ANNEXINS MEDIATED GENE TRANSFER IN TOBACCO PLANT (*Nicotiana tabacum* L.): A REVIEW

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Abstract

Agrobacterium tumefaciens is a remarkable species of soil - dwelling bacteria that has the ability to infect plant cells with a piece of its DNA. Plant transformation mediated by Agrobacterium tumefaciens, a soil plant pathogenic Gram negative bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. Tobacco (Nicotiana tabacum L.) is a tall leafy annual plant that belongs to the Solanaceae family. Every part of the plant except the seed contains nicotine, but the concentration was related to different factors such as species, type of land, culture or weather conditions. Annexins are soluble, hydrophilic proteins capable of binding to the negatively charged membrane. Annexins activities and conformations can be affected by post translational modifications. These modifications are mechanistically linked to the different annexins functions. The present review is focused on Annexins mediated gene transfer in Tobacco. This present review composed of the following topics: Tobacco and Transigenic Tobacco, Annexins, Molecular biology of Annexins, Involvement of Annexins in the responses of plants to stresses, Agrobacterium tumefaciens, Agrobacterium tumefaciens mediated Gene transformation in plants, Direct and Indirect Gene transfer by Agrobacterium tumefaciens, Ti plasmids, Transfer DNA (T-DNA) of Ti Plasmid, Pili formation, Binary vectors of Ti plasmids, Integration of Agrobacterium tumefaciens T-DNA into plant genome, Agrobacterium

tumefaciens mediated gene transfer in plants, Establishment of Transformation protocol by Agrobacterium tumefaciens and Future developments in producing Transgenic plants.

Key words: Tobacco, *Agrobacterium tumefaciens*, Annexins, Transgenic plants, Gene transfer, Plasmids and Transformation.

A transgenic crop plant contains a gene or genes, which have been artificially inserted instead of the plant acquiring them through pollination. The inserted gene sequence (known as the transgene) may come from another unrelated plant, or from a completely different species. Plants containing transgenes are often called “Genetically Modified” or “GM Crops”. Although in reality, all crops have been genetically modified from their original wild state by domestication, selection and controlled breeding over long periods of time. At this point, we will use the term transgenic to describe a crop plant which has transgene inserted.

The underlying reason that transgenic plants can be constructed is the universal presence of DNA (Deoxyribonucleic acid) in the cells of all living organisms. This molecule stores the organism's genetic information and orchestrates the metabolic processes of life. Genetic information was specified by the sequence of four chemical bases (adenine, cytosine, guanine and thymine) along the length of the DNA molecule. Genes are discrete segments of DNA that encode the information necessary for assembly of a specific protein. The proteins then function as enzymes to catalyze biochemical reactions, or as structural or storage units of a cell, to contribute to expression of a plant trait.

The transcription and translation processes are controlled by a complex set of regulatory mechanisms, so that a particular protein was produced only when and where it was needed. Even species that are very different have similar mechanisms for converting the information in DNA into proteins. Thus, a DNA segment from bacteria can be interpreted and translated into a functional protein when inserted into a plant. Among the most important tools in the genetic engineer's tool kit, enzymes are important agents that perform specific functions on DNA. Two enzymes are used in the Genetic engineering techniques. They are: a) Restriction enzyme (EcoR1): It recognizes and cuts the DNA at its specific region and b) Ligases: It joins the ends of two restricted DNA fragments. Identifying and locating genes for an agriculturally important traits was currently the most limiting step in the transgenic process. We still know relatively little about the specific genes required enhancing yield potential, improving stress tolerance, modifying chemical properties of the harvested product or otherwise affecting plant characters. Usually, identifying a single gene involved with a trait was not sufficient. Scientists must understand how the gene was regulated, what other effects it might have on the plant and how it interacts with other genes active in the same biochemical pathway. Public and private research programs are investing heavily into

new technologies to rapidly sequence and determine functions of genes of the most important crop species. These efforts should result in identification of a large number of genes potentially useful for producing transgenic varieties.

Transformation is the heritable change in a cell or organism brought about by the uptake and establishment of introduced DNA. There are two main methods of transforming plant cells and tissues. The "Gene Gun" method is also known as micro projectile bombardment or biolistics. This technique has been especially useful in transforming monocot species like corn and rice. The *Agrobacterium* method, which was described below. Transformation *via Agrobacterium* has been successfully practiced in dicots (broadleaf plants like soybeans, tobacco and tomatoes) for many years, but only recently has it been effective in monocots (grasses and their relatives). In general, the *Agrobacterium* method was considered preferable to the gene gun, because of the greater frequency of single-site insertions of the foreign DNA, making it easier to monitor.

Agrobacterium tumefaciens is a remarkable species of soil - dwelling bacteria that has the ability to infect plant cells with a piece of its DNA. When the bacterial DNA is integrated into a plant chromosome, it effectively hijacks the plant's cellular machinery and uses it to ensure the proliferation of the bacterial population. Many gardeners and orchard owners are unfortunately familiar with *Agrobacterium tumefaciens*, because it causes crown gall diseases in many ornamental and fruit plants.

The DNA in an *Agrobacterium tumefaciens* cell is contained in the bacterial chromosome as well as in another structure known as a Ti (tumor-inducing) plasmid. The Ti plasmid contains a stretch of DNA termed T-DNA (~20 kb long) that was transferred to the plant cell in the infection process. A series of *vir* (virulence) gene's that direct the infection process. *Agrobacterium tumefaciens* can only infect a plant through wounds. When a plant root or stem is wounded it gives off certain chemical signals. In response to those signals, the *vir* genes of *Agrobacterium tumefaciens* become activated and direct a series of events necessary for the transfer of the T-DNA from the Ti plasmid to the plant's chromosome.

Tobacco and Transigenic Tobacco

Tobacco (*Nicotiana tabacum* L.) is a tall leafy annual plant that belongs to the Solanaceae family. It is an annual shrub, herb or small tree from 0.9 to 1.5 m tall according to the variety. Tobacco includes numerous species, which are grown throughout the world. One species (*Nicotiana tabacum* L.), common tobacco is the main source of commercial tobacco used today. *Nicotiana tabacum* is a native of Tropical and Sub Tropical America but it is now commercially cultivated worldwide. It is referred to as a cash crop because it is grown for sale and not for use on the farm. Other varieties are cultivated as ornamental plants or grow as a weed.

Every part of the plant except the seed contains nicotine, but the concentration was related to different factors such as species, type of land, culture or weather

conditions. The concentration of nicotine increases with the age of the plant. Tobacco leaves contain 2 to 8 % of nicotine combined as malate or citrate. The distribution of the nicotine in the mature plant was widely variable: 64 % of the total nicotine exists in the leaves; 18 % in the stem, 13 % in the root and 5 % in the flowers. The main uses of tobacco plant are listed below.

a) The nicotine of tobacco is used as an insecticide.
b) Instillation of tobacco enemas for treatment of intestinal worms or constipation.
c) Dried tobacco leaves for chewing, snuffing or smoking.

Globally, the hectares of genetically improved crops are increasing. In 2015, the global area of transgenic crops continued to grow for the seventh consecutive year at a sustained double-digit growth rate of 15 % compared with 12 % in 2014. The estimated global area of Genetically Modified (GM) crops for 2015 was 67.7 million hectares. It was noteworthy that a double - digit rate of 10 % growth in GM crops was sustained in 2015. Nearly, 7 million farmers in 18 countries grew the 67.7 million hectares of GM crops in 2015, equivalent to 167 million acres, an increase from 6 million farmers in 16 countries in 2014. The increase in area between 2014 and 2015 of 15 % was equivalent to 9 million hectares or 22 million acres. In India, genetically modified or transgenic crops are still creating a scare and are talked of as manipulations of a few multinational companies.

Tobacco is processed into cigarettes, cigars, snuff, and chewing tobacco. Tobacco is widely regarded as being highly addictive due to the presence of high (20000 ppm to 30000 ppm) levels of nicotine in conventional varieties. It was generally agreed that reducing the level of nicotine delivered by tobacco products will reduce dependence on tobacco, which is commonly regarded as large contributor to cancerous diseases and premature mortality worldwide. The Central Tobacco Research Institute, Rajahmundry, is doing researches with Bt toxin gene to generate plants resistant to two specific pests.

Annexins

Annexins are soluble, hydrophilic proteins capable of binding to the negatively charged membrane (Gerke and Moss, 2002). They have a conserved structural element called annexin repeat, a segment of between 70 - 75 amino acids (Clark and Roux, 1995; Gerke and Moss, 2002). Annexins have two domains, a conserved C-terminal protein core and the N-terminal region (Jost *et al*., 1994). Annexins are able to bind to all cell membranes, including plasma membrane, vacuoles, nucleus, mitochondria, golgi apparatus, mitochondria, peroxisome and chloroplast (Seigneurin Berny *et al*., 1999; Lee *et al*., 2004; Mortimer *et al.*, 2008; Eubel *et al*., 2008). They bind to the membranes in calcium – dependant or – independent manner (Dabitz *et al*., 2005).

Annexins have two calcium binding sites: the high affinity type II site and low affinity type III site (Weng *et al.*, 1993). Besides the calcium binding sites, several other sequences are relatively conserved in several plant annexins such as the His40 key

peroxidase residue and the sulfur cluster (Hofmann *et al*., 2003; Mortimer *et al*., 2008). The sulfur cluster is usually involved in posttranslational modifications and is presented in all eight annexins (Hofmann *et al*., 2003; Dorota *et al*., 2009).

Since, the discovery of the first plant annexin in tomato (Boustead *et al*., 1989), various other studies have emerged reporting their identification in other plant species (Clark *et al*., 2001; De Carvalho-Niebel *et al*., 2002; Lee *et al*., 2004; Dabitz *et al.*, 2005; Cantero *et al*., 2006; Vandeputte *et al*., 2007). Plant annexins occur in diverse cell and tissue types and are highly abundant in roots and ripening fruits (Blackbourn *et al*., 1992). Annexins have been identified in more than 65 species, including protists, fungi, plants and vertebrates including zebra fish (Moss and Morgan, 2004). Animal annexins were discovered in 1970 (Creutz *et al*., 1978). They are two main differences observed between plant and animal annexins. Firstly, the endonexin sequence of plant annexins is present only in the first and fourth repeat regions, while it is well conserved in at least three of the four repeat regions of animal annexins (Mortimer *et al*., 2008). Secondly, the N- terminal region of plant annexins is short (10 amino acids), while that of animal annexins is significantly longer (40 amino acids) (Gerke *et al*., 2005; Mortimer *et al*., 2008). Animal annexin N-terminal tail is bound at its C-terminal domain when it is free, and when combined with phospholipids, its N- terminal tail is detached from its C-terminal domain (Gerke and Moss, 2002).

Annexins activities and conformations can be affected by post translational modifications (O'Brian and Chu, 2005; Dorota *et al*., 2009). These modifications are mechanistically linked to the different annexins functions. The N-terminal region is the main site of posttranslational modifications, including phosphorylation, *S*-glutathiolation, *S*- nitrosylation and *N* – myristoylation (Gerke and Moss, 2002; Gerke *et al*., 2005; Mortimer *et al*., 2008). These modifications are commonly observed in protein- effector involved in cell signalling (O'Brian and Chu, 2005) and highlight the regulatory importance of N-terminal tail in post translation modifications (Gerke and Moss, 2002). A number of tyrosine, histidine and Serine/Threonine kinases that phosphorylate human annexins A1 and A2 have been described (Sarafian *et al*., 1991; Biener *et al*., 1996; Muimo *et al*., 2000). Plant annexins AnnAt1, AnnGh2 and p33 possess phosphorylation sites that are similar to those observed in human annexins A1 and A2 (Delmer and Potikha, 1997).

The *S*-glutathiolation and *S*- nitrosylation (consisting of the cysteine residues oxidation) in plants are induced by abiotic stress through the mediation of ROS system (reactive oxygen species) (Gould et *al.*, 2003; Apel and Hirt, 2004). The cysteine residues of S3 cluster is usually involved in these mechanisms (Dorota *et al*., 2009). Annexin *AnnAt1* has *S* – glutathiolation which has been observed *in vitro* and *in vivo*, that is induced after treatment with ABA and reduced by half of their Ca^{2+} affinity (Dorota *et al*., 2009). *S*- nitrosylation of *AnnAt1* has also been described (Lindermayr *et al*., 2005).

Annexins have been reported to regulate the activity of the cell surface enzyme involved in β-glucan (callose) (Gidrol *et al.,* 1996). Callose synthesis interacting with annexins was found in purified annexin from cotton fiber (Andrawis *et al*., 1993). Purified annexin p35 has been shown to have different catalytic activities in different plant species including binding to F-actin in calcium-dependent manner in tomato (Calvert *et al*., 1996; Lim *et al*., 1998), ATPase activity in maize (Mc Clung *et al*., 1994), GTPase activity in cotton (Shin and Brown, 1999) and peroxidase activity in maize (Gidrol *et al*., 1996; Laohavisit *et al*., 2009). Annexin p35 also plays a role in phospholipid binding in tomato (Lim *et al.,* 1998).

Molecular Biology of Annexins

That all annexin display similar properties regarding Calcium and phospholipids are due to a common primary structure. Each annexin was constituted of two different regions, the unique N-terminal domain, also called the 'tail' and the C-terminal domain, named 'core'. The 34 kDa C-terminal domains is the conserved part of the molecule and strictly defines the annexin family. With some exceptions, it is always composed of 4 repeats of a~70 amino acid sequence containing an increased homology region. In contrast to the core domain, the sequence of the N-terminal domain was extremely variable. The length varies from a few amino acids to more than 100. In addition the annexin N-terminal domain was considered as the regulatory region of the protein since it contains the major sites for phosphorylation, proteolysis or even interactions with other proteins.

Members of the known annexin subfamilies are encoded by paralogous genes with common genomic organization, regulatory properties, and protein structural features. The 5'coding DNA sequence (CDS) of each annexin exhibits sequence identity and structural homology limited to its respective sub-family. Hence, the sequence length and identity and structural homology limited to its respective sub-family. Hence, the sequence length and identity of the corresponding protein amino termini provide a reliable means of inferring subfamily identity except where those amino-termini are short or unknown. The 3'- CDS region contains four internally homologous repeats of 68 - 69 codons linked by repeat - specific segments of four to 16 codons.

Involvement of Annexins in the responses of plants to stresses

Expression of annexin in *Arabidopsis* is affected by light. For example the expression of *AnnAt5* and *AnnAt6* in the hypocotyls and cotyledons increases with red light but decreases with far-red light (Cantero *et al*., 2006). In *Mimosa pudica*, the expression and localization of *Annexin p35* are linked to nyctinastic movements of the pulvinus. The p35 protein was abundant at night and less abundantly during the day. It is mainly cytosolic (Hoshino *et al*., 2004).

Low temperature of 4 °C significantly induced *AnnAt1* and *AnnAt3* expression, and significantly suppressed *AnnAt2* expression (Cantero *et al*., 2006). Temperature of 37 °C has been shown to significantly induce *AnnAt2*, *AnnAt6* and *AnnAt7* expression and significantly suppresser *AnnAt3* and *AnnAt4* expression (Cantero *et al*., 2006). Four annexins in a particular variety of wheat resistant to cold were found to accumulate during cold two of these annexin p39 and annexin p22.5 in membranes during wheat acclimation to cold at a temperature of 4 °C. Annexins may play a role as sensors or transducers of calcium signal linked to cold (Breton *et al*., 2000).

Salt stress significantly induced expression of *AnnAt1, AnnAt4, AnnAt5, AnnAt6, AnnAt7* and *AnnAt8* and significantly represses *AnnAt2* expression (Lee *et al*., 2004). Salt stress induces translocation of AnnAt1 and AnnAt4 proteins from cytosol to membranes (Lee *et al.*, 2004). In *M. sativa*, salt stressed cells revealed over expression of annexin *AnnMs2* (Kovacs *et al*., 1998). Drought significantly induces expression of genes *AnnAt1*, *AnnAt3*, *AnnAt6* and *AnnAt8* and significantly represses *AnnAt2* expression (Cantero *et al*., 2006). When Annexin gene from Arabidopsis was engineered into plants using *A. tumefaciens*, transgenic plants maintained significantly higher photosynthesis rates than did the non-transgenic control under drought condition, suggesting that *AnnAt1* gene induced a mechanism that protect plant from dehydration (Dorota *et al.*, 2009).

Annexins p33 and p35 from *Bryonica dioica* was found to change in response to mechanical stress in injured plants (Thonat *et al*., 1997). These annexins are localized in the cytoplasm of parenchyma cells of the internodes and accumulate at the plasma membrane of these cells following an injury. The relocation of these annexins could govern the radial expansion of the cell after stress or preparing the plasma membrane to undergo further stress (Mortimer *et al*., 2008).

Phytohormones control the growth and development of plants (Paciorek *et al*., 2006). The expression of pepper annexin *Ca32* and strawberry annexin *RJ4* was observed to increase during ripening of fruits (Proust *et al*., 1996; Wilkinson *et al*., 1995), indicating a hormonal control of expression of these genes (Mortimer *et al*., 2008). Salt stress and water stress act through the ABA, which also causes an increased expression of genes involved in these stresses (Lee *et al*., 2004; Hoshino *et al*., 2004; Cantero *et al*., 2006). *S*-glutathiolation of *AnnAt1 in vitro* and *in vivo* was induced by over expression of ABA (Dorota *et al*., 2009).

Agrobacterium tumefaciens

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic Gram negative bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. *Agrobacterium tumefaciens* naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumors. The first evidences indicating this bacterium as the causative agent of the crown gall goes back to

more than ninety years (Smith and Townsend, 1907). Since, that moment, for different reasons a large number of researches have focused on the study of this neoplastic disease and its causative pathogen. During the first and extensive period, scientific effort was devoted to disclose the mechanisms of crown gall tumor induction hoping to understand the mechanisms of oncogenesis in general, and to eventually apply this knowledge to develop drug treatments for cancer disease in animals and humans.

Agrobacterium tumefaciens has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease. T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation; and the genes encoding for the synthesis of opines. These compounds, produced by condensation between amino acids and sugars, are synthesized and excreted by the crown gall cells and consumed by *Agrobacterium tumefaciens* as carbon and nitrogen sources. Outside the T-DNA are located the genes for the opine catabolism, the genes involved in the process of TDNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995).

The first record on transgenic tobacco plant expressing foreign genes appeared at the beginning of the last decade, although many of the molecular characteristics of this process were unknown at that moment (Herrera-Estrella, 1983). Since, that crucial moment in the development of plant science, a great progress in understanding the *Agrobacterium*-mediated gene transfer to plant cells has been archived. However, *Agrobacterium tumefaciens* naturally infects only dicotyledonous plants and many economically important plants, including the cereals, remained accessible for genetic manipulation during long time. For these cases, alternative direct transformation methods have been developed (Shillito *et al*, 1985; Potrykus, 1991) such as polyethyleneglycol-mediated transfer (Uchimiya *et al*., 1986), microinjection (De la Pena *et al*., 1987), protoplast and intact cell electroporation (Fromm *et al*., 1985, 1986; Lorz *et al*., 1985; Arencibia 1995) and gene gun technology (Sanford, 1988). However, *Agrobacterium tumefaciens* - mediated transformation has remarkable advantages over direct transformation methods. It reduces the copy number of the transgene, potentially leading to fewer problems with transgene co-suppression and instability (Koncz *et al*., 1994, Hansen *et al*., 1997). In addition, it is a single-cell transformation system not forming mosaic plants, which are more frequent when direct transformation is used (Enriquez-Obregon *et al*., 1997; Enriquez-Obregon *et al*., 1998).

Agrobacterium tumefaciens mediated gene transfer into monocotyledonous plants was not possible until recently, when reproducible and efficient methodologies were established on rice (Hiei *et al*., 1994; Cheng *et al*., 1998), banana (May *et al*., 1995), corn (Ishida *et al*, 1996), wheat (Cheng *et al*., 1997) and sugarcane (Enríquez-Obregón 1997, 1998, Arencibia *et al*, 1998). Reviews on plant transformation using

Agrobacterium tumefaciens and the molecular mechanisms involved have been published during the last years (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995). A thorough analysis of the strategies for practical application of this methodology has been published recently (Birch, 1997).

***Agrobacterium tumefaciens* mediated Gene transformation in plants**

Currently, *Agrobacterium* and particle bombardment - mediated genetic transformation of target tissues followed by regeneration of plants either through somatic embryogenesis or organogenesis is widely used for the development of transgenic tobacco. Regeneration of plants from transformed tissues of tobacco *via* embryogenesis is restricted to a limited number of cultivars and efforts made to increase the range of cultivars that can produce somatic embryos, have met with limited success (Wilkins *et al*., 2004; Sakhanokho *et al*., 2005). Problems such as prolonged culture periods resulting in the appearance of somaclonal variation, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets and high genotype-dependency have further restricted the application of this technique for production of transgenics (Mishra *et al*., 2003; Wilkins *et al*., 2004; Sun *et al*., 2006).

Somaclonal variations resulting from prolonged culture periods are undesirable when production of true-to-type transgenic plant is the ultimate objective. Methods for direct regeneration of transformed shoots have some advantages of being more genotype independent than somatic embryogenesis besides allowing the speedy recovery of transgenic plants (Wilkins *et al*., 2000; Divya *et al*., 2008). However, constraints like low rooting efficiency of the shoots and low frequency of stable germline transformants need to be overcome (Gould *et al*., 1991; Hemphill *et al*., 1998; Luo and Gould, 1999). Different transformation techniques available have merits as well as certain limitations (Showalter *et al*., 2009).

The mechanism of gene transfer between *Agrobacterium* and the plant kingdom has facilitated the insertion of beneficial alien genes into diverse plant genomes (John, 1997). *Agrobacterium*-mediated transformation has been the most widely used and preferred method of transferring genes into plants (Wilkins *et al*., 2000). For the first time, successful *Agrobacterium* mediated transformation and regeneration of transgenic cotton plants from hypocotyls and cotyledons *via* somatic embryogenesis were reported by Firoozabady *et al*. (1987) and Umbeck *et al*. (1987). Later, Perlak *et al*. (1990) developed insect-resistant transgenic plants by introducing *cry1A* (*b*) and *cry1A* (*c*) genes of *Bacillus thuringiensis* into the cotton cv. Coker 312 employing the *Agrobacterium* strain A208. Transgenic cotton plants resistant to *Helicoverpa armigera* and *Sitophilus litura* were developed by introducing *cry* genes through A*grobacterium* - mediated genetic transformation using different explants, such as hypocotyl, cotyledon and embryogenic calli (Singh *et al*., 2004; Wu *et al*., 2005; Kumar *et al*., 2009).

Besides widely used techniques, attempts were also made for genetic transformation of cotton using various alternative approaches. The process of producing

transgenic cotton plants by *Agrobacterium* - mediated transformation, direct introduction of exogenous DNA into tobacco embryos and pollinating the flowers with pre-transformed pollen *via* pollen-tube pathway in a tissue culture independent manner were reported. Initially, introduction of exogenous DNA into embryos of self pollinated cotton flowers through the pollen-tube pathway was reported by Zhou *et al*. (1983). Adopting similar approaches, Huang *et al*. (1999) and Lu *et al*. (2002) produced transgenic tobacco plants expressing green fluorescent protein gene (*gfp*) and cellulose synthesizing genes (*acsA*, *acsB, acsC* and *acsD*) of *Acetobacter xylinum*.

Wang *et al*. (2004) produced *Verticillium* wilt resistant transgenic tobacco plants by applying 0.1 – 0.2 µg of DNA of *Gastrodia* antifungal protein (*gafp*) and bialaphos resistance (*bar*) genes on the floral stigmatic region. Herbicide-tolerant transgenic cotton plants were produced by direct inoculation of *Agrobacterium* culture on the pistils of pollinated flowers (Tian *et al*., 2010). Transformation of tobacco was also reported by manual pollination of flowers using transformed pollen. Li *et al*. (2004) reported cotton transformation by fertilizing the flowers using the pollen co-cultivated with *Agrobacterium* harbouring *acsA, acsB, gusA* and hygromycin phosphotransferase (*hpt*) genes.

Chemical-induced tobacco transformation was carried out by exposing cotyledon-derived embryogenic suspension cultures in Mannitol Salt medium containing spermidine and polybrene along with the DNA carrying *hpt* and *gusA* genes (Sawahel, 2001). Beringer *et al*. (2004) obtained herbicide-tolerant transgenic tobacco plants by agitating cotyledon-derived embryogenic calli in a solution containing mannitol and sorbitol together with needle-like silicon carbide microfibers coated with DNA containing the *bar* gene. Gounaris *et al*. (2005) reported the generation of transgenic cotton plants after fertilization of flowers employing the pollen bombarded with *Arabidopsis thaliana* 3′-hydroxylmethyl glutaryl coenzyme A reductase (*hmgr*) gene, along with the *nptII* gene.

Asad *et al*. (2008) produced salt tolerant transgenic tobacco plants expressing *Arabidopsis* vacuolar pyrophosphatase proton pump (*AVP1*) gene. Keshamma *et al*. (2008) reported in plant genetic transformation of tobacco by pricking the meristematic regions and dipping tobacco seedlings in the *Agrobacterium tumefaciens* culture. However, these techniques were not repeatable and involved screening of a large number of plants for identifying the stable transformants. Furthermore, plants selected as transgenics failed to transfer the transgene into subsequent generations (Showalter *et al*., 2009; Obembe *et al*., 2011).

Mao *et al*. (2011) obtained insect-resistant transgenic tobacco plants expressing *dsCYP6AE14* using *Agrobacterium* mediated genetic transformation of hypocotyl and cotyledon explants. Although, certain tobacco varieties have been transformed through *Agrobacterium* and plants have been subsequently regenerated through embryogenesis, commercially important cultivars proved recalcitrant to regeneration due to their inability to develop embryogenic tissues. Regeneration of transgenic tobacco plants

from shoot apex explants infected with *Agrobacterium* harboring *β*-glucuronidase (*gusA*) and neomycin phosphotransferase (*nptII*) genes were reported by Gould and Cedeno (1998) and Zapata *et al*. (1999). Direct regeneration of plants from the infected explants facilitated the development of transgenic plants in genotype-independent manner within a short time span. Subsequently, transgenic cotton plants have been produced in diverse cotton varieties through *Agrobacterium* - mediated transformation of apical meristems (Lv *et al*., 2009; Nandeshwar *et al*., 2009; Liu *et al*., 2011).

Direct and Indirect Gene transfer by *Agrobacterium tumefaciens*

Genetic transformation has become an important tool for crop improvement. At the same time, gene transfer by *Agrobacterium tumefaciens* is the established method of choice for the genetic transformation of most plant species compared to direct gene transfer methodologies (particle bombardment, electroporation and others). *Agrobacterium* - mediated transformation offers several advantages such as the possibility to transfer only one or a few copies of DNA fragments carrying the gene of interest at higher efficiencies with lower cost and the transfer of very large DNA fragment with minimal rearrangements (Hiei *et al.*, 1997; Hansen and Wright, 1999; Negrotto *et al.*, 2000). The most important advantage, however, is the possibility of producing transgenic plants which are free of marker gene (Komari *et al.*, 1996; Matthews *et al.*, 2001).

Selectable marker gene was added to the gene "construct" in order to identify plant cells or tissues that have successfully integrated the transgene. This is necessary because achieving incorporation and expression of transgenes in plant cells is a rare event, occurring in just a few percent of the targeted tissues or cells. Selectable marker genes encode proteins that provide resistance to agents that are normally toxic to plants such as antibiotics or herbicides. As explained below, only plant cells that have integrated the selectable marker gene will survive when grown on a medium containing the appropriate antibiotic or herbicide. As for other inserted genes, marker genes also require promoter and termination sequences for proper function. Once a gene has been isolated and cloned (amplified in a bacterial vector), it must undergo several modifications before it can be effectively inserted into a plant. For the proper functioning of transgene in a host cell, it requires a) Marker gene, b) Promoter gene and c) Termination sequence.

Promoter sequence

A Promoter sequence must be added for the gene to be correctly expressed (i.e., translated into a protein product). The promoter is the on/off switch that controls when and where in the plant the gene to be expressed. To date, most promoters in transgenic crop varieties have been "constitutive", i.e., causing gene expression throughout the life cycle of the plant in most tissues. Osmotin promoter shows a very specific pattern of temporal and spatial regulation of the osmotin promoter during normal plant development and after adaptation to NaCl. Various researchers found that the osmotin

promoter has a very high natural level of activity in mature pollen grains during anther dehiscence and in pericarp tissue at the final, desiccating stages of fruit development.

Cloned Gene

Sometimes, the cloned gene was modified to achieve greater expression in a plant. For example, the *Bacillus thuringiensis* (Bt) gene for insect resistance is of bacterial origin and has a higher percentage of A-T nucleotide pairs compared to plants, which prefer G-C nucleotide pairs. In a clever modification, researchers substituted A-T nucleotides with G-C nucleotides in the *Bacillus thuringiensis* (Bt) gene without significantly changing the amino acid sequence. The result was enhanced production of the gene product in plant cells.

Termination sequence

The Termination sequence signals to the cellular machinery that the end of the gene sequence has been reached.

Selectable marker gene

Selectable marker gene was added to the gene "construct" in order to identify plant cells or tissues that have successfully integrated the transgene. This is necessary because achieving incorporation and expression of transgenes in plant cells is a rare event, occurring in just a few percent of the targeted tissues or cells. Selectable marker genes encode proteins that provide resistance to agents that are normally toxic to plants such as antibiotics or herbicides. As explained below, only plant cells that have integrated the selectable marker gene will survive when grown on a medium containing the appropriate antibiotic or herbicide. As for other inserted genes, marker genes also require promoter and termination sequences for proper function.

Ti Plasmids

Tumour-inducing (Ti) Plasmid of *Agrobacterium tumefaciens* is necessary for transferring the piece of bacterial DNA into the plant cell. One component is the chromosomal virulence A (*chv*A) gene, which is on the *Agrobacterium* chromosome and activated by sugars. ChvA protein triggers bacteria to bind to the wounded plant tissue and to respond to a specific chemical (chemotaxis). The Ti plasmid in bacteria contains the other main components, which are generated or activated efficiently for causing crown gall in host plants after bacteria attach to the plant wound site.

The first is T-DNA, which is actually integrated into the plant cell chromosome. The second is the 35 kb virulence (*vir*) region, which is composed of seven loci (*vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*G, and *vir*H). Expression of *vir* genes is triggered by a phenolic compound, which is secreted from the wound site of the host plant. The main functions of *vir* proteins are to mediate the T-DNA excision from the Ti plasmid, export of the T-DNA piece from the bacteria, and insert it into the host plant chromosome

(Gelvin, 2003). These two components are essential for a successful gene transfer. The Ti plasmid also has other components, an opine catabolism region, a conjugal transfer region, and a vegetative origin of replication of the Ti plasmid (*ori*V) and these *vir* regions have sequence conservation between the octopine and nopaline Ti plasmids.

Ti plasmids are classified according to the opines, which are produced and excreted by the tumors they induce. During infection the T-DNA, a mobile segment of Ti or Ri plasmid is transferred to the plant cell nucleus and integrated into the plant chromosome. The T-DNA fragment is flanked by 25 bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. The 30 kb virulence (*vir*) region is a regulon organized in six operons that are essential for the T-DNA transfer (*virA, virB, virD* and *virG*) or for the increasing of transfer efficiency (*virC* and *virE*) (Hooykaas and Schilperoort, 1992; Zupan and Zambryski, 1995, Jeon *et al*., 1998).

Transfer DNA (T-DNA) of Ti Plasmid

The process of gene transfer from *Agrobacterium tumefaciens* to plant cells implies several essential steps: (1) bacterial colonization (2) induction of bacterial virulence system, (3) generation of T-DNA transfer complex (4) TDNA transfer and (5) integration of T-DNA into plant genome. A hypothetical model depicting the most important stages of this process is presented, supported by the most recent experimental data and accepted hypothesis on T-DNA transfer.

Transfer DNA (T-DNA) is the DNA segment transferred into the plant cell. The T-DNA is present on the Ti-plasmid of the wild type *Agrobacterium*, and its size is an average of 25 kb, ranging from 10 to 30 kb. The T-DNA region is flanked and delineated by two 25 bp direct repeats, known as the right border and left border. These border sequences are highly homologous and are targets of the border-specific endonuclease (VirD1/VirD2). The excised single strand of T-DNA from the Ti plasmid was exported from the bacterial cell to the plant cell by the activity of the other *Agrobacterium* Vir proteins.

Deletion of the right border leads to a reduction of virulence, whereas the left border does not. These data suggested that the right border is essential for *Agrobacterium* pathogenecity. They concluded that the transfer of T-DNA was directed from the right to left border by the polarity. Additional evidence, that the right border is more important than left border, is that the VirD2 protein can alone bind to the single stranded right border sequence and cleaves a single -stranded T-DNA. The VirD2 protein remained on the right border, of the resulting single stranded T-DNA molecule, termed the T-strand. T-DNA of octopine-type Ti plasmid has an overdrive sequence

near the right border, but not left border, which may enhance the functional polarity of right border and left border (Gelvin, 2003).

Wild type T-DNA also has genes that are involved in plant hormone synthesis in the host plant. They are the *tml*, *tms*, and *tmr* regions for leafy tumor, shooty tumor and rooty tumor, respectively, in the plant wound site. The opine synthase region was also located within the T-DNA. *Agrobacterium* strains are classified based on opine type, and components of the T-DNA are different in different opine types. If the Ti plasmid is a nopaline type, all components on T-DNA are a contiguous stretch, whereas the octopine type T-DNA consists of three individual parts, left (13 kb), central (1.5 kb) and right (7.8 kb). Each segment has T-DNA border repeat sequences (Sheng and Citovsky, 1996); therefore, these segments are transferred separately to the other organism due to the sequence. After T-DNA is integrated into the host plant, opine was synthesized, then secreted out and then imported into *Agrobacterium*. The absorbed opine molecule is catabolized by a specific enzyme in *Agrobacterium*. Opine is degraded into amino acid and the sugar moieties, which can be used as carbon and energy sources for bacterial growth.

Gene transfer starts from tight binding between *Agrobacterium* and the host cell. This process was caused by bacterial chromosomal proteins ChvA, ChvB and PscA. Expressions of these proteins are triggered by substances secreted from wound site. Characterized substances were sap with acidic pH (5.0 to 5.8) or phenolic compounds, such as acetosyringone (Winans, 1992), lignin or flavonoid precursors. It was found that monocyclic phenolics, such as acetosyringone (AS), are the most effective *vir* gene inducers. Uninjured plants do not produce these phenolic compounds or produce them at low levels, but production is dramatically increased in the wounded plant. Addition of artificial phenolic compounds during bacterial infection of the plant increased gene transfer efficiency. Sugars also assist activation of the major phenolic-mediated, wound-signaling pathway when small amounts of phenolic compounds are secreted from wounded cells.

Citovsky *et al*. (1992) reported when AS concentration was low or not detectable, *vir* gene expression was significantly increased by monosaccharides, such as glucose or galactose. Certain types of sugars can induce VirA protein activation with acetosyringone (AS) synergistically (Shimoda *et al*., 1990). After bacteria and plant cell binding, the protein in bacteria, which was activated by signal molecules and secreted from the plant wound was VirA (Sheng and Citovsky, 1996).

VirA can amplify the transformation system by detection of monosaccharide in the presence of low concentrations of phenolic compounds. Vir proteins are involved in signal recognition, transcriptional activation, conjugal DNA metabolism, intercellular transport, nuclear import and probably T-DNA integration into the plant nucleus. The major roles of the VirA and VirG proteins are activation of other *vir* genes. VirA activates VirG, which is a cytoplasmic DNA binding protein and works as a transcriptional activation factor to induce the expression of other *vir* genes. The

autophosphorylated histidine residue of VirA by a signal molecule phosphorylates an aspartate residue of VirG. These two proteins initiate the process of T-DNA transport (Winans, 1992). The phosphorylated VirG protein recognizes the *vir* genes containing a *vir* box, a conserved 12-bp sequence, and induces the expression of *vir* genes. This conserved region is located at a promoter region of the *vir* genes (Citovsky *et al*., 1992). VirD1 and VirD2, a heterologous system, act like endonucleases that cut between the third and fourth base pairs of 24 bp right and left border repeats of the T-DNA bottom strand.

A linear single-stranded copy of the T-DNA region, named T-strand, is generated in *vir*-induced *Agrobacterium* cells. The T-strand is produced from the 5 to 3 direction, initiating at the right T-DNA border and terminating at the left border, by the endonuclease activity of the VirD protein. VirD2 covalently binds to the right border of the T-strand, and to the 5 end of the remaining bottom strand of the Ti plasmid after the cleavage. The resulting single-stranded gap was repaired after the T-DNA strand is removed. VirD2 in the remaining strand may participate in ligating the left border nick. structural model of the T-strand when it was transferred out of the bacterium and into the plant cells, which was a protein-nucleic acid complex, called a T-complex. This T-DNA transport intermediate has at least three components: a T-strand, a VirD2, and VirE2 single strand DNA-binding protein. The VirE coats the single stranded DNA and forms a strong, stable, unfolded VirE2- ssDNA complex that is protected from external nucleolyic activity. The T-complex is 3600 nm long and 2 nm wide, and it seems to contain about 600 molecules of VirE2 and one molecule of VirD2. The predicted molecular mass was 50,000 kD. The structural model suggested that VirD2 and VirE proteins are transported to the plant cell with the T-strand. Another protein from the octopine Ti plasmid, VirC1, helps generate a T-strand when VirD1 and VirD2 are limiting.

Pili Formation

Several putative mechanisms of gene transfer from bacteria to plants have been proposed. One mechanism involves the conjugation machinery to transfer T-DNA into the plant cells. Eleven *vir*B genes in the Ti-plasmid make proteins that seem to be involved in T-DNA transfer (Lai *et al*., 2000). VirB proteins are primarily linked with the cytoplasmic and periplasmic membranes and are a part of putative trans-membrane pore or channel. The amino acid sequence of the VirB protein has high homology to the Tra proteins in *Escherichia coli*. Tra proteins are directly involved in the synthesis and assembly of the flagella (F pilus), the way that the genetic material is transferred between individual bacterial cells during conjugation (Shirasu and Kado, 1993). The structure of the F pilus is mainly composed of TraA protein, and TraA has to be processed from 12.7-kDa propilin into 7.2-kDa pili to be a structural subunit of the F pilus. The VirB protein sequence has high homology with TraA. VirB2 also has to be processed from a 12.3-kDa protein into a 7.2-kDa protein.

High amino acid sequence similarity, similar size of both proteins and post-processing into a small molecule strongly suggest that VirB2 is the propilin. pili are required for bacteria virulence, and the formation of pili in *Agrobacterium* required expression of the *vir* genes of the Ti-plasmid. Mutant experiments of *vir* genes demonstrated that *vir*A, *vir*G, *vir*B1 to *vir*B11, and *vir*D4 are the only genes that are necessary for pili formation. Experiments with various loss or gain of function mutants of these genes for pili formation showed that T-DNA transfer between bacteria highly depends on the expression of these Vir proteins. This implies that *Agrobacterium* pili are required for transferring DNA to plant cells in a process similar to that of conjugation of *E. coli*. VirB2 protein expression in the exocellular space of the *Agrobacterium* is directly correlated with pili formation. The correlation of high exocellular VirB2 protein expression and VirB2 filaments was determined by western blotting and electron microscopy. In the *vir*B2 or other *vir* gene mutants, VirB2 protein was not detected, and also pili were not detected. It was concluded that VirB2 is a majorcomponent of pili formation.

Jones *et al*. (1996) also confirmed that VirB2 is a major component of the *Agrobacterium tumefaciens* transfer pilus (T pilus). The main proteins for pili formation were identified, and they have been studied to find the mechanism for pili formation (Mushegian *et al*., 1996). Three VirB proteins (VirB1, VirB2, and VirB5) were proposed as the main pilus components.

Mushegian *et al*. (1996) reported that VirB1 was highly homologous with bacterial transglycosylases in its N-terminal domain, and it may facilitate assembly of the pili by lysis of the cell wall. The C-terminal region of the VirB1 was localized in the extracellular space, and electron microscopy studies showed that VirB1-cross-reactive material was on the surface of *Agrobacterium*. Since VirB1, among other members of VirB protein family, has a weak similarity with TraA, which is major flagella component in *E. coli*, they concluded that VirB1 may have an important role in plant cell interaction.

Schmidt-Eisenlohr *et al*. (1999) reported that VirB1, VirB2 and VirB5 needed each other for the stability and extracellular localization. Although VirB1 is not related to VirB2 induction, the expression level of VirB5 was highly regulated by VirB1 and VirB2 expression. High amounts of VirB1 and VirB2 were detected when abundant pili formation was observed. It was concluded that VirB1 and VirB2 are major pili components. VirB5 also cofractionated with VirB2, and it was concluded that VirB5 is directly involved in the pili assembly as a minor component.

The T-complex (protein-nucleic acid complex), which was ssDNA bound with VirD2 and VirE2 proteins, is transported through the *Agrobacterium* channel, composed of the VirB protein family, into the cytoplasm of the host plant cell. T- strands along with VirD2 and VirE2, moves to the plant cell from the bacteria cell. VirD2 and VirE2 are also known as the main factors for T-DNA insertion into the plant chromosome. These proteins have specific Nuclear Localization Signals (NLS) to move into the

nucleus. NLS is composed of a first active domain of two adjacent basic residues, linker and second activator domain, containing at least three out of five basic amino acids. The nuclear-imported *Agrobacterium* T-strand is integrated into the host plant cell chromosome. Both T-strand-associated proteins (VirD2 and VirE2) have been implicated in the integration process. A short amino acid sequence downstream of VirD2 NLS, designated the ω domain, is necessary for T-DNA integration.

Regarding the conversion of ssDNA into dsDNA, there are two different models. Tinland (1996) proposed that double strand synthesis would be performed by the plant cell DNA repair machinery following T-strand integration. The successful expression of the transgene depends on the position within the chromosome where the T-DNA integrates. T-DNA can be inserted near or far from transcriptional activating elements or enhancers, resulting in success or failure of activation of T-DNA carried transgenes. The failure of transgene expression (gene silencing) can also be caused by methylation or post-transcriptional gene silencing of multiple copies of transgenes. RNAs from these transgene copies may interfere with each other and then be degraded.

Binary Vectors of Ti Plasmids

The *Agrobacterium* - mediated transformation method was improved by developing modern binary Ti vectors after the removal of all the genes for tumor induction and opine synthesis. Ti plasmids without the tumor-inducing function are called disarmed plasmids (nononcogenic Ti plasmid). Ti plasmids have been engineered to separate T-DNA and *vir* regions into two distinct plasmids, resulting in a binary vector and a *vir* helper plasmid, respectively. Since disarmed binary plasmids, containing the T-DNA region, do not have the ability to move a T-DNA into the plant, they need the help of another separate plasmid containing the *vir* genes. Many *Agrobacterium* strains containing non-oncogenic *vir* helper plasmids (LBA 4404, GV3101 MP90, AGL0, EHA101, and its derivative strain EHA 105) have been developed (Gelvin, 2003). Binary vector plasmids are small and easy to handle in *Escherichia coli* and *Agrobacterium* when the wild type Ti plasmid is around 200 kb. The sizes of the processed binary vectors from wild type Ti plasmids have been reduced to less than 10 kb.

The binary vector has a replication origin for both *Escherichia coli* and *Agrobacterium*, an antibiotic selectable marker for bacteria and plants, a reporter gene and a T-DNA region containing a multi-cloning site for insertion of genes of interest. The binary vector is transformed to *Agrobacterium* harboring a disarmed Ti-plasmid, called the helper plasmid, providing *vir* gene functions. The T-DNA region from the binary vector is transferred to the plant by expression of the *vir* gene in the helper plasmid. Selectable markers are a convenient method to distinguish between transformed and non-transformed tissues.

Plants do not have an antibiotic resistance gene naturally. Transformed tissues contain selectable markers and survive on selective media containing antibiotics, while

non-transformed cells, which do not have an antibiotic resistance gene, are killed on the antibiotic media. Antibiotic-resistance genes, usually the kanamycin (neomycin phosphotransferase II) resistance gene have been used as selectable markers. Reporter genes, such as ß-glucuronidase (GUS), luciferase or green fluorescent protein (GFP), are important components of the T-DNA region. Expression of reporter genes is visualized in the transformed tissue through staining or auto-fluorescence. Transformed tissue can be distinguished from non-transformed tissue by reporter gene expression. ß-glucuronidase (GUS) is a reporter gene isolated from *E. coli* and has been used extensively in plant molecular biology studies. Efforts to increase GUS gene expression have been conducted by adding an intron or optimizing codon efficiency. It was found that a GUS gene isolated from *Staphylococcus* resulted in 10 times higher expression in rice tissues.

Integration of *Agrobacterium tumefaciens* T-DNA into plant genome

Inside the plant cell, the ssT-DNA complex is targeted to the nucleus crossing the nuclear membrane. Two Vir proteins have been found to be important in this step: VirD2 and VirE2, which are the most important, and probably VirF, which has a minor contribution to this process (Hooykaas and Schilperoort, 1992). The nuclear signals (NLS) of VirD2 and VirE2 play an important role in nuclear targeting of the delivered ss-TDNA complex, as early described. VirD2 has one functional NLS. The ssT-DNA complex is a large (up to 20 kb) nucleoprotein complex containing only one 5'end covalently attached VirD2 protein per complex. But, the complex is coated by a large number of VirE2 molecules (approximately 600 per a 20 kb T-DNA), and each of them has two NLS. The two NLS of VirE2 have been considered important for the continuos nuclear import of ss-T-DNA complex, probably by keeping both sides of nuclear pores simultaneously open. The nuclear import is probably mediated also by specific NLS-binding proteins, which are present in plant cytoplasm.

The final step of T-DNA transfer is its integration into the plant genome. The mechanism involved in the T-DNA integration has not been characterized. It was considered that the integration occurs by illegitimate recombination (Gheysen *et al*., 1991, Lehman *et al*., 1994; Puchta, 1998). According to this model, paring of a few bases, known as micro-homologies, are required for a pre-annealing step between T-DNA strand coupled with VirD2 and plant DNA. These homologies are very low and provide just a minimum specificity for the recombination process by positioning VirD2 for the ligation. The 3´-end or adjacent sequences of T-DNA find some low homologies with plant. DNA resulting in the first contact (synapses) between the T-strand and plant DNA and forming a gap in 3'-5' strand of plant DNA. Displaced plant DNA is subsequently cut at the 3'-end position of the gap by endonucleases, and the first nucleotide of the 5' attaches to VirD2 pairs with a nucleotide in the top (5'-3') plant DNA strand. The 3' overhanging part of T-DNA together with displaced plant DNA are digested away, either by endonucleases or by 3'- 5' exonucleases. Then, the 5' attached

to VirD2 end and other 3'-end of T-strand (paired with plant DNA during since the first step of integration process) joins the nicks in the bottom plant DNA strand. Once the introduction of T strand in the 3'-5' strand of the plant DNA is completed, a torsion followed by a nick into the opposite plant DNA strand was produced. This situation activates the repair mechanism of the plant cell and the complementary strand was synthesized using the early inserted T-DNA strand as a template (Tinland *et al*., 1995). VirD2 has an active role in the precise integration on T - strand in the plant chromosome. The release of VirD2 protein may provide the energy containing in its phosphodiester bond, at the Tyr29 residue, with the first nucleotide of T-strand providing the 5'-end of the T-strand for ligation to the plant DNA. This phosphodiester bond can serve as electrophilic substrate for nucleophilic 3'-OH from nicked plant DNA (Jayaram, 1994). When the mutant VirD2 protein is transferred attached to the T-strand, the integration process take place with the loss of nucleotides at the 5'-end of the T-strand (Tinland *et al*., 1995).

Agrobacterium tumefaciens mediated gene transfer in plants

Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants (Birch, 1997), using *Agrobacterium* - mediated or direct transformation methods. The idea that some species cannot accept the integration of foreign DNA in its genome and lack the capacity to be transformed is unacceptable under the increasing number of species that have been transformed. However, efficient methodologies of *Agrobacterium tumefaciens* mediated gene transfer have been established mainly for dicotyledoneous plants. These plants have been considered to be outside the *Agrobacterium* host range and other gene-transfer methods were developed for them. To develop these methodologies for a monocotyledoneous plant it is important to take into consideration the critical aspects in the *Agrobacterium tumefaciens*-plant interaction, the cellular and tissue culture methodologies developed for that species. The suitable genetic materials (bacterial strains, binary vectors, reporter and marker genes, promoters) and molecular biology techniques available in the laboratory are necessary for selection of the DNA to be introduced. This DNA must be able to be expressed in plants making possible the identification of transformed plants in selectable medium and using molecular biology techniques to test and characterize the transformation events has become one of the most important targets for plant genetic engineering. Transgenic tobacco plants expressing *Bacillus thuringiensis* d-endotoxin gene cry1Ab (B) successfully resists the attack of *Heliothis virescens* larvae. Non-transgenic plant (A) exhibits the damages of pest attack.

The optimization of *Agrobacterium tumefaciens* - plant interaction is probably the most important aspect to be considered. It includes the integrity of the bacterial strain, its correct manipulation and the study of reaction in wounded plant tissue, which may develop in a necrotic process in the wounded tissue or affect the interaction and

release of inducers or repressors of *Agrobacterium* virulence system. The type of explant is also an important fact and it must be suitable for regeneration allowing the recovery of whole transgenic plants. The establishment of a method for the efficient regeneration of one particular species was crucial for its transformation.

It was recommended to work firstly on the establishment of the optimal conditions for gene transfer through preliminary experiments of transient gene expression using reporter genes (Jefferson *et al*., 1987). This opinion is supported by the fact that *Agrobacterium*-mediated gene transfer is a complex process and many aspects of the mechanisms involved remain unknown. The transient expression experiments help to identify also the explants, which may be used as targets for gene transfer, providing evidence of successful transformation events and correct expression of transgene.

The preliminary studies also include the use of histologically defined tissues of different explants and regeneration of the whole plant. Transient expression experiments may be directed to the regenerable tissue and cell. Optimization of transient activity is a waste of time if experiments are conducted on non-regenerable tissues or consider conditions inhibiting regeneration or altering the molecular integrity of the transformed cell. *Agrobacterium* mediated transfer introduce a small number of copies of foreign DNA per cell compared with particle bombardment or electroporation, but high efficiencies of stable transformants may be obtained even from cells without positive results in transient expression assays. These aspects are important to establish a transformation procedure for any plant, especially for those species categorized as recalcitrant. Cereals, legumes and woody plants, which are very difficult to transform or remain untransformed, can be included in this category. Many species, originally considered in this category, has been transformed in recent years.

Establishment of Transformation protocol by *Agrobacterium tumefaciens*

Optimization of the *Agrobacterium tumefaciens* - mediated DNA transfer to various agricultural crops has recently been reported by Enriquez - Obregon *et al*. (1998). They hypothesize that the problems of transferring *Agrobacterium tumefaciens* – mediated genes to plants are related with the poor survival rate of the target cells. They attempted to overcome necrosis by using a set of antioxidant compounds, allowing cell survival and the development of competence to gene transfer.

The design of an adequate artificial environment to favor the interaction of *Agrobacterium tumefaciens* with the plant tissue was critical for the success of genetic transformation experiments. Oxidative burst, penalization and the subsequent cell death have been described as frequent phenomena during the interaction of *Agrobacterium tumefaciens* with monocotyledonous plant cells. Various researchers treated the explants with a set of antinecrotic compounds in order to minimize the *Agrobacterium tumefaciens* - induced hypersensitive response. In a previous report of Enriquez - Obregon *et al*. (1997) they showed that the co-culture of explants with *Agrobacterium*

tumefaciens induces rapid necrosis in the tissue. They tested several antinecrotic treatments on the *Agrobacterium tumefaciens* - mediated DNA transfer and callus formation, demonstrating that genetically transformed calli could be obtained only when a mix of antinecrotic compounds with remarkable antioxidative activity was added. Thus, this may be a reliable approach to overcome the *Agrobacterium tumefaciens* - induced hypersensitive cell death. As the physiological role of each compound during plant-microbial interaction has not been established already, they prefer to use the term "antinecrotic compounds" instead of "antioxidant compounds". The application of such compounds in a method for *Agrobacterium* - mediated transformation of grape was recently published by Perl *et al*. (1996); however, we do not know of any report on stable genetic transformation of monocotyledonous plants using antinecrotic treatments.

Ishida *et al*. (1996) reported that the enrichment of the media with sugars and a low pH were both important factors in achieving efficient transformation of rice and maize by *Agrobacterium tumefaciens*. They performed the infection step in a medium supplemented with glucose and sucrose at the concentrations suggested in the literature, but the observed transformation efficiencies were lower than those obtained when the pre-coculture was performed in conventional low-sugar medium (Enriquez - Obregon *et al*., 1997). Good transformation efficiencies were obtained without infecting the explants in low-pH media. They assume that osmotic conditions affect the process of recognition of plant cells by the *Agrobacterium*. These results are in agreement with those reported by Rashid *et al*. (1996), who stated that neither high sugar concentrations nor low pH are required in the genetic transformation process.

Research works of Torisky *et al*. (1997) on *Agrobacterium tumefaciens* - mediated genetic transformation of monocotyledonous plant species are focused on the use of the so-called "super-binary" vector systems, i.e. binary vectors carrying a DNA fragment from the *Agrobacterium tumefaciens* virulence region. Our procedures, however, are based on conventional genetic vectors; in addition, aceto syringone - a commonly used inductor of *Agrobacterium tumefaciens vir* genes was not required during infection. They concluded that in our system the *vir* genes naturally carried by *Agrobacterium tumefaciens* are sufficient to initiate the infection and transformation processes; their induction occurs normally without adding chemical activators. In their study, they investigated the regeneration abilities of the obtained calli (Deblaere *et al*., 1985). The results were similar to those observed under normal conditions and were not affected by the duration of the antinecrotic treatment. They obtained a classical insertion pattern usually observed in *Agrobacterium tumefaciens* - transformed plants without any discernible genetic rearrangement. The number of independent insertion patterns per individual plant genome was one or two. The results are in good agreement with those reported for other systems of *Agrobacterium tumefaciens* - mediated transformation; they also contrast with the complex multicopy insertion patterns described for the "first-generation" transgenic sugarcane plants produced by intact-cell

electroporation (Arencibia *et al*., 1995). The latter phenomena are clearly undesirable due to the lack of control, the possibility of gene silencing, cosuppression, etc.

The *Agrobacterium tumefaciens* - mediated transformation protocols differ from one plant species to other and, within species, from one cultivar to other. In consequence, the optimization of *Agrobacterium tumefaciens* - mediated transformation methodologies requires the consideration of several factors that can be determined in the successful transformation of one species. Firstly, the optimization of *Agrobacterium tumefaciens* - plant interaction on competent cells from different regenerable tissues. Secondly, the development of a suitable method for regeneration from transformed cells.

Future developments in producing Transgenic plants

New techniques for producing transgenic plants will improve the efficiency of the process and will help resolve some of the environmental and health concerns. Among the expected changes are the following:

- More efficient transformation, that is, a higher percentage of plant cells will successfully incorporate the transgene.
- Better marker genes to replace the use of antibiotic resistance genes.
- Better control of gene expression through more specific promoters, so that the inserted gene will be active only when and where needed.
- Transfer of multi-gene DNA fragments to modify more complex traits.

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