GENETIC TRANSFORMATION OF *Bar* GENE AND ITS INHERITANCE AND SEGREGATION BEHAVIOR IN THE RESULTANT TRANSGENIC COTTON GERMPLASM (BR001)

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Abstract

The *Agrobacterium*-mediated genetic transformation technique in cotton using two genotype independent approaches were exploited. The segregation and inheritance of the *Bar* gene as well as various agronomic and fiber quality traits of the resultant transgenic cotton germplasm (BR001) and its background germplasm (Coker 312) were also studied. PCR and Southern blot analyses showed successful integration of the foreign gene in case of both approaches. Although the transformation efficiency of shoot apex culture method was higher than Pollen Tube Pathway *via* ovarian injection method, the number of plants developed in the soils was almost the same. Moreover, the Mendelian inheritance and segregation studies confirmed that a single nuclear dominant gene governed the herbicide resistance characters in BR001. The present study suggests that both genotype independent approaches can be utilized in order to save time as well as to avoid chimeras.

Introduction

Gluphosinate (phosphinothricin; glufosinate) is a potent inhibitor of glutamine synthetase (Devine *et al*., 1993). It is a contact herbicide and acts by inhibiting photosynthesis, apparently due to glyoxylate accumulation. Gluphosinate does not persist in the environment and is toxicologically benign. Two genes i.e., *bar* gene from *Streptomyces hygroscopicus* and the *pat* (phosphinothricin-acetyl transferase) gene from *S. viridochromogenes* (Droge *et al*., 1992; Strauch *et al*., 1998) have been used, which confer resistance to gluphosinate. More than 20 crops, including cotton (Keller *et al*., 1997), have now been transformed with one or the other of these two genes through a number of *in vitro* and *in vivo* transformation methods.

The *Agrobacterium*-mediated transformation is one of the most commonly used methods to transform valuable foreign genes. The basic principle behind this transformation technique is the use of the bacterium (*Agrobacterium tumefaciens*) and its natural transformation process to insert foreign DNA into host genomic DNA of plant tissue. The first transgenic tobacco plant produced by *Agrobacterium*-mediated transformation was developed in 1983 (Herrera-Estrella, 1983), which was a revolutionary step in developing a protocol for inserting foreign DNA into numerous crops such as cotton, rice, soybean, wheat, maize etc.

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In the past more than one decade, extensive research efforts have been focused on cotton and a number of transgenes have been introduced either through *Agrobacterium* (Lyon *et al.*, 1993; Thomas *et al.*, 1995), particle bombardment (Finer *et al.*, 1990; Rajasekharan *et al.*, 1996) or by a combination of both methods (Majeed *et al.*, 2000). In most of the transformation techniques using tissues as explants, 'Coker' highly regenerable germplasm (i.e. Coker 312) and the related lines have been used (Wilkins *et al.*, 2004). But problems to the "Coker Method" are that these lines are obsolete and a number of crossings, backcrossings and selections are required to identify lines suitable for commercialization. Also in tissue culture methods, a long time is required in order to get regenerated plants *via* somatic embryogenesis, hence the probability of occurrence of soma clonal variations is high.

In order to avoid these limitations, the genotype-independent approaches have been proposed (Gould *et al.*, 1998). These transformation techniques target either ovaries, meristems or other tissues that ultimately give rise to gametes (Chee *et al.*, 1995; Birch, 1997). Although the overall transformation efficiency is very low, the sheer number of seeds recovered for screening and the ease of methods make them an extremely attractive alternative (Bechtold *et al.*, 1993; Clough *et al.,* 1998; Bent, 2000; Tjokrokusumo *et al.*, 2000; Li *et al.*, 2004).

A similar approach utilizing application of naked DNA / recombinant *Agrobacterium* directly on the severed styles of flowers after self-pollination or pollination with the pollen imbibed by naked DNA or attached by recombinant *Agrobacterium* to pollen to predestined emasculated flowers was introduced, which is known as Pollen Tube Pathway (PTP) method (Zhou *et al.*, 1983). Exogenous DNA had been introduced into cotton, rice and wheat by the pollen tube pathway technique (Luo *et al.*, 1988; Zeng *et al*., 1994).

In the present study, we utilized two genotype-independent approaches for the insertion of *Bar* gene into cotton genome. They are Pollen Tube Pathway *via* ovarian injection and Shoot Apex Culture approaches using shoot apices as explants. Our main objectives were to obtain stably transformed plants, to study the nature of inheritance in the transgenes and to compare both approaches regarding time and labor needed, their transformation efficiencies as well as pattern of Mendelian law of segregation.

Materials and Methods

Plant materials: Seeds of Coker 312 were obtained from the Cotton Research Institute, Chinese Academy of Agricultural Sciences, Anyang, Henan, China. For the Pollen Tube Pathway *via* ovarian injection method (Method-I), the seeds were sown in the field at the end of April, 2005. For the shoot apex culture method (Method-II), the seeds were surface disinfected with concentrated Sulphuric acid (H_2SO_4) for 10-15 min., and thoroughly washed with running tap water. They were surface sterilized first with 70% (v/v) ethyl alcohol for 3 min., followed by 0.1% (w/v) aqueous Mercuric chloride ($HgCl_2$) solution for 10 min., and washed subsequently with sterilized distilled water. The sterile seeds were then inoculated on MS (Murashige & Skoog, 1962) basal medium supplemented with 1.5% (w/v) sucrose and 0.8% (w/v) agar for germination. Seeds were cultured at $28 \pm 2°C$ in the dark for 3 days and then transferred to the culture room ($28 \pm 2°C$) under a 14: 10 hours day: night photoperiod with light provided by cool-white fluorescent lamps at an irradiation of 135 lmol/m2/s) for 5–7 days.

***Agrobacterium* strain and plant species:** *Agrobacterium tumefaciens* strain LBA4404 carrying a binary plasmid pCB4 was used in this experiment (Fig. 1). The plasmid contained *Bar* gene for herbicide resistance controlled by *CaMV 35S* constitutive promoters. The *A. tumefaciens* was cultured in YEP medium (10 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 15 g/L agar, pH 7.0, autoclaved). A two-day-old bacterial suspension was used at an OD_{600} of 0.80.

Transformation methods

Pollen tube pathway via ovarian injection method (method-I): Plasmid DNA possessing *Bar* gene was extracted from host bacteria by using alkaline lysis with SDS and stored in TE solution at pH 8.0. Such exogenous DNA solution was used to inject into the freshly pollinated ovaries (2nd day after flowering, identified by seeing to the withered petals). Style was cut, placed the needle point in the exactly center top, pierce the needle and took back little and then released the DNA solution taken in the syringe 5.0~6.0 µl. We placed few drops (1-2) of gibberrlic acid (GA_3 20ppm) over the injured part of the ovary.

Shoot apex culture method (Method-II)

Preparation of shoot apex explants: Shoot apices were prepared and isolated according to a method described by Gould *et al.*, (1998). Briefly 9 to 11-day old healthy germ free seedlings were selected. The shoot apex was exposed by pushed down one cotyledon until it broke away. Additional tissue was removed to expose the base of the shoot apex.

Agrobacterium co-cultivation: The *Agrobacterium* strains were cultured in YEP medium (contains 10g/L Bacto-Tryptone, 5g/L Yeast extract and 10g/L NaCl). 20ml of YEP medium plus antibiotics (50 mg/L kanamycin for strain LBA 4404) was inoculated with *Agrobacterium* and incubated in a 100ml flask overnight (about 17 hrs) on a shaker set for 180 to 220 rpm at 28°C. Then few drops of the overnight culture medium were withdrawn and used to inoculate 50 ml of YEP medium without antibiotics. Equal numbers of shoot apices were randomly distributed to two independent treatments, one with and another without *Agrobacterium* co-cultivation. Shoot apices were inoculated with *Agrobacterium* solution in a co-culture medium (MS + 100 µM acetosyringone) and incubated at 28 ± 2°C under dark conditions for 1 to 4 days.

Selection of transgenic plants: After co-cultivation, explants were treated with bactericide (homomycin) to remove the *Agrobacterium* and then washed three times with sterile distilled water. Cleaned apices were blotted using a sterile paper towel and cultured on the selection medium consisting of MS + 0.3 ml/L basta. Shoot apices not inoculated with *Agrobacterium* were planted on the selection medium as a negative control. The petri dishes were incubated at a temperature of 28 ± 2°C under 16hrs photoperiod and sub-cultured after every three week. The process was repeated until controls, not co-cultivated with *Agrobacterium*, were totally dead.

Putative transgenic plants: The surviving shoot apices were transferred to non-selective MS medium. They were then placed on shoot elongation and rooting medium (MS medium+0.1mg/L Kinetin) for two weeks to induce shooting and rooting (Gould *et al.*, 1991). After three weeks, rooted plants were transferred to soil and grown to maturity in the greenhouse.
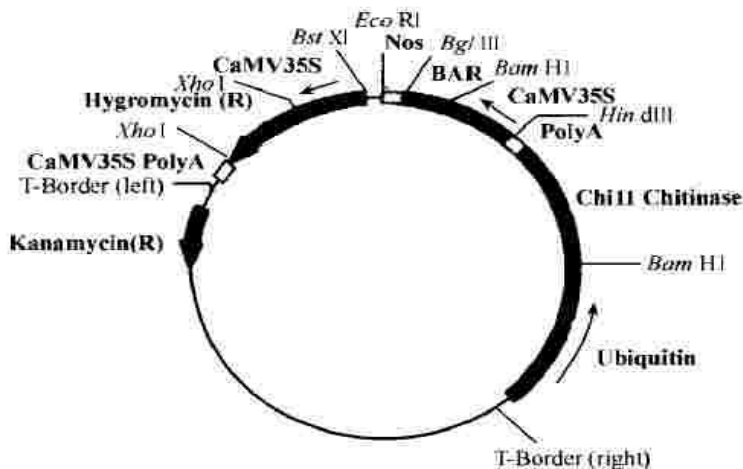
Fig.1. Gene map of the recombinant binary vector pCB4 carrying the gluphosinate herbicide-resistant gene (*Bar*) and *npt*II gene driven by CaMV35S promoter (P35S). LB: left border, RB: right border, *npt*II, neomycin phosphotransferase, Lines show restriction sites, *NOS* represents the polyA signal of CaMV35S as terminator.

Selection and screening of putative transgenic seedlings: In the putative transgenic plants, expression of *npt*II and *Bar* genes were also analyzed by kanamycin and basta tests. For the presence of *npt*II gene, leaves of both non-transformed and transformed were wet with cotton having kanamycin (50mg/L). For the presence of *Bar* gene, the lowest concentration of basta that would kill untransformed plants was established. The lowest level (0.3 mg/L) of basta was used to evaluate resistance to gluphosinate. Leaves of the putative transgenic plants were applied basta by cotton swab. Plants were evaluated for resistance 7 days after leaf application of basta.

Polymerase chain reaction (PCR) analysis : Genomic DNA was isolated from young leaves of putative transgenic plants by using cetyl-trimethyl ammonium bromide (CTAB) method as described by Paterson *et al.,* (1993). The DNA samples were tested for the presence of T-DNA region using a pair of *Bar gene* specific primers (primer 1: *5'-CAG GAA CCG CAG GAG TGG A -3'* and primer 2: *5'- CCA GAA ACC CAC GTC ATG CC-3*) to amplify the 470 bp fragments. The PCR reaction mixture was prepared as described by Altaf *et al.*, (1997). The 25 µL amplification mixture contained 2.5 µL 10x PCR II buffer (50mM Tris (pH 8.3) 500 mM KCl);15 mM MgCl$_2$; 1.0 mM dNTP mix (*Sangon Biotech (P) Ltd*); 0.2 µM primer; 1.0 unit of AmpliTaq DNA polymerase (*Sangon Biotech (P) Ltd*); and 100 ng of genomic DNA as template.

DNA was amplified in a eppendorf thermocycler, programmed for a first denaturation step of 2 minutes at 94ºC followed by 35 cycles at 94ºC for 1 minute, at 59ºC for 1 minute, and at 72ºC for 1 minutes. A final extension at 72ºC was carried out for 10 minutes. The PCR products were then stored at 4ºC until electrophoresis was done. PCR products were separated on a 1.2 % agarose gel prepared with 0.5x TBE buffer. The sample were subjected to electrophoresis at 120V for 1 hr in 0.5x TBE buffer. The gel was stained with sybr gold and visualized under ultraviolet light.

Southern blot hybridization analysis: Southern blot hybridization analysis was carried out using standard procedures (Sambrook *et al*., 1989). Total genomic DNA was isolated from young leaves of putative transgenic plants and untransformed (control) plants as mentioned earlier and completely digested with *EcoRI*. Based on the construct of the plasmid, *EcoRI* digested genomic DNA will result in a 1.62 Kb fragment in transformed plants. Fifteen µg of genomic DNA was digested with *EcoRI* kept overnight at a 37ºC. The digested DNA fragments were electrophoresed on 0.8% agarose gel in 0.5x Tris-Borate-EDTA (TBE) buffer, and transferred to a nylon membrane (*Sangon (P) Ltd*) by the alkaline transfer method (Reed & Mann, 1985). The $[^{32}P]$ - labeled probes for transformed plants were made from a 0.59-kb polymerase chain reaction (PCR) product containing the *Bar* coding region. The band was excised from agarose gel and purified using 5x Denhardt's reagent at 65ºC for 2 hrs. Then hybridized *Bar* gene probe labeled with $[^{32}P]$ dCTP by random priming method. After hybridization, the membrane was washed twice for 15 min each in 2x SSC+0.1% Sodium Dodecyl Sulfate (SDS) at room temperature; once in 1x SSC + 0.1% SDS at 55ºC for 30 min and twice with 0.1x SSC without SDS for 10 min. The blots were exposed to *Kodak BiomaX-Omat* film at -75ºC with two intensifying screens for autoradiography after 24 h exposure in the cassette.

Progeny tests: To test the functional expression of the *Bar* gene in the T_0 progeny, a germination test was performed. At least 20 seeds collected from T_0 plants of each of the two methods after selfing and those from untransformed plants were germinated on medium containing 0.3 ml/L of basta. After 3 generations of selection for herbicide resistance, a pure and stable upland cotton germplasm with herbicide resistant *Bar* gene was obtained in 2006 and was named BR001.

Identification of herbicide resistance: For the identification of herbicide resistance of BR001, basta was applied @ 20 ml/L and 30 ml/L on both putative transgenic BR001 and its parent line at fourth leaf stage.

Inheritance of the herbicide resistant trait in transgenic cotton germplasm (BR001): The inheritance behavior was studied in the basta resistant transgenic cotton cultivar (BR001) in two steps: In one step, BR001 was crossed with a genetic standard line (TM-1) to produce F_1, then selfed to produce F_2 and backcrossed with TM-1 to produce BC1. And in another step, BR001 was crossed with its parent line (Coker 312), then selfed and backcrossed to produce F_1, F_2 and BC_1 as mentioned above. Basta (0.3 ml/L) was applied at the seedling stage. Fourteen days after treatment, dead and surviving seedlings were counted and were subjected to segregation analysis.

Agronomic characters and fiber quality of the resultant transgenic germplasm: Experiments were conducted in 2007 in Hangzhou. A well designed field experiment was conducted to analyze the agronomic characters and fiber quality of BR001, using Coker 312 as control. The experiment was in a complete random manner with three replications. A sample of 30 bolls was collected from each plot. These samples were weighed and ginned to determine boll weight and lint percentage. The lint samples were sent to TCCQ (Test Center of Cotton Quality, Ministry of Agriculture, Anyang, Henan, China) for determination of fiber quality such as 2.5% staple length (Len), uniformity index (UI), micronaire (Mic), elongation (El), and fiber strength (Str).

Data analysis: Chi-square goodness-of-fit test was applied on data of the F_1, F_2 and BC_1 populations derived from crosses between basta resistant cultivar (BR001), its parent line (Coker 312) and genetic standard line (TM-1). Simple one-way analysis of variance (ANOVA) was performed using SAS V.9.0 package to determine the statistical significance at 5% probability level. All the results were expressed as means ± SE. Means were separated by Least Significant Difference (LSD) test.

Results

Kanamycin test for the presence of *npt*II gene: The putative transgenic plants developed using both the methods were also tested for the presence of *npt*II gene (Table 1). The leaves of both non-transformed and transformed showed clear symptoms of kanamycin application after one week of application. The leaves of non-transformed plants wilted and withered, while those of transformed plants remained healthy.

Bioassay test for basta® and comparative analysis of the two transformation methods: The bioassay test for herbicide tolerance in both transgenic and non-transgenic field grown plants was evaluated after one week of application of basta® @ 0.3ml/L. The leaves of untransformed plants turned mottled, while those of transformed plants were having no such symptoms. Furthermore, the comparative analysis of both transformation methods revealed that both methods could be opted for genetic transformations in cotton, however, regarding basta selection, transformation efficiency, and percentage of plants established *Agrobacterium*-mediated shoot apex culture method proved to be highly efficient method as compared to Pollen Tube Pathway via ovarian injection method (Table 2).

PCR analysis: PCR analysis of the putative transgenic cotton plants of the cultivar Coker 312 from both methods of transformation revealed that the expected 430bp product was successfully integrated into the cotton genome (Fig. 2).

Southern blot hybridization: Fig. 3 shows the southern blot hybridization analysis of the leaf samples from putative transgenic plants, non-transgenic plants and plasmid pCB4. Hybridization of the *bar* with 1.62kb fragment was detected in four transgenic plants. This was consistent with the restriction map of pCB4, which has two EcoRI sites, separated by 1.62 kb, which flank the 35S-bar-NOS gene. This result also confirmed the PCR results and indicated the integration of T-DNA region in the transgenic plant genome. No variation in number of copies of the *bar* gene was observed between the two transgenic plants examined. No hybridization was detected in the non-transgenic control plants.

In lane 3, the dot was much darker and bigger than the others, it could be suggested that there might be more than one copy of the foreign gene in this plant. Due to two site of *EcoR I* in the plasmid, the *EcoR I* is not useful in southern blotting experiment as it cannot express the copy number in the transgenic plant.

Analysis of T_1 plants: The seed germination test of the T_1 generation was done for both non-transformed and transformed plants on basta® containing medium for both transformation methods (Table 3). The test revealed that none of the seeds from non-transformed plants germinated on medium containing basta®. For the transformed lines tested, the ratio of resistant to sensitive was found to be approximately 3:1, which is the expected ratio for a single dominant gene in a self-pollinated population. All χ2 values indicated significant fit to 3:1 ratio tested at 5% probability level.

Table 1. Screening of T_1 generation plants for kanamycin resistance.

*Methods	**Flowers/ explants	***Kn^r selection	*NptII* gene transformation efficiency	Total plants established in soil	% Age of plants established in soil
Method-I	714	27	3.78	25	3.50
Method-II	720	356	49.44	33	4.58
Control	94	0	0	0	0

*Method-I: Pollen Tube Pathway *via* ovarian injection, Method-II: Shoot apex culture; ** Total number of flowers/explants treated with kanamycin; *** No. of seeds/explants selected after 3 cycles of kanamycin®

Table 2. Comparative analysis of two transformation methods regarding transformation efficiency and the number of plants established in soil.

*Methods	**Flowers/ explants	***Basta® selection	Transformation efficiency	Total plants established in soil	Plants established in soil (%)
Method-I	923	34	3.68	33	3.57
Method-II	916	463	50.54	37	4.03
Control	90	0	0	0	0

*Method-I: Pollen Tube Pathway via ovarian injection, Method-II: Shoot apex culture; ** Total number of flowers/explants treated with Basta®; *** No. of seeds/explants selected after 3 cycles of Basta® selection;

Table 3. Segregation analysis of T_1 transformed plants.

*Methods	Seeds tested	**Seeds germinated on Basta® medium		**$\chi 2$ value
		(-)	(+)	
Method-I	27	23	4	0.28
Method-II	29	19	10	0.26
Control	25	0	25	/

*Method-I: Pollen tube pathway *via* ovarian injection, Method-II: Shoot apex culture; **Basta concentration of 0.3 ml/L was used in the medium; ***All X^2 values indicate significant fit to 3:1 ratio tested at *P*=0.05 level.
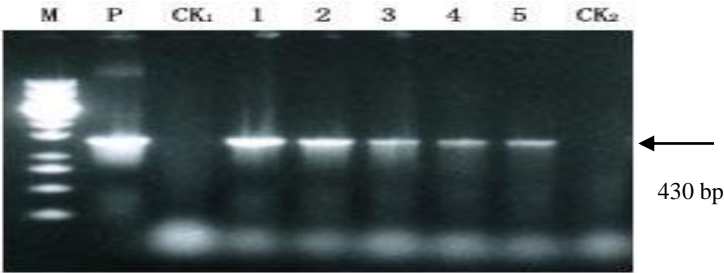

Fig. 2. PCR analysis of putative transgenic plants showing integration of *Bar* gene. M: Marker of 1kb, P: Positive control; CK1, CK2: Negative control from non-transgenic control plants. 1-5: Integration of *bar* gene.
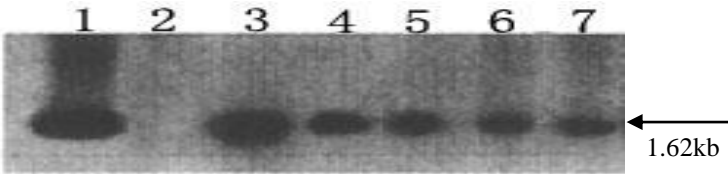

Fig. 3. Southern blot analysis of transgenic plants for integration of the *bar* gene. Lane 1: undigested plasmid DNA (positive control); Lane 2: DNA sample from non-transgenic control plant; Lanes 3-7: DNA samples from putative transgenic plants. Lane 3: DNA samples from putative transgenic plants resulted from method-II, Lanes 4-7: DNA samples from putative transgenic plants resulted from method-I.

Inheritance and segregation of exogenous *Bar* gene in transgenic cotton germplasm (BR001): To elucidate the inheritance of herbicide trait of BR001, a genetic design and analysis for the trait was performed (Table 4). The F_1 plants from the crosses of BR001×TM-1 (75 plants) and TM-1×BR001 (49 plants), BR001×Coker 312 (41 plants) and Coker 312×BR001 (45 plants) were all tolerant to gluphosinate, which indicated that this trait was a dominant one without the effect of cytoplasm. The number of tolerant plants was 215, and that of susceptible ones was 80 in F_2 plants population derived from the combination of BR001×TM-1, which were consistent to 3:1 segregation ratio (χ^2=0.706, p>0.10). The segregation of the tolerant and susceptible in F_2 plants from Coker 312×BR001 was confirmed to the ratio 3:1 as well (χ^2=0.292, p>0.10). The ratio from backcross of (BR001×TM-1)×TM-1 and (BR001×Coker 312)×Coker 312 were consistent with 1:1 segregation ratio, according to the χ^2 statistics. Moreover, the table further reveals that all kind of F_2 population derived from various crosses showed monogenic pattern of inheritance.

Identification of herbicide resistance: For the identification of herbicide resistance in BR001, basta was applied @ 20 and 30 ml/L on both putative transgenic BR001 and its parent line at fourth leaf stage. The mean data in Table 5 showed that when basta was sprayed @ 20ml/L, the leaves appeared normal and were quite resistant in BR001, while when it was applied @ 30ml/L, the leaves wilted but recovered tolerance. However, in case of Coker 312, the leaves became wilted irrespective of the rate of application of basta.

Agronomic characters and fiber quality of BR001: The agronomic and fiber quality traits of BR001 are shown in Table 6. According to the gathered information all the agronomic traits and fiber quality of BR001 were similar to its genetic background germplasm (Coker 312). The results further revealed that the herbicide resistant gene (i.e. Bar) did not significantly affect the yield, agronomic characters and fiber quality.

Discussion

In any transformation method, the ultimate goal is to obtain fertile transgenic plants with desirable foreign gene(s). However, the main problem with different transformation methods is variable transformation efficiency. Presently, a number of transformation techniques/ methods are used in plants, which utilize both *in vitro* and *in vivo* means. The *in vivo* methods of transformation are genotype-independent and are time saving and cost effective . In our present study, we implied two genotype-independent approaches i.e., Pollen Tube Pathway *via* Ovarian Injection and Shoot Apex Culture. Our main goal was to obtain basta resistant transgenic cotton cultivar, to compare the two approaches regarding the number of transgenic plants obtained and as well as to study the inheritance and segregation behavior of the exogenous gene (*Bar*) in the resultant transgenic cotton cultivar (BR001).

The main advantages of both methods are that transgenic plants can be obtained easily and quickly. The choice of the shoot apex culture is based on the fact that the meristematic cells are rapidly dividing and the gene of interest can be easily inserted into the genome of cotton using *Agrobacterium,* which cause infection. Also, it has been reported that the incidence of genetic mutations and somaclonal variation was very low in plants regenerated from shoots (Gould *et al*., 1998). The choice of pollen tube pathway transformation stems from the underlying assumption that at fertilization stage the egg cell accepts the donation of entire genome from the sperm cell and it might thus be appropriate stage to deliver transgenes. This concept was demonstrated by the successful transformation method developed for *Arabidopsis* (Bechtold *et al.*, 1993). In cotton, pollen tube pathway *via* ovarian injection was first reported by Zhou *et al*., (1983). However, it did not gain so much importance because of the low transformation efficiency.

Table 4. Inheritance of herbicide resistance in BR001 14 days after treatment with 0.3ml/L basta

Cross	Tolerant plants	Susceptible plants	χ^2	Probability
(BR001×TM-1)F$_1$	75	0	/	/
(BR001×TM-1)F$_2$	215	80	0.706(3:1)	0.5~0.75
(BR001×TM-1) ×TM-1	81	77	0.101(1:1)	0.75~0.9
(TM-1×BR001)F$_1$	49	0	/	/
(TM-1×BR001)F$_2$	225	84	0.786(3:1)	0.25~0.5
(BR001×Coker312)F$_1$	41	0	/	/
(BR001×Coker312)F$_2$	215	66	0.343(3:1)	0.5~0.75
(BR001×Coker312)×Coker 312	225	215	0.227(1:1)	0.5~0.75
(Coker312×BR001)F$_1$	45	0	/	/
(Coker312×BR001)F$_2$	189	68	0.292(3:1)	0.5~0.75

Table 5. The results of the identification for the resistance to BASTA in BR001.

Materials	Year	Concentration (ml/L)	Results
BR001	2005	20	Normal, resistant
BR001	2005	30	Wilted but recovered tolerance
BR001	2006	20	Normal
Coker 312	2005	20	Wilted, defoliation, susceptible
Coker 312	2005	30	Wilted, defoliation, susceptible
Coker 312	2006	20	Wilted, defoliation, susceptible

Table 6. Agronomic and fiber quality traits of BR001 and its parent line (Coker 312).

Traits	BR001	Coker 312 (CK)
Seed yield (Kg/ha)	3663.33 ± 125.65a	3798.00 ± 171.56a
Lint yield (Kg/ha)	1494.63 ± 31.29a	1524.50 ± 91.00a
Lint (%)	40.83 ± 0.64a	40.10 ± 0.91a
Boll weight (g)	5.53 ± 0.03a	5.60 ± 0.12a
Seed index (g)	10.67 ± 0.24a	11.00 ± 0.64a
Staple length (mm)	31.03 ± 0.12a	29.93 ± 0.30b
Fiber uniform (%)	49.27 ± 0.56a	50.37 ± 0.49a
Fiber strength (cN/tex)	23.17 ± 0.52a	22.33 ± 0.39a
EI (%)	7.70 ± 0.06a	7.13 ± 0.20a
MIC	4.77 ± 0.15a	4.87 ± 0.13a

Values are the means ± SE of the three replicates. Variants possessing the same letter are not statistically same at 5% probability level.

In our transformation study, we were able to obtain transgenic cultivar, which was named as BR001. The PCR and Southern blot hybridization analyses revealed the successful integration of this exogenous gene in the genome of Coker 312. The kanamycin and basta bioassays also showed that putative transgenic plants were highly resistant against these chemicals.

We further studied various agronomic and fiber quality traits in both BR001 and its parent line. The studies revealed that there were no significant differences regarding these traits between BR001 and its background parent line. Also, the classical studies like

segregation analysis and inheritance behavior confirmed that *Bar* gene segregated as simple Mendelian traits. This finding is in line of Perlak *et al*., (1990) and Zhang *et al*., (2005). However, Zeng *et al*., (1998) reported no evidence of marker genes in progeny of transgenic wheat obtained using the pollen tube method.

Conclusions

The use of cotton transformation in breeding programs requires the production of multiple transgenic homozygous plants that will display stable transgene inheritance. Based on our findings, we can conclude that both methods were having some advantages and disadvantages over each other. For example, as shown in the present experiment, the early steps of the pollen tube pathway are very simple but a large number of homozygous transformants are needed for screening . For the shoot apex culture based transformation approach, though much time and resources are required, the transformation efficiency and number of plants that were successfully established in the field were higher than the pollen tube pathway method. However, regarding the transgenic expression analysis via PCR and southern blot hybridization and Mendelian law of segregation, it is evident that both methods of transformation were almost at par with each other.

Furthermore, our field studies demonstrated that the agronomic and fiber quality traits were almost the same in both BR001 and its parent line (Coker 312). Also the *Bar* gene was inherited in a Mendelian fashion and only a single dominant nuclear gene was involved. There could be found no role of cytoplasm in inheritance of this trait. Moreover, conventional breeding could be exercised in order to introgress the exogenous resistance genes of the transformant into conventional cotton varieties, and thus new cotton cultivars carrying desired characters could be bred.

Acknowledgements

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