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*Review*

# Transgenesis: An efficient tool in mulberry breeding

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**Genetic engineering is the most potent biotechnological approach dealing 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, 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 of resistance genes on the basis of amino acid sequence, conservation enables plant breeder to monitor 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 tolerant corn, cotton, soyabeen and papaya; virus resistant corn, potato, cotton among others. 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*).**

**Key words:** Transgenic plant, mulberry, resistance, salinity.

## INTRODUCTION

Genetic engineering is the most potent biotechnological approach and 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 transgenoesis. The combined use of recombinant DNA technology, gene transfer methods and tissue culture techniques has led to the efficient transformation

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**Abbreviations: CaMv,** Cauliflower mosaic virus; **TGMv,** tomato golden mosaic virus; **DHFR,** dihydrofolate reductive; **PEG,** polyethylene glycol; **PAT,** phosphinothricin acetyl transferase; **PDR,** pathogen derived resistance; **CMV,** cucumber mosaic virus; **AOS,** active oxygen species; **cP-MR**, coat protein mediated resistance; **UPLC,** ultra-performance liquid chromatography; **GUS**, β-gluconidas.

and production of transgenic in a wide variety of crop plants.

## 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.

### Advantages of transgenic plants

The following are the advantages of transgenic plants:

1. Agronomic traits can be improved by producing plants with increased resistance to pest,
stress along with increased vigor, yield.
2. Enhanced efficiency of physiological process like photosynthesis and improved nutritional qualities.
3. Transgenic plants are also used as an analytical tool to explore aspects of gene regulation.
4. Major advantages 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 large number of cultivars of different crops.

## HOW TO MAKE TRANSGENIC PLANT

The whole process of introduction, integration and expression of foreign genes in the host is called genetic transformation (Figure 1). 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. *Agrobacterium*-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.

***Agrobacterium*-mediated gene transfer:** Among the various vectors used in plant transformation , the Ti plasmid of *Agrobacterium tumefaciens* has been widely used . This bacterium is known as “natural genetic engineer” of plants because it has natural ability to transfer T-DNA of its plasmids into plant genome upon infection of cells at the wound site and causes 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. To transfer the foreign DNA in host plants, leaf discs, embryogenic callus or other suitable explants are collected and infected with *Agrobacterium* carrying recombinant disarmed Ti plasmid vector. In general, briefly the vector tissue is then cultured on a shoot regeneration medium for 2-3 days during which the transfer of T-DNA along with foreign genes takes place. After this, the transformed tissues are transferred onto a regeneration medium supplemented with usually lethal concentration of an antibiotic to selectively eliminate non-transformed tissues. After 3-5 weeks, the regenerated shoots are transferred to root -inducing medium, root shoots are acclimatized and after 3-4 weeks, complete plants are transferred to soil. Molecular techniques like polymerase chain reaction (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.

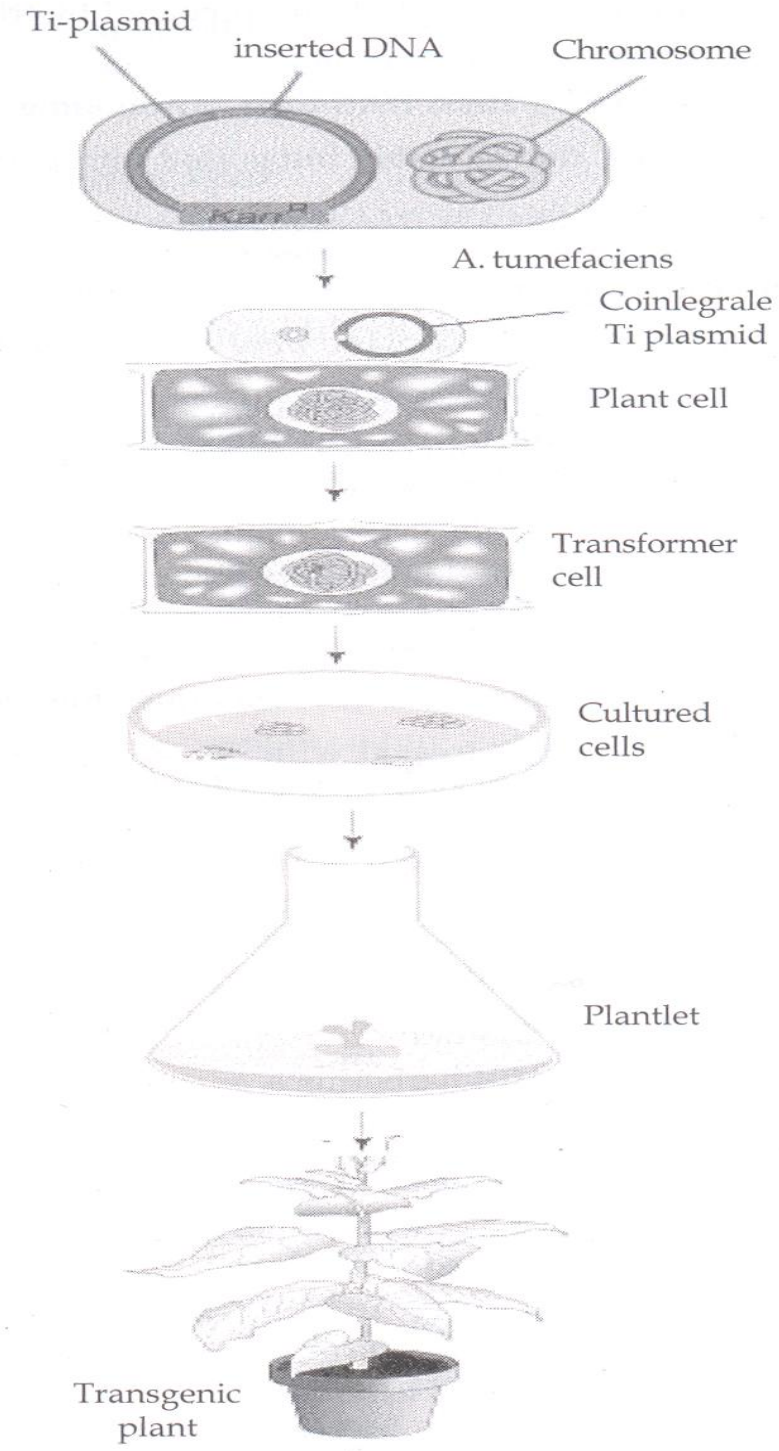


**Figure 1.** Schematic representaion of transfer of gene using ti-plasmid. Source: Rukamet et al., 2010.

**Direct uptake of DNA:** Direct DNA uptake by protoplasts can be stimulated by chemicals like polyethylene glycol (PEG) and dextran sulphate. PEG is used to improve the efficiency of electroporation. PEG at high concentration (15-25%) precipitates ionic macromolecules such as DNA and stimulate their uptake by endocytosis without any gross damage to protoplasts. This step is followed by cell wall formation and initiation of cell division. Now these cells can be plated at low density on selection medium.

However, there are problems in using this method for getting transgenic plants mainly related to plant regeneration from protoplasts and particularly in woody species.

**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 makes transient (temporary) pores in the plasma membrane which facilitate the uptake of foreign DNA, if the DNA is in direct contact with the membrane. The electroporation pulse is generated by discharging a capacitor across the electrodes in a specially designed electroporation chamber. Either a high voltage (1.5 kV) rectangular wave pulse of short duration or a low voltage (350 V) 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, gene(s) associated with a suitable promoter sequence was transferred. Transformation frequencies can be further improved by using field strength of 1.25 kV/cm, adding PEG after adding DNA, heat shocking protoplasts at 45°C for 5 min before adding DNA and by using linear instead of circular DNA. For delivery of DNA to protoplasts, electroporation is one of the techniques most used 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 also into walled cells.

**Microinjection:** The DNA is directly injected into plant protoplasts or cells (especially 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 is 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/s).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 could 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. This technique has been used to produce stable genetic transformation in soybean, tobacco, maize, rice, wheat, among others. Transient expression of genes transferred in cells by this method has also been observed in onion, maize, rice, and wheat (Table 1).

## ENGINEERING INSECT RESISTANCE

The most known approaches to develop insect resistant transgenic plants are introduction of bacterial gene Bt synthetic Bt and 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 *B. 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) and transfer of gene whose enzyme product becomes insensitive to herbicide (target modification).

**Table 1.** Genetic transformation in monocotyledonous plants.

| Work | Reference |
|---|---|
| Few transgenic rice plants by inoculating immature embryos 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) |

**Table 2.** 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.

### 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 (Table 2). 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.

## 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 1$^{st}$ 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.

1. Antifungal protein-mediated resistance.
2. 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 $H_2O_2$ generating fungal gene for glucose oxidase were found to have elevated levels of $H_2O_2$ and enhanced levels of resistance 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 (Table 3).

**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**

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

**GENETIC ENGINEERING**

Genetic engineering has recently made some intervention into mulberry research. Efficient protocols have been developed for direct plant regeneration from explants and insertion of desired genes into plant genome via *A. tumefaciens* and particle bombardment mediated methods (Bhatnagar et al., 2003). Functional evidence indicate that expression of Hva1 has shown landmark achievements in combating stress response (Chauhan and Khurana, 2011; Fu et al., 2007; Maqbool et al., 2002; Sivamani et al., 2000). Transgenic mulberry plants were therefore generated by over expression of barley Hva1 using *Agrobacterium* mediated transformation (Table 4) (Lal et al., 2008). Detailed physiological, biochemical, and molecular evidence indicated increased performance of transgenic mulberry plants when subjected to simulated salinity and drought conditions. Transgenic and non transgenic lines behave differentially under stress conditions and show better cell membrane stability, photosynthetic yield, less photo oxidative damage, and high relative water content under salinity and water stress than non-transgenic lines.

**Table 3.** Foreign genes expressed in transgenic plants.

| 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 | Drought and freezing |

Transgenic Plants by Kakralya/Ahuja.

**Table 4.** Transgenesis in mulberry for abiotic stress tolerance.

| Gene | Expression profile | Reference |
|---|---|---|
| WAP21 | Cold tolerance | Ukaji et al.(1999) |
| COR | Cold tolerance | Ukaji et al.(2001) |
| AlaBlb | Salinity tolerance | Wang et al. (2003**)** |
| OC | Insect resistance | Wang et al. (2003) |
| SHN1 | Drought tolerance | Aharoni et al. (2004) |
| HVA1 | Drought and salinity stress | Lal et al.(2008) |
| Bch | Drought and salinity stress | Khurana (2010) |
| NHX | Drought and salinity stress | Khurana (2010) |
| Osmotin | Drought and salinity | Das et al. (2011) |

WAP21, Water collection plan; COR, cold on regulation; AlaBlb, soybean glycine gene; OC, osteocalcin; SHN1, schnurri from *Drosophila melanogaster*; HVA1, *Hevea braziliensis*, abiotic stress gene; bch-L inhibitor 2, aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; NHX, Na+/H+ exchanger;Osmotin-Osmotic stress induced gene. Adopted from Vijayan et al., 2011.

Hva1 thus confers broad spectrum of tolerance against various a biotic conditions in transgenic plants. Preliminary studies regarding suitability of these transgenic plants for silkworm rearing was also performed. However, transgenic mulberry plants with barley hva1 gene under the control of CaMV35S promoter displayed growth retardation under normal conditions. Therefore, stress-inducible rd29A promoter was used instead of constitutive CaMV35S promoter for the overexpresssion of Hva1 to minimize the negative effects on plant growth. Results indicate that a combination of rd29a and hva1 is useful for tolerance against diverse stresses and minimizing negative effects on plant growth (unpublished data). Subsequently, efforts have been initiated for field trials of these transgenic mulberry lines for assessing their growth under field conditions. Similar success was achieved in genetic transformation of mulberry with tobacco osmotin under the control of a constitutive (CaMV 35S) as well as a stress-inducible promoter rd29A. Osmotin and osmotin-like proteins are stress proteins belonging to plant PR-5 group of proteins in several plant species in response to various types of biotic and a biotic stresses. Physiological analysis of transgenic plants under simulated salinity and drought stress as well as fungal challenge was undertaken to test the effect of the integrated gene. Transgenic plants with stress-inducible promoter were able to tolerate salt and drought stress efficiently than those with constitutive promoter, but in case of fungal tolerance 35S: osmotin transgenic plants performed better. Therefore, transformation of mulberry with the osmotin gene would confer tolerance against drought, salinity, and fungal pathogens (Das et al., 2011b). These transgenic plants were also well accepted by the silkworms—the ultimate users of mulberry leaves. Genetic transformation of *Morus indica* cv K-2 has also been achieved with bch1 (b-carotene hydroxylase-1), and screening of the transgenic mulberry revealed better tolerance of transgenic mulberry for high temperatures, high light, and UV radiation stress. These transgenic plants accumulate higher levels of xanthophylls under stress conditions than non-transgenic plants as revealed by ultra-performance liquid chromatography (UPLC). This is the first attempt of manipulating the carotenoid biosynthesis pathway in mulberry by over expressing b-carotene hydroxylase-1 gene (Das, 2009). Efficient screening method for mulberry was developed by Vijayan et al. (2003). In this method axillary buds were cultured *in vitro* saline conditions based on growth and development of shoots and roots under different salt concentration, salinity tolerance level of accessions were determined. Seed germination in a saline gel on petri-plates was also

used for identification of salt tolerant maternal parents (Vijayan et al. 2004).

## Genetic transformation of mulberry

*Agrobacterium* 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 (Figure 2).

## 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 abcissic acid (ABA) and water deficit conditions. Over expression of HVA1 in mulberry under a constitutive promoter via *Agrobacterium* 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 (Lal et al., 2008).

## 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 in 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 (Table 5).

Electroporation was conducted in various combinations of capacitance and pulse voltages, protoplasts stained by histochemical assay were observed constantly in pulse voltage ranging from 500-750 V/cm with a capacitance of 330 µFd. 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 750 V/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 (Table 6). 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.

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 0.7 M manitol pre to bombardment tended to decrease the number of cells that transiently expressed the GUS gene (Table 7).

## Bio-safety and risks of disease resistance transgenic plants

There is 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

**Figure 2.** Drought and salinity tolerant transgenic mulberry.

**Table 5.** 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).

**Table 6.** Difference of bombardment efficiency among leaves precultured for various periods.

| Experiment | Preculture period (days) | No. of blue spots/cm$^2$ |
|---|---|---|
| 1 | 0 | 98.7±40.00 |
| | 3 | 86.5±29.3 |
| | 6 | 70.0±33.5 |
| | 9 | 17.0±8.1 |
| 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).

**Table 7.** Improvement in bombardment efficiency by various treatment of target leaves.

| 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)

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, later it can be used in a variety of cultivars of different crops.

## 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.

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