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AGROBACTERIUM MEDIATED TRANSFORMATION OF BAR GENE IN COTTON

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ABSTRACT

Cotton (*Gossypium hirsutum*) is one of the largest source of natural fiber that's why its production is required in enormous quantity, but different environmental stresses cause the lost in the yield. Genetic engineering has enabled us with various techniques to overcome the losses and fulfilling the human needs through modifying the genetics of plant cells. In this study a variety of cotton plant was modified using *Agrobacterium tumefaciens* with BAR gene. BAR gene was inserted to resist the effect of herbicide bialaphos, which affects the primary growth of plants. The whole study was conducted at Four Brothers genetics. The Bar gene cassette, seeds and all the necessary material was obtained from Four Brother genetics. The construct was already made and it was just transformed to *A. tumefaciens*. The seed were delinted, soaked and BAR (Bialaphos resistant gene) was transformed to germinated embryos. After the step by step growth of embryos into healthy plants, molecular analysis for transformation of BAR gene was confirmed through leaf sample of stable transformed plants. Transformation was determined by PCR and gel electrophoresis. The entire study confirms that transgenic cotton variety of eagle-2 shows better results of transformation for BAR gene than other varieties.

Keywords: Cotton, *A. tumefaciens*, herbicide resistance, bialaphos, BAR gene, PCR

INTRODUCTION

Cotton is a well-known natural fiber and one of the foremost and outspreading natural crop covering one of the world's largest industries. Its common name is related to an Arabic word "quotn" which generally means of spinnable fiber (lint) on their seed coat. The other common names are upland cotton, American or Mexican cotton. The biological name of cotton is *Gossypium* was first named by Linneaus in the mid-18th century on the basis of its hairy structure. It belongs to genus *Gossypium*, family Malvaceae, order Malvales and population Gossypieae. Pakistan is the fourth largest cotton yielding country and approximately 1.7 million of farmers are working for its cultivation **[1, 2]**. The estimated area coverage by cultivation of cotton is 2.79 million/ha with 1200 km of belt along with the river Indus. The major farming is done in the two provinces of Pakistan, they are Sindh and Punjab. Pakistani farmers are working for high quantity and good quality yield of lint to fulfill the demand of cotton lint, fabric and oil etc. inside the country and also for exporting outside **[3-6]**. Cotton (*Gossypium hirsutum*) is one of the world's important source of natural fiber that's why its production is required in enormous quantity, but different environmental stresses like heat, drought, pests, herbicides and pathogens etc., cause the lost in the yield **[7]**. Traditional breeding processes are effective in overcoming the strains but they are slow procedures with various hurdles **[8]**. Fortunately nowadays, the field of genetic engineering has enabled us to reproduce this crop by modifying its genetic makeup and introducing stress resistant genes in cell's DNA. Genetically modified cotton is being reproduced with desirable yield as well as enhanced quality and resistance to different environmental stresses **[8, 9]**. Generally transfer of genes or genetic transformation is the variation in a living cell at genetic level. This modification can occur due to direct uptake as well as assimilation of external genetic material from surrounding system via membranes. This transformation can occur naturally and artificially through different techniques in all type of living cells like animal, plant or bacterial cells **[10]**. The recombinant DNA technology has opened many routes for the improvement in the field of crops by genetically engineering plants **[11]**. Many genes are being recognize, characterized, isolate, altered and transformed in plants cells to form genetically modified plants or crops for enhanced quality of crop or for better

resistant against pests, drought or other stresses **[12]**. Advancement in different techniques in the field of genetic engineering to regenerate plants with improved genetic system in order to enhance resistance against different stresses is more beneficial than usual propagation methods **[13,14]**. Traditional breeding procedures are usually slow and they take years to enhance the quality of crops whereas, DNA recombinant technology provides improved yield, better nutritional value, increased shelf life as well as resistance to insects, pests, drought, frost or other stresses **[15]**. This study focuses on indirect transfer of BAR gene into cotton germplasm using *Agrobacterium tumefaciens* as vector. BAR gene is being genetically inserted in cotton plants to resist the effect of bialaphos. Bialaphos also known as bilanaphos **[16]** is a natural herbicide which when applied to crops it disturbs its primary growth. Bialaphos is produced naturally by bacteria *Streptomyces viridochromogenes* and *Streptomyces hydroscopicus* **[17]**. The bar gene was initially replicated from bacteria *Streptomyces hygroscopicus* and then after several researches and experiment its nucleotide sequence was determined. Now it is being used as herbicide resistant gene to resist the effect of bialaphos on primary growth of plants **[18]**. The aim of this study is to transfer BAR gene into cotton plant to obtain stably transformed plants and to study the effectiveness of transformation.

MATERIALS AND METHODS

The study was conducted at Four Brother Genetics, Lahore under the supervision of Dr. Arfan Ali. All the materials chemicals, selection drugs, instruments and gene construct and cotton seeds were obtained from Dr. Arfan Ali, Four Brother Genetics Lab.

GENE TRANSFORMATION INTO *AGROBACTERIUM TUMEFACIENS*

Cultured colonies of *Agrobacterium tumefaciens*

Readily available culture of *Agrobacterium tumefaciens* in YEP broth was incubated on shaker incubator at 28^{o}C for one hour. Than 100 µl of this culture was spread on YEP agar containing kanamycin as selection drug with sterilized spreader. The whole procedure was performed under laminar flow cabinet to avoid any contamination. These culture plates were incubated at 28^{o}c for 48 hours. After incubation colonies are formed which will be used for making competent cells later.

Competent cells of *Agrobacterium tumefaciens*

Competent cells of *Agrobacterium tumefaciens* were prepared from the

previously cloned culture colonies. For this purpose properly sterilized toothpick was used to pick the single colony from the culture plate of the bacteria and inoculated into 50ml of YEP (Yeast Extract Peptone) broth containing 100µg/ml rifampin as selective drug. Then this culture was incubated overnight at 28°C temperature on orbital shaker with speed of approximately 300 rpm. Next day dilution was made by adding few drops of this culture into 25 ml of YEP broth and incubated again on orbital shaker for few hours. Than it is placed on ice until the culture is chilled properly. Than 1.5 ml of culture was transferred to sterilized eppendorf tubes and placed on ice for 10 minutes and then centrifuged for 15 minutes at 4°C temperature and 13550 rpm speed. After centrifugation the supernatant was discarded. Then 1.5 ml of $CaCl_2$ of 0.1 M concentration was added into the eppendorfs to resuspend the cells and place it on ice for 10 minutes, this whole process was done under laminar flow cabinet to avoid any contamination. In next step washing was given to cells for removing any possible impurity from the cells. For the purpose the cell suspension was again centrifuged for 15 minutes at 4°C temperature and 13550 rpm speed, supernatant was discarded and pellet was resuspanded with 100 µl mixed solution of 0.1M $CaCl_2$ and 100% glycerol (90ul of 0.1M $CaCl_2$ was mixed with 10 µl of 100 % glycerol). These competent cells were later used for the transformation of BAR gene.

Transfer of Bar Gene into *Agrobacterium tumefacians*

For transformation of BAR gene into *Agrobacterium tumefaciens,* the already cloned plasmid containing BAR gene was used and transformed into the competent cells using heat shock method. 3 µl of plasmid was inoculated into previously prepared competent cells and incubated for one hour at 28°C. Then the eppendorf was placed in liquid nitrogen (-70°C) for 10 minutes. Then the sample was placed in water bath at 37°C for 5 minute to give heat shock. Again transfer the sample into liquid nitrogen for 3 minutes. Then remove the eppendorf tubes from liquid nitrogen and transfer the complete sample into sterilized culture tube. 500 µl of YEP broth was added into the culture tube and mixed properly. The culture was then incubated on orbital shaker for two hours at 28°C temperature.

Confirmation of transformation through Colony PCR

After the above incubation, to confirm the transformation the colony PCR was run and confirmed through gel electrophoresis. 100ul of culture was

transferred to eppendorf tube and centrifuged for 10 minutes, the supernatant was discarded and pellet was resuspended with 50 µl of TE (Tris EDTA buffer) buffer. This solution was shifted to PCR tubes and PCR was run at 98oc for 12 minutes. Then the tubes were centrifuged on short spin centrifuge machine for 10 minutes. This prepared solution was then used as template for colony PCR for confirming the transformation of BAR gene. 4ul of the supernatant was picked as a template and transferred to another PCR tubes. Some quantity of readily available PCR master mix along with forward and reverse primers and nuclease free water was added to the template to make total of 20ul solution and PCR was run. The PCR product was then confirmed through gel electrophoresis, the sample was run on 1% agarose gel for 30 minutes at 120 volts and bands were observed under UV light.

GENE TRANSFORMATION INTO COTTON SEEDS

Preparation of variety of cotton seeds for transformation

Cotton seeds were obtained from Four Brothers genetics lab and prepared for further process. The Eagle-2 variety of seeds was used for the purpose. Delinting was the first process done to remove all the lint (cotton) from the seed coat. An appropriate amount of seeds was taken and added in a beaker containing solution of sulphuric acid H_2SO_4 (20 ml) and distilled water (80 ml). The seeds were mixed with spatula until the lint was removed. Than the seeds were washed with tap water 5 to 7 times until all the acid was removed. Only the sinker seeds were selected for the further process and floater seeds were discarded. The delinted seeds were then dried properly for 24 hours or more.

Screening of seeds

This process is important for avoiding any decontamination in further procedures. Damaged seeds and any debris were removed and only healthy seeds were taken.

Soaking and washing of seeds

15 to 20 grams of screened seeds were taken in 1L conical flask and soaked with some autoclaved water, a drop of 10% SDS and few drops of 5% $HgCl_2$. Shake the flask and remove all the liquid carefully. Wash the seeds again with some amount of autoclaved water step by step to remove all the SDS and $HgCl_2$ in the flask. Once the SDS is removed aluminium foil was used to cover the mouth of flask. Than the flask was covered with paper and incubated at 30^oC for overnight incubation. Next day the seeds were washed with few drops of 5% $HgCl_2$ and water. And then again washed with water

until all the $HgCl_2$ was removed. The flask was covered again and incubated at 30°c for overnight. Next day germinated embryos were obtained from seed for further procedure.

Embryo isolation

Under the completely sterilized laminar flow cabinet the germinated seeds were taken out from the flask and embryos were isolated from the seed coat gently. For the purpose, sterilized scalpel and forceps were used to press the seed coat and embryo was removed gently and a small incision at the tip was made with the help of surgical blade. In this way all the mature and healthy embryos were isolated and inoculated in bacterial culture containing transformed agrobacterium in YEP broth.

BAR gene transformation into cotton plant (isolated embryos)

The prepared and BAR gene transformed culture of *Agrobacterium tumefaciens* in YEP broth was centrifuge for 5 minutes at 5000 rpm, supernatant was discarded and pellet was resuspanded in 10 ml of MS media broth. The isolated embryos were transferred and inoculated in this culture suspension. The culture was then incubated on orbital shaker at 28°C for half an hour.

FURTHER PROCESSESING OF TRANSFORMED EMBRYOS

MS media agar preparation

For further processing of transformed embryos MS media was prepared in plates and tubes. MS media was prepared according to laboratory manual and autoclaved to avoid any contamination, and then cooled till 50°C temperature and cefotaxime was added as selective drug. Then the media was poured in plates and tubes carefully under sterilized laminar flow cabinet. Then media was left to cool down until it solidifies for future use.

Shifting of embryos in MS agar plates

The culture with embryos was removed from shaker and embryos were shifted on sterilized filter paper and dried and transferred to the plates of MS agar with the help of sterilized forceps. The culture plates containing transformed embryos were then sealed with flexible thin film and incubated in suitable growth environment in the lab at room temperature for few days for further growth.

Shifting of plantlets into tubes

After cultivation of approximately 72 hours the healthy plantlets that grown on MS media plates were removed and shifted to tubes containing MS agar with kanamycin and cefotaxime as selective drugs. The tubes were covered properly with sterilized cotton

plugs and incubated again in plant growth room with appropriate conditions for approximately 8 weeks for further growth of plantlets. The condition of tubes was regularly examined within every 14 days of the 8 weeks.

Shifting of plants into pots

After 8 to 10 weeks of growth the plantlets were shifted from tubes to pots with sterilized soil along with fungicide spray. The small plants were removed from tubes and media attached to roots was washed with water and then roots were dried properly with tissue paper. The roots were dipped once in growth hormone IBA (Indole-3-butyric acid) before planting in soil for better growth of transgenic cotton plants. The plant containing pots were than covered with plastic bags to maintain proper environment for plant growth in plant room. The plants were then kept in a room with temperature 25±2°C and photoperiod time of 16 hours light and 8 hours dark was maintained.

Shifting of plants to field

The plants that were shifted to pots were maintained properly in climatic plant room and observed for growth. After first week some plants were lost, remaining healthy growing plants were shifted to field (Behria farm) of Four Brothers after 15-20 days. In the field plants were grown under natural environmental conditions.

DNA EXTRACTION

Following the growth of transgenic cotton plants for few months, some plants couldn't survive in the environmental conditions but several plants survived and remain healthy. These transgenic plants were than tested further for the expression of BAR gene in their DNA. For the purpose, firstly the DNA was extracted from the leaves of healthy cotton plants. The leaves taken from plants were stored in liquid nitrogen with proper labeling. Than the leaves were grinded into powder using pestle and mortar and powder was transferred into eppendorf tubes. 500 µl of extraction buffer, 400 µl of CTAB and 10 µl of mercaptoethanol were added to the leaf powder. The tubes were vortexes properly to mix the solution and incubated in a water bath at 65oC for 90 minutes. Than 500 µl of (CIA) chloroform:isoamylalcohol (24:1) was added and the tubes were vortexes to mix the solution properly. Then the tubes were centrifuged at 13550 rpm for 15 minutes. Supernatant was separated carefully and transferred to new eppendorf tubes and pellet was discarded. If the supernatant is not clear properly than add CIA again and centrifuge until the solution is cleared. Than 60%

volume of isopropanol was added as compared to the total volume of supernatant and incubated for half an hour. Than the solution was centrifuged and supernatant was discarded. Pellet would contain the DNA. Then 200 µl of washing solution (70% ethanol) was added and pellet was resuspended to eliminate any possible impurity in DNA. The tubes were centrifuged again for 10 minutes at 13550 rpm. The supernatant was discarded and pellet was air dried properly. The DNA pellet was eluted with 20-25µl of nuclease free water. 10 µl of RNAase was also added and tubes were incubated in water bath at 37oC for 3 minutes. Than the quality of DNA was determined by using agarose gel electrophoresis.

GEL ELECTROPHORESIS

The extracted DNA was determined using gel electrophoresis. For the purpose, 1% of agarose gel with 5 µl of ethidium bromide (dye) was prepared and casted into caster plate and dipped in 1X TAE buffer (Tris Acetate EDTA buffer). DNA samples were loaded in wells made on gel with the PCR help of comb and run at 120V for 30 minutes and then the gel was examined in gel doc.

PCR (POLYMERASE CHAIN REACTION)

The transformed DNA samples were than amplified and run on PCR to determine the transformation of BAR gene into the DNA. ThermoScientific Dream Taq green PCR master mix (2X) was used for making the reaction mixture for PCR. Dream Taq green PCR master mix contains Dream Taq DNA polymerase, optimized buffer, $MgCl_2$ and dNTPs and two tracking dyes. Dream taq Green master mix was vortexes and added to PCR tubes. The reaction mixture was made by adding 10 µl of master mix, 2 µl of extracted DNA, 1 µl of forward primer, 1 µl of reverse primer and 6 µl of nuclease free water to complete the reaction mixture of total 20 µl. Then the PCR tubes were placed in thermocycler PCR machine and the procedure was started **(Figure 1)**. After amplification was completed the products were determined on gel electrophoresis using 1% agarose gel for band size of BAR gene.

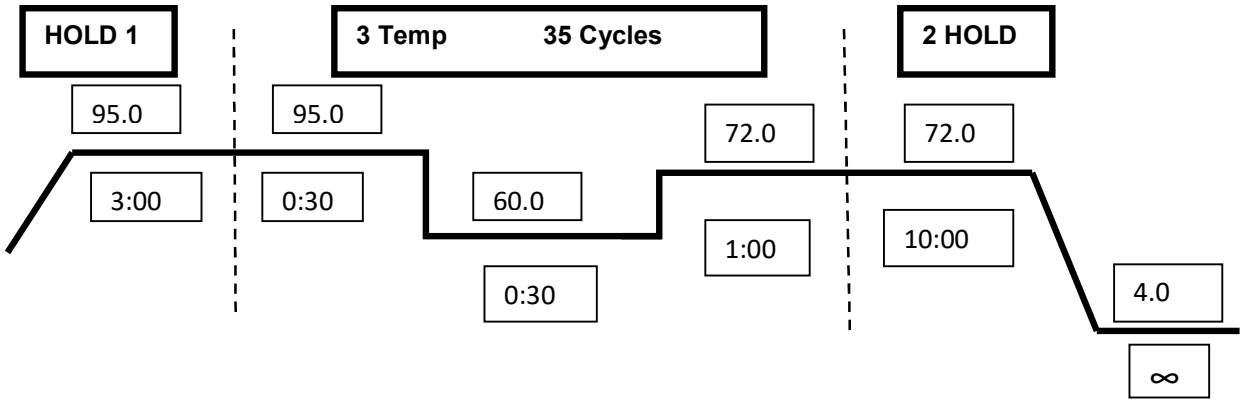

Figure 1: Conditions for BAR gene amplification

RESULTS

CONFIRMATION OF TRANSFORMATION THROUGH COLONY PCR

The BAR gene was transformed to *Agrobacterium tumefaciens* competent cells and later the transformation was confirmed through colony PCR and gel electrophoresis. The picture of colony PCR is given below **(Figure 2)**. After confirmation of transformation through colony PCR the stock culture of transformed agrobacterium was applied on YEP broth and agar, and colonies were saved for future use.

Transformation of BAR gene to cotton plant

Once the embryos were germinated they were isolated and inoculated in transformed culture of BAR gene in *Agrobacterium tumefaciens* and incubated on shaker, after that embryos were transferred to MS agar plates and then to tubes, pots and field step by step. In the field plants were subjected to natural environment also the herbicide was sprayed to determine the stability of plants, some plants was sensitive to the spray and some were resistant and growing healthily. After few months of growth leaf sample from healthy and stable plants was taken and tested further **(Figures 4-7)**.

Delinting and soaking of seeds

The seeds covered in lint were treated with sulphuric acid and washed properly to obtain delinted and clean seed for further transformation. The seeds were then screened, soaked, washed and incubated for 48 hours to obtain embryo germination **(Figure 3)**.

DNA extraction and gel electrophoresis

During the entire process, approximately 550-600 embryos were shifted to MS agar plates and after step by step growth of transformed plants in the process DNA of 11 plant samples was extracted using CTAB method. The DNA samples were than determined using gel

electrophoresis technique 1% agarose gel **(Figure 8)**.

PCR confirmation of BAR gene transformation

After DNA extraction, the DNA samples from transformed plants were amplified with PCR using Dream Taq Green master mix, and forward and reverse primers of BAR gene on thermocycler PCR machine. The PCR product was then determined by Gel electrophoresis using 1% agarose gel and 50 bp ladder to resolute the band of about 230 p for BAR gene. The PCR results showed that five plants were successfully transformed with BAR gene **(Figure 9)**.

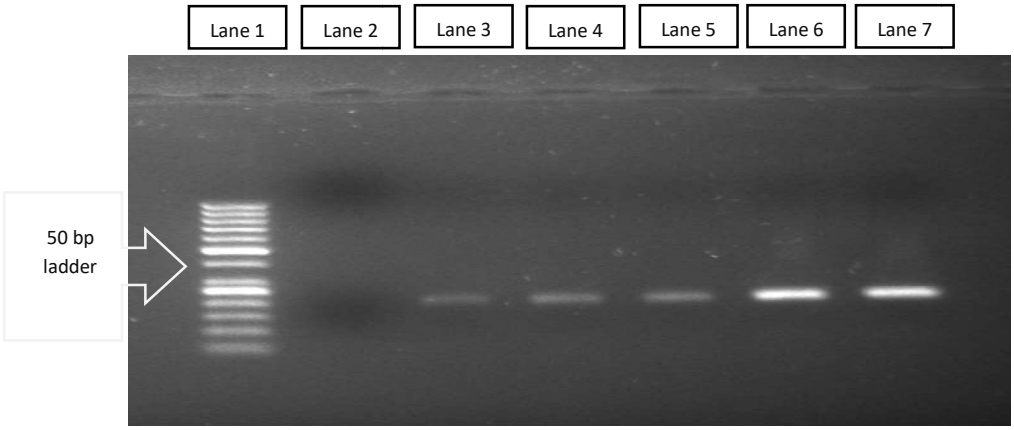

Figure 2: The lane 1: 50 bp ladder, lane 2: negative control, lane 3: positive control, lane 4, 5, 6, 7: positive transformation of BAR gene into agrobacterium (product size of BAR gene is 230 bp).

Seeds with lint **De lint seed** **Germinated seeds**

Figure 3: Seed conditions

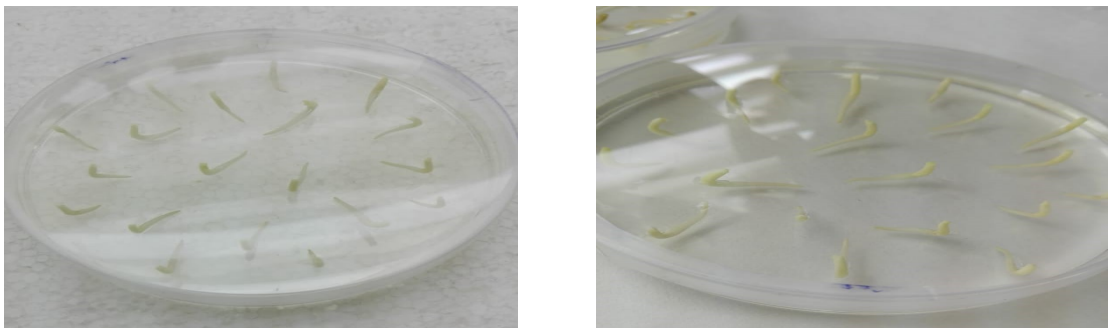

Figure 4: Bar gene transformed embryos on MS agar plates

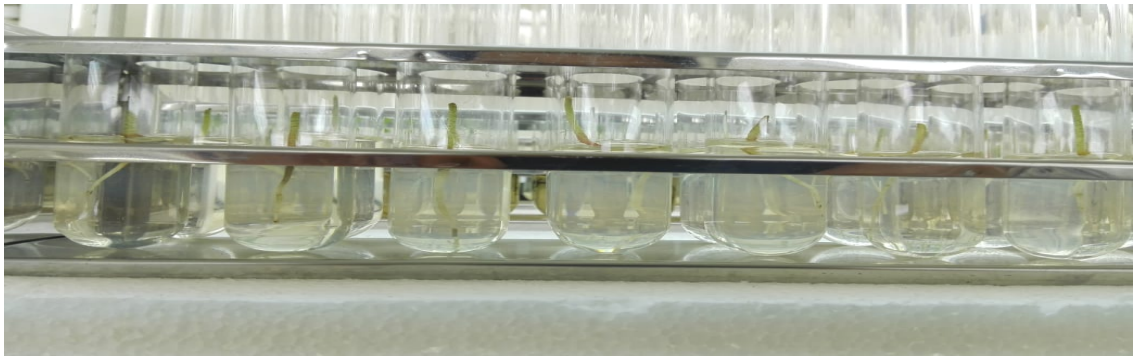

Figure 5: Healthy plantlets has been shifted from plates to tubes with MS agar

Figure 6: BAR gene transformed plants after few weeks of growth

Figure 7: Stable plant has been shifted from tubes to soil containing pots

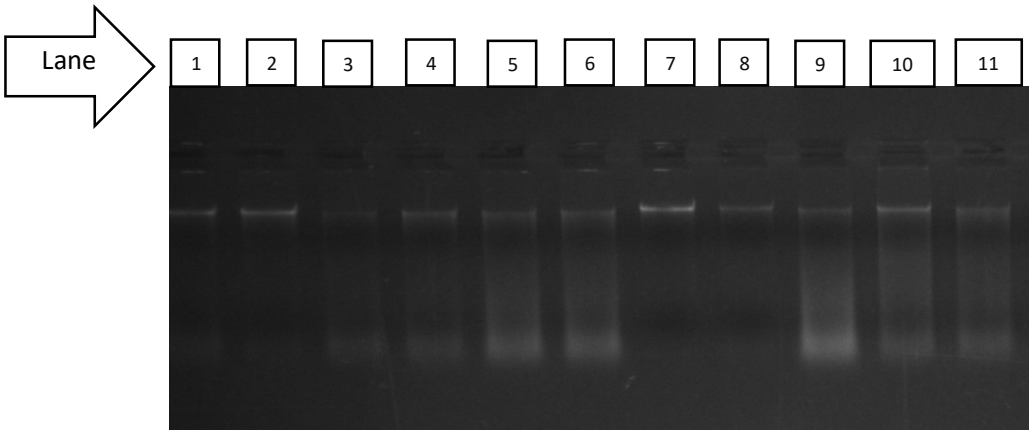

Figure 8: Gel doc picture of 11 DNA samples from transformed plants

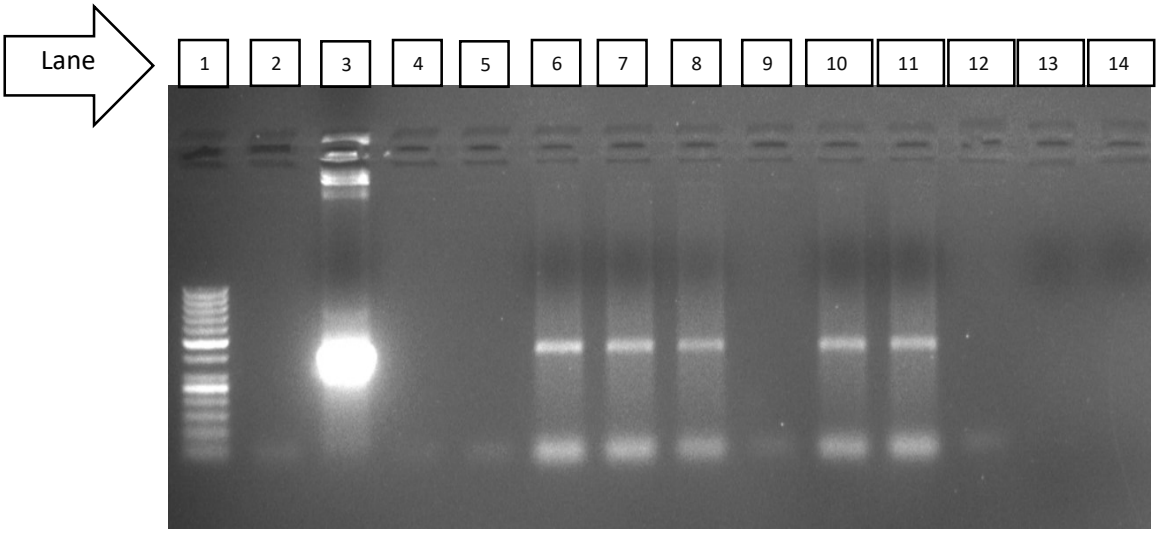

Figure 9: The lane 1: 50bp ladder, lane 2: negative control, lane 3: positive control, lane 4, 5, 9, 12, 13, 14: negative transformation, lane 6, 7, 8, 10, 11: positive transformation of BAR gene into agrobacterium (product size of BAR gene is 230 bp)

DISCUSSION

Bar gene is being genetically inserted in cotton plants to resist the effect of bialaphos. Bialaphos also known as bilanaphos **[19]** is a natural herbicide which when applied to crops it disturbs its primary growth. Bialaphos is produced naturally by bacteria *Streptomyces viridochromogenes* and *Streptomyces hydroscopicus* **[20]**. The bar gene was initially replicated from bacteria *Streptomyces hygroscopicus* and then after several researches and experiment its nucleotide sequence was determined. Now it is being used as herbicide resistant gene to resist the effect of bialaphos on primary growth of plant **[21]**. *Agrobacterium tumefaciens* is a plant pathogen which infect plants at wound sites and develop Crown gall disease and during this process it also transfer T-DNA from bacterial cell to plant's

cell. Using this property of *A. tumefaciens* various plants have been genetically modified with different types of stress resistant genes. With the advancement of molecular biology and genetics *A. tumefaciens* is now being used very commonly in the field of genetic engineering for generating genetically modified organisms especially plants. For instance, *Arabidopsis* the most studied plant nowadays is readily transformable by *A. tumefaciens* for steady transformations **[17, 22]**. Cotton is well known and important natural fiber being cultivated, but different environmental stresses are creating hindrance in its growth. To avoid these stresses many traditional and scientific techniques are being used worldwide. In this study herbicide bialaphos resistant gene (BAR gene) was transformed into cotton germplasm using *Agrobacterium tumefaciens* mediated transformation.

This whole study was conducted at four brother's genetic lab. For the study cotton seeds of variety eagle-2 were used. Cotton seeds were first delinted using sulphuric acid, washed and dried. Then the seeds were soaked and incubated for 2 days to obtain the germination. After the germination of seeds embryos were isolated from seeds carefully and co-cultivated with BAR gene transformed *A.tumefaciens* culture in YEP broth. After incubation of half an hour on orbital shaker at 28^{o}C the embryos were washed with plain MS broth, dried and transferred to MS agar media plates containing selective drug. The plates were then incubated for 72 hours and then healthy embryos were shifted to tubes with MS agar. After growth of few weeks the healthy grown plants were shifted to soil in pots and acclimatized and maintained under optimal conditions **[23-25]**. After few weeks the healthy and stable plants were shifted to bahria farm (plant field of four brothers). Plants were grown under natural condition in the field and sprayed with herbicide bialaphos to determine the strength and expression of transformed BAR gene. Some plants were lost in this entire process but some plants show healthy growth and stability despite of whole procedure. 550-600 embryos were first isolated and processed of which Leaf samples from 11 healthy transformed plants were taken and their DNA was extracted using CTAB method and determined through gel electrophoresis. After DNA extraction PCR was applied to confirm the transformation of BAR gene. The PCR product was than resolute through gel electrophoresis on 1% agarose gel for band size of 230 bp for BAR gene and five plants were found positive with BAR gene

transformation **[26-29]**. The result of overall transformation shows that eagle-2 variety of cotton shows successful transformation of BAR gene phenotypically and genetically as compared to other varieties **[23, 30-39].**

CONCLUSION

Cotton is a gold crop in the agriculture industry and of main importance throughout the world. Pakistan is one of the largest producing countries of cotton. Farmers are working efficiently to increase the yield of cotton plant. But there are many hindrances which farmers have to face during the cultivation of cotton. To avoid those stresses many traditional and conventional methods are being used since years. Genetic engineering has opened many routes for the better growth of plants. Many techniques have been established to avoid the danger of environmental stress and to increase the yield of cotton. Agrobacterium mediated genetic transformation is one of the most effective method in which the desired gene is transformed to plant germplasm using A.tumefaciens. In the current study we use this technique to transfer herbicide bialaphos resistant BAR gene into the cotton germplasm. For the purpose, cotton seeds were delinted, soaked, washed and incubated for embryo germination. Healthy embryos were than isolated and inoculated with BAR gene transformed culture of *A.tumefaciens* in YEP broth for half an hour. The culture was prepared from previously available agrobacterium colonies on YEP agar (competent cells were prepared) and BAR gene cassette obtained from Four brothers genetics lab. After incubation embryos were shifted to MS agar plates, then to tubes and pots step by step. In the plant room, plants were acclimatized regularly and shifted to field after few weeks of growth. In the field plants were grown under natural conditions and observed for their growth. To observe the resistance of plants to herbicide and weeds and expression of BAR gene, herbicide was also sprayed. The leaf sample was taken from plants which remain healthy, stable and show successful growth to determine the transformation of BAR gene. From the plant's leaf samples, DNA was extracted and transformation of BAR gene was confirmed through gel electrophoresis after PCR amplification. The result shows the successful transformation of BAR gene into cotton plant.

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whole work was conducted at The University of Lahore, Lahore, Pakistan.

CONFLICT OF INTEREST

Authors declare no conflict of interests.

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