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EFFECT OF ACETOSYRINGONE ON *Agrobacterium*-MEDIATED TRANSFORMATION OF COTTON

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ABSTRACT

Genetic transformation method using *Agrobacterium tumefaciens* was developed for cotton plant species. Following the efficient regeneration of three cotton varieties, the effect of inclusion of acetosyringone in co-cultivation medium was measured. Subsequently, transformation was obtained by co- cultivation of 3 weeks old cotton apical shoot and *A. tumefaciens* strain LBA 4404 containing a plasmid harboring neomycin phosphotransferase and β-glucuronidase encoding genes. PCR analyses performed to identify the presence of marker gene (npt II) in the transgenic plants using primers used for amplification of a 700 bp fragment of the npt II gene showed that 87% of the transgenic plants selected for kanamycin resistance were transformed with the gene encoding β-glucuronidase. Routine transformation efficiency of cotton was established at 1.3%. The mean number of GUS positive apices was 67% higher when acetosyringone was included in the medium.

Keywords: acetosyringone, *Agrobacterium tumefaciens*, mediated transformation, cotton.

INTRODUCTION

Transformation efficiency is influenced by several factors, including Agrobacterium strain, addition of phenolic compounds (e.g. acetosyringone) in the co-cultivation medium, wounding treatment of target tissue (Godwin *et al*., 1991; Norelli *et al*., 1996) and appropriate selection of transformed cells or tissue from majority of untransformed tissue. Inducers if the *Vir* genes include monocyclic phenolic compounds, such as acetosyringone and related acetophenones, syrigaldehyde and other benzaldehydes, sinapinic acid and other benzonic acids, and cinnamic acid derivatives (Ashby, *et al*., 1988; Bolton *et al*., 1986 and Stachel *et al*., 1985). Acetosyringone is a phenolic compound secreted by wounded plant tissue and is known to be a potent inducer of Agrobacterium vir genes (Stachel *et al*., 1985). In the presence of low concentration of acetosyringone, sugars such as glucose and galactose act synergistically to induce the vir genes (Cangelosi *et al*., 1990). Induction of the vir gene by acetosyringone is enhanced by opines (Veluthambi *et al*., 1989) and antagonized by a growth inhibitor from corn (Sahi *et al*., 1990). 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; Sunikumar *et al*., 1999). McCormac *et al*. (1998) used 100 µM of Acetosyringone and found that presence of Acetosyringone increase the efficiency of transformation. They reported a transformation efficiency of 46.15% and an efficiency of 6.66% in the absence of Acetosyringone. Ke *et al*. (2002) added 100µM Acetosyringone to inoculation and co-cultivation media and observed development of GUS loci within the shoot and root structures. Amoah *et al*. (2001) added Acetosyringone to final concentration of 200 µM and obtained increased number of explants producing blue spots. Here we report the effect of inclusion of 100uM Acetosyringone during the final stage of Agrobacterium growth and during co-cultivation with apical shoot of cotton explants used in Agrobacterium-mediated transformation of cotton.

METHOD

Transgenic plants regeneration

Transgenic cotton lines were prepared as described by Afolabi-Balogun *et al*., 2011.

Agrobacterium co-cultivation

The *Agrobacterium* strains were cultured in LB medium (contains 10g/L Bacto Tryptone, Bacto, 5g/L Yeast extract and 10g/L NaCl). 20 ml of LB medium plus antibiotics (50mg/L kanamycin) was inoculated with *Agrobacterium* and incubated in a 100ml Erlenmeyer flask overnight (about 17 hours) on a shaker set for 150 rpm at 28°C. Then 2ml of the overnight culture was withdrawn and used to inoculate 50ml of LB medium without antibiotics. Acetosyringone was added to the culture at a final concentration of 100 ìM. After incubation for 3 hours at 28°C with shaking, the cultures were diluted with additional LB medium (containing 100 ìM acetosyringone) to a concentration (OD600 0.6) for transformation. Different concentrations of acetosyringone (0, 20, 50 and 100µM) were used at the time of co-cultivation and in the co-cultivation plates. 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 ìM acetosyringone) and incubating at 28ºC 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 28ºC under an 18 hours photoperiod and sub-cultured every 3 weeks.

Control transformation was performed by omitting acetosyringone from every step. Thirty apical shoot were used in each treatment and the experiment replicated three times. The numbers of GUS positive apices were recorded after 3 days co-cultivation.

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.

Post-transformational analysis

The histochemical assay for β-Glucuronidase (GUS) gene expression was initially investigated 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 NaH_2PO_4, 0.1% Triton X-100 and 50% methanol, pH 8.0) at 37ºC. The number of apices that stained with blue spots was noted. Young leaves of putative transgenic plants were also collected for GUS staining and glucuronidase gene amplification using primer to confirm the transformation event.

In the putative transgenic plants, expression of the *lectin* gene was confirmed by PCR amplification of the gene using primers designed based on the insecticidal gene The pH of all medium was adjusted to 5.8 before autoclaving, and all medium were solidified with 8.0g/L agar (Sigma).

Statistical analysis

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

RESULT

Histochemical GUS assays indicated that transformation had occurred at specific zones, and each spot represented an independent transformation event. When the explants were transferred to selection medium immediately after inoculation with *Agrobacterium*, no transformation was observed. GUS activity was observed from the explants co-cultivated for 2 days. It was observed that explants in which co-cultivation for 4 to 5 days also showed GUS activity; the tissues were however adversely affected due to the overgrowth of bacteria.

The result in Table-1 shows that acetosyringone improved significantly the transformation efficiencies. The mean number of GUS positive apices was 67% higher when acetosyringe was included in the medium. This suggests that acetosyringone can be used to obtain significant improvements in transformation of cotton.

PCR assays of the transgenic plantlet using *npt* II coding sequences in DNA extracted from kanamycin resistant shoots expressing GUS activity, showed a 770 bp fragment amplicon in the case of putative transformants, confirming transformation. No amplification was obtained in non-transformed (control) plantlets (Figure-1).

Table-1. Transformation efficiency of wheat cultivars using mature embryos as explant source with different concentrations of acetosyringone.

Acetosyringone conc.	Total no. of explant	GUS positive leaf			Mean
0uM	30	12	14	16	13.0
20uM	30	14	18	15	15.67
50uM	30	18	22	20	20.0
100uM	30	22	24	24	23.3

AC = Acetosyringone concentration in Mm, TNE = total number of explants, CSGA = calli selected for GUS activity, PGA = percentage of GUS analysis, SEH = selected explants on hygromycin, GPC = GUS positive calli, TCSH = total calli for selection on hygromycin, and TE = transformation efficiency (%).

DISCUSSIONS

It is clear from the result that the co-cultivation time with *Agrobacterium* needed was 2 days to obtain efficient expression of GUS in cotton. In rice also it was reported that co-cultivation for 2 to 3 days with *Agrobacterium* was required to obtain efficient expression of GUS (Rashid *et al*., 1996). Although prolonged co-cultivation periods more than two days have been successfully used for certain plants (Mourgues *et al*., 1996), 2 to 3 days co-cultivation has been routinely used in most reported transformation protocols, since longer co-cultivation periods frequently result in *Agrobacterium* overgrowth (Cervera *et al*., 1998).

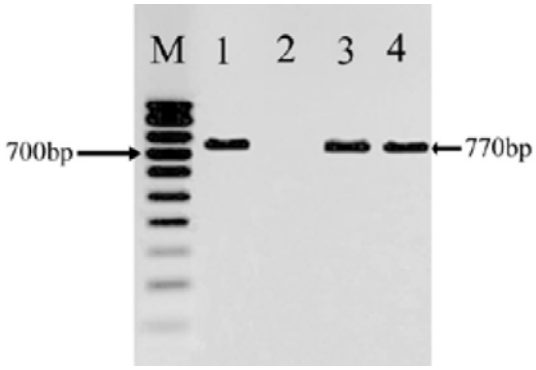

Figure-1. PCR analysis of transgenic plants for integration of the NPTII gene. Lanes: M 1Kb marker; Lane 1: Plasmid DNA (positive control); Lane 2: DNA sample from non-transgenic control plant; Lanes 3, 4: DNA samples from putative transgenic plants. Arrow shows the expected 770 bp product.

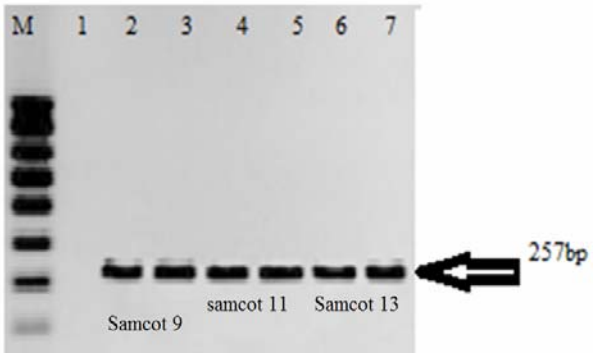

Figure-2. PCR analysis of transgenic plants for integration of the *BLEC1* gene. Lane: M marker; Lane 1: DNA sample from non-transgenic control plant; Lanes 2-7: DNA samples from putative transgenic plants. Arrow shows the expected 257bp product.

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