Expression of Mannose-Binding Insecticidal Lectin Gene In Transgenic Cotton (Gossypium) Plant

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Abstract Cotton (Gossypium spp) is an important world crop. Despite the efforts made through traditional breeding methods, cotton breeders still faced with many problems, i.e., narrow genetic base, inability to use alien genes and difficulty in breaking gene linkages. Breeders attempted genetic transformations analyses tools to overcome these problems with very little success, hence the need for transgenic intervention. In this report, an optimized cotton regeneration system from shoot apices used to transform cotton wit insecticidal lectin gene from Allium sativum.

Background

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.

Cotton (Gossypium) is an important cash crop, a major agricultural and industrial crop in Nigeria, providing employment and means of livelihood to about 2 million Nigerian families. A total of 24 states of the Federation produce cotton namely: Katsina, Zamfara, Gombe, Kaduna, Kano, Sokoto, Kebbi, Niger, Plateau, Jigawa, Yobe, Bauchi, Borno, Adamawa, Kwara, Taraba, Nasarawa, Kogi, Benue, Ekiti, Oyo, Ondo, Osun and Ogun. The average annual production is about 250,000 metric tonnes against a total world production of 20.5 million metric tones. Depending on the season of aphid attack on a cotton field the yield loss may be between 10-80%. The economic effect of which may be deterrent to a cotton producing nation. Transgenic attempt to address this problem has been...
encouraging recently. MMBLs have been reported to have strong insecticidal properties, hence effective delivery of the MMBL gene into the cotton seed may lead to a decrease in yield loss of crop due to sap-sucking insect pest. It has been reported that when the gene for monocot mannose-binding ASAL is expressed, mustard can partially withstand aphid attack (Dutta et al 2005a). There are obvious bioactivity and resistance as plant defense proteins to insects and/or nematodes in different Monocot mannose-binding lectins (MMBL).

In this study, a gene encoding the mannose-binding insecticidal lectin was cloned from *Allium sativum* (garlic) bulb and transgenic cotton plants were obtained via *Agrobacterium*-mediated transformation using shoot apices as explants. The study was based on the report that characterization and cloning of more genes with the super family of MMBL will be helpful for extending gene resources used in genetic engineering for development of insect-resistant transgenic crop plants and for more understanding of plant agglutinins with diverse functions.

1 Results and Analysis

1.1 Preliminary test

At day 14, there was almost no aphid on the *A. sativum*. In contrast, numerous aphids were observed on the bolts of each cotton plant. Both *A. sativum* and cotton were still healthy at this stage. However, accumulation of anthocyanin (a purple color) was observed on the stem of cotton which is usually a sign of stress and senescence.

At day 21, there was almost no aphid on *A. sativum*.

In contrast, although not many aphids were observed on the cotton plant, the plants showed obvious senescence, such as yellow wilting on the leaf edges. However, the *A. sativum* was still healthy.

1.2 Seed surface disinfection

The results show that the method used was effective (number of contaminated seed is zero). From the germination results, germination was observed to be about 89%.

1.3 Effect of age of explant

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 2).

The isolated shoot tips (Figure 1) 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.

1.4 Root efficiency of three cotton varieties on ms medium

The number of rooted shoot tips was noted. The results are shown in Figure 1. The rooting efficiency (Figure 2) of the three varieties were from 36% to 47%. Samcot 9 had the highest rooting efficiency (47%).

### Table 1 Mean number of explants elongated on elongation medium from 3 cotton varieties at 4 different ages

| Cotton variety | 5 days | 7 days | 9 days | 11 days | Mean |
|---------------|--------|--------|--------|---------|------|
| Samcot 9      | 11.00±2.00++ | 25.33±2.08 | 28.67±0.57 | 30±0.0 | 23.75a |
| Samcot 11     | 13.33±3.06 | 26.70±0.57 | 28.00±1.0 29 | 33±0.57 | 24.33a |
| Samcot 13     | 14.67±3.21 | 26.67±2.08 | 28.67±0.57 | 30±0.0 | 25.00a |
| Mean          | 12.75±c+  | 25.75b | 28.41a | 29.75a |

Note: + different letter label significant at p=0.05 level using LSD method; ++ Mean ± Std.
### 1.5 Production of putative transgenic plants

Under kanamycin selection pressure, most of the shoots appeared to be bleached (Figure 4 B), and some of the shoots that were initially green bleached out gradually, leaving only a few green shoots (Figure 4 A). Rooting of the transformed shoot apices occurred when they were transferred from kanamycin selection medium to kanamycin free medium. Rooted plantlets were first transferred to Magenta boxes (Figure 4 C) for two weeks and were grown in a green house (figure 4 D). The morphological features of the transgenic plants did not differ from those of non-transgenic plants. Out of a total of 300 *Agrobacterium* treated shoot apices placed on kanamycin selection, four (0.9%) regenerated plants (T0), grew. In contrast, for the 80 apices not treated with *Agrobacterium*, all died on kanamycin selection. Rooting of the transformed shoot apices occurred when they were transferred from kanamycin selection medium no kanamycin free medium.
1.6 Confirmation of transformation event

1.6.1 Leaf gus assay

Histochemical staining revealed that the leaves of these transgenic A gene. Leaf samples from non-co-cultivated plants did not stain blue (Figure 5).

1.6.2 Kanamycin leaf-spotting test

The putative transgenic plants were tested using a kanamycin leaf-spotting test on the young leaves. Based on the primary experiment of kanamycin leaf test, the concentration of 2% was used in this experiment. Kanamycin solution (2%) plus 0.1 mg/L Tween 20 was painted to fully expand young leaves. Kanamycin resistance activity in the leaves was variable after one week. Leaves of non transgenic plants (control) turned mottle in one week, while leaves from putative transgenic plants did not have the symptom (Figure 6).

1.6.3 Molecular analysis of transformed cotton

Reverse transcription (RT) PCR using ORF F and R Primers shows that BLEC 1 has been expressed in the transgenic cotton at RNA level (Figure 7).

2 Discussion

To fully take advantage of gene transfer techniques, it is important to develop a reliable and efficient regeneration system for cotton. Cotton seeds from the field are highly contaminated as they contain large numbers of small hairs that can hold spores of fungi and bacteria. Delinting with H₂SO₄ is a highly effective way to remove the hairs and reduce the risk of contamination in the cultures. For any tissue culture study, the surface of explants must be fully sterilized. In previous research, different sterilization methods were used to sterilize delinted cotton seeds surface (Gould et al., 1991; Chen et al., 1987; Zhang, 1994). Delinted seeds were disinfected as described above and the disinfected seeds were then cultured on MS medium for 7 days. The number of visually contaminated seeds and the number of germinated seeds (shoot elongation) observed after 7 days. The reason for the results obtained may be that the residual of Clorox, specifically, chlorine, suppressed the germination of cotton seeds, while the residual of hydrogen peroxide is water and CO₂, which did not affect the germination of cotton seeds In recent years, there has been a focus in the development of regeneration systems through shoot apices. Regeneration from the shoot apex was direct and simple. 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 recent years, protocols involving proliferation of cotton shoots (Agrawal et al., 1997; Hemphill et al., 1998) have been published.
The rooting efficiency ranged from 38% to 58% in their reports. In this experiment, sterilizing seed surface with 40% hydrogen peroxide greatly lowered the chance of contamination. Removal of the seed coat also lowered contamination rates of this method. The regeneration was carried out without a callus phase. Cotton plants rooted in an MS medium without hormones for a period of 3 to 6 weeks, and they could be transferred directly to soil without further steps. Two weeks later they could be transferred to the greenhouse and all plants were fertile and grown to set seed. Efforts have been made to couple this regeneration procedure with Agrobacterium mediated transformation for rapid introduction of value-added traits directly into high-fiber-yielding cotton germplasm.

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. Pretreated shoot apices were co-cultivated with Agrobacterium at concentration of OD$_{600}$ 0.6 for 3 days with addition of 100 μM acetosyringone. Under 50 mg/L kanamycin selection pressure, a total of eight transgenic plants were recovered, by Agrobacterium LBA4404 transformation. The overall transformation rate was 1.2%, which is higher than that of Smith et al. (1997) and 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 mm to 1.5 mm had a greater chance of surviving under experimental conditions as shown in Figure 5. 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.

Agrobacterium strains play an important role in the transformation process, as they are responsible not only for infectivity but also for the efficiency of gene transfer. Acetosyringone is one of the phenolic compounds secreted by wounded plant tissue and is known to be a potent inducer of Agrobacterium vir genes (Stachel et al. 1985). Several reports suggest that acetosyringone pre-induction of Agrobacterium and/or inclusion of acetosyringone in the co-cultivation medium can enhance significantly Agrobacterium mediated transformation (Yao, 2002; Samuels, 2001; Sunikumar et al. 1999). In our experiments, acetosyringone was included at a final concentration of 100 μM during the final stage of Agrobacterium growth and during co-cultivation. The results suggest that acetosyringone can be used to obtain significant improvements in transformation of cotton. All of the other experiments were performed with acetosyringone treatment during the final stage of Agrobacterium growth and during cocultivation.

The data show that GUS expression rate was always lower in 1 day co-cultivation than 2 days co-cultivation at different Agrobacterium concentrations. Increasing the Agrobacterium concentration did not always increase the transformation rate. This may be because at high Agrobacterium concentration overgrowth of the bacterium occurs. The highest observed GUS positive rate was 38%, which occurred at OD$_{600}$ 0.6 and 3 days co-cultivation. These conditions were used in the transformation system.

3 Material and Method
3.1 Material
Cotton varieties SAMCOT-9, 11 and 13 used for in this study were obtained from Institute of Agricultural Research (IAR) Ahmadu Bello University, Zaria.

3.2 Preliminary analysis
A brief test was performed to see the choice of M. persicae between cotton and garlic. At day 0, one Amaranthus infested with M. persicae was placed
between three healthy cotton plants and one pot of *A. sativum* plant.

### 3.3 Preparation of explant materials

The seeds were surface sterilized by a series of steps including; soaking of seeds in tap water for 1 hour before being treated with 40% hydrogen peroxide for 30 minutes. 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 minutes changing the Clorox every 10 minute 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 liter, plus 3% sucrose and 0.8% agar (Sigma, USA). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 min.

Three seeds were placed in each germination bottle. The seeds were incubated in the dark at 28°C overnight and then in the light for 5 days. Upon removal from incubation, the number of elongated shoots was 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.

### 3.3 *Agrobacterium* co-cultivation and transgenic plants regeneration

The *Agrobacterium* strains were cultured in LB medium (contains 10 g/L Bacto Tryptone, Bacto, 5 g/L Yeast extract and 10 g/L NaCl). 20 mL of LB medium plus antibiotics (50 mg/L kanamycin) was inoculated with *Agrobacterium* and incubated in a 100 mL Erlenmeyer flask overnight (about 17 hours) on a shaker set for 150 r/min at 28°C. Then 2 mL of the overnight culture was withdrawn and used to inoculate 50 mL of LB medium without antibiotics. Acetosyringone was added to the culture at a final concentration of 100 μM. After incubation for 3 hours at 28°C with shaking, the cultures were diluted with additional LB medium (containing 100 μM acetosyringone) to a concentration (OD600 0.6) for transformation. Equal numbers of shoot apices were randomly distributed to two independent treatments, one with *Agrobacterium* co-cultivation and one without *Agrobacterium* co-cultivation. Shoot apices were inoculated by placing one drop of *Agrobacterium* solution onto each shoot apex in co-culture medium (MS+100 μM acetosyringone) and incubating at 28°C under dark conditions for approximately 2 days. After co-cultivation, explants were washed three times with sterile distilled water. Cleaned apices were blotted dry using a sterile paper towel and cultured on the selection medium consisting of MS with 400 mg/L timentin and 50 mL/L kanamycin. Shoot apices not inoculated with *Agrobacterium* were plated on the selection medium as a negative control. Timentin was included in the selection medium to suppress the *Agrobacterium* growth. The Petri dishes were incubated at a temperature of 28°C under an 18 hours photoperiod and sub-cultured every 3 weeks.

The process was repeated until the controls, that were not co-cultivated with *Agrobacterium*, were totally dead. After this period the surviving shoot apices were transferred to an MS medium without kanamycin to root the plants. Rooted plants were then transferred to soil and grown to maturity in a greenhouse.

### 3.4 Post-transformational Analysis

The histochemical assay for β-Glucuronidase (GUS) gene expression was performed by established methods (Jefferson, 1987; Kosugi et al., 1990). Following co-cultivation, apices were harvested for GUS staining. The apices were incubated overnight in a solution containing 25 mg/L X-gluc, 10 mM EDTA, 100 mM NaH₂PO₄, 0.1% Triton X–100 and 50%
methanol, pH 8.0) at 37°C. The number of apices that stained with blue spots was noted. Young leaves of putative transgenic plants were also collected for GUS staining to confirm the transformation event.

In the putative transgenic plants, expression of the transgene (NPT II) or lectin gene was analyzed by first establishing the lowest concentration of Kanamycin that would kill untransformed plants. Leaves of control plants were painted with a cotton swab when they had two totally opened true leaves using 0%, 0.1%, 1%, 2%, or 3% (W/V) of kanamycin controls was used to evaluate for resistance to kanamycin in the greenhouse. Plants were evaluated for resistance 7 days after leaf application of kanamycin.

For shoot elongation and root development, isolated shoot apices from the three different cotton varieties: SAMCOT-9, 11 and 13, were placed on MS medium+0.1 mg/L Kinetin (Gould et al., 1991) for two weeks to induce shoot elongation. The shoots were then transferred to MS medium for rooting. After three weeks, the number of rooted shoots was noted.

The rooted shoots were then transferred to Magenta boxes containing MS medium and incubated in a culture chamber (27°C) for four weeks and then transferred to the greenhouse. The number of rooted plants was noted and the rooted plants were transferred to Magenta boxes containing MS medium and incubated in a culture chamber for four weeks before being transferred to the greenhouse.

The pH of all medium was adjusted to 5.8 before autoclaving, and all medium were solidified with

3.5 Statistical analysis

The data were analyzed via Proc Mixed in SAS 9.0 (SAS Institute, Cary, NC).

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