TRENDS IN ANIMAL AND PLANT SCIENCES
https://doi.org/10.62324/TAPS/2025.089
www.trendsaps.com; editor@trendsaps.com
E-ISSN: 3006-0559; P-ISSN: 3006-0540

RESEARCH ARTICLE

Comprehensive Genomic Characterization and Stress-responsive Expression Profiling of *ASR* Gene Family

Fariha Qadir[1], Abdul Razzaq[1, 2], Ghulam Rasool[1] and Abdul Hafeez[3]

[1]Institute of Molecular Biology and Biotechnology, the University of Lahore, Pakistan
[2]Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences, Haikou, China
[3]Department of Agronomy, Sindh Agriculture University Sub-Campus Umerkot 69100
***Corresponding author:** biolformanite@gmail.com

Article History: 25-046 Received: 25-Aug-2025 Revised: 05-Oct-2025 Accepted: 10-Oct-2025

ABSTRACT

Cotton (*Gossypium*) is considered a white gold. It is grown in different countries such as China, United States of America, India, Pakistan, Brazil and Mexico. Pakistan is ranked 5th in cotton producing countries. It shares significantly in GDP of a country. It contributes to strengthen the economy of a country. Development of salt tolerant genotypes has been a major bottleneck issue to grow them on salt affected area in order to enhance the productivity of cotton. Extensive research is being carried out to cater the problems associated with the cotton production and its quality. A comprehensive genome-wide analysis of the cotton ASR gene family is presented in this work, encompassing three species and fully identifying and categorizing 20 ASR genes. We looked into how these genes expressed themselves in different cotton organs and growth stages, analyzed their evolutionary traits, and evaluated their responses to abiotic stress. Our analysis included the building of evolutionary tree among three major species of cotton and Arabidopsis thaliana. Different clades were formed which determined the evolutionary relationship between the closely related genes. Chromosomal locations of the ASR genes were also found. Conducting motif analysis, and characterizing their promoter regions through different software made it more concise to understand the integrity and intactness of the ASR gene family. Expression profile, through heat map and co-expression networking, analysis indicates that the ASR gene family of cotton (*Gossypium hirsutum L.*), is important for stress responses. Notably, we identified *GH_D10G0498* as promising candidate for enhancing salt stress tolerance. These discoveries offer insightful information about the biological processes of ASR genes in cotton growth and development, facilitating future research aimed at improving cotton's resilience to environmental stresses

Key words: Gossypium, Genome-wide analysis, ASR gene family, Salt stress, Abiotic stress.

INTRODUCTION

Introduction to cotton

Cotton is a cash crop that is grown worldwide (Nadeem et al., 2021). It belongs to the mallow or Hibiscus family (Malvaceae) (Wendel et al., 2009). The textile industry primarily views cotton as a potential revenue crop due to its natural fiber status. The high rate of consumption and significant marketability of the textile industry are driving up demand. It is cultivated in more than 80 nations worldwide. Just four of the 52 Gossypium species that are now recognized are widely grown worldwide: *G. hirsutum, G. barbadense, G. arboreum,* and *G. herbaceum* (Fryxell, 1992). Compared with other types of agriculture, cotton farming has higher yields, is more adaptable to different areas, and can supply approximately 95% of the world's food supply (Chen et al. 2007). Allotetraploid cotton was produced by crossing *Gossypium herbaceum* (A1) or *Gossypium arboreum* (A2), the ancestor of *Gossypium raimondii* (D5) (Wendel, 1989). Highland cotton (*G. hirsutum*) was first domesticated four to five thousand years ago. Later, through domestication and evolutionary research, modern highland cotton varieties, highland varieties, and subtropical varieties were developed (Zafar et al., 2024) (Fang et al., 2017). There are seven types of semi-wild highland cotton (Hutchinson, 1951).

Cite This Article as: Qadir F, Razzaq A, Zafar MM, Rasool G and Hafeez A, 2025. Comprehensive genomic characterization and stress- responsive expression profiling of *ASR* gene family. Trends in Animal and Plant Sciences 6: 154-163. https://doi.org/10.62324/TAPS/2025.089

There are 45 diploid species (2n = 2× = 26) and 7 tetraploid species (2n = 4× = 52) in the genus *Gossypium*. Eight genomic groups comprise the diploid species (A–G and K) (Wang et al., 2019). In America, interspecific hybridization produced allotetraploids, which led to the polyploidization of the A and D subgenomes. These two sub-genomes reassembled during the geographic shift about 1-2 million years ago. Genome D is most likely contributed by *G. raimondii* (D5), and genome A's ancestral donors are most likely *G. herbaceum* (A1) and *G. arboreum* (A2) (Hu et al., 2019; Zafar et al., 2024). Spinnable fibers are typically produced by A genome species, whereas D genome species are not grown. Globally, *G. hirsutum* accounts for over 90% of annual cotton production (AD) 1-Upland cotton (USDA, 2019). Fiber strength and length are the most important characteristics of fiber quality, followed by micronaire and uniformity (Yang et al., 2016). The relationship between fiber processing and dyeing is influenced by fiber quality, which is important for the spinning technology used in the quality textile production. However, it remains important for local economies and traditional textile industries in regions where it is cultivated. *Gossypium raimondii*, although not cultivated for its fibers, plays a crucial role in the genetic study and breeding of cotton. This species is native to textile industry (Rodgers et al., 2017). Yield and fiber quality are many characteristics. Fiber quality is determined solely by additive gene effects and is less affected by the environment, while fiber quality is controlled by many genes and influenced by epigenetics (Mingbao et al., 2008; Zhang et al., 2017).

It was reported that in response to changes in the environment, ASR1 adopts different conformations such as polyproline type II or the α-helix. The polyproline type II conformation is promoted by low pH and low temperatures (PII). PEG and glycerol have a significant stabilizing effect on α-helix conformation, whereas NaCl raises PII content and slightly destabilizes it. Low micromolar Zn2+ binding encourages α-helix folding, whereas excess Zn2+ causes homo-dimerization. Zn2+ is necessary for the sequence-specific binding of *ASR1* to DNA. The addition of Zn2+ causes structure induction, but ASR1 chaperone activity remains unchanged. Additionally, trehalose has synergistic *ASR1*-induced heat resistance to the reporter enzyme citrate synthase (CS), but is ineffective in the *ASR1*-only model. These findings led to the development of a FRET reporter to detect *ASR1* folding in vivo. Its efficacy was validated in saline and osmotic stress conditions in Escherichia coli, signifying a potential probe for application in plant cells. All things considered, this research lends credence to the idea that *ASR1's* plasticity is a crucial characteristic that enables it to react to drought stress and interact with particular targets (Wetzler et al., 2018).

The aim of this study is to explore the genes of ASR gene family to find out the candidate genes of salt tolerance in cotton.

MATERIALS AND METHODS

Selection of Material

Phytozome database was employed for the sequence retrieval of Arabidopsis thaliana At ASR. The genome sequences of *Gossypium hirsutum* (ZJU), *Gossypium raimondii* (JGI), and *Gossypium arboreum* (CRI) were obtained from the Cotton Genome Database Cotton FGD. The whole genome sequence of *G. barbadense, G. hirsutum, G. raimondii,* and *G. arboreum* were used to find homologues of the *ASR* domain. (https://www.cottongen.org).

The sequences such as CDS sequence, peptide sequence, and genome sequence were retrieved from Cotton fgd (www.cottonfgd.org). Clustal X software was used for sequence alignment. A phylogenetic tree was built using Mega 11 software. Gene structure display server (GSDS) was used for the gene structure analysis.

Physical Location of ASRs Genes on the Chromosome

The Complete genomes of *G. hirsutum*, *G. arboreum* and *G. raimondii* species were retrieved from Cotton fgd in the format of GTF/GFF. The genomic files of all species were uploaded on TB tool along with the list of gene IDs for chromosomal mapping.

Gene Structure Visualization

Gene Structure Display Server v.2.0 was used to analyze display the structure**.**

Identification of Protein Domains

HMMERV3.3.2 (https://www.ebi.ac.uk/Tools/hmmer/) was used to find out homologs and sequence alignment by using Pfam35.0 database. Using HMMER, we evaluated peptide sequences from the cotton species and Arabidopsis thaliana to determine whether a collection of protein sequences had similar domains. The pfam database, similar to Myb/SANT, was used for this purpose.

Phylogenetic Tree

ASR protein sequences were aligned using Clustal X, and a neighbor-joining phylogenetic tree was constructed with 1000 bootstrap replicates using MEGA 11.

Analysis of *Cis*-acting Regulatory Elements in Promoter Regions

Genes were retrieved from the *Gossypium hirsutum* genome database and analyzed using the PlantCARE online program (https://bioinformatics.psb.ugent.be/webtools/plantcare/html) to predict cis-acting mechanisms. The processed results from PlantCARE were then analyzed using TBtools version V2.012.

The upstream sequence 2000 was retrieved from cottonfgd (www.cottonfgd.org) and submitted to PLANTCARE database. The obtained file was then prepared and using TB tools, conserved motifs of ASR

gene family were identified. TB Tool version V2.012 was used to analyze the PLANTACRE results.

Expression Analysis

HeatMap was generated for *G. hirsutum* by the using of relevant expression data from NCBI. HeatMap was used to find out the expression of genes.

Motif Analysis

Motif analysis for find out the functional proteins was conducted through MEME suite (Multiple Em for Motif Elicitation) v5.5.4

Micro-circos Analysis

TB tool was used to create the gene circular view in gene linked information.

DNA Extraction

DNA extraction was the first step of wet lab.

Agarose Gel Electrophoresis

Agarose gel electrophoresis did for visualize the DNA bands. Either the DNA was present or not.

PCR (Polymerase Chain Reaction)

The PCR was performed (Fig. 1), and results were checked on 1% agarose gel electrophoresis.

The following (table 1) volumes were added to prepare the reaction volume:

Table 1: *Reaction volume for PCR*

Component	Volume
MasterMix	16 µL
DNA-ase free water	6 µL
Forward Primer	1 µL
Reverse Primer	1 µL
Cotton genomic DNA	1 µL

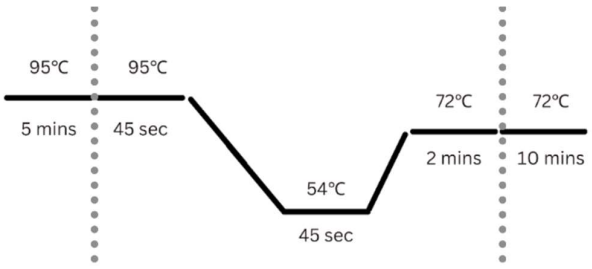

Fig. 1: Condition for PCR.

RESULTS

Identification, Sequence Analysis and Phylogenetic Tree of *ASR* Genes in *G. arboreum*, *G. raimondii* and *G. hirsutum*

12, 6 and 5 genes were identified in *G. hirsutum, G. raimondii* and *G. arboreum*, respectively. To discover the *ASR* gene, the *ASR* sequence (BLAST) was compared with the complete genome of *G. arboreum, G. raimondii* and *G. hirsutum*. All *ASR* genes are derived from all species. Use Pfam software to identify the amino acid sequences of 23 *ASR* genes to verify that they are reliable and contain *ASR* names. Genes without *ASR* domains or genes that were truncated during the protein coding process were removed, as well as genes that were not transcribed in their genomes. To determine the relationship between the amino acid sequences of ASR proteins in *G. hirsutum, G. raimondii,* and *G. arboreum* and 18 genes of Arabidopsis thaliana, a phylogenetic tree (neighbour sharing) was designed to determine the relationship between ASR genes. The phylogenetic tree divided the ASR genes into 6 partitions with well-supported bootstrap values (Fig. 2).

Physical Location of ASRs Genes on the Chromosome

According to the gene data of the linked genome data, each *ASR* gene is mapped to its own chromosome and the distribution of the *ASR* gene on the chromosome is determined. The discovery of WGDs, or segment and tandem duplications, in genomic duplication events allowed researchers to better understand the evolution of the *ASR* gene in four different cotton species. Five ASR genes were dispersed throughout the 13 chromosomes of *G. arboreum*. Five genes were found on Chr01, Chr05, Chr06, Chr10, and tig00017379, according to the results. Every gene was dispersed uniformly across every chromosome. In *G.hirsutum*, 9 genes were unevenly localized on 7 chromosomes. 4 genes were located on A genome and 5 genes were located on D genome. Five ASR genes were dispersed throughout the 13 chromosomes of *G. raimondii*. Five genes were found on Chr02, Chr09, Chr10, and Chr11, according to the results. Every gene was evenly spaced, with the exception of two genes that were localized on Chr02 (Fig. 3a-3c).

Gene Structure Visualization

Based on the evolutionary tree, *G. arboreum* genes can be divided into three groups. All of the genes present in this gene structure are intact which represents integrity and completeness of the gene family (Fig. 4).

Motif Analysis

To learn more about the evolution and structural diversity of each cotton species in the *ASR* family, we analyzed the conserved motif and gene structure of the *ASR* genes. Every *ASR* gene in every species contained every motif (Fig. 5).

Analysis of Putative *Cis*-acting Elements in *Ghi-ERFs* Promoters

To investigate additional biological functions of *Ghi-ASRs*, a 1.5 kb upstream promoter region of each *Ghi-ASR* was obtained and screened for cis – acting regulators using the PlantCARE database. ASR gene promoter sites in *G. hirsutum* included a range of components. The binding regions, also known as cis-acting elements, of transcription factors are essential for controlling the expression of particular genes. Many cis-acting genes

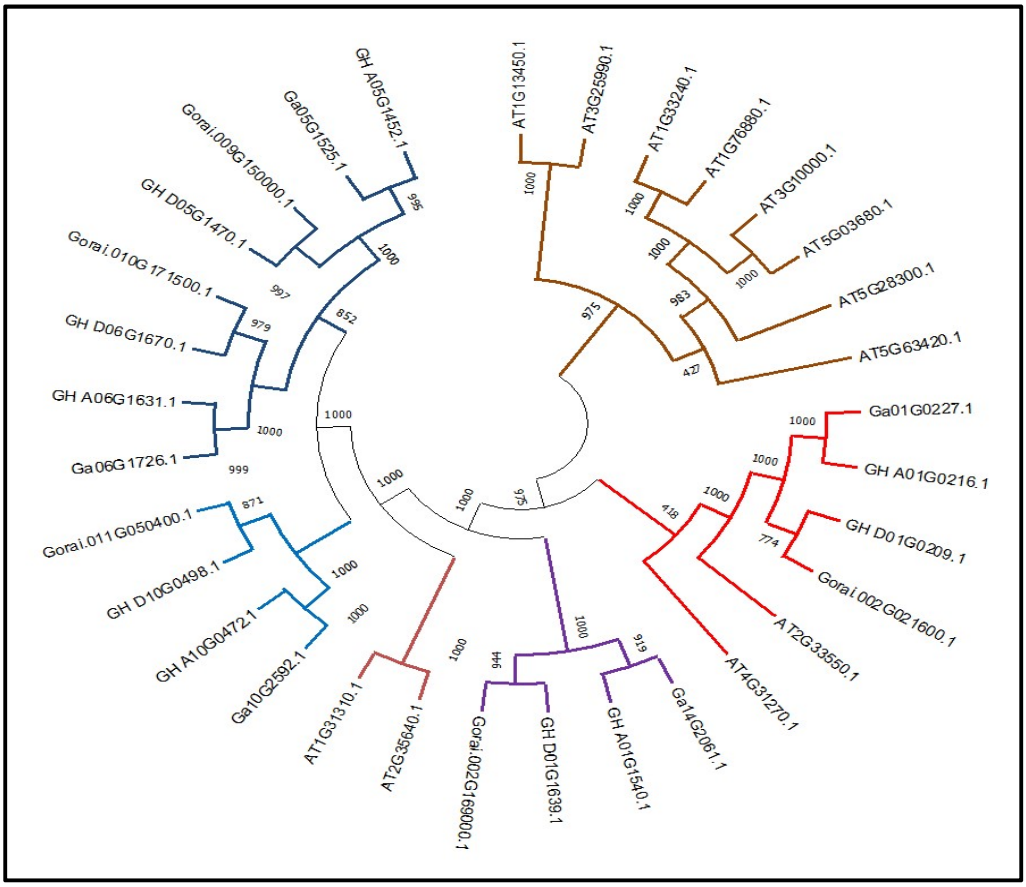

Fig. 2: Construction of Phylogenetic tree.

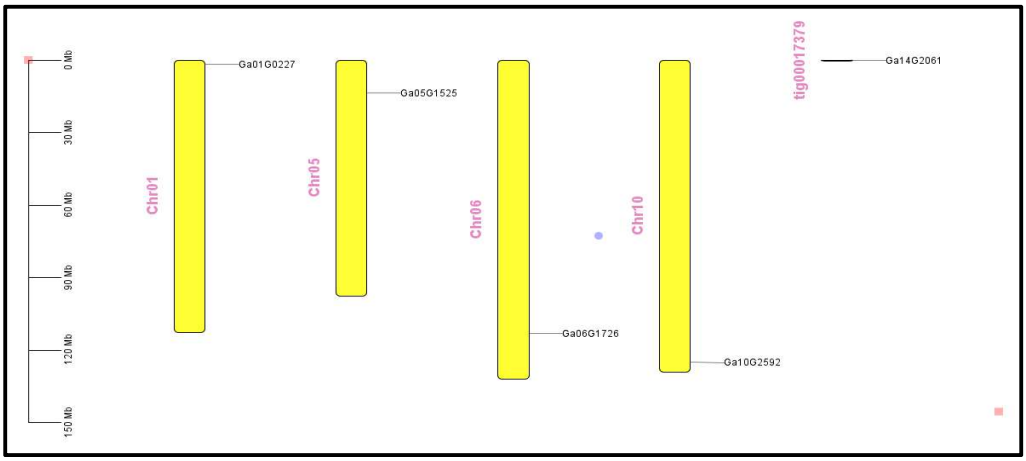

Fig. 3a: Chromosomal mapping of *G. arboretum.*

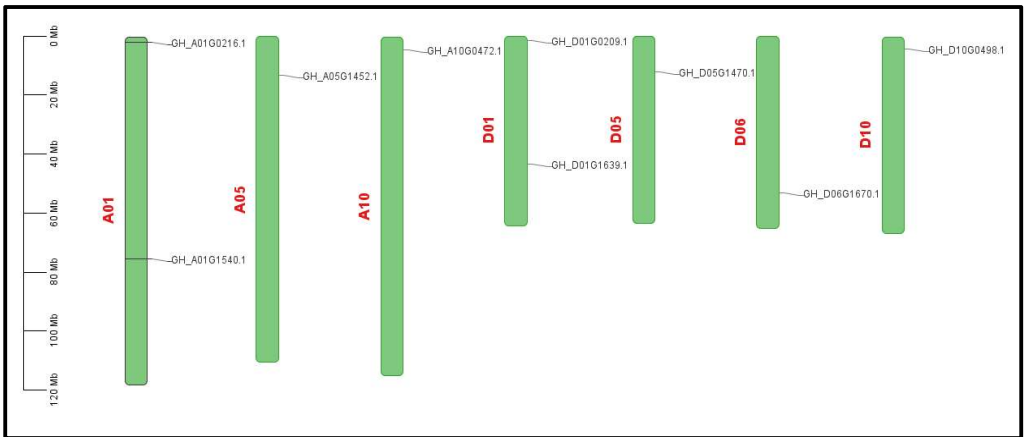

Fig. 3b: Chromosomal mapping of *G. hirsutum.*

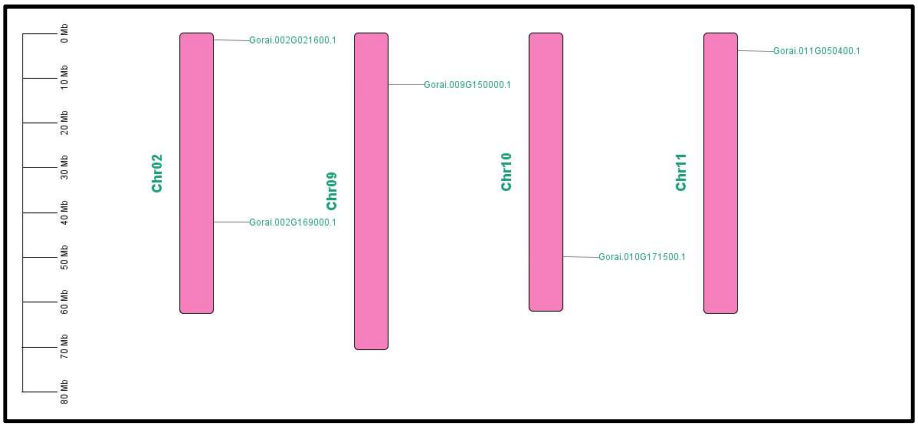

Fig. 3c: Chromosomal mapping of *G. raimondii*

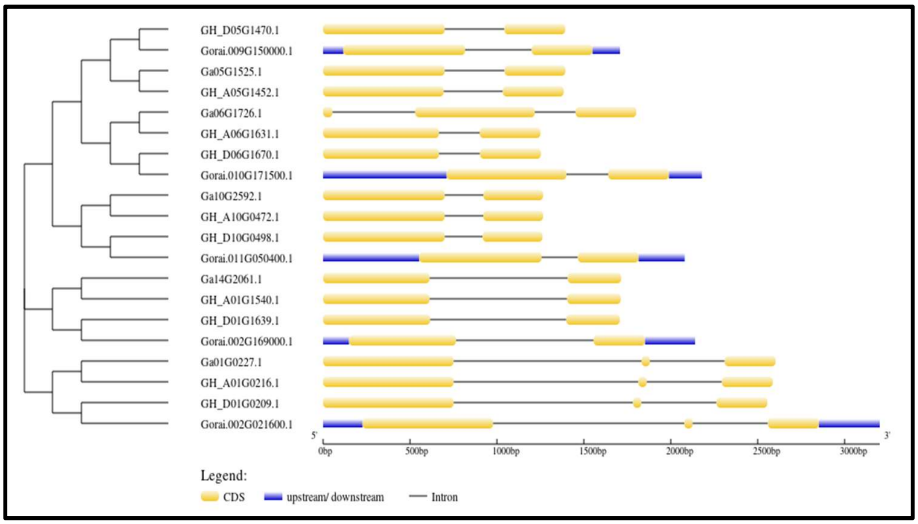

Fig. 4: *Gene structure of the ASR gene family of Cotton.*

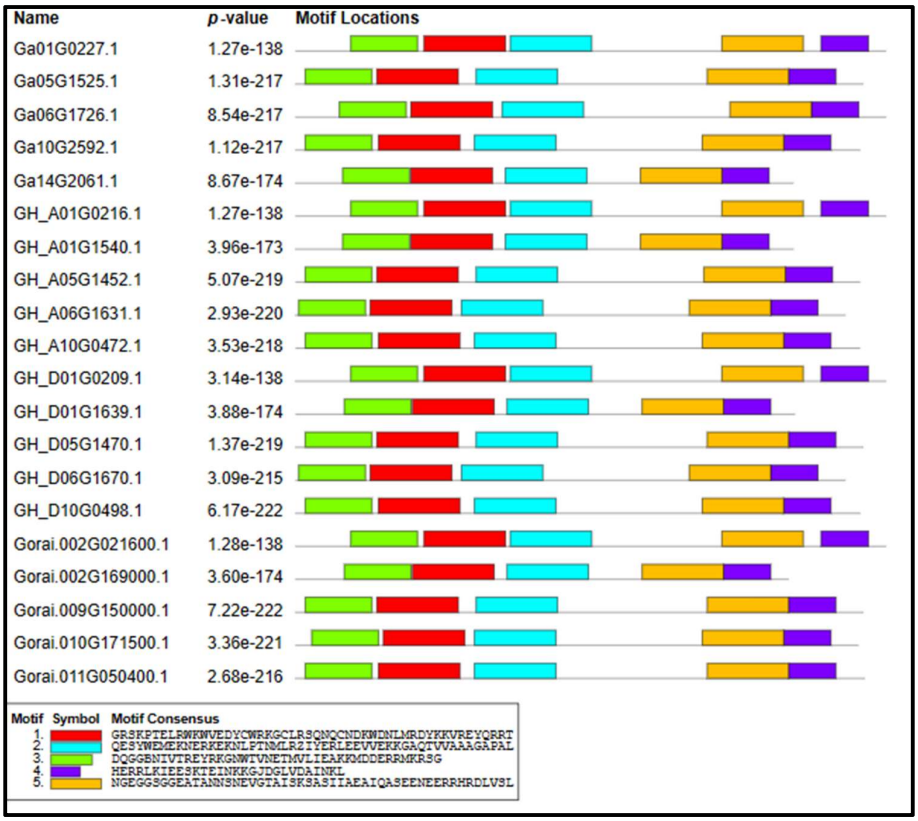

Fig. 5: Motif analysis *of the ASR gene family of Cotton.*

are predicted to play roles in metabolism, hormones, stress response, cell cycle, and development, although their distributions vary across genes. Various phytohormone response elements that have been discovered include ABRE4 response elements (ABRE4), ethylene response elements (EREs), and AAGAA motif elements. These findings indicate that *Ghi-ASR* expression is regulated by different hormones. The findings indicate that *Ghi-ASR* plays an important role in the growth and development of plants as well as in the response of plants to environmental stress (Fig. 6).

Micro-circos Analysis

All 20 out of 20 genes ASR genes had WGD or segment duplication. The chromosomes AT01, DT01 of *G. hirsutum* contained the highest WGD or segmentally duplicated genes. In *G. raimondii*, the highest number of WGD or segmentally duplicated genes was found on chromosome D02. Additionally, all ASR homologs genes were identified *G. hirsutum* and *G. raimondii* (Fig. 7).

Expression Analysis through Heatmap

Gh_A05G1452 gene showed high expressions in the heatmap results. On the other hand, *Gh_D10G0498* gene showed results in second number and *Gh_A10G0472* gene showed third highest expression (Fig. 8).

Co-expression Network

These genes *GH_D05G1470*, *GH_D10G0498*, *GH_A10 G0472* and *GH_A06G1631* were identified as a hub genes (Fig. 9).

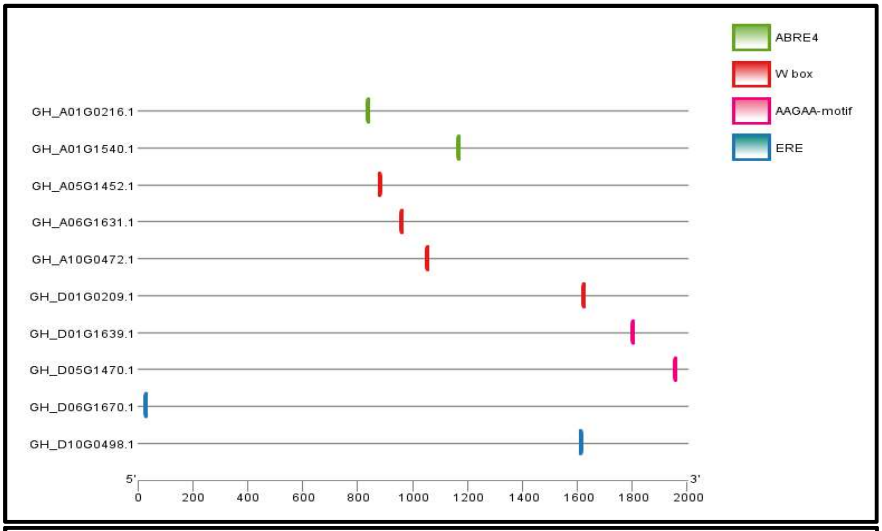

Fig. 6: *Cis* regulatory element *of the ASR gene family of Cotton.*

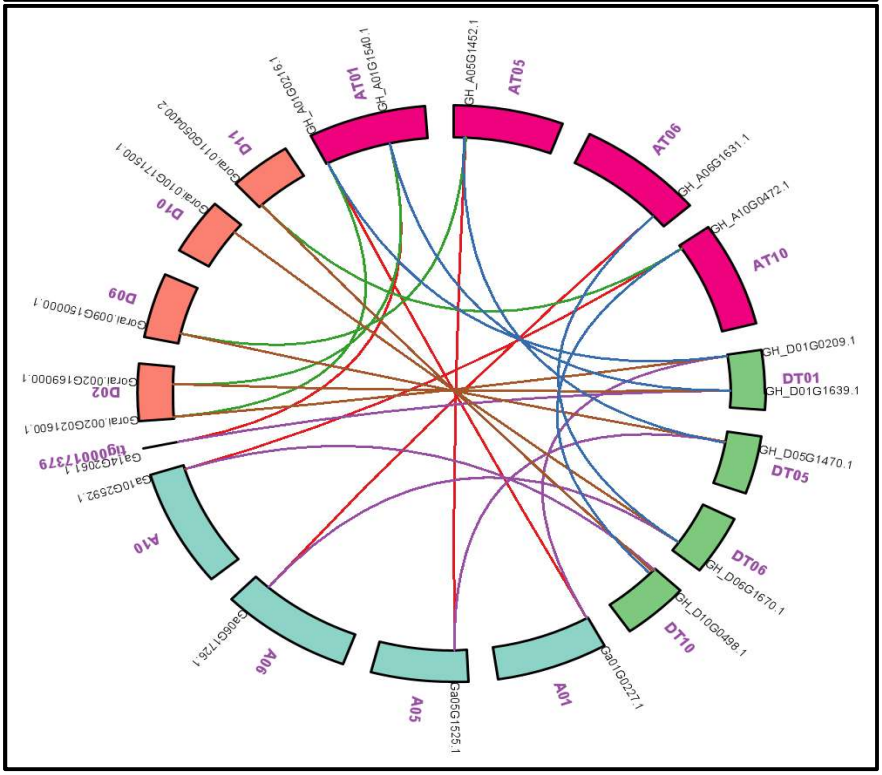

Fig. 7: Micro circus analysis of ASR gene family of cotton.

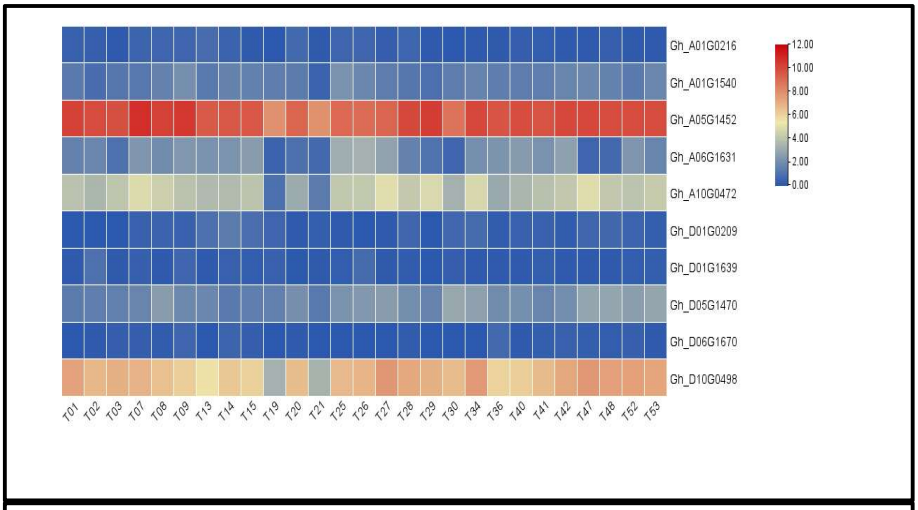

Fig. 8: HeatMap of *G.hirsutum ASR* expression data.

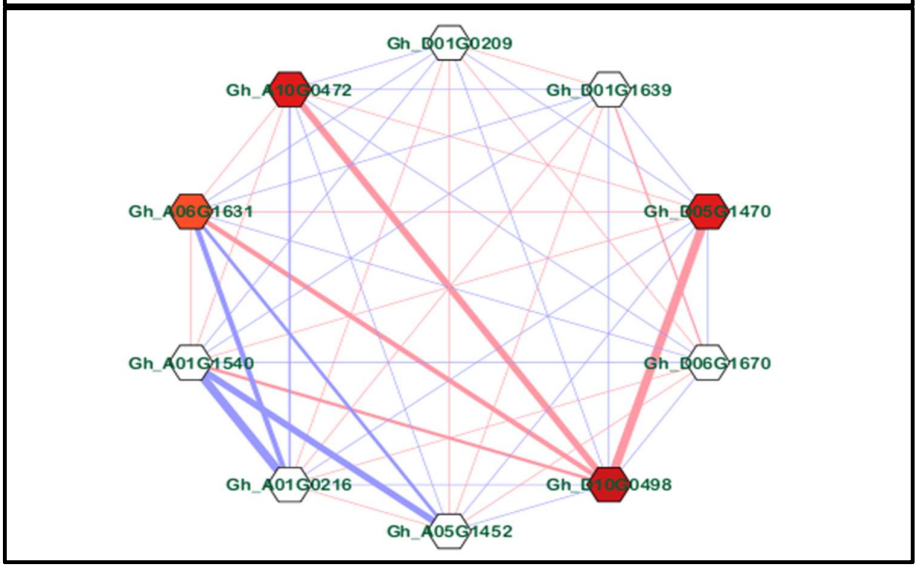

Fig. 9: Co-expression networking results of *G.hirsutum ASR* gene family.

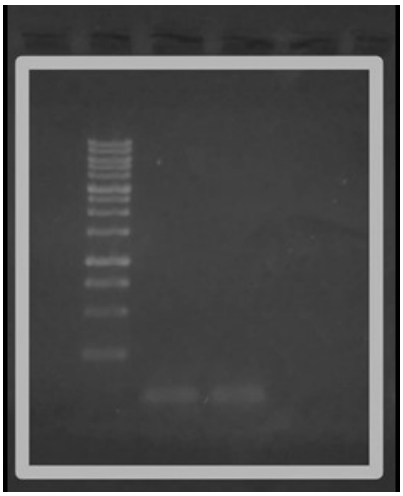

Fig. 10: *Gel electrophoresis of PCR products visualized in a UV transilluminator.*

DNA Extraction and Polymerase Chain Reaction

The agarose gel electrophoresis was performed for the DNA extraction results. The gel showed bright bands of DNA. The bands of the PCR Product gel electrophoresis, on the other hand, showed unexpected positions (Fig. 10).

DISCUSSION

Abiotic stress responses in plants are mediated by ASR (abscisic acid-, stress-, and ripening-induced) genes, which are normally upregulated by ABA exposure, injury, cold, light deprivation, heavy metals and water deficits (Schneider et al., 1997; Huang et al., 2000; Maskin et al., 2001; Jeanneau et al., 2002; Kalifa et al., 2004).

ASR genes have been detected in a variety of cereal crops since they were first discovered in tomatoes in 1993 (Golan et al., 2014). For instance, the genome-wide identification and characterization of 5, 6, 6, and 10 *ASR* genes were found in *Brachypodium distachyon*, foxtail millet, rice, and maize, respectively (Wang et al., 2016; Feng et al 2016; Philippe et al., 2010; Virlouvet et al., 2011; Zhang et al., 2019). The *ASR* gene family's genomic information is still lacking in cotton. Here, a thorough investigation of the cotton genome-wide *ASR* gene family was carried out.

We found 20 cotton *ASR* genes in the current study, which spans three different cotton species and has a conserved Myb-DNA-bind-4;Myb/SANT-like DNA-binding domain. Furthermore, a phylogenetic tree with well-supported bootstrap values separated the cotton ASR genes into six clades.

In *G. arboreum* five genes were found on Chr01, Chr05, Chr06, Chr10, and tig00017379, according to the results. Four genes were found on the A genome and five on the D genome of *G. hirsutum*. The results showed that five genes were located on Chr02, Chr09, Chr10, and Chr11 in *G. raimondii*.

G. hirsutum's chromosomes AT01 and DT01 had the highest proportion of segmentally duplicated or WGD genes. In *G. raimondii*, the highest number of WGD or segmentally duplicated genes was found on chromosome D02. Independent evolution and duplication events of homologous chromosomes may be responsible for this. In general, the main reason for family expansion is gene duplication (Zhang, 2003). Additionally, essential genes may change in copy due to duplication; this suggests that similar genes may diverge over long periods of evolution and contribute to development and changes in gene families (Conant & Wolfe, 2008; Grassi et al., 2008).

We examined the conserved motif and gene structure of the *ASR* genes to discover more about the evolution and structural diversity of every cotton species in the *ASR* family. All five motif types were preserved across the three cotton species.

Transcriptional patterns are regulated by cis-regulatory elements (CREs), which are DNA sequence motifs that specific transcription factors (TFs) target. Cis-regulatory modules (CRMs) are frequently found to contain clusters of CREs. CRMs control transcription kinetics, cell-type/spatial patterning, and developmental staging and stress tolerance through the coordinated action of cognate DNA-binding proteins (Marand et al., 2023). Additionally, different genes have different cis-acting elements, and many of these are predicted to play roles in transcription, hormones, stress responses, cell cycle, and development. Various phytohormone response elements that have been discovered include ABRE4 response elements (ABRE4), ethylene response elements (EREs), and AAGAA motif elements. According to TF binding site analysis, most TaASR genes are associated with various processes during growth and development. As expected, most rice ASR genes are expressed in different tissues and developmental stages, suggesting they may be crucial for cotton growth and development.

ASR genes are generally positively regulated and have been shown to be extensively involved in the transcriptional responses of plants to a variety of abiotic stresses. Numerous genes with high expression under abiotic stress were found using HeatMap and co-expression network analysis. Following a thorough examination of these findings, it was concluded that *GH*_D10G0498 and *GH*_A10G0472 were the best options.

*GH*_D10G0498, a PRIMERS-specific tool, was created; nevertheless, agarose gel electrophoresis revealed unanticipated bands that suggested dimer formation. Given that the primer design and optimization steps were meant to prevent such problems, these results were unexpected. These unexpected dimers may have resulted from a variety of causes, including faulty primer design, contamination, or variations in the PCR conditions (due to a possible fault in a thermal cycler machine).

Nevertheless, a comprehensive reassessment failed to find any anomalies. Since the problem could not be resolved by standard troubleshooting, the possibility of errors in the production of other reagents was taken into consideration. Although we get our primers from a commercial supplier, there is a slight chance that the primers were not synthesized correctly.

After talking with other lab members, it became clear that everyone was experiencing the same problems with their PCR product, which was that all of the bands showing up on the gel electrophoresis were fewer than 100 base pairs. This might be a sign that the PCR apparatus wasn't operating at the intended level.

Conclusion

This work involved a genome-wide analysis of the ASR genes in cotton. Three species' 20 ASR genes were fully identified and categorized. We looked at the ASR genes' expression patterns in different cotton organs and growth stages, their evolutionary traits, and how they responded to abiotic stress. For the ASR gene family, chromosomal location, motif analysis, and promoter region characterization were done. According to the expression profile analysis, upland cotton's ASR gene family may play a significant role in stress responses. We demonstrated that GH_D10G0498 and GH_A10G0472 are excellent choices for tolerance to salt stress. Understanding the biological function of the ASR genes in cotton growth and development will be made easier by these findings.

DECLARATIONS

Funding: There was no funding to carry out this research.

Acknowledgement: The authors gratefully acknowledge the Institute of Molecular Biology and Biotechnology, the University of Lahore, Pakistan, for providing the necessary facilities, resources, and technical support to carry out this research.

Conflict of Interest: The authors declare that there is no known conflict of interest associated with this publication.

Data Availability: The data will be available upon request to the corresponding authors.

Ethics Statement: All animal studies have been approved by the relevant ethics committee or institutional supervisory board and have been conducted in accordance with the ethical standards.

Author's Contribution: Fariha Qadir, Abdul Razzaq, conducted experiments on quails, analysis and generalization of experimental data. Ghulam Rasool conducted chemical and amino acid analysis of feed reactions. Abdul Hafeez developed a methodological part of the studies. Conducted experiments on the analysis of the raw material and enzymatic hydrolysis of lupine, analyzed the experimental data, designed the article. The final draft manuscript was revised by all authors. All authors edited, read, and approved the final 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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