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Transgenic Indian Cotton (*Gossypium hirsutum*) Harboring Rice Chitinase Gene (Chi II) Confers Resistance to Two Fungal Pathogens

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Abstract: Problem statement: The present investigation described a simple and reproducible protocol for transgenic cotton regeneration and characterization of chitinase (Chi II) gene expression against two different fungal pathogens in cotton. **Approach:** Transgenic cotton (*Gossypium hirsutum* cv. SVPR2) plants were produced by pCambia-bar-Chi II (13.8 kb) under the control of the CaMV 35S promoter, harbored in the strain LBA 4404 *Agrobacterium tumefaciens* by using shoot tip explants. **Results:** Finally, from the 10 experiments, 21.8% of transformation frequency was recorded. Segregation ratio of 3:1 was recorded in the T_0 plant seeds. Polymerase chain reaction and southern blotting analysis were used to confirm the integration of Chi II transgene in the T_0 plants genome of putative transgenics. Quantitiave and qualitative (SDS-PAGE) analyses were also carried out to confirm the expression of chitinase enzyme in T_0 plants. Further, randomly selected transgenic plants (T_0) were analyzed for disease tolerance by evaluating them with spores of *Fusarium oxysporum* and *Alternaria macrospora*. All the selected PCR positive plants showed enhanced disease resistance against *Fusarium* wilt. The plants selected randomly showed an enhanced survival rate compared with the control when they were grown in earthen pots inoculated with 1×10^5 spores 100^{-1} g of soil mixture. Another four randomly selected plantlets were sprayed with spores of *Alternaria macrospora* in order to test their tolerance to Alternaria leaf spot disease. After 20 days of culture, the number of lesions per leaf and the lesion length per leaf spot in non-transferred leaves increased. In the case of transgenic plantlets, lesion formation was completely absent. **Conclusion:** The disease resistance against *Fusarium* wilt and *Alternaria* leaf spot in cotton strains would serve as good breeding materials for producing fungal disease resistant cotton varieties.

Key words: Cotton, transformation, shoots tip culture, phosphinothricin, chitinase gene, *Fusarium* wilt, alternaria leaf spot, disease resistance

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) has been estimated that 180 million people depend on cotton fiber production. Apart from the fiber production, cotton cultivation was also used for the production of gossypol due to its wide range of biological properties including anti-cancer, antimicrobial, anti-HIV, anti-oxidative and male contraceptive activities[1]. Among the cotton producing countries, India ranks first in cultivation, with 32 of the world's total area followed by USA (23) and China (20%)[2]. Unfortunately, in the production rate, India ranks third. This is due to the non-availability of genetically modified superior genotypes with desired traits. In Indian varieties, the yield of cotton was significantly affected by several biotic and abiotic factors, particularly by insect pests and fungal pathogens. However, the chemical control of pests and diseases has not always been effective, resulting in crop failure or heavy reduction in yield. In the cotton market, before 1996, a 29% loss in the total income was accounted for by these insect pests and

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diseases per year. After the introduction of Bt cotton, the yield loss caused by insect pests was significantly controlled[3]. In India also Bt cotton is being field tested for insect pest control.

Apart from the insect pests, the fungal pathogens cause an 8-12% loss in the annual total yield. The fungal diseases like *Fusarium* wilt (*F. oxysporum*), Verticillium wilt (*Verticillium dahliae*) and Alternaria leaf spot (*Alternaria macrospora*) of cotton, causes wilt and lesions on whole plant[4]. Hence, heavy loss in the yield of fiber is regularly observed. While the insect resistant transgenic cotton has made a great impact on cotton cultivation around the world, fungal disease resistant transgenic cotton has not reached the world market yet. The above concerns have led to genetic engineering of cotton for improved fungal resistance.

In the present investigation, we planned to use rice chitinase gene for the fungal resistant cotton production. Among various fungal resistant genes, chitinase genes are potentially most promising as the enzymes degrade the substrate chitin found in the fungal cell wall. Hence, genetic engineering of plants with chitinase gene is attractive for fungal disease control mechanism. Several reviews and research articles have also stressed the advantages of using chitinases for plant protection because these enzymes are fungicidal and part of the plant defense system and not harmful to plants[5]. Various protocols have been explored for the transformation of cotton such as meristem transformation[6], particle bombardment[7] and *Agrobacterium*-mediated transformation[8]. Of these methods, only the *Agrobacterium*-mediated and microprojectile bombardment methods are routinely used in cotton transformation studies[9]. *Agrobacterium*-mediated transformation *via* somatic embryogenesis has been the most common method for transgenic cotton development. It is a multi-step process involving labor-intensive work over a 10-12 month period starting from co-cultivation of *Agrobacterium* culture with explants followed by production and maintenance of hundreds of calli derived from independent transformation events, induction of somatic embryos and development of somatic embryos into normal plantlets[10]. In this procedure, the transformation efficiencies are generally low due to the low frequency of embryogenesis and the difficulty in germination of transformed embryos[11]. Compared with somatic embryogenesis, the shoot tip mediated regeneration techniques are easy and less time-consuming process[12]. In recent years, there has been increasing focus on the use of meristems and shoot axes as the source of tissue explants for transgenic cotton production[13-18]. Hence, in the present investigation, we targeted to produce the cotton plantlets with chitinase gene through *Agrobacterium*-mediated transformation protocol by using shoot tip culture technique.

MATERIALS AND METHODS

Plant regeneration protocol: Plant regeneration was achieved by our earlier method by using shoot tip culture[12]. In this protocol, the influence of different forms of cytokinins, auxins and polyamines were tested for mass multiplication and regeneration of cotton. In the above protocol, media fortified with MS salts; B5 vitamins; 30 g L^{-1}, glucose; 2.0, 2iP; 2.0, IAA 20 mg L^{-1} putrescine and 0.7% agar showed a superior response for multiple shoot induction from shoot tip explants. Elongation of shoots was achieved on multiple shoot induction medium itself. Significant numbers of roots were initiated in the medium supplemented with MS salts, vitamin B5, IBA (2.0) and PVP (10 mg L^{-1}). These plantlets were hardened by using sand, soil and vermiculate in 1:1:1 ratio. The hardened plants were transferred to the environmental growth chamber for proper acclimatization. The hardened plants were then transferred to field for boll yielding and they exhibited 95% survival rate.

Transformation experiments:

Pre-culture of explants: Pre-culture is an important step involved in *Agrobacterium*-mediated transformation. The process of pre-incubation makes the explant tissue competent enough to withstand the bacterial infection and other related stress caused during the preculture period *in vitro*. The shoot tip explants were pre-cultured on the multiple shoot induction medium, for 1-6 days prior to selection on the Phosphinothricin (PPT) containing medium. The sensitivity of the shoot tip explants to PPT was determined by culturing the explants in multiple shoot induction medium along with PPT at different concentrations (1-10 mg L^{-1}).

Agrobacterium strain and plasmid: Construct pCambia-bar-Chi II (13.8 kb) harbored in strain LBA 4404 of *Agrobacterium tumefaciens* was used (Fig. 1). Selectable markers were the phosphinothricin acetyl transferase gene (bar) and the hygromycin phosphotransferase (hpt). Both selectable marker genes were driven by the cauliflower mosaic virus (CaMV) 35S promoter. The chitinase gene, Chi 11 (1.1 kb), was controlled by the maize ubiquitin 1 (ubi1) promoter (2.0 kb). The ubi-chi11 fragment (3.1 kb) was released by digesting the construct with Hind III.

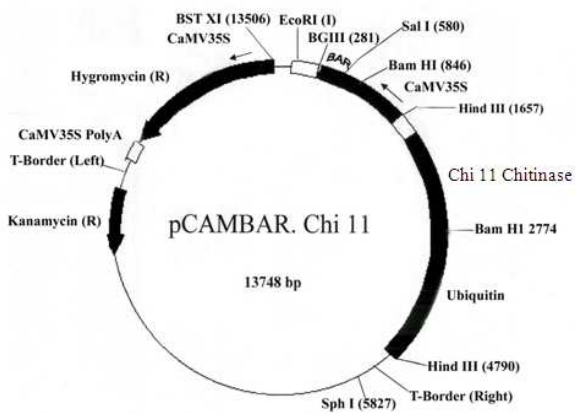

Fig. 1: Diagrammatic representation of complete map of pCAMBIA Bar = Ubi-Chi 11 transforming vector used in this study for fungal disease resistant cotton production

The remaining part of the construct was 10.7 kb in size. After 6 h, the cultures were collected and used for transformation experiments.

Co-cultivation and selection of stable transformants: The *Agrobacterium* strains were cultured in LB medium (contains 10 Bacto Tryptone, Bacto, 5 Yeast extract and 10 g L^{-1} NaCl). Twenty mL of LB medium plus antibiotics (50 kanamycin and 50 mg L^{-1} cefotaxime for strain LBA 4404) was inoculated with *Agrobacterium* and incubated in a 100 mL Erlenmeyer flask overnight (about 8 h) on a shaker set for 180 to 220 rpm at 28°C. Then 2 mL of the overnight culture was withdrawn and used to inoculate 50 mL of LB medium without antibiotics. After incubation for 4-6 h at 28°C with shaking, those cultures were diluted with additional LB medium to a concentration (OD600 1.0) for transformation. Shoot apices were inoculated by placing one drop of *Agrobacterium* solution onto each shoot apex in co-culture medium and incubating at 28°C under dark conditions for 1-6 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 1-6 mg L^{-1} PPT. Shoot apices not inoculated with *Agrobacterium* were plated on the selection medium as a negative control. The cultures were incubated at a temperature of 28°C under an 18 h photoperiod and sub-cultured every week. The process was repeated until controls (not co-cultivated with *Agrobacterium*) were died. The surviving shoot apices were transferred to an MS medium without PPT to root the plantlets. Rooted plants were then transferred to soil and grown to maturity in an environmental growth chamber[12]. These plantlets were selected for further characterization of transgene expression.

Analysis of T_0 plants: To test the functional expression of the Chi II gene in the T_1 progeny, a germination test was performed. Twenty to thirty T_0 seeds were collected from self-pollinated transgenic lines (T_0) as well as non-transgenic plants (control). T_0 seeds were germinated on MS basal medium supplemented with 5.0 mg L^{-1} PPT.

PCR amplification of transgene: The total DNA was extracted from leaves of cotton plant WITH CTAB (cetyl trimethyl ammonium bromide). The isolated precipitate form of nucleic acid contains both DNA and RNA. The RNA was removed by RNAse solution (0.2 mg mL^{-1}) at room temperature for 15 min. DNA was further purified with phenol/chloroform extraction and ethanol precipitation methods. The total DNA was isolated from *A. tumefaciens* and estimated by using calf thymus DNA as a standard. The plasmid DNA samples were digested with *Hind III* for 2-4 h and the plant genomic DNA samples were digested for 8-10 h: Double digestions were carried out by second digestion after the contents of the first reaction were extracted with neutral phenol/chloroform water saturated ether and precipitated with ethanol. DNA isolated from young leaves of putative transgenic plants was used for the PCR analysis. The DNA samples were tested for the presence of the T-DNA region using a pair of chi II specific primers (F) 5N-GCTTCTACACCTACGACGCCTT-3N, (R) 5N-GTAGCGCTTGTAGAACCCGATC-3NO to yield a 584-bp fragment to amplify the 584- bp nptII fragments. DNA was amplified in a Eppendorf PCR System, programmed for a first denaturation step of 2 min at 94°C followed by 45 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 2 min. After the completion of 45 cycles, a final extension at 72°C was carried out for 5 min. The completed reactions were then held at 4°C until electrophoresis was done. PCR products were separated by loading 12 of sample and 2 μL of loading buffer on a 1.2% agarose gel prepared with 1.0X TBE buffer. Electrophoresis was preformed at 4-8 v cm^{-1} in 1×TBE or buffer and upon completion of the run: DNA in the gel was stained with ethidium bromide (0.5 Jg mL^{-1}) and viewed under UV (PDQuest-BioRad).

DNA blot analysis: DNA was extracted as described earlier and polysaccharides were removed with 2 M NaCl and ethanol. The DNA was digested with HindIII and electrophoresed on a 0.7% agarose gel[19]. DNA

fragments were transferred to a nylon membrane (Hybond-N+, Boehringer, Laval, Quebec, Canada) and hybridized with a digoxigenin-labeled ubi1-Chi11 fragment to detect presence of the Chi11 gene. The remainder of the construct (pCambia-bar-chi11), after the release of ubi1-Chi11, was labeled with[32] to hybridize with the other part of the DNA sequence in T-DNA.

Quantitative and qualitative measurements of chitinase: Total proteins were isolated from the young leaves of 30-day-old putative transgenic and control plants[20] and stored at-20°C until use. Analysis by SDS-PAGE was carried out by using 1 mm thick macro gels. Ten micrograms of total proteins were loaded and electrophoresed for approximately 2 h at 150 V. The gels were then stained with colloidal Coomassie blue (Himedia India Ltd, Mumbai, India) and photographed using a Kodak digital camera[21]. Quantitative analysis of chitinase was determined by the leaf specific method[22]. In this method, 500 mg of young leaves (30-day-old plants) of each selected plantlets were homogenized on 0.02 M citric acid/0.04 M sodium phosphate buffer (pH 6.8) and centrifuged at 15,000 rpm for 15 min. For the chitinase reaction, 2 mL of 0.05 M citric acid/0.1 M sodium phosphate buffer (pH 6.8) containing 20 mg of carboxymethyl chitin (Himedia India Ltd, Mumbai, India) was mixed with 1 mL of the crude enzyme solution, incubated with shaking at 37°C for 1 h and the reaction stopped by the addition of 1 mL of trichloroacetic acid. After centrifugation at 15,000 rpm for 10 min, the concentration of reducing sugars in the supernatant was measured by the Schales method. One unit was defined as the amount of chitinase which produces 1 μmoL of reducing sugars as N-acetyl- D-glycosamine min^{-1}.

Bioassay for fungal disease resistance:

Test for *Fusarium* resistance: Macrospores of *Fusarium oxysporum* were produced by culturing the fungus, on Czapek yeast extract agar medium. After 14 days of growth, macrospores were harvested by washing the culture surface with 10-20 mL of distilled water per petri dish using a pressurized hand sprayer. The suspension of spores and mycelial fragments was filtered through one layer of cheesecloth and spore concentrations adjusted to 1×10^5 spores mL^{-1}. Thirty-day-old fully regenerated plants with tertiary roots were hardened in the earthen pots containing soil mixture and fungal spores (1×105 spores/100 g of soil mixture)[23]. Each plantlet was covered with the plastic bags to prevent inadvertent spore dispersal. The *Fusarium* wilt of the plants was identified by yellowing of leaves and vascular browning.

Alternaria leaf spot bioassay: Plantlets regenerated through normal tissue culture conditions were selected as control. The resistance of selected and regenerated control plants to *A. macrospora* was assessed by spraying of spores (2×10^5 spores mL^{-1}) on leaves and maintained under green house conditions without disturbance[24]. During maintenance, each plantlet was covered with a polythene bag without touching the leaves. These plants were incubated under normal green house condition. For every 12 h, pathogenecity of *A. macrospora* was recorded by measuring the lesion area and numbers. After a week, variations in lesion number and size were carefully measured.

Statistical analysis of data: Means and standard errors were used in presenting the data here and the values were assessed by using a parametric Moods median test[25]. The data were analyzed for variance by Duncan's Multiple Range Test (DMRT) using the SAS program (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Factors analyzed for high efficiency transformation:

Pre-culture of explants: The transformation efficiency can be increased by manipulating either by the explant and/or by the bacterium to enhance virulence. Such manipulations are based on either increasing the number of competent plant cells for transformation by pre-culturing explants or improving the induction of the vir genes by using pre-culture of explants[26]. Hence pre-culture of explants must be examined to improve the transformation frequency. During transformation, pre-culture of explants on shoot multiplication and regeneration medium prior to co-cultivation was considered as one of the essential processes. In this experiment, pre-culture of explants in the regeneration medium for 3 days was found to be good for high percentage of response (72.4%). Whereas, the explants, which are not pre-cultured, showed only a 24% response (Fig. 2a).

The impact of selection agent: The use of proper type and concentration of a selection agent in the selection medium is essential to enhance transformation frequency, in which the selection agent that allows only transformed cells or plants to survive. PPT has been extensively used as a selective antibiotic in transformation experiments, mainly because several plant transformation vectors include bar gene as selectable marker. Initially the explants were placed on shoot tip multiplication medium, which contained different concentrations of PPT (1-6 mg L^{-1}). The

cultures, which are not supplemented with any selection agents, were considered as controls. Our results obtained showed that increased concentrations of PPT led to a gradual decline in the shoot regeneration frequency. The lowest frequency of shoot regeneration was noticed on 4 mg L^{-1} of PPT (4.8%). In 5 mg L^{-1} of PPT treatment, absence of shoot formation and explant growth was noticed. Hence, 5 mg L^{-1} of PPT treatment was considered for selection procedures. The bar gene encodes Phosphinothricin Acetyl Transferase (PAT) that detoxifies PPT by catalyzing the addition of an acetyl group to the free amino group. The efficacy of PPT as a selection marker and bar as a resistance mechanism has resulted in bar being widely exploited in the construction and selection of transgenic plant lines. Similar to our observations, bar gene, which provides resistance to herbicide PPT, has been used successfully as a selectable marker in several plants[27].

Effect of co-cultivation conditions: Transformation efficiency of any explants can be enhanced by co-cultivation on shoot regeneration medium at different period (0-6 days). Among them, 3 days co-cultivated shoot tip explants produced significantly higher rate of transformation (60.1%) when compared with 0 days and 6 days of co-cultivation. The explant viability was affected after 3 days co-cultivation period (Fig. 2b). Influence of co-cultivation period on *Agrobacterium*-mediated transformation has also been reported in a number of plant species[28]. Similar to our results, in these studies co-cultivation of explants for 2-3 days generally provided the best transformation frequency. However, prolonged co-cultivation periods of 6-7 days increased transformation efficiency in flax[29] and a 5 day co-cultivation was the most effective for transient GUS expression in citrange plants[30]. The effectiveness of prolonged co-cultivation in some plant species might be due to the increased number of induced bacteria attaching to plant cells and/or the increased number of plant cells competent for *Agrobacterium*-mediated transformation[29]. During co-cultivation, we also evaluated the effect of different concentrations of bacterial concentration (0.75-1.3 OD cultures) on transformation frequency. From the different concentrations, 1.0 OD culture showed a superior response (70.2%) and unfortunately above 1.1 OD culture showed a decline in the percentage of response and the explants turned to necrotic and devoid of shoot regeneration due to excessive growth of the bacteria (Fig. 2c). Although cotton transformation rates have been significantly improved, increasing its efficiency on plant recovery is still needed[30]. Many factors such as plant genotype, explants type, pH, co-cultivation media,

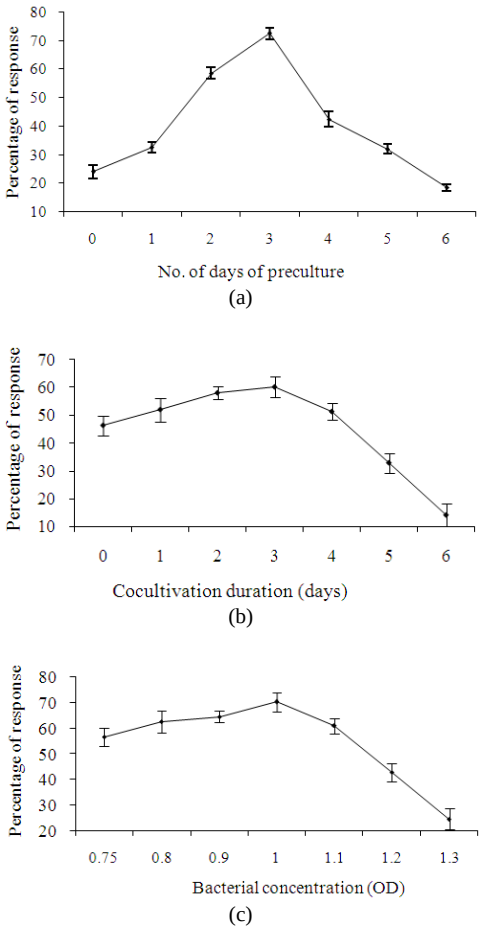

Fig. 2: The effect of pre-culture days (a): Co-culture duration; (b): And bacterial concentration; (c): On growth response and transformation frequency of shoot tip explants

temperature and period influence the gene transfer efficiency from *Agrobacterium* to plant cells and the above external factors play a critical role in T-DNA transfer mechanism in particularly vir gene activation[31].

Selection and characterization of transformants: The shoot apices were co-cultivated with *A. tumefaciens* LBA4404 for 3 days. After co-cultivation, the shoot apices were transferred to shoot bud regeneration medium with 5 mg L^{-1} PPT. Under PPT selection (bar gene), the most of the shoots appeared to be bleached and some of the shoots that were initially green and bleached out gradually, leaving only a few green shoots. After 2 weeks, these green shoot apices were transferred to fresh media for every 2 weeks (Fig. 3a and b). In the PPT supplemented media,

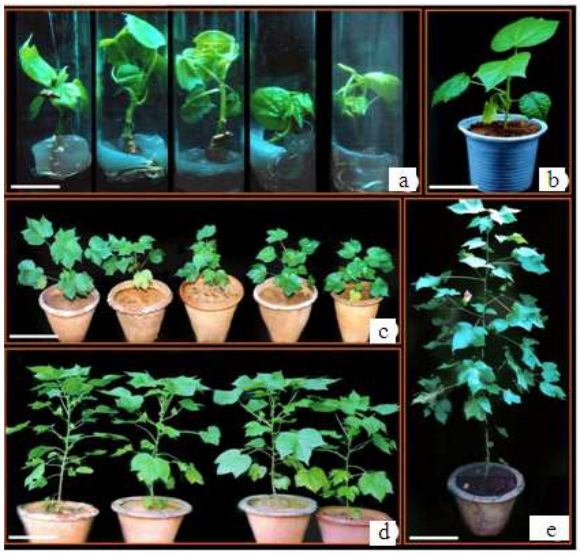

Fig. 3: Different developmental stages of transgenic cotton through shoot tip-mediated direct regeneration; (a): Rooted transgenic shoots containing the secondary and tertiary roots. Bar = 2.5 ㎝; (b): Hardened transgenic plantlet grown in plastic pot. Bar = 5 cm; (c): One month old well developed transformed plantlets established in earthen pots. Bar = 15 cm; (d): Two months old transformed plantlets. Bar = 20 cm; (e): Transgenic plantlet with flowers and bolls. Bar = 20 cm

declines trend was recorded for multiple shoot induction percentage, when compared with control cultures (Fig. 3c). After 10 weeks of selection, surviving shoots were transferred to MS media without PPT to induce rooting (Fig. 3d). Rooted plantlets were first transferred to plastic pots and grown in an environmental growth chamber (Fig. 3e-h).

During selection procedure, 10 independent experiments were carried out. After the completion of transformation procedure, the plantlets harvested in the PPT containing selection media were considered as putative transgenic plants. The morphological features of the transgenic plants did not differ from those of non-transgenic plants. After root induction, in the 10 experiments the transformation efficiency was 21.4%. In contrast, for the 25 shoot apices not treated with *Agrobacterium*, died on PPT selection (5 mg L^{-1}). The *Agrobacterium* strain used had a major effect on transformation efficiency of cotton. It was found that the use of strain LBA4404 yielded a higher degree of transformation efficiency in cotton. This is in agreement with the previous report on cotton transformation[8].

Table 1: Transformation efficiency of regenerated plantlets on shoot tip regeneration medium fortified with selection marker (PPT 5 mg L^{-1})

Sample	Total Number of explants tested (10 experiments)	Total number of plantlets showing resistant to selection marker	Transformation efficiency
Non-transformed	25	0	0
Transformed	392±5.72	84±2.37	21.4±075

Table 2: Segregation analysis in progeny of the primary transformants (T_0) of the three cotton varieties as assessed by the PPT sensitivity test (5.0 mg L^{-1})

Sample seeds	No. of seeds tested	No. of seeds germinated	Death of seedlings	Growth ratio on selection media (transformed/ non-transformed)
Non-transformed	24	0	0	0
SVPR2-Chi-1	26	20	6	3.3:1
SVPR2-Chi-15	21	16	5	3.2:1
SVPR2-Chi-31	16	12	4	3:1
SVPR2-Chi-45	20	15	5	3:1
SVPR2-Chi-62	28	21	7	3:1

The transformed shoots grew up to 7-8 mg L^{-1} of PPT treatment and further increase led to shoot death (Table 1). Finally, 84 plantlets were germinated on the selection medium. These lines (SVPR2-Chi-1, SVPR2-Chi -2, -3-SVPR2-Chi-84) were used for further analyses. Transformants had similar morphological characteristics to non-transformants, except that SVPR2-Chi-24, -42 and -59 had somewhat smaller phenotypes.

Analysis of T_0progeny: Seeds obtained from T_0 were germinated to raise the T_1 plants and these were tested for the presence of the Chi II gene. The inheritance of the introduced genes in the T_1 generation in the variety was studied using *in vitro* seed germination test on seed germination media containing 5 mg L^{-1} PPT. The untransformed plant seeds showed nil response for germination on selection media. For the transformed lines tested; SVPR2-Chi-1, SVPR2-Chi- 15, SVPR2-Chi-31, SVPR2-Chi-45 and SVPR2-Chi-62, the ratio of resistant to non-resistant plants was equal or close to 3:1 (Table 2). The presence of transgene was further confirmed in the T_1 generation by PCR amplification. In previous reports on progeny analysis of transgenic cotton, a segregation ratio of 3:1 was reported.

This ratio was expected for a single gene trait. The gene was the CryIA (b) gene from *Bacillus thuringiensis* (Bt) for insect resistance, regulated by the CaMV 35S promoter. Like our experiments, the T_1 progeny expressed the Bt gene as a single dominant Mendelian trait in 3:1 ratio[3].

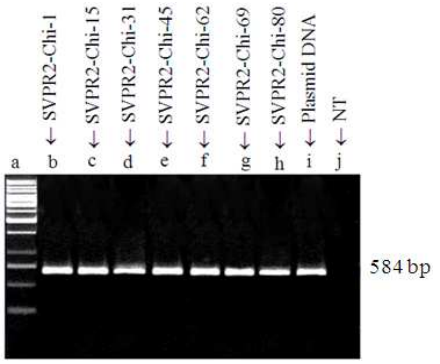

Fig. 4: PCR analysis of DNA isolated from leaves of independent transformants of cotton and non-transformed control plant. Agarose gel electrophoresis of PCR amplification was performed with primers for the Chi II gene; (Lane a): Molecular size marker (Lanes b-h): DNA from transformed plants showing the expected 584 bp; (Lane i): Positive control (DNA from plasmid DNA of *A. tumefaciens*); Lane (j): DNA from untransformed plant

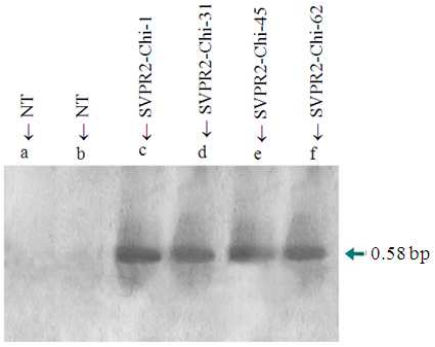

Fig. 5: Southern blot analysis of DNA isolated from leaves of untransformed control and independent transformants; (Lane a and b): Total genomic DNA of non-transformed plants (Lane c-f): DNA from transformed plants showing 0.58 Kb (λ-DNA digested with HindIII and probed with 0.58 Kb Chi II)

Confirmation of transformants:

PCR analysis: Leaf DNA isolated from 7 randomly selected putative T_0 plants (SVPR2-Chi-1, SVPR2-Chi-15, SVPR2-Chi-31, SVPR2-Chi-45, SVPR2-Chi-62, SVPR2-Chi-69 and SVPRChi-80) and non-transformed control plants were analyzed for PCR amplification of the Chi II. The presence of a band at 584 bp in samples from transformed plants confirmed the integration of the chitinase gene (Fig. 4 Lanes b-h). Amplification of this fragment (584 bp) was not observed in non-transformed control plants (Fig. 4: Lane j). These observations indicated that the Chi II gene had been integrated into the genome of the transformed shoots of T_0 progeny thereby confirming transformation. Some reports proved that the shoot tip/meristem transformation protocols, albeit genotype-independent, are extremely laborious and generate a high frequency of chimeras[18,32]. Our experiments on PCR analysis proved that chimera formation significantly reduced and the randomly selected 6 plantlets showed PCR positive results. T_0 support our PCR experiments, low percentage of chimera formation was noticed by using shoot tip explants[33].

Southern blot analysis: The southern hybridization was done by using restricted DNA and probed with 0.58 kb Chi II gene and results showed the presence of chi II gene in the T_1 plants (SVPR2-Chi-1, SVPR2-Chi-31, SVPR2-Chi-45 and SVPR2-Chi-62) (Fig. 5: Lanes c-f). The Chi II gene was not detectable in the non-transformed plants (control) (Fig. 5 Lane a and b).

In the Hind III digestion, a positive band was observed in all the lines as expected. This result also confirmed the PCR results and indicated the integration of the T-DNA region in the transgenic plants of T_1 genome. No variation in number of copies of the chi II gene was observed between the transgenic plants examined. In this study, a significant number of cotton plants carrying the chitinase (Chi11) gene have been produced using the *Agrobacterium* transformation system. These results represent a convincing confirmation of *Agrobacterium*-mediated transformation of cotton and showed the potential reproducible protocol for genetic engineering of Indian cotton. The frequencies of transformation obtained in this study seem to be equal or slightly higher than other systems of transformation, although genotypic differences were observed[18].

Expression of chitinase: Qualitative and quantitative analysis were also performed for chitinase gene expression. Differences in extracellular protein profile between the transformed and non-transformed control was observed on SDS-PAGE gels at 36 kDa levels (Fig. 6). In the selected two lines SVPR2-Chi-1 and SVPR2-Chi-69 over expression of 36 kDa was recorded and it was due to the integration of Chi11 in cotton genome (Fig. 6 Lanes c and d). In the case of control non-transformed plantlets very low level of 36 kDa protein was observed (Fig. 6 Lanes a and b). Based on SDS-PAGE experiments, quantitative analysis showed an 18-21-fold increase in chitinase enzyme activity in the transformed lines (SVPR2-Chi-1, SVPR2-Chi-15, SVPR2-Chi-31, SVPR2-Chi-45, SVPR2-Chi-62 and SVPR2- Chi-69). In all the selected transformed lines,

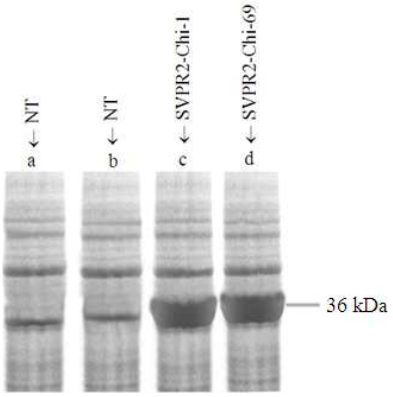

Fig. 6: Variations in the extracellular leaf proteins of 30-day-old plants by SDS-PAGE on transformed and non-transformed cotton plantlets. The gels were stained with coomassie blue; (Lanes a and b): Leaf protein profile of control plants; (Lanes c and d): Leaf protein profile of transformed cotton plantlets showing expected 36 kDa chitinase activity

Table 3: Analysis of chitinase activity in 30-day-old leaves of transformed lines of cotton

Sample	Glu NAc* (mU g^{-1} fresh weight)
Non-transformed	6±1.2
SVPR2-Chi-1	135±4.2
SVPR2-Chi-15	135±3.8
SVPR2-Chi-31	125±4.0
SVPR2-Chi-45	145±5.1
SVPR2-Chi-62	140±5.4

*: N-Acetyl-D-glucosamine, values are means ± SE of four repeated experiments

enhanced expression of chitinase was observed (Table 3). Enhanced expression of chitinases in plants is commonly observed under the stressed conditions as well as in pathogen infection. These plant chitinases degrade chitin in fungal cell walls and can inhibit the infectious fungal growth[34]. Extracellular chitinases may directly block the growth of the hyphae invading intercellular spaces and possibly release fungal elicitors, which then induce additional chitinase biosynthesis and further defense reactions in the host[35].

Bio-assay for disease resistance in transformed plantlets:

Analysis for *Fusarium* wilt resistance: For *Fusarium* wilt resistance analysis, the survival percentage of control (non-transformed plantlets) was significantly affected by the inoculation of *Fusarium* macrospores (1×105 spores/100 g of soil mixture) during hardening (Table 4). All the 7 PCR positive plants showed healthy regeneration in fungal spore inoculated soil (SVPR2-Chi-1, SVPR2-Chi-15, SVPR2-Chi-31, SVPR2-Chi-45,

Table 4: Survival rate of PCR positive plantlets and control plants on earthen pots inoculated with 5×10^5 spores mL^{-1} of *Fusarium oxysporum*

No. of days after inoculation of spores	Survival rate of T_1 plantlets		
	C (25)	Ci (25)	PCR positive plantlets (6)
4	25a	25a	6a
8	23b	12b	6a
12	23b	2c	6a
16	23b	0	6a
20	23b	0	6a
40	23b	0	6a

C: Non-transformed plantlets without spore inoculation; Ci: Control plantlets inoculated with macroconidia; Means within a column followed by the same letters are not significant at p = 0.05 according to DMRT

Table 5: Variations in lesion size and number and wilted area of transformed and non-transformed plants sprayed with *Alternaria macrospora* spores (5×10^5 spores 10^{-1} mL)

No. of days after inoculation of spores	Leaf spots /leaf		Lesion length (mm)	
	PCR positive Ci(Nt)	plantlets	PCR positive Ci(Nt)	Plantlets
4	8.5±0.96e	0	2.2±0.21c	0
8	25.8±1.85d	0	5.4±0.18b	0
12	27.2±2.5c	0	5.8±0.29b	0
16	32.8±2.4b	0	5.8±0.32b	0
20	34.9±3.12a	0	5.8±0.10a	0

Ci: Control plantlets inoculated with macroconidias; Nt: Non-transformed Values are means ± SE of four repeated experiments; Means within a column followed by the same letters are not significant at p = 0.05 according to DMRT

SVPR2-Chi-62 and SVPR2-Chi-69). These plants were finally selected as *Fusarium* wilt tolerant plants. Consistent with the above results, vascular browning was observed only in control cultures inoculated with spores of *F. oxysporum*. At the same time vascular browning was completely absent in the transformed plants. The regenerated control plantlets showed 95% survival rate under normal environmental conditions. All the selected disease tolerant plants showed equal regeneration potential when compared with controls.

Alternation leaf spot assay: Randomly selected two PCR positive plantlets (50 days old) (SVPR2-Chi-1 and SVPR2-Chi-69) showing resistance to *Fusarium* wilt were also analyzed for *Alternaria* leaf spot resistance. The control and PCR positive plantlets were sprayed with *Alternaria* spores. All the 10 control plants showed signs of susceptibility; they developed a larger number of lesions with greater size compared to the transformed plantlets. However, selected transformed plantlets showed 100% disease resistance for up to 30 days. In the non-transformed plantlets, the lesion number and length increased to 34.9 and 5.8, respectively after 20 days of inoculation (Table 5). The bioassay of the T_0 PCR and Southern-positive plants

with *Fusarium oxysporum* and *Alternaria macrospora* showed that the infection level was significantly controlled in transgenic progenies having a higher expression of PR-proteins than in the non-transgenic control plants, indicating enhanced resistance to *Fusarium* wilt and *Alternaria* leaf spot diseases. Usually, in the transformed plants, variations in disease resistant among individual T_1 plants were recorded. This is most probably due to different amounts of chitinase production. Our experiments showed that there is not significant variation in disease resistance among the T_1 plants obtained.

The rapid development of transgenic cotton production technology not only provides a valuable method for introducing useful genes into cotton to improve important agronomic traits, but also helps in the evaluation of mechanism, function and regulation of gene(s). The present investigation was aimed to standardize the simple and reproducible protocol in transgenic cotton regeneration for fungal disease tolerance. In this study, a large number of cotton plants carrying the rice chitinase (Chi11) gene have been produced using the *Agrobacterium* transformation system. These results represent a convincing confirmation of *Agrobacterium*-mediated transformation of cotton and show the potential of this technique for genetic engineering of Indian cotton. The frequencies of transformation obtained in this study seem to be equal to other systems of transformation (protoplast and biolistic) used to obtain transgenic cotton, although genotypic differences must be considered[9]. Selection markers mediated inheritance studies on T_1 progeny showed that the chitinase gene is transmitted to the T_1 progeny, demonstrating stable incorporation of T-DNA into the cotton nuclear DNA and the 3:1 segregation ratio suggested that the chitinase gene was integrated at a single locus. Previously, three reports were published on transgenic cotton with chitinase gene. Those studies, they used tobacco chitinase[36]: Fungal chitinase of *Trichoderma virens*[37] and bean chitinase[9] to control the fungal pathogens *Verticillium dahliae*, *Rhizoctonia solani*, *Alternaria macrospora* and *A. alternate* respectively[38]. These reports are relevant to American and Turkey cotton varieties due to genotype dependent responses. Unfortunately, in Indian varieties no reports were published for transgenic cotton production for fungal disease tolerance. Hence, the present study on production of transgenic cotton with chitinase gene is important. In cotton, like chitinase gene transformation, different antifungal genes were also transferred for fungal disease tolerance. Transgenic cotton expressing the chloroperoxide or D4E1 genes showed antifungal activity against *Aspergillus flavus* and *Verticillium dahliae*[39]. The expression of 14-kDa Corn trypsin inhibitor gene was also showed resistance to *A. flavus*[40]. Similar to the above attempt, transgenic cotton plants harboring *Talaromyces flavus* GO gene results notable resistance against root pathogen Verticillium dahliae[41].

Similar to our observations, the enhanced resistance against fungal pathogens was obtained when the plants were engineered with chitinase genes. In all the economically important plants, the yield was directly affected by the fungal pathogens[11]. Mostly chitinases causes hyphal tips lyse *in vitro*. Some chitinases are having isozymal activities and can hydrolyze the peptides in bacterial cell wall, whereas others have exohydrolytic activity[42]. Rice was the first plant species transformed with chitinase gene, (Chi II). A chitinase gene isolated form rice genomic library was placed under the control of CaMV 35S promoter in rice protoplasts under PEG-mediated transformation[43]. They identified one to six copies of chitinase transgene in rice plant through Southern blot analysis. The level of expression varied over 15-fold range between the each selected individual transgenic plants. Expression of the chitinase was observed in almost all the part of transgenic plants (root, sheath and leaves). Several crop plants were also transformed with chitinase gene for improved disease tolerance. For example, transgenic cucumber plants with Chi-II gene showed enhanced disease resistance against gray mold[44]. In strawberries also increased resistance to the *Sphaerotheca humuli* was observed[22]. Similarly, 13-43% reduction in the development of block spots in transgenic rose plants was observed by basic class I chitinase gene[24]. A very few transgenic plant species expressing chitinases have been evaluated in field trails and it was proved that disease incidence was reduced[45]. On the other hand, some reports showed that transgenic plants over expressing chitinase do not have resistance to some fungal diseases. For example, transgenic tobacco (*Nicotiana sylvestris*) containing a tobacco class I chitinase gene does not have increased resistance to *Cercospora nicotianae*, although it has increased chitinase enzyme activity[46]. Transgenic tobacco (*Nicotiana benthamiana*) harboring a class III chitinase from sugar beet also did not have increased resistance against *Cercospora beticola*[47]. The resistance in these transformants most likely depends on several factors, including the catalytic specificity or localization of chitinase and the characteristic state or infection behavior of the fungi. Advances in regeneration and transformation protocols have led to the successful development of transgenic cotton with improved

agronomic characters. There are several types of fungal resistance genes that were used for disease resistance of plants[48,49]: Among them chitinase was proved as one of the best fungal resistant genes.

CONCLUSION

In our studies, we obtained a significant improvement in the percentage of plant regeneration for transformed plants and recovery of transgenic plants resistant to two fungal pathogens. Limitations associated with somatic embryogenesis protocol make the present protocol more suitable for rapid development of transgenics in recalcitrant system-like cotton and the method will be useful for genetic engineering of cotton for various agronomical traits including fungal resistance.

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