Year	Sem.	Subject Code	Title of the paper	Hours/ Week
2018 -2019 onwards	IV	18MBO43E	ELECTIVE PAPER – VI BIOTECHNOLOGY	7

Unit–IV

Agrobacterium and crown gall tumors; Mechanism of T-DNA transfer; Disarmed Ti Plasmid vectors (Co-integrate and Binary vectors); plant viral vectors; Direct gene transformation methods (Particle gun bombardment, Electroporation, $CaCl_2$, PEG and Liposome mediated transformation); Selectable markers and promoters used in plant genetic engineering; transgenic plants (Herbicide resistance and pest resistance).

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Notes

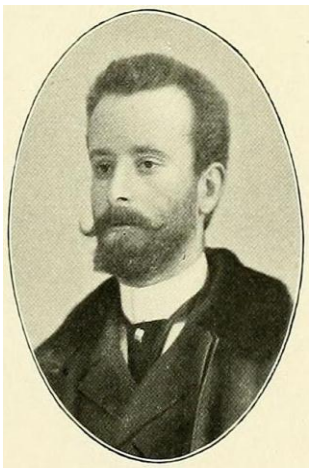
Agrobacterium vectors and crown gall tumors

Agrobacterium

Agrobacterium system was historically the first successful plant transformation system, marking the breakthrough in plant genetic engineering in 1983. The breakthrough in gene manipulation in plants came by characterizing and exploiting plasmids carried by the bacterial plant pathogens *Agrobacterium tumefaciens* and *A. rhizogenes*. These provide natural gene transfer, gene expression and selection systems. In recent times, *A. tumefaciens* has been treated as nature's most effective plant genetic engineer.

Crown gall tumors

Smith and Townsend showed in 1907 that a bacterium is the causative agent of crown gall tumors. Experiments of Braun and his coworkers showed that the continued presence of viable bacteria is not required for tumor maintenance (Braun and Stonier, 1958). The bacteria do not penetrate into the plant cells that are converted into tumor cells. Rather, bacteria penetrate into the intercellular spaces and into injured cells and attach themselves only to the wall of healthy plant cells. Attention thus focussed on the identification of a putative ´tumor inducing principle'. Zaenen et al. (1974) first noted that virulent strains of A. tumefaciens harbor large plasmids and the virulence trait is plasmid borne. Virulence is lost when the bacteria are cured of the plasmid.



Fridiano Cavara (1897) found that a bacterium causes crown gall in grape

A. tumefaciens induces tumors called crown galls, while A. rhizogenes causes hairy root disease. Large plasmids in these agrobacteria are called tumor-inducing plasmid

(Ti plasmid) and root inducing plasmid (Ri plasmid) respectively which confer on their hosts the pathogenic capacity. Both sorts of diseases result from transfer and functional integration of a particular set of the Ti or Ri plasmid into plant chromosomes. It was later revealed that only a small part of the Ti plasmid, the T-DNA or transferred DNA, is transferred to and integrated into the host plant nuclear genome.

Crown gall disease caused by A. tumefaciens is characterized by unlimited plant cell proliferation (gall formation). Naturally transformed crown gall tumor tissue differs from nontransformed tissue in its ability to grow autonomously on synthetic media in the absence of hormones, i.e. auxins and cytokinins. The plant cells in the tumor acquire two new properties:

1. These show phytohormone independent growth.
2. These contain one or more of unusual amino acid derivatives known as opines. Opine synthesis is a unique characteristic of tumor cells; normal plant tissues do not usually produce these compounds. The ability to produce these opines was specified by the transferred bacterial DNA and not by the host plant genome. They do not appear to have any known useful function in the plant cells and are not directly responsible for the tumorous state of the cells. However, opines are useful to the bacterium since they serve as a source of carbon and nitrogen. In addition, some of the opines also induce conjugation between bacteria. Opines distinguish agrobacteria from other bacteria in the rhizosphere. Agrobacteria are able to utilize opines, thus providing a competitive advantage in the crown gall rhizosphere to the inciting agrobacteria over other soil organisms. This phenomenon has been termed genetic colonization.

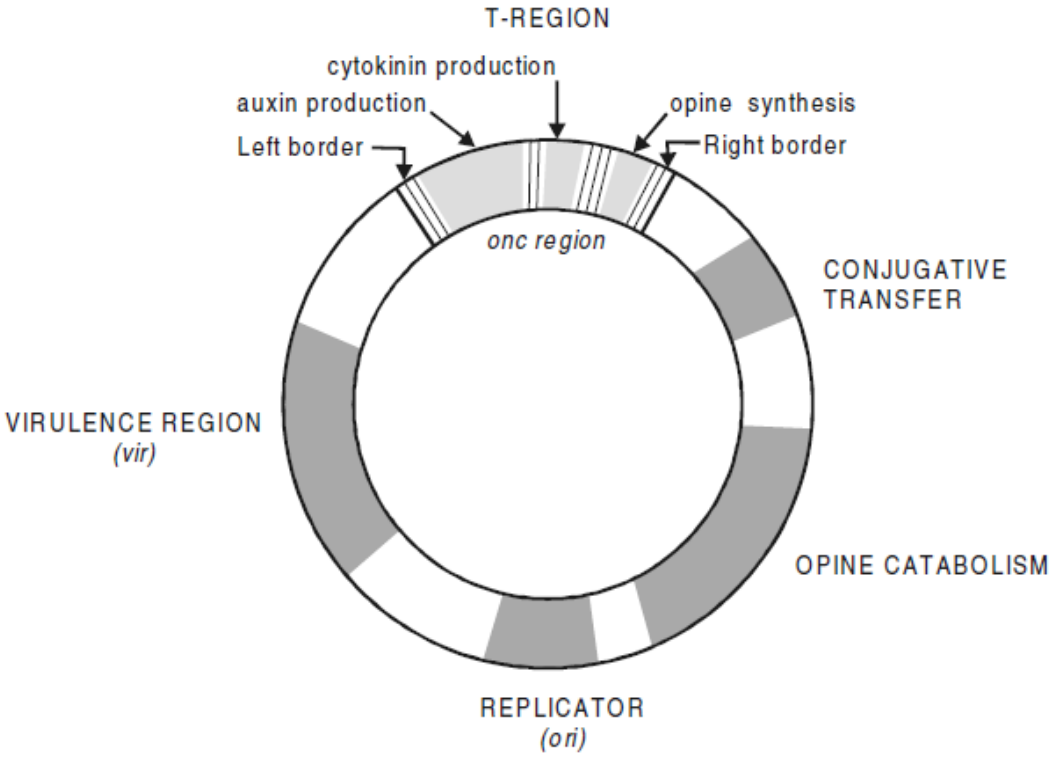


Fig. 1: Genetic map of an octopine Ti plasmid.

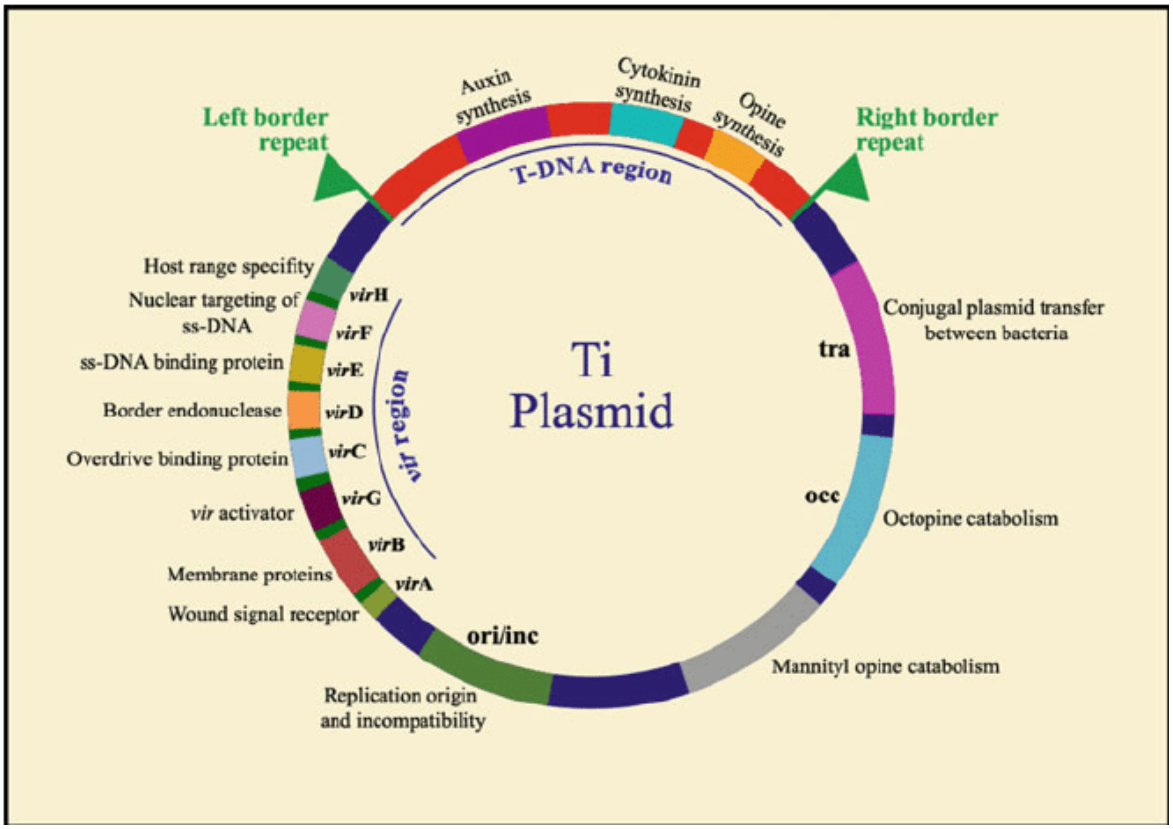
It was earlier observed that there were two families of bacteria and the crown galls they established differed by producing two different amino acid derivatives, subsequently called octopine and nopaline. The octopine family has carboxyl-ethyl derivatives of arginine while nopaline family has related compounds that are dicarboxypropyl derivatives of arginine. For the synthesis of octopine and nopaline, the corresponding enzyme octopine synthase (ocs). and nopaline synthase (nos) are coded by T-DNA.

Now, Ti plasmids are defined as octopine, nopaline, succinamopine, agropine or leucinopine depending on the type of opine produced in the tumors.

Organization of Ti plasmid: The Ti plasmids of Agrobacterium are large circular DNA molecules, up to 200kb in length with molecular weights of about 1.2 x 108 (3 to 8%) of the Agrobacterium chromosome. They exist in the bacterial cells as independent replicating genetic units. The Ti plasmids have major regions for virulence, origin of replication, conjugation, oncogenicity and catabolism of opines. Genetic organization of these regions **has been shown in octopine Ti plasmid DNA (Fig. 1).**

Mechanism of T-DNA transfer

T-DNA transfer begins with the introduction of bacteria into a plant wound. Wounding is a necessary event in the process and may, at least in part, be required for the synthesis by the plant certain compounds that induce the expression of the vir genes.



Two of the most active substances identified are acetosyringone and β-hydroxy-acetosyringone. The vir A product is a periplasmic membrane protein that senses specific phenolic compounds (such as acetosyringone and related molecules) synthesized by wounded plant cells. It is most likely that Vir A protein also interacts with another protein encoded by chvE gene present on bacterial chromosome and is important for the binding of sugar coinducers. The presence of these phenolic and sugar inducers results in the autophosphorylation of Vir A, which then transfers the phosphate group to Vir G protein, thus activating Vir G. The activated Vir G protein acts as a transcriptional activator of the other vir genes (most likely by binding to specific DNA sequences, the "vir box" that precedes the vir genes). An early event in the T-DNA transfer is the nicking of Ti plasmid **(Fig. 2).** Two of the proteins coded by vir D, Vir D1 and D2, provide an endonuclease that initiates the transfer process by nicking T-DNA at a specific site.

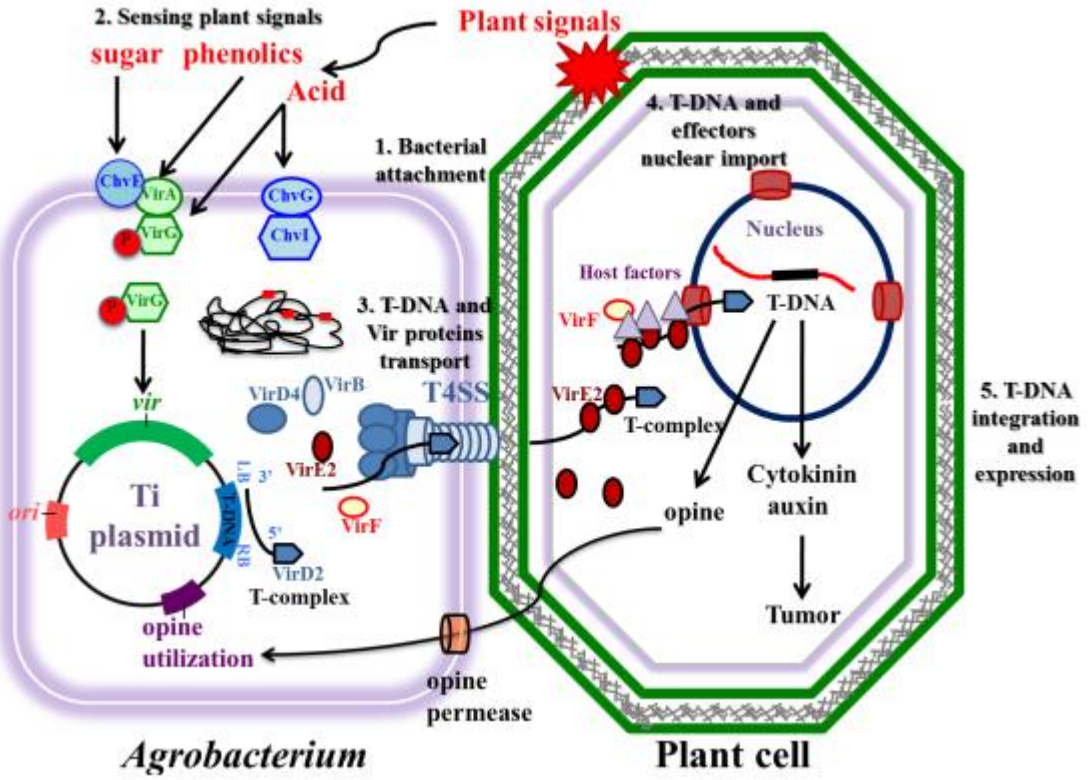


Fig. 2:. Major steps of the Agrobacterium tumefaciens-mediated plant transformation process.

(1) Attachment of A. tumefaciens to the plant cells. (2) Sensing plant signals by A. tumefaciens and regulation of virulence genes in bacteria following transduction of the sensed signals. (3) Generation and transport of T-DNA and virulence proteins from the bacterial cells into plant cells. (4) Nuclear import of T-DNA and effector proteins in the plant cells. (5) T-DNA integration and expression in the plant genome.

Nicking takes place between the third and fourth base of the bottom strand of each 25-bp repeat. A nick at the right border 25bp repeat provides a priming end and synthesis of new DNA strand begins from this nick in 5′→ 3′ direction. New strand displaces the old T-DNA single strand. Recent evidence indicates that T-strand retains the Vir D2 protein covalently attached to the 5′ terminus. The presence of Vir D2 makes the 5′ end of the T-strand less vulnerable to an attack by exonuclease. Besides, the Vir D2 protein may also act as a pilot to direct the T-strand to the nucleus of the transformed cell, since it contains a nuclear targeting sequence.

Outside the T-DNA, but immediately adjacent to the right border, is another short sequence called overdrive, which greatly stimulates the transfer process. Overdrive functions like an enhancer. Vir C1 and Vir C2 may act at the overdrive sequence. Vir E2 is a 69 kDa protein that binds single stranded T-DNA and is involved in transporting T-DNA into plant cell nucleus. The remaining Vir proteins are not involved in regulation of expression or T-strand expression; only those encoded by the vir B operon are essential for virulence. Most of the proteins predicted for the vir B operon are located in the membrane and these proteins may form a conjugal pore or pilus structure in the membrane through which the T-DNA is delivered into the plant cell. The vir B11 gene has an ATP binding site and the protein has an ATPase activity. It may therefore be involved in delivering energy required for T-DNA transfer. The virH operon consists of two genes and it has a role in the detoxification of certain plant compounds that might otherwise adversely affect the growth of Agrobacterium. Enhanced tumorigenicity was observed for bacteria having the virH genes as compared to those lacking these in certain hosts. vir F plays a role in T-

DNA delivery rather than symptom formation. Presence of vi rF gene in octopine Ti strains makes them vastly superior in transferring DNA.

The transfer of T-DNA closely resembles the events involved in bacterial conjugation when the E. coli chromosome is transferred from one cell to another in single stranded form. A difference is that the transfer of T-DNA is usually limited by the boundary of the left repeat, whereas transfer of bacterial DNA is indefinite. It is still not very clear how the transferred DNA is integrated into the plant genome. At some stage the newly generated single strand must be converted into duplex DNA.

Disarmed Ti plasmids

Most cloning vectors for plants are based on the Ti plasmid, which is not a natural plant plasmid, but belongs to a soil bacterium Agrobacterium tumefaciens. This bacterium invades plant tissues, causing a cancerous growth called a crown gall. During infection, a part of the Ti plasmid called the T-DNA is integrated into the plant chromosomal DNA.

It is a fact that the Ti plasmid is a natural vector for genetically engineering plant cells because it can transfer its T-DNA from bacterium to the plant genome. However, wild type Ti plasmids are not suitable as general gene vectors because they cause disorganized growth of the recipient plant cells owing to the oncogenes in the T-DNA. The tumor cells which result from integration of normal T-DNA (wild-type) have proved recalcitrant to attempts to induce regeneration, either into normal plantlets, or into normal tissue which can be grafted onto healthy plants. The oncogenic function of the T-DNA is responsible for inhibiting the regeneration ability of a cell to a complete plant. So it must use vectors in which the T-DNA has been disarmed by making it nononcogenic. This is most effectively achieved simply by deleting all its oncogenes and substituting them with an insert (desirable gene) between the regions of the left and right border.

Thus, the Agrobacterium Ti plasmid system exploits the elements of the Agrobacterium transformation mechanism. But the following properties of Ti plasmids do not allow their direct use: (i) large size; (ii) tumor induction property and (iii) absence of unique restriction enzyme sites. Agrobacterium plasmids are disarmed by deleting naturally occurring T-DNA encoded oncogenes and replacing them with foreign genes of interest. Besides, restriction enzyme sites are also added into the plasmids.

Advantages of T-DNA mediated mutagenesis:

1. Results in fewer (1–2) insertions per line.
2. Insertions are stable.
3. Easy to maintain and does not show strong insertional biases.

Limitations of T-DNA mediated mutagenesis:

1. It is more difficult to achieve saturation with T-DNA insertional mutagenesis.
2. Most of the transformation systems developed for Arabidopsis involve tissue culture, which often induces high frequencies of somaclonal variation.

Cointegrate vectors

Vectors that recombine via DNA homology into a resident Ti plasmid are often referred to as integrative or cointegrate vectors.

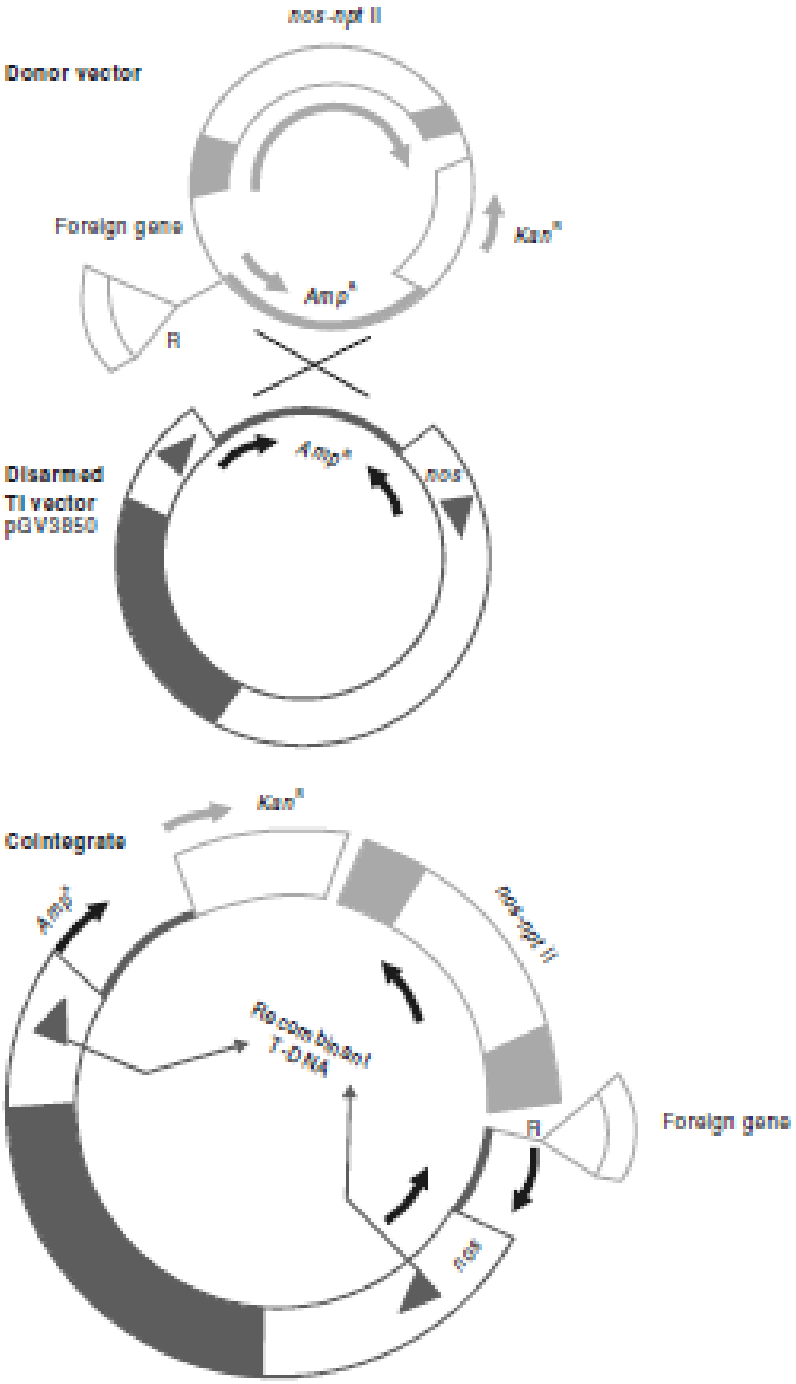


Fig. 3: Producing a recombinant T-DNA by cointegrate formation. The donor vector recombines with the disarmed vector by homologous recombination to produce the cointegrate

In a cointegrate vector, the disarmed Ti vector is covalently linked to donor vector with gene of interest-T-DNA border sequences present in a A. tumefaciens strain to act as one unit. In a cointegrate vector, much of the wild type T-DNA (especially the hormone biosynthetic genes and not the border regions) is replaced with a segment of DNA common to many E. coli cloning vectors. Homology between the E. coli plasmidbased segment of the modified T-DNA and the donor vector with identical sequences provides a site for recombination to occur which is catalyzed by native Agrobacterium rec functions, resulting in the formation of a hybrid or cointegrate Ti plasmid. Because, most cointegrates result from a single recombination event, the ensuing product places the gene of interest along with the entire donor vector with its marker genes between the native T-

DNA borders of the disarmed Ti plasmid. The formation of cointegrate vector using the disarmed vector pGV3850 is illustrated in **Fig. 3.**

This vector has pBR322 DNA in the T-DNA region, which provides a region of homology with most other cloning vectors. In this example, donor vector pLGVneo1103 carries a plant selectable marker nos-npt-II hybrid gene and a bacterial selectable marker, the kan gene. Recombination between the homologous pBR322 DNA in the two plasmids produces the co-integrate.

The standard cointegrate vector contains: (i) convenient sites for insertion of the gene of interest, (ii) antibiotic selectable marker gene or genes active in both E. coli and A. tumefaciens, (iii) a plant functional selectable marker gene, and (iv) E. coli functional origin of replication that does not operate in Agrobacterium. Research has not demonstrated any specific advantage to retaining the T-DNA and vir functions on a single replicon.

Binary vectors

The binary vector system consists of two autonomously replicating plasmids within A. tumefaciens: a shuttle (more commonly referred to as a binary) vector that contains gene of interest between the T-DNA borders and a helper Ti plasmid that provides the vir gene products to facilitate transfer into plant cells. Disarmed helper Ti plasmids have been engineered by removing the oncogenic genes while still providing the necessary vir gene products required for transferring the T-DNA to the host plant cell.

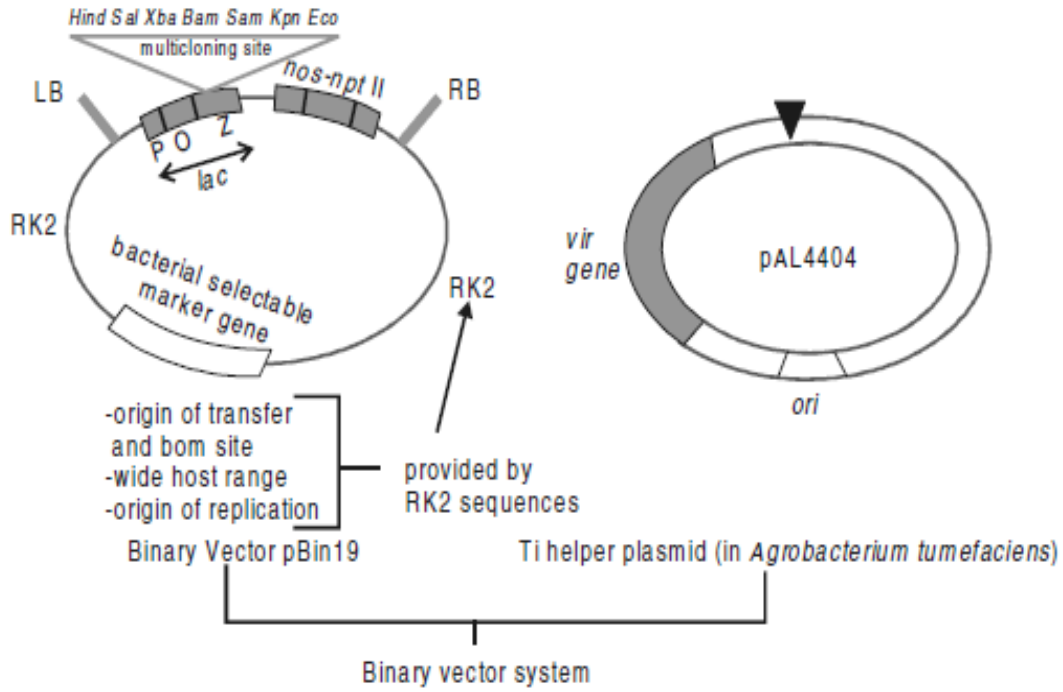


Fig. 4: A typical binary Ti vector system.

The standard components of a binary vector are: (i) multiple cloning site, (ii) a broad host range origin of replication functional in both E. coli and A. tumefaciens (e.g. RK2) (iii) selectable markers for both bacteria and plants, and (iv) TDNA border sequences (although only the right border is absolutely essential).

A typical binary vector system is shown **in Fig. 4.**

The helper Ti plasmid pAL4404 is a derivative of the octopine plasmid pTiAch5 that has the complete T-DNA region deleted but contains vir genes. The mini T-DNA donor vector pBin19 contains a truncated T-DNA from the nopaline plasmid pTiT37 comprising the left and right border sequences and a nos-nptII hybrid gene as a selectable marker. A lacZ polylinker is inserted in the mini T-DNA to provide unique cloning sites and a color test for insertion. The linker carries the galactosidase that produces blue

colonies on IPTG/X-GAL indicator plates and white colonies if it is disrupted by an insertion of foreign gene, i.e. pBin 19.

There are a number of advantages associated with using a binary system as compared to cointegrate vector:

1. Binary vectors do not need in vivo recombination whereas cointegrate vectors require a recombinational event for stable maintenance within the target A. tumefaciens strain.

2. Binary vectors require only that an intact plasmid vector be introduced into the target bacterium, making the process of bacterial transformation both more efficient and quicker (2–3 d versus 4–7d).

3. Binary vector systems with plant ready genes in agrobacteria are easily and efficiently obtained.

4. In binary system, the binary plasmids exist as separate replicons, thus copy number is not strictly tied to that of Ti plasmid. Because of this, in most cases, confirmation of the transformation event is accomplished via agrobacteria mini preps as compared to Southern hybridization used for cointegrate plasmids.

Most of the recently developed plant transformation vectors are binary, largely due to the ease of both in vivo and in vitro DNA manipulation and their higher transformation efficiencies, allowing the use of direct Agrobacterium transformation techniques.

Plant viral vectors

Vectors based on viruses are desirable because of the high efficiency of gene transfer that can be obtained by infection and the amplification of transferred genes that occurs via viral genome replication. Also, many viral infections are systemic so that gene can be introduced into all the cells in a plant. Viruses provide natural examples of genetic engineering since viral infections of a cell results in the addition of new genetic material which is expressed in the host.

1. Caulimovirus group (Cauliflower mosaic virus)

Among the plant viruses type, the virus of the caulimovirus group, cauliflower mosaic virus (CaMV), is cited as the most likely potential vector for introducing foreign genes into the plants. This is mainly because caulimoviruses are unique among plant viruses in having a genome made of double stranded DNA, which of course lends itself more readily to the manipulations. They were the first plant viruses to be manipulated by the use of recombinant DNA technology. The caulimoviruses group consists of 6–19 viruses, each of which has a limited host range. The commonest are carnations etched virus (CERV), cauliflower mosaic virus (CaMV), dahlia mosaic virus (DaMV), mirabilis mosaic virus, strawberry vein banding virus. The best known CaMV infects many members of Cruciferae and Datura stramonium. The virus is naturally transmitted by aphids but can very easily be transmitted mechanically. It has been found that virion DNA alone or cloned CaMV DNA are infectious when they are simply rubbed on the surface of susceptible leaves. Virus particles can be found at a high copy number (up to 106 virions per cell) and spread rapidly in 3–4 weeks in a systemic manner throughout the entire plant. Virus accumulates in the cytoplasm as inclusion bodies, which consist of a protein matrix with embedded virus particles. The virus particle is spherical, isometric, about 50 nm in diameter, and may be isolated from the inclusion body using urea and nonionic detergents. The DNA exists in linear, open circular and twisted or knotted forms; however, none of the circular forms is covalently closed due to the presence of site-specific single stranded breaks (Fig 5).

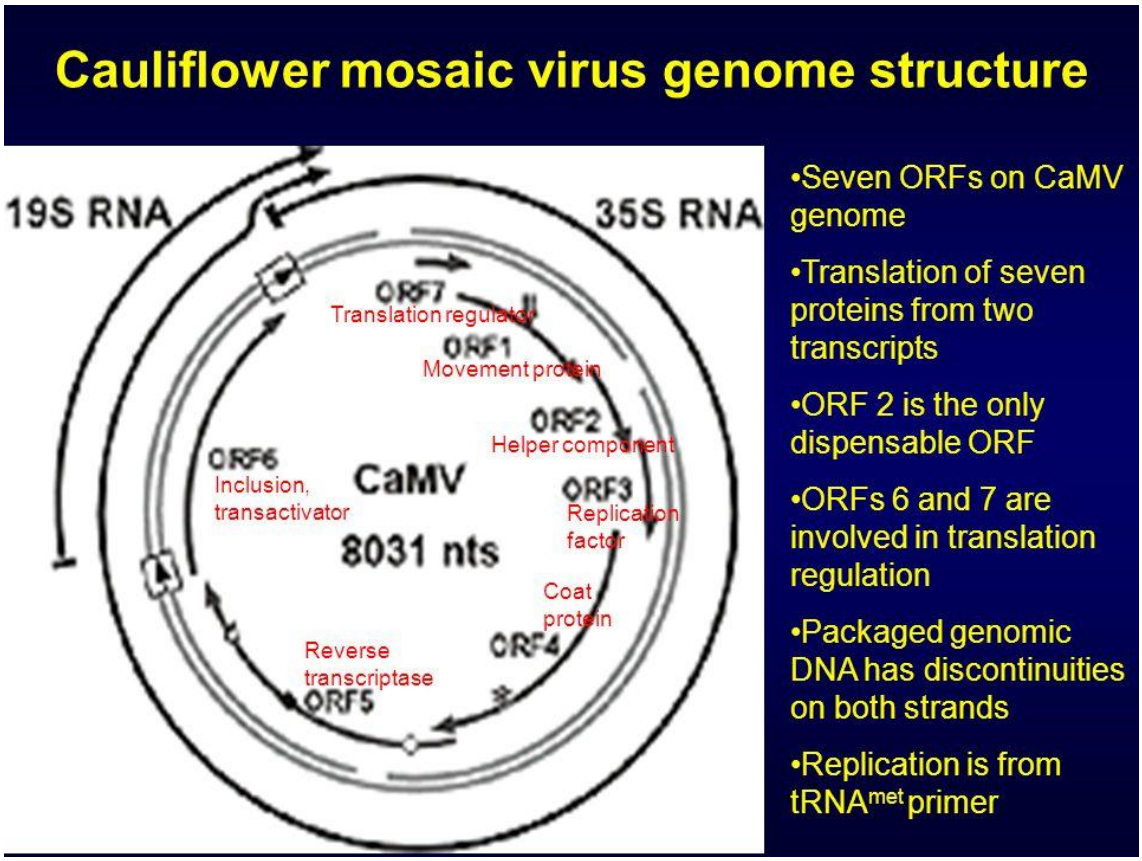


Fig. 5: A functional map of the cauliflower mosaic virus genome. The coding regions are shown as dark boxes. The different reading frames are indicated by the position of the boxes. The thin line in the centre is the DNA and outside is 35S transcript. Concept of the figure was taken from Applied Molecular Genetics

The genome of CaMV consists of a relaxed circular molecule of 8 kb. DNA sequence analysis revealed that six major and two minor open reading frames (ORF) are present on one coding strand in a very tightly packed arrangement. The caulimoviruses as well as badnaviruses replicate via reverse transcription of a more than genome length RNA. The mechanism of replication is as follows: The infecting CAMV DNA enters the plant nucleus, where the single stranded overlaps are digested and gaps ligated to give a supercoiled minichromosome. The function of this minichromosome is to act as a template for plant nuclear RNA polymerase II. The transcript thus formed is transported to the cytoplasm where it is either translated or replicated by reverse transcription. The RNA transcript is then copied into the minus strand DNA. Synthesis of the plus strand DNA starts at two primer binding sites near gaps 2 and 3. From gap 2 synthesis proceeds to the 5′ end of the minus strand DNA, whereas synthesis from gap 3 continues to gap 2. This DNA molecule gets packaged into virus particles or re-enters the nucleus and undergoes another round of transcription and/or translation/replication.

Two genomic regions, ORF II that codes the insect transmission factor and ORF VII with unknown function, can be dispensed with and replaced by the gene of interest. There is one report on the insertion of a foreign gene into the plant describing the use of CaMV. Brisson et al. (1984) achieved the expression of foreign protein encoded by the bacterial dihydrofolate reductase gene in turnip plant cells by a CaMV vector.

Uses as a vector

1. Naked DNA is infective, being able to enter plant cells directly if rubbed on to a leaf with a mild abrasive.

2. As a DNA virus whose genome is known to be packaged in nucleosomes and transcribed by RNA polymerase II, it is more suited for exploitation as an experimental tool than any other plant virus.

Problems

1. The genome is so tightly packed with the coding regions that there is little room for insertion of foreign DNA. Most deletions of any significant size destroy virus infectivity. The theoretical packaging capacity is of 1000 nucleotides, but attempts to propagate and stably express genes larger than 500 bp have not been successful.

2. CaMV-derived vectors are restricted to members of Cruciferae that can be infected by viral DNA. Recently, some mutant strains of CaMV infecting species of Solanaceae have also been described.

3. CaMV DNA has multiple cleavage sites for most of the commonly used restriction endonucleases. This would limit the usefulness of wild isolates of CaMV.

4. Infectivity with CaMV is another problem because once established it becomes systemic, spreading throughout the whole plant. This lack of inheritance through the germline may be advantageous in that the CaMV DNA and any inserted gene sequence would be highly amplified in the host plant cells, potentially permitting the expression of large quantities of the foreign gene product. However, it appears that to propagate CaMV and to allow its movement throughout the vasculature of the plant, the DNA must be encapsidated, and this would impose serious constraints on the size of foreign DNA that can be inserted into the viral genome

2. Gemini Viruses

The gemini viruses replicate and cause diseases in a wide variety of plant species, including many of agricultural importance. These viruses have some properties that make them ideal vectors for the expression of foreign genes in plants. The potential of gemini viruses as gene cloning vectors for plants stems from work on several plant diseases now recognized as being caused by these agents. Both the curly top virus (CTV) and maize streak virus (MSV) cause important diseases.

The gemini viruses are characterized on the basis of their unique virion morphology and possession of single stranded (ss) DNA. The gemini virus group takes its name from the unusual twin icosahedral capsid structure of its members. These have a small capsid size, 18– 20 nm × 30 nm, and geminate (paired particles) morphology, which makes them different from all other classes of viruses. They have covalently closed circular (ccc) topography of the ssDNA which is in the molecular weight range of 7 × 105–9 × 105. All gemini viruses have a single major coat protein subunit in the range 2.7–3.4 × 104 daltons.

The genome of Gemini viruses consists of either one or two circular, single stranded DNA molecules. The single stranded viral DNA, 2.6– 3.0 kb long, is converted into a double stranded replicative form in the nucleus of plant cells. Many copies of the replicative form of a Gemini virus genome accumulate inside the nuclei of infected cells. There is no evidence to date for a reverse transcription step in Gemini virus replication. Bean golden mosaic virus (BGMV) DNA was found to be 2510 nucleotides long, and if this was the complete genome it would be less than half the length of any other known autonomously replicating plant virus. By comparing the ssDNA of the virus particles with the viral dsDNA found in infected plants, it was found that the nucleotide sequence had twice the complexity expected on the physical size of the viral DNA. This indicates that BGMV DNA is heterogeneous, the virus having a divided genome consisting of two DNA molecules of approximately the same size but different genetic content.

The genomic ssDNA replicates in the nucleus through doublestranded DNA (dsDNA) intermediates, most likely by a rolling circle mechanism. Despite an overall similarity in capsid and genome structure, the Gemini virus group is diverse and can be divided into at least three subgroups.

The genomes of all group members contain two elements that must be part of any functional Gemini virus vector system, although these elements need not be physically present on the same molecule. These elements are an origin of replication and an essential viral replication protein.

Most Gemini virus vectors described to date are relatively primitive and simply permit the investigator to replace the coat protein coding sequence with a reporter or other gene of interest. Gemini virus expression vectors may be used to deliver, amplify and express foreign genes in several different systems of protoplasts and cultured cells, leaf discs, and plants.

The cereal Gemini virus, wheat dwarf virus, is under development as a vector for introducing genes into cereals. It is capable of accommodating and replicating gene inserts up to about 3 kb in length. Three bacterial genes inserted into the wheat dwarf virus genome have been successfully replicated and expressed after transfer into cultured cells of Triticum monococcum and Zea mays.

Uses as a cloning vector

1. These viruses contain single stranded DNA that appears to replicate via a double stranded intermediate and thus makes in vivo manipulation in bacterial plasmids more convenient.

2. An attractive feature is the ability of bipartiteGemini viruses to contain a deletion or a replacement of virus coat protein sequences by foreign genes without interfering with the replication of the virus genome.

Problems

1. These are not readily transferred bymechanical means from plant to plant, but transmitted in nature by insects in a persistent manner.

2. The small particle size may present packaging problems for modified DNA molecules and any useful genetic modifications will have to solve the problems of a vector which in its natural state causes severe disease in susceptible plants.

3. RNA viruses

RNA viruses have advantages and disadvantages, which make them the ideal choice for certain applications as vectors. Their assets include ease of use, perhaps the highest levels of gene expression, and the vector's ability to infect and spread in differentiated tissue. There are two basic types of single stranded RNA viruses. The monopartite viruses have undivided genomes containing all the genetic information and are usually fairly large, e.g. tobacco mosaic virus (TMV). The multipartite viruses, as the name suggests, have their genome divided among small RNAs either in the same particle or separate particles, e.g. brome mosaic virus (BMV) contains four RNAs divided between three separate particles. The RNA components of multipartite genomes are small and appear to be able to self-replicate in plants. With some members of the group, the genes encoding the coat protein may be dispensable, as their loss does not affect viral DNA multiplication.

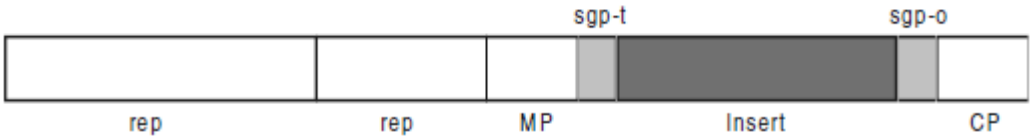
The second group, subgenomic RNAs (e.g. RNA IV of BMV), is unlikely to find application as cloning vectors as it is unable to self-replicate in infected plants.

The third group, satellite RNAs has perhaps the greatest potential as it is totally dispensable to the virus. Satellite RNAs vary in size from 270 bases (tobacco ringspot virus satellite) to 1.5 kb (tomato black ring virus satellite). These satellites appear to share little homology with the viral genomic RNAs, templates for their own replication, and utilize the machinery for replication set up by the virus. They are not required for virus replication, but are capable of altering the pathogenicity of the viral infection. These satellite RNAs have a number of other unusual properties, including the ability to code for proteins and stability in the plant in the absence of other viral components.

Plant viruses have only few genes, but these are expressed to high levels by a variety of means. High gene product yields and activities are associated with RNA viral vectors. Plant viral genomes contain a gene for movement through the plant, a gene for the protein making up the protective coat and a gene for replication. Coat proteins are the most abundant viral gene product. One example of a gene replacement construction is the

replacement of RNA viral vector coat protein gene of BMV by chloramphenicol acetyl transferase gene (French et al., 1986). Expression of cDNA in barley protoplasts suggests that RNA virus may be a useful vector for gene manipulation.

A second common type of vector construction is the insertion of a foreign gene into an intact viral genome. An example is the TMV vector TB2 that was the first plant RNA viral vector able to spread systemically in whole plants (Donson et al., 1991). In TB2, the foreign gene is inserted at 3′ end of the movement protein, which is the normal position for the TMV coat protein ORF (Fig 6). Thus, foreign gene expression is driven by the native coat protein subgenomic promoter (sgp) and a sgp from related virus ORSV—odontoglossum ringspot tobamovirus drives the expression of ORF of coat protein.



Insert : [DHFR, II, trichosanth in] ; **rep :** viral replicase

sgp-t & sgp-o: sgp from TMV and ORSV (Odontoglossum ringspot tobamovirus)
CP : viral coat protein; **MP** : viral movement protein

Fig. 6: Genomic map of TMV: TB2 plant RNA viral vector.

Most RNA viral vectors carrying large or small inserts replicate stably in protoplasts and/ or inoculated leaves. Thus, experiments can be carried out in either protoplasts or differentiated tissue using the present generation of plant viral vectors.

A variety of DNA and RNA viral vectors can be used to express genes in protoplasts but the choice of vector is limited for use in inoculated leaves of whole plants. The other disadvantages are limitation of insert size and the induction of symptoms in the host.

Vectorless or Direct DNA transfer

Direct gene transfer has proved to be a simple and effective technique for the introduction of foreign DNA into plant genomes. It has been further sub-divided into three categories:

1. Physical gene transfer methods.
2. Chemical gene transfer methods.
3. DNA imbibition by cell, tissue and organs.

1. Physical gene transfer methods.

a) Particle gun bombardment/microprojectile/biolistics

The technique of particle bombardment also known as biolistics, microprojectile bombardment, particle acceleration, etc. has been shown to be the most versatile and effective way for the creation of many transgenic organisms, including microorganisms, mammalian cells and plant species. The procedure in which high velocity microprojectiles were utilized to deliver nucleic acids into living cells was described by Klein et al. (1987) and Sanford et al. (1987).

The basic system that has received attention employs PDS 1000 (gun powder driven device) or the PDS-1000/He (helium driven particle gun). The DNA bearing tungsten or gold particles (1– 3 μm in diameter), referred to as microprojectiles, is carried by a macroprojectile or macrocarrier and is accelerated into living plant cells. The DNA bearing particles (microprojectiles) are placed on the leading surface of the macrocarrier and released from the macrocarrier upon impact with a stopping plate or screen. The stopping plate is designed to halt the forward motion of macroprojectile while permitting the passage of the microprojectiles. In this procedure when helium gas is released from the tank, a disc known as rupture disc blocks its entry to the chamber. These discs are

available with various strengths to resist the pressure of gas, which varies from 500 to 1700 p.s.i. When the disc ruptures, compressed helium gas is suddenly released, which accelerates a thin plastic sheet carrying microprojectiles into a metal screen. Upon impact with a stopping plate or screen, the macroprojectile movement is stopped, but this permits the passage of microprojectiles through the mesh screen. The microprojectiles then travel through a partial vacuum until they reach the target tissue. The partial vacuum is used to reduce the aerodynamic drag upon the microprojectiles and decrease the force of the shock wave created when the macrocarrier impacts the stopping plate. See Fig. 7 for a schematic diagram of the PDS–1000/He.

The use of particle bombardment requires careful consideration of a number of parameters. These can be classified into three general categories:

Physical parameters

Nature, chemical and physical properties of the metal particles utilized to carry the foreign DNA: Particles should be of high mass in order to possess adequate momentum to penetrate into the appropriate tissue. A variety of metals such as tungsten, gold, platinum, palladium, rhodium, iridium and possibly other second and third row transition metals can be used. Size range of the particles is ca. 1 µm. Tungsten particles of 1.2 µm diameter and gold particles of 1.0 to 1.6 µm diameter have been most commonly used. Metals should be chemically inert to prevent adverse reactions with the DNA or cell components.

Nature, preparation and binding of DNA on to the particles: The nature of DNA, i.e. as single versus double stranded, may be important under some conditions but this may not be a significant variable in specific cases. In the process of coating metal particles with DNA, certain additives such as spermidine and calcium chloride appear to be useful.

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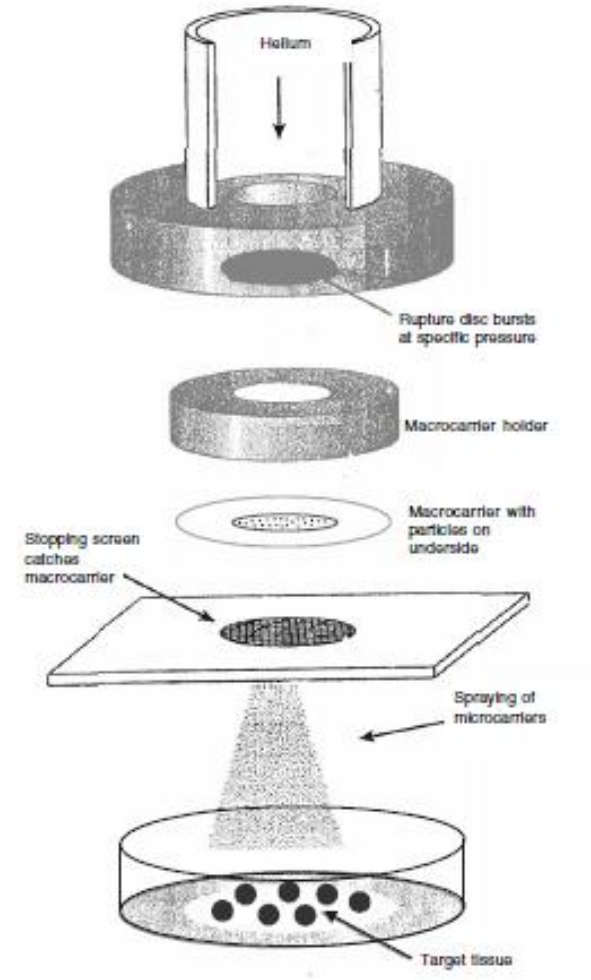


Fig. 7: Schematic representation of the particle bombardment process.

Target tissue: It is very important to target the appropriate cells that are capable of cell division and are competent for transformation. It is apparent that different tissues have different requirements, thus extensive studies need to be performed in order to ascertain the origin of regenerating tissue in a particular transformation study. Penetration of microprojectile (DNA coated to inert metal of gold or tungsten) is one of the most important variables and the ability to tune a system to achieve particle delivery to specific layers may result in success or failure in recovering transgenic plants from a given tissue. Generally the cells near the center of the target are injured and thus cannot proliferate

Environmental parameters: These include variables such as temperature, photoperiod and humidity of donor plants, explants and bombarded tissues. These parameters effect the physiology of the tissues, influence receptiveness of target tissue to foreign DNA delivery and also affect its susceptibility to damage and injury that may adversely affect the outcome of the transformation process. Some explants may require a healing period after bombardment under special regimes of light, temperature and humidity.

Biological parameters: Choice and nature of explants, and pre- and post-bombardment culture conditions determine whether experiments utilizing particle bombardments are successful. The explants derived from plants that are under stress or infected with bacteria or fungi, over- or under-watered will be inferior material for bombardment. Osmotic pre- and posttreatment of explant with mannitol has been shown to be important in transformation. Experiments performed with synchronized cultured cells indicate that the transformation frequencies may be also influenced by cell cycle stage.

Advantages

Several advantages make this technique a method of choice for engineering crop species.

1. It is clean and safe.
2. Transformation of organized tissue: The ability to engineer organized and potentially regenerable tissue permits introduction of foreign genes into elite germplasm.
3. Universal delivery system: Transient gene expression has been demonstrated in numerous tissues representing many different species.
4. Transformation of recalcitrant species: Engineering of important agronomic crops such as rice, maize, wheat, cotton, soybean, etc. has been restricted to a few noncommercial varieties when conventional methods are used. Particle bombardment technology allowed recovery of transgenic plants from many commercial cultivars.
5. Study of basic plant development processes: It is possible to study developmental processes and also clarify the origin of germline in regenerated plants by utilizing chromogenic markers.

Disadvantages

1. In plants gene transfer leads to nonhomologous integration into the chromosome, and is characterized by multiple copies and some degree of rearrangement.
2. The emergence of chimeral plants.
3. Lack of control over the velocity of bombardment, which often leads to substantial damage to the target cells.

b) Electroporation

Electroporation is the process where electrical impulses of high field strength are used to reversibly permeabilize cell membranes to facilitate uptake of large molecules, including DNA. The electroporation method is based on Neumann et al. (1982) for animal cells. It uses a relatively high initial field strength (1–1.5 kV) with a low capacitance and therefore a short decay time. Other methods with low initial field strength and long decay time have been described. In this procedure, a sample of protoplasts is pulsed with

high/low voltage pulses in the chamber of an electroporator. The chamber is cylindrical in form with a distance of 1cm between parallel steel electrodes. The pulse is applied by discharge of a capacitor across the cell. It has been reported that using linear DNA rather than circular DNA, a field strength of 1.25 kV/cm and employing polyethylene glycol (PEG) can increase protoplast transformation frequency. PEG is believed to assist the association of the DNA with the membrane.

Electroporation has been used for a long time for transient and integrative transformation of protoplasts. Only recently, conditions under which DNA molecules can be delivered into intact plant cells of sugarbeet and rice that are still surrounded by a cell wall have been standardized. Further, transformability of intact plant cells or tissues depends on pretreatment of the cells or tissues to be transformed, either by mechanical wounding or by treating the cells or tissues with hypertonic or enzyme (e.g. 0.3% macerozyme) containing solutions.

The range of tissues that can be transformed by electroporation seems to be narrower. For tissues that are susceptible to DNA uptake by electroporation, this method is convenient, simple, fast, low cell toxicity and inexpensive to obtain transient and stable transformation in differentiated tissues. The disadvantage of the technique is the difficulty in regenerating plants from protoplasts.

c) Liposome-mediated transformation

Liposomes are artificial lipid vesicles surrounded by a synthetic membrane of phospholipids, which have been used in mammalian tissue culture to deliver drugs, proteins, etc. into the cells. These can be induced to fuse with protoplasts using PEG and have therefore been used for gene transfer. In this method, DNA enters the protoplasts due to endocytosis of liposomes and it involves the following steps:

i) Adhesion of liposomes to the protoplast surface.

ii) Fusion of liposomes at the site of adhesion.

iii) Release of plasmids inside the cell.

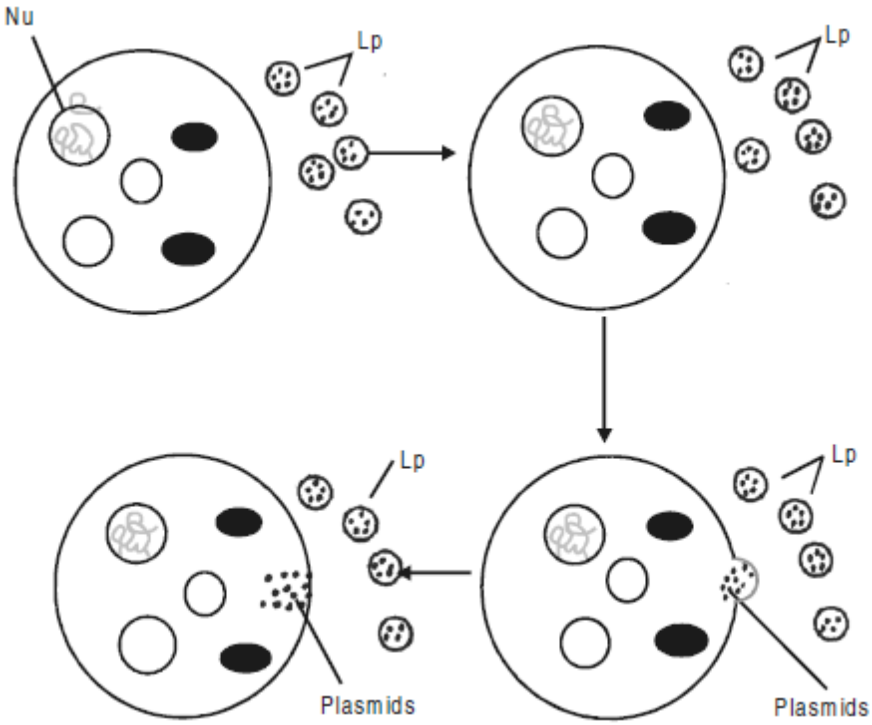


Fig. 8: Fusion of plasmid filled liposomes with protoplast. Nu–nucleus; Lp–liposomes.

Uptake of liposomes containing DNA molecules has been demonstrated in protoplasts of tobacco and carrot (Fig. 8). Liposomemediated transformation showed higher efficiency when it was used in conjunction with PEG. Liposomes offer many advantages for protoplast transformation including

(i) Protection of nucleic acids from nuclease digestion prior to introduction into cellular environment,

(ii) Reduced levels of cell toxicity,

(iii) Applicability to a wide variety of cells types,

(iv) High degree of reproducibility, and

(v) Stability and storage of nucleic acids once encapsulated.

2. Chemical gene transfer methods

This involves plasma membrane destabilizing and/or precipitating agents. Protoplasts are mainly used which are incubated with DNA in buffers containing PEG, poly L-ornithine, polyvinyl alcohol or divalent ions. The chemical transformation techniques work for a broad spectrum of plants.

i) PEG mediated gene transfer

The first conclusive demonstration of uptake and integration of isolated Ti plasmid DNA into plant protoplasts was reported in Petunia and tobacco in the presence of poly L-ornithine or polyethylene glycol (PEG). The presence of Ti DNA in the plant genome was demonstrated both by the phenotype of hormone auxotrophic growth, the production of the expected opine and by Southern blot analysis of DNA from the transformants.

In a general procedure protoplasts are isolated and a particular concentration of protoplast suspension is taken in a tube followed by addition of plasmid DNA (donor and carrier). To this 40% PEG 4000 (w/v) dissolved in mannitol and calcium nitrate solution is slowly added because of high viscosity, and this mixture is incubated for few minutes (ca. 5 min). As per the requirements of the experiment, transient or stable transformation studies are conducted.

Among the most important parameters that affect the efficiency of PEG-mediated gene transfer, are the concentration of magnesium or calcium ions in the incubation mixture, and the presence of carrier DNA. The linearized double stranded plasmid DNA molecules are more efficiently expressed and integrated into the genome than supercoiled forms. Integration of foreign DNA into the nuclear genome occurs predominantly at random sites.

The main application of the technique, apart from analyzing the transformation process itself, is in introducing foreign genes to plant cells. This can be accomplished by constructing a molecule containing a selectable marker and the gene of interest, or more easily by simply mixing DNA of the gene of interest with the selectable marker plasmid in a molar ratio of approximately 3:1 to 10:1, transforming, selecting for the marker and analyzing transformants for the presence of the second gene. The method can also be applied with DNA from different sources. Manipulation of nucleic acids prior to transformation is also possible and there are no host range limitations.

The advantage of the method is that the form of the DNA applied to the protoplasts is controlled entirely by the experimenter and not by an intermediate biological vector. The main disadvantage is that the system requires protoplasts and a functional system for regeneration of these protoplasts to calluses and whole plants. It is therefore not applicable to many plant systems. In addition, the relatively random way in which the DNA is integrated into the genome means that, for the introduction of nonselectable genes, a thorough characterization of the transformants by Southern blot analysis is necessary to confirm the nature of the integration event.

ii) Calcium Chloride

Calcium chloride transformation technique is the most efficient technique among the competent cell preparation protocols. It increases the bacterial cell's ability to incorporate plasmid DNA, facilitating genetic transformation. Addition of calcium chloride to the cell suspension allows the binding of plasmid DNA to LPS. Thus, both the negatively charged DNA backbone and LPS come together and when heat shock is provided, plasmid DNA passes into the bacterial cell. Prepare 2000 ml of 50 mM Calcium

chloride stock solution by adding 14.701 g of CaCl2.2H2O in 2 l of milli-Q water, autoclave, and store at 4 °C.

Materials
LB broth: Yeast extract 0.5%, NaCl l%, tryptone 1%.
2 LB agar: As above, plus 2% agar prior to autoclaving.

0.1M $CaCl_2$.
Antibiotics are added to the above media after autoclaving. Tetracycline to a final concentration of 15 pg/mL and ampicillin to 50 kg/mL Solutions of these antibiotics are prepared with ampicillin at 50 mg/mL m slightly alkaline distilled water and tetracycline at 15 mg/mL in ethanol.

Method
Prepare a small, overnight culture of the bacteria in LB broth. Grow at 37°C without shaking.
About 2 h before you are ready to begin the main procedure, use 1.0 mL of the overnight culture to inoculate 100 mL of fresh LB broth. This culture is grown with rapid shaking at 37°C until it reaches roughly 5 x 107 cells/ml. Thus corresponds to an OD650 for our cultures, but you should calibrate this for each of your own strains
Take a 5 mL aliquot of each transformation reaction and transfer to sterile plastic centrifuge tubes. Cool on ice for 10 mm.
Pellet the cells by spinning for 5 mm at 5000g. It is necessary for the centrifugation to be performed at 4°C. We have found a refrigerated bench centrifuge ideal for this.
Pour off the supernatant and resuspend cells in 25 mL of cold 0.1M $CaCl_2$. Leave on ice for at least 20 min.
Centrifuge as in Step 3. You should observe a more diffuse pellet than previously. This is an indication of competent cells.
Resuspend the cells in 0.2 mL of cold 0.1M $CaCl_2$.
Transfer the suspensions to sterile, thin-walled glass bottles or tubes. The use of glass makes the subsequent heat shocks more effective.
To each tube add up to 0.1 mg of DNA, made up in a standard DNA storage buffer such as TE to a volume of 100 mL. Leave on ice for 30 min.
Transfer to a 42°C water bath for 2 min and return briefly to ice.
Transfer the contents of each tube to 2 mL of LB broth in a small flask. Incubate with shaking at 37°C for 60-90 min.
Plate 0.1 mL aliquots of undiluted, 10-1 and 10-2 dilutions onto LB plates to which the antibiotics to be used for selection have been added.
Incubate overnight at 37°C.
Notes

This method generally gives 104-106 transformants/mg of closed circle plasmid DNA. Do note that the relationship between amounts of DNA added and yield is not totally linear. Greater than 0.1 mg of plasmid DNA per tube will decrease transformation efficiency.
It is essential that the cells used are in a rapid growth phase when harvested. Do not let them approach stationary phase.
Cells can be stored at 4°C once competent. Holding cells in CaCl2 at 4°C will, in fact, increase transformation efficiency although this declines with more than 24 h storage. Long periods of storage can be achieved by freezing the competent cells.
The revival step is necessary both to allow the plasmid establishment and to allow expression of the resistance genes.
One problem encountered on plating on ampicillin is that resistant colonies will often be surrounded by a region of secondary growth. This is caused by the p-lactamase activity of the resistant cells hydrolyzing the surrounding antibiotic and thus allowing surviving sensitive cells to begin to grow. This problem can be avoided by using freshly made ampicillin plates and removing plates from the incubator promptly after the period of overnight growth.

Selectable markers and promoters used in plant genetic engineering.

Selectable markers used in plant genetic engineering.

The Selection marker genes impart resistance to phototoxic compounds like antibiotics and herbicides. It is a stable dominant gene and is integral part of transformation vector. The criterion is positive selection for a resistance gene without which untransformed cells die when a selection agent is applied. Ideally, the selection agent must not affect the transformed cells and should be efficient even at lower concentrations. The sensitivity of plant cells to the selection agent depends on the genotype, explant type, developmental stage, and tissue culture conditions. Five different selection markers, cat, nptII, hpt, bar, and manA are used in sorghum transformation.

Neomycin phosphotransferase II (nptII) gene isolated from Escherichia coli confers resistance to the kanamycin, an antibiotic. It is effective in tobacco and carrot but has proven to be less effective in monocots. In monocotyledons, growth is not significantly inhibited by kanamycin. Battraw and Hall (1991) employed nptII in sorghum transformant selection, and later many workers corroborated the utility of nptII as a selectable marker in sorghum

Hygromycin phosphotransferase (hpt, hph, aphIV) is similar to nptII, derived from E. coli conferring resistance to the antibiotic, hygromycin. Hagio et al. (1991) reported limited utility of hygromycin resistance as a selectable marker where adequate exposure to light is important like meristem cultures. It is photosensitive and not suitable for selection of regenerated plants.

The most widely used selectable marker is **bar gene isolated from Streptomyces hygroscopicus.** It codes for **phosphinothricin acetyl transferase (PAT) proteins** of 183 amino acids and shows 85% DNA sequence homology with another marker gene pat isolated from Streptomyces viridochromogenes. It imparts resistance to phosphinothricin (~PPT), an analogue of glutamate, an inhibitor of glutamine synthetase (GS) activity, resulting in inhibition of amino acid biosynthesis

Tetracycline resistance, based on the efflux of the antibiotic from the cells rather than its destruction, and thus the concentration in the medium is unaffected.

Promoters used in plant genetic engineering.

An obvious requirement for any genes that are to be expressed as transgenes in plants is that they are expressed correctly. ¬ the major determinant of gene expression (level, location and time) is the region upstream of the coding region, termed Promoter. A promoter sequence is the site to which RNA polymerase first binds during the initiation of transcription. Affinity of RNA polymerase to the promoter sequences is several order of magnitude higher than that for other DNA sequences.

Types of Promoters used to regulate gene expression

1. Constitutive promoters are commonly used type of promoter.

They are capable of expressing linked DNA sequences in all tissues of a plant throughout normal development. Monocot promoters- Plant ubiquitin promoter (Ubi), Rice actin 1 promoter (Act-1), Maize alcoh dehydrogenase 1 promoter (Adh-1). In addition to promoters obtained from plant genes, there are also promoters of bacterial and viral origin which have been used to constitutively express novel sequences in plant tissues. Examples of such promoters from bacteria include the octopine synthase (ocs) promoter, the nopaline synthase (nos) promoter and others derived from native Ti plasmids. The 35S and 19S promoters of cauliflower mosaic virus are commonly used examples of viral promoters. Plant pathogen/Dicot promotersOpine promoters, CaMV 35S promoter.

2. Tissue-specific promoters

They are those promoters that are capable of selectively expressing heterologous DNA sequences in certain plant tissues. Eg., anther specific promoter for induction of male sterility, LEA protein promoters for gene expression during embryonic development. promoter region from the ethylene regulated genes E4 and E8 and from the fruit-specific polygalacturonase gene have been used to direct fruit specific expression of a heterologous DNA sequence in transgenic tomato plants.

3. Inducible promoters

A very popular way to regulate the amount and the timing of protein expression is to use an inducible promoter. An inducible promoter is not always active the way constitutive promoters are (e.g. viral promoters). Their performance is not conditioned to endogenous factors but to environmental conditions and external stimuli that can be artificially controlled.

Transgenic plants (Herbicide resistance and pest resistance).

Transgenic plant products have now been released as commercial varieties in a range of crops. By all accounts, 1996 was a critical year in the history of plant biotechnology. For the first time farmers planted large areas under transgenic/biotech plants. In 1995 the variety of tomato named as Flavr Savr was given regulatory approval for commercial cultivation which had delayed ripening gene. In the following year, cotton varieties expressing the Bt crystal protein toxic to a number of lepidopteran pests including the tobacco budworm and cotton bollworm were introduced. At the same time Bt corn hybrids with improved resistance to European corn borer became available to the farmers. All these commercial introductions have taken place in USA. Transgenics for herbicide tolerance was the dominant trait in 1997. Now the transgenics have been released for various traits from virus resistance to quality and for industrial use.

Insect resistant plants through transgenic approach

Insecticides based on the crystalline toxin from *Bacillus thuringiensis* (B.t) vividly illustrate the influence of molecular biology and genetic engineering on traditional crop protection. The isolation of insect toxin genes from B.t has opened up an area relevant to plant protection.

Plant Biotechnology has become a source of agricultural innovation, providing new solutions to age-old problems of crop protection from insect pests. The simple reason for this interest is that crop damage inflicted by phytophagous insects is staggering despite the use of sophisticated crop protection measures, chiefly chemical pesticides. This brings to the conclusion why transgenics for insect resistance. The cost associated with management practices and chemical control of insects approaches $ 10 billion annually, yet global losses due to insects still account for 20% to 30% of the total production. Every year individual farmers face the possible devastation of their crops by insect infestations. Insecticides to control insects are mainly chemically synthesized and they have a negative or adverse effect on host as well as on environment apart from the cost incurred in their use. Chemical pesticide used for protection from insects has very hazardous effect because:

i. Highly inefficient.

ii. Most of the applied chemicals is wasted either in runoff or in subsequent washing from the plant surfaces, and it has been estimated that as much as 98% of the sprayed pesticides end up in the soil.

iii. It is difficult to deliver pesticides to the most vulnerable part of the plant e.g. roots or inside of stem or fruit.

iv. Pesticides and their residues are often highly toxic to beneficial organisms.

v. A plant species in its natural environment is in balance with the various organisms including insects that depend upon it for food. These insects in turn provide food for predatory insects, birds, fish and mammals.

vi. The natural order is often overlooked when considering crop plants and use of pesticides that may be toxic to non-target organisms.

vii. Soil condition may decline.

viii. Human toxicity is a major concern.

ix. Very strong selection pressure on insect populations is imposed by insecticides causing resistance to such compounds to be rapidly acquired.

x. Overuse of pesticides and herbicides can decrease the vigour of the crop and actually make it more susceptible to insect attack.

In view of this, genetic engineering of insect resistance into crops represents an attractive opportunity to reduce insect damage.

Insect resistance.

Spraying crops with insecticides is a very costly and hazardous procedure. Insecticides are often more toxic to humans than are herbicides because insecticides target species closer to our own. Many insect biochemical pathways are found not only in humans, but also in rodents or birds that may inhabit crop fields. Luckily, naturally occurring toxins exist that are lethal to insects but harmless to mammals.

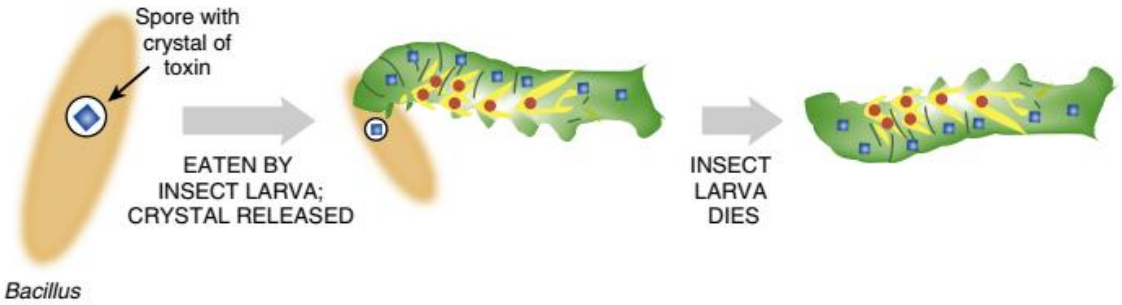
The prime example is the toxin from a soil bacterium called Bacillus thuringiensis. Bt toxin has been sprayed on crops (including organic crops) to prevent insects such as the cotton bollworm and European corn borer from destroying cotton and corn, respectively. Damage from the European corn borer plus the cost of insecticides to control it cost farmers about $1 billion annually. Damage by the corn borer also makes corn plants susceptible to infection with Aspergillus, a fungus that produces aflatoxin that can harm humans if ingested.

Bacteria of the genus Bacillus produce spores that contain a crystalline, or Cry protein. When insects eat Bacillus spores, the Cry protein breaks down and releases the Bt

toxin. This toxin binds to the intestinal lining of the insect and generates holes, which cripple the digestive system, and the insect dies. Different species of Bacillus produce a family of related Cry proteins that exhibit toxicity toward various groups of insects.

Instead of spraying crops with Bt toxin, scientists have used transgenic technology to insert the cry genes directly into plants. When a cloned toxin gene was inserted into tomato plants, for instance, it partially protected against tobacco hornworm. However, the plants made only low levels of the toxin because the bacterial gene was not optimized to express well in plants. Therefore, scientists removed the latter 506 of the protein's 1156 amino acids, which allowed the plant to produce a truncated (but still effective) toxin using less energy. The toxin gene was also placed under the control of a promoter that gives constant high-level expression in plants. Certain promoters from plant viruses, such as cauliflower mosaic virus, are commonly used to increase transgene expression and often provide a 10-fold boost in toxin production.

When genes from one organism are expressed in a very different host cell, codon usage also becomes a problem. The genetic code is redundant in the sense that several different codons can encode the same amino acid. Different organisms favor different codons for the same amino acid, a phenomeon known as codon bias, and have different levels of the corresponding tRNAs. If a bacterial transgene uses codons that require tRNA molecules that are rare in the plant, the rate of protein synthesis will be limited. This is particularly a problem for transgenes, which often need to be expressed at high levels. Therefore, the Bt toxin gene was altered by changing many of the bases in the third position of redundant codons. Almost 20% of its bases were altered to make the gene more plant-like in codon usage. Because such tweaks do not change the amino acids encoded, the toxin protein sequence was not affected by the procedure. However, the rate at which plant cells made the protein greatly increased and gave another 10-fold increase in toxin production.



Insect Larvae Are Killed by Bt Toxin.*Bacterial spores of Bacillus are found on food eaten by caterpillars. The crystalline protein is released by digestion of the spore, and its breakdown produces a toxin that kills the insect larvae.*

An alternative to toxins is to combat insects using pheromones or other compounds that induce changes in behavior. (E)-β-farnesene (EBF) is released by aphids as an alarm pheromone. Aphids avoid plants that express EBF and, simultaneously, aphid predators, such as ladybugs, are attracted to the plants (Fig. 15.21). One group is field-testing a variety of wheat that was transformed with synthetic genes whose encoded enzymes produce EBF. Compared to Bt toxin, the likely benefits of the pheromone approach are that the target insects are unlikely to develop resistance and nontarget insects will not be harmed.

Advantages

1. More effective targeting of insects protected within plants.
2. Greater resilience to weather conditions.
3. Season long protection.
4. Insects are always treated at the most sensitive stage.

5. It affords protection of plant tissues which are difficult to treat using insecticide. For example control of larvae of corn root worm (Diabrotica spp.) which attack underground tissues of maize and the cabbage seed weevil (Centrorhynchus assimilis) which is the major pest of oil seed rape attacking the developing ovules with immature pods.

6. Only crop eating insects are exposed.

7. Fast biodegradability.

8. No need of skilled hand.

9. Reduced exposure of farmers, laborers, and non-target organisms to the pesticides.

10. Net financial benefit to farmers.

11. Increased production of a particular crop.

12. Increased activities of natural enemies of pests, because of reduction in insecticide sprays.

13. Reduced amount of pesticide residues in the food and food products.

14. A safer environment to live because of reduction in pesticide use.

Limitations/Apprehensions

1. The secondary pests will no longer be controlled in the absence of sprays for the major pests.

2. Cost of production of transgenic plant is very high.

3. Cost of transgenic seed sold to the farmers is very high with the result that there is increased burden on the farmer.

4. Development of resistance in insect population may limit the usefulness of transgenic crops for pest management.

5. Evolution of new insect biotypes: Due to deployment of transgenic crops there is danger of evolution of new biotypes of insects.

6. Insect sensitivity: There are many species of insects that are not susceptible to currently available Bt proteins. There is need to broaden the pool of genes, which can be effective against insects that are not sensitive to currently available genes.

7. Gene escapes into the environment.

8. Secondary pest problem: Most crops are not attacked by a single pest species, but a complex of insect pests attack on it. In the absence of competition from major pests, secondary pests may assume a major pest status. Effective and timely control measures should be adopted for the control of secondary pests on transgenic crops.

9. Effect on non-target organisms: One of the major concerns of transgenic crops is their effects on non-target organisms, about which little is known at the moment. Bt proteins are rapidly degraded by the stomach juices of vertebrates. Most Bt toxins are specific to insects as they are activated in the alkaline milieu of insect gut. However, Bt proteins can have harmful effects on the beneficial insects. Although, such effects are much less severe than those of the broad spectrum insecticides.

Herbicide tolerant plants through transgenic approach

Herbicide tolerance has been the dominant trait since 1996 when GM crops were released commercially. However, there have been arguments against and favor of this trait in transgenic crops which have been summarized below.

Arguments in favor of herbicide tolerance in crop plants

1. Use of highly specific and powerful herbicides in combination with transgenic crops tolerant to specific herbicides will lead to more effective control of weeds and reduced herbicide use.

2. Would promote use of environmentally more benign chemicals that are less likely to leach into ground water.

3. Farmers would get a greater option for weed control and more flexibility in the choice of crops for rotation or for double crop planting.

4. Environmental gains of GM crops: Three independent teams of CAST (Council of Agricultural Science and Technology) reviewed the available scientific literature to compare the environmental impacts of biotechnology derived and traditional crops.

The study involved various criteria and was based on herbicide tolerant soybeans, the most widely planted crop and further studies showed similar benefits for corn also. These are:

i. Soil quality: No till soybean acreage in US has increased significantly since the introduction of herbicide tolerant soybeans. Use of no till farming results in less soil erosion, dust and pesticide run off as well as increased soil moisture retention.

ii. Water quality: Use of biotechnology derived soybeans enables farmers to use a more benign herbicide that rapidly dissipates in soil and water.

iii. Air quality: Greenhouse gas emissions from some farm operations decreases by an estimated 88% as a result of biotech soybeans planted in a no-tillage system, which may help slow global warming.

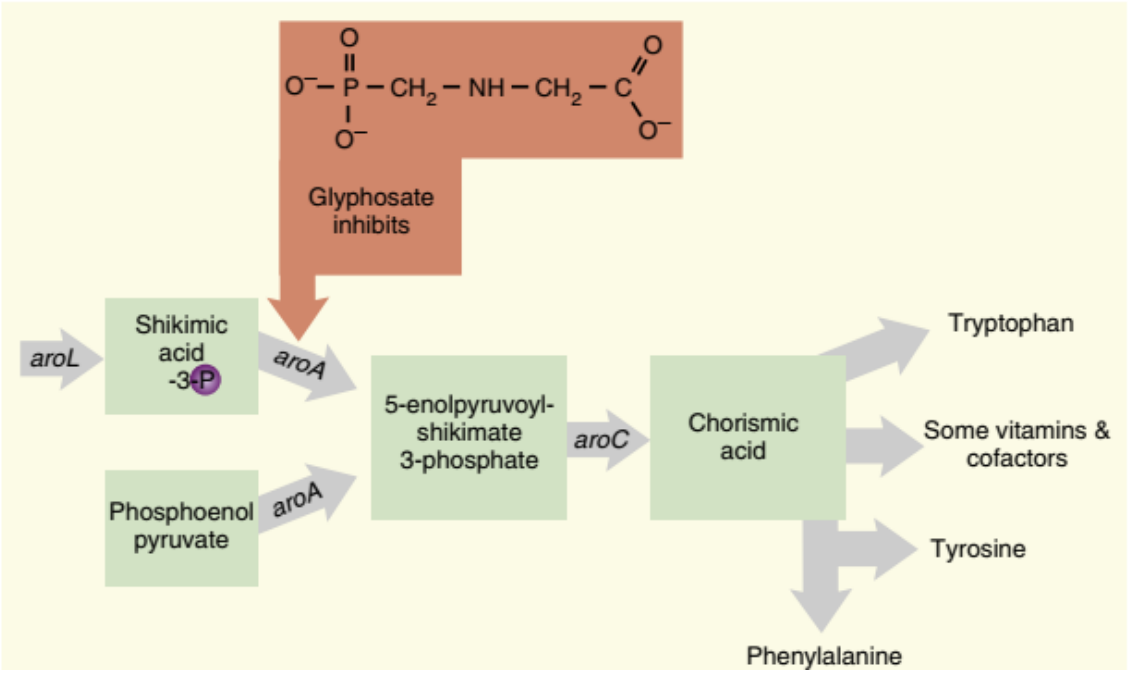
iv. Biodiversity: The no-till practices commonly associated with biotech soybeans provide a more favorable habitat for birds and other wildlife. No-tillage systems provide food and shelter for wild life such as pheasants and ducks.

v. Land use efficiency: Biotechnology derived soybeans may lead to increased yields through improved weed control and the adoption of narrow row spacing.

Technique for Herbicide resistance

Herbicides cost the world's farmers more than $14 billion each year. Despite this massive investment, around 10% of crops is lost due to weeds. One problem isthat many of the herbicides used do not discriminate between crops and weeds. One solution is to make the crops resistant to the herbicide by genetic engineering. Therefore, whenthe herbicide is sprayed on the weeds and crop, only the crop will survive.

One of the best herbicides on the market is glyphosate. Glyphosate is environmentally friendly because it quickly breaks down to nontoxic compounds in the soil. The glyphosate molecule is a phosphate derivative of the amino acid glycine. Glyphosate kills plants by blocking the synthetic pathway for the aromatic amino acids phenylalanine, tyrosine, and tryptophan by inhibiting one particular enzyme, EPSPS (5-enolpyruvoylshikimate- 3-phosphate synthase), which is the product of the aroA gene and localized to the chloroplast (Fig. 15.17). This target enzyme is found naturally in all plants, fungi, and bacteria, but not in animals. Aromatic amino acids are therefore essential to the diets of all animals, including humans, because those organisms cannot produce them. When glyphosate is sprayed onto plants, the herbicide penetrates the chloroplasts and binds to EPSPS, blocking the pathway for aromatic amino acids. The plant essentially starves to death.



Glyphosate Inhibits EPSPS in the Aromatic Pathway

The enzyme 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS) is the product of the aroA gene and makes 5-enolpyruvoylshikimate-3-phosphate, a precursor in the pathway to aromatic amino acids and cofactors. Glyphosate, an analog of phosphoenolpyruvate, inhibits EPSPS.

Developing herbicide resistance directly in plants is difficult, so scientists isolated a glyphosate-resistant EPSPS enzyme from bacteria. Mutant strains of Agrobacterium that are resistant to glyphosate can be directly selected by plating onto medium containing glyphosate. Such mutants produce an EPSPS enzyme that is resistant to glyphosate but still enzymatically active. A glyphosate-resistant version of the aroA gene was then cloned and modified for expression in plants. The bacterial promoter and terminator sequences were replaced with plant promoters and terminators. An antibiotic resistance gene was also added to the construct to allow for selection. Finally, because EPSPS is localized to the chloroplast, DNA encoding a small chloroplast transit peptide was added to the front of the gene. The chloroplast transit peptide, present at the N-terminus of the protein, targets EPSPS to the chloroplast but is cleaved off while crossing the chloroplast membrane.

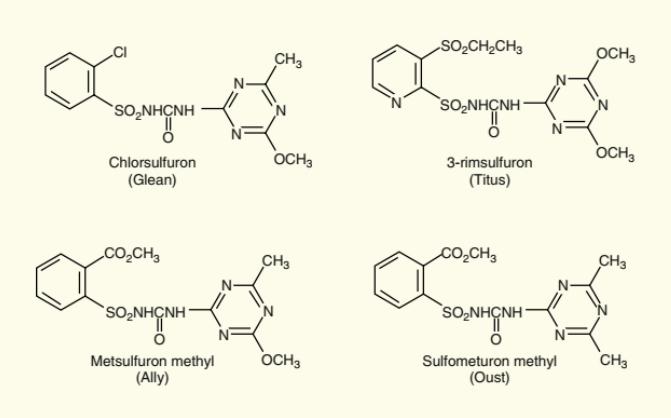
Expression of the Agrobacterium aroA Gene in Plants

The bacterial aroA gene must be placed under control of a promoter active in plants. Correct localization of the AroA protein (EPSPS) into the chloroplast requires addition of a chloroplast transit peptide at the N-terminus of the protein.

Only the functional enzyme enters the chloroplast. The glyphosate-resistant aroA gene from Agrobacterium has been transformed into several different crops, including soybean, cotton, and canola Comparison of the mutant bacterial aroA gene with the sensitive wild-type version revealed which amino acid changes were needed for glyphosate resistance. Because bacterial and plant aroA genes are homologous, equivalent

changes should result in glyphosate resistance in plant aroA genes as well. Indeed, this information allowed the aroA gene from corn to be engineered by altering its DNA sequence in vitro. The altered corn aroA gene provided glyphosate resistance after being introduced back into corn plants with the gene gun.

Other herbicide tolerance genes have been used to make transgenic crops, although these are not as widely used as the glyphosate-resistant aroA gene. For example, plants can be made resistant to sulfonylureas and imidazolinones, which inhibit an enzyme in the pathway that synthesizes the branched amino acids leucine, isoleucine, and valine.



Various Sulfonylureas and Their Trade Names Sulfonylureas can be used as herbicides

Plants resistant to these herbicides are quite common because resistance results from a single amino acid substitution in the appropriate enzyme. Another example is resistance to glufosinate, an herbicide that blocks synthesis of glutamine. Glufosinate was originally discovered as an antibiotic produced by Streptomyces. Scientists identified the enzyme that prevented Streptomyces from being poisoned by its own antibiotic and transformed it into crops.

Arguments against herbicide tolerant transgenics in crop plants

1. Use of herbicide tolerant transgenic crops can lead to transfer of herbicide tolerance genes to sexually compatible wild relatives or weeds, which can be a major potential threat to environment.
2. Transgenic crops can create ‘super weeds’.
3. It would actually increase the dependence on a few herbicides rather than reducing herbicide usage.
4. It may increase the problem of weed control if weeds develop resistance to such herbicides through gene flow from transgenic crops.
5. Herbicide tolerance is being sought not only for environmentally comparatively acceptable herbicides but also for older, more toxic and persistent products.
6. Non-chemical means of weed control, such as crop rotation, dense plantings, cover cropping, ridge tillage, and others, however labour intensive for farmers, are preferable than the use of any herbicide at all.
7. Gene flow is the primary risk in releasing transgenic plants.

However, genetically improved crops show no sign of turning into super-weeds according to a 10-year study by a team of scientists in Britain. Researchers planted genetically improved varieties of oilseed rape, potato, maize and sugar beet alongside conventional crops in 12 different habitats in the UK to see whether the plants could

invade natural habitats. But the research team found that native wild plants displaced both genetically improved plants and ordinary crops and that conventional crop actually outlived the biotech ones. Scientists said most of the crops died out after 4 years, and after ten years the only survivor was one type of non-biotech potato. When the study began, the kind of biotech plants available were varieties engineered to make them better able to withstand spraying with herbicides or insect attack, not crops engineered to withstand drought or natural pests. It was not expected that herbicide tolerance would give plant an ecological advantage. The results do not mean that other genetic modifications could not increase weediness or invasiveness of crop plants, but they do indicate that arable crops are unlikely to survive for long outside cultivation.