PRINT: ISSN 0970-9274 ONLINE: 2456-6608
J. Hum. Ecol., 8 (1) : 21-31 (1997)
DOI: 10.31901/24566608.1997/08.01.03

Gene Manipulation : Its Impact on Tree Improvement

Maitreyee Kundu and Rupnarayan Sett*

Institute of Rain and Moist Deciduous Forests Research, Jorhat 785001, Assam, India

KEY WORDS Gene. Genetic Engineering. Recombinant DNA. Protoplast Fusion. Tree improvement.

ABSTRACT By the application of basic genetic knowledge, the artificial regeneration of woody plants is a very important component in the reforestation programmes in recent years. Large gaps of knowledge still exist regarding the arrangement and functions of plant chromosomal DNA. In this review, priority has been offered to the need and use of plant genetic material in the management of natural forests deploying the knowledge of gene expression and their successful manipulation. The basic principles, scope of application, prospects and probable shortcomings in the use of nucleic acid hybridisation, protoplast fusion and gene transfer by recombinant DNA technology have been focussed.

GENE MANIPULATION AND FOREST MANAGEMENT

In many tropical zones, reforestation of dry or moist degraded lands is the most important management objective. If there is going to be a reforestation programme, why not plant the best trees possible ? The same is true when an artifical regeneration is an important component of the management of natural forests. A proven method of increasing forest productivity is the careful application of basic genetic knowledge. When properly established and carried out, such genetic activities are standard forest management practice for increasing productivity of timber, fiber and fuel.

Terrestrial ecology is directly concerned with forestation and plantation programmes which require extensive research in tree improvement. The knowledge regarding the understanding of nuclear metabolism and plant genetics is growing rapidly in recent years. The arrangement of DNA sequence in chromosomes in various plant species has been revealed. An insight concerning the number and molecular arrangement of plant genes is understandable from the general manner in which the genetic material is arranged in plant chromosomes. In plant species as a whole, large gaps of knowledge still exist concerning the arrangement of plant chromosomal DNA. To understand the functional meaning of the pattern of sequence organisation, we must have information concerning which DNA sequences are transcribed in plant nuclei and the extent of differential transcription in plant development. Breaking the riddle of cellular and molecuar basis of controlling functions in trees will be a breakthrough in forest genetics. The most likely technologies which will aid the understanding of tree genome and its functions are gene manipulation, gene transfer and recombinant DNA technology, and the most desirable application is the insertion of markers in tree species, as this would help the forest geneticist in regulating tree breeding programmes over a short rotation period.

* *Correspondence to* : Dr. Rupnarayan Sett, Ph. D.

THE NEED AND USE OF PLANT GENETIC MATERIAL

Understanding the expression of genes and their successful manipulation is one of the frontier area of research in recent science. The functional chord of DNA (deoxyriboncleic acid) is termed as gene, and the quality and quantity (C-value) of the DNA of haploid chromosome complement is termed as genome. The quality of the DNA is characterised by the number and frequency of the nucleotide bases. The quantity of DNA is very important in many aspects of cell function such as its direct correlation with the period of mitotic (Jones and Brown, 1976) and meiotic (Bennett, 1971) cell division. Perennial species have higher nuclear DNA con-

tent than annuals and it has been hypothesized that in plants minimum generation time is correlated with nuclear DNA content (Bennett, 1972)

Eukaryotic genome differs from the prokaryotic one by its larger size, more imformation content as well as non-coding repetitive DNA and association of DNA with acidic and basic proteins. As revealed by renaturation kinetics, eukaryotic DNA is composed of single and repetitive sequences. The active genes are present in the single copy fraction of DNA where many non-coding sequences may remain inserted. Generally the reiterated fraction contains intermediately repetitive sequences, highly repetitive sequences, and inverted repeats or palindromes. The intermediately repetitive sequences comprise varying degrees of reiteration such as gene regulatory function coding for r RNA, t RNA and a few m RNAs. Upto an excess of 10^6 copies of simple or highly repititive sequences may occur per genome which play a structural role in chromosomes. When centrifuged in a CsCI gradient, the repetitive DNA fraction exhibits one or more satellite bands from nuclear DNA main band.

Genetic engineering is the isolation of useful genes from a donor organism or tissue and their incorporation into an organism that does not normally possess them. Purified nucleic acid samples can be used to study a number of important questions about tree genomes. This chapter describes some modern techniques and instances on reannealing, hybridisation and recombinant manipulation or engineering of nucleic acid organisation that are applicable to forest genetics research. Recombination studies can be aimed at different aspects of tree improvement and hybridisation studies can be aimed at elucidating genetic relatedness of tree species at molecular level.

Population studies and genetic field tests are practiced for only a relatively few of the important tropical tree species (Zobel and Talbert, 1984). In spite of this lack of actual field experience in tree improvement, the basic genetic principles still apply (Namkoong et al., 1980). The list of tropical species undergoing genetic improvement will increase as more countries decide to grow their native species.

GENE MANIPULATION: PROCEDURES AND FINDINGS

NUCLEIC ACID HYBRIDISATION

Hybridisation of nucleic acids is potentially valuable in the determination of relationship between organisms and in the estimation of the percentage of a genome that produces a particular RNA. The method consists of uniting strands of DNA and RNA and quantifying the degree to which the nucleic acid strands reassociate or are complementary. Two complementary strands should from a double helix and the degree to which the strands do not reassociate should indicate the degree to which the two strands differ in base pair composition and genetic relatedness. In this way, it is possible to determine the amount of difference in the genetic message coded by two different species or the percentage of DNA that is comlementary to a particular RNA. Using this technique, promising results in molecular hybridisation of DNA have been found among *Picea glauca, P. sitchensis* and *P. abies.* Greater complementarity of the nucleotide bases was demonstrated between *P. glauca and P. sitchensis* (Miksche and Hotta, 1977).

DNA - RNA Hybridisation

The formation of duplex DNA-RNA hybrid molecules is accomplished by exposure of denatured DNA to a RNA solution under annealing conditions. The DNA may be immobilised on a filter (Gillespie and Spigelman, 1965); in an agar column (Bolton and McCarthy, 1962); or free in solution (Marmur et al., 1968). The conditions that facilitate annealing are high cation concentration (0.01M Na^+), elevated temperatures, approximately 25° C below the thermal profile or

Tm of DNA to weaken intrastand secondary structure, and sufficient concentrations and incubation times to permit collisions for hybridisations to occur. Filter formed hybrids (Gillespie and Spigelman, 1965) are assayed by radioactivity scintillation methods. In case of agar column, the amount of RNA bound to the column is measured (Bolton and McCarthy, 1962). Hybrids formed in free solution can be assayed by CsCL density gradients (Hall and Spigelman, 1971), MAK chromatography (Hayashi et al., 1965) and filtration on nitrocellulose filters (Nygard and Hall, 1963). Hotta and Miksche (1974) utilised CsCI density gradient method to ascertain that for several conifer species the 28 S and 18 S RNA genes are located next to each other on a relatively short DNA strand.

DNA - DNA Hybridisation

The nitrocellulose filter technique may be used here also, but method must be used that facilitate the removal of added DNA which has not annealed with the initially bound DNA. The single stranded DNA is removed with a buffer of low ionic strength and a high pH after hybridisation is complete (Warnaar and Cohen, 1966).

The amount of labeled DNA that has annealed to the DNA on the filter is determined by liquid scintillation counting. Hybridisation is quantified as the percentage of membrane-bound DNA that has become associated with labeled DNA.

PROTOPLAST FUSION AND GENE MANIPULATION

The genetic manipulation using protoplasts as a tool has proved very promising in tree science (Kirby, 1982). As the protoplasts are totipotent and are able to regenerate entire plant, this should enable studies on gene transfer and uptake of foreign genes through plasmids. In trees, protoplasts are obtained from young parts and callus cultures. Since now, regeneration of complete plant has been obtained in citrus (Vardi et al., 1975).

Somatic hybridisation through protoplast fusion opens up avenues for synthesizing characteristics. Somatic hybridisation is an alternative to sex in order to combine the entire genomes from incompatible parents, and result in hybrids which are otherwise impossible to obtain (Fig. 1).

Cytoplasmic hybrids or cybrids possess nuclear genes from only one parent and cytoplasm from both. Male sterility is thus transferred by chloroplast DNA (Cp DNA) and mitochondrial DNA (mt DNA). The protoplast fusion at interspecific and intergeneric level offers a mechanism for genetic exchange between species that is not possible using other transformation techniques (Cocking et al., 1981). The transfer of disease resistance and herbicide resistance by protoplast fusion have been reported in tobacco (Evans et al., 1981) and potato (Gressel et al., 1984), respectively. As haploids possess only one set of alleles, it is possible for recessive characters to be exhibited and mutants can be easily detected in haploid protoplasts (Bajaj, 1983).

The potentiality of protoplast fusion technology in the synthesis of novel germplasm, therefore, seems to be highly promising in tree improvement.

GENE TRANSFER IN PLANTS

Besides the conventional methods of gene transfer by hybridisation experiments (Stebbins, 1950), transgenic plants have been produced in a number of plant species by employing *Agrobacterium* mediated or direct gene transfer. Dominant selectable markers like kanamycin resistance, herbicide tolerance and insecticidal genes have been cloned in bacteria and transferred and expressed in plants. Unexpected genetic variability may be associaed with foreign gene transfer in plants.

As gene transfer has been described mainly in herbaceous plants than in woody plants, it seems convenient to discuss general principles of gene transfer by drawing references from

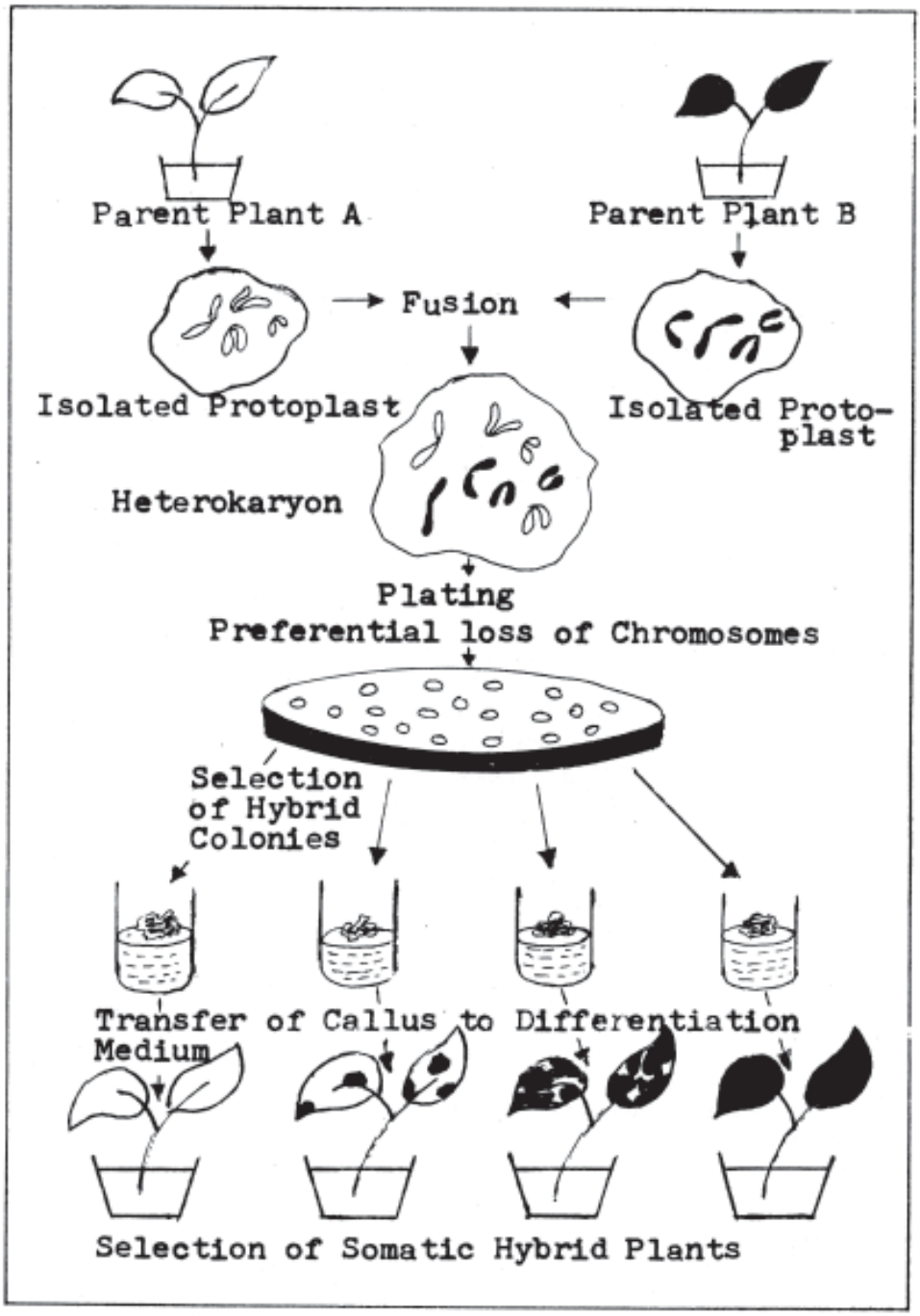

Fig. 1. Diagrammatic representation of protoplast fusion and cell hybridisation from two different plant species and formation of somatic hybrids

work on herbaceous plants.

Direct Gene Transfer

Genes can be transferred directly into totipotent protoplasts by a vector or delivery system possessing protection against cellular endonucleases. Plasmid DNA has been fused with bacterial spheroplast and then transferred to *Nicotiana* protoplasts (Hain et al., 1984). Liposomes, the artifically made lipid vesicles, have also been used for targeting DNA in plant protoplasts (Caboche and Deshayes, 1984; Ohgawara et al., 1983).

Besides the development of stable transgenic *Nicotiana* plant, direct gene tranfer has also described in *Lolium multiflorum* (Potrykus et al., 1985), *Triticum monococcum* (Loorz et at., 1985) and *Oryza sativa* (Uchimiya, 1986).

Direct gene transfer has also been tried by microinjection of DNA into protoplasts. Random portions of plasmid DNA have been found to be integrated into tobacco protoplasts following microinjection (Crossway, 1986). In tree species, the protoplasts are generally recalcitrant where chance of integration and expression of foreign DNA is very less; therefore, the method of direct DNA microinjection might possess limited efficiency in tree improvement.

Microcell or Chromosome Mediated Gene Transfer

A portion of nuclear genome is transferred from a donor to a recipient cell line. A gene present in the donor genome is selected as a marker and some other as beneficial genes. Mitotic division is arrested in the donor cell line to produce metaphase chromosome or mirconuclei. These arrested cells are lysed and the selected gene bearing particular chromosomes or micronuclei are isolated. The purified chromosomes or micronuclei are then mixed and fused chemically with the recipient cell line. After incubating, the fused cells are plated and the fusion products containing the marker gene are identified by subsequent growth, and the colonies are cultured and are observed for stability of the donor characters (Fig. 2). Griesbach (1987) transferred genes from *Petunia alpicola* into *Petunia hybrida* by injecting isolated chromosomes into protoplasts. Hervas et al. (1985) and Schlarbaum (1987) have been successful to induce micronuclei in *Allium* and *Pinus strobus*, respectively. Saxena et al. (1987) have well described the recent and modified procedures for isolation of microcells.

Gene Transfer by Recombinant DNA Technology

Agrobacterium- mediated plasmid vector system:

Plasmids in *Agrobacterium* :

Ti plasmid is carried by *Agrobacterium tumefaciens* which induces tumor formation in different plant species. The Ri plasmid is carried by *A. rhizogenes* and causes hairy root disease in plants. The Ti plasmid of *A. tumefaciens* has been largely exploited for gene transfer in plants. The T-DNA of Ti plasmid is transferred to the genome of plant cell and causes tumor formation. After integration to the genome, the T-DNA is stably maintained in the lineage of transformed cells (Chilton, 1977; Lemmers, 1980).

For the initiation and maintenance of tumorigenic state, the Ti plasmid contains several genes (Schell, 1984; Schilperoort, 1986), especially genes involved in the biosynthesis of phytohormones, auxins and cytokinins. The genes *iaaM* and *iaaH* encode for tryptophan monoxygenase and indole-acetamide hydrolase (Thomashow et al., 1984) enzymes and are involved in indole-acetic acid synthesis. The *ipt* gene encodes isopentenyl transferase (Akiyoshi, 1984) which is involved in cytokinin (isopentenyl adenosine) synthesis.

For the maintenance of tumorigenic state, the T-DNA controlled biosynthetic machinery produces high levels of phytohormones in the transformed cells (Braun, 1958). In addition to the phytohormone genes or *onc* genes, T-DNA also contains genes coding for opines (nopaline, octopine and agropine) which are utilised by

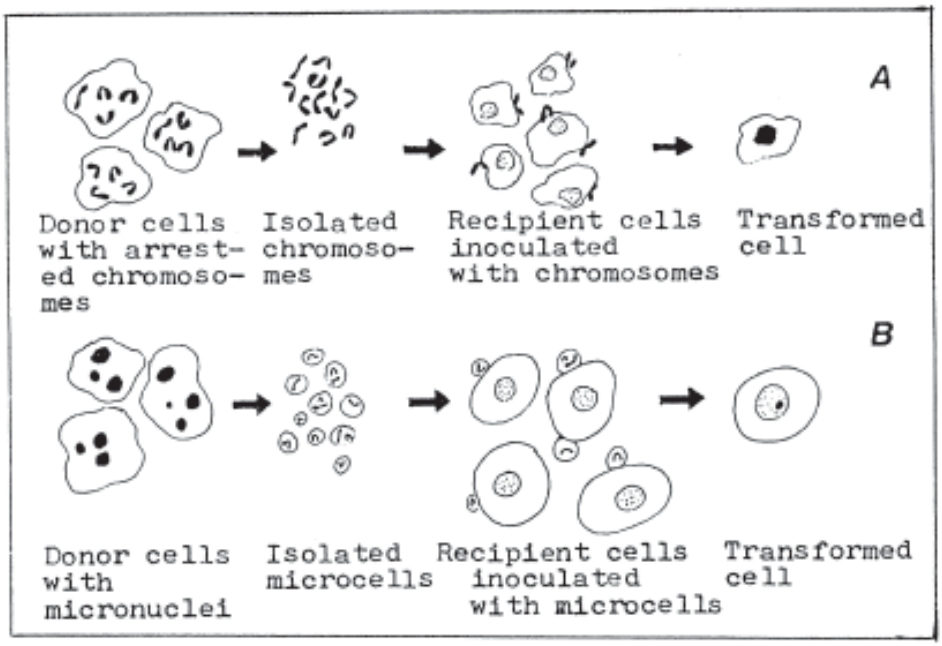

Fig. 2. Schematic representation of A. Chromosome-mediated gene transfer, and B. Microcell-mediated gene transfer

bacteria for their growth. The opines are amino-acid derivatives which can not be easily utilised by plants.

The presence of virulence region (*vir*) is another part of Ti plasmid, activation of which by some specific phenolic compounds like acetosyringone is required for the transfer of T-DNA from Ti plasmid to the plant cell genome (Stachel et al., 1985).

The borders of T-DNA containing 25 base pair imperfect repeats (Schell, 1984; Nester et al., 1980) are very important in gene transfer. All genes originally present in T-DNA can be removed and any genetic sequence may be inserted between the borders of T-DNA and this engineered gene sequence can be successfully integrated in the plant genomes following transformation with *Agrobacterium* (Schell, 1984; Schilperoort, 1986). By employing disarmed Ti plasmid, gene transfer and recovery of normal plants have been demonstrated in a number of plant species (Hain, 1985; Horsch, 1984, 1985; Lloyd, 1986; Ooms et al., 1987) (Fig. 3).

Vector Systems for Gene Transfer

Two types of vector systems have been employed for gene transfer mediated by *Agrobacterium viz.* the cointegrating vectors and the binary vectors.

The essential prerequisite in the cointegrating vector system is a homologous region between the vector plasmid carrying the selectable marker gene and the Ti plasmid; this homology allows the integration of the vector into the Ti plasmid. By employing recombinant DNA technology, two types of cointegrating vectors have been constructed.

1. The phytohormone genes have been replaced in the *Agrobacterium* Ti plasmid pGV3850 (Zymbryski, 1983) by vector plasmid pBR322 sequences. Here the cointegrating vector is placed between the T-DNA borders alongwith the nopaline synthase gene from the Ti plasmid (Herrera and Strella, 1985).

2. Another construction of a cointegrating vector has involved the removal of the right

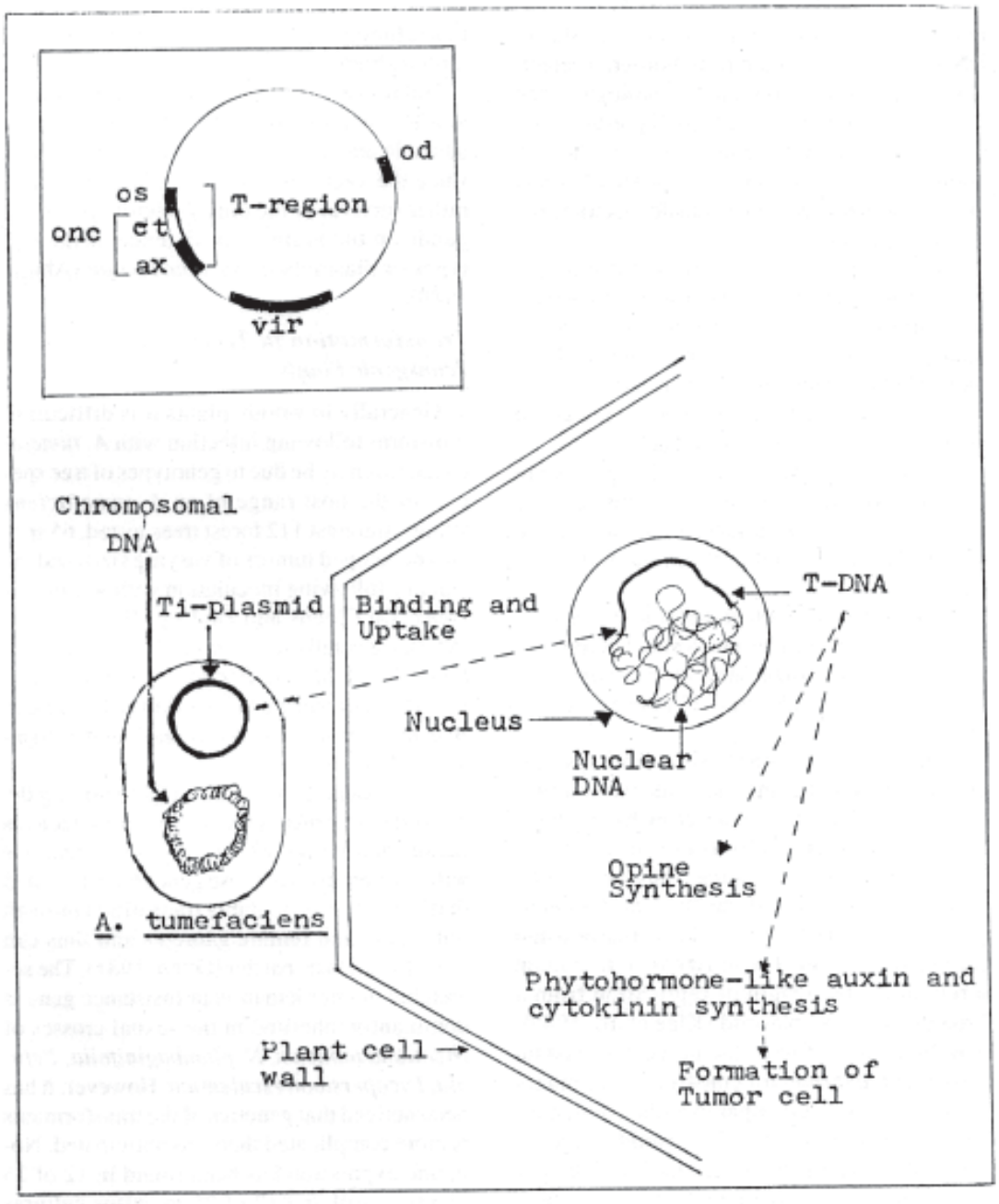

Fig. 3. Schematic representation of the events that occur during the infection of plant cell by octopine type virulent *Agrobacterium tumefaciens*, the integration of T-DNA and the subsequent transformation of the plant cell into a tumor cell. Inset: Schematic representation of some important markers on the octopine-type Ti-plasmid of *A. tumefaciens*. onc. Tumor forming region; os. Octopine synthetic gene; ax. Auxin synthetic gene; ct. Cytokinin synthetic gene; od. Octopine degrading gene; vir. Genes causing virulence

border and phytohormone genes from the T-DNA. A vector with the right border, a selectable marker, and the region of homology is then cointegrated into the disarmed Ti plasmid containing the left border (Fraley, 1985). The new cointegrating vector has both T-DNA borders and is functional as a gene-transfer vector in the acceptor plant cell.

The selectable marker genes in *A. tumefaciens* Ti plasmid are 1. the phytohormone genes; the transformed cells can grow on hormone-free media *in vitro*, whereas untransformed cells require phytohormone for their growth.

2. the opine genes; the presence of genes can be detected in the transformed cells.

As this tumorigenic selectable genes are undesirable traits, their is a pressing need for the search for other non-virulent selectable markers which should perform the following qualities:

1. should allow a clear distinction between the transformed and untransformed cells;

2. growth *vs* growth inhibition *in vitro;*

3. presence or absence of an enzyme or a chemical.

Some of the available selection systems employ chemical compounds like antibiotics, which exclusively inhibit the growth of untransformed cells or gradually kill them.

In contrast to the cointegrating vector system, binary vector systems have no region of genetic homology between the Ti plasmid and the vector plasmid. The binary vectors contain a region for the origin of replication from a broad host-range plasmid (Klee et al., 1985). The frequency of introducing vector plasmid into *Agrobacterium* for construction of binary vector is higher as compared to the cointegrating vector. Here the vector plasmid is not dependent on a specific Ti plasmid for a binary vector construct, and therefore, these vectors carrying specific genes may be introduced in any *Agrobacterium* strain containing a disarmed or an tumorigenic Ti or Ri plasmid. Therefore a wide range of binary vector may be constructed for plant species where transformation is limited by the host range of the *Agrobacterium* strain.

But in contrast to the stability of the cointegrating vector system, the binary vectors are unstable and may be lost from *Agrobacterium* since the vector plasmids do not cointegrate; rather survival of the binary vector system depends on the mutual coexistence of the two types of plasmids in *Agrobacterium* (Ahuja, 1988).

Transformation in Trees and Genetics of Transgenic Plants

Generally in woody plants it is difficult to transform following infection with *A. tumefaciens;* this may be due to genotypes of tree species or the host range of an *A. tumefaciens* strain. Amongst 112 forest trees tested, 65 species developed tumors of varying sizes and incidence following inoculation with *A. tumefaciens* (De Cleens and De Ley, 1976; Ahuja, 1967), especially in *Pinus teads, Pinus sylvestris, Picea abies, Picea nordmanniana, Pseudotsuga menziesii, Betula verrucosa* and *B. mandsehurica, Populus tremula* and *P. tremula* x *P. tremuloides.*

It was found earlier that T-DNA carrying the tumorigenic genes is excluded during meiosis (Braun and Wood, 1976). Later investigations with the deletions of those genes have revealed that T-DNA may be stably transmitted through both male and female gametes and thus can cross the meiosis barrier (Otten, 1981). The selectable marker kanamycin resistance gene is dominantly inherited in the sexual crosses of *Nicotiana tabacum, N. plumbaginifolia, Petunia, Lycopersioon esculentum.* However, it has been noticed that genetics of the transformants is more complicated than was anticipated. Nopaline expression has been found in 12 of 25 plants in soil and 17 of 25 in callus cultures (Horsch, 1985).

In *N. tabacum* transgenic plants have been produced to carry the highly toxic insecticidal gene *bt2* originally coined from *Bacillus thurigiensis* (Vaeck, 1987). A total of 85 transgenic

tobacco genes transformed by four types of chimeric *bt2* genes expressed various levels of active insect toxin when tested on the lepidopteran larvae of *Manducta sexta*. The toxin levels of the F1 progeny varied between 20 to 50 ng/mg of total protein, as compared to 30 ng/mg in the two parental transgenic plants.

GENE TRANSFER IN WOODY PLANTS: PROBLEMS AND PROSPECTS

A number of approaches have been employed for the transfer of foreign genes into different plant species. Besides the very effective hybridisation experiment in plants, most of the gene transportation designs in the sophisticated area have been innovated by recombinant DNA technology. Genes with promoter region for recognition of transcriptional signal and with terminator region can now be isolated, sequenced, cloned, put in transporting vectors and transferred from one plant to another at will. A number of transgenic plants have been produced in *Nicotiana* species. The herbicide tolerance gene *Aro a* has been transferred and expressed in transgenic *Populus* plants (Fillatti, 1987). In future, like in agriculture research today, there remains the possibility of transfer of nitrogenase gene system (Mazur et al., 1980); Ruvkun and Ausubal, 1980) (responsible for nitrogen fixation) from prokaryotes to trees.

There are many limitations, of course. Most of the economically important traits such as growth, vigour, yield, height and biomass are the quantitative traits that are controlled by polygenic inheritance. These complex systems have been poorly understood at the molecular level upto the recent research scenario. We would be limited to single or few gene transfer at the present time, since genetic engineering technology offers better prospects for a single or a block of closely linked gene transfer.

We should continue to search for new variants by gene technology methods in the forest tree species. But how far it is really necessary to introduce foreign gene in the tree species? The question finds its reality in the fact that we have not so far effectively utilised the enormous existing variability in forest trees. In addition, forest trees have long generation cycles with an extended vegetative phase ranging from ten to hundred years or more. The transferred foreign genes may not be immediately expressed or may remain inactive for a long time; again they may cause genetic changes by imposing position effect. This detection is critical if the milestone for the gene expression takes a long time in the vegetative phase or during the flowering phase, *i.e.* after maturity. Therefore, there remain genuine hesitations and concerns in the application of genetic engineering to forest trees.

ACKNOWLEDGEMENT

Authors are grateful to Mr. S.N. Kalita and Dr. S. Chakrabarty for encouragement.

REFERENCES

Ahuja, M.R. : Gene transfer in forest trees pp. 50-68. In : *Genetic Manipulation of Woody Plants*. J. Hanover and D. Kiethley (Eds.). Plenum Press, New York. (1987).

Ahuja, M.R. : Gene transfer in woody plants; perspectives and limitations. pp. 83-101. In : *Somatic Cell Genetics of Woody Plants*. M.R. Ahuja (Ed.). Kluwer Academic Publishers, Dordrecht (1988).

Akiyoshi, D.E. : T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proc. Natl. Acad. Sci. USA*, **81**: 5994-5998 (1984).

Bajaj, Y.P.S. : Haploid protoplasts pp.113-114. In : *Plant Protoplasts*. K.L. Giles (Ed.). Academic Press, London (1983).

Bennett, M.D.: The duration of meiosis. *Proc. R. Soc. Lond. B.*, **178**: 259-275 (1971).

Bennett, M.D.: Nuclear DNA content and minimum generation time in herbaceous plants. *Proc. R. Soc. Lond B.* **181**: 109-135, (1972).

Bolton, E.T. and Mc Carthy, B.J. : A general method for the isolation of RNA complementary to DNA. *Proc. Natl. Acad. Sci. USA*, **48**: 1390-1397 (1962).

Braun, A.C.: A physiological basis of autonomous growth of crown gall tumor cell. *Proc. Natl. Acad. Sci. USA*, **44**: 344-349 (1958).

Braun, A.C. and Wood, H.N. : Suppression of the neoplas-

tic state with the acquisition of specialised functions in cells, tissues and organs of crown gall teratomas of tobacco. cell. *Proc. Natl. Acad. Sci. USA*, **73**: 496-500 (1976).

Caboche, M. and Deshayes, A.: Utilization de liposome pour la transformation de protoplasts de mesophylle de tabac par plasmide recombinant de *E. Coli* leur conferant la resistance a la Kanamicine. *C.R. Acas. Sci. (Paris)*, **229**: 663-669 (1984).

Chilton, M.D. : Stable incorporation of plasmid DNA into higher plants. The molecular basis of crown gall tumorignesis. *Cell*, **11**: 263-271 (1977).

Cocking, E.C., Davey, M.R., Pental, D. and Power, J.B.: Aspects of plant genetic manipulation. *Nature*, **293**: 263-270 (1981).

Crossway, A. : Micromanipulation techniques in plant manipulation. *Biotechniques*, **4**: 320-334 (1986).

De Cleene, M. and De Ley, L.: The host range of crown gall. *Bot. Rev.*, **42**: 389-466 (1976).

Evans, D.A., Flick, C.E. and Jenson, R.A.: Incorporation of disease resistance into sexually incompatible somatic hybrids of the genus *Nicotiana. Science*, **231**: 907-909 (1981).

Fillantti, J.J.: *Agrobacterium* mediated transformation and regeneration of *Populus Mol Gen. Genet.*, **206**: 192-199 (1987).

Fraley, R.T. : Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. USA*, **80**: 4803-4807 (1983).

Gillespie, D. and Spigelman, S.: A quantitative assay for DNA-RNA hybrids with DNA immobilised on a membrane. *J. Mol Biol.*, **12**: 829-842 (1965).

Gressel, J., Cohan, N. and Binding, H.: Somatic hybridisation of an atrazine resistant biotype of *Solanum nigram* with *Solanum tuberosum*, 2. Segregation of plastomes. *Theor. Appl. Genet.*, **67**: 131-134 (1984).

Griesbach, R.J.: Chromosome-mediated transformation *via* microinjection. *Plant Sci.*, **50**: 69-77 (1987).

Hain, R.: Uptake, integration expression and genetic transmission of a selectable chimeric gene by plant protoplasts. *Mol. Gen. Genet.*, **199**: 161-168 (1985).

Hain, R., Steinbiss, H.H. and Schell, J.: Fusion of *Agrobacterium* and *E. coli* spheroplasts with *Nicotiana tabacum* protoplasts : Direct gene transfer from microorgnisms to higher plants. *Pl. Cell Rep.*, **3**: 60-64 (1984).

Hall, B.D. and Spiegelman, S.: Sequence complementary of T2-DNA and T2 Specific RNA. *Proc. Natl. Acad. Sci. USA*, **47**: 137-146 (1971).

Hayashi, M., Hayashi, M.N. and Spiegelman, S.: Chromatographic separation of annealed and enzymatically synthesized RNA-DNA hybrids. *Biophys. J.*, **5**: 231-246 (1965).

Herrera Estrella, L.: *Agrobacterium* as a vector for the introduction of genes into plants. pp. 61-93. In : *Plant Genetic Engineering*, J.H. Dodds (Ed.). Cambridge University Press, Cambridge (1985).

Hervas, J.P., Lopez-Safz, J.F. and Gimenez-Martin, G.: Proliferation of multinucleate cells with unbalanced nuclei. *Cell. Biol. Int. Rep.*, **9**: 91-101 (1985).

Horsch, R.B. Inheritance of functional foreign genes in plants. *Science*, **223**: 496-498 (1984).

Horsch, R.B.: A simple and general method for transferring genes into plants. *Science*, **227**: 1229-1231 (1985).

Hotta, Y and Miksche, J.P.: Ribosomal RNA genes in four conifers. *Cell. Differ.*, **2**: 199-305 (1974).

Jones, R.N. and Brown, L.M.: Chromosome evaluation and DNA variation in *Crepis. Heredity*, **36**: 91-104(1976).

Kirby, E.G.: The use of *in vitro* techniques for genetic modification of forest trees. pp. 369-386. In : *Tissue Culture in Forestry*. J.M. Bonga and D.J. Durzan (Eds.). Nijhoff, The Hague (1982).

Klee, H.J., Yanofsky, M.F. and Nester, E.W.: Vectors for transformation of higher plants. *Bio/Technology*, **3**: 637-642 (1985).

Lemmers, M. : Genetic identification of functions of TL-DNA transplants in octopine crown gall. *EMBO J.*, **1**: 147-152 (1980).

Lloyd, A.M.: Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens. Science*, **234**: 464-466 (1986).

Loorz, H., Baker, B. and Schell, J.: Gene transfer to cereal cells mediated by protoplast transformation. *Mol. Gen. Genet.*, **199**: 178-182 (1985).

Marmur, J., Rowand, R. and Schildkraut, C.L. : Denaturation and renaturation of deoxyribonucleic acid. *Prog. Nucleic Acid Res.*, **1**: 231-300 (1963).

Mazur, B.J. Rice, D. and Haselkorn, R.: Identification of blue-green algal nitrogen fixation genes by using heterologous DNA hybridisation probes. *Proc. Natl. Acad. Sci. USA*, **77**: 186-190 (1980).

Miksche, J.P. and Hotta, Y.: The application of DNA reassociation kinetics for the evaluation of *Picea* crossability patterns. *Silv. Genet.*, **26**: 90-95 (1977).

Namkoong, G., Barnes, R.D. and Burley, J.: A philosophy of breeding strategy for tropical forest trees. *Tropical Forestry Papers*, No. 16, Commonwealth Forestry Insitute, University of Oxford, Oxford (1980).

Nester, E.W., Gordon, M.P., Amasino, R.M. and Yanofsky, M.F.: Crown gall: a molecular and physiological analysis. *Ann. Rev. Plant Physiol.*, **35**: 387-413 (1984).

Nygaard, A.P. and Hall, B.D.: A method for the detection of RNA-DNA Complexes. *Biochem. Biophys. Res. Comm.*, **12**: 98-104 (1963).

Ohgawara, T., Uchimiya, H. and Harada, H.: Uptake of liposome encapsulated plasmid DNA by plant protoplasts and molecular fate of foreign DNA. *Protoplasma*, **116**: 145-148 (1983).

Ooms, G., Burrel, M.M., Karp, A. and Hille, J. : Genetic transformation in two potato cultivars with T-DNA from disarmed *Agrobactoerium. Theor. Appl. Genet.*, **73**: 744-750 (1987).

Otten, L.: Mendelian transmission of genes introduced into plants by the Ti-plasmid of *Agrobacterium tumefaciens. Mol. Gen. Genet.*, **183**: 209-213 (1981).

Potrykus, I., Saul, M., Petruska, I., Paszkowski, J. and Schillito, R.D.: Direct gene transfer to cells of monocots. *Mol. Gen. Genet.*, **199**: 178-182 (1985).

Ruvkun, G.B. and Ausubel, F.M. : Interspecies homology of nitrogenase genes. *Proc. Natl. Acad. Sci. USA*, **77**: 191-195 (1980).

Saxena, P.K., Liu, Y. and King, J.: Nuclear transplantation into plant-protoplasts: Optimal conditions for induction and determination of nuclear uptake. *J. Plant Physiol.*, **128**: 451-460 (1987).

Schlarbaum, S.E.: Cytogenetic manipulation in forest trees through tissue culture. pp. 330-352. In: *Tissues Culture in Forestry. I. General Principles and Biotechnology*. J. Bonga and D.J. Durzan (Eds.), Martinus Nijhoff Publishers, Dordrecht (1987).

Schell, L.: Transfer of foreign genes in plants and its use to study developmental processes. pp. 113-128. In: *Cell Fusion, Gene Transfer and Transformation*. R.F. Beers and E.G. Bassett (Eds.) Raven Press, New York (1984).

Schilperoort, R.A.: Intergration, expression and stable transmission of genes through seeds of foreign origin in plants. pp. 837-858. In: *Genetic Manipulation and Plant Breeding*. W. Horn, C.J. Jensen, W. Odenbach and O. Schieder (Eds.). Walter de Gruyter & Co., Berlin (1986).

Stachel, S.C., Timmermann, B. and Zymbryski, P. : Generation of single stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens*. *Nature*, **322**: 706-712 (1985).

Stebbins, G.L. In: *Variation and Evolution in Plants*. Columbia University Press, New York (1950).

Thomashow, M.F., Hugly, S., Buchholz, W. and Thomashow, L.S.: Molecular basis for the auxin independent phenotype of crown gall tumor tissues. *Science*, **231**: 616-618 (1986).

Thomashow, L.S., Reeves, S. and Thomashow, M.F. : Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiAG encodes an enzyme that catalyzes synthesis of indoleacetic acid. *Proc . Natl. Acad. Sci. USA*, **81**: 5071-5075 (1984).

Uchimiya, H.: Expression of foreign gene in callus derived from DNA-treated protoplasts of rice (*Oryza sativa* L). *Mol. Gen. Genet.*, **204**: 204-207 (1986).

Vaeck, M.: Transgenic plants protected from insect attack. *Nature*, **328**: 33-37 (1987).

Vardi, A. Spiegel-Roy, P. and Galun, E.: Citrus cell culture: isolation of protoplasts, planting densities, effect of mutagens and regeneration fo embryo. *Plant Sci. Lett.*, **4**: 231-236 (1975).

Warnaar, S.O. and Cohen, J.A.: A quantitative assay for DNA-DNA hybrids using membrane filters. *Biochem. Biophys. Res. Comm.*, **24**: 554-558 (1966).

Zobel, B.J. and Talbert, J.T.: In: *Applied Forest Tree Improvement*. John Wiley and Sons, New York (1984).

Zymbryski, P.: Ti plasmid vector for the introduction of DNA into plant cells without alternation of their normal regeneration capacity. *EMBO J.*, **2**: 2143-2150 (1983).