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ISSN: 2320 – 7051
Int. J. Pure App. Biosci. SPI: **6 (1):** 47-55 (2018)

International Journal of Pure & Applied Bioscience

Research Article

OPEN ACCESS

Genetic Engineering of Tomato (*Lycopersicon esculentum*) Plant Using Annexin Gene through Agrobacterium Mediated Transformation

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Received: 7.05.2018 | Revised: 12.06.2018 | Accepted: 19.06.2018

ABSTRACT

Drought and high salinity are two major abiotic stresses that significantly affect agricultural crop productivity worldwide. Annexins are a multigene family that plays an essential role in plant stress responses and various cellular proceses. The present study involves the development of genetically engineered tomato, (Lycopercicum esculantum) plant with annexin gene. E. coli bacteria (resistant to kanamycin) carrying pGPTV/ annexin, E. coli carrying helper plasmid pRK2013 and A. tumefaciens (MTCC431 strain) carrying disarmed Ti plasmid (resistant to Streptomycin) were used for tri-parental mating,. The annexin gene construct was transferred into plant genomic DNA by using leaf disc prepraration, co- cultivation and selection media transfer. The selection media consists of Kenamycin, Augumentin and Cefatoxime. Leaf disc containing gene construct survives on selection media and were allowed to grow in the form of callus that is regenerated into transgenic plantlets, indicating the presence of gene of interest, which was further confirmed by extraction of genomic DNA and PCR analysis. Study revealed that Agrobacterium- mediated transformation in tomato was successful method for transfer of annexin gene. The methodology may be used for the commercial production of transformed tomato plant with salt and Drought tolerance character.

Key words: *A. tumefaciens, Lycopersicon esculentum, Annexin gene, E. coli DH5α, pUC119, pGPTV,*

INTRODUCTION

Annexins are a multigene, multifunctional family of Ca^{2+}- dependent membrane binding proteins found in both animal and plant cells, where they serve as important components of Ca^{2+} signaling pathways[24]. All annexins display similar properties calcium and phospholipids is due to a common primary structure. Each annexin is 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 define the annexin family.

Cite this article: Sharma, B.K. and Islam, M.S., Genetic Engineering of Tomato (*Lycopersicon Esculentum*) Plant Using Annexin Gene through Agrobacterium Mediated Transformation, *Int. J. Pure App. Biosci.* SPI: **6(1):** 47-55 (2018).

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 is extremely variable, albeit with few exceptions. The length varies from a few amino acids to more than 100. In addition the annexin N-terminal domain is considered as the regulatory region of the protein since it contains the major sites for phosphorylation, proteolysis or even interactions with other proteins. Member of the known anneixn subfamilies are encoded by paralogous genes with common genomic organization, regulatory properties and protein structural features. There are 160 distinctive annexin proteins which have been recognized in excess of 65 different species ranging from fungi and protists and higher vertebrates. Among high plants annexins have been identified in maize[1] and in pea[4]. 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[2]. Many reports have been shown annexin signalling to many different physiological processes in plants[9]. Annexin plays a major role in control in both biotic and abiotic stresses in plants[21]. Annexins are an evolutionarily conserved multigene family of calcium-dependent phospholipid binding proteins that play important roles in stress resistance and plant development. They have been relatively well characterized in model plants Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), but nothing has been reported in hexaploid bread wheat (*Triticum aestivum*) and barely (*Hordeum vulgare*), which are the two most economically important plants[35]. 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[19]. AnnSp2 gene was cloned from drought-resistant wild tomato (Solanum pennellii) and functionally characterized in cultivated tomato. AnnSp2 protein was localized in the nucleus and had higher expression in leave, flower and fruit. It was induced by several phytohormones and some abiotic stresses. Tomato plants overexpressing AnnSp2 had increased tolerance to drought and salt stress, as determined by analysis of various physiological parameters. AnnSp2-transgenic plants were less sensitive to ABA during the seed germination and seedling stages. However, under drought stress, the ABA content significantly increased in the AnnSp2-overexpressing plants, inducing stomatal closure and reducing water loss, which underlay the plants' enhanced stress tolerance. Furthermore, scavenging reactive oxygen species (ROS), higher total chlorophyll content, lower lipid peroxidation levels, increased peroxidase activities (including APX, CAT and SOD) and higher levels of proline were observed in AnnSp2-overexpressing plants. These results indicate that overexpression of AnnSp2 in transgenic tomato improves salt and drought tolerance through ABA synthesis and the elimination of ROS[16]. Several reports implicate a role for annexins specifically in salt and drought tolerance[10]. reported that an annexin is highly expressed in drought stressed seedlings of the drought-resistant rice (Oryza sativa) 'Nagina 22'; drought also affects annexin expression in loblolly pine[32]. Recently, Jami *et al*[17]. showed that ectopic expression of an annexin from Brassica juncea confers drought and salt tolerance to transgenic tobacco (Nicotiana tabacum) plants. Additional evidence that certain plant annexins can exhibit peroxidase activity in vitro was obtained for maize (Zea mays) annexins[22]. Different annexins include diverse enzymatic activities, such as peroxidase and ATPase/GTPase activity, as well as calcium channel activity[14,11]. Some subsequent reports focused on the roles of plant annexins involved in stress signaling[32,20,6]. Several reports indicate a role for annexin genes in the actions of plant hormones such as abscisic acid[34], gibberellic acid[23], Jasmonic acid[36], and salicylic acid[20]. Proline is an important osmolyte that stabilizes

macromolecules and membranes in cells exposed to osmotic stress[33,25]. Both drought and salinity can lead to oxidative stress in plant cells, in turn leading to the accumulation of osmoprotective solutes[25]. The present study was intended at the transfer of annexin gene in tomato (*Lycopercicum esculantum*) plant and verifies the transformation.

MATERIAL AND METHODS

The experiments were carried out from February 2007 – May 2007 at SBL, Bangalore. Annexin gene was obtained from Genbank. *Lycopersicon esculentum* was available at Shreedhar Bhat's Biotechnology Laboratory, Bangalore, India. Glycerol stocks of E. coli containing pUC119 with annexin gene and pGPTV were used. The plasmid DNA from E. coli was isolated by alkaline lysis method (Brinboim and Dolly, 1979). Plasmid DNA was eluted from gel by gel elution method and 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 by T_4 DNA ligase enzyme. Competent cells were prepared by calcium chloride method and recombinant pGPTV/ annexin were transferring into E. coli competent cells and spreaded on LB Kanamycin plate. The bacterial colonies were screened for recombinant pGPTV by using plasmid isolation and restriction digestion process. For tri-parental mating, E. coli bacteria (resistant to kanamycin) carrying pGPTV/ annexin, E. coli carrying helper plasmid pRK2013 and A. tumefaciens (MTCC431 strain) carrying disarmed Ti plasmid (resistant to Streptomycin) were used. All the three strains were mixed and inoculated on YEP plate and allowed to grow at 28°C for 16 h. After serial dilution, spreaded on AB-KRS plate (Antibiotics Kanamycin-50 µg/mL; Streptomycin-100 µg/mL) and selected colonies carrying Ti plasmid / annexin gene construct was used for transformation method.

Transformation of Ti plasmid / annexin gene into plant genomic DNA

The annexin gene construct was transferred from Ti plasmid to plant genomic DNA. In this method, the surface sterilized Tomato 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 Recombinant gene (Ti plasmid /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 200 µg/ml cefatoxime, 50 µg/ml kanamycin and 250 µg/ml augmentin and incubated for about 2½ weeks under 12 h photoperiod for the induction of callus.

PCR conformation test

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. 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 tomato callus was subjected to PCR amplification in order to confirm the presence of the annexin gene. The presence of annexin gene construct was confirmed by PCR reaction using isolated genomic DNA and primers specific to gene construct. Each 25 mL reaction mixture contained 1X PCR buffer,3.5mM MgCl2, 25pmol of each Forward primer (5'-TGAATGATCTGCAGGACGAGG-3'; 21 mer) and reverse primer (5'-CCAACGCTATGTCCCGATAGC-3'; 21 mer) with 0.2 mM dNTPs and 1 U of Taq DNA Polymerase (Bangalore Genei Private Ltd. Bangalore, India). The PCR conditions were as follows: initial denaturation at 95°C for 5 min, denaturation at 95°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 analyzed in 1.2%

agarose gel and stained with ethidium bromide and observed under UV light.

RESULTS

Plasmids pGPTV and pUC119/annexin (Fig. 1) were digested by using XbaI and EcoRI enzyme and cross checked by using 0.8% agarose gel (Fig. 2). pGPTV and annexin gene recuperate from agarose gel by ammonium acetate gel elution method (Fig. 3) and ligated by using T4 DNA ligase. The recombinant plasmid were transformed into DH5α competent cells (prepared by calcium chloride method) and spreaded on LB kanamycin plate containing 50 µg/mL concentration (Fig. 4). The basic strain such as DH5a were tested for contamination by growing on LB plain and LB Kan plates the results shown in Table 1. The bacterial colonies were screened for recombinant pGPTV/ annexin by plasmid isolation and restriction digestion. These transformed colonies, which were found to be positive for the presence of genes, were then used in tri-parental mating. In this process the three strains pGPTV/annexin, pRK2013 and Agrobacterium tumefaciens mixed and grow on YEP plate. The colonies from YEP plate were plated on kanamycin, refampicin, streptomycin plate and after overnight incubation of bacterial colonies further confirmation of the gene of interest was carried out. After confirming recombinant pGPTV / annexin plasmid in E. coli bacteria, the annexin gene construct was transferred into Agrobacterium tumefaciens carrying Ti plasmid with the help of pRK2013 plasmid in E. coli (Fig. 5). The annexin gene construct 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). The survived leaf disc has formed callus because only those leaf disc containing gene construct survived on selection media and remaining were degraded. The leaf discs were then transferred to selection media and allowed to grow into calli. 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 (Fig. 6 and Fig. 7).

Table 1: Results of DH5a (*E. coli*) grown on LB plain and LB Kan plates

No.	Media	Inoculum	Observation
1	LB Plain	150 µl competent cells	Lawn type of growth
2	Lb Kan	150 µl competent cells	No growth
3	Lb Kan	150 µl competent cells + diluted pGPTV	Growth present
4	Lb Kan	150µl competent cells + eluted pGPTV	No growth
5	Lb Kan	150 µl competent cell +ligated DNA (pGPTV / Annexin)	Growth present

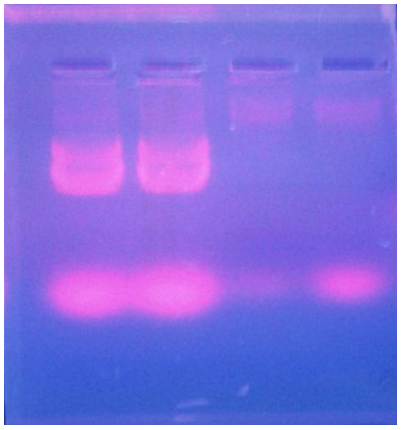

Figure 1: Isolated pGPTV and pUC119/ annexin

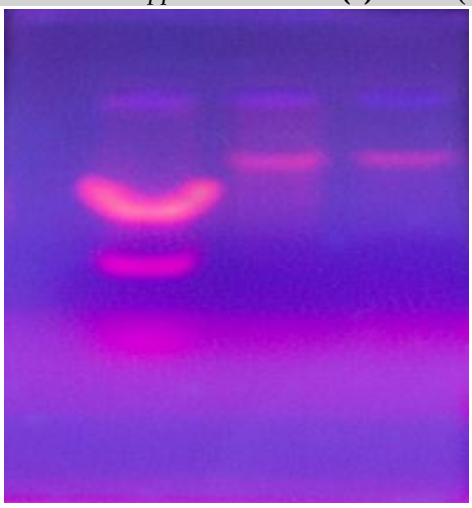

Figure 2: XbaI and EcoRI digested pGPTV and pUC119/ annexin

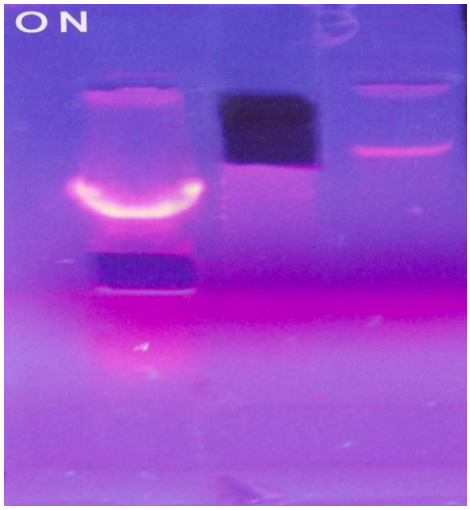

Figure 3: Recuperate pGPTV and annexin gene from agarose gel

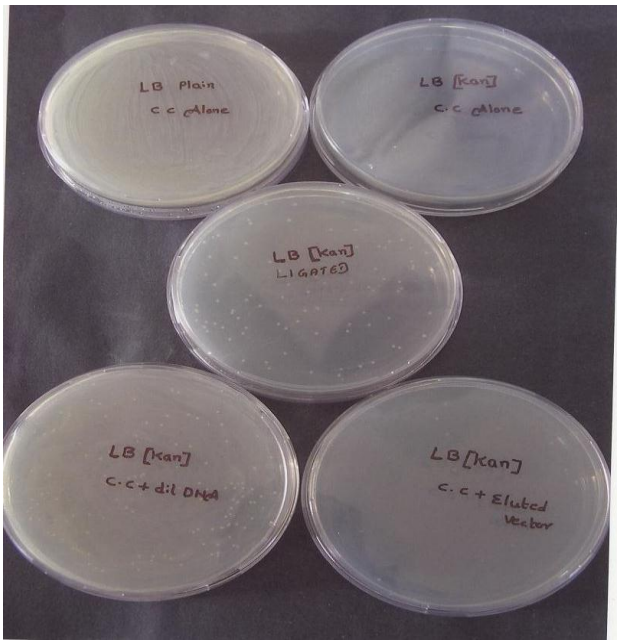

Figure 4: Transformation. Plate 1: Competent cell alone on LB plain plate. Plate 2: Competent cell alone on LB Kanamycin plate. Plate 3: Competent cell + ligated DNA (p GPTV with Annexin) on LB (Kan) plate. Plate 4: Competent cell + diluted DNA (p GPTV) on LB (Kan) plate. Plate 5: Competent cell + eluted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

Figure 5: Triparental mating master plate

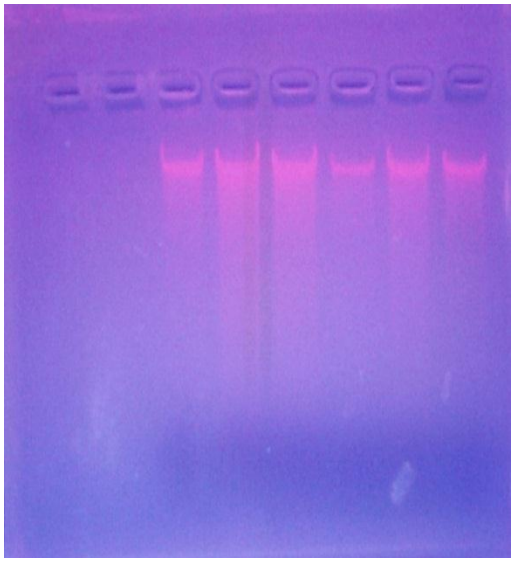

Figure 6: Genomic DNA of Transformed Callus

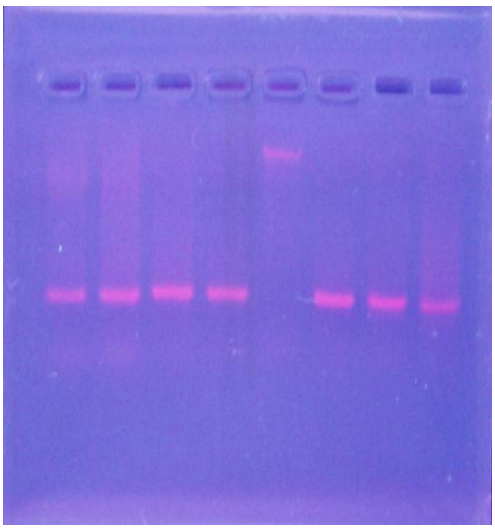

Figure 7: Profile of PCR amplified DNA.
Lane 1, 2,3,4,6,7 and 8 (from left to right):
Amplified PCR product of Callus Genomic
DNA. Lane5: Callus Genomic DNA of non- tranformant sample

DISCUSSION

Several different direct DNA transfer methods for plants have been described, including particle bombardment[26,5], microinjection[7] transformation of protoplasts mediated by polyethylene glycol or calcium phosphate[28], electroporation[31,13] and transformation using silicon carbide whiskers[8]. *A. tumefaciens* mediated transfer of DNA has been used for the production of transgenic tomatoes expressing a variety of heterologous proteins like the beta amyloid protein for vaccine against Alzheimer`s disease and human Factor IX. The transgenic plants obtained in this study will further have to be subjected to field trials to prove their efficacy. The use of *A. tumefaciens* is less labour intensive, does not require sophisticated equipment and is more cost effective. *Agrobacterium tumefaciens* is seen as such a useful gene delivery system because it is able to carry any gene of interest within the T-complex, and insert the gene into the target plants DNA with a high degree of success. High efficiency of ligation was seen with vector with insert vector ratio of 1:2. A number of economically important cereals have now been transformed using A. tumefaciens[27], working alongside other, more traditional gene transfer methods. One of the main reasons for favouring transformation by A. tumefaciens is that it allows delivery of a well-defined piece of DNA into the plant genome, although the success rate is not 100%[12]. Several different plant species have already been successfully transformed, including Lettuce, Rice[15] and Tomato[30]. This proves that direct gene transfer methods are no longer the only avenue of approach for transforming important crop plants[27]. Recent studies such as Broothaerts *et al*.[3], have shown that non agrobacterium species-Rhizobium sp. NGR234, Sinorhizobium meliloti and Mesorhizobium loti are capable of genetically transforming different plant tissues and plant species but their advantage over agrobacterium is not yet confirmed and agrobacterium remains the most favoured tool for plant genetic engineering. Thus, we attempted transfer of Annexin gene through agrobacterium in tomato. Transgenic tomato plants have been 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[29]. GhAnn1, which encodes a putative annexin protein, was isolated from a cotton (Gossypium hirsutum L. acc 7235) cDNA library. Tissue-specific expression showed that GhAnn1 is expressed at differential levels in all tissues examined and strongly induced by various phytohormones and abiotic stress[37]. *MYB49*-overexpressing tomato plants showed significant resistance to *Phytophthora infestans*and tolerance to drought and salt stresses. This finding reveals the potential application of tomato *MYB49* in future molecular breeding[18]. We utilized in vivo grown tomato plants 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 and excessive growth of agrobacterium and allows only the kanamycin resistant ones to grow. From the result of the present study, it may be concluded that the Agrobacterium-mediated transformation in tomato was a successful method for transfer of annexin gene. The methodologies can also be used for the commercial production of transformed tomato with salt and drought tolerance.

Acknowledgements

The authors express thanks to Shreedhar G. Bhat, Director, Shreedhar Bhat's Laboratory, Bangalore for providing facilities to carry out the research work and suggestions.

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