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Transgenic Plants

Conventionally the genetic variations are necessary in plant cells and tissue for the production of improved plant varieties and crop improvement. This would be achieved by manipulating the characteristics of plants using genetic engineering. Various transformation techniques are involved in production of transformed cells and tissue of plants. The transformation is achieved by genetic engineering of plants by biotechnologists or Genetic engineers. The plants obtained through genetic engineering contain a gene or genes usually from an unrelated organism; such genes are called transgenes, and the plants containing transgenes are known as transgenic plants. Transgenic plants are those transformed varieties of plants which contain a foreign gene or gene of interest incorporated into the genome of its cells or tissue.

The first transgenic plant was produced in 1983, when a tobacco line expressing kanamycin resistance was produced. Till date number transgenic plant varieties have been developed which shows resistance against herbicides, insects,viruses many stress conditions like high and low salt concentration, drought resistance, and many more. Transgenic varieties also developed to improve the plant product in terms of increasing yields and long shelf life of fruits and flowers.

Transgenic plants are produced by manipulation in DNA of plant cells by genetic engineering. Many techniques are used to produce a transformed cell of a plant and to develop a new improved transgenic plant variety. Some of the important techniques involves: vector mediated transgenic plant production, Direct Gene transfer, chemical based transfer of foreign DNA, Electroporation technique, particle gun bombardment method, lipofection, Microinjection etc.

Vector mediated gene transfer:

Plant cells do not have any endogenous plasmids. The plasmid vectors used for plant cell transformation are mostly based on pTi (tumour inducing plasmid) of *Agrobacterium tumefaciens*, but some are derived from pRi (root inducing plasmid) of *A. rhizogenes*. These are plant pathogenic gram negative soil bacteria known to cause crowngall (*A. tumefaciens*) and hairy root (*A. rhizogenes*) dieases of dicot plants. They infect plant cell near wounds, usually at the crown of roots at the soil surface.

Among the various vectors used in plant transformation, the Ti plasmid of ***Agrobacterium tumefaciens*** has been widely used. This bacterium is known as "**natural genetic engineer**" of plants because these bacteria have natural ability to transfer T-DNA of their plasmids into plant genome upon infection of cells at the wound site and cause an unorganized growth of a cell mass known as crown gall. Ti plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues. The foreign gene is cloned in the T-DNA region of Ti-plasmid in place of unwanted sequences.

Molecular biology of Agrobacterium infection:

The process of infection by *A. tumefaciens* involves the transfer of small part of pTi plasmid

into the plant cell genome; this DNA sequence is called T-DNA (transfer DNA).The infection process is governed by both chromosomal and plasmid borne genes of *A. tumefaciens.* Attachment of bacteria to plant cell begins the infection, governed by chromosomal virulence genes (chv); which are expressed constitutively. Some of the chromosomal genes and their function in *A. tumefaciens* attachment to plant cell and its infection are listed in the figure1.

Chromosomal gene	Function
chvA	Encodes an inner membrane protein essential for the transport of β-1,2-glucan from cytoplasm to periplasm
chvB	Encodes an inner membrane protein most likely involved in the synthesis of β-1,2-glucan
chvD and *chvE*	Needed for an optimal expression of *vir* genes of *p*Ti
exo locus genes	Biosynthesis of attachment polysaccharides
exoC	Encodes an enzyme directly involved in the biosynthesis of β-1,2-glucan
cel genes	Cellulose fibril synthesis especially during the early phases of infection so that the bacterial cells become firmly adhered to plant cells

Figure: 1- The chv gene name and their specific functions for attachment of bacterial plasmid to the surface of plant cell (Reference: 1)

Crowngall cells:

Infection by *A. tumefaciens* produces tumour like growth from which roots or shoots may sometimes be produced. But infection by A. rhizogenes give rise to hairy roots, which may often show negative geotropism. The crowngall and hairy root cells also synthesize unique nitrogenous compounds by normal plant cells but not utilize them. These compounds are called opines. The agrobacterium use the opines as their carbon and nitrogen source. The bacteria usually present in the intercellular spaces of the crowngalls. There are different types of opines depends on agrobacterium strain. For instance, *Agrobacterium tumefaciens* generally involves in the production of octopine and nopaline, while the *A rhizogenes* are involves in the production of agropine and mannopine. The concerned gene for the catabolism of opines are present on pTi and pRi plasmid itself.

The Ti plasmid:

A **Ti plasmid** or **tumour inducing** plasmid is a circular plasmid that often, but not always, is a part of the genetic equipment that *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* use to transduce its genetic material to plants. The Ti plasmid is lost when *Agrobacterium* is grown above 28°C. Such cured bacteria do not induce crown galls, i.e. they become avirulent. pTi and pRi share little sequence homology but are functionally rather similar. The Ti plasmids are classified into different types based on the type of opine produced by their genes.

The different opines specified by pTi are octopine, nopaline, succinamopine and leucinopine.

The pTi and pRi plasmid are unique bacterial plasmid as they contain some genes (located within T –DNA segment), which have regulatory sequences recognised by plant cell so these genes only shows expression inside plant cells and the remaining genes have regulatory sequences and expressed only in prokaryotic bacterial cell. Thse plasmids naturally transfer their T- DNA into the host plant genome, which makes Agrobacterium a natural genetic engineer.

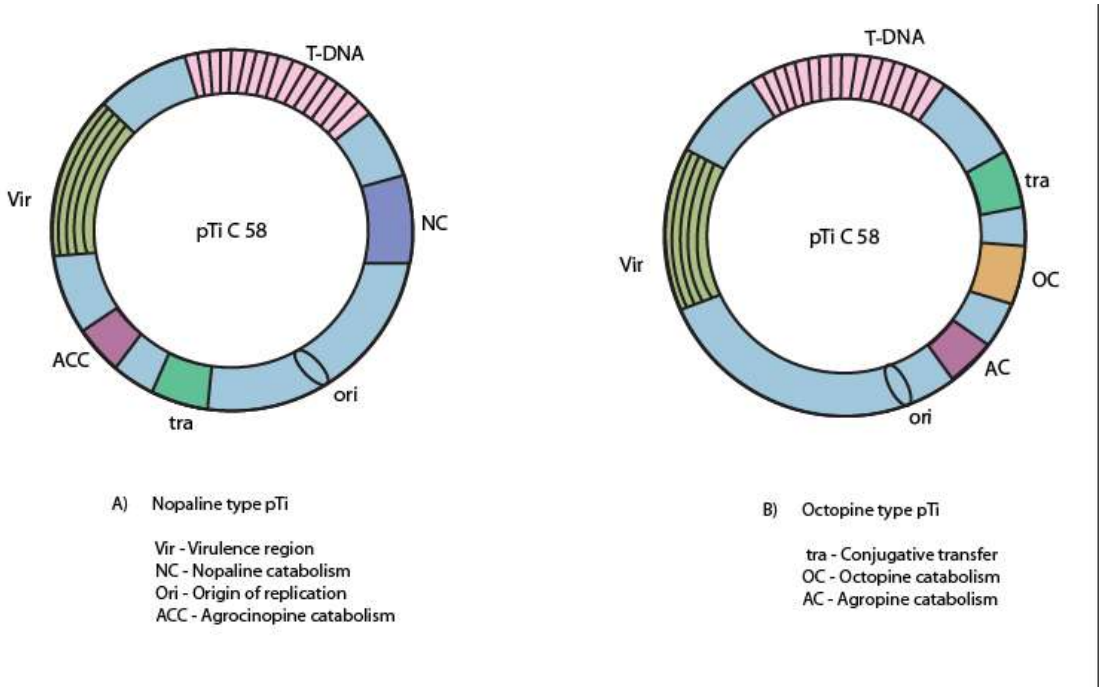

Figure 2: General organisation of Nopaline and octopine type of Ti plasmid (Reference: 1)

In figure 2 the general organisation of Nopaline and octopine type of Ti has been shown. The Ti plasmid contains different functional regions including T-DNA segment contains oncogene and opine synthesis genes, and is transferred into the host plat genome, vir region, which express number of genes to regulates the transfer of T- DNA into the plant cells, Opine catabolism regions (NC and OC). Which produce enzymes necessary for the utilization of opines by agrobacterium. OriT and Tra genes for the origin and initiation of replication and transfer of plasmid by the process of conjugation.

T-DNA organisation:

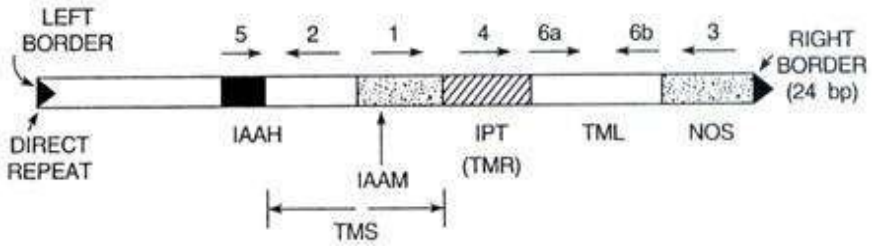

Figure 3: Organisation of T DNA segments present in Ti plasmid (Reference: 1))

The **transfer DNA** (abbreviated **T-DNA**) is the transferred DNA of the tumor-inducing (Ti) plasmid of some species of bacteria such as *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. The T-DNA is transferred from bacterium into the host plant's nuclear DNA genome. The T-DNA is bordered by 24-base-pair repeats on each end and contains the gene for tumours/hairy root induction and those for opine biosynthesis .

pTi has three genes:Two of which (iaaM,iaaH) encode enzyme to convert tryptophan into IAA (indole acetic acid). Both of these genes are involved in Auxin biosynthesis. And the thirds gene IPT encodes enzyme to produce zeatin type cytokine isopentenyl adenine for cytokine biosynthesis. The deletion of these genes from the sequence results in Shooty and rooty crowngalls so designated as TMS and TMR respectively. Another locus involve in tumour production is TMl locus. In addition T DNA also contains genes involved in opine biosynthesis; these genes are located near the right border of T- DNA. NOS gene is also present which involves in the synthesis of enzyme nopaline synthase. In case of octopine type Ti, it contains OCS gene for octopine synthase.

All the genes present in T-DNA contain eukaryotic regulatory sequences. As a result, these genes are expressed only in plants and not expressed in Agrobacterium. Transfer is initiated at the right border and terminated at the left border and requires the *vir* genes of the Ti plasmid to transfer T DNA into plant cell.

vir Region:

The genes of vir region are not transferred themselves; they only induce the transfer of T-DNA. The vir region of a nopaline type Ti plasmid contains 8 operons (designated as vir A, virB, virC, virD, virE, virF, virG and virH), which are about 40 kb of DNA and have 25 genes (Fig.4). This region mediates the transfer of T-DNA into plant genomes, and hence is essential for virulence, that is, production of crowngall/hairy root disease; therefore, it is called the virulence region or vir region.

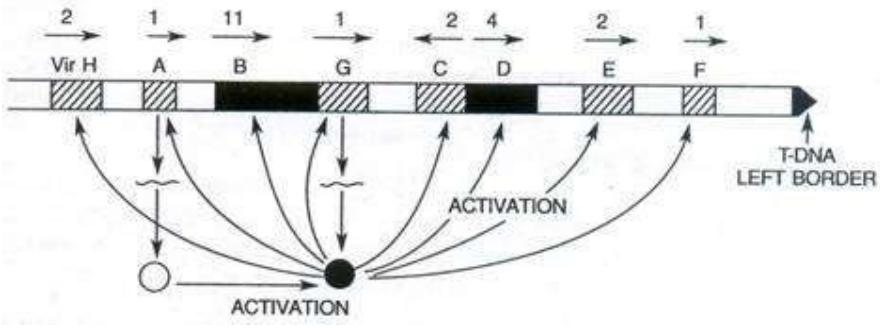

Figure 4: Organisation of the vir region of a nopaline type pTi. The arrows, above the letters designating the operons, indicate the direction of transcription, and the numbers **above the arrows represent the numbers of genes present in the different operons (Reference: 1).**

Of the 8 vir operons, 4 operons, viz., virA, virB, virD and virG, are essential for virulence, while the remaining 4 operons play an accessory role. The operons virA and virG are constitutive, encode one protein each, and are concerned with the regulation of all the vir operons. The other vir operons encode various proteins involved in the T-DNA transfer.

Table: The important genes/sequences of pTi (nopaline type) and their functions (Reference: 1):

Gene/Operon	Function
	T-DNA
iaaM (*aux1*, *tms1*)*	Auxin biosynthesis; encodes the enzyme tryptophan-2-mono-oxygenase, which converts tryptophan into indole-3-acetamide (IAM).
iaaH (*aux2*, *tms2*)	Auxin biosynthesis; encodes the enzyme indole-3-acetamide hydrolase, which converts IAM into IAA (indole-3-acetic acid)
ipt (*tmr*, *Cyt*)	Cytokinin biosynthesis; encodes the enzyme isopentenyl transferase, which catalyzes the formation of isopentenyl adenine
nos	Nopaline biosynthesis; encodes the enzyme nopaline synthase, which produces nopaline from arginine and pyruvic acid
24 bp left and right	*Sites of endonuclease action during T-DNA transfer; the only sequences of T-DNA essential*
border sequences	*for its transfer*
	vir *Region* (vir *Regulon*)**
virA (1)*	Encodes a sensor protein; receptor for acetosyringone and functions as an autokinase; also phosphorylates VirG protein; *constitutive expression*
virB (11)	Membrane proteins; combine with VirD4 to form a channel for T-DNA transport (conjugal tube formation); VirB11 has ATPase activity
virC (2)	Helicase; binds to the overdrive region just outside the right border; involved in unwinding of T-DNA
virD (4)	VirD1 has topoisomerase activity; it binds to the right border of T-DNA; VirD2 is an endonuclease; it nicks the right border
virE (2)	VirE2 is a single-strand binding protein (SSBP); it binds to T-DNA during its transfer; VirE1 stabilizes VirE2
virF (1)	Presumed to mark some host plant proteins for proteolysis
virG (1)	DNA binding protein; probably forms dimer after phosphorylation by VirA, and induces the expression of all *vir* operons (operons *A* to *H*); *constitutive expression*
virH (2)	Detoxification of the phenolics produced by plant cells at the wound site

Transfer of T-DNA:

The exact mechanism of transfer of T-DNA is not clearly understood. T-DNA transfer is brought about by the vir region, also called vir regulon. The vir regulon is activated by the phenolic signal molecules acetosyringone and α-hydroxyaceto- syringone, which are produced by wounded tissues of virtually all dicot plant species, and constitute the wound response. Operons virA and virG show low levels of constitutive expression. In response to plant phenolic signal molecules, ViA protein phsophorylates itself and VirG protein; the VirG now induces all the 8 operons of vir regulon.

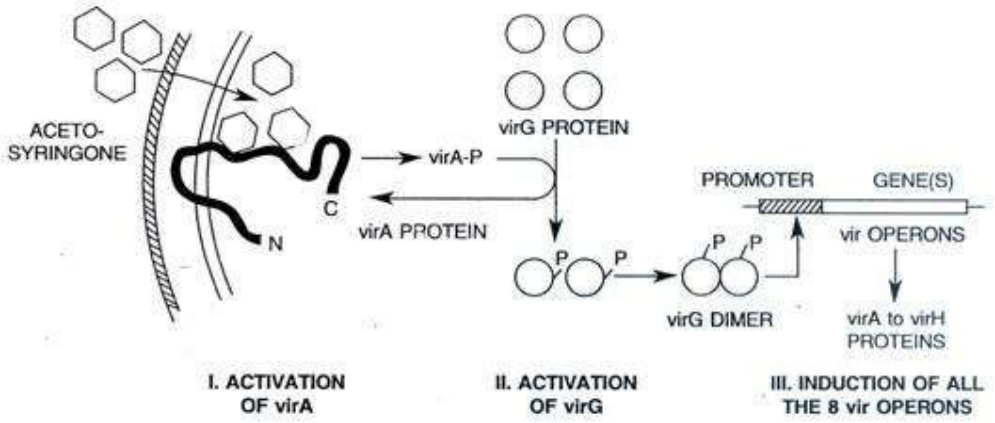

Figure 6. Activation of vir operons by plant phenolic signal molecules acetosyringone/α-hydroxyacetosyringone. I. VirA protein is activated by the phenolics to autophosphorylate (VirA-P). II. Phosphorylated VirA protein phosphorylates VirG protein, and VirG forms.dimers. III (Reference: 1).

The mechanism for transfer of T DNA into plant cell involves the action of Vir DI and VirD2. The virDl gene product, VirDl protein, has topoisomerase activity; it binds to the right border sequence, and relaxes supercoiling, which facilitates the action of protein VirD2. VirD2 is an endonuclease; it nicks at the right border and covalently binds (and remains bound during the T-DNA transfer) to the 5′-end so generated. The 3′-end produced at the site of nick serves as a primer for DNA synthesis in the 5’—>3′ direction as a result of which a single strand of T-DNA is displaced from the DNA duplex (Fig.7).

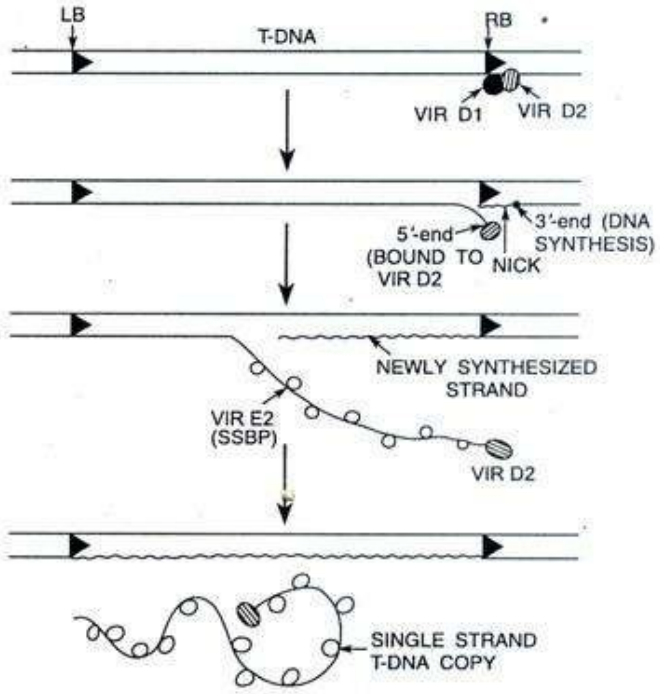

Figure.7: A schematic representation of the production of T-DNA copy (single-stranded) for transfer into plant cells (Reference: 1).

The T-strand is again nicked at the left border to generate a single- strand copy of T-DNA. VirE2 protein is a single-strand binding protein (SSBP); about 600 copies of it bind to the single-strand T-DNA and protect it from nuclease action. The virB operon has 11 genes, which encode mostly membrane-bound proteins; virB is essential for virulence. Most likely VirB proteins, together with VirD4 protein, participate in conjugal tube formation between the bacterial and plant cells, which provides a channel for T-DNA transfer. VirB 11 has ATPase activity and generates energy needed for the delivery of T-DNA into the plant cells. The endonuclease VirD2, which nicks the right border and remains covalently bound to the 5′-end of the single-strand T-DNA copy, has a signal sequence, which drives, it towards the nucleus of the transformed plant cell (after the delivery of T-DNA into the plant cell). The T- DNA most likely enters the nucleus through nuclear pore complex. The transferred single stranded T DNA is immediately converted into a double-stranded form in the nuclei. The double-stranded T-DNA integrates at random sites in the host plant genome by homologous recombination.

Vectors derived from pTi:

Agrobacterium is known as a natural genetic engineer, which can transfer a part of its DNA into plant cell, keeping that in mind many vectors derived by slight modifications in Agrobacterium Ti and Ri plasmids. Wild Type Ti could not be used as a plant vector because of the Presence of oncogenes (iaaM, iaaH and ipt) in T-DNA, which causes a disorganised growth and a loss of regeneration potential of the cells having T-DNA in their genomes, large size, lack of unique cloning sites within the T-DNA, which are needed for the insertion of DNA segments to be cloned. These problems have been resolved by deleting the oncogenes from the T-DNA (disarming) and by developing intermediate vectors and binary vectors to facilitate gene cloning procedures.

Disarming:

The deletion of genes governing auxin and cytokinin production (the oncogenes) from T- DNA of a Ti plasmid is known as disarming. Agrobacterium containing this disarmed plasmid still transferred this modified T-DNA into plant cells. The cells containing the modified T-DNA were nontumorous, produced nopaline and readily regenerated plantlets. Since then it has been shown that only the LB and RB

sequences of T-DNA are necessary for the transfer of any DNA insert placed between them.

Co-integrate pTi Vectors:

The genes of interest to be transferred into plants are initially cloned in E. coli for obvious reasons of ease in the cloning procedures. A cointegrate vector is produced by integrating the modified E. coli plasmid (used for cloning of and containing the gene construct to be transferred) into a disarmed pTi. The cointegration of the two plasmids is achieved within Agrobacterium by homologous recombination. Therefore, the E. coli plasmid, e.g., pBR322, and the disarmed pTi must have some sequences common to both for recombination to occur. A common approach for ensuring this is to disarm a pTi by replacing its oncogenes with sequences from the E. coli plasmid to be used for cloning the DNA insert/gene to be transferred. For example, pTiC58 was disarmed by replacing its oncognes with E. coli plasmid pBR322 sequences; this disarmed pTi is designated as pGV3850 (Fig.8).

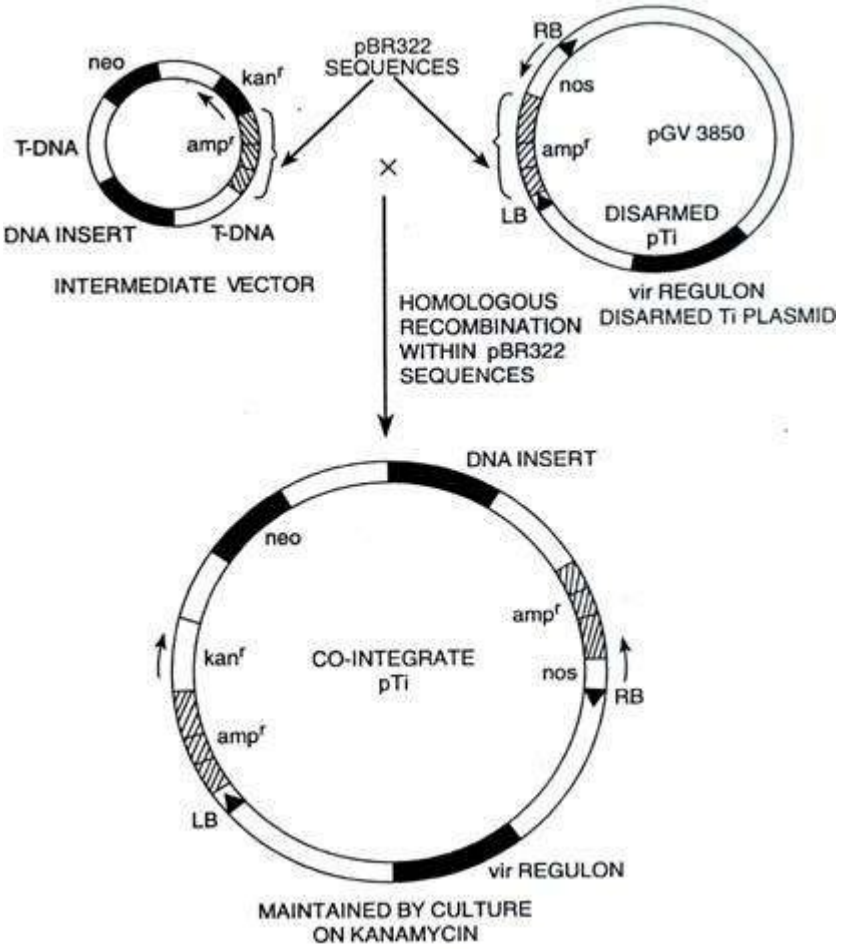

Figure 8: Homologous recombination between a suitably disarmed pTi and a recombinant IV (intermediate vector) containing the desired DNA insert to produce a cointegrate vector (Reference: 1).

The pBR322 is suitably modified to produce an intermediate vector (IV). The IV must contain origin for replication in E. coli, the pBR322 sequences present in the T-region of the disarmed pTi, T-DNA (without the borders) from pTi and appropriate selectable markers, e.g., neo gene for selection of plant cells containing the recombinant T-DNA (T-DNA containing the DNA insert) and kan^r (kanamycin resistance) for the selection of cointegrate vector in Agrobacterium. The DNA inserts can be readily and conveniently inserted within the T-DNA present in it to yield a recombinant IV. The transfer of recombinant IV from E. coli into the Agrobacterium is usually achieved by conjugation. Since IV is nonconjugative, an E. coli strain containing a conjugation-proficient plasmid, called helper plasmid, to mobilize the transfer of IV is used. An example of a conjugation-proficient helper plasmid is pRK2013, which has the tra genes of the naturally occurring plasmid pRK2. The plasmid pRK2013 also does not have an origin for replication in Agrobacterium. Homologous recombination between the recombinant IV and the disarmed pTi will integrate the former into the T-region (i.e., between the left and right borders of T-DNA) of the latter.
The resulting plasmid is a cointegrate pTi and is used as a vector for transferring the DNA insert present in it into the plant genomes.

Binary Vector:

Binary vector is a combination of two vectors together present in a single cell and T DNA present in one vector is transferred to the host cell by the expression of number of genes of Vir region present in another vector inside the same cell. The vir region of Ti plasmid need not be present in the same plasmid for an efficient transfer of T-DNA. Vir region is essential to transfer T DNA to the host cell. A binary vector consists of a pair of plasmids of which one plasmid contains disarmed T-DNA sequences (at least the left and right borders of T-DNA must be present), while the other contains the vir region, and ordinarily lacks the entire T- DNA including the border. The plasmid containing disarmed T-DNA is called mini-Ti or micro-Ti, e.g., Bin 19, and has the origins for replication in both E. coli and Agrobacterium. The DNA insert is integrated within the T-region of mini-Ti, and the recombinant mini-Ti is cloned in E. coli. Transfer of recombinant mini-Ti from E. coli into Agrobacterium is achieved either by conjugal transfer or direct

transformation.

Mini-T Bin 19 has kan^r (kanamycin resistance) gene for the selection of Agrobacterium cells containing Binl9, and neo gene for the selection of transformed plant cells. It also has a polylinker site (a DNA sequence having unique restriction sites for several restriction endonucleases) within the α peptide gene of E. coli lacZ locus. Integration of a DNA insert in the polylinker site disrupts lacZ-α enabling the selection of E. coli colonies containing the recombinant mini-Ti as they form white colonies on X-gal + IPTG medium as against the normal blue colonies. The polylinker and the neo gene are placed within the LB and RB sequences of T-DNA (Fig 9). Binl9 is capable of replication both in E. coli and Agrobacterium, as it is based on the broad host range plasmid pRK252.

The 'helper' plasmid is a Ti plasmid having a functional vir region but lacking the T-DNA region, including the border sequences. pAL4404 helper Ti plasmid is derived from the wild type pTiAch5 by deletion of the entire T-region. The vir genes present in 'helper' pALAAOA induce the transfer of T-DNA (containing the DNA insert) of the mini-Ti Binl9 into the plant cells. The transformed plant cells can be selected on kanamycin medium due to the gene neo present within the T-DNA.

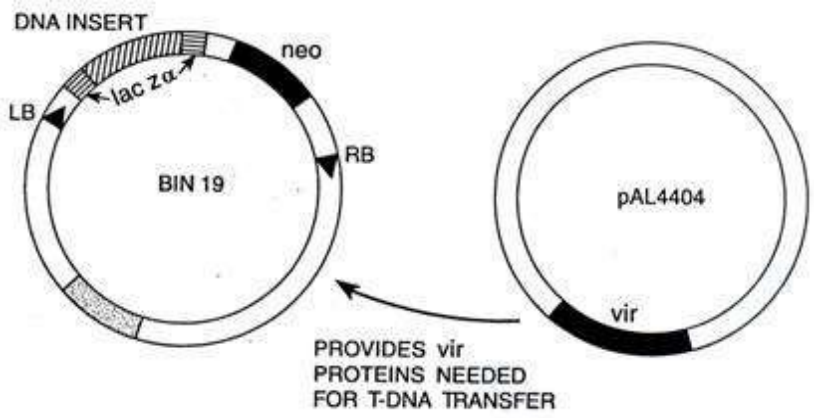

Figure. 10: A schematic representation of the binary vectors Bin19 and pAL4404 of pTi
(Reference: 1)**.**

The binary system avoids the transfer into plant cells/genomes of unnecessary sequences, which occurs in the case of cointegrate vectors. BIBAC2 is the first such vector; it contains F-plasmid origin of replication and transforms tobacco with high efficiency. The other examples of constructed binary vectors are BIBAC2 and TAC.

Genomic libraries have been constructed for several plant species using BIBAC2

and TAC vectors, and a number of novel genes have been isolated. These vectors can also be used to transfer multiple genes, e.g., genes encoding sequentially acting enzymes of a metabolic pathway.

Plant Virus Vectors:

Viruses have the following attractive features as vectors: (i) viruses infect cells of adult plants, (ii) they produce large number of copies per cell leading to gene amplification, and production of the recombinant protein in large quantities and (iii) some viruses are systemic in that they spread throughout the plant.

Plant virus genomes do not integrate into plant genome. As a result, they cannot be used to produce stable and heritable transformations. But they can be used to express transgenes with a view to either improve the phenotypic performance of host plants or to produce large quantities of valuable proteins.

Most plant viruses have RNA genomes; two such viruses that have great potential as vectors are, brome mosaic virus (BMV) and tobacco mosaic virus (TMV). But the greatest progress has been made with the two groups of viruses having DNA genomes, viz., caulimoviruses and gemini viruses.

Cauliflower Mosaic Virus (CaMV):

CaMV is the best studied member of the caulimovirus group. It contains about 8 kb double- strands DNA genome, and produces spherical particles. The caulimovirus group infects a wide range of dicot crops. An example of use of CaMV as vector pertains to the bacterial dhfr (dihydrofolate reductase) gene. This gene was inserted in the CaMV genome in the place of gene II, and was successfully expressed in plants. However, CaMV is of limited interest as a vector although studies with CaMV have provided some very useful promoters for use in plants.

Gemini Viruses:

These viruses infect a variety of monocot and dicot plants, and have circular single-stranded DNA genomes. Maize streak virus (MSV) is a member of this group, causes yellow streaks on maize leaves, and is able to produce infection only when transmitted by its natural insect vector (leaf-hopper). MSV multiplies to a high copy number in the nuclei of dividing cells.

MSV genome has been successfully introduced into plant cells with the help of

Agrobacterium (agroinfection). In this approach, MSV genome (native as well as cloned) was inserted within the T-DNA of pTi in form of a tandem dimer.

Agrobacterium containing this recombinant pTi was used to infect maize plants, which developed streaks on their leaves within 2 weeks of inoculation.

Tobacco Mosaic Virus (TMV):

TMV has an RNA genome, which also serves as mRNA. Its genome has 4 genes of which the coat protein (cp) gene seems to be nonessential (although it is necessary for the systematic spread of the infection in a plant), and can be the site of integration of a transgene.

In one study, the bacterial gene cat was inserted just downstream of the cp gene initiation codon, and the RNA transcripts of this recombinant genome were used to infect tobacco plants. Gene cat was expressed in the infected leaves, but there was no systemic spread of the infection. TMV CP is produced in large amounts; hence its promoter is suitable for an efficient expression of transgenes.

Brome Mosaic Virus (BMV):

BMV infects several species of Graminae, including barley. It has three genomic segments (1, 2 and 3) each packaged into a separate particle. The CP gene is located on the RNA segment 3, and is the only target site for DNA insertion, although this prevents the formation of virus particles. The bacterial gene cat was inserted in the cp gene of RNA 3, using its cDNA, and its RNA transcripts were used together with RNAs 1 and 2 to infect barley protoplasts. The infected protoplasts showed a high cat activity. This indicates that placing a transgenes downstream to the regulatory sequences of the cp gene of BMV will give high yields of the protein encoded by it. So the main steps involved in plant transformation using vectors includes: Propagate vector in *E. coli*, Isolate vector from *E.coli* and engineer (introduce a foreign gene), Re-introduce engineered vector into *E. coli* to amplify, Isolate engineered vector and introduce into *Agrobacteria*., Infect plant tissue with engineered *Agrobacteria* (T-DNA containing the foreign gene gets inserted into a plant cell genome), In each cell T-DNA gets integrated at a different site in the genome.

Vectorless or Direct gene transfer

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

transfer in plants are:

Chemical mediated gene transfer

e.g. chemicals like polyethylene glycol (PEG) and dextran sulphate induce DNA uptake into plant protoplasts. Calcium phosphate is also used to transfer DNA into cultured cells. In this method the coprecipitate of Calcium phosphate and DNA has been prepared and the cell can take up these coprecipitate by the process of phagocytosis.

Microinjection

where the DNA is directly injected into plant protoplasts or cells (specifically into the nucleus or cytoplasm) using fine tipped (0.5 - 1.0 micrometerdiameter) glass needle or micropipette. This method of gene transfer is used to introduce DNA into large cells such as oocytes, eggs, and the cells of early embryo.

Electroporation

It involves a pulse of high voltage applied to protoplasts/cells/ tissues to make transient (temporary) pores in the plasma membrane which facilitates the uptake of foreign DNA. The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.

Particle gun/Particle bombardment

In this method, the foreign DNA containing the genes to be transferred is coated onto the surface of minute gold or tungsten particles (1-3 micrometers) and bombarded onto the target tissue or cells using a particle gun (also called as gene gun/shot gun/microprojectile gun).This gun contains a cylinder in which the helium gas is present, and by the very high acceleration of gas the DNA coated on the microprojectiles are forcefully embedded on the tissue placed just in front of the gun. The microprojectile bombardment method was initially named as biolistics by its inventor Sanford (1988). Two types of plant tissue are commonly used for particle bombardment- Primary explants and the proliferating embryonic tissues.

Transformation

To enhance transformation the artificial competent cells are prepared. Copetent ceels are those cells which have the capability to take naked DNA directly from the environment and become competent. This method involves the introduction of foreign DNA into bacterial cells
e.g. E. Coli. The transformation frequency (the fraction of cell population that can be transferred) is very good in this method. E.g. the uptake of plasmid DNA by E. coli is carried out in ice cold CaCl2 (0-50C) followed by heat shock treatment at 37-450C for about 90 sec. The transformation efficiency refers to the number of transformants per microgram of added DNA. The CaCl2 breaks the cell wall at certain regions and binds the DNA to the cell surface.

Lipofection

Liposomes are circular lipid molecules with an aqueous interior that can carry nucleic acids. Liposomes encapsulate the DNA fragments and then adher to the cell membranes and fuse with them to transfer DNA fragments. Thus, the DNA enters the cell and then to the nucleus. Lipofection is a very efficient technique used to transfer genes in bacterial, animal and plant cells.

Transgenic plants are produced with improved productivity and performance. Many resistant and stress tolerant transgenic plants have been developed till date. Some of them are listed below:

Stress tolerance
Biotechnology strategies are being developed to overcome problems caused due to biotic stresses (viral, bacterial infections, pests and weeds) and abiotic stresses (physical actors such as temperature, humidity, salinity etc).

Abiotic stress tolerance

The plants show their abiotic stress response reactions by the production of stress related osmolytes like sugars (e.g. trehalose and fructans), sugar alcohols (e.g. mannitol), amino acids (e.g. proline, glycine, betaine) and certain proteins (e.g. antifreeze proteins). Transgenic plants have been produced which over express the genes for one or more of the above mentioned compounds. Such plants show increased tolerance to environmental stresses. Resistance to abiotic stresses includes stress induced by herbicides, temperature (heat, chilling, freezing), drought, salinity, ozone and intense light. These environmental

stresses result in the destruction, deterioration of crop plants which leads to low crop productivity. Several strategies have been used and developed to build ressitance in the plants against these stresses (6).

Herbicide tolerance

Weeds are unwanted plants which decrease the crop yields and by competing with crop plants for light, water and nutrients. Several biotechnological strategies for weed control are being used e.g. the over-production of herbicide target enzyme (usually in the chloroplast) in the plant which makes the plant insensitive to the herbicide. This is done by the introduction of a modified gene that encodes for a resistant form of the enzyme targeted by the herbicide in weeds and crop plants. Roundup Ready crop plants tolerant to herbicide-Roundup, is already being used commercially. The biological manipulations using genetic engineering to develop herbicide resistant plants are: (a) over-expression of the target protein by integrating multiple copies of the gene or by using a strong promoter., (b) enhancing the plant detoxification system which helps in reducing the effect of herbicide., (c) detoxifying the herbicide by using a foreign gene., and (d) modification of the target proteinbymutation.

Some of the examples are: Glyph
pyruvylshikimate 3- phosphate synthase (EPSPS) in the shikimic acid pathway. Due to it's structural similarity with the substrate phosphoenol pyruvate, glyphosate binds more tightly with EPSPS and thus blocks the shikimic acid pathway. Certain strategies were used to provide glyphosate resistance to plants.

Other abiotic stresses

The abiotic stresses due to temperature, drought, and salinity are collectively also known as water deficit stresses. The plants produce osmolytes or osmoprotectants to overcome the osmotic stress. The attempts are on to use genetic engineering strategies to increase the production of osmoprotectants in the plants.

Insect resistance

A variety of insects, mites and nematodes significantly reduce the yield and quality of the crop plants. The conventional method is to use synthetic pesticides, which also have severe effects on human health and environment. The transgenic technology uses an innovative and eco- friendly

method to improve pest control management. About 40 genes obtained from microorganisms of higher plants and animals have been used to provide insect resistance in crop plants.

The first genes available for genetic engineering of crop plants for pest resistance were Cry genes (popularly known as Bt genes) from a ***bacterium Bacillus thuringiensis***. These are specific to particular group of insect pests, and are not harmful to other useful insects like butter flies and silk worms. Transgenic crops with Bt genes (e.g. cotton, rice, maize, potato, tomato, brinjal, cauliflower, cabbage, etc.) have been developed. This has proved to be an effective way of controlling the insect pests and has reduced the pesticide use. The most notable example is Bt cotton (which contains CrylAc gene) that is resistant to a notorious insect pest Bollworm ***(Helicoperpa armigera).*** There are certain other insect resistant genes from other microorganisms which have been used for this purpose. Isopentenyl transferase gene from Agrobacterium tumefaciens has been introduced into tobacco and tomato. The transenic plants with this transgene were found to reduce the leaf consumption by tobacco hornworm and decrease the survival of peach potato aphid.

Certain genes from higher plants were also found to result in the synthesis of products possessing insecticidal activity. One of the examples is the Cowpea trypsin inhibitor gene (CpTi) which was introduced into tobacco, potato, and oilseed rape for develping transgenic plants. Earlier it was observed that the wild species of cowpea plants growing in Africa were resistant to attack by a wide range of insects. It was observed that the insecticidal protein was a trypsin inhibitor that was capable of destroying insects belonging to the orders Lepidoptera, Orthaptera etc. Cowpea trypsin inhibitor (CpTi) has no effect on mammalian trypsin, hence it is non-toxic to mammals.

Virus resistance

There are several strategies for engineering plants for viral resistance, and these utilizes the genes from virus itself (e.g. the viral coat protein gene). The virus-derived resistance has given promising results in a number of crop plants such as tobacco, tomato, potato, alfalfa, and papaya. The induction of virus resistance is done by employing virus-encoded genes- virus coat proteins, movement proteins, transmission proteins, satellite RNa, antisense RNAs, and ribozymes. The virus coat protein-mediated approach is the most successful one to provide virus resistance to plants. It was in 1986, transgenic tobacco plants expressing tobacco mosaic virus (TMV) coat protein gene were first developed. These plants exhibited high levels of res cucumber mosaic virus (CMV), potato virus X (PVX) , potato virus Y (PVY) etc.

Resistance against Fungal and bacterial infections

As a defense strategy against the invading pathogens (fungi and bacteria) the plants accumulate low molecular weight proteins which are collectively known as pathogenesis- related (PR) proteins. Several transgenic crop plants with increased resistance to fungal pathogens are being raised with genes coding for the different compounds. One of the examples is the Glucanase enzyme that degrades the cell wall of many fungi. The most widely used glucanase is beta-1,4-glucanase. The gene encoding for beta-1,4 glucanase has been isolated from barley, introduced, and expressed in transgenic tobacco plants. This gene provided good protection against soil-borne fungal pathogen Rhizoctonia solani. Lysozyme degrades chitin and peptidoglycan of cell wall, and in this way fungal infection can be reduced. Transgenic potato plants with lysozyme gene providing resistance to Eswinia carotovora have been developed.

Delayed fruit ripening

The gas hormone, ethylene regulates the ripening of fruits, therefore, ripening can be slowed down by blocking or reducing ethylene production. This can be achieved by introducing ethylene forming gene(s) in a way that will suppress its own expression in the crop plant. Such fruits ripen very slowly (however, they can be ripen by ethylene application) and this helps in exporting the fruits to longer distances without spoilage due to longer-shelf life. The most common example is the 'Flavr Savr' transgenic tomatoes, which were commercialized in U.S.A in 1994. The main strategy used was the antisense RNA approach. In the normal tomato plant, the PG gene (for the enzyme polygalacturonase) encodes a normal mRNA that produces the enzyme polygalacturonase which is involved in the fruit ripening. The complimentary DNA of PG encodes for antisense mRNA, which is complimentary to normal (sense) mRNA. The hybridization between the sense and antisnse mRNAs renders the sense mRNA ineffective. Consequently, polygalacturonase is not produced causing delay in the fruit ripening. Similarly strategies have been developed to block the ethylene biosynthesis thereby reducing the fruit ripening. E.g. transgenic plants with antisense gene of ACC oxidase (an enzyme involved in the biosynthetic process of ethylene) have been developed. In these plants, production of ethylene was reduced by about 97% with a significant delay in the fruit ripening. The bacterial gene encoding ACC deaminase (an enzyme that acts on ACC and removes amino group)

has been transferred and expressed in tomato plants which showed 90% inhibition in the ethylene biosynthesis.

Male Sterility

The plants may inherit male sterility either from the nucleus or cytoplasm. It is possible to introduce male sterility through genetic manipulations while the female plants maintain fertility. In tobacco plants, these are created by introducing a gene coding for an enzyme (barnase, which is a RNA hydrolyzing enzyme) that inhibits pollen formation. This gene is expressed specifically in the tapetal cells of anther using tapetal specific promoter TA29 to restrict its activity only to the cells involved in pollen production. The restoration of male fertility is done by introducing another gene barstar that suppresses the activity of barnase at the onset of the breeding season. By using this approach, transgenic plants of tobacco, cauliflower, cotton, tomato, corn, lettuce etc. with male sterility have been developed.

MOLECULAR FARMING

Plants can be used as cheap chemical factories that require only water, minerals, sun light and carbon dioxide to produce thousands of sophisticated chemical molecules with different structures. By transferring the right genes, plants can serve as bioreactors to modified or new compounds such as amino acids, proteins, vitamins, plastics, pharmaceuticals (peptides and proteins), drugs, enzymes for food industry and so on. The transgenic plants as bioreactors have some advantages such as the cost of production is low, there is an unlimited supply, safe and environmental friendly and there is no scare of spread of animal borne diseases. Tobacco is the most preferred plant as a transgenic bioreactor because it can be easily transformed and engineered. Tobacco is an excellent biomass producer with about 40 tons of fresh leaf production as against e.g. rice with 4 tons. The seed production is very high (approx. one million seeds per plant) and it can be harvested several times in a year. Some of the uses of transgenic plants are:

Improvement of Nutrient quality

Transgenic crops with improved nutritional quality have already been produced by introducing genes involved in the metabolism of vitamins, minerals and amino acids. A transgenic Arabidopsis thaliana that can produce ten-fold higher vitamin E (alpha- tocopherol) than the native plant has been developed. The biochemical machinery to produce a compound close in structure to alpha-tocopherol is present in A. thaliana. A gene that can finally produce alpha-tocopherol is also present, but

is not expressed. This dormant gene was activated by inserting a regulatory gene from a bacterium which resulted in an efficient production of vitamin E. Glycinin is a lysine-rich protein of soybean and the gene encoding glycinin has been introduced into rice and successfully expressed. The transgenic rice plants produced glycinin with high contents of lysine. Using genetic engineering Prof Potrykus and Dr. Peter Beyer have developed rice which is enriched in pro-vitamin A by introducing three genes involved in the biosynthetic pathway for carotenoid, the precursor for vitamin A. The aim was to help millions of people who suffer from night blindness due to Vitamin A deficiency, especially whose staple diet is rice. The presence of beta-carotene in the rice gives a characteristic yellow/orange colour, hence this pro-vitamin A enriched rice is named as GoldenRice The genetic engineering is also being used to improve the taste of food e.g. a protein 'monellin' isolated from an African plant (Dioscorephyllum cumminsii) is about 100,000 sweeter than sucrose on molar basis. Monellin gene has been introduced into tomato and lettuce plants to improve their taste The genetic engineering is also being used to improve the taste of food e.g. a protein 'monellin' isolated from an African plant (Dioscorephyllum cumminsii) is about 100,000 sweeter than sucrose on molar basis. Monellin gene has been introduced into tomato and lettuce plants to improve their taste.

Improvement of seed protein quality

The nutritional quality of cereals and legumes has been improved by using biotechnological methods. Two genetic engineering approaches have been used to improve the seed protein quality. In the first case, a transgene (e.g. gene for protein containing sulphur rich amino acids) was introduced into pea plant (which is deficient in methionine and cysteine, but rich in lysine) under the control of seed-specific promoter. In the second approach, the endogenous genes are modified so as to increase the essential amino acids like lysine in the seed proteins of cereals. These transgenic routes have helped to improve the essential amino acids contents in the seed storage proteins of a number of crop plants. E.g. overproduction of lysine by de-regulation. The four essential amino acids namely lysine, methionine, threonine, and isoleucine are produced from a non-essential amino acid aspartic acid. The formation of lysine is regulated by feedback inhibition of the enzymes aspartokinase (AK) and dihydrodipicolinate synthase (DHDPS). The lysine feedback- insensitive genes encoding the enzymes AK and DHDPS have been respectively isolated from E. Coli and Cornynebacterium. After doing appropriate genetic manipulations, these genes were introduced into soybean and canola plants.

The transgenic plants so produced had high quantities of lysine.

Diagnostic and therapeutic proteins

Experiments are going on to use transgenic plants in diagnostics for detecting human diseases and therapeutics for curing human and animal diseases. Several metabolites and compounds are already being produced in transgenic plants e.g. the monoclonal antibodies, blood plasma proteins, peptide hormones, cytokinins etc. The use of plants for commercial production of antibodies, referred to as plantbodies, is a novel approach in biotechnology. The first successful production of a functional antibody, namely a mouse immunoglobulin IgGI in plants, was reported in 1989. This was achieved by developing two transgenic tobacco plants- one synthesizing heavy chain gamma- chain and other light kappa- chain, and crossing them to generate progeny that can produce an assembled functional antibody. In 1992, C.J. Amtzen and co-workers expressed hepatitis B surface antigen in tobacco to produce immunologically active ingredients via genetic engineerir produced in transgenic plants (maize) are avidin and beta- glucuronidase and are used in diagnostic kits.

Edible vaccines

Edible vaccines are those vaccines, in which the antigen is expressed in the dibble part of the plant so this can be directly eaten orally. The mechanism involves the isolation of a specific gene encoding the particular protein/antigen isolated from the concerned pathogen, and then the gene express in the edible part of the plant. Such transgenic plants or their tissues producing antigens can be eaten for vaccination/immunization (edible vaccines). The expression of such antigenic proteins in crops like banana and tomato are useful for immunization of humans since banana and tomato fruits can be eaten raw. Transgenic plants (tomato, potato) have been developed for expressing antigens derived from animal viruses e.g. rabies virus, herpes virus. In 1990, the first report of the production of edible vaccine (a surface protein from Streptococcus) in tobacco at 0.02% of total leaf protein level was published in the form of a patent application under the International Patent Cooperation Treaty (Mason and Arntzen,1995).The first clinical trials in humans, using a plant derived vaccine were conducted in 1997 and were met with limited success. This involved the ingestion of transgenic potatoes with a toxin of E. coli causing diarrhoea.

The process of making of edible vaccines involves the incorporation of a plasmid carrying the antigen gene and an antibiotic resistance gene, into the bacterial cells e.g. Agrobacterium tumefaciens. The small pieces of potato leaves are exposed to an antibiotic which can kill the cells that lack the new genes. The surviving cells with altered genes multiply and form a callus. This callus is allowed to grow and subsequently transferred to soil to form a complete plant. In about a few weeks, the plants bear potatoes with antigen vaccines.

The bacteria E.coli, V. cholerae cause acute watery diarrhea by colonizing the small intestine and by producing toxins. Chloera toxin (CT) is very similar to E.Coli toxin. The CT has two subunits, A and B. Attempt was made to produce edible vaccine by expressing heat labile enterotoxin (CT-B) in tobacco and potato.

Another strategy adopted to produce a plant-based vaccine, is to infect the plants with recombinant virus carrying the desired antigen that is fused to viral coat protein. The infected plants are reported to produce the desired fusion protein in large amounts in a short duration. The technique involves either placing the gene downstream a subgenomic promoter, or fusing the gene with capsid protein that coats the virus.

Biodegradable plastics

Polythenes and plastics are one of the major environmental hazards. Efforts are on to explore the possibility of using transgenic plants for biodegradable plastics. Transgenic plants can be used as factories to produce biodegradable plastics like polyhydroxy butyrate or PHB. Genetically engineered ***Arabidopisis*** plants can produce PHB globules exclusively in their chloroplasts without effecting plant growth and development. The large-scale production of PHB can easily be achieved in plants like ***Populus***, where PHB can be extracted from leaves.

Molecular breeding

The term molecular breeding is frequently used to represent the breeding methods that are coupled with genetic engineering techniques. Up till now, conventional breeding methods have been used to meet the food demands of the growing world population and the challenges of poverty and improved crop production and yields. Molecular breeding involves breeding using molecular (nucleic acid) markers. A molecular marker is a DNA sequence in the genome which can be located and

identified therefore molecular markers can be used to identify particular locations in the genome.

Due to mutations, insertions, deletions, etc. the base composition at a particular location may be different in different plants. These differences, termed ***polymorphisms***, allow DNA markers to be mapped in a genetic linkage group. Generally, there are three types of markers used in screening/selection: Morphological marker based on visible character (phenotypic expression) e.g. flower color, seed color, height, leaf shapes, etc. , Biochemical marker: The proteins produced by gene expression are also used as markers in plant breeding programmes, Molecular marker based on DNA polymorphism detected by DNA probes or amplified products of PCR, e.g.Restriction fragment length polymorphism (RFLP), Randomly Amplified polymorphic DNA (RAPD), variable Number Tandom Repeats (VNTR), Microsatellites, etc.

Advantages of Transgenic plants:

Transgenic plants have significant potential in the bioproduction of therapeutic agents due to ease of genetic manipulations, lack of potential contamination, low cost of biomass production, short time duration of production and conservation of eukaryotic cell machinery mediating protein modification.

Disadvatages:

Different in codon usage between plants and prokaryotes can lead to inefficient expression of prokaryotic protein in plants.some plants may produce allergic compounds.

Transgenic plants can work as a bioreactor to produce number valuable products related pharmaceuticals, therapeutic proteins, vaccines, many industrial enzymes, antibodies, and many other secondary metabolites. By the use Transgenic technology, productivity and performance of plants have been increased. Transgenic plants and their products are also used at commercial level.