

Plant Physiology and Biochemistry

GENETIC ENGINEERING AND BIOTECHNOLOGY

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A. GENETIC ENGINEERING

Genetic engineering is a technique in which, a gene of interest isolated and cloned from any source e.g. virus, bacteria, plant or animal or even a synthetic or artificial gene sequence synthesized or modified in laboratories, is introduced to a host organism e.g. microbe, plant or animal, through a vector or through a physical facilitator, in which, it is expressed and produced a desired character. The target plant to be modified for the new character is the host in plant genetic engineering, in which the gene donors may be either of any source mentioned above (e.g. plant, animal, bacteria, virus, or a synthetic gene sequence prepared in laboratory). The technique used in the genetic engineering is often called as recombinant DNA technology (r- DNA technology).

Tools and Techniques of Recombinant DNA Technology: Recombinant DNA Technology (r-DNA Technology)-

Recombinant DNA technology, which is also called gene cloning or molecular cloning, is an umbrella term that encompasses a number of experimental protocols, leading to the transfer of genetic information (DNA fragments i.e. gene) from one organism to another. There is no single set of methods that can be used to meet this objective; however, a recombinant DNA experiment often follows the following steps.

Step 1:

A foreign DNA fragment (gene) from a donor organism is extracted, enzymatically cleaved (cut /digested) and joined (ligated) to another DNA entity (a cloning vector) to form a new, recombinant DNA molecule (cloning vector–insert DNA construct).

Step 2:

This cloning vector-insert DNA construct is transferred into and maintained within a host cell by a desired method. This process is called transformation.

Step 3:

Those host cells, which have successfully inserted the new DNA fragment in their genome (transformed cells), are identified and selected (separated / isolated), from those who have not been transformed by this effort.

Step 4:

The integration of foreign DNA in the host cells are ensured by various methods e.g. amplification by polymerase chain reaction (PCR), southern blotting of DNA against a known probe etc. and blotting of the protein product that is encoded by the cloned DNA sequence by the western blotting etc. It is also confirmed by northern blotting techniques which elucidate synthesis of mRNA to ensure the expression on the introduced foreign gene (s) in the transformed host cells.

Step 5:

The modification in the character of the transgenic plant (produced from the transformed cells), which is an outcome of the genetic engineering is verified and steps for the application /use of new product with its commercial, social, environmental health risk assessments and ethical aspects are established.

Molecular Cloning

Through several discoveries in the areas of molecular biology, nucleic acid enzymology and the molecular genetics of bacterial, virus and bacterial extra chromosomal DNA elements (plasmids), as well as of the other eukaryotic organisms, made it possible to develop recombinant DNA technology as such a revolutionary technique in the manipulating living organisms in desired manner. This technology would have not existed without the availability of enzymes (restriction enzyme; restriction endonucleases) that recognize specific double-stranded DNA sequences and cleave the DNA in both strands at these sequences.

Restriction Endonucleases

For molecular cloning of a foreign gene into a cloning vector, it is necessary to cut the DNA fragment at a specific site containing the target sequences, both in the source DNA that contain the largest sequences and in the cloning vector. The cut sites in the both kinds of DNA must be consistent for each time into discrete and reproductive fragments. Subjecting isolated DNA to passage through a small-bore needle or to sonication produces double stranded pieces of DNA that may range from 0.3 to 5 kilo basepair (Kb), in length, but these fragments are produced by the random breaking of DNA and each time we may end up with DNA with different sequences. So by these simple procedures we can't cut the DNA at desired site with the targeted sequences.

Box.1. Some simple facts about r-DNA Technology

Nucleic acid is an universal genetic material in all organisms.

DNA can be broken at specific desired positions by the restriction enzymes. (endonucleases) to isolate a specific segment and it can be inserted in another DNA molecule at a desired position (the product thus obtained is called recombinant DNA and the technique often called genetic engineering).

Using the technique, we can isolate and clone single copy of a gene or a DNA segment into an indefinite number of copies, all identical.

The discovery of bacterial enzymes, that cut DNA molecules internally at the specific base pair sequences, called type II restriction endonucleases, made it feasible to obtain DNA sequences of desired nature from a source DNA and to insert it in the genome of another organism between the enzymatic cut sites which can accommodate the new insert / foreign DNA. One of the first of these type II restriction endonucleases characterized from the bacterium *Escherichia coli*, and it was designated *Eco RI*. This enzyme binds to a DNA region with a specific palindromic sequence (the two strands are identical in this

region when either is read in the same polarity, i.e. 5' to 3') of 6 base pairs (bp) and cuts between the guanine and adenine residues on each strand.

Eco RI enzyme specifically cleaves the internucleotide bond between the oxygen of the 3' carbon of the sugar of one nucleotide and the phosphate group attached to 5' carbon of the sugar of the adjacent nucleotide. The symmetrical staggered cleavage of DNA by *Eco* RI produces two single-stranded, complementary cut ends, each with extensions of four nucleotides. Each single-stranded extension, in this case, ends in a 5' -phosphate group and the 3' – hydroxyl group from the opposite strand is recessed.

Eco RI type enzymes are not the only restriction endonucleases, which have been isolated and used for gene isolation and cloning. Hundred of other type II restriction endonucleases are known which have been isolated from the various bacteria. For naming them, as in *Eco* RI, genus of the source bacteria is the capitalized letter and the first two letters of the species name are in lowercase letters. The strain designation is often omitted from the name and roman numerals are used to designate the order of characterization of different restriction endonucleases from the same organisms. For example, *Hpa* I and *Hpa* II are the first and second type II restriction endonucleases that were isolated from *Haemophilus parainfluenzae*.

Plant Genomes; Genomic and cDNA Libraries

The genetic information which controls the entire function of a plant is stored in the form of a polymer called deoxyribonucleic acid (DNA), in the cells as in the other eukaryotes. The instructions that control all the activities of a plant are stored in the DNA as genes, which are the DNA sequences making the functional ribonucleic acid (RNA) and proteins. In plants, each gene codes for one protein or functional RNA, so each plant contain a large number of gene which vary species to species and genus to genus. The total amount of DNA in the nucleus of a cell, or in organelles, is called “the genome”.

In plant cells the genes may be organized in nuclei, mitochondria and chloroplasts. The nuclear genome is contained in large linear DNA molecules called chromosomes, which varies in size and number in different plant species, consequently the size of the genome also varies between the plant species (Table-1). The mitochondrial and chloroplast genome are, on the other hand, contained in the circular DNA in multiple copies in each organelle. Though the majority of genetic informations in green plants are contained in the nuclear genome, the mitochondria and the chloroplasts also share a significant amount of the genetic information that controls the functional biology of plants.

The significance of genome size and organization

The size of nuclear genome which represents an unreplicated DNA content (C-value) in the cells of organisms reflects the complexity of the organism. The genome of higher order organisms are generally bigger than those of lower order organism, for example, the C-value vary from $\sim 10^7$ to 10^{11} bp in eukaryotic organisms, having a trend of bigger size of genome in order of fungi, animals and plants as compared to bacteria.

Table 1. Genome size of various plants

Plant	Genome size (Mb)	Relative genome size compared with <i>Arabidopsis</i>
Arabidopsis	120-130	1
Rice	389-430	3.0
Maize	2500	20
Barley	5000	38
Wheat	15000-16960	128
Oilseed rape	1200	10
Garden pea	3947	33
Soybean	1115	9
Potato	840	14
Tomato	950	8
Source: Nancy Federspiel (2000) <i>Pl. Physiol.</i> 124:1456-1459, Slater, A. <i>et al.</i> (2003) <i>Plant Biotechnology</i> , Oxford University Press Oxford, <i>Nature</i> (2005); 436 (11 Aug 2005) pp 793-800, www//teosinte.agron. Missouri-edu/moulon.moulon.inra/imgd/www.staff.or.jp/		

However, this simple relationship does not always hold true, a situation known as ‘the C value paradox’. We can see that in higher plants, for example, plants of similar size and similar groups can have a genome size that vary by several orders of magnitudes (see rice and wheat in table 1), and many amphibian have C-values much larger than that of humans.

Surprisingly only a small percentage of the genome is known to actually encode proteins which lead to the development of a character in terms of function or structure. It means a vast majority of DNA components in a genome in certain organisms are either non-coding and apparently functionless or unrevealed yet by the known tools and techniques of plant biology and biotechnology.

Arabidopsis: A Model Plant for Understanding of Molecular Biology and Plant Biotechnology

Arabidopsis thaliana is a small dicotyledonous cruciferous weed plant that has been considered as a model plant due to its smaller genome and shorter life span, being a flowering plant, easy to handle and maintain in the laboratory conditions. Though it does not have commercial value as such, it belongs to the same family related to many important commercial oilseed crops e.g. Indian mustard and oilseed rape and vegetables e.g. cabbage, cauliflower and radish etc.

Its short life cycle, small structure and producing a large number of offspring are major characters that suited for genetic and mutational analysis. It has a good *in vitro* regeneration and genetic transformation ability and over all its smallest genome size (120-130 Mb) amongst the higher plants, made it a model plant and the first plant to have its complete genome sequenced in December, 2000.

The international /multinational collaborative ‘*Arabidopsis* Genome Initiative’ (AGI) began sequencing the genome in 1996. A total of 115.4 million bases were sequenced with a previously unmatched accuracy of between 99.999%. The remaining 10Mb represents repeats and / or difficult to sequence. The sequences are available in public domain for researcher and learners throughout the world as the entire sequence was achieved by publicly funded resources with global efforts. There are 25,498 genes in the *Arabidopsis* genome which are distributed on five chromosomes (1-5) of 29,105 (chr1), 19,647 (chr2), 23,173 (chr3), 17,550 (chr4) and 25,953 (chr5) Kbp length counting a total length of 115,410 Kbp (The *Arabidopsis* Genome Initiative, 2000, *Nature* 408:796-815). *Arabidopsis* genome is having more genes than some other multicellular eukaryotes (for example, a nematode, *Caenorhabditis elegans*, has about 19,000 genes and the fruit fly *Drosophila melanogaster* has about 13,600 genes) but less than 30,000 - 40,000 genes predicted for humans. It appears that *Arabidopsis* possess extensive gene duplication, as the total number of distinct protein types is only about 11,600, a similar number to that estimated for the nematode, *C. elegans* and the fruit fly *D. melanogaster*.

Functional analyses of *Arabidopsis* genes have shown that the 12% of them are associated with transcription, 3% with protein synthesis and 16% with metabolism. 7% of the genes are associated with signaling process, 3% with transport across the cells and another 6% with intracellular transport mechanisms. About 8% of the identified genes of *Arabidopsis* are associated with plant defense and another 8% are with growth. 7% genes are found to be associated with protein modification and about 30% have been left unclassified due to their unknown functions.

Rice, a monocotyledonous cereal is one of the most important food crops, which may differ from the model plant *Arabidopsis* (a dicot) in certain aspects of development and reproduction, next to *Arabidopsis* sequencing a major multinational effort (the IRSGP- the International Rice Genome Sequencing Project) to sequence the genome of rice has been another significant effort to studies on plant genomics, in which Indian laboratories (Department of Plant Molecular Biology Delhi University South Campus and NRC in Plant Biotechnology at Indian Agricultural Research Institute under the leadership of Prof. Akhilesh K. Tyagi and Dr. N. K. Singh) have also participated. The rice genome is reported to contain 37,544 genes on 389 Mb genome size the non-transposable genes only (International Rice Genome Sequencing; *Nature* (2005), 436:793-800), whereas maize contains 50,000 genes on 2500 Mb genome size (teosinte.agron.missouri.edu).

CLONING VECTORS

DNA cloning is a technique to produce large quantities of a specific DNA segment. The DNA segment to be cloned is first linked to a vector DNA, which is a vehicle for carrying foreign DNA into a suitable host cell, such as the bacterium *E.coli*. The vector contains sequences that allow it to be replicated within the host cell.

The r-DNA technology allows the cloning of random DNA or cDNA segments, often used as probes as well as cloning of the specific genes, which has either been isolated from the genome or synthesized in laboratory or obtained as cDNA from specific mRNA sequences.

The vectors; vehicles for genetic engineering

Genetic engineering become possible because vectors like plasmids and phages reproduce in a host (e.g. *E. coli*) in their usual manner even after insertion of foreign DNA; the inserted DNA also replicate faithfully with the parent DNA (The technique is called gene cloning and the vectors used for this purpose are called cloning vectors). Using a variety of cloning, gene can be isolated, cloned and characterized and new characters can be inserted vector beyond the taxonomic boundaries. The vectors can also manipulate the expression of the inserted genes in the host; expression vectors. Various kinds of vectors are available e.g. plasmids, (often used for cloning DNA segments of small size (upto 10 kilobases), phages (20-25 Kbp), cosmids (40-50 Kbp DNA segment), bacteriophage P1 system and F-factor based vectors (BACs= bacterial artificial chromosomes), YACs, MACs etc. can allow cloning of DNA segments, as large as 100 to 1000 Kbp (or 1 Mp=10⁶ bp) length (preferred when fragments bigger than 50-100 Kbp are to be cloned), phagemids (combine desirable features of both plasmids and bacteriophages), BACs and PACs (100-300 Kbp), YACs (100-2000 Kbp), MACs (mammalian artificial chromosomes (> 1000Kbp)).

Plasmids and vectors

Plasmids are self replicating circular (rarely linear) duplex DNA molecules, which are maintained in a bacterial cell, yeast cell or eukaryotic cell organelles e.g. chloroplasts and mitochondria in a definite number of copies (characteristic to the specific organism or organelle). The number can range from as small as 1 to as large as 1000 copies per cell. Plasmids are a preferable source as cloning vectors, due to their increased yield potential. The concept of cloning a foreign DNA segment in plasmid is as follows

A plasmid (pBR322) confers resistance to both ampicillin and tetracycline.

The restriction endonuclease enzyme can cut it at ampicillin site at which a foreign DNA can get inserted.

After insertion this foreign gene ampicillin resistance will be ineffective, whereas the tetracycline resistance will be maintained intact.

By the differential resistance capability of the plasmids wild type and recombinant type can be separated.

Plasmid vectors are often used for cloning segments of small size (upto 10 kilobases). Commonly used *E.coli* plasmid vectors are pBR322 and pBR 327 vectors. Some details of *Agrobacterium* plasmid vectors which are most widely used in plant transformation will be discussed later in this chapter. Under the subheading biotechnology

Lambda phage (λ) vectors

for preparing genomic libraries of the eukaryotes, cloning of larger DNA segments are required. Phage lambda (λ) vectors can permit cloning of 20-25 Kbp long segments. Working with phage lambda considered easier and more efficient for making genomic and cDNA libraries.

Cosmids as vectors

Cosmid vectors can also permit cloning of DNA segments upto 45 Kbp long. They are plasmid particles with cos sites, allow the packing of DNA into phage particles *in vitro*. Certain specific DNA sequences, those for cos sites are inserted easily into cosmids. It is highly efficient vector to produce a complete genomic library of 10^6 - 10^7 clones from a mere 1 µg of insert DNA.

Cosmids are unable to accept more than 40-50 Kbp of DNA, which can be facilitated by bacteriophage P 1 system and F-factor based vectors as described below.

Yeast Artificial Chromosomes (YACs)

YACs, are capable of accepting fragments of 100-2000 Kbp, most commonly used for human genome project, but following difficulties have been encountered while using YACs 1) In YAC libraries, a fraction of cloned genes result due to co-cloning events giving single clones with non-contiguous fragments. They are described as chimeric vectors. 2) YAC clones exhibit some degree of instability due to deletions/rearrangements in the cloned insrts. 3) YACs are similar to yeast normal chromosomes in size and thus making it difficult to separate them by simple method.

YACs can allow cloning of sequences that are several hundred Kbp (upto 1000 Kbp=1Mbp). A YAC vector mimics a chromosome because it has a sequence that acts as an origin of DNA replication (autonomous replicating sequence), a yeast centromere sequence, sequence that appear at both ends after linearization of the DNA and acts as chromosome telomeres to maintain the chromosome stability.

Bacterial Artificial Chromosomes (BACs):

In order to overcome the difficulties associated with YACs, bacterial cloning systems based on *E.coli* F-factor was designed, which was capable of cloning fragments of upto 300-350 Kbp. These were 'user friendly' being a bacterial systems, known as BACs and are superior to other bacterial systems, based on high to medium copy number of replicons. They show structural instability of inserts, deleting or rearranging portions of cloned DNA, the *F* factor has regulatory genes that regulate its own replicon and controls its copy number. these regulatory genes include 1) *ori S* and *rep E* which meiate unidirectional replication and 2) *par A* and *par B*, which maintain the copy number 1 or 2 per *E.coli* genome. These essential genes of *F* factor are incorporated in every BAC vector (pBAC), which also has a chloramphenicol resistance gene as a marker and a cloning segment.

Mammalian Artificial Chromosomes (MACs):

To clone large DNA segments in mammalian cells MACs, have been produced with the isolation of mammalian telomere and centromere. MACs are designed to be replicate, segregate and express in a mammalian cell like any other mammalian chromosome along with other chromosomes. Since it will be an independent chromosome, with all the functional elements (telomeres, origins of replication, centromere etc.), MAC will not be integrated with the genome and can be used as a vector maintaining a single copy per cell. It could carry large fragments of DNA (upto 1000 Kbp) representing an intact eukaryotic

split gene with exons and introns permitting its normal expression regulated by the associated promoter sequences. In April, 1997 issue of Nature Genetics, successful production of human artificial chromosome was reported. These human artificial chromosome (HACs) are 1/5th to 1/10th size of a normal human chromosome and are already being used for the study of regulation of gene activity and also for gene therapy.

Plant and Animal viruses as vectors

Cauliflower mosaic virus (CaMV), Tobacco mosaic virus (TMV) and Gemini viruses are those groups of plant viruses which have been used as vectors for cloning DNA segments. Due to their high potential of fast replication in the appropriate hosts, they can multiply the inserted foreign DNA very fast and in very large numbers of copies

A number of animal viruses are also used as vectors, either for the delivery of DNA into the host genome or for the fast and higher level amplification of foreign genes using the virus based promoters.

Transposons as vectors

Transposons are mobile DNA segments that are able to move and integrate throughout an organism's genome. Certain transposons of higher plants (e.g. Ac/Ds or Mn 1 of maize) and P element of *Drosophila* are the common transposons used as cloning vectors. Transposons possess short terminal repeats enclosing a long DNA segment containing the gene for transposase enzyme responsible for transposition. Part of this region can be deleted and the transposon can be used for cloning of foreign DNA segments as it occurs in other cases.

Genomic and cDNA Libraries

Genomic DNA is the genetic material of an organism stored in its genetic pool, whereas cDNA is DNA sequence derived from mRNA isolated from a specific metabolically active tissue of an organism. A mixture of clones each carrying DNA sequences derived either from the genomic DNA or from cDNA are called as genomic or cDNA libraries respectively. These libraries are constructed and used for various steps involved in r-DNA technology.

Genomic Library

Cloning of a complete genome as library of random genomic clones is also called as a shotgun experiment. In this protocol, genomic DNA is extracted and then broken into fragments of reasonable size by restriction endonucleases and subsequently inserted into a cloning vector to generate a population of chimeric vector molecule. The DNA fragments cloned in this manner are known as genomic library. Once prepared, the clones can be put into the plasmid vector and retrieved whenever required for various purposes e.g. identification and isolation of genes, source genes for genetic engineering, genetic studies etc.

Various restriction endonucleases can cut the fragments of varying sizes, which facilitate the fragmentation of genome for library making depending on the genome size and vector type. For a probability level of 99% that all the sequences are present in a genomic library

of a species about 1,500 cloned fragments are needed for *E.coli*, 4,600 for yeast, 48,000 for *Drosophila melanogaster* and 8, 00,000 for human being.

cDNA library from mRNAs

cDNA (complementary DNA) libraries are prepared by the help of activated mRNA, isolated from the cells actively synthesizing proteins (for example meristems, roots and leaves in plants). The cDNA is obtained as a reverse transcriptase induced copy of mRNA.

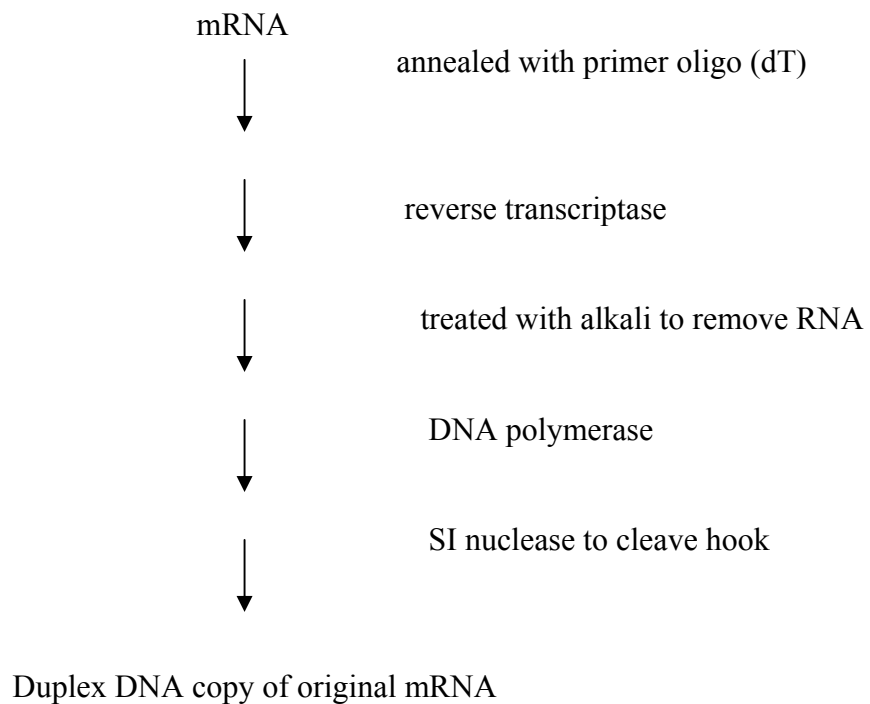
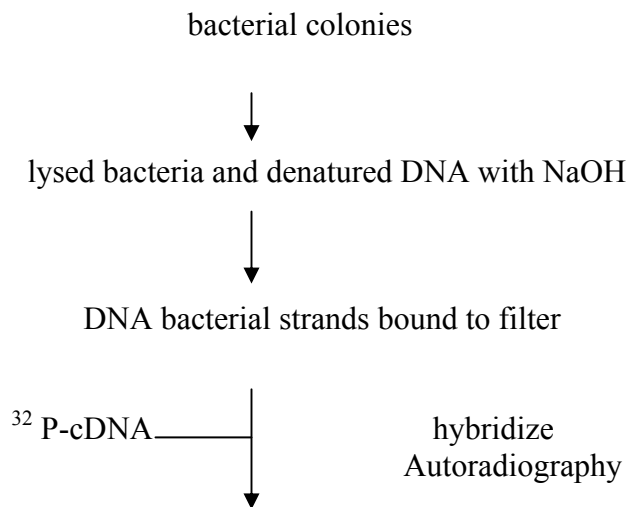


Figure 1 Schematic presentation synthesis of cDNA from mRNA, using reverse transcriptase enzyme.

Though cDNA molecules can be made double stranded (fig.1) it differ from genomic clones in lacking the introns present in split genes. The advantage of cDNA libraries is being capable to be expressed in bacteria, which do not have the machinery to process the eukaryotic split gene Hn RNA into mRNA.

Screening of genomic and cDNA libraries

These libraries can be processed with colony hybridization technique (fig . 2) for isolation of a gene sequence.



Specific colonies with DNA sequences related to radioactive cDNA probe

Figure 2. Colony hybridization technique for selection and isolation of DNA fragment having sequence complimentary to a radioactively labeled probe.

Transposable Elements and Gene Walking

A transposable element (TE) is a DNA sequence that is able to move and integrate throughout an organism's genome. In contrast to homologous recombination processes that require at least some degree of sequence homology. Thus, the mechanism of integration of TE into chromosomes are considered as non homologous recombination and is highly useful in r-DNA technology.

Discovery of transposable elements began in the 1940s with the experimental work of Marcus Roades and Barabara McClintok during their classical work on maize genetics. They indicated that genomes may contain unstable and possibly mobile components as they found the appearance of unexpected phenotypes amongst the progeny of certain strains of maize. Later it was confirmed in bacteria and higher organisms that such unusual genetic results are consequence of the insertion of mobile DNA pieces, known a transposable elements (also called as jumping genes).

Though the findings of Roades and McClintock was the first clear indication that movable DNA sequences existed in any genome, the first evidence for occurrence existed in any genome, isolation and characterization of transposable element was obtained from *E.coli* after development of molecular techniques up to the late 1970s.

Table 2 Some Transposable elements (Insertion Sequences; IS) in *E. coli*

Element name	Length (bp)	Size of direct repeats at target site (bp)	Number in typical <i>E. coli</i> strain
IS 1	768	9	5-8
IS 2	1327	5	5
IS 4	1428	11 or 12	1-2
IS 5	1195	4	
IS 10 R	1329	9	
IS 903	1057	9	

Insertion sequences are the simplest class of bacterial transposable elements encoding only a transposase. This enzyme function to excise the IS element from its existing chromosomal site and splice it into a new chromosomal position within the host chromosome.

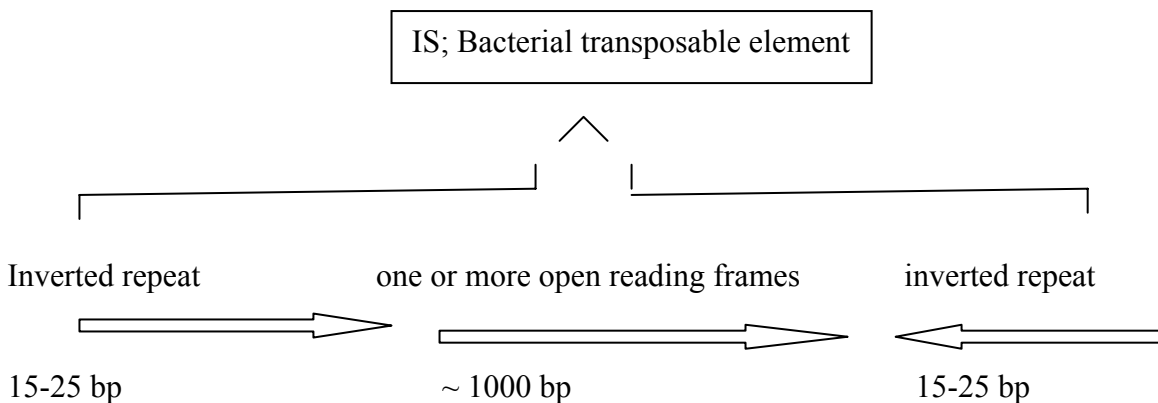


Figure 3 Structure of a bacterial insertion sequence (the simplest transposable elements).

Chromosome Walking

During probing a gene sequence in a genomic library, the probe may hybridize with a number of clones, each carrying a part of large gene fragmented during preparation of genomic library. The partial digests may give fragments with overlapping sequences, because sites cleaved in different genomes of the same organisms will differ being random. These overlapping sequences may be used to construct the original genomic sequence by the technique of chromosome walking.

This technique involves following steps.

a clone of interest (identified by a probe) is selected from the genomic library and a small fragment is subcloned from one end of the clone.

The subcloned fragments of the selected clone is hybridized with other clones in the library and a second clone hybridizing with the second clone of the first clone is identified due to the presence of overlapping region.

The end of second clone is subcultured after that and used for hybridization with other clones to identify a third clone having overlapping region with the subcloned end of the second clone.

The third clone identified is also subcultured and hybridized with other clones in the same manner and procedure may go on.

The restriction maps of selected overlapping clones may be prepared and compared to know the region of overlapping.

The identification of few overlapping restriction sites will amount along the chromosome or along a long chromosome segment.

BIOTECHNOLOGY

Functional Definition:

Various attempts have been made to define biotechnology, however, no one can claim to derive a complete definition of biotechnology due to its wide integration of techniques, disciplines and usage. It ranges from fermentation to process modification, to high precision commercial drugs and biochemicals, to agroproducts as well as waste management and so on. It involves simple adsorption, fermentation, shoot tip culture to recombinant DNA technology, genomics and proteomics. Some authoritative agencies/groups have defined biotechnology with their own words. A few placed below as examples:

US National Science Foundation defines biotechnology as the controlled use of biological agents, such as microorganisms or cellular components, for beneficial use;

Office of Technology Assessment of the United States Congress defines biotechnology as “any technique that uses living organisms or substances from these organisms, to make or modify a product, to improve plants or animals, or to develop microorganisms for specific uses;

European Federation of Biotechnology define biotechnology as “the integrated use of biochemistry, microbiology and engineering sciences in order to achieve technological (industrial) application of the capabilities of microorganisms, cultured tissue cells and parts thereof;

British biotechnologists have given a definition of biotechnology as “the application of biological organisms, systems or process to manufacturing and service industries; whereas *Japanese biotechnologists* define it as a technology using biological phenomena for copying and manufacturing various kinds of useful substances;

A book entitled “Biotechnology: Building on Farmers’ Knowledge 1996” define biotechnology as “the application of indigenous and/or scientific knowledge to the management of (parts of) microorganisms, or of cells and tissues of higher organisms, so that these supply goods and services of use to human beings, emphasize on significance of indigenous knowledge for betterment and also the rights of tribals and farmers in the

regime of intellectual property rights and patent laws, which has a large share in biotechnology.

A functional definition can be drawn from these various perceptions as simple as “uses of living forms, natural or modified for obtaining better medicines, high quality and cost effective industrial products, qualitatively and quantitatively improved food and a clean environment for sustainable and comfortable human life something more technically defined and detailed to cover various processes, products and usage at one place. The students may try to develop their own functional definitions in the way and words they want to perceive biotechnology with the perspectives described above and in this chapter.

Plant Tissue Culture; Basic Aspects

The Major Events; A Brief Historical Perspective

The first event which led to the beginning of plant cell and tissue culture is the classical work of a German Botanist Gottlieb Haberlandt (1854-1945), who is regarded as the father of plant tissue culture. Haberlandt was the first person to culture isolated, fully differentiated cells of many plants as early as in 1898, which was presented in 1902 to the scientific community. Little progress was made in cell culture research for up to three decades after the pioneering work of Haberlandt. The first successful report of continuously growing cultures of tomato root tips was made by Philip R. White (1901-1968) in 1934. White added three B-vitamins namely pyridoxine, thiamine and nicotinic acid in place of yeast extract along with inorganic salts and sucrose in his culture media (White, 1937). The two important discoveries made the mid 1930s significant in development of plant tissue culture techniques were 1) use of auxin as a natural growth regulator and 2) application of B-vitamins in the culture media. Two groups 1) Roger J. Gautheret and 2) White with Nobecourt independently reported the establishment of continuously growing cultures of carrot in the same year in which an auxin(IAA) was also used in addition to B-vitamins, inorganic salts and sucrose. The induction of divisions in isolated mature and differentiated cells were reported by Skoog in (1944) and by Skoog and Tsui (1951) by addition of adenine (a cytokinin) in tobacco pith tissue cultures. By early 1960s, the method of *in vitro* cultures of plant cells, tissues and organs were reasonably well developed. i.e. inorganic salts, B-vitamins Though many other substances, in addition to the basic media components, auxins and cytokinins, e.g. certain amino compounds e.g. polyamine, and L-proline, ethylene inhibitor silver nitrate, herbicides with strong auxin like activity; 2,4-D, 2,4,5-T, defoliant like thidiazuron (TDZ) etc. have been recognized time to time to regulate various stages and pathways of *in vitro* morphogenesis in plants, many species and cultivars of *in vitro* morphogenesis in plants are yet considered as recalcitrant as do not effectively respond to the known regeneration stimuli. The adequate understanding of signaling system and molecular mechanisms precisely involved in cell division, differentiation and regeneration of plants by a specific morphogenetic pathway are yet to be elucidated though many up-regulated genes and gene products involved in the process have been recently identified.

During 1980 onwards, plant tissue culture research became popular again due to the renewed emphasis on plant genetic engineering which is a strong technique to manipulate plants for new characters do not exist within the taxonomic boundaries(within the sexually compatible species), and can not be introduced or improved by the conventional plant

breeding. *In vitro* regeneration of genetically engineered plant cells or tissues is a prerequisite for the success of the plant genetic engineering protocols.

Totipotency

The ability of plants to regenerate from a single somatic cell or tissue pieces (explants) in *in vitro* conditions in test tubes or other culture vessels in the artificially controlled laboratory conditions occurs possibly due to their plasticity and totipotency. Plants due to their sessile (non-mobile) nature and long life span survive under the extreme conditions than that in animals. Many of the growth and development related plant processes are regulated by the environmental conditions, showing a plasticity that allows plants to alter their metabolism, growth and development. To best suit to the survival in a pertaining environment. The capability initiate cell division from almost any tissue of the plant and to regenerate lost organs or undergo different developmental pathways in response to particular stimuli, have been particularly significant in achieving regeneration of a whole plant from a somatic cell/tissue in the culture vessels providing appropriate chemical and environmental signals. When plant cells and tissues are cultured *in vitro* they generally exhibit a very high degree of plasticity, which allows one type of cell, tissue or organ to redifferentiate into another type. In this way, whole plants can be subsequently regenerated.

This potential of regeneration of a complete plant from any kind of plant cell or tissue, given the correct stimuli, which can express the total genetic potential of the parent plant, as a concept, is known as totipotency. (Figure 4) “Totipotency” refers to this maintenance of genetic potential *in toto*. Plant cell culture and regeneration do, in fact, provide the most compelling evidence for totipotency. Identifying the adequate culture conditions and correct stimuli required to manifest this totipotency from each somatic cell in each plant can be practically extremely difficult and it is still a largely empirical process, till we are able to reveal the molecular signals and mechanisms involved in each step of differentiation and growth with all the possible interactions of external stimuli, tissue metabolites and environmental conditions. Though a great success has been achieved in developing the successful protocols for *in vitro* regeneration and genetic transformation in several plants, a long way still to go to achieve 100% totipotency in plants.

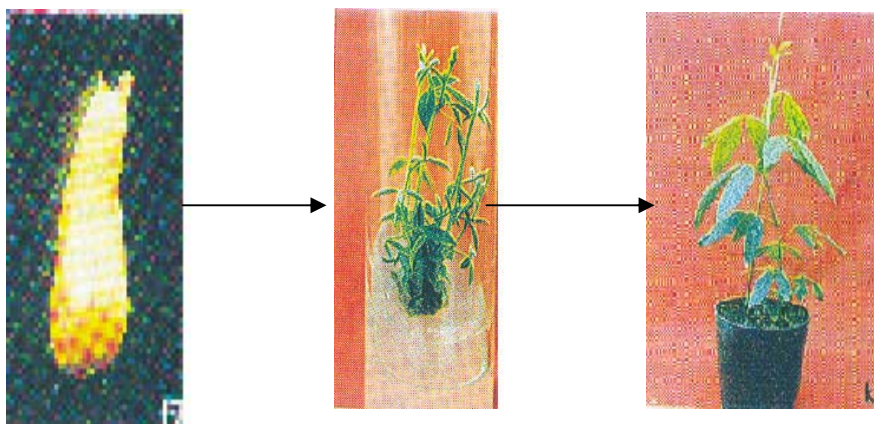


Figure 4: The regeneration of totipotency *in vitro*

Certain Common Terms Used in PTC

Explant- Though in principle, a somatic cell can regenerate a whole plant, generally an organ or a piece of differentiated tissue, meristematic or non-meristematic is used as a source of inoculation. This piece of differentiated tissue is known as explant. For example, a piece of cotyledon, or entire cotyledon, petiole tissue, epicotyle, hypocotyle, leaf discs, immature and mature embryos, cotyledonary node, shoot tip, axillary node etc. can be used as explant for initiating a tissue culture study.

Callus- A mass of undifferentiated cells which are derived in certain cases of *in vitro* morphogenesis as an intermediary phase in which the already differentiated tissue achieve a meristematic state by the reversion of mature cells is called callus.

Dedifferentiation- Production of callus from mature fully differentiated explant cells is called dedifferentiation.

Redifferentiation- The cells of an explant, given to an appropriate stimulus may directly produce shoot buds or somatic embryos which can subsequently develop into a whole plant or it can enter into a callus phase first by the process of dedifferentiation and subsequently develop shoot buds or somatic embryos leading to the formation of an entire plant from the callus is called redifferentiation.

Laboratory Requirements for Plant Tissue Culture

Laboratory space

Tissue culture laboratory needs:

Media preparation room to wash and store glass wares, plastic wares, chemicals, water purification system, and an autoclave. This room should be furnished with the working benches, a laminar air flow cabinet, a deepfreeze, a refrigerator, a hot plate-cum-magnetic stirrer, a pH meter, weighing balance and a vacuum pump and a bench centrifuge.

A culture room, with controlled temperature, diurnal illuminations in racks where culture vessels can be placed and humidity control is required. The room temperature is maintained by air-conditioners (in summers) and heat blowers (in winters), attached to thermostat controller to maintain a temperature around $25 \pm 2^{\circ}\text{C}$ throughout. The cultures are generally grown in diffuse light (less than 1 Klx), however, provisions may be made for maintaining higher light intensities (5-10 Klx), and total darkness in certain racks for specific experiments. Diurnal control of illumination of lamps can be achieved by automatic time-clock devices. A relative humidity between 50-60% is required to maintain the cultures more properly. The area of culture room should be clean and should not have any contact with the outside. The paints on the walls and the flooring should be well suitable for the regular cleaning and being dust free. Air curtains and /or double door systems should be preferred for a tissue culture room.

An acclimatization room along with a green house are required for initial acclimatization of the tissue culture derived plants, and then for maintaining them for the initial phase of life cycle in a semi controlled environment of the green house. Later, either these plants can be maintained in the green house for entire growth phase (especially in case of transgenic plants or elite species, cultivars for the first generation and seeds can be used

for field plantation or can be transformed to the normal field conditions in case of common micropropagation/vegetatively cultivated plants. The acclimatization room needs to have controlled temperature ($25 \pm 2^{\circ}\text{C}$), diffused light with diurnal control (light: dark cycle) system and a higher relative humidity (of around 70-80%). The green house should have a microenvironment with semi controlled temperature ($30-35^{\circ}\text{C}$) maintained by wet pad panels or air circulation devices, a relative humidity of about 60-70%, maintained by sprinklers and diffused light usually as per the seasonal variations (avoiding extremes). The controlled green houses are also becoming popular to cultivate plants of elite properties (in isolation) as well as of commercial value in the off seasons for higher sale values.

Plant Cell and Tissue Culture Media

The culture media for the *in vitro* cultivation of plant cells are composed of three basic components.

- a) Essential nutrients supplied as inorganic salts (macronutrients, micronutrients and an iron source)
- b) An organic supplement e.g. vitamins, amino acids, polyamines etc.
- c) A source of carbon; usually sucrose
- d) A gelling agent

For solid media only and not in liquid media used for certain experiments e.g. cell suspension cultures. Agar-agar is the most commonly used gelling agent e.g. one of the agar-agar, Merck agar, Bacto agar, Phyta agar, TC agar, Bitek agar, gelrite (gelrite gellan) etc.

The various components of the inorganic salts perform different functions in plant growth and development (Table 3 & 4). Though the initial widely used media were Gautheret medium (1939) and White's medium (1943), which were evolved from the Knop's (1865) salt solution and Upenski and Upenskaia's medium (1925) for algae respectively, many other workers kept modifying the composition of various media components to suit the culture differentiation and growth in different plants. Different genotypes and even tissues from different parts of a plant may have different requirements for satisfactory growth a medium formulated by Murashige and Skoog (1962), (Table) popularly known as MS medium has widely been used by the plant tissue culture scientists worldwide with minor modifications/additions. Though the concentrations of media constituents have been expressed as mg l^{-1} , ppm and μg , μmol etc, the International Association for Plant Physiology has recommended the use of mole values.

Box 2.

Mole is an abbreviation for gram molecular weight, which is the formula weight of a substance in grams, which is equal to the sum of weights of the atoms in the formula of a substance. One liter of solution containing one mole of a substance can be presented as 1M or 1mol/L = 1000 or 10^3 m mol/L = 1000000 or 10^6 μ mol/L. The association recommends to use m mol/L for macronutrients and organic nutrients and μ mol/L for micronutrients, plant growth regulators, vitamins and other constituents used in very little quantity. Use of mole values are advantageous as the number of molecules per mole is constant for all compounds

Sterilization of Media and Explants:

Cleanness and sterilization are amongst the prime concerns maintained throughout for the tissue culture laboratory and experiments. The glasswares and plastic wares are washed thoroughly by detergents, rinsed with tap water and then with distilled water.

The culture media, distilled water and all the glasswares, plastic wares and other autoclavable tools are autoclaved at 121°C for about 25-30 min. (if autoclave is not available, a big pressure cooker may be used). Certain compounds, for example, growth factors, such as GA₃, zeatin, ABA, amino acids, polyamines, urea etc (which are thermolabile) should not be autoclaved but sterilized separately by membrane filtration. The culture media excluding the heat labile compounds are autoclaved in a flask and kept in the sterilized hood to cool down. The thermolabile substances to be added in the culture media (already autoclaved) after the filter sterilization of the solution. The bacteria-proof filter membranes of pore size 0.45 μ m or less are used. The sterilized membranes are fitted into filter holders of appropriate size and by help of a sterilized syringe (without needle), the solution to be filtered, is pushed through the membrane carefully within the laminar hood. The sterilized membranes fixed in the sterilized holders are placed at the place of needle in the syringe. The sterilized solution dripping out from the filter is added to the cooled medium preferably at the room temperature in the desired quantity.

The plant materials (explant) are surface sterilized using any one or two of the (70%) ethanol, calcium(9-10%) or sodium hypochlorite(0.2-2%), hydrogen peroxide (10-12%), bromine water (1-2%), silver nitrate (1%), mercuric chloride 0.1-1%, antibiotics 4-5-mgl⁻¹ etc. Most often the explant is rinsed with 70% alcohol for about 5-20 min with continuous stirring provided often by magnetic stirrer followed by a rinsing with (0.3-0.6%) sodium hypochlorite for 15-30 min. The sterilization time is generally decided based on the size and softness of the explant and penetration efficiency/toxicity of the surface sterilizing agent. It should be optimized for maximum removal of the surface microbes, keeping in view that a minimum damage occur to the explant due to the toxicity of antimicrobial agent to the plant tissue.

The Culture Environment

For *in vitro* cultures, all the needs of the differentiating plant cell both chemical and physiological, have to meet by the culture vessel, the growth medium and the external environment (light, temperature, etc.). The growth medium has to supply all the essential mineral ions required for growth and development since all the biosynthetic capability of cells cultured *in vitro* may not replicate that of the parent plant, supply of additional

organic supplements such as amino acids and vitamins are also often required. The plant cell cultures, being non photosynthetic to certain stages, also require the addition of a fixed carbon source in the form of a sugar (most often sucrose). One other vital component that must also be supplied is water, the gaseous environment and light in defined quality and duration. The osmotic pressure also should have to be maintained within acceptable limits.

Table-3. Function of certain elements used as inorganic salts in culture media

Element	Function
Nitrogen	Required for biosynthesis of amino acids, proteins, nucleic acids and some coenzymes etc.
Potassium	Regulates osmotic potential, used in certain metabolic activities
Calcium	Cell wall synthesis, membrane function, cell signaling
Magnesium	Enzyme cofactor, component of chlorophyll
Phosphorus	Component of nucleic acids, energy transfer, component of intermediates in metabolic activities including respiration and photosynthesis
Sulphur	Component of some amino acids (methionine, cysteine), antioxidants(glutathione), chelators(metal chelators;phytochelatins) and some cofactors
Chlorine	Required for photosynthesis
Iron	Electron transfer as a component of cytochromes
Manganese	Enzyme cofactor
Cobalt	Component of some vitamins
Copper	Enzyme cofactor, electron-transfer reactions
Zinc	Enzyme cofactor, chlorophyll biosynthesis
Molybdenum	Enzyme cofactor, component of nitrate reductase

Table- 4. Composition of one of the most widely used tissue culture medium (MS-medium)^a

Essential Components	Concentration in Stock Solution(mg/L)	Concentration in medium (mg/L)
<i>Macronutrients</i> (50ml of stock solution used per litre of medium)		
NH ₄ NO ₃	33000	1650
KNO ₃	38000	1900
CaCl ₂ .2H ₂ O	8800	440
MgSO ₄ .7H ₂ O	7400	370
KH ₂ PO ₄	3400	170
<i>Micronutrients</i> (5ml of stock solution used per litre of medium)		
KI	166	0.83
H ₃ BO ₃	1240	6.2
MnSO ₄ .4H ₂ O	4460	22.3
ZnSO ₄ .7H ₂ O	1720	8.6
Na ₂ MO ₄ .2H ₂ O	50	0.25
CuSO ₄ .5H ₂ O	5	0.025
CoCl ₂ .6H ₂ O	5	0.025
Iron Source ^c		
FeSO ₄ .7H ₂ O	5 560	27.8
Na ₂ EDTA.2H ₂ O	7 460	37.3
<i>Organic Supplement</i> (5ml of stock solution used per litre of medium)		
Vitamins* and Amino acid		
Myoinositol*	20 000	100
Nicotinic Acid*	100	0.5
Pyrodoxine-HCL*	100	0.5
Thiamine-HCL*	100	0.5
Glycine	400	2
<i>Carbon Source</i> (Added as solid)		
Sucrose	Added as solid	30 000

Based on Murashige,T. and Skoog F., 1962) *Physiol. Plant.*15:473-497;Slater, A., Scott, N. and Fowler M (2003), *Plant Biotechnology* (Oxford University Press, Oxford, U K, p-38)

^a Many other commonly used plant tissue culture media (such as Gamborg's B5 and Schenk and Hildebrandt (SH) medium) are similar in composition to MS medium and can be thought of as high-salt media. MS is an extremely widely used medium and forms the basis for many other media formulations.

Media Components

Macronutrients

Supplies of certain nutritional elements are required in large amounts for plant growth and development. Nitrogen, phosphorus, potassium, magnesium, calcium and sulphur and

carbon (which is added separately) are usually regarded as macronutrients (Table 3 & 4). These elements usually comprise at least 0.1% of the dry weight of plants.

Micronutrients

These elements are required in trace amounts for plant growth and development, and have many and diverse roles (Table 3 & 4). Copper, cobalt, boron, molybdenum, iron, manganese and iodine etc. though other elements such as nickel and aluminium are frequently found in most of the media formulations.

Organic supplements

The two vitamins, thiamine (vitamin B1) and myoinositol (considered a B vitamin) are considered essential for the culture of plant cells *in vitro*. However, other vitamins are often added to plant cell culture media though their role need to be examined carefully.

Amino acids are commonly included in the organic supplement. The most frequently used one is glycine. Arginine, asparagines, aspartic acid, alanine, glutamic acid, glutamine and proline are also used in specific cases. Amino acids provide reduced nitrogen and, like ammonium ions, its uptake causes acidification of the medium. Casein hydrolysate can be used as a relatively cheap source of amino acids. L-proline and polyamines e.g. putrescine, spermidine and spermine etc. have recently been found beneficial for *in vitro* differentiation and development in many cases.

Carbon source

Sucrose is cheap, easily available, readily assimilated and relatively stable and is therefore the most commonly used carbon source. Other carbohydrates (such as glucose, maltose, galactose and sorbitol) can also be used and in certain circumstances may prove superior to sucrose.

Gelling agents

Media for *in vitro* plant cell culture can be used in liquid or 'solid' forms, depending on the type of culture being grown. For the culture types which require the plant cells or tissues to be grown on surface of the medium, it must be solidified (gelled). Agar, produced from seaweed, is most common type of gelling agent, and is ideal for routine applications. For more range of purity more expensive gelling agents are available. Purified agar or agarose or a variety of gellan gums are available in the market.

Plant growth regulators

Plant growth regulators are the critical media components in determining the differentiation of explants and the developmental pathway of the plant cells and tissues *in vitro* (as well as *in vivo*). The plant growth regulators used most commonly in plant tissue culture are plant hormones or their synthetic analogues.

There are five main classes of plant growth regulator used in plant cell culture, namely: auxins; cytokinins; gibberellins; abscisic acid and ethylene.

Auxins

Auxins promote both cell division and cell growth. The most important naturally occurring auxin is IAA (indole-3-acetic acid), but its use in plant cell culture media is limited because it is unstable to both heat and light. The amino acid conjugates of IAA (such as indole-acetyl-L-alanine and indole-acetyl-L-glycine, which are more stable, are also used in certain cases. 2,4-Dichlorophenoxyacetic acid (2,4-D) which is also used as a herbicide is the most commonly used auxin and is extremely effective in most circumstances. Other auxins are 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), Dicamba (2-methoxy-3,6-dichlorobenzoic acid), IBA (Indole-3-butyric acid), MCPA (2-methyl-4-chlorophenoxyacetic acid), NAA (1-naphthoxyacetic acid), Picloram (4-amino-2,5,6-trichloropicolinic acid) and some may be more effective or 'potent' than 2,4-D in some instances.

Cytokinin

Cytokines also promote cell division. Naturally occurring cytokinins are a large group of structurally related (purine derivatives) compounds. Naturally occurring cytokinins, used in plant tissue culture media are zeatin and 2iP (2-isopentyl adenine). They are relatively expensive (particularly zeatin) and therefore used less frequently. Non-purine-based chemicals, such as substituted phenylureas can also substitute for cytokinin in some culture systems. 6-benzyl amino purine (BAP) is most commonly used cytokinin in plant tissue culture.

Gibberellins

Numerous, naturally occurring, structurally related compounds termed 'gibberellins', are involved in regulating cell elongation, and are agronomically important in determining plant height and fruit-set. Only a few gibberellins are used in plant tissue culture media, GA₃ being the most common.

Abscisic acid

Abscisic acid (ABA) inhibits cell division. It is most commonly used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis especially for the maturation of somatic embryos.

Ethylene

Ethylene is a gaseous, naturally occurring, plant growth regulator most commonly associated with controlling fruit ripening in climacteric fruits, and its use in plant tissue culture is not widespread. Some plant cell cultures produce ethylene, which, if it builds up sufficiently, can inhibit the growth and development of the culture. The type of culture vessel and its means of closure affect the gaseous exchange between the culture vessel and the outside atmosphere and thus the levels of ethylene present in the culture. Silver nitrate an inhibitor of ethylene production is, sometimes, beneficial for plant tissue cultures. Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and are usually used together. The ratio of the auxin to the cytokinin determine the differentiation of the regenerants in vitro shoots, roots, callus, somatic embryos etc. A high auxin to cytokinin ratio generally favours root formation, whereas a high cytokinin to auxin ratio favours shoot formation. An intermediate ratio favours callus production.

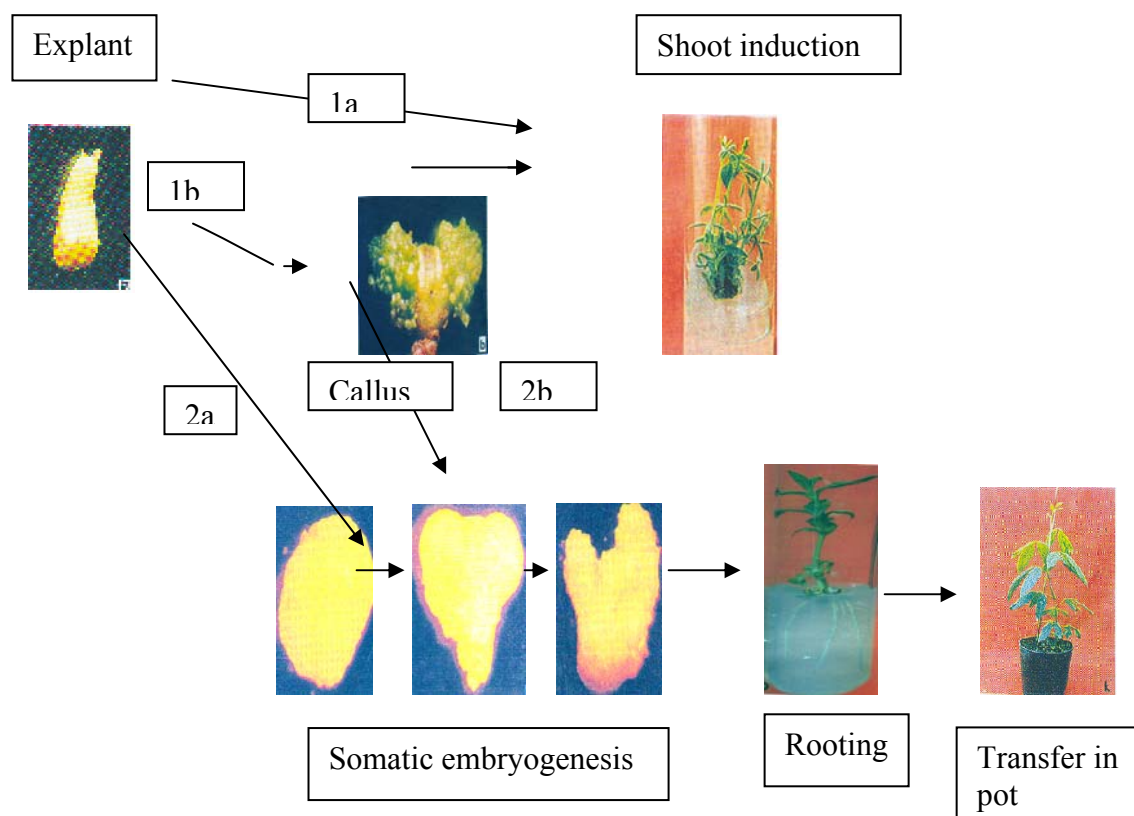


Figure 5. A summary of *in vitro* regeneration of plants via organogenesis (both direct and indirect; 1a&1b) and somatic embryogenesis (both direct and indirect 2a& 2b).

Cultures are raised from explants (sterile pieces of a whole plant) e.g. leaves or roots, or may be specific cell types, such as pollen or endosperm. Younger, explant and more rapidly growing tissue (or tissue at an early stage of development) are considered most effective for differentiation *in vitro*.

The plant tissue cultures are of various types. A suitable culture type is maintained for specific purposes e.g. micropropagation, somatic hybridization, haploid production, genetic transformation or production of commercial metabolites etc.

Callus

Explants, when cultured on the medium supplemented with an auxin and a cytokinin, or other induction stimuli give rise to an unorganized, growing and dividing mass of cells. In culture this proliferation can be maintained more or less indefinitely, by subculturing callus onto fresh medium periodically. There is some degree of dedifferentiation (i.e. the changes that occur during development and specialization are, to some extent, reversed), both in morphology (callus is usually composed of unspecialized parenchyma cells) and metabolism during callus formation. One major consequence of this dedifferentiation is that most plant cultures lose the ability to photosynthesize. This requires the addition of other components-such as vitamins and, most importantly, carbon source-to culture medium, in addition to the usual mineral nutrients. Callus culture can be performed in the dark (the lack of photosynthetic capability being no drawback) since light can encourage

differentiation of the callus. For long term maintenance of callus cultures induction factor e.g. auxin/cytokinin etc can be avoided.

A change in auxin to cytokinin ratio in the medium may lead subsequently to the development of shoots, roots or somatic embryos from which whole plants can be produced via an intermediary callus phase. Such kind of *in vitro* morphogenesis is known as indirect or callus mediated morphogenesis. Callus cultures can also be used to initiate cell suspension, which are used in plant transformation studies and for producing commercially valuable metabolites from plant cell cultures.

Cell Suspension Cultures

Callus cultures, can be compact or friable. In compact callus the cells are densely aggregated, whereas in friable callus the cells are loosely associated and the callus is soft and breaks apart easily. Friable callus provides the inoculum to form cell-suspension cultures. Explants from some plant species or particular cell types do not form friable callus, making cell-suspension initiation a difficult task. The friability of callus can be improved either by manipulating the medium components, or by repeated subculturing or by culturing it on 'semi-solid' medium (medium with low concentration of gelling agent).

When friable callus is cultured onto a liquid medium (the same composition) with continuous agitation, the cells released grow and divide, can produce a cell-suspension culture. An adequate volume/size of inoculum is optimized to initiate adequately growing cell suspension cultures in required amount.

Protoplasts for Culture

Protoplasts, plant cells without the cell wall are most commonly isolated from either leaf mesophyll cells or cell suspensions. Two general methods are employed to remove the cell wall without damaging the protoplast 1) Mechanical isolation 2) Enzymatic isolation.

1) Mechanical isolation involves plasmolysis of plant cell and removal of the cell wall with the help of knife. It results in low yields, poor quality and poor performance in culture due to substances released from damaged cells.

2) Enzymatic isolation of protoplast is usually carried out in a simple salt solution with a high osmoticum, plus the cell wall degrading enzymes (pectinase + cellulase). The isolated and purified protoplast can be used for genetic manipulation e.g. genetic engineering or somatic hybridization and a successful product can be subjected to regeneration into whole plant with a modified character. The regeneration of plants from the naked protoplasts has been found, however, extremely difficult.

Root Cultures

Root cultures can be established *in vitro* from explants of the root tip of either primary or lateral roots and can be cultured on simple media. The growth of roots *in vitro* is potentially unlimited, as roots are indeterminate organs. Root cultures are often does not produce shoots or somatic embryos and thus are rarely used in plant tissue cultures except for the use of transformed roots (hairy roots) for metabolite production.

Shoot Tip and Meristem Culture

The tips of shoots (which contain the shoot apical meristem) can be cultured *in vitro*, producing clumps of shoots from either axillary or adventitious buds. This method can be used for clonal propagation. Shoot meristem cultures are potential alternatives to the more commonly used methods for cereal regeneration as they are less genotype-dependent and more efficient.

Embryo Culture

Embryo can be used as explants to generate callus cultures or somatic embryos. Both immature and mature embryos can be used as explants. Immature, embryo-derived embryogenic callus is the most popular method of monocot plant regeneration.

Microspore Culture

Haploid tissue can be cultured *in vitro* by using pollen or anthers as an explant. Pollen contains the male gametophyte, which is termed the 'microspore'. Both callus and embryos can be produced from pollen.

Anthers (somatic tissue that surrounds and contains the pollen) can be cultured on solid medium (agar should not be used to solidify the medium as it contains inhibitory substances). Pollen-derived embryos are subsequently produced via dehiscence of the mature anthers. Anthers can also be cultured in liquid medium, and pollen released from the anthers can be induced to form embryos, although the efficiency of plant regeneration is often very low.

Plant Regeneration

Irrespective of culture type maintained regeneration of a whole plant *in vitro* and its establishment into a mature plant is the major objective of plant tissue culture (except for those cultures used for commercial metabolite production using cell suspension cultures or callus tissues). The explant or callus can produce plants by two morphogenetic pathways namely 1) organogenesis 2) somatic embryogenesis

Organogenesis

Organogenesis relies on the production of organs, either directly from an explant or from a callus culture. Organogenesis relies on the inherent plasticity of plant tissues, and is regulated by altering components of the medium. In particular, it is the auxin to cytokinin ratio of the medium that determines which development pathway the regenerating tissue will take. It is usual to induce shoot formation by increasing the cytokinin to auxin ratio of the culture medium. These shoots can then be rooted relatively simply.

An explant cultured on the media with plant growth regulators to produce multiple shoots can directly induce shoots in 2-3 weeks time. On acquiring a length of 2-3 cm, these shoots can be excised and can be transferred to rooting medium (often contain $\frac{1}{2}$ MS, 1-2 μ M IAA or IBA), and subsequently the plantlets can be transferred to pots acclimatization in green house and then in fields. This pathway of *in vitro* morphogenesis is called as direct

organogenesis (Figure 5). Alternatively, shoot buds can be produced from a callus produced by dedifferentiation of explants and can be developed into a mature plant in similar way. This pathway is known as indirect callus mediated *in vitro* organogenesis (figure 6).

In broad terms two methods of plant regeneration are widely used in plant transformation studies.i.e. oraganogenesis and somatic embryogenesis.

Somatic embryogenesis

In somatic (asexual) embryogenesis, embryo-like structures, which can develop into whole plants in away to analogous zygotic embryos, are formed from somatic tissues. These somatic embryos can be produced either directly or indirectly. In direct somatic embryogenesis, the embryo is formed directly from a cell or small group of cells without the production of an intervening callus. Though common from some tissues (usually reproductive tissues such as the nucellus, styles or pollen), direct somatic embryogenesis is generally rare in comparison with indirect somatic embryogenesis.

In indirect somatic embryogenesis, callus is first produced from the explant. Embryos can be then be produced from the callus tissue or from a cell suspension produced from that callus. Somatic embryogenesis from carrot is the classical example of indirect somatic embryogenesis (Fig 7.).

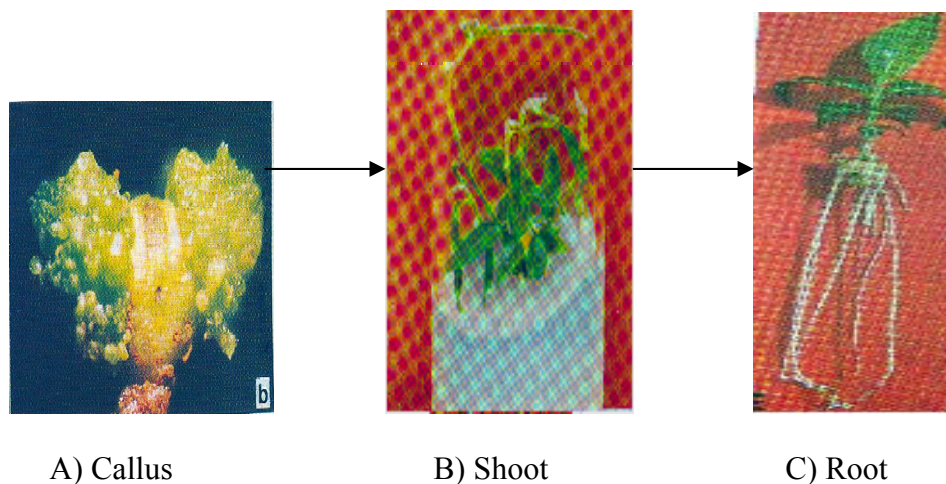


Figure 6. *In vitro* regeneration of plants via callus mediated organogenesis.

Types of Plant Tissue Cultures

Somatic embryogenesis usually proceeds in two distinct stages. In the initial stage (embryo initiation), a high concentration of 2,4-D is used. In the second stage (embryo production) embryos are produced in a medium with no or very low levels of 2,4-D.

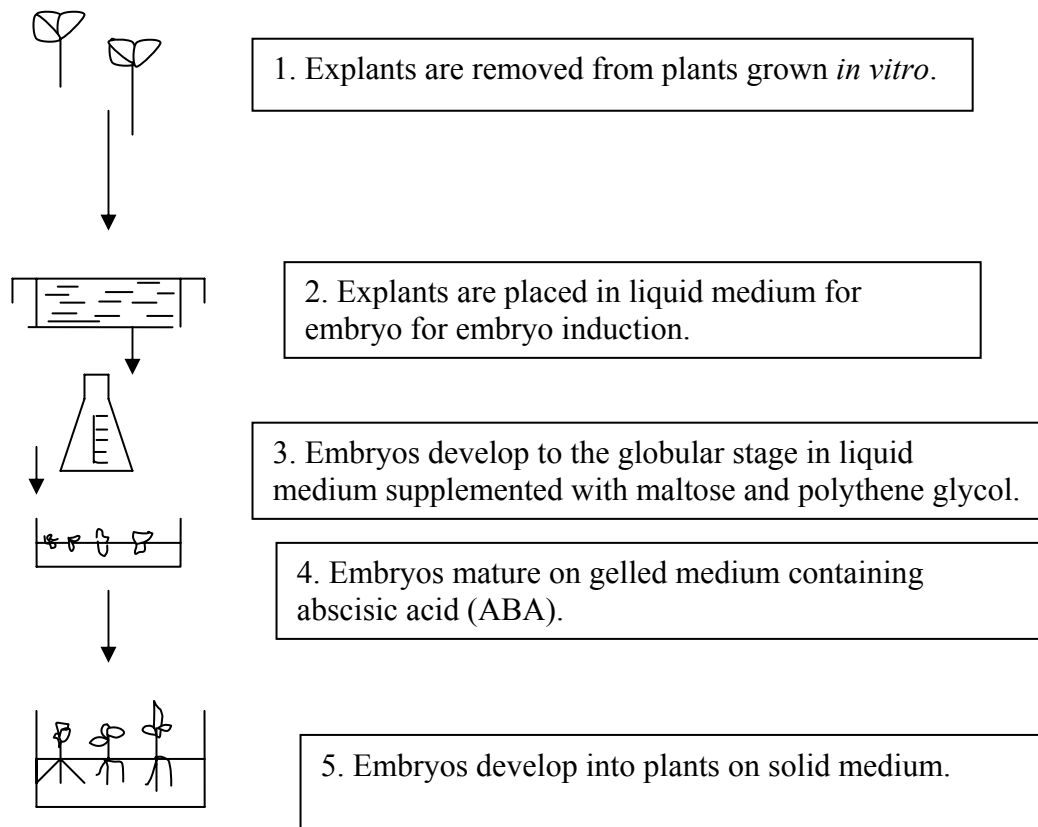


Figure 7. A schematic representation of somatic embryo production using cell suspension cultures in plants.

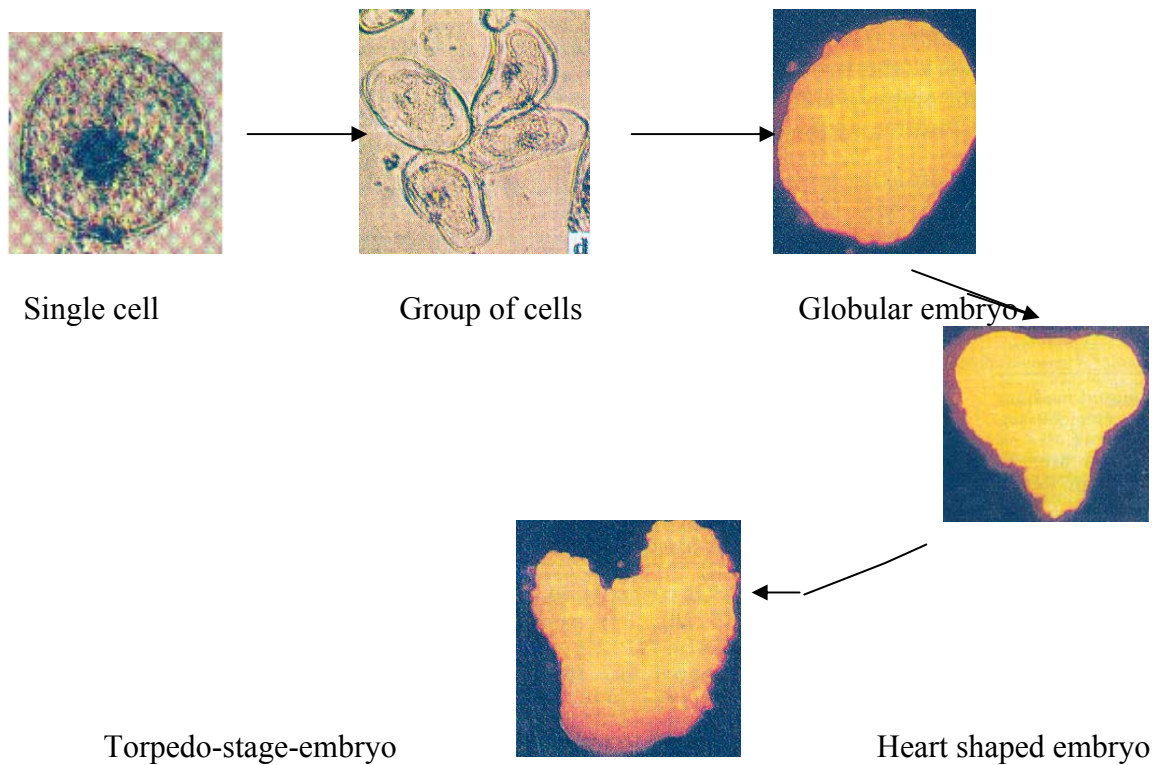


Figure 8. A photographic representation of somatic embryo production in liquid cell cultures. The sequential stages of somatic embryo development. Somatic embryos may develop from single cells or from a small group of cells. Repeated cell divisions lead to the production of a group of cells that develop into an organized structure known as a 'globular-stage embryo'. Further development results in heart and torpedo stage embryos, from which plants can be regenerated. Zygotic embryos undergo a fundamentally similar development through which plants can be regenerated. Polarity is established early in embryo development. Signs of tissue differentiation become apparent at the globular stage and apical meristems are apparent in heart stage embryos

Box 3.	Examples of Somatic Embryogenesis
<i>Indirect somatic embryogenesis in carrot (<i>Daucus carota</i>)</i>	
A callus can be established from explants from a wide range of carrot tissues by placing the explant on solid medium (e.g. Murashige and Skoog (MS) containing 2,4-D (1mg/l). this callus can be used to produce a cell suspension by placing it in agitated liquid MS medium containing 2,4-D (1mg/l). this cell suspension can be maintained by repeated subculturing into 2,4-D-containing medium. Removal of the old 2,4-D-containing medium and replacement with fresh medium containing abscisic acid (0.025 mg/l) results in production of embryos. The production of embryos is fundamentally a two step process. The initial medium, which contains 2,4 D is replaced with a medium that does not contain 2,4 D.	
<i>Direct somatic embryogenesis from Alfalfa (<i>Medicago falcata</i>)</i>	
Young trifoliolate leaves are used as the explant. These are removed from the plant and chopped into small pieces. The pieces are washed in a plant growth regulator-free medium and placed in liquid medium (B5) supplemented with 2,4-D (4mg/l), kinetin (0.2mg/l) and glutathione(10mg/l). The cultures are maintained in agitated liquid medium for about 10-15 days. Washing the explants and replacing the old medium with B5 medium containing abscisic acid.	

Various methods of plant regeneration are available to the plant biotechnologist. Some plant species may be amenable to regeneration by a variety of methods, but some may only be regenerated by one method. Not all plant tissues are suited to every plant transformation method, and not all plant species can be regenerated by every method. There is, therefore, a need to find both a suitable plant tissue culture/regeneration regime and a compatible plant transformation methodology for biotechnological improvement of plants.

vectors for gene delivery and marker genes

in last one decade, a number of techniques have been developed for the transfer of genes into plants. these techniques can be divided into two broad groups:

- (1) those employing a vector, such as *agrobacterium* or cauliflower mosaic virus or gemini virus.
- (2) non-biological techniques- which employ physical or chemical means of transferring genes into cells/protoplasts or intact plants.

biology of agrobacterium

agrobacterium are gram negative ubiquitous soil phyto-pathogen that genetically transforms plant cells. in nature this transformation results in crown gall tumors (cancerous growth) or hairy roots (proliferic root formation) at the infection sites in a range

of plant species especially dicots and gymnosperms. crown gall formation or hairy roots is the consequence of transfer, integration and expression of a particular segment of dna, the t-dna (transfer dna) from the tumor inducing (ti) or root inducing (ri) plasmid within the bacterium to plant cell genome. over the last one decade, the basic principle involved in this transformation process has led to the design of modified non-oncogenic *agrobacterium* strains that can be used to transfer any dna of interest to plant cells without interfering with their normal growth and regeneration property.

(1) ti plasmid and t-dna

1. all tumor forming (virulent) strains of *agrobacterium* harbour a large plasmid (140-235 kb) called ti or ri plasmid. a discrete segment (t-dna) of this plasmid which is bordered by 25bp conserved repeats and ranges in size from 14-24 kb (approximately 1/10th of plasmid) is transferred to the plant cell and stably integrated to plant nuclear dna.

2. most of the genes that are located within t-dna do not express in bacteria, but express only after t-dna is inserted into the plant genome, because these genes possess typical eukaryotic promoter and poly-adenylation signals. the products of t-dna are responsible for oncogenicity (crown gall) formation. the three genes of t-dna region *tms1* (*iaam*), *tms2* (*iaah*) and *tmr* (*ipt*) direct the constitutive synthesis of the phytohormones, auxin and cytokinins which are responsible for rapid proliferation of plant cells resulting into tumorous growth such as crown gall. the first two genes (*tms1* and *tms2*) encode enzymes that synthesize the plant hormone auxin (indole-3- acetic acid).

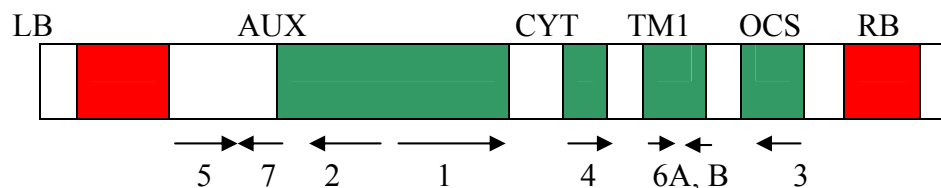
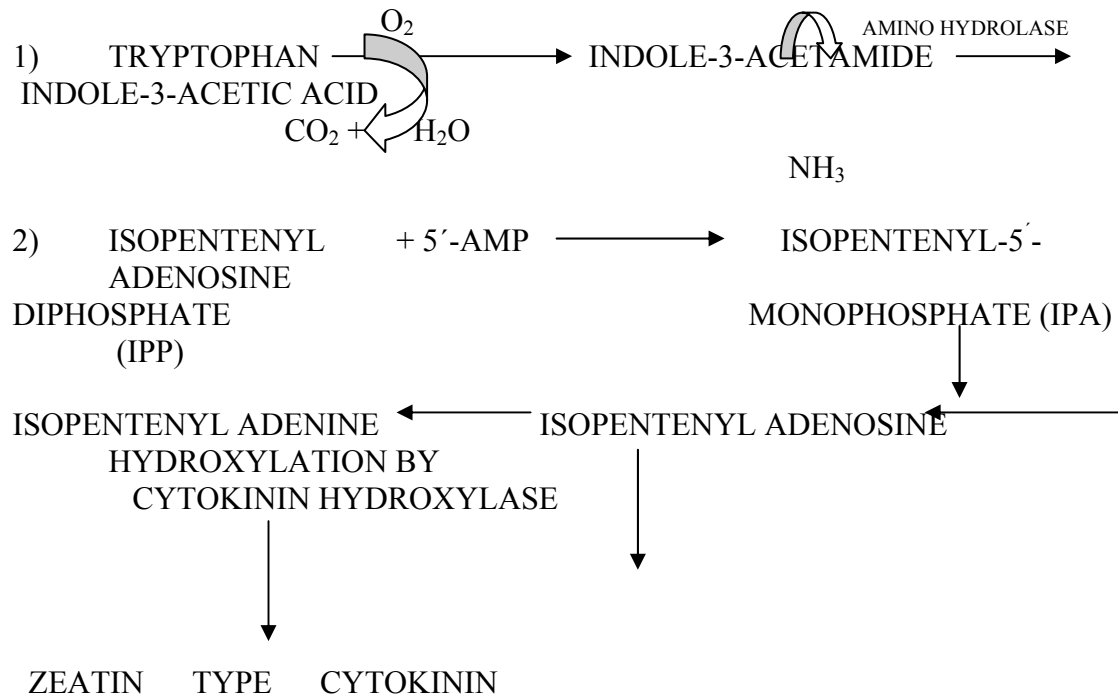


figure.9. the genetic organization of the t1 t-dna of an octopine-type ti plasmid. only the t1 region is shown as this has homology with the t-dna of nopaline-type ti plasmids. eight open reading frames (orfs) are indicated (1-7), although orfs 5 and 7 are not discussed in this text. regions of import are shaded light grey, and include the aux genes (which encode enzymes involved in auxin biosynthesis), cyt which encodes isopentenyl transferase (an enzyme involved in cytokinin production), tm1 which is involved in regulating tumour size and ocs (octopine synthase) which encodes opine synthesis. (hughes, m.a. (1996).

specially *tms1* codes for the enzyme tryptophan-2-mono-oxygenase which converts tryptophan to indole-3-acetamide and gene *tms2* contains the information for indole-3-acetamide hydrolase, which converts indole-3-acetamide to indole-3-acetic acid, in addition, the third gene *tmr* encodes isopentenyl transferase enzyme, which adds 5'-amp to an isoprenoid side chain to form the cytokinin isopentenyl adenine and isopentenyl adenosine.

TRYPTOPHAN MONO-OXYGENASE

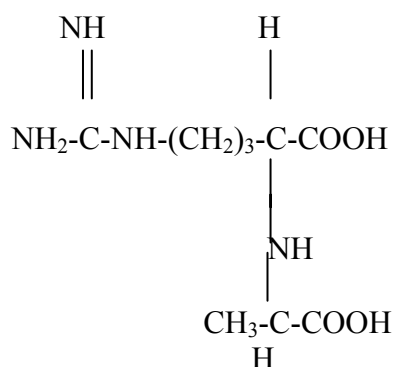


hydroxylation of these two molecules by plant enzyme generates the zeatin like cytokinins called trans-zeatin and transribosylzeatin, respectively.

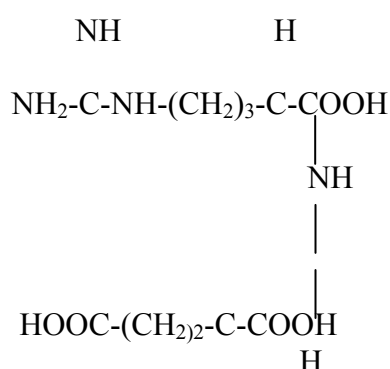
in addition to auxin and cytokinin biosynthesis genes, the t-dna also codes for one or more enzymes that direct the synthesis of a molecule unique to tumor cells only (not found in normal cells), called opines. these are unusual amino acids or sugar derivatives formed by the condensation of an amino acid and a ketoacid or an amino acid and a sugar. originally two groups of opines were recognized: octopine (the condensation product of arginine and pyruvic acid) family and nopaline (condensation product of arginine and α -ketoglutaraldehyde). two other f groups of opines i.e. agropine (a bicyclic sugar derivatives of glutamic acid) and agrocinosins (phosphorylated sugars) have been classified more recently.

OCTOPINE	=	ARGININE PYRUVIC ACID
NOPALINE	=	ARGININE + α -KETO GLUTARALDEHYDE
AGROPINE	=	SUGAR DERIVATIVE OF GLUTAMIC ACID
AGROCINOPINS	=	PHOSPHORYLATED SUGAR

opines are secreted from transformed plant cells into the intercellular regions of a tumor or rhizosphere of hairy roots where *agrobacterium* lives. these compounds cannot be metabolized by plant cells but serve as a carbon and nitrogen source for the bacteria. the genes for catabolism of opines are present on ti-plasmid but is not the part of t-dna.



OCTOPINE



NOPALINE

the opine synthesis genes carried on t-dna of each ti and ri plasmid determine the opine produced by tumor or hairy root cells. ti or ri plasmids are often classified on the basis of opine types. nopaline and succinoaminopine ti plasmid and mannopine ri plasmid have one continuous t-dna flanked by two border sequences. in contrast, octopine ti plasmid and agropine ri plasmid have non-continuous t-dnas known as a left t-dna (tl), a central t-dna (tc) and a right t-dna (tr) which are each bound by two border sequences.

the tl contains oncogenic functions for tumor initiation and maintenance, tr contains several opine synthesis genes and tc does not specify a phenotype in transformed plant cells.

four main regions of homology, designated as a,b,c, and d have been identified between the octopine and nopaline plasmids. the main functions attributed to these regions are: (a) it is internal to t-dna region and determines the oncogenic properties and opine synthesis; (b) plasmid replication and incompatibility; (c) conjugative, function and (d) *vir*- region.

T-DNA transfer

extensive genetic and molecular biology studies have revealed that three genetic components of *agrobacterium* are involved in t-dna transfer.

(1) *agrobacterium* chromosomal genes: the initial step toward gene transfer by *agrobacterium* is the attachment of bacterium to plant cell at wound sites. the nature of

plant cell receptors to which *agrobacterium* binds is unknown. four different bacterial chromosomal virulence loci *chv* (a and b), *cel*, *psc a* and *att* are involved in the binding of bacteria to plant cells. the *chv a* and *chv b* are linked loci that synthesis and excrete β , 1-2 glucan, the *cel* locus synthesizes cellulase fibrils, the *psc a* (exoc) even affects both cyclic glucan and acidic succinoglycan synthesis and the *att* even affects cell surface protein. the chromosome loci are constitutively expressed and potentially reflect a general role of surface components in mediating a bacterial plant cell interaction.

Now it is believed that the bacteria respond to certain low mol. weight phenolic compounds such as acetosyringone and hydroxyacetosyringone which are secreted by susceptible wounded plants. these wound -response compounds resemble some of the products of phenylpropanoid metabolism, which is the major plant pathway for the synthesis of plant secondary metabolites such as lignins and flavanoids. these small molecules (i.e. acetosyringone, hydroxyl acetosyringone) act to induce the activity of the virulence (*vir*) genes.

THE T-DNA BORDER SEQUENCES:

the structure and organization of the integrated t-dna in tumour cells has been studied in detail. the main conclusions of these studies are listed below.

1. none of the t-dna encoded genes are required for t-dna transfer.
2. t-dna does not influence the site of insertion since t-dna inserts were found to be at random locations in the genome and present at a range of copy numbers (averaging 2-3) within individual transformed cell lines.
3. t-dna is a discrete unit which is inserted into the plant genome without modification.
4. t-dna regions on all ti or ri plasmids are flanked by almost 25 bp direct repeat or border sequences. these 25 bp repeat sequences particularly those on the right border to t-dna are absolutely required for t-dna transfer and that they function in a cis-acting and polar fashion. any dna sequence placed between these borders can be transferred into plant cell.
5. detection of the first 6 bp or the last 10 bp of the 25 bp sequence blocks t-dna transfer.

BOX 4	AGROBACTERIUM RHIZOGENES
although <i>agrobacterium rhizogenes</i> also infects plants, it differs from <i>a. tumefaciens</i> in that the resulting pathology is not crown galls but a phenomenon as 'hairy roots'. at the site of infection there is a proliferation of roots. plasmids in <i>agrobacterium rhizogenes</i> (ri plasmids) strains have been characterized, and it has been shown that there are a number of different types, which can be classified based upon opine usage. hormone biosynthetic genes <i>tms1</i> and <i>tms2</i> have been identified on the agropine-strain ri plasmids. however, despite the well-established link between auxin and rooting, the genes are unnecessary for virulence. rather, a series of other open reading frames have been identified within the t-dna of the plasmids, including the <i>rol b</i> and <i>rol c</i> genes that are involved in the metabolism of plant growth regulators and lead to the plant being sensitized to endogenous auxin. it is this increased sensitivity	

that leads to the root formation.as with *a. tumefaciens*, vectors have been constructed that can be used in *a. rhizogenes* as binary systems.hairy roots are important in some areas of plant biotechnology as they can be cultured *in vitro*. for many years they have been used as a source of secondary metabolites, but more recently they have been used as a source of secondary metabolites, but more recently they have been used for the production of pharmaceutical proteins.*a. rhizogenes* transformation was, at one stage, considered an alternative strategy to *a. tumefaciens* for gene transfer as it led to the production of defined tissues (hairy roots) that could be regenerated into whole plants. this strategy seems to have been discarded; however, as more efficient *a. tumefaciens* systems have been developed.

THE VIRULENCE REGION OF TI OR RI PLASMID

the 30 kb vir region on ti plasmid occurs outside the t-dna and is responsible for t-dna transfer from agrobacterium to plant cells. however, physical separation of t-dna and virulence regions onto different plasmids did not affect t-dna transfer provided both plasmids are present in same agrobacterium cell, this indicating that the virulence functions were trans-acting.

The virulence region is organized into six operons (a,b,c,d,e,g) that are either absolutely essential for (*vira*, *virb*, *vird*, and *virg*) for virulence that enhance the efficiency of (*vir*c and *vire*) plant cell transformation. all the operons are polycistronic except *vira* and *virg*. the *vira* and *virg* loci are expressed constitutively under all conditions

All other vir loci (b,c,d,e) are exclusively plant inducible i.e. their expression is greatly enhanced when bacteria come in contact with plant cell or plant cell exudates. it has been shown that small phenolic compounds such as acetosyringone or hydroxyacetosyringone present in plant exudates induce the expression of vir genes. the products of *vira* and *virg* gene are required for the expression of the other vir loci. the *vira* product (*vira*) is located on the inner membrane of *agrobacterium* cell and is probably a chemoreceptor which senses the presence of inducer molecule (acetosyringone) and transmits this information to the inside of the bacterium, potentially by activation (possibly phosphorylation) of *virg* protein (which is transcriptional regulator) which then stimulates transcription by binding to operator sequences in the promoters of the plant inducible vir genes (*virb*, c, d and e).

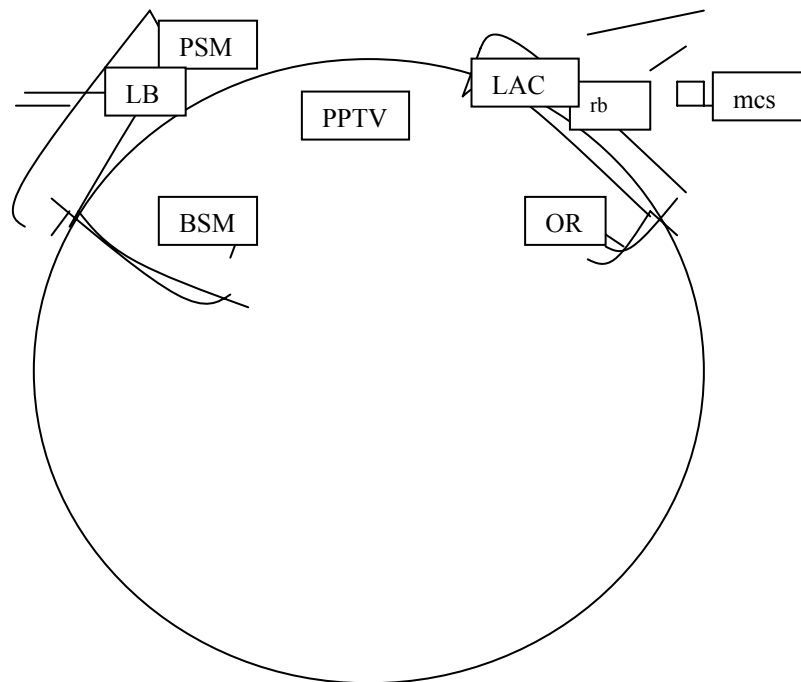


FIGURE 10 A Simplified Representation Of A Plasmid Plant Transformation Vector (Pptv) Showing The Essential Features Of A Binary-Type Plant Transformation Vector. The Dna That Is Transferred To The Plant Genome (The T-Dna) Is Situated Between, And Defined By, The Left (Lb) And (Rb) Borders. It Contains A Multiple Cloning Site (Mcs) In Lac Z A To Facilitate Cloning Of Transgene Into The Vector. The T-Dna Also Contains A Selectable Marker Gene To Enable Selection Of Transformed Plant Cells (Psm). Outside The T-Dna Is Bacterial Selectable Marker Gene (Bsm) To Allow Selection Of Transformed Plant Cells (Psm). Outside

T-DNA TRANSFER PROCESS

two proteins encoded by the *virD* operon, *virD1* and *virD2*, act as a site specific endonuclease which produce nicks between 3 or 4 base pairs on the bottom strand of each 25 bp repeat. the *virD2* protein attaches to the 5' terminus of the nicked right border t-dna and replicative process synthesizes a single stranded dna from the bottom strand of t-dna. the *virE2* product coat the ssdna.

the *virB* operon encodes at least 11 proteins throughout to form membrane associated structures that may form a channel(s) through which t-dna strand-protein complex is exported.

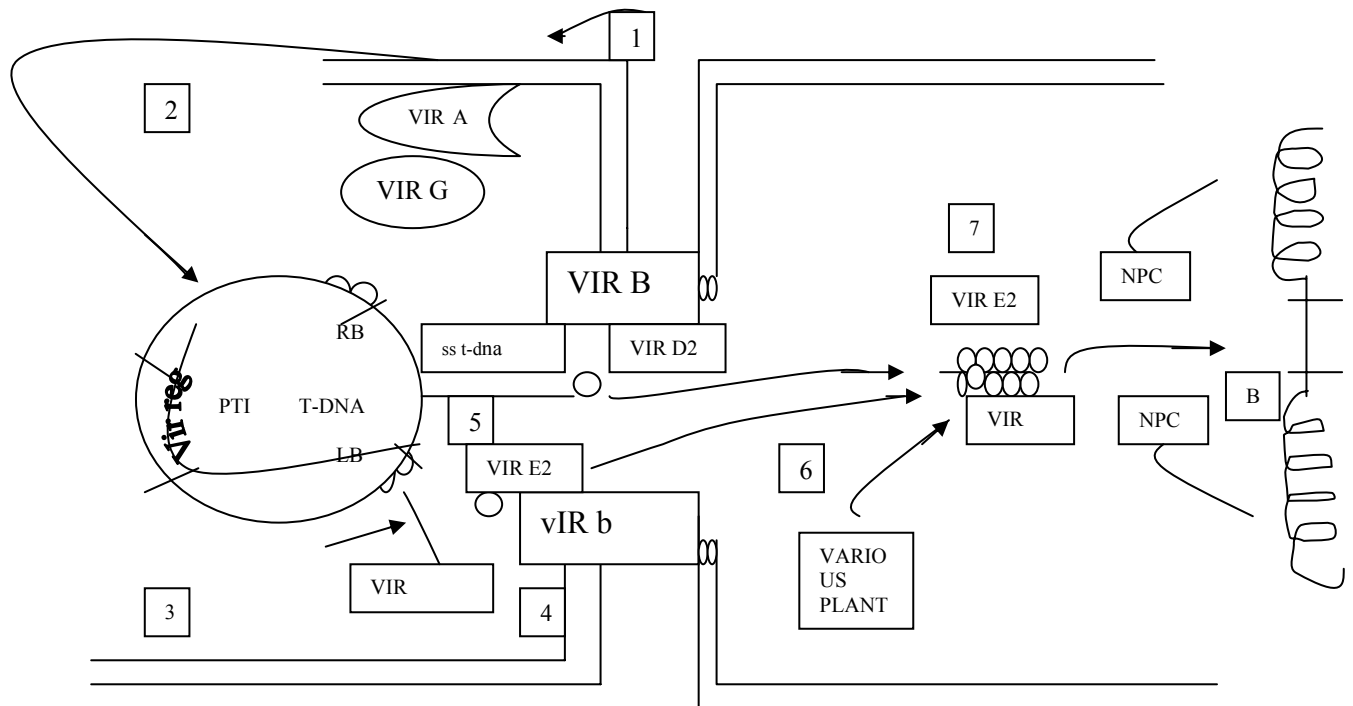


figure 11 a simplified representation of the t-dna transfer and integration process. wounded plant cells release phenolic substances and sugars (1) that are sensed by vir a, which activates vir g by phosphorylation. virg induces the expression of all the genes in the *vir* region of ti plasmid (2) gene products of the *vir* genes (3) are involved in a variety of processes. vir d1 and vir d2 are involved in single-stranded t-dna production, protection and export (4) and (5) vir b products form the transfer apparatus. the single-stranded t-dna production (associated with vir d2) and vir e2 are exported through the transfer apparatus (vir f may also be exported) (6) in the plant cell the t-dna becomes coated with vir e2 (7) various plant proteins interact with either the vird2 and vir e2, which are attached to the t-dna and influence transport and integration. the t-dna/vird2/vire2/plant protein complex enters the nucleus through the nuclear pore complex. integration into the plant chromosome (8) occurs via illegitimate recombination. (lb, left border; rb, right border; pti, ti plasmid; ss, single stranded; npc, nuclear pore complex). adopted from

AGROBACTERIUM AS A VECTOR SYSTEM

agrobacterium plasmids have been exploited as vectors for biological delivery of foreign dna to plants, this is the most wide spread transformation strategy in use today. however, (wild type ti plasmids) have several serious limitations as routine cloning vector.

the phytohormone biosynthetic genes encoded on t-dna of wild-type ti plasmids interfere with the regeneration of transformed cells growing in culture. therefore, the phytohormone (auxin and cytokinin) genes completely removed (disarmed plasmid) for t-dna to regenerate complete plants from transformed plant cells.

a gene encoding opine synthesis is not useful to a transgenic plant and may lower the final plant yield by diverting plant resources into opine production. therefore, the opine synthesis gene should be removed.

ti plasmids are large (approximately 200 kb). therefore they are difficult to handle experimentally. moreover it is impossible to find adequate unique restriction sites in the t-region. for recombinant dna experiments, however, a much smaller version is preferred, so large segments of dna that are not essential for a cloning vector must be removed. because ti plasmid does not replicate in *e.coli*, the convenience of perpetuating and manipulating ti plasmids carrying inserted dna sequences in this bacterium does not exist. therefore, in developing ti plasmid - based vectors, an origin of replication that can be used in *e.coli* must be added.

To overcome these constraints, many non-oncogenic transformation vectors with different features have been constructed. they fall into two broad categories, the cis and the trans vectors.

cis vectors contain both the border sequences and the vir region on the same replicon (co-integration) whereas in trans vectors both the border and vir functions are on two replicons (binary vectors).

CO-INTEGRATE VECTORS AND OTHER VECTORS FOR GENE TRANSFER TO PLANTS:

The co-integrating system involves two independent plasmids. (1) a non-oncogenic (disarmed) ti plasmid (in which majority of t-dna is removed and replaced by a section of dna homologous to small *e. coli* cloning vector) in *agrobacterium* and (2) an intermediate vector which can't replicate in *agrobacterium*, is used for cloning and manipulation of the gene which are to be introduced in *e. coli*. since both the plasmids have a region of homology which undergoes recombination to form a large, co-integrated plasmid after conjugation between *agrobacterium* and *e. coli*.

There are two main methods for their construction :

1. zambryski et al (1983) has developed one of the first co-integrative vectors, *pgv3850* from nopaline type ti plasmid (c58), where almost all t-dna were deleted (except for the border sequences and nopaline synthase gene) and replaced by *pbr 322*, a common small *e. coli* cloning vector. the intermediate vector (e.g. *pgv1103*) based on *pbr322* is conjugated into *pgv3850* at the region of *pbr322* homology. after co-integration, *pgv3850* : *pgv1103* t-dna contains the which intermediate vector including unwanted dna sequences which are also transferred to transformed plants along with the gene intended to be transferred.

2) SPLIT -END VECTOR (SEV) SYSTEM

in this system, the left and the right border sequences are present on separate plasmids. these plasmids form the co-integrate following a single recombination event whereas other co-integrating systems may involve one or two. the sev system eliminates the presence of unwanted bacterial genes between the border sequences, a feature in the zambryski system.

agrobacterium plasmid *pti b6s3-se* is a derivative of the octopine type ti plasmid *pti b6s3* containing only left border and left inside homology (lih) sequences to allow recombination with intermediate vector.

the intermediate vector *pmon200* consists of (i) the kanamycin selectable plant marker neomycin phosphotransferase ii (*nptii*) with regulation sequences of nos (ii) a multiple cloning site for insertion of foreign dna and (iii) a function right border sequences. *pmon200* when transferred to *A. tumefaciens* by conjugation, can recombine with the resident plasmid pti b6s3-se via homology lih regions. the resulting plasmid pti b6s3-se:: *pmon200* contains an *nptii* and *nos* genes for monitoring foreign dna in plant cells. transfer of sev t-dna into plant cells, utilizes the *nos* right border sequences.

the main advantage of the co-integrate vectors is their high stability in *agrobacterium*. however, two disadvantages are the detailed knowledge required of the ti plasmid before it can be manipulated and, the relatively low rates of co-integrate formation (about 10^{-5}).

BINARY VECTOR

the binary vectors are based on the principle that vir gene products can function in trans configuration. these vectors (binary vectors) contain t-dna border repeats as well as both *E. coli* and *agrobacterium* origin of replication but no vir genes, it is actually an *E. coli*-*agrobacterium* shuttle vector. all the cloning steps are carried out in *E. coli* before the vector conjugatively transferred into *agrobacterium* which contains a disarmed ti plasmid lacking the entire t-dna region, but an intact *vir* region (helper ti plasmid, e.g. *pal4404*).

many binary vectors have been developed which differ in size, source of 25 bp repeat sequence, plant selection marker, bacterial selection marker and cloning sites for the insertion of dna for transfer to plants. *pbin19* - a broad host range (*prk252*) binary vector which was designed in 1984. this vector contains: prokaryotic kanamycin resistant gene (*aph-1*) for selection of bacteria, t-dna border derived from pti t37, adjacent to the right border a plant selectable transformation marker, *nptii* isolated from transposon tn5 (under the control of the nopaline synthase (*nos*) promoter and polyadenylation signals) and on left border a multiple cloning site derived from *puc19*, housed within lac z of which contains seven unique restriction enzyme sites for the insertion of passenger dna. bacterial colonies containing *pbin19* are recognized by loss of blue colour on iptg/x-gal plants.

unlike co-integrative vectors, binary vectors need not have any homology with the resident ti plasmid and are capable of autonomous replication, usually in multiple copies within *agrobacterium*. this gives the binary a considerable advantage over the co-integrative system since any binary can be used in conjunction with any *vir* helper strain even with wild type oncogenic strains of *agrobacterium*. non-oncogenic virulence helper plasmids have been developed from several common ti plasmids including nopaline (t 37-se), octopine (*pal 4404*) and succinamopine strain (*eha t01*). binary vectors are usually smaller than co-integrating vectors and consequently are easier to maintain and manipulate in *E. coli*. these plasmids have a 10,000 fold greater frequency of transfer from *E. coli* to *agrobacterium* than co-integrating plasmids, since there is no t-dna integration step. the presence of genes encoded in the t-dna of a binary plasmid in *agrobacterium* is confirmed easily by plasmid restriction digests, rather than by southern hybridization or pcr, which is required to detect large co-integrated plasmids. as a result of these features, binary vectors have virtually excluded co-integrate vectors.

VIRUSES AS VECTOR FOR PLANT GENE TRANSFER:

Plant viruses are attractive as vector for introducing genes into plants due to the following reasons. many viruses or their isolated genomes are capable of infecting cells of intact plant. many plant viruses have the ability to systematically spread their genome throughout the whole plant. if the viral genome includes a foreign gene, then that too would spread systematically throughout the plant, thus eliminate the need of growing plants from infected protoplasts or cell cultures. plant viruses replicate and express at a high rate in plant cells, leading to the production of large amounts of foreign protein from recombinant viruses. multicopy amplification and systemic spread of engineered viruses could be exploited to produce large quantities of gene/gene products.

Viruses are known which infect plant for which current alternative technology is limited. most of the plant viruses, about 78% have genomes comprising of single stranded rna in the '+' or messenger sense. about 13 % contain single stranded rna in the '-' sense, double stranded rna, double stranded dna and single stranded dna. however, only three kinds of viruses containing double stranded dna (e.g. caulimoviruses), single stranded dna (e.g. gemini viruses) and single stranded "plus rna" (e.g. tobacco mosaic virus) are capable of transferring genes into intact plant tissues where they are expressed.

CAULIFLOWER MOSAIC VIRUS (CAMV).

camv, caulimovirus is spherical and contain a circular double stranded dna of 8 kb. these viruses cause important diseases in cultivated crops and have a limited host range. the virus dna is infections and spread is systemic. In infected cells, refractile, round inclusions form, which consist of many virus particles embedded in a protein matrix. the matrix protein is virus coded.

the duplex viron dna has three single stranded discontinuities, one in one strand and two in the other. these discontinuities are regions of sequence overlapping. the sequences of camv dna reveal eight closely packed reading frames. functions have been ascribed to three of these regions with reasonable certainty. region ii codes for protein that is associated with aphid transmission of virus. region iv codes for precursor to the coat protein.. region vi codes for the inclusion body matrix protein. the product of region v has been suggested to be associated with viral replication. coding regions i, ii and v appears to be absolutely essential for viral replication and spread throughout the plant. coding region ii does not appear to be essential for replication and sizable part of it can be removed without deleterious effects on virus replication.

there are only two intergenic regions:

one large intergenic region of about 600 bp between coding regions in 6th and 7th; other short intergenic region of 60 bp between coding sequence 5th and 6th. the only non-essential genes are the two small genes ii and vii. another unusual features of camv dna is the presence of ribonucleotides covalently attached to the 5'-termini of the discontinuities. these and other observations suggest that camv replication involved reverse transcription like those of retroviruses.

two major camv specific polyadenylated rna transcript are found in infected cells. the 19s transcript is the mrna for gene product 6th the other major rna, the 35s transcript, is a more than full-length transcript.

CAMV AS VECTOR:

One feature of camv which makes it attractive as a vector is that viruses spread systematically throughout the plant. in order for camv to be transmitted through the vascular system of plant, the dna must be assembled within virion. the strategy for delivering foreign genes using camv has to replace a small section of genome, not required for virus propagation, with foreign dna small enough not to interfere with packing of genome into the virion particle. the foreign dna is inserted at a unique *xho*i site which lies in the non essential gene ii. if dna longer than a few hundred nucleotides was inserted, the infectivity was destroyed. this packaging limitation and the absence of long non-essential sequences which can be deleted in gene severally limited the use of camv dna as vector.

However, small foreign dna, comprising methotrexate resistant dihydrofolate reductase (dhfr) gene of *e.coli*, replacing the gene ii coding sequence of camv has been successfully expressed in plants.

The other limitations of camv as vector are

progeny of the transformed plant cannot carry the introduced trait since; neither the gene gets incorporated into plant nuclear genome nor is the virus transmitted through seed. camv exhibit a very limited host range than that of ti plasmid. these viruses naturally infect only a small number of species primarily members of brassicaceae such as cauliflower and turnip.

The high rate of recombination, which is thought to be a feature of replication by reverse transcription, is also considered to be an obstacle to the introduction of foreign genes into camv dna.

DIRECT GENE TRANSFER METHODS

Table 5 in addition to the vector mediated gene transfer methods, these are certain direct gene transfer methods has been used for genetic transformation a brief account of these methods has been given in table

direct gene transfer method	comments
particle bombardment	very successful method. risk of gene rearrangements and high copy number. useful for transient expression assays
electroporation	transgenic plants obtained from a range of cereal crops. low efficiency. requires careful optimization
dna uptake into protoplasts	used for all major cereal crops. requires optimization with a regenerable cell suspension that may not be available
silicon carbide fibres	requires regenerable cell suspensions. transgenic plants obtained from a number of species

Promoters and terminators

An obvious requirement for any gene that is to be expressed as transgene in plants is that it is expressed correctly (or at least in a predictable fashion). It is known that the major determinant of gene expression (level, location and timing) is the region upstream of the coding region. This region, termed 'the promoter', is therefore of vital importance. Any gene, that is to be expressed in the transformed plant must have to possess an eukaryotic promoter that will function in plants. This is an important consideration as many of the genes that are to be expressed in plants, Bt gene, reporter genes, and selectable marker genes etc are bacterial in origin. They, therefore, have to be cloned with a promoter that will drive their expression in plants. Transgenes also need to have suitable terminator sequences at their 3' terminus to ensure that transcription ceases at the correct position. Failure to stop transcription can lead to the production of aberrant transcripts and can result in a range of deleterious effects, including inactivation of gene products and increased gene silencing.

In addition to the basic need for the promoter to be capable of driving expression of the gene in plants, there are other considerations that need to be taken into account, such as promoter strength, tissue specificity and developmental regulations etc.

Agrobacterium derived promoter and terminator sequences

The genes from the Ti plasmid of *Agrobacterium* that code for opine synthesis, and in particular the nopaline synthase (*nos*) gene, are widely used as a source of both promoters and terminators in plant transformation vectors. Although derived from bacterial genes, their presence on the T-DNA means they are adapted to function in plants. The *nos* promoter is usually considered to be constitutive.

The 35S promoter

The most widely used promoter used to drive expression of genes in plant transformation vectors is the promoter of the cauliflower mosaic virus 35 S RNA gene (35S promoter). This promoter is considered to be expressed in all tissues of transgenic plants (though not necessarily in all cell types). In dicots it drives expression at high levels, although in monocots the level of expression is not so high. This makes the 35S promoter ideal for driving the expression of selectable marker genes, and in some cases of reporter genes, as expression is more or less guaranteed. In monocots, alternatives, such as the maize *ubiquitin 1* promoter or the rice actin promoter/first intron sequence, are often used to drive the high level expression of transgenes.

Tissue specific promoters

Considerable effort has been made in isolating promoters that can be used to drive expression in a tissue specific manner. The expression of any potentially harmful substances can be limited to tissues that are not consumed by animals or humans, and genes involved in specific processes can be limited to tissues in which that process occurs. In certain cases the promoters have been found not to function, or not to drive expression

in the predicted pattern, in heterologous systems. Therefore considerable care has been taken with the use of promoters.

Inducible promoters

Inducible expression systems can be divided into three categories: 1) non-plant-derived systems; 2) plant derived systems 3) plant-derived systems based on developmental control of gene expression.

Non-plant-derived systems are independent of the normal plant processes, requiring use of inducers on agricultural scale. While the plant derived systems do not have the advantage of independence from normal plant processes. This makes their use potentially simpler as the application of an inducer is not required.

Marker Genes

During the genetic transformation of plants, often the success in integration of introduced foreign gene(s) is a very-low frequency event. It will be, otherwise wastage of time, energy and resources to maintain a large number of regenerants (shoots or somatic embryos) obtained from the initial transformation efforts. Therefore it is vital that some means for selecting the transformed tissue/plantlets at initial stages should be devised.

To achieve the above target some marker genes are also cloned along with the 'gene of interest' in the cloning vectors. The marker genes are broadly two types: Selectable markers 2) Reporter genes.

Selectable markers

The selectable marker gene cloned within the vector confers resistance that is toxic to plants. The selection in such cases is based on the inclusion of a substance toxic to the plants in the culture media. The transformed cells /tissues/plants expressing the bacterial genes showing resistance to such toxic substances are survived onto such culture media, whereas other normal (wildtype) non transformed cells/tissues/plants get die. Table..... list ceratin selectable markers often used in plant genetic engineering.

Table: 6 selectable markers used in plant transformation.

Selectable marker gene	abbreviation	Source of gene	Selection mechanism	Selective agent
Hygromycin phosphotransferase	<i>hpt/aphiv/byg</i>	<i>E.coli</i>	Antibiotic resistance	hygromycin
Neomycin phosphotransferase II	<i>nptII/neo</i>	<i>E.coli</i>	Antibiotic resistance	Kanamycin Geneticin (G 418)
Neomycin phosphotransferase III	<i>nptII</i>	<i>Streptococcus faecalis</i>	Antibiotic resistance	Kanamycin Geneticin (G 418)
Glyphosphate oxidoreductase	<i>gox</i>	<i>Achromobacter</i> LBAA	Herbicide resistance	Glyphosate
Phophinothricin	<i>bar/pat</i>	<i>Streptomyces</i>	Herbicide	Bialophos

acetyltransferase		<i>hygroscopicus</i>	resistance	Glufosinate
Mannose-6-phosphate isomerase	<i>bmi/man A</i>	<i>E.coli</i>	Alternative carbon source	Mannose
Betaine aldehyde dehydrogenase	<i>badh</i>	Spinach	Detoxication	Betain aldehyde

Public concerns are growing on recently on expression of antibiotic or herbicide resistant genes in transgenic plants as a negative factor against genetically modified crops. Attempts are being made to develop plant based endogenous marker genes often for health and environment.

Reporter genes

In addition to the selectable markers or as an alternative to them, reporter genes are used as markers in many plant transformation vectors. At present, only a small number of repoter genes in widespread use in lant transformation vectors (table.3) the reporter genes should be, ideally, easy to assay, preferably with a non-destructive assay system, and there should be little or no endogenous activity in the plant to be transformed.

Table 7. Certain reporter genes used in plant transformation.

Reporter gene	Abbreviation	Source of gene	Detection/assay
B-glucuronidase	<i>gus/uid A</i>	E. coli	Fluorimetric (quantitative) or historical (in situ), non-radioactive
Green fluorescent protein	<i>gfp</i>	<i>Aequorea victoria</i> (jelly fish)	Fluorescence, non-destructive
Chloromphenicol acetyltransferase	<i>cat</i>	E.coli	Radioactive assay of plant extract, sensitive, semi-quantitative
Luciferase	<i>luc</i>	<i>Photinus pyralis</i> (firefly)	Luminscence
Luciferase	<i>Lux A, Lux B</i>	<i>Vibrio barveyi</i>	Luminscence

SALIENT ACHIEVEMENTS IN CROP BIOTECHNOLOGY

The world's population has increased from 2.5 billion to 6.1 billion in the last 50 years and it is unlikely to stabilize before 2100 by which time another 3 billion people will inhabit the earth. The "green revolution" enabled the world's food supply to be tripled during he last three decades of the 20th Century, however, it has lead to certain environmental concerns e.g. monoculture, water crisis and extreme uses of agrochemicals and chemical fertilizers causing environmental and health hazards. Despite of these problems, there is a desperate need to produce more food from less land, with less water and reduced agrochemical inputs to feed the burgeoning population and to save people from hunger and malnutrition. The majority of agricultural scientists, including Norman Borlaug, Monkombu Swaminathan and Gurudev Khush, the leaders of "green revolution" are convinced that the required food should be obtained with low cost and low environmental

impact by exploiting the new techniques of plant biotechnology and molecular breeding for the development of new crop varieties.

Transgenic crops for high yield, better quality of food, resistant to diseases, and tolerant to the environmental stresses to a great extent have been developed and adopted by farmers of the several countries. In addition to food plants, several new possibilities have emerged for improvement in commercial plants for sweeteners, oils, medicines, timber, fibers and flowers etc. Transgenic plants are produced by techniques of genetic engineering and biotechnology in which new characters can be incorporated by introducing the genes from any source, even from a distant relative across the taxonomic boundaries. For example, a gene from bacteria can successfully be used to express a new character of immense importance and utility in plants or animals and vice-versa. The success in producing plants have been achieved widely because of the totipotency in plants and availability of the plant tissue culture protocols, which can be coupled easily with genetic engineering protocols.

Recent advances in crop biotechnology indicate a possibility of 'gene revolution' in plants after the so called green revolution. The major achievements include

The production of new transgenic cultivars of many crop plants. For example, cotton, tomato, sugarcane, wheat, rice, potato, pulses, oilseeds, various kinds of fruits and vegetables etc. for various kinds of qualitative and quantitative agronomic traits, e.g. for disease resistance (*Bt* cotton), delayed fruit ripening (tomato), abiotic stress tolerance (brassicas), improved oil quality (oilseeds), better flower and fruit qualities, better timber qualities, better medicinal values etc.

Gene knockout, gene silencing, antisense technology, identification and transfer of pathway regulatory genes (transcription factor genes) etc. have been the new approaches in recent past to counter the limitations in the technology. A great success is achieved on this front.

New tissue culture media and systems/protocols for low cost, high efficiency *in vitro* regeneration and genetic improvement protocols are in progress in various laboratories to amend the existing protocols for better output.

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