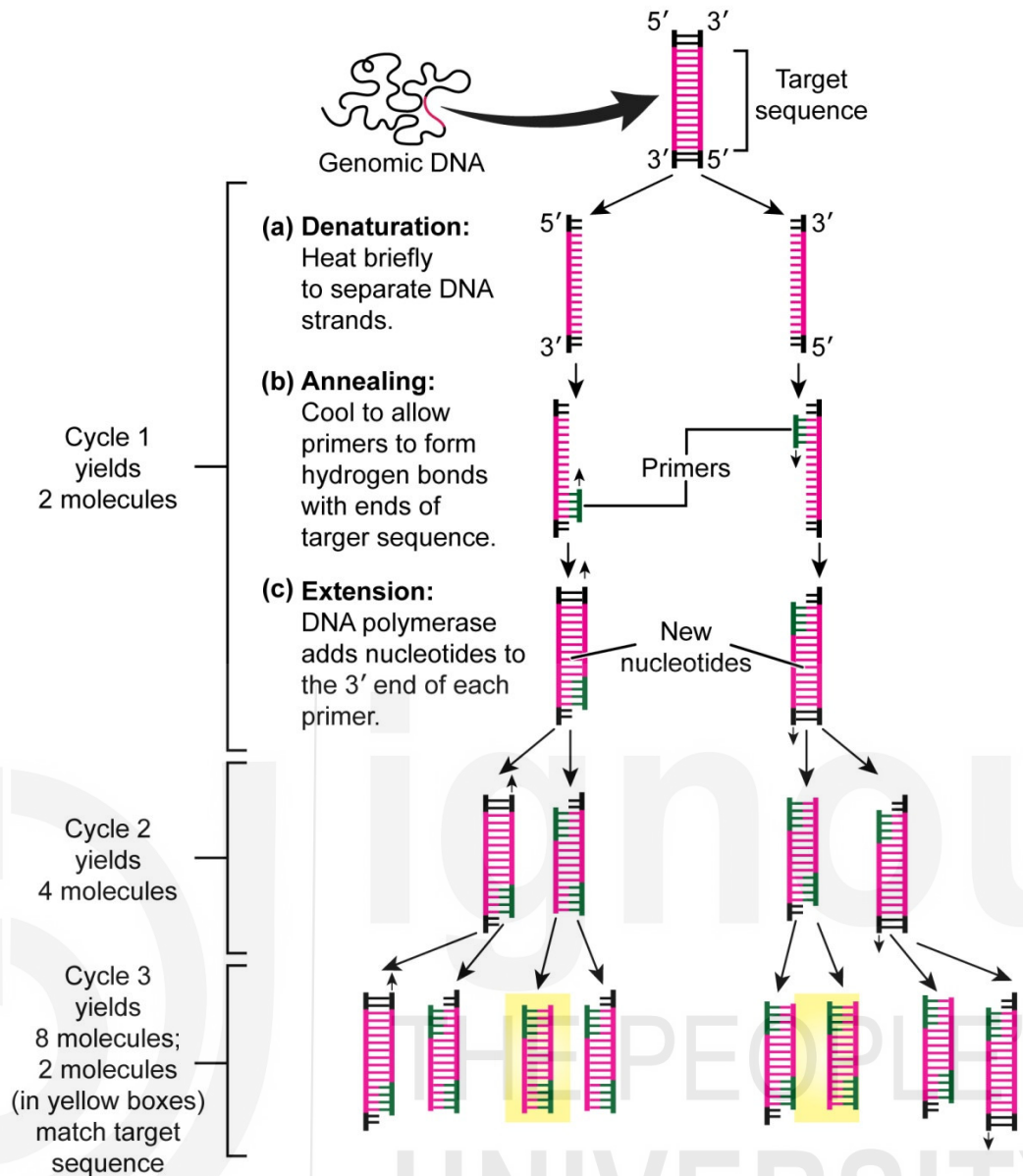


Fig.13.9: Various steps in the PCR technique.

Reverse Transcriptase-PCR

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is sensitive *in vitro* method to amplify RNA sequence. RT-PCR is used to detect and compare mRNA levels and the surface proteins and a crucial role in medical science.

Reverse transcription coupled to the polymerase chain reaction (RT-PCR) is commonly used to detect the presence of mRNAs, pre-mRNAs, or other types of RNA such as non-coding RNAs. The method involves transcribing a portion of the RNA genome into complementary DNA (cDNA), then amplified through PCR. The method involves using a primer annealed to the RNA of interest. The primer is usually a synthetic oligo(dT)15-18, a random hexamer mixture (dN)6, or a synthetic DNA oligonucleotide that is complementary to a specific transcript (a gene-specific primer) (Fig.13.10). The DNA-RNA hybrid serves as a template during reverse transcription. The reverse enzyme transcriptase (RT) generates a single-stranded cDNA copy of a portion of the target RNA molecule. cDNA sequences obtained from mRNAs can be used as a template for PCR. The use of gene-specific primers produces DNA fragments corresponding to a portion of the RNA of interest.

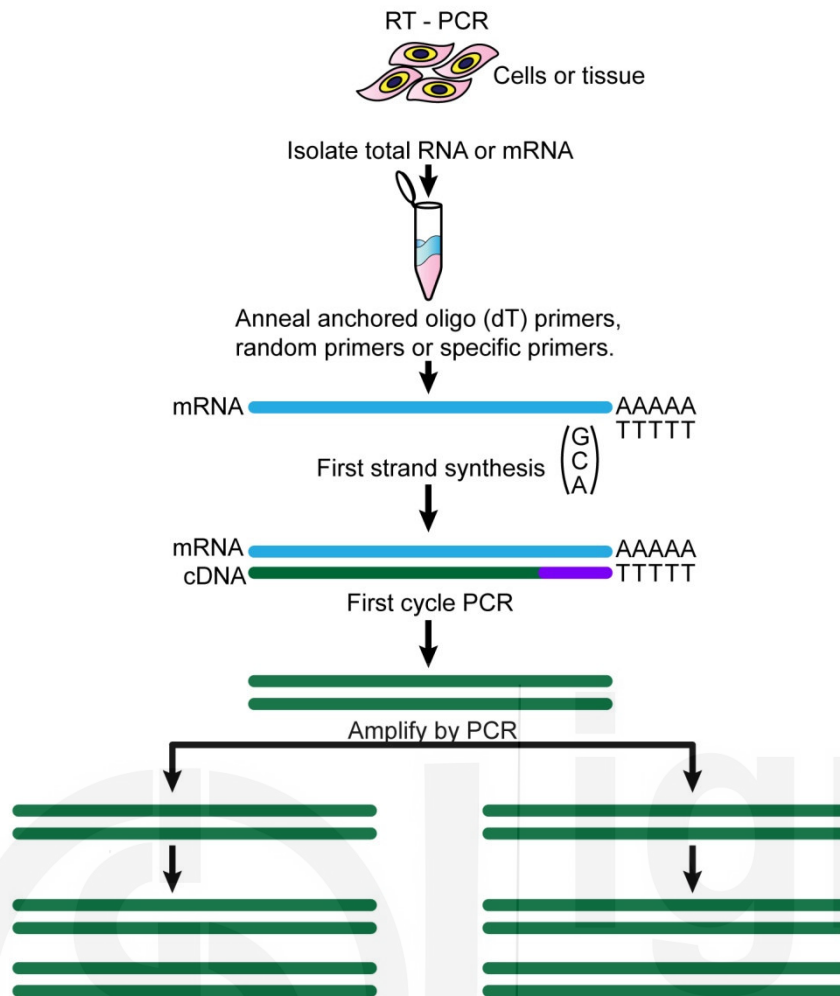


Fig. 13.10: Various steps in the RT-PCR technique.

SAQ 3

- a) Answer in one word.
- Technique by which unlimited copies of DNA can be produced
 - Single-stranded DNA fragments.
 - Heat stable DNA polymerase enzyme.
 - The technique used for the detection and comparing mRNA levels
 - The enzyme that generates a single-stranded cDNA copy of a part of the RNA molecule
- b) What is the difference between PCR and RT-PCR?

13.6 HYBRIDOMA AND MONOCLONAL ANTIBODIES

Antibodies are the proteins produced by lymphoid tissues in response to foreign materials or antigens. Antibodies are specific in their mode of action.

Antibodies bind to selective molecules within the cell, having a small part that fits into the antigen-antibody binding sites of the antibody. In 1975, **Cesar Milstein and Gorges Kohler** carried out experiments related to preparing antibodies against specific antigens. Antibody producing cells derived from a single lymphocyte synthesizes antibodies having identical antigen combining sites. When a single purified antigen is injected into the animal, many different lymphocytes are activated, and each antibody has an affinity for different parts of the antigen. If an animal is injected with pure antigen, antibodies are produced after a week. The spleen or the lymphoid organs are removed from the animal, and isolates of the single suspension of cells produce the desired antibodies (Fig.13.11). If these cells are grown as separate colonies, large quantities of the particular immunoglobulin can be obtained. This procedure can obtain antibodies produced by a single colony of cells (clone). The antibodies produced by this procedure are called monoclonal antibodies.

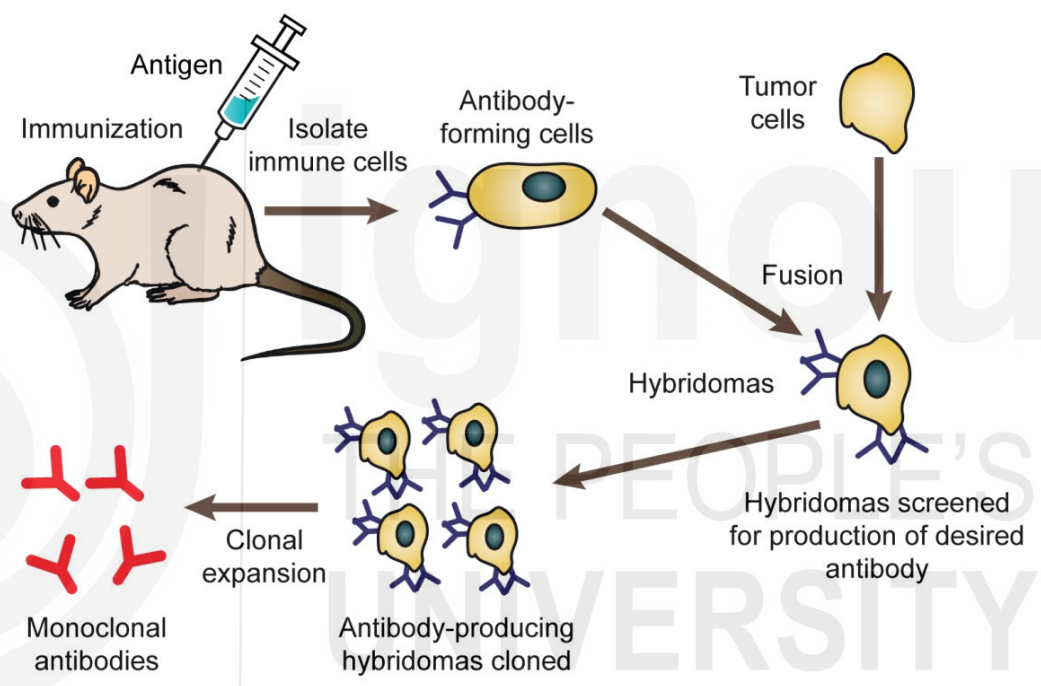


Fig.13.11: Hybridoma technology for production of monoclonal antibodies.

For the production of monoclonal antibodies, the antigen is injected into the mouse to cause the proliferation of specific antibody-forming cells. After several weeks, the spleen is removed and dissociated into single cells. The antibody-forming lymphocytes are fused with a population of malignant myeloma cells. It makes the hybrid cells immortal, which show capacity for limited division. Hybrid cells, i.e. hybridomas, are selected from the non-fused cells by placing the cells in the medium in which only hybrid cells can survive. Hybridomas are grown clonally in separate wells. They are individually screened for the production of antibodies against the antigen studied. Hybrid cells containing the antibody are then cloned *in vitro* or *in vivo* to get monoclonal antibodies.

Malignant myeloma cells are cancer cells that proliferate in culture and produce large quantities of antibodies. The antibodies are not formed in response to an antigen in these cells. Instead, Myeloma cells are formed by the random conversion of normal lymphocytes to a malignant state. They form

antibodies that were produced from the lymphocyte before turning into the myeloma cells. Milstein and Kohler combined the properties of two types of cells, i.e. normal antibody-producing lymphocyte cells and the immortal myeloma cell, to produce hybrid calls called a hybridoma. They grow and proliferate indefinitely and produce large monoclonal (single-cell) antibodies.

Monoclonal antibodies find their use in medical diagnostics to test or determine the concentration of specific proteins in the blood or urine. For example, the monoclonal antibodies form the basis of pregnancy confirmation tests to monitor the presence of a protein—chorionic gonadotropin that appears in the urine after a few days of conception. Monoclonal antibodies have also become useful as therapeutic agents in treating various diseases.

13.7 ELISA AND IMMUNODETECTION

Enzyme-linked immunosorbent assay (ELISA) is used to detect specific antigens or proteins in a biological sample. It is also used to detect viruses, fungi, bacteria, mycotoxins and hormones. The assay is based on the ability of low molecular weight antibodies to combine with enzymes to produce immunological conjugates. The immune reaction is detected with histochemical staining techniques. The antibody is involved in the immune reaction, and the conjugated enzyme is used for staining reaction using the specific substrate. ELISA technique was developed by **E. Engvall and P. Perlmann** in 1972. **Clarks and Adams** (1977) used this technique to detect viruses by using antibodies. **Voller and his colleagues** (1980) explored using a polystyrene or polyvinyl chloride microtitre plate with 96 wells for the assay. The plate provides a solid phase of the immune reaction. The antigens/antibodies get stuck on the inner surface of ELISA wells. The molecular event of the antigen-antibody binding can be visualized by adding an enzyme to label the antigen or the antibody (Fig. 13.12). The enzymes such as horseradish peroxidase alkaline phosphatase have been used for this purpose. The addition of the substrate for the enzyme produces a colored product that indicates antigen-antibody binding. The optical densities are measured using an ELISA reader. In DAS (Double Antibody Sandwich) ELISA, the wells are coated with antibodies or immunoglobulins (Ig). The sample is added, and time is provided to allow the antigen to bind to the antibody. The virus antigen gets trapped in the coated antibody. The enzyme labelled antiviral, i.e., Ig, is added after that. It gets attached to the trapped antigen. Finally, an enzyme substrate is added to produce the colored product. Generally, the enzyme peroxidase or alkaline phosphatase is used for the reaction.

In DAC ELISA, the antigen coating is applied directly to the wells. Diluted unfractionated antiserum is added. Antibodies or immunoglobulins (Ig) get attached to the virus antigen can be detected with the help of an enzyme –Ig-conjugates. In another type of ELISA called PAC-ELISA, the wells are coated with protein A to which highly diluted unprocessed specific antisera is added. Test samples and antiserum are added. Finally, the substrate is added to get the colored reaction. The procedure has been used to detect mycoplasma like organisms (MLOs) in crude leaf extracts. DAS-LISA has been used for the quantitative estimation of bacterial populations. ELISA has been used for the detection of aflatoxins in the seed samples.

The ELISA technique is used widely to detect the antigens, antibodies in the blood, pregnancy, hormone levels, and adulterants in food. In addition, ELISA diagnostic kits have been created to detect various diseases. It has also been used to detect mycorrhizal fungi and gibberellins through the use of monoclonal antibodies.

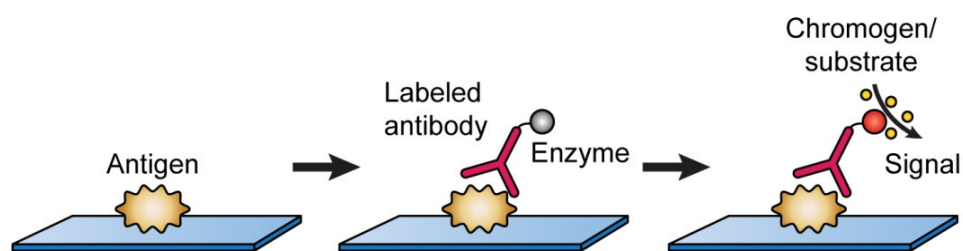


Fig.13.12: Diagrammatic representation of ELISA technique.

SAQ 4

Define

- i) Hybridoma
- ii) Monoclonal antibodies
- iii) Primer
- iv) Probe

13.8 SUMMARY

- DNA, RNA and protein fragments can be separated by gel electrophoresis. The bands obtained after separation can be hybridized using a labelled probe. The bands are transferred on the nitrocellulose membrane. "Blotting" describes the process of transferring bands to a membrane and then detecting their presence using a probe. The technique in which DNA bands are blotted is called Southern blotting. The transfer of RNA bands is called Northern blotting. The transfer of protein bands is done by the technique called Western blotting. Southern blot hybridization helps in the detection of specific DNA fragments. In contrast, Northern blotting helps characterize one or more specific mRNA transcripts in a sample of total RNA. Western blotting is used for the detection of proteins of particular specificity.
- DNA fingerprinting technique is used to identify an individual using a sample of DNA. DNA sequences provide many visual markers that can be used to map their locations in the chromosome of various species.
- Molecular markers are used to detect changes among different species and strains. The molecular markers that are extensively used in the preparation of genome maps include RFLP, RAPD and SNPs. RFLP markers are based on specific restriction enzymes and molecular probes.

- RAPD markers are DNA fragments from PCR amplification of random segments of genomic DNA with a single primer of an arbitrary nucleotide sequence.
- SNPs represent sites where DNA sequences differ by a single base. They are also referred to as simple nucleotide polymorphisms. RFLP or AFLP can detect the presence of SNPs conducted on PCR products. However, number of methods for SNP detection uses gel-based assays.
- Identical template molecules obtained from PCR or cloned DNA fragments are taken and used as the template along with primer complementary to the 3' end of one strand of the region to be sequenced. The nucleotides incorporated in the complementary strands are identified using various procedures. The strands synthesized by this procedure are short in length. Therefore, these DNA sequencing technologies are best suited for studying individual genomes from a species.
- The amplifying DNA fragments are known as polymerase chain reactions (PCR). It allows the production of unlimited copies of a DNA segment quickly. Many copies of the specific DNA fragment can be created using this technique. Kary Mullis developed the PCR technique in 1983. Reverse transcriptase PCR (RT-PCR) was developed to amplify RNA targets. The method involves using a primer annealed to the RNA of interest.
- The normal antibody-producing lymphocyte cells and the immortal myeloma cell is fused to produce hybridoma cells. They grow and proliferate indefinitely and produce large monoclonal (single-cell) antibodies. First, the antigen is injected into the mouse, where it causes the proliferation of specific antibody-forming cells. Next, the antibody-forming lymphocytes are fused with a population of malignant myeloma cells. Hybrid cells containing the antibody are then cloned *in vitro* or *vivo* to get monoclonal antibodies.
- Enzyme-linked immunosorbent assay (ELISA) technique is used to detect specific proteins present in a biological sample. It is also used to detect viruses, fungi, bacteria, mycotoxins and hormones. The low molecular weight antibodies combine with enzymes to produce immunological conjugates.

13.9 TERMINAL QUESTIONS

1. Differentiate between Northern and Southern blotting.
2. Explain the technique of Western blotting.
3. Explain the significance of the DNA fingerprinting technique.
4. Enumerate the role of a few molecular markers.
5. Describe the significant steps involved in the amplification of DNA using the PCR technique.
6. What is the significance of hybridoma technology?
7. How is ELISA technology important for medical diagnostics?

13.10 ANSWERS

Self-Assessment Questions

1. a) Southern blotting b) Northern blotting
c) Western blotting d) Molecular probe
2. a) i) False; ii) True; iii) True; iv) False; v) True
b) Refer to Section 13.4
3. a) i) Polymerase chain reaction ii) Templates DNA
iii) Taq Polymerase iv) Reverse transcriptase PCR
v) Reverse transcriptase
b) Refer to Section 13.5.
4. a) Refer to Section 13.6.
b) Refer to Section 13.6.
c) Primer- Primers are single strands of DNA used for initiating the synthesis of DNA. These sequences are about 20 to 30 bases in length.
d) Probe- A single-stranded DNA or RNA fragment corresponding to a gene or DNA sequence of interest is called a probe. It is a nucleotide sequence that is complementary to the target sequence.

Terminal Questions

1. Refer to Section 13.2.
2. Refer to Section 13.2.
3. Refer to Section 13.3.
4. Refer to Section 13.4.
5. Refer to Section 13.5.
6. Refer to Section 13.6.
7. Refer to Section 13.7.

Acknowledgement

Fig. 13.8 : Source:

<https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fwww.researchgate.net%2Fprofile%2FMichel-Gauthier-4%2Fpublication%2F234248746%2Ffigure%2Ffig2%2FAS%3A669425925623808%401536614986157%2FThe-Sanger-sequencing-method-in-7-steps-1-The-dsDNA-fragment-is-denatured-into-two.png&imgrefurl>

UNIT 14

RECOMBINANT DNA TECHNOLOGY

Structure

14.1	Introduction	14.5	Construction of Genomic and cDNA Libraries
	Objectives		
14.2	Restriction Enzymes (Endonucleases Type I-IV)	14.6	Colony Hybridization
14.3	Vectors	14.7	Methods of Genetic Engineering
	Cloning Vectors		<i>Agrobacterium</i> -Mediated
	Expression Vectors		Direct (Electroporation, Microinjection, Microprojectile Bombardment)
	(Prokaryotic, Eukaryotic, Shuttle, Lambda, Cosmid)		Crispr-Cas-9 Technology
14.4	Gene Cloning	14.8	Summary
	Recombinant DNA	14.9	Terminal Questions
	Transformation and Selection of Recombinant Clones	14.10	Answers
	Pcr Mediated Gene Cloning		

14.1 INTRODUCTION

Traditional approaches, such as plant breeding, have been used for many years to increase agricultural productivity, combat various abiotic/biotic stresses, and develop resistance to insects and pests. However, genetic engineering has provided an alternative to traditional plant growth and productivity strategies. To achieve reliable products, genetic engineering employs modern tools such as molecular cloning, transformation, and expression of genes, which are less time-consuming than traditional plant breeding approaches. Recombinant DNA technology is based on modifying an organism's genetic material to achieve enhanced expression of a gene of interest. Manipulation of an organism's genome occurs through the

introduction of genes or regulatory elements, as well as the inhibition of endogenous gene expression. A suitable vector, such as a plasmid, is used to insert the desired gene sequence into the genome of an organism. The recombinant DNA is formed when the desired DNA fragment or a gene of interest is introduced into the host genome. The model microorganism is being transformed with the recombinant plasmid, It replicates and passes it on to its offspring, creating copies of the DNA. Paul Berg, Herbert Boyer, Annie Chang, and Stanley Cohen of Stanford University and the University of California, San Francisco created the first recombinant DNA (rDNA) molecule in 1972. Several products such as hormones, vaccines, therapeutic agents, and diagnostic tools were developed in the mid-1980s with the help of recombinant DNA technology. Furthermore, genetic engineering strategies have been used to develop biofuels in order to reduce pollution caused by fossil fuels. By developing new vaccines and pharmaceuticals, recombinant DNA technology helps to improve health conditions. In recent years, genetic engineering has demonstrated its applications in plants through the development of crops that are resistant to fungal, viral, and plant disease infections. Figure 14.1 summarises various applications of genetic engineering technology.

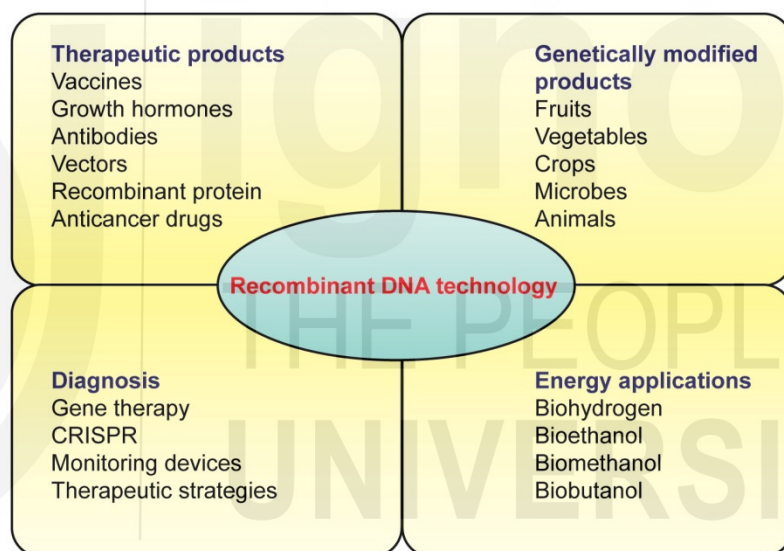


Fig. 14.1: Applications of Recombinant DNA Technology.

In the present unit, you will be studying the role of various enzymes and methods involved in developing recombinant DNA.

Objectives

After studying this unit, you would be able to:

- ❖ describe the concept of recombinant DNA technology;
- ❖ enlist various enzymes involved in the formation of recombinant DNA;
- ❖ describe the role of vectors used in the cloning of DNA;
- ❖ describe the process of construction of DNA (genomic and cDNA) libraries; and
- ❖ enumerate the methods of genetic engineering.

14.2 RESTRICTION ENZYMES (ENDONUCLEASES TYPE I - IV)

A restriction enzyme is a type of nuclease enzyme that cleaves DNA at random or specific recognition sites known as restriction sites, it is very useful to obtain clones or copies of DNA.

Classification of Restriction Endonucleases

On the basis of subunit composition, cleavage positions, cofactor requirements, and sequence specificity, restriction enzymes are classified as I, II, III, and IV.

Type I restriction enzymes: These enzymes are multi-enzyme complex, with 15-bp recognition sequences, and capable of both restriction and modification. These enzymes cut DNA far from its recognition sequences. Cleavage happens about 1000 bp away from the recognition site. The recognition site is asymmetrical and is made up of two distinct sections, one with 3-4 nucleotides and the other with 4 - 5 nucleotides. A non-specific spacer of about 6 - 8 nucleotides separates the two parts. The methylation of the target DNA determines restriction. For activity, these enzymes require S-adenosylmethionine (SAM), ATP, and magnesium ions (Mg^{2+}). These enzymes are made up of three subunits: a specificity subunit that determines the DNA recognition site, a restriction subunit that cleaves at sites other than the recognition site, and a modification subunit that determines the cleavage site, for example, Eco B.

Type II restriction enzyme: These enzymes cut at specific locations near or within their restriction sites. Palindromic sequences are recognized by these enzymes. Because they bind to DNA as homodimers, most recognize symmetric DNA sequences, but a few (e.g., BbvCI: CCTCAGC) recognize asymmetric DNA sequences because they bind as heterodimers. For activity, they only need magnesium, and the corresponding modification enzymes only need S-adenosylmethionine. They're usually small, with subunits ranging from 200 to 350 amino acids. There are over 350 different types of type II restriction endonucleases. These enzymes are employed in gene cloning, mapping, and DNA reconstruction. example Hind III and EcoRI.

Type III restriction enzymes are a bridge between type I and type II restriction enzymes. These enzymes recognize and methylate the same DNA sequence but approximately 24–26 bp away, i.e. they cleave DNA near the recognition site. These are divided into two subunits. One subunit (M) recognizes and modifies the DNA sequence, while the other (R) acts as a nuclease. DNA cleavage necessitates the presence of Mg^{2+} , ATP, and SAM, for example, EcoPI.

Type IV restriction enzymes, such as McrBC, target modified DNA such as methylated, hydroxymethylated, and glucosyl-hydroxymethylated DNA.

Based on their mode of action, restriction enzymes have been classified into two types:

- 1) **Exonucleases** - The enzymes that catalyze the hydrolysis of terminal nucleotides from the end of DNA or RNA molecule either 5'to 3' direction or 3' to 5' direction. For example, *Exonuclease I*, *Exonuclease II*, etc.

- 2) Endonucleases - The enzymes that recognize specific base sequence (restriction site) within DNA or RNA molecule and cleave internal phosphodiester bonds within a DNA molecule. For example, *Eco* R I, *Hind* III, *Bam* HI.

A restriction endonuclease recognizes a sequence and cuts DNA molecules by catalyzing hydrolysis (breaking a chemical bond with the addition of a water molecule) between adjacent nucleotides. The sugar-phosphate backbone of the DNA molecule is cut twice. The restriction enzymes recognize **palindrome sequences**, which are short DNA sequences ranging in length from 4 to 8 base pairs. Palindromes are the same nucleotide sequences on the two complementary strands of DNA in the 5' to 3' direction. For example, if one strand's sequence is 5' GAATTC 3', the opposite strand's sequence is 3' CTTAAG 5'. When both strands are read in the 5' → 3' direction, the sequence remains the same. The palindrome is written as

5' GAATTC 3'

3' CTTAAG 5'

Different enzymes recognize different and specific sequences in DNA.

When the same restriction enzyme cuts DNA on both ends, it forms ends with one strand overhanging the other, resulting in a short single-stranded segment. The overhang easily re-attaches to other similar ends and is thus referred to as "sticky ends." Even if the overhangs come from different parent DNA molecules, they hybridize ("anneal"). Sticky ends, as a result, facilitate the ligation of diverse DNA segments and the formation of novel DNA constructs. Other restriction endonucleases generate a "blunt end," which means that there are no unpaired bases or overhangs at the end of the fragments. Because these DNA fragments cannot anneal to each other, they are difficult to clone.

Some enzymes, in addition to cleavage, bring about modification in the form of methylation. These enzymes are referred to as modification enzymes (also called methylases). In bacteria, restriction enzymes form a combined system (restriction + modification system), which means that they cleave the bacterial DNA as well as methylate it. Methylation of bacterial DNA protects the bacteria's own DNA from cleavage by a restriction enzyme.

Restriction endonucleases are named after the organism that discovered them. The enzymes are identified by letters and numbers. Roman letters are commonly used and italicised. Each enzyme is named after the bacterium that produced it. Specific enzymes are identified using Roman numerals. *Hind* III, for example, was discovered in *Haemophilus influenza*, and *Eco* RI was derived from *E. coli*. In the name *Eco*RI, E stands for the genus *Escherichia*, co stands for the species *coli*, R stands for strain RY13, and I stands for the order of identification in the bacterium. *Hind* II, the first restriction endonuclease enzyme, was isolated in 1970 from *Haemophilus influenzae* Rd strain II. Daniel Nathans, Werner Arber, and Hamilton O. Smith isolated and characterized restriction endonucleases in 1978. They were awarded the Nobel in physiology and medicine for developing the restriction enzymes.

Applications of Restriction Enzymes

- DNA fragments can be manipulated using restriction enzymes. These enzymes create single-stranded DNA overhangs by making staggered cuts in the DNA molecule around the point of symmetry. Some enzymes, on the other hand, cut straight across the molecule at the symmetrical axis, resulting in blunt ends.
- Each enzyme recognizes the same unique base sequence and helps DNA molecules establish fixed landmarks. Gel electrophoresis can separate a long DNA molecule into fragments (you have studied this technique in the course BBYET 141: Cell and Molecular Biology). Each fragment generated is used for further analysis, such as sequencing or vector cloning. Using DNA fragments, the location of a specific gene can be easily tracked.
- Restriction enzymes can also be used to specifically distinguish gene alleles.
- Recognizing single base changes in DNA, also known as single nucleotide polymorphisms (variables) (SNPs).
- Restriction enzymes are employed in the treatment of Restriction Fragment Length Polymorphism (RFLP) analysis.
- This method is used to identify individuals or strains of a specific species.

SAQ 1

Answer in one word:

- The cleavage of DNA is brought by specific enzymes known as
- The short sequences of DNA recognized by restriction enzymes are known as
- The first restriction endonuclease enzyme isolated was
- Which type of restriction enzyme recognizes a specific base sequence (restriction site) and cleaves internal phosphodiester bonds within a DNA molecule?
- While naming restriction enzyme *EcoRI*, what does E stand for?

14.3 VECTORS

A vector is a DNA fragment that can replicate with newly inserted DNA. When inserted into a host cell, vectors act as vehicles for transporting a foreign DNA fragment, resulting in multiple clones of recombinant DNA. A suitable vector should have a replication origin, one or more selection genetic markers, and multiple cloning sites (MCS) where foreign DNA can be inserted. A plasmid, bacteriophage, cosmid, phagemid, or even a virus can serve as the vector. A cloning vector usually has a site where foreign DNA can be inserted without interfering with any essential functions.

Characteristics of an ideal vector are as follows:

- It should be compact in size (less than 10 KB)
- It must be capable of autonomous replication, i.e. self-replicating within the host cell.
- It should be simple to isolate and purify from the host cell.
- It should be simple to incorporate into the host cell. Furthermore, it should be able to incorporate itself or the DNA insert into the host cell's genome.
- It should have a selectable marker gene for identifying recombinant cells (usually an antibiotic resistance gene that is absent in the host cell).
- The vector should have distinct target sites as well as recognition sites for various restriction enzymes. The presence of distinct restriction sites allows a specific restriction endonuclease to cleave specific sequence restriction sites. The introduction of donor DNA fragments must not interfere with the vector's replication property.

Vectors are divided into two types based on their role in recombinant DNA technology.

- Expression vector
- Cloning vector

Cloning vectors are DNA molecules that deliver a specific gene of interest into a host cell. Their primary goal is to replicate the inserted gene as many times as possible. They serve as a foundation for the DNA insert to reproduce and spread in bacteria. Cloning vectors include a replication origin, distinct restriction sites, and a selectable marker. Plasmids, cosmids, phages, BACs, YACs, and MACs are all examples of cloning vectors.

Expression vectors are linked to the gene's actual expression into mRNA and protein in the target organism. An expression vector must contain all of the elements required for a host cell to transcribe and translate the gene. The expression vector contains enhancers, a promoter region, a termination codon, a transcription initiation sequence, a translation initiation sequence, etc. Furthermore, the plasmid serves as an expression vector. Cloning vectors and expression vectors can both replicate on their own within the host cell.

Shuttle vector This type of vector has the ability to reproduce in two different host species. As a result, inserted DNA can be tested and manipulated in two distinct cell types. These vectors are applicable to both eukaryotes and prokaryotes. The main advantage of these vectors is that they can be modified in *E. coli* before being used in a more difficult or slower system. Shuttle vectors are used to rapidly replicate the gene in *E. coli*. In-vitro experiments and modifications such as mutagenesis and PCR can also be performed using them. The yeast shuttle vector is one of the most common shuttle vectors, as it contains components that allow replication and selection in both *E. coli* and yeast cells. A yeast shuttle vector's *E. coli* component includes the origin of replication and a selectable marker, such as an antibiotic resistance gene-like

β -lactamase. A yeast shuttle vector's yeast component consists of an autonomously replicating sequence (ARS), a yeast centromere (CEN), and a selectable yeast marker.

Table 14.1: Summary of the properties of some well-known vectors.

Vector name	Preferred host cell	Insert size	Unique feature	Example
Plasmid	<i>E.coli</i>	up to 10 kb DNA fragment.	Self-replicating, high copy number, and possess antibiotic-resistant genes.	pBR322, pUC18, F-plasmid
Bacteriophage	<i>E.coli</i>	fragment of size up to 30 kb	It can accept very large pieces of foreign DNA.	Phage λ , M13 phage
Phagemid		fragment of size ranging from 100- to 300-kb	They possess multiple cloning sites and an inducible lac gene promoter	pEMBL, pBluescript plasmids
Bacterial Artificial Chromosomes (BAC)	<i>E.coli</i>	Fragments in sizes ranging from 150 to 300 kb.	Marker like sights such as antibiotic resistance genes and a very stable origin of replication (ori). They can hold much larger pieces of DNA.	
P1-derived artificial chromosomes (PACs)	<i>E.coli</i>	fragment of size ranging from 100–300kb	It is a DNA construct derived from the DNA of P1 bacteriophages and Bacterial artificial chromosomes. It can carry large DNA sequences.	
Yeast Artificial Chromosomes (YAC)	<i>Saccharomyces cerevisiae</i>	Fragments ranging from 200 to 2000kb	It is used in the mapping and sequencing of genomes.	

Cosmids		inserts of up to about 40 kb	They are capable of incorporating the bacteriophage λ DNA segment.	Col EI cosmid, pHC 79, pJB8, pWE cosmid, c2RB
Human Artificial Chromosomes (HAC)	Cultured Human Cells	fragment size >2000kb	Help in the transfer of new genes, studying their expression and chromosomal function.	

14.3.1 Types of Cloning Vectors

Different types of cloning vectors are used for different cloning experiments, i.e. gene amplification, ligation, transformation, and expression. The choice of vector also depends on the type of host cell (prokaryotic/eukaryotic), size, and type of DNA to be cloned (smaller/larger fragments). The prokaryotic vectors include plasmids, bacteriophages, phagemids, and fosmids. Eukaryotic vectors comprise yeast, animal, and plant cells.

Plasmid

Plasmids are self-replicating, double-stranded, circular DNA molecules found in bacteria that are extrachromosomal entities. The size of plasmids is about <1kb to > 500kb, it is single or multicopy plasmids. Each plasmid has an origin of replication (ori) that aids in its replication within the host cell (Fig. 14.2). The plasmid can clone relatively large (100 bp to 10 kbp) foreign DNA fragments. The pSC101, pSF2124, pSC101, pSF2124, Col E1, etc. are the most basic cloning vectors used in recombinant DNA technology.

Naturally occurring plasmids frequently lack several important features required for cloning, and must therefore be modified. Size reduction, deletion of non-essential and toxin-producing genes, insertion of selectable marker genes (e.g., antibiotic resistance genes), and addition of restriction enzymes (RE sites) such as MCS or polylinker are examples of modifications. pBR322, pBR327, pUC8, pUC18, pUC19, etc. plasmids are restructured by inserting antibiotic resistance genes.

When naming a vector, for example, 'pBR322', the 'p' stands for plasmid, the 'BR' stands for Bo-liver and Rodriguez, the two researchers who developed it, and the '322' stands for plasmid as opposed to others (such as pBR325 and pBR327) developed in the same laboratory. The pBR322 vector is derived from *E. coli* and contains antibiotic resistance genes for ampicillin and tetracycline. pUC is yet another cloning vector series. The plasmid is denoted by the letter 'p,' and the letter 'UC' stands for the University of California, where J. Messing and his colleagues first developed it. This vector is found in pairs and has reverse order restriction sites relative to the lacZ promoter; one such pair is pUC8 and 9.

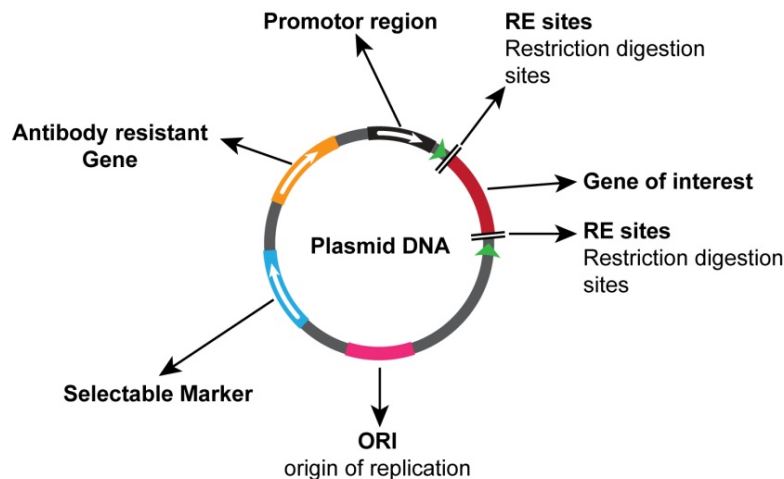


Fig. 14.2: A schematic representation of plasmid containing different regions.

Properties of plasmid

1. Typically small in size (<10 kb).
2. It is simple to purify and handle.
3. Draw two antibiotic resistance genes (Amp^R and Tet^R) that aid in the selection of transformed or recombinant cells (Fig. 14.2).
4. Display a high copy number. For example, a transformed *E. coli* cell contains 15 molecules, but the copy number can be increased to 1000-3000 by plasmid amplification in the presence of chloramphenicol.

Bacteriophage

Another source of the cloning vector is bacteriophage. A single break in a phage's linear DNA molecule generates two fragments, which are later joined with the help of foreign DNA to form a chimeric phage particle (Fig. 14.3). Because the capacity of the phage head is limited, only small amounts of foreign DNA can be inserted into it. Before using phage, segments of DNA that do not contain essential genes are usually removed. In phage lambda, this technique was used to create a smaller vector genome with a single restriction site for the enzyme *EcoRI*.

Takagi and colleagues (1976) discovered a cohesive end site (*cos*). *Cos* from bacteriophage lambda DNA in a plasmid allows it to be packaged into virus particles *in vivo*. *In vivo* packaging aids in the selection of DNA molecules from the lambda genome's total size (48.5 kb). As a result, a lambda cloning vector can be created by removing (restriction deletion) a portion of the nonessential region. In the nonessential region, a segment of foreign DNA can be cloned in a unique restriction site. These vectors are known as 'insertion vectors.' The lambda has the following properties that make it suitable for use as a vector:

- Approx.50% of the lambda genes are required for host cell replication and lysis
- Lambda genome is packaged inside the phage head by what is known as the 'head-full mechanism'. It means an upper limit of the amount of DNA packaged inside the phage head.

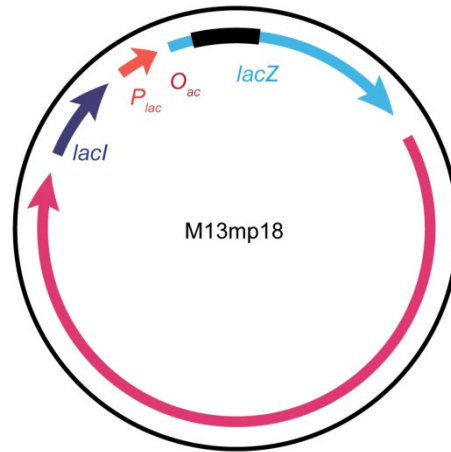


Fig. 14.3: Diagrammatic representation of a bacteriophage used as a vector.

Phagemids

It is a cloning vector that is created by combining the filamentous phage M13 with plasmids to create a vector that can both grow as a plasmid and it can be packaged as single-stranded DNA in viral particles (Fig. 14.4). The phagemid contains the replication origins of both the plasmid and the coliphage. The pBluescript vector is a common phagemid vector. The origins of the pBluescript phagemids are both ColE (pMB9-like) and filamentous phage f1. The pBluescript II KS is 2691 base pairs long and descended from pUC19.

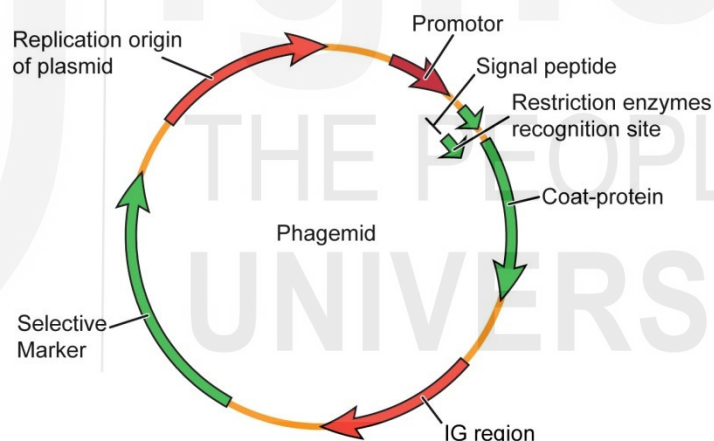


Fig. 14.4: Diagrammatic representation of a Phagemid as a vector.

Cosmids

The cosmids vector is made up of the plasmids and bacteriophage λ sequences. It contains DNA sequences with *cos* sites inserted into them that enable DNA to get packed into the lambda phase (Fig. 14.5). Cosmids allow the packaging of phage DNA into phage particles *in vitro*. Cosmid vector was first developed in 1978 by J. Collins and co-workers to facilitate the cloning of larger DNA fragments than plasmids. An example of a commonly used cosmid is pHV79 is pBR322. It contains cohesive end site *cos* λ , accommodating up to 45 kb-sized inserts.

The commonly used cosmid pLFR-5 (6kb size) contains two bacteriophage *cos* sites (*cos* ends) separated by a restriction endonuclease site, a multiple

cloning sequence with six distinct sites (Hind III, PstI, SalI, BamHI, SmaI, and EcoRI), an origin of DNA replication (ori), and a tetracycline resistance (TetR) gene. This cosmid carries about 40 kb of cloned DNA.

The *cos* ends get cleaved during packaging of phage. As a result, the *cos* ends base pair forms a circular DNA molecule inside the bacteria. This circular form is stable, so the cloned DNA can be maintained as a plasmid-insert DNA construct.

The advantages of using a cosmid vector are

- A study of intact genes and linkage can be carried out at the molecular level.
- They have a high frequency of transformation than plasmids.
- Cosmids are constructed by joining a linearized plasmid DNA with DNA fragments of bacteriophage that have *cos*-ends. For example, a DNA of 85kbp can be packaged into the head of a P1 phage with a genome size of 115 kbp.
- These cosmids help to clone large genes and gene clusters in bacteria. Cosmid picks up relatively larger DNA fragments than plasmid and hence are used to establish gene libraries.
- Cosmids help in the study of non-sense sequences in the genome of organisms.

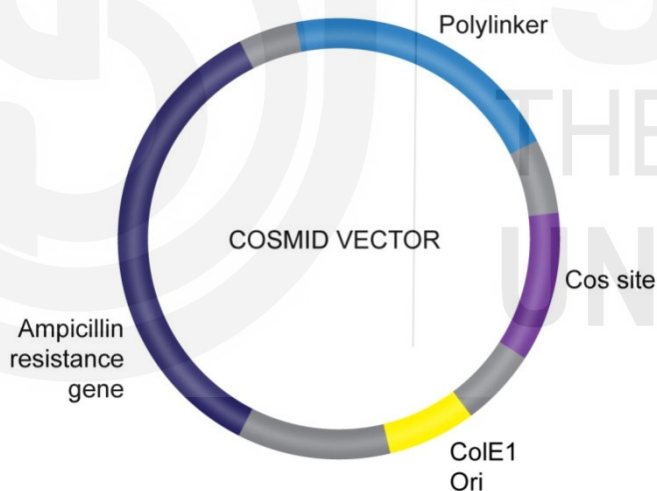


Fig. 14.5: Diagrammatic representation of a cosmid vector.

Fosmid Vectors

Fosmid vectors are similar to cosmids but are based on the bacterial F-plasmid. The cloning potential is limited as the host (usually *E. coli*) contains one fosmid molecule. Fosmid is about 40 kb of random genomic DNA. It may aid in the construction of sustainable libraries from complex genes such as *Hi53*, *leo2*, *trpI*, and *ura3*.

Phasmid

This vector contains several tandem copies of plasmid, which makes it larger than 38 kb. This size is suitable for packaging in λ phage particles. These are λ insertion vectors that consist of short linear λ genomes containing DNA replication.

14.3.2 Eukaryotic Vectors

Yeast, animal, and plant vectors are all considered eukaryotic vectors. Yeast artificial chromosomes (YACs) and mammalian artificial chromosomes (MACs) based on the yeast and human centromeres, telomere sequences, and autonomously replicating sequences have also been developed for use as vectors.

Eukaryotic vectors help in cloning to control or improve the synthesis of a metabolic product or change the properties within an organism. For example, they are introducing resistance into a crop plant. Various plant and animal viruses have also been used as vectors for introducing foreign genes into cells. Plant viruses used as vectors are cauliflower mosaic virus (CaMV), tobacco mosaic virus (TMV), and geminivirus. Animal viruses used as vectors include baculovirus, adenovirus, papovavirus, herpesvirus, and retrovirus.

Ti plasmid of *Agrobacterium tumefaciens* and Ri plasmid of *Agrobacterium rhizogenes* are the vectors present in higher plants. *Agrobacterium* species act as pathogens and induce various diseases in higher plants. Ti (tumor-inducing) plasmids are produced by *Agrobacterium tumefaciens*, while *Agrobacterium rhizogenes* produce Ri (root-inducing) plasmids. Both Ti and Ri plasmids consist of a part of the plasmid DNA known as T-DNA that is transferred into the plant genome with virulence (vir) genes. Ti plasmid induces tumor/crown gall in dicots, whereas Ri plasmid induces hairy roots. Due to their ability to infect plants, Ti and Ri plasmids are widely used as vectors to produce transgenic plants (Fig. 14.6). The size of both Ti and Ri plasmids are around 200 kb.

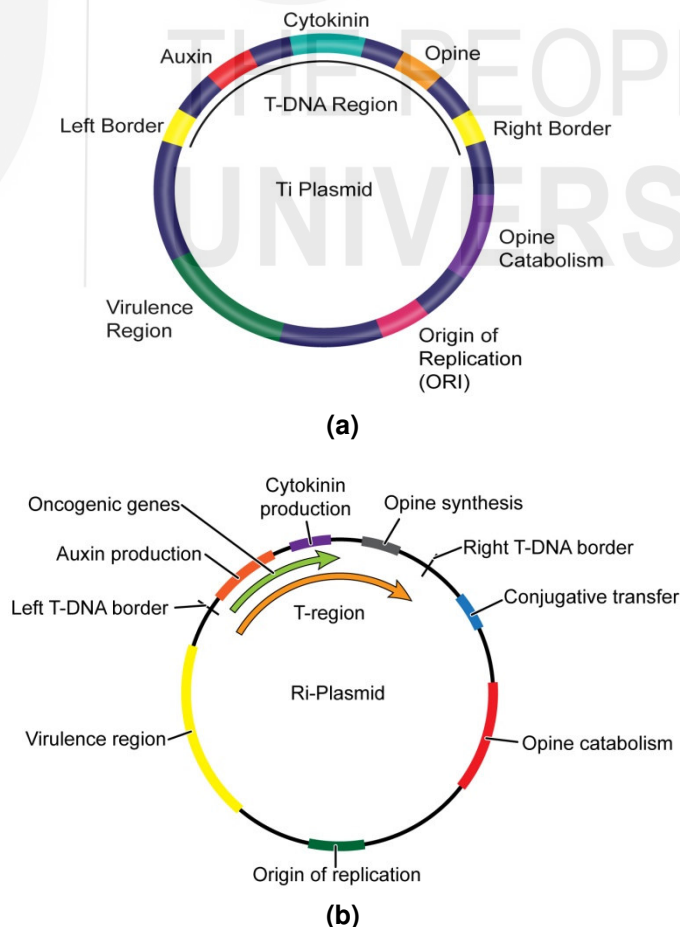


Fig. 14.6: Diagrammatic representation of an a) Ti plasmid; b) Ri plasmid.

Ti plasmid consists of the *vir* region, which encodes for virulence genes, and the transfer DNA (T-DNA) region, which is a section of the Ti plasmid that is transferred via conjugation into host plant cells. These regions have features that allow the delivery of T-DNA into host plant cells and can modify the host plant cell to cause the synthesis of molecules like plant hormones and opines and the formation of crown gall tumours. Because of their ability to infect plant cells, both Ti and Ri plasmids are used in genetic engineering to produce transgenic plants.

Therefore, T-DNA from Ti or Ri plasmid of *Agrobacterium* is a potential vector for cloning in higher plants. Foreign DNA has first cloned into T-DNA of Ti or Ri plasmid, which is then transferred to *Agrobacterium*. Recombinant Ti plasmids can be obtained by cloning a large fragment of Ti plasmid into pBR322.

Yeast Vectors

A plasmid present in most yeast strains (*Saccharomyces cerevisiae*) also plays a role of a cloning vector. The advantages of using these vectors are:

1. They contain unique target sites for several restriction endonucleases.
2. They are high copy number vectors that can replicate in *E. coli*.
3. All of them use markers to select recombinants.

Most cloning experiments are carried out with *E. coli* as the host. Several cloning vectors have been designed with genetic elements that allow for the expression of foreign genes. These elements usually include the signals for transcription and translation initiation. Plasmid and bacteriophage cloning vectors have been widely used for the isolation and analysis of individual genes or multigene families.

14.3.3 Shuttle Vectors

Vectors are designed to replicate in cells of two species as they contain two origins of replication that are known as shuttle vectors (Fig. 14.7).

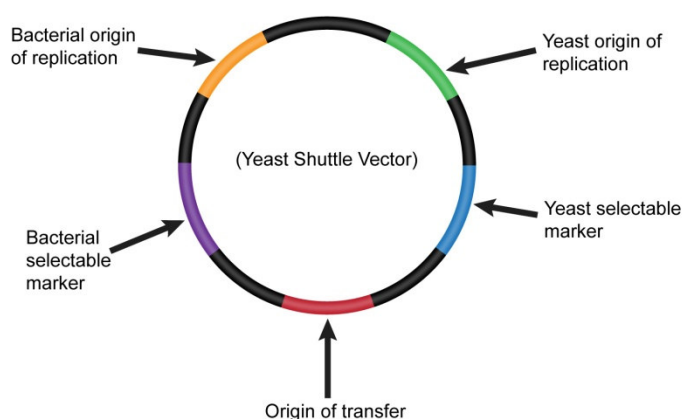


Fig. 14.7: Diagrammatic representation of a shuttle vector.

Each origin of replication is specific to species and possesses genes necessary for their replication. Shuttle vectors multiply into two different unrelated species. Shuttle vectors allow an efficient transfer of recombinant

DNA into yeast cells. These vectors include most of the eukaryotic vectors. For example, the yeast shuttle vector contains components allowing for the replication and selection in both *E. coli* cells and yeast cells. The *E. coli* component of the vector includes an origin of replication and a selectable marker, such as an antibiotic resistance like β -lactamase. In contrast, the yeast component includes an autonomously replicating sequence (ARS), a yeast centromere (CEN), and a selectable yeast marker.

SAQ 2

- a) Differentiate between:
 - i) Prokaryotic and eukaryotic vectors
 - ii) Plasmid and cosmid vectors
- b) Fill in the blanks:
 - i) molecules act as vehicles for carrying a foreign DNA fragment when inserted into a host cell to produce multiple clones of recombinant DNA.
 - ii) A vector should be able to show replication inside a host cell.
 - iii) Self-replicating, double-stranded circular DNA molecules present in bacteria as independent extrachromosomal entities are called
 - iv) Vector 'pBR322' is derived from
 - v) Plasmids generally possess two resistance genes which help in the selection of transformed cells from non-transformed cells.
 - vi) Cosmid contains DNA sequences having sites inserted into them.
 - vii) are the vectors similar to cosmids.
- c)
 - i) Give names of a few commonly used prokaryotic vectors.
 - ii) Give names of a few commonly used eukaryotic vectors.

14.4 GENE CLONING

The production of exact copies (*clones*) of a particular gene or DNA sequence using genetic engineering techniques is called cloning. Multiple copies of genes are produced using gene cloning technique. After gene cloning, the expression analysis of the cloned genes can be done. The basic steps of gene cloning include isolation, insertion, transformation, and multiplication/ expression. Various steps involved in gene cloning are:

1. Isolation of DNA (gene of interest) fragments.
2. Insertion of isolated DNA into the suitable vector.

3. Transformation of recombinant DNA into a suitable host cell.
4. Selection of transforming host cells that contain the cloned gene of interest.
5. Multiplication/Expression of the cloned gene in the host cell.
6. Isolation of multiple copies of gene/proteins expressed in microorganisms by the cloned gene.

A. Isolation of the DNA fragment or gene

The target DNA or gene is isolated using restriction endonuclease, which cuts at specific recognition sites of DNA known as restriction sites towards the inner region, resulting in blunt or sticky ends.

B. Selection of suitable cloning vector

The DNA fragment/GOI is first inserted into a vector, then after, the vector is inserted into a host cell to replicate the GOI, and the vector makes its multiple copies. A suitable vector is selected depending on the size and the application of the insert.

C. Formation of Recombinant DNA

The vector is cut by the same restriction enzyme present on the flanking ends of the DNA fragment. The foreign DNA is then inserted and joined (ligated) to another DNA entity (a cloning vector or plasmid DNA) to form a new DNA molecule (recombinant DNA molecule) using DNA ligase. Base pairing of foreign DNA fragment and plasmid vector occurs in DNA ligase's presence. The resulting DNA molecule is a combination of two DNA molecules. Hence, the combined DNA molecule is called a recombinant DNA molecule, and the technology is referred to as **recombinant DNA technology**. The transformation process requires the incorporation of modified DNA into the suitable host cell (Fig. 14.8).

D. Transformation of the recombinant vector into a suitable host cell

The recombinant DNA is transformed into a suitable host cell to replicate the recombinant DNA molecule to get the multiple copies of the GOI and also allows the expression GOI if cloned into an expression vector to obtain the protein product. Some bacteria such as *Bacillus*, *Haemophilus*, and *Helicobacter pylori* have naturally transformable (competent) to incorporate the foreign DNA without any special treatment. On the other hand, some bacteria, such as *E.coli*, require incorporating artificial methods such as Ca^{++} ion treatment, electroporation, etc.

E. Isolation of Recombinant Cells

The cloning vector–insert DNA construct is transferred into a host cell. The introduction of foreign DNA into a host cell is called **transformation**. The transformation process generates a mixed population of transformed (host

cells that take up the foreign DNA construct) and non-transformed host cells (host cells that do not take up the foreign DNA construct). The selection process involves filtering the transformed host cells only. For the selection of the recombinant cells from non-recombinant cells, a selection marker gene of the plasmid vector is employed. For example, the pBR322 plasmid vector contains different selection marker genes, viz. Ampicillin-resistant and tetracycline-resistant genes. When *pst1* RE is used, it knocks out Ampicillin resistant gene from the plasmid so that the recombinant cell becomes sensitive to ampicillin.

F. Multiplication of Selected Host Cells

The transformed host cells are provided with optimum conditions to grow and multiply. The transformed host cells are introduced into fresh culture media. The host cells divide and re-divide, along with replicating the recombinant DNA carried by them. Replication of the host cell is allowed for obtaining numerous copies of GOI.

G. Isolation and Purification of the Product

The next step involves isolating the multiplied GOI cloned into the vector or the protein encoded by the GOI if cloned into an expression vector. It is followed by the purification of the isolated gene/protein.

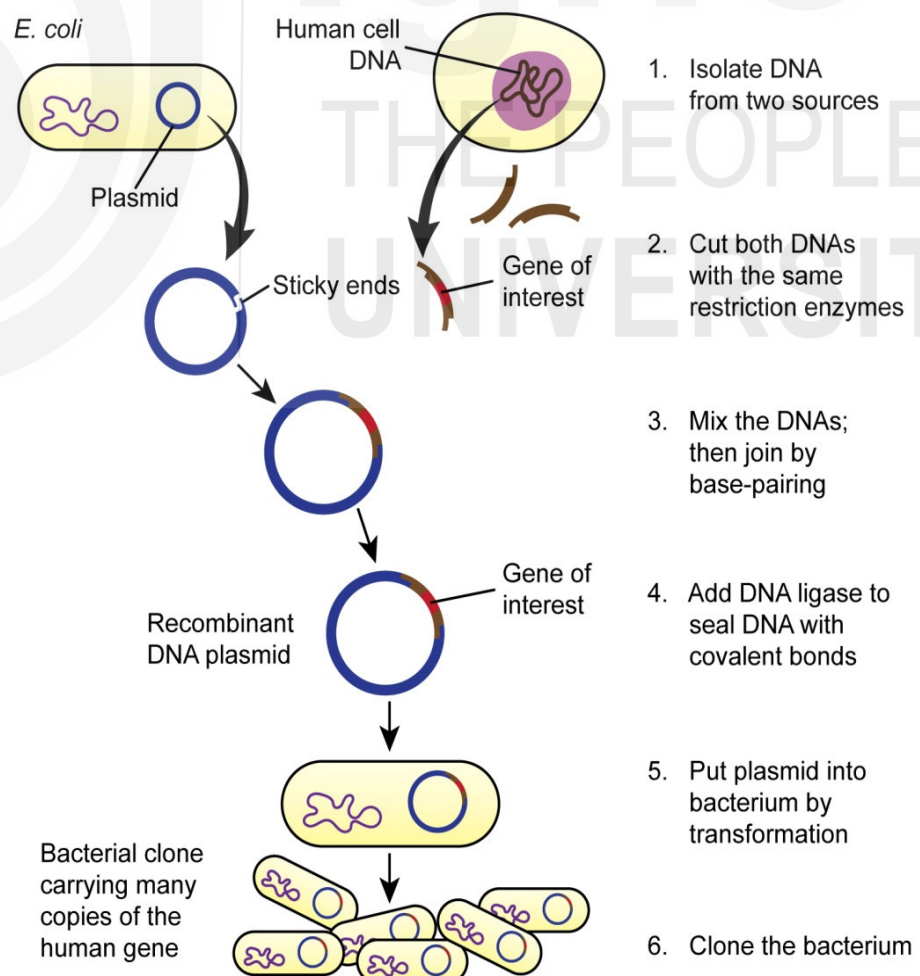


Fig. 14.8: Various steps involved in molecular cloning.

14.4.1 Recombinant DNA

The recombinant DNA (rDNA) technology helps develop species with characteristics or properties not naturally found in these species. The preparation involves four main steps:

1. DNA isolation
2. DNA splicing
3. Ligation of DNA
4. Transformation
5. Amplification of rDNA

1. Isolation of DNA

Both vector and donor DNA are isolated and purified using lysozymes, cellulase, chitinase, ribonuclease, and proteases.

2. DNA splicing

The purified DNA is incubated with the selected restriction enzyme. Restriction enzymes cut DNA. The restriction enzyme recognises the specific DNA sequence at specific locations to get the desired fragment. The cleavage occurs at these sites leading to the formation of sequences known as palindromes. Circular DNA opens up. The sticky or cohesive ends (points where the cut was made) of the donor DNA are compatible with the sticky ends of the vector DNA. The digested DNA fragments are separated using electrophoresis. The vector DNA is also processed using the same procedure.

3. Ligation of DNA

The sticky ends formed in both DNA fragments after using the restriction enzyme are complementary. Therefore, the two DNA fragments can be combined to form the new rDNA. The process of forming hydrogen bonds between complementary sequences on single strands to form double-stranded DNA is called **annealing**. Enzyme *DNA ligase* joins the DNA fragments. DNA ligase creates phosphodiester linkages between the fragments. The process of joining these two pieces together using the enzyme *DNA ligase* is '**ligation**'. The resulting DNA molecule is a hybrid of two DNA molecules (Fig. 14.9). The hybrid DNA molecule is also called a recombinant DNA molecule, and the technology is referred to as recombinant DNA technology. Proteins that are produced from recombinant DNA molecules are called recombinant proteins.

3. Transformation

The rDNA is inserted into a recipient host cell (bacterial cell). This process is called '**transformation**'. The transformation process generates a mixed population of transformed and non-transformed host cells. Marker gene of plasmid vector is employed in the isolation of recombinant cell from the non-recombinant cell.

4. Amplification

Before the transformation, DNA fragments that are not available in large amounts can be multiplied using amplification. A PCR reaction amplifies the sequence of DNA that can be ligated to a vector via a blunt or single-base overhang ligation. A single copy of DNA can be used to get millions of copies of DNA.

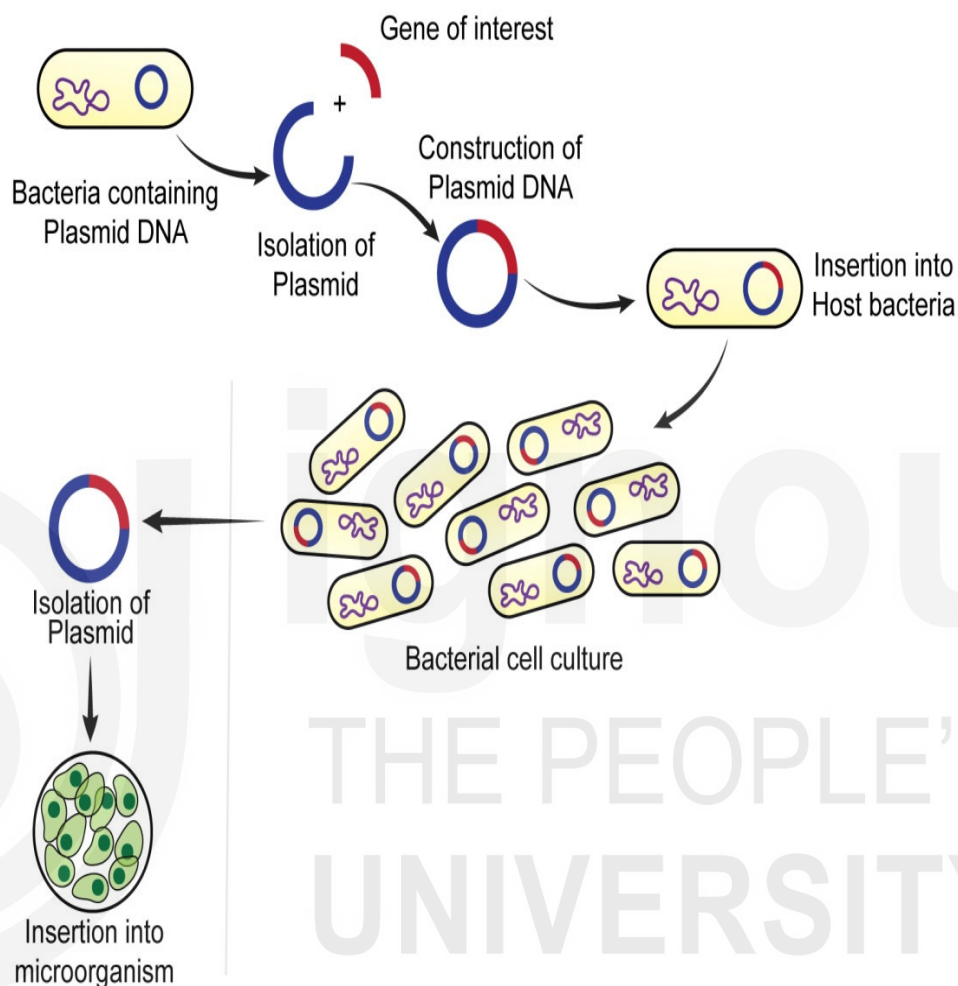


Fig. 14.9: Various steps are involved in formation of recombinant DNA.

Box 14.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) helps generate a large amount of DNA from a single DNA molecule. A rapid increase in the number of copies of specific regions of DNA can be obtained through PCR. Kary Mullis developed the technique in 1988. DNA polymerase enzyme replicates DNA with the help of short nucleotide sequences called primers that base pair for a specific portion of the replicated DNA (Fig. 14.10). With each round of replication, the number of DNA molecules increases exponentially. As a result, a high quantity of DNA can be obtained from a small number of template copies. For example, if amplified through 30 cycles of replication, a single DNA molecule gives about 2^{30} (Approx. 1 billion) DNA molecules.

PCR is used to identify the owner of a DNA sample left at a crime scene, perform paternity analysis, and comparison of small amounts of ancient DNA with modern organisms.

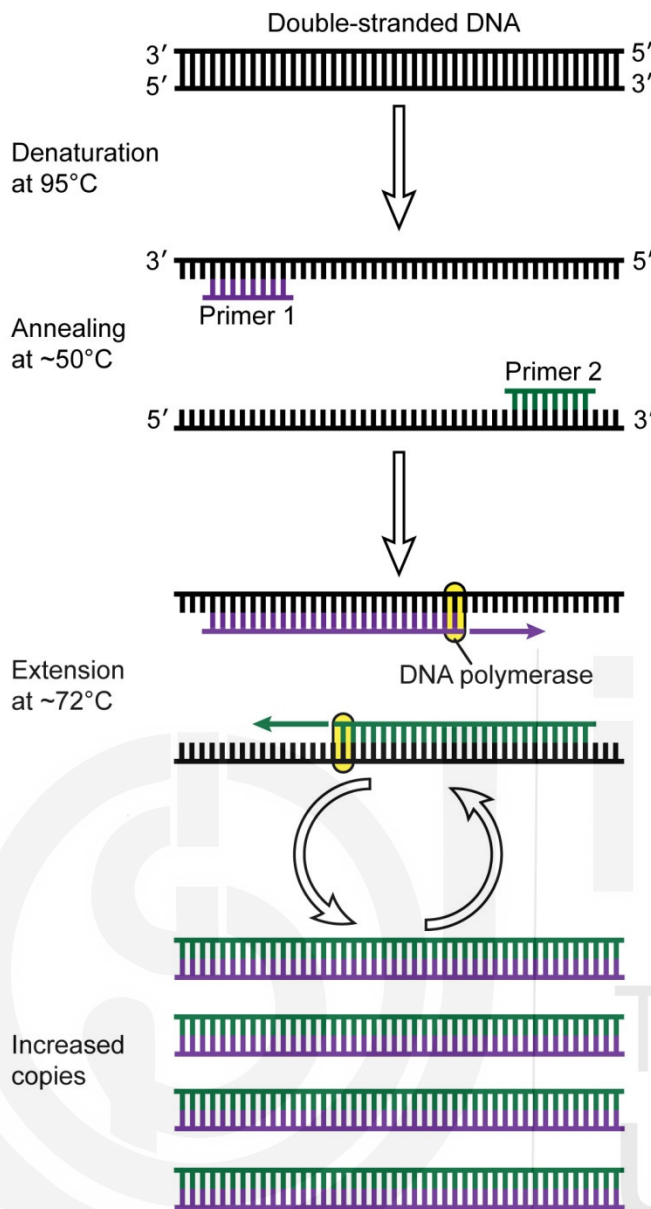


Fig. 14.10: Diagrammatic representation of various steps involved in Polymerase Chain Reaction.

Application of Recombinant DNA technology

- Recombinant DNA technology is widely used in medicine and research.
- The technology is used to identify, map and sequence genes and determine their function.
- This technology is also used to detect the presence of diseases such as HIV in a person.
- The technology can be applied in developing genetically engineered crops.
- The technique is useful in gene therapy for correcting gene defects that give rise to heredity diseases.
- Recombinant proteins generate antibody probes for examining protein synthesis within cells and organisms.

Limitations of Recombinant DNA technology

- Recombinant organisms contaminate the natural environment. Cross-contamination and migration of proprietary DNA between organisms can result in “gene pollution”. In addition, recombinant organisms are vulnerable to diseases, pests, and other agents that quickly wipe out the entire wild population.
- The ‘superweeds’ develop over time because hybridization transfers new genes to wild plants and introduces herbicide resistance, stress tolerance, and insect resistance traits.
- Human or animal consumption of rDNA modified plants may produce a toxic secondary metabolite or protein toxin, especially if the plants are engineered to resist an insect pest.

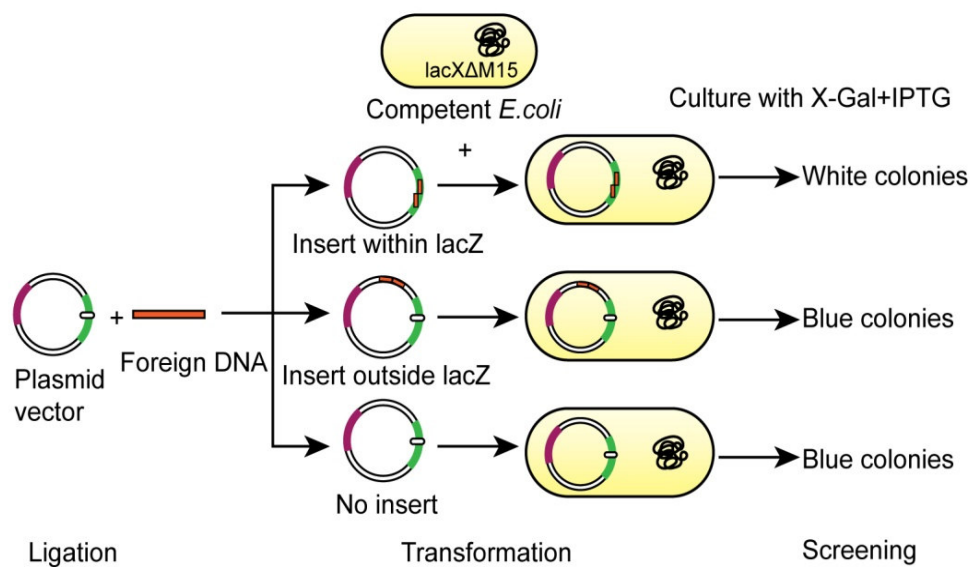
14.4.2 Transformation and Selection of Recombinant Clones

Bacterial transformation is a process of horizontal gene transfer that occurs when bacteria take up foreign DNA fragments. The foreign DNA fragments enter the cell and get integrated into the bacterial chromosome by non-reciprocal recombination. A stable transformation occurs when recombination is successfully working, and the host organism may express the inserted DNA fragment. The mixture of all cells is plated on a suitable medium, and the clone containing the recombinant DNA is selected. The transformed clone contains foreign DNA insert having the foreign DNA insert is identified on the selection medium. A marker gene produces a phenotype that helps quickly identify and select cells in which desired cloned insert is present. A selectable marker enables only such cells to survive that possess the cloned DNA insert and selection marker genes such as the kanamycin resistance gene under defined conditions. Scorable markers produce different phenotypes that allow easy identification of cells, for example-*gus* (β -galactourinidase) and *nos* (nopaline synthase).

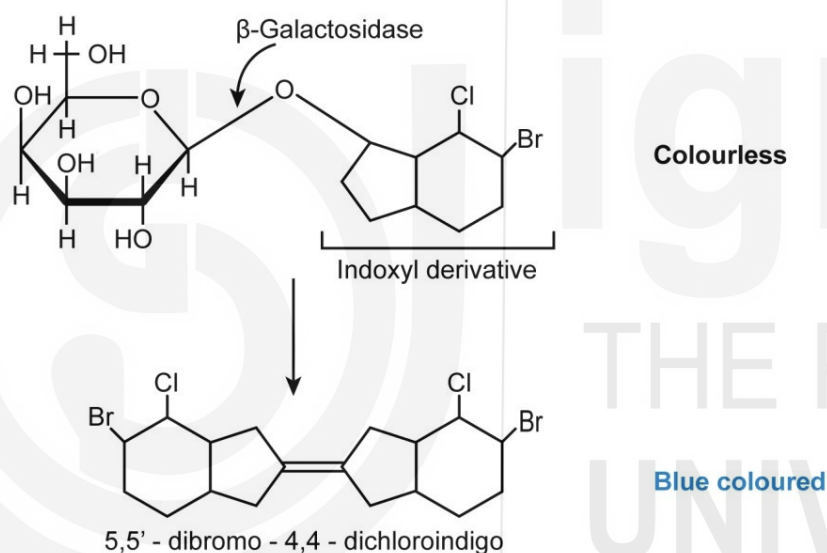
Various methods followed for the screening of transforming colonies have been listed below.

1. Blue-white screening

It is a rapid and efficient technique for identifying recombinant bacteria that depend on the activity of the enzyme β -galactosidase found in *E. coli*, this enzyme cleaves lactose into glucose and galactose. which is the product of the lac Z gene of the lac operon, For screening the clones containing recombinant DNA, a chromogenic substrate known as the X-gal is added to the agar plate. The chromogenic substrate detects enzymatic activity. The colourless compound X-gal or 5-bromo-4-chloro-3-indolyl- β -D-galactoside is used in the screening. It is a substrate for β -galactosidase (Fig. 14.11). If β -galactosidase is produced, X-gal is hydrolysed to form 5-bromo-4-chloro-indoxyl and galactose, which spontaneously dimerizes (oxidizes in the air) to produce an insoluble blue pigment known as 5,5'-dibromo-4,4'-dichloro-indigo. Therefore, the colonies formed by non-recombinant cells appear blue, while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured.



(a)



(b)

Fig. 14.11: a) Diagrammatic representation of Blue-white screening and; b) reaction involved in the chemical conversion of X-gal.

Isopropyl β -D-1-thiogalactopyranoside (IPTG) is used along with X-gal for blue-white screening. IPTG is a non-metabolizable analogue of galactose that induces the expression of the lacZ gene. IPTG is not a substrate for β -galactosidase but only an inducer. Therefore, a chromogenic substrate like an X-gal is required for visual screening purposes.

ii) Antibiotic sensitivity

A circular plasmid containing an antibiotic resistance gene can replicate the host cell plated on antibiotic-containing media. Cloning a fragment into this plasmid involves cutting the plasmid with restriction enzymes and ligating a fragment to create a circular plasmid with an insert. The only circularized clone will give colonies, whereas the cut plasmid will not grow as it has lost the antibiotic resistance gene (Fig. 14.12).

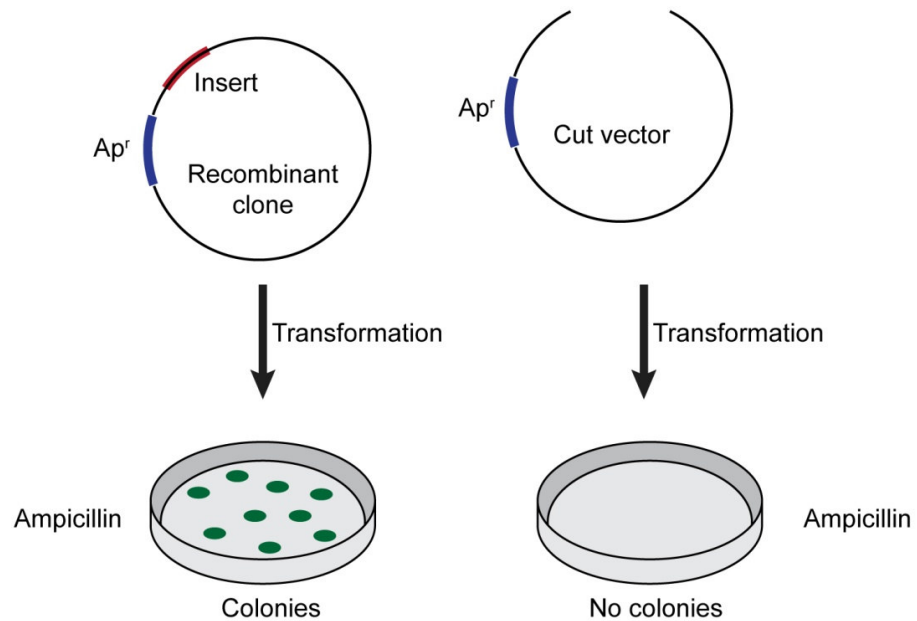


Fig 14.12: Diagrammatic representation of a selection of transforming colonies using Antibiotic sensitivity assay.

iii) Autotrophic yeast strain

The yeast vector has 4 different genes, His3, Leu2, Trp1 and Ura3, as a selectable markers. Yeast hosts with a mutation in these genes can be used with the yeast vector to screen the recombinant clone. Ura3 and Lys2 markers offer positive and negative selection (Fig. 14.13).

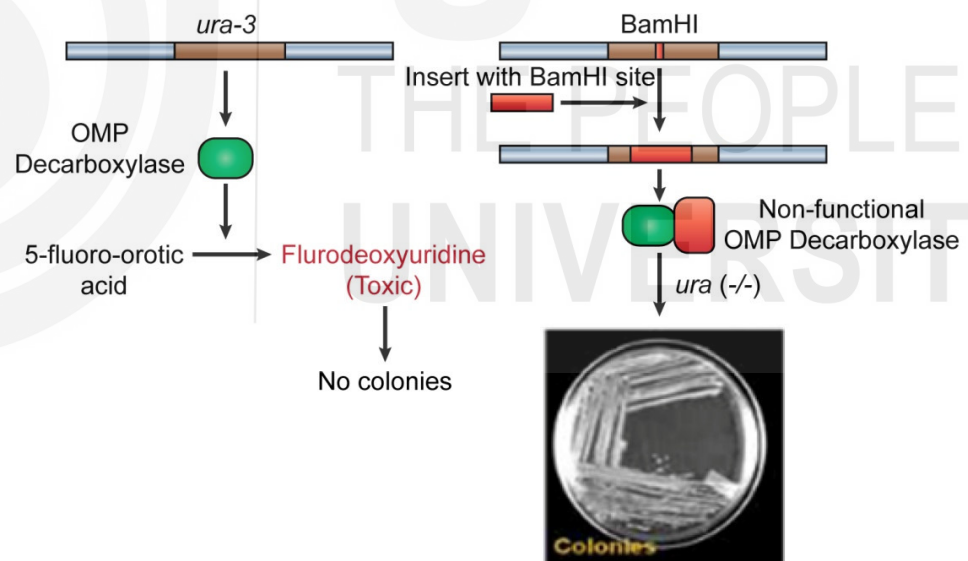


Fig 14.13: Diagrammatic representation of a selection of transformed colonies using growth on minimal medium.

Positive selection: In this type of selection, the host strain does not grow on the media lacking the functional gene, but the host transformed with the recombinant clone can supply the gene product required to grow in the media.

Negative selection-: In the negative selection, a chemical compound is added to the media, which will be converted to the cytotoxic agent in the presence of the gene product. Ura3 codes for orotidine-5'-monophosphate (OMP) decarboxylase and an active enzyme processes the 5-fluoro-orotic acid to the toxic fluorodeoxyuridine. The generation of this cytotoxic agent kills the cells carrying functional Ura3 gene products.

iv) Selection based on growth on minimal medium

An auxotrophic mutant with a non-functional *leu2* gene is used as a host in yeast. Such a mutant can survive if leucine is supplied in the growth medium. However, transformants can grow on a minimal medium (which contains no added leucine) due to the presence of the *leu2* gene in the vector.

A suitable vector possesses two marker genes, one of which is a selectable marker. For example- vector pBR 322 has genes for tetracycline (tet^R) and ampicillin (amp^R) resistance. The non-transformed cells get eliminated by plating them on a medium containing a selection agent.

iv) Reporter gene assay

This assay system creates a chimeric construct by cloning an enzyme gene and the gene of interest. Then, the green fluorescent protein is used to screen cells containing recombinant protein fluorescently tagged with the GFP at their c-terminus. The cell receiving recombinant DNA will give green fluorescence.

v) PCR based method

Polymerase chain reaction (PCR) based procedure helps select recombinant clones. First, the DNA insert is to be cloned and ligated to an antibiotic resistance marker gene. Then, the ligation gene construct is amplified by PCR, followed by cloning into a suitable bacterial vector. The selection of the antibiotic resistance coded by the PCR product ensures 100% insertion frequency.

SAQ 3

State whether these statements are 'True' or 'False'.

- a) The endonuclease enzyme cuts DNA at restriction sites producing blunt or sticky ends.
- b) The foreign DNA is then inserted into another DNA entity (a cloning vector) to form a new recombinant DNA molecule using enzyme DNA polymerase.
- c) Polymerase chain reaction helps in getting an increase in the number of copies of specific regions of DNA.
- d) Recombinant organisms are always safe for our environment.
- e) In the Blue-white screening method used to identify transformed colonies, the transformed colonies appear blue.

14.5 CONSTRUCTION OF GENOMIC AND CDNA LIBRARIES

The process of dividing genomic DNA into clonable elements and inserting them into host cells creates a library (gene bank, or genomic library). Gene libraries are of two types - genomic libraries and cDNA libraries. These two libraries have the same principles but are created differently and have many different aspects.

14.5.1 Genomic Library

A genomic library contains all the DNA sequences present in an organism's genome. In other words, the genomic library is representative of the organism's entire genome. The desired sequence (target DNA, or gene of interest) is a small portion of the total chromosomal DNA in a prokaryote. Vectors are used to create genomic libraries that accommodate large inserts. These libraries are significant as they contain both coding and non-coding regions.

Various steps involved in the construction of a genomic library include:

a) Isolation of target DNA

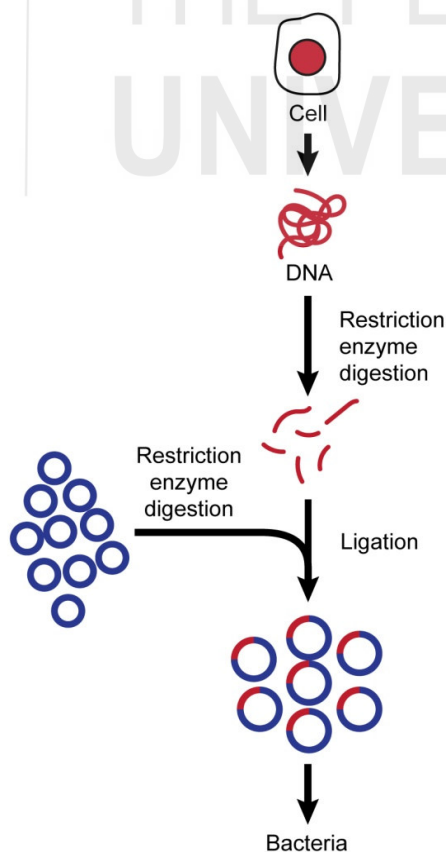
Genomic libraries can be constructed by isolating complete DNA from bacteria, viruses, plants, and animals. The isolated DNA is purified by using cesium chloride and other available methods.

b) Restriction Fragments

DNA is fragmented using mechanical shearing or restriction enzymes. Specific restriction endonuclease enzyme cleaves the DNA approximately once in every 256 bp. The resulting DNA fragments of suitable size are inserted in the appropriate cloning vector. Once the DNA is located within the vector, the vector is inserted into a bacteriophage to be amplified.

c) Cloning the fragments in a vector

The digested DNA sample is subjected to electrophoresis. Target DNA fragments are then cloned in suitable vectors like lambda or cosmid vectors and maintained as a library. Finally, the cloned DNA is isolated again, and the process is repeated until the organism's entire genome gets inserted into the genomic library (Fig. 14.14).



d) Screening of Genomic library

The genomic library can be screened for clones by hybridisation with a probe, western blotting to detect protein products, and a screening of protein activity.

Genomic libraries offer many advantages, such as studying gene regulation or targeting a particular mutation. In addition, the large amounts of data allow researchers to understand better how mutations located outside of the coding region of a gene affect the organisms.

14.5.2 cDNA

A DNA that is complementary to a given RNA serves as a template for synthesising the DNA in the presence of reverse transcriptase. The enzyme reverse transcriptase synthesises double-stranded DNA, a complimentary copy of the mRNA. The addition of linker sequences to the end of this DNA, containing the restriction site, followed by treatment with a restriction enzyme, produces a cDNA with cohesive ends ready for insertion into a vector. cDNA molecules to be cloned are only a few kilobases long and easily inserted into a plasmid cloning vector. cDNA library is representative of the organism's exonic regions, meaning that only the coding regions of the genome are recorded and stored in the library.

Procedure for creating a cDNA library

Various steps involved in the formation of the cDNA library are as follows;

- mRNA is isolated and subjected to reverse transcriptase treatment to generate the double-stranded cDNA corresponding to the mRNA sequence.
- The ends of DNA are cleaved, and the single-stranded DNA is then inserted into a selected vector using DNA ligase.
- The vector is then inserted into a bacteriophage and amplified. After amplification, the cDNA is isolated and collected in the cDNA library (Fig. 14.15).

The cDNA library is much smaller than that of genomic libraries as it only represents the exonic portion of the organism's genome. cDNA libraries offer a unique approach to studying variant mutations present within coding regions of a gene. In addition to studying variant mutations located within a gene, cDNA libraries also offer a more convenient approach to studying protein function, interaction and expression.

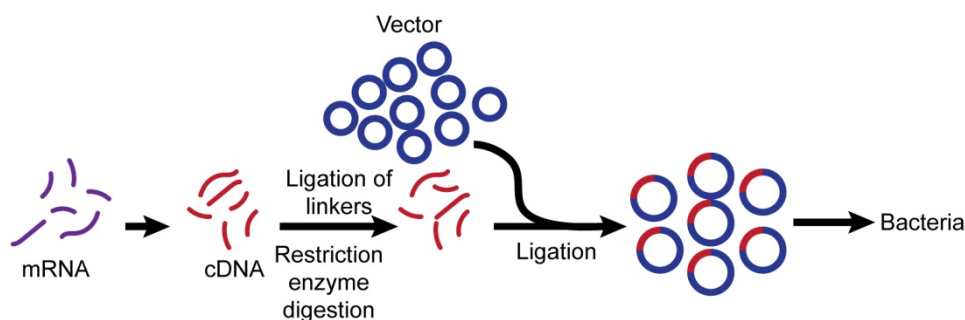


Fig. 14.15: Diagrammatic representation of various steps in the formation of the cDNA library.

Screening of cDNA library

The cDNA libraries consist of probably many thousand clones in the form of either plaques or colonies on a plate. Screening of libraries can be done by detecting a particular DNA sequence or gene expression. Various techniques are used to screen the gene libraries of interest.

Nucleic acid hybridisation is the most commonly used method of library screening. Grunstein and Hogness, in 1975, developed the technique to detect DNA sequences in transformed colonies using radioactive RNA probes. It is based on a single-stranded DNA molecule, used as a probe that can hybridise to its complementary sequence and identify the specific sequences. This method is quick and can handle a large number of clones. It is used to identify cDNA clones that are not full-length (cannot be expressed). Two hybridisation methods are recognized: (i) Colony hybridization; (ii) Plaque hybridisation.

Other techniques of screening include the use of labelled oligonucleotide probes. This technique relies on the peptide sequence rather than the DNA sequence. Subtractive screening (also called differential screening) helps examine this unique expression of DNA. This method is capable of identifying novel gene expression of mRNA. Finally, PCR screening is employed to identify rare DNA sequences in complex mixtures of molecular clones by increasing the abundance of a particular sequences. It is possible to identify any clone by PCR only if there is available information about its sequence to design suitable primers.

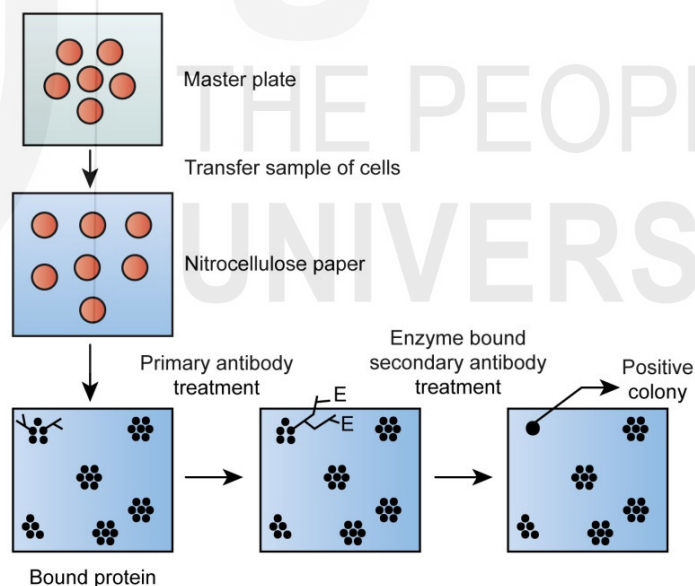


Fig. 14.16: A schematic representation of the immunological screening of colonies.

Immunological screening is another method of screening a gene library. In this method, primary and secondary antibodies are used to identify colonies of interest. The process involves using antibodies that specifically recognise antigenic determinants on the polypeptide. The samples are first treated with a primary antibody followed by a radiolabelled secondary antibody. The cells are grown as colonies on master plates, transferred to a solid matrix and subjected to lysis, releasing the proteins which bind to the matrix (Fig. 14.16). Next, the proteins are treated with a primary antibody that specifically binds to

the protein (acts as antigen), encoded by the target DNA. The unbound antibodies are removed by washing. The secondary antibody recognises the region of the primary antibody. It is conjugated to a simple enzyme (horseradish peroxidase or alkaline phosphatase) that can be measured using a colorimetric change or light emission using an X-ray film. After the binding of the secondary antibody to the primary antibody, a colourimetric reaction occurs. The secondary antibody carries an enzyme label that converts the colourless substrate to a coloured product. A colony that shows a positive response is identified and subcultured. The positive colony carries insert DNA and encodes the protein that binds the primary antibody.

In this method, raising a specific antibody for each protein to be detected is difficult. Moreover, it is a lengthy and costly procedure and can only be carried out successfully with proteins produced in reasonably large amounts.

14.6 COLONY HYBRIDISATION

A method that helps identify many colonies carrying different hybrid plasmids containing a specified DNA sequence of genes is called colony hybridisation. It is also known as replica plating, which allows screening colonies plated at high density using radioactive DNA probes. The colonies are to be screened on nitrocellulose filters. This method can be used to screen plasmid or cosmid-based libraries. This technique identifies the DNA of interest using a radiolabelled probe that binds to the target sequence.

During DNA fragmentation, cutting DNA with restriction endonucleases results in the formation of DNA fragments. The cloning of the fragments is done with the help of a cloning vector. The bacteria carrying the DNA fragments in a chimeric plasmid are seeded at low density on agar plates. The plating colony of host cells is plated onto solid medium containing antibiotics. The presence of antibiotics in the medium ensures the growth of only transformed cells due to the antibiotic resistance gene in their plasmid. When a discrete colony is formed on the plate, it is transferred onto nitrocellulose or nylon membrane (commonly referred to as solid matrix). A replica plate is established from the master plate when the colonies are established by pressing a membrane filter. A nylon filter paper replicates a master plate containing colonies (Fig. 14.17). The cells from the colonies get transferred from the master plate onto the nylon paper. The filter paper is treated with an alkaline solution to lyse the cells and denature the DNA.

Lysed and deproteinised cells release DNA. After denaturation of the DNA on the nitrocellulose filter, it is hybridised with a labelled DNA or RNA probe. The probes hybridise with DNA from the lysed cells. The labelled DNA probe facilitates hybridisation between the target DNA and DNA probe. The unbound probe is washed off from the matrix. The matrix is then subjected to autoradiography to determine the location of cells containing hybridised DNA. The filter is autoradiographed, and the colonies containing the desired DNA molecules are identified. The filter paper is exposed to X-ray, which helps to visualise the target. The development of a dark spot on the X-ray film indicates the presence of target DNA (gene). The dark spot on the X-ray film corresponds to the cell colony on the master plate. Once a transformed cell

colony is identified, it is then sub-cultured and maintained as it carries cloned DNA of interest. In this way, screening of colonies is done to identify and isolate the colony which contains the desired gene.

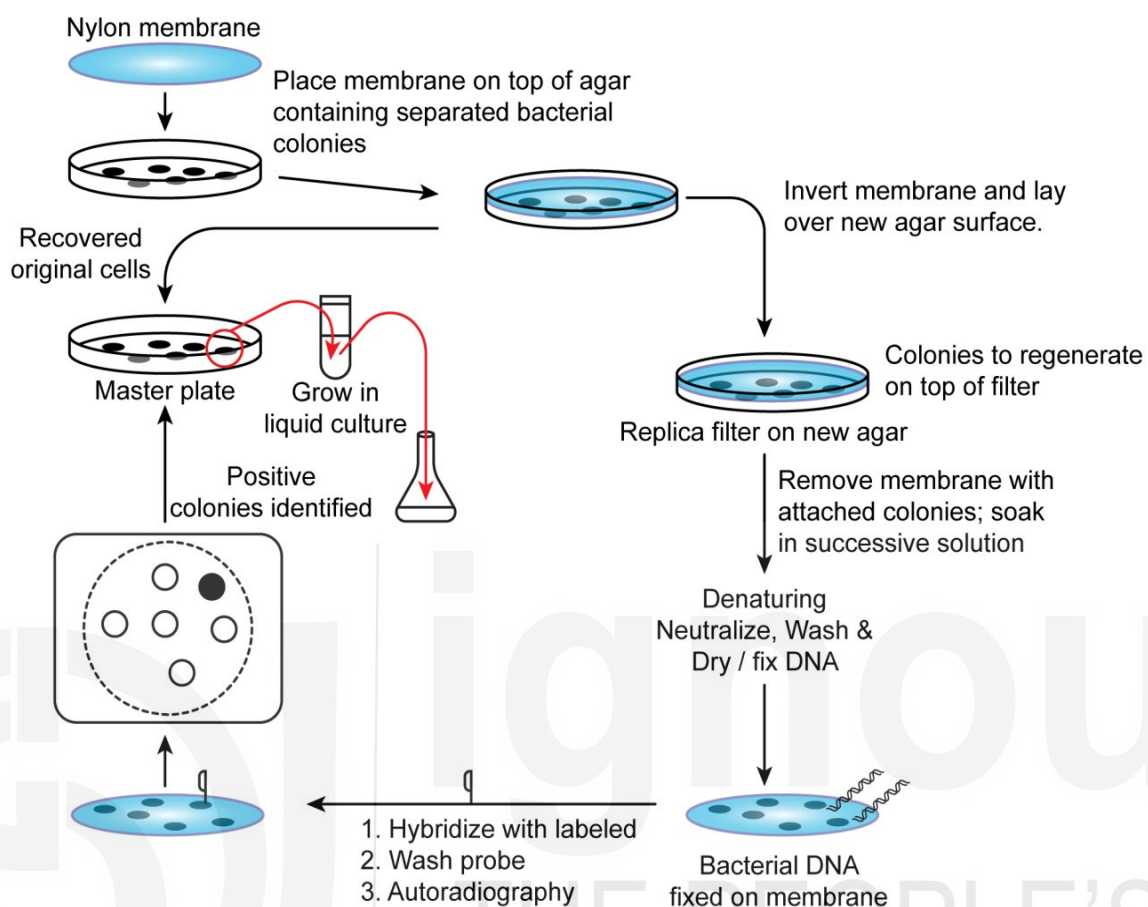


Fig. 14.17: Diagrammatic representation of various steps in a screening of colonies via colony hybridization.

Plaque Hybridisation

A genomic library is screened by infecting phage viruses containing inserted DNA on a lawn of bacteria. Plaque hybridisation, also known as Plaque lift, was developed by Benton and Davis in 1977 and employs a filter lift method applied to phage plaques. This procedure is successfully applied to isolate recombinant phage by nucleic acid hybridisation and probably is the most widely applied library screening method.

The nitrocellulose filter is applied to the upper surface of agar plates, making direct contact between plaques and the filter, the plaques containing phage particles and recombinant DNA bind to the filter. The DNA is denatured, fixed to the filter, hybridised with radioactive probes and assayed by autoradiography (Fig. 14.18). A large number of recombinant clones can be screened for the presence of sequences homologous to the probe by this method. Instead of RNA probes, DNA or synthetic oligonucleotide probes are used to identify a clone from a genomic library. The labelling of probes is done by incorporating radioactive or other markers into the molecule. Chemical labels like digoxigenin or biotin are also incorporated into the probe and can be detected with a specific antibody or the ligand streptavidin

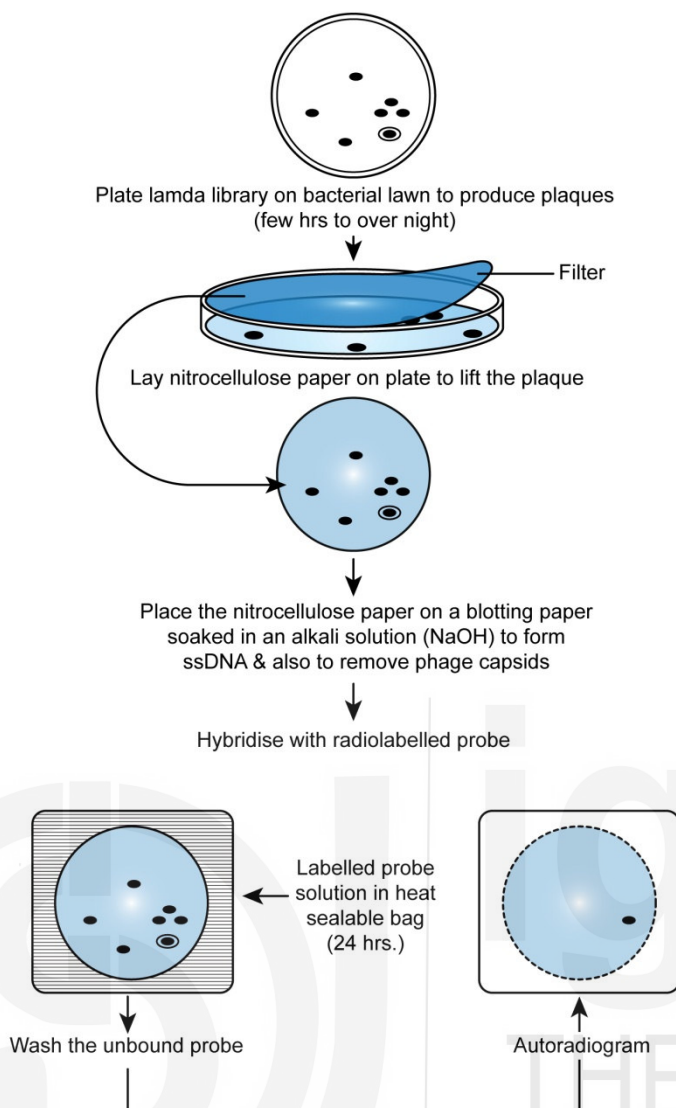


Fig. 14.18: Diagrammatic representation of various steps in the screening of colonies via plaque hybridization.

SAQ 4

Fill in the blanks:

- DNA that is complementary to a given RNA is called
- Short nucleotide sequences of DNA are used in the screening of genomic libraries are called as
- The technique involves using antibodies that specifically recognise antigenic determinants on the polypeptide, called
- A method successfully applied to isolate recombinant phage by nucleic acid hybridisation is called
- An alternate name for colony hybridisation is

14.7 METHODS OF GENETIC ENGINEERING

The process by which an organism's genetic material (genotype) is modified to get the desirable characteristic (phenotype) changes is referred to as genetic

engineering. The new fragments of DNA are inserted or deleted, or existing DNA is edited by targeting a specific sequence and random mutagenesis using chemicals, UV radiation, or transposons. In other words, genetic engineering uses recombinant DNA technology to modify an organism's DNA to achieve desirable traits. An organism that receives recombinant DNA is a genetically modified organism (GMO). The host organism with foreign DNA introduced into it is called transgenic. Bacteria, plants, and animals have been genetically modified since the early 1970s.

The procedure of genetic modification includes the following steps:

- i) First, identify the characteristic desired for the organism.
- ii) Then, isolate the characters from the organism that displays that trait.
- iii) Next, insert that characteristic into the desired organism.
- iv) Propagate the organism.

14.7.1 Transformation

The methods used for the transfer of genes include:

- 1) Vector-mediated gene transfer, which includes *Agrobacterium*-mediated plant transformation.
- 2) Direct (or vector less) DNA transfer such as particle bombardment

A gene of interest is isolated from the organism using restriction digestion or amplified by the polymerase chain reaction. Recombinant DNA is constructed by inserting a gene of interest into the plasmid (such as Ti plasmid). The T-plasmid is inserted into the *Agrobacterium*. The recombinant DNA can also be integrated into the genome directly via electroporation and microinjection. After that, the infected and transformed plant species are cultured.

***Agrobacterium*-mediated transformation**

This method uses a 'vehicle' or vector to transfer foreign genetic material from one cell to another. *Agrobacterium tumefaciens* is used to insert recombinant DNA into the plant cell. *Agrobacterium* leads to the development of tumors in plants through a process known as **horizontal gene transfer**. *Agrobacterium tumefaciens* is responsible for causing a disease called crown gall disease. The bacterium transfers DNA or T-DNA from the bacterium into the plant. The T-DNA makes its way into the nucleus of the plant and gets randomly integrated into the plant genome. A gene of interest is inserted into the Ti-plasmid of the *Agrobacterium*. Once integrated, the genes located within the T-DNA region express, causing prolific cell division, resulting in the formation of a tumor (Fig. 14.19). This bacteria culture infects the plant cells, and the transformed cells are regenerated using the plant tissue culture methods.

In 1977, Zambryski and his coworkers engineered the tumour-inducing (Ti) plasmid (the circular genetic material that contains the T-DNA fragment in *Agrobacterium tumefaciens* by replacing the natural T-DNA fragment with a different gene of interest. They demonstrated that it was possible to transfer the engineered T-DNA into a plant cell. It was the first instance where scientists showed cloning a GOI into the T-DNA region and transforming plant

cells under laboratory conditions. For example, the cytochrome P450 gene is introduced into pineapple, allowing the plant to degrade persistent organic pollutants from the environment. This method has been applied across many different species of plants.

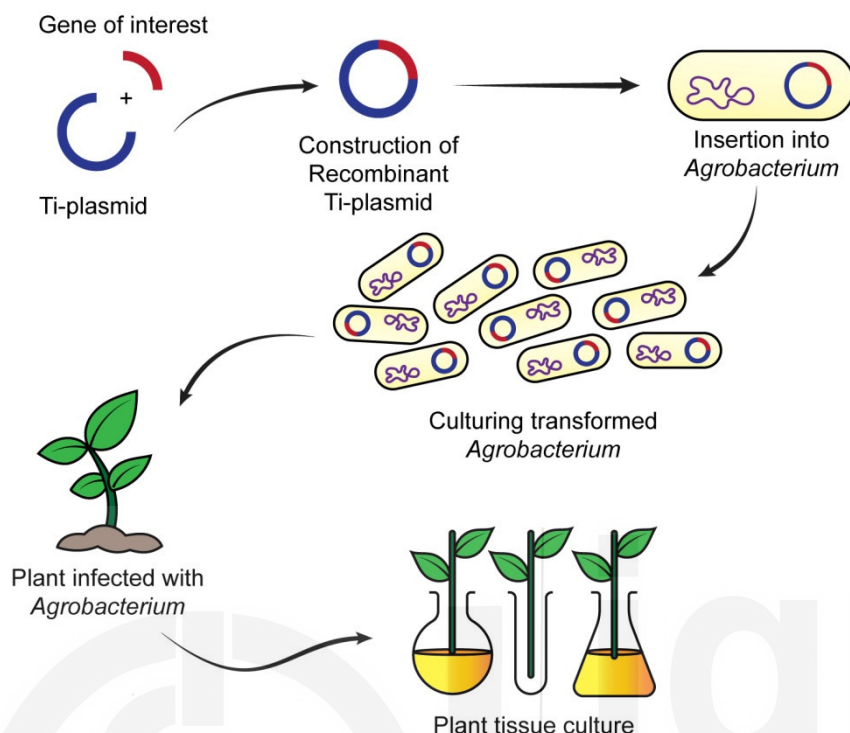


Fig. 14.19: A diagram showing the various steps of *Agrobacterium*-mediated transformation.

Direct transfer

Transporting the recombinant DNA into the recipient cell or the host genome is done via various other methods. The direct method is based on introducing DNA into plant cells without the use of a biological agent. Physical methods such as heat or electrical shock transfer plasmid DNA into bacteria.

Microinjection is a process in which a sharp needle is used to insert DNA directly into the nucleus of a cell. The protoplasts are used in this process. This method can achieve a transformation frequency of about 14 to 66%. Another method for inserting recombinant DNA into the host genome is electroporation, which involves permeabilising the cell with an electrical current.

Sonication is yet another suitable method in which ultrasonic waves are used to transfer genes in which the recombinant DNA is inserted into the target cell. In liposome-mediated gene transfer, an artificial cell-like outer coat known as a liposome is used to insert recombinant DNA into the host genome. Gene transfer using bacterial infection is one of the popular methods used in plant genetic engineering. In this method, the plant species is infected with the transformed bacteria to insert a gene of interest.

In Particle Bombardment (“biolistics”) method, a gene gun is used to insert DNA particles into a cell. Tungsten or gold particles (1-2µm) called microprojectile coated with DNA are used for transformation at accelerated velocities to enable the entry of DNA into plant cells. Acceleration is provided

by explosive charge or shock waves generated by high voltage electric discharge. DNA can adhere to microscopic particles of metal, such as gold, silver, or tungsten. The DNA-coated metal particles are placed inside the gene gun, and these particles are shot into the targeted cell at a very high speed (~1,000-2,000 feet/second). The technique was developed in 1987. This approach bombards plant cells with DNA-coated particles. Once inside the plant cell, the particles release the DNA, which can be integrated into the genome. The excised tissues are transformed and placed on selective nutrient media after that. The engineered T-DNA fragment or DNA fragment bombarded into a plant cell is a selectable marker gene. This can include genes that confer antibiotic resistance, such as kanamycin (*nptII*) or hygromycin (*hpt*), or herbicide resistance, such as glufosinate (*bar*). Adding the selecting agent (antibiotic or herbicide) to the media allows for selecting transformed (or transgenic) plants.

Specific chemical agents such as PEG (Polyethylene glycol), polyvinyl alcohol, and calcium phosphate helps in the uptake of DNA by plant protoplasts. PEG and calcium phosphate precipitate DNA on the outer surface of the plasma membrane.

Application of genetic engineering

The technique of genetic engineering has been applied in various areas. Some of the major ones have been listed below.

Medicine

Low-cost drugs, hormones, enzymes, and vaccines are created using genetic engineering tools. Insulin is a classic example of a therapeutic protein designed using genetic engineering technology. Using recombinant DNA technology, scientists have created a unique type of vaccine that only contains the DNA for viral coat protein; thus, the pathogen can never be activated again. Vaccines against smallpox, herpes simplex virus and hepatitis are produced using genetic engineering.

Gene therapy

The technique of gene therapy can be used to treat inherited genetic disorders. A faulty, non-function or mutated gene is replaced with the wild type in the technique. Gene therapy has proved helpful in treating both hereditary and infectious diseases. Some include cancer, cystic fibrosis, heart disease, diabetes, haemophilia, muscle disorder called spinal muscular dystrophy and AIDS.

Agriculture

Genetically modifying economically important food crops is another application of genetic engineering technology. The crops are genetically modified to increase their nutrient, protein-content imparts disease, stress resistance and tolerance against abiotic stresses. For example, Flavr Savr tomato, a genetically modified tomato species, has significant economic value as it can easily be transported from one place to another. In addition, the insecticides resistance plant species such as tobacco, potato, corn, and cotton have been developed by genetic engineering. The quality of other crops such as cotton,

corn, and soybeans has been improved using recombinant DNA technology. In agriculture, America, about 71.5 million hectares of land were planted with GM crops in 2019. In addition, plants capable of generating their fertilisers have also been developed using genetic modification techniques.

Environment

Genetic engineering is being used to exploit the potential of microorganisms and plants to restore the environment. Several genetically engineered microorganisms (GEMs) are developed that assist in the degradation of waste materials. For example, Chakrabarty and coworkers developed a strain of *Pseudomonas* named "Superbug" in the 1970s. This superbug produced enzymes that helped degrade several hydrocarbons present in petroleum. Recombinant strains of *Pseudomonas* have been developed, which can treat/transform several toxic chemicals such as hydrocarbons, chlorinated compounds, solvents, polychlorobiphenyls and metals.

Efforts are being made to reduce atmospheric CO₂ levels by improving the efficiency of the enzyme ribulose biphosphate carboxylase (RUBP-case), which is primarily involved in CO₂ fixation. Genetically modified organisms are used to clear up oil spills, a major environmental hazard. Attempts have been made to develop bacterial and viral pesticides, which will help in reducing the use of chemical pesticides.

SAQ 5

Fill in the blanks with suitable words:

- helps in the identification of a large number of colonies carrying different hybrid plasmids containing a specified DNA sequence or genes.
- filters are used to screen recombinant colonies.
- The organism having foreign DNA introduced into it is called.....
- bacterium leads to the development of tumors in plants.
- The bacterium transfers..... into the plant.

14.7.2 CRISPR-Cas-9

CRISPR-Cas is a new technology of genetic engineering. CRISPR is a powerful tool for editing genomes. It is used to alter DNA sequences and modify gene function. The genetic material of viruses, bacteria, cells, plants, and animals can be altered in a relatively simple, accurate and efficient way by using this technology. The technology works by changing characteristics through genetic alterations or adding entirely new genetic information.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) include DNA sequences or specialised stretches of DNA found within the genomes of prokaryotic organisms such as bacteria. Cas-9 is a protein - Cas stands for

"CRISPR-associated" enzyme — that acts like a pair of molecular scissors capable of cutting DNA strands. CRISPR describes a region of DNA made up of short, repeated sequences with so-called "spacers" sandwiched between each repeat. When the components of CRISPR are transferred into other, more complex organisms, those components can then manipulate genes, a process called "gene editing."

Crispr sequences are derived from DNA fragments of viruses that have infected prokaryotes. The bacteria capture leftover DNA from invading viruses and use them to create segments known as CRISPR arrays. These arrays allow the bacteria to produce RNA segments that target virus DNA. The bacteria use the CRISPR-associated protein 9 (Cas-9) enzyme to cut DNA and disable the virus. Cas 9 protein cuts foreign DNA. It uses CRISPR sequences as a guide to recognise and cleave specific strands of DNA complementary to CRISPR sequences. Crisper sequences detect and destroy DNA from similar viruses after infection.

In 1987, a Japanese team of scientists at Osaka University noticed a strange pattern of DNA sequences in a gene belonging to *E. coli*. The gene had five short repeating segments of DNA separated by short non-repeating 'spacer' DNA sequences. All five repeating segments had identical sequences composed of 29 bases. By contrast, each of the 'spacer' sequences had its unique sequence, composed of 32 bases. Such a pattern was not seen before. By the 1990s, they discovered that this pattern was prevalent in many different microbial species. This typical pattern was given the name: 'clustered regularly interspaced short palindromic repeats or CRISPR'. The term was coined by a team of Dutch scientists Rudd Jansen in 2002. He noted that another set of sequences always accompanied the CRISPR sequence. This second set of sequences they dubbed 'Cas genes'. The Cas genes were found to code for enzymes that cut DNA. CRISPR technology was first discovered by Danisco researchers Rodolphe Barrangou and Philippe Horvath in 2007. Erik Sontheimer and Luciano Marraffini thought that CRISPR could act as an all-purpose genome-editing tool at Northwestern University.

CRISPR technology was adapted from the natural defense mechanisms of bacteria and archaea. These organisms use CRISPR-derived RNA and Cas proteins to tackle attacks by viruses. The organisms chop up the viral DNA and then put away bits of DNA in their genome, which is used as a weapon against the foreign invaders that can attack bacteria again. Eugene Koonin and colleagues 2008 demonstrated the working of the CRISPR/Cas-9 mechanism for the first time. Whenever bacteria confront an invader, they copy and incorporate its DNA segments into their genome as 'spacers' between the short DNA repeats in CRISPR. The segments in the 'spacers' provide a template for the bacteria's RNA molecules to recognise any future DNA of an incoming virus and help guide the Cas 9 enzyme to cut it up to disable the virus. In August 2012, Jennifer Doudna and Emmanuelle Charpentier published a paper showing how to harness the natural CRISPR-Cas-9 system as a tool to cut any DNA strand in a test tube. Later on, many researchers demonstrated the CRISPR/Cas-9 system's ability to edit genomes in human cells. In 2013, Feng Zhang and George Church discovered the therapeutic applications of CRISPR in disorders of the nervous system using mice and human cells.

In 2017, a team of researchers led by Mikihiro Shibata of Kanazawa University in Japan and Hiroshi Nishimasu of the University of Tokyo showed the action of CRISPR. They found that a set of repeating DNA segments found in this bacterium contains an immune system that records copies of DNA segments from viruses from first contact. When they come into contact with the virus a second time, the spacers between the bacterial segments effectively cut up the DNA segments of the virus, rendering that virus useless. The findings concluded that these repeating spacers could be programmed to alter genetic material from most living things, whether humans, animals, or plants. CRISPR finds the genetic data wish to alter and replaces it with new material. The importance of the CRISPR/Cas-9 was recognised with the Nobel Prize in Chemistry award to Jennifer Doudna and Emmanuel Charpentier in 2020.

The three key components that allow CRISPR to work effectively are **Guide RNA**. A small fragment of RNA with a short guide sequence binds to a specific target sequence of DNA in a genome. RNA also binds to the enzyme Cas-9. The modified RNA is used to recognise DNA sequence and the Cas-9 enzyme cuts DNA at the targeted location (Fig. 14.20).

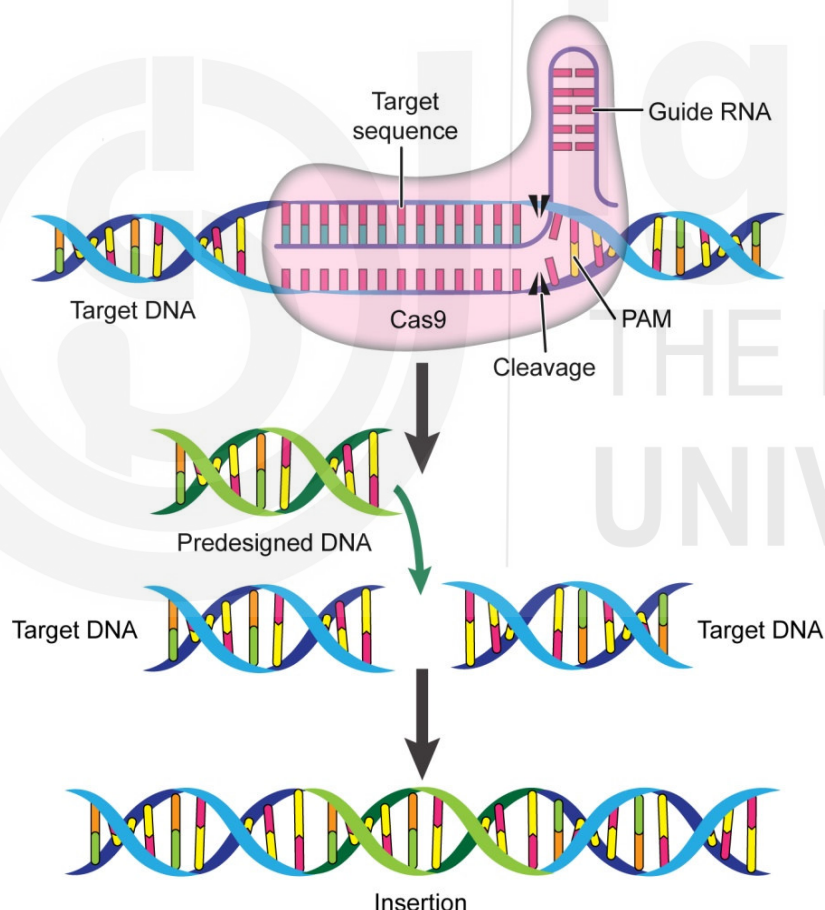


Fig. 14.20: Schematic representation of CSIPR-Cas technique.

Importance and application

CRISPR/Cas-9 is one of several gene-editing techniques that exhibit a high degree of flexibility and accuracy while also being very cost-effective and time-efficient. In addition, it allows for the introduction or removal of more than one gene at a time. Hence, it is possible to quickly manipulate many different genes in a cell line, plant or animal, reducing the process from years to weeks.

The technique is already being explored for many applications in fields ranging from agriculture to human health. The technology has been applied for basic biological research, development of biotechnology products and treatment of diseases. For example, Danisco applied the CRISPR/Cas-9 system in 2008 to improve the immunity of bacterial cultures against viruses. Many food manufacturers now use the technology to produce cheese and yoghurt. Since then, the technology has been used to delete, insert and modify DNA in human cells and other animal cells grown in Petri dishes. Scientists are also using it to create transgenic animals such as mice, rats, zebrafish, pigs and primates. Between 2014 and 2015, scientists successfully used the technique in mice to eliminate muscular dystrophy, cure rare liver diseases, and make human cells immune to HIV. It is also being investigated with pluripotent stem cells to provide human organs from transgenic pigs. Such work is directed towards helping solve some of the shortage of human organs for transplant operations and overcome some of the side-effects caused by organ transplantation, such as graft-versus-host disease. The technology is also being investigated to genetically engineer insects to wipe out insect-borne diseases such as malaria, which is transmitted by mosquitoes, and Lyme disease, which is transmitted by ticks. In addition, it could cover the way to the development of new treatments for rare metabolic disorders and genetic diseases ranging from haemophilia to Huntingdon's disease. The technology is also being investigated for gene therapy. Such therapy aims to insert normal genes into the cells of people who suffer from genetic disorders such as cystic fibrosis, haemophilia or Tay Sachs.

In agriculture, it could help design new grains, roots and fruits. Its potential applications include correcting genetic defects, treating and preventing the spread of diseases, and improving the growth and resilience of crops.

Limitations

CRISPR/Cas-9 is a potent tool, but it has important limitations:

- First, it is challenging to deliver the CRISPR/Cas-9 material to mature cells in large numbers, which remains a problem for many clinical applications. As a result, viral vectors are the most common delivery method.
- It is not 100% efficient and “off-target” edits, so even the cells that take in CRISPR/Cas-9 may not have genome editing activity.
- Chinese group reported the first application of CRISPR/Cas-9 to (non-viable) human embryos. A major bioethical debate is how CRISPR/Cas-9 should be used to alter 'germ-line' cells - eggs and sperm - responsible for passing genes on to the next generation.
- The issue is safety. Many scientists caution that the technology still needs much work to increase its accuracy and make sure that changes made in one part of the genome do not introduce changes elsewhere which could have unforeseen consequences. Nevertheless, it is a critical issue when using technology for applications directed toward human health. Another critical issue is that once an organism, such as a plant or insect, is modified, it is difficult to distinguish from the wild-type and could endanger biodiversity once released into the environment.

SAQ 6

Answer in one word.

- a) The term is used for the manipulation of genes.
 - b) In 2020 Jennifer Doudna and Emmanuel Charpentier were given the Nobel Prize in Chemistry for the importance of this technology.
 - c) They demonstrated the working of the CRISPR/Cas 9 mechanism for the first time.
 - d) The full form of CRISPR.
 - e) They discovered CRISPR technology.
-

14.8 SUMMARY

- The genetic engineering technique is used in molecular cloning and transformation.
- Recombinant DNA technology includes alteration in the genetic material of an organism to get enhanced and desired characteristics in living organisms or their products. Manipulation in an organism's genome is mainly carried out by introducing one or several new genes and regulatory elements. When introduced into another DNA segment, the desired DNA fragment leads to the formation of recombinant DNA. The technique of getting recombinant DNA is known as genetic engineering.
- The cloning of DNA is possible with the help of another DNA molecule capable of replicating inside the host, known as a vector or cloning vehicle. The vector can be a plasmid, a bacteriophage, a cosmid, a phagemid or even a virus. For getting clones or copies of DNA, the DNA needs to be cut at a different site. The cleavage of DNA is brought by specific enzymes known as restriction enzymes.
- Different types of cloning vectors are used. The choice of vector also depends on the type of host cell (prokaryotic/eukaryotic) and the size and type of DNA to be cloned (smaller /larger fragments). The prokaryotic vectors include plasmids, bacteriophages, phagemids, cosmid and fosmid vectors. Eukaryotic vectors comprise vectors for yeast, animal and plant cells.
- The production of exact copies (*clones*) of a particular gene or DNA sequence using genetic engineering techniques is called cloning. First, isolate DNA [gene of interest] fragments to be cloned. Various steps in gene cloning include insertion of isolated DNA into a suitable vector to form recombinant DNA, the introduction of recombinant DNA into a suitable organism (host), selection of transformed host cells, identification of the clone containing the gene of interest and multiplication/expression of the introduced gene in the host.

- Plasmids with foreign DNA inserted into them are called recombinant DNA (rDNA) molecules. The preparation of rDNA involves four main steps: DNA isolation, DNA splicing, ligation of DNA and amplification of rDNA.
- The genomic library is representative of the organism's entire genome. The process of dividing genomic DNA into clonable elements and inserting them into host cells creates a library (gene bank, or genomic library). Vectors are used to create genomic libraries that accommodate large inserts.
- Various methods make the selection of recombinant clones. These methods mainly include blue-white screening, antibiotic sensitivity, and selection based on growth on a minimal medium.
- Genetic engineering uses recombinant DNA technology to modify an organism's DNA to achieve desirable traits. An organism that receives recombinant DNA is a genetically modified organism (GMO). The host organism with foreign DNA introduced into it is called transgenic. The gene transfer can occur through *Agrobacterium*-mediated or vector-mediated gene transfer or direct (or vector less) DNA transfer such as particle bombardment.
- CRISPR-Cas is a genetic engineering technology used as a powerful tool for editing genomes. It is used to alter DNA sequences and modify gene function. The transfer of components of CRISPR into complex organisms manipulates genes. The technology has been applied for basic biological research, development of biotechnology products and treatment of diseases.

14.9 TERMINAL QUESTIONS

1. What is the importance of endonucleases in recombinant DNA technology?
2. Define the palindromic sequences? How are they different from typical sequences?
3. Enlist the major characteristics of a vector.
4. Enumerate various gene cloning steps with the help of a well-labelled diagram.
5. Define the recombinant DNA? How is it formed?
6. Enlist the major applications of recombinant DNA technology.
7. What are genomic libraries? How are they created?
8. List a few methods used in the screening of cDNA libraries.
9. Discuss the significant steps involved in plant genetic engineering.
10. Genetic engineering is a beneficial technology. Justify the statement by giving a suitable explanation.
11. Define CRISPR-Cas-9 techniques? How does it work?
12. List the applications of CRISPR technology.

14.10 ANSWERS

Self-Assessment Questions

1.
 - a) Restriction enzymes (also called restriction endonucleases).
 - b) Palindrome sequences
 - c) *Hind II*
 - d) Endonucleases
 - e) genus *Escherichia*
2.
 - a)
 - i) Refer to Section 14.3.
 - ii) Refer to Section 14.3.
 - b)
 - i) Vectors
 - ii) autonomous/self
 - iii) plasmid
 - iv) *E. coli*
 - v) antibiotic
 - vi) cos
 - vii) Fosmid
 - c)
 - i) Refer to Section 14.3.
 - ii) Refer to Section 14.3.
3. a) True; b) False; c) True; d) False; e) False
4.
 - a) cDNA
 - b) Probe
 - c) Immunological Screening
 - d) Plaque hybridisation
 - e) replica plating
5.
 - a) colony hybridisation
 - b) nitrocellulose
 - c) transgenic
 - d) *Agrobacterium*
 - e) DNA or T-DNA
6.
 - a) gene editing
 - b) CRISPR/Cas9

- c) Eugene Koonin and coworkers
- d) Clustered regularly interspaced short palindromic repeats
- e) Rodolphe Barrangou and Philippe Horvath in 2007

Terminal Questions

1. Refer to Section 14.2.
2. Refer to Section 14.2.
3. Refer to Section 14.3.
4. Refer to Section 14.4.
5. Refer to Section 14.4.
6. Refer to Section 14.4.
7. Refer to Section 14.5.
8. Refer to Section 14.5.
9. Refer to Section 14.7.
10. Refer to Section 14.7.
11. Refer to Section 14.7.
12. Refer to Section 14.7.

UNIT 15

APPLICATIONS OF PLANT BIOTECHNOLOGY

Structure

15.1	Introduction	15.4	Biosafety Studies for Genetically Engineered Crops
	Objectives		
15.2	General Account	15.5	Biosafety Regulation of GMOs in India
15.3	Genetically Engineered Crops	15.6	Summary
	Resistance Against Biotic Stresses	15.7	Terminal Questions
	Herbicide, Pest, Disease-Resistant Crops	15.8	Answers
	Improved Quality Traits (Golden Rice, the Flavr Savr Tomato)		

15.1 INTRODUCTION

An introduction to plant biotechnology has been given to you in the previous units. In this unit, you will be studying the various applications of plant biotechnology. The advent of plant biotechnology has opened new vistas for improvement in the growth of crop plants. It has been possible to develop plants that can grow in stress conditions imposed due to heat, cold or drought, or insect/pest/pathogen attack. Biotechnology has also found its applications in increasing the nutritional quality of crops, getting enhanced amounts of compounds, and improving the floral properties of plants. The Food and Agriculture Organization of the United Nations (FAO) states that biosafety allows us to analyze and manage risks related to food safety. Biosafety is defined as the requirement to protect the environment and human health from the potential adverse effects of genetically modified organisms (GMOs) and products resulting from modern biotechnology in national and international environmental law and policy.

Objectives

After studying this unit, you would be able to:

- ❖ describe the role of plant biotechnology in the development of crops resistant to abiotic and biotic stress;
- ❖ discuss the role of plant biotechnology in the development of crops with improved nutritional quality;
- ❖ enlist the biosafety issues related to genetically modified crops; and
- ❖ comprehend the biosafety regulation of GMOs in India.

15.2 GENERAL ACCOUNT

Plant biotechnology is having a significant impact on agriculture, horticulture, and forestry. It is a powerful tool for developing new plant traits and varieties. The techniques such as genetic engineering and molecular biology have proved very useful in producing new agricultural products. Micropropagation offers an excellent practical approach for the large-scale production of plant species and the commercialization of new varieties. Production of secondary metabolites, genetics improvement in plants, germplasm conservation, and production of large numbers of disease, pathogen-free, and resistant varieties have been considered significant plant biotechnology applications. Production of biopharmaceuticals, recombinant or other therapeutic proteins, transgenic plants, and plant-made vaccines or antibodies (plantibodies) has also been made possible through the technique of plant biotechnology. Regeneration of plant cells for rapid production of elite varieties with superior genotypes on a large scale has been made through a plant tissue culture technique.

15.3 GENETICALLY ENGINEERED CROPS

Plant biotechnology has enabled the transfer of genes for the desired trait from other plants or other organisms into the plant. The gene transfers in plants have been done to develop plants having resistance against various traits such as abiotic, biotic stress (herbicide, pest, and disease) and getting enhanced nutritional content. In addition, the overproduction of biochemical compounds is another aspect that is considered while developing transgenic plants.

15.3.1 Resistance Against Biotic Stresses

Plant protection against various diseases and pests is an issue of great concern because they lead to substantial crop losses. Plant biotechnology has produced very useful in developing plants showing resistance against insects, pests, viruses, and pathogens.

Herbicide-resistant transgenic plants

Herbicide contamination in the environment increases due to their excessive use in crop protection. The development of transgenic plants for herbicide resistance was the first achievement made via plant biotechnology technique. Plants showing resistance against herbicides such as glyphosate, sulfonyleurea, and imidazolinones have been developed using biotechnology.

Several enzymes such as *glutathione S-transferase* (GST) involved in the detoxification of the degradation of the herbicides have been identified in plants. The transgenic plants have been developed following two approaches: overproducing the target protein or detoxifying the herbicide.

Glyphosate herbicide kills plants by blocking the EPSPS enzyme which is involved in the biosynthesis of aromatic amino acids, vitamins, and many secondary plant metabolites. Crops can be modified to be glyphosate-tolerant species by incorporating a soil bacterium gene that produces a glyphosate-tolerant form of EPSPS or by a different soil bacterium gene that produces a glyphosate degrading enzyme. A transgenic *Petunia* resistant to glyphosate was developed by transferring a gene to overproduce enzyme *EPSPS* (*5 enol-pyruvyl- shikimate-3-phosphate synthase*) (Fig. 15.1). The enzyme detoxifies the herbicide atrazine, nitrilase and bromoxynil. Glufosinate herbicides contain phosphinothricin which kills plants by blocking the enzyme responsible for nitrogen metabolism and detoxifying ammonia. Crops showing tolerance to glufosinate contain a bacterial gene that produces an enzyme that detoxifies phosphinothricin. Similarly enzyme *phosphinothricin acetyltransferase* (PAT) that helps in the detoxification of herbicide L-phosphinothricin (PPT) has been introduced in plants. Transgenic plants expressing both enzymes have also been developed. Transgenic tobacco that possesses genes for resistance against, an active ingredient of herbicide 'Basta' has also been produced.

Basta is a herbicide that irreversibly inhibits the *glutamine synthetase* (GS) enzyme resulting in the accumulation of ammonia in the cell and subsequent plant death.

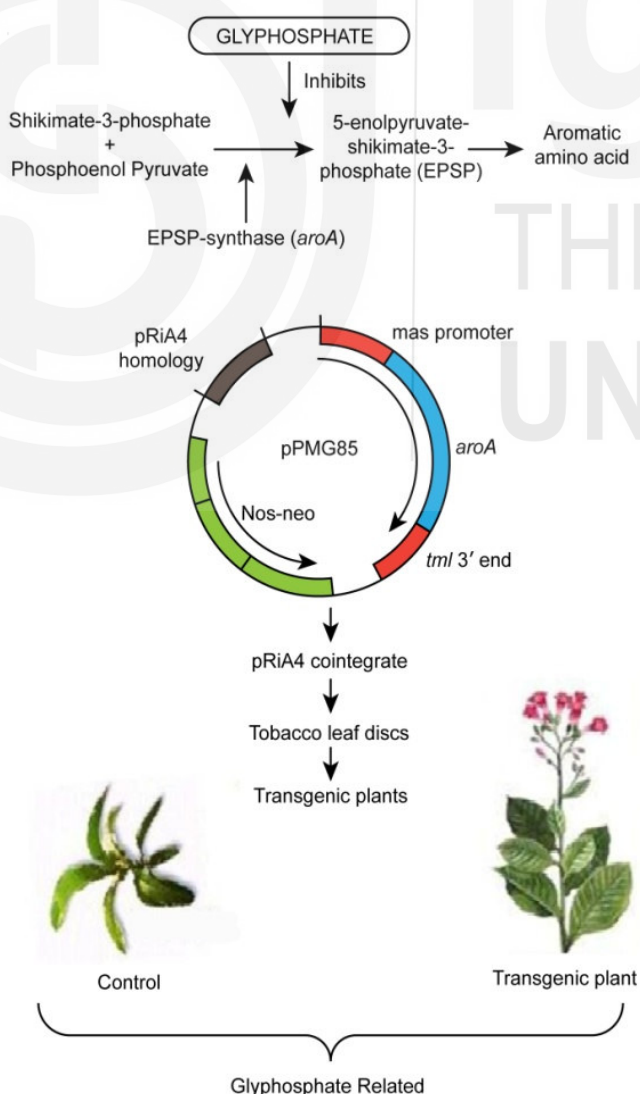


Fig. 15.1: The process involved in development of herbicide resistant transgenic plant.

Insect-resistant transgenic plants

Transgenic plants with resistance against insects have been developed via plant biotechnology. Transgenic crops having **Bt toxin** genes that provide resistance against insects have been developed. *Bacillus thuringiensis* (*Bt*) is a gram-positive soil bacterium. The strains of Bt kill insects belong to families such as Lepidopteran, Coleoptera, and Diptera. It is due to an insecticidal protein called δ - endotoxin. This protein disrupts the function of the digestive system of insects. Formulations of Bt toxins consist of Bt protein, spores, and crystalline inclusions released on lysis of bacterium.

Bt endotoxins or Cry (crystalline) proteins are classified into four types: Cry I, active against Lepidoptera. Cry II is active against Lepidoptera and Diptera; Cry III, which is active against Coleoptera, and Cry IV, is active against Diptera. Other classes of proteins include Cry V, Cry Vi, and Cry IX. These are active against nematodes and other pests. Till date, 50 Cry proteins have been isolated. The significant Bt genes include *Cry1A(b)*, *Cry1A (c)*, *CryIII (a)*. Transgenic tobacco plants transformed via adding genes expressing *Cry 1A (b)* and *Cry1A (c)* developed at International Maize and Wheat Improvement Center (CIMMYT) in Mexico showed resistance to *Manducta sexta* (pest of tobacco). The leaves of transgenic plants showed a 75% -100% rate of mortality of the larvae. The use of transgenic plants has proved effective in reducing crop losses due to pests by about 50%.

A strain of Bt that kills the targeted insect is identified. The gene that produces the lethal protein is targeted. That gene is taken out from the Bt bacterium. The Bt gene with the resistance gene attached is inserted into plant cells. The genetically transformed plant cells are then grown into whole plants by a process called tissue culture. The modified plants produce the same lethal Bt protein produced by Bt bacteria as the gene responsible for the production of the toxin has been inserted into it (Fig. 15.2). Bt transgenic crops have been developed for cotton, corn and rice.

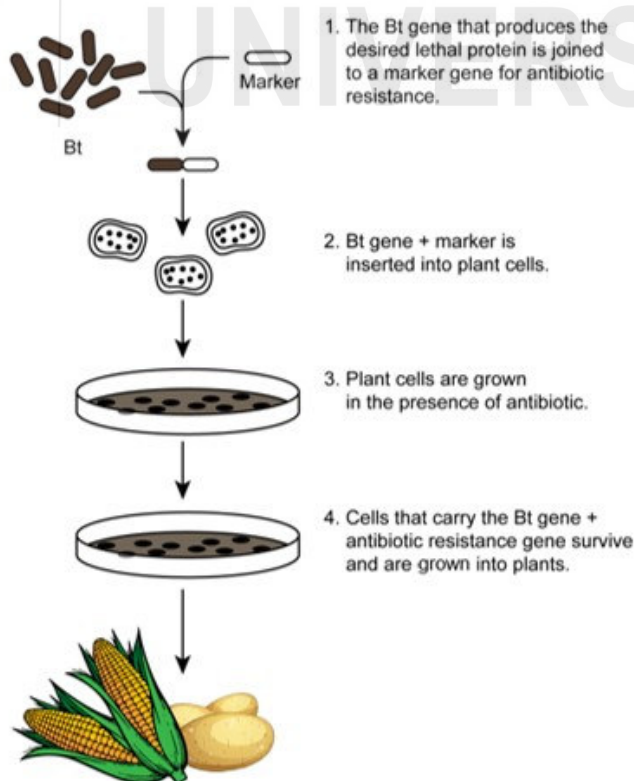


Fig. 15.2: The process of development of insect resistant transgenic crops.

Insect-resistant plants contain innovative proteins and VIPs (vegetative integrated proteins) that show toxicity to insects through protease activity. The genes coding for protease inhibitors have been used in development of insect-resistant crops. Most of these genes encode trypsin inhibitors, *alpha-amylase* inhibitors and *cysteine proteinase* inhibitors. Transgenic tobacco expressing a high level of Trypsin inhibitor gene *CpTI*, isolated from cowpea (*Vigna unguiculata*), has been developed using a binary vector and a CaMV 35S promoter. Transgenic tobacco showed production of protein at high levels. The gene provides resistance to attack by major storage pests of seeds. Gene for alpha-amylase inhibitor (α AI-Pv) isolated from common bean (*Phaseolus vulgaris*) provide adequate protection to the seeds during storage. Transgenic plants having the gene α AI-Pv showed resistance to cowpea weevils.

Phytocystatin genes namely oryzastatin which is *cysteine proteinase* inhibitor has been discovered in various plants. Transgenic plants with a high level of expression of phytostatins, namely Oryza cystatin and corn cystatin have been developed. High expression level of the genes coding for these proteins provides protection against various insects.

Resistance against virus

Plant viruses are one of the main problems affecting the agricultural production worldwide. Genetic resistance has proved to be the most effective way to control plant viruses. An enormous number of transgenic plants resistant to viruses were produced using viral genes. This genetically engineered resistance was called as pathogen-derived resistance (PDR) and was divided into two categories; protein mediated and RNA-mediated. In protein mediated resistance the transformation cassette is designed in such a manner that the introduced viral gene either of the coat protein, *replicase* would be able to get translated and expressed into the plant and interfere with the disassembly, the replication or the movement of the intruding virus. The coat protein gene of *Tobacco mosaic virus* (TMV) was expressed in plants to develop transgenic plants conferring resistance to TMV. The virus coat or capsid protection (C.P.) from TMV was transferred to tobacco. The transgenic tomato plant with resistance against tomato mosaic virus (TMV), potato with resistance against spindle tuber viroid and citrus plants showing resistance against citrus Tristeza virus has been developed. The capsid protein gene from the tomato leaf curl virus has been introduced into tomato plants. The transgenic plants show expression of nucleocapsid protein (N) which provided resistance against negative-strand RNA virus.

RNA-mediated resistance is related to RNA-silencing which is probably the most important and common strategy for engineered resistance to plant viruses. RNA-silencing is a sequence specific RNA degradation mechanism. The RNA-silencing acts as an antiviral weapon supported by the fact that every known plant virus species encodes for at least one protein with RNA-silencing suppression activity. Tobacco plants transformed with the AC2 gene of tomato golden mosaic virus in the antisense orientation induced resistance against TGMV infection.

The satellite RNA depends upon the virus for its replication and transmission. Transgenic tobacco plants carry a DNA fragment that, when transcribed, give

satellite RNA. Transgenic tobacco showing resistance against cucumber mosaic virus (CMV) has been produced using satellite RNA. The presence of satellite RNAs in transgenic plants leads to a reduction in disease symptoms.

DNA coding for *TMV replicase* enzyme, which confers tolerance against TMV, has been transferred to tobacco plants. Tobacco plants transformed with a tandem repeat of the sub-genomic DNA of African Cassava Mosaic virus has also been developed.

The antisense gene SAHH (*S-adenosyl homocysteine hydrolase*) has been introduced into plants to provide resistance against a wide range of viruses. Inhibition of this enzyme affects the methylation at the 5' end of the viral mRNA reaction leading to inhibition of virus replication.

Besides the PDR strategy, alternative biotechnological approaches for the manufacturing of plants resistant to viruses include the expression of plant virus-resistance genes in other plants than those from which they were isolated and the expression of peptides or antibodies. Satellite RNAs (sat-RNA) have been used for developing resistance against specific viruses.

Various crop plants such as tomato, alfalfa, potato, melons and rice showing resistance against a broad range of positive RNA viruses such as potato virus X, alfalfa mosaic virus, Gemini viruses and potato leafroll virus have been developed.

Resistance against bacterial and fungal pathogens

Disease resistance in plants is controlled by resistance genes (R genes) that recognize pathogen strains containing complementary avirulence genes (*Avr*). Hypersensitive reaction (HR) depends upon the interaction between the R gene of the host and the *Avr* gene of the pathogen. It induces broad-spectrum resistance in plants. The resistance and corresponding avirulence genes have been isolated from the pathogens and cloned in plants to produce disease-resistant transgenic crops. The plants respond to pathogen attack via signal transduction pathways by producing R proteins (Resistance) and secondary metabolites such as phytoalexins and phytoanticipins.

Plant defense responses are triggered by the recognition of Pathogen Associated Molecular Patterns (PAMPs). PAMPs are recognized by the plant using extracellular receptor-like kinases (RLK) that can perceive the PAMP and rapidly trigger a signaling cascade through MAP kinases resulting in basal immunity. Pathogens that develop *avr* genes can override the basal immunity of the plant by either blocking perception of a particular PAMP or through the inhibition of the MAP kinase signaling cascade. Plants develop immunity to the pathogens through expression of distinct R-genes targeted against individual *avr* genes. R-genes from unrelated plant species are transferred to plants through genetic engineering. The R-gene Rxo1 from maize was successfully introduced into rice and conferred resistance against bacterial streak disease caused by *Xanthomonas oryzae* pv. *oryzicola*. Other examples include expression of R-gene RCT1 from *Medicago truncatula* in alfalfa. It conferred resistance to *Colletotrichum trifolii*. The gene RPI-BLB2 from wild potato *Solanum bulbocastanum* conferred resistance to *Phytophthora infestans* in cultivated potato.

Another approach for engineering fungal and bacterial resistance in plants is through the expression of antimicrobial peptides, PR-proteins and proteins involved in the production of antimicrobial metabolites. Naturally occurring PR proteins are constitutively expressed at low levels and are induced to high levels during pathogen challenge or application of either salicylic acid or jasmonic acid. They perform various functions including degradation of fungal cell walls, membranes, RNA or production of secondary metabolites.

Overexpression of both chitinases and glucanases (hydrolytic enzymes that break structural components of fungal cell walls such as chitin and laminarin) has been done in variety of plant species. Chitinase overexpression has been successful in increasing tolerance to diseases caused by both biotrophic and necrotrophic fungal pathogens. Combined expression of chitinase and β -1, 3 glucanase results in a synergistic effect, further enhancing the resistance in several plant species. Overexpression of genes encoding for other proteins such as defensins and thionins have been used as peptides for overexpression within transgenic plants. This leads to development of moderate levels of resistance to a wide range of pathogens.

Antimicrobial peptides, including thaumatin-like proteins (TLP) and lipid-transfer proteins (LTP) have been investigated in a range of transgenic plants. TLP affects the plasma membrane of potential pathogens that can lead to membrane “leakiness.” The phenylpropanoid biosynthetic pathway that is responsible for the biosynthesis of a large number of low molecular weight polyphenolic compounds such as flavonoids has also attracted interest from the perspective of genetic engineering to enhance disease resistance. Phenylpropanoid metabolism is controlled primarily through the action of phenylalanine ammonia lyase (PAL), and therefore Overexpression of PAL has been investigated extensively as a source for increased genetic resistance. Tobacco plants overexpressing endogenous or exogenous PAL cDNA had increased resistance to *Cercospora nicotinae* and *Phytophthora parasitica* pv. *nicotianae* corresponding to increased accumulation of the antimicrobial phenolic compounds.

Some of the biotic stress resistant crops developed via genetic engineering technology have been listed below (Table 15.1).

Table 15.1: List of major biotic stress resistant crops.

Plant	Stress	Gene
Squash	Resistance to Cucumber Mosaic Virus	<i>Viral coat protein</i>
Papaya	Resistance to Papaya Ring Spot Virus	<i>Viral coat protein</i>
Soybean	Soybean dwarf virus	<i>Viral coat protein</i>
Rice	Bacterial blight resistance	<i>Class of Xa21 genes</i>
Rice	Fungal disease resistance	<i>Chitinase</i>
Soybean	<i>Phytophthora</i>	<i>Rps1-k</i>
Cotton	Fungal disease resistance	<i>Bean chitinase</i>
Rice	Stem borer	<i>Cawpea serin PI</i>
Eggplant	<i>Leptinotarsa decemlineata</i>	<i>crvIIIB (Bt toxin)</i>

Maize	European corn borer	<i>cry1H (Bt toxin)</i>
Potato	Potato aphid	<i>Snow drop lectin</i>
Rice	Insect resistance	<i>Barley trypsin inhibitor</i>
Soybean	Insect resistance	<i>cry1a (Bt toxin)</i>
Chickpean	Insect resistance	<i>cryIAc</i>
Cotton	Cotton bollworm	<i>crv1Ac (Bt toxin),</i>
Alfalfa	Insect resistance	<i>cry3a (Bt toxin)</i>
Maize	Bromoxynil (herbicide) resistance	<i>Nitrilase</i>
Soybean	Herbicide resistance	<i>Glnfosinate N-acetyltransferase</i>
Soybean	Dicamba (herbicide) resistance	<i>Glnfosinate N-acetyltransferase</i>
Corn	2,4-D (herbicide) resistance	<i>Arylalkanoate dioxygenase enzymes</i>
Soybean	Dicamba (herbicide) resistance	<i>aad-I</i>

SAQ 1

a) Answer in one word:

- i) The first transgenic development showed resistance against this biotic stress.
- ii) Most of the transgenic plants developed herbicide resistance.
- iii) The transgenic plants showing insect resistance has the genes for these toxins inserted into them.
- iv) The number of cry proteins isolated to date
- v) The most common promoter used in the development of transgenic plants

b) Fill in the blanks:

- i) The Cry proteins refer to
- ii) Cry proteins have been classified into main classes.
- iii) The presence of RNAs in the transgenic lead to a reduction in disease symptoms.
- iv) The disease resistance in plants is controlled by genes.

c) Answer in short:

- i) Name major Bt genes.
- ii) Name a few transgenic plants developed for virus resistance.

15.3.2 Resistance Against Abiotic Stresses

Several genes providing resistance against stresses such as heat, cold, salt, heavy metals have been identified and inserted in plants to get stress-resistant plants. The introduction of these genes into plants help to overcome stress conditions by enhancing the synthesis of specific compounds called compatible solutes (proline, betaines), antioxidants (such as carotenoids, ascorbate) and triggering the synthesis of antioxidant enzymatic machinery. Plants exposed to abiotic stress activate genes coding for synthesis of specific proteins such as late embryogenesis abundant (LEA) proteins, antifreeze proteins and chaperones. These proteins provide protection against desiccation or dehydration caused due to abiotic stresses.

Drought resistant plants

Plants exposed to drought stress activate genes coding for production of certain compounds that protect the plant from harmful effects of drought or detoxification enzymes. The genes that encode for trehalose and proline biosynthesis via enzymes *trehalose 6-phospho synthase* (TPS), *trehalose phosphatase*, *pyrroline-5-carboxylate synthetase* (P5CS) have been introduced in plants.

Drought tolerant transgenic rice, wheat, soybean, cotton have been developed. A gene named *P5CS* encoding of proline biosynthetic pathway has been introduced in tobacco. The plant show 8-10 fold higher proline content. The genes for the glycine betaine biosynthetic pathway have been introduced into plants. These enzymes convert choline into betaine aldehyde, which gets converted to glycine betaine. Transgenic tobacco expressing genes for these enzymes [*bet A* encoding *choline dehydrogenase* (CDH) and *bet B* encoding *betaine aldehyde dehydrogenase* (BADH)] have been developed. Transgenic plants having genes encoding the polyamine biosynthesis enzymes viz. *arginine decarboxylase* (ADC), *ornithine decarboxylase* (ODC), and SAMDC (*S-adenosyl methionine decarboxylase*) have been developed. Accumulation of polyamines confers tolerance in plants.

ERA gene controls ABA (abscisic acid) biosynthesis in plants. The downregulation of the gene imparts drought tolerance through stomatal closure. The plants expressing this gene show high resistance to drought. ERA gene has been introduced in canola and corn.

Plants become drought tolerant by activating specific drought-responsive genes. Several regulatory genes including transcription factors (TFs) play essential role in multiple abiotic stress responses via regulating downstream stress-responsive genes. A cis-acting promoter element called dehydration response element (DRE) plays an essential role in regulating gene expression in drought stress. DRE element is responsible for ABA-independent induction of many genes in response to osmotic and cold stress in many plants including *Arabidopsis*. DREBs (dehydration responsive element binding) are important plant transcription factors (TFs) that regulate the expression of many stress-inducible genes and play a critical role in improving the abiotic stress tolerance of plants by interacting with a DRE/CRT cis-element present in the promoter region of various abiotic stress-responsive genes. The transcription factors DRE1A and DRE2B bind specifically to DRE and activate genes containing DRE.

Resistance of plants against chilling stress

Biotechnology offers new strategies that can be used to develop transgenic crop plants with improved tolerance to cold stress. A number of genes have been isolated and characterized that are responsive to freezing stress.

The plants showing sensitivity to chilling stress possess lipids with saturated fatty acids. The presence of double bonds in one of the 16 or 18 carbon saturated fatty acids found in lipid imparts resistance against injury at low temperatures. It is widely assumed that *glycerol-3-phosphate acyltransferases* (GPATs) play an essential role in plants' ability to withstand cold temperatures. The introduction of GPAT gene from a cold-sensitive pumpkin into tobacco increased the saturation degree of fatty acids in the membrane lipids of transgenic tobacco plants.

The genes that increase the expression of antioxidant enzymes such as superoxide dismutase, which improves resistance to abiotic stress has been expressed in plants. The genes responsible for biosynthesis of osmoprotectants such as trehalose, glycine betaine, proline have been introduced in plants. These compounds facilitate the intake water retention, protect organelles, and help stabilize macromolecules. In addition, genes such as Rab proteins and osmotin have also been introduced into plants.

Low temperature inducible genes isolated from plants are supposed to be involved in tolerance to cold stress. The C-repeat binding factor (CBF) genes play an important role in adaptation to low temperature and signal transduction. The expression of some of them is regulated by C-repeat binding factor/dehydration-responsive element binding (*CBF/ DREB1*) transcription factors.

Resistance of plants with against heat stress

Heat-shock proteins (Hsps) and antioxidant enzymes play an important role in encountering heat stress in plants (Fig. 15.3). Hsps play a role in stress signal transduction, protecting and repairing damaged proteins and membranes, protecting photosynthesis as well as regulating cellular redox state. Genes encoding heat shock proteins (HSPs) have been introduced into plants to impart resistance or tolerance against heat/high-temperature stress.

Expression of various Hsps is controlled at both the transcriptional and the translational level. A cis-acting DNA sequence, the heat-shock element (HSE), has been found to be necessary for heat-induced transcription.

Plants under HT stress usually accumulate more reactive oxygen species (ROS) in both chloroplasts and mitochondria, which can severely damage DNA and cause cell membrane lipid peroxidation (LPO). Thus, increased capacity of scavenging and detoxifying the ROS is required to protect plant against HT. Induction of thermotolerance is ascribed to the maintenance of better membrane thermostability, and low level of ROS accumulation due to improved antioxidant capacity. Thus genes encoding for increased expression of antioxidant enzymes have also expressed in plants.

The accumulation of osmolytes such as proline, glycine betaine and trehalose is a well-known adaptive mechanism in plants and play a major role in imparting heat tolerance. The plants showing high thermotolerance have been developed by inducing synthesis of synthesis of compatible solutes.

Membrane lipid saturation is considered an important element in HT tolerance. HT causes an increase in fluidity of membranes which can lead to disintegration of the lipid bilayer. The silencing of chloroplast localized *fatty acid desaturase* has also proved helpful in imparting heat resistance in plants. The transgenic plants developed via this strategy show resistance to high temperatures.

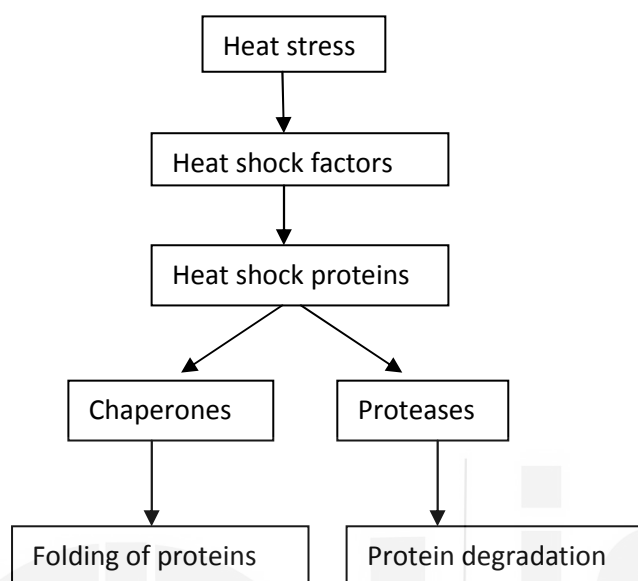


Fig. 15.3: Role of heat shock proteins in resistance against heat stress in plants.

SAQ 2

a) Answer in short:

- i) Name the proteins that protect against heat stress in plants.
- ii) Name the genes involved in inducing resistance against dehydration stress in plants.
- iii) Name the compatible solutes that play a role in inducing drought tolerance in plants.

b) Write full form of :

- i) DRE
- ii) LEA
- iii) BADH
- iv) ABA

15.3.3 Transgenic Plants for Improved Quality Traits

Transgenic crops for improved quality and productivity, such as harvest index, biomass, have been developed. Change in the nutritional quality, edible oil composition, and cereals grain quality has been done in crop plants following a transgenic approach.

Transgenic tobacco showing overexpression of PhyA has been produced. PhyA exerts stem elongation as a shade response to far-red light. A high level of PhyA has been suggested to suppress shade avoidance response.

The development of transgenic plants overexpressing the gene coding for the bacterial haemoglobin molecule (VHb) for high chlorophyll content has been developed. These plants show a high growth rate, alteration in metabolic pathways and enhancement in productivity. About 80-100% more biomass production has been noted in these plants.

Transgenic crops for the production of nutraceuticals have been developed. Nutraceuticals are compounds isolated from foods that act as health supplements. Efforts have been made to increase the oil and protein content of oil crops such as soybean, palm, rapeseed and sunflower.

A transgenic approach can alter the proportion of saturated and unsaturated fatty acids in edible oils. Transfer of the gene encoding for *sn-2 acyltransferase* leads to an increase in fatty acid content in rapeseed. An increase in glucosinolate level in crop plants increases the nutritional value. The linoleic acid content in flax has been reduced, the stearic acid content in safflower and soybean has been increased following the transgenic approach. *Stearoyl-ACP desaturase* (ACP) activity in seeds alters the ratio of saturated to unsaturated fatty acids. The enzyme catalyzes the first desaturation step in seed oil biosynthesis. It converts stearoyl-ACP to oleoyl ACP. Transgenic plants have been produced using seed-specific antisense gene constructs of *Brassica stearoyl-ACP desaturase* (ACP). *Desaturase* activity decreases, consequently increasing the stearate levels in seeds, and 30% of the seed oil is a polyunsaturated fatty acid (PUFA). The transfer of genes responsible for the increase in PUFA in rapeseed increases the nutritional and economic value of the crop plant. The increased fatty acid content of 60% has been noted in such crops.

Transgenic plants have been raised to improve the quality of seeds by changing the composition of amino acids and the quality of seed starch. Efforts have been made to improve the composition of essential amino acids in grains, cereals, legumes and oilseeds. Lysine rich transgenic '*Brassica*' and soybean (*Glycine max*) has been produced. The lysine content of the seed grains has been improved by manipulating the biosynthetic pathway that leads to the conversion of aspartic acid into lysine, threonine and methionine. Methionine rich seeds can be produced by increasing the content of albumin protein in legumes and prolamins in cereals. Generally, the amylopectin and amylase levels are high in the crops such as potato, corn and rice. Therefore, the ratio of amylopectin and amylase has altered plants to produce resistant starch. Transgenic potatoes having high amylase content have been produced.

Golden rice

Transgenic crops like golden rice and golden mustard rich in vitamin A have been developed. Golden rice has been developed by inserting the genes from *Narcissus* and *Erwinia*. The developed rice variety possesses a high content of β carotene and carotenoids, which are the precursors of vitamin A. The

work was initiated by the International Rice Research Institute (IRRI).

Agrobacterium tumefaciens-mediated transformation of embryogenic rice calli with the introduction of the *phytoene synthase* (*psy1*) gene from *Zea mays* (*Zmpsy 1*) and the *carotene desaturase I* (*crtl*) gene from *Pantoea ananatis* resulted in GR2E rice. High levels of provitamin A (β -carotene) in rice endosperm get converted to vitamin A when metabolized by the human body. The Golden Rice Project was introduced in 1999 by Ingo Potrykus and Peter Beyer. Similarly, golden mustard was developed by introducing a gene that elevated the β carotene levels.

Transgenic crops rich in iron content have also been developed. The gene targeted in these plants is ferritin, which provides iron for synthesizing iron proteins such as ferredoxin and cytochromes. The synthesis of ferritin is regulated at the transcriptional level. The animals can use ferritin as a rich source of iron if the content gets increased in the cereal grain. For this, the genes for ferritin protein were isolated from the soybean, *Phaseolus* and transferred into the rice. As a result, the plants produced seeds rich in ferritin. The experiments were conducted in Japan in 1999 and Europe in 2001. Later on, the International Rice Research Institute developed a rice variety rich in Fe and Zn by crossing these two varieties.

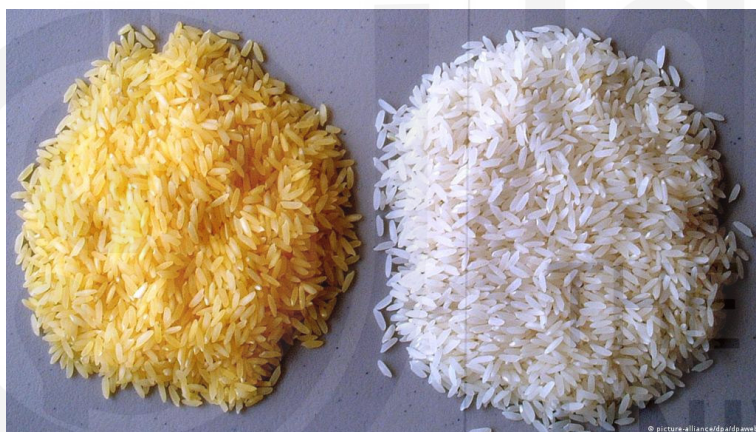


Fig. 15.4: Photograph showing golden rice (left) and normal rice (right).

Flavr Savr tomato

Antisense RNA regulates the expression of the enzyme *polygalacturonase* (PG) involved in the ripening of tomato fruit. This enzyme is abundantly found in ripe tomato fruit and has been responsible for softening the fruit skin by producing ethylene. *Polygalacturonase* dissolves cell wall pectin. The tomatoes expressing antisense RNA against PG have been developed. Accumulation of PG in ripening tomatoes can be suppressed by introducing a reverse-orientation copy of the gene (an "antisense" copy). Insertion of PG antisense gene reduced the amount of PG produced in the tomato. The transgenic tomato was not able to produce PG.

A gene named *ACC deaminase* that degrades 1-aminocyclopropane 1 carboxylic acid (ACC) is an immediate precursor of ethylene. The tomato expressing antisense *ACC synthase* or *ACC oxidase* gene exhibits delayed ripening in fruits (Fig. 15.5). Tomatoes showing expression of the gene coding for enzyme ACC synthase were called **Flavr Savr**. The fruits accumulate more amounts of sugars hence having better flavour. The Flavr Savr tomatoes have a long shelf life. This technique has been developed tomatoes and melon fruits.

Tomatoes with higher sucrose and lower starch content could be created by introducing the *sucrose phosphate synthase* or *isopentenyl transferase (ipt)* genes from *Agrobacterium tumefaciens*.

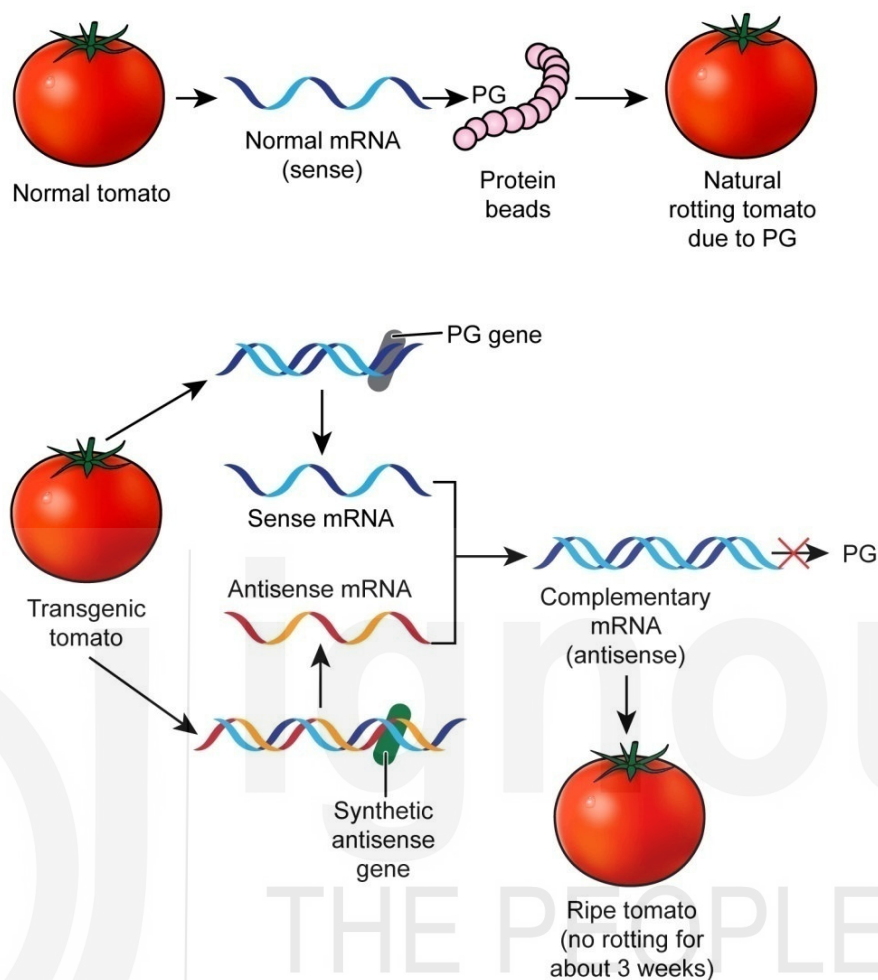


Fig. 15.5: Comparison between normal and Flavr Savr tomato.

Some of the transgenic crops improved for their nutritional quality has been listed below (Table 15.2).

Table 15.2: Transgenic crops developed for improvement in nutritional quality.

Nutrient	Plant transformed	Genes used
Vitamin A	Maize	<i>Zmpsy1, PacrtB, ParctI</i>
	Wheat	<i>Zmpsy1</i>
	Potato	<i>EuCrtB, EuCrtI, AtZEP, PacrtB</i>
	Rice	<i>Zmpsy1, EuCrtI</i>
	Cassava	<i>PacrtB</i>
Vitamin C	Corn	<i>Osdhr</i>
	Tomato	<i>Acgpp</i>
	Potato	<i>StVCT2A</i>
Folic acid	Rice	<i>Atadcs</i>
	Tomato	<i>Atgch</i>

Iron	Rice	<i>Osnas2</i>
	Corn	<i>Gm ferritin, Af phytase</i>
	Cassava	<i>Crfea1</i>
Zinc	Rice	<i>Osnas2, Gm ferritin</i>
	Cassava	<i>Atzat1, Atzip</i>
Calcium	Carrot	<i>scax1</i>
	Lettuce	<i>scax1</i>
	Potato	<i>scax1</i>

SAQ 3

Name two ways by which transgenic plants has been raised for change in nutritional quality.

Use of plant biotechnology in floriculture

The development of flowering plants with variations in flower color has been made using biotechnology. The colour of flowers is caused by a group of compounds known as anthocyanidins and their glycosylated derivatives, anthocyanins. Anthocyanidins code for white and yellow color, while anthocyanins are red and blue. The genes involved in synthesis of anthocyanidins bring alteration in flower color. Transcriptional activators of a gene of the anthocyanin biosynthetic pathway have also increased the intensity of flower color. Flowers with changing colours have been created in the rose, tulip, and carnation by modifying genes to synthesize delphinidin (3'5' hydroxylated anthocyanins). Expression of the gene coding for delphinidins produces blue color in flowers.

The genes for flavonoids have also been manipulated in these plants; for example, the enzyme *chalcone synthase* has produced white and variegated flowers in *Petunia*, *Chrysanthemum*, *Gerbera*, and roses. In addition, the *chalcone synthase* gene was used to alter the color of the flower. For example, the expression of this gene introduces the production of white and pink flowers in *Petunia*.

The genes that increase the synthesis of flavonoids by blocking the flavonoid pathway at a specific step (using RNA antisense) have been introduced into plants. For example, another gene named the *dihydroflavanol-4-reductase gene* (DFR) has been expressed in *Petunia*. The expression of the gene produces an orange compound-pelargonidin.

The life period of flowers can be increased by introducing genes *aco* and *acFs* encoding for enzymes *ACC synthase* and *ACC oxidase* involved in the biosynthesis of ethylene. The genes encoding enzymes such as ACC synthase and *ACC oxidase* have been cloned in many species including carnation. As a result, the flowers produced exhibit longer shelf life.

Besides these, the genes for monoterpenes responsible for inducing fragrance in plants have also been introduced in other plants. It has also been done by manipulating gene *S-linalool synthase* coding for monoterpenes.

SAQ 4

Fill in the blanks:

- gene was used in *Petunia* to alter the color of the flower.
- Expression of the gene produces orange coloured compound in *Petunia*.
- The life period of flowers can be increased by introducing genes *aco* and *acs* encoding enzymes and involved in the biosynthesis of ethylene.
- are responsible for giving red and blue colour to the flower.

15.3.4 Use of Plant Biotechnology in the Production of Biochemical in Plants

Plants synthesize a large number of bioactive secondary metabolites including alkaloids, flavonoids and other phenolics, saponins, terpenoids, steroids, glycosides, tannins, volatile oils, etc. These compounds are used as drugs (pharmaceuticals), flavors, perfumes (fragrances), pigments (dyes), agrochemicals, cosmetics, food additives, etc. Most of these metabolites are obtained from plants. Biotechnology offers a valuable tool to produce these compounds of interest in a desired amount. In vitro plant cell culture technique, in which plant cells, tissues, and organs are cultivated under aseptic conditions (independent of geographical and climatic factors), offers alternatives for producing important metabolites. The process includes mainly four main approaches, viz. callus, suspension, immobilized cells, and differentiated cultures (Fig. 15.6).

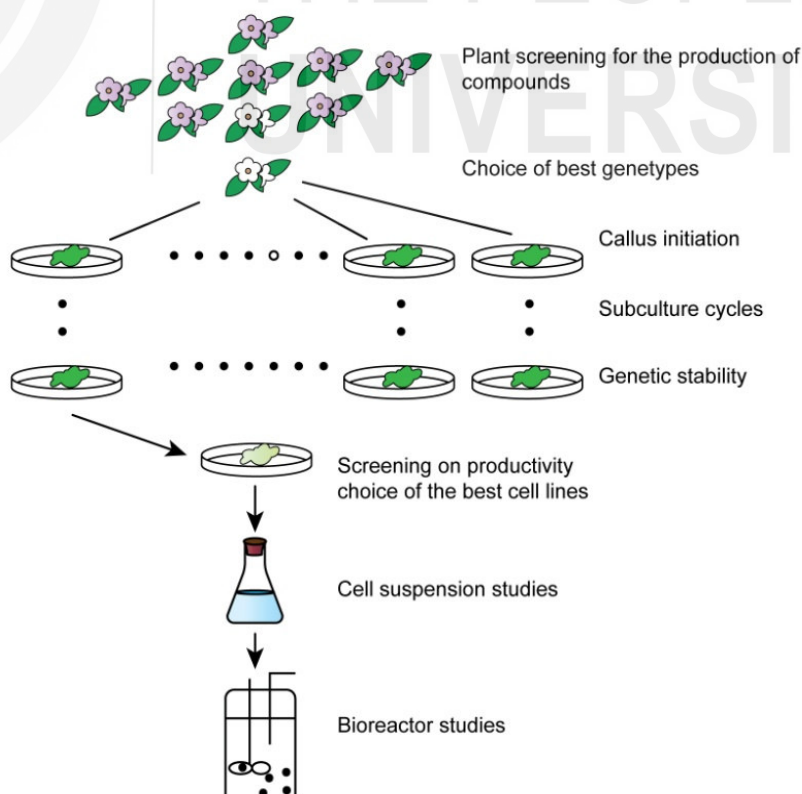


Fig. 15.6: Use of plant biotechnology in getting enhanced production of useful compounds from plants.

It is possible to regulate the biosynthetic pathway of plants in order to enhance/decrease the synthesis of a particular compound via the use of biotechnological techniques. Metabolic engineering offers new perspectives for improving the production of compounds of interest. This approach can be used to improve production in the cell culture, in the plant itself or even production in other plant species or organisms. Recombinant DNA techniques can be used to manipulate metabolic pathways and produce protein pharmaceuticals such as antibodies, and protein hormones. Overexpression of regulatory genes cause an upregulation of enzymes involved in the synthesis of alkaloid/compound.

Advances in plant biotechnology have been successful in producing edible vaccines. Research is now conducted to produce transgenic plants that produce therapeutic agents such as hormones (example -insulin, somatotropin, erythropoietin), blood components, coagulation factors and various interferons. Plants are primarily modified by the introduction of novel gene sequences which drive the production of 'designer' proteins or peptides. These proteins or peptides possess therapeutic value and show properties that allow them to be used as precursors in the synthesis of medicinal compounds, or may serve as technical enzymes in pharmaceutical production.

Cyclodextrins are compounds that are helpful for pharmaceutical delivery systems, flavour odour enhancement and removing undesired compounds. The gene coding for cyclodextrin has been introduced in tomatoes. It has been done by expressing the enzyme *cyclodextrin glucosyltransferase*. The bacterial gene encoding the enzyme *cyclodextrin glucosyltransferase* (CGTase) from *Klebsiella* has been successfully transferred in potatoes. It increased the production of cyclodextrins in potato tubers.

The genes that encode the enzymes *acetoacetyl CoA reductase* and *polybutyrate synthase* which catalyze the biosynthesis of polyhydroxy butyrate (PHB), have been successfully transferred from *Arabidopsis* to other plants. In addition, genes for starch biosynthesis, namely *ADP glucose pyrophosphorylase* (AGPase), *soluble starch synthase* (SSS) and branching enzyme, have been isolated and introduced into plants to get high starch content.

Genes responsible for synthesis of peptide epitopes of pathogens can be introduced into plants. This results in production of vaccines against various human and animal diseases. In 2002, the transgenic carrot that has the potential as an edible vaccine against hepatitis B was also developed. Transgenic tobacco, potato, banana, carrot has been developed as edible vaccines. The diseases targeted by these vaccines include measles, polio, diphtheria, yellow fever, hepatitis B. A vaccine developed in the maize plant has been found effective in providing protection against gastroenteritis virus (TGEV). Transgenic plants expressing the antigen for hepatitis B (HbsAg) has been developed. Transgenic plants have the potential for the production of monoclonal antibodies has also been developed. These are called plantibodies. These antibodies can be targeted into seeds or tubers.

Glucocerebrosidase is a lysosomal *hydrolase* enzyme that has been expressed in transgenic plants. Its deficiency causes 'Gaucher disease' in humans. The

production of this drug in transgenic tobacco was done in 1999 by scientists at Virginia State University. Hirudin is another drug used as an anticoagulant for the treatment of thrombosis. Transgenic plants expressing the hirudin have been produced. Transgenic oilseed rape expressing hirudin is commercially available in Canada.

15.4 BIOSAFETY STUDIES FOR GENETICALLY MODIFIED (GM) CROPS

Efforts have been made to get the commercialization of transgenic crops. Concerns have been raised regarding the various possible risks associated with them. The significant concerns related to transgenic crops include

- The risk of the possible gene escape of the transgene (gene introduced for various stresses)
- The risk effect of toxins (Bt toxin) produced in transgenic crops on non-target insects.
- Risk effects of selectable markers, particularly those providing antibiotic resistance on human health.
- There is a risk of developing insects, pests resistant against insecticides, pesticides, and superweeds resistant against herbicides.
- Risk of creation of deadly virus due to recombination between the viral DNA expressed by a transgenic plant and viral genome infecting the virus.

Because of the safety concerns, transgenic crops have been subjected to biosafety regulations. Biosafety regulations are prescribed for conducting laboratory or greenhouse and field experiments. Field experiments are conducted using transgenic plants to ensure the safe health of humans and the environment. Various authorities have issued various guidelines for the regulation of transgenic lines. According to recent guidelines, two categories have been formed:

vertical regulation (the USA and Canada) and ii) horizontal regulation (European Union). Vertical regulation is based on regulating a particular product or characteristic of the crop. Therefore, the products from transgenic crops need not be regulated. On the other hand, a horizontal system is a process base system and involves regulating all the products of the transgenic crops.

The regulation of the field trials of transgenic crops was first developed in the USA. First, keys were issued to get the step-by-step evaluation of the transgenic crops. Then, APHIS (Animal and Plant Health Inspection Service) of USDA (U.S. Department of Agriculture) introduced a 'Notification system' for transgenic crops (corn, tomato, soybean, potato, cotton and tobacco). If APHIS does not have any objection, the person or authority can conduct field trials. In 1993 the 'Notification system' was developed in which all field trials are permitted.

Regulations were also issued for the commercialization of transgenic crops. The commercialization can be done the independent approval have been obtained from several agencies, depending upon the nature of the crop, the trait changed via genetic engineering and finally, the use of the product (food etc.). In the USA, any crop containing the gene for pesticide requires approval through EPA (Environmental Protection Agency). If the crop is used as food, additional clearance from the Food and Drug Administration (FDA) is required. In European Union (E.U.), the regulation is complex, and the approval is required at the individual country level and the level of the Union.

The data obtained after testing transgenic crops have been included in the database with the Green Industry Biotechnology Platform (GIBP). In addition, the International Service for the Acquisition of Agri-Biotech Applications (ISAAA) maintains the complete data on field testing and commercialization of transgenic crops.

The first transgenic crop to be sold in the market was Flavr Savr tomato in 1994. The USA granted the commercialization of the crop. Other transgenic crops approved for commercial cultivation included sugar beet, sweet pepper, alfalfa, flax and papaya.

Area occupied by transgenic crops has reached 2.7 billion hectares in 2019. In 2019, biotech crops were planted by up to 17 million farmers in 29 countries (Fig. 15.7). Year 2019 has reported a ~112-fold increase in plantation of transgenic crops (Table 15.3). The crops mainly include soybean, corn, cotton, canola, insect, herbicide and virus resistant crops.

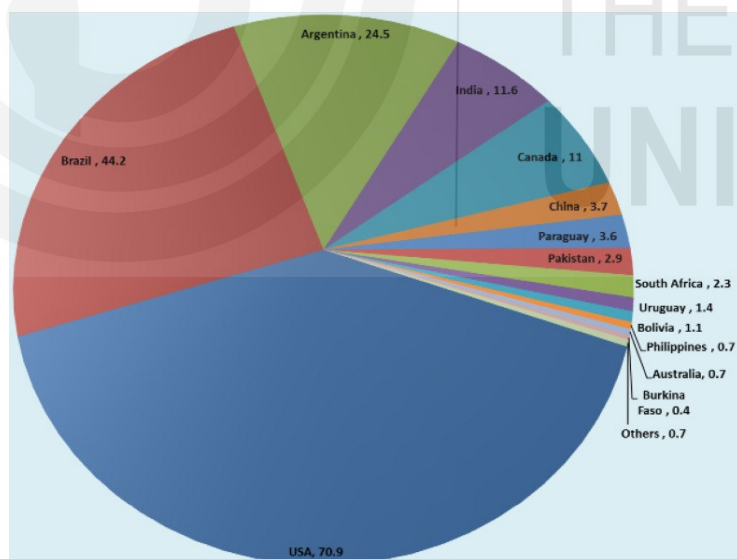


Fig. 15.7: Proportion of the transgenic crop production by various countries of the world.

Table 15.3: Global Area of Biotech Crops in 2018 and 2019: by Country (million hectares)

Rank	Country	2019
1.	USA*	71.5
2.	Brazil*	52.8

3.	Argentina*	24
4.	Canada*	12.5
5.	India*	11.9
6.	Paraguay*	4.1
7.	China*	3.2
8.	South Africa*	2.7
9.	Pakistan*	2.5
10.	Bolivia*	1.4
11.	Uruguay*	1.2
12.	Philippines*	0.9
13.	Australia*	0.6
14.	Myanmar*	0.3
15.	Sudan*	0.2
16.	Mexico*	0.2
17.	Spain*	0.1
18.	Colombia*	0.1
19.	Vietnam*	0.1
20.	Honduras	<0.1
21.	Chile	<0.1
22.	Malawi	<0.1
23.	Portugal	<0.1
24.	Indonesia	<0.1
25.	Bangladesh	<0.1
26.	Nigeria	<0.1
27.	Eswatini	<0.1
28.	Ethiopia	<0.1
29.	Costa Rica	<0.1
	Total	190.4

The commercialization of transgenic crop plants was encouraged because of various benefits. These include

- Improved and efficient weed control,
- Decrease in the use of pesticides, insecticides or viruses due to effective pest, insect and virus control
- Decrease in post-harvest losses due to better shelf life
- Increase in nutritional quality
- Increased production of hybrid seeds.

15.5 BIOSAFETY REGULATION OF GENETICALLY MODIFIED ORGANISMS (GMOS) IN INDIA

Biosafety regulations include policies and procedures that need to be followed to ensure that biotechnology applications are not harmful to the environment. It also includes assessment of the risk associated with the development and use of genetically modified organisms. Indian regulatory framework has set regulations and guidelines for transgenic crops in the following sections:

a) Rules and policies

1. Rules, 1989 under Environment Protection Act (1986)
2. Seed Policy, 2002

b) Guidelines

1. Recombinant DNA guidelines, 1990
2. Guidelines for research in transgenic crops, 1998

Government agencies such as Ministry of Environment, Forests, and Climate Change and the Department of Biotechnology, Government of India are identified as the primary agencies responsible for the implementation of guidelines related to genetically modified organisms biosafety into effect (Fig 15.8).

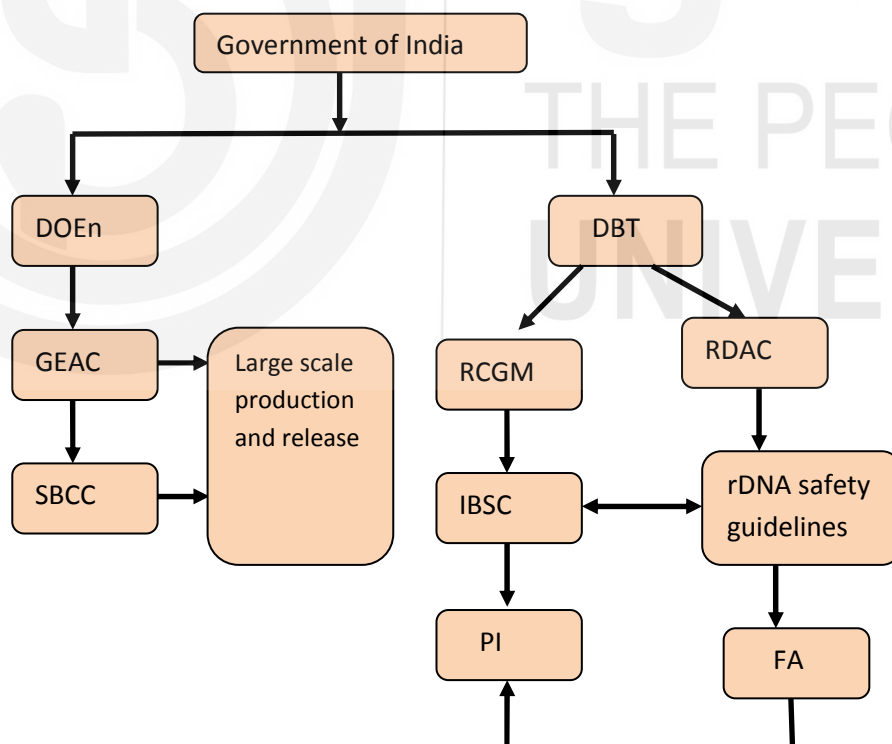


Fig.15.8: Institutional mechanism as proposed for implementation of Biosafety rule for GMOs.

DBT- Department of Biotechnology; SBCC- State Biotechnology Coordination Committee; DoEn-Department of Environment; RDAC-Recombinant DNA Advisory Committee; IBSC-Institutional Biosafety Committee; RCGM- Review Committee on Genetic Manipulation; GEAC-Genetic Engineering Approval Committee; FA-Funding Agency.

The competent authorities and the composition of such authorities have also defined to deal with the various rules. According to the regulations, there are six competent authorities.

1. Recombinant DNA Advisory Committee (RDAC)
2. Review Committee on Genetic Manipulation (RCGM)
3. Genetic Engineering Approval Committee (GEAC)
4. Institutional Biosafety Committees (IBSC)
5. State Biosafety Coordination Committees (SBCC)
6. District Level Committees (DLC).

The three organizations that are responsible for the approval of new transgenic crops are:

1. The IBSC is an institutional level committee responsible for monitoring institute-level research in genetically modified organisms.
2. The RCGM established at DBT monitors the ongoing research activities related to GMOs and small-scale field trials.
3. The Genetically Engineered Organisms Advisory Committee (GEAC) function in the Ministry of Environment, Forests, and Climate Change (MoEF & CC) to authorise large-scale trials and release of GMOs.

The Recombinant DNA Advisory Committee (RDAC) established by the Department of Biotechnology (DBT) review the developments in biotechnology at the national and international level and makes appropriate recommendations as well as safety regulations for India. State Biotechnology Coordination Committees (SBCCs) are established in each state where the research and application of GMOs are considered. SBCCs are responsible for coordinating the activities related to GMOs in the state with the federal government. In addition to monitoring functions, SBCCs have the authority to inspect, investigate, and take disciplinary action when there is a violation of these rules and regulations. District Level Committees (DLCs) are also established at the district level to ensure that the safety regulations are followed in facilities that are engaged in GMOs related research and its application in the environment.

SAQ 5

Write the full form of the abbreviation.

- a) APHIS
- b) FDA
- c) EPA
- d) ISAAA

15.6 SUMMARY

- Biotechnology has been used to produce genetically engineered crops with specific traits such as increased resistance to pests, diseases and abiotic factors such as heat cold.
- The first achievement of the plant biotechnology technique was the development of transgenic plants for herbicide resistance. The plants were developed by overproducing the target protein or detoxification of the herbicide. As a result, plants developed resistance to herbicides such as glyphosate, sulfonylurea, and imidazolinones.
- Plant biotechnology has been used to develop transgenic plants having resistance to insects. Transgenic crops having Bt toxin genes possess insect resistance. The strains of Bt are effective against different insects and kill insects belonging to families such as Lepidopteran, Coleoptera and Diptera. The insecticidal protein called δ - endotoxin or Cry proteins (Cry or crystalline proteins) disrupt the digestive system of insects. Bt transgenic crops have been raised mainly for cotton, corn and rice.
- Various crop plants having developed resistance against a broad range of positive RNA strand plant viruses such as potato virus X, alfalfa mosaic virus, and potato leafroll virus have been developed. In addition, DNA coding for TMV replicase enzyme, which confers tolerance against TMV, has been transferred to tobacco plants.
- Several resistance genes from hosts and corresponding avirulence genes from the pathogens have been isolated and cloned. These genes have been used for the development of disease-resistant transgenic crops.
- Genes providing resistance against stresses such as heat, cold, salt have been identified and inserted in plants. The transgenic plants overcome stress conditions by synthesizing compatible solutes (proline, betaines) and antioxidants (carotenoids, ascorbate) or triggering the antioxidant enzymatic machinery. In addition, the synthesis of proteins such as late embryogenesis abundant proteins (LEA), heat shock proteins (HSPs), antifreeze proteins, chaperones also get triggered in plants exposed to abiotic stress conditions.
- Crops plants with improved nutritional value such as golden rice and delayed maturation, such as flavr savor tomatoes have been developed. Plant biotechnology has also found applications in flowering plants with variations in flower color and longer shelf life.
- Because of some ecological and ethical issues, a strict regulatory network needs to be followed for field trials and experimentation of transgenic crops. Various agencies at the state and national level regulate the biosafety rules related to use of genetically modified organisms.

15.7 TERMINAL QUESTIONS

1. Describe in brief how plant biotechnology is applied to develop plants resistant to abiotic and biotic stresses.
2. Discuss in brief the concept of developing transgenic plants for improved quality traits.
3. Write a note on golden rice.
4. What is Flavr Savor tomato? How is it developed?
5. How has plant biotechnology been applied in floriculture?
6. Plant biotechnology can be used to increase the production of valuable chemicals. Explain.
7. Discuss the significant concerns related to the use of genetically modified crops.

15.8 ANSWERS

Self-Assessment Questions

1. a) i) herbicide
ii) glyphosate
iii) Bt
iv) 50
v) CaMV35S
b) i) crystalline proteins
ii) four
iii) satellite
iv) resistance (R)
c) i) *Cry1A(b)*, *Cry1A (c)*, *CryIII (a)*
ii) tomato, potato, citrus, rice
2. a) i) heat shock proteins (HSPs)
ii) DRE1A, DRE2
iii) proline, glycine betaine, trehalose
b) i) Dehydration response element
ii) Late embryogenesis abundant
iii) Betaine aldehyde dehydrogenase
iv) abscisic acid

3. 1) Tomatoes and melon fruits expressing antisense ACC synthase or an ACC oxidase gene for the delay in ripening of fruits. 2) Transgenic grain cereals, legumes and oilseeds to improve the quality of seeds by changing the composition of amino acids.
4. a) Chalcone synthase
b) dihydroflavanol-4-reductase gene (DFR)
c) ACC synthase, ACC oxidase
d) anthocyanins
5. a) Animal and Plant Health Inspection Service
b) Food and Drug Administration
c) Environmental Protection Agency
d) Service for the Acquisition of Agri-Biotech Applications

Terminal Questions

1. Refer to Section 15.3.
2. Refer to Subsection 15.3.3.
3. Refer to Subsection 15.3.3.
4. Refer to Subsection 15.3.3.
5. Refer to Subsection 15.3.4.
6. Refer to Subsection 15.3.5.
7. Refer to Section 15.4.

Acknowledgements

Fig. 15.4 : Source:

https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fstatic.dw.com%2Fimage%2F17392379_101.jpg&imgrefurl=https%3A%2F%2Fwww.dw.com%2Fen%2Fgenetically-modified-golden-rice-variety-stirs-up-controversy%2Fa-17396082&tbnid

GLOSSARY

Adventitious	:	Arising from another source and not inherent or innate.
Agar	:	Natural gelling agent made from algae.
Anneal	:	The process by which two single strands of DNA hydrogen bond at complementary nucleotides to form a double-stranded molecule.
Axenic culture	:	A culture without foreign or undesired life forms but may include the deliberate co-culture with different types of cells, tissues or organisms.
Autoradiography	:	A photograph showing the distribution of a radioactive substance in a specimen after exposure to radiation.
Biotinylated	:	The process of covalently attaching biotin to a protein, nucleic acid or other molecule.
Cell culture	:	Culture of single cells or their maintenance <i>in vitro</i> conditions.
Chemically defined medium	:	A nutritive solution or substrate for culturing cells in which each component is specified.
Clones	:	A group of genetically uniform plants propagated from vegetative parts.
Clonal propagation	:	Asexual multiplication of plants from a single individual or explant.
Contamination	:	Infected by unwanted microorganisms in controlled environment.
Chromogenic	:	Anything producing colour.
Cryopreservation	:	Storage of cells, tissues, embryos and seeds at ultra-low temperature.
Culture	:	A plant growing <i>in vitro</i> in a sterile environment.
Cultivar	:	A variety of a plant that was produced from a natural species and is maintained by cultivation.
Denature	:	Change nature of a protein or compound by chemical or physical means.
Diagnostics	:	The act of identifying a disease, illness, or problem by examining someone or something.
Differentiated	:	Cultured cells that maintain all or much of the specialized structure and function typical of the cell type <i>in vivo</i> .
Dystrophy	:	Body disorder or degeneration in health caused by defective nutrition, or muscular decline and weakness.

Explant	:	An excised piece or part of a plant used to initiate a tissue culture.
Exponentially	:	Growing or increasing very rapidly.
Genetic engineering	:	alteration of the genetic makeup of an organism using the molecular methods of biotechnology.
Genetically modified organism (GMO)	:	An organism whose genome has been artificially changed.
Laminar Flow Hood	:	An enclosed work area where the air is cleaned using HEPA filters.
Medium	:	A solid or liquid nutritive solution used for culturing cells.
Meristem	:	A group of undifferentiated cells situated at the tips of shoots, buds and roots, which divide actively and give rise to tissue and organs.
Micropropagation	:	Multiplication of plants from vegetative parts by using tissue culture.
Morphogenesis	:	The origin and development of morphological characteristics.
Primers	:	Small, chemically synthesized oligonucleotides that are complementary to a region of the DNA.
Propagule	:	A portion of an organism (shoot, leaf, callus, etc.) used for propagation.
Recombinant DNA	:	Combination of DNA fragments generated by molecular cloning that does not exist in nature.
Recombinant protein	:	A protein that is expressed from recombinant DNA molecules.
Somatic embryos	:	Non-zygotic bipolar embryo-like structures obtained from somatic cells.
Subculture	:	Transfer of a culture or portion of culture to a fresh synthetic media.
Tandem	:	One following or behind the other.
Template	:	DNA to be amplified.
Therapeutics	:	A branch of medical science dealing with the application of remedies to diseases.
Undifferentiated	:	Cells that have not transformed into specialized tissues.
Vaccine	:	Chemical that stimulates the body's immune system to better fight pathogens when they attack the body.