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Full Length Research Paper

Agrobacterium mediated transformation of annexin gene in tobacco (*Nicotiana tabacum*)

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The present study involves the development of genetically engineered tobacco plants with annexin gene. The plasmid pUC 119 with the Annexin gene and pGPTV vector were isolated from the *Escherichia coli.* These plasmids were subjected to restriction digestion with EcoRI and XbaI where the Annexin gene is released from the pUC 119 as a linearised band was eluted from the gel. The recombinant PGPTV plasmid with the annexin gene in *Agrobacterium tumefaciens* MTCC 431 was mobilized and transferred to plant system through the mobilization helper plasmid pRK2013. The kanamycin resistance gene (NPT II) was used as a selective marker. The calli used for isolating the genomic DNA which was then amplified for confirmation of annexin gene. The nptII gene of 800 bp serves as a selectable marker system in plants and its amplification confirmed the presence of annexin gene in transgenic plants by PCR method.

Key words: Annexin gene, ***Agrobacterium tumefaciens***, ***Nicotiana tabacum***, pGPTV vector, pUC119, transgenic tobacco.

INTRODUCTION

Annexins represent a highly defined family of proteins that all share a characteristic peptide sequence of 70 amino acids, consists of four or eight repeats of various proteins. All annexins share a common property, namely, the Ca^{2+}–dependent interaction with membrane phospholipids. The N-terminal region of the annexins is more diverse and confers the specific properties associated with the individual members of the family (Calvert et al., 1996). Annexin gene is present in almost all plants, animals, and human beings. They are mainly involved in a wide variety of essential cellular processes such as membrane ion–channel activity, membrane–cytoskeletal linkage, membrane trafficking, mitotic signaling, cytoskeletal rearrangement and DNA replication (Gerke and Moss, 1997; Lecat et al., 1999). Annexin protein has two important characteristics. Primarily it must be capable of binding to a Ca^{2+}-dependent negatively charged phospholipids and it must have a conserved structural element the so-called annexin repeat which is a segment of 70 amino acid residues (Boustead et al., 1989). Structurally, annexin domain consists of four annexin repeats paired into a highly α-helical disc that acts as a general membrane binding modules. The annexin fold is one of three main types of Ca^{2+} regulatory motifs found in plants (Kopka et al., 1998). Many reports have been linking annexin signaling to many different physiological processes in plants. Annexin gene expression in plants also appears to be regulated by developmental and environmental signals and the changes in expression of plant annexin have been observed during fruit ripening and cell cycle progression and in response to stress and abscisic acid (Kovacs et al., 1998). There are 160 unique annexin proteins which have been identified in more than 65 different species ranging from fungi and protists and higher vertebrates. The amino acid sequence of plant annexins show 40% similarity with the vertebrate annexins, where as the plant members share up to 97% similarity with each other (Morgan and Fernandez, 1997; Morgan et al., 1999). Soil salinity is one of the most significant abiotic stresses, especially for crop plants, leading to reductions in productivity. Annexin gene plays a main role in both biotic and abiotic stress tolerance, which is used to produce drought tolerant transgenic

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plants (Lee et al., 2004). Soil salinity is one of the most significant abiotic stresses, especially for crop plants, leading to reduction in productivity, which induce ionic and osmotic stress in plants. Annexin plays a major role in control in both biotic and abiotic stresses in plants (Lee et al., 2004).

The annexins have been identified in tomato (Smallwood et al., 1990), maize (Blackbourn, et al., 1992) and pea (Clark et al., 1992). The annexins present in the plant members show 97% similarity with animal and vertebrate sequences. The partial and complete cDNA clones for annexins have now been isolated from alfalfa (Prick et al., 1994), soybean (Shi et al., 1995), strawberry (Wilkinson et al., 1997), cotton (Potikha and Delmer, 1997), corn (Genbank listings x98244 and x98245) and the small crucifer ***Arabidopsis thaliana*** that have became the model genetic system of higher plants (Gidrol et al., 1996).

Plants annexins may be involved in secretory processes (Raynal and Pollard, 1994). The plant annexins interact with phospholipids in a Ca^{2+}-dependent manner. In the case of corn, the annexins were also shown to induce aggregation of liposomes or plant secretory vesicles (Blackbourn et al., 1993). Annexins interact in a calcium-dependent manner with membrane phospholipids. Although their exact function is not known, annexins have been proposed to be involved in a variety of cellular processes. Proust et al. (1997) proposed that Solanaceae annexins constitute a distinct type, called Sp32 annexins. The Sp32 annexin proteins accumulate during the cell cycle and peak at the end of mitosis. Immunolocalization shows that the majority of Sp32 annexins is present in intercellular junctions, forming a ring structure under the plasma membrane. Their localization at cell junctions suggests that annexins could be involved in cell wall maturation (Proust et al., 1997).

Lee et al. (2004) performed a comparative proteomic analysis of the ***A. thaliana*** of salt stress signaling. Among the salt-responsive microsomal proteins, two spots that increased upon salt treatment on a two-dimensional gel were identified as the same protein, designated annexin 1 (Ann At1).

Annexins comprise a multigene family of Ca^{2+}-dependent membrane binding proteins and have been extensively studied in animal cells. Ann At1 is strongly expressed in root but rarely in flower tissue. In their study, the results suggest that salt stress induces translocation from the cytosol to the membrane and potential turnover of existing protein. This process is blocked by EGTA treatment, implying that AnnAt1 functions in stress response are that tightly associated with Ca^{2+}. T-DNA insertion mutants of Ann At1 and a different isoform ann At4, displayed hypersensitivity to osmotic stress and abscisic acid (ABA) during germination and early seedling growth. The results collectively suggest that Ann At1 and Ann At4 play important roles in osmotic stress and ABA signaling in a Ca^{2+} dependent manner. Lim et al. (1998) were analyzed the cDNA sequence of p^{35} reveals that the annexin has two typical endonexin folds, corresponding to repeats IV and I. Expression of recombinant p^{35} in ***Escherichia coli*** confirmed both phospholipids binding and a nucleotide phosphatase activity that could be inhibited on interaction of the recombinant annexin with phospholipids. Site-directed mutagenesis in which the acidic residues Glu-68 (repeat I), and Asp-297 (repeat IV) were changed to Asn, generated two mutant forms, E68N and D297N, respectively. Both the mutant forms of the annexin continued to express catalytic activity. Changing repeat I had little effect on phospholipid binding, whereas the change to repeat IV abolished this property. These data shows that in this plant annexin, repeat IV plays a more critical role in calcium-dependent phospholipid binding than repeat I, and that the catalytic and phospholipid binding activity of the protein can be separated experimentally. Smallwood et al. (1990) reported that peptide sequence data derived from a plant annexin P^{34} was used to design amplimers for PCR. A unique fragment of 95bp, amplified from tomato (***Lycopersicon esculertum***) genomic DNA, was used in Northern analyses and demonstrated a differential pattern of expression in vegetative tissues of tomato, potato (***solanum tuberosum***) and barley (***Hoodeum vulgare***). This tissue-specific abundance of annexin transcript was found to correlate closely with abundance of annexin proteins as revealed by their partial purification and analysis with antisera specific for annexins isolated from tomato suspension–culture cells. It was hypothesized that proteins such as annexins, capable of forming Ca^{2+} channels ***in vitro***, may be involved in the LT (low temperature) signal transduction pathway (Jost et al., 1997). Recently two new isoform of wheat annexin protein with molecular mass of 39 and 22.5 KDa have been identified by and the level of both proteins increased rapidly in response to low temperature (Breton et al., 2000).

Tobacco (***Nicotiana tabacum***) belongs to the family ***Solanaceae***, that usually after aging and processing used in various ways namely, smoking, chewing, snuffing and also for the extraction of nicotine which is the principle alkaloid of tobacco. Tobacco holds an unparalleled position among crop plants in the world. It is one of the most widely grown commercial non-food plant. It holds a high importance in financial and economic policies in many countries. The global production of tobacco is estimated at 6.265 million kg from an area of 4.2 mha. Almost all continents are capable of growing tobacco but the United States, China, India and Brazil are the leading countries to grow tobacco (Smallwood et al., 1990)

Members of the genus ***Agrobacterium*** are soil-borne, Gram-negative bacteria, which belongs to the same family as ***Rhizobium***. ***Agrobacterium*** is an oncogenic agent, which genetically transforms plant cells and directs the resulting tumours to synthesize special nutrients that support the growth of the bacteria. ***Agrobacterium*** Ti plasmid is being used as a vector for plant genetic engineering. The transformation process involves transfer of genetic information from the bacteria to the plant stimulated by

compounds exuded from wounded plant cells.

The present study was aimed at the transfer of annexin gene in tobacco plants and verifies the transformation. The experiments includes, isolation of plasmid from ***E.coli,*** restriction digestion of pUC119 with Annexin gene and pGPTV (Vectors), Gel elution, DNA ligation and transformation in the competent cells (DH5α), Triparental mating for the transfer of plasmid to ***Agrobacterium tumefaciens***, ***Agrobacterium*** mediated transformation in tobacco and isolation of genomic DNA from transformed callus and amplification by PCR.

MATERIALS AND METHODS

The experiments were carried out from December 2005 – October 2007. Annexin gene was obtained from Genbank. ***Nicotiana tabacum*** was available at Shreedhar Bhat's Biotechnology Laboratory, Bangalore, India. Glycerol stocks of ***E. coli*** containing pGPTV and pUC119 with Annexin gene were used.

Construction of pGPTV plasmid/annexin plasmid

The plasmid DNA was isolated by alkaline lysis method (Brinboim and Dolly, 1979). The pGPTV and pUC119/Annexin plasmid from ***E. coli*** bacterial culture. Annexin gene/pUC119 and linear pGPTV vector were prepared by digesting respective plasmids with EcoRI and XbaI. The pGPTV/annexin construct was prepared by ligating annexin gene into pGPTV vector. The recombinant pGPTV/annexin were replicated by transferring into ***E. coli*** competent cells and spreaded on LB Kanamycin plate. The ***E. coli*** colonies were screened for recombinant pGPTV by using plasmid isolation and restriction digestion process.

Construction of Ti plasmid/annexin

Ti plasmid/annexin construct was prepared by performing triparental mating. In this method, ***E. coli*** bacteria (resistant to kanamycin) carrying pGPTV/annexin, ***E. coli*** carrying helper plasmid pRK2013 and ***A. tumefaciens*** MTCC 431 carrying disarmed Ti plasmid (resistant to Streptomycin and Rifampicin) were used. All the three starins were mixed and inoculated on YEP plate and allowed to grow at 27°C for 18 h. After serial dilution, spreaded on AB-KRS plate (Antibiotics Kanamycin-50 µg/ml; Rifampicin-10 µg/ml; Streptomycin-100 µg/ml) and selected colonies carrying Ti plasmid / annexin gene construct (annexin + Npt II gene).

Transformation of annexin gene construct into plant genomic DNA

The annexin gene construct was transferred from Ti plasmid to plant genomic DNA. In this method, the surface sterilized Tobacco leaves (treated with Tween 20 and Mercuric chloride) were used for preparation of leaf disc. The prepared leaf discs were placed on MS media for overnight under light. Then co-cultivated with ***Agrobacterium tumefaciens*** MTCC 431 carrying Ti/Annexin gene construct were placed on regeneration medium containing 0.1 mg/L IAA and 2.5 mg/L BAP. The co-cultivated leaf discs were incubated in the regeneration medium for about 2 days under dark condition to avoid photosynthesis. The transformed leaf discs were selected from non-transformed by transferring them to selection media containing 50 µg/ml kanamycin, 250 µg/ml augmentin and 200 µg/ml cefatoxime and incubated for about 2½ weeks under 12 h photoperiod for the induction of callus.

Screening of callus samples

The developed callus was screened for annexin gene construct by isolating genomic DNA by modified CTAB method. The isolated genomic DNA was analysed by using 0.8% agarose gel.

Polymerase chain reaction

Forward primer (5'CTG AAT GAT CTG CAG GAC GAG G 3'; 22 mer) and reverse primer (5' GCC AAC GCT ATG TCC CGA TAG C 3'; 22 mer) were used. The extracted DNA was taken in small specific amounts to check the quality and presence of annexin gene. The genomic DNA that was extracted from the transformed tobacco callus was subjected to PCR amplification in order to confirm the presence of the annexin. The presence of annexin gene construct was confirmed by PCR reaction using isolated genomic DNA and primers specific to gene construct. The PCR conditions were as follows: initial denaturation at 92°C for 5 min, denaturation at 92°C for 1 min, annealing at 55°C for 30 s, extension at 72°C for 1 min for about 30 cycles, final extension at 72°C for 3 min and final hold at 10°C. The PCR amplified DNA was analysed in 0.8% agarose gel and stained with ethidium bromide and observed under U.V. light.

RESULTS

By using EcoRI and XbaI enzyme, the pGPTV and pUC119/annexin plasmids were digested and cross checked by using 0.8% agarose gel. The ammonium acetate gel elution method was used to recover linear pGPTV and annexin gene from agarose gel and ligated by using T4 DNA ligase. The ligated pGPTV / annexin recombinant plasmid were transformed into DH5α competent cells and spreaded on LB kanamycin plate containing 50 µg/ml concentration (Figure 1). The bacterial colonies were screened for recombinant pGPTV/annexin by plasmid isolation and restriction digestion. The plasmids were subjected to restriction digestion by using the EcoRI and XbaI and subjected to agarose gel electrophoresis. Linear shaped bands were observed (Figure 2). Both the vector pGPTV and the insert (annexin) bands in agarose gel were eluted and purified by gel elution. The basic strain such as DH5α were tested for contamination by growing on LB plain and LB Kan plates the results shown in Table 1.

A 25 ng of pUC 119 DNA gave 1217 colonies and 1ng gave colonies (1 × 1217)/25 = 48.68 cfu per ng of DNA4.868 × 10^4 colony forming units (cfu/µg of DNA). These transformed colonies, which were found to be positive for the presence of genes were then used in triparental mating. In this process the three strains pGPTV annexin, pRK2013 and ***Agrobacterium tumefaciens*** MTCC431 mixed and grow on YEP plate (Figure 3). The colonies from YEP plate were serially diluted from 10^{-1} to 10^{-6} and then plated on AB kanamycin, refampicin, streptomycin (AB KRS) plate (Figure 4). More number of colonies was observed on 10^{-1} and less number in subsequent dilution. The plasmid DNA preparation from 10^{-3} was found to be of good quality and then further confirmation of the gene of interest was carried out. After

Table 1. Results of DH5α (***E. coli***) grown on LB plain and LB Kan plates.

No.	Media	Inoculum	Observation
1	LB Plain	150 µl Competent cell	Lawn type of growth present
2	LB Kan	150 µl competent cell	No growth
3	LB Kan	150 µl competent cell + Ligated DNA (pGPTV annexin)	Growth present
4	LB Kan	150 µl competent cell + diluted DNA (pGPTV)	Growth present
5	LB Kan	150 µl competent cell + eluted vector DNA (pGPTV)	No growth

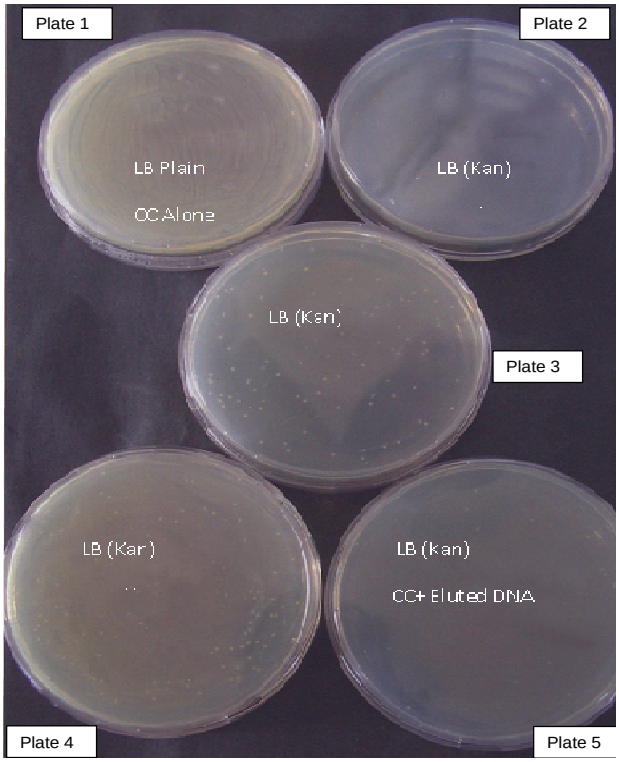

Figure 1. Transformation. Plate 1: Competent cell alone on LB plain plate. Plate 2: Competent cell alone on LB Kanamycin plate. Plate 3: Competent cell + ligated DNA (p GPTV with Annexin) on LB (Kan) plate. Plate 4: Competent cell + diluted DNA (p GPTV) on LB (Kan) plate. Plate 5: Competent cell + eluted Vector DNA (p GPTV) on LB (Kan) plate.

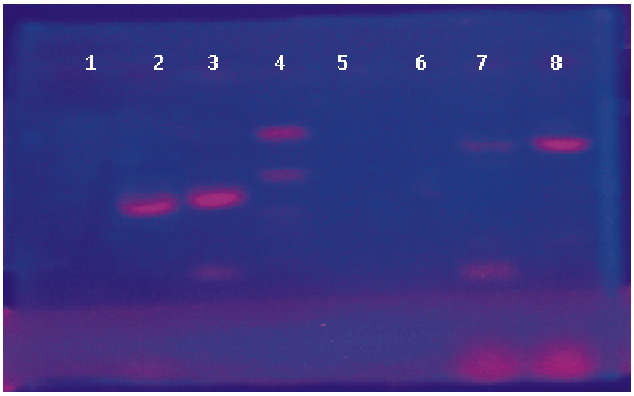

Figure 2. Screening of transformants. Lane 2: Undigested pUC119 with annexin gene. Lane 3: pUC119 with annexin gene digested with EcoRI and Xba1. Lane 4: λ DNA digested with HindIII. Lane 7: pGPTV with annexin gene digested with EcoR1 and XbaI. Lane 8: Undigested pGPTV

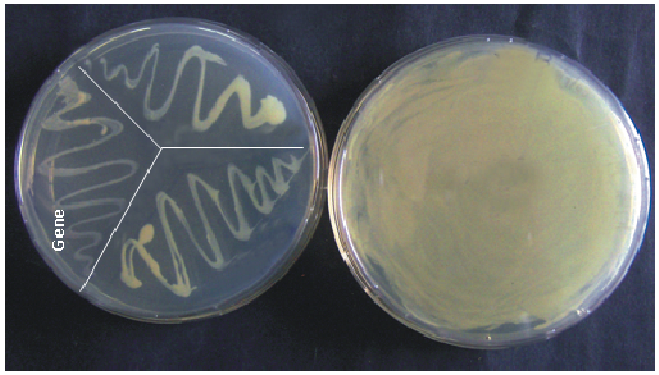

Figure 3. Triparental mating master plate.

confirming recombinant pGPTV/annexin plasmid in ***E. coli*** bacteria, the annexin gene construct was transferred into ***Agrobacterium tumefaciens*** MTCC 431 carrying disarmed Ti plasmid with the help of pRK 2013 plasmid in ***E. coli.*** The annexin gene construct (annexin +Npt II gene) was transferred into plant Genomic DNA by using leaf disc preparation, co-cultivation and selection media transfer. The selection media consists of kanamycin (to select transformed leaf sample), augmentin (to prevent excess multiplication of ***Agrobacterium*** and cefatoxime (to prevent growth of contaminated bacteria). Only those leaf disc containing gene construct survived on selection media and remaining were degraded. The survived leaf disc has produced callus. The leaf discs were then transferred to selection media and allowed to grow into calli and shoot initiation was observed. Some calli were found to degenerate and some produced shoots. The growing calli indicates the presence of our gene of interest. This was further confirmed by total extraction of genomic DNA and PCR analysis. The presence of transferred DNA in Tobacco plants was confirmed by Plant genomic DNA isolation followed by comparison with non-transgenic plants genome DNA samples, PCR analysis of plant genomic DNA, plasmid DNA and its combinations. The

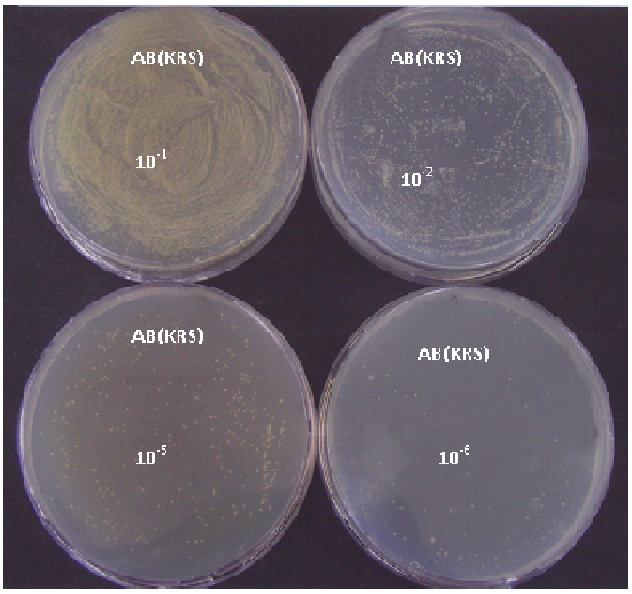

Figure 4. Triparental mating serial dilution.

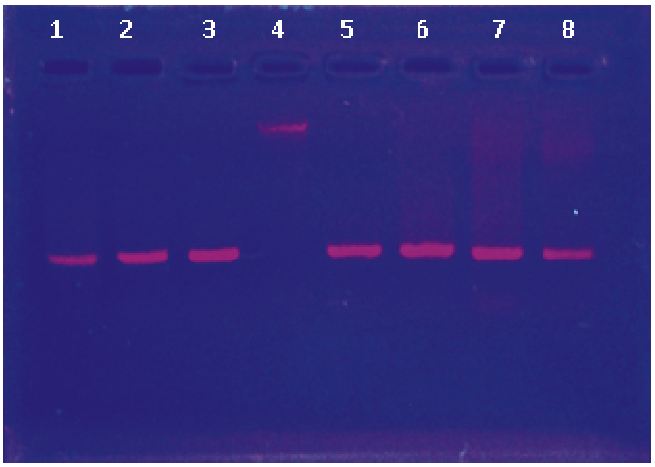

Figure 5. Profile of PCR amplified DNA. Lane 1: Genomic DNA sample 1. Lane 2: Genomic DNA sample 2. Lane 3: Genomic DNA sample 3. Lane 4: Genomic DNA from non-transformants. Lane 5: Genomic DNA sample 4. Lane 6: Genomic DNA sample 5. Lane 7: Genomic DNA sample 6. Lane 8: Genomic DNA sample 7.

results were interpreted based on the band thickness and clarity (Figure 5). Lane 1, 2, 3, 4, 5, 6, 7, 8 that were loaded with the amplified genomic DNA from callus showed the presence of amplified band that were very clearly visible. The npt II gene of 800 bp serves as selectable marker system in plants and its amplification confirmed the presence of Annexin gene in the recombinant plants. For the purpose of comparison the genomic DNA samples (non-amplified) were also loaded along with the amplified samples.

DISCUSSION

The present study was carried out in tobacco plant, ***N. tabacum*** because it is a plant that can grow quite easily in the sunny, hot and humid climates of ours. The plant can be easily maintained in both ***in vivo*** and ***in vitro*** conditions, due to its growth conditions and various factors. It was found that enriching the media with low sugars and low pH were both important factors in achieving efficient transformation in plants. The human cytochrome p450 (CYP) 2A6 was used to modify the alkaloid production of tobacco plants, cDNA is made and is transferred into ***N. tobaccum*** through ***Agrobacterium*** mediated transformation. Transgenic tobacco plants were produced that express an anti ***Salmonella enterica*** single chain variable fragment (ScFv) antibody that binds to lipopolysaccharide of ***S. enterica*** Paratyphi B was used in diagnosis and detection, as a therapeutic agent, and in applications such as water system purification.

In the present study, the leaf discs of ***in vivo*** grown tobacco plants was used for co cultivation with ***Agrobacterium*** and the transformed callus tissue was obtained by applying cefatoxin, kanamycin and augmentin which prevents the growth of other bacterial cells, prevent excessive growth of ***Agrobacterium*** and allows only the kanamycin resistant ones to grow. Plant transformation vectors and methodologies have been improved to increase the efficiency of plant transformation and to achieve stable expression of transgenes in plants. Due to simplicity of the transformation system and precise integration of transgenes, ***Agrobacterium*** Ti Plasmid – based vectors continue to offer the best system for plant transformation. Binary vectors have been improved by the incorporation of super virulent vir genes, matrix attachment regions (MAR) and the insertion of introns in marker genes and reporter genes. With these improvements and with the use of acetosyringone transformation in plants using ***Agrobacterium*** has almost become a routine process (Veluthambi et al., 2003). ***Agrobacterium***-mediated co-transformation, a very simple and elegant approach for marker elimination, was simultaneously evaluated in tobacco and rice. Using annexin genes through ***Agrobacterium*** mediated transformation we can produce drought, salt tolerance plants such as tobacco, rice, etc. (Kovacs et al., 1998).

From the result of the present study, it may be concluded that the ***Agrobacterium***-mediated transformation in tobacco was a successful method for transfer of annexin gene. The methodologies can also used for the commercial production of transformed tobacco with salt and drought tolerance.

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