Australian Journal of Crop Science AJCS
AJCS 7(2):206-212 (2013) ISSN:1835-2707

Expression of *Calotropis procera* expansin gene *CpEXPA3* enhances cotton fibre strength

Kamran Shehzad Bajwa[1*], Ahmad Ali Shahid[1], Abdul Qayyum Rao[1], Muhammad Sarfraz kiani[1], Muhammad Aleem Ashraf[1] , Abdelhafiz Adam Dahab[1], Allah Bakhsh[3], Ayesha Latif[1], Muhammad Azmat Ullah Khan[1], Agung Nugroho Puspito[1], Asiya Aftab[2], Aftab Bashir[2] and Tayyab Husnain[1]

[1]Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, 53700, Pakistan
[2]National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan
[3]Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey

[*]Corresponding author: kamrancembian@yahoo.com

Abstract

Cotton fibre is naturally occurring pure cellulose that plays an important role in the textile industry. In addition to traditional breeding, genetic modification can improve fibre quality. Many multigenic traits and genes involved in various developmental stages of cotton fibre have been identified in wild plants, including *Calotropis procera*, which despite its different evolutionary origin shares some fibre traits with cotton. To obtain better fibre strength and micronaire values, *Calotropis procera* genes can be used in the genetic modification of crop plants such as cotton. In the present study, the *CpEXPA3* gene taken from *Calotropis procera* was transformed into local cotton (*Gossypium hirsutum* variety NIAB-846) using *Agrobacterium* strain LBA 4404. The integration and expression of the *Calotropis procera* fibre gene *CpEXPA3* in cotton plants was confirmed by southern blot analysis, real-time PCR and cellulose assay. Data from three years of field performance of the transformed cotton plants indicate that fibre strength was significantly improved as compared to a control. From this study, it is clear that improvement of fibre quality can be achieved by adding traits via transformation; such methods have the potential to significantly improve the agricultural and textile industries of Pakistan.

Keywords: Agrobacterium, *Calotropis procera*, *CpEXPA3*, Cellulose, Wild Ancestor.
Abbreviations: *CpEXP*-calotropis procera expansin.

Introduction

Cotton fibre is a naturally occurring source of pure cellulose and the foundation of the textile industry (Stewart, 1975). Important members of the Malvaceae family include cotton, hibiscus and okra. Cotton bolls produce soft, staple fibre that grows around the seeds. Cotton of the Gossypium spp grows in tropical and subtropical regions. Conventionally, the reported staple length of cotton fibre is near 32 inches. There are four important classes of staple length: short fibre (<21 mm), medium fibre (22-25 mm), medium-long fibre (26-28 mm) and long fibre (29-34 mm). short fibre content is defined as less than 12.7mm (Cui et al., 2003). *Calotropis procera* belongs to the Asclepiadaceae family (Carruthers et al., 1984; Cheema et al., 2010). The species is highly cross-pollinated and has 22 (2n) chromosomes (Cheema et al., 2010; Eisikowitch, 1986; Raghavan, 1957). *Calotropis procera* is a small bushy wild plant with a height of 2.5 m, a branched stem, and woody character, with corky greyish bark. Wild *Calotropis* spp plants have succulent branches and compact white tomentose. *Calotropis procera* plants have opposite leaves with oblong obovate to nearly orbicular shape. The blade colour of *Calotropis procera* leaves is light green to dark green, with nearly white veins (Abbas et al., 1992; Varshney and Bhoi, 1988). *Calotropis procera* has been characterised on the basis of physical, chemical and tensile properties, with good staple length, fibre strength, fibre uniformity ratio, fibre fineness and moisture absorption (Sakthivel et al., 2005). The primary cell wall of *C. procera* fibre is mainly composed of cellulose, lipids, proteins and polysaccharides, similar to the primary cell wall of cotton fibres. *C. procera* exhibits a three-stage fibre development process that includes i) initiation, ii) elongation, and iii) maturation. The lack of secondary cell wall deposition in *C. procera* (stage iii in cotton) is correlated with fewer metabolic pathways involved in secondary cell wall deposition (Cheema et al., 2010). Mature fibre characteristics including fibre length, fineness, uniformity/maturity and strength are better or equivalent to the finest grades of cotton fibre. *Calotropis procera* exhibits high-quality fibre characteristics—micronaire value 2.09, fibre length (mm) 42.0, fibre strength (g/tex) 29.5 and uniformity index (%) 81.6—as compared to *G. hirsutum* with micronaire value 4.5-4.9, fibre length (mm) 28-32, fibre strength (g/tex) 28-32 and uniformity index (%) 80-85 (Cheema et al., 2010). *Calotropis procera* fibres are 3-3.5 cm long and the fibre quality is strong white silk, used for mattresses and pillows as well as for weaving into strong cloth (Abbas et al., 1992; Varshney and Bhoi, 1988). Numerous methods for exogenous gene introduction into plant genomes have been described as part of plant genetic engineering and can be classified into two groups: indirect gene transfer, where exogenous DNA is introduced by a biological vector, and direct gene transfer, where physical and chemical processes are used (Kohli et al., 1998). Gene transformation is a highly successful tool for plant breeding and genetic, physiological or biochemical and biotechnological research. *Agrobacterium*-mediated transformation is a novel molecular technique used for genetic transformation and incorporation of fibre

improvement genes (Li et al., 2004). In this study, a wild plant expansin gene responsible for fibre development, i.e., *CpEXPA3* was transformed into local cotton variety NIAB 846, and fibre quality was compared with control parent lines after molecular analysis.

Results

Screening and detection of putative transgenic plants

After incorporating the *CpEXPA3* gene in *Gossypium hirsutum* var. NIAB 846, screening of putative transgenic plants revealed that out of 8,500 transformed embryos, only 106 were putative transgenic plants (Sunilkumar and Rathore, 2001). The orientation PCR of these putative transgenic plants confirmed that at least four plants contained the *CpEXPA3 g*ene. The gene and promoter orientation primers (5'GACTTTTCAACAAAGGGTAATTTCG 3' and 3' TTTATGGACACGGAATGAACG 5') were successfully amplified as a 925 bp fragment (Fig 1).

Integration of CpEXPA3 fibre gene in cotton

DNA gel blot analysis was used to detect stable incorporation of the *CpEXPA3* fibre gene (Tohidfar et al., 2005). Plasmid DNA and plant genomic DNA were digested with restriction enzyme HindIII and integrated into the *CpEXPA3* fibre gene in putative transgenic cotton plants after hybridisation with a gene-specific probe (Fig 2). DNA extracted from local cotton variety NIAB-846 was used as a negative control.

Quantification of CpEXPA3 fibre gene mRNA in transgenic cotton plants

Quantitative real-time PCR was used to check the expression levels of the *CpEXPA3* fibre gene in leaf samples of four transgenic lines. The GAPDH gene was used as the reference gene to normalise expression levels. In Fig. 3, all lines show different levels of *CpEXPA3* mRNA expression. Plant line 1(*CpEXPA3*-1) produced some improvement in expression (2.5 fold), as did line number 2 (*CpEXPA3*-4) (2 fold). Plant line 3 (*CpEXPA3*-5) exhibited a much higher level of expression (7 fold) and transgenic cotton plant line 4 (*CpEXPA3*-9) also showed high expression levels (3 fold). Plant line 3 stands out due to its excellent fibre quality parameters.

Cellulose content assay

Cellulose content in cotton fibre increased from 15-20 days post-anthesis (DPA), with high variability between transgenic fibre and control fibre. The cellulose content of fibre in transgenic and non-transgenic lines was compared. Plant line 2 (*CpEXPA3-4*) produced a higher level (2-fold) of expression as compared with control plants; plant line 3 (*CpEXPA3-5*) produced 3-fold higher expression, and line number 4 (*CpEXPA3-9*) showed a 2.5 fold increase in expression over controls. The expression level of transgenic line 1 (*CpEXPA3-1*) was quite low, near control levels, as compared with other transgenic lines with fibre improvement gene *CpEXPA3.* Transgenic plant lines 3 and 4 had improved cellulose content that resulted in improvement of one or more fibre qualities as compared to control lines (Fig 4).

Fibre quality analysis

The constitutive up-regulation of fibre characteristics was produced after examination of more than 40 independent

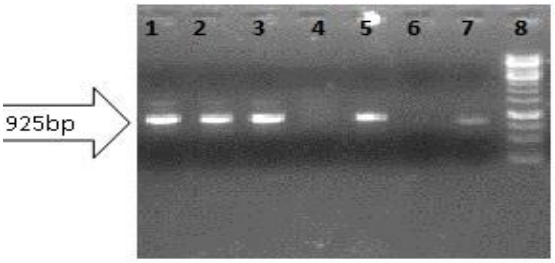

Fig 1. PCR Amplification of Transgenic Cotton Plants (Fiber Gene *CpEXPA3)*: Lane 1-5 show putative transgenic plants in which lane 1, 2, 3, and 5 show positive transgenic plants having gene amplification from orientation primers resulting in band of 925 bp whereas Lane 4 shows negative transgenic plant and lane 6 and 7 shows negative control & positive control respectively while 1 kb Marker in run in lane 8. Whereas arrow indicate the detection of gene in transgenic cotton plants.

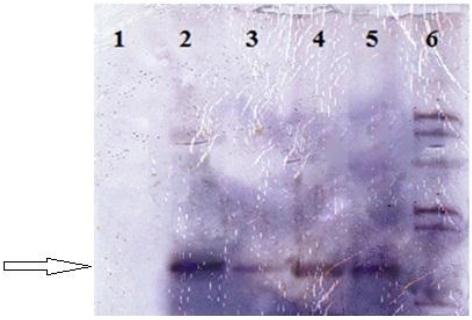

Fig 2. Southern blot analysis of putative transgenic cotton plants for fiber gene *CpEXPA3.* Lane 1 contain negative control plant, in lane 2-5 arrow indicate the integration of fiber gene *CpEXPA3* in selected Transgenic Cotton Plant Samples and 1kb ladder for comparison was run in Lane 6.

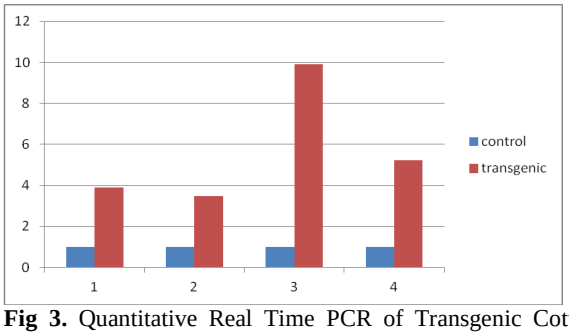

Fig 3. Quantitative Real Time PCR of Transgenic Cotton Plants (Fiber Gene CpEXPA3*)*: Group 1-4: described the different levels of expression of transgenic and control mRNA samples, blue color represent the expression level of control plant samples (*Gossypium hirsutum* var. NIAB 846), red color indicated the putative transgenic plant expression, GAPDH was used as a internal contol for normalization.

transgenic plants. By evaluating transgenic cotton plants for improved fibre quality, four transgenic lines were chosen for their response over three generations. Transgenic cotton plants exhibited elevated qualitative yield; a *Gossypium hirsutum* parent, an elite line of NIAB-846, was used as a control. Fibre samples were collected from each transgenic plant and sent to Cotton Research Station Multan (CRS) and APTMA, Pakistan for fibre testing using high-volume

instruments. Analysis of fibre quality parameters of transgenic cotton plants showed representative positive effects in all three generations (2009-2011). Improvement in fibre quality was observed for fibre length, fibre strength and micronaire value. Fibre length increased (up 10% to 1.14 in) only in transgene line *CpEXPA3-5*; all other lines showed lower response (Fig 5). There was a large impact from all four transgenes on fibre strength in all three generations. Putative transgenic cotton plants also improved fibre strength (a) up to 12% to 31.77 g/tex (line *CpEXPA3-4*), (b) up 28% to 34.80 g/tex (line *CpEXPA3-5*), and (c) up 13% to 32.04 g/tex (line *CpEXPA3-9*) (Fig 6). The micronaire values of transgenic plants were superior to control plants: (a) increased micronaire up 8.13% to 5.04 (line *CpEXPA3-4*), (b) increased micronaire up 11.47% to 4.87 (line *CpEXPA3-5*), and (c) increased micronaire up 10.38% to 4.92 (line *CpEXPA3-9*) (Fig 7).

Progress of fibre characteristics in different generations

The transgenic cotton plants showed significant improvement in two generations (F_0 and F_1) but not in the F_2 generation. A critical examination of fibre strength data indicated that the transgene improved fibre strength, with significant results in all three generations. Statistical analysis of micronaire values indicated greater experimental mean values than the control in all three generations, but these results were not in an acceptable range to meet textile standards.

Growth characteristics of transgenic plants

Morphological and agronomic characteristics are very important in the evaluation of crops in field conditions. We analysed transgenic cotton plants for their delinted seed weight, fibre weight per seed and fibre ginning out turn percentage. Improvement in these characters was observed: (a) improvement in delinted seed weight (Fig 8, up 18% to 0.109 g); (b) up regulation of fibre weight per seed (Fig 10, up 23% to 0.056 g); and (c) improved fibre ginning out turn percentage (Fig 9, up to 25%). Overall, there were large differences between the transgenic and untransformed cotton plants, a highly desirable outcome for textile communities in Pakistan because farmers can cultivate cotton only for quantity, not quality. Similarly, other morphological characteristics such as number of bolls, plant height and average yield indicated significant changes.

Improvement in morphological and agronomic characteristics

Statistical analyses of morphological characteristics were performed according to the least significant difference test. The LSD values obtained, 0.018 (2009) and 0.024 (2011), were greater than all control values. The LSD value 0.066 (2010) was less than all control values. As indicated in the figure subscripts, the values differed significantly at the P>0.05 level in two generations (2009, 2011) but not in 2010. For yield parameters, results were significant in 2011 (F_2 generation) but not in 2009 or 2010 (F_0, F_1 generations). For ginning out turn percentage, statistical analysis of the data represented a significant change in all three generations (2009-2011).

Discussion

The understanding of cotton fibre initiation and development is dependent upon the understanding of plant cell

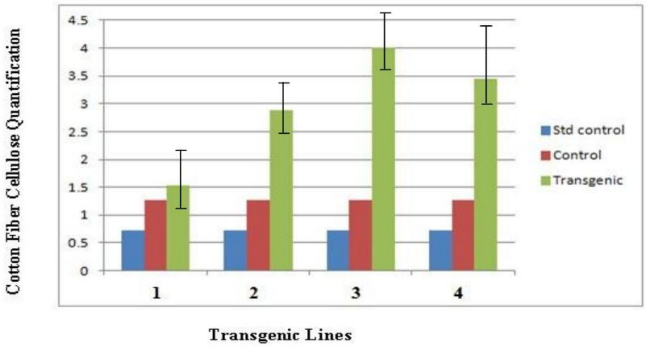

Fig 4. Comparison of Cellulose Contents of Transgenic (Fiber Gene *CpEXPA3*) and control Cotton Fiber along with Standard Control. Plants in groups 1 to 4 were showed three different samples of cellulose. Blue color represent the amount of cellulose from Avicel PH-101pure cellulose, red color represent the amount of cellulose obtained from control plant (*Gossypium hirsutum var*. NIAB-846), green color indicated the depicts the presence of cellulose in transgenic plants.

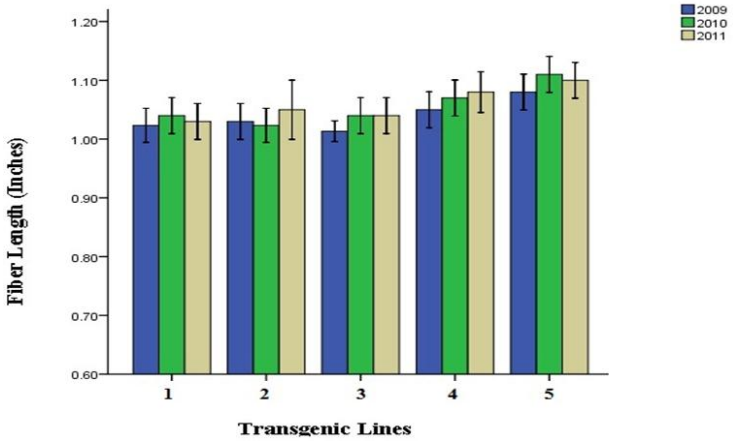

Fig 5. Comparison of Fiber Length of Transgenic Cotton Plants Fiber Gene *CpEXPA3* with Control (T0, T1, and T2 Generations 2009, 2010 and 2011): group 1 indicates the fiber length of controlled plant in three consecutive years. Group 2-5 depicts the fiber length variation of four different transgenic cotton Plants for T_0, T_1, and T_2 in Generations of 2009, 2010 and 2011.
Note: Bar Determined the Standard Error

differentiation in model plants such as Arabidopsis, which has advanced over the past decade due to remarkable advancements in research. Recent advances in genome studies have provided innovative pathways for a complete understanding of basic procedures in cotton fibre expansion. Many types of signal transduction and transcriptional regulation are involved in the complex process of cotton fibre development (Lee et al., 2007). The high global demand for cotton is economically significant; development of new cotton varieties with improved fibre characteristics can have a lasting effect on the world economy. The key factors that play an important role in cotton transformation are the discovery of genes of interest from plant genomes and suitable mechanisms of transfer into cotton plants. The use of *Agrobacterium*-mediated transformation of cotton has been limited to specific cultivars because cotton cannot be regenerated from callus (Zhang et al., 2011). It is well known that gene transformation will be critical to increasing

cellulose synthesis. Here, we used transformation to improve cotton fibre qualities (Wang et al., 2011). Molecular analysis indicated that 4 out of 50 putative transgenic cotton plants were PCR-positive (Fig 1) and all expressed *CpEXPA3*, as indicated by Southern blot analysis (Fig 2). The expression level of *CpEXPA3-5* and *CpEXPA3-9* in lanes 4 and 5 is much higher than other *CpEXP A3* transgenic plants, possibly due to transgene copy number, the positional effect of the gene, or the nucleotide sequence of the gene or promoter, insertion, internal cell environment, or other external environmental factors as determined by (Rao et al., 2011). The results of real-time PCR indicated that *CpEXPA3-9* exhibited a higher level of mRNA expression as compared to non-transgenic plants where mRNA expression was quite low (Fig 3). Cellulose content data clearly illustrated a 50% improvement in transgenic plant *CpEXPA3-5* and *CpEXPA3-9* over the control plants (Fig 4). Transgenic plant *CpEXPA3-4* produced 15-20% greater cellulose content than the control plants as in figure 4, as represented by (de Morais Teixeira et al., 2010; Wang et al., 2009). Qualitative analysis of fibre generated data for the transgenic plants with respect to fibre length, fibre micronaire values, and fibre strength. In the case of fibre length and micronaire values, the transgenic lines were not consistent across the three generations (2009-2011). Transgenic cotton lines showed only 10% up-regulation for fibre length. (Fig 5) and (Fig 7) illustrate the improvement in fibre micronaire values (Jiang et al., 2000). All transgenic plants showed excellent improvement in fibre strength (22%) during this period, with consistently higher fibre strength in all three generations (Fig 6), similar to the findings of (Luo et al., 2007; Machado et al., 2009; Qin and Zhu, 2011; Zhang et al., 2010). On the basis of fibre analysis, we concluded that the transgenic plants with fibre gene *CpEXPA3* were greatly improved in fibre strength after genetic modification. Other qualitative fibre characteristics of the transgenic plants showed some improvement in one generation but not in the next. These results correlate with previous studies (Li et al., 2005; Pu et al., 2008; Wang et al., 2009). Morphological characteristics also indicated that this gene had a significant effect on ginning out turn percentage, an essential parameter for textiles. The results from the present study suggest that transgenic cotton with *Calotropis* gene is necessary for improvement of fibre strength as compared to other fibre qualitative characteristics. In this study, we sought to improve fibre quality by modifying the cotton plant genome with gene transformation. We found that transformation allowed for new features and improved cellulose synthesis, suggesting that expression of the wild plant *CpEXPA3* gene in cotton may be a useful way to genetically engineer cotton traits for high fibre quality.

Materials and methods

Transformation of cotton variety NIAB 846

The cotton variety *Gossypium hirsutum* var. NIAB 846 was selected for transformation on the basis of its high seed germination and low fibre quality response. The mature embryos (kernels from mature seeds) of *Gossypium hirsutum* var. NIAB 846 were transformed with *CpEXPA3* using *Agrobacterium* LBA-4404. The cotton seed surface was sterilized by 1 g/l $HgCl_2$ and 1 g/l of 10% sodium dodecyl sulfate (SDS). Mature embryos were isolated from germinating seeds and a cut was made at the apex of the shoot with a sterilized blade. Afterwards, the embryos were co-cultivated for 1 h with an *Agrobacterium* strain containing *CpEXPA3* gene. The embryos were than dried on sterilized

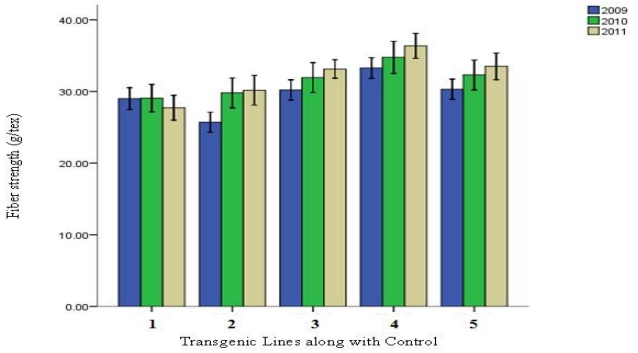

Fig 6. Comparison of Fiber Strength of *CpEXPA3* cotton plants with control (T0, T1, and T2 Generations 2009, 2010 and 2011). Group 1 shows fiber strength of control plant for three generations. Group 2-5 indicate fiber strength of different transgenic cotton plants for T0, T1, and T2 Generations of year 2009, 2010 and 2011 respectively.
Note: Bar Determined the Standard Error.

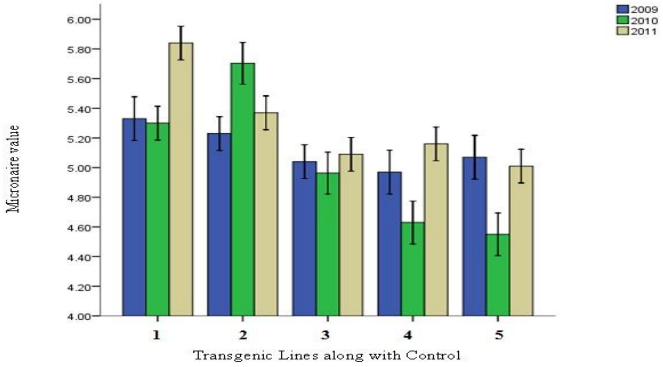

Fig 7. Comparison of Fiber Micronaire Value of *CpEXPA3* cotton plants with control (T0, T1, and T2 Generations 2009-2011). Group 1 shows Micronaire Value of control plant for three generations. Group 2-5 indicate Micronaire Value of different transgenic cotton plants for T0, T1, and T2 Generations of year 2009, 2010 and 2011 respectively.
Note: Bar determined the standard error

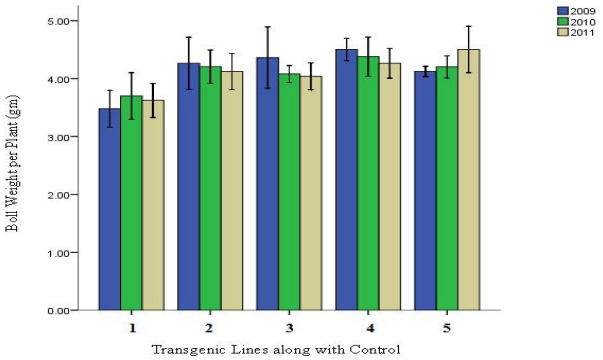

Fig 8. Comparison of Boll Weight per Plant of Transgenic plants with fiber gene *CpEXPA3* and Parental plant (T0, T1, and T2 Generations 2009-2011). Group 1: Control Plant, Group 2-5: Boll Weight per Plant of putative transgenic cotton plants with *CpEXPA3* fiber gene for T0, T1, and T2 Generations in year 2009, 2010 and 2011. Note: Bar determined the standard error.

filter paper and cultured on MS medium for 3 d at 28 °C. After 3 d, embryos were subcultured on MS medium containing kanamycin 50 g/l optimized for cotton plants for selection. After two months of kanamycin selection, the putative transgenic plants were shifted to soil containing sand, silt and clay in the ratio called loamy soil, in pots. The stable putative transgenic plants were subjected to molecular analysis after 15–20 d following shifting (Jin et al., 2005; Rao et al., 2009).

Stable gene integration and detection

Confirmation of stable transformation of *CpEXPA3* in all three generations of transgenic cotton plants of NIAB 846 were performed using PCR with orientation primers and DNA gel blot analysis or Southern blot analysis (Zhao et al., 2006).

Polymerase chain reaction and DNA gel blot analysis

Newly emerging portions of the fibre transgenes and parental lines were used for the isolation of genomic DNA as describe by (Paterson et al., 1993) with some modification. Transgenes with fibre gene *CpEXPA3* were detected using polymerase chain reaction, which amplifies the 925 bp PCR product with orientation primers designed from both the promoter region and gene region to confirmation the presence of the transgene (Cronn et al., 2002). Orientation primers for this amplification were: 5'-GACTTTTCAACAAAGGG-TAATTTCG-3' and 3'- TTTATGGACACGGAATGAACG-5'. The stable integration of transgenes with the fibre gene *CpEXPA3* was detected by performing a DNA gel blot analysis or southern blot analysis. The restriction enzyme HindIII was used to digest transgenes containing the fibre improvement gene, with the parental lines used as a negative control (Feinberg and Vogelstein, 1983; Liu et al., 2006; Rao et al., 2011; Southern, 1975).

RNA extraction and quantitative Real-time PCR (qtr.-PCR)

Total RNA was extracted from the young leaves of transgenic cotton according to the protocol described by (Jaakola et al., 2001). Quantitative real time PCR was performed on the total transgenic RNA using gene specific primers. Forward Primer: 5' GAGATGCAGCTCTTCTG-CTG 3' and Reverse Primer: 5' GATCGAAGTGCTGA-AGTGGA 3'. RNA samples of transgenes were normalised using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The thermal profile used for all PCR was 94 ºC for 3 minutes, 40 cycles of 45 seconds at 94 ºC, 45 seconds at 53.4ºC, and 45 seconds at 72 ºC. Each PCR assay generated three replicates (Rao et al., 2011).

Cellulose quantification studies

Cellulose content was measured using the acetic-nitric assay. In this method, fibre impurities other than the cellulose, such as lignin, hemicellulose, and xylosan, were removed by dissolving cotton fibre in an acetic acid-nitric acid reagent according to the method described by (Shu et al., 2009). Cellulose content from transgenic cotton fibre and parental lines was calculated after the digestion of fibre in this reagent, followed by the addition of an anthrone reagent, using a spectrophotometer (Shu et al., 2009; Thygesen et al., 2005; Updegraff, 1969).

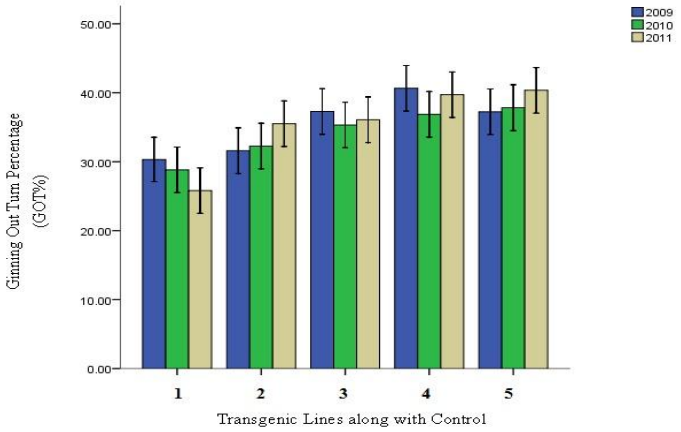

Fig 9. Comparison of Ginning Out Turn percentage (GOT%) of Transgenic with *CpEXPA3* and Control Lines (T0, T1, and T2 Generations 2009-2011), Lane 1: Control Plant, Lane 2-5: Ginning out Turn %age (GOT %) of selected transgenic plants with *CpEXPA3* fiber gene, Note: Bar determined the standard error.

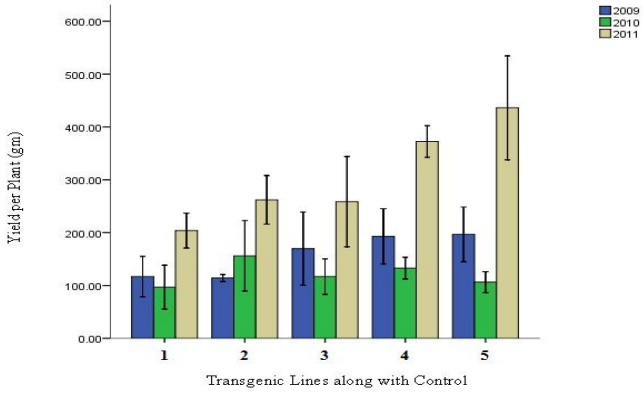

Fig 10. Comparison of Yield per Plant Transgenic with *CpEXPA3* and Control Lines (T0, T1, and T2 Generations 2009-2011): Lane 1: Control Plant, Lane 2-5: yield per Plant of Different *CpEXPA3* Plants.
Note: Bar determined the standard error.

Measurements of fibre characteristics

To measure fibre characters, 4 transgenic lines and a control were selected, and samples were collected from the F_0, F_1, and F_2 generations during 2009, 2010, and 2011 during the boll-opening period. The fibre quality parameters were tested according to ICC standards at the Test Centre of Cotton Fibre Quality at the APTMA, Pakistan. Fibre quality data such as fibre length, fibre strength, fibre uniformity and micronaire value for 2009-2011 was collected in replicate. The instrument used for determining the increase in fibre length (fiberogram) measured the length of the fibre at 25% and 50%. The other high volume instrument was used to calculate the fibre strength and micronaire value.

Morphological and agronomic characteristics

Morphological characteristics are very important with respect to crop performance in field conditions (Allah Bakhsh, 2009). Various morphological and agronomic characteristics were recorded, including plant height, number of bolls, boll weight per plant, number of sympodial and monopodial branches,

average yield and ginning out turn (GOT %). The most important agronomic data, including yield of putative transgenic and control plants, were calculated for the period of 2009-2011.

Statistical analysis

Fisher's analysis is a well-renowned technique for analysis of variance used for these data. The experimental and control plants were compared using the least significant difference test (LSD) at 5% probability (Petersen, 1994). Cotton fibre length, fibre strength and fibre micronaire values of transgenic cotton were calculated using classical fibre analysis.

Acknowledgements

We are highly thankful to the Director, Nuclear Institute of Agriculture and Biotechnology (NIAB), Faisalabad for providing seeds of cotton variety NIAB-846 and also grateful to APTMA office, Lahore for allowing us to use fiber analysis equipment. We are also thankful to Higher Education Commission (HEC) for providing research funding for this project.

References

Abbas B, El Tayeb AE, Sulleiman YR (1992) Calotropis procera: feed potential for arid zones. Vet Rec 131:132.

Allah Bakhsh AQR, Ahmad Ali Shahid, Tayyab H and Riazuddin S (2009) Insect Resistance and Risk Assessment Studies in Advance Lines of Bt Cotton Harboring Cry1Ac and Cry2A Genes. Am-Eurasian J Agric Environ. Sci 6 10.

Carruthers IB, Griffiths DJ, Home V, Williams LR (1984) Hydrocarbons from Calotropis procera in northern Australia. Biomass 4:275-282.

Cheema HMN, Bashir A, Khatoon A, Iqbal N, Zafar Y, Malik KA (2010) Molecular characterization and transcriptome profiling of expansin genes isolated from Calotropis procera fibers. Electron J Biotechnol. 13:10-11.

Cronn R, Cedroni M, Haselkorn T, Grover C, Wendel JF (2002) PCR-mediated recombination in amplification products derived from polyploid cotton. Theor Appl Genet. 104:482-489.

Cui X, Calamari TA, Robert KQ, Price JB, Watson MD (2003) Measuring the Short Fiber Content of Cotton. Text Res J 73:891-895.

de Morais Teixeira E, Corrêa A, Manzoli A, de Lima Leite F, de Oliveira C, Mattoso L (2010) Cellulose nanofibers from white and naturally colored cotton fibers. Cellulose 17:595-606.

Eisikowitch D (1986) Morpho-ecological aspects on the pollination of Calotropis procera (Asclepiadaceae) in Israel. Plant Syst Evol. 152:185-194.

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6-13.

Jaakola L, Pirttilä A, Halonen M, Hohtola A (2001) Isolation of high quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. Mol. Biotechnol. 19:201-203.

Jiang C-X., Chee PW, Draye X, Morrell PL, Smith CW, Paterson AH (2000) Multilocus Interactions Restrict Gene Introgression in Interspecific Populations of Polyploid Gossypium (Cotton). Evolution 54:798-814.

Jin S, Zhang X, liang S, Nie Y, Guo X, Huang C (2005) Factors affecting transformation efficiency of embryogenic callus of Upland cotton (*Gossypium hirsutum*) with Agrobacterium tumefaciens. Plant Cell Tiss Org 81:229-237.

Kohli A, Leech M, Vain P, Laurie DA, Christou P (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc Natl Acad Sci U S A 95:7203-8.

Lee JJ, Woodward AW, Chen ZJ (2007) Gene Expression Changes and Early Events in Cotton Fibre Development. Ann Bot-London 100:1391-1401.

Li X, Wang XD, Zhao X, Dutt Y (2004) Improvement of cotton fiber quality by transforming the acsA and acsB genes into Gossypium hirsutum L. by means of vacuum infiltration. Plant Cell Rep. 22:691-697.

Li XB, Fan XP, Wang XL, Cai L, Yang WC (2005) The cotton ACTIN1 gene is functionally expressed in fibers and participates in fiber elongation. Plant Cell 17:859-75.

Liu D, Zhang X, Tu L, Zhu L, Guo X (2006) Isolation by suppression-subtractive hybridization of genes preferentially expressed during early and late fiber development stages in cotton. Mol Biol (Mosk) 40:825-34.

Luo M, Xiao Y, Li X, Lu X, Deng W, Li D, Hou L, Hu M, Li Y, Pei Y (2007) GhDET2, a steroid 5alpha-reductase, plays an important role in cotton fiber cell initiation and elongation. Plant J 51:419-30.

Machado A, Wu Y, Yang Y, Llewellyn DJ, Dennis ES (2009) The MYB transcription factor GhMYB25 regulates early fibre and trichome development. Plant J 59:52-62.

Paterson A, Brubaker C, Wendel J (1993) A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis. Plant Mol Biol Rep 11:122-127.

Petersen RG (1994) Agricultural Field Experiments: Design and Analysis. Marcel Dekker Inc., NY, USA, p.205-260.

Pu L, Li Q, Fan X, Yang W, Xue Y (2008) The R2R3 MYB transcription factor GhMYB109 is required for cotton fiber development. Genetics 180:811-20.

Qin YM, Zhu YX (2011) How cotton fibers elongate: a tale of linear cell-growth mode. Curr Opin Plant Biol. 14:106-11.

Raghavan R (1957) Chromosome numbers in Indian medicinal plants. Plant Sci. 45:294-298.

Rao AQ, Bakhsh A, Kiani S, Shahzad K, Shahid AA, Husnain T, Riazuddin S (2009) The myth of plant transformation. Biotechnol Adv 27:753-63.

Rao AQ, Irfan M, Saleem Z, Nasir IA, Riazuddin S, Husnain T (2011) Overexpression of the phytochrome B gene from Arabidopsis thaliana increases plant growth and yield of cotton (*Gossypium hirsutum*). J Zhejiang Univ Sci B 12:326-34.

Sakthivel JC, Mukhopadhyay S, Palanisamy NK (2005) Some Studies on Mudar Fibers. J Ind Text. 35:63-76.

Shu H, Zhou Z, Xu N, Wang Y, Zheng M (2009) Sucrose metabolism in cotton (*Gossypium hirsutum* L.) fibre under low temperature during fibre development. Eur J Agron. 31:61-68.

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol. 98:503-517.

Stewart JM (1975) Fiber Initiation on the Cotton Ovule (*Gossypium Hirsutum*). Am J Bot. 62:723-730.

Sunilkumar G, Rathore KS (2001) Transgenic cotton: factors influencing Agrobacterium-mediated transformation and regeneration. Mol Breeding 8:37-52.

Thygesen A, Oddershede J, Lilholt H, Thomsen AB, Ståhl K (2005) On the determination of crystallinity and cellulose content in plant fibres. Cellulose 12:563-576.

Tohidfar M, Mohammadi M, Ghareyazie B (2005) Agrobacterium -mediated transformation of cotton (*Gossypium hirsutum*) using a heterologous bean chitinase gene. Plant Cell Tiss Org. 83:83-96.

Updegraff DM (1969) Semimicro determination of cellulose inbiological materials. Anal Biochem. 32:420-424.

Varshney AC, Bhoi KL (1988) Cloth from bast fibre of the Calotropis procera (Aak) plant. Biol Waste 26:229-232.

Wang HY, Wang J, Gao P, Jiao GL, Zhao PM, Li Y, Wang GL, Xia GX (2009) Down-regulation of GhADF1 gene expression affects cotton fibre properties. Plant Biotechnol. J 7:13-23.

Wang F, Gong Y, Zhang C, Liu G, Wang L, Xu Z, Zhang J (2011) Genetic effects of introgression genomic components from Sea Island cotton (*Gossypium barbadense* L.) on fiber related traits in upland cotton (*G. hirsutum* L.). Euphytica 181:41-53.

Zhang F, Zuo K, Zhang J, Liu X, Zhang L, Sun X, Tang K (2010) An L1 box binding protein, GbML1, interacts with GbMYB25 to control cotton fibre development. J Exp Bot. 61:3599-3613.

Zhang F, Liu X, Zuo K, Sun X, Tang K (2011) Molecular cloning and expression analysis of a novel SANT/MYB gene from Gossypium barbadense. Mol Biol Rep. 38:2329-36.

Zhao F-Y, Li Y-F, Xu P (2006) Agrobacterium -mediated transformation of cotton (*Gossypium hirsutum* L. cv. Zhongmian 35) using glyphosate as a selectable marker. Biotechnol Lett. 28:1199-1207.