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Research Article

Genome-wide Characterization and Expression Analysis of the SPY Gene in *Gossypium hirsutum* under Salt Stress

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ABSTRACT

The *SPINDLY* (*SPY*) gene has garnered attention because of its role in plant stress responses, namely salt stress. It was first identified as a suppressor of gibberellic acid (GA) signaling. This study offers a comprehensive analysis of the whole genome and the expression patterns of the *SPY* gene in *Gossypium hirsutum* under situations of salt stress. To investigate *SPY* expression patterns and their impact on *G. hirsutum* salt stress response pathways, we used transcriptome analysis. Our study's findings show that *SPY* is essential for controlling salt stress tolerance. Reduced salt tolerance is caused by mutations in *SPY* (-3), while plants that overexpress *SPY* (*SPY*-OX) have reduced survival rates when exposed to salt stress. Stress-responsive genes, including Sensitive to Dehydration20 and AREB1-1-like transcription factor, were discovered to be up-regulated in *SPY*-3 mutants, which increased their ability to withstand stress, according to transcriptome analysis. Conversely, *SPY*-OX plants showed decreased expression of genes that respond to stress, which suggests that salt stress is negatively affecting their capacity to withstand it. Moreover, the increase of the CKX3 gene in *SPY* mutants, which suggests a reduction in cytokinin signaling, highlighted the importance of *SPY* in cytokinin signaling pathways. Our research provides significant understanding of how the *SPY* gene controls pathways related to *G. hirsutum's* salt stress response. This demonstrates the significance of the gene for agricultural resilience and plant stress physiology.

Keywords: Transcriptome Analysis, *Gossypium hirsutum*, Salt Stress, *SPINDLY* (*SPY*) gene.

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INTRODUCTION

Cotton is a major cash crop in Pakistan, crucial for the country's economic survival (Nazeer et al., 2023). Cotton and cotton products contribute 0.8% to the country's GDP and account for 60% of its foreign currency profits. Over 40% of the industrial sector is directly or indirectly involved in the cotton industry (Raza et al., 2023). In Pakistan, the provinces of Punjab and Sindh are the primary cotton-producing regions, with Punjab alone contributing 70% of the overall output. Pakistan has the potential to produce 30 million bales from 10 million acres of land (Sharma et al., 2023). However, recent cotton production has averaged about 10 million bales, which is below the country's potential, particularly after achieving a record-breaking 14.8 million bales in 2011–2012 (Jdanov et al., 2023). Since 2015, cotton output has seen a decline, producing 5.6 million bales—a significant decrease compared

to previous decades (Rana et al., 2023). Several factors contribute to this decline, including climate change (characterized by high temperatures and erratic rainfall), and outdated Bt. Bollgard-I technology, poor seed quality, pest issues (especially whiteflies), bollworm infestations, cotton leaf curl
virus (CLCuV) disease, insecticide resistance, high weed infestation, labor shortages for picking, low mechanization adoption, and reduced cotton profitability (Sharma et al., 2023). Pakistani cotton experts have been striving to address these issues since 1992 (Nasereldin et al., 2023).

In response to these challenges, heat-tolerant cotton genotypes (MNH-1020 and FH-Super) have been commercialized for widespread planting in Punjab Province by researchers at the Cotton Research Institute in Multan and other research facilities (Punjab Province) (Nazeer et al., 2023). Additionally, new cultivars with glyphosate and bollworm resistance genes are expected to be available for general cultivation in the next two years. The future of Pakistan's cotton industry appears promising, with advances such as transgenic cotton (Bt) and dual herbicide-resistant cultivars being marketed globally to manage weeds and pests.

Moreover, high-throughput genetic analysis and genotyping methods, including miRNA, re-sequencing, RNA-sequencing, and gene expression association analyses, have been applied to evaluate fiber quality, as well as resistance to salt, drought, and disease (Abdullah et al., 2022). The main drivers of genetic advancement in cotton include genome size, polyploidy, genetic multiplication, a lengthy development cycle, and resistance to transgenic plants (Abbas et al., 2022). A quick and efficient identification technique is necessary to understand the molecular functions and regulatory processes of the various genetic variants for high-yield cotton breeding.

The *SPY* gene is a significant component in various organisms. In *Salmonella enterica*, *SPY* is induced during envelope stress, making it a potential biosensor for monitoring stress levels in the bacterial envelope. In eukaryotes, a new group of DNA transposons called *SPY* has been identified, characterized by terminal inverted repeats (TIRs) and a DDE motif-containing transposase, which transposes precisely between specific host nucleotides without creating target site duplications (Rhee et al., 2022). In Schizosaccharomyces pombe, *SPY*1, a histidine-containing phosphotransfer protein, plays protective roles against nitrosative and nutritional stress, being up-regulated in response to these stresses in a Pap1-dependent manner. Additionally, in *Streptomyces pristinaespiralis*, the *SPY*1 gene encodes a serine/threonine protein kinase involved in pristinamycin production (Bhinda et al., 2022). In Arabidopsis, *SPY* is expressed throughout the plant and plays a role in root development, with the majority of *SPY* protein localized in the nucleus, suggesting involvement in gibberellic acid (GA) signaling regulation.

The SPINDLY (*SPY*) gene was initially identified as a negative regulator of GA signaling in plants, with mutations in this gene mimicking the effects of excessive GA and conferring resistance to GA inhibitors during seed germination (Bhinda et al., 2022). Beyond its role in GA-related functions, *SPY* also influences salt stress tolerance in *Arabidopsis thaliana*. Studies on *SPY* mutants (-3) revealed decreased salt tolerance, suggesting a positive role of *SPY* in the salt stress response. Transcriptome analysis of *SPY*-3 mutants showed up-regulation of stress-inducible genes such as Sensitive to Dehydration20 and AREB1-1-like transcription factor, along with genes encoding late embryogenesis abundant proteins, likely contributing to enhanced stress tolerance in *SPY*-3 plants (Rhee et al., 2022). Furthermore, the increased expression of the CKX3 gene, involved in cytokinin degradation, in *SPY* mutants indicates a reduction in cytokinin signaling, highlighting *SPY*'s involvement in cytokinin signaling mechanisms.

Conversely, transgenic plants overexpressing *SPY* (*SPY*-OX) exhibited decreased survival under salt stress conditions, opposing the phenotype observed in *SPY* mutants. Gene expression analysis revealed decreased expression of stress-responsive genes such as SNH1/WIN1 and DREB1E/DDF1 in *SPY*-OX plants, which were enhanced in *SPY*-3 mutants. This research indicates that *SPY* reduces abiotic stress tolerance in plants, likely due to its role in integrating environmental stress signaling through GA and crosstalk (Lin et al., 2022). Given that salt and drought often cause similar water stress in plant cells, testing that considers water scarcity situations has improved plant survival.

MATERIAL AND METHODS

Sequence identification and characteristics of *SPY* gene

Four cotton species, including *Gossypium arboreum*, *Gossypium herbaceum*, *Gossypium raimondii*, *Gossypium hirsutum*, *Gossypium barbadense*, was taken from CottonFGD (https://cottonfgd.org/) (Zhu *et al*., 2017) Arabidopsis protein sequences were found from TAIR (https://www.arabidopsis.org/index.jsp). Further, the ProtParam tool (https://web.expasy.org/protparam/) was used for the determination of biophysical properties of *SPY* genes, including chromosome, start, end, strand, amino acid length, molecular weight (Da) & isoelectric point (pl).

Phylogenetic analysis

A maximum likelihood phylogenetic tree was constructed using the amino acid sequences of *Gossypium hirsutum* (Upland cotton), *Gossypium barbadense* (Pima cotton), *Gossypium arboreum* (Tree cotton), *Gossypium raimondii* (Wild cotton), *Arabidopsis thaliana* (Thale cress), *Sorghum bicolor* (Sorghum), *Oryza sativa* (Rice), *Helianthus annuus* (Sunflower), *Glycine max* (Soybean), *Glycine soja* (Wild soybean), *Zea mays* (Maize), *Pisum sativum* (Pea), and *Theobroma cacao* (Cacao). The sequences were aligned using MUSCLE and subjected to phylogenetic analysis using MEGA X 10.0.5 (Kumar *et al*., 2018), with a bootstrap value set at 1000. The resulting tree was visualized using iTOL v5 (https://itol.embl.de/itol.cgi) (Letunic and Bork, 2018).

Gene structure and protein motif analysis

The MEME tool (https://meme-suite.org/meme/index.html) was employed to identify protein motifs by utilizing the full-length protein sequences of *SPY* genes (Bailey *et al*., 2008) For gene structure analysis, the Gene Structure Display Server 2.0 was utilized, employing both genomic and coding sequences of *SPY* genes (Guo *et al*., 2008).

Chromosomal localization and cis-regulatory elements

A physical map was created using MapCart 2.2 (Voorrips, 2002), and chromosome position information for *SPY* genes was obtained from a cotton genomic file. The PlantCare database was utilized to assess the cis-regulatory elements within the 2 kb upstream promoter sequence (Lescot *et al*., 2008) (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Sequence logos

The analysis of sequence logos utilized the protein sequences of *SPY* genes from Gossypium hirsutum. Sequence logos were generated using the WebLogo tool (https://weblogo.berkeley.edu/README) (Crooks *et al*., 2004).

Expression Analysis under Salinity

To investigate the expression level of the *SPY* gene under salinity stress, *Gossypium hirsutum* cultivars were planted in pots under greenhouse conditions with temperatures ranging from 22°C to 26°C and a 12-hour light/12-hour dark cycle. Upon the development of true leaves, seedlings were subjected to peg solution treatment, with tap water serving as a control. Leaf, stem, and root samples were collected at 0 hours, 3 hours, 6 hours, and 12 hours post-treatment. Expression patterns of *SPY* genes under salinity stress were visualized using the online tool Heatmapper (http://www.heatmapper.ca/).

RNA Extraction and Quantitative Real-Time PCR Analysis

RNA Isolation

Total RNA was isolated from all samples using the EASYspin RNA plant kit (Cat#DR103-03) following the manufacturer's instructions.

DNaseI (RNase-free) was used to eliminate genomic DNA contamination in the RNA samples.

RNA Concentration and Purity Check

The concentration and purity of RNA samples were checked using the Thermo Fisher Scientific NanoDrop One and run on a 1% agarose gel.

First Strand cDNA Synthesis

Total RNA (5 µg) was taken as a template for first-strand cDNA synthesis using the iScript™ Reverse Transcription Supermix for RT-qPCR (BIO-RAD, Hercules, CA, USA).

Real-Time PCR Setup

BIO-RAD's CFX Connect Real-Time PCR Detection System was utilized to study the relative expression level of the *Gossypium barbadense* and *Gossypium hirsutum* NHX genes. iTaq™ Universal SYBR Green Mix (BIO-RAD) with gene-specific primers was used for the real-time PCR reactions. Each gene expression was normalized using the Actin genes as internal controls (Artico *et al*., 2010).

Thermal Cycling Conditions

The thermal cycler conditions were set as follows: Initial denaturation at 95 °C for 3 minutes. Amplification with 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 1 second, and extension at 72 °C for 30 seconds. Melting curve analysis at 95 °C for 10 seconds, 65 °C for 1 minute, and 97 °C for 1–5 seconds.

RESULTS

Properties of *SPY* gene

In our investigation of the cotton species *Gossypium hirsutum* genome, we uncovered a total of four *SPY* genes. These genes exhibited specific biophysical characteristics which we thoroughly examined. The details we gathered include

the locus ID, coding sequence (CDS) length in base pairs (bp), protein length in amino acids (aa), predicted protein molecular weight (MW), projected cellular localization, isoelectric points (pI), and presence of transmembrane domains. Our analysis revealed that these *SPY* genes are predominantly localized in the cytoplasm and chloroplast, with amino acid counts ranging from 926 to 927. This comprehensive data is summarized in Table 1.

Table 1. Characteristics of the *G. hirsutum SPY* Gene.

Gene ID	gene family	chromosome no	Localization	A. A	pI	M.w
GH-*SPY*-D01	*SPY*	*SPY*-D01	Cytoplasm	927	5.63	103050.47
GH-*SPY*-A01	*SPY*	*SPY*-D01-1	Cytoplasm, Chloroplast	927	5.49	103188.64
GH-*SPY*-A11	*SPY*	*SPY*-D01-2	Cytoplasm, Chloroplast	927	5.63	103050.47
GH-*SPY*-D11	*SPY*	*SPY*-D01-3	Chloroplast	926	5.50	103024.24

Phylogeny of *SPY* Genes with Different Species

To investigate the evolutionary relationship among the *SPY* genes, we gathered protein sequences from 10 diverse plant species, including four Gossypium species (*G. hirsutum, G. barbadence, G. arboreum, and G. raimondii*), *Arabidopsis thaliana* (At), *Sorghum bicolor* (Sb), *Oryza sativa* (Os), *Glycine max* (Gm), *Zea mays* (Zm), and *Triticum aestivum* (Ta). These sequences were utilized to construct a maximum likelihood phylogenetic tree. The resulting tree exhibited a clear correlation with the subcellular localization of the *SPY* genes.

The phylogenetic analysis grouped the *SPY* genes from different species into three distinct clades based on their subcellular localization: VAC (vacuolar membrane-bounded), ENDO (endomembrane-bounded), and PM (plasma membrane-bounded). This clustering pattern indicates a potential evolutionary conservation of *SPY* gene function within specific cellular compartments across various plant species. The constructed phylogenetic tree depicting these relationships is illustrated in Figure 1.

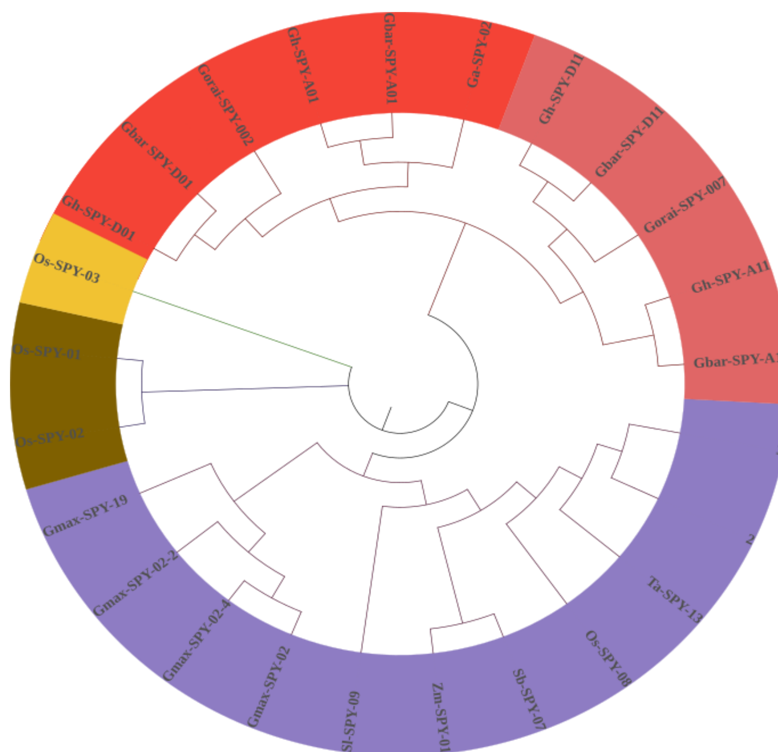
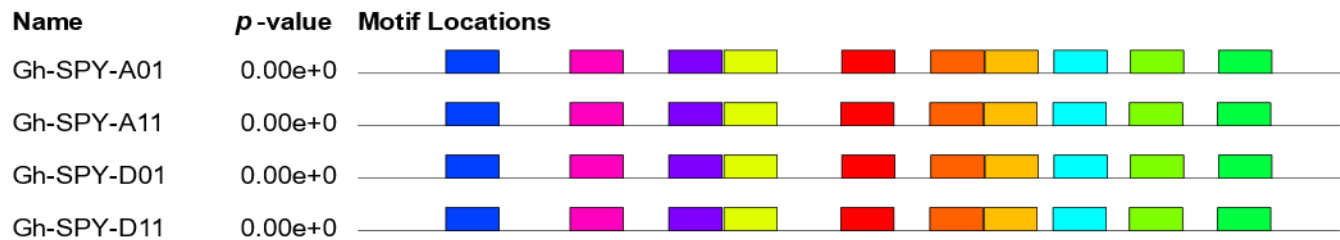


Figure 1. Phylogenetic tree of *SPY* between 11 plant species by the neighbor-end joining method using MEGAX 10.0.5. The tree divides all the *SPY* genes into three groups based on their subcellular localization. The amino acid sequences used in phylogenetic analysis are provided.

Protein motif analysis & Physical Genome Mapping of SPY Genes in *G. hirsutum*

A motif prediction carried out by MEME with 0–10 motif sites showed that all of the *G. hirsutum SPY* genes have a binding motif. The physical mapping of the *SPY* genes on the equivalent chromosomal loci in four Gossypium species showed that the *SPY* genes are scattered on both the A and D genomes. In the case of *G. hirsutum*, the at sub-genome has 2 and the Dt sub-genome has 2 *SPY* genes (Figure 3.)



Motif	Symbol	Motif Consensus
1.		FDAHRDWGRRFMRLYPQYDSWDNPKDPERPLVIGYISPDYFTHSVSYFIE
2.		GFITFGSFNNLAKITPKVLQVWARILCAVPNSRLVVKCKPFCCDSVRQKF
3.		NHDHMQAYSLMDISLDTFPYAGTTTTCESLYMGVPCVTMAGSVHAHNVGV
4.		YNWHYADAMYNLGVAYGEMLKFDMAVVYYELAFHFNPHCAEACNNLGVIY
5.		QVTWIGYPNTTGLPTIDYRITDSFADPPGTKQKHVEELVRLPECFLCYTP
6.		QNLRASLRDLMSKSPVCDGQNFISGLEATYRGMWRRYCKGDVPSLRYMET
7.		HIGKGICLQMQNMGRPAFESFAEAIKLDPQNACALTHCGILYKDEGRLVD
8.		SEMMQYDTALSCYEKAALERPMYAEAYCNMGVIYKNRGDLESAIACYERC
9.		KKGGLWRDIYGIDEKKVASMIRDDKIDILVELTGHTANNKLGTMACRPAP
10.		RDNLDKAVECYQLALSIKPNFSQSLNNLGVVYTVQGKMDAAASMIEKAII

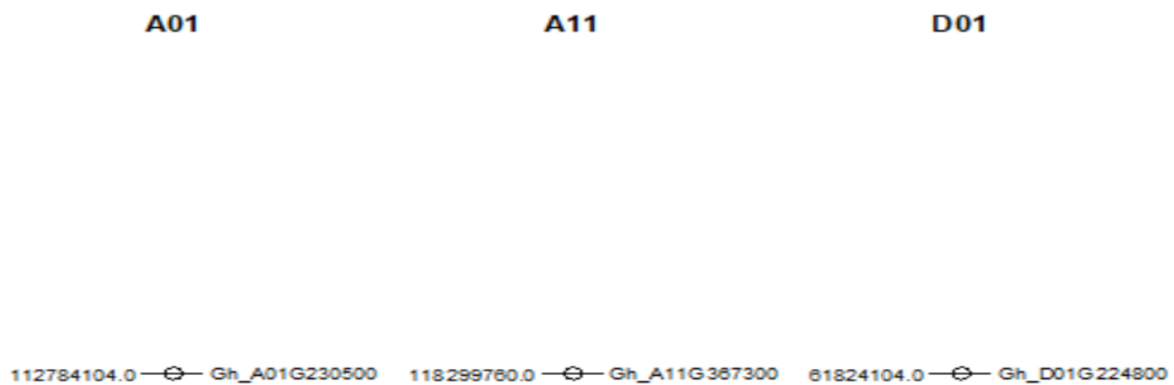


Figure 2. Representation of the conserved motifs in *SPY* Genes. Each colored box represents a motif in the gene., with the motif name indicated in the box along the bottom.

Figure 3. Chromosomal location of the *SPY* genes in the *Gossypium* species.

Gene Structure Analysis of *SPY* Gene Family

The gene structure analysis of the cotton species (*G. hirsutum*) along with the phylogeny results showed that the genes with a similar intron/exon pattern clustered near each other in the same groups (Figure 4) The exons and introns of *G. hirsutum* were analyzed and compared by the online program GSDS.

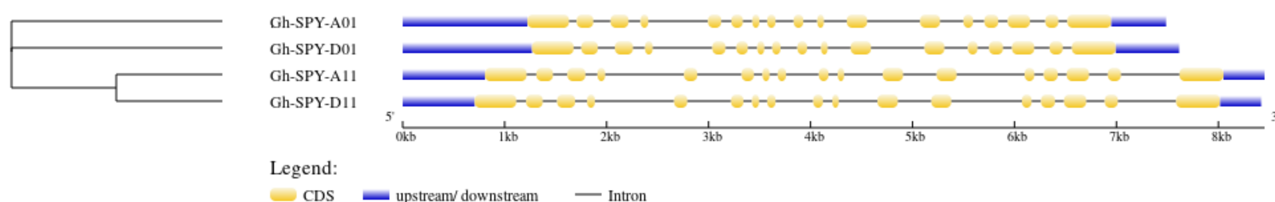


Figure 4. Gene Structure of the *G.hirsutum SPY* genes. The yellow box represents the exons and the black lines represent the introns.

Promoter Analysis of *G. hirsutum* SPY Genes

To assess the transcriptional potential of the SPY genes, we analyzed the cis-elements within the 2000 bp promoter regions upstream of the start codon. Alongside the common core promoter/enhancer elements such as CAAT-box and TATA-box, we identified a range of elements associated with stress, light, and hormone responses.

Remarkably, our analysis revealed that the SPY genes harbored a greater abundance of cis-elements related to light response compared to stress and hormone responses, suggesting their involvement in light-mediated regulatory processes. Among the stress-responsive elements, MYB (CAACCA/TAAC/TAACTG) and MYC (CAATTG/TCTCTTA/TCTCTTA) elements were the most prevalent, constituting approximately 12% and 10% of all elements, respectively. Additionally, elements such as AREs and STREs were present in GhSPY genes, with AREs found in 15 genes and STREs in 12 genes. Moreover, the WUN motif and W-box were identified in 9 and 7 GhSPY genes, respectively. Notably, the promoter regions of Gb-SPY7-1A (Gbar_A03G012870) and Gb-SPY-1D (Gbar_D02G014810) exhibited the highest number of stress-responsive cis-elements among all SPY genes analyzed. This comprehensive analysis provides insights into the potential regulatory mechanisms governing the transcriptional activity of SPY genes in response to various environmental cues and stresses.

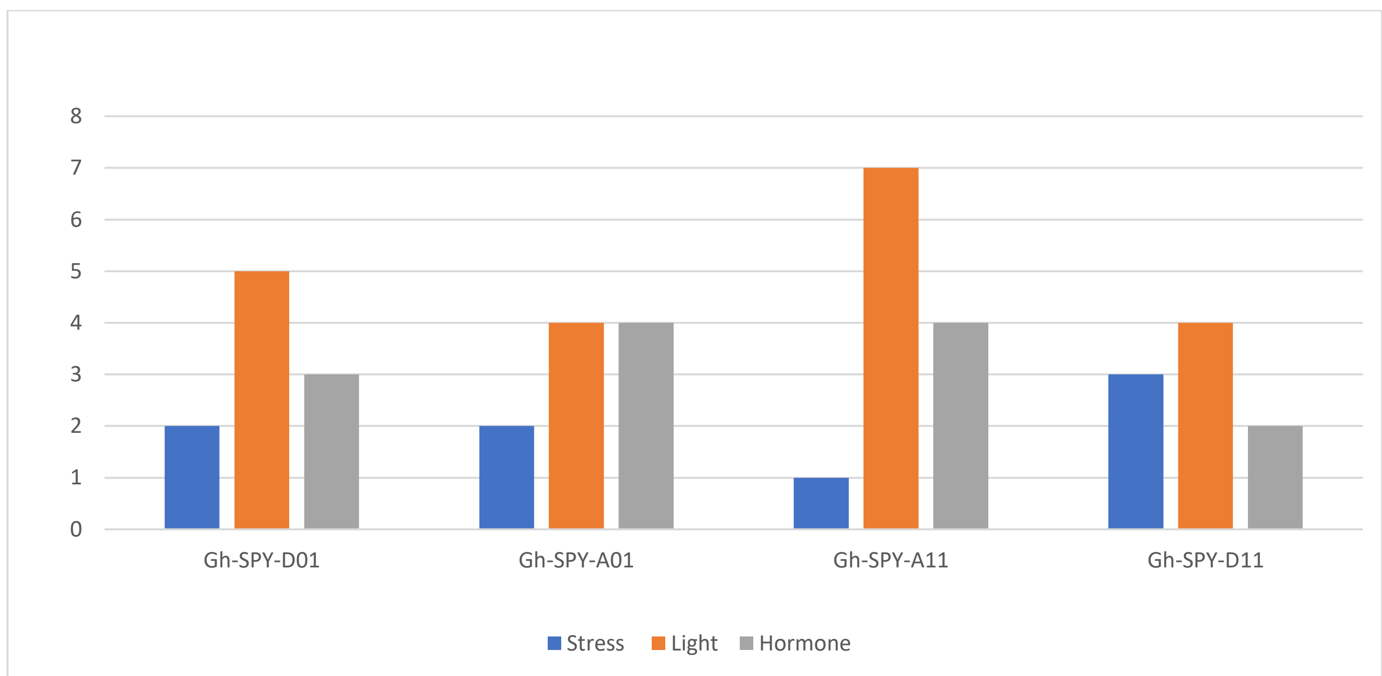


Figure 5. Cis-elements of the *SPY* genes. The vertical axis represents the number of Cis-elements, and the horizontal axis shows the genes' names.

Protein-Protein Interaction Prediction GhSPY Genes

Glycosyltransferase family 41 protein (domain architecture ID 11420499). glycosyltransferase family 41 protein containing tetratricopeptide (TPR) repeats, similar to Oryza sativa probable UDP-N acetylglucosamine--peptide N-acetylglucosaminyltransferase *SPINDLY,* an O-linked N acetylglucosamine transferase (OGT) that catalyzes the addition of nucleotide-activated sugars directly onto the polypeptide throughO-glycosidic linkage with the hydroxyl of serine orthreonine (Szklarczyk *et al.*, 2023).

Expression Pattern of *G. hirsutum* SPY genes under Salt Stress

The expression pattern of *SPY* genes under salinity stress was investigated to elucidate their potential role in salinity tolerance in *Gossypium hirsutum*. Notably, *G. hirsutum* was found to exhibit greater tolerance to salinity stress, which correlated with its increased development of lateral roots under stress conditions. In our study, we employed qRT-PCR to analyze the expression of all *SPY* genes in *G. hirsutum* across root, stem, and leaf tissues at various time points (0, 3, 6, and 12 hours) following exposure to salinity stress. Our findings unveiled distinct expression patterns across tissues and time intervals.

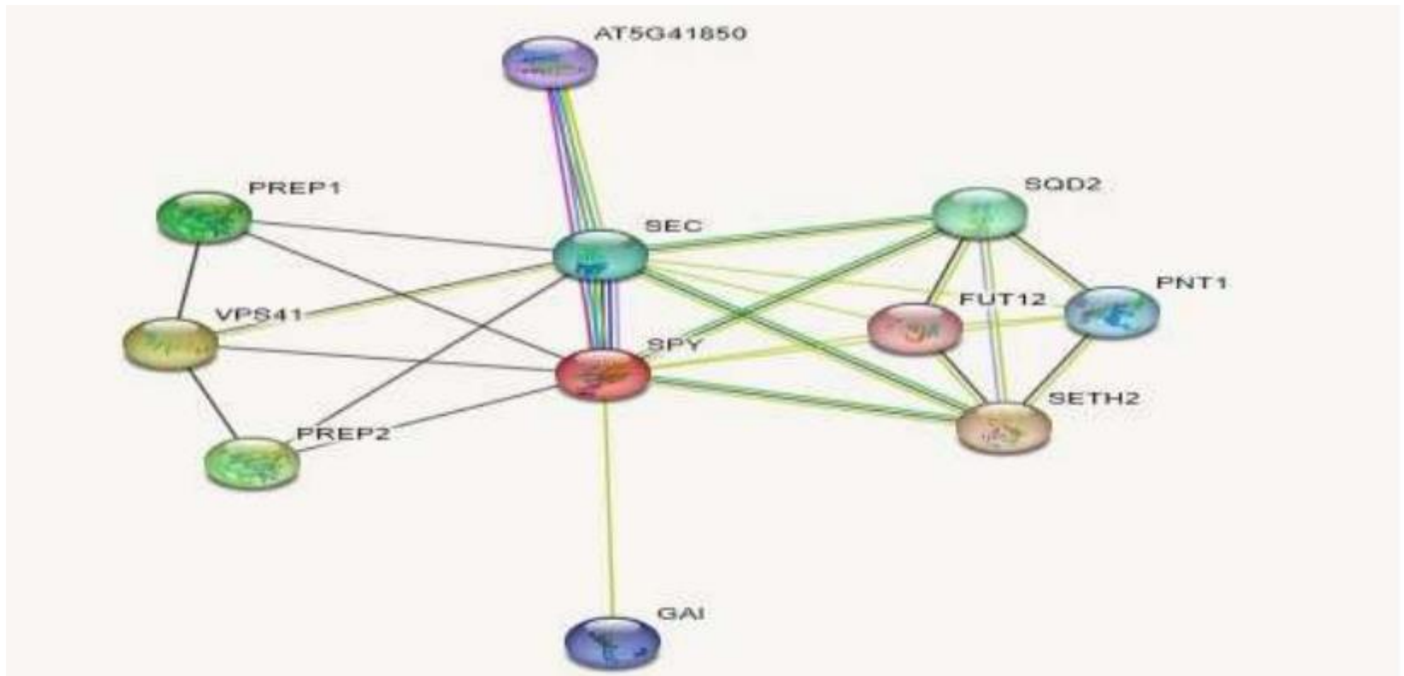


Figure 6. Multiple-TPRmotif proteins would fold into a right-handed super-helical structure.

Firstly, we observed that most *SPY* genes exhibited higher expression levels in stem tissue compared to root and leaf tissues. However, a greater number of genes were expressed in roots and leaves, with a comparatively lower expression in stems under stress conditions compared to the control. Further analysis focused on the *Gh*-*SPY*-D01 gene, which displayed notably higher expression levels. Differential expression patterns were observed among tissues, with nearly all genes exhibiting maximal expression at the 12-hour time point across various tissues.

This dynamic expression pattern suggests a temporal regulation of *SPY* genes in response to salinity stress, with a peak in expression occurring at the 12-hour mark. These findings shed light on the tissue-specific and temporal dynamics of *SPY* gene expression in *G. hirsutum* under salinity stress, providing valuable insights into their potential roles in salinity tolerance and stress response mechanisms in cotton plants. Figure 7 depicts the expression profiles of *SPY* genes across different tissues and time points.

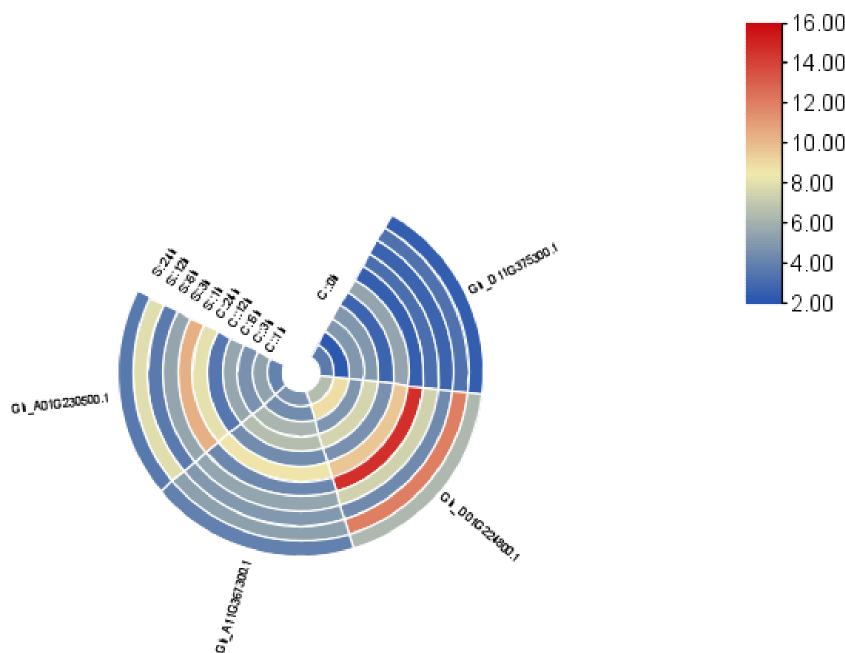


Figure 7. Relative expression level analysis of *SPY* in *Gossypium Hirsutum*. Relative expression of different *SPY* is shown under controlled conditions and salinity stress in different tissues at different time intervals.

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RNA Extraction

As previously described, RNA was extracted from plant leaves using Trizol (Invitrogen). A Nanodrop 2000 was used to determine the amount of RNA (Thermo Scientific, USA). Isolated RNA (2lg) was synthesized using the Revert Aid first strand cDNA synthesis kit (Thermo Scientific, USA) and oligo d (T) primers following the manufacturer's procedure.

RNA extraction by TRIZOL method

For RNA extraction, the following steps were taken, the homogenized samples were left for five minutes at 15 to 30°C so that nucleoprotein complexes could break up completely. For every 1 ml of TRIZOL Reagent, 0.2 ml of chloroform has been added. The specimen tubes' tops have been closed. The tubes were shaken by hand for 15 seconds at temperature levels between 15°C and 30°C, and then they were put in an incubator for 2 to 3 minutes. The specimens were spun in a centrifuge between 2 and 8°C for 15 minutes, but not more than 12,000 times. After centrifuging, the solution was split into the white aqueous medium at the top, interphase, as well as a red phenol-chloroform phase at the bottom. Only in the watery phase can you find RNA. About 60% of the volume of the TRIZOL Reagent used for homogenization is made up of the water phase.

The organic layer was kept, while the watery phase was put into a new tube. To get the RNA to stick together, isopropyl alcohol was added to the water phase. During the initial homogenization, 0.5 ml of isopropyl alcohol was added to every 1 ml of TRIZOL reagent. After being kept at 15 to 30°C for 10 minutes, specimens were spun at a maximum of 12,000 rpm for 10 minutes at 2 to 8°C. Before samples were centrifuged, the RNA precipitate formed a gel-like pellet on the side and bottom of the tube, which is often not visible.

The supernatant was removed. For every 1 ml of TRIZOL reagent utilized during the initial homogenization, approximately 1 ml of 75 percent ethanol was added to fully rinse the RNA pellet. The material was centrifuged at 7,500 rpm for 5 minutes between 2 and 8°C while vortexed.

The RNA pellet was dried for a brief period (air-dry for 5-10 minutes). The RNApellet was not allowed to dry fully since this will significantly reduce its solubility. By many times passing the solution through a pipette tip and then incubating it at 55 to 60°C for 10 minutes, RNA was dissolved in RNase-free water.

qRT-PCR

The abovementioned procedure was used to extract total RNA. The CDC gene was amplified using particular primers for qRT-PCR. Gbpolyubiquitin-1 expression was utilized as an internal control to normalize the RNA sample for every qRT-PCR.

Table 2. Concentrations of various reagents for RT-PCR reaction.

Sr. No.	Reagents	Volume (µL)
1	SYBR green super mix	10
2	Forward primer	1
3	Reverse primer	1
4	Template (cDNA)	1
5	U.P water	11
6	Total Volume	26

Table 3. RT-PCR profile.

Sr. No.	Step	Temperature (ºC)	Duration (seconds)	Cycles
1	First denaturation	95	180	1
2	Denaturation	95	15	40
3	Annealing	60	15	
4	Extension	72	45	
5	Final extension	72	180	1
6	Melt curve analysis	94	30	1

In *Gossypium spp.*, understanding the optimal environmental and biological conditions for studying gene expression and silencing is crucial, especially when investigating the role of the *SPY* gene in stress responses. The *SPY* gene plays a significant role in regulating plant responses to gibberellic acid (GA), and its modulation could have profound effects on how plants cope with environmental stresses such as salinity.

Researchers have assessed various factors that impact the effectiveness of gene expression studies in cotton, including temperature, light cycle, light intensity, and the age of the plant. These factors are particularly relevant when exploring how the *SPY* gene contributes to stress tolerance. For instance, studies have shown that temperature has a significant impact on the physiological responses of cotton plants. When temperatures were maintained around 35 to 38 degrees Celsius, optimal conditions were observed for gene expression related to stress tolerance, including the *SPY* gene. However, at temperatures as high as 40 degrees Celsius, plants did not exhibit the expected stress response, indicating that extreme heat may inhibit the normal functioning of stress-related genes.

Light conditions also play a crucial role in plant stress responses. An extended photoperiod of 16 hours of light resulted in more pronounced stress-related responses, such as increased bleaching, compared to a shorter 8-hour light/dark cycle. Additionally, plants exposed to moderate light conditions showed better physiological responses to stress than those placed in either intense light or complete darkness. This suggests that the *SPY* gene, along with other stress-related genes, may be more actively regulated under specific light conditions that mimic natural environmental stresses.

The developmental stage of the plant is another critical factor influencing gene expression. Studies have indicated that younger cotton plants, particularly those between 7- and 10-days post-germination, are more responsive to environmental cues and show stronger stress-related gene expression. This stage of development appears to be the most effective for studying the impact of gene modulation on stress tolerance. In contrast, as plants mature, their responsiveness diminishes, making it harder to observe significant changes in gene expression.

These insights are directly applicable to the study of the *SPY* gene in *Gossypium hirsutum* under salt stress. By understanding the optimal conditions for gene expression, researchers can more effectively explore the role of *SPY* in mediating stress responses. The *SPY* gene is known to negatively regulate GA signaling, which plays a key role in plant growth and stress tolerance. Altering the expression of *SPY* under controlled environmental conditions allows for a deeper understanding of how this gene influences plant resilience, particularly in response to salinity.

Optimizing the environmental and developmental parameters for studying gene expression in cotton is crucial for unraveling the complex roles of genes like *SPY*. This knowledge not only enhances our understanding of plant stress physiology but also provides valuable tools for developing cotton varieties with improved stress tolerance. As climate change continues to pose challenges to agriculture, such insights are essential for ensuring sustainable cotton production.

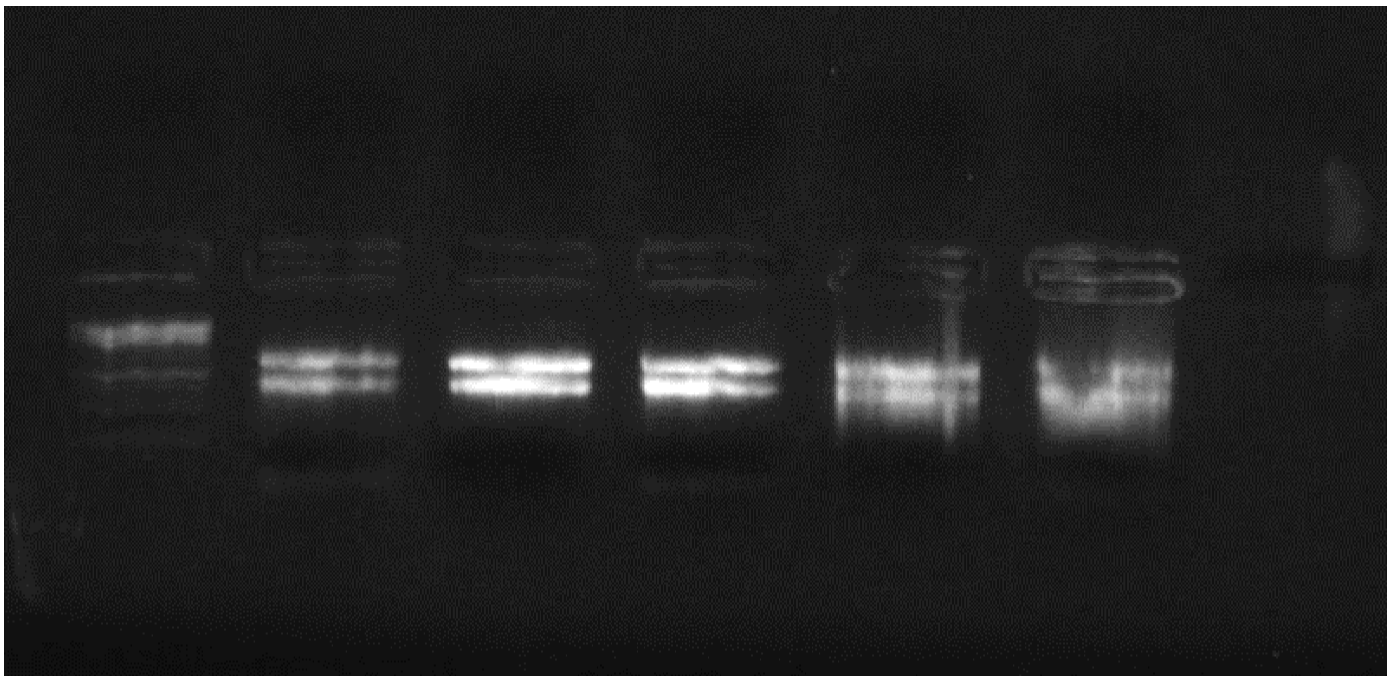

Figure 8. Total RNA run on 2% agarose gel. Lane 1 (starting from left) is showing ladder while Lanes 2-6 are showing RNA

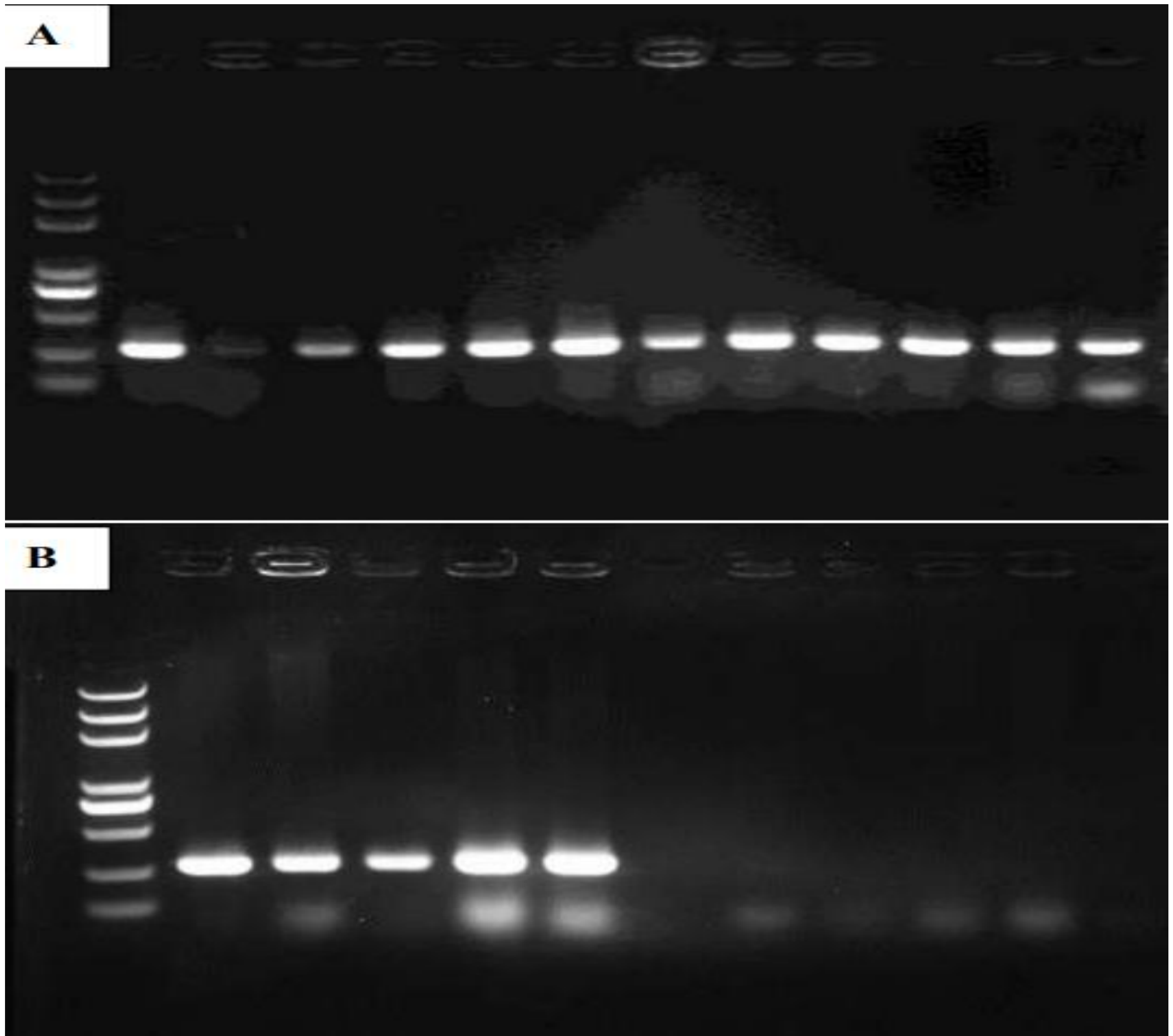

Figure 9. Semi-qPCR results of *SPY* gene expression. A) Showing amplification of internal control (18S rRNA) in all samples. Lane 1 (starting from left) is a ladder. Lanes 7-13 show amplification of the internal control gene. (Starting from left) B) Lane 1 (starting from left) shows a ladder. Lanes 2-6 showing gene expression in the control plants. Lanes 7-13 show no expression of the *SPY* gene in the infiltrated plants.

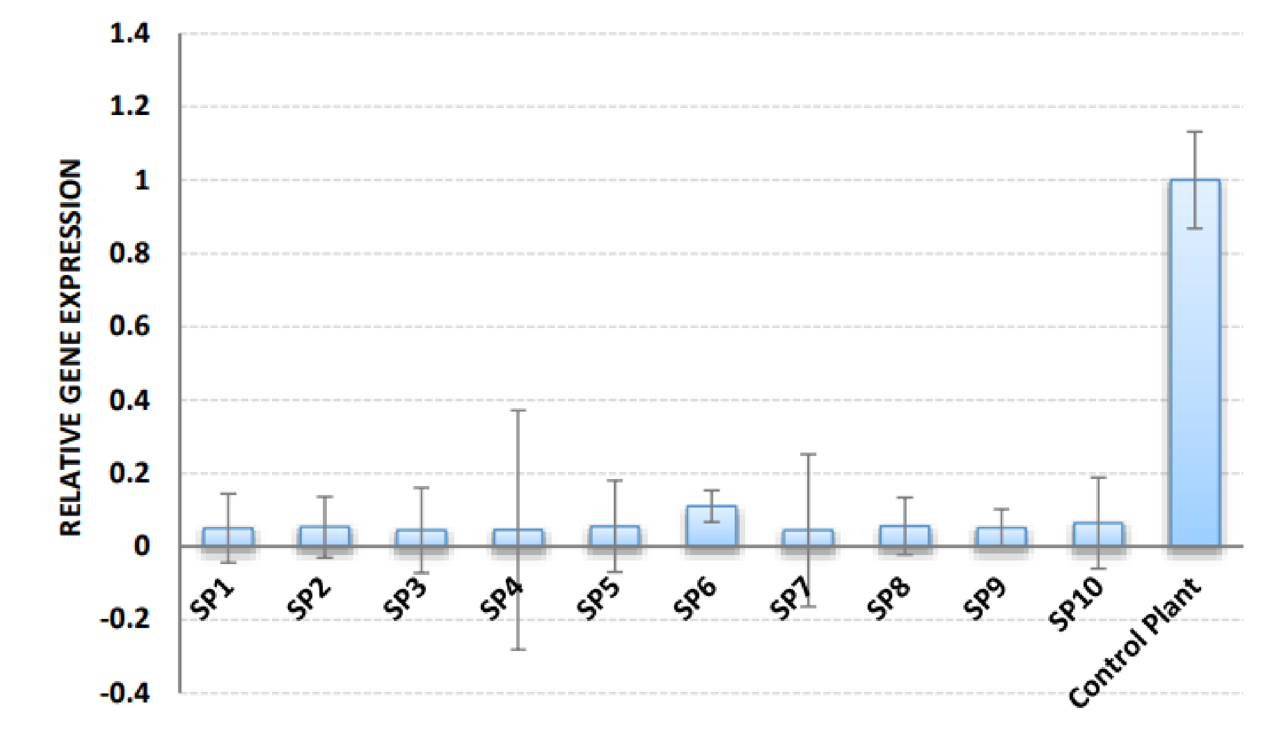

Figure 10. Results of RT-PCR showing gene silencing in infiltrated plants.

DISCUSSION

The *SPY* gene in *Arabidopsis* encodes O-GlcNAc transferases, which play a crucial role as negative regulators of gibberellic acid (GA) signaling. Mutations or deletions in the *SPY* gene lead to enhanced GA responses, indicating that *SPY* acts as a suppressor within the GA signaling pathway. Conversely, loss-of-function alterations in the *SPY* gene result in diminished GA reactions, further emphasizing its role in modulating plant growth and development (Qin et al., 2011). Although *SPY* is well-established as a negative regulator of GA signaling, there is still much to learn about its interaction with GA signaling pathways and the broader functions of plant OGTs (Sarwar et al., 2022). The *SPY* gene is also associated with various other cellular processes, including unusual phyllotaxy, cytokinin (CK) responses, light responses, and altered growth patterns in the hypocotyl and rosette, none of which are directly correlated with GA responses (Bouré et al., 2021).

Recent research has provided insights into how the *SPY* gene might influence plant responses to environmental stress. GA, a critical phytohormone, is known to modulate plant responses to various stressors, including salt, oxidative, and heat stresses, by overcoming growth inhibition through the degradation of DELLA proteins in the nucleus (Altaf et al., 2022). Mutations in DELLA that impair its function increase plant susceptibility to high salinity stress, suggesting a protective role for *SPY* in modifying DELLA's function or stability, potentially through its O-GlcNAc modification activity (Amist et al., 2022). Additionally, *SPY* may act to inhibit GA signaling while simultaneously promoting CK responses, further influencing plant adaptability to stress conditions.

The *SPY* gene's involvement in stress tolerance is further supported by its influence on the expression of key transcription factors and stress-responsive genes. For instance, in Arabidopsis, the *SPY*-3 mutant exhibits up-regulated expression of genes involved in drought response, such as AREB1, which in turn enhances the expression of late embryogenesis abundant (LEA) proteins, contributing to improved stress resilience (Wani et al., 2016). Similarly, the upregulation of CKX3 in *SPY*-3 mutants, both under normal and stressed conditions, underscores the gene's role in modulating CK signaling pathways, which may further contribute to stress tolerance (Burch-Smith et al., 2004). Conversely, overexpression of *SPY* suppresses these stress-related pathways, potentially leading to reduced stress tolerance, as seen in the downregulation of drought-responsive genes like SHN1/WIN1 and DREB1E/DDF1 in *SPY*-OX plants (Peleg et al., 2011).

These findings are particularly relevant in the context of cotton, a crop of significant economic importance that faces challenges due to climate change and its associated stresses. With increasing temperatures and unpredictable rainfall patterns threatening cotton production, enhancing the crop's stress tolerance through genetic engineering becomes

imperative (Lu et al., 2022; Sadaf et al., 2022). Understanding the role of negative regulators like *SPY* is crucial for developing new breeding strategies aimed at improving stress resistance. Genetic engineering techniques, such as CRISPR/Cas9, can be employed to precisely target and modulate genes like *SPY* to enhance cotton's resilience to environmental stresses (Amist et al., 2022).

The *SPY* gene's regulation of GA signaling and its broader role in stress responses provide a valuable target for genetic improvement in cotton. By leveraging functional genomics and modern genetic tools, it is possible to develop cotton varieties with enhanced stress tolerance, contributing to the sustainability of cotton production in the face of climate change.

CONCLUSIONS

This study presents a comprehensive analysis of the *SPY* gene in *Gossypium hirsutum*, focusing on its role in salt stress tolerance. Through a combination of transcriptome analysis, phylogenetic studies, and expression profiling under salinity conditions, the research highlights the dual role of the *SPY* gene in modulating stress responses. Specifically, the study reveals that mutations in the *SPY* gene enhance the expression of stress-responsive genes, thereby improving salt tolerance in cotton, while overexpression of the *SPY* gene leads to reduced survival under stress conditions.

These findings suggest that the *SPY* gene plays a critical role in balancing growth and stress responses in cotton, with potential implications for improving crop resilience through genetic manipulation. The insights gained from this research could inform future strategies aimed at developing salt-tolerant cotton varieties, contributing to more sustainable agricultural practices in salt-affected regions.

Future research should explore the molecular mechanisms underlying *SPY*-mediated stress responses in greater detail, as well as investigate the potential for combining *SPY* gene manipulation with other genetic or agronomic approaches to further enhance stress tolerance in cotton and other crops.

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AUTHOR CONTRIBUTIONS

All authors contributed equally to this research.

COMPETING OF INTEREST

No conflicts of interest have been disclosed by the authors.

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