Swayam Course - Analytical Techniques

Week 12, Tutorial 31 - Introduction to Recombinant DNA Technology

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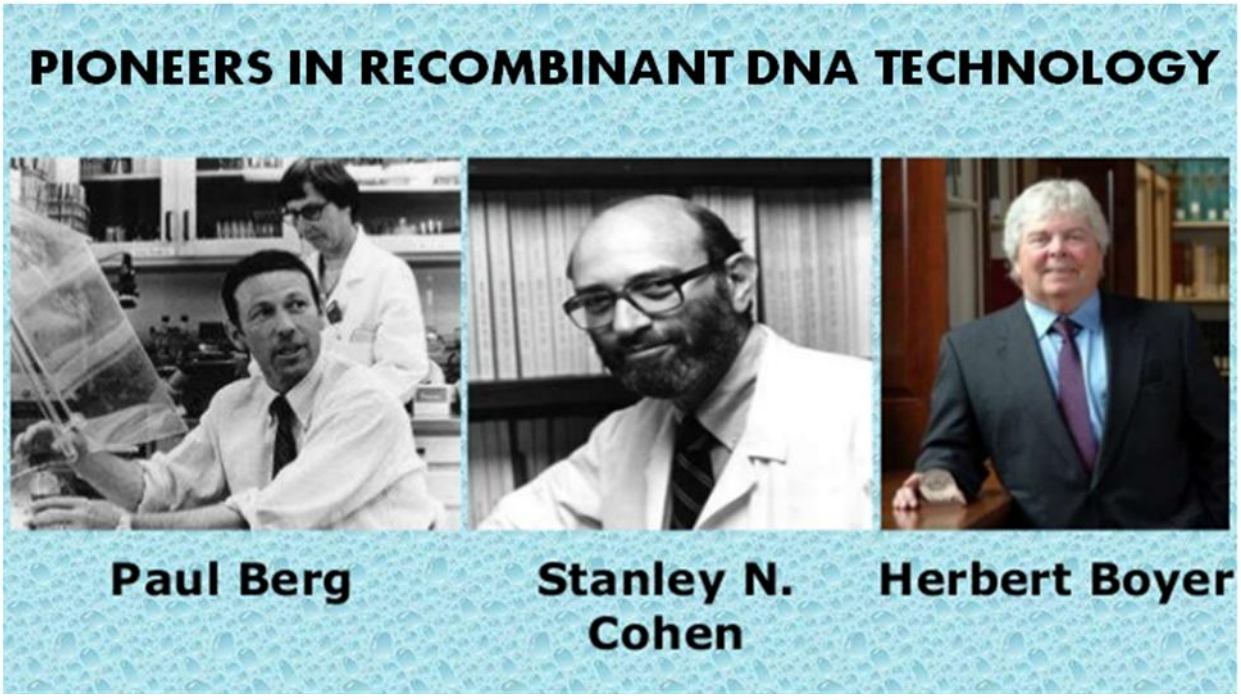
1. INTRODUCTION

In recent years, a remarkable development has been made in the manipulation of prokaryotic and eukaryotic DNA using the advent of Genetic Engineering. One such mean of genetic engineering is Recombinant DNA Technology which involves the generation of recombinant DNA molecules which are then introduced into suitable host cells where they can be propagated and multiplied. These recombinant DNA molecules are generated by joining of two DNA molecules from same or different species, hence, are not found otherwise in nature.

Recombinant DNA Technology was first discovered by Paul Berg, Herbert W. Boyer and Stanley N. Cohen in 1977 when they first successfully expressed somatostatin in bacteria.



The way by which genetic material from one organism is artificially introduced into the genome of another organism and then replicated and expressed by that other organism has tremendously helped in the generation of certain clinically significant proteins. This chapter therefore will

discuss about the introduction of recombinant DNA technology with emphasis being given on the steps involved in RDT, the components required for RDT and their applications in medical science.

2. OBJECTIVES

The following sections would be covered in this chapter:

- basis of the technique Recombinant DNA Technology (RDT) or gene cloning
- various steps involved in RDT
- detailed description of various components of RDT
- transfer of clone into host cells
- selection of clone by different methods
- various applications of this technique in different fields

3. STEPS OF RECOMBINANT DNA TECHNOLOGY

Recombinant DNA Technology involves the generation of recombinant DNA followed by its propagation in an appropriate host cell in order to produce many copies of the sequence which is also known as gene cloning. The basic steps involved in the process of cloning include:

(i) Isolation of DNA fragment commonly known as 'insert' that is to be used or manipulated along with the isolation of a carrier DNA molecule termed 'vector' that can self replicate within the host cell.

(ii) Both insert and vector were treated individually with specialized cutters or enzymes called restriction endonucleases followed by their ligation to generate recombinant DNA (rDNA) molecule.

(iii) Transfer of these rDNA molecules into an appropriate host cell.

(iv) Selection of transformed host cells that carry the desired rDNA molecule followed by their replication to create a population of genetically identical cells or 'clone'. Each cell in the clone contains multiple copies of the insert.

The schematic representation of recombinant DNA technology or gene cloning has been shown in Figure 1. The various steps of cloning and the components involved will be discussed in detail in subsequent sections in this chapter.

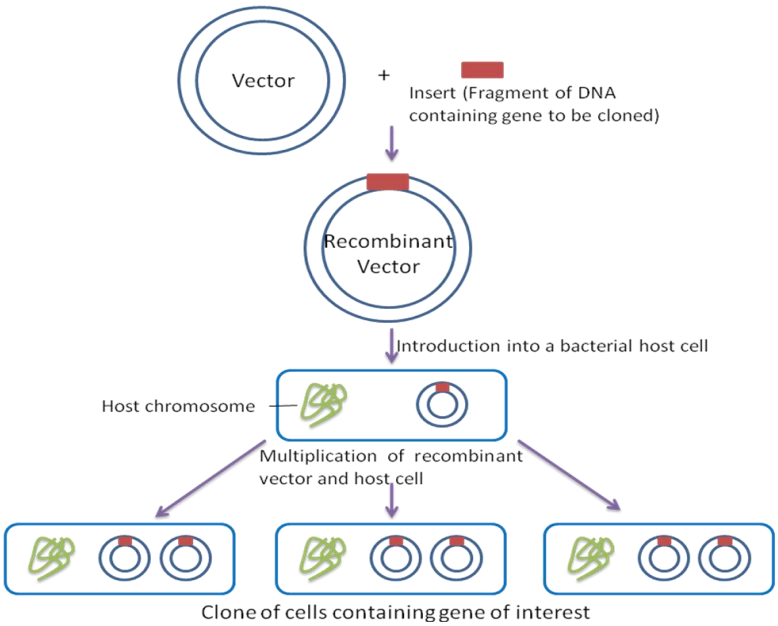


Figure 1: Schematic representation of basic steps of cloning

4. CLONING VECTORS

Initiation of the process of cloning requires the use of specialized vehicles termed as "vectors" or more appropriately "cloning vectors" which are the DNA molecules that can carry a foreign DNA segment and replicate within the host cell. In order to serve as a cloning vehicle, a vector must possess the following characteristics:

1. It must possess an origin of replication (ori site) that enables it to replicate autonomously within the host cells.
2. It should contain a selectable marker which will help in selection of transformed cells from non-transformed cells.
3. The vector must comprise of atleast one unique restriction endonuclease cleavage site in one of the marker genes that enable the insertion of foreign DNA into the vector. Certain vectors possess a small region called polylinker or multiple cloning sites (MCS) which contains several unique recognition sites for various restriction enzymes. This provides flexibility in the choice of restriction enzymes in the process of cloning.
4. It should be relatively small in size since large molecules tend to break down during purification and are also difficult to manipulate.
5. When expression of the foreign DNA is desired, the vector should contain suitable control elements such as promoter, operator, terminator, ribosome binding sites, etc.

Cloning was first performed using *Escherichia coli*, and cloning vectors in *E. coli* include plasmids, bacteriophages, cosmids, and bacterial artificial chromosomes (BACs). However, very large DNA fragment cannot be maintained in prokaryotes, hence, yeast may be used to clone these segments. Cloning vectors in yeast include yeast artificial chromosomes (YACs). Although all of these vectors satisfy the above criteria, plasmids and bacteriophages are most commonly used vectors nowadays.

5. RESTRICTION ENDONUCLEASES

Restriction endonucleases (REs) are enzymes that selectively recognize a specific DNA sequence and cleave at or near that sequence. REs digestion produces two types of ends- **Blunt ends or cohesive (sticky or staggered) ends**. Sticky ends has protruding single stranded overhangs which can base pair with another complementary overhang while ligation. On the other hand, blunt ends are non-cohesive ends generated by linear cut on the double stranded DNA molecule. As depicted in Figure 2, EcoRI produces sticky ends whereas SmaI produces blunt ends.

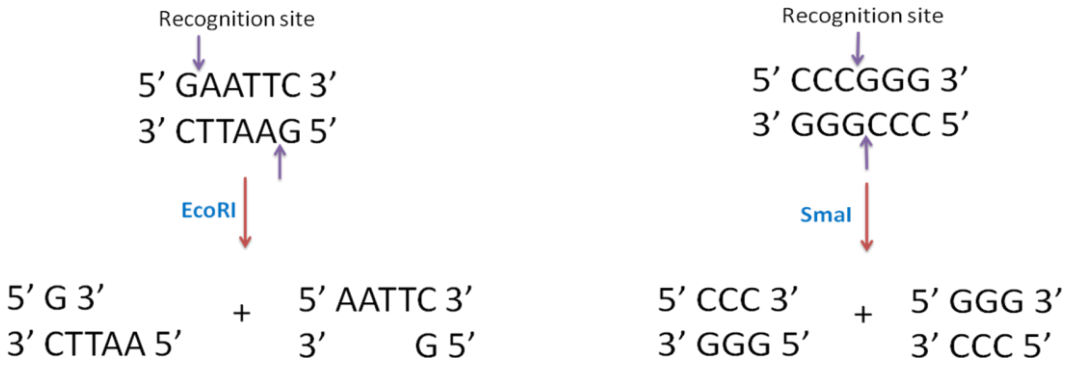


Figure 2: Sticky (cohesive) and blunt ends produced by digestion with restriction endonucleases

Ligation of complementary sticky ends is much more efficient because compatible sticky ends can form a relatively stable structure by base pairing with one another via hydrogen bonding. These stable base-paired structure increases the efficiency of ligation by increasing the length of time the ends are in contact with one another. Therefore, sticky ends are favored over blunt ends in recombinant DNA technology.

Cloning vector and the insert that is to be cloned needs to be treated with same restriction endonuclease in order to produce similar ends that can be joined to each other with the help of an enzyme called "**DNA Ligase**" to form recombinant vector (Figure 3). The DNA ligases used for cloning are mainly of two types: *E.coli* DNA Ligase and T4 DNA Ligase. *E.coli* DNA ligase is encoded by *E.coli*, requires NAD+ as a cofactor and catalyzes the ligation of cohesive ends only. Contrary to this, T4 DNA ligase is encoded by phage T4 and requires ATP as a cofactor. It catalyzes the ligation of cohesive ends along with blunt ends.

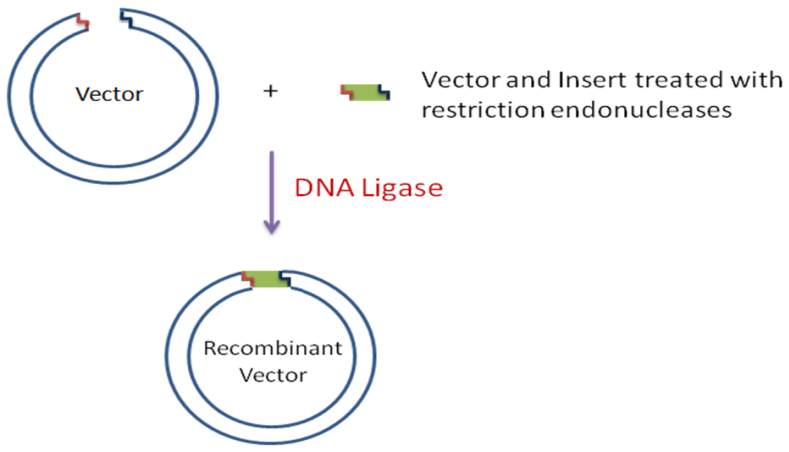


Figure 3: Schematic representation of generation of recombinant vector

The treatment of vector with single restriction enzyme promotes the recircularization of vector which can be prevented by treating the linearized vector DNA with alkaline phosphatase enzyme to remove phosphate groups from the 5'-terminal of the vector. Hence, circularization of vector can occur only by insertion of non-phosphatase treated foreign DNA (or insert) which provides one 5' terminal phosphate at each join leaving behind an unligated nick which can be repaired after transformation.

As mentioned earlier, sticky ends can base pair easily with the complementary overhangs, however, the ligation efficiency of blunt ends is very low. Blunt ended DNA molecule, therefore, are quite difficult to ligate but can be joined by the following approaches:

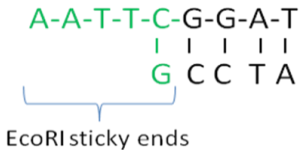
(i) **T4 DNA ligase** catalyzes the formation of phosphodiester bonds between blunt-ended fragments.

(ii) **Use of linkers or adaptors**

Ligation of adaptors or linkers to the blunt ended insert creates cohesive end terminal that assist in joining.

Linkers are short blunt-ended oligonucleotides which contain site for one or more restriction endonucleases. These are ligated to the blunt-ended foreign DNA followed by treatment with restriction endonuclease to generate sticky ends and then, allowed to ligate to the vector. Linker ligation involves treatment with endonuclease, thus, might also cleave the target DNA at internal site.

Adaptors are short synthetic double stranded DNA molecules with a preformed cohesive end and one blunt end. These are ligated to the blunt-ended foreign DNA followed by ligation to the vector. Adaptor ligation did not involves treatment with endonuclease, thus, excludes the possibility of cleavage of foreign DNA to be cloned.



Treatment of linker ligated foreign DNA molecule with restriction endonuclease might also cleave the foreign DNA if site for that enzyme exists in it. Hence, adaptors with preformed cohesive ends would be advantageous for ligation.

(iii) **<u>Use of 'terminal deoxynucleotidyl transferase'</u>** that synthesize 3′ homopolymer tails at the ends of fragments to be joined. Homopolymer tailing involves addition of homopolymer tails at the 3' end of insert along with vector with the help of an enzyme known as "Terminal deoxynucleotidyl transferase". These homopolymers facilitate the joining of insert and vector using DNA ligase as described in the figure 4 below.

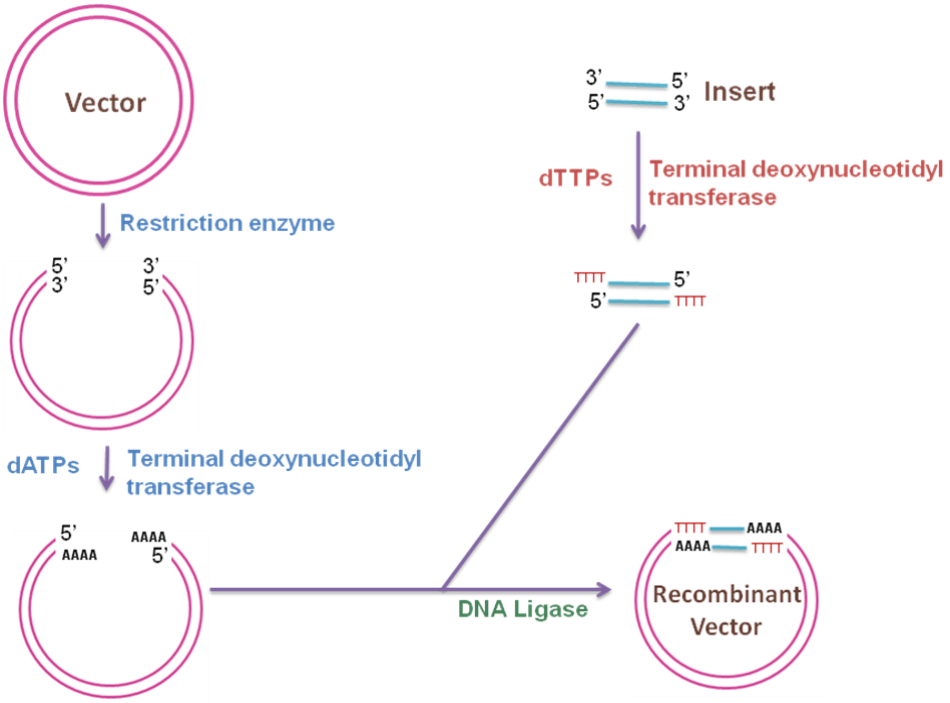


Figure 4: Homopolymer tailing for joining blunt ended DNA molecule

6. DIFFERENT VECTORS USED IN CLONING

Vectors used in cloning include plasmids, bacteriophages, cosmids, phasmids, Bacterial Artificial Chromosomes (BACs), Yeast Artificial Chromosomes (YACs). The respective insert size has been mentioned in Table 1.

CLONING VECTOR	INSERT SIZE (Kb)
Plasmid	0.5-8
Bacteriophage	5-25
Cosmid	35-45
BAC	≤300
YAC	200-1000

Table 1: Various types of vectors and their insert size

6.1 Plasmids

Plasmids are extra-chromosomal, self-replicating, closed and circular double-stranded DNA molecules, found naturally in bacteria and also in some yeast. It is capable of behaving as a carrier of DNA fragment in the process of cloning.

pBR322 was the first vector developed and generated by Bolivar & Rodriguez and on whom the plasmid was named. This plasmid consists of the following characteristics:

- pBR322 is small in size, i.e., 4363bp, thus, it can be purified with ease.
- It carries two sets of antibiotic resistance genes (ampicillin and tetracycline) that allows simple identification of transformants.
- It has a plausibly high copy number. In most cases, there are about 15 molecules present in a transformed *E.coli* cell which can be increased up to 1000–3000 by plasmid amplification.

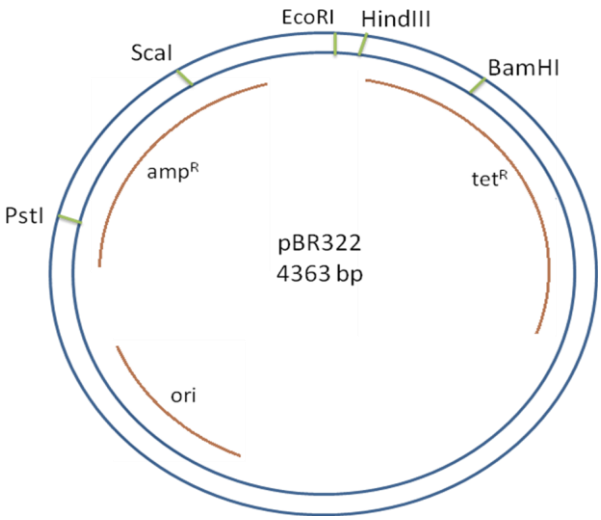


Figure 5: Overview of pBR322 plasmid

pUC is an off-shoot of pBR322 with various advantages which includes:

(i) It consists of polylinker or multiple cloning site (MCS) which carry sites for several restriction endonucleases extending the range of enzymes that can be used to generate a restriction fragment suitable for cloning.

(ii) It also contains DNA sequence for lac Z' coding for β-galactosidase that permits rapid visual detection of an insert via blue-white selection method that will be discussed later in the chapter.

(iii) pUC has high copy number in contrast to pBR322 as the former can be maintained at 500-700 copies per cell before amplification.

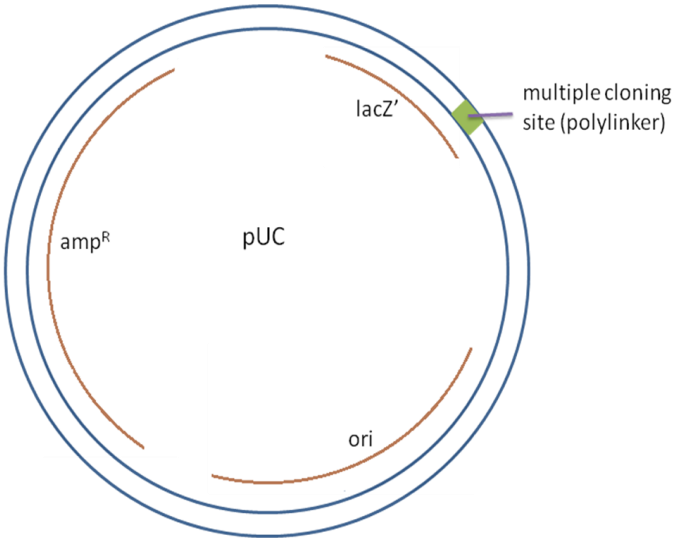


Figure 6: Overview of pUC plasmid

6.2 Bacteriophages

Bacteriophage or λ phage is a virus that infect bacterial cell and replicate using the machinery of those infected cell. They are advantageous over plasmids as:

(i) They infect cells with high efficiency than transformation by plasmids; hence, the quantity of clones yielded with phage vectors is usually greater.

(ii) They can carry a larger fragment of size upto 25kb in contrast to limit of 8 kb in plasmids.

M13 phage has the ability to isolate single stranded form of the cloned gene which is of utmost importance in both sequencing and mutagenesis.

6.3 Cosmids

Cosmids are plasmids that incorporate cohesive end site (cos) of bacteriophage λ which contains elements required for packaging DNA into λ particles. It is used to clone large DNA fragments between 35 to 45 Kb.

6.4 Phagemids

Phagemids are plasmids containing properties of plasmid and phage. They contain f1 origin of replication (ori) from filamentous 'f1' phage. They can be maintained as plasmids as well as can be packaged as single stranded DNA in viral particles.

6.5 Bacterial Artificial Chromosomes (BACs)

Bacterial Artificial Chromosomes (BACs) are engineered vectors based on Fertility plasmid (or F plasmid) used for transformation in bacteria. F-plasmids contain partition genes which promote the even distribution of plasmids after bacterial cell division. BACs can carry a large gene of interest of size upto 300kb, hence, are often used to sequence the genome of organisms.

6.6 Yeast Artificial Chromosomes (YACs)

Yeast Artificial Chromosomes (YACs) are genetically engineered chromosomes derived by the ligation of yeast DNA into a bacterial plasmid. These vectors have large insert carrying capacity of upto 1000kb. The components of YACs include:

(i) Autonomously Replicating Sequence (ARS) for plasmid replication.

(ii) Centromere (CEN) for uniform distribution of chromosome to daughter cells during cell division.

(iii) Telomeres (TEL) for protection of ends of chromosomes.

(iv) TRP1 and URA3 for selection of recombinants.

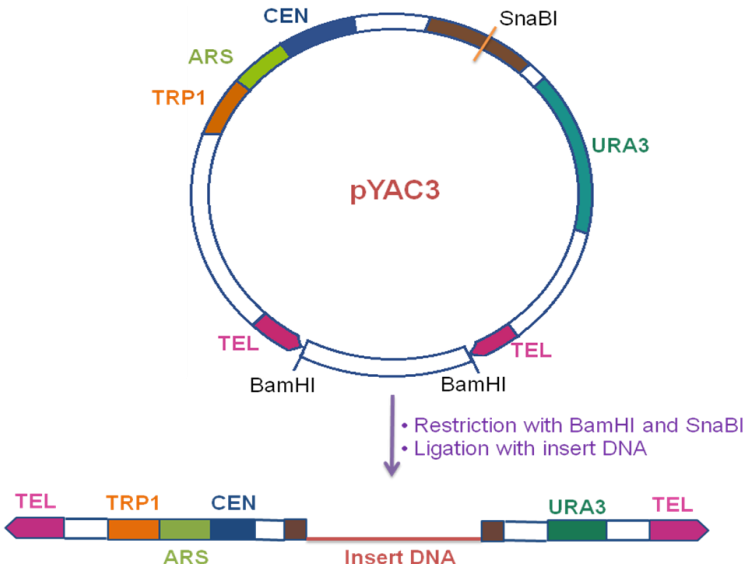


Figure 7: Overview of YAC vector

6.7 ***Agrobacterium tumefaciens***

Agrobacterium tumefaciens is the natural genetic engineer of plants. It is a soil bacterium consisting of a tumor inducing (Ti) plasmid. It causes crown gall disease in dicot plants due to its natural ability to transfer a portion of its plasmid called T-DNA which then, integrates into the plant chromosome. This inherent property of bacteria can be exploited in cloning wherein T-DNA can be replaced with gene to be cloned and then can be delivered efficiently to the plants.

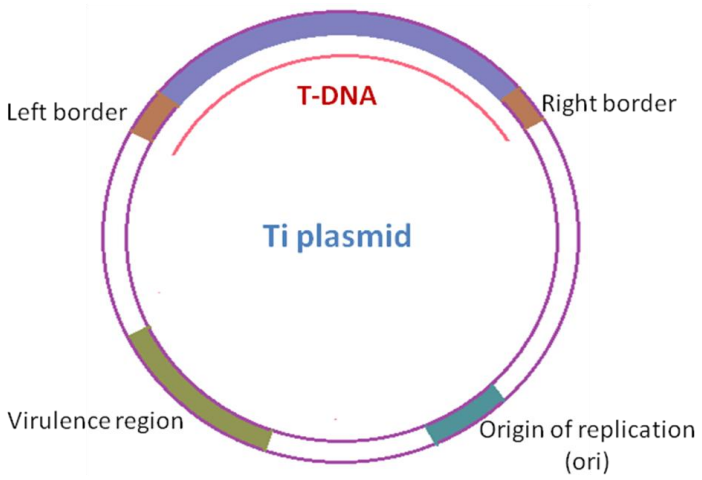


Figure 8: Ti plasmid of *Agrobacterium tumefaciens*

7. EXPRESSION VECTORS

Expression vectors are the cloning vectors containing suitable expression signals required to have maximal expression of the gene product. These vectors incorporate signals necessary for

initiation and termination of transcription and translation adjacent to the cloning site. These vectors aid in facile synthesis of large quantities of protein. Continuous high level synthesis of gene product exerts a metabolic drain on the cell leading to slower growth along with some toxicity to the cell. Hence, presence of regulated promoter might control the production of gene product.

The construct of an expression vector is shown in Figure 9.

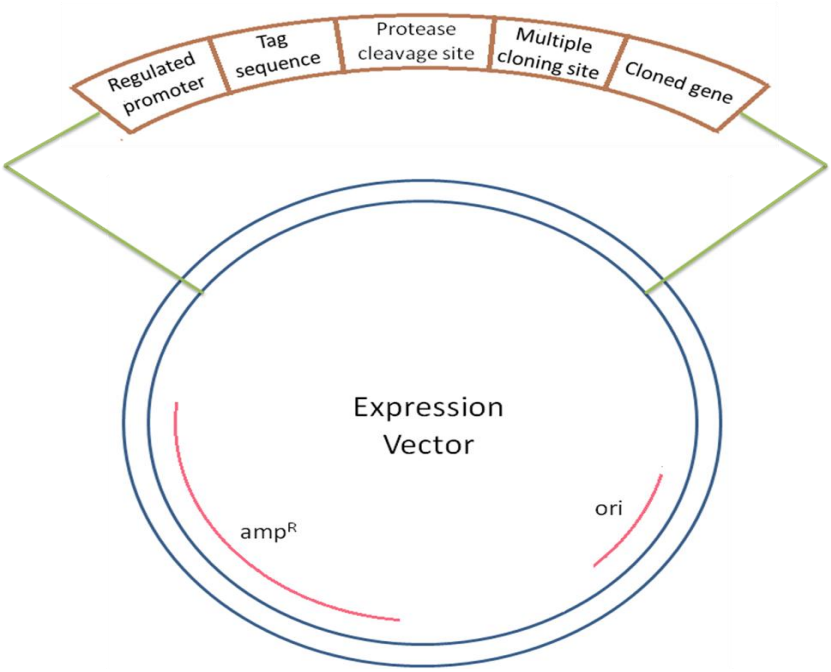


Figure 9: Schematic representation of an expression vector

Various regulated promoters such as λP_L, T7, trc (tac), BAD promoters are used in the expression vector and one of them has been explained below to understand the mechanism of the regulation.

λP_L promoter system

This system combines very tight transcriptional control with high levels of gene expression. This is achieved by putting the cloned gene under the control of the P_L promoter carried on a vector, while the P_L promoter is controlled by a cI repressor gene in the *E. coli* host. This cI gene is itself under the control of the tryptophan (trp) promoter.

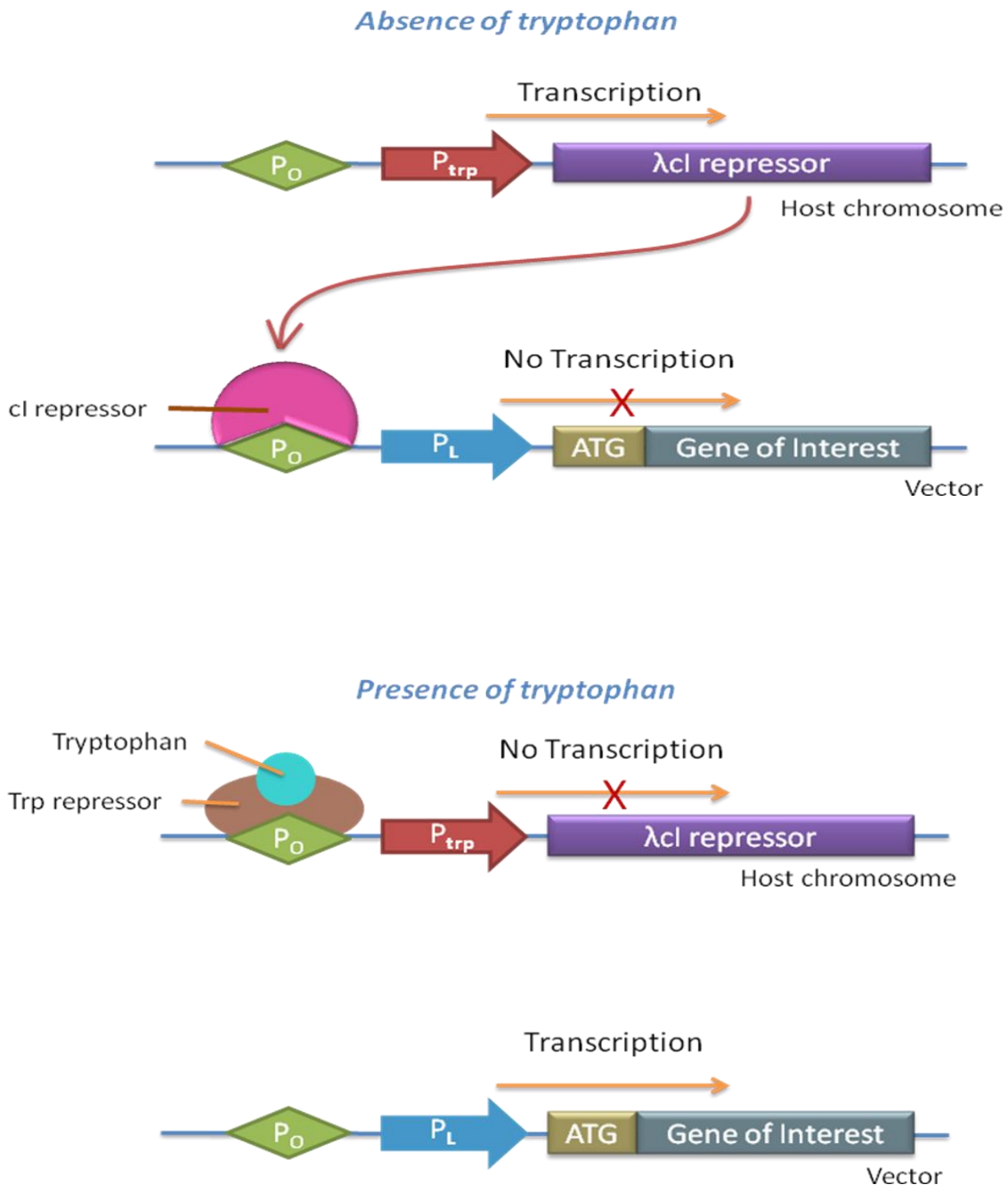


Figure 10: Regulation of gene expression using λP_L promoter system

Purification of a cloned gene product

This can be facilitated by use of short peptide sequences coined "tag" which is fused to the protein being expressed. This tag includes multiple histidine residues, biotin, etc.

The tag used for purification of protein should comprise of following properties:

- It should be easily assayed.
- It should be easily separable from the protein of interest. This can be achieved by inserting a protease cleavage site between the tag and the gene being expressed.
- It should easily be purified by methods like affinity chromatography.

8. TRANSFER OF CLONED GENE INTO HOST CELL

The process of transferring foreign DNA into the host cell is known as transformation. The host cell can be prokaryotes or eukaryotes (plant cell or animal cell). The transformation requires host cell to be competent which could be defined as the ability of host cell to take up foreign DNA from outside. The ability to transform can be natural or induced.

Natural competence: an inherited ability of bacteria to transform under natural conditions and also in the laboratory.

Induced competence: arises when host cells are treated with certain chemicals to make them transiently permeable to DNA.

The transform ability can be induced artificially by various methods depending upon the host cell as discussed below:3

8.1 Bacterial cells

8.1.1. Calcium chloride ($CaCl_2$) method

This method was developed by Mandell & Higa. In this, bacterial cells are treated with cold $CaCl_2$ which make the cell competent for transformation. Ca^{2+} being positively charged bind to the negatively charged membrane and when DNA is added, it bind to the membrane through these calcium ions. After addition, cells are given heat shock treatment at 42°C for 90s which transiently create pores in the cell membrane allowing entry of foreign DNA.

8.1.2. Transfection

It is the transfer of foreign DNA into cultured host cell mediated through charged chemicals (cationic liposomes, calcium phosphate, DEAE dextran) which are taken & mixed with DNA molecules. The recipient host cell is overlayered by this mixture & foreign DNA is taken up by the host cell.

Bacterial cells (mainly *E.coli*) are widely used in genetic engineering but there are certain disadvantages of using *E. coli* for production of eukaryotic proteins such as:

- *E.coli* lacks eukaryotic post-translational machinery and chaperones resulting in protein misfolding.

- Proteins might precipitate into insoluble aggregates known as inclusion bodies.
- Codon utilization is biased among the organisms causing translation error which results in improper biological activity of protein.

Keeping this in mind, eukaryotic cells such as animal cells or plant cells are also being used in genetic engineering and method of transformation varies in these cells as mentioned below:

8.2 Animal cells

8.2.1. Microinjection

It is a technique of delivering foreign DNA into a living cell (a cell, egg, oocyte, embryos of animals) through a glass micropipette.

8.2.2. Direct transformation

In this, foreign DNA fragment is precipitated with calcium phosphate & mix with the cells to be transformed. DNA molecule passes through cell membrane & integrates with the mammalian chromosome.

8.3 Plant cells

8.3.1. Vector-mediated or indirect gene transfer method

This is mediated by Ti plasmid of a soil bacterium, Agrobacterium tumefaciens which is known as natural genetic engineer of plants. This has already been discussed above in cloning vectors section.

8.3.2. Electroporation

This involves application of a pulse of high voltage (~350 V) to protoplasts/ cells/ tissues which induce the formation of transient (temporary) pores in the plasma membrane which facilitates the uptake of foreign DNA.

8.3.3. Particle bombardment method (biolistics)

The foreign DNA is coated onto the surface of minute gold or tungsten particles & bombarded (shot) onto the target tissue/ cells using a particle gun (gene gun/ shot gun/ microprojectile gun).

8.3.4. Microinjection

It is similar method as for animal cells.

9. SELECTION OF TRANSFORMED CELLS

The recombination and transformation efficiency is not absolute, hence, could result in both recombinant cells containing plasmid wit gene of interest and non-recombinant cells containing plasmid without insert. Non-transformed cells would not have any kind of plasmid. To select only the recombinant transformed cells, certain methods are being employed nowadays which are listed below:

9.1. Insertional inactivation method

Insertion of gene of interest in any region of antibiotic resistance gene leads to interruption of antibiotic resistivity of plasmid. As shown in Figure 11 below, plasmid contains ampicillin resistance gene (amp^R) and tetracycline resistance gene (tet^R). The gene of interest is inserted in tetR region leading to disruption of tetracycline resistance. Recombinant cells with desired gene inserted at tetracycline coding region can grow only in ampicillin containing medium, whereas non-recombinant cells with unaltered vector can grow in both ampicillin and tetracycline medium. Hence, recombinant cells can then be selected by comparing replica plate with master plate. In addition, non-transformed cells would not have any plasmid, hence, these cells could not grow in presence of any antibiotic.

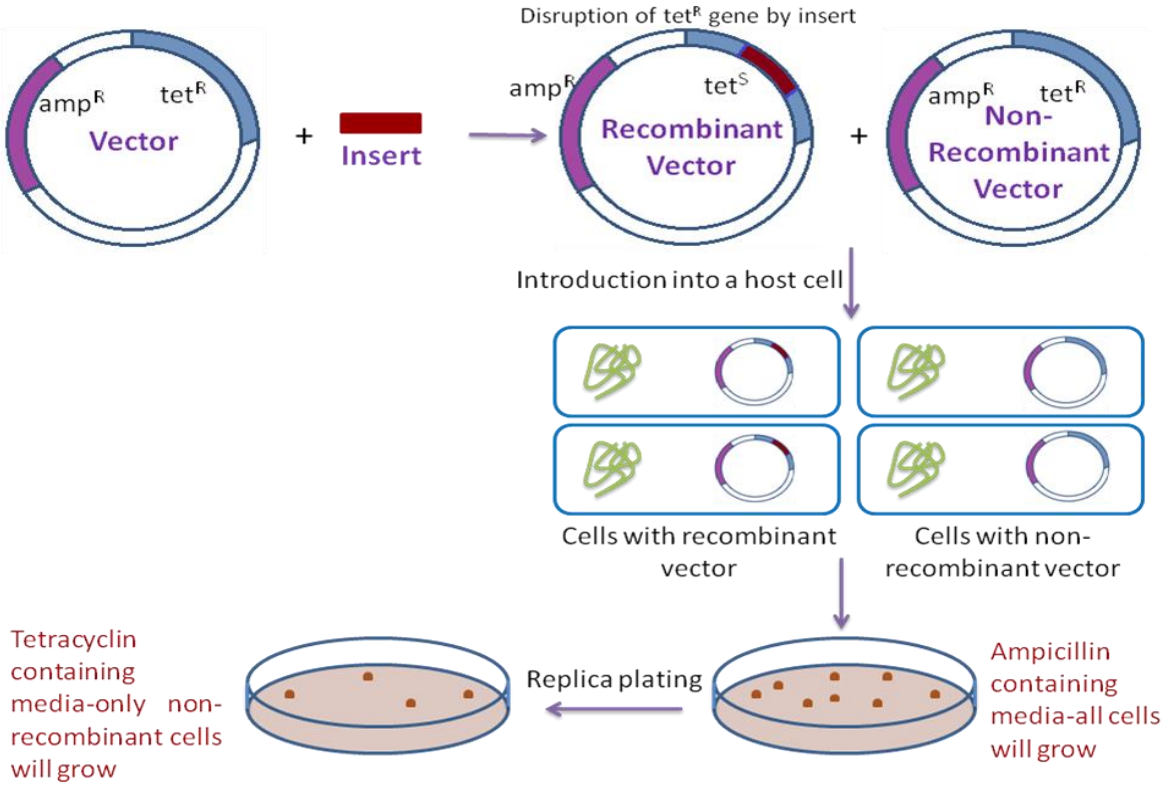


Figure 11: Selection of recombinant plasmid using Insertional Inactivation method

9.2. Blue-white selection method

As mentioned earlier, pUC vector contains lacZ gene which encodes for the protein β-galactosidase which converts X-Gal substrate into blue coloured product as shown in Figure 12a. Insertion of gene in lacZ region would disrupts the formation of β-galactosidase, hence, no blue coloured product will be formed. Therefore, recombinant cells will be differentiated from non-recombinant cells by this blue-white selection method with recombinant cells giving blue colonies while white colonies by non-recombinant cells (Figure 12b).

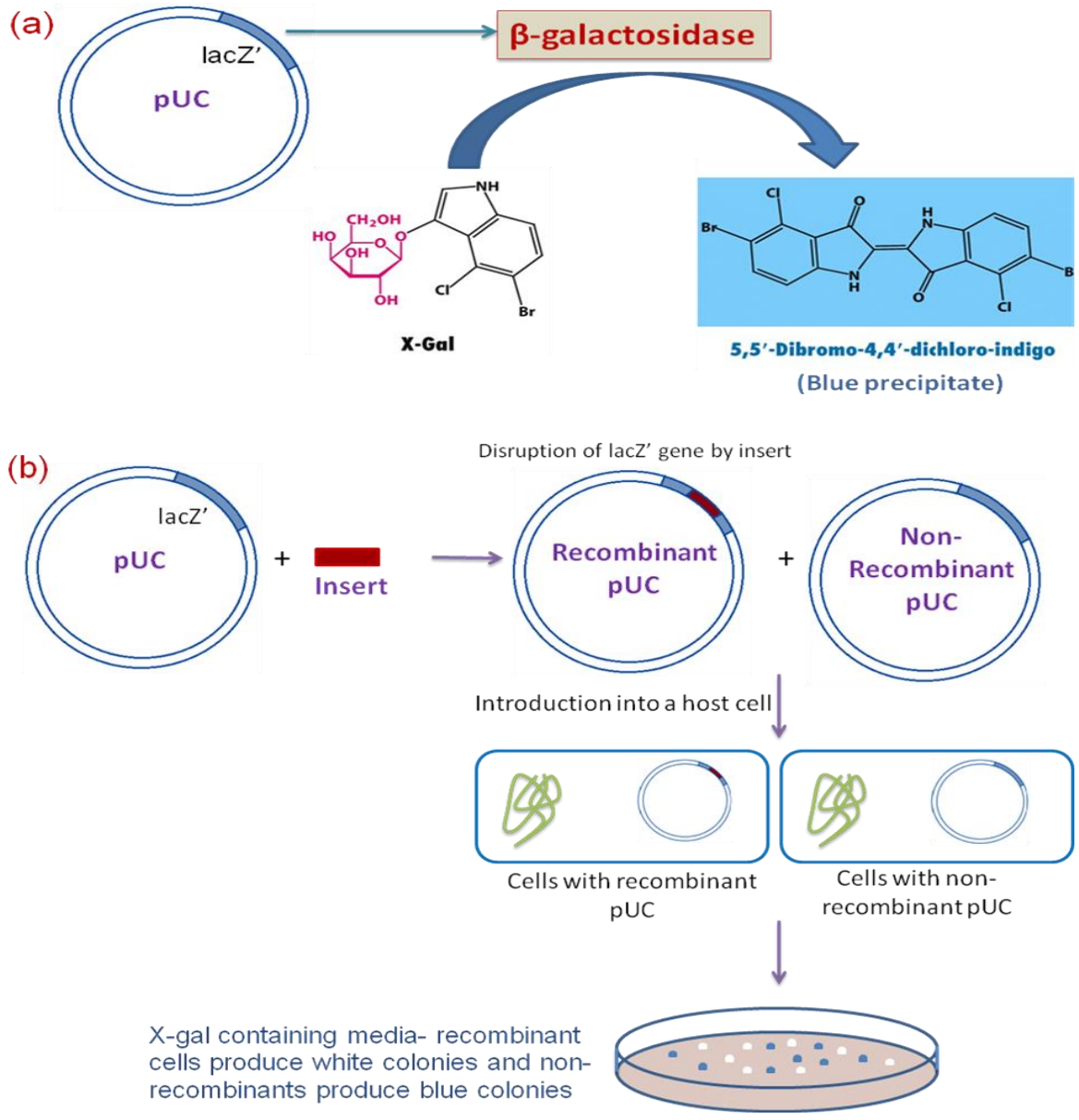


Figure 12 (a) & (b): Blue-white selection method for identification of pUC recombinants

10. APPLICATIONS OF RDT

Recombinant DNA Technology has applications in various fields listed below:

1. Medicine: This technology is nowadays used in medicine for production of insulin (for diabetes patients), growth hormones, antibiotics, vaccines, interferons, etc. Moreover, genetic engineering is also employed in diagnosis of HIV and hepatitis, etc. The short segments of DNA have been cloned by RDT and labeled with fluorescent marker to behave as probe to test the presence of DNA of the infectious pathogen. The transgenic animals are being produced such as transgenic cows or goats containing human proteins in their milk and transgenic fish containing human growth hormone.

2. Agriculture: RDT is widely used in the field of agriculture to generate genetically-modified organisms that could produce genetically-modified crops. The first genetically modified food licensed for human consumption was Flavr Savr tomato. The ripening process in these tomatoes is slowed down by inhibition of an enzyme polygalacturonase which resulted in delayed ripening and resistant to rotting. In addition, genetic modifications in crops have made them resistant to herbicide, insects, virus, drought and thus resulted in development of many such products such as Bt cotton, Bt brinjal, golden rice, etc as shown in Figure 13.

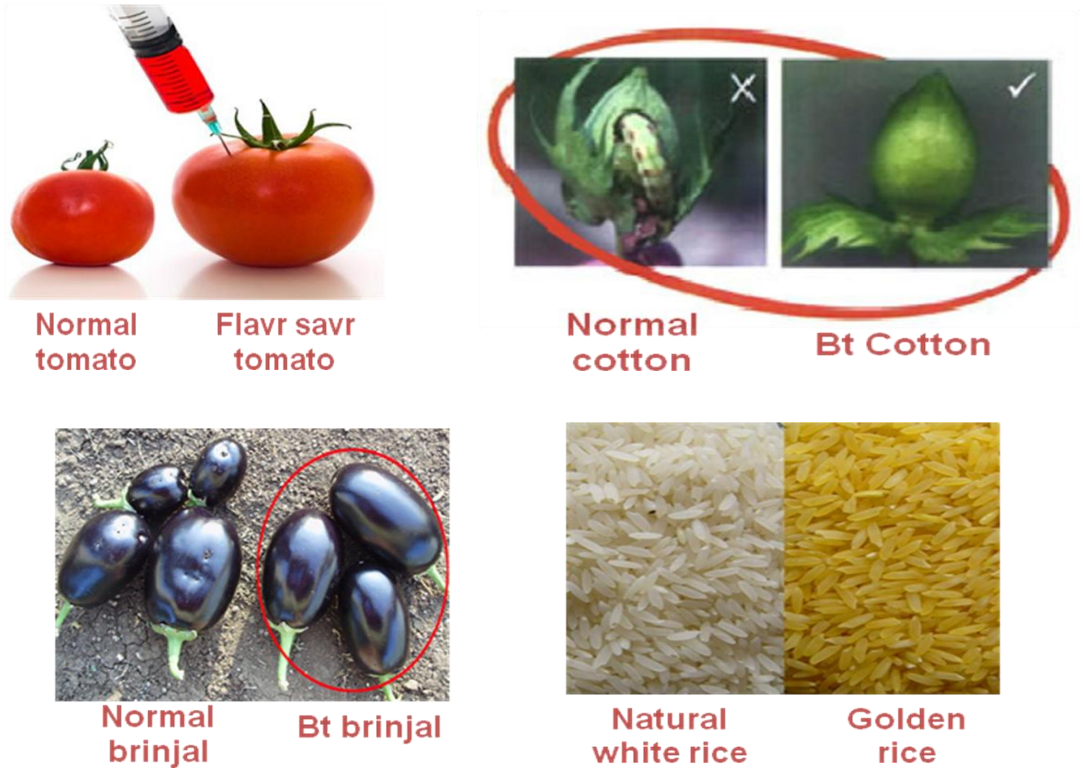


Figure 13: Few genetically modified products

3. Research: This technology has significant contribution in field of research where in RDT has make it effortless to study the functionality of a particular protein. The purification followed by identification of role of any protein could be performed in physiology or pathology conditions. For example, p53 protein can be cloned and transfected into any cancer cells to over-express it and afterward, the downstream effects of p53 could be identified in any particular condition.

11. SUMMARY

To summarize, Recombinant DNA Technology is one of the better acceptable technology which could join two different DNA molecules which are not found otherwise in nature. The vector and gene of interest are first digested by restriction endonucleases and then joined together by DNA ligase. This recombinant DNA then transformed into a host cell for further propagation and multiplication. The efficiency of recombination and transformation is not 100%, hence, recombinants were selected by various selection strategies like insertional inactivation method and blue-white selection method. RDT is of utmost importance in numerous fields like medicine, agriculture, research, etc. This chapter has therefore discussed each and every aspect of RDT in detail, its various components, steps and applicability in various areas. This method has a great potential for future outcomings in various fields.