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OVEREXPRESSION OF GENE *ΔGH_A07G1537* ASSOCIATED WITH FIBER QUALITY IN UPLAND COTTON (*GOSSYPIUM HIRSUTUM* L.) THROUGH POLLEN TUBE TRANSFORMATION METHOD

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ABSTRACT

Cotton is an important textile fiber and a significant oil seed crop. Molecular interventions helped explore the genes involved in fiber strength and length. RNA-seq data on the differential expression of the *Gh_A07G1537* gene reveals its role in enhancing fiber quality parameters. Fiber length is an important characteristic of cotton that affects its quality and suitability for different textile products. Fiber quality plays a significant role in determining the value and marketability of cotton as a textile raw material. In this study, the gene construct was prepared in *Agrobacterium* for transformation. The pollen tube method was employed for the transformation of a gene. A total of 2,250 samples of buds were injected with gene construct with varied concentrations (1-10µL) at different intervals (7:00AM–11:00AM). Leaf samples were taken for transgene integration and validated through PCR. The differential expression of (*Gh_A07G1537*) gene was evaluated through real-time qPCR. It was found that the gene construct injected into cotton variety Eagle-2 at a concentration of 5µL at 9:00AM showed higher expression of -5.9folds followed by 7µL concentration -5.7folds as compared to the non-transgenics. The transformation efficiency was 1.3% at 5µL followed by 1.25% at 7µL concentration of the construct.

Keywords: *Gossypium,* Gene transformation, Pollen tube, *Agrobacterium,* Fiber Quality

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1. INTRODUCTION

Cotton belongs to the Malvaceae family, is composed of cellulosic fiber used in fiber products, and is also known as “King of fibers” or White gold (Zafar et al. 2022; Zafar et al. 2024a). Cotton fibers are seed trichomes that is developed around the seed and protected by the cotton bolls (Razzaq et al. 2022; Ijaz et al. 2024). Cotton contains 52 genomic species which are differentiated into eight genome groups A-G and K (Zafar et al. 2023a; Anwar et al. 2023). Only four species are used in cultivation: two Asiatic A-genome diploid species, *Gossypium arboreum* and *Gossypium herbaceum* (2n=26), and two allotetraploid AD-genome species, *Gossypium hirsutum* and *Gossypium barbadense* (Wilkins and Arpat, 2005; Zafar et al. 2024b). *Gossypium hirsutum*, commonly known as upland cotton, is the most widely grown species, accounting for about 90% of global cotton production, while *Gossypium barbadense*, known for its superior fiber quality, contributes to the remaining 10%. These species have been cultivated and selectively bred for their fiber characteristics, adaptability, and resistance to environmental stresses, making them essential to the cotton industry worldwide (Iqbal et al. 2022; Zafar et al. 2023b).

Cotton fiber is composed of 88-96.5% cellulose, with non-cellulosic components present in the lumen or cuticle. They include proteins (1.0-1.9%), pectin (0.4-1.2%), waxes (0.4-1.2%), inorganic materials (0.7-1.6%) and substances (0.5- 8.0%) (Bowling et al. 2011). Cotton fiber is a seed trichome that can be used in the textile industry and expressed genes at different developmental stages. There are four successive stages of development for cotton fiber: initiation, elongation, synthesis of secondary wall and maturation. Prior to the day of anthesis, initiation begins and then lasts for 5 days after anthesis and elongates rapidly until 20-day post anthesis (DPA) and the fiber length increases from 25-25mm resulting in a thin primary cell wall (PCW) layer (Lee et al. 2007; Tian and Zhang,

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2021). The secondary cell wall (SCW) synthesis begins between 15-22 and then lasts for 30-40 days. At maturity between 40-45 DPA cotton boll dehisce, fiber is exposed to air and sunlight (Liu, 2017; Kim, 2015). However, mature fiber contains a thicker SCW as compared to immature fiber (Liu and Kim, 2015). China produces the highest raw cotton followed by India, Pakistan and Brazil with production of 30.50, 20.90, 8.25 and 6.50 million bunches (Abdelraheem et al. 2019).

China is the leading producer of cotton, contributing approximately 24% of the world's total production, followed closely by India, which accounts for 23%. The United States, a major player in the cotton industry, produces 15% of the global supply, while Brazil contributes 11%, and Pakistan accounts for 5% (Zafar et al. 2021; Zhang et al. 2023). These countries play a crucial role in supplying raw cotton to meet global demand, contributing to the textile and fabric industries (Baffes, 2005). Cotton production, particularly in these regions, is influenced by factors such as climate, soil conditions, and advancements in agricultural practices, all of which impact the fiber quality and yield (Zhang et al. 2023).

Pollen tube pathway-mediated transformation is the process of introducing foreign genes into embryo sacs, which are then integrated into the plant genome during self-pollination. Pollen is transferred to the stigma during pollination (Wang et al. 2019; Waheed et al. 2022). After germination, the pollen forms a tube that passes through the style's tissues and into the ovary. This procedure creates a lengthy pollen tube channel that can be used to insert foreign DNA into embryo sacs (Geitmann and Palanivelu, 2007; Zheng et al. 2018). The first successful use of this technique was for genetically modifying cotton in 1978. By utilizing the pollen tube method to introduce foreign genomic DNA into cotton embryo sacs, Zhou and colleagues successfully generated a significant number of transgenic plants (Zhou et al. 1983; Aslam et al. 2020). Transformation of hygromycin and kanamycin resistance gene is achieved through pollen tube transformation (Bibi et al. 2013). A 5-7µL of LB-hpt/nptIIRB (pCAMBIA1301/2301) was used for transformation and 1.9- 15.3% efficiency was found through PTT (Rajasekaran, 2012).

Differentially expressed gene *Gh_A07G1537* belongs to CCC-H zinc finger gene superfamily that regulates the primary cell wall synthesis which was located on chromosome 7. Cotton fiber length was improved due to overexpression of gene. Stable integration of the transgene *ΔGh_A07G1537* was validated by tracking its expression in different generations (T_0, T_1, and T_2) of transformed cotton plants. Transgenic cotton plant shows -2.97, -2.86- and -2.92-folds higher expression than non-transgenic cotton plants, which improved cotton fiber parameters (Razzaq et al. 2021). The gene contributes significantly to the improvement of fiber length, similar to other transgenes such as expansin, cellulose synthase, sucrose synthase, and actin (Bajwa et al. 2015; Ahmed et al. 2018).

By using the shoot apex method, following gene differentially expressed *Gh_A07G1537* was incorporated into cotton by *Agrobacterium* mediated transformation method. It demonstrated -2.97, -2.86- and -2.92-folds expression, which enhanced the fiber quality. The same gene *Gh_A07G1537* was transformed by another method-pollen tube mediated transformation method to observe the overexpression of gene. The purpose of this research is to study the transformation efficiency and overexpression of the gene *Gh_A07G1537* in cotton using pollen tube transformation method.

2. MATERIALS AND METHODS

2.1. Selection of Gene

The Institute of Cotton Research, Chinese Academy of Agricultural Sciences (ICR-CAAS) developed Chromosomal Segment Substitution Lines (CSSLs)-MBI7747 from CCRI45 and CCRI36. Both line and parents were subjected to transcriptome sequencing for differential expression of the genes. A candidate gene (*Gh_A07G1537*) was selected for further functional validation.

2.2. Preparation of Cassette

The gene sequence was retrieved and synthesized by Bio Basic Co. Canada after codon optimization. The arrangement of genes in a cassette have been shown in (Fig. 1). The Nos terminator and the CaMV35S promoter regulate the gene. Primer 3 (Ver.3) was used to design the primers.

2.3. Amplification of Gene in Gene Cassette

The gene cassette was received in puc57 vector, and gene was detected using gene specific primers (Fig. 3 and Table 2). A 25µL was the total volume that was prepared for the PCR reaction. Following an initial denaturation at 95°C for 3min, denaturation at 95°C for 45s, annealing at 65.4°C for 45s, extension at 72°C for 2min, and a final extension at 72°C for 10min were the conditions of the PCR. The amplified gene band was seen on 1% agarose gel.

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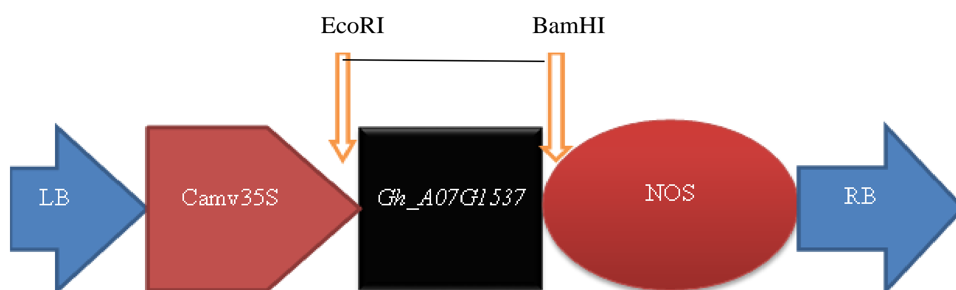

Fig. 1: Synthesis of gene (*Gh_A07G1537*) cassette

Fig. 2: Schematic flow of pollen tube transformation in cotton

2.4. Cloning of Genes into the Plant Expression Vector pCAMBIA2300

Gene cassette and PCAMBIA2300 were digested with *EcoRI* and *BamH1* and visualized on 1% agarose gel. The rapid ligation kit was used to ligate the digested products that had been purified. The purified product was cloned into *Agrobacterium tumefaciens* using the liquid nitrogen technique. To initiate the transformation process, 100µL of competent cells were treated with 3µL of plasmid and incubated in liquid nitrogen for 10min. After that, the cells were quickly placed in an incubator and left there for five minutes at 37°C. The cells were again incubated for three minutes in liquid nitrogen following the first incubation. After that, 500µL of YEP broth was added, and the combination was left for two to three hours at 28°C in a shaker incubator. After two days of incubation, the cells were plated onto YEP agar plates containing 50mg/mL of rifampin, and colony development was observed. Then, using PCR, discrete *Agrobacterium tumefaciens* colonies were chosen for gene confirmation.

2.5. Pollen Tube Transformation

The construct was prepared about 50µL in *Agrobacterium tumefaciens*. During the flowering season, flowers were selected that would bloom the next day. The upper part of the bud was closed with nail paint that kept the self-pollination to avoid the potential effects of cross-pollination. After blooming, the petals and style were gently and carefully removed to minimize damage to the ovary. The micro injector was cleaned and 10µL construct were taken and then punctured vertically with the micro injector into the ovary about 5mm deep from the top of the ovary then drew back about 2mm and slowly injected 5–10µL construct into the ovary. The microinjector was removed carefully from the ovary. After completing the injection, all vegetative parts of that specific branch were removed. During harvest season, the cotton bolls were harvested individually. The seeds were taken out of each boll and then sown in the pots. After germination, cotton plants were shifted into the field. When the bolls formed, first-generation plants (T_1) were sown in the field again to produce second-generation plants (T_2). The schematic description of the process transformation efficiency is shown in Fig. 2 and Table 1.

2.6. DNA Extraction and PCR Identification

The 2nd leaf of 1st generation plants was taken and placed in the ice box to avoid wilting and sample was used for the detection of gene. The sample of leaves was placed in pestle and mortar and 700µL of buffer A was added to grind the sample with liquid nitrogen. A 500µL concentration of buffer B was added to the mixture,

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and the sample was separated into a 2mL Eppendorf tube. The sample was vortexed thoroughly and kept them in an incubator at 65°C for 30min. After incubation, centrifuged the sample for 10min at 11,000rpm then supernatant was taken in another Eppendorf tube and discarded the debris. About 500µL of CIA was added in the Eppendorf tube then centrifuged for 10min at 11,000rpm. The supernatant solution was carefully collected and again 500µL CIA was added and centrifuged at 11,000rpm for about 10min, and supernatant was separated. About 300µL chilled isopropanol was added in the supernatant solution and kept on -20°C for 20min. Sample was centrifuged for 15min at 14°C at 13,500rpm. The supernatant was discarded and washed the pellet with 70% ethanol of 400-500µL, then centrifuged for 2-3min. Pellet was dried for two minutes and dissolved in 40µL of autoclaved water. RNase was added to the final concentration of 5µL to the DNA. After a short spin, the samples were kept at 37°C overnight or 30min. The quality of DNA was further ascertained by resolving DNA on agarose gel. The gene in transgenic plants was amplified through PCR using full length and short length primers (Fig. 4 and 5).

Table 1: Genetic transformation of gene in pollen tubes of cotton variety Eagle 2

Genetic transformation						
Exp.	Time (AM)	Concentration(µL)	Buds injected	Bolls survived	Detected as positive	Transformation Efficiency
1		1	180	4	1	0.5
2		2	190	5	1	0.52
3		3	200	7	1	0.5
4		4	210	11	1	0.4
5		5	220	15	3	1.3
6		6	230	13	2	0.8
7		7	240	17	3	1.25
8		8	250	10	2	0.8
9		9	260	12	1	0.3
10	7-11	10	270	7	0	0

Table 2: list of primers used for PCR amplification

Primers	Sequences (5' - 3')
Forward	CCATGGATGCCT GATAATCGGCAAGTTCAGAAC
Reverse	AGATCTTCAATCATCATGTGAGGTTTTCGAAGA ACCC

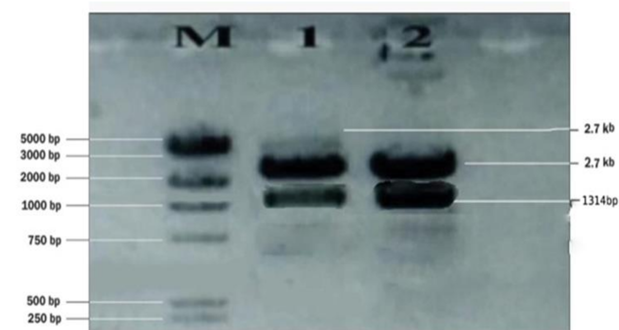

Fig. 3: Detection of gene (*Gh_A07G1537*) through restriction digestion; Lane M: Molecular weight markers; Lane 1-2: lower band shows the gene (*Gh_A0 7G1537*) size (1314bp) and the upper bands contain the PUC57 (2.7kb) vector.

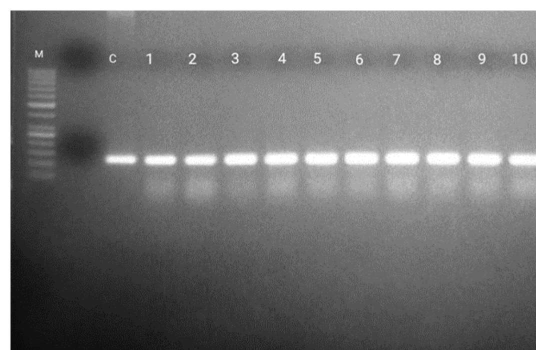

Fig. 4: PCR confirmation of *Gh_A07G1537* gene; Lane M: Molecular weight markers; Lane C: Non-transgenic plants; lane 1-10: transgenic plants containing gene (*Gh_A07G1537*)

2.7. RNA Extraction and qRT-PCR

Using Agilent RNA extraction kit (Agilent Technologies, Santa Clara, USA, Cat # 5185-6000), total RNA was extracted from both young and mature leaves of transgenic cotton. A NanoDrop 2000 spectrophotometer operating at 260 and 280nm wavelengths was used to measure the RNA concentration (ng/µL). After being synthesized, the cDNA was kept at -20°C using the Thermo Scientific cDNA synthesis kit (Cat # K1632). The qRT-PCR was used to measure relative gene expression. Gene specific primers were used, and each reaction was run in triplicate (Thermo Scientific, Cat # K0221). Maxima SYBR Green/ROX qPCR Master Mix (2x), 1µL of cDNA (50ng/µL), and 1 µL of forward and reverse primers (10pmol) were combined to create a 20µL reaction mixture. The internal reference gene *GAPDH* was used in this study.

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3. RESULTS

3.1. Detection of Gene in Gene Cassette

Gene cassette was received in the PUC57 vector that contained the *Gh_A07G1537* gene and it was detected through restriction digestion. The product size of gene *Gh_A07G1537* was observed to be 1314bp as shown in (Fig. 3).

3.2. Confirmation of Genes through PCR using Short-length Primers

Leaf samples were taken and then amplified through PCR. The PCR product was corresponding a size of 128bp. The gene amplification can be shown in (Fig. 4).

3.3. Detection of a Gene through PCR using Full-length Primers

Leaf samples were taken and then amplified through PCR. The PCR product was found to be a size of 1314bp. The gene amplification can be shown in (Fig. 5).

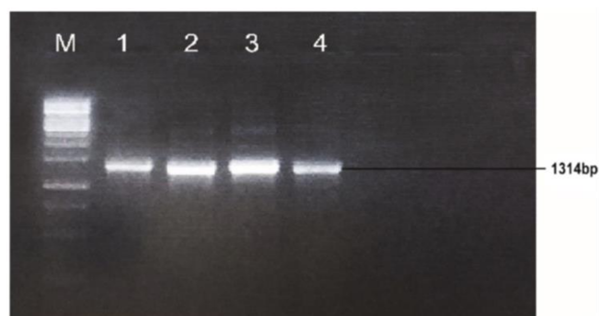

Fig. 5: PCR confirmation of *Gh_A07G1537* gene using full length primers; Lane M: Molecular weight marker; Lane 1-4: transgenic plants containing gene (*Gh_A07G1537*)

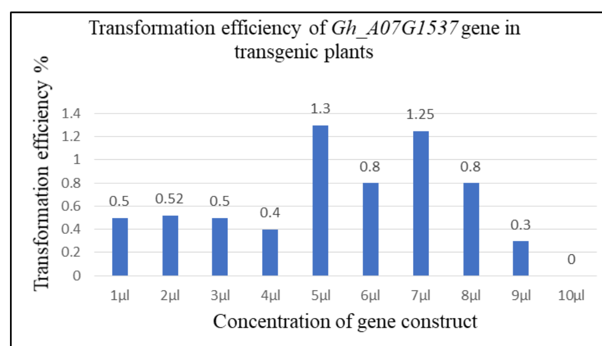

Fig. 6: Transformation efficiency of *Gh_A07G1537* gene in transgenic plants

3.4. Genetic Transformation of Cotton through Pollen Tube Transformation

About 1µL construct was injected at three different time intervals i.e., 7:00AM., 9AM. and 11AM., in 180 self-pollinated buds, resulting in transformation rate of 0.5%. On the second day, at the same time intervals about 2µL was injected in 190 buds and the transformation efficiency was 0.52%. A 3µL was injected on the 3rd day at 7AM., 9AM. and 11AM. and 0.5% transformation efficiency was recorded. On 4th day, the transformation efficiency was 0.4% at 4µL concentration at 7AM, 9AM, and 11AM. On the 5th day, at same time intervals about 5µL construct was injected and the transformation efficiency was turned out to be 1.3%. About 6µL was injected on 6th day and the rate of transformation was 0.8%. The transformation efficiency was 1.25% at 7µL concentration using same time intervals. On the day 8th, with 8µL construct the transformation efficiency was found to be 0.8%. On days 9th and 10th, 9µL, and 10µL constructs were injected and transformation was found to be 0.3% and 0% respectively. The graphical and tabular representation is shown in Table 1 and Fig. 6. The injection of sample through pollen tube in the field can be seen in Fig. 7.

3.5. Quantitative Expression of a Gene through qRT-PCR

The expression analysis of transgenic cotton plants was performed by qRT-PCR. The cotton transgene expression compared to non-transgenic plants was 3.5, 3.9, 3.5, 4.9, 5.9, 4.4, 5.7, 4.4, 3.2 and 0 folds. Transgenic plants injected with a concentration of 5µL showed higher expression of -folds 5.9 at 9:00AM followed by -folds 5.7 at 7µL concentration as shown in Fig. 8.

4. DISCUSSION

Cotton (*Gossypium hirsutum* L.) was the first plant species to be used for the pollen tube transformation (PTT) approach, which involved transferring exogenous DNA into the plant embryo (Zhou et al., 1983; Nkaa and Okpe, 2021). Using the gramineous expression vector *pGU4ABBar* and the pollen-tube pathway, the *CryIa* gene, a synthetic insecticidal crystal protein gene from *Bacillus thuringiensis*, was introduced into the wheat types Xinong 2208 and Xinong 132. The *CryIa* gene was obtained in 27 transgenic plants using PCR and Southern blotting investigation. The protein was expressed in the transgenic plants, according to a western blotting examination.

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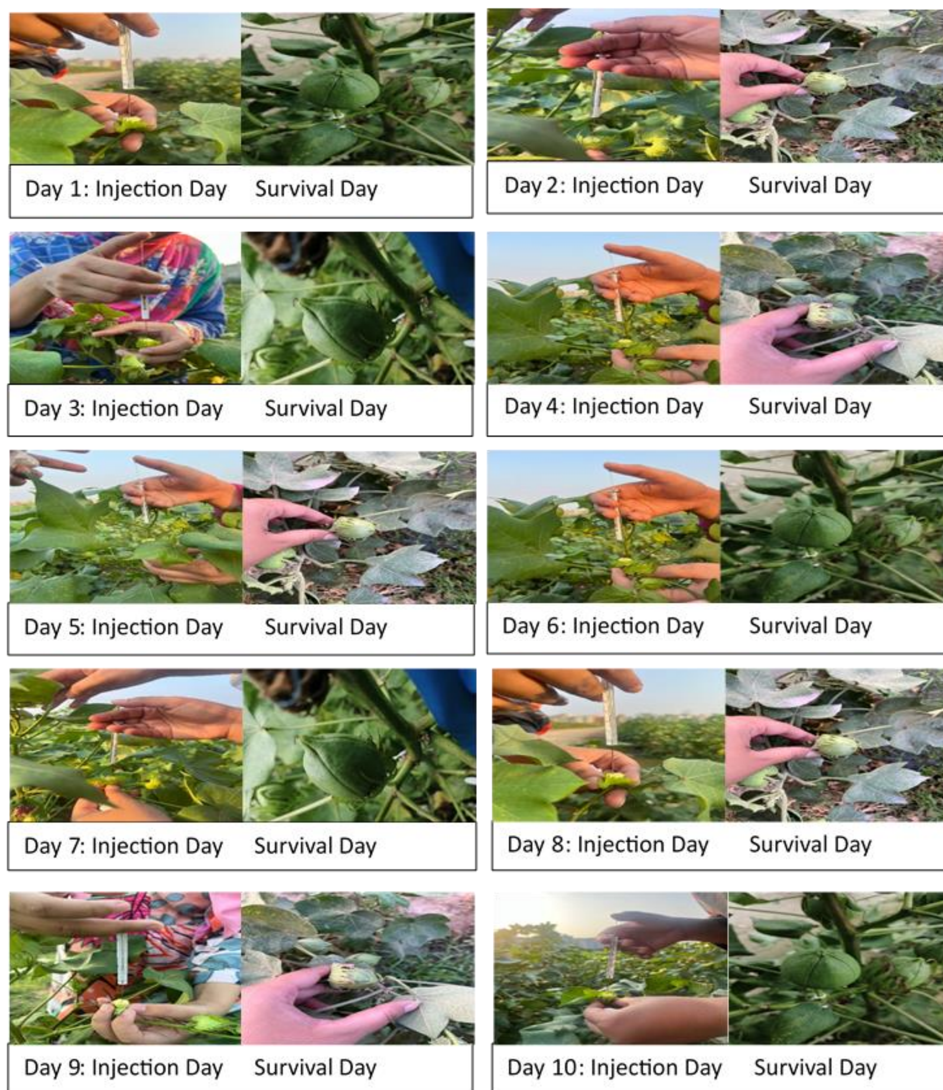

Fig. 7: A schematic flow diagram of gene transformation

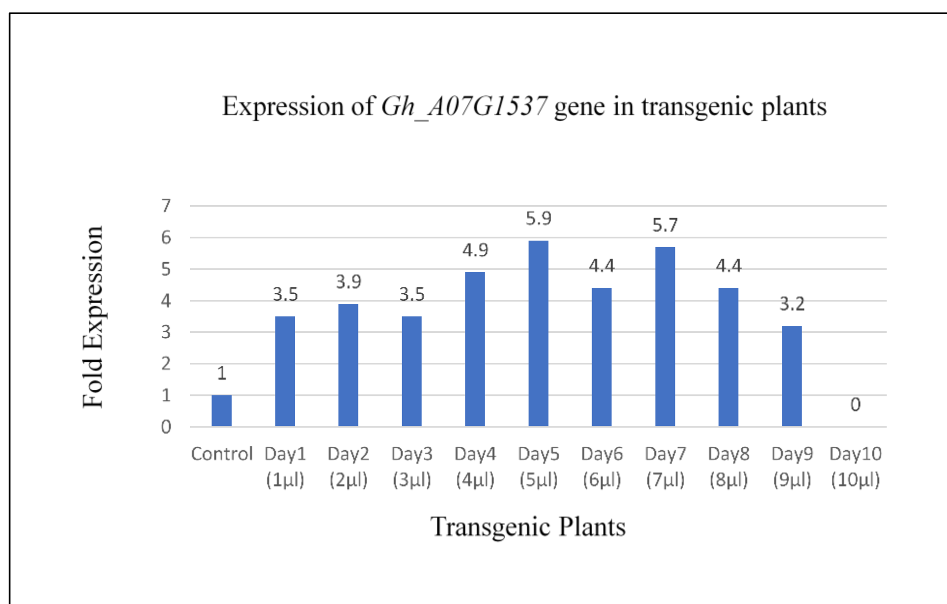

Fig. 8: Relative expression of gene through qRT-PCR

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The frequency of transformation ranged between 1.13 and 1.21% (Hou et al. 2003). The gramineous expression vectors *pGU4AGBar* and *pGBIU4AGBar* were used respectively. The *GNA* gene, a synthetic *agglutinin* gene from *Galanthus* species, was inserted into the winter wheat types Xinong 2208 and Xinong 132 via pollen tube method. The PCR and southern blotting studies showed a total of 20 transgenic plants expressing the *GNA* gene. A western blotting analysis revealed that the transgenic plants expressed the target protein. The transition frequency ranged from 0.28 to 0.84% (Hou and Guo, 2003). Regardless of the type of agriculture, whether solid or liquid, pollination took place in the evening, resulting in a low boll set of 3-5% (Woodcock, 2012). Pollination during the morning hours resulted in effective fertilization which led to boll set of 21 to 28.5% despite the pollen not being treated with pollen germination media and cotton stigma receptivity being low during evening hours. *Agrobacterial* culture with *PCAMBIA AC*, *cry1 Ia5*, *cry1 Aa3*, and *cry1 F* gene constructs was applied to 5619 flowers in total. The boll set has a range of 23.1 to 29.9%. Insect damage and incomplete pollination had a combined effect on the low rate of boll set. The solium selection of these kanamycin-resistant cotton transformants resulted in the production of 521 kanamycin resistant plants of the T1 generation. These plants produced 5692 healthy seeds, which were transferred to the T2 generation. Of these seeds, only 1199 germinated positively. The sprouting plants were then intensively in-solium kanamycin screened in the transgenic greenhouse. The gene integration studies made it clear that just seven plants had PCR positive results. The change's efficiency was 0.30 percent (Mogali et al. 2013).

The *pCAMBIA3301* plasmids were employed as a binary expression vector to transform cotton using the pollen-tube pathway. The CaMV35S (Cauliflower Mosaic Virus 35S) promoter was used to drive the selection and reporter genes in this cassette, including *bar* and *beta-glucuronidase* (*Gus*). The downstream regions of the genes contributed 35S polyA and NOS polyA, respectively, for the polyA tailing of the *bar* and *Gus* genes. At various concentrations of DNA injection, boll setting rates were observed in the ovaries of the investigated cultivars 33B and 99B. Regardless of the DNA injection concentrations, there was often little difference in boll setting effectiveness between the two cultivars. On the other hand, the success of boll setting was significantly influenced by the position of the bloom within the plant. After injecting exogenous DNA, it was easier to locate the boll in the flowers on fruit branches 5-7 compared to fruit branches 2-4 or fruit branches 8-10. The selection of the flower location in the plant and the appropriate concentration of target DNA are crucial for optimizing the efficiency of genetic transformation through the pollen-tube pathway. From 1.04 to 3.63%, the transformation rates dramatically increased, exceeding those previously observed in cotton (Li et al. 2005). Comparable to the boll setting rate, ovaries from fruit branches 5-7 showed a higher transformation efficiency compared to those from fruit branches 2-4 and 8-10. The concentration of DNA injection had a significant impact on the efficiency of transformation. In the experimental setting, this effect increased as the concentration of DNA increased (Zhang et al. 2009).

Chalcone synthase-A (*CHSA*) was a key enzyme in the biosynthesis of all classes of flavonoids, and variation of its expression might affect the colour of flowers (Wang et al. 2006; Ohta et al. 2022). Petunia (*Petunia hybrida*) flower petals were used to clone the *CHSA* gene, which was then placed into the expression vectors *pBI121* and *pWM101* which contain the sense-oriented CaMV35S promoter. Pollen-tube method was used for germline transformation to transfer *Cyclamen persicum*. Over 4400 seeds were obtained. Transgenic plants among them have changed blossom color. In 8 plants with white blooms, some petals developed yellow or light-yellow patches or even the entire petal turned yellow. Three plants with white blooms' half-petals even the entire flower of a few turned peach (Wanling et al. 2005).

In our study, *Gh_A07G1537* gene is used for the transformation in cotton variety using pollen tube transformation. Almost 2,250 buds injected with construct containing gene *Gh_A07G1537* with the help of microinjecter. The expression of transgene is 5.9 and 5.7 folds than in non-transgenic plants. The transformation efficiency was found to be 1.3%.

5. CONCLUSION

Genetic engineering is a breeding technique that enables the introduction of a plant with a variety of foreign genes simultane. Due to its favorable economics, cotton is farmed all over the world and is extensively used on an industrial scale for clothes and other home items. The research showed that cotton fiber parameters can be improved due to overexpression of the *Gh_A07G1537* gene. In comparison to non-transgenics, the gene construct injected into cotton variety Eagle-2 at a concentration of 5µL at 9:00 AM exhibited increased expression of 5.9 folds, followed by 5.7 folds at a concentration of 7µL. At a 5µL concentration of the construct, the transformation efficiency was found to be 1.3% using pollen tube method.

Author's Contribution: TF, ZH and FQ wrote the initial draft of the manuscript. RF, RM, AA and SS provided the space and helped to conduct the experiment. AI SS, MM and AI Conceptualization, writing—review and editing;

AG, ZA, and FQ data curation and helped in statistical analysis. AR, AI, FM, AI and GR helped in writing-review and editing, and AR and AA reviewed and supervised the experiment. All authors approved final version of the manuscript.

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