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GENETIC MANIPULATION OF TOMATO PLANT WITH ANNEXIN GENE THROUGH *AGROBACTERIUM* MEDIATED TRANSFORMATION

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ABSTRACT
The present study of genetic manipulation by incorporation of annexin gene has been attempted in tomato plant, *Lycopercicum esculantum* through *Agrobacterium tumefaciens*. 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, augmentin *and* cefatoxime. Leaf disc containing gene construct survives on selection media and were allow growing in calli, 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 a successful method for transfer of annexin gene. The methodologies may be used for the commercial production of transformed tomato with salt and drought tolerance.

INTRODUCTION

Annexins comprise a relatively large family of calcium and phospholipid binding proteins, which provide an exemplary model for studying the prototype and process of molecular evolution in multigene families. Annexins are defined structurally by a conserved C-terminal region that contains four or eight repeating units of about 70 amino acids each. There are 160 distinctive annexin proteins which have been recognized in excess of 65 different species ranging from fungi and protists and higher vertebrates. There are at least 10 distinct annexins in vertebrates (Annexins I-VIII, XI and XIII), and additional members of this family have been identified in Drosophila (annexins IX and X) and Hydra (annexin XII). Annexins have been identified in other lower organisms, such as slime molds (annexin VII), sponges (annexin I) and sea urchins (annexin VI). Among high plants annexins have been identified in maize (Blackbourn *et al*., 1992) and in pea (Clark *et al*., 1992). 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) 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).

Many reports have been shown annexin signalling to many different physiological processes in plants (Greg *et al*., 2001). Annexin plays a major role in control in both biotic and abiotic stresses in plants (Lee *et al*., 2004). 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). 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). The present study was intended at the transfer of annexin gene in tomato (*Lycopercicum esculantum*) plant and verifies the transformation.

MATERIALS AND METHODS

Glycerol stocks of *E. coli* containing pGPTV and pUC119 with annexin gene 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. 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 triparental mating, *E. coli* bacteria (resistant to kanamycin) carrying pGPTV/ annexin, *E. coli* carrying helper plasmid pRK2013 and *A. tumefaciens* 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 25°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 gene of interest (annexin gene) was transferred from Ti plasmid to plant genomic DNA. In this method, the Tomato leaves were surface sterilized with Tween 20 and Mercuric chloride before preparation of leaf disc. The prepared leaf discs were placed on MS media for overnight under light. Then co-cultivated with *Agrobacterium tumefaciens* carrying Recombinant gene (Ti plasmid / annexin gene) were placed on regeneration medium containing 2.5 mg/L BAP and 0.1 mg/L IAA. The co-cultivated leaf discs were incubated in the regeneration medium for about 2 days under dark condition to shun photosynthesis. The transformed leaf discs were selected from non- transformed by transferring them to selection media containing 250 μg/mL augmentin,50 μg/mL kanamycin and 200 μg/mL cefatoxime and incubated for about 1½ 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,3.5 mM $MgCl_2$, 25 pmol 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 analysed in 1.2% agarose gel and stained with ethidium bromide and observed under UV light.

RESULTS

Plasmids pGPTV and pUC119annexin (Fig. 1) were digested with 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 DH5± competent cells (prepared by calcium chloride method) and spreaded on LB kanamycin plate containing 50 μg/mL concentration. 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 XbaI and EcoRI and subjected to agarose gel electrophoresis. Linear shaped bands were observed. Both the vector pGPTV and the insert (annexin) bands in agarose gel were eluted and purified by gel elution. These transformed colonies, which were found to be positive for the presence of genes, were then

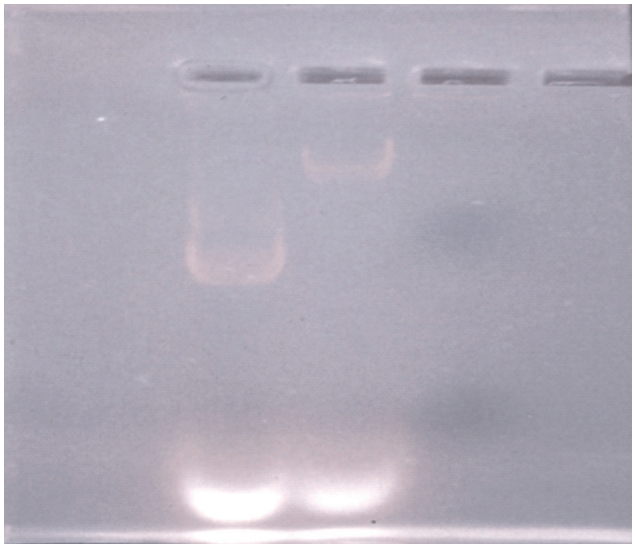

Figure 1: Isolated pGPTV and pUC119/ annexin

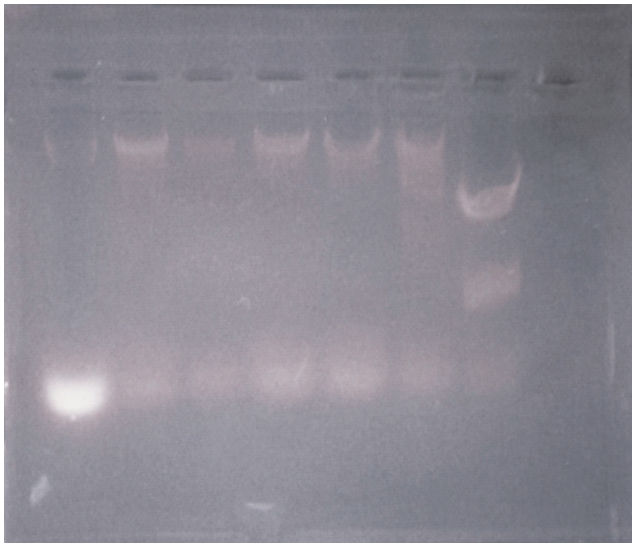

Figure 2: XbaI and EcoRI digested pGPTV and pUC119/ annexin

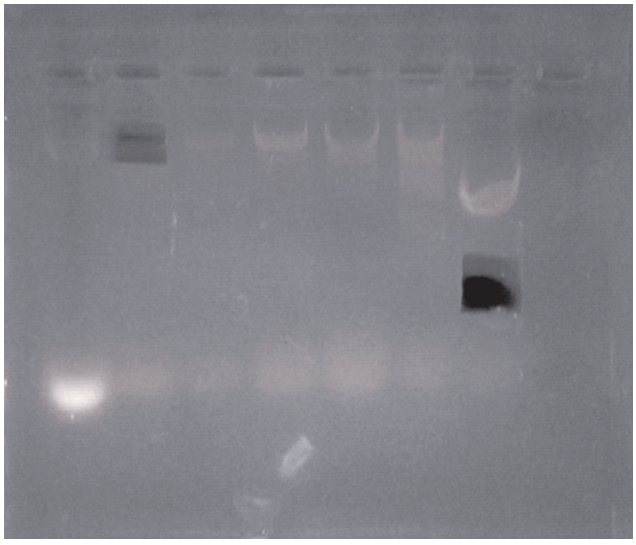

Figure 3: Recuperate pGPTV and annexin gene from agarose gel

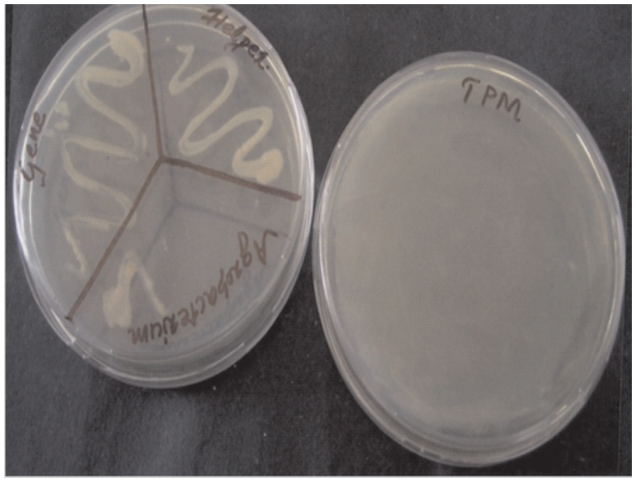

Figure 4: Triparental mating master plate

used in triparental 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 pRK 2013 plasmid in *E. coli* (Fig. 4). 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

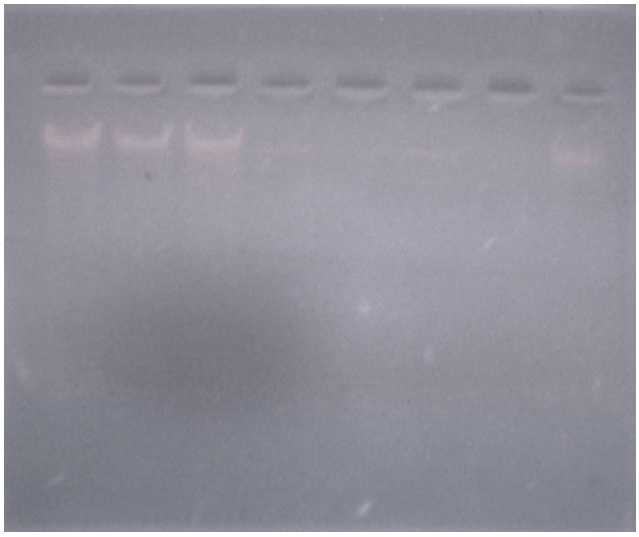

Figure 5: Genomic DNA of Transformed Callus

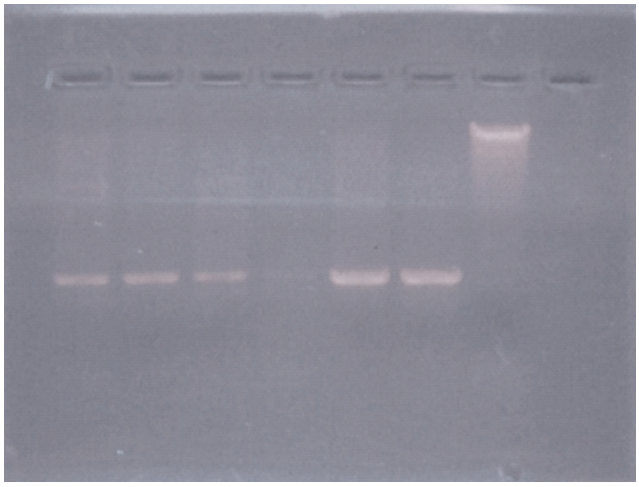

Figure 6: Profile of PCR amplified DNA. Lane 1, 2,3,4,5 and 6 (from left to right): Amplified PCR product of Callus Genomic DNA. Lane 7: Callus Genomic DNA sample

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. 5 and Fig. 6).

DISCUSSION

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. A number of economically important cereals have now been transformed using *A. tumefaciens* (Newell, 2000), 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% (Gheysen *et al*., 1998). Several different plant species have already been successfully transformed, including Lettuce (Curtis, 1995), Rice (Hiei *et al.,* 1997) and Tomato (Tzfira *et al*., 2002). This proves that direct

gene transfer methods are no longer the only avenue of approach for transforming important crop plants (Newell, 2000). Recent studies such as Broothaerts *et al.,* (2005) 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 (Richard *et al*., 2007). 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.

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