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Tobacco (*Nicotiana tabacum* L.)—A model system for tissue culture interventions and genetic engineering

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Tobacco (*Nicotiana tabacum* L.) has become a model system for tissue culture and genetic engineering over the past several decades and continues to remain the '*Cinderella of Plant Biotechnology*'. An *in vitro* culture medium (Murashige and Skoog, 1962), based on the studies with tobacco tissue cultures, has now been widely used as culture medium formulation for hundreds of plant species. Studies with tobacco tissue culture have shed light on the control of *in vitro* growth and differentiation. Further, induction of haploids, microspore derived embryos and selection of mutant cell lines, have been achieved successfully. Tobacco has also been employed for the culture and fusion of plant protoplasts, providing invaluable information on way to explore the potential of somatic hybridization in other crops. Optimization of genetic transformation, using *Agrobacterium tumefaciens* and *A. rhizogenes*, has been central to the cascade of advances in the area of transgenic plants. Developments in the field of molecular farming for the expression and/or production of recombinant proteins, vaccines and antibodies are gaining significance for industrial use and human healthcare.

Keywords: genetic transformation, molecular farming, plant biotechnology, plant cell and tissue culture, recombinant proteins, tobacco

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Introduction

Advances in plant biotechnology have made a significant impact in the area of *in vitro* culture, genetic manipulation and newer approaches in experimental plant biology[1-6]. In the past 2-3 decades, plant tissue culture has undergone an exciting development, providing knowledge about totipotency, differentiation, cell division, cell nutrition, metabolism, radiobiology, mutations and cell preservation. While microbial systems have been successfully used for the production of antibiotics and other useful compounds, it is now becoming possible to use plant tissue culture system to produce a wide range of biologically active compounds, like alkaloids, steroids, phenolics, vitamins and other useful chemicals. Further, cell culture could be used for the purposes of specific biotransformation of organic molecules. Other more sophisticated approaches of tissue culture include *in vitro* selection, protoplast fusion and plant genetic engineering, which have shown tremendous potential for application in crop improvement.

Majority of the discoveries in the field of plant cell, tissue culture and molecular biology have originated from the experimentation with tobacco plants[1,7]. As a result, tobacco has become a model system as the '*Cinderella of Plant Biotechnology*'. This plant has been found to be an extremely versatile system for all aspects of cell and tissue culture research. Working with tobacco tissue cultures, Murashige and Skoog[8] devised an *in vitro* culture medium that has become the widely used nutrient formulation for an ever-growing range of plant species. *In vitro* studies with tobacco tissue culture, using different physical and chemical factors, have provided insight into the control of growth and differentiation. Induction of haploids and selection of mutant cell lines, owing to the experiments with tobacco tissue cultures, have become useful tools. Isolation, culture and regeneration of plants from protoplasts as well as somatic hybridization, all have been accomplished using tobacco system, which has provided invaluable

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information on ways to explore the potential of somatic hybridization in other crops.

Tobacco (*Nicotiana tabacum* L.; 2n=4x=48) is a natural allotetraploid formed through hybridization between two diploid (2n=24) progenitors, *N. sylvestris* and *N. tomentosiformis* approximately 6 million years ago[9]. Within 3 months of time, a tobacco plant goes from seed to next generation seed and generates up to a million seed per plant. Scaling up to hundred or thousands of acres is very rapid. From the early experiments with the uptake of naked DNA to the recent vector mediated gene transfer, tobacco has remained as the most sought after research system. The first transgenic plants are produced in tobacco. Also, the experiments related to plant transformation, gene expression and gene stability have all been worked out using tobacco. All the first achievements of plant genetic engineering are mostly based on the work with tobacco. Currently, this plant is being employed in studies on the production of useful recombinant proteins, antibodies and special chemicals for use in medicine and industry. In the area of plant tissue culture and genetic manipulation, the work on tobacco is so enormous and vast that it cannot be summed up in a single compilation. In this article, therefore, only those aspects of plant tissue culture research have been described in which tobacco has been a role model and paved the way for improvement in other crop systems.

Studies on *in vitro* Cultures

In a series of publications, Skoog and co-workers[10-12] reported on the *in vitro* shoot bud induction in *Nicotiana* with a systematic approach. They demonstrated that differentiation into shoot and root could be induced by the manipulation of the balance of an auxin (IAA) and a cytokinin (Kn). The presence of adenine or Kn in the medium resulted in the promotion of shoot bud differentiation, while the effect of cytokinin was modified by other components in the medium particularly by IAA or NAA. A higher level of auxin induced rooting from tobacco callus cultures. The other physical factors, such as temperatures, *p*H, photoperiod and sucrose concentration, influenced the differentiation from callus cultures. For instance, Skoog[13] studied the effect of a range of temperatures (5-33°C) on tobacco callus growth and differentiation. He observed that growth of the callus increased with the rise in temperatures up to 33°C but for shoot-bud formation, 18°C was optimum. High light intensity was found to be inhibitory for shoot-bud formation in tobacco[14]. Shoot-bud regeneration was observed in the presence of blue light, whereas rooting occurred in red light.

Extensive studies were carried out on various parameters controlling flower bud formation and induction from epidermal cells of tobacco dihaploids, which were obtained from fertile flowers raised from peels of male sterile plants[15]. Thus, hypohaploids with less than (dihaploids) chromosomes of epidermal cell culture were obtained, which were free from endogenous hormonal influence[15], making it easy to study the changes of single cell leading to differentiation. Hicks and Sussex[16] cultured flower primordia of tobacco (Wisconsin 38). Then, buds with only sepal primordia were cultured, leading to the petal, stamen and carpel primordia in acropetal sequence on the apex. Organ primordia of the flower buds of tobacco were also subjected to surgical manipulations.

There have been many reports on organogenesis in tobacco tissues and calli. Regeneration of plantlets was reported from the cultured leaf explants of tobacco[17]. Bud formation was induced in tobacco petiole cultures[18]. Plants were regenerated from callus obtained form cotyledons of a hybrid (*N. suavelons x N. tabacum)* and brown spot resistance was transferred from *N. suavelons* to *N. tabacum*[19]. Cellular and ultra structural changes in regenerating shoots in tobacco have also been studied[20]. Similarly, experimental data on the effect of growth regulators, temperature and light on tobacco tissue cultures have contributed to the basic knowledge about the role of several factors on differentiation that have been extended to other crops.

Studies on Anther and Pollen Culture

A survey of literature pertaining to anther culture represents that this technique has been successfully utilized for the production of haploids and evoked considerable interest among plant breeders and geneticists[1,21]. The major advantage was the recovery of homozygous diploids for breeding and for establishing haploid cell cultures for mutational studies and genetic manipulation experiments. Extensive studies conducted on/with anther culture of tobacco has led to the identification of critical factors for successful development of haploids. Nitsch[22] reported haploid plantlets, for the first time, from *N. tabacum*. This was followed by investigations on the regeneration of

haploid plantlets from anther culture of several species of *Nicotiana*. Three promising lines of tobacco were raised through anther culture of hybrids (line MC-1610 × Coker 139)[23]. The new lines exhibited higher resistance to bacterial wilt and black shank without loosing the agronomic and chemical features of MC-1610. Similarly, double haploids of tobacco were raised using anther culture[24], which showed high yield, good quality of curved leaves and disease resistance characters, achieved in a much shorter period as compared with the conventional breeding practice. In China, new varieties of tobacco (Tangu 1, 2 and 3) have been released using anther culture by Shangtung Institute of Tobacco and are being cultivated in about 20,000 acres.

First pollen mitosis was reported to be the critical stage for *Nicotiana* anther culture[25]. The physical factors, like light, temperature and *p*H, also influenced the pattern of response. For example, anthers of tobacco kept in the dark at the beginning of the experiment responded better[26]. The nitrogen starvation of anther donor plants increased the anther response and embryo yield[27]. Chilling of anthers prior to culture or a cold treatment of flower buds also enhanced embryogenesis in tobacco[26,28]. Further, Deaton *et al*[29] studied the vigour and variation expressed by pollen plantlets of tobacco and conducted their evaluation trials. However, Zeppernick *et al*[30] studied the relationship between ploidy level, morphology and concentration of nicotine of haploid and doubled haploids raised through anther culture.

Depending upon species and other factors, pollen grains either developed into embryos or formed callus, which then differentiated into embryos upon transfer to an appropriate medium. Normal embryogenic process was usually observed, showing globular, heart and torpedo stages of embryos[31]. Haploid plants produced large number of flowers and these were generally smaller in size. However, in tobacco anther cultures of *Nicotiana*, the pollen grains directly produced haploid plantlets without intervention of a callus mass. Moreover, besides the development of normal embryos, numerous abnormal embryos in various stages of differentiation were detected[25]. Albino plants have also been observed in tobacco anther cultures[32].

In general, success in the production of embryos from anther cultures depends to a large extent on various factors such as method of culture, nutritional and hormonal composition of the medium, developmental stage of microspore and ontogeny of pollen embryos as well as the growth condition of the donor plants. The usefulness of haploids is based on the assumption that homozygous inbred lines can be readily and rapidly achieved. Haploids could offer as supplementary breeding lines for breeding programmes and the knowledge gained about culture method, treatment and growth regulators can be applicable for anther culture in other crops.

Studies on Genetic Variation and Mutant Selection

Mutant selection through tissue culture has become possible due to the occurrence of a high degree of variability (somaclonal variation) in cell cultures. The fact that millions of cells can be cultured in shake flasks has permitted the selection for specific mutations at cellular level and to regenerate plants from selected cell lines for specific mutations at cellular level, and to regenerate field plants[33]. Various types of chemical and physical mutagens have been applied to callus, cell suspensions or protoplasts in attempts to isolate desirable mutants.

Plant cell and tissue cultures have been shown to cause or allow many genetic changes to take place. Variability in DNA content and nuclear volume was noticed in regenerating cultures of tobacco[34]. Increase in ploidy, chromosomal rearrangements, and abnormal chromosomes and altered DNA content in regenerating plantlets have been effectively used in tobacco improvement programmes. The fate and possible role of aberrations (dicentric chromosomes) were studied on the development of the different embryonic phases[35]. Extensive chromosomal chimeras were observed in callus derived regenerants of tobacco and transmission of chromosomal chimeras was reported to first and second selfed progeny plants[36].

For mutant selection, tobacco cells are a favourite material due to the regenerative ability coupled with the capability of tobacco callus to form fine cell suspensions that grow rapidly. Carlson[37] isolated auxotrophic mutants of tobacco using selection technique developed for animal cell culture. The nucleotide 5′ bromodeoxyuridine was used to select six tobacco clones showing partial growth requirements[37]. Clones were selected in *N. plumbaginifolia* requiring isoleucine, leucine and uracil[38]. Temperature sensitive variants of *N. tabacum* were also isolated successfully[39]. Of the specific selection

agents, chlorate has been found to be the most effective with plant cells. Nitrate reductase converted chlorite that kills plant cells. The cells lacking nitrate reductase survived and could be isolated. This system was so effective that Muller and Grafe[40] isolated double recessive mutants in *N. tabacum*.

Like auxotrophic mutants, there are many reports wherein tobacco cell lines resistant to various compounds have been isolated. Widhlom[41] utilized tryptophan analogue, 5 methyltryptophan to select *N. tabacum* resistant cell lines. In another case, suspension cultures resistant to ethionine were isolated[42]. Selection for resistance to several antibiotics, initially with the streptomycin, produced organelle encoded mutations in *Nicotiana*[43].

Tobacco cell lines tolerant to high NaCl levels (0.88%) have been isolated[44,45]. The regenerated plants from these line also retained tolerance through two successive sexual generations with the enhanced levels of tolerance. Dix[46] obtained NaCl tolerant plants in *N. sylvestris* and observed that the character was transmitted to the next generation. Different cell lines of *N. tabacum* resistant to various herbicides, such as Amitrole[47], Bentazone[48], Paraquat[49], Picloram[50] have also been isolated. Moreover, glycerol and lactose were successfully utilized for detecting mutant cells of tobacco[51].

Other application of mutant selection includes selection for disease resistance. Plant resistance to virus and its transmission to the progeny of tobacco has been reported[52]. Single clones of tobacco tissue varied in their susceptibility to virus and fungal infections and their multiplications. Population of mutagenized haploid cells of tobacco was plated in a medium containing inhibitory concentrations of methionine since *Pseudomonas tabacii*, causal bacteria of wild fire disease, produces methionine. *In vitro* survived cells, produced on inhibitory levels of methionine, were further used for generating disease resistant varieties[53]. In callus derived plants of an interspecific hybrid of tobacco, variability for plant height, number of days to flower, number of leaves and leaf area were also reported[54].

Studies on Isolation, Culture and Fusion of Protoplasts

Isolation, culture, fusion of plant protoplasts and regeneration has generated great hopes for plant improvement. Absence of cell wall allows the fusion between protoplasts, derived from two diverse plants that are sexually incompatible, as well as uptake of a foreign genetic material, such as organelle or DNA, into genome. For the first time, Nagata and Takebe[55] described the regeneration of whole plants from mesophyll protoplasts of *N. tabacum*. Later, others also reported regeneration of entire plants from mesophyll protoplasts of tobacco[56,57]. Protoplasts derived from haploid tobacco have also shown the regeneration potential[58]. Leaves are considered an ideal source of protoplasts for tobacco because they can be kept in abundant supply by *in vitro* shoot tip culture or from greenhouse grown plants. Thus, a large quantity of mesophyll protoplasts of tobacco can be isolated from diploid or haploid plants. Moreover, high percentage of the protoplasts can reform cell wall and divide to produce a callus. Protoplasts have also been isolated from epidermis, cell suspensions and stem callus, beside mesophyll cells. Further, parameters affecting the initiation of protoplast division of haploid and diploid tobacco have been evaluated[59].

Somatic hybridization has assumed great significance among many applications of protoplast technology. The experiment to select somatic hybrids with the aid of albino mutant was first performed on tobacco haploids[60]. Similar results were obtained with the interspecific combinations of non-allelic light sensitive albino mutants of *N. sylvestris*[61]. Non-allelic albino mutants were also used for *N. tabacum* and *N. knightiana*, which was unable to produce shoots forming potential of tobacco and chlorophyll synthesis of *N. knghtiana*. The first somatic hybrids plant was reported after a fusion of *N. glauca x N. langsdorffii* protoplasts with the help of sodium nitrate[62]. Protoplasts from cell line of *N. sylvestris* resistant to kanamycin that have no ability to form shoots were fused with the protoplasts from *N. knightiana* that do not form shoots *in vitro*. Somatic hybrids were obtained on the basis of kanamycin resistance and on the capacity to regenerate shoots[63]. Vigorous growth patterns of hybrid colonies were also used to isolate hybrids from *N. glauca* and *N. langsdorfii*[64]. Several fusion experiments on tobacco was conducted using plastome mutants in relation to chlorophyll synthesis and cytoplasmic male sterility (CMS) and segregation of mixed cytoplasm into mutant and wild type plastome were observed[65,66]. Gamma irradiated protoplasts, which were carrying a functional *npt*II gene, were fused with un-irradiated kanamycin-sensitive recipient protoplasts to yield asymmetric hybrids [67].

The somatic hybrid plants generally have chromosome number more than 4n due to the fusion of more than one protoplast. The hybrid nature of most of the somatic fusion products could be demonstrated by their chromosome analysis, isoenzymes, morphological comparisons or growth characteristics. Prat[68] examined mutations arised following protoplasts culture of highly inbred line of *N. sylvestris* and also a line derived from it after five consecutive cycles of androgenesis and chromosome doubling. A system was devised in *N. tabacum*[69] and *N. sylvestris*[70] where somatic embryos were directly produced from cultured protoplasts without callus formation. The feasibility of this technique at the application level will further determine the practicability of somatic hybrids as to be complementary to classical plant breeding methods. Besides protoplast fusion, extensive experiments have been conducted on tobacco protoplasts for various other aspects of genetic manipulation.

Studies on Secondary Metabolites and Biotransformation

Plant cell cultures synthesize secondary metabolites (biochemical totipotency) and this has significance not only for basic research but also for industrial processes. Ohta and Yatazawa[71] reviewed the work on nicotine production in tobacco tissue cultures. Nicotine in the range of 0.1-1 mg/mg dry wt was detected in cell cultures of tobacco; whereas, 29 mg/mg of dry wt was found in the roots of intact plants[72]. It was observed that nicotine synthesis in tobacco could be regulated by exogenous supply without recourse to organogenesis[73]. A close relationship has also been demonstrated between cell organization and nicotine production in tissue cultures of tobacco[74]. By obtaining single cell clones, high yielding strains of nicotine were isolated. The technique of single cell plating was used and a number of cell colonies were isolated from cell cultures of *N. cellrustica*, which showed wide variations in their growth characteristics and ability to synthesize nicotine[75]. Further, the nicotine content in tobacco showed relationship with the ploidy level of the plant[30]. Besides nicotine, a number of other secondary products, such scopoletin, esculetin, bergapten, cycloartenol, citrostradienol, citroastradiol, cycloeulenol, obtusifoliol[76] and aliphatic alkanes[77], have been detected in tobacco cell cultures. *Nicotiana* cell cultures also have the ability to transform organic compounds and referred to as biotransformation, for example stereospecific reduction of codeine[78] and conversion of N-diphenylurea into D-glucose[79]. Microsomes from tobacco tissue cultures were also found to convert squalene 2, 3 epoxide into cycloartenol[80]. These examples opened up the possibilities of identifying a high yielding nicotine cell line. The work on biotransformation in *Nicotiana* pertaining to several compounds has shown the possibilities of utilizing plant tissue and cell cultures for the isolation of several useful compounds.

Studies on Transgenic Tobacco and Applications

Studies undertaken by Uchimiya and Murashige with tobacco DNA and tobacco protoplasts showed uptake of homologous DNA[81]. Suzuki and Takebe demonstrated the insertion of viral DNA into mesophyll protoplasts of tobacco[82]. *Agrobacterium tumefaciens,* a soil bacterium, has been known to induce crown gall disease in many plants. During infection process, the bacterial plasmid integrates into plant genome and influences the plant tissue to form galls. Using *A. tumefaciens*, tobacco cell suspensions were transformed[83,84] and showed the presence of nopaline, the plasmid DNA encoding amino acid. The initial experiments with tobacco generated interest in the use of *A. tumefaciens* system for transformation and, in recent years, its plasmid has become an important vector for gene transfer. Venkateswarlu and Nazar[85] presented evidence by using tobacco chloroplasts that *Agrobacterium*-mediated transformation could be used to introduce foreign genes into higher plant chloroplasts by site-specific homologous recombination.

An unusual approach to transfer genes was demonstrated in *Nicotiana* using irradiated pollen[86]. Seeds were produced in *N. forgetiana* by pollinating it with irradiated pollen of *N. alta*. Although, the irradiated pollen produced pollen tube but failed to fertilize the ovule. However, most of the plants produced showed flower colour and other characters of *N. alta*. In another approach, it has been shown that swelling of germinating pollen grains could take up DNA or bacteriophage[87]. The progenies of *N. glauca*, derived from *N. glauca* pollen treated with *N. langsdorffi* DNA, produced tumours. The tobacco protoplasts could also be transformed with disarmed Ti plasmid vector pG 3850. Further, PN CAT containing a chimeric PNOS CAT gene construct gave rise to chloroamphenicol resistant calli.

A simple and general method for transformation, using tobacco leaf disks, was developed[88], which has become the standard method for producing transgenic tobacco. Leaf disks (1 cm^2) were excised from *in vitro* shoot cultures for preculture on MS medium with BA and NAA. After 2 days, the leaf disks were co-cultured with overnight grown *A. tumefaciens* culture. After 30 min of co-infection, the leaf disks were blotted to remove excess bacteria and transferred to fresh culture plates of MS medium with BA and NAA. Following three days of co-cultivation, the leaf disks were transferred to selection medium with kanamycin and cefotaxime or carbenicellin. The regenerated shoots were rooted on rooting medium containing NAA with high levels of kanamycin, usually 100 mg/l. Those plants that rooted on this medium were mostly considered to be transgenic and could be studied further. Fig. 1 shows different stages in the leaf disk transformation of tobacco var. Havana 425. A wide range of selectable markers are employed in transformation experiments, however, kanamycin has been the most extensively used.

Table 1 presents different transformation methods used for the first time in tobacco[89-94]. Employing tobacco as the experimental system, transfer of useful genes have also been made, for example insect resistance, herbicide tolerance, stress and disease resistance. For developing herbicide tolerance, *Arabidopsis csr-1-1* gene was transferred into Canadian flue-cured variety, Delgold[95]. Resistance to wildfire disease was introduced into transgenic tobacco plants by expressing toxin resistance gene[96]. Very significant resistance to tobacco mosaic virus has also been obtained by the expression of viral replicase[97]. Genetically modified, virus (CMV, TMV) resistant tobacco yielded an average 5-7% more leaves for processing and saved 2-3 applications of insecticide applications. Virus resistant tobacco has also been field tested in China since 1991 and is now being used in industrial manufacturing for national consumption. Using the codon-optimized δ-endotoxin gene from *Bacillus thuringiensis*, under the control of CaMV 35S promoter, four lines were developed based on the toxicity to tobacco hornworms in greenhouse trials[98]. By expressing *E. coli* mannitol dehydrogenase gene, increased mannitol accumulation in tobacco has been demonstrated[99].

Transgenic tobacco, expressing satellite RNA and coat protein gene from cucumber mosaic virus (CMV), showed resistance to CMV under both

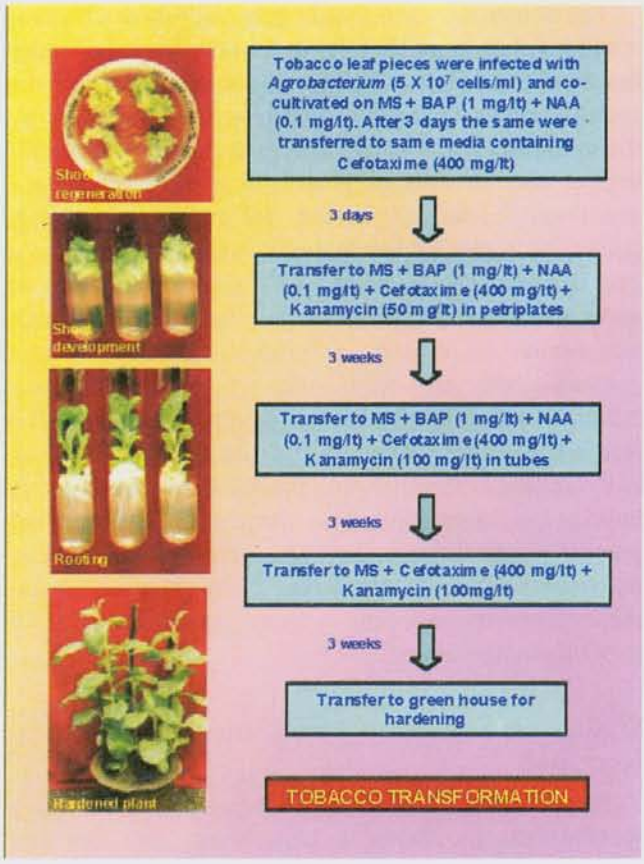

Fig. 1—Different stages in the *Agrobacterium*-mediated leaf disk transformation of tobacco var. Havana 425

Table 1—Genetic transformation methods used in tobacco

S.No.	Method	Reference
1	Direct DNA uptake	Paszokowski *et al*[89]
2	*Agrobacterium* mediated	Horsch *et al*[90]
3	Electroporation	Shillito *et al*[91]
4	Liposome mediated	Deshayes *et al*[92]
5	Microinjection	Crossway *et al*[93]
6	Particle bombardment	Klein *et al*[94]

natural and mechanical inoculations. Potato virus Y coat protein gene was cloned and sequenced from an Indian isolate[100]. The coat protein gene was further sub-cloned into plant expression vector pBINPLUS and leaf discs of tobacco var. Havana 425 were transformed using *A. tumefaciens*. Molecular analysis confirmed the integration of the PV'Y' coat protein gene. All the transgenic plants were morphologically similar to non-transformed controls with respect to appearance, flowering and seed set. The resistance was assayed by infecting the control and transgenic

plants with the virus and the virus accumulation was analysed through ELISA. All the transgenic lines showed good amount of resistance against PV'Y' accumulation upon infection. The data clearly demonstrate that the distinct Indian isolate of PV'Y' can be used in imparting coat protein gene-mediated resistance in tobacco and the level of resistance is not correlated to the level of expression of the PV'Y' coat protein in transgenic tobacco[100].

A synthetic substitution analogue of magainin, MSI-99 was expressed in tobacco[101]. Magainin is one of the earliest reported anti-microbial peptide isolated from skin secretions of the African clawed frog, *Xenopus* laevis. MSI-99 was sub-cloned into plant expression vector pMSI168, wherein the peptide was targeted to extracellular spaces. Tobacco plants transformed with pMSI168 showed enhanced resistance against *Sclerotinia sclerotiorum*, *Alternaria alternata* and *Botrytis cineria* pathogens. Tobacco leaves (control and transgenic) were infected with actively growing fungal mycelia of these pathogens and lesion diameter was measured after three days of infection. The leaves of transgenic tobacco plants showed the significant reduction in lesion diameter compared to control leaves, indicating the usefulness of this peptide in enhancing the disease resistance in tobacco plants.

Rol C gene was transferred into tobacco using *A. rhizogenes* and transgenic clones for flowering, leaf and flower size and height were isolated[102]. As a result, such transfer system is suggested for modifying horticulture crops. However, *rol*A gene was reported to stimulate nicotine production directly[103]. A cDNA and corresponding promoter region, for a naturally occurring feedback insensitive anthranilate synthase α subunit gene, has been isolated from unselected but 5 methyl tryptophan resistant tobacco cell line[104]. In an another experiment, the tobacco rdcS promoter fused to the *GUS* reporter gene was delivered to black spruce *via* microprojectile DNA bombardment and its regulation was studied. The results showed that tissue specific regulation of the rbcs promoter might be conserved between tobacco and black spruce[105]. The DNA binding domain of yeast transcriptional activator (GAL 4) was demonstrated expressing in the transgenic tobacco plants in order to attempt specific repression of reporter genes[106]. Transgenic tobacco plants were obtained from tissues infected either with a disarmed or a virulent root forming *Agrobacterium* containing a binary vector[107]. Transgenic tobacco plants were also achieved through microtargeting[108]. Further, the cells of *Escherechia coli* and *A. tumefaciens* were used as microprojectiles to deliver DNA into suspension cultured tobacco cells, using helium powdered biolistic device, and obtained hundreds of transient transformants per bombardment but did not get any stable transformants[109].

Phytoremediation, i.e. bioremediation using plants, is becoming a reality for safe removal of organic compounds from contaminated water and soil. The natural ability of certain plant species to take up heavy metals and radioactive elements is being exploited for bioremediation efforts. Transgenic tobacco plants, genetically engineered to express a bacterial enzyme, were developed to detoxify TNT (2,4-6 trinitrotoluene)[110]. A wide variety of naturally occurring soil bacteria possess a wide array of enzymes capable of degrading insecticides and herbicides. Genes encoding such enzymes can be cloned and expressed in transgenic tobacco plants for use in phytoremediation.

Tobacco plant has been extensively used for transgenic research and continues to remain as the model plant of choice[111]. However, as the development proceeded in the area of genetic transformation, *Arabidopsis* and other economically important plants (rice) became the other choice. The first field trial of transgenic crops was conducted with tobacco in France and USA in 1986. Tobacco was also the first crop to be commercialized in China and USA in 1991 and 1994, respectively. Transgenic plants resistant to TMV were grown in almost 1 million ha in China in 1994, which yielded 5-7% more leaves[112].

Studies on Molecular Farming

Several recombinant proteins are being produced in transgenic plants as bioreactors for the large-scale production of commercially important compounds of pharmaceutical and industrial importance (Table 2)[113-118]. Tobacco has served as the choice plant system for the production of potential therapeutic proteins in plants[1119,120]. Tobacco is an ideal plant-bioreactor for molecular farming because the pharmaceutical protein is produced in its leaves (about 40 tons of leaves/acre) and the production system does not require flowering. The leaves contain 10% protein and about 2000 kg protein/acre/yr can be produced. Tobacco is also a prolific seed producer with about 1 million seeds/plant. The first transgenic

Table 2—Production of biopharmaceuticals for human health in transgenic tobacco plants

Protein	Application	Expression level	Reference
Human Protein C	Anticoagulant	0.01% SLP	Crammer *et al*[113]
Human granulocyte macrophage calony stimulating factor	Neutropenia		Giddings *et al*[114]
Human somatotropin	Growth hormone	7% SLP (chloroplasts)	Staub *et al*[115]
Human erythropoietin	Anemia	0.01%SLP	Kusnadi *et al*[116]
Human epidermal growth factor	Wound repair and cell proliferation	0.01%SLP	Crammer *et al*[113]
Human interferon beta	Hepatitis B and C	0.01% FW	Kusnadi *et al*[116]
Human haemoglobin alpha, beta	Blood substitute	0.05% SP	Crammer & Weissenborn[117]
Human homotrimeric collagen	Collagen	0.01%FW	Ruggiero *et al*[118]
Angiotensin converting enzyme	Hypertension		Giddings *et al*[114]
Alpha Trichosanthin from TMV-U1 subgenomic coat protein	HIV therapy	2% SLP	Giddings *et al*[114]
Glucocerebrosidase	Gauchers disease	1-10% SLP	Crammer *et al*[113]

FW: fresh weight, SLP: soluble leaf protein, SP: seed protein, -: not reported

plant synthesized product is a tobacco derived antibody targeting gum disease. Most of the tobacco types secrete sticky compounds on the outer leaf surface as a first line of defence and some varieties produces 16% of the leaf dry weight as gum. Scientists of the University of Kentucky isolated a promoter to control the type of compounds that are secreted by tobacco leaf hair. Targeting the recombinant proteins in tobacco gum offered several advantages and purification would be easier and much cheaper[121].

Various types of antibodies are used in human medicine for diagnostic as well as for therapeutic purposes[122]. Recombinant antibodies have been produced in transgenic tobacco plants. The expression and assembly of immunoglobulin (lgG heavy and light chains) led to the production of other antibodies, like IgG-IgA antibody against a surface antigen of *Sterptococcus mutans* designed to prevent tooth decay. Estimates of production costs for an antibody in plants indicate as much as 10 to 20-fold lower costs per gram compared to produced by cell culture. Since plant based expression levels are low, chloroplast transformation may offer as an alternative. As there are 10,000 copies of chloroplast genomes per cell, this can facilitate the introduction of 10,000 copies of foreign genes per transformed cell and subsequently can boost several hundred-fold increase in gene expression compared to nuclear transformation[123].

Vaccines are of prime significance for the human health. However, in many developing countries, the incidence of infectious diseases and the expense of immunization programme limit the use of available vaccines for large segments of population. Vaccine/ antigen coding genes for traveller's diarrhoea, hepatitis B, gastroenteritis, foot and mouth disease, mink enteritis, swine fever, hog cholera, rabies, swine transmissible gastroenteritis, dental caries, auto-immune diabetes and cholera have been expressed in different plant systems including tobacco (Table 3)[124-137]. The first study on the production of plant-based vaccine was done in tobacco with the hope of developing a less expensive product[130]. It was also shown that rHBsAg self-assembled into sub viral particles, identical to the plasma and yeast derived HBsAg specific antibodies in mice[122]. The expression of cholera toxin B was demonstrated in transgenic tobacco chloroplast, resulting in the accumulation of 4.1% of total soluble leaf protein as functional CTB oligomers[122]. Further, binding assays confirmed the correct folding and disulfide bond formation of the plant derived CTB pentamers. The expression of C-terminal region of merozite surface protein ($PfMSP1_{19}$), a potential malaria vaccine candidate, was reported in tobacco[128]. Immunoblot assay indicated that transformed plant expressed $MSP1_{19}$ displayed structural and immunological characteristics identical to the *E. coli* expressed protein. This presents a significant step towards the development of low-cost subunit vaccine against malaria.

The tobacco cell line (NT-1) was transformed with hepatitis B virus '*s*' gene, coding for surface

Table 3—Vaccines produced in transgenic tobacco plants

Disease/Antigen	Origin	Expression level	Reference
Traveller's Diarrhoea (Heat labile enterotoxin B)	Enterotoxigenic *E. coli*	0.001% SLP	Haq *et al*[124]
Hepatitis B (HBsAg)	Hepatitis B virus	0.0066% SLP	Mason *et al*[125]
Gastroenteritis (Norwalk virus capsid protein)	Norwalk virus	0.23% SLP	Mason *et al*[125]
Immunocontraception (ZP 3)	Murine	-	Fitchen *et al*[126]
Malaria (epitopes derived from sporozoites)	*Plasmodium* sporozoites *Plasmodium falciparum*	0.0035% SLP	Turpen *et al*[127] Ghosh *et al*[128]
Swine fever (Hog cholera, E0, E1 & E2)	Swine fever virus	-	Kapusta *et al*[129].
Dental caries (SpA antigen)	*Streptococcus mutans*	0.02% SLP	Mason & Arntzen[130]
Cholera(CT-B)	*Vibrio cholerae*	-	Arakawa *et al*[131]
Colon cancer	-	-	Verch *et al*[132]
Influenza	Influenza virus	-	Beachy *et al*[133].
Lymphoma (Tumour derived ScFv epitopes)	-	-	Mc Cormick *et al*[134],
Post-surgical/burn infections Epitope of outer membrane protein F	*Pseudomonas aeruginosa*	-	Stackzek *et al*[135]
Human cytomegalo virus	Cytomegalo virus	0.02% SLP	Tackaberry *et al*[136]
Swine Transmissible gastroenteric virus	Corona virus	0.20% SLP	Tuboly *et al*[137]

SLP: soluble leaf protein, -: not reported

antigen[138]. Two plant transformation vectors pHER100 and pHBS100 with and without endoplasmic reticulum retention signal, respectively were used for transformation. The integration of the transgene was analysed by PCR and southern blot hybridization, and expression level was determined by ELISA. The maximum expression of 2 µg/g fresh weight and 10 ng/ml of spent medium was reported in pHER100 transformed cells. Western blot analysis confirmed the presence of 24 kDa band specific to HBsAg in the transformed cells. The buoyant density in CsCl of HBsAg derived from pHBS100 transformed tobacco cells was determined and found to be 1.095 gm/ml. The secretion of HBsAg particles by plant cells into the cell culture medium was reported for the first time.

The availability of genetic transformation methods of plants has broadened the type of experimental problems, the diversity of cell types that can be approached and the transfer of useful genes. These investigations promise to make the plant based systems extremely attractive and powerful for crop improvement, besides studying gene regulation and developmental biology in higher plants.

Conclusions

Tobacco has become the plant system of choice for almost all the aspects of cell and tissue culture research. Majority of the experimental discoveries in the field of plant cell, tissue culture and plant molecular biology owe their inception to the studies with tobacco. Trends in plant biotechnology research show a substantial increase in research publications on tobacco from 123 to 1396 from 1980-1990 to 1990-2000. Next to tobacco, arabidopsis has been now the model plant for molecular research. However, researchers all over the world still continue to use tobacco for a wide variety of plant research programmes. Tobacco as a model system has played a major role in the advancement of plant science and has been used as a tool in gaining fundamental knowledge in diverse areas of plant biology. Recent advances in the field of molecular farming have used tobacco as a 'plant factory' for the purposes of developing production system for recombinant proteins, pharmaceuticals, vaccines, industrial enzymes and antibodies. Several companies are already using this technology for commercial production. Vector Tobacco Inc., Durham, USA has developed a variety of genetically engineered tobacco

plants. One of them is nearly nicotine-free. Based on the present developments, it appears that, in future, tobacco being a non-food crop and having apathy from non-cigarette lovers, will assume the role of a most useful crop for molecular farming, leading to better industrial and human healthcare options.

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