International Journal of Agriculture and Biosciences 2026 15(1): 168-174.
https://doi.org/10.47278/journal.ijab/2025.143

RESEARCH ARTICLE
eISSN: 2306-3599; pISSN: 2305-6622

Genome-wide Identification and Functional Analysis of Kunitz-Type Trypsin Inhibitor Gene Family in Cotton against Pest Resistance

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ABSTRACT

The proteinase inhibitors, such as Kunitz-type inhibitors (KTI), play a vital role in increasing pest resistance in genetically engineered crops. The KTI genes encode proteinase inhibitors known to confer resistance to major pests such as cotton bollworm and whiteflies. This study aims to comprehensively investigate the cotton genome-wide Kunitz Trypsin Inhibitor (KTI) gene family and assess its role in contributing to improving insect pest resistance. A total of 18 KTI genes were identified across three cotton species: *Gossypium hirsutum*, *Gossypium arboreum*, and *Gossypium raimondii*. The genomic structure, evolutionary relationships, and functional implications of these genes were analyzed using advanced bioinformatics tools. The agarose gel electrophoresis confirmed the integrity of the extracted DNA, ensuring the reliability of the analyses. The key genes with differential expression were identified using qRT-PCR. A qRT-PCR analysis was performed on selected genes, including *GH_A05G4127.1, GH_D04G0257.1, GH_A05G4126.1* and *GH_D04G0256.1* for computational predictions. The results showed that the *GH_A05G4127.1* gene exhibited 5-fold relative expression, whereas the other three genes were downregulated compared with the control. This research represents a significant step in characterizing KTI gene function in cotton and demonstrates its potential in conferring insect resistance. The findings contribute to the development of pest-resilient cotton cultivars, offering promising applications for sustainable agriculture.

Keywords: KTI genes, Pest-resistant, Kunitz Trypsin Inhibitor

Article History
Article # 25-337
Received: 13-Jun-25
Revised: 02-Jul-25
Accepted: 13-Aug-25
Online First: 09-Sep-25

INTRODUCTION

Cotton (*Gossypium hirsutum*) is one of the most significant crops in global agriculture, cultivated in approximately 55 countries and plays a central role in the textile industry (Razzaq et al., 2022). About 50 species of Gossypium are known to exist globally, four of which are grown primarily. Among these, *G. hirsutum* and *G. barbadense* are tetraploids, but *G. arboreum* and *G. herbaceum* are diploids (Razzaq et al., 2021; Zafar et al., 2025). Over 80% of the world's cotton-growing regions are dominated by tetraploid cottons, with *G. hirsutum* being the most extensively grown variety, especially in the Americas, Africa, and some regions of Asia (Ribeiro et al., 2021; Zafar et al., 2022). On the other hand, diploid species are mostly farmed in Asia and the Middle East; India is the only country that commercially farms almost all cultivated cotton species and hybrids (Wang et al., 2023; Anwar et al., 2023; Ijaz et al., 2024).

In 1753, Linnaeus made the first contribution to the taxonomic categorization of *Gossypium*. Later contributions included the cytological studies and research on both wild and farmed cotton (Shad et al., 2022). The *Gossypium* is a dicotyledonous genus in the Malvaceae

Cite this Article as: Shehzad S, Hafeez A, Chang MS, Suthar V, Khaskheli RA, Fatima S, Binish S, Rasool G, Mehmood A, Ali A, Khan AI and Razzaq A, 2026. Genome-wide identification and functional analysis of Kunitz-Type Trypsin Inhibitor gene family in cotton against pest resistance. International Journal of Agriculture and Biosciences 15(1): 168-174. https://doi.org/10.47278/journal.ijab/2025.143

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family, consisting of 45 diploid species divided into three subgenera: Sturtia, Houzingenia, and Gossypium. The tetraploid species have Old World A and New World D genomes as part of the subgenus Karpas. Among them are five allotetraploid species. The Tetraploid cottons, especially *G. hirsutum*, are most economically significant among these species in the world's cotton industry (Zafar et al., 2024).

Pakistan is one of the earliest regions to cultivate cotton. It ranks as the fifth largest producer and third largest exporter of raw cotton, alongside being a significant exporter of yarn. Approximately 60% of Pakistan's foreign exchange profits and 10% of its agricultural GDP come from cotton, with the Punjab province being the country's main cotton-producing region. Agriculture remains crucial to Pakistan's economy, providing employment for 38.5% of the population and contributing 19.2% to the national GDP (Zafar et al., 2025).

Worldwide cotton agriculture has a significant challenge in the form of pest infestations (Fan et al., 2025). To combat pests, especially the cotton bollworm (*helicoverpa armigera*), genetically modified (GM) insect-resistant crops, such as Bt cotton. It generates insecticidal proteins derived from *Bacillus thuringiensis* (Bt), which have been widely employed (Wang et al., 2023; Oliveira et al., 2024; Zhu and Luo 2024). Insect pests can have their genes silenced to limit their growth and feeding using RNA interference (RNAi) technology, which has also drawn interest as a pest management method (Ren et al., 2019; Zafar et al., 2020; Razzaq et al., 2023; Akram et al., 2025). Furthermore, it has been reported that proteinase inhibitors, such as Kunitz-type inhibitors (KTI) can play a vital role to increase pest resistance in genetically engineered crops (Long et al., 2024; Santos et al., 2025).

In cotton, Agrobacterium-mediated transformation has shown itself to be a dependable and effective technique for introducing foreign genes into plant genomes. One special ability of the soil bacterium *Agrobacterium tumefaciens* is to insert a part of its DNA, called T-DNA, into the genome of plant cells (Rahman et al., 2024). The Production of genetically modified cotton cultivars with enhanced characteristics, such as pest resistance, is made possible using Agrobacterium, which enables stable gene integration and expression (Shreni Agrawal, 2022).

This study focuses on the identification and validation through qRT-PCR of pest-resistant genes of KTI gene family, which encodes proteinase inhibitors known to confer resistance to major pests such as cotton bollworm and whiteflies. The research will explore the potential of the KTI gene family to enhance pest resistance and contribute to more sustainable cotton production.

MATERIALS & METHODS

Sequence Retrieval and Analysis of the KTI Gene

The nucleotide and amino acid sequences of the Kunitz Trypsin Inhibitor (KTI) gene were obtained from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/). The presence of the Kunitz domain (PF00197) in the obtained protein sequence was checked using the Pfam database (http://pfam.xfam.org/) for domain confirmation. The protein sequence was analyzed using HMMER3.3.2 (https://www.ebi.ac.uk/Tools/hmmer/) to further confirm the conserved domains. The profile Hidden Markov Models of Pfam was used to ensure the structural and functional integrity of the KTI domain.

Phylogenetic Tree

Peptide sequences of the cotton species were used to construct the phylogenetic tree. ClustalX (http://www.ebi.ac.uk/Tools/clustalw2/) software was used for alignment, and a Bootstrap N-J file was generated. The MEGA.11 software was used to visualize the tree.

Chromosomal Location of KTI Genes

The KTI gene location on the chromosomes of the cotton species was observed using TBtool. The Gene Structure Display Server v2.0 (http://gsds.gao-lab.org/) was used to display the gene structure.

Motif Analysis

The motif analysis for the KTI proteins was conducted through MEME (Multiple Em for Motif Elicitation) v5.5.4 (https://meme-suite.org/meme/tools/meme).

Cis-acting Regulatory Elements in Promoter Regions

A promoter upstream sequence (2.0 kb) of G. hirsutum was obtained from a database. The PlantCare online software (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was employed to analyze and predict the putative Cis-regulatory elements.

Plant Material and PCR Amplification

The cotton seeds of the Ghauri genotype were obtained from FB Genetics, Four Brothers Group, Lahore. The seeds were carried to the laboratory and stored at room temperature.

RNA Isolation and cDNA Synthesis

The RNA was isolated from cotton seeds using the Trizol method. The chloroform was added, followed by vortex and centrifugation at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was collected, and RNA was precipitated with isopropanol. Following a 10-minute incubation period, samples underwent another centrifugation, a 70% ethanol wash, and air drying. The RNA pellet was resuspended in RNase-free water. The quantity and integrity of isolated RNA were confirmed using a nanodrop spectrophotometer (Thermo-Scientific, United States) at A260: A280 ratio, and visualized using 1.5% gel electrophoresis. The RNA was stored at -80°C. The quantified RNA was used for cDNA synthesis in a reaction volume of 20µl, and final dilution was prepared in 100µl using the cDNA MIX recipe (Thermoscientific).

PCR Amplification

The cDNA template was taken for PCR amplification using optimum conditions set at: initial denaturation was 94°C for 5min, denaturation 94°C for 30s, annealing at 62.5°C for 35s, extension 72°C for 30s, and final extension

Table 1: List of primers

Gene IDs	F' primer	R' primer	Product length (bp)
GH_A05G4126.1	GGTCAAGAGCTCCGAACAGG	ACCGTCGGGCAGTAAACAAG	395
GH_A05G4127.1	TGGAGGTGTCAAACGGTCTC	TGAGAGCCAGACGCCTTACA	351
GH_D04G0256.1	TCCGGTAAAATGCTCCGCAC	AACACCCACGTCCCTACACA	442
GH_D04G0257.1	TAATCCGCGGTAGAGGTGGT	ACCGTCGGGCAGTAAACAAG	352

was 72°C for 5min. The total reaction volume was prepared in 25µl. A 1.5% agarose gel (Thermoscientific top vision agarose Ref# R0491; and Electrophoresis power supply model 500/200; JY-SPCT Horizontal electrophoretic tank) was used to visualize the PCR bands (Fig. 5). The gene-specific primer sequences were used for the four identified genes in Table 1. A 100 base pair DNA Ladder of (Thermoscientific cat# SM0323) was used in this study.

Relative Gene Expression

The relative expression of four genes was performed by qRT-PCR (Applied Biosystem Gene Amp PCR system 2700) following the SYBR Green master mix protocol (Thermoscientific cat# K1081 and Genedirecx). The qRT PCR was optimized at the following conditions with 35 cycles; initial denaturation was 94°C for 5min, denaturation 94°C for 30s, annealing at 62.5°C for 35s, extension 72°C for 30s, and final extension was 72°C for 5min. The reaction volume was prepared in 25µl with the following components: 12.5 µL SYBR Green master mix, 1 µL Forward Primer, 1 µL Reverse Primer, 9.5 µL PCR water, and 1 µL cDNA template. The relative expression of all four genes was determined using Histidine as an internal control to normalize the reaction.

RESULTS

Evolutionary Relationship of KTI Genes in Cotton

The phylogenetic tree analysis was conducted among the selected species of Gossypium and divided into KTI (blue) and ASP groups (red). The KTI group and ASP groups exhibited 18 and 28 genes, respectively. A phylogenetic tree was constructed through the neighbor-joining method to explore the evolutionary relationship among the genes of the KTI gene family (Fig. 1). The analysis showed a high bootstrap value among the genes of the KTI group, but a lower bootstrap value across the genes of the ASP group. The high bootstrap value indicates and validates the strong evolutionary relationship, whereas a lower bootstrap value shows a weak evolutionary relationship.

Physical Location of Genes on Chromosomes

In *G. arboreum*, a total of 9 KTI genes were located on three chromosomes. Out of the total, 2, 1, and 6 genes were located on chr04, chr09, and chr10, respectively. A total of 8 KTI genes were distributed on two chromosomes of *G. raimondii*, with 6 genes on chr11 and 2 genes on chr12. However, a larger number of KTI genes were distributed on chromosomes of *G. hirsutum* as compared to other *Gossypium* species. A total of 18 genes were unevenly distributed on 6 chromosomes along with a scaffold. The chromosomes A10 and D10 have the maximum number of 6 genes on each. The chrA05 and D04 are carrying 2 genes each, whereas chrA09 and D09 are carrying 1 gene each, as shown in Fig. 2.

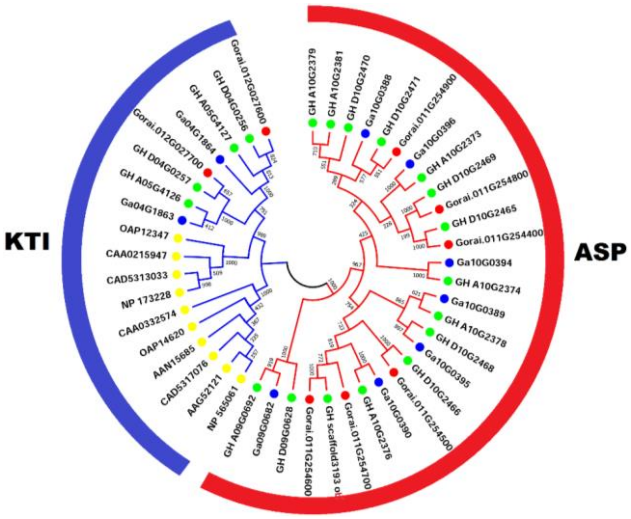

Fig. 1: Phylogenetic tree of the KTI gene family of cotton.

Conserved Motifs of the Cotton KTI Gene Family

The analysis of conserved Motifs was conducted to observe the evolution and structural diversity among the genes of the KTI gene family. This analysis helped to understand the functional role of KTI genes across selected cotton species. For example, motif 1 was found to be conserved in all KTI genes across all the selected Gossypium species. Motif 2 was present in all the KTI genes except *Ga10G0390* and *Ga10G0395*. Similarly, motif 3 was found to be absent in *GH_A10G2373* and *Ga10G0389*. The motif 4 was found to be absent in 4 KTI genes (*GH_D10G2468*, *GH_A10G2378*, *Ga10G0389*, and *Ga10G0395*. However, motif 5 was found to be absent in a greater number of KTI genes compared to other motifs. It was found to be absent in *GH_A10G2378, GH_A10G2373, GH_D10G2468, Ga10G0395, Ga10G0390, Ga10G0389, Gorai.011G254500, Gorai.011G254600, Gorai.011G254700*, and *Gorai.011G254800* (Fig. 3).

Putative CREs in *Ghi- KTI* Promoter Region

A promoter upstream sequence (2.0 kb) of *G. hirsutum* was obtained from a database and analyzed through PlantCare online software (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The analysis was performed to understand the biological activity of Ghi-KTI in the upstream (2.0kb) of promoter region. Several genes were found to be present in the promoter site of KTI genes in the *G. hirsutum* (Fig. 4). The distribution of cis-acting elements was found to be different in different genes. A large number of unevenly distributed genes are associated with various biological functions like transcription, hormonal release, cell cycle, and stress responses. For example, the presence of ABREs

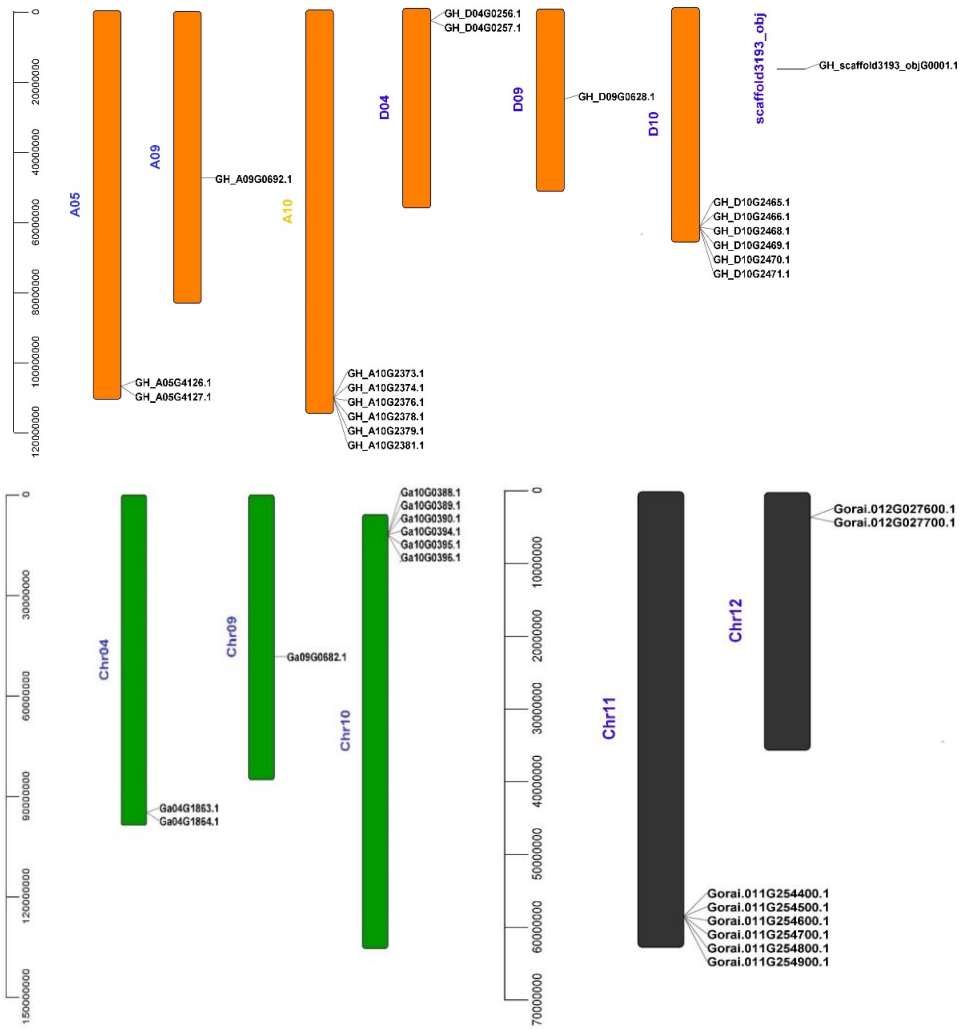

Fig. 2: Location of genes on chromosomes of cotton species

Fig. 3: MEME motif analysis of KTI gene family in cotton species

and TCA elements shows that the expression of Ghi-KTI is regulated by different phytohormones. Various abiotic stress-responsive CREs, such as DRE, LTR, and STRE, are also found. However, wound-responsive CREs like WRE and WUN associated with biotic stress were also found across the promoter region.

PCR Amplification

The synthesized cDNA of four KTI genes from isolated RNA was subjected to PCR amplification using short-length primers listed in Table 1. The optimum PCR conditions were used, and a reaction volume was prepared in 25µl. The sizes of the PCR products of the four genes

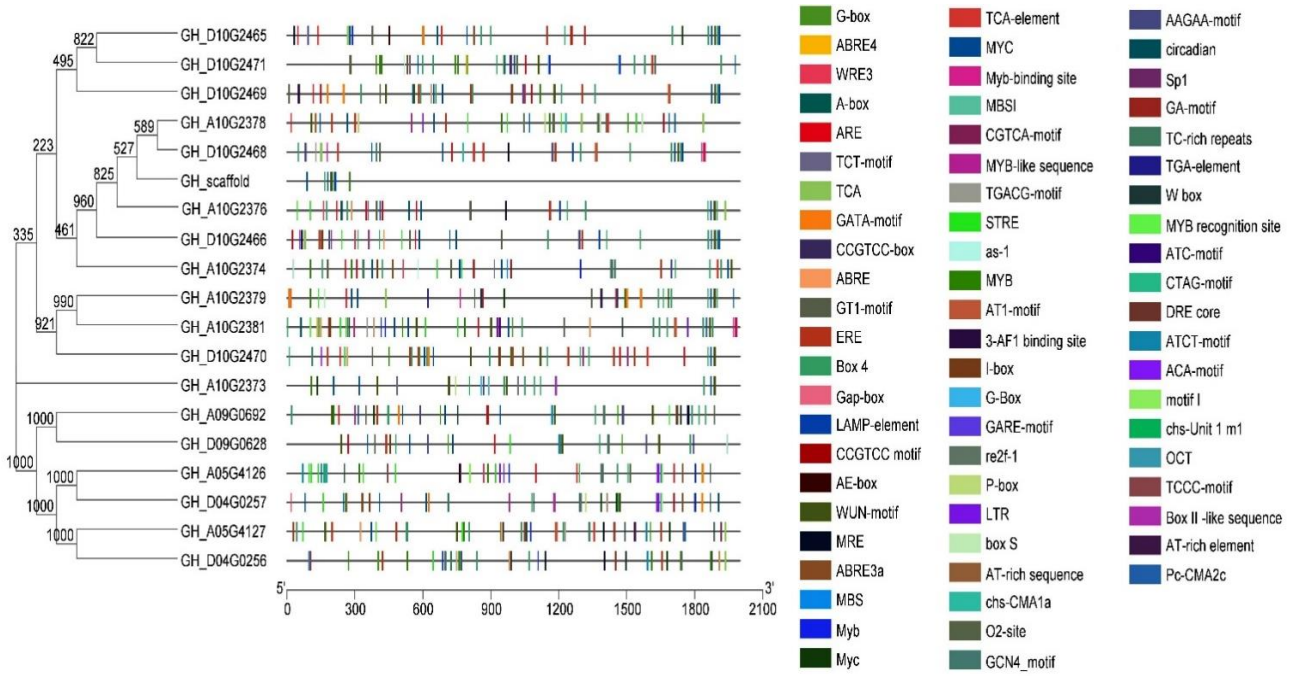

Fig. 4: Analysis of putative CREs in the Ghi-KTI promoter region.

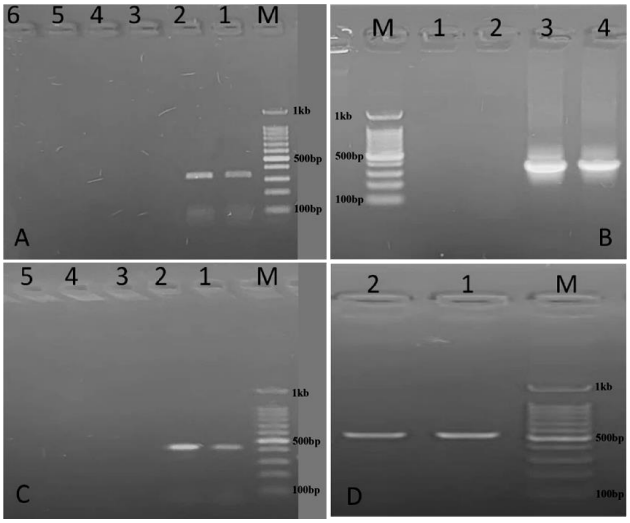

Fig. 5: Gel electrophoresis of PCR products: 5a: M shows molecular weight marker of 1kb, lane 1 and 2 show product size of 351 bp, Lane 3, 4, 5 and 6 are empty; 5b: M shows molecular weight marker of 1kb, Lane 3, 4 and show product size of 352 bp, Lane 1 and 2 are empty; 5c: M shows molecular weight marker of 1kb, Lane 1 and 2 show product size of 395 bp, Lane 3,4 and 5 are empty; 5d: M shows molecular weight marker of 1kb, Lane 1 and 2 show product size of 442 bp.

were visualized as (*GH_A05G4127.1*) 351 (A), (*GH_D04G0257.1*) 352 (B), (*GH_A05G4126.1*) 395 (C), and (*GH_D04G0256.1*) 442 bp (D) using a 1.0% agarose gel (Fig. 5). The visuals of these sizes showed the integrity of the KTI genes.

Relative Expression of Genes Through qRT-PCR

The qRT-PCR was performed against four selected genes. The ΔΔCq method was used to calculate the relative fold expression. The histidine was used as an internal control to normalize the expression, and sample 1 was taken as a reference control (sample 1.00). The relative expression of each gene was successfully measured, supporting the pest resistance development. All of the genes (*GH_A05G4126.1, GH_D04G0256.1, GH_D04G0257.1*) exhibited downregulation as compared to the control, whereas the *GH_A05G4127.1* gene exhibited strong upregulation. The fold expression of all genes was <1, whereas the *GH_A05G4127.1* gene showed a ~5 folds higher expression than the control (Fig. 6). The fold expression of the *GH_A05G4127.1* gene confirms its functional activity, suggesting its potential role in enhancing pest resistance in transgenic cotton varieties.

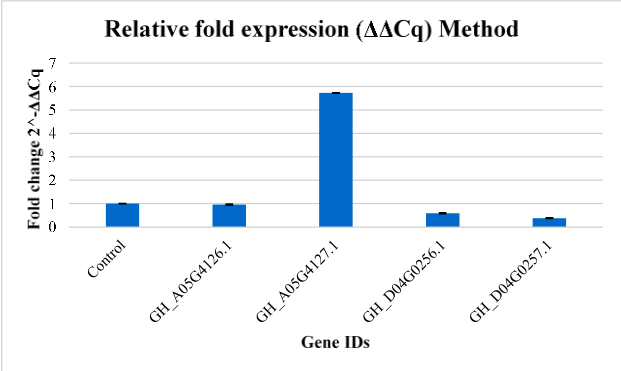

Fig. 5: Expression of genes through qRT-PCR.

DISCUSSION

Cotton (*Gossypium hirsutum* L.) is a globally important fiber crop whose productivity is frequently compromised by various insect pests. In this study, *Agrobacterium tumefaciens*-mediated transformation was used to introduce Kunitz Trypsin Inhibitor (KTI) genes, which produce protease inhibitors that can interfere with insect pests' digestive enzymes, and produce pest-resistant cotton plants. These inhibitors are important for plant defense and molecular breeding to increase insect

resistance requires a thorough examination of their genomic distribution, evolutionary relationships, and functional characterization (Yang et al., 2023).

The genome-wide characterization of the KTI (Kunitz-type inhibitor) gene family in cotton revealed varying numbers of gene family members across different *Gossypium* species. This gene family displayed diverse structural characteristics, suggesting potential functional diversity. The genes were analyzed for various factors, including their identities, gene structure, chromosomal locations, phylogenetic relationships, cis-regulatory elements (CREs), and conserved motifs. The phylogenetic analysis categorized the KTI family genes into distinct groups based on genetic similarities, which likely reflect their evolutionary relationships and functional roles within the species. This categorization offers insights into the roles of these genes and their diversification over time. The subgroups of KTI genes exhibited conserved domains and similar exon-intron structures, indicating close evolutionary relationships within each subgroup, while functional diversification was observed across different groups. While functional diversification was observed among the various groups, the subgroups of KTI genes showed conserved domains and comparable exon-intron structures, suggesting strong evolutionary relationships within each group (Ahmed et al., 2024). The KTI gene family in cotton expanded and became more diverse as a result of both whole-genome duplications (WGD) and segmental duplications, according to the gene duplication analysis. The presence of various cis-regulatory elements, such as ABREs and TCA, showed that the expression of Ghi-KTI is regulated by different phytohormones. Various abiotic stress-responsive CREs, such as DRE, LTR, and STRE, are also found. However, wound-responsive CREs like WRE and WUN associated with biotic stress were also observed across the promoter region. In cotton, whole-genome and segmental duplications led to the expansion of the KTI (Kunitz-type inhibitor) gene family. The uneven distribution of KTI genes on different chromosomes indicates the functional diversification, which enables the cotton crop to adapt to various environmental and developmental challenges, as shown by Siddiqui et al. (2023) and Farooq et al. (2024). Agarose gel electrophoresis showed distinct DNA bands of predicted sizes (395, 351, 441, 352 bp) for genes like *GH_A05G4126.1, GH_A05G4127.1, GH_D04G0256.1,* and *GH_D04G0257.1*, confirming the successful amplification and integrity of KTI gene sequences. This confirmed the precision of the study's PCR settings, primer specificity, and DNA extraction. The non-specific amplification or contamination was ruled out by distinct band patterns. Additionally, uniform DNA quality was demonstrated by consistent band intensities across samples (Zafar et al., 2024; Majumder et al., 2025).

The significant upregulation of *GH_A05G4127.1* indicates robust transcriptional activation. However, *GH_D04G0257.1*, *GH_D04G0256.1*, and *GH_A05G4126.1* genes were downregulated, suggesting selective gene suppression, which could be brought on by epigenetic mechanisms, post-transcriptional regulation, or the positional consequences of gene insertion in the genome. These expression patterns of genes revealed the significance of the KTI gene family. They also imply that the genes actively contribute to increased pest resistance, most likely by blocking insect pests' digestive proteases, which disrupts nutrient absorption and results in growth retardation or death (Egan et al., 2024; Zafar et al., 2022).

Conclusion

The genome-wide characterization and in silico analysis of the KTI gene family in cotton revealed their roles in pest resistance, an area not previously explored. The study identified and characterized KTI genes, examining their phylogenetic relationships, conserved motifs, and gene structures. The gene GH_A05G4127 exhibited significant differential expression, according to the expression profiling of the control. This suggests its potential functions in strengthening resistance against pest invasions and its possible involvement in the plant's defense response. The qRT-PCR analysis confirmed that *GH_A05G4127.1* exhibits elevated expression levels, suggesting its potential involvement in pest control mechanisms. These findings suggest that KTI genes could be important targets for developing cotton varieties with enhanced pest resistance. This research provides a solid basis for future functional studies and potential applications in breeding programs to improve cotton's resilience to pest resistance.

DECLARATIONS

Funding: This study did not get any financial support from any organization.

Acknowledgement: We acknowledge the University of Lahore and Sindh Agriculture University on providing space and expertise to accomplish this research.

Conflict of Interest: The authors declare no conflict of interest.

Consent for Publication: All authors declare their consent for publication in this journal.

Data Availability: The data is available from the corresponding author(s) based upon a reasonable request.

Ethics Statement: Not Applicable.

Author's Contribution: SS, MSC, and AR wrote the initial draft of the manuscript. VD and AR provided the space and helped to experiment. AH, MSC, and VS Conceptualization, writing—review, and editing; AH and AIK data curation and helped in statistical analysis. RAK, SF, SB, and GR helped in writing-review and editing, and AH and AR reviewed and supervised the experiment. All authors approved the final version of the manuscript.

Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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