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SONICATION ASSISTED *AGROBACTERIUM* MEDIATED TRANSFORMATION (SAAT): AN ALTERNATIVE METHOD FOR COTTON TRANSFORMATION

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Abstract

We report here a new procedure for cotton transformation based on cavitations caused by sonication which results in thousands of micro wounds on and below the surface of plant tissue and allow *Agrobacterium* to travel deeper and completely throughout the tissue. This wounding fashion increases the probability of infecting plant cells lying deeper in tissue. Many parameters were optimized for the enhancement of GUS transient expression in cotton using mature embryos as explant. GUS was first detected 24h following incubation of the explants and by 48h, GUS expression was very intense which served as a useful indicator of successful transformation of the cotton explant following sonication assisted *Agrobacterium* mediated transformation (SAAT) procedure. The study also showed the competitive advantage of this procedure over other transformation procedures being routinely used.

Introduction

Cotton is grown on 2.5% of the arable land in five continents (Anon., 1991). Nearly, 50% of the total textile fibre consumption depends on cotton fibre (Anon., 1993). A rich genetic reservoir is available in wild and wild relatives of cotton but genetic improvement through interspecific hybridization is hampered by incompatibility barriers (Peeters *et al*., 1994). Genetic engineering can be used in a variety of ways to improve plants and can produce results that cannot be imagined using conventional breeding techniques. The availability of this technique to researchers to bring changes at the molecular level is an additional tool in the hands of breeders who have been limited by the available genetic material with which they could work.

Although several novel methods for the transformation of plants are available yet presently, methods based on the use of the soil bacterium *Agrobacterium tumefaciens* is still preferred in many instances. *Agrobacterium* is capable of transferring new genes to a wide variety of plant species but this can be limited by both host specificity and inability of the bacterium to reach the proper cells in the target tissue. Therefore, a new and efficient *Agrobacterium* based transformation method that overcomes these barriers and enhance DNA transfer is required (Trick & Finer, 1998). However, modifications have made in the method of wounding and the way tissues are infected.

Recently, sonication assisted *Agrobacterium* mediated transformation (SAAT) procedure has shown promising for the transformation of plants. This method has the potential for uniform transformation of meristematic tissues. Furthermore, the SAAT procedure produced a 100-1400 fold increase in gene expression in a variety of tissues

(Trick & Finer, 1997). The present study reports the transformation of meristematic tissue of local cotton cultivar using an integrated sonication and *Agrobacterium* system. This method combines the advantages of *Agrobacterium* with ability of sonication to generate thousands of micro wounds on and below the surface of the tissue, thus enhancing the movement of *Agrobacterium* deeper and more completely throughout the tissue.

Materials and Methods

Delinting of seeds: For delinting of seeds, concentrated commercial H_2SO_4 was used @ 100 ml Kg^{-1} of seeds. The seeds were continuously stirred with the help of spatula for 10-15 minutes until shiny surface of seeds appeared. Then some water was added and the stirring was continued for a few seconds. Seeds were washed 5 times with tap water to remove the acid completely. At that point the seeds, which floated at the surface of water, were discarded.

Seed sterilization: Seeds of local cotton variety CIM-446 were sterilized using autoclaved magenta boxes. After adding water, few drops of Tween 20 were added and the seeds were washed by giving vigorous shaking followed by three washings with autoclaved water. Surface sterilization was done by using 0.1% $HgCl_2$ for 20 minutes followed by 5 washings with autoclaved distilled water. The seeds were soaked in autoclaved distilled water for one hour. After that, the excess water was removed and the seeds were kept in the dark at 30°C for germination. All the work of sterilization was performed in a laminar airflow cabinet.

Preparation of explant: Delinted seeds were sterilized and soaked in sterile water overnight for germination. The embryos were isolated from the germinating seeds by carefully dissecting the seeds. Testa of the seeds was removed and cotyledonary leaves were excised. About 50 mature embryos were placed on MS medium (Murashige & Skoog, 1962) in the magenta boxes for each replication. Cultures were maintained on culture shelves at 28 ± 2°C using a photoperiod of 16-hour light/8 hour dark. The same procedure was followed for the other two replications.

1. *Agrobacterium* mediated transformation: Single colony of *Agrobacterium* strain LBA4404 (pBINGUSINT) or LBA4404 was cultured in LB (Luria-Bertani) broth at 28^0 C till the O.D. reached 1.0 at 696 nm. Bacterial suspension (10 ml) was centrifugated @ 3000 rpm for 5 minutes. The pellet was washed twice with MS (Murashique & Skoog, 1962) liquid medium, suspended in 10 ml MS liquid medium.

Isolated mature embryos were cultured with *Agrobacterium* suspension for 1 hour. Blotted dry on sterile filter paper and cultured on MS medium in 9 cm Petri plates.

2. Bombardment mediated transformation: Tungsten particles were prepared according to Finer & McMullen (1990) with some modifications. Twenty five µl of 2.5 M $CaCl_2$ followed by 10µl of 0.2 M spermidine were added to 50 ml of tungsten particles suspended in distilled water. After 5 minutes on ice, 50µl of the supernatant were removed. The pellet was resuspended using a vortex, immediately before use, and 20 µl were transferred to filter assembly. No DNA was added to the mixture, when bombardment followed inoculation with *Agrobacterium*. Following bombardment, the

explants were inoculated and incubated for one hour into the bacterial suspension. Inoculated explants were blotted on sterile filter paper and GUS activity was judged after 24 and 48 hours.

3. SAAT transformation: Isolated mature embryos were placed in polystyrene ultra clear centrifuge tube (Beckman) containing 2 ml MS broth. Tubes were placed in ice bucket during sonication. The sonicator was controlled by an electric timer (W-375 model, heat systems. Ultrasonics Inc. USA). After sonication, the mature embryos were immediately co-cultivated with *Agrobacterium* suspension for one hour. After co-cultivation, embryos were cultured on MS medium containing 250µg/ml cefotaxime to kill *Agrobacterium*. Control explants were also treated under same conditions except that *Agrobacterium* did not carry pBINGUSINT.

GUS Histochemical Assay: Histochemical staining of GUS activity was performed first 24h and then 48h after transfer of the explants to MS medium. Explants were placed in GUS assay mixture (Jefferson, 1987) and incubated overnight at 37^0C. GUS mixture was removed and tissues were rinsed twice with 70% ethanol. GUS activity was then determined by placing the tissues on a grid and estimating the %age of the tissue surface that showed blue foci under a dissecting microscope.

Results

The study was conducted with the idea in mind that micro-wounds caused by sonication and co-cultivation with *Agrobacterium* suspension would result in thorough transformation of whole tissue as described by Hooykaas *et al*., (1991) and Droste *et al*., (2000). These micro-wounds facilitate *Agrobacterium* attachment and gene transfer to the cells lying deep into the tissue due to chemical signals (Hooykaas *et al*., 1991). The "Super binary" vector used in this study contains a gus A-intron gene, certifying that any GUS activity detected should be from eukaryotic cells. For the selection of transformed tissues, T-DNA contains a kanamycin resistance gene.

All the pre and post transformation conditions were kept same except the transformation procedure. Four transformation procedures viz. *Agrobacterium* mediated transformation, Biolistic method, a combination of these two procedures and a recently described SAAT procedure were compared. Each experiment consisted of four treatments with two replications and a total of three experiments were conducted. Statistical analysis (ANOVA) has shown that there is no significance difference between the replications but highly significance difference exists between treatments (Table 1). LSD test has also clearly shown that first three procedures are not statistically different but SAAT has highly significant difference over other three procedures used in this study (Table 2). One important phenomenon noticed in this study that almost all the GUS activity was found towards the shoot apex (Fig. 1). Similarly embryo growth kinetics was also noted in all four procedures but no growth inhibition or abnormality in growth was observed. When embryos were sonicated for longer periods, many embryos were broken into pieces.

Table 1. Analysis of Variance (ANOVA).

Source of variation (SOV)	Degrees of freedom (DF)	Mean squares (MS)
Replication	2	1376.0833^{NS}
Procedure	3	82194.972**
Error	6	1175.972^{NS}

Table 2. Means followed by the same letter are not significantly different (p=0.05) according to least significant difference test.

Procedure	Mean value	LSD Grouping
Agro-mediated	7.33	a
Bombardment	17.00	a
Agro + Bombardment	33.67	a
Sonication + Agro	349.67	b

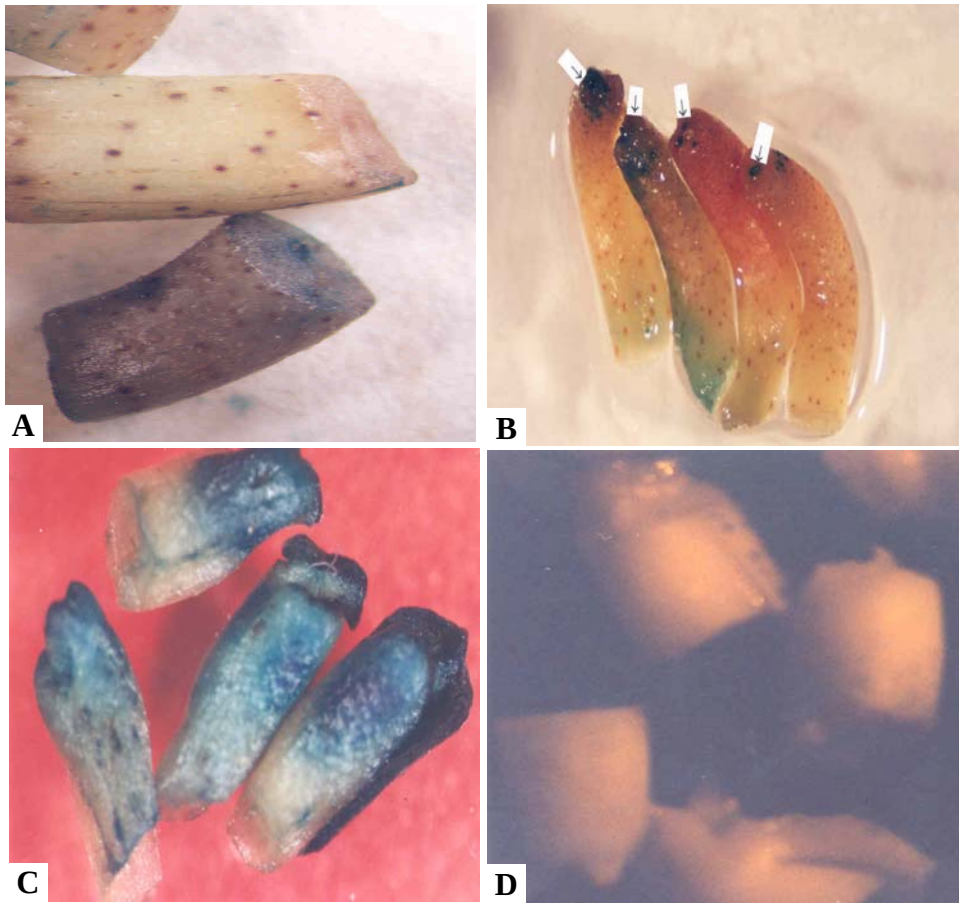

Fig. 1. Gus expression in mature embryos.
A- *Agrobacterium* mediated transformation, B- Biolistic transformation, C- Sonication assisted *Agrobacterium* mediated transformation, D- Control

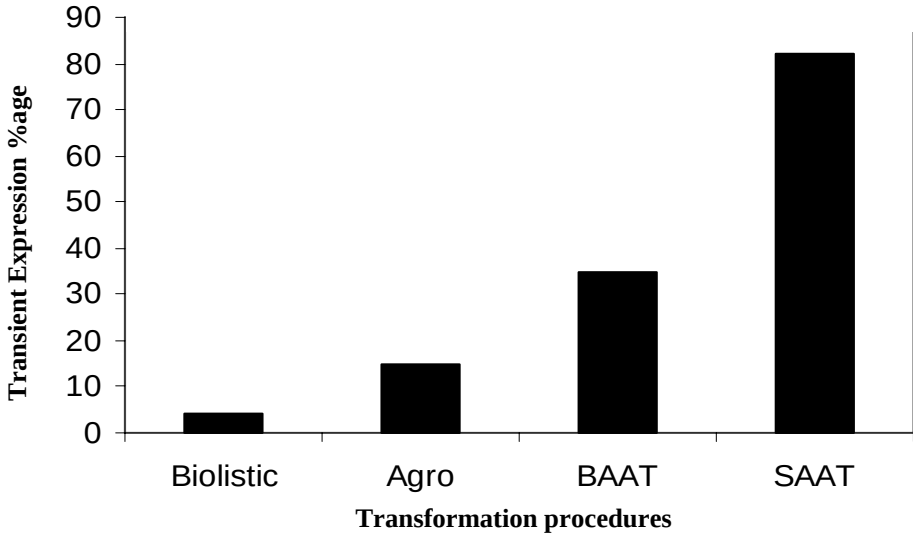

Fig. 2. Choice of transformation procedure on the basis of transient expression of GUS.

Discussion

Key to success for transformation is rapid and normal plant development and efficient transformation system best suited to the crop plants being transformed. Previously, callus, leaf discs, cotyledonary pieces, hypocotyle and shoot apices (Gould *et al*., 1998; Chlan *et al*., 1995; Srivastava *et al*., 1991; Kolgenova *et al*., 1991; Firoozabady *et al*., 1987; Umbeck *et al*., 1987; Horsch *et al*., 1985) had been transformed but there is inconsistency in results. In this study, mature embryos were used as explant for the transformation of cotton. The main advantage of mature embryo transformation is the normal plant development of even recalcitrant crops like cotton (Majeed *et al*., 2000). Similarly, consistent results have been obtained when mature embryos were used as an explant (Haris *et al*., 1998).

Agrobacterium mediated transformation is most commonly used method for cotton transformation but difficulties exist due to low efficiency of regeneration and low transformation efficiency of routinely used transformation procedures (Firoozabady *et al*., 1987; Grieson & Covey, 1988). Similarly, plant transformation via *Agrobacterium* can be limited by both host specificity and the inability of *Agrobacterium* to reach the proper cells in the target tissue so a new and efficient *Agrobacterium* based transformation method that overcomes these barriers and enhances DNA transfer was required. Biolistic transformation was initially welcomed as an alternative method for generating transgenic plant species. This method offers a mean to bypass genetic barriers to genotype independent transformation, direct transformation of organized tissues and relatively rapid recovery of transgenics (Christou, 1996a, b; John, 1997). Particle gun bombardment requires the identification and selection of stable germline events from among a population of chimeric transformants and is yet another long, drawn out process that requires the maintenance of a large number of plants and requires considerably more plants than *Agrobacterium* mediated transformation. But biolistic is still woefully inefficient in terms of penetration to germline layers and secondary damage to target tissue (Wilkins *et al*., 2000).

Particle bombardment methods using *Agrobacterium* were previously used to transform tobacco leaves and sunflower meristems (Bidney *et al*., 1992) banana meristems (May *et al*., 1995), common & tepary bean meristems (Brasileiro *et al*., 1996) and cotton tissues (Finer & McMullen, 1990; Majeed *et al*., 2000). A potential problem associated with biolistic is the delivery of DNA coated particles to only one side of the target tissue and limited penetration (Trick & Finer, 1998), thus restricting *Agrobacterium*'s ability to transform the cells lying deeper into the tissues.

Since *Agrobacterium* is still a method of choice for transformation, so a new and efficient *Agrobacterium* based transformation method that overcomes these barriers and enhances DNA transfer (Trick & Finer, 1998) was used in comparison of above described procedures (Fig. 2). This new method is called sonication assisted *Agrobacterium* mediated transformation (SAAT). The strength of this method is that the cavitation caused by sonication results in thousands of microwounds on and below the surface of the plant tissue. This wounding fashion permits *Agrobacterium* to travel deeper and more completely throughout the tissue than conventional microscopic wounding, increasing the probability of infecting plant cells (Trick & Finer, 1997; Santarem *et al*., 1998; Trick & Finer, 1998).

Our results of comparative studies using all the above-mentioned transformation procedures clearly demonstrated the efficacy of SAAT as highly significance difference exists in statistical analysis (Table 1). Similarly LSD test also clearly showed no significance difference in first three procedures but SAAT has highly significance difference ($p>0.01$) over these procedures (Table 2). Similarly growth inhibition or abnormal growth of embryos was not observed in all the procedures. Any how, longer exposure of embryos to sonication results in breakening of the tissue.

Although high intensity ultrasound results in immediate cell lysis (Joersbo & Brunstedt, 1992), sublethal doses result in temporary suppression of RNA and protein synthesis as well as moderate rupture of cell walls (Joersbo & Brunstedt, 1992). The cell wall disruption caused by the low energy ultrasonic frequency utilized in the present study is apparently very useful for *Agrobacterium* mediated transformation. Secondly, the wounding may aid in the production of signal phenolics (Stachel *et al*., 1985) and enhance the accessibility of putative cell wall binding factors to the bacterium (Lippencott & Lippencott, 1969). For stable expression, the transformation of subsurface tissue is critical when attempting to transform other tissue such as meristems that are buried several layers deep. The basis for the increase in GUS transient expression from SAAT is believed to be caused by cavitation induced micro wounds, which facilitate the infection by *Agrobacterium* of the target plant tissue (Trick & Finer, 1997).

The microwounds caused by sonication can significantly enhance the *Agrobacterium* mediated transformation frequency in different target tissues. Although the positive GUS assays only demonstrate transient expression of the introduced gene, the SAAT procedure holds much promise to enhance *Agrobacterium* infection and to obtain stable transformation events. With such efficient delivery of *Agrobacterium* into cotton tissues, SAAT should be a valuable and alternative method for demonstrating the stable cotton transformation.

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