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Effect of Age of Explant on Transgenic Cotton (*Gossypium*) Plant Due to Expression of Mannose-Binding Lectin Gene from *Allium sativum*

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Abstract: Cotton is the most important textile plant in the world and is one of the most important crops for the production of oilseed. Because of its worldwide economic importance, new cultivars are constantly being released in the world. Although great improvements have been achieved through traditional breeding methods, cotton breeders are facing many problems, i.e., narrow genetic base, inability to use alien genes and difficulty in breaking gene linkages. Genetic transformations analyses are main tools used by breeders to overcome these problems. The aim of the study reported in this paper is to determine the effect of age of explant on regeneration response of apical shoot for tissue culture and gene transfer systems of cotton. This enabled us evaluate it effects on cotton transformation. The age of explants was observed to have significant effect on shoot tip elongation. The elongation rates of the three varieties studied were not significantly different from each other ($p = 0.1573$) and was observed to be affected by the size of isolated tips. It was observed that if the starting size of the apex was less than 1mm, the tips would not grow at all. Insecticidal lectin gene from *Allium sativum* was transferred into the transgenic cotton plants via *Agrobacterium*-mediated transformation using shoot apices as explants. Putative transgenic plants were confirmed by leaf GUS assay, kanamycin leaf test and molecular analysis of plantlet.

Key words: *Allium sativum*, Blec 1, cotton regeneration, explant regeneration, insecticidal lectin, transgenic plant

INTRODUCTION

Genetic engineering offers a directed method of plant breeding that selectively targets one or a few traits for introduction into the crop plant. The development and commercial release of transgenic cotton plants relies exclusively on two basic requirements. The first one is a method that can transfer a gene or genes into the cotton genome and govern its expression in the progeny. The two main gene delivery systems for achieving this end are *Agrobacterium* - mediated transformation and particle gun bombardment. The other requirement is the ability to regenerate fertile plants from transformed cells. This is achieved by regenerating plants via somatic embryogenesis or from shoot meristems.

Plant tissue culture is an important tool in both basic and applied studies. It is founded upon the research of Haberlandt, a German plant physiologist, who in 1902 introduced the concept of totipotency. That all living cells containing a normal complement of chromosomes should be capable of regenerating the entire plant.

Although regeneration efficiency has been improved via somatic embryogenesis or organogenesis, genotype dependent regeneration, prolonged culture period, high frequency of abnormal embryo into plantlets, lack of shoot elongation, difficulties of rooting and browning which causes dealth of tissues are the problems associated with cotton tissue culture systems (Kumari *et al*., 2003). Some of these problems are related to the plant materials such as explant age or genotype and others to the culture conditions such as hormones, medium composition, or other physical culture conditions (Ikram-ul-haq, 2004). Some studies have shown that explant characteristics such as type, source, genotype and history affect the success and commercial viability of tissue culture systems (Bhau and Waklu, 2001; Chan and Chang, 2002; Hoy *et al*., 2003). Plants regenerated from shoot apices are true to phenotype with low incidence of Somaclonal variation and chromosomal abnormalities (Bajaj, 1998). On the other hand, development of transgenics via somatic embryogenesis requires 6-12 months to obtain mature transgenic plants and an addition of 6-10 years are

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necessary to backcross the added value traits into the desired agronomic lines (Bajaj, 1998).

In this study, the effect of age of explant on the regeneration of apical shoot was monitored to determine the most effective age for regeneration in cotton tissue culture as well as the most suitable age of explant for gene transfer.

MATERIALS AND METHODS

Allium sativum Bulbs and three improved Cotton (*Gossypium* sp.) SAMCOT 9, 11 and 13 were collected from Institute for Agricultural Research Ahmadu Bello University Zaria, Kaduna-State.

Experimental design: All analysis was done at the Plant Breeding Division of Vegetable and Ornamental Plant Institute, Agriculture Research Council, Roodeplaat, Pretoria, South African September 2010. All experiments were conducted as a Randomized Complete Block Design (RCBD) with three or four replications.

Method generation of plant expression cassette plasmid construction: Inseclec gene was inserted into plant expression cassette, comprising of CaMV 35S promoter and nos terminator by excising the gene from pGEMT Easy vector using restriction endonuclease reaction and cloning it in between CaMV 35S promoter and nos terminator of intermediary vector. The recombinant vector was then ligated into *E. coli* HB101 cells and mobilized into *Agrobacterium tumefaciens* LB4404 by triparental mating using PBK 2013 as helper vector. The resulting co-integrate vector was used for transformation

Triparental mating: The cells were sedimented in a bench centrifuge for 10 min and the supernant carefully pour off. 10 mL LB broth was added to resuspend the cells with gentle vortexing. The following test tubes were then set up using 0.2 mL of the appropriate strain;

Tube	Strain	
A	1 (*Agrobacterium)*	
B	2 (PRK 2013)	
C	3 (Lectin gene)	
D	1+2	(MIX GENTLY!)
E	1+3	
F	2+3	
G	1+2+3	

0.2 mL from each tube was spread onto prelabelled LB plates (no drug selection) and incubate overnight at 30ºC upside down. Selection on minimal T+kan plates for *Agrobacterium* LBA4404 containing lectin gene was done followed by further streaking from single colonies to help separate *Agrobacterium* from any *E. coli* cells that survive by cross feeding since pRK2013 is unstable in *Agrobaterium.*

A loopful of bacteria from LB plate was streak onto a Minimal T, Kan 50, Step 500 plate and incubate for three days at 30ºC. on day 3 a single colony from plate G (Day 2) onto was restreak on two minimalT plates and repeated two times to ensure purity. On day ake a loopful of *Agrobacterium* from plate G (Day 4) was inoculated into 10 mL LB broth containing kanamycin (25 μg/mL) and incubated at 30ºC in orbital incubator.

Preparation of explant materials: Cotton varieties SAMCOT-9, 11 and 13 were used in this study. The seeds were surface sterilized by a series of step including; soaking of seeds in tap water for 1 h before been treated with 40% hydrogen peroxide for 30 min. The seeds were then rinsed three times with double-distilled water. They were then treated with a 50% Clorox® (5.25% NaOCl) solution on a rotary shaker at 50 rpm for 30 min changing the Clorox every 10 min and rinsed at least three times with sterile double-distilled water. The seeds were left in the final rinse water overnight on a rotor shaker at 100 rpm. After removing the seed coat, the seeds were placed on seed germination medium.

The seed germination medium contained 4.3 g Murashige and Skoog (MS) salts (Sigma, Product No. M2909) (Murashige and Skoog, 1962) per L, plus 3% sucrose and 0.8% agar (Sigma, USA). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121ºC for 20 min. Three seeds were placed in each germination bottle. The seeds were incubated in the dark at 28ºC overnight and then in the light for 5 days. Upon removal from incubation, the number of elongated shoots were counted. Contamination was determined by visual inspection for fungal and/or bacterial growth.

Shoot apices were isolated from 3 to 11 days old seedlings with the aid of a dissecting microscope. The seedling apex was exposed by pushing down on one cotyledon until it broke away, exposing the seedling shoot apex. The apex was removed just below the attachment of the largest unexpanded leaf. Additional tissue was removed to expose the base of the shoot apex. The unexpanded primordial leaves were left in place to supply hormones and other growth factors. The isolated shoot apex was then placed on shoot elongation and rooting medium.

RESULTS

Isolation and characterization of trans gene from garlic: A gene encoding the mannose-binding insecticidal lectin was cloned from *Allium sativum*. A putative

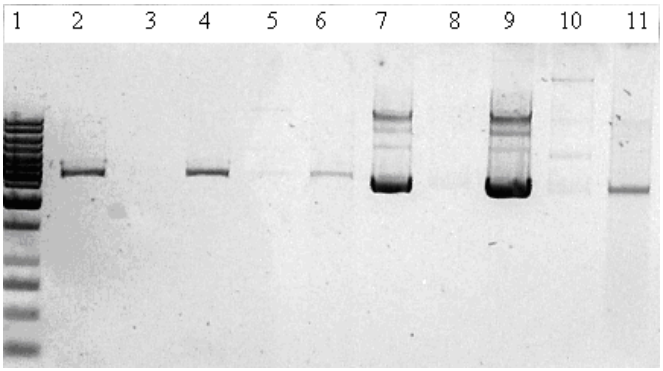

Fig. 1: Electrophoregram of genomic DNA from the result of the triparental mating. It was observed that gene was successfully inserted into the *Agrobacterium.* Lane 7 and 9

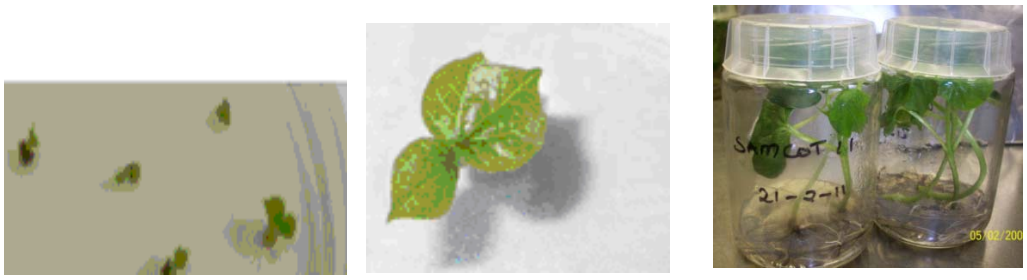

Fig. 2: Isolated shoot apices growing on elongation medium after two weeks. A: shoot tip growing on petri dish. B: close up of elongated shoot tip. C: Regenerated plantlet

Table 1: Percentage numbers of explants elongated on elongation medium from 3 Cotton varieties at 4 different ages %age of Explant

Cotton variety	5 Days	7 Days	9 Days	11 Days
Samcot 9	3.60	8.43	9.55	9.99
Samcot 11	4.33	8.89	9.32	10.99
Samcot 13	4.67	8.88	9.55	9.99

conserved domain in the deduced amino acid sequence was detected and the blast program predicts the gene is a bulb-type mannose-binding lectin (b-lectin). The full-length cDNA was 362 bp, and had a 327 bp Open Reading Frame (ORF) encoding 108 aa. Multiple alignments of the trans gene amino acids with those of other MMBLs revealed four highly conserved domains for sugar-binding [Gln (Q), Asp (D), Asn (N) and Tyr (Y)] namely QDNY. The "BLEC 1" gene was engineered into the expression vector pCAMBIA 2301 and then into *Agrobacterium tumefecien* LBA 4404 by triparental mating (Fig. 1).

The age of explants has a significant effect on shoot tip elongation (Table 1). On average, 42.5 % of shoot tips from 5 day-old explants had elongated; 85.5% of shoot tips from 7 day-old had elongated; 94.7% of shoot tips from 9 day-old explants had elongated and 99.2% of shoot tips from 11 day-old explants have elongated. The elongation rates between 9 days of age and 11 days of age were not significantly different. The elongation rates of the three varieties were not significantly different from each other ($p = 0.1573$) (Table 1).

The isolated shoot tips (Fig. 2a, b) began to grow in one week. The elongation rate was also affected by the size of isolated tips. It was observed that if the starting size of the apex was less than 1mm, the tips would not grow at all.

DISCUSSION

A gene encoding a novel monocot mannose-biding lectin from *Allium sativum* bulb as been cloned. The gene encoded a protein with high similarity with many MMBLs. The comparison shows single base pair mutation with the exception of threonine replacement in motif II which is due to two base pair mutation. The mutation are also mainly transitional, hence are easier than the transversion mutation. This means that the mannose-binding motifs are well protected and are important for Allium sativum. Since the agglutinating properties of lectin are likely to be responsible for insecticidal properties of lectin, it will be logical to conclude that the trans gene "BLEC" would have the ability to induce insecticidal effect if successfully transformed into other plants.

Since the development of an efficient transformation system is an important tool for gene manipulation. In this

research, we optimized a shoot apex based *Agrobacterium* mediated transformation system. The overall transformation rate was 1.3%, which is higher than that of Zapata *et al.* (1999) (0.8%). It is possible that the slightly higher transformation rate achieved in this study was also due to the slicing of the shoot apex prior to the co-cultivation step and bearing the fact that the varieties used were already improve from the Institute of Agricultural Research, Ahmadu Bello University, Zaria, Nigeria. The plants obtained by the present procedure were phenotypically normal, and in contrast to an embryogenesis-based transformation system, which takes one year or more to obtain fertile plants, we obtained transgenic plants in 5-6 months. The effect of age on explant indicates that the elongation of shoot tips on elongation medium was not genotype-dependent. This may be because there was too much leaf tissue removed and/or the tips themselves were damaged. Shoot tips sizes between 1.0 to 1.5 mm had a greater chance of surviving under experimental conditions. It was also observed that some tips with small size grew into callus; this may be because the kinetin was used in the medium to promote cell division and aid in growth. No multi shoot formation was observed in this experiment. It may be because of apical dominance.

CONCLUSION

To achieve a successful gene transfer technique, the development of a standard cotton regeneration procedure is definitely inevitable. Several works have been reported on the development of a regeneration technique for cotton. Theoretically, each excised apex should develop into a rooted plant; however, the yield of shoots *in vitro* from isolated apices depends on the incidence of contamination and rooting efficiency (Gould *et al*., 1991). In this report, seed sterilization with hydrogen perioxide and clorox greatly lowered contamination. Regeneration was carried using apical shoot of different ages. The shoot elongation was observed to be dependant of age and length of the explant rather than genotype. Cotton plants rooted in MS medium without hormones within a period of 6 weeks. Insecticidal lectin gene was successfully transferred into the regenerated plantlet by *Agrobacterium*-mediated transformation.

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