



## Identification of Differential Expression of the FTSH Gene Family to Produce Heat-resilient Cotton Cultivar (Upland Cotton) for Sustainable Agriculture

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### ABSTRACT

Cotton is one of the major crops, and is crucial for the textile industry. Cotton is susceptible to various abiotic stresses, including heat stress. The Filamentous Thermosensitive (*FTSH*) gene family in plants encompasses a group of genes that are integral to the cellular response to heat stress. A total of 110 *FTSH* 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. Agarose gel electrophoresis confirmed the integrity of the extracted DNA, ensuring the reliability of the analysis. The key genes with differential expression were identified using quantitative real-time PCR (qRT-PCR). To validate the identified candidate genes (*GH\_A01G0236.1*, *GH\_A01G1045.1*, *GH\_DO1G0225.1*, and *GH\_DO1G1085.1*), a qRT-PCR analysis was performed. The results showed that all of the genes were upregulated, but the *GH\_DO1G0225.1* gene exhibited higher differential expression. The fold expression of the *GH\_DO1G0225.1* gene was found to be 8-fold higher than the control. This research represents a significant step in characterizing *FTSH* gene function in cotton and demonstrates its potential in conferring heat resistance. The findings contribute to the development of heat-resilient cotton cultivars, offering promising applications for sustainable agriculture.

**Keywords:** Cotton, *FTSH*, Heat stress, Abiotic stress, Sustainable agriculture.

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### INTRODUCTION

Cotton is a crop of immense global importance, with an annual production nearing 25 million tons. In the 2022-2023 season, China, India, the United States, and Brazil emerged as the top producers (Farooq et al., 2024; Zafar et al., 2025). It is projected that global cotton production will increase by 1.5% annually, reaching 28 million tons by 2030 (Gürsoy, 2024; Kamal et al., 2024a; Zafar et al., 2024a). In Pakistan, cotton plays a vital role in the economy as a key cash crop. Cotton and related products contribute

0.8% to the GDP and account for 60% of the country's foreign exchange earnings. Moreover, over 40% of Pakistan's industrial sector is either directly or indirectly linked to the cotton industry (Nazeer et al., 2023; Zafar et al., 2023a).

Belonging to the genus *Gossypium* in the family Malvaceae, cotton is crucial for the textile industry (Razzaq et al., 2022; Zafar et al., 2023b; Zafar et al., 2024b). The genus *Gossypium* includes around 50 species, with only a few being commercially significant. Among these, *Gossypium hirsutum*, *Gossypium arboreum*, and

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*Gossypium raimondii* stand out for their unique traits and contributions to cotton diversity (Razzaq et al., 2021; Zafar et al., 2022). *Gossypium hirsutum*, or upland cotton, is the most widely cultivated species, making up about 90% of the world's cotton production. This species is favored for its high yield, adaptability to various environmental conditions, and excellent fiber quality (Wang et al., 2018; Kamal et al., 2024b; Firdous et al., 2025).

The Filamentous Thermosensitive (Fts) gene family in plants encompasses a group of genes that are integral to the cellular response to heat stress. These genes are primarily known for their roles in maintaining cellular functions and integrity under elevated temperatures. The FtsH gene family includes several members, among which FtsH is one of the most extensively studied (Xiao et al., 2021; Wang et al., 2024). FtsH proteins are ATP-dependent metalloproteases found in the membranes of chloroplasts, mitochondria, and other organelles. They play a critical role in the quality control of membrane proteins by degrading damaged or misfolded proteins. This proteolytic activity is essential for preventing the accumulation of dysfunctional proteins that could disrupt cellular processes under stress conditions. The FtsH proteins are involved in the maintenance of photosystem II in chloroplasts by degrading the damaged D1 protein, a core component of the photosystem that is particularly susceptible to heat-induced damage. This degradation allows the replacement of the damaged D1 protein, ensuring continued efficiency of the photosynthesis (Wagner et al., 2012; Xiao et al., 2021).

Unlike various abiotic stress proteins (Ali et al., 2024; Ummer et al., 2024; Perveen et al., 2025), FtsH proteins are vital for abiotic stress tolerance, particularly heat tolerance. The sustained high temperatures can cause protein denaturation, resulting in the loss of protein activity. Under heat stress, defense mechanisms unite to restore and hydrolyze enzymes (when referring to proteins that act as biological catalysts) through regulatory systems, repairing proteins that have lost functionality to maintain normal metabolic processes (Luo & Kim, 2021; Xiao et al., 2021). A filamentation temperature-sensitive H (FtsH), is a member of the ATP-dependent protease family, which is involved in protein hydrolysis, featuring an N-terminal region with a transmembrane domain followed by an AAA domain. The FtsH possesses both ATPase and molecular chaperone activities. It breaks down proteins that are misfolded or incorrectly translated, serving as a crucial regulatory mechanism for eliminating malfunctioning proteins and maintaining cellular energy metabolism (Ito & Akiyama, 2005). This study focuses on the identification and validation through qRT-PCR of climate-resilient genes of the FtsH gene family. This gene family is known to confer resistance to one of the major abiotic stresses, such as temperature. The research will explore the potential of the FtsH gene family to enhance temperature resistance and contribute to more sustainable cotton production.

## MATERIALS & METHODS

### Sequence Retrieval and Analysis of the FTSH Gene

The protein sequence of the FTSH genes was obtained from the NCBI GenBank database

(<https://www.ncbi.nlm.nih.gov/>). The presence of the FTSH 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.

### Phylogenetic Tree

The phylogenetic tree was constructed using peptide sequences from cotton species. The ClustalX (<http://www.ebi.ac.uk/Tools/clustalw2/>) software was used for alignment, and a Bootstrap N-J file was created. The tree was visualized using the MEGA.11 program.

### Chromosomal Location of FTSH Genes

The FTSH gene position on the cotton chromosomes was determined using TBtool. The Gene Structure Display Server v2.0 (<http://gsds.gao-lab.org/>) was used to show the gene structure.

### Motif Analysis

The MEME (Multiple Em for Motif Elicitation) v5.5.4 was used to do the motif analysis on FTSH proteins.

### Cis-acting Regulatory Elements in Promoter Regions

The PlantCare online software (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) was used to analyze and predict the potential Cis-regulatory elements after obtaining a 2.0kb upstream sequence of *G. hirsutum*'s promoter from a database.

### Gene Structure Prediction

An online tool MEME (Multiple Em for Motif Elicitation) version 5.3.0, and TBtools software were used to analyze and predict gene structure integrated with a phylogenetic tree.

### Circos Plot Analysis in *G. arboreum*, *G. raimondii*, *G. barbedense* and *G. hirsutum* L

The complete genome sequences of four cotton species were subjected to TBtool to visualize the results. Advanced Circos was used to identify the orthologs and paralogs among the species.

### Heat Map Analysis

The expression pattern of different FTSH genes was examined at different time intervals of 0-, 3-, 6-, and 12-hours using Gene Expression Omnibus under accession number GSE119184 (Hu et al., 2019).

### Plant Material and PCR Amplification

The cotton seeds of the Ghauri genotype were obtained from the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Faisalabad, Pakistan. The seeds were carried to the laboratory and stored at room temperature.

### RNA Isolation and cDNA Synthesis

The RNA was extracted from cotton seeds using the Trizol technique. The chloroform was added, then vortexed

and centrifuged at 12,000rpm for 15min. at 4°C. The aqueous phase was collected and the RNA was precipitated using isopropanol. After a 10-min. incubation period, samples were centrifuged again, then washed with 70% ethanol and air dried. The RNA pellet was then resuspended in RNase-free water. A nanodrop spectrophotometer (Thermo-Scientific, United States) at an A260:A280 ratio was used to confirm the quantity and integrity of the recovered RNA, which was then observed using 1.5% gel electrophoresis. The RNA was kept at -80°C. The measured RNA was utilized for cDNA synthesis in a reaction volume of 20µl, with a final dilution of 100µl using the cDNA MIX formula (Thermoscientific).

### PCR Amplification

The cDNA template was used for PCR amplification under optimal conditions: initial denaturation at 94°C for 5 min, denaturation at 94°C for 30s, annealing at 62.5°C for 35s, extension at 72°C for 30s, and final extension at 72°C for 5min. The total reaction volume was prepared in 25µl. The PC was visualized using a 1.5% agarose gel (Thermoscientific top vision agarose Ref# R0491; and Electrophoresis power supply model 500/200; JY-SPCT Horizontal electrophoretic tank). The list of primers is given in Table 1.

### Relative Gene Expression

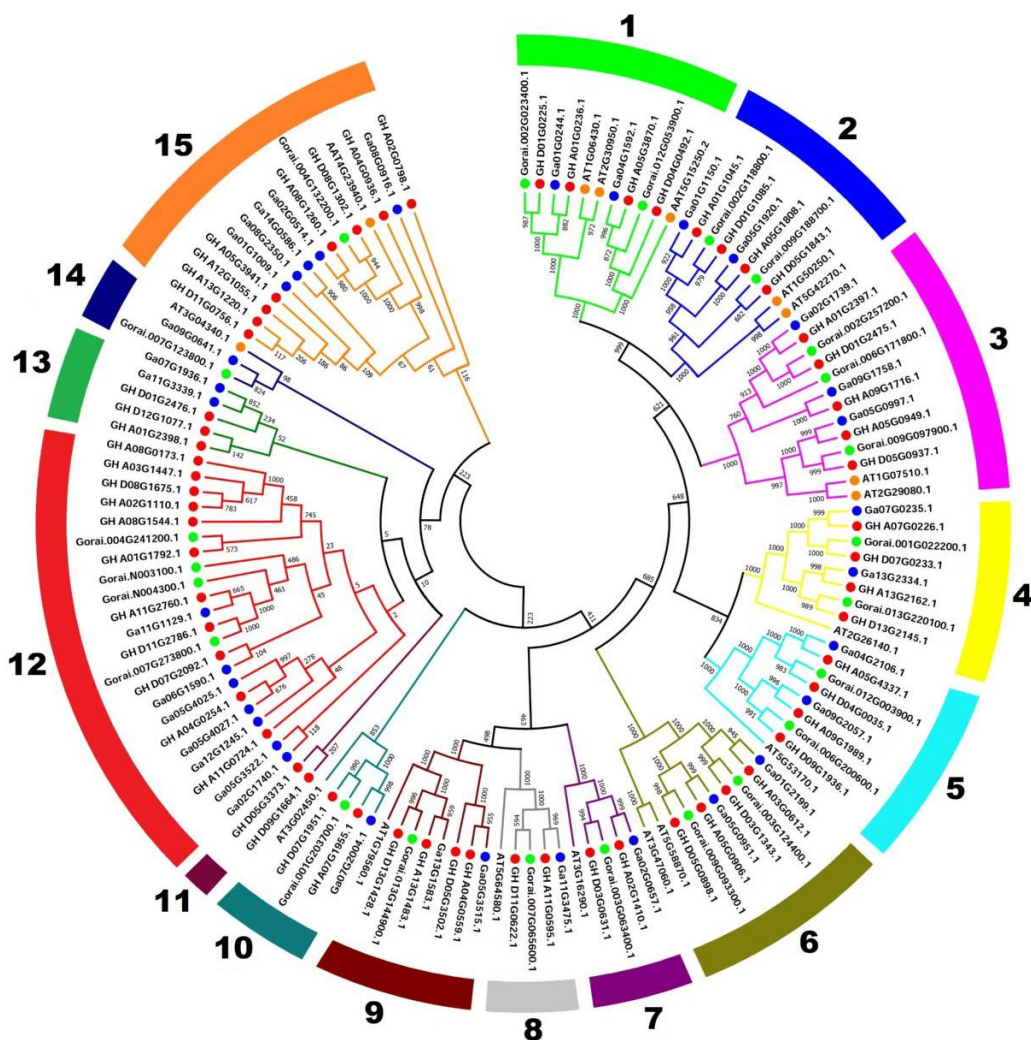
The relative expression of four genes was determined

by qRT-PCR (Applied Biosystem Gene Amp PCR system 2700) using the SYBR Green master mix methodology (Thermoscientific cat# K1081 and Genedirectx). The qRT PCR was optimized for 35 cycles using the following conditions: initial denaturation at 94°C for 5min., denaturation at 94°C for 30s, annealing at 62.5°C for 35s, extension at 72°C for 30s, and final extension at 72°C for 5 min. The reaction volume was made in 25µL with the following components: 12.5µL SYBR Green master mix, 1µL Forward Primer, 1µL Reverse Primer, 9.5µL PCR water, and 1µL cDNA template. To normalize the response, the relative expression of all four genes was evaluated with histidine serving as an internal reference.

## RESULTS

### Evolutionary Relationship of FTSH Genes in Cotton

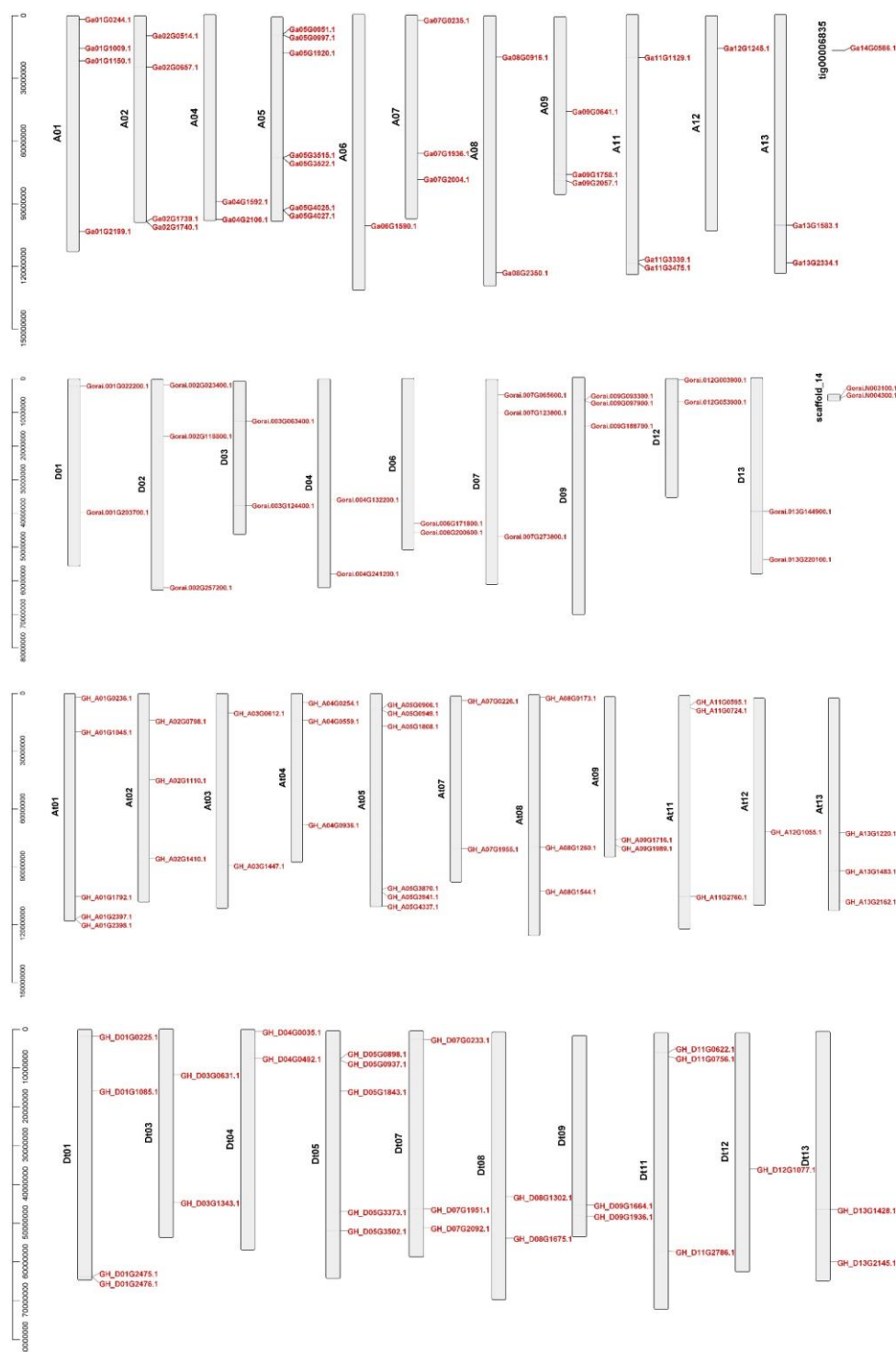
The phylogenetic tree analysis was performed on the selected *Gossypium* species, and separated into 15 clades ranging from 1 to 15, and represented in different colors. To investigate the evolutionary relationship between the FTSH gene family's genes, a phylogenetic tree was created using the neighbor-joining method (Fig. 1). The tree analysis revealed a high bootstrap value in most of the clades. A high bootstrap value suggests and supports a strong evolutionary relationship, whereas a low bootstrap value implies a weak evolutionary relationship.



**Fig. 1:** Phylogenetic tree of the KT1 gene family of cotton.

**Table 1:** List of primers

Gene IDs	F' primer	R' primer	Product length (bp)
GH_DO1G0225.1	GTGGCGTATCATGAAGTGGG	AAATTCATCGCCGCTCATGG	537
GH_A01G1045.1	CGAATTTCTGAACGCGGTGA	CCACATCGCTAAAGGTCACG	375
GH_A01G0236.1	CCATGATCCCGTGCAGAAAG	TTTCCAGCAGCACTTCCAC	462
GH_DO1G1085.1	CGAATTTCTGAACGCGGTGA	CCACATCGCTAAAGGTCACG	377

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

### Physical Location of Genes on Chromosomes

In *G. arboreum*, a total of 33 FTSH genes were distributed unevenly on 11 chromosomes. Out of the total, 2, 1, and 6 genes were located on chr04, chr09, and chr10, respectively. A total of 21 FTSH genes were distributed on 9 chromosomes of *G. raimondii*, with 2 genes on a scaffold. However, a large number of genes were found on chromosomes of *G. hirsutum* A and D

genomes as compared to other *Gossypium* species. A total of 30 genes were unevenly distributed on 11 chromosomes of the A genome, whereas the D genome carries 26 genes across 10 chromosomes. The chromosomes AT01 and AT05 have the maximum number of 5 and 6 genes, respectively. The chromosome DT05 carries the maximum number of 5 genes, as shown in Fig. 2.



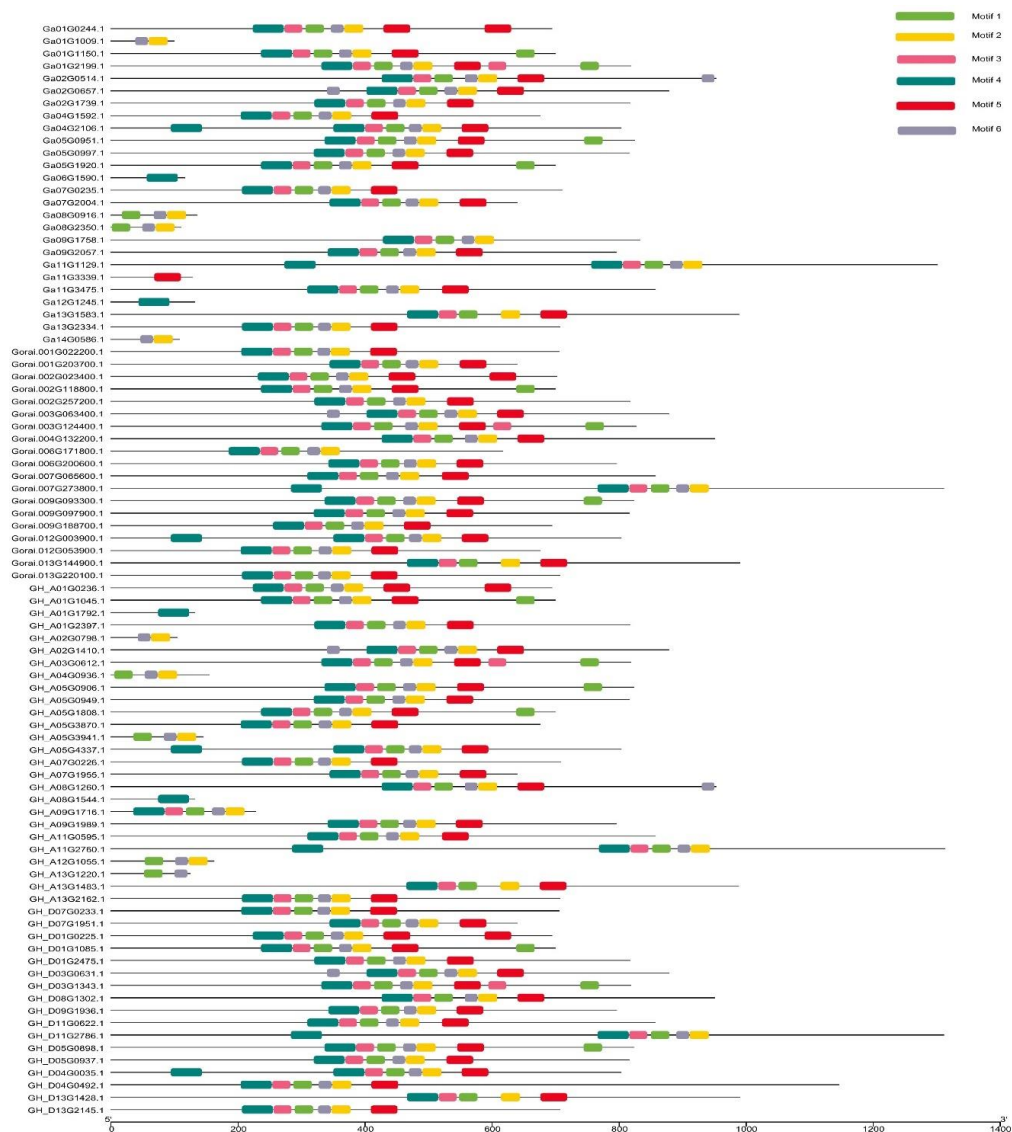
### Conserved Motifs of the Cotton FTSH Gene Family

The analysis of conserved Motifs was conducted to observe the evolution and structural diversity among the genes of the FTSH gene family. This analysis helped to understand the functional role of FTSH genes across selected cotton species. For example, motif 1 was found to be conserved in all FTSH genes across all the selected *Gossypium* species except a few genes, *Ga01G009.1*, *Ga11G3339.1*, *Ga12G1245.1*, *GH\_A02G0798.1*, *GH\_A13G1220.1*, etc. Motif 2 was present in all the FTSH genes except *Ga11G3339.1*, *Ga12G1245.1*, *Ga06G1590.1*, *GH\_A08G1544.1*, *GH\_A01G1792.1*, etc. Similarly, motif 3 was found to be present in all except *Ga01G009.1*, *Ga11G3339.1*, *Ga12G1245.1*, *GH\_A02G0798.1*, *GH\_A01G1792.1*, etc. The motif 4 was found to be present across all genes except (*Ga01G009.1*, *Ga08G0916.1*, *Ga14G0586.1*, *GH\_A02G0798.1*, *GH\_A04G0936.1*, *GH\_A13G1220.1*), etc. However, motif 5 was found to be absent in a greater number of FTSH genes, including *Ga01G009.1*, *Ga06G1590*, *Ga08G2350.1*, *Gorai.006G17800.1*, *Gorai.007G273800.1*, *GH\_A01G1792.1*, *GH\_A08G1544*, *GH\_A12G1055.1* etc., compared to other motifs. Similarly, motif 6 was absent in genes *Ga06G1590.1*, *Ga11G3339.1*, *Gorai.013G144900.1*, *GH\_A01G1792.1*, *GH\_D13G1428.1*, etc. It was observed that

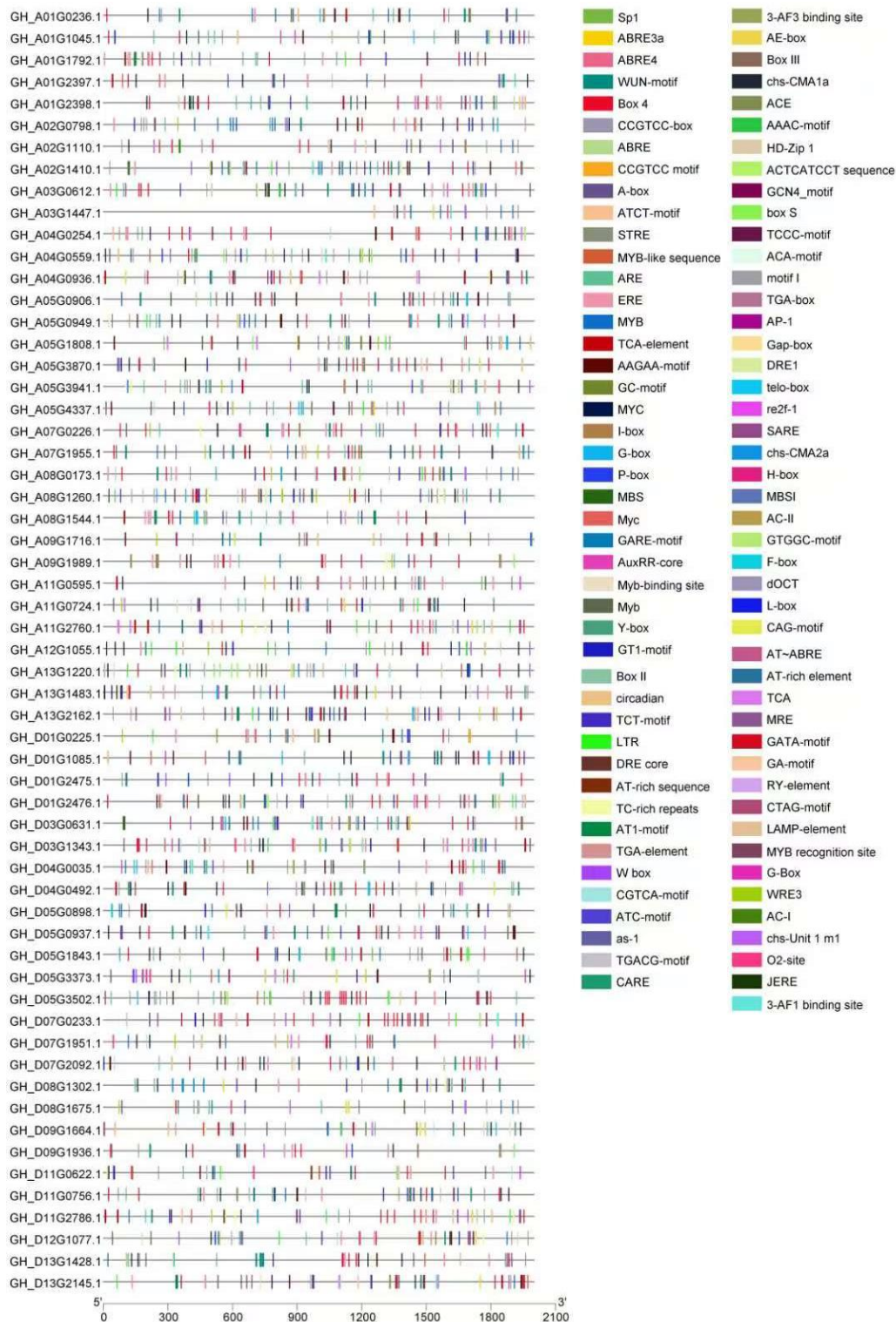
all six motifs were found in all of the candidate genes *GH\_A01G0236.1*, *GH\_A01G1045.1*, *GH\_DO1G0225.1*, and *GH\_DO1G1085.1* (Fig. 3).

### Putative CREs in *Ghi*- FTSH 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-FTSH in the upstream (2.0kb) of promoter region. Several genes were found to be present in the promoter site of FTSH 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 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.



**Fig. 3:** Analysis of the motif across *Gossypium* species.



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

### Gene Structure Display Server

The gene structure display server analysis was performed to understand the structural and functional diversity of the FTSH gene family more comprehensively, and their evolutionary relationship. All of the motifs were found in all of the genes of all *Gossypium* species, except for a few genes. The motifs were found to be conserved across all the species. Only 17 genes carry introns, whereas all other genes are intronless, which makes them more conserved. All of the other genes carry a conserved region of exons, as shown in Fig. 5.

### Circus Plot Analysis

In the circos plot analysis, two sub-genomes, A and D, were taken and analyzed. In this analysis, two sets of chromosomes, A01-A13 and D01-D13, were taken, which are presented in different colors. The connecting lines in different colors of yellow, green, blue, and orange represent different groups. The connecting lines inside the circle illustrate the homology, collinearity, duplication, and translocation of genes. The connecting lines also represent ortholog and paralog relationships. This analysis shows a relationship between closely related species of sub-genomes A and D, as shown in Fig. 6.

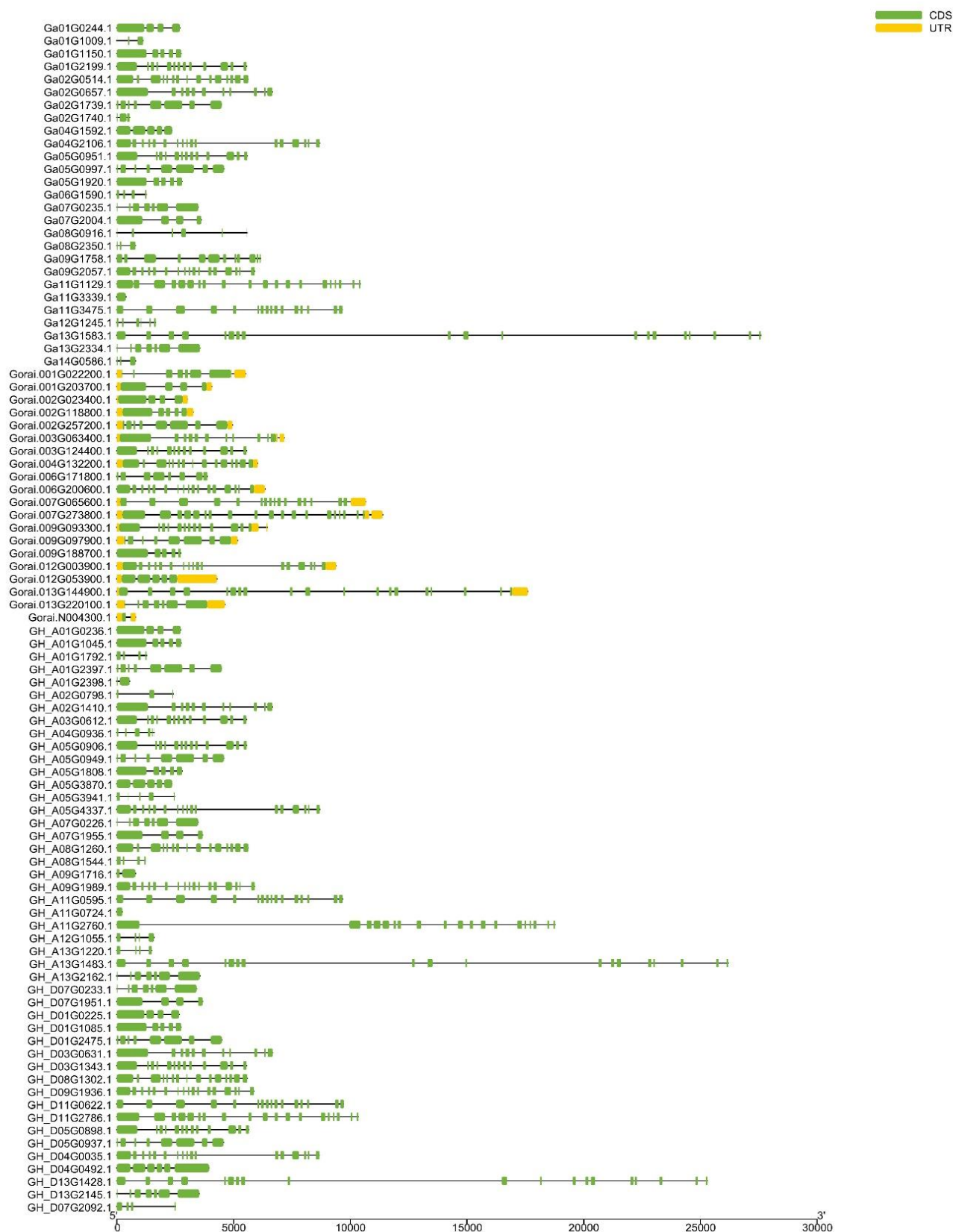


Fig. 5: Analysis of gene structure display server.

### Heat Map Analysis

The expression pattern of different FTSH genes was examined at different time intervals of 0, 3, 6, and 12 hours. The differential expression of *GH\_DO1G0225.1* was found to be higher among all the other genes, as shown in Fig. 7.

### PCR Amplification

The samples of selected genes were subjected to PCR using short-length primers listed in Table 1. The PCR was performed under optimum conditions, and the reaction volume was prepared in 25 $\mu$ l. The size of all four genes was visualized on 1.0% agarose using a 1.0 kb molecular



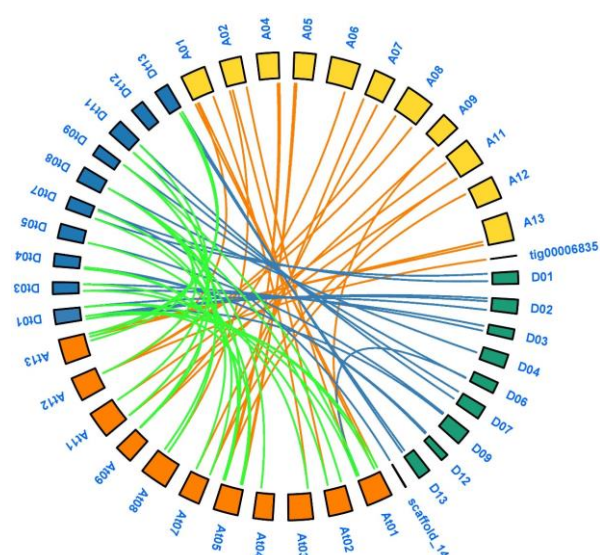


Fig. 6: Circos plot analysis comparing two sub-genomes A and D.

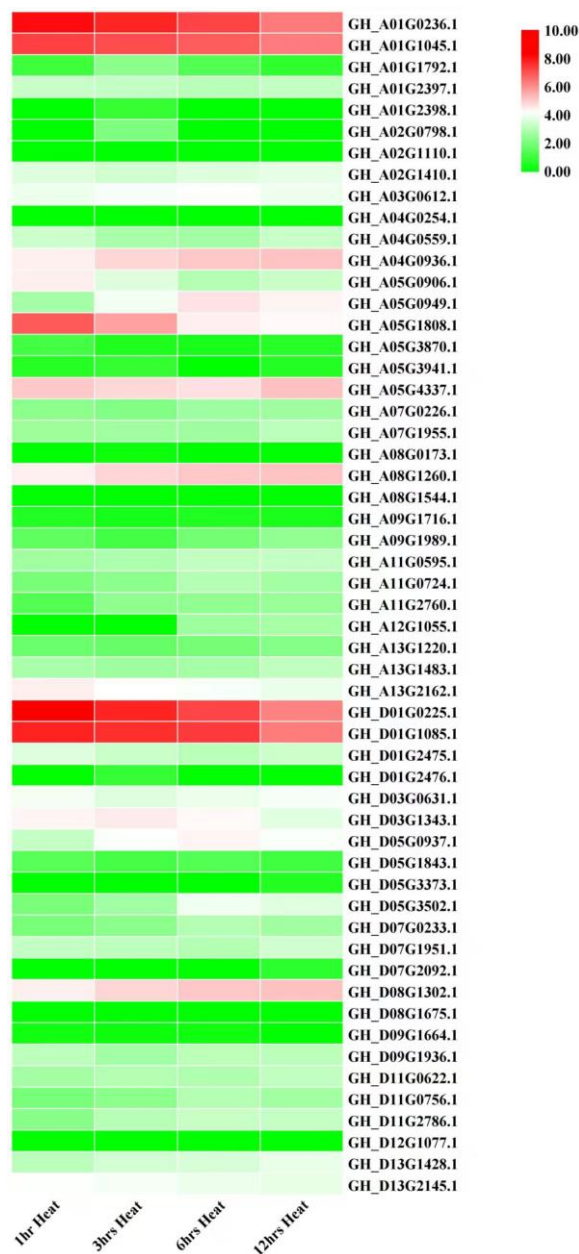


Fig. 7: Heat map analysis for expression pattern.

weight marker. The size of each gene corresponded to 537 (A) (*GH\_DO1G0225.1*), 375 (B) (*GH\_A01G1045.1*), 462 (C) (*GH\_A01G0236.1*), and 377 (D) (*GH\_DO1G1085.1*) bp, respectively (Fig. 8).

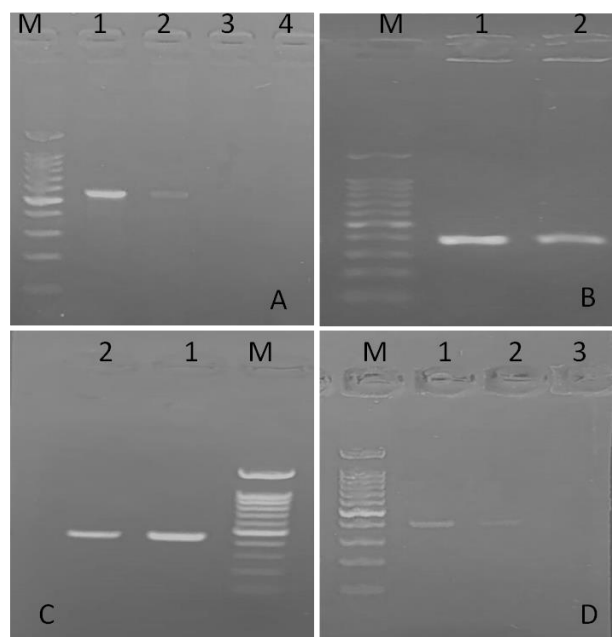


Fig. 8: Gel electrophoresis of PCR products: 8a: M shows molecular weight marker of 1kb, lane 1 and 2 show product size of 537 bp, Lane 3, 4 are empty; 8b: M shows molecular weight marker of 1kb, Lane 1, 2 show product size of 375 bp; 8c: M shows molecular weight marker of 1kb, Lane 1 and 2 show product size of 462 bp; 8d: M shows molecular weight marker of 1kb, Lane 1 and 2 show product size of 377 bp, Lane 3 is empty.

### Relative Expression of Genes Through qRT-PCR

The qRT-PCR was performed against four selected genes. The relative fold expression was measured using the  $\Delta\Delta C_q$  method. 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 measured successfully. All of the genes *GH\_A01G0236.1*, *GH\_A01G1045.1*, *GH\_DO1G0225.1* and *GH\_DO1G1085.1* exhibited upregulation, whereas *GH\_DO1G0225.1* showed a very high differential expression compared with the control (Fig. 9). The gene *GH\_DO1G0225.1* exhibited an 8-fold higher expression than the control. The relatively higher expression of the *GH\_DO1G0225.1* gene suggests its potential role in enhancing temperature resistance in transgenic cotton varieties.

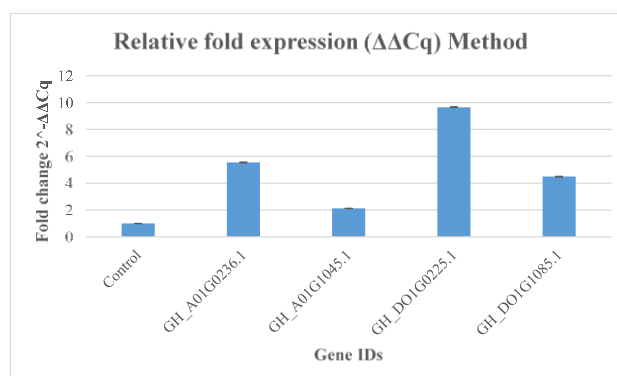


Fig. 9: Expression of genes through qRT-PCR.



## DISCUSSION

The Filamentous Thermosensitive (FTSH) gene family plays a critical role in plant responses to abiotic stress, particularly heat stress. In this study, a genome-wide analysis of FTSH genes in four cotton species (*Gossypium hirsutum*, *G. arboreum*, *G. raimondii*, and *G. barbadense*) revealed insights into their structural conservation, evolutionary dynamics, and functional relevance under elevated temperatures.

Phylogenetic analysis categorized FTSH genes into 15 distinct clades, with high bootstrap values in several clusters indicating strong evolutionary conservation (Xiao et al., 2021). This suggests that a maximum number of genes are sustaining the core function across the species. Contrarily, clades with lower bootstrap values indicate gene or species diversification. Chromosomal mapping showed uneven distribution of FTSH genes, particularly A05, A01, and D05, suggesting possible gene clustering for stress adaptation (Razzaq et al., 2022).

Motif and gene structure analyses confirmed the presence of highly conserved motifs (motifs 1–4) in most genes, crucial for ATPase and protease functions (Ito & Akiyama, 2005). The predominance of intronless genes aligns with the notion that stress-responsive genes often favor simpler structures to enable rapid transcription (Luo & Kim, 2021). The conserved exon regions further reinforce the potential of these genes as stable markers for stress resilience breeding. It was also observed that all of six motifs were found in all of the candidate genes *GH\_A01G0236.1*, *GH\_A01G1045.1*, *GH\_DO1G0225.1*, and *GH\_DO1G1085.1*.

The Circos plot analysis illustrated extensive collinearity, gene duplication, and syntenic relationships between the A and D sub-genomes. This level of orthology and paralogy underscores the evolutionary robustness and redundancy of the FTSH family, allowing functional compensation under heat stress (Wang et al., 2018). Moreover, such genomic conservation highlights candidate gene regions for targeted breeding and genetic engineering.

Promoter analysis revealed numerous cis-regulatory elements, including ABRE, DRE, and WUN, associated with abiotic and biotic stress (Hu et al., 2019; Luo & Kim, 2021). These elements suggest that FTSH gene expression is modulated by a complex interplay of hormonal and environmental stimuli, supporting their functional versatility under stress conditions.

qRT-PCR results validated the differential expression of four key FTSH genes under heat stress. The standout candidate, *GH\_DO1G0225.1*, showed a remarkable 8-fold upregulation, suggesting a central role in heat stress response. This is consistent with findings in other crops, where upregulated FTSH homologs confer thermotolerance by maintaining protein homeostasis in chloroplasts (Wagner et al., 2012; Xiao et al., 2021; Hajibarat & Saidi, 2022). However, all other genes also exhibited upregulation, indicating gene-specific functional roles within the FTSH family.

Together, these results reinforce the functional

significance of FTSH genes in maintaining cellular integrity under thermal stress. The integration of structural, expression, and evolutionary data provides a holistic view of FTSH gene utility in stress tolerance breeding. Future studies should focus on functional validation through gene overexpression or knockout approaches, particularly targeting *GH\_DO1G0225.1*. These efforts will support the development of heat-resilient cotton varieties, a critical need in the face of climate change and for ensuring sustainable agriculture (Manan et al., 2021; Nazeer et al., 2023; Gürsoy, 2024; Luqman et al., 2025).

## Conclusion

This study reveals the vital role of the FTSH gene family in protecting cotton against heat stress. Through genomic, evolutionary relationship, chromosomal location, and expression analyses, it was found that key candidate genes, particularly *GH\_DO1G0225.1*, demonstrate a potential role in heat stress in cotton, making it a heat-resilient crop. The presence of conserved motifs and cis-regulatory elements further underscores the functional role of the FTSH gene family in cotton. The findings of this study are promising, contributing to developing heat-resilient crop varieties for sustainable agriculture in the midst of several challenges of climate change. Further functional studies and field trials are recommended for validation.

## DECLARATIONS

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

**Data Availability:** Not applicable

**Ethics Statement:** No live animals or humans were used in the study; thus, it is not required.

**Author's Contribution:** SI and AH wrote the initial draft of the manuscript. AR provided the space and helped to experiment. FQ, LTZ, VD, MAK, and GR Conceptualization, writing, review, and editing; AH, MSC, and MMZ data curation and statistical analysis. AR, HN, HB, PA and HP helped in writing-review and editing, and AH and AR reviewed and supervised the experiment. All authors approved the final version of the manuscript.

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