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BIOTECHNOLOGICAL APPROACHES IN MULBERRY

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ABSTRACT

Mulberry (genus *Morus*) a perennial tree or shrub in an economically important plant used for sericulture and is the sole food plant for the domesticated silkworm, *Bombyx mori*. Mulberry is widely distributed in Asia, Europe, North and South America and it is cultivated extensively in East, central and South Asia for silk production. Genetic engineering is the most potent biotechnological approach deals with transfer of specially constructed gene assemblies through various transformation techniques. Tools of recombinant DNA technology facilitated development of transgenic plants. The plants obtained through genetic engineering contain a gene or genes usually from an unrelated organisms, such genes are called transgenes and are known as transgenic plants. The combined use of recombinant DNA technology, gene transfer methods and tissue culture techniques has led to the efficient transformation and production of transgenics in a wide variety of crop plants. In fact transgenesis has emerged as a novel tool for carrying out "single gene breeding" or transgenic breeding of crop plants. Identification, isolation and cloning of resistant genes is the prerequisite for development of transgenic plants for disease resistance. Identification for a variety of resistance genes on the basis of amino acid sequence, conservation enables plant breeder to moniter resistance gene segregation using appropriate DNA probe intend of testing progeny for disease resistance and susceptibility. Significant developments in plant genetic modification have been achieved in the last 15 years. Some of the success include herbicide tolerent corn, cotton, soyabeen and papaya; virus resistant corn, potato, cotton etc. In mulberry little work has been carried out at Delhi University (south campus). They have developed drought and salinity tolerent transgenic mulberry through *Agrobacterium* mediated transformation. The overexpression of HVA1 gene from barley generates tolerence to salinity and water stress in transgenic mulberry (*Morus indica*). Genetic characterization of germplasm resources is necessary for their effective management and efficient utilization, especially for species like mulberry in which the available germplsm exhibits rich phenotypic diversity with almost no information about its genetic base. Molecular markers are useful complements to morphological and phenological characters because they are plentiful, independent of tissue or environmental effects and allow accession identification in the early stages of development. Such techniques reveal polymorphisms at the DNA level and are very powerful tool for characterization and genetic diversity estimation. Many molecular markers such as RAPD, SSR, AFLP and SRAP have been successfully used in identification and genetic diversity analysis in mulberry.

Keywords: Transgenic plant, Molecular markers**,** Genetic diversity, *Morus* spp.,

INTRODUCTION

Mulberry (*Morus*) is an important crop plant in Sericulture. Its foliage is the exclusive food of domestic silkworm (*Bombyx mori* L.) which produces the natural silk. The queen of textiles. Genetic engineering is the most potent biotechnological approach deals with transfer of specially constructed gene assemblies through various transformation techniques. Tools of recombinant DNA technology facilitated development of transgenic plants. Transgenic plants carry additional, stably integrated and expressed, foreign genes from trans species. The whole process involving introduction, integration and expression of foreign genes in the host is called genetic transformation or transgenesis. The combined use of recombinant DNA technology, gene transfer methods and tissue culture techniques has led to the efficient transformation and production of transgenic in a wide variety of crop plants.

Mulberry is believed to have originated at the feet of Himalayan ranges. According to watt (1873) certain forms of *Morus* were truly wild in India, but according to Vavilov (1926) the primary centre of origin of mulberry was the China, Japan gene center, which includes East China, Korea and Japan. Presently, *Morus* species are grown in warm and moist climates between lat. 500 N and 100 S, which includes the Southeastern tip of Asia and Japan. Taxonomically, the genus was placed in Urticales under the family Moraceae. A number of species have been reported within this genus. Initially, linneaus (1753) divided the genus *Morus* into seven species: *Morus alba* L; *Morus nigra* L; *Morus rubra* L; *Morus tarterica* L; *Morus indica* L; *Morus papyrifera* and *Morus tinetoria*. But the last two where later shifted to the genera Broussonetia and chlorophora respectively. However, Brandis (1874) described four species and classified the genus into two section based on style length and subdivided both the sections again according to the length and shape of the syncarp and a few leaf characters. Koidzumi (1923) identified 25 species and classified them into 2 sections, dolichostyle and macromorus based on the length of the style each section was then subdivided into two subsections pubescentae and papillosae based on stigma hairiness on the

basic of leaf anatomical and wood characters. Shah and Kachroo (1979) classified the genus into two sections (i) *Morus nigra* L; (ii)*Morus alba* L; *Morus bombycis koidz*., and *Morus latifolia* P. However, after assessing the protein and Allozyme profiles of *M. alba*, *M. bombycis* and *M. latifolia*, Hirano (1982) suggested grouping all three spices together. The confusion over the classification of *Morus* into species remains a major bottleneck for formulating appropriate strategies to conserve the previous materials for future breeding programs. Genetic characterization of germplasm resources is necessary for their effective management and efficient utilization, especially for species like mulberry in which the available germplasm exhibits such phenotypic diversity with almost no information about its genetic base. Genetic diversity in the genus *Morus* have been developed using morphological characters and molecular techniques. Molecular markers are useful complements to morphological and phenological characters because they are plentiful, independent of tissue or environmental effects and allow accession identification in the early stages of development. Such techniques reveal polymorphisms at the DNA level and are a very powerful tool for characterization and genetic diversity estimation. Many molecular marker techniques have been successfully used in identification and genetic diversity analysis in mulberry, such as RAPD (Xiang *et al*., 1995; (Zhao and Pam, 2004), SSR (Aggarwal *et al*., 2004), ISSR (Zhao *et al*., 2006) and AFLP (Sharma and Sharma 2000). RAPD in simple, convenient and inexpensive but poor consistency and low reproducibility limit in its utilization (Roodt et al., 2002). AFLP technology is now widely used for genomic fingerprinting (Karaca *et al*., 2002) due to its high polymorphism (Vos *et al*., 1995). However AFLP is complex, requires multiple steps and shows pseudo-polymorphism when methylation- sensitive restriction enzymes are used. SSRs are stable, abundant, highly polymorphic and reproducible, but they require the development of working primers for each species which makes the method laborious and costly (Xiao *et al*., 2008). Sequence- related amplified polymorphism (SRAP) is a novel molecular marker technique based on two primer amplification that preferentially amplifies open reading frames (Li and Quiros, 2001). The observed polymorphism originates in the variation in the length of extrons, introns, promoters and spacers both among individuals and species. SRAP markers are more powerful for revealing genetic diversity among closely related cultivars than SSR, ISSR or RAPD markers.

Need of Biotechnological tools in mulberry

The main focus of mulberry breeding is to improve leaf productivity as it alone contributes more than 38.2% to the sericulture productivity (Banerjee, 1998).However improving leaf productivity is difficult being a multifactorial trait and determined by a number of associated characters such as plant height, number of branches, leaf retention capacity, nodal length, leaf size and weight and total biomass(Doss *et al*.,2011).High heterozygosity and inbreeding depression hinder the development of inbreed, hence directional breeding failed to make much progress. Therefore, the heteriozygous parents are used to generate F1 progenies, which are then subjected to different evolutions and selection procedures to identify the best one. This type of breeding systems bars the possibility of introgression gene of desirable traits from wild relatives/species due to genetic drag and subsequent difficulty in eliminating the undesirable traits that come along. Under such circumstances, the feasible means of improving specific traits without disturbing the current trait combination is adoption of biotechnological tools like transgenssis which enable introduction and over expression of desirable genes (Vijayan *et al*., 2011a) or knocking out undesirable genes through RNA interference technology (Vijayan *et al*., 2011b).Mulberry being a tree with high heteriozygosity, possess difficulties on improving traits of economic importance through

conventional breeding and selection. Environmentally less influenced and developmentally stable molecular markers provide reliable tools for the breeders to characterize the germplasm and select parents and off springs through marker assisted selection. Thus it would be prudent to use biotechnological tools to harness the vast benefit mulberry offers to mankind.

Transgenic Plants

The plants obtained through genetic engineering contain a gene or genes usually from an unrelated organism, such genes are called transgenes and the plants containing transgenes are known as Transgenic Plants. Due to limitations of conventional breeding for attaining the desirable traits, the use of recombinant DNA technology has been taking advantage and development of transgenics started.

Advantages of Transgenic plants

Agronomic traits can be improved by producing plants with increased resistance to pest, Stress along with increased vigor, yield.

- Enhanced efficiency of physiological process like photosynthesis and improved nutritional qualities.
- Transgenic plants are also used as an analytical tool to explore aspects of gene regulation.
- Major advantages of molecular breeding is that when a particular gene has been isolated and reconstructed it can be first in model plants and later it can be used in a variety of different crops.

Other uses

Environment

Transgenic plants may be used as a means of bioremediation i.e. decontamination of polluted land. Transgenic *Liriodendrom tulipifera* (yellow poplar) expressed bacterial mercuric reductase gene (Rugh *et al*. 1988). These plants survive in high Hg levels and release elemental Hg.

Industry

Biomass/fuel: Biomass producing crops include rapidly growing trees used for fuel or products (ethanol). Transgenic plants for this aim either over express cellulose synthase gene (*Arioli et al*.1998) or cellulases that convert cellulose to ethanol (Label *et al.*1998).

Lignin/Paper: Cellulose is raw material for paper. Lignin is separated from cellulose fibers during pulping. Transgenic plants with modified lignin ease of lignin solubilization and fragmentation. The only difference is the red brown colored wood.

House hold *(clothes and ornaments)*

Transgenic approach on cotton includes introduction of pigment compounds and synthesis of plastics like PHB in same cells. Coloured fibers avoid dying process whereas plasticity lowers thermal conductance. Genetic manipulation of ornamentals can produce colored flowers that donor occur naturally.

Food and drink

Protein, sugars, starch, oil and vitamins: Proteins can be modified either by changing the amino acids in a protein or by adding entirely new protein. Another application is expression of human milk proteins in edible plants. For example, gene for beta casein protein can be introduced in potato under the control of an amino auxins inducible promoter.

Allergens/Toxin*:* Allergens can be successfully reduced in transgenic plants e.g. level of allergenic proteins (e.g. AraH1) in peanut can be reduced.

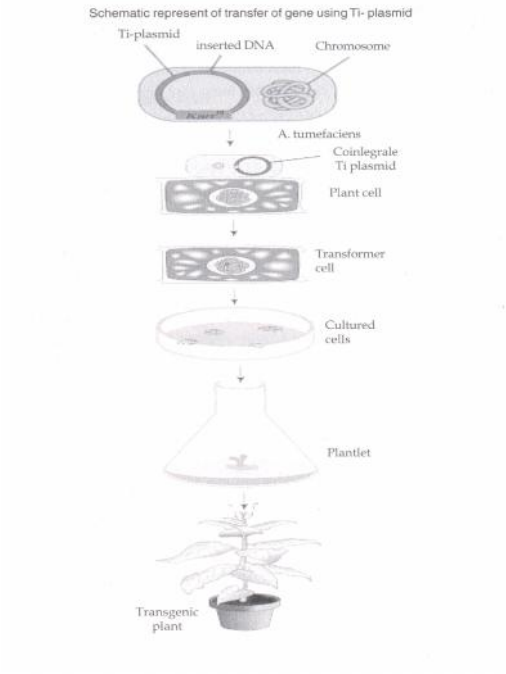
Beer

Transgenic barely is generated involving the modification of the glucanase enzyme as means of improving the efficiency of brewing process.

Medical application

Vaccines/antigens/antibiotics

Production of plant based vaccines and other medically related compounds. Transgenic potato have been successful in expressing human insulin (auto antigen) either alone or as a protein in which insulin was linked to terminus of cholera toxin B subunit (CTB).



(Source: Rukamet et al. 2010)

How to make transgenic plant

The whole process of introduction, integration and expression of foreign genes in the host is called genetic transformation. The understanding of the R gene function and resistance reaction have helped in designing the strategy for the development of transgenic plants resistant to different pathogens.

Gene Transfer Methods

Vector-mediated gene transfer

A. Agro bacterium-mediated gene transfer
B. DNA viruses as vectors

Vector less gene transfer

A. Direct uptake of DNA
B. Electroporation
C. Micro injection
D. Micro projectile bombardement

Vector-mediated gene transfer

Foreign genes are transported into recipient cells, protoplast or intact plant through a vector. It is a DNA molecule capable of replication in a host organism, into which a gene is inserted to construct a recombinant DNA molecule. This is also called indirect method of gene transfer. A vector could be either DNA virus such as caulimovirus or plasmid.

Agro bacterium-mediated gene transfer

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

The foreign gene is cloned in the T-DNA region of Ti plasmid in place of unwanted places. To transfer plants, leaf discs, (in case of dicots) or embryogenic callus (in case of monocots) are collected and infected with Agro bacterium carrying recombinant disarmed Ti plasmid vector.

The vector tissue is then cultured on shoot regeneration medium for 2-3 days during which time the transfer of T-DNA along with foreign genes takes place. After this, the transformed tissues (leaf discs/calli) are transformed onto selection cum plant regeneration medium supplemented with usually lethal concentration of an antibiotic to selectively eliminate non-transformed tissues. After 3-5 weeks, the regenerated shoots (from leaf discs) are transferred to root -inducing medium, and after another 3-4 weeks, complete plants are transferred to soil. Following the hardening of regenerated plants. The molecular techniques like PCR and southern hybridization are used to detect the presence of foreign genes in the transgenic plants.

DNA viruses as vectors

The genomes of caulimoviruses such as cauliflower mosaic virus (CaMv) and germinivirus such as tomato golden mosaic virus (TGMv) are double stranded DNA which makes these viruses as potential transformation vectors. Methotrexate resistant dihydrofolate reductive (DHFR) gene of *E. coli* has been successfully cloned into an intergenic region of CaMv. This engineered CaMv was used to infect turnip plants.

Vector less gene transfer

This is non-biological method for introduction of foreign genes into plants. Gene transfer in monocotyledonous plants is done by this method. Chemical and physical means are used to facilitate the entry of DNA into plant cells.

Direct uptake of DNA

Direct DNA uptake by protoplasts can be stimulated by chemicals like polyethylene glycol (PEG) and dextran sulphate. The technique is so efficient that virtually every protoplast system has proven transformable.

PEG is also used to stimulate, the uptake of liposomes and to improve the efficiency of electroporation. PEG at high concentration (15-25%) will precipitates ionic macromolecules such as DNA and stimulate their uptake by endocytosis without any gross damage to protoplasts. This is followed by cell wall formation and initiation of cell division. These cells can now be played at low density on selection medium.

However, other plant systems (rice, maize, etc.) were also successfully used later. In these methods, PEG was used in combination with pure Ti plasmid, or calcium phosphate precipitated Ti plasmid mixed with a carrier DNA. Transformation frequencies up to 1 in 100 have been achieved by this method. Nevertheless, there are serious problems in using this method for getting transgenic plants and all these problems relate to plant regeneration from protoplasts.

Electroporation

This method is based on the use of short electric impulses of high field strength; a pulse of high voltage applied to protoplast/cells/tissues to make transient (temporary) pores in the plasma membrane which facilitate the uptake of foreign DNA, If the DNA is in direct contact with the membrane. In view of this, for delivery of DNA to protoplasts, electroporation is one of the several routine techniques for efficient transformation. However, since regeneration from protoplasts is not always possible, cultured cells or tissue explants are often used.

Consequently, it is important to test whether electroporation could transfer genes into walled cells. The electroporation pulse is generated by discharging a capacitor across the electrodes in a specially designed electroporation chamber. Either a high voltage (1.5kV) rectangular wave pulse of short duration or a low voltage (350V) pulse of long duration is used.

Using electroporation method, successful transfer of genes was achieved with the protoplast of tobacco, maize, rice, wheat and sorgum. In most of these cases cat gene associated with a suitable promoter sequence was transferred.

Transformation frequencies can be further improved by

1. Using field strength of 1.25kV/cm,
2. Adding PEG after adding DNA,
3. Heat shocking protoplasts at 45^0C for 5 minutes before adding DNA and by using linear instead of circular DNA.

Microinjection

Where the DNA is directly injected into plant protoplasts or cells (specially into the nucleus or cytoplasm) using fine tipped (0.5-1.0 micrometer diameter) glass needle or micropipette. This method of gene transfer is used to introduce DNA into large cells such as oocytes, eggs and the cells of early embryo. The process is observed and controlled under the microscope. The DNA is then integrated into the plant genome-probably during the cells own DNA repair processes.

The advantages of microinjection are that, the target gene, which confers a new trait, is introduced directly into a single cell. The cells transformed in this way are easy to identify if a dye is injected along with the DNA. If the process works, it will no longer be necessary to select the transformed cells using antibiotic resistance or herbicide resistance markers.

Micro projectile bombardment

In recent years, it has been shown that DNA delivery to plant cells is also possible, when heavy micro particles (tungsten; or gold) coated with the DNA of interest are accelerated to a very high initial velocity (1,400 ft per; sec).These micro projectiles, normally 1-3 pm in diameter , are carried by a macro projectile or the bullet and are accelerated into living plant cells (target cells can be pollen, cultured cells in differentiated tissues and meristems) so that they can penetrate cell walls of intact tissue.

The acceleration is achieved either by an explosive charge or by using shock waves initiated by a high voltage electric discharge.

The design of two particles gun used for acceleration of microprojectiles. Transformed plants using the above technique have been obtained on many cases including soybean, tobacco, maize, rice, wheat, etc. Transient expression of genes transferred in cells by this method has also been observed in onion, maize, rice, and wheat.

There is no other gene transfer approach, which has met with so much of enthusiasm. Consequently considerable investment has been made in experimentation and manpower for development of this technique.

Work	Reference
Few transgenic rice plants by inoculating immature embryo with a strain of *A. tumefaciens*	Chua *et al.* (1993)
Reported a method for efficient production of transgenic rice plants from calli of japonica cultivars that had been co-cultivated with *A. tumefaciens*	Hiei *et al.* (1994)
The successful application of calli of japonica cultivars method to Basmati cultivars of indica rice	Rashid *et al.* (1996)
Achieved efficient transformation of maize by *A. tumefaciens*	Irshida *et al.* (1996)
Transformation of monocots and cereals	Heii *et al.* (1997)

Genetic transformation in monocotyledonous plants Engineering insect resistance

Two are approaches to develop insect resistant transgenic plants; Introduction of bacterial gene Bt synthetic Bt. Introduction of plant gene(s) for insecticidal proteins.

Introduction of bacterial gene Bt synthetic Bt

Bacillus thuringiensis synthesizes an insecticidal crystal protein which resides in the inclusion bodies produced by the Bacillus during sporulation. This crystal protein when ingested by insect larvae is solubilized in the alkaline conditions of the midgut of insect and processed by midgut proteases to produce a protease resistant polypeptide which is toxic to the insect. *Bt,* lepidopteran specific from *Bacillus thuringiensis* subsp.Kurstaki has been widely and successfully used in tobacco, tomato, potato, cotton, rice and maize for developing resistance against several lepidopteron insect pests. The use of redesigned synthetic *Bt* genes has also been used in some of these crops and in several instances the synthetic versions have exhibited up to 500- fold increase in the expression. Some of the transgenic have been released in the field for commercial cultivation.

Introduction of plant gene(s) for insecticidal proteins

Several insecticidal proteins of plant origin such as lectins, amylase inhibitors and protease inhibitors can retard insect growth and development when ingested at high doses. Some genes like CpTi, P1N-1, P1N11, A-1 and GNA have been cloned and being used in the transformation programme aiming at the insect resistance.

Engineering for herbicide resistance

There have been two approaches to develop herbicide resistant transgenic plants. Transfer of gene whose enzyme products detoxify the herbicide (detoxification) Transfer of gene whose enzyme product becomes insensitive to herbicide (Target modification).

Transfer of gene whose enzyme product detoxify the herbicide (Detoxification)

Using this approach the introduced gene, produces an enzyme which degrade the herbicide sprayed on the plants. Introduction of bar gene cloned from bacteria *Stroptomyces hygroscopicus* into plants, make them resistant to herbicides based on phosphinothricin.

Bar gene produces an enzyme, phosphinothricin acetyl transferase (PAT) which degrades phosphinothricin into a non-toxic acetylated form. Plants engineered with bar gene were found to grow in ppt at levels 4-10 times higher than normal field application.Bxn gene of *Klebsiella ozaenae* which produces nitrilase enzyme imparts resistance to plants against herbicide bromoxynil. Other genes including tfdA for 2,4-D tolerance and GST gene for Atrazine tolerance have also been used.

Transfer of gene whose enzyme product becomes insensitive to herbicide (Target modification)

In this approach a mutated gene is introduced which produces modified enzyme in the plant which is not recognized by the herbicide, hence the herbicide cannot kill the plant. A mutant aroA gene from bacteria *Salmonella typhimurium* has been used for developing tolerance to herbicide; glyphosate.Tolerance to herbicides has been achieved by engineering the expression of the mutant herbicide ALS gene derived from plant.

Herbicide resistant transgenic plants

Species modified	Transgene source	Transgene product
Target modifications : *Betavulgaris, Nicotiana tobaccum*	*Arabidopsis thaliana, A. thaliana*	Acetolactate synthase, Acetolactate synthase
Enzyme overproduction : *Glycine max*	*Plant and microbial genes*	Analogue of EPSP synthase
Enzyme detoxification : *Brassica napus*	*Streptomyces hygroscopicus*	Phosphinothricin

(Transgenic Plants by Kakralya/Ahuja)

Engineering virus resistance

The genetic engineering of virus resistant plants has exploited new genes derived from viruses themselves in a concept referred to as pathogen derived resistance (PDR).

1. Coat protein mediated resistance (cP-MR)
2. Satellite RNAs mediated resistance
3. Antisense mediated protection

Coat protein mediated resistance (cP-MR)

Introduction of viral coat-protein gene into the plant, make the plant resistant to virus from which the gene for the cP was derived (Shah *et al*. 1995). It was 1st demonstrated for TMV in tobacco. Virus resistant transgenic have been developed in tomato, melon, rice, papaya, potato and sugar beet. Several cP-MR varieties of potato, cucumber and tomato are under field evaluation.

Satellite RNAs mediated resistance

Satellite RNAs are molecules which show little sequence homologies with the virus to which they are associated, yet are replicated by the virus polymerase and appear to affect the severity of infection produced by virus. It has been demonstrated that engineering cucumber, using cucumber mosaic virus (CMV) satellite RNA lead to transgenic resistant to CMV. This approach has been extended to several other crops.

Antisense mediated protection

It is now established that gene expression can be controlled by antisense RNA. cDNAS representing viral RNA genome were cloned in an antisense orientation to a promoter and transferal though the protection was not as effective as with coat protein. This approach has been effective against TMV gene.

Engineering for fungal resistance

There have been two approaches to develop fungal resistant transgenic plants.

A. Antifungal protein-mediated resistance
B. Antifungal-compound mediated resistance

Antifungal protein-mediated resistance

Introduction of chitinase gene in tobacco and rice has been shown to enhance the fungal resistance in plants. Chitinase enzymes degrade the major constituents of the fungal cell wall (chitin). Coexpression of chitinase gene in tobacco and tomato plants confers higher level of resistance alone. A radish gene encoding antifungal protein 2(Rs-AFP2) was expressed in transgenic tobacco and resistance to *Alternaria longipes* was observed.

Antifungal-compound mediated resistance

The low molecular weight compounds such as phytoalexins possess antimicrobial properties and have been postulated to play an important role in plant resistance to fungal and bacterial pathogens. Expression of a stilbene synthase gene from grapevine in tobacco, resulted in the production of new phytoalexin (resveratrol) and enhanced resistance to infection by *Botrytis cinerea*. Active oxygen species (AOS) including hydrogen per oxide also play an important role in plant defense responses to pathogen infection. Transgenic potato plants expressing an H2O2 generating fungal gene for glucose oxidase were found to have evelvated levels of H2 O2 and enhanced levels of resistence both to fungal and bacterial pathogens particularly to *Verticillium wilth.*

Bacterial resistance

Genetic engineering for bacterial resistance has relatively met with little success. The expression of a bacteriophage T4 lysozyme in transgenic potato tubers led to increased resistance to *Erwinia caroto* Vora. Besides, the expression of barley a –thionin gene significantly enhanced the resistence of transgenic tobacco to bacteria *Pseudomonas syringae*. Advances in the cloning of several new bacterial resistance genes such as the *Arabidopsis*. RPS2 gene, tomato Cf9 and tomato P to gene may provide better understanding in the area of plant bacterial inter reactions.

Engineering for a biotic stress tolerance

Transfer of cloned genes has resulted in the transgenic which are tolerant to some a biotic stresses. For frost protection, an antifreeze protein gene from fish has been transferred into tomato and tobacco. Likewise, a gene coding for glycerol-3-phosphate acyltransferase from Arabidopsis has been transferred to tobacco for enhancing cold tolerance

Engineering for male sterility

- The introduction of barnase gene results into male sterility whereas the introduction of barstar gene into another plant results into development of restorer line.
- The resulting hybrid is fully fertile.This system has been commercially exploited in maize and oilseed rape.

Engineering for food processing/quality

1. Using antisense RNA technology and inhibiting polygalacturonase, shelf- life can be extended in vegetable and fruits.
2. High protein ‘phaseolin’ and Ama-1 genes have been introduced to heterologous systems.
3. Introduction of provitamin A and earotene genes have resulted into the production of ‘golden rice’

Gene	Origin	Host	Stress
Bet A	*E. coli*	Tobacco	Salinity
Bet A	*E. coli*	Potato	Freezing
Mltd	*E. coli*	Arabidopsis	Salinity
Fad7	*Arabidopsis*	Tobacco	Chilling
HVA-1	Barley	Rice	Salinity and drought
Mn-SoD	*N.plumbaginif* olia	Alfalfa	Droughtand freezing

(Transgenic Plants by Kakralya/Ahuja)

Molecular approaches to develop salt tolerance in mulberry

The efficient regeneration of plants from tissue culture is of great value as it can be applied in direct cultivar improvement and is a prerequisite for genetic manipulation and transformation studies. Over the past 40 years numerous reports on reliable and efficient regeneration protocols of commercially important genotypes of mulberry have been published. Auxiliary and apical buds are most commonly used explants for in vitro propagation of mulberry although successful regeneration has also been obtained from leaf, cotyledon, and hypocotyls explants (Bhatnagar *et al*. 2000; Kapur *et al*. 2001; Raghunath *et al*. 2008; Tewari *et al*. 1999). Improvement of mulberry by somatic hybridization via protoplast fusion has been demonstrated in temperate and tropical mulberry, but practical realization of this technology is yet to be achieved (Umate *et al*. 2005; Wei *et al*. 1994). Another area of production of haploid plants in mulberry has also been an arduous process. Despite initial progress on mulberry androgenesis (Jain *et al*. 1996; Katagiri and Modala 1993; Sethi *et al*.1992; Shoukang *et al*. 1987; Tewari *et al*. 1994), significant success has not yet been achieved. Similarly, although gynogenic plants of mulberry were reported by Lakshmi Sita and Ravindran (1991) and Thomas *et al*. (1999), these have not been translated into practical ventures. Tissue culture studies in mulberry have been reviewed in detail earlier (Khurana *et al*. 2003; Thomas 2002; Wakhlu and Bhau 2000).

Although mulberry is a hard to deal woody recalcitrant tree species, several protocols have been optimized to transform *Morus indica* using particle bombardment (Bhatnagar *et al*. 2002) and via *Agro bacterium tumefaciens* (Bhatnagar and Khurana 2003). These protocols are particularly efficient on cultivar K-2 but can be extended to other cultivars. Although seedling hypocotyls and cotyledon explants are regenerative (Bhatnagar *et al*. 2002, 2003; Lal *et al*. 2008), mature leaf-based transformation via *Agro bacterium tumefaciens* proved to be a rapid and efficient transformation system in this heterozygous plant allowing the transformation efficiency of 90% (Das *et al*.2011b).

Absence of significant achievements in traditional plant breeding for tolerance to a biotic stresses and the economic losses incurred due to a biotic stresses in recent years have paved the way for molecular tailoring of crops through transgenic approaches. Genomic technologies generating valuable information on molecular basis of stress tolerance is the starting point for targeted gene-based transgenic approaches for introgression of beneficial genes, conferring stress tolerance in mulberry.

There are reports of multiple stresses being simultaneously regulated by using single gene encoding stress-inducible transcription factor (Kasuga *et al*. 1999). Putative transgenic plant should be tested in range of relevant environments to study the effect of inserted gene at whole plant level if impact of powerful transgenic approach has to be achieved. Further, to have enduring effect by these contentious transgenic strategies in this cross pollinated crop, proper discretion is definitely required.

Genetic transformation of mulberry

Agro bacterium mediated transformation has opened up several opportunities to develop mulberry transgenic. Progress made in developing transgenic at Delhi University (South campus) is quite impressive and has paved the way to initiate transgenic programmes in mulberry in other centres such as CSRTI, Mysore and UAS, Bangalore. Delhi University group has developed transgenic over expressing HVA1, a LEA_3 group stress responsive gene.

Drought and salinity tolerant transgenic mulberry

Over expression of HVA1 gene from barley generates tolerance to salinity and water stress in transgenic mulberry

The HVA1 gene from barley encodes a group 3LEA protein and is induced by ABA and water deficit conditions. Over expression of HVA1 in mulberry under a constitutive promoter via Agro bacterium mediated transformation. Molecular analysis of the transgenic plants revealed the stable integration and expression of the transgenic in the transformants. The transgenic plants showed better cellular membrane suitably, photosynthetic yield, less photo-oxidative damage and better water use efficiency as compared to non transgenic plants under both salinity and drought stress. Amongst the lines analyzed for stress tolerance transgenic lines ST8 was relatively more salt tolerant, ST30 and ST31 was more drought tolerant. (Source: Lal *et al*. 2007)

Gene transfer by electroporation into protoplasts isolated from mulberry calli

For callus induction, explants were excised from the roots of mulberry seedlings grown in a medium containing benzylaminopurine, dichlorophenoxy acetic acid and thidiazuron. The isolated calli were repeatedly subcultured in the liquid medium resulting a faster growing callus line. Protoplasts were enzymatically isolated from clumped cells and transfer of the β-gluconidas (GUS) gene by electroporation was carried out at various pulse voltages. Observation showed that successful transient expression of the GUS gene was accomplished in 20-30% of protoplasts at specific pulse voltage.

Transient expression of GUS gene in electroporated protoplasts

Pulse voltage[1] (v/cm)	Survival (%)		Stained protoplasts (%)[2]
	Initial	After electroporation	
500	92	78	21
750	92	68	32
1000	92	52	0

[1]Capacitance; 330 µFd.

[2]Percentage of blue-stained protoplasts in 150-200 surviving protoplasts counted

(Source : Yukio *et al*. 1999)

] , protoplasts stained by histochemical assay were observed constantly in pulse voltage ranging from 500-750 V/cm with a capacitance of 330µFd .A typical result showed that survival rate of protoplasts decreased with increasing pulse voltage in electroporation.Transient expression of the GUS gene was operative in 20 -30% of protoplast electroporated at 500 and 750V/cm. It was clearly demonstrated that the protoplasts from the callus line established by the study have the capability to permit successful transient expression of the GUS genes under the control of 35 S CaMV promoters, which is the first time this has been done in mulberry

protoplasts. This success may open new possibilities in the analysis of various constructs using genes isolated from mulberry trees and in the production of transgenic mulberry clones as well.

Major improvements in gene delivery into mulberry leaf cells by particle inflow gun

The efficiency of gene delivery into mulberry leaf cells using a particle inflow gun for the production of b-glucronidase. Two days following bombardment with plasmid-coated micro projectiles, transiet expression of the b-glucuronidase was detected by forming a blue precipitate visually detectable within transformed cells. Bombardment efficiencies were determined by counting the number of blue spots that appeared in bombarded leaf tissue

Difference of bombardment efficiency among leaves precultured for various periods

	Preculture period (days)	No. of blue spots/cm^2
Expt. 1	0	98.7±40.00
	3	86.5±29.3
	6	70.0±33.5
	9	17.0±8.1
Expt. 2	5	112.0±31.3
	10	11.3±6.6
	15	11.8±5.1

Fifteen leaves were harvested at different preculture periods and bombarded
(Source Yukie *et al*. 2000)

To survey the optimal tissue conditions for efficient bombardment, leaf tissue was precultured for various periods prior to bombardment with one µm micro projectiles. High levels of bombardment efficiency were obtained in day 0 – day 5 tissues after they were precultured. The prolonged preculture period markedly reduced bombardment efficiency.

Treatment	No. of blue spots/cm^2
Control	89.6±49.1
0.7 M mannitol	59.3±41.5
Heat shock	79.1±15.8
1% DMSO	167.0±40.4

Twelve leave which were precultured for 5 days were used for each treatment and bombarded
(Source Yukie *et al*.2000)

Various treatments that could potentially enhance microprojectile penetration and gene expression were examined.

Treatment with elevated osmoticum concentration may work in protecting the cells from leaking and bursting damage caused by micro projectile penetration. This idea was applied to the bombardment of mulberry leaf tissues. Treatment with 0.7M manitol pre to bombardment tended to decrease the number of cells that transiently expressed the GUS gene.

Bio safety and risks of disease resistance transgenic plants

Possibility of selectable marker gene (npt11) product to be toxic or allergic to human or animals. Mutation of sat RNAs may result in severe strain of the specific virus. Heteroencapsidation in case of cPMR may also result in development of virulent strain. Recombination between engineered and challenged viruses.

Advantages of transgenic plants

Agronomic traits can be improved by producing plants with increased resistance to pest, disease, stress along with increased vigour and yield. Enhanced efficiency of physiological process like photosynthesis and improved nutritional qualities. Major advantage of molecular breeding is that when a particular gene has been isolated and reconstructed in model plants and later it can be used in a variety of cultivars of different crops.

Molecular marker technology

In order to acquire thorough knowledge of the total genetic make-up of the germplasm bank, environmentally insensitive, developmentally stable, reproducible, easy to define, unbiased, numerous and ubiquitous molecular markers have been used for germplasm characterization in mulberry (Vijayan *et al*.,2006).Several types of molecular markers which have been developed and used in plants are restriction fragment length polymorphism (RFLP), sequence tagged sites (STS), Expressed sequence tags (ESTS), Simple sequence repeats (SSRs) or microsatellites, randomly amplified polymorphic DNA (RAPDs), sequence characterized amplified regions (SCARs), amplified fragment length polymorphic (AFLP) and Sequence-related amplified polymorphism (SRAP) markers.

Hybridization based markers

Restriction Fragment length polymorphism (RELPs)

The variation at DNA level is assessed by shearing the entire DNA with restriction enzymes. Such enzymes are available in bacteria for use as defense mechanism against viruses. Each restriction enzyme identifies a specific site of DNA usually 4-8 base pairs at which the enzymes act upon to cut the both stands of DNA. The restriction sites for a particular enzymes are present at several places throughout the entire genome with the result that a large number of DNA are produced. The length of each segment depends upon the distance between two adjacent restriction sites. The electrophoresis of stained DNA can detect fragments with different lengths. But the number of all fragments being very large gives a continuous smear which makes it very difficult to observe individual fragments. The number of fragments is thus reduced by screening through probes which hybridized with only some of the fragments. Only the fragments complementary to the sequences of probe hybridize with it which are observed through autoradiography. Each individual genotype has a fixed pattern of distribution of fragments for a given enzyme and probe. Another individual with difference at DNA level will differ for restriction sites as a result of which a different pattern of fragment distribution is observed. The term restriction fragment length (RFLP) has been coined to describe this variation for length of fragments from digestion of DNA form two or more organism with same endonuclease (Helentjaris *et al*., 1985). The presence of polymorphism depends on the restriction enzyme used and nucleotide sequence of the probe. So a number of probes and restriction enzymes are used. All the individuals in the mapping population like F2 are evaluated for each enzyme/probe combination. Banding pattern among individuals is compared and variation in one DNA fragment obtained with a specific enzyme is treated as one RFLP. Probes may be obtained from either cDNA or genomic DNA libraries but probes with unique sequence or interspersed low copy number portion of the genome are most useful as these produce only resolvable number of bands. The sequences with high copy number will detect a large number of fragments which are difficult to interpret and may even give a smear on autoradiograph. A unique sequence probe will hybridize to a single restriction fragment and low copy number may hybridize to as many as five loci. Technical complexity of performing RFLP analysis

and difficulties with the use of short-lived radio- isotopes have led to the development of some other, mostly PCR based techniques. However (RFLPs) has not been used in mulberry due to some limitations.

- The assay is time consuming and labour intensive.
- Requirement of radioactive isotopes makes the analysis expensive and hazardous.
- Satellite and highly respective sequences are inaccessible with naturally occurring probes.

Polymerase chain reaction (PCR) based markers Sequence tagged sites (STS)

The RFLP probes linked to desirable traits can be converted to polymerase chain reaction (PCR) based markers. In this the RFLP probes are end-sequenced and complementary primers are synthesized. These primes (generally 20 mers) are then used for amplifying specific genomic sequences using PCR. One major limitation of these markers is the reduced polymorphism hence they are not used in mulberry.

Expressed sequence tags (EST)

These markers are developed by end sequencing of random cDNA clones. The cDNA markers are first mapped as RFLP markers and then partially sequenced to convert them into PCR based markers. These can be used for synteny mapping and cloning of specific genes.

Randomly Amplified Polymorphic DNAs (RAPDs)

Williams *et al.* (1990) originally developed the technique. This is a PCR based technique where a single short oligonucleotide primer which binds to many different loci, is used to amplify random sequences from a complex DNA template such as a plant genome. For most plants the primes that are 9-10 nucleotide long are expected to generate 2-10 amplification products. The primers are generally of random sequence, based to contain at least 50 GC content and to lack internal repeats. The products are easily separated by standard electrophoretic techniques and visualized by UV illumination of ethidium bromide stained gels. Polymorphism results from changes in either the sequence of the primer binding site (e.g. point mutation) or from changes which alter the size or prevent the amplification of target DNA (e.g. insertions, deletions, inversions) in inheritance studies, the amplifications products are transmitted as dominant markers(Waugh and Powell, 1992).

A total of 12 oligonucleotide random primers were used for RAPD analysis against the 12 collections of mulberry of each of Mysore local and V-I. All the primers produced distinct polymorphic banding pattern between the two cultivators. A total of 73 markers were generated of which 40 were monomorphic and rests 33 were polymorphic (45%). The size of the amplified markers ranged from 500- 2500 bp with 3-10 markers per primer as expected of asexually propagated material, no difference in banding pattern was observed within the collections of the same cultivator (Naik *et al*., 2002). Similar result was obtained by Mulcahy *et al*. (1993) in vegetatively propagated apple and concluded that the different accessions of same cultivar yielded identical fingerprints.

RAPD analysis showed the genetic similarity among the different collection ranged from 76% (between v-1 and RFs-175/Anantha) to 100% (among duplicate collections). However the third suspected group of duplicate collection namely, Kousen and Xuan-10 did not

show complete similarity in DNA amplifications profiles (Naik et al., 2006).

Thirty RAPD markers generated a total of 179 alleles across 17 genotypes and the number of alleles per locus varied from 4 (OPA 13) to 9 (OPBB 13) with an average of 5.96 per locus. The similarity coefficient ranged from 0.74 to 0.93 with an average of 0.84. The highest similarity value 0.93 was recorded between SKM- 33 and KNG and SKM -33 and Goshoerami, lowest similarity coefficient (0.74) was found between local mulberry and Kokuso - 20. Cluster analysis by Unweight Pair Group Method using Arithmetic average (UPGMA) grouped 17 mulberry genotypes/selections into 8 different clusters. Cluster-2 contain highest (6) genotypes (wani *et al*., 2014).

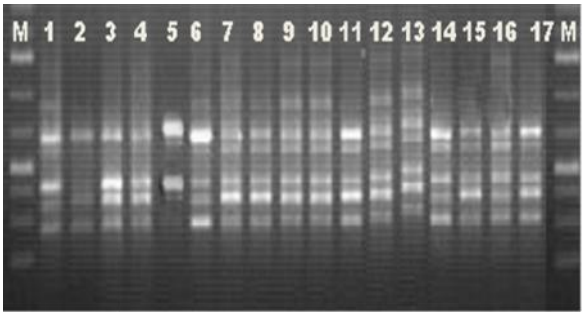


Fig.1 Ethidium bromide stained DNA amplification of 17 genotypes of mulberry (*Morus spp*), M = ladder (OPA4).

Simple Sequence Repeats (SSRs)

In multicellular organisms about 90% of DNA is redundant which comprises 'satellite' or repeat regions which may be present at one or more sites throughout the geneome. The tandem repeats of sequences ranging from 9 to 100 bp are called minisatellites or variable Number of Tandem Repeats (VNTR). The tandem repeated sequences of DNA with a repeat size ranging from 1-6 bp are called microsatellites or simple sequence Repeat (SSR). DNA sequences containing SSRs can be amplified by PCR and SSR variants can be detected by gel electrophoresis of the amplified fragments (Bligh *et al*., 1195).

The analysis of six microsatellite across 43 genotypes revealed significant ($P<0.05$) deviation from HWE and significant LD for two pairs of markers (MULSTR1/MULSTR2 and MULSTR2/MULSTR3) using ARLEQUIN and four pairs (MULSTR1/ MULSTR2, MULSTR2/ MULSTR3, MULSTR1/MULSTR5 and MULSTR2/MULSTR5) with GENEPOP (Aggrawal *et al*., 2004).

Six SSR primers were utilised which generates 17 alleles among the genotypes. The polymorphism information content (PIC) value varied from 0.260 (MulSTR3) to 0.623 (MulSTR4), with an average of 0.438 per locus. The highest similarity value of 0.92 was observed between Lemoncina and Kanva-2, as compared to the lowest similarity coefficient of 0.15 was between SKM-48 and Chinese white. Clustering of the genotypes was done with Unweight pair group method using arithmetic average (UPGMA) which generates five clusters. Cluster-2 contained maximum (six) genotypes (wani *et al*., 2013).

Inter-simple sequence repeats (ISSR)

ISSR marker has been used for the genetic study of mulberry germplasm characterization, crop improvement and molecular systematics. Genetic variability of clonal mulberry within field and tube seedling. Fifteen ISSR primers were used with five mulberry individuals, eight primers generated bright amplification products, six primers failed to generate any

amplification products and one generated weak or ambiguous amplification products. The 8 ISSR primers produced amplification products that were monomorphic within the population of mulberry field and tissue culture. Only using the primer (GAG) GC, one polymorphic band was present within two populations. Within the natural cultivated mulberry population, a total of 33 reliable fragments were obtained, one bands were polymorphic with a polymorphic ratio of 3.03 per cent, while within the population of *in vitro* culture plant, 35 bands were amplified among 27 individuals with the polymorphic ratio of 2.89 per cent, indicating the polymorphism detected by ISSR markers (Zhao *et al*., 2007).

Sequence characterized Amplified Region Markers(SCAR)

A scar marker represents a specified genomic region that is amplified by PCR using a pairs of specific oligonucleotide primers. 'Scar' markers are considered better than RAPD because these are identified as distinct single bands in agarose gels and some of these show codominance which differentiates heterozygotes from both types of homozygotes (Paran *et al*., 1993).

Amplified Fragment Length Polymorphism (AFLP)

This technique was developed by Vos *et al*. (1995). In this technique restriction fragments generated by a frequent (4 base) and a rare (6 base) cutter are anchored with oligonucleotide adapters of a few oligonucleotide base. This method generates a large number of restriction fragments facilitating the detection of polymorphism. The number DNA fragments amplified can be controlled by choosing different base numbers and composition of nucleotides in the adapters. This technique is more reliable since stringent reaction conditions are used for primer annealing.

This technique thus shows an ingenious combination of RFLP and PCR techniques and is extremely useful in detection of polymorphism even between closely related genotypes. Characterization of 43 *Morus* accessions originating from distinct regions of Turkey using fluorescent dye amplified fragment length polymorphism (AFLP) markers and capillary electrophoresis. The accessions belonged to *M. alba, M. nigra* and *M. rubra*, *M. alba* consisted of white and purple fruited samples. Eight primer combinations generated a total of 416 bonds, 337 of which were polymorphic (80.5%). Resolving powers of the AFLP primers ranged from 0.410 to 0.924 making a total of 5.015, where as the polymorphic information content ranged from 0.662 to 0.898 with an average of 0.812. Unweighted pair group method of arithmetic mean (UPGMA) clustering of the accessions showed three major groups representing to *M. nigra, M. rubra*, and *M. alba* accessions. The *M. alba* group had two subgroups that were not correlated with fruit colour. The UPGMA dendrogram of average taxonomic differences confirmed these results (Salih *et al*., 2008).

Sequence related amplified polymorphism (SRAP)

SRAP is a novel molecular marker technique designed to amplify open reading frames (ORFs) SRAP was used to identify mulberry germplasm. Twenty three mulberry accessions were screened using SRAP techniques, with 12 primer combinations selected for their reproducibility and polymorphism. Out of 83 PCR fragments scored, 59 (71.1 %) were polymorphic, with an average of 4.9 polymorphic bands and 6.9 bands per primer combination. Mean gene diversity and PIC were 0.161 and 0.1353 respectively. The genetic similarly coefficient ranged from 0.6905 to 0.9524with an average of 0.8330 (Weigh *et al*.,

2009).

Combined RAPD and ISSR marker analysis

The genetic similarity obtained by combined analysis of RAPD and ISSR marker was more similar to the one obtained through RAPD marker analysis alone and ranged from 0.689 (between G.No.4 and Mysore local) to 0.896 (between RFS-135 and RFS-175). Clustering obtained by UPGMA analysis of the combined marker data was identical to the one obtained through RAPD analysis alone. In all the three analysis AR-11, S—1635, G.NO.2 and G.NO.4 clustered distinctly. AR-11 was considered as an out group in all the three dendrograms. The pattern of clustering was slightly different when compared with that of the one based on ISSR marker analysis alone (Naik and Dandin, 2005).

Genetic diversity was observed among the genotypes based on the DNA markers generated by both types of primers. The band profiles generated by the RAPD primer OPA-11 and UBC-826 have clearly revealed such variability among the genotypes. The ten ISSR primers generated a total of 58 bands of which 43 were polymorphic, thus generating 74.13% polymorphism. Similarly, out of 80 bands generated by the RAPD primers, 49 were polymorphic, thus generating 60.75% polymorphism among the 11 mulberry genotypes. The results clearly showed that ISSR primers were more efficient in revealing DNA polymorphism among the genotypes than the RAPD primers (Srivastava *et al*., 2004). The genetic similarity coefficient among genotypes estimated on the basic of Nei and Li (1979) varied from 0.904 to 0.544 with an average genetic similarity of 0.728 in ISSR markers. The same was in the range of 0.923 to 0.617 with a mean coefficient of 0.756 when the pooled data of both marker systems were used. The Dice coefficients (Sneath and Sokal, 1973) among the genotypes also showed considerable variation. In ISSR, it varied from 0.475 to 0.352 and in RAPD it was between 0.490 to 0.379. Incase of pooled data, the maximum genetic similarity was 0.480 and the minimum was 0.382. The similarity coefficients among the genotypes estimated on the basis of Jaccard (1901) was between 0.921 and 0.440, 0.825 and 0.374 and 0.825 and 0.272 in ISSR , RAPD and in the pooled data of ISSR + RAPD markers respectively. The Pearson's correlation coefficient between different matrices subjected to the Mantel test (Mantel 1967) were found to be highly significant (r =0.435, 0.998, p = 0.000).

The dendrograms realized from the above matrices of ISSR, RAPD and the pooled data from both marker system using UPGMA method grouped the 19 mulberry genotypes, into four clusters. The first group comprised four genotypes, two from *M. alba* and one each from *M latifolea* and *M. bombycis* .In the 2nd cluster , seven genotypes from *M. alba*, *M. latifolea* and *M. bombycis* were again grouped together. The third cluster contained only four genotypes, all from *M. indica*. The fourth cluster included all *M. laevigata* genotypes. Another important points noticed from the dendrogram is the high genetic distances enjoyed by *M.. laevigata* from the others (Vijayan *et al*., 2003).

CONCLUSION

Genetic engineering offers a very promising alternative to the chemical disease management practices and a good supplement to the conventional plant breeding methods. Integration of transgenic technology in a total system approach will result in ecofriendly and sustainable means of diseases and insect pest management. Species identification in mulberry (*Morus*) is a great debate among scientists despite the number of criteria such as floral character, wood, leaf anatomy and biochemical characters used to identify the species within this genus. Thus it is concluded from this revealed study identification of taxa based on morphofloral

characters alone often generates misleading results. Thus, an approach integrating morphological and genetic molecular parameter is required to resolve the problems pertaining to the taxonomic position of species in mulberry.

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