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Virus-induced gene silencing of *SPY* gene related to drought stress in cotton

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ABSTRACT

Cotton (*Gossypium spp.*) is the most important crop in the world, producing natural fiber for the textile industry as well as a source of feed and fuel. Cotton yield, fiber quality, and stress tolerance have all improved because of genetic engineering and molecular breeding of valuable genes/traits into the crop. Rapid and effective transient techniques to evaluate cotton gene function have been developed to assist high-throughput functional genomics studies in cotton. Virus-mediated gene silencing is an efficient approach to inhibit viral growth, which relies on host post-transcriptional gene silencing (PTGS). Tobacco rattle virus (TRV) is one of multiple viral VIGS vectors, infects a broad range of hosts, and can propagate widely throughout the plant while causing very mild disease within the host. The SPINDLY (*SPY*) gene encodes an O-linked N-acetylglucosamine transferase and is a known negative regulator of drought tolerance in Arabidopsis thaliana. The objective of the study was to use VIGS for *SPY* gene silencing. Drought stress triggers the expression of stress-responsive genes. *SPY* homolog in cotton was designed and cloned, and put into the binary vector pYL156 (TRV RNA2) for VIGS-mediated gene silencing. The agroinfiltration method is used to introduce VIGS vectors into plant leaves. The gene expression was quantified through qPCR in both stressed and normal plants. Low gene expression observed from 7-14 days post-infiltration (dpi). The study could help in the functional analysis of the *SPY* gene and explore its role in drought responsiveness in upland cotton that can be further exploited to develop drought-tolerant varieties.

Keywords: Tobacco rattle virus (TRV), *SPINDLY (SPY)* gene, Virus-mediated gene silencing, and Drought stress.

INTRODUCTION: Pakistan relies on cotton for its economic success. Cotton and its products account for 0.8% of GDP and 60% of foreign currency revenues. About 40% of the industry is involved in cotton. Pakistan produces cotton in Punjab and Sindh. Punjab produces 70% (Shi *et al.*, 2022). Pakistan can produce 30 million bales from 10 million acres. Pakistan's cotton output has remained around 10 million bales since 2011, significantly below its potential. Since 2015, cotton output has declined to 5.6 million bales, the lowest in 35 years (Li et al., 2022). Once the Cotton Research Institute, Multan, and other Punjab Province research institutions developed extremely heat-tolerant cultivars (MNH-1020 and FH-Super cotton) for commercial production. The bollworm- and glyphosate-resistant types will be available for cultivation in two years. Pakistan's cotton industry has several prospects. This paper should help Pakistan and Punjab restore their cotton crops (Manchur *et al*., 2022). Climate change endangers agriculture and the environment. Extreme weather due to climate change reduces agricultural yield. Previous research suggested that air temperature had climbed by 0.74°C and would continue to rise by 1.8°C–4.0°C by the end of the century (van Rees *et al*., 2022). Human-produced greenhouse gases cause this temperature to rise. Most cotton-growing areas lack rainfall and evapotranspiration forecasts. Temperature and rainfall fluctuations reduce crop duration and increase insect populations, mineralization, and evapotranspiration (Dong *et al*., 2022).

The cotton output will drop by 40%–50%. Additionally, higher carbon dioxide levels boost photosynthesis, biomass production, and cotton output. Cotton output increases with carbon dioxide but decreases with warmth (Samantara *et al*., 2022). Therefore, adaptive techniques are required to lessen the effect of climate change. Climate change may be mitigated by developing cultivars that withstand biotic and abiotic stress and react to increasing carbon dioxide and temperature (Biswas *et al*., 2021). Output technology changes enlighten producers, academics, and politicians and will help maintain cotton production in a changing environment. Plant growth and development are hampered by drought stress, which alters metabolic processes. Drought stress affects photosynthesis and the amount of photosynthetic available, which causes square and boll shed as well as reduced lint production (Anu *et al*., 2021). Numerous physiological processes are impacted by drought, including photosynthesis, stomatal control, and decreased root-shoot development, decreased leaf area expansion, decreased transpiration, and osmoregulation. Effects of drought stress are mediated through plants' physiological, molecular, and biochemical processes for responding to stress as well as stress detection and communication (Yue *et al*., 2022). Drought stress increases the formation of reactive oxygen species, Ca2+, mitogen-activated protein kinase, and hormone-mediated signaling at the cellular level.

The stimulation of abscisic acid-dependent and independent stress signaling pathways in cotton is caused by transcription factors activated by drought. Apart from breeding drought-tolerant cultivars, other methods for enhancing tolerance against drought stressors include the use of primary and secondary nutrients, osmoprotectants, plant growth regulators (PGRs), and plant growth-promoting rhizobacteria (Chen *et al*., 2022). New cotton varieties with longer fibers, better yields, and alleles/genes that are more resistant to biotic and abiotic challenges have been developed because of recent developments in cotton genomics and genetics. The process of creating engineered viruses with sequences matching to the host gene that must be silenced is known as virus-induced gene silencing, or VIGS (Mácová *et al*., 2021). In functional genomics, VIGS is a potent technique for quick and extensive gene analysis. It has been used to several significant plants, including Arabidopsis thaliana, tomato, wheat, and numerous species of legumes (Senthil-Kumar *et al*., 2011). The foundation of VIGS is RNA interference (RNAi), a method of gene silencing those silences target genes by acting on sequences. The enzyme DICER breaks down double-stranded (ds) RNA into small interfering RNAs (siRNAs) of 21–25 nucleotides in the RNAi pathway (Bhattacharjee *et al*., 2022). The guide and passenger strands of the siRNA are separated. The passenger strand is damaged while the guide strand is integrated into the RNA-induced silencing complex (RISC) to degrade the single-stranded RNA complementary to that of the guide RNA (Killiny *et al*., 2022).

Both the Tobacco rattle virus (TRV) and Cotton leaf crumple virus (CLCrV) are *begomoviruses,* and both mechanisms cause the target gene to be silenced while increasing the production of *Gossypium spp* (Singh *et al*., 2022). TRV is a member of the *Tobravirus* genus (family *Virgaviridae*). The positive sense single-stranded RNA genome of the virus is made up of two parts, RNA 1 and RNA 2. Genes for viral replication and movement are encoded by RNA 1, while the coat protein and several structural proteins that may be deleted to accommodate foreign sequences are encoded by RNA 2 (McPherson *et al*., 2021).By silencing the *chloroplastos alterados 1* (*SPY*) gene in *G. hirsutum* and *G .barbadense,* the TRV vector has shown its value. The chloroplast development gene *SPY*, whose silencing results in a bleached phenotype, may be used as a helpful marker to assess silencing (Mahfuz *et al*., 2022). The *KATANIN* and *WRINKLED* genes' roles in the production of fiber in *G. hirsutum* have since been studied using the TRV vector. By silenceing the *SPY* gene, the work described here has expanded the TRV VIGS system to include *G. arboreum* and *G. herbaceum*(Liu *et al*.,2002). Furthermore, it has been demonstrated that not all G. hirsutum types respond identically to TRV-mediated silencing, which is an important consideration for future gene function research (Pasin *et al*., 2021).

The *SPINDLY* (*SPY*) gene was first discovered as a negative regulator of plant gibberellic acid (GA) signaling because a mutation of this gene mimics plants that have received an excessive amount of bioactive GA and makes them resistant to a GA inhibitor during seed germination (Yu *et al*., 2022). The O-linked N-acetylglucosamine transferase that the *SPY* gene produces may alter the target protein and control the function of the protein in cells. Along with its GA-related characteristics, Arabidopsis (Arabidopsis thaliana) *SPY*-3 mutants have significant salt and drought resistance traits. It was discovered that salt stress and drought stress both modestly altered *SPY* gene expression (Abdullah *et al*., 2022). Numerous GA-

responsive genes were found to be up-regulated in *SPY*-3 after transcriptome analysis, which may help to explain *SPY*-3's phenotype of GA overdose (Taliansky *et al*., 2021).

Some stress-inducible genes, including those producing late embryogenesis abundant proteins, Responsive to Dehydration20, and AREB1-like transcription factor, were discovered to be up-regulated in *SPY*-3 (Tuo *et al*., 2021). These genes may provide *SPY*-3 with a higher level of stress tolerance. In *SPY*-3, the gene for CKX3, which is involved in cytokinin (CK) catabolism, was upregulated. This upregulation suggests that the mutant has decreased CK signaling, which is consistent with a beneficial function for *SPY* in CK signaling (Tiedge *et al*., 2022). Additionally, contrary to *SPY*'s phenotype, transgenic (*SPY*-OX) plants with the SPINDLY (*SPY*) gene overexpressed had decreased plant tolerance to drought stress (Fareed *et al*., 2022). Several genes, including *DREB1E/DDF1* and *SNH1/WIN1*, have reduced expression in *SPY*-OX but elevated expression in *SPY*-3 (Jiang *et al*., 2022). When combined, these studies show that *SPY* has a deleterious impact on a plant's ability to withstand abiotic stress, most likely through integrating environmental stress signals via GA and crosstalk (Feng *et al*., 2022).

OBJECTIVES: (1) *In silico* characterization of *SPINDLY (SPY)* gene. (2) Isolation and cloning of *SPINDLY (SPY)* gene in VIGS vectors.(3) Transient expression of the VIGS vectors in cotton.(4) Molecular analysis of *SPY* -silenced cotton plants.

MATERIALS AND METHOD: The fundamental structural and operational component of heredity is a gene. DNA is a component of genes. Because of the complexity of the gene's sequence and the fact that it is not just a collection of codons, it is essential to break the gene to comprehend its structure and function before using TRV-based VIGS to alter it. Exons and introns are the two components of the gene (Li *et al*., 2022). Exons are conserved DNA and RNA nucleotide sequences that are necessary to produce mature RNA. Transcription is the process of creating mRNA by utilizing DNA as a template. To create proteins, mRNA works with ribosomes and transfer RNA (tRNA), both of which are in the cytoplasm, during a process known as translation. Exons typically include the 5' and 3' untranslated sections of mRNA, which include start and stop codons, in addition to every protein-coding sequence (Chen *et al*., 2022).

Retrieval of SPY nucleotide sequence from cotton FGD: The very first step in the construction of a vector is to obtain its sequence from the database. The most used and efficient database is the Cotton Functional Genomics Database (Cotton FGD). The complete coding sequence of the gene was retrieved from Cotton FGD using descriptive words to search for the *SPY* gene present in cotton. *SPY* is a member of the protein superfamily that regulates drought stress negatively (Lee *et al*., 2022).

Domain analysis of SPY protein: For the selection of the target site, domain analysis of the protein was done using the Pfam tool. The mRNA sequence was retrieved to select the specific target site. The protein sequence was used to find the protein domain to specify the target (Rhee *et al*., 2022).

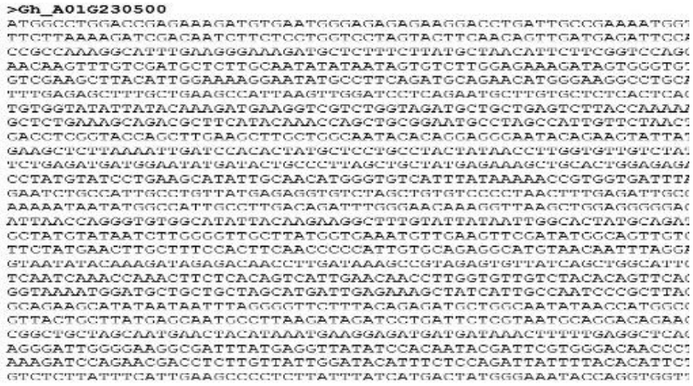
TPR-mediated protein-protein interactions of SPY: Glycosyltransferase family 41 protein (domain architecture ID 11420499) containing tetratricopeptide (TPR) repeats, similar to Oryza sativa probable UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase *SPINDLY*, an O-linked N-acetylglucosamine transferase (OGT) that catalyzes the addition of nucleotide-activated sugars directly onto the polypeptide through O-glycosidic linkage with the hydroxyl of serine or threonine (Vikas *et al*., 2022).

Expression vector (TRV) sequence retrieval: Vector was required to deliver the VIGS construct into the target organism. The vector was designed using Snap Gene V. 6.1.

Structure analysis of gene: A gene is the basic physical and functional unit of heredity. Genes are made up of DNA. Because the gene is not simply a bunch of codons and it has complexities in its sequence, so, when we want to mutate any gene through VIGS, it's compulsory to break down the gene and understand its function and structure by analyzing its sequence. The gene has two parts, the exons, and the introns (Verma *et al*., 2022). Exons are conserved nucleotide sequences in DNA and RNA that are used to make mature RNA. The process of using DNA as a template to make mRNA is known as transcription. In a process known as translation, mRNA collaborates with ribosomes and transfer RNA (tRNA), both of which are found in the cytoplasm, to produce proteins. In addition to any protein coding sequences, exons normally include both the 5' and 3' untranslated regions of mRNA, which contain start and stop codons (Bhinda *et al*., 2022). Introns are nucleotide regions in DNA and RNA that do not code for proteins and are deleted by RNA splicing during the precursor messenger RNA (pre-mRNA) stage of mRNA maturation. Introns are found in a wide variety of genes that create RNA in most living species, including viruses, and can range in size from 10 to 1000 base pairs (Zhu *et al*., 2022).

RESULTS AND DISCUSSION: A gene is the basic physical and functional unit of heredity. Genes are made up of DNA. Because the gene is not simply a bunch of codons and it has complexities in its sequence, so, when we want to mutate any gene through VIGS, it's compulsory to break down the gene and understand its function and structure by analyzing its sequence. The gene has two parts, the exons, and the introns (Verma *et al*., 2022). Exons are conserved nucleotide sequences in DNA and RNA that are used to make mature RNA. The process of using DNA as a template to make mRNA is known as transcription. In a process known as translation, mRNA collaborates with ribosomes and transfer RNA (tRNA), both of which are found in the cytoplasm, to produce proteins. In addition to any protein coding sequences, exons normally include both the 5' and 3' untranslated regions of mRNA, which contain start and stop codons (Bhinda *et al*., 2022). Introns are nucleotide regions in DNA and RNA that do not code for proteins and are deleted by RNA splicing during the precursor messenger RNA (pre-mRNA) stage of mRNA maturation. Introns are found in a wide variety of genes that create RNA in most living species, including viruses, and can range in size from 10 to 1000 base pairs (Zhu *et al*., 2022).

Retrieval of SPY nucleotide sequence from Cotton FGD: The nucleotide sequence for SPY was retrieved from cotton FGD. The sequence was 2.7kb long. Other features have been provided. Whereas a complete CDS of the SPY gene has been provided in figure 1.



Complete cds of *Gossypium hirsutum* cultivar spindly gene.

Protein-protein interactions of SPY: SPY -Probable UDP-N-acetylglucosamine—peptide N-acetylglucosaminyltransferase SPINDLY; Probable O-linked N-acetylglucosamine transferase (OGT) involved in various processes such as gibberellin (GA) signaling pathway and circadian clock. OGTs catalyze the addition of nucleotide-activated sugars directly onto the polypeptide through O-glycosidic linkage with the hydroxyl of serine or threonine (Gautam *et al*., 2022). Probably acts by adding O-linked sugars to yet unknown proteins. Acts as a repressor of GA signaling pathway to inhibit hypocotyl elongation. Functions with GIGANTEA (GI) in pathways controlling flowe (Lin *et al*., 2022). Multiple-TPR motif proteins would fold into a right-handed super-helical structure with a continuous helical groove suitable for the recognition of target proteins, hence defining a novel mechanism for protein recognition. The spatial arrangement of α-helices in the PP5–TPR domain is like those within 14-3-3 proteins.14-3-3 proteins are a family of conserved regulatory molecules expressed in all eukaryotic cells (Kumar et al., 2022) (figure 2).

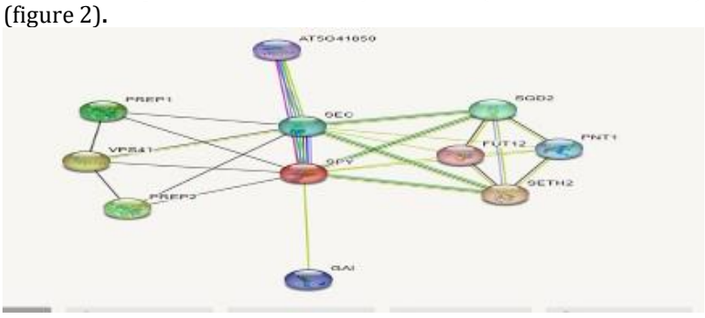


Figure 2: Multiple-TPR motif proteins would fold into a right-handed super-helical structure.

Structure analysis of SPY gene: It's critical to delete the introns accurately, as any leftover intron nucleotides or exon nucleotide

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deletion could result in a defective protein being created (Muoz - López *et al*., 2002). This is because codons, which are made up of three nucleotides, are used to link the amino acids that make up proteins. As a result, an incorrect intron removal could cause a frameshift, causing the genetic code to be read wrongly (Xie *et al*., 2022) (figure 3).

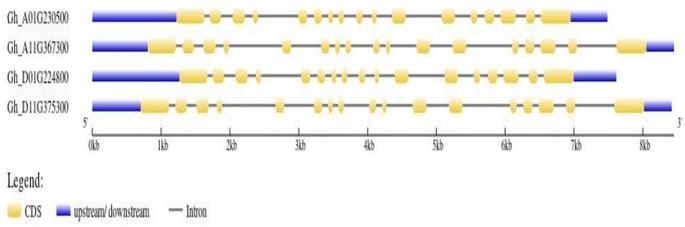

Figure 3: The structural analysis of the *SPY* gene was done through gene structure display server 2.0. To understand the gene structure, it's necessary to understand the mRNA sequence. The different elementsare highlighted through the different colors. Like yellow color indicates the CDS region, and upstream and downstream introns are represented by blue color

Expression vector designing using snap gene: The vector designed using the Snap Gene V. 6.1 was 16437 bp in size (figure 4).

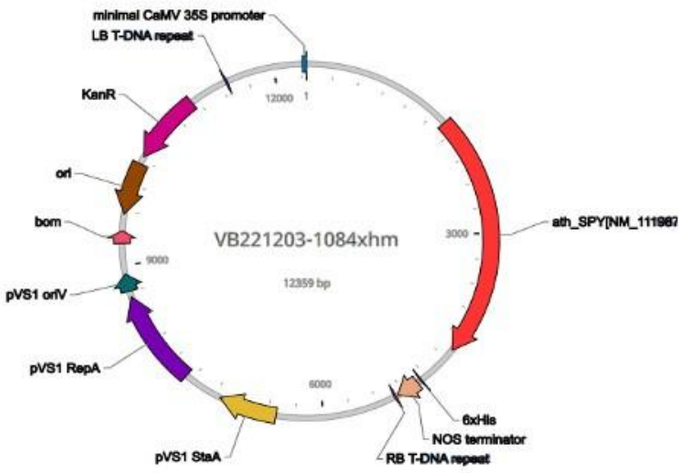

Figure 4: Expression vector pTRV2-ath_*SPY* [NM_111987.4]: The pTRV2-ath_*SPY* [NM_111987.4] is a Plant binary vector that is 12359 bp in size. It has the ability to carry *SPY* gene to target the specific sequence(37). It is synthetic in nature and has kanamycin resistance with neomycin selectable marker and AtU6-26 promoter. This plant binary vector contains the AtU6-26 promoter.

Expression analysis: The SPY gene was expressed throughout the plant and can be detected not only in all organs where the phenotypes of SPY mutants have been observed but also in the roots, indicating a role for the gene in root development. The SPY protein is predominantly localized to the nucleus, where it modifies components of the GA signaling pathway. Cytosolic SPY activity was reported to promote cytokinin (CK) responses and to repress GA signaling. The SPY protein consists of 915 amino acids, including multiple tetratricopeptide repeats (TPRs) at the N terminus and a Ser and Thr O-linked GlcNAc (O-GlcNAc) transferase (OGT) domain at the C terminus. Each TPR motif consists of a highly degenerate sequence of 34 amino acids with eight loosely conserved residues. The TPR motifs and OGT domain are crucial for mediating protein-protein interactions and facilitating correct assembly, respectively, to enable enzyme activity.

Like animal OGTs, in vitro-expressed SPY protein possesses OGT activity. OGTs transfer a GlcNAc monosaccharide to the O-linkage of Ser or Thr of cytosolic and nuclear proteins. In animals, more than 1,000 O-GlcNAc-modified proteins have been identified, and this kind of protein modification is believed to regulate many basic cellular and disease processes. In some cases, O-GlcNAc modification and phosphorylation occur at the same site on the substrate protein, leading to the hypothesis that these two modification processes compete to fine-tune the activity of the substrate protein under different circumstances. In Arabidopsis, there are only two genes encoding O-GlcNAc transferases, SPY.

Preparation of Plant Material: Before getting rinsed in deionized, cotton seeds were disinfected with 3% H2O2 for 16 hours. Seeds were put on moist filter paper for two days at 28°C to encourage germination. Seedlings were put in soil-filled containers and raised at a temperature of 30°C 2. Pots on a tray were put in a growing chamber with a 12-hour light/12-hour dark photoperiod, a polyethylene dome covering, and 120 E m-2 S-1 light at 23 °C. After two cotyledons appeared, the dome was therefore taken off. The plants were employed for the VIGS experiment with two completely grown cotyledons around two weeks later. The real leaves had not yet developed at this point.

VIGS-vector: Both the creation of a recombinant TRV vector for the silencing of *SPY* and the creation of a TRV-based VIGS vector suited for Agrobacterium-mediated inoculation have been publicly reported. Agrobacterium tumefaciens strain GV3101 was electroporated with binary plasmids carrying the TRV RNA 1 (pTRV-RNA1), TRV RNA 2 (pYL156), and the transgenic RNA 2 including *SPY* sequences (pYL156) *GhSPY*, designated here as TRV *GhSPY* for clarification. The process of infiltrating cotton and preparing bacterial cultures for injection.

Construct of VIGS vector carrying the GhSPY gene: Both the creation of a recombinant TRV vector for the suppression of *SPY* and the creation of a TRV-based VIGS vector suited for Agrobacterium-mediated inoculation has indeed been previously described. Binary plasmids harboring TRV RNA 1 (pTRV-RNA1), TRV RNA 2 (pYL156), and the recombinant RNA 2 containing *SPY* sequences (pYL156) (*SPY*, referred to here as TRV *SPY* for clarity) were electroporated into Agrobacterium tumefaciens strain GV3101 and selected on plates containing kanamycin (50 lg/mL) and rifampicin (12.5 lg/mL). The process of infiltrating cotton and preparing bacterial cultures for injection.

GhSPY gene amplification: The *SPY* gene of cotton (*GhSPY*) was Amplified by PCR with primers *GhSPY-F*, 5'-GCCCTTTGTGCATCTTC-3' and *GhSPY-R*, 5' CTCTAGGGGCATT GAAG-3' from a cDNA library of *TM1* leaf tissues.

Digestion and cloning of *GhSPY*: The PCR products of *GhSPY* were digested with *EcoRI* and *KpnI* and inserted into pYL156 (pTRV-RNA2) vector by ligation.

Transformation into Agrobacterium: The plasmid was electroporated into Agrobacterium tumefaciens GV3101, and the bacteria were allowed to recover in LB liquid medium at 28°C. The transformants were selected on LB plates with Gentamycin (25 µg/mL) and Kanamycin (50 µg/mL). For long-term use, the bacteria can be stored in glycerol containing 25% and kept at –80°C.

***VIGS* Inoculation:** The Agrobacterium tumefaciens strains pYL192 (TRV): RNA1, pYL156 (TRV): RNA2 (vector alone), and pYL156 (TRV): RNA2-Gh*SPY* were streaked on LB agar plates containing 50 g/mL of Kanamycin and 25 g/mL of Gentamycin three days before inoculation. For 24 hours, the plates were incubated at 28 °C. A single colony from each of the preceding plates was taken two days before VIGS induction, and it was inoculated into 5 mL of LB medium containing 50 µg/mL of Kanamycin and 25 µg/mL of Gentamycin. The bacterial culture was then grown overnight at 28 °C in a roller drum at 50 rpm. The culture was transferred into a flask, along with 10 mM MES (2-(4-morpholino) ethanesulfonic acid) and 20 mM acetosyringone, in 50 mL of LB medium supplemented with 50 µg/mL of Kanamycin and 25 µg/mL of Gentamycin. The culture was cultivated overnight in a shaker at 50 rpm while maintaining a temperature of 28 °C. The next day, the agro-bacterial cells were spun down at 4000 rpm for 5 minutes. Then the culture was re-suspended in an infiltration buffer containing 200 µM acetosyringone, 10 mM MgCl2, and 10 mM MES. The culture's OD 600 was changed to 1.5. The culture was left on the bench at room temperature for 3 hours. Before Agrobacterial invasion, the cotton plants' cotyledons were pinched with a 25-gauge needle without piercing them. On each cotyledon portion, one or two holes were drilled. A 1:1 ratio of the agrobacterial culture suspension of pYL192 (TRV): RNA1 and pYL156 (TRV): RNA2 or pYL156 (TRV): RNA2-Gh*SPY* was combined, and the mixture was manually injected into the wounding sites from the underside of cotyledons.

The plants were penetrated, covered with a plastic dome, and left at room temperature with low lighting overnight. The plants were transferred to a growth chamber with a temperature of 23°C, a light intensity of 120 µmol m-2 s-1, and a 12-hour cycle of light and dark. Seven to eight days after infiltration, the silencing phenotype was assessed. Plants silenced by pYL156 (TRV): RNA2-Gh*SPY* began to exhibit the albino phenotype in their true leaves. As a control, plants infected with Agrobacteria expressing pYL156 (TRV): RNA2 were used. RNA extracted from control and silenced cotton plants was used to verify the effectiveness of gene silencing by measuring the

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expression levels of endogenous genes using reverse transcription polymerase chain reaction (RT-PCR) (figure 5).

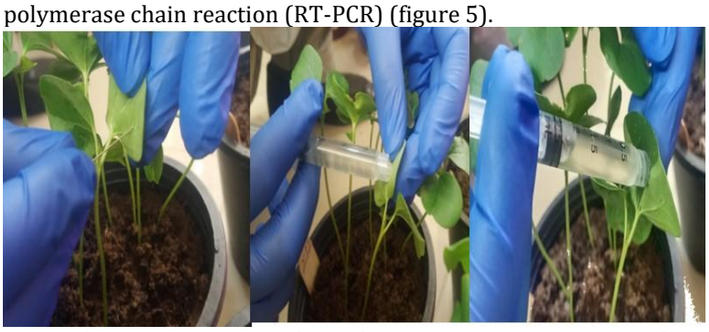

Figure 5: TRV2 vector inoculates the *SPY* gene in cotton to produce drought stress tolerance. The efficiency of gene silencing was evaluated by measuring the levels of expression of endogenous genes using reverse transcription polymerase chain reaction (RT-PCR) with RNA taken from control and silenced cotton plants. This was done to determine whether gene silence was successful.

Optimization of Conditions for TRV-based VIGS in Cotton: To determine the impact of various photoperiods on photobleaching brought on by *SPY* gene silencing, the agro-infiltrated plants were cultivated under long photoperiods of 16/8 and short photoperiods of 8/16 h day/night. Using the same experiment as previously, different light intensities of 100, 300, and 500mol/ (m2 s) were assessed (Zhou *et al*., 2023). To ascertain the *SPY* gene's effectiveness in silencing at various stages (7, 10, 15, 20, and 30 days), plant leaves underwent testing. Plants were injected with four different amounts of agrobacterium (cotyledon stage) to examine the impact on photobleaching (OD600 value 0.5, 1.0, 1.5, and 2). The photobleaching impact of the *SPY* gene was examined at various temperature ranges (25–40 C) (Ui-Tei *et al*., 2008). In these studies, which were carried out three times, a group of twenty plants came together. The proportion of gene silencing was calculated using ImageJ (http://imagej.net). Three leaves from each plant were tested to see how photobleaching affected them and how they compared them to control leaves (Lopez-Gomollon *et al*., 2022).

RNA Extraction: RNA was extracted from leaf tissue as specified using Trizol (Invitrogen). Using Nanodrop 2000, the concentration of RNA was measured (Thermo Scientific, USA). Revert Aid first strand cDNA synthesis kit (Thermo Scientific, USA) was used to transcribe purified RNA (2 lg) in accordance with the manufacturer's instructions using oligod(T) primers.To completely dissociate nucleoprotein complexes, homogenized materials were treated for 5 minutes at 15 to 30°C. Each 1 mL of TRIZOL Reagent was mixed with 0.2 mL of chloroform. Closed sample tubes. After aggressively shaking the tubes by hand for 15 seconds at 15°C–30°C, they were incubated for 2–3 minutes. The samples were centrifuged 12,000 times for 15 minutes at 2–8°C. The mixture was split into an upper, white aqueous phase, an interphase, and even a lower, red, phenol-chloroform phase following centrifugation. Only aqueous RNA exists. 60% of the homogenization TRIZOL Reagent is aqueous. The organic phase was kept, and the aqueous phase was shifted to a new tube kept, The aqueous phase and isopropyl alcohol precipitated RNA. The initial homogenization used 0.5 mL of isopropyl alcohol per 1 mL of TRIzol. Samples were centrifuged at a maximum of 12,000 rpm at 2 to 8°C for 10 minutes after being incubated for 10 minutes at 15 to 30°C. The RNA precipitate forms a gel-like pellet on the tube's side and bottom before centrifugation and is sometimes undetectable. Collecting supernatant. For every 1 mL of TRIzol reagent used for the initial homogenization, 1 mL of 75% ethanol was added to wash the RNA pellet. Vortexing the mixture, the sample was centrifuged at 7,500 rpm for 5 minutes at 2 to 8°C.RNA pellets were briefly dried (air-dry for 5-10 minutes). Since drying reduces solubility, the RNA pellet was not entirely dried. RNA was dissolved in RNase-free water by repeatedly pipetting the solution and incubating it at 55 to 60°C for 10 minutes.

The PCR (qRT-PCR): The procedure extracted total RNA. *SPY* gene primers were used for quantitative real-time PCR (qRT-PCR). The internal control for each qRT-PCR was Gbpolyubiquitin-1 expression. Optimizing TRV VIGS Conditions in Gossypium spp. To maximize TRV-based VIGS of the *SPY* gene via Agrobacterium infiltration, temperature, photoperiod, light intensity, plant age at inoculation, and inoculum concentration were evaluated. As a negative control, equal numbers of plants were infiltrated with TRV Gh*SPY* and the empty vector (TRV RNA1: TRV RNA2). Control plants showed no symptoms after 10–15 days in all studies. Compared to 35–38°C plants, which bleached 20–30%, 25–30°C plants silenced 90–95%. No cotton species bleached at 40°C.TRV VIGS in all three species demonstrated maximum bleaching (up to 99%) during an extended photoperiod (16 h light) compared to a short one (8 h light; 30–40% silencing). Plants were placed in dark, bright, and low-light conditions to study the effects of light intensity. In contrast, plants in shadow or low light bleached (Nakano *et al*., 2022).Bacterial culture concentration affected gene silencing. Compared to plants injected with OD 1.5 cultures, all species inoculated with 0.5–1 culture bleached 10–30% less.

However, plants inoculated with 2.0 OD and higher developed necrosis at the inoculation site, resulting in withering and death. To determine how the plant development stage affects bleaching effectiveness, plants were infected at 7 days after germination (with fully grown cotyledons), 10–15 days (first true leaves), 20 days (two true leaves), and maturity (30 days after germination). Bleaching was more effective for inoculating plants 7–10 days after germination compared to two genuine leaf plants (20 days). No bleaching was detected when mature plants were injected (Kernodle *et al*., 2022). Cotton plants showed complete bleaching within 30 days as evident from the complete photobleaching in the upper, newly developing leaves. For all plants, bleaching was only evident in tissues developing at the time of, or after, inoculation (Zhao *et al*., 2022). These results together show that the VIGS is a powerful tool for cotton gene suppression. The ploidy level may also have an impact on its efficacy; for example, TRV-based VIGS was much more effective in the diploid *G. arboreum* and *G. herbaceum* than in *G. hirsutum* (figure 6).

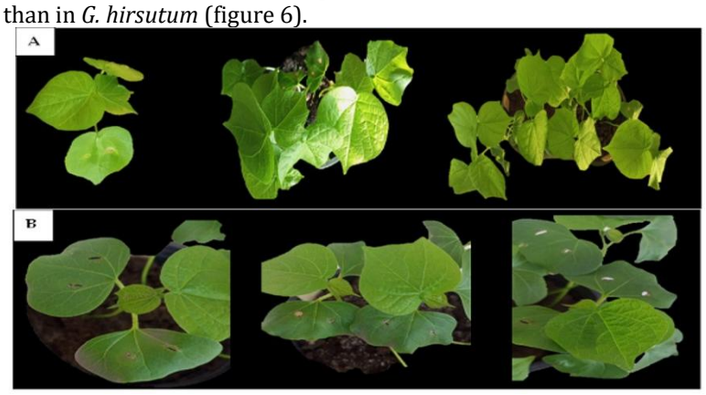

Figure 6: VIGS-based Silencing of the SPY Gene in cotton. A) at 7 dpi. B) at 14 dpi.

VIGS-based silencing of the *SPY* gene in *Gossypium* species: All cotton plants were treated with injections seven to ten days after germination in line with the results of the optimization experiments. TRV *GhSPY* was found to be present in ten cotton plants. Three times each trial was carried out. After exposure to TRV *GhSPY*, various cultivars of *G. hirsutum* showed varying degrees of bleaching. Gene suppression was determined by semi-qPCR and RT-PCR, the results are given in figure 7.

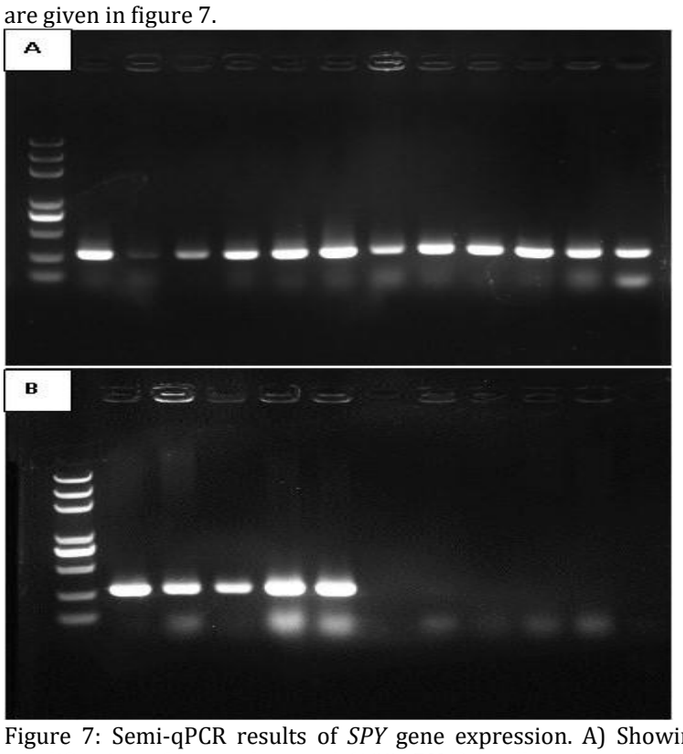

Figure 7: Semi-qPCR results of *SPY* gene expression. A) Showing amplification of internal control (18S rRNA) in all samples. Lane 1 (starting from left) is ladder. Lane 7-13 showing amplification of

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internal control gene. (Starting from left) B) Lane 1 (starting from left) shows ladder. Lane 2-6 showing gene expression in the control plants. Lane 7-13 showing no expression of *SPY* gene in the infiltrated plants.

Some plant species have limited ways to regenerate, which keeps them from changing in a stable way. Cotton is one of the plants that are least able to adapt and survive. Furthermore, its large genome size and long growth cycle add to the complexity of this species (Berry *et al*., 2022). Somatic embryogenesis had also been demonstrated as a universal way to change, but the rate of change in cotton is not very high. Still, two cotton varieties, Coker 5110 and Coker 312, showed a significant difference even though embryogenesis is constrained and takes 8–12 months (Manu *et al*., 2022).For this reason, gene function research in cotton is especially interested in finding a quick way to change things temporarily. Using the TRV-based VIGS technology made here, it may be possible to turn off any gene in cotton within 30 days (Ligot *et al*., 2022). In this research, the TRV-based VIGS was set up to work with *G. arboreum*, *G. herbaceum*, and *G. hirsutum*. Depending on the species or variety, the *SPY* gene could either turn off the gene completely or partially. PCR and qPCR tests on TRV-*GhSPY* -infected plants showed lower levelsof *SPY* transcripts, which could mean that genes are being turned off.Previous research showed that the best conditions for silencing were long photoperiods and cold temperatures. Also, TRV-based silencing worked better in diploid species (*G. arboreum*, *G. herbaceum*) than in tetraploid species (*G. tetraploides*) (*G. hirsutum*) (Zheng *et al*., 2022).

In a previous study, *G. hirsutum* was treated with RNAi technology. To stop the d-cadinine synthase gene from working so that less gossypol is made in cottonseed tissue. The d cadinene synthase RNAi cassette was run by the seed-specific b globulin promoter. As a result, the amount of gossypol in cottonseed dropped by a lot. Systemic silencing was shown to only happen in seeds (Kumar *et al*., 2022). Even though gossypol was found in leaf and flower parts, the amount of gossypol in these tissues did not change. The main reason for this was that siRNA (the "silencers") didn't get from the seeds to other parts of the plant well enough.The limited spread of TRV or, more likely, the insufficient delivery of the suppressing signal could be to blame for the uncertainty in this study's results. The whole genome sequences of *G. raimondii*, *G. arboretum*, and *G. hirsutum* are now available to the public (Vig *et al*., 2022). Combining genome sequencing with TRV VIGS is a powerful way to figure out how the genes in these plant species work. So, the TRV-based VIGS method that has been described could help researchers learn more about how cotton works (Wang *et al*., 2022). To use the TRV VIGS system to find out how cotton and the viruses that cause cotton leaf curl disease, which is the biggest problem for growing cotton in Pakistan, can develop a long-lasting, all-around resistance to these diseases. Originally, the rates of plant survival in various soil containers have been assessed.Most Col plants died when the water supply was turned off for two weeks, but almost all *SPY*-3 mutants survived.

The differences in transpiration and soil water content across pots may be explained by the fact that the rosette leaves of the 4-week-old mutant plants were smaller than those of the wild-type plants (Yadav *et al*., 2022). After depriving the plants of water, we worked to dry them out equally by making sure that the soil moisture level in each soil pot fell to the same level. Four-week-old plants growing in numerous pots had their water supply turned off. The soil's moisture content was monitored every day for 12 days after that. Less water was present in the soil that was home to wild-type plants than in the soil that was home to *SPY* -3 plants, which may indicate Positive impact of NEFR integration was also visible on per acre yield compared to yields of previous year and Non-NEFR cotton fields (current season). Although, a significant increased level of productivity was not achieved in first season but, consistent production without application of pesticides reduced input costs that can be added as profit/productivity. Through NEFRs, not only conservation of the parasitoids was done, but also destruction of millions of males and females of *P. gossypiella* was an additional advantage that probably could not be achieved by use of pesticides in field. Moreover, consistent production without application of traditional control tactics like chemical control, is an achievement of NEFR Technology, that not only successfully replaced all previous practices to suppress population of pink bollworms within a short period of time, but also demonstrated as an environment friendly, economical, and sustainable pest control mechanism which could be that the wild-type plants used more water or transpired more. Watering was resumed on day 16 when the overall water content in the pots housing wild-type plants had dropped to around 10% of the beginning level. When extremely wilted plants were restored, around 28% did. After the soil water content in these pots had decreased to the same level as in the pots of the control plants after 19 days of dehydration, watering was restarted for the *SPY* -3 plants. A wildcard 88.0% of *SPY* -3 plants survived despite being dehydrated for three days longer than wild-type plants.

In similar trays, plant viability was evaluated. In this study, *SPY* -3 mutants and wild-type plants were grown next to each other in a single soil plot where the soil's water content was much more uniform than it would be in different pots. As compared to wild-type plants, *SPY* -3 mutant plants had early blooming and bolting traits that were indicative of GA-elevated characteristics. Water was often removed from the growing *SPY* -3 mutant during our drought studies. Water intake was assessed after 2.5 hours of dehydration in Petri dishes. As a contrast to their wild-type counterparts, *SPY* mutants showed much-reduced ion loss. Considering all of the information, we were able to determine that a loss-of-function mutation of the SPY gene increased the ability of plants to withstand water stress in comparison to their wild counterparts. This was probably due to reduced leaf water loss and managed to improve membrane integrity under drought stress. It was investigated whether the elevated GA reaction was relevant to drought resistance, as SPY mutants exhibit enhanced GA signalling and a phenotype indicative of GA overload (Aragonés et al., 2012). We sprayed the wild-type plants with either water or a bioactive GA3 solution. The usual GA phenotype, which is characterized by quicker blooming and light green coloring, corresponding to SPY -3 mutants, was produced as expected because of this treatment. The subsequent scarcity of water strained the vegetation (Paudel et al., 2022). The GA-treated plants, in contrast to the SPY -3 mutants, were considerably more susceptible to water stress and had a lower survival rate. This result demonstrated that, despite SPY 's involvement in the GA reaction, the salt and drought conditions tolerance observed in the SPY mutation was probably not due to an increase in GA signaling (Singh et al., 2022).

CONCLUSION: The future of this technique will presumably lie in the generation of high-throughput gene function analysis systems for species highly susceptible to VIGS, like the *Solanaceae* or California poppy. Ligation-free vector systems are already available and inserting partial sequences of the target gene in VIGS vectors is sufficient for VIGS to be effective. Forward genetic screens may be achieved in non-model plants for which large insertion mutant collections and genome maps are unavailable, but EST sequencing projects have provided sufficient information on the transcriptome. High quality genome or transcriptome data provides an excellent foundation to select the most promising regions of a transcript to be silenced, either to avoid or to induce simultaneous silencing of genes closely related in sequence. Published examples of high throughput approaches include cloning of normalized or subtractive cDNA libraries in VIGS vectors and subsequent plant inoculation with the aims to reduce nicotine levels, to analyze genes required for Pto-mediated resistance in tobacco, and to screen for developmental defects in tomato. These high-throughput platforms would be particularly desirable for expanding our knowledge, and they will be useful for assessing target genes related to abiotic stress resistance, pathogen interaction, and yield improvement in crop breeding programs.

Declaration: We hereby certify that the current study has neither been published elsewhere, nor is it under consideration elsewhere, and will not be submitted elsewhere.

Certify from authors and co-authors: We certify that all co-authors have been informed and agree with the submission of this paper.

Ethics approval: Not needed for this study.

Consent for publications: Not applicable.

Conflict of interest: The authors declare that they do not have any personal relationships that can affect the work reported in this review.

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