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MOLECULAR BIOLOGY AND PHYSIOLOGY

Tissue Culture-independent *In Planta* Transformation Strategy: an *Agrobacterium tumefaciens*-Mediated Gene Transfer Method to Overcome Recalcitrance in Cotton (*Gossypium hirsutum L.)*

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ABSTRACT

The major constraint in cotton improvement has been the recalcitrance of cotton varieties to tissue culture. Alternate methods that avoid/ minimize tissue culture would be beneficial for the improvement of cotton. In this report, transgenic cotton plants have been produced by a tissue-culture independent *Agrobacterium tumefaciens* – mediated transformation procedure. *Agrobacterium* strain LBA4404 harboring the binary vector pKIWI105 that carries the genes for β-glucuronidase (GUS) and neomycin phosphotransferase (*npt* II) was used for transformation. Apical meristem of the differentiated embryo of the germinating seedling is infected with *Agrobacterium.* Since the transgene is integrated into the cells of already differentiated tissues, the T_0 plants will be chimeric and stable integration can be seen only in the T_1 generation. The first proof of transformability in the T_0 generation was indicated by the GUS histochemical analysis of the seedlings, five days after co-cultivation and subsequently in the pollen and lint. T_1 transformants were identified by PCR analysis and subsequently confirmed by Southern. Three plants (T_1) with single copy insertions were selected for continuing into the next generations. Molecular characterization and GUS expression analysis (histochemical and fluorimetric) of the T_1, T_2 and T_3 generation suggested the feasibility of the method to generate transgenic plants in cotton

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INTRODUCTION

Cotton is an economically important crop that is grown throughout the world. It is grown as a source of fiber, food and feed. Lint, the most economically important product from the cotton plant, provides a source of high quality fiber of the textile industry. Cotton seed is an important source of oil and cotton seed meal is a high protein product used as livestock feed. Although significant progress has been made in the field of cotton improvement with conventional breeding methodology, it has limitations to introduce new alleles. Genetic engineering offers a directed method of plant breeding that selectively targets one or a few traits for introduction into the crop plant. The first transgenic cotton plants were obtained in 1987 (Umbeck et al., 1987). Since then, many laboratories have obtained insect resistant (Perlak et al., 1990; Cousins et al., 1991; Xie et al., 1991; Thomas et al., 1995; Jenkins et al., 1997; Li et al., 1998a Li et al., 1998b; Ni et al., 1998) or herbicide resistant (Bayley et al., 1992; Lyon et al; Chen et al., 1994; Rajashekaran et al., 1996; Keller et al., 1997) transgenic cotton plants. Transgenic cotton has been mainly obtained by *Agrobacterium*-mediated transformation (Perlak et al., 1990; Thomas et al., 1995; Bayley et al., 1992; Satyavathi et al 2002; Leelavathi et al., 2004) and particle bombardment (McCabe and Martinelli, 1993; Finer and McMullen, 1990; Rajasekaran et al., 2000). Transgenic plants have also been generated by regeneration from shoot apex tissues in both *Gossypium hirsutum* and *G. barbadense* (Gould and Magallanes-Cedeno, 1998; Gould et al., 1991). More recently a high efficiency, embryogenic calli based method of obtaining transgenic cotton has been standardized in two Chinese cultivars (Wu et al., 2005). Another method followed to generate transgenic plants in cotton has been the pollen tube pathway transformation. This method is used to transfer

DNA into zygotic embryos via pollen tube pathway. This is a widely used method in China (Zhou et al., 1983; Huang et al., 1999). Nevertheless, one of the major drawbacks of the *Agrobacterium*-mediated transformation is the recalcitrance of cotton and its difficult-to-regenerate nature (John, 1997). *In vitro* regeneration in cotton from callus is limited to non-indigenous Coker cultivars or closely related genotypes. Even in the case of Coker cultivars, the low efficiency of somatic embryogenesis, elaborate culture procedures, relatively long time period required for regeneration and high seed to seed variation in response, collectively pose serious technical difficulties and restrict progress in cotton biotechnology. Hence this necessitates development of easy, reliable and efficient transformation protocols for cotton transformation for improvement, particularly in the Indian cultivars which are adapted to local conditions.

To tackle the problems pertaining to regeneration in cotton and certain other recalcitrant crops, alternate methods to minimize or eliminate the steps of regeneration are being standardized. These are called the *in planta* transformation protocols. Research with *Arabidopsis* has benefited from the development of high throughput transformation methods that avoid plant tissue culture (Azipiroz-Leehan and Feldmann, 1997). In particular, the development of the *Agrobacterium tumefaciens*-mediated vacuum infiltration method (Bechtold et al., 1993) has had a major impact on *Arabidopsis* research. *In planta* transformation methods have also been standardized for rice (Supartana et al., 2005), buckwheat (Kojima et al., 2000), kenaf (Kojima et al., 2004) and mulberry (Ping et al., 2003). In all the crops, *Agrobacterium* is directed towards either the apical meristem or the meristems of axillary buds. One such viable *in planta* transformation protocol has also been standardized for other crops (Sankara Rao and Rohini, 1999; Rohini and Sankara Rao, 2000a; Rohini and Sankara Rao, 2000b; Rohini and Sankara Rao, 2001). The strategy essentially involves *in planta* inoculation of embryo axes of germinating seeds and allowing them to grow into seedlings *ex vitro*. These *in planta* transformation protocols are advantageous over other methods because they do not involve regeneration procedures and therefore the tissue culture-induced somaclonal variations are avoided. This paper presents successful transformation of cotton by following *in planta* transformation protocol.

MATERIAL AND METHODS

Plant material. Seeds of a breeding line of cotton viz., NC-71 were soaked overnight in distilled water and were surface sterilized first with 1% Bavastin for 10 mins and later with 0.1% $HgCl_2$ for few seconds and washed thoroughly with distilled water after treatment with each sterilant. The seeds were later allowed to germinate on petriplates at 30 °C in dark. Two-day old seedlings were taken as explants for *Agrobacterium* infection.

Bacterial strain and vector. *Agrobacterium tumefaciens* strain LBA4404, harbouring the binary vector pKIWI105 (Janssen Gardner, 1989), was used for transformation. The vector contains the *uid* A reporter gene driven by the CaMV 35S promoter and a neomycin phosphotransferase II (*npt* II) gene driven by the nopaline synthase promoter. The reporter gene of pKIWI105 is a version of *uid* A that lacks the bacterial ribosome binding site and shows no expression in *Agrobacterium* but good expression in plant cells.

Transformation and recovery of transformants. *Agrobacterium* strain LBA4404/pKIWI105 was grown overnight at 28 °C in LB medium (pH 7.0) containing 50 $\mu g ml^{-1}$ kanamycin. The bacterial culture was later resuspended in 100 ml of Winans' AB medium (Winans et al., 1988) (pH 5.2) and grown for 18 h. For *vir* gene induction treatments wounded tobacco leaf extract (4 ml in 50 ml Winans' medium) (Cheng et al., 1996; Rohini and Rao, 2000b) was added separately to the *Agrobacterium* suspension in Winans' AB medium, 5 h before infection. The seedlings with just emerging plumule were infected by separating the cotyledons without damaging them such that the meristem is visible, and then pricked at the meristem with a sterile sewing needle and subsequently dunked in the culture of *Agrobacterium* for 60 mins. Following infection, the seedlings were washed briefly with sterile water and later transferred to autoclaved soilrite (vermiculite equivalent) moistened with water for germination under aseptic conditions in the growth room in wide mouth capped glass jars of 300 ml capacity, 5 seedlings per jar. After 5 to 6 days, the seedlings were transferred to soilrite in pots and were allowed to grow under growth room conditions for at least 10 days before they were transferred to the greenhouse. The growth chamber was maintained at 26-28 °C under a 14 h photoperiod with florescent light of intensity 35 $\mu mol\ m^{-2}\ s^{-1}$.

Expression of β-glucuronidase. For analysis of the transformants, tissues that were tested and found free of residual *Agrobacterium* were used. The persistence of *Agrobacterium* in the putative transformants was largely controlled by a brief agitation (30s) of the co-cultivated embryos with 0.1% sodium hypochlorite followed by thorough washes with distilled water.

The method of Jefferson (1987) was used to assess histochemical *uid* A gene expression in the tissues of primary transformants, 4 days post co-cultivation, in the pollen and in the lint. The tissues were incubated overnight at 37 ˚C in a solution containing 0.1M phosphate buffer, pH 7.0, 2 mM X-Gluc, 5 mM each of potassium ferricyanide and ferrocyanide and 0.1% Triton X-100. The tissues were later soaked with 75% ethanol to clear chlorophyll.

For GUS activity in the pollen, flowers with undehisced pollen were selected. After subjecting them to GUS histochemical analysis in an eppendorf tube, the flowers were crushed gently with a glass rod to release the pollen and the pollen was allowed to settle. A Pasteur pipette was used to take in most of the pollen that would have settled down and put on a glass slide. A drop of glycerol was placed on the slide and a cover slip was placed such that the pollen spread evenly on the glass slide. The slides were then observed under the microscope at 40X magnification and photographed.

The GUS enzyme activity was quantified by measuring the hydrolysis rate of the fluorescent substrate 4-methyl umbelliferyl-β-D-glucuronide (MUG) as described by Jefferson (1987). The leaves of T_3 generation cotton plants along with the wild type plants were used for the fluorogenic assay.

The fresh leaf material (100 mg) was homogenized using a pestle and mortar in 1 ml of GUS extraction buffer (100 mM Potassium phosphate buffer, pH 7.4; 10 mM β-mercaptoethanol; 10 mM EDTA; 0.1% (w/v) triton x-100; 0.2 mM PMSF and 0.1% (w/v) Sodium lauryl sarcosine). The homogenates were centrifuged at 10,000 rpm for 10 min and supernatant was collected and stored on ice until further use. The protein concentration in the supernatant was measured as described by Bradford (1976).

The fluorogenic reaction was carried out in GUS assay buffer (2 mM MUG in 100 ml of GUS extraction buffer). The reaction was set up in 96-well black Nunc microtiter plates. About 2 µg of total protein was taken in 25 µl of extraction buffer to which 25 µl of GUS assay buffer was added and incubated at 37 ˚C for 1 h after sealing the plate with aluminium foil. The reaction was stopped by adding 350 µl of 0.2 mM Na_2CO_3 (stop buffer). Fluorescence was measured with excitation and emission filters set at 365 nm and 455 nm using fluoromax-3 with micromax reader (Jobin-Yvon-Spex, Horiba group), with slit width set at excitation 2 nm and emission 3 nm. The GUS activity was quantified using freshly prepared 4-MU (4-methyl umbelliferone sodium salt) standard of 10nM, 20 nM, 40nM, 80 nM, 120 nM, 160 nM, 200 nM, 240 nM, 280 nM and 320 nM and enzyme blank in the stop buffer (0.2 M Na_2CO_3). The GUS activity was expressed as pmol of 4-MU/mg protein/min.

Molecular analysis for the presence and integration of the marker genes. DNA for polymerase chain reaction (PCR) and Southern analysis from leaf tissue was isolated following the procedure of Dellaporta et al. (1983).

Polymerase chain reaction. PCR was performed to amplify a 450 bp *uid* A gene fragment in the putative transformants. In order to amplify the *uid* A gene fragment, PCR was initiated by a hot start at 94 ˚C for 4 min followed by 32 cycles of 1 min at 94 ˚C, 1 min 30 s at 60 ˚C and 1 min at 72 ˚C. Annealing temperature was at 58 ˚C for the amplification of a 750 bp *npt* II gene. PCR was performed with both the primers in the same reaction mix to check for the co-integration of both the transgenes in the transformants. The conditions for the reaction was the same as above, accept that the annealing temperature was at 59 ˚C. The products were run on a 1% agarose gel.

Southern analysis. In order to analyze the total genomic DNA for transgene integration of *npt* II gene, 15 µg of total genomic DNA was digested with the appropriate restriction enzyme. The digested DNA samples were electrophoresed on a 0.8% agarose gel. The separated fragments along with the uncut DNA were transferred onto a nylon membrane. Hybridization was performed at 65 ˚C in Church buffer for 18 h. Membranes were washed for 30 min each in 2X SSC, 0.1 % SDS; 0.1X SSC, 0.1% SDS at 65 ˚C (Sambrook et al., 1989). Membranes were wrapped, placed on x-ray film and exposed at -70 ˚C. For probe preparation, *uid* A gene was amplified using the gene specific primers, the fragment was random prime labeled and used for Southern hybridization.

RESULTS

Plant transformation and recovery of transformants. Optimization of the conditions for transformation was largely based on the protocol

standardized earlier for sunflower, groundnut and safflower (Sankara Rao and Rohini, 1999; Rohini and Sankara Rao, 2000a; Rohini and Sankara Rao, 2000b). The feasibility of the transformation strategy adopted in the study was initially evaluated by the number of cotton embryo axes germinating into normal seedlings following wounding with a needle at the intercotyledonary region, infection with *Agrobacterium* and transplantation to the greenhouse. The seedlings could withstand infection for a period of 60 min and about 60% of the seedlings survived after infection and developed into plantlets. Out of 50 seedlings that were subjected to *Agrobacterium* infection, 37 plants that survived the infection process were transferred to the greenhouse. Among them, 25 plants survived, attained maturity, flowered and set seed. However, the growth of these seedlings was slow when compared to that of untransformed controls.

Integration and expression of the transgene in T_0 plants. Infection of the already differentiated embryonic tissue with *Agrobacterium* may result in the gene integration randomly and the T_0 plants will be chimeric. However, the feasibility of gene integration in some tissues by *in planta* transformation protocol can be ascertained based on the GUS histochemical assay.

Fig 1A(a) shows the GUS expression in the shoot apex of the primary transformants 5 days after infection whereas endogenous GUS-like activity was not seen in the non-transformed controls. Sections of the GUS-stained tissues revealed the expression of the *uid* A gene within the cells and not in the apoplastic region (Fig. 1Aa b, c and d). Figure 1A(d) clearly shows the transgene expression in the cells of the pericycle and not the parenchymatous cells indicating the possibility of stable transformants in the next generation. The section of the wild type tissue did not show any staining (Fig. 1Ae). Based on these indicative experiments, the infected seedlings were transferred to pots in the greenhouse.

GUS histochemical analysis of the reproductive structures can provide leads about the possibility of generating stable T_1 transformants. Among the large number of plants screened by histochemical GUS assay for expression in the pollen and lint, few plants showed expression in some of the flowers and developing bolls. Two plants, plant no. I and IV out of 25 T_0 plants showing consistent GUS expression were chosen for further analysis in the subsequent T_1 generation.

Figure 1A

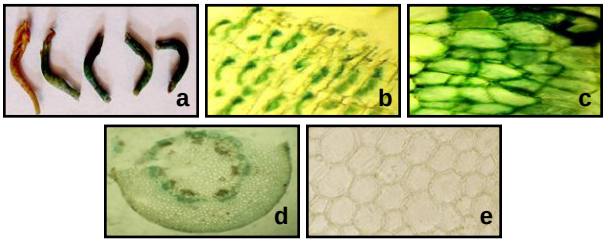

Figure 1B

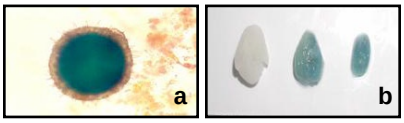

Figure 1. (A). GUS histochemical analysis of transformed T_0 plants.
(a) GUS histochemical analysis of the seedlings 5 days after infection.
(b), (c) and (d), Sections of the meristamatic region showing GUS expression within the cell and (e) Section of the negative control showing no expression.
(B). GUS histochemical analysis of the pollen and lint of T_0 plants.

Integration and expression of the transgene in T_1 plants. Approximately 75 seeds were sown from each of the the two selected primary transformants. From plant I, 26 seeds germinated whereas 59 seeds germinated from plant IV. PCR analysis for *uid* A gene revealed the presence of a 450 bp amplicon in a total of 13 plants, 3 from I and 10 from IV (Fig. 2). Further confirmation of the integration and inheritance of the transgene in the progeny plants was obtained by genomic southern analysis. Fig. 3a (lanes 1-10) shows the hybridization signal in the uncut DNA of the PCR positive plants which were probed with a 450 bp *uid* A gene fragment. The variation in the hybridization pattern of the *Sma*I digested DNA confirms the inheritance and the integration of the *uid* A gene in the genome of the T_1 plants (Fig. 3a; lanes 11-20). Three plants, I-E and I-S belonging to plant I of T_0 and IV-14 belonging to plant IV of T_0 showed the single copy insertions. These progeny plants were phenotypically normal, flowered and set seed in a normal fashion. GUS expression in the pollen (Fig. 3b) of these plants confirmed stability of the transgene.

Stability of the transgene in T_2 plants. From the selected plants, fifty, forty and sixty seeds were selected randomly from each of the plants and were sown in pots in the greenhouse. PCR was performed using primers for uidA gene in all the 78 plants that germinated i.e., 26 from I-E, 20 from I-S and 32 from IV-14. Amplification of the 450 bp amplified

fragment of the *uid* A gene (Fig. 4a) in all the plants confirm the stable integration of the transgene. Expression of GUS in the pollen (Fig. 4b-a representative picture) supported the PCR analysis.

Analysis of the T3 generation plants for the integration of transgenes.. Following T_2 analysis, 13 plants were randomly selected for further analysis into the T_3 generation. A total of 130 plants were obtained from these 13 plants (refer legend for Fig. 5). They were subjected to both molecular and expression analysis.

PCR with both *uid* A and *npt* II primers separately and both the primers (Fig. 5) in the same reaction confirmed the stability of the gene in all the selected transformants.

Among the T_3 generation plants, eleven plants (T_3) were randomly selected for fluorimetric analysis of GUS activity. Retainment of GUS activity in these ten plants indicated continued inheritance and expression of the gus (*uid* A) gene (Fig.6). Approximately 16 fold increase was seen in the transgenics when compared to the untransformed negative control.

Southern analysis of ten plants that exhibited high GUS activity confirmed the transgenic status further. In pKIWI105, *Hind* III releases a 4.7 kb fragment with the gus (*uid* A) gene in it. Fig. 7 gives the hybridization signal at the expected 4.7 kb position reconfirming the transgenic nature.

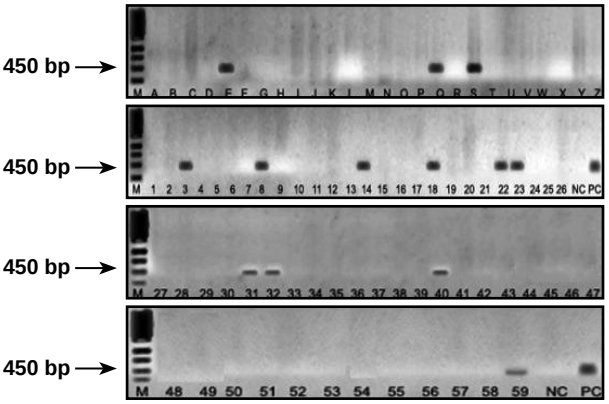

Figure 2. PCR analysis of the T_1 generation cotton plants obtained from 2 events (no. I and IV). 26 plants were developed from plant no. I and 59 plants were developed from plant no. IV. These were analysed by PCR using primers for *uid* A (gus) gene. Lanes A-Z: DNA from the T_1 progeny of the plant no. I (T_0); Lanes 1-59: DNA from the T_1 progeny of the plant no. IV (T_0); Lane M: DNA ladder; Lane PC: positive control (pKIWI105 DNA); Lane NC: DNA from the untransformed control.

Figure 3a

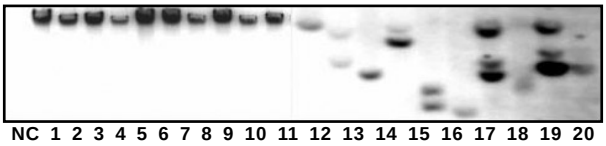

Figure 3b

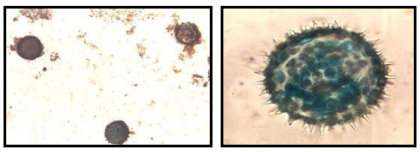

Figure 3. (a). Genomic Southern analysis of the T_1 generation plants.The Southern analysis was carried out for 10 PCR positive T_1 plants (3 from T_0 plant no.I and 7 from T_0 plant no. IV). 10 µg of uncut genomic DNA and 15 µg DNA digested with *Sma*I was probed with 450 bp *uid* A gene fragment. Lane 1: uncut DNA from untransformed plant. Lanes 2-11: uncut DNA from 10 PCR positive T_1 generation plants (I-E, I-Q, I-S, IV-3, IV-8, IV-14, IV-18, IV-22, IV-23 and IV-31). Lanes 12-21: Digested DNA from 10 PCR positive T_1 generation plants (I-E, I-Q, I-S, IV-3, IV-8, IV-14, IV-18, IV-22, IV-23 and IV-31).
(b). GUS expression in the pollen of flowers in the T_1 generation plants

Figure 4a

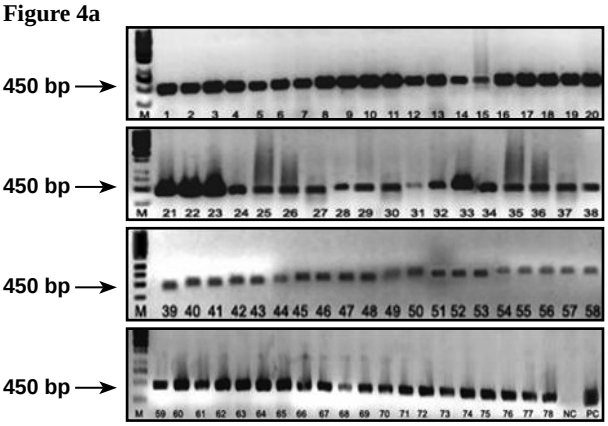

Figure 4b

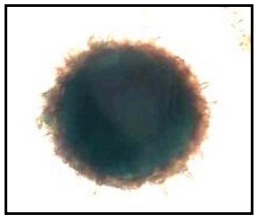

Figure 4. (a). PCR of the DNA of transgenic plants in the T_2 generation cotton plants using primers for *uid* A (gus) gene. Lanes 1-26: DNA from the transgenic plants raised from the T_1 plant I-E; Lanes 27-46: DNA from the transgenic plants raised from the T_1 plant I-S; Lanes 46-78: DNA from the transgenic plants raised from the T_1 plant IV-14; Lane M: DNA ladder; Lane NC: DNA from the untransformed control; Lane PC: positive control (pKIWI105 DNA).
(b). GUS expression in the pollen of flowers in the T_2 generation plants

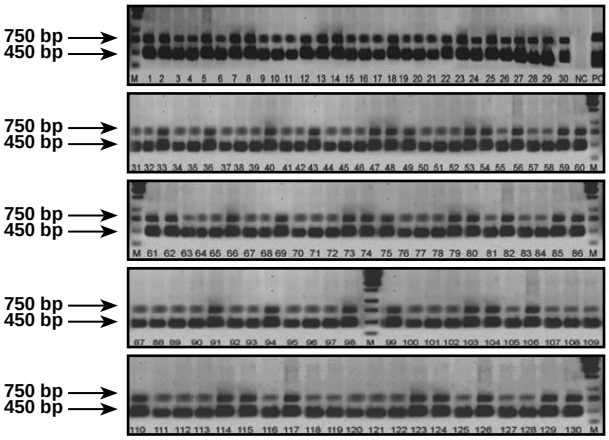

Figure 5. PCR of the DNA of transgenic plants in the T_3 generation cotton using primers for both *uid* A (gus) and *npt* II genes. Lanes 1-12: DNA from the transgenic plants raised from T_2 plant no. I-E-1; Lanes 13-22: DNA from the transgenic plants raised from T2 plant no. I-E-8; Lanes 23-30: DNA from the transgenic plants raised from T2 plant no. I-E-16; Lanes 31-41: DNA from the transgenic plants raised from T_2 plant no. I-E-24; Lanes 42-50: DNA from the transgenic plants raised from T_2 plant no. I-S-1; Lanes 51-59: DNA from the transgenic plants raised from T_2 plant no. I-S-4; Lanes 60-71: DNA from the transgenic plants raised from T_2 plant no. I-S-8; Lanes 72-84: DNA from the transgenic plants raised from T_2 plant no. I-S-16; Lanes 85-94: DNA from the transgenic plants raised from T_2 plant no. IV-14-1; Lanes 95-102: DNA from the transgenic plants raised from T_2 plant no. IV-14-6; Lanes 102-109: DNA from the transgenic plants raised from T_2 plant no. IV-14-12; Lanes 110-118: DNA from the transgenic plants raised from T_2 plant no. IV-14-16; Lanes 119-130: DNA from the transgenic plants raised from T_2 plant no. IV-14-24; Lane M: DNA ladder; Lane NC: DNA from the untransformed control; Lane PC: positive control (pKIWI105 DNA).

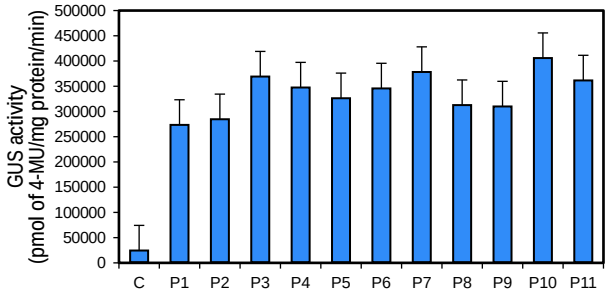

Figure 6. Fluorimetric analysis of GUS by using MUG as substrate. About 2 µg of total protein was mixed with 25 µl of GUS assay buffer and incubated at 37 ˚C. The reaction was stopped and fluorescence was measured with exitation and emission filters set at 365 nm and 455 nm. C: protein extract from the untransformed control; P1-P11: protein extracts from the different transgenics T3 transgenics.

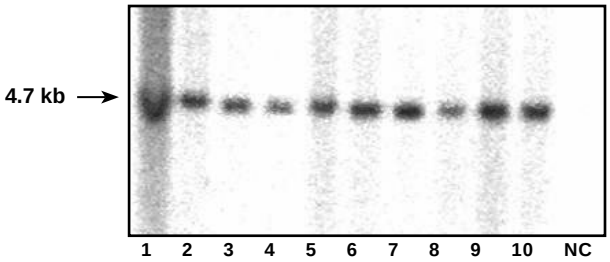

Figure 7. Genomic Southern analysis of the T_3 generation plants. 15 µg DNA was digested with *Hind* III and was probed with the 450 bp *uid* A gene fragment. In pKIWI105, *Hind* III releases a 4.7 kb fragment with the gus (*uid* A) gene in it. Lane 1-10: Digested DNA from PCR positive T_3 generation plants. (lane 1: I-E-1-1; lane 2: I-E-8-1; lane 3: I-E-16-1; lane 4: I-E-24-1; lane 5:I-S-1-1; lane 6: I-S-4-1; lane 7: I-S-8-1; lane 8: IV-14-1-1; lane 9: IV-14-12-1; Lane 10: IV-14-16-1; lane NC: DNA from non transformed plants.

DISCUSSION

Recent advances in transgenic technology now make it possible to transfer and express various genes in agriculturally important species like cotton. The rapid development of cotton transformation technology not only provides a valuable method for introducing useful genes into cotton to improve important agronomic traits, but also helps in the study of gene function and regulation. Although transformation rates have been significantly improved since the first report of success in the transformation of cotton (Firoozabady et al., 1987; Umbeck et al., 1987), increase in transformation efficiency is still needed.

Transformation techniques that evade tissue culture (Graves and Goldman, 1986) therefore become important in recalcitrant crops such as cotton. In the present study, a tissue culture-independent *in planta* transformation protocol was used to develop transformants (Rohini and Sankara Rao, 2000a; Rohini and Sankara Rao, 2000b; Rohini and Sankara Rao, 2001). Such *in planta* transformation techniques have also been standardized in other crops like, buckwheat (Kojima et al., 2000), mulberry (Ping et al., 2003), kenaf (Kojima et 2004), soybean (Chee et al., 1989) and rice (Supartana et al., 2005) etc. In this method, *Agrobacterium* is targeted to the wounded apical meristem of the differentiated seed embryo. Therefore, *Agrobacterium tumefaciens* transfers the gene into the genome of diverse cells which are already destined to develop into specific organs and the meristematic cells still to be differentiated. This results in the primary

transformants (T_0) being chimeric in nature. This is the reason for the analysis of the transgenic plants to be carried out in the T_1 generation. Nevertheless, analysis of the T_0 generation plants was carried out with an objective to check for chimeras. *Uid* A gene used in the study that expresses only upon transfer to plant system facilitated the identification of the chimeras. The first indication of the transformability and chimeric nature in T_0 plants was obtained by the GUS histochemical analysis of the seedlings. In the indicative experiment to check the feasibility of the transformation, at least 60% of the T_0 seedlings showed GUS expression indicating transgene integration and also chimeric nature of the T_0 seedlings. The chimeric plants producing the stable transformants in the T_1 generation depends on the type of cells that were transformed in the T_0 plants. If the transgene is integrated into undifferentiated meristematic cells which are destined to develop sympodial branches, seeds obtained from these reproductive structures of these branches would produce stable transformants in T_1. Therefore, evaluation of the reproductive tissues in T_0 is necessary. GUS expression in the lint and pollen of some of the plants clearly suggested that some of the T_1 seeds from these plants would produce stable transformants.

In the analysis of T_1 generation plants, transgenic nature was ascertained first by PCR and later by Southern analysis. Three plants (I-E, I-S, IV-14) were selected to be the basis for further generations because of the single copy insertion of the transgene as revealed by the Southern analysis. The progeny of these plants in the T_2 generation showed the amplification of the transgene in all the randomly selected plants showing transgene inheritance. GUS expression analysis by using the fluorimetric assay in T_3 generation plants also supported the transgenic nature as upto 16 fold increase in the GUS activity was seen in the transformants when compared to the controls. Southern analysis of these samples for the release of the gus (*uid* A) gene reconfirmed the transgenic nature of these selected plants.

Our results indicate that transgenic cotton plants can be obtained by *Agrobacterium* –based transformation strategy and transgene stability confirmed for three generations. The method therefore is advantageous because it avoids the need for tissue culture. Nevertheless, transformability depends on the susceptibility of the variety to *Agrobacterium.* A number of factors affect transformability by *in planta* transformation.

- First, the method depends on the number of T_0 seedlings that survive and develop into normal mature plants after infection with *Agrobacterium*
- Second, chimeras have to be generated suggesting that transformation has occured.
- Third, transgene integration has to occur in the cells that eventually develop into reproductive structures that subsequently produce stable transformants.

With the presumption that 60% of the T_0 plants would be chimeric based on an indicative experiment (data not shown), the surviving 25 T_0 plants were maintained in the greenhouse. Nevertheless, analysis of GUS expression in the pollen and lint revealed that 8% of the primary transformants, i.e., 2 plants out of 25 were positive and showed consistent expression and therefore the probability of producing stable transformants. However, the number of stable transformants varied between the T_0 plants considered for T_1 generation. Plant no. I produced 3 transformants out of 24 plants based on PCR analysis for the *uidA* gene and 11 transformants from 59 plants belonged to plant no. IV. The transformation efficiency therefore in our study varies between 8.3% and 19%. Similar transformation efficiency based on PCR analysis has also been reported in other crops (Putu Supartana *et al.,* 2006, Supartana *et al.,* 2005) where *in planta* transformation methodology has been used.

In summary, this study provides experimental evidences for stable transformation by *in planta* transformation technique. It opens options to exploit transgene technology in cotton since efficient transformation in diverse genotype is still a constraint.

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