MODULE 5- LECTURE 1

GENE TRANSFER TECHNIQUES: BIOLOGICAL METHODS

5-1.1. Introduction

The main gene transfer methods using biological means are as follows:

- Bacterial gene delivery i.e. bactofection.
- Delivery using a viral vector i.e. transduction

5-1.2. Bactofection

It is a method of direct gene transfer using bacteria into the target cell, tissue, organ or organism. Various bacterial strains that can be used as vectors in gene therapy are listed in Table 5-1.2. The genes located on the plasmids of the transformed bacterial strains are delivered and expressed into the cells. The gene delivery may be intracellular or extracellular. It has a potential to express various plasmid-encoded heterologous proteins (antigens, toxins, hormones, enzymes etc.) in different cell types. Strains that are invasive and having better cell to cell spread are more efficient.

Vector	Target gene	Disease	Model
L. monocytogenes	IL-12	*L. major*-infection	*Mus musculus*
L. monocytogenes	CFTR	Cystic fibrosis	CHO-K1 cells
S. typhimurium	VEGFR-2 (FLK-1)	Various carcinomas	*Mus musculus*
S. choleraesuis	Thrombospondin-1	Melanoma	*Mus musculus*
S. typhimurium	IFNγ	Immunodeficiency	*Mus musculus*
S. typhimurium	CD40L	B-cell lymphoma	*Mus musculus*

Table 5-1.2. Bactofection in various disease models.

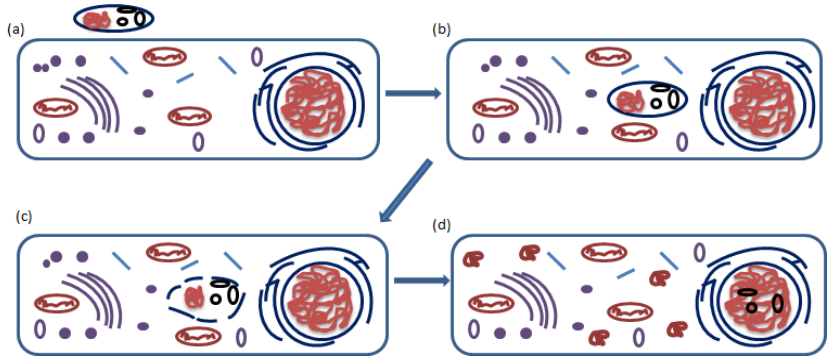

Figure 5-1.2. The process of bactofection (a) the transformed bacterial strain with plasmid containing transgene is transferred to target cell (b) genetically engineered bacteria penetrates into the cell (c) In the cytoplasm, the vector undergoes lysis and get destructed releasing plasmids (d) The released plasmids enter into the nucleus where the transgene is expressed by eukaryotic transcription and translation machinery.

(Adpapted from Palffy R, Gardlik R, Hodosy J, Behuliak M, Resko P, Radvansky J, Celec P. 2006. Gene Therapy 13: 101-105)

The efficiency of bactofection mediated gene transfer can be increased using integrin receptors. Integrin receptors are the transmembrane surface receptors present on the mammalian cell surface. Another method, lipofectamine-mediated bactofection has also been employed for enhancing the gene transfer efficiency in *E. coli* strains, particularly in the transfer of large intact DNA for gene expression. This method is also effective on various widely used bacterial vectors such as *L. monocytogenes* and *S. typhimurium*.

Uses

- Bactofection can be used for DNA vaccination against various microbial agents such as viruses, fungi, protozoans and other bacteria.
- It can be used in the treatment of several tumours like melanoma, lung carcinoma and colon carcinoma in mice.

Advantages

- Simple, selective and efficient transfection.
- Low synthesis cost and can be administered easily.

Disadvantages

- Unwanted side effects associated with host-bacteria interaction. This can be reduced by using genetically modified bacteria which contain suicide genes that ease the bacterial destruction and thus reduces the risk of clinical infections.

5-1.3. Transduction

This method involves the introduction of genes into host cell's genome using viruses as carriers. The viruses are used in gene transfer due to following features-

- Efficiency of viruses to deliver their nucleic acid into cells
- High level of replication and gene expression.

The foreign gene is packaged into the virus particles to enter the host cell. The entry of virus particle containing the candidate gene sequences into the cell and then to the nuclear genome is a receptor- mediated process. The vector genome undergoes complex processes ending up with ds-DNA depending on the vector that can persist as an episome or integrate into the host genome followed by the expression of the candidate gene (Figure 5-1.3).

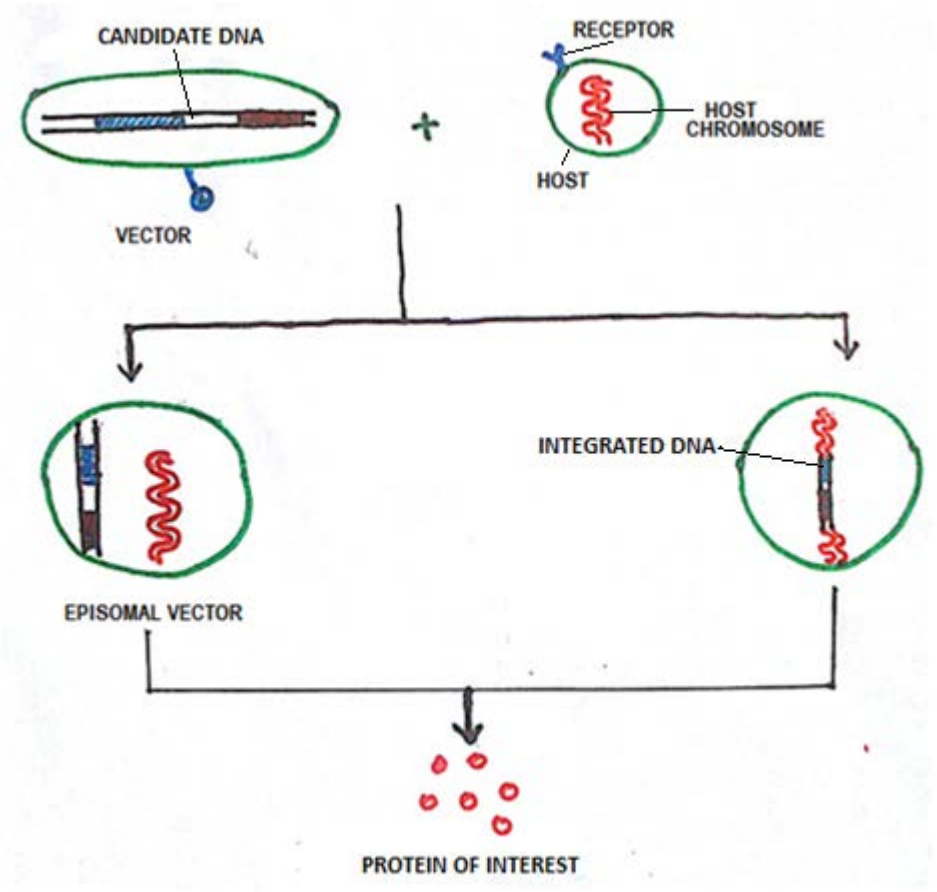

Figure 5-1.3 Transduction of host cell.

5-1.3.1 Viral vectors as therapeutic agents

Viruses have paved a way into clinical field in order to treat cancer, inherited and infectious diseases. They can be used as vectors to deliver a therapeutic gene into the infected cells. They can be genetically engineered to carry therapeutic gene without having the ability to replicate or cause disease.

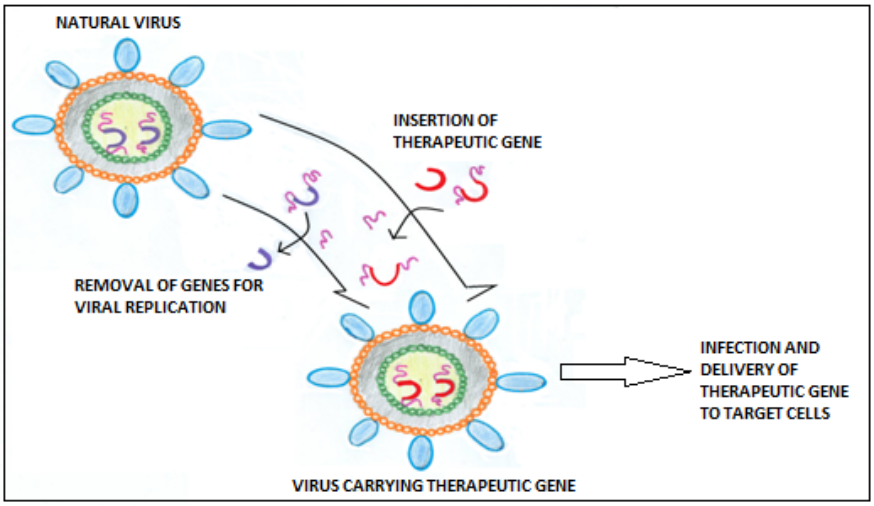

Figure 5-1.3.1 Virus as therapeutic agent.

5-1.3.2 Strategy for engineering a virus into a vector:

The strategy for engineering a virus into a vector requires the following-

- **Helper virus**

  It contains all the viral genes essential for replication but lack the sequence coding for packaging domain (ψ) making it less probable to be packaged into a virion. It can be delivered as helper virus or can stably integrate into the host chromosomal DNA of packaging cell. Some vectors also possess the helper DNA lacking additional transfer functions to increase safety.

- **Vector DNA**

  It contains non-coding *cis*-acting viral elements, therapeutic gene sequences (up to 28–32 kb) and the normal packaging recognition signal allowing the selective packaging and release from cells. Some vectors comprise relatively inactivated viral genes as a wide type infection due to lack of other viral genes. The viral proteins essential for replication of the vector DNA are produced which

then synthesize multiple copies of the vector genome (DNA or RNA, depending upon the type of vector). These structural proteins recognize the vector (psi plus) but not the helper (psi negative) nucleic acid resulting in the packaging of the vector genome into viral particles.

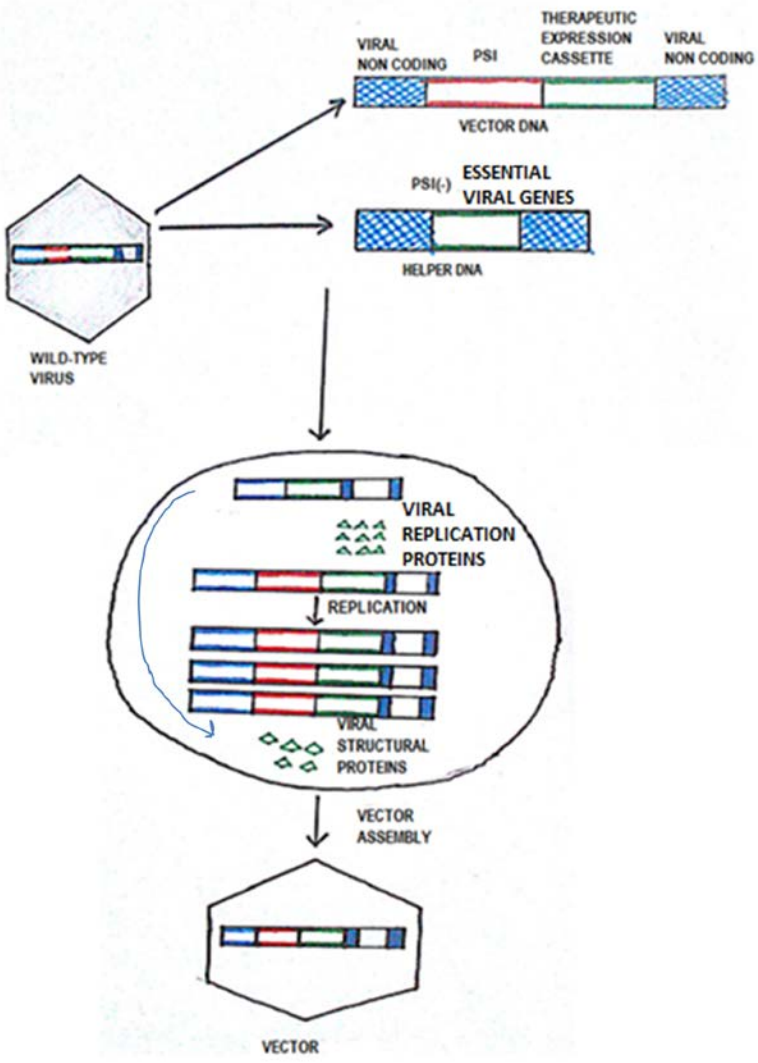

Figure 5-1.3.2(a) Strategy for engineering a virus into a vector.

(Adapted from Kay MA, Glorioso JC, Naldini L. 2001. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nature Medicine, 7(1).)

Transgene may be incorporated into viral vectors either by addition to the whole genome or by replacing one or more viral genes which can be generally achieved by ligation or homologous recombination.

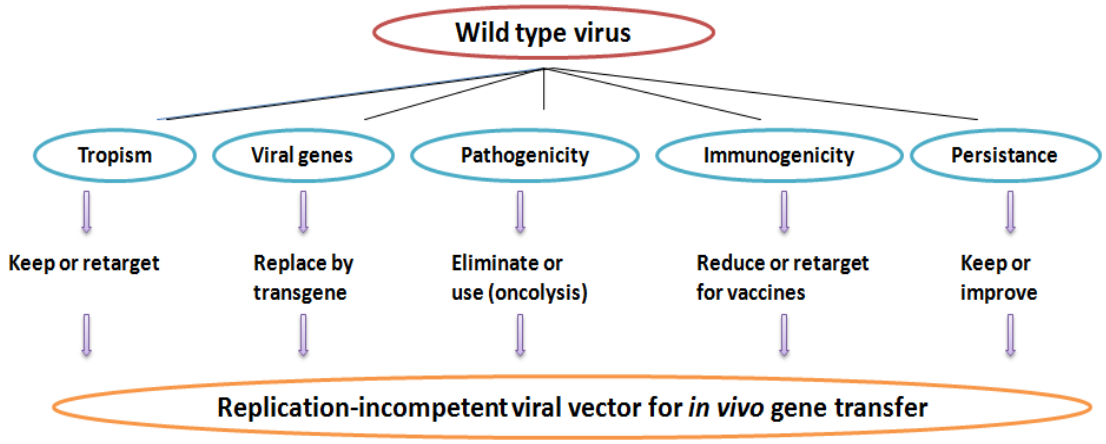

Figure 5-1.3.2(b) Modifications required for the generation of replication-defective viral vectors from wild type virus for *in vivo* gene transfer.

(Adapted and modified from Bouard D, Alazard-Dany N, Cosset F-L. 2009. Viral vectors: from virology to transgene expression. British Journal of Pharmacology, 157: 153–165.)

- If the transgene is added to the genome or replaces one or more non-essential genes for the infection cycle in the expression host, the vector is described as **replication-competent or helper-independent**, as it can propagate independently *e.g.* helper independent adenoviral vectors.
- However, if the transgene replaces an essential viral gene, this renders the vector **replication-defective or helper-dependent**, so that missing functions must be supplied *in trans.* This can be accomplished by co-introducing a helper virus or transfecting the cells with a helper plasmid, each of which carry the missing genes *e.g.* helper dependent retroviruses (Figure 5-1.3.2(b)).
- An alternative to the co-introduction of helpers is to use a complementary cell line, which is transformed with the appropriate genes called as ‘packaging lines’.
- The vectors from which all viral coding sequences have been deleted and depend on a helper virus which can provide viral gene products *in trans* for packaging and vector DNA replication are known as *`gutless vectors’*.

Advantages

- High capacity for foreign DNA
- The vector has no intrinsic cytotoxic effects.

5-1.4. Viral vectors

Various kinds of viruses can be used as viral vectors, but five classes of viral vector are used in human gene therapy-

1. Adenovirus
2. Adeno- associated virus (AAV)
3. Herpes virus
4. Retrovirus
5. Lentivirus

Vector	Host cells	Entry pathway	Vector genome forms	Transgene expression	Uses
Retrovirus	Actively dividing cells	Receptor-binding, membrane fusion	Integrated	Long term (years)	SCID, Hyperlipedemia, solid tumors
Lentivirus	Dividing and non-dividing cells	Receptor-binding, membrane fusion	Episomal	Stable	Hematopoetic cells, muscles, neuron, hepatocytes
Adeno virus	Most cells	CAR (Coxsackie and Adenovirus Receptor)-mediated endocytosis endosomal escape	Episomal	Transient (short term for weeks)	CNS, hepatocytes, pancreas

Adeno-associated virus	Most cells	Receptor-mediated endocytosis endosomal escape	Episomal (90%) Integrated (10%)	Medium to long term (year)	lung , muscle, heart, CNS
Herpes virus	Most cells	Endocytotic or membrane fusion	Episomal	Transient	Suitable particularly for nervous system

Table 5-1.4. Viral vectors and their properties.

5-1.4.1. Adenoviruses

- Adenoviruses are medium-sized (90-100 nm), non-enveloped, icosahedral viruses containing linear, double-stranded DNA of approximately 36 kb.
- 57 immunologically distinct types (7 subgenera) of adenoviruses cause human infections.
- They are unusually stable to physical or chemical agents and adverse pH conditions for long-term survival outside the body.
- There are six early-transcription units, most of which are essential for viral replication, and a major late transcript that encodes capsid.
- They result in transient expression in dividing cells as they do not integrate efficiently into the genome, but prolonged expression can be achieved in post-mitotic cells, like neurons.
- Adenovirusesare mostly attractive as gene therapy vectors, because the virions are taken up efficiently by cells *in vivo.* Adenovirus-derived vaccines have been used in humans with no reported side-effects.

The adenovirus infection cycle comprises two phases-early and late phase, separated by viral DNA replication. The first or "early" phase involves the entry of the virus into the host cell and virus genome to the nucleus. The late genes are transcribed from the major late promoter. The “late” phase involves the formation of gene products related to production and assembly of capsid proteins.

Wild Type Adenovirus Genome

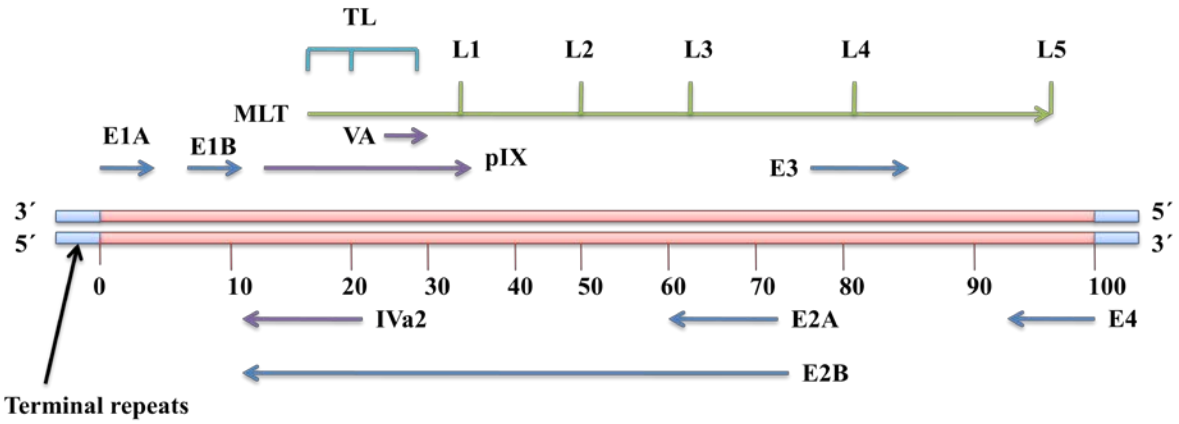

Figure5-1.4.1(a). A wild type adenovirus genome. (E1A, E1B, E2A, E2B, E3, E4- early genes; L1 to 5- late genes; MLT- major late transcript; TL-tripartite leader; other genes are represented by pIX, IVa2, VA)

Adenoviral genes	Function
Early genes: E1A, E1B, E2, E3, E4	Transcription, replication, host immune suppression, inhibition of host cell apoptosis.
Delayed early genes: pIX, IVa2	Packaging
Major late gene (L)	Assembly

Table 5-1.4.1. Different types of adenoviral genes and their function.

Construction of Adenoviral vectors

First generation adenoviral vectors were *replication deficient*, lacking the essential E1A and E1B genes and often the non-essential gene E3 and were called **'E1 replacement vectors'.** They had a maximum capacity of about 7 kb and were propagated in the cell lines transfected with DNA containing E1 genes e.g. human embryonic kidney line 293 (HEK 293).

Drawback

- These vectors caused **cytotoxic effect** due to low-level expression of the viral gene products, and chances of recombination between the vector and the integrated portion of the genome, resulting in the recovery of replication-competent viruses.

Higher-capacity vectors lacking the E2 or E4 regions in addition to E1 and E3 provide a maximum cloning capacity of about 10 kb but still allow low level of transgene expression. These must be propagated on complementary cell lines providing multiple functions. The use of E1/E4 deletions is a sound strategy as the E4 gene is responsible for many of the immunological effects of the virus.

To overcome the above limitations, an alternative strategy employs insertion of 'stuffer DNA' into the nonessential E3 gene as part of the vector backbone so to maintain optimum vector size. Helper dependent adenoviral vectors (HDAd) are favoured for *in vivo* gene transfer due to deletion of all viral coding sequences.

Advantages of HDAd

- Large cloning capacity (up to 37 kb)
- High transduction efficiency
- Long term transgene expression
- Lack of immune response and cytotoxicity.

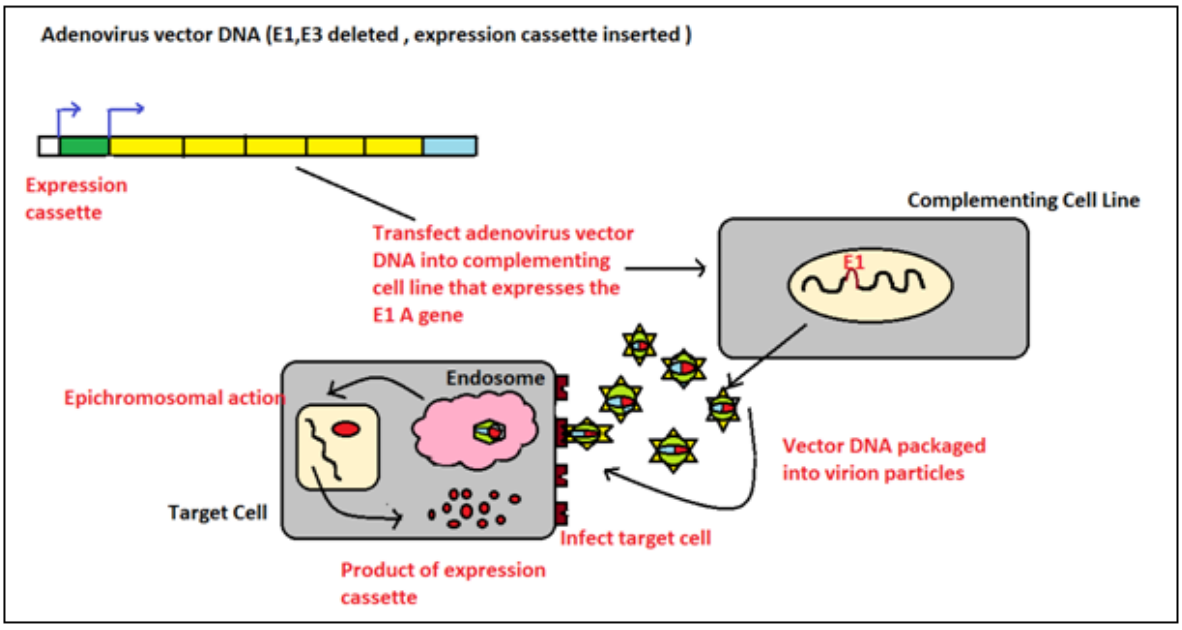

Figure 5-1.4.1(b). Adenovirus vectors in gene transfer.

Role in gene therapy

- Mainly used for cancer treatment. Gendicine, a recombinant adenovirus, is the first gene therapy product to be licensed for cancer treatment.

Advantages of Adenoviral vectors

- High transduction efficiency
- Insert size up to 8 kilobases
- Generation of high virus titres
- High level of expression in a wide variety of cell types
- No mutagenic effects due to lack of random integration into the host genome.

Disadvantages of Adenoviral vectors

- Transient expression due to lack of integration into the host.
- Pathogenic to humans.

5-1.4.2. Adeno-associated virus

- It was first discovered as a contaminant in an adenoviral isolate in 1965.
- It is a small, non-enveloped virus packaging a linear single stranded DNA belonging to Parvovirus family.
- It is naturally replication defective thus requiring a helper virus (usually adenovirus or herpes virus) for productive infection.
- In human cells, the provirus integrates predominantly into a 4-kb region (AAVS1) on chromosome19. Subsequent infection by adenovirus or herpes virus can ‘rescue’ the provirus and induce lytic infection.

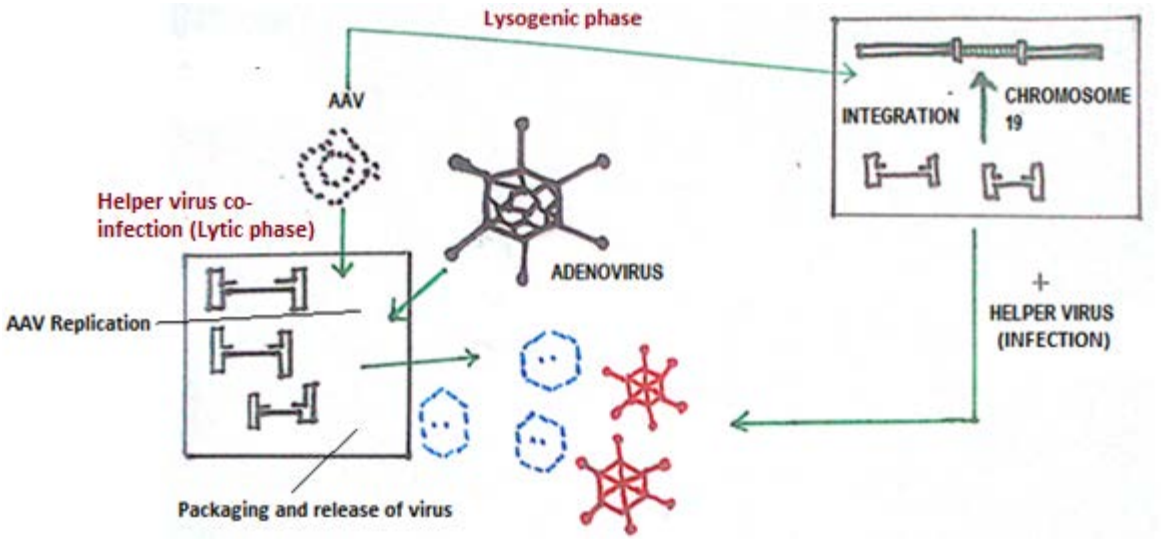

Figure 5-1.4.2(a). AAV life cycle.

(Adapted from Daya S, Berns KI.2008. Gene Therapy Using Adeno-Associated Virus Vectors.Clin.Microbiol. Rev, 21(4): 583.)

AAV life cycle

- AAV life cycle comprises two phases-lytic and lysogeny.
- In the presence of helper virus, AAV undergoes lytic phase comprising genome replication, expression of viral genes and production of virions (Figure 5-1.4.2(a).).
- In the absence of helper virus, it undergoes lysogenic phase and integrates into the host cell's genome as a latent provirus. This latent genome undergoes replication by subsequent infection with helper virus.
- Both the stages of life cycle of AAV are controlled by complex interactions between the AAV genome and helper virus, AAV and host proteins.

Adeno-associated viral genome

The AAV genome is small (about 5 kb) and comprises a central region containing rep (replicase) and cap (capsid) genes flanked by 145 base inverted terminal repeats (ITRs). The rep gene is involved in viral replication and integration whereas cap gene encodes viral capsid proteins. ITRs are required for replication, transcription, proviral integration and rescue.

In earlier AAV vectors, foreign DNA replaced the cap region and was expressed under the control of an endogenous AAV promoter. The transgene expression was inefficient using heterologous promoters due to inhibition of their activity by Rep protein.

Rep interference with endogenous promoters resulted in cytotoxic effects of the virus. To overcome the above limitations, such vectors in which both genes were deleted and the transgene was expressed from either an endogenous or a heterologous promoter, were developed.

In vitro manipulation of AAV is facilitated by cloning the inverted terminal repeats in a plasmid vector and inserting the transgene between them. Transfection of this construct into cells along with a helper plasmid produced recombinant viral particles.

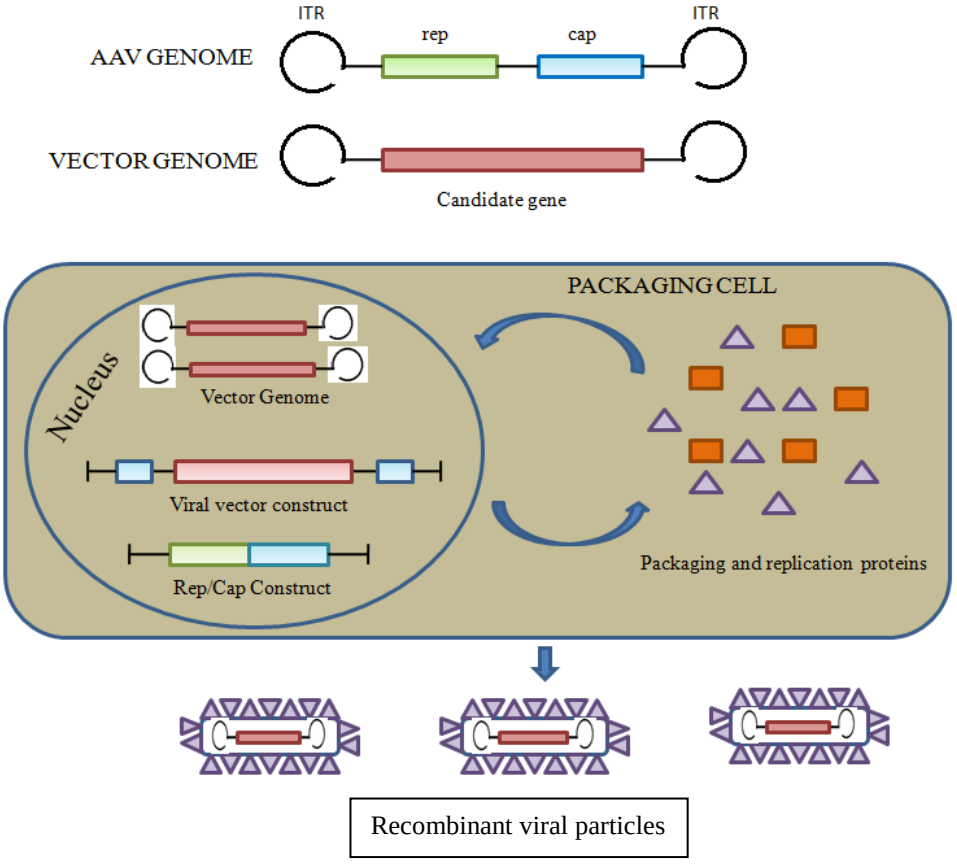

Figure 5-1.4.2(b). AAV Genome, Vector genome and Packaging coil.

(Adapted from Somia N, Verma IM. 2000. Gene therapy: trials and tribulations. Nature Reviews Genetics, 1: 91-99)

Recombinant AAV (rAAV) is used as an expression cassette containing a reporter or candidate gene of interest. The foreign gene replaces all of the viral genes present in a wild type virus. Only the inverted terminal repeats are left to function as the essential replication/packaging signal.

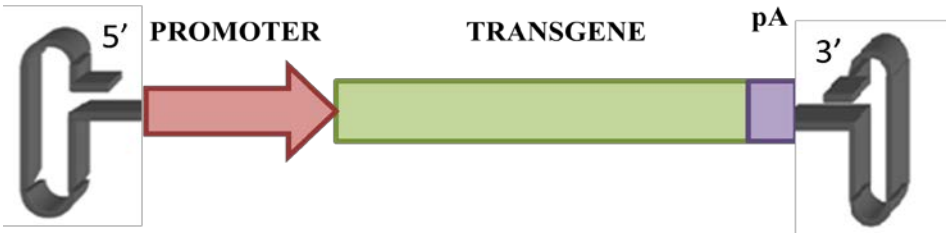

Figure 5-1.4.2(c). Organization of a typical recombinant AAV (rAAV) genome. pA represents Poly A tail.
(Adapted from http://aavvectors.tripod.com/id3.html)

Advantages

- Stable and have a wide host range
- Lack of initiating an immune response
- The dependence of AAV on a heterologous helper virus provides higher control over vector replication, making AAV vectors safer for use in gene therapy
- Potential of targeted/site-specific integration
- Non-pathogenic

Disadvantages

- AAV uses concatemeric replication intermediates
- They must be closely screened as they are often contaminated with adenovirus or Herpes Virus.
- Insert size is limited (4Kb)
- Difficult generation of high virus titres

5-1.4.3. Herpes virus vectors

- The herpes viruses are linear ds-DNA viruses of approximately 150 kb size *e.g.* EBV (Epstein–Barr virus) and the HSVs (Hepatitis B virus, e.g. HSV-I, varicella zoster).
- Most HSVs are transmitted without symptoms (varicella zoster virus is exceptional) and cause prolonged infections.
- With the help of two viral glycoproteins, gB and gD, the virus binds to cells through an interaction with heparan sulphate moieties on the cell surface.
- Unlike EBV as a replicon vector (contains both *cis* and *trans* acting genetic elements required for replication), HSV-I have been developed as a transduction vector for purpose of gene transfer and can efficiently transduce a wide range of cell types.
- HSV virus is remarkably neurotropic and thus HSV vectors are particularly suitable for gene therapy in the nervous system. HSV can also be transmitted across neuronal synapses during lytic infections which can be used to trace axon pathways.
- Generation of recombinants in transfected cells takes place by homologous recombination. These viral vectors may be replication competent or helper dependent.
- The plasmid based amplicon vectors carrying only the *cis*-acting elements required for replication and packaging can be constructed. These vectors require packaging systems to provide the missing functions in *trans*.

Role in gene therapy

Most promised use of HSV vectors involves gene transfer to neural cells where it can cause a latent infection (e.g. spinal cord, brain, and peripheral nervous system).

Advantages

- Infects a wide range of cell types
- Insert size up to 50 kb due to large viral genome size

- Natural tropism to neuronal cells
- Stable viral particles allow generation of high virus titres (10^{12}pfu/ml)

Disadvantages

- No viral integration into host genome and transient transgene expression
- High level of pre-existing immunity
- Cytotoxicity effects
- Risk of recombination with latently HSV-infected cells

5-1.4.4. Retroviral vectors

Retroviruses are RNA viruses that replicate via a ds-DNA intermediate. The infection cycle begins with the interaction between viral envelope and the host cell's plasma membrane, delivering the particle into the cell. The capsid contains two copies of the RNA genome, as well as reverse transcriptase/integrase. After infection, the RNA genome is reverse transcribed to produce a cDNA copy, a DNA intermediate, which integrates into the genome randomly.

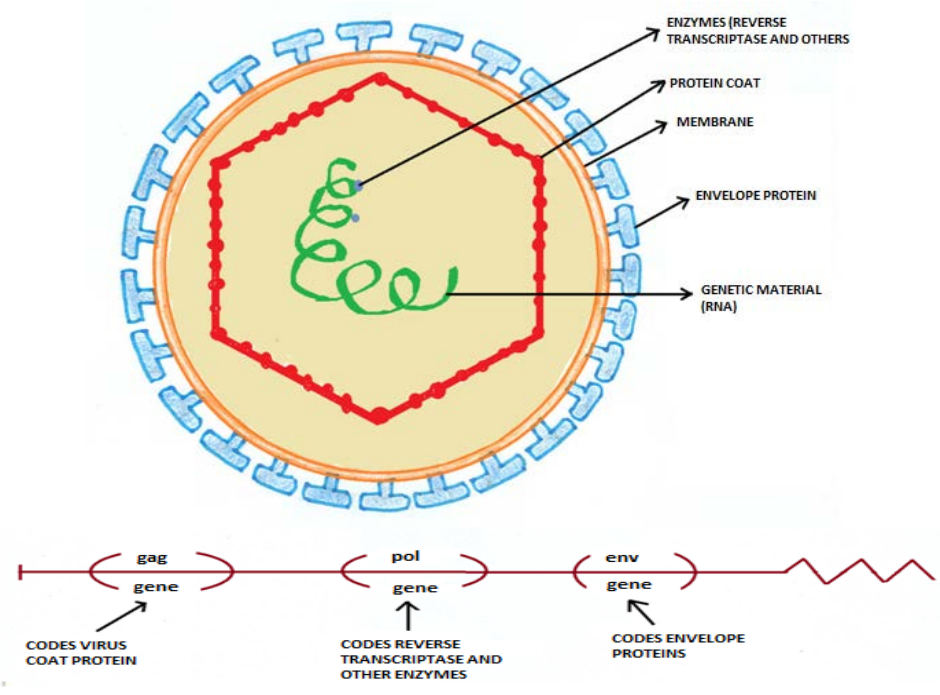

Figure 5-1.4.4(a). Structure of a Retrovirus vector. RNA showed in the figure is single stranded.

Life cycle of retroviruses

A retrovirus, on binding to a cell surface receptor, enters the cell where it reverse transcribes the RNA into double-stranded DNA. Viral DNA gets integrated into the cell chromosome to form a provirus. Cellular machinery transcribes, processes the RNA and undergoes translation into viral proteins. The viral RNA and proteins are then assembled to form new viruses which are released from the cell by budding (Figure 5-1.4.4(b).).

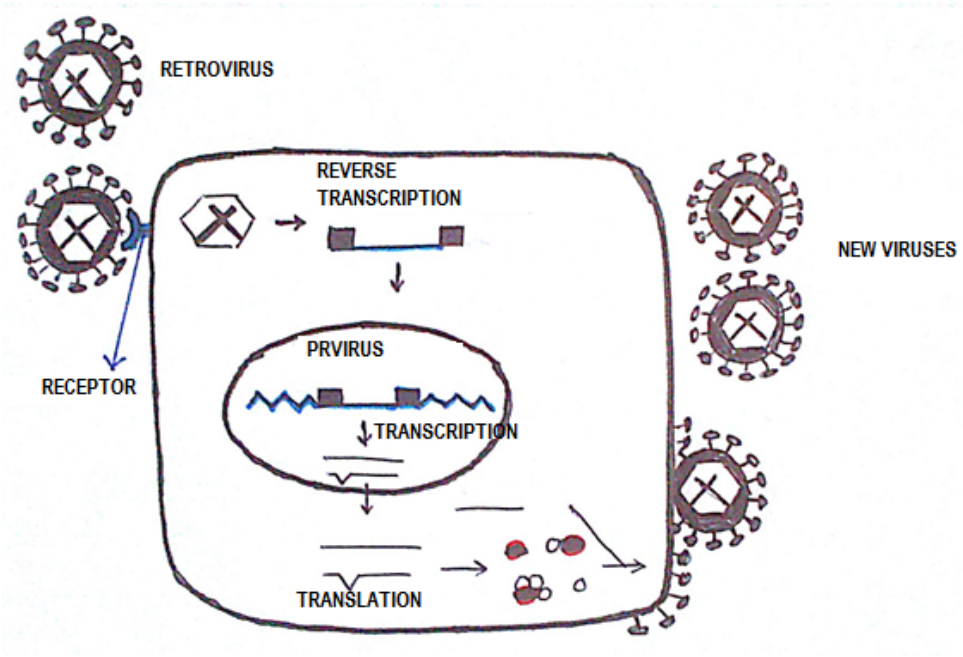

Figure 5-1.4.4(b). Replication cycle of retroviruses.

(Adapted from Hu W-S, Pathak VK. 2000. Design of Retroviral Vectors and Helper Cells for Gene Therapy. Pharmacol Rev, 52: 493–511)

Retroviral genome

The integrated provirus comprises three genes (*gag*, *pol* and *env*). The *gag* gene encodes a viral structural protein, *pol* encodes the reverse transcriptase and integrase and *env* gene encodes viral envelope proteins. Retrovirus can be classified as oncoviruses, lentiviruses, and spuma-viruses. Oncoviruses are simple whereas lentiviruses and spuma-viruses are complex retroviruses.

Viral genomic RNA is synthesized by transcription from a single promoter located in the left LTR and ends at a poly-A site in the right LTR. Thus, the full-length genomic RNA is shorter than the integrated DNA copy and lacks the duplicated LTR structure. The genomic RNA is capped and polyadenylated, allowing the *gag* gene to be translated. The *pol* gene is also translated by read through, producing a Gag–Pol fusion protein, which is further processed into several distinct polypeptides. Some of the full-length RNA also undergoes splicing, eliminating the *gag* and *pol* genes and allowing the downstream *env* gene to be translated. Two copies of the full-length RNA genome are incorporated into each capsid requiring a specific *cis*-acting packaging site termed ψ. The reverse transcriptase/ integrase are also packaged.

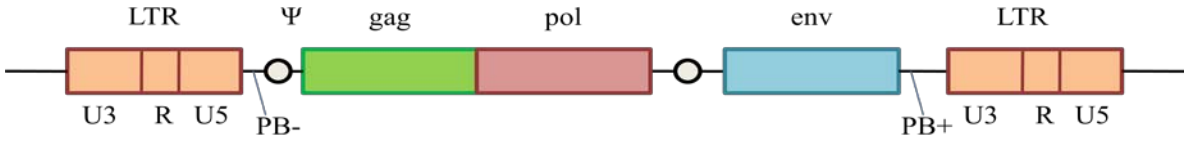

Figure 5-1.4.4(c). An oncoretrovirus genome comprising long terminal repeats (LTRs) enclosing the three open reading frames *gag, pol* and *env*. PB represents primer binding sites in the viral replication cycle, ψ is the packaging signal and small circles represent splice sites.

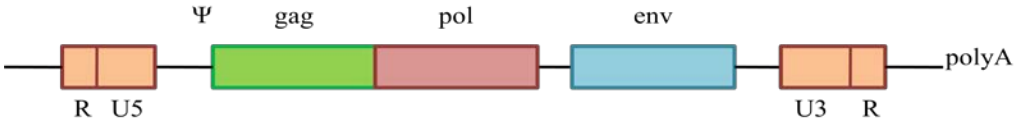

Figure 5-1.4.4(d). Structure of a packaged RNA genome having a poly (A) tail but lacking the LTRs.

Construction of a retroviral vector and propagation in helper cell

The retroviral construct involved in gene delivery comprises two constructs-

- A vector consisting of all *cis*-acting elements required for gene expression and replication (Figure 5-1.4.4(f).)
- A helper cell expressing all the viral proteins (*gag*, *pol*, *env*) lacking in vector and support the replication of vector. Helper cell lacks RNA containing packaging signal which is required for formation and release of infectious particles but not for non-infectious viral particles.

When the vector DNA is introduced into a helper cell, helper cell produces the viral proteins which help in the assembly of viral particles containing RNA transcribed from the viral vector. These viral particles on infecting the target cell, reverse transcribe the vector RNA into ds-DNA which gets integrated into the host genome forming a provirus which encodes the gene of interest. Target cells do not express viral proteins and cannot generate infectious viral particles containing the vector RNA and thus cannot infect other target cells (Figure 5-1.4.4(e).).

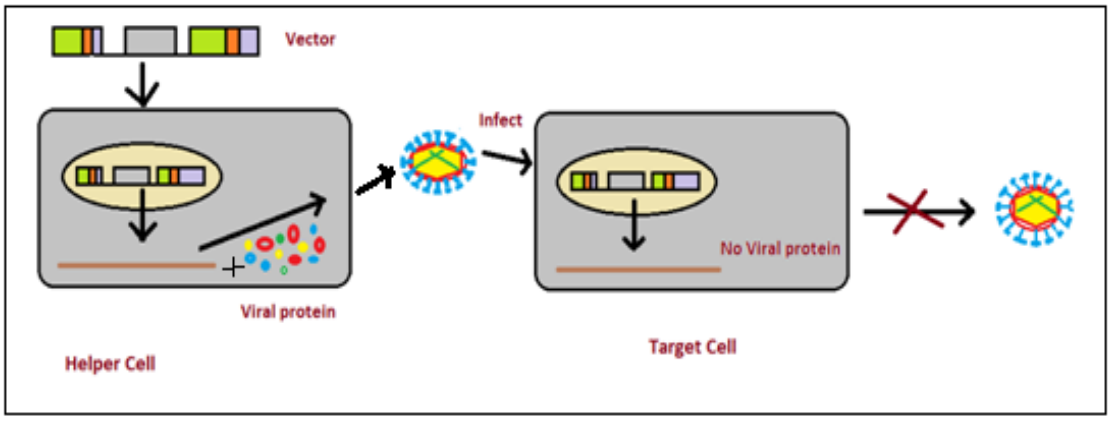

Figure 5-1.4.4(e). Propagation of retroviral vectors in helper cells.

(Adapted from Hu W-S, Pathak VK.2000.Design of Retroviral Vectors and Helper Cells for Gene Therapy.Pharmacol Rev, 52: 493–511)

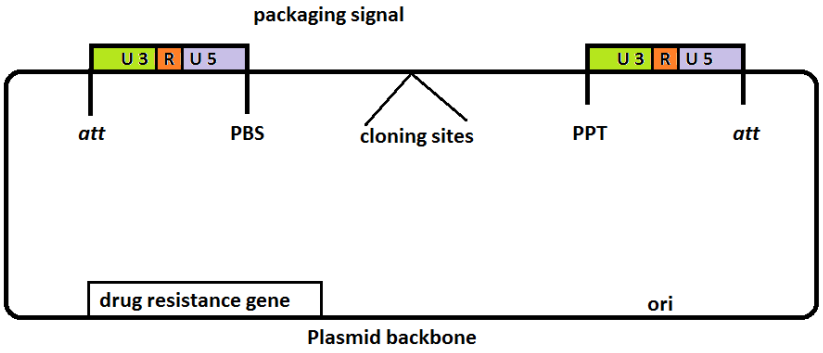

Figure 5-1.4.4(f). *cis*-acting elements required by a prototypical retroviral vector. The plasmid backbone contains a bacterial origin of replication (ori) and a drug resistance gene.

(Adapted from Hu W-S, Pathak VK. 2000. Design of Retroviral Vectors and Helper Cells for Gene Therapy.Pharmacol Rev, 52: 493–511.).

Advantages

- Well studied system having high transduction efficiency
- Insert size up to 8 kb
- Integration into host genome resulting in sustained expression of the vector
- Vector proteins are not expressed in host

Disadvantages

- Infection by retrovirus requires dividing cells
- Low titres (10^6-10^7)
- Random integration
- Poor *in vivo* delivery

5-1.4.5 Lentivirus

- They are subclass of retroviruses.
- They are more efficient and advantageous for gene transfer than other vectors due to following reasons-
    - Unlike retroviruses which can infect only dividing cells, lentiviruses can be used as vectors due to their ability to infect both dividing and non-dividing cells.
    - Low immunogenicity
    - Stable, long term transgene expression

5-1.5. Properties of viral vectors used in gene transfer

The properties of different viral vectors are listed in Table 5-1.5.

Viral vectors	Genome	Insert capacity (kb)	Specific integration	Long-term maintenance	RNA intermediate
Retroviruses	ssRNA with DNA intermediate	1-7	Y	Y	RNA with DNA intermediate
Adenovirus	dsDNA	2-38	N	N	N
Adeno-associated virus	ssDNA	4.5	Y	Y	N
Herpes simplex virus	dsDNA	50	N	Y	N
Lentivirus	RNA with DNA intermediate	7-18	Y	Y	RNA with DNA intermediate

Y-Yes; N-No.

Table 5-1.5. Various viral vectors used in gene transfer

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MODULE 5- LECTURE 2

GENE TRANSFER TECHNIQUES: CHEMICAL METHODS

5-2.1. Introduction

Cell membrane is a sheet like assembly of amphipathic molecules that separate cells from their environment. These physical structures allow only the controlled exchange of materials among the different parts of a cell and with its immediate surroundings. DNA is an anionic polymer, larger molecular weight, hydrophilic and sensitive to nuclease degradation in biological matrices. They cannot easily cross the physical barrier of membrane and enter the cells unless assisted.

Various charged chemical compounds can be used to facilitate DNA transfer directly to the cell. These synthetic compounds are introduced near the vicinity of recipient cells thereby disturbing the cell membranes, widening the pore size and allowing the passage of the DNA into the cell.

An ideal chemical used for DNA transfer should have the ability to-

- Protect DNA against nuclease degradation.
- Transport DNA to the target cells.
- Facilitate transport of DNA across the plasma membrane.
- Promote the import of DNA into the nucleus.

The commonly used methods of chemical transfection use the following,

1. Calcium phosphate
2. DEAE dextran
3. Cationic Lipid
4. Other polymers - poly-L-lysine (PLL), polyphosphoester, chitosan, dendrimers

5-2.1.1. Calcium phosphate mediated DNA transfer

5-2.1.1.1. Historical perspective

The ability of mammalian cells to take up exogenously supplied DNA from their culture medium was first reported by Szybalska and Szybalski (1962).

They used total uncloned genomic DNA to transfect human cells deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT). Rare HPRT-positive cells with fragments of DNA containing the functional gene were identified by selection on HAT medium. Till then, the actual mechanism of DNA uptake was not understood. It was later found that successful DNA transfer takes place by the formation of a fine DNA/calcium phosphate co-precipitate, which first settles onto the cells and is then internalized. This technique was first applied by Graham and Van Der Eb in 1973 for the analysis of the infectivity of adenoviral DNA.

5-2.1.1.2. Calcium phosphate transfection

This method is based on the precipitation of plasmid DNA and calcium ions by their interaction.

Inthis method, the precipitates of calcium phosphate and DNA being small and insoluble can be easily adsorbed on the surface of cell. This precipitate is engulfed by cells through endocytosis and the DNA gets integrated into the cell genome resulting in stable or permanent transfection.

Uses

- This method is mainly used in the production of recombinant viral vectors.
- It remains a choice for plasmid DNA transfer in many cell cultures and packaging cell lines. As the precipitate so formed must coat the cells, this method is suitable only for cells growing in monolayer and not for suspension cultures.

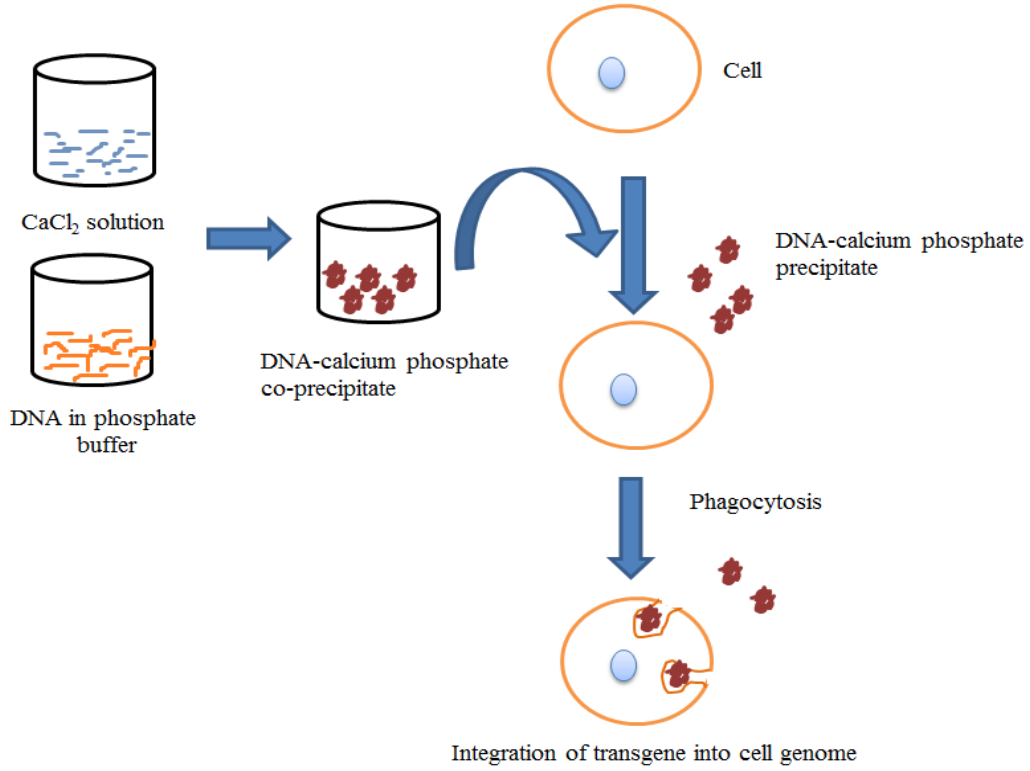

Figure 5-2.1.1.2. A schematic representation of transfection by Calcium Phosphate Precipitation.

Advantages

- Simple and inexpensive
- Applicability to generate stably transfected cell lines
- Highly efficient (cell type dependent) and can be applied to a wide range of cell types.
- Can be used for stable or transient transfection

Disadvantages

- Toxic especially to primary cells
- Slight change in pH, buffer salt concentration and temperature can compromise the efficacy
- Relatively poor transfection efficiency compared to other chemical transfection methods like lipofection.
- Limited by the composition and size of the precipitate.

- Random integration into host cell.

Optimal factors (amount of DNA in the precipitate, the length of time for precipitation reaction and exposure of cells to the precipitate) need to be determined for efficient transfection of the cells.

This technique is simple, expensive and has minimal cytotoxic effect but the low level of transgene expression provoked development of several other methods of transfection.

5-2.1.2. DEAE-Dextran (Diethylaminoethyl Dextran) mediated DNA transfer

- This method was initially reported by Vaheri and Pagano in 1965 for enhancing the viral infectivity of cell but later adapted as a method for plasmid DNA transfer.
- Diethylaminoethyl dextran (DEAE-dextran) is a soluble polycationic carbohydrate that promotes interactions between DNA and endocytotic machinery of the cell.
- In this method, the negatively charged DNA and positively charged DEAE – dextran form aggregates through electrostatic interaction and form apolyplex. A slight excess of DEAE – dextran in mixture results in net positive charge in the DEAE – dextran/ DNA complex formed. These complexes, when added to the cells, bind to the negatively charged plasma membrane and get internalized through endocytosis. Complexed DNA delivery with DEAE-dextran can be improved by osmotic shock using DMSO or glycerol.
- Several parameters such as number of cells, polymer concentration, transfected DNA concentration and duration of transfection should be optimized for a given cell line.

Advantages

- Simple and inexpensive
- More sensitive
- Can be applied to a wide range of cell types
- Can be used for transient transfection.

Disadvantages

- Toxic to cells at high concentrations
- Transfection efficiency varies with cell type
- Can only be used for transient transfection but not for stable transfection
- Typically produces less than 10% delivery in primary cells.

Another polycationic chemical, the detergent Polybrene, has been used for the transfection of Chinese hamster ovary (CHO) cells, which are not amenable to calcium phosphate transfection.

5-2.1.3. Lipofection

- Lipofection is a method of transformation first described in 1965 as a model of cellular membranes using liposomes.
- Liposomes are artificial phospholipid vesicles used for the delivery of a variety of molecules into the cells. They may be multi-lamellar or unilamellar vesicles with a size range of0.1 to 10 micrometer or 20-25 nanometers respectively.
- They can be preloaded with DNA by two common methods- membrane-membrane fusion and endocytosis thus forming DNA- liposome complex. This complex fuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are susceptible to lipofection method.
- Liposomes can be classified as either cationic liposome or pH-sensitive.

5-2.1.3.1. Cationic liposomes

- Cationic liposomes are positively charged liposomes which associate with the negatively charged DNA molecules by electrostatic interactions forming a stable complex.

Neutral liposomes are generally used as DNA carriers and helpers of cationic liposomes due to their non-toxic nature and high stability in serum. A positively charged lipid is often mixed with a neutral co-lipid, also called helper lipid to enhance the efficiency of gene transfer by stabilizing the liposome complex (lipoplex). Dioleoylphosphatidyl ethanolamine (DOPE) or dioleoylphosphatidyl choline (DOPC) are some commonly used neutral co-lipids.

- The negatively charged DNA molecule interacts with the positively charged groups of the DOPE or DOPC. DOPE is more efficient and useful than DOPC due to the ability of its inverted hexagonal phase to disrupt the membrane integrity.
- The overall net positive charge allows the close association of the lipoplex with the negatively charged cell membrane followed by uptake into the cell and then into nucleus.
- The lipid: DNA ratio and overall lipid concentration used in the formation of these complexes is particularly required for efficient gene transfer which varies with application.

5-2.1.3.2. Negatively charged liposomes

- Generally pH-sensitive or negatively-charged liposomes are not efficient for gene transfer. They do not form a complex with it due to repulsive electrostatic interactions between the phosphate backbone of DNA and negatively charged groups of the lipids. Some of the DNA molecules get entrapped within the aqueous interior of these liposomes.
- However, formation of lipoplex, a complex between DNA and anionic lipidscan occur by using divalent cations (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ba^{2+}) which

canneutralize the mutual electrostatic repulsion. These anionic lipoplexes comprise anionic lipids, divalent cations, and plasmid DNA which are physiologically safe components.

- They are termed as **pH sensitive** due to destabilization at low pH.

The efficiency of both *in vivo* and *in vitro* gene delivery using cationic liposomes is higher thanthat of pH sensitive liposomes. But the cationic liposomes get inactivated and unstable in the presence of serum and exhibit cytotoxicity. Due to reduced toxicity and interference from serum proteins, pH-sensitive liposomes are considered as potential gene delivery vehicles than the cationic liposomes.

5-2.1.3.3. Liposome Action

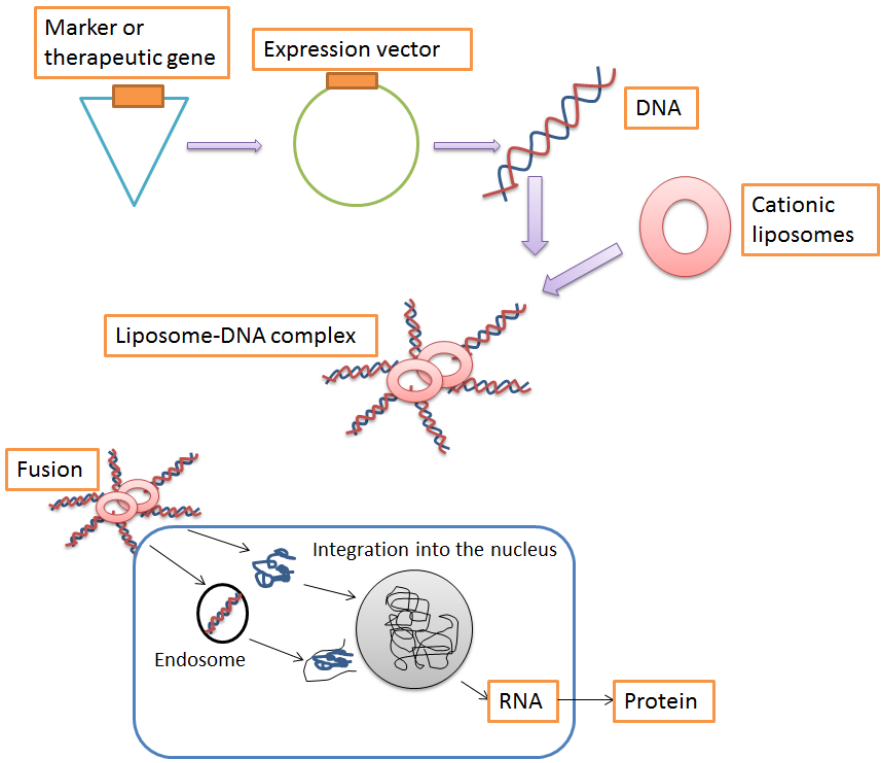

Figure5-2.1.3. Schematic representation of liposome action in gene transfer. ***(Source: Pleyer U, Dannowski H. 2002. Delivery of genes via liposomes to corneal endothelial cells. Drug News Perspect, 15(5): 283)***

In addition, liposomes can be directed to cells using monoclonal antibodies which recognize and bind to the specific surface antigens of cells along with the liposomes. Liposomes can be prevented from destruction by the cell's lysosomes by pre- treating the cells with chemicals such as chloroquine, cytochalasin B, colchicine etc. Liposome mediated transfer into the nucleusis still not completely understood.

Advantages

- Economic
- Efficient delivery of nucleic acids to cells in a culture dish.
- Delivery of the nucleic acids with minimal toxicity.
- Protection of nucleic acids from degradation.
- Measurable changes due to transfected nucleic acids in sequential processes.
- Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

Disadvantages

- It is not applicable to all cell types.
- It fails for the transfection of some cell lines with lipids.

5-2.1.4. Other Methods

Other methods of chemical transfection involve the use of chemicals such as polyethylenimine, chitosan, polyphosphoester, dendrimers.

5-2.1.4.1. Polyethylenimine

- Polyethylenimine (PEI) is a non-degradable, high molecular weight polymer which may accumulate in the body.
- PEI, due to its polycationic nature, condenses with the DNA molecule resulting in the formation of PEI-DNA complex which enters the cell by endocytosis, thus mediating gene transfer.
- PEI exhibit cytotoxicity due to its ability to permeabilize and disrupt cell membranes leading to necrotic cell death.

- The cytotoxicity may be reduced using various methods e.g. PEGylation and conjugation of low molecular weight polyethylenimine with cleavable cross-links such as disulfide bonds in the reducing environment of the cytoplasm.

5-2.1.4.2. Chitosan

- Chitosan, a biodegradable polysaccharide is composed of D-glucosamine repeating units and can be used as a non-viral gene carrier.
- It can efficiently bind and protect DNA from nuclease degradation.
- The biocompatibility and low toxicity profile makes it a safe biomedical material for clinical applications.
- Chitosan DNA nanoparticles can transfect several different cell types with relatively low transfection efficiency.
- Modified chitosans such as trimethylated chitosan and chitosan conjugated with deoxycholic acid have been developed to increase the solubility of chitosan at neutral pH which can efficiently transfect COS-1 cells.
- Chitosans with different molecular weights exhibit different DNA binding affinities. The efficiency of transfection is determined by the particle stability which is one of the rate-limiting steps in the overall transfection process.

5-2.1.4.3. Polyphosphoester

- Polyphosphoesters (PPE) are biocompatible and biodegradable, particularly those having a backbone analogous to nucleic acids and teichoic acids and used in several biomedical applications. They may result in extracellular persistent release of the DNA molecules thus enhancing the expression of transgene in the muscle as compared to naked DNA intake.
- Several polyphosphoesters with positive charges both in the backbone and in the side chain can be used as non-viral gene carriers.
- They can efficiently bind and protect DNA from nuclease degradation.
- They exhibit a significantly lower cytotoxicity than Poly-L-Lysine or polyethylenimine both *in vitro* and *in vivo.*

- It is a cell type dependent transfection method the efficiency of which can be enhanced using chloroquine.
- The transfection using polyphosphoestersis found to be effective in many cell lines, with some of them comparable to Liposome-mediated transfection.

5-2.1.4.4. Dendrimers

- Dendrimers are a new class of polymeric materials that are highly branched and monodisperse macromolecules. Due to their unique behaviour, they are suitable for a wide range of biomedical applications.
- They have positively charged amino groups (termini) on their surface which interact with the negatively charged phosphate groups of the DNA molecule to form a DNA-dendrimer complex.
- This DNA-dendrimer complex has an overall net positive charge and interacts with negatively charged surface molecules of the cell membrane thus allowing the entry of complex into the cell through non-specific endocytosis.
- Once inside the cell, these complexes are then transported to the endosomes where these are protected from nuclease degradation by being highly condensed within the DNA-dendrimer complex.
- The unprotonated amino groups on the dendrimers at neutral pH can become protonated in the acidic environment of the endosome leading to buffering of the endosome and thus inhibiting pH-dependent endosomal nucleases.

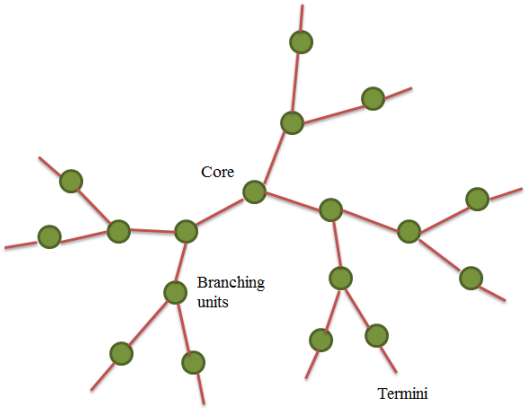

Figure 5-2.1.4.4. Structure of a dendrimer.

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MODULE 5- LECTURE 3

GENE TRANSFER TECHNIQUES: PHYSICAL OR MECHANICAL METHODS

5-3.1. Gene transfer techniques

It has been discussed earlier that due to amphipathic nature of the phospholipid bilayer of the plasma membrane, polar molecules such as DNA and protein are unable to freely pass through the membrane. Various physical or mechanical methods are employed to overcome this and aid in gene transfer as listed below-

1. Electroporation
2. Microinjection
3. Particle Bombardment
4. Sonoporation
5. Laser induced
6. Bead transfection

5-3.1.1. Electroporation

- Electroporation is a mechanical method used for the introduction of polar molecules into a host cell through the cell membrane.
- This method was first demonstrated by Wong and Neumann in 1982 to study gene transfer in mouse cells.
- It is now a widely used method for the introduction of transgene either stably or transiently into bacterial, fungal, plant and animal cells.
- It involves use of a large electric pulse that temporarily disturbs the phospholipid bilayer, allowing the passage of molecules such as DNA.

The basis of electroporation is the relatively weak hydrophobic/hydrophilic interaction of the phospholipids bilayer and ability to spontaneously reassemble after disturbance. A quick voltage shock may cause the temporary disruption of areas of the membrane and allow the passage of polar molecules. The membrane reseals leaving the cell intact soon afterwards.

5-3.1.1(a). Procedure

The host cells and the DNA molecules to be transported into the cells are suspended in a solution. The basic process inside an electroporation apparatus is represented in a schematic diagram (Figure 5-3.1.1(a).).

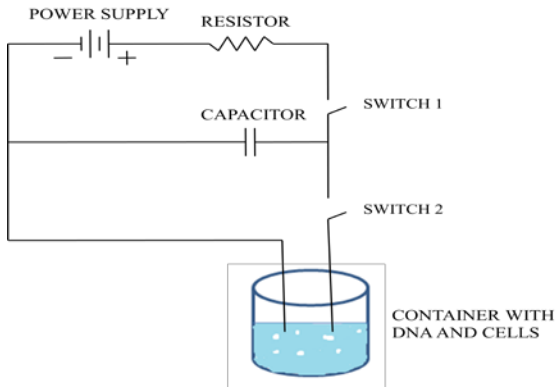

Figure 5-3.1.1(a). The basic circuit setup of the electroporation apparatus.
(Adapted from http://o]pbs.okstate.edu/~melcher/MG/MGW4/MG431.html)

- When the first switch is closed, the capacitor charges up and stores a high voltage which gets discharged on closing the second switch.
- Typically, 10,000-100,000 V/cm in a pulse lasting a few microseconds to a millisecond is essential for electroporation which varies with the cell size.
- This electric pulse disrupts the phospholipid bilayer of the membrane causing the formation of temporary aqueous pores.
- When the electric potential across the cell membrane is increased by about 0.5-1.0 V, the charged molecules e.g. DNA migrate across the membrane through the pores in a similar manner to electrophoresis.
- The initiation of electroporation generally occurs when the transmembrane voltage reaches at 0.5-1.5 V. The cell membrane discharges with the subsequent

flow of the charged ions and molecules and the pores of the membrane quickly close reassembling the phospholipid bilayer.

5-3.1.1(b). Applications

Electroporation is widely used in many areas of molecular biology and in medical field. Some applications of electroporation include:

- **DNA transfection or transformation**

  Electroporation is mainly used in DNA transfection/transformation which involves introduction of foreign DNA into the host cell (animal, bacterial or plant cell).
- **Direct transfer of plasmids between cells**

  It involves the incubation of bacterial cells containing a plasmid with another strain lacking plasmids but containing some other desirable features. The voltage of electroporation creates pores, allowing the transfer of plasmids from one cell to another. This type of transfer may also be performed between species. As a result, a large number of plasmids may be grown in rapidly dividing bacterial colonies and transferred to yeast cells by electroporation.
- **Gene transfer to a wide range of tissues**

  Electroporation can be performed in vivo for more efficient gene transfer in a wide range of tissues like skin, muscle, lung, kidney, liver, artery, brain, cornea etc. It avoids the vector-specific immune-responses that are achieved with recombinant viral vectors and thus are promising in clinical applications.

5-3.1.1(c). Advantages

- It is highly versatile and effective for nearly all cell types and species.
- It is highly efficient method as majority of cells take in the target DNA molecule.
- It can be performed at a small scale and only a small amount of DNA is required as compared to other methods.

5-3.1.1(d). Disadvantages

- Cell damage is one of the limitations of this method caused by irregular intensity pulses resulting in too large pores which fail to close after membrane discharge.
- Another limitation is the non-specific transport which may result in an ion imbalance causing improper cell function and cell death.

5-3.1.2. Microinjection

- DNA microinjection was first proposed by Dr. Marshall A. Barber in the early of nineteenth century.
- This method is widely used for gene transfection in mammals.
- It involves delivery of foreign DNA into a living cell (e.g. a cell, egg, oocyte, embryos of animals) through a fine glass micropipette. The introduced DNA may lead to the over or under expression of certain genes.
- It is used to identify the characteristic function of dominant genes.

5-3.1.2(a). Procedure

- The delivery of foreign DNA is done under a powerful microscope using a glass micropipette tip of 0.5 mm diameter.
- Cells to be microinjected are placed in a container. A holding pipette is placed in the field of view of the microscope that sucks and holds a target cell at the tip. The tip of micropipette is injected through the membrane of the cell to deliver the contents of the needle into the cytoplasm and then the empty needle is taken out.

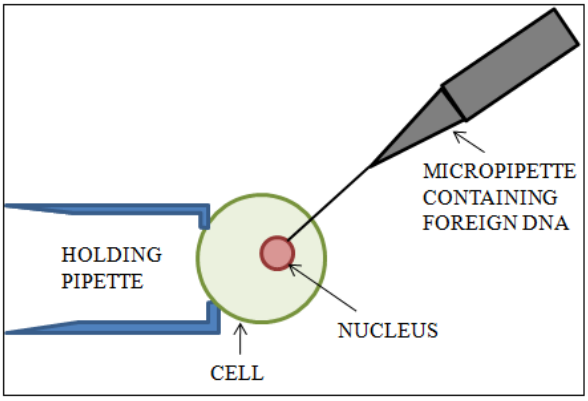

Figure 5-3.1.2(a). Delivery of DNA into a cell through microinjection.

(Adapted from http://www.eplantscience.com/index_files/biotechnology/Genes%20&%20Genetic%20Engineering/Techniques%20of%20Genetic%20Engineering/biotech_microinjection.php)

5-3.1.2(b). Advantages

- No requirement of a marker gene.
- Introduction of the target gene directly into a single cell.
- Easy identification of transformed cells upon injection of dye along with the DNA.
- No requirement of selection of the transformed cells using antibiotic resistance or herbicide resistance markers.
- It can be used for creating transgenic organisms, particularly mammals.

5-3.1.3. Particle bombardment

- Prof Sanford and colleagues at Cornell University (USA) developed the original bombardment concept in 1987 and coined the term “biolistics” (short for “biological ballistics”) for both the process and the device.
- Also termed as particle bombardment, particle gun, micro projectile bombardment and particle acceleration.
- It employs high-velocity micro projectiles to deliver substances into cells and tissues.

5-3.1.3(a). Uses

- This method is commonly employed for genetic transformation of plants and many organisms.
- This method is applicable for the plants having less regeneration capacity and those which fail to show sufficient response to *Agrobacterium*- mediated gene transfer in rice, corn, wheat, chickpea, sorghum and pigeon-pea.

5-3.1.3(b). Apparatus

The biolistic gun employs the principle of conservation of momentum and uses the passage of helium gas through the cylinder with arrange of velocities required for optimal transformation of various cell types. It consists of a bombardment chamber which is connected to an outlet for vacuum creation. The bombardment chamber consists of a plastic rupture disk below which macro carrier is loaded with micro carriers. These micro carriers consist of gold or tungsten micro pellets coated with DNA for transformation.

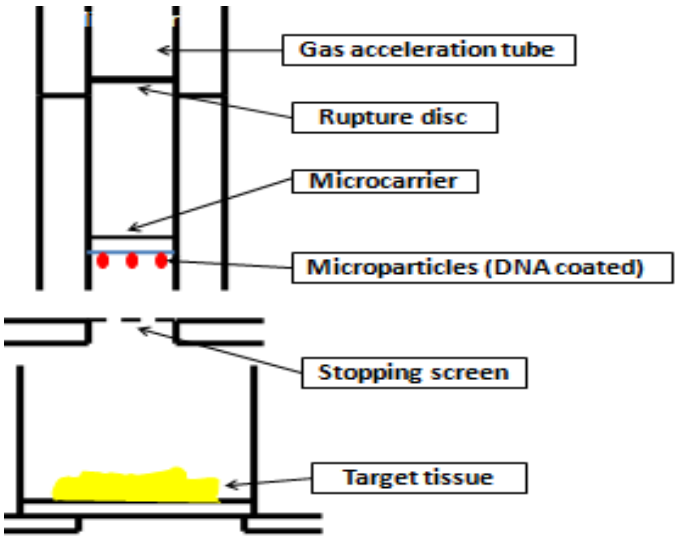

Figure 5-3.1.3(b). Working system of particle bombardment gun.

The apparatus is placed in Laminar flow while working to maintain sterile conditions. The target cells/tissue is placed in the apparatus and a stopping screen is placed between the target cells and micro carrier assembly. The passage of high pressure helium ruptures the plastic rupture disk propelling the macro carrier and micro carriers.

The stopping screen prevents the passage of macro projectiles but allows the DNA coated micro pellets to pass through it thereby, delivering DNA into the target cells.

5-3.1.3(c). Advantages

- Simple and convenient method involving coating DNA or RNA on to gold microcarrier, loading sample cartridges, pointing the nozzle and firing the device.
- No need to obtain protoplast as the intact cell wall can be penetrated.
- Manipulation of genome of sub-cellular organelles can be done.
- Eliminates the use of potentially harmful viruses or toxic chemical treatment as gene delivery vehicle.
- This device offers to place DNA or RNA exactly where it is needed into any organism.

5-3.1.3(d). Disadvantages

- The transformation efficiency may be lower than *Agrobacterium- mediated transformation*.
- Specialized equipment is needed. Moreover the device and consumables are costly.
- Associated cell damage can occur.
- The target tissue should have regeneration capacity.
- Random integration is also a concern.
- Chances of multiple copy insertions could cause gene silencing.

5-3.1.4. Sonoporation

- Sonoporation involves the use of ultrasound for temporary permeabilization of the cell membrane allowing the uptake of DNA, drugs or other therapeutic compounds from the extracellular environment.
- This method leaves the compound trapped inside the cell after ultrasound exposure.

- It employs the acoustic cavitation of micro bubbles for enhancing the delivery of large molecules like DNA. The micro bubbles form complex with DNA followed by injection and ultrasound treatment to deliver DNA into the target cells.
- Unlike other methods of transfection, sonoporation combines the capability to enhance gene and drug transfer.

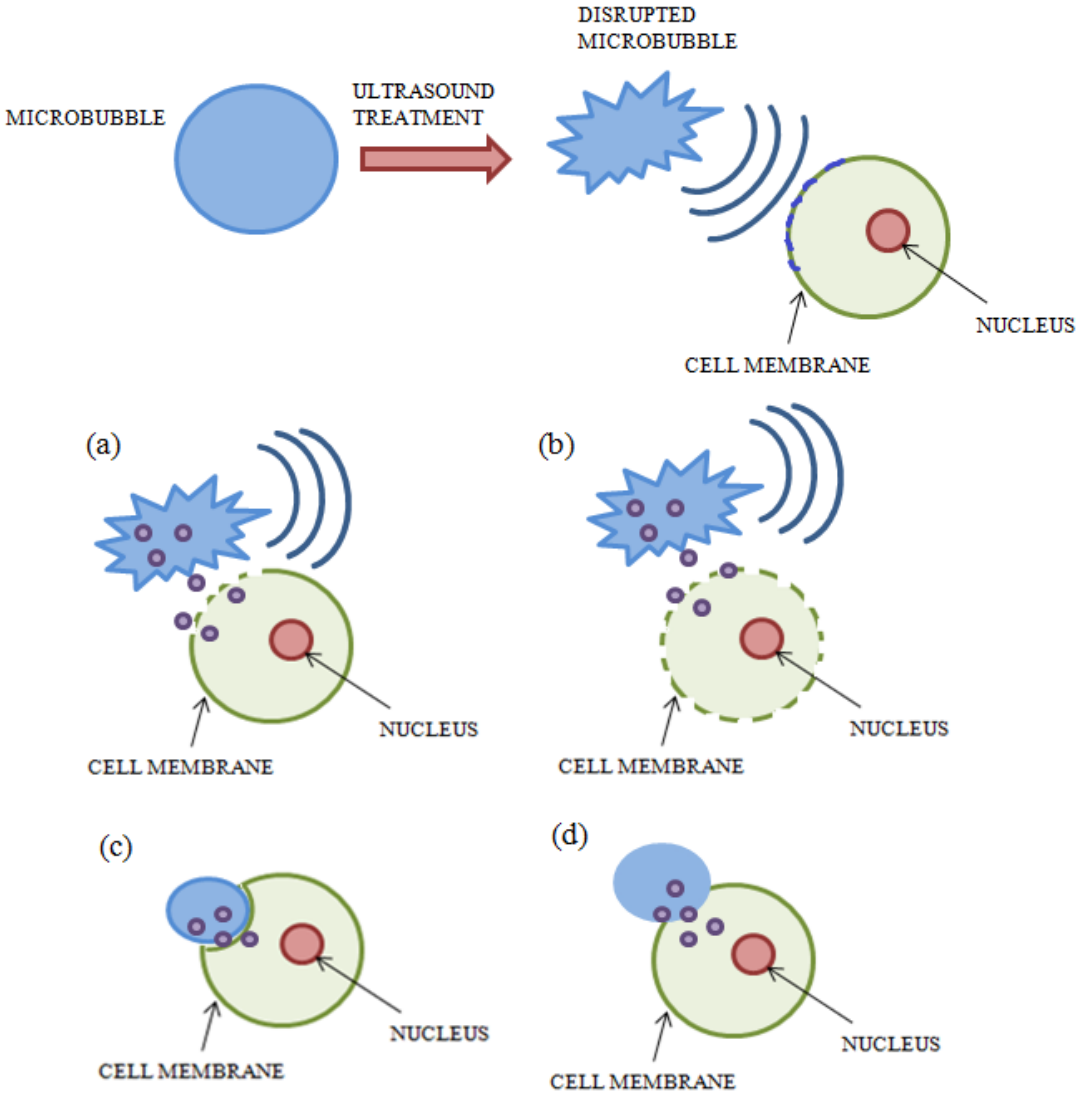

Figure 5-3.1.4. Rupture of microbubbles by ultrasound resulting in enhanced membrane permeability caused by shear stress, increased temperature and activation of reactive oxygen species. Drug delivery by microbubbles by (a) transient holes induced by shear stress for drug delivery (b) increase in membrane fluidity (c) endocytosis of microbubbles (d) microbubble- cell membrane fusion.

(Adapted from http://88proof.com/synthetic_biology/blog/archives/192)

5-3.1.4(a). Advantages

- Simple and highly efficient gene transfer method.
- No significant damage is cause to the target tissue.

5-3.1.4(b). Disadvantages

- Not suitable for tissues with open or cavitated structures.
- High exposure to low-frequency (<MHz) ultrasounds result in complete cellular death (rupture of the cell). Thus cellular viability must be taken into consideration while employing this technique.

5-3.1.5. Laser induced transfection

- It involves the use of a brief pulse of focused laser beam.
- In this method, DNA is mixed with the cells present in the culture and then a fine focus of laser beam is passed on the cell surface that forms a small pore sufficient for DNA uptake into the cells. The pore thus formed is transitory and repairs soon.

5-3.1.6. Bead transfection

- Bead transfection combines the principle of physically producing breaks in the cellular membrane using beads.
- In this method, the adherent cells are incubated for a brief period with glass beads in a solution containing the DNA.
- The efficiency of this rapid technique depends on:
    - Concentration of DNA in a solution.
    - Timing of the addition of DNA.
    - Size and condition of the beads and the buffers utilized.

Immunoporation is a recently developed transfection process involving the use of new type of beads, Immunofect^{TM} beads, which can be targeted to make holes in a specific type of cells.

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MODULE 5- LECTURE 4

AGRO- BACTERIUM MEDIATED GENE TRANSFER IN PLANTS

5-4.1. Introduction

Agrobacterium is considered as the nature's genetic engineer. *Agrobacterium tumefaciens* is a rod shaped, gram negative bacteria found in the soil that causes tumorous growth termed as crown gall disease in dicot plants (Figure 5-4.1.). The involvement of bacteria in this disease was established by Smith and Townsend (1907).

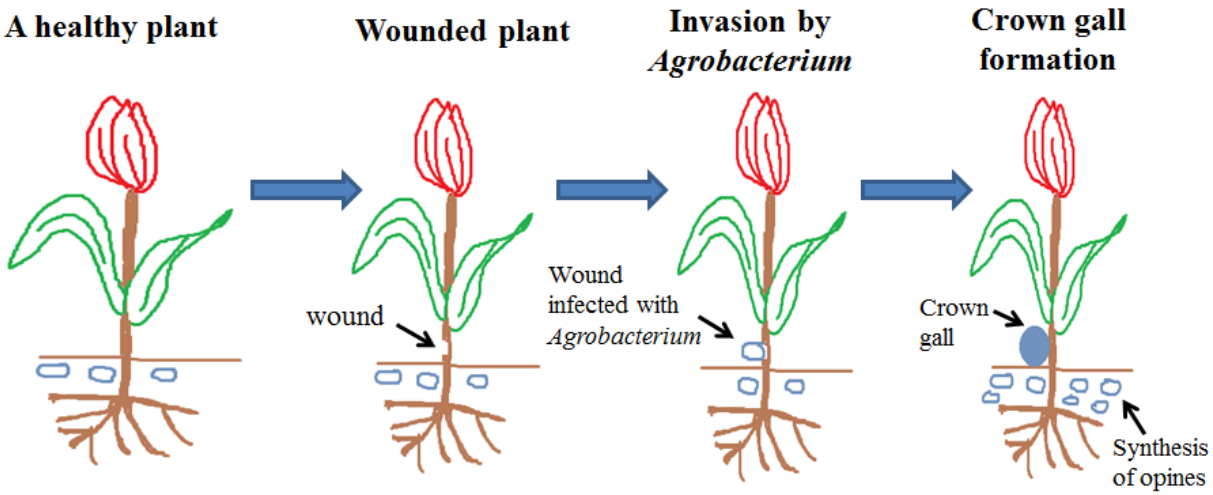

Figure 5-4.1. Tumor induced by *Agrobacterium* in plants

Agrobacterium contains a transfer DNA (T-DNA) located in its tumor-inducing (Ti) plasmid that is transferred into the nucleus of an infected plant cell. The T-DNA gets incorporated into the plant genome and is subsequently transcribed. The T-DNA integrated into the plant genome carries not only oncogenic genes but also opine synthesizing genes.

Agrobacterium "Species" And Host Range

The genus *Agrobacterium* has been divided into a number of species on the basis of symptoms of disease and host range. *A. radiobacter* is an "avirulent" species, *A. tumefaciens* causes crown gall disease, *A. rubi* causes cane gall disease, *A. rhizogenes* causes hairy root disease and *A. Vitis* causes galls on grape and a few other plant species.

Agrobacterium can transform a remarkably broad group of organisms including dicots, monocots and gymnosperms. In addition, it can also transform fungi, including ascomycetes, basidiomycetes and yeasts.

5-4.2. Molecular basis of *Agrobacterium*-mediated transformation

5-4.2.1. Ti-plasmid

The virulent strains of *A. tumefaciens* harbor large plasmids (140–235 kbp) known as tumor-inducing (Ti) plasmid involving elements like T-DNA, vir region, origin of replication, region enabling conjugative transfer and o-cat region (required for catabolism of opines).

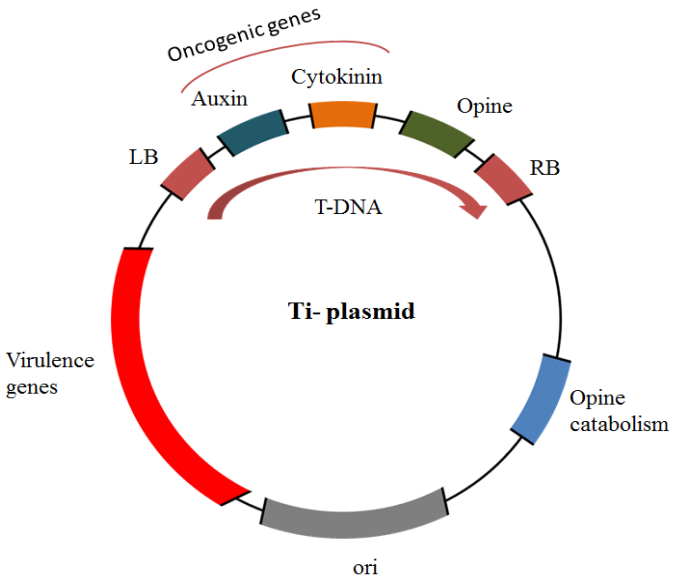

Figure 5-4.2.1. Ti-plasmid of *Agrobacterium*

T- DNA

It is a small, specific segment of the plasmid, about 24kb in size and found integrated in the plant nuclear DNA at random site. This DNA segment is flanked by right and left borders.

Genes on T-DNA

The T-DNA contains two groups of genes, which possess the ability to express in plants as follows-

- **Oncogenes** for synthesis of auxins and cytokinins (phytohormones). The over-production of phytohormones leads to proliferation of callus or tumour formation.
- **Opine synthesizing genes** for the synthesis of opines (a product from amino acids and sugars secreted by the crown gall infected cells and utilized by *A. tumefaciens* as carbon and nitrogen sources). Thus opines act as source of nutrient for bacterial growth, e.g. Octopine, Nopaline.

The functions of T-DNA genes are listed in the Table 5-4.2.1.

Gene	Product	Function
ocs	Octopine synthase	Opine synthesis
nos	Nopaline synthase	Opine synthesis
trns1 (iaaH, auxA)	Tryptophan-2-mono-oxygenase	Auxin synthesis
trns2 (iaaM, auxB)	Indoleacetamide hydrolase	Auxin synthesis
trnr (ipt, cyt)	Isopentyltransferase	Cytokinin synthesis
trnL	Unknown	Unknown, mutations affect tumor size
frs	Fructopine synthase	Opine synthesis
mas	Mannopine synthase	Opine synthesis
ags	Agropine synthase	Opine synthesis

Table 5-4.2.1. Function of various T-DNA genes

T- DNA:Border Sequences

- T-regions are defined by direct repeats known as T-DNA border sequences (Right and Left Border i.e. RB and LB of25 bp each).
- These are not transferred intact to the plant genome, but are involved in the transfer process.
- TheRB is rather precise, but the LB can vary by about 100 nucleotides.
- Deletion of the RB repeat abolishes T-DNA transfer, but the LB seems to be non-essential. The LB repeat has little transfer activity alone.

Virulence genes (vir genes)

Virulence genes aid in the transfer of T-DNA into the host plant cell. Ti plasmid contains 35 *vir* genes arranged in 8 operons.

5-4.3. DNA transfer into the plant genome

The schematic representation of *Agrobacterium*-mediated transformation method is shown in figure 5-4.3. T-DNA transfer and integration into the plant genome involves following steps-

Recognition and vir gene induction

- *Agrobacterium* perceives signaling molecules (phenolic compounds and sugars) released by the wounded plant cells. These signaling molecules act as chemotactic attractants for *Agrobacterium*. This is followed by recognition by the bacterial VirA/VirG2-component signal transduction system.
- Vir A, a kinase present in the inner bacterial membrane senses these phenolics (e.g. acetosyringone), gets autophosphorylated and then activates Vir G. *VirG* protein is a transcriptional activator of other vir genes and is inactive in non-phosphorylated form. The activation of *Vir G* thus induces the expression of the other vir genes.

Formation of T-DNA complex

Vir D1/D2 border-specific endonucleases recognize the left and right borders of T-DNA. Vir D2 induces single stranded nicks in Ti plasmid causing the release of the ss-T DNA. Vir D2 then attaches to the 5'-end of the displaced ss-T DNA forming an immature T-complex.

Transfer of T-DNA and integration into the plant cell

- The transfer of T-DNA to the plant cell is mediated by Type-IV secretion system composed of proteins encoded by *Vir B* and *Vir D4*that form a conjugative pilus (T-pilus). VirD4 serves as a "linker" that helps in the interaction of the processed T-DNA/VirD2 complex with the VirB- encoded pilus. Other vir genes (*Vir E2*, *Vir E3*, *VirF*, *Vir D5*) also pass through this T-pilus to aid in the assembly of T-DNA/vir protein complex in the plant cytoplasm forming a mature T-complex.
- Most VirB proteins help in the formation of the membrane channel or act as ATPases to provide energy for assembly and export processes of channel. VirB proteins, including VirB2, VirB5 and VirB7 help in the formation of the T-pilus. VirB2 is the major pilin protein that undergoes processing and cyclization.
- Ss-TDNA is coated with VirE2, a non-sequence specific single stranded DNA binding protein.
- Vir D2 and Vir E2 protect the ss-T strand from nucleases inside the plant cytoplasm by attaching to the 5'end. Both VirD2 and VirE2 proteins have nuclear localization signals (NLS) which serves as pilot proteins to guide the mature T-complex to the plant nucleus.
- The efficiency of transfer is enhanced by VirC2 proteins, which recognize and bind to the overdrive enhancer element.
- Some additional proteins like importins, VIP1 and VirF may interact with the T-strand, either directly or indirectly, to form larger T-complexes in the plant cell. Vir F directs the proteins coating T-complex (VIP1 and Vir E2) for destruction in proteasome.

- Inside the nucleus, ss-TDNA is converted into ds-TDNA which gets integrated into the plant genome via process called illegitimate recombination.

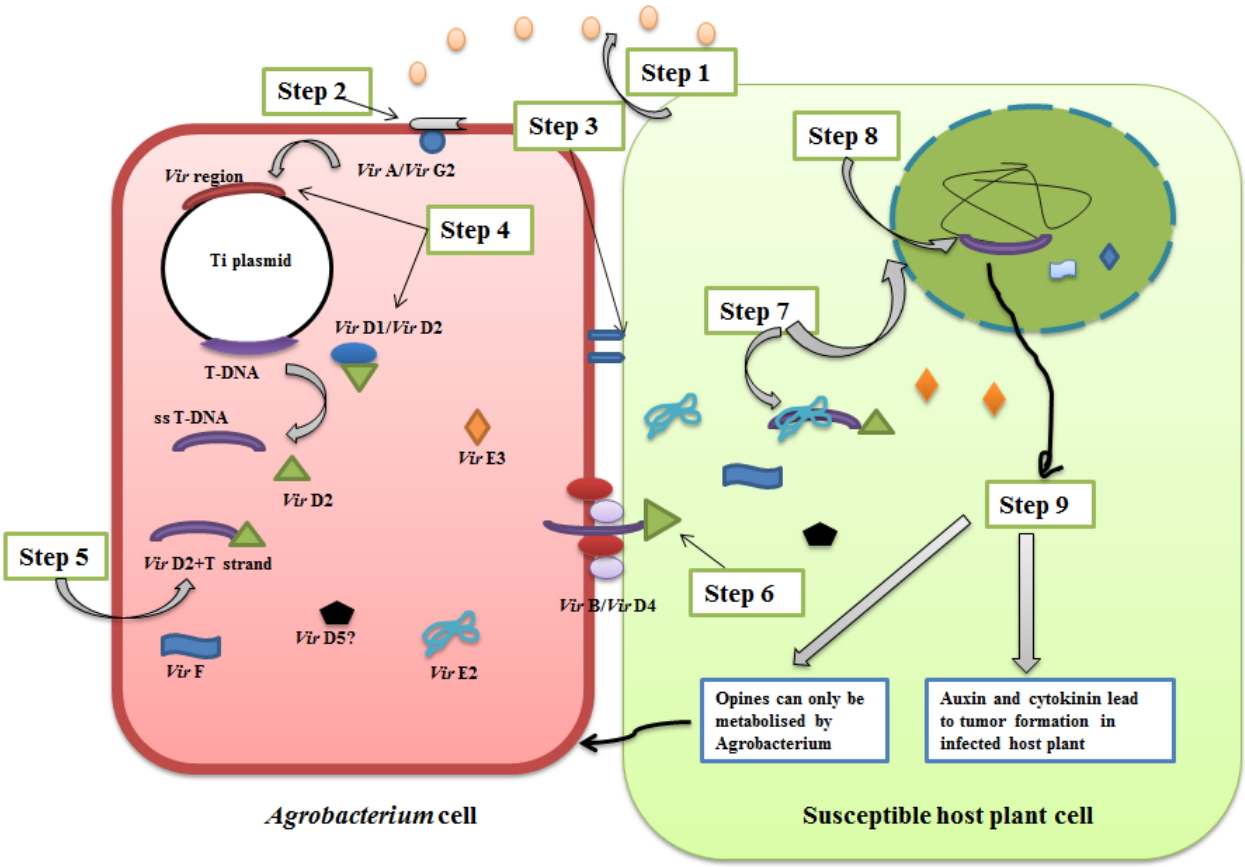

Figure 5-4.3. Schematic representation of *Agrobacterium*- mediated transformation. Step 1: Production of signal molecules form wounded plant cell; Step 2: Recognition of signal molecules by bacterial receptors; Step 3: Attachment of *Agrobacterium* to plant cell; Step 4: Activation of *Vir* proteins which process ss-TDNA; Step 5: Formation of immature T-complex; Step 6: T-DNA transfer; Step 7: Assembly of mature T-complex and Nuclear transport; Step 8: Random T-DNA integration in the plant genome; Step 9: Expression of bacterial genes and synthesis of bacterial proteins.

(Adapted and modified from Pacurar DI, Christensen HT, Pacurar ML, Pamfil D, Botez C, Bellini C. 2011. Agrobacterium mediated transformation: From crown gall tumors to genetic transformation. Physiological and Molecular Plant Pathology, 76: 76-81)

5-4.4. Disarmed Ti-plasmid derivatives as plant vectors

5-4.4.1. Prototype disarmed Ti vector

Ti plasmid is a natural vector for genetically engineering plant cells due to its ability to transfer T-DNA from the bacterium to the plant genome. But wild-type Ti plasmids are not suitable as vectors due to the presence of oncogenes in T-DNA that cause tumor growth in the recipient plant cells. For efficient plant regeneration, vectors with disarmed T-DNA are used by making it non-oncogenic by deleting all of its oncogenes. The foreign DNA is inserted between the RB and LB and then integrated into the plant genome without causing tumors.

For example, Zambryski *et al.* (1983) substituted *pBR322* sequences for almost all of the T-DNA of *pTiC58*, leaving only the left and right border regions and the *nos* gene. The resulting construct was called *pGV3850.*

No tumour cell formation takes place when modified T-DNA is transferred from *Agrobacterium* carrying *pGV3850* plasmid. The evidence of transfer is done by screening the cells for nopaline production.

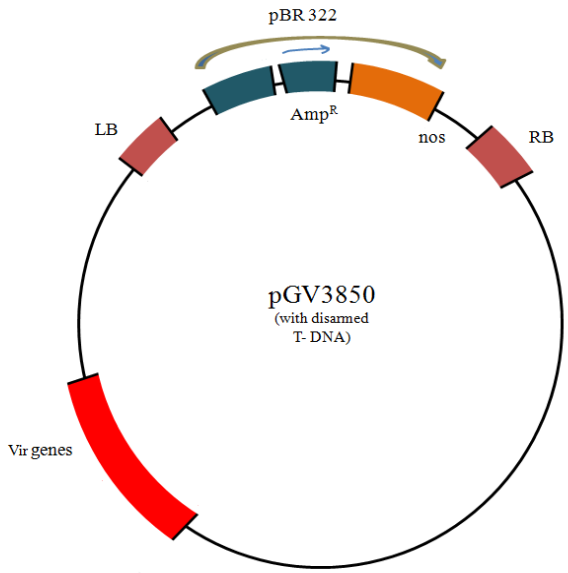

Figure 5-4.4.1. Structure of the Ti-plasmid pGV3850 with disarmed T-DNA.

The creation of disarmed T-DNA is an important step forward, but the absence of tumor formation makes it necessary to use an alternative method for the identification of transformed plant cells. Opine production using *pGV3850* was exploited as a screenable phenotype, and the *ocs* and *nos* genes are now widely used as screenable markers.

Drawbacks

Several drawbacks are associated with disarmed Ti- vector systems as discussed below;

- Necessity to carry out enzymatic assays on all potential transformants.
- Not convenient as experimental gene vectors due to large size.
- Difficulty in *in vitro* manipulation and
- Absence of unique restriction sites in the T-DNA.

Several approaches are employed to overcome the limitations associated with disarmed Ti vectors. The transformed plant cells can be identified on the basis of drug or herbicide resistance by inserting selectable markers (Table 5-5.7.1. of Lecture 5 of Module 5) into the T-DNA.

5-4.4.2. Co- integrate vectors

Co-integrate vectors are the deletion derivatives of Ti-plasmids. The DNA to be introduced into the plant transformation vector is sub cloned in a conventional *Escherichia coli* plasmid vector for easy manipulation, producing a so-called *intermediate vector*. These vectors are incapable of replication in *A. tumefaciens* and also lack conjugation functions. Transfer is achieved using a 'triparental mating' in which three bacterial strains are mixed together:

(i) An *E. coli* strain carrying a helper plasmid able to mobilize the intermediate vector in *trans*;

(ii) *The E. coli* strain carrying the recombinant intermediate vector;

(iii) *A. tumefaciens* carrying the Ti plasmid.

Conjugation between the two *E. coli* strains transfers the helper plasmid to the carrier of the intermediate vector, which in turn is mobilized and transferred to the recipient *Agrobacterium.* Homologous recombination between the T-DNA sequences of the Ti plasmid and intermediate vector forms a large co- integrate plasmid resulting in the transfer of recombinant T-DNA to the plant genome.

In the co- integrate vector system, maintenance of the recombinant T-DNA is dependent on recombination which can be enhanced byextensive homology between the two plasmids e.g. Ti plasmid *pGV3850* carrying a segment of the *pBR322* backbone in its T-DNA.

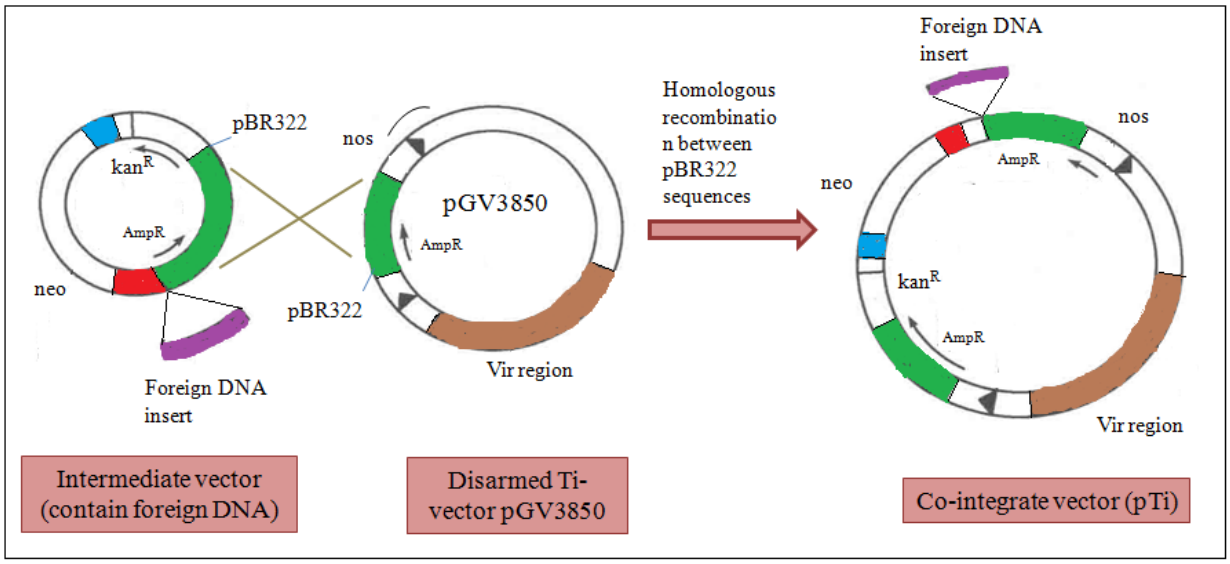

Figure 5-4.4.2. Construction of a Co-integrate vector (foreign gene cloned into an appropriate plasmid is integrated with a disarmed Ti-plasmid through homologous recombination).

5-4.4.3. Binary vector

- Binary vector was developed by Hoekma *et al* (1983) and Bevan in (1984).
- It utilizes the trans- acting functions of the vir genes of the Ti-plasmid and can act on any T-DNA sequence present in the same cell.
- Binary vector contains transfer apparatus (the vir genes) and the disarmed T-DNA containing the transgene on separate plasmids.

5-4.4.3.1.Advantages of Binary vector

- Small size due to the absence of border sequences needed to define T-DNA region and vir region.
- Ease of manipulation

5-4.4.3.2. Binary vector system

A plasmid carrying T-DNA containing LB and RB, called mini-Ti or micro-Ti can be sub- cloned in a small *E. coli* plasmid for ease of manipulation (Figure 5-4.4.3.2(b).).

The T-DNA of mini-Ti can be introduced into an *Agrobacterium* strain carrying a Ti plasmid from which the T-DNA has been removed but contains vir region (Figure 5-4.4.3.2(a).). The vir genes function *in trans*, causing transfer of the recombinant T-DNA to the plant genome.

The T-DNA plasmid can be introduced into *Agrobacterium* by triparental mating or by a more simple transformation procedure, such as electroporation.

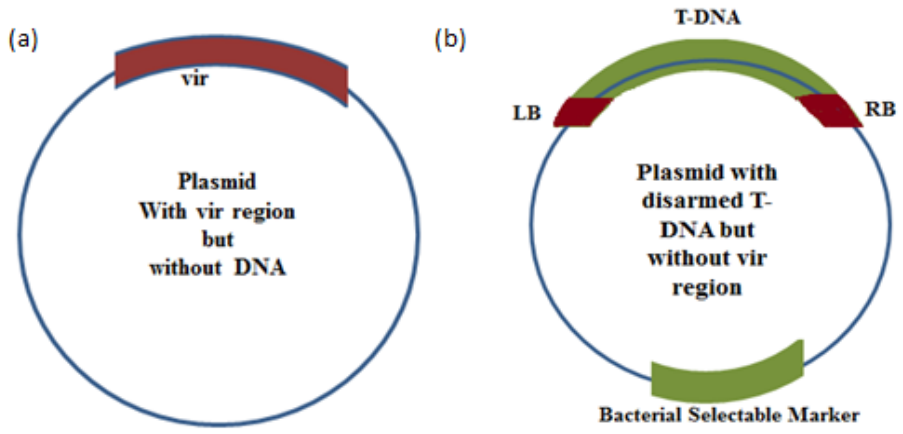

Figure 5-4.4.3.2. A binary vector system (a) A plasmid containing vir region but no T-DNA, therefore no T-DNA transfer takes place in plant genome. (b) Another plasmid containing T-DNA with Right border (RB) and Left border (LB) but no vir genes. Vir function is supplied *in trans* by former plasmid.

In Ti-plasmid transformation system, the T-DNA is maintained on a shuttle vector with a broad host range origin of replication, such as *RK2* (which functions in both *A. tumefaciens* and *E. coli*), or separate origin for each species.

An independently replicating vector is advantageous because maintenance of the T-DNA does not rely on recombination. The copy number of binary vector is not determined by the Ti plasmid, making the identification of transformants much easier.

All the conveniences of bacterial cloning plasmids are incorporated into binary vectors, such as multiple unique restriction sites in the T-DNA region to facilitate subcloning, the *lacZ* gene for blue–white screening and a *λ cos* site for preparing cosmid libraries.

Examples of Binary vector system

pBIN19- one of the first binary vectors developed in 1980s and was widely used.

pGreen- A newly developed vector with advanced features than pBIN19.

Both the vectors contain *Lac Z* gene for blue-white screening of recombinants. The reduction of size of *pGreen* is due to the presence of *pSa* origin of replication. An essential replicase gene is housed on a second plasmid, called *pSoup* which functions *in trans*. All conjugation functions have also been removed, so this plasmid can only be introduced into *Agrobacterium.*

Features	*pBIN19*	*pGreen*
Size	Large (11777bp)	Small (<5bp)
Position of Selectable marker	Situated near RB. Due to origination of transfer from RB, transfer of selectable marker before gene of interest will result in plants expressing selectable marker but not with transgene or truncated version.	Situated next to LB
Restriction sites	Limited	Much larger MCS with many restriction sites.

Table 5-4.4.3. Comparison between Binary vectors *pBIN19* and *pGreen*

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MODULE 5- LECTURE 5

CHLOROPLAST TRANSFORMATION

5-5.1. Introduction

Chloroplast transformation is an important tool for biotechnological applications and has gained much interest in the regulation of gene expression and plant physiology.

5-5.2. Chloroplast

Chloroplasts are sub cellular organelles (plastids) of plant cells generally considered to be derived from the symbiotic cyanobacteria. They are present in shoots and leaves of green plants and contain pigment called chlorophyll. They are also present in several forms as colorless plastids (amyloplasts) in roots and as colored plastids (chromoplasts) in fruits. A cell comprises variable number of plastids, each plastid containing many copies of genome (50 to 100).

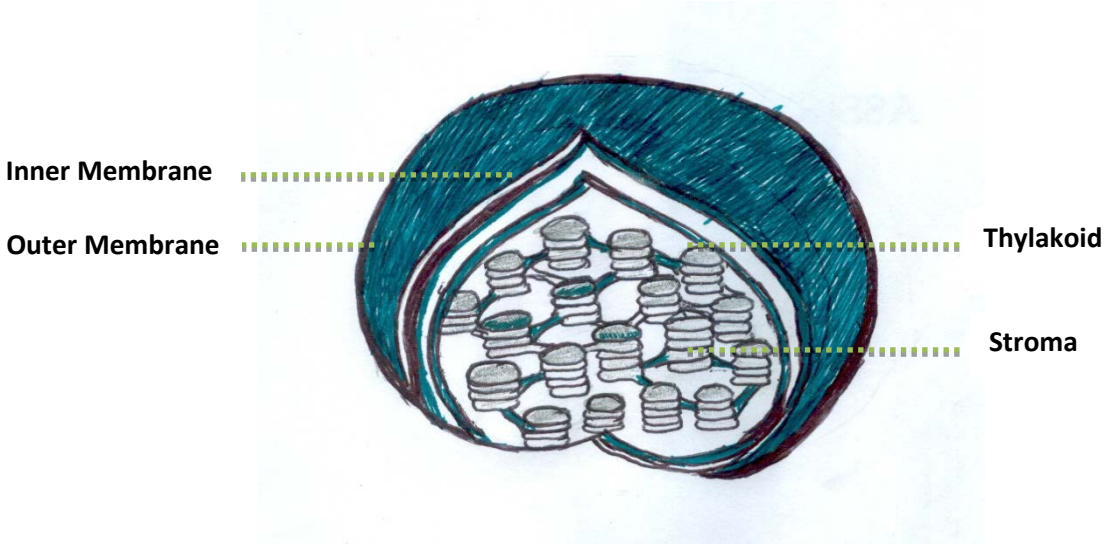

Figure 5-5.2.1. Schematic diagram of Chloroplasts

(Adapted from http://www.nature.com/scitable/topicpage/plant-cells-chloroplasts-and-cell-walls-14053956)

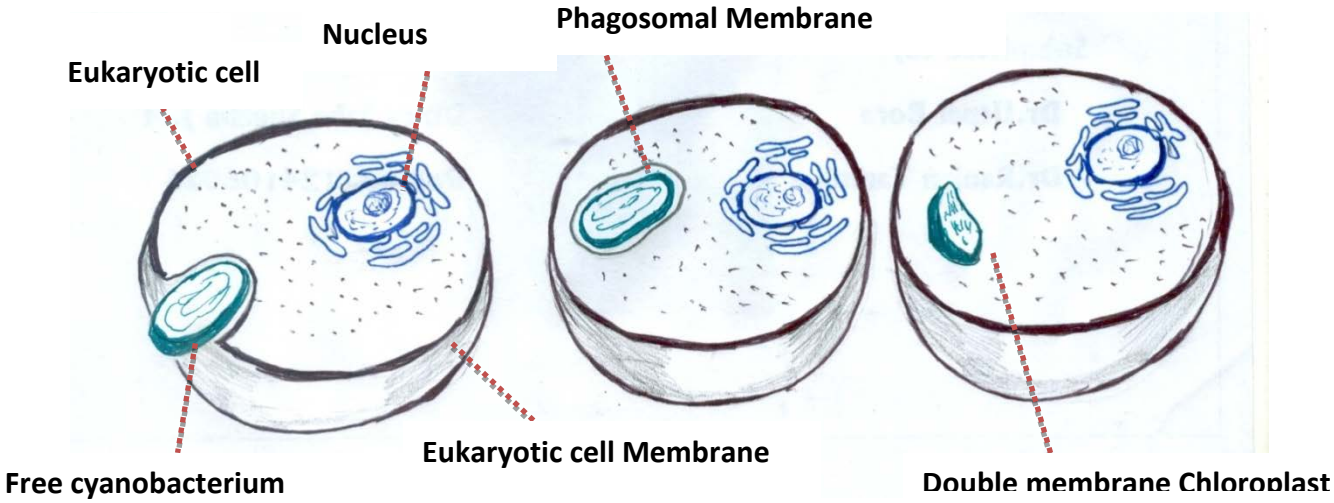

Figure 5-5.2.2. Chloroplasts derived from endosymbiotic cyanobacteria.

(Adapted from http://en.wikipedia.org/wiki/Chloroplast)

Plastid genomes resemble bacterial genomes in many aspects and also contain some features of multicellular organisms, such as RNA editing and split genes.

Most of the proteins are encoded in the plant nucleus, synthesized and then imported into the chloroplast. However, chloroplast genome encodes for transcription and translation machinery and numerous proteins.

5-5.3. Chloroplast transformation: historical perspective

First stable chloroplast transformation was achieved in the alga *Chlamydomonas reinhardtii*. In addition, the *aadA* marker and methods for removal of marker were first demonstrated. In higher plants, Tobacco due to its ease of culture and regeneration, gained significant attention for chloroplast transformation.

Tobacco protoplasts were co-cultivated with *Agrobacterium* but the resulted transgenic lines showed the unstable integration of foreign DNA into the chloroplast genome.

The candidate genes were introduced in isolated intact chloroplasts and then into protoplasts resulting in transgenic plants. Gene gun, a transformation device, was developed by John Sanford to enable the transformation of plant chloroplasts without using isolated plastids.

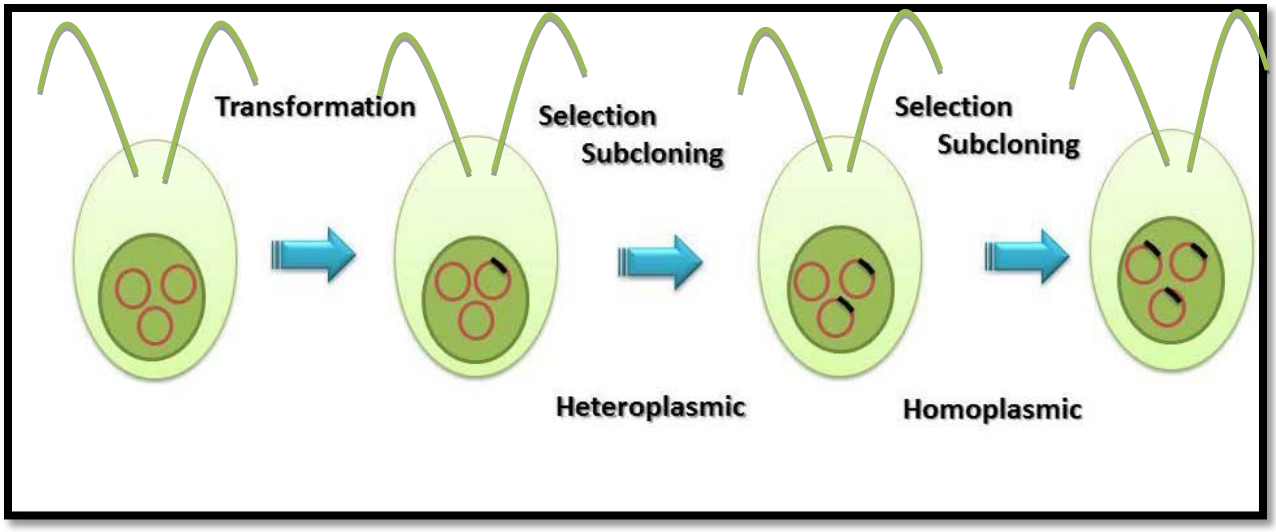

Figure 5-5.3.1. Chloroplast transformation in *Chlamydomonas*: *Chlamydomonas* comprises a single large chloroplast with about hundreds of copies of its genome. Initial integration occurs in only one copy of the polyploid plastome resulting in heteroplasmic. Repeated sub-cloning and selection result in recovery of homoplasmic clones.
(Adapted from Day A and Clermont MG. 2011. The chloroplast transformation toolbox: selectable markers and marker removal. Plant Biotechnology Journal 9, 540–553)

5-5.4. Advantages of chloroplast transformation

Chloroplast transformation offer several advantages compared with nuclear transformation which are as follows-

5-5.4.1. Risk of transgene escape

Chloroplast genome is maternally inherited and there is rare occurrence of pollen transmission. It provides a strong level of biological containment and thus reduces the escape of transgene from one cell to other.

5-5.4.2. Expression level

- It exhibits higher level of transgene expression and thus higher level of protein production due to the presence of multiple copies of chloroplast transgenes per cell and
- Remains unaffected by phenomenon such as pre or post-transcriptional silencing.

5-5.4.3. Homologous recombination

- Chloroplast transformation involves homologous recombination and is therefore precise and predictable.
- This minimizes the insertion of unnecessary DNA that accompanies in nuclear genome transformation.
- This also avoids the deletions and rearrangements of transgene DNA, and host genome DNA at the site of insertion.

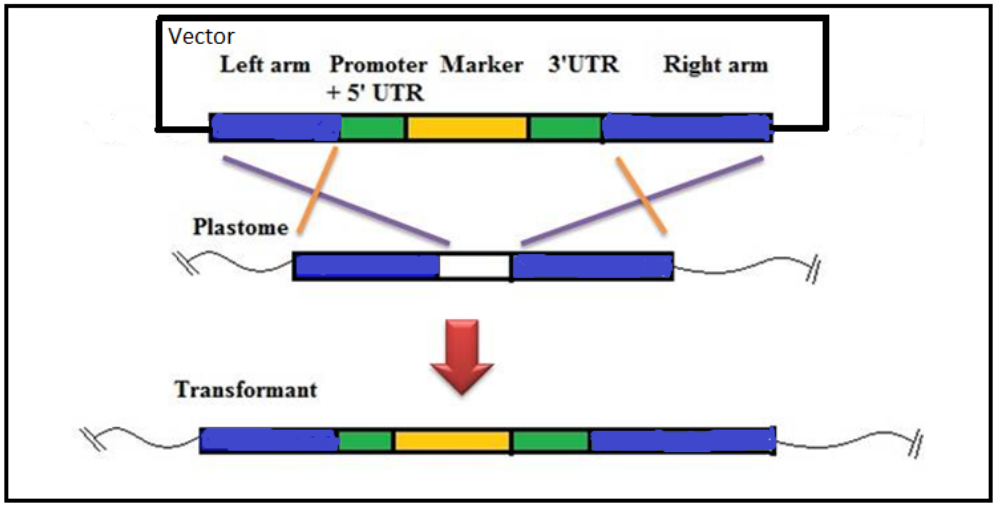

Figure 5-5.4.3. A vector containing a selectable marker (yellow) under the control of plastid expression signals (promoter, 5´ UTR and 3´ UTR , shown in green) flanked by chloroplast sequences (shown in purple). Homologous recombination takes place between the flanking targeting arms and recipient plastid genome (plastome).

(Adapted from Day A and Clermont MG. 2011. The chloroplast transformation toolbox: selectable markers and marker removal. Plant Biotechnology Journal 9, 540–553)

5-5.4.4. Gene silencing/ RNA interference

Gene silencing or RNA interference does not occur in genetically engineered chloroplasts.

5-5.4.5. Position effect

- Absence of position effect due to lack of a compact chromatin structure and efficient transgene integration by homologous recombination.
- Avoids inadvertent inactivation of host gene by transgene integration.

5-5.4.6. Disulphide bond formation

Ability to form disulfide bonds and folding human proteins results in high-level production of biopharmaceuticals in plants.

5-5.4.7. Multiple gene expression

Multiple transgene expression is possible due to polycistronic mRNA transcription.

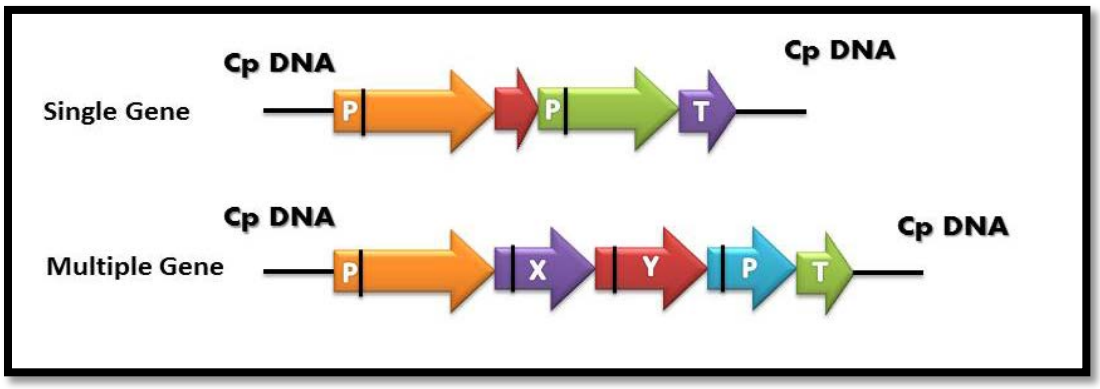

Figure 5-5.4.7. Multigene expression

(Adapted from Daniell H, Khan MS, Allison L. 2002. Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends in Plant Science 7 (2): 84-91.)

5-5.4.8. Expression of edible vaccine

High level of expression and engineering foreign genes without the use of antibiotic resistant genes makes this compartment ideal for the development of edible vaccines.

5-5.4.9. Codon usage

Chloroplast is originated from cyanobacteria through endosymbiosis. It shows significant similarities with the bacterial genome. Thus, any bacterial genome can be inserted in chloroplast genome.

5-5.4.10. Expression of toxic proteins

Foreign proteins observed to be toxic in the cytosol are non-toxic when accumulated within transgenic chloroplasts as they are compartmentalized inside chloroplast.

5-5.5. Comparison of chloroplast and nuclear genetic engineering

Transgenic	*Chloroplast Genome*	*Nuclear Genome*
Transgene copy number	***10-100 plastid genome***	***Species - Specific***
Level of gene expression	***High***	***Limited***
Gene arrangement	***Occurs often***	***Transgene is independently inserted***
Position effect	***No***	***Yes***
Gene silencing	***No***	***Yes***
Gene Containment	***Yes***	***No***
Disulphide bond formation	***Yes***	***No***
Toxicity of foreign proteins	***Low***	***High***
Transgenic lines	***Uniform gene expression***	***Variable gene expression***
Homogenecity	***Homoplasmic***	***Heterozygous or homozygous***

Table 5-5.5. Differences between chloroplast and nuclear genetic engineering

5-5.6. Transformation methods for chloroplast transformation

- Biolistic/Particle bombardment method involves the introduction of *Escherichia coli* plasmids containing a gene of interest and marker gene into chloroplasts or plastids. The insertion of foreign genes into plasmid DNA occurs by homologous recombination via the sequences flanking at the insertion site. First successful chloroplast transformation was performed in *Chlamydomonas reinhardtii* by particle bombardment method. Simple operation and high transformation efficiency makes it a favorable way for plastid or chloroplast transformation.

- PEG-mediated and *Agrobacterium- mediated* transformation method was also employed in the early days.

After the first chloroplast transformation in *Chlamydomonas reinhardttii*, the stable plastid transformation has also been established in higher plants, *Nicotiana tobacum, Arabidopsis*, rape, *Lesquerella*, rice, potato, lettuce, soybean, cotton, carrot and tomato.

However, plastid transformation is routinely performed only in tobacco because of higher efficiency of transformation in tobacco than in other plants.

5-5.7. Vector design for chloroplast transformation

5-5.7.1. Selectable marker genes

Due to the presence of multi-copy plastid DNA, selectable marker genes are very important to achieve uniform transformation of all genome copies. The non-transformed plastids are gradually left out during an enrichment process using a selective medium. Initially plastid *16S rRNA* (*rrn16*) gene was used as a selection marker in chloroplast transformation. The transgenic lines were selected by spectinomycin resistance but with low efficiency.

Various selectable markers for chloroplast transformation have been developed based on various features like dominance, cell-autonomy or portability (listed in Table 5-5.7.1.).

- Dominant markers confer high transformation efficiency due to the expression at early stages although present in a minority of the plastomes. e. g. *aadA* (aminoglycoside 3′ adenylyltransferase) gene confers resistance to streptomycin and spectinomycin by inactivation of antibiotics.

- Recessive markers confer lower transformation efficiency. They confer resistance only when enough transformed plastome copies are produced by random segregation resulting in a selectable phenotype. e. g. point mutation in the

ribosomal RNA, *rrnS* and *rrnL* genes, confer antibiotic resistance by relieving the sensitivity of individual ribosome.

- Plastid- or cell-autonomous markers confer their phenotype only to the organelle or the cell in which they reside. Some markers may integrate at a specific locus of the plastid genome e.g. *rrnS* or *rrnL* genes.
- Autonomous and portable markers can be inserted virtually in any locus of the plastome e.g. *aadA* gene.
- Some markers have a property to confer a phenotype strong enough for direct selection of transformants.

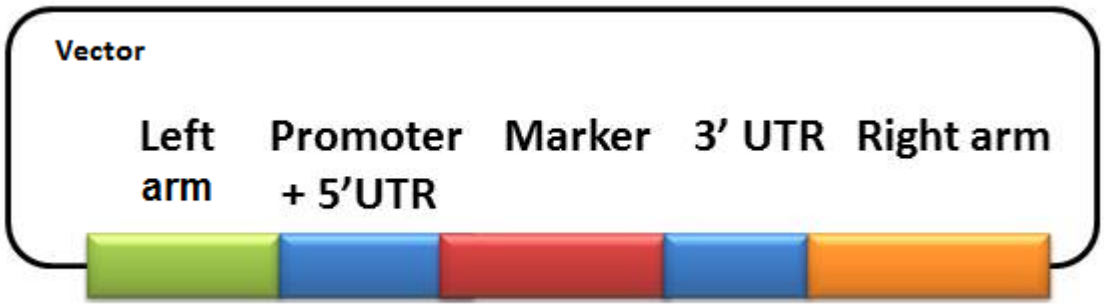

Figure 5-5.7.1. Selectable marker (in case when it is a foreign gene to be inserted into plastid genome shown in red) is under the control of expression signals such as promoter, a 3´ UTR and a 5´ UTR (shown in blue).
(Adapted from Day A and Clermont MG. 2011. The chloroplast transformation toolbox: selectable markers and marker removal. Plant Biotechnology Journal 9, 540–553)

Marker	Selection	Organism
Photosynthesis		
atpB	Photo autotrophy	*Chlamydomonas*
tscA	Photo autotrophy	*Chlamydomonas*
psaA/B	Photo autotrophy	*Chlamydomonas*
petB	Photo autotrophy	*Chlamydomonas*
rbcL	Photo autotrophy	Tobacco

Drug resistance		
rrnS	Spectinomycin, Streptomycin	*Chlamydomonas*, Tobacco, Tomato
rrnL	Erythromycin	*Chlamydomonas*
nptII	Kanamycin	Tobacco (low transformation efficiency), Cotton
aphA-6	Kanamycin, Amikacin	*Chlamydomonas*, Tobacco, Cotton
aad (preferred for chloroplast transformation)	Trimethoprim, Spectinomycin, Streptomycin, Sulphonamides	*Solanum tuberosum* (Potato)
ble	Bleomycin	Tobacco
dhfr	Methotraxate	Rice
hpt	Hygromycin	Rice
gat	Gentamycin	Tobacco
Herbicide resistance		
psbA	DCMU, metribuzin	*Chlamydomonas*
bar (not good enough for transformation) and ***pat***	Phosphinothricin	Tobacco
AHAS	Sulfometuron methyl	*Porphyridium* sp.
EPSP	Glyphosate	Tobacco
HPPD	Diketonitrile	Tobacco
Csr 1-1	Chlorsulphuron	Rice
dhps	Sulphonamides	Tobacco
Metabolism		
BADH	Betaine aldehyde	Tobacco
coda	5-fluorocytosine	Tobacco
ARG9	Arg autotrophy	*Chlamydomonas*

ASA2	Trp analogues	Tobacco

Table 5-5.7.1. Various selectable markers used for chloroplast transformation

5-5.7.2. Insertion sites

- Plastid expression vectors possess left and right flanking sequences each with 1–2 kb in size from the host plastid genome, which facilitates foreign gene insertion into plastid DNA via homologous recombination.
- The insertion site in the plastid genome is determined by the choice of plastid DNA segment flanking the marker gene and the gene of interest. The foreign DNA is inserted in intergenic regions of the plastid genome.
- Most commonly used insertion sites are *trnV-3'rps12, trnI-trnA and trnfM-trnG.*

The *trnV-3'rps12* and *trnI-trnA* sites are located in the *25 kb* inverted repeat *(IR)* region of plastid DNA and insertion of gene into these sites results in the rapid copying into two copies in the IR region.

The *trnfM-trnG* site is located in the large single copy region of the plastid DNA. The insertion of gene between *trnfM* and *trnG* should contain only one copy per plastid DNA. *pSBL- CTV2* was the first vector developed in the Daniell laboratory for expression of several proteins. This vector inserts the foreign gene in *trnI-trnA* intergenic region.

5-5.7.3. Regulatory sequences

- The level of gene expression in plastids is predominately determined by regulatory sequences such as promoter as well as 5′ UTR elements.
- Strong promoter is required to ensure high mRNA level for high-level of protein accumulation e.g. rRNA operon (*rrn)* promoter *(Prrn).* Most commonly used promoter is *CaMV* 35S promoter of cauliflower mosaic virus which drives high level of transgene expression in dicots.

- In plastid expression vectors, a suitable 5′ untranslated region (5′-UTRs) containing a ribosomal binding site (RBS) is an important element.

- Stability of the transgenic mRNA is ensured by the 5′ UTR and 3′ UTR sequences flanking the transgene. Protein accumulation from the transgene depends on the 5′-UTR inserted upstream of the open reading frame encoding the genes of interest.

5-5.8. Applications of chloroplast engineering

Chloroplast transformation can be used in the production of transgenic plants with herbicide resistance, insect resistance, viral resistance, fungal resistance, abiotic and biotic stress tolerance, production of biopharmaceuticals etc. (described in detail in Lecture 1 of Module 6)

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