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RESEARCH ARTICLE

Trends in Biotechnology and Plant Sciences

Genome-wide Identification, Characterization and Expression Analysis of *GLX* Gene Family in Upland Cotton (*Gossypium hirsutum* L.) under Salt Stress

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Abstract

Cotton is a soft, delicate fibre that is a member of the *Gossypium genus* and family *Malvaceae*. It grows inside a protective shell known as a boll. Only four of the more than 50 species are farmed commercially: two tetraploid (*G. hirsutum and G. barbadense*) and two diploid (*G. herbaceum and G. arboretum*). With over 90% of production going to *G. hirsutum*, or American cotton, it commands a dominant share of the global market. Cotton's lengthy maturation cycle and big genome make it difficult to analyse its genes, matching its significant economic impact. *Glyoxalases* (*GLX*) are vital plant enzymes that improve resistance to abiotic stresses by detoxifying methylglyoxal, a hazardous compound that builds up during stressful conditions. Environmental cues control the *GLX* gene family, which includes *GLXI, GLXII*, and *GLXIII*, and is essential in lowering cellular toxicity. For plant growth, resilience to stress, and agricultural productivity, this system is essential. This study characterized the *GLX* gene family in three cotton species, identifying 8 genes in *G. hirsutum,* 4 in *G. arboreum*, and 4 in *G. raimondii*. Phylogenetic and structural analyses revealed three groups of GLX genes, each with distinct motifs and conserved domains important for DNA binding and stress responses. A study on the cotton *GLX* gene family discovered that *GLX* genes, in particular *GH_A10G1518,* contribute to resistance to abiotic stressors such as exposure to salt. Under salt stress, this gene has significantly altered expression, indicating that it may be able to increase cotton's resistance to these kinds of contaminants. The study backs up the use of *GLX* genes for genetic enhancement to create cotton kinds resistant to stress, which will help the textile sector. Among these, the gene *>GH_A10G1518* emerged as a key candidate due to its notable response to salt stress. PCR validation confirmed the integration of *GH_A10G1518* into the cotton genome, and qRT-PCR demonstrated its successful overexpression. This overexpression improved cotton's performance under salt stress, highlighting the gene's potential for enhancing stress resistance. 1t was found -2.6 folds higher than control *GH_D04G0124.* These findings validate the potential of candidate gene identified through different in silico analysis. This work establishes the foundation for further research on *GH_A10G1518* and could lead to the development of more resilient cotton cultivars, which would promote agricultural sustainability in high-stress settings.

KEYWORDS
Gossypium, Abiotic stress, Basic PentaCysteine, Salt toxicity.

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1 | INTRODUCTION

Cotton, a soft and economically valuable fiber, is produced within protective bolls and belongs to the genus *Gossypium* in the Malvaceae family. Among more than 50 *Gossypium* species, only four are widely cultivated, with *G. hirsutum* (upland or American cotton) dominating global production due to its high yield, accounting for over 90% of cotton output. However, the large genome size of cotton has made gene function analysis labor-intensive using traditional methods. Climate variability significantly affects cotton growth, with drought and salinity posing severe challenges. Drought stress, predicted to worsen in the future, causes extensive crop losses, while soil salinity, affecting about 6% of the global landmass, results primarily from sodium chloride accumulation (Gu et al., 2014). This salinity disrupts plant growth by causing nutrient deficiencies and ionic imbalances, ultimately reducing agricultural productivity and threatening global cotton yields (Ahmad et al., 2013). Cotton (*Gossypium spp.*) is a vital global economic resource, supplying around 35% of the world's fiber consumption and generating significant revenue, often referred to as "white gold" in some regions. Major cotton-producing nations contribute nearly two-thirds of global production, supporting a textile industry valued at approximately $600 billion annually, with cotton output reaching 25 million tonnes. Pakistan ranks as the fifth-largest cotton producer and the seventh-largest cloth manufacturer, with cotton serving as a key cash crop, contributing to 60% of the country's export earnings (Kim et al., 2015).

Abiotic stresses such as drought, salinity, and extreme temperatures significantly hinder plant growth and modern agriculture. These stresses can lead to the excessive production of toxic aldehydes, including *methylglyoxal* (MG), glyoxal (GO), and 3-deoxyglucosone (DOG), through enzymatic and non-enzymatic processes. Elevated MG levels can damage biomolecules, cause carbonyl stress, and react with amino acids to form irreversible advanced glycation end products (AGEs). MG also stimulates reactive oxygen species (ROS) production, leading to oxidative stress that harms proteins, DNA, RNA, lipids, and biofilms. Despite its harmful effects, MG acts as a signaling molecule at low concentrations, interacting with compounds like Ca^{2+}, H_2O_2, NO, and H_2S to enhance stress tolerance and regulate physiological functions such as seed germination and plant development (Mehari et al., 2021). To counteract MG's toxicity, plants employ detoxifying systems, primarily the glyoxalase system, which clears about 99% of MG. This system involves three enzymes: glyoxalase I (GLXI), glyoxalase II (GLXII), and glyoxalase III (GLXIII). These mechanisms are critical for mitigating MG's negative effects and maintaining plant resilience under stress conditions (Turan et al., 2009).

Glutathione (GSH) is essential for the glyoxalase system, serving as a cofactor for glyoxalase I (*GLXI*) to detoxify excess methylglyoxal (MG) and form S-D-lactoylglutathione (SLG). *Glyoxalase II (GLXII)* converts SLG into lactic acid, regenerating GSH to maintain redox balance and homeostasis. *Glyoxalase* III (*GLXIII*) independently converts MG to lactic acid without requiring GSH or other cofactors (Maryum et al., 2022). Cotton, a vital natural fiber crop also used for edible oil and biofuel, faces various biotic and abiotic stresses throughout its lifecycle. Among these, salinity is a significant global challenge, threatening sustainable cotton production. Cotton, a moderately salt-tolerant crop with a salinity threshold of 7.7 dS m^{-1}, faces significant challenges under salt-induced stress, which disrupts osmotic and ionic balance, hinders photosynthesis, and causes redox imbalances, ultimately impairing plant growth. Salinity affects over half of the world's countries, particularly in arid and semiarid regions. Asia, the Pacific, and Australia are among the most impacted regions, with 6% of their total land affected. In Pakistan, 6.28 million hectares of agricultural land are salt-affected, with saline sodic soil constituting 60.5% of this area (Shi and Sheng 2005).

Abiotic stresses like drought, salinity, extreme temperatures, and heavy metal toxicity severely limit plant productivity and sustainable agriculture. These stresses disrupt physiological and biochemical processes, including nutrient uptake, photosynthesis, respiration, and cellular organelle function (Zafar et al., 2025). Plant responses to stress involve signaling events mediated by phytohormones and reactive molecules such as reactive carbonyl species (RCS), reactive oxygen species (ROS), and reactive nitrogen species (RNS). Emerging research suggests that RCS, often produced through ROS-mediated lipid peroxidation, also play a role in signal transduction under stress conditions (Zhang et al., 2016). Methylglyoxal (MG), a reactive carbonyl species (RCS), acts as both a signaling molecule and a toxin in plants, depending on its concentration. Produced during carbohydrate, protein, and lipid metabolism, MG levels increase under biotic and abiotic stresses, causing damage to biomolecules like proteins, DNA, RNA, and lipids. This can lead to advanced glycation end products (AGEs) and glycated DNA adducts, linked to various human diseases (Alam et al., 2000).

In plants, MG-induced protein glycation primarily targets arginine, forming hydroimidazolones like MG-H1, the most common AGE. Cotton (*Gossypium hirsutum*), while moderately salt-tolerant, exhibits varying levels of tolerance among cultivars. Research on NaCl-induced salt stress during the seedling stage highlights cotton's sensitivity to salinity despite its overall resilience (Mostofa et al., 2018).

2 METERIAL AND METHOD

2.1. Accessing Databases and Retrieving Sequences

Cotton FGD was used to get genomic, CDS, and peptide sequences of the GLX gene family in G. hirsutum (ZJU), G. arboreum (CRI), and G. raimondii (JGI). BLASTP was performed using Phytozome and obtained sequences from Arabidopsis thaliana and G. hirsutum.

2.2. Domain of Proteins

To make sure the collection of protein sequences had identical domains, the homology of the previously listed cotton species and *Arabidopsis thaliana* was assessed using the HMMER3.3.2 online programme (https://www.ebi.ac.uk/Tools/hmmer/). The Pfam35.0, GAGA binding protein-like family, database was utilised for this.

2.3. Construction of Phylogenetic Trees

A file containing the peptide sequences of Arabidopsis thaliana and the previously indicated cotton species was developed and provided into the ClustalX programme. Following alignment, a Bootstrap N-J file was created. Next, MEGA.11 software was used to open this N-J (Neighbor-Joining) file. Ultimately, by recognizing clades, a phylogenetic tree was produced.

2.4. Physical Location of GLX Genes on Chromosomes

We used TB Tool to generate a visualisation of the GLX gene positions on the chromosomes of our selected cotton species.

2.5. Gene Structure Visualization

The gene structure was displayed using the Gene Structure Display Server v2.0 (http://gsds.gao-lab.org/). The interface's format was changed to use the FASTA format. The genomic sequence of the cotton species's data and the FASTA files from CDS were uploaded. The phylogenetic tree's NEWICK file was also exploited. An evaluation was made of the gene structure. The number of exons was recorded along with the genes having different architectures.

2.6. Motif Analysis

By using MEME (Multiple Em for Motif Elicitation) v5.5.4 (https://meme-suite.org/meme/tools/meme), motif analysis for the GLX proteins was carried out.

2.7. Analysis of CREs in Promoter Regions

The *G. hirisutum*'s upstream sequence (2.0 kb) was submitted to Plant CARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) . Once the findings are loaded into the Excel sheet, remove duplicates from the data. PlantCARE results were analyzed by using the TB tool.

2.8. Heatmap Analysis

Using relevant expression data from NCBI (https://www.ncbi.nlm.nih.gov/gds), TBtools was used to generate a HeatMap for G.hir.

2.9. Micro-circos Analysis

TBtools was used to construct a circular gene view based on gene-linked data.

2.10. Expression Co-networking

Expressional data was processed using Correlation Calculator, and expression co-networking was visualized using Metscape, a Cytoscape extension, once the output file had been normalized.

2.11. Cotton seed Delinting

The seed surface was cleared of cotton lint, commonly referred to as cotton fuzz, in order to facilitate the efficient and seamless extraction of DNA.

2.12. DNA Extraction

DNA was isolated from the delinted cotton seeds using the CTAB procedure.

2.13. Agarose Gel Electrophoresis for extracted DNA

Agarose Gel Electrophoresis at 1% was carried out to ensure that the genomic DNA was successfully extracted.

2.14. Polymerase Chain Reaction

GH_A10G1518 was the selected candidate gene following in silico research. The NCBI Primer tool was used to design and validate primers with the following specifications.

Table 1: Sequences of primers.

Sequence (5'-3')	F\R	Length	Tm (℃)	GC%
ATGCTCTCTAAAACCTCACC	F	20	54.75	45
CTAGAAATTATCCTTTGCTCTGC	R	23	55.46	39.13

To maximize the PCR conditions using a 2720 Thermal Cycler, the experiment was run at multiple annealing temperatures, starting at 5°C below the average melting temperature of the forward and reverse primers (Fig. 1).

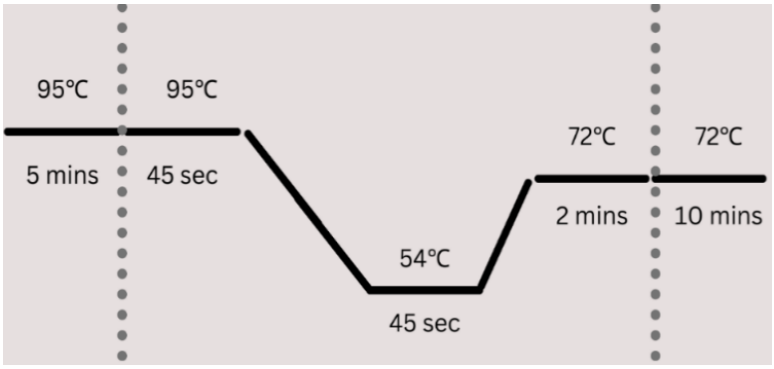


Fig. 1: *Conditions for PCR.*

The reaction volume was prepared by adding the following volumes:

2.15. PCR Product Agarose Gel Electrophoresis

The same process for agarose gel electrophoresis was followed for PCR validation as was followed for gel electrophoresis after DNA extraction.

Table 2: *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

2.16. RNA Extraction and cDNA Preparation

Using Primer3 Input Version 4.0, primer pairs were designed for the >GH_A10G1518 gene. RNA was isolated from the leaves of suspected transgenic cotton using the Agilent RNA Isolation Kit. Quantification of RNA (in ng/µl) was performed using a Thermo Scientific NanoDrop 2000 spectrophotometer (USA), by measuring absorbance at 260 and 280 nm. DNase-treated total RNA was reverse transcribed into cDNA with the PrimeScript® RT Reagent Kit (Perfect Real Time, Takara Biotechnology Co., Ltd., Dalian, China), and the resulting cDNA was stored at -20°C.

A qRT-PCR assay was used to analyze gene expression in transgenic cotton, utilizing Thermo Scientific's Maxima SYBR Green/ROX technology. The reaction mixture, totaling 20 microliters, included 5 µl of Maxima® SYBR Green/ROX qPCR Master Mix (2x), 1 µl of cDNA (50 ng/µl), and 1 µl each of the 10 pmol forward and reverse primers. GAPDH primers served as an internal control for normalization during the calculation of relative expression levels. All experiments were performed in triplicate to ensure accuracy.

3 RESULTS

3.1. Gene identification, sequence analysis, and phylogenetic tree of GLX genes in cotton

G. arboreum, G. raimondii, and G. hirsutum were found to contain 8, 8, and 14 genes, respectively. The GLX domain sequence was used in a BLAST search against the complete genome sequences of these three species in order to identify the GLX genes. Every species' non-redundant GLX gene was gathered. The authenticity and presence of GLX domains were subsequently confirmed by analyzing the amino acid sequences of these GLX genes

using Pfam software. Excluded from the analysis were genes that were truncated, lacked annotation in their respective genomes, or lacked GLX domains in their encoded protein sequences.

The uneven distribution of *GLX* genes across clades in the three cotton species and *Arabidopsis thaliana* indicates that the *GLX* gene family has undergone varied evolutionary paths. This variety suggests that the *GLX* gene family has undergone substantial evolutionary diversification, which may be the cause of the various roles observed in other species (Fig. 2).

3.2. Chromosome distribution of GLX Gene Family in cotton

1. No *GLX* genes were found on chromosomes 2, 3, 9, or 10 in *G. arboreum*, while four GLX genes were unevenly dispersed throughout these chromosomes.
2. Four *GLX* genes were sporadically distributed over chromosomes 5, 6, 11, and 12 in *G. raimondii*.
3. In eight of the fourteen chromosomes in *G. hirsutum*, the distribution of eight GLX genes was asymmetric. In the *G. hirsutum* genome, chromosomes A02, A05, and A10 in the A sub-genome have the highest number of *GLX* genes, but chromosome A10 in the Dt sub-genome has the highest amount of *GLX* genes (Fig. 3a-c).

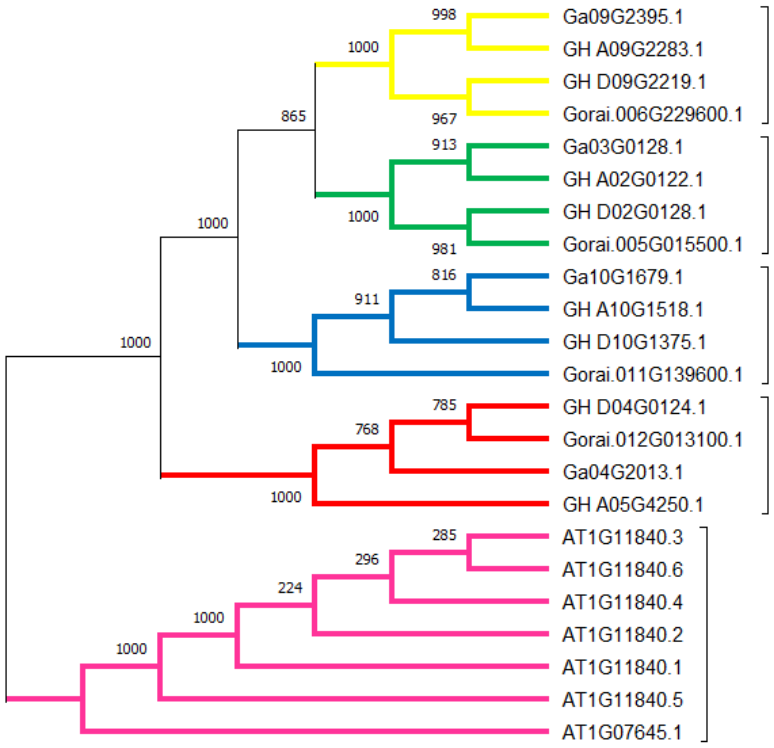


Fig. 2: Phylogenetic tree of the GLX gene family of cotton and Arabidopsis.

3.3. Gene Duplication Analysis of *GLX* Gene Family in Cotton

1. Due to whole genome duplications (WGD) and segmental duplications, the *GLX* gene family has experienced substantial diversification and expansion.
2. The genes in *Gossypium arboreum* are dispersed irregularly throughout several chromosomes.
3. Four of the eight *GLX* genes in *Gossypium raimondii* are distributed irregularly on chromosomes and have undergone segmental duplications or WGD.
4. The eight *GLX* genes in *Gossypium hirsutum* exhibit an irregular distribution across chromosomes and signs of WGD or segmental duplications (Fig. 4).

3.4. Gene Structure of the Cotton *GLX* Gene Family

Based on the evolutionary tree, *G.arboreum* genes can be divided into three groups, the first group contain Ga03G0128 and Ga04G2013 both are CDS or exon. The second group Ga09G2395 contains an upstream or downstream. The third group Ga10G1679 CDS or exon (Fig. 5).

The gene structure of G.raimondii displays 2 three groups based on its evolutionary tree where Group I has two genes. They contain Gorai.005G015500 and Gorai.006G229600 which contain an upstream or downstream with high bp. The second group has 2 genes as well, Gorai.011G139600 and Gorai.o12G013100 contain upstream or downstream with high 3000bp-3500bp.

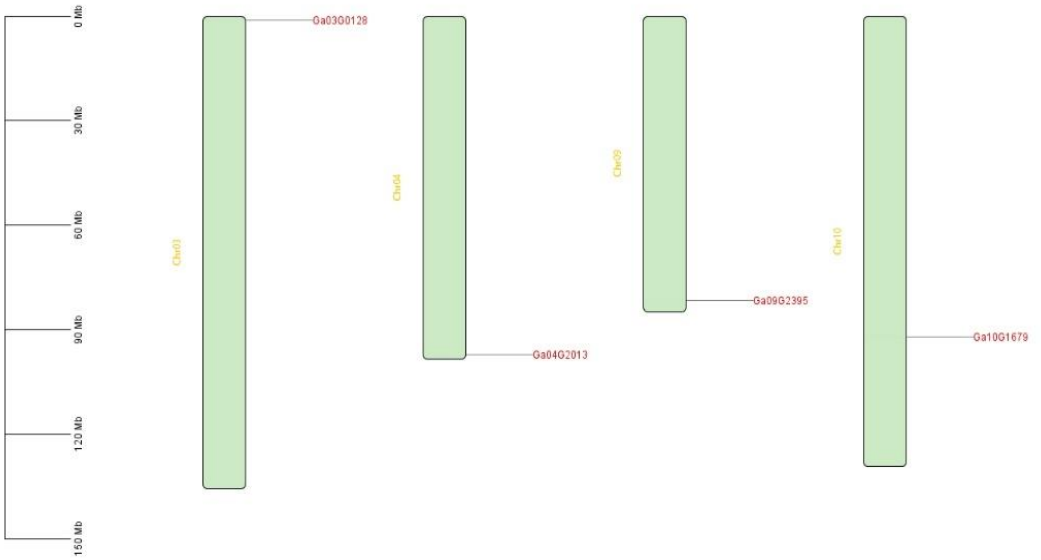


Fig. 3 (a): *GLX* gene's location on chromosome of *G. arboretum.*

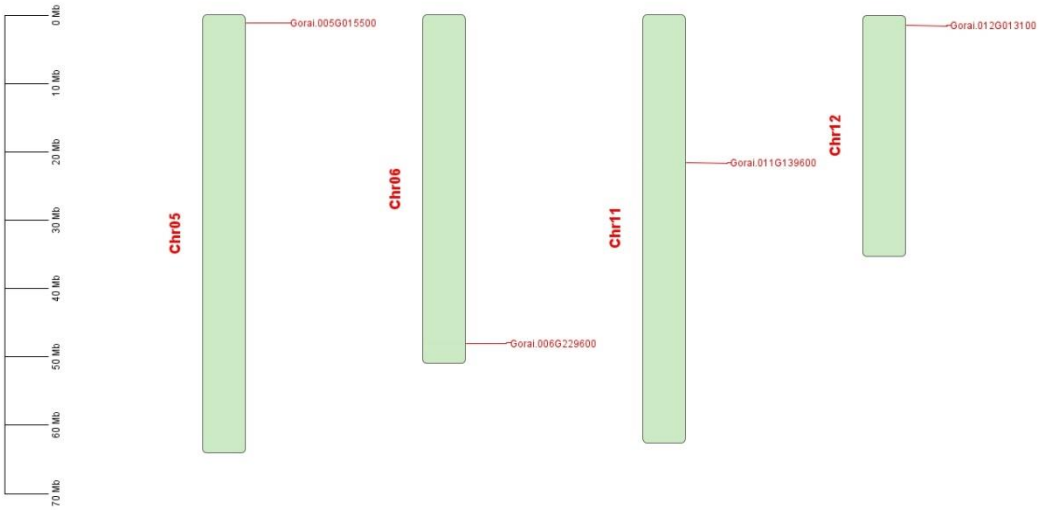


Fig 3 (b): *GLX* genes' location on chromosome of *G. raimondii.*

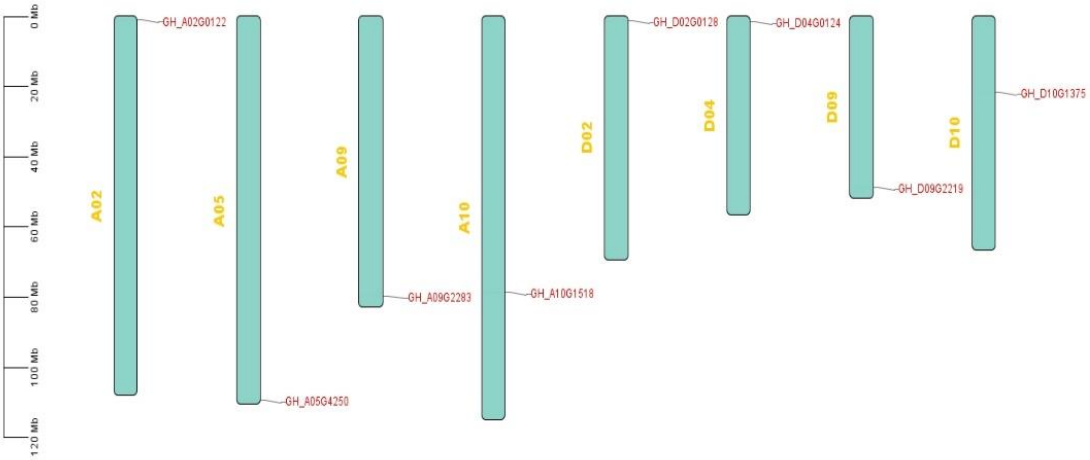


Fig 3 (c): *GLX* genes' location on chromosome of *G. hirsutum.*

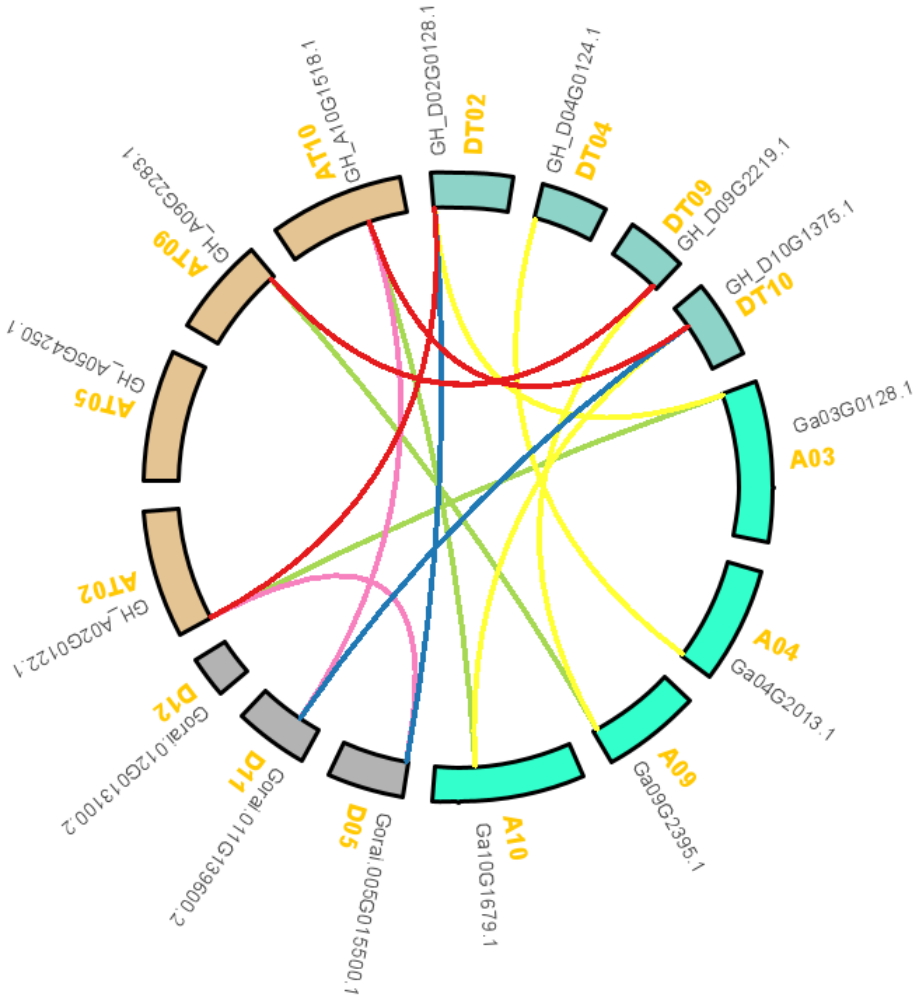


Fig. 4: Micro-circos analysis of the *GLX* gene family across cotton species.

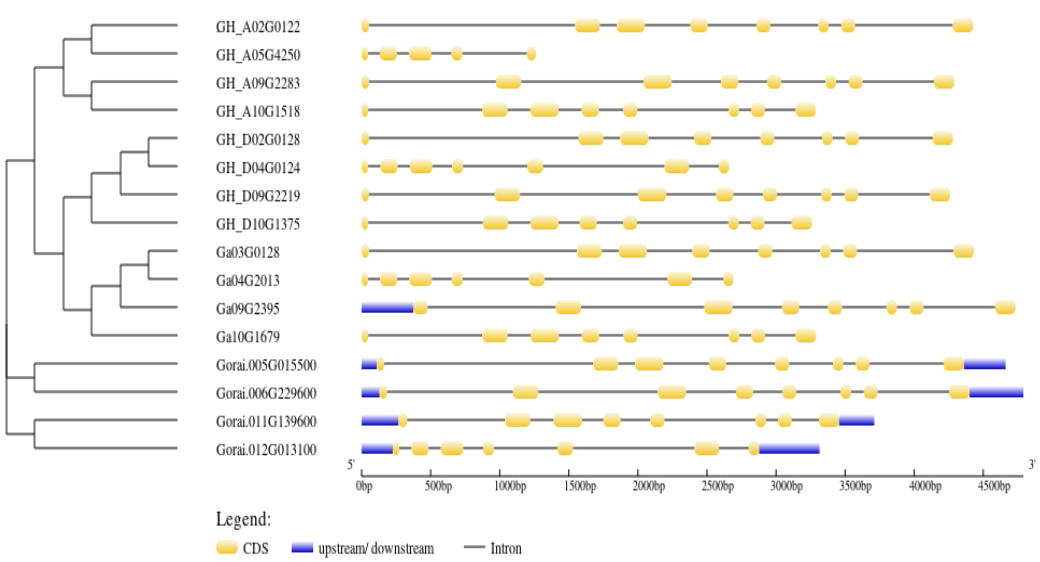


Fig 5: Gene structure of the GLX gene family of Cotton.

Gene Structure Display Server divided the G. hirsutum genes into two groups. This group have further divided into two sub groups. Which contain high introns.

3.5. Conserved Motifs of the Cotton *GLX* Gene Family

Analyses of the conserved motifs were done in order to investigate the structural variation and evolutionary history within the *GLX* gene family. The purpose of this study was to identify the evolutionary patterns and functional roles of these genes in various cotton species (Fig. 6).

3.6. CREs in *G. hirsutum GLX* Promoter Region

The promoter regions of *GLX* genes in *Gossypium hirsutum* contain numerous cis-regulatory elements (CREs) associated with various biological and metabolic processes. These CREs can be broadly categorized into three types: those involved in stress responses, those linked to hormone regulation, and those associated with growth and development. Notably, seven CREs in *GLX* promoters are responsive to stress, including drought inducibility, anaerobic induction, defense and stress response, light response, and low-temperature response. This pattern suggests that GLX genes may play a role in enhancing tolerance to abiotic stresses (Fig. 7).

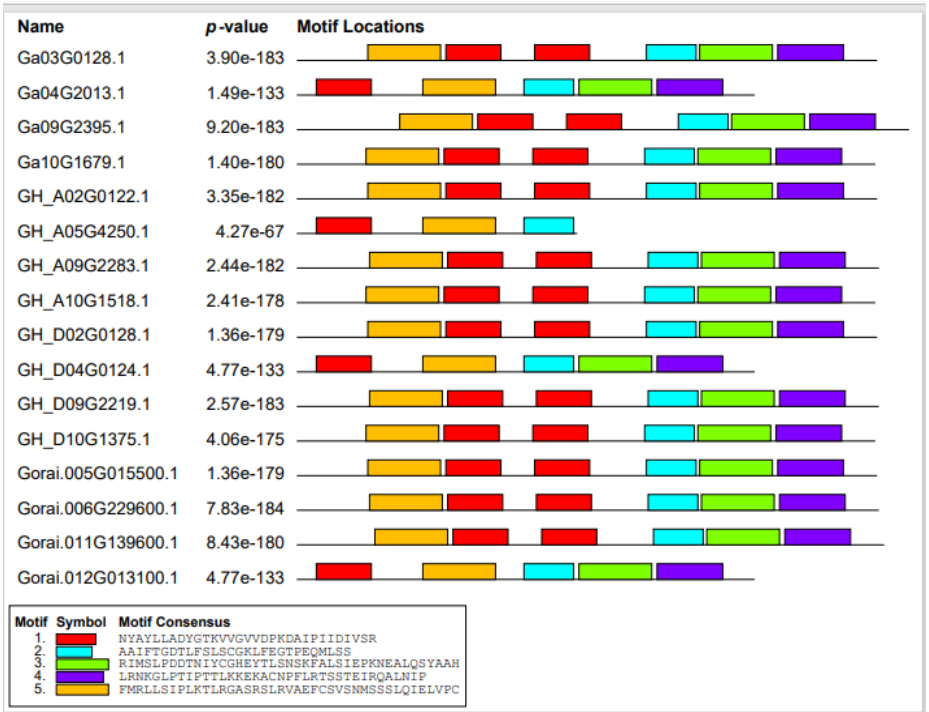


Fig. 6: *MEME motif analysis of GLX gene family in cotton species.*

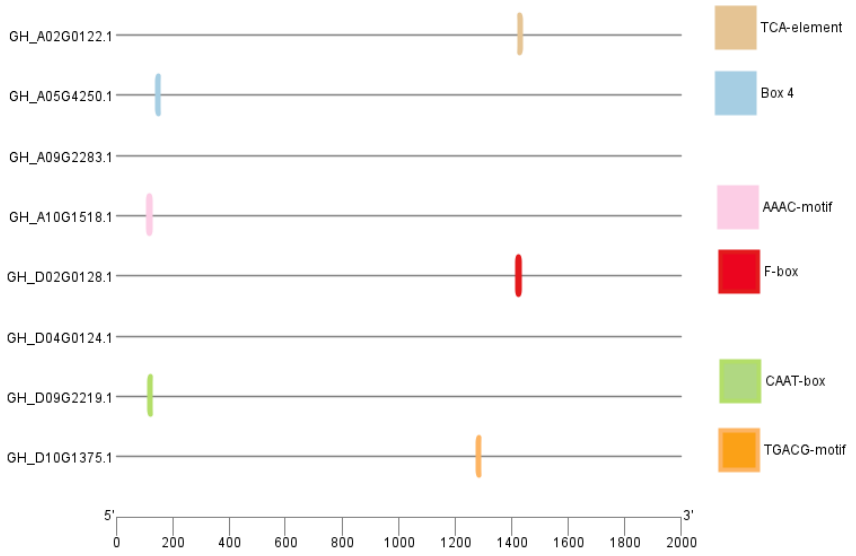


Fig. 7: CREs of *G.hirsutum GLXI* genes.

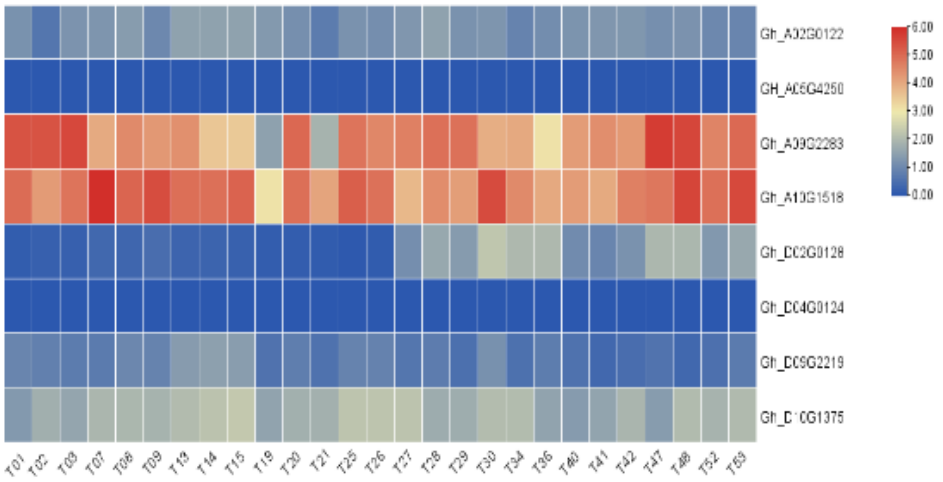


Fig. 8: HeatMap of *G. hirsutum* GLX expression data under abiotic stress.

3.7. Heat map of the *G.hirsutum GLX* Gene Family

The expression patterns of *GLX* genes in *Gossypium hirsutum* under different stress situations are depicted in the heatmap. This heatmap shows a different stress treatment or time point for each row, which corresponds to a particular *GLX* gene. On the right, there is a color scale that shows the different expression levels: blue denotes low expression, yellow represents moderate expression, and red denotes high expression (Fig. 8).

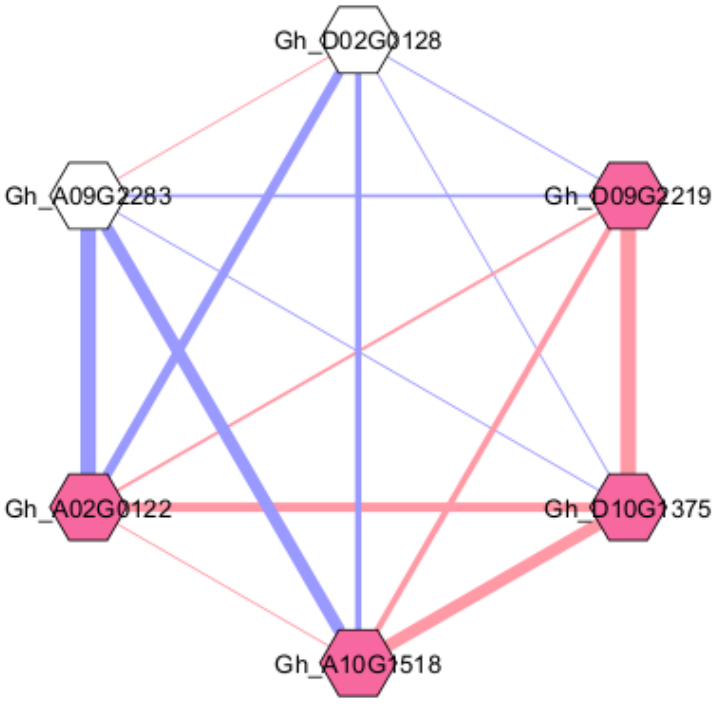


Fig. 9: Co-expression networking results of *G. hirsutum* GLX gene family.

3.8. Co-expression Network Analysis of the *G. hirsutum GLX* Gene Family

The co-expression network exhibits a scale-free structure, characterized by many peripheral genes and a few highly connected hub genes, which is a typical feature of biological networks (Fig. 9). In the cotton gene family, several hub genes have been identified, potentially playing critical roles in regulating abiotic stress tolerance and coordinating gene expression. These hub genes include *Gh_D09G2219, Gh_D10G1375, Gh_A10G1518*, and *Gh_A02G0122.*

3.9. DNA Extraction

Agarose gel electrophoresis was used to assess the purity of the isolated DNA. The outcomes of the gel electrophoresis showed how effectively the DNA from cotton seeds was extracted. Sharp, distinct bands showed that

the extracted DNA is of a high caliber and appropriate for additional molecular testing. Further evidence of the effectiveness of the extraction procedure and the DNA's purity came from the bands' intensity and lack of smearing.

3.10. Polymerase Chain Reaction

The agarose gel electrophoresis of PCR amplified product was performed and observed the required band of gene with a size of 981bp. The PCR product agarose gel electrophoresis showed some clear bands (Fig. 10).

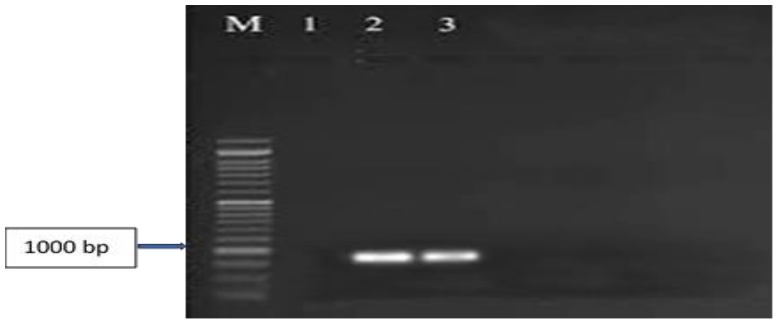
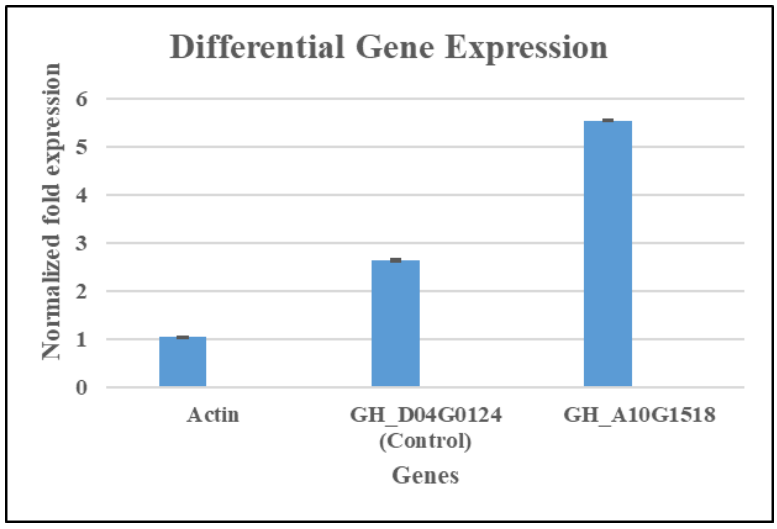


Fig. 10: Gel electrophoresis of PCR products visualized in a UV transilluminator.

3.11. Tracking Expression of the Transgene *GH_A10G1518* in Cotton

Three transgenic cotton plants were subjected to an expression study using qRT-PCR (Thermo Scientific, United States) and gene-specific primers in triplicate using the Maxima SYBR Green/ROX protocol. The actin gene regulated reaction normalisation internally. >*GH_A10G1518*.1 gene's fold expression *GH_D04G0124*, the control, with a value -2.6 times lower than this one. These results confirm that the candidate gene found by various in silico analyses has potential (Fig. 11).



Graph 1: Expressions of qRT-PCR

Normalize the target gene expression to housekeeping genes (e.g., GAPDH, β-actin) using the ΔΔCt method.

4 | DISCUSSION

Throughout their lives, plants encounter a range of environmental obstacles that can seriously impair their development and yield. According to Wei et al. (2020), transcription factors (TFs) are essential for increasing plant resistance to abiotic stress. Among these, the *GLX* TF family is essential for environmental adaptation and plant development (Morgenstern et al*.,* 2020; Mohanan et al*.,* 2021). Several species, including Chinese cabbage, Arabidopsis, and cucumber, have been found to possess these genes (Abdelrahman et al., 2018; Zhang et al*.,* 2023). *GLX* family genes in cotton have not yet been thoroughly investigated, nevertheless.

Eight, four, and four members of *Gossypium hirsutum*, *Gossypium arboreum*, and *Gossypium raimondii*, respectively, were found in a genome-wide investigation of the cotton *GLX* gene family. The structural diversity of these genes points to possible functional variety within the family. Phylogenetic linkages, cis-regulatory elements (CREs), conserved motifs, chromosomal locations, gene architectures, and identities were all thoroughly examined.

Using phylogenetic analysis, the *GLX* genes were categorised into three different clades according to their genetic similarities. The fact that this grouping supports the results of earlier studies emphasises the reliability of these classifications. In order to provide insight on the evolutionary divergence and biological importance of the *GLX* genes, the observed groupings most likely indicate evolutionary links and shared functional roles (Sun et al., 2022; Waadt et al., 2022). Similar conserved domains and structural characteristics, including intron and exon compositions, were shown by the *GLX* gene subgroups. A deeper evolutionary link within each subgroup is suggested by this structural consistency, although possible functional diversity is highlighted by the observed differences between groupings.

In *GLX* proteins, five different motifs have been found; motifs 1 and 2 are particularly notable because of their highly conserved C-terminal regions. These domains are important for DNA binding because they contain five cysteine residues that are necessary for the formation of structures that resemble zinc fingers. These structures allow certain DNA sequences, such GAGA motifs, to be recognised and bound. A complex process of DNA binding is highlighted by the conservation of these cysteine residues, highlighting the functional importance of these motifs in the regulatory activity of *GLX* proteins (Liu et al., 2021; Ma et al., 2022). The remaining motifs that were found displayed different degrees of distribution and conservation among the *GLX* proteins. Other functional domains that might be involved in subcellular localisation, protein-protein interactions, or other regulatory processes are probably represented by these patterns. A possible functional specialisation and diversification within the gene family, enabling cotton plants to successfully adapt to a range of developmental and environmental challenges, is indicated by the difference in motif composition among various *GLX* proteins.

The structural and evolutionary dynamics of the GLX gene family in cotton were better understood thanks to the micro-circos plot. These genes are highly selected to maintain their roles, as seen by the conserved syntenic links between them, which highlight their evolutionary relevance. Given that GLX genes are involved in a number of regulatory pathways, this conservation suggests that they are essential to basic physiological functions, most likely including stress responses. These kinds of results are essential for determining which genes to target for genetic alteration or breeding initiatives aimed at enhancing cotton's resistance to stress.

A DNA sequence located upstream of a gene that serves as a binding site for particular proteins that start the transcription process is called a promoter. Through proper transcription machinery assembly to begin converting the gene into RNA, this region plays a critical role in controlling gene expression (Rhaman et al., 2021). CREs, which are found in promoter regions, offer information about the function and regulatory mechanisms of the gene. Stress-responsive elements like MBS, TCA-element, ARE, and TGACG-motif are found in GLX genes, which implies that these genes are involved in the plant's reaction to a number of abiotic stressors, such as drought, salicylic acid signalling, anaerobic conditions, and methyl jasmonate (Xu et al., 2023; Zhang et al., 2023). Given that these genes are known to be important for plant development and stress responses, the discovery of MYB and MYB-like sequences raises the possibility that MYB transcription factors may control them (Wang et al., 2021). Furthermore, the fact that multiple promoters contain light-responsive elements (Box II, Box 4, ACE) suggests that light plays a significant regulatory role in these genes, possibly connecting them to photosynthetic and photomorphogenic processes. Cotton plants' flexibility and resilience are greatly influenced by the variety of cis-regulatory elements (CREs) found in the promoter regions of *GLX* genes. These CREs are implicated in a wide range of biological processes and regulatory pathways.

According to recent research on cucumbers, *GLX* increases the activity of antioxidant enzymes, hence improving tolerance to NaCl stress. In this way, oxidative stress indicators such as malondialdehyde (MDA) and reactive oxygen species (ROS) are less likely to build up (Li et al., 2019; Zafar et al., 2024). Likewise, under salt stress, transient overexpression of GLX in cabbage regulated the expression of genes for antioxidant enzymes and stimulated growth. (Zhang et al., 2023).

Heatmap and co-expression network data integration provided a solid foundation for choosing *GH_A10G1518* as a potential gene. The distinct expression pattern seen in the heatmap suggests that it might be involved in systems of stress tolerance. A vital functional role is also suggested by its prominent location in the co-expression network. The hypothesis was that *GH_A10G1518* would be important for abiotic stress tolerance based on these in-silico results. Cotton seeds' DNA was effectively extracted using a modified CTAB technique. Agarose gel electrophoresis was used to evaluate the freshness and purity of the isolated DNA. Without any discernible smearing, the gel electrophoresis results showed distinct, high-molecular-weight bands, indicating that the DNA was undamaged and intact. These findings confirm the effectiveness of the extraction protocol, yielding high-quality DNA suitable for downstream applications, particularly PCR. Obtaining high-quality DNA is essential for ensuring the reliability and accuracy of subsequent molecular biology experiments.

Primers specific to *GH_A10G1518* be carefully designed however, agarose gel electrophoresis of PCR products displayed unexpected bands indicating dimer formation. These results were surprising as the primer design and optimization steps were intended to avoid such issues.

Additionally, quantitative expression analysis using qRT-PCR was performed to evaluate the differential

expression of the gene. This real-time PCR analysis, conducted on both transgenic and non-transgenic cotton plants, highlighted significant differences in gene expression. Leaves from three generations of transgenic plants (T0, T1, and T2), all from the same cotton variety CCRI24, were collected, revealing variations in relative gene expression across these generations. The transgene was overexpressed 2.97-, 2.86-, and 2.92-fold higher in the T0, T1, and T2 generations, respectively, compared to non-transgenic plants. Similar improvements in cotton fiber quality were observed. The minimal differences in gene expression among the three generations may be due to environmental stresses or handling conditions, but overall, this suggests stable integration of the transgene into the host genome (Fang et al., 2021).

The study's findings show a considerable increase in the overexpression of the *GH_A10G1518* gene. This implies that encouraging *GH_A10G1518* overexpression may result in the creation of cotton cultivars in the future that are more abundant and of higher quality when exposed to salt stress. Furthermore, by focusing on the *GH_A10G1518* gene, we anticipate that the knowledge gathered from this study will be helpful in applying gene-editing technologies to improve cotton yield even more.

5 | Conclusion

The genome-wide characterization and in silico expression analysis of the *GLX* gene family in cotton have provided novel insights into their roles in abiotic stress tolerance, a previously unexplored area. The study identified and characterized *GLX* genes, elucidating their phylogenetic relationships, conserved motifs, and gene structures. Expression profiling under various abiotic stresses, including salt exposure, revealed distinct expression patterns, highlighting the potential involvement of *GLX* genes in stress response mechanisms. Candidate *GH_A10G1518* emerged as particularly promising, demonstrating significant differential expression under salt stress conditions. This gene may play crucial roles in enhancing cotton's resilience to salt toxicity, which poses a serious threat to agricultural productivity and environmental health. Co-expression network analysis further supported the importance of these candidate genes, identifying key regulatory pathways and potential interacting partners that may contribute to salt tolerance.

The findings underscore the potential of *GLX* genes as targets for genetic improvement strategies aimed at developing cotton varieties with enhanced abiotic stress tolerance, including resistance to salt pollution. This research lays a robust foundation for future functional validation studies and the potential application of *GLX* genes in breeding programs to mitigate the impacts of environmental stresses on cotton production.

The conclusion of PCR results is that we obtained the required band of gene with a size of 981bp through the process of agarose gel electrophoresis PCR. We concluded from the expression that was carried out via qRT-PCR (Thermo Scientific, United States) that the candidate genes screened through in silico analysis can be the potential genes. The fold expression of gene > *GH_A10G1518*. 1t was found -2.6 folds higher than control *GH_D04G0124* These findings validate the potential of candidate gene identified through different silico analysis. This overexpression led to enhance production of cotton under salt stress.

Declarations

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Data Availability: The data collected for this article are included in the article.

Ethics Statement: No prior study was conducted on live animals/humans; thus, it did not require any ethical approval.

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