



Cloning, Genetic Transformation and Cellular Localization of Abiotic Stress Responsive Universal Stress Protein Gene (GUSP1) in *Gossypium hirsutum*

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Background: Drought stress seriously affects the cotton fiber development. Universal stress protein gene isolated from native species *Gossypium arboreum* has the promising tolerance role against these stresses.

Objectives: This study aimed to clone, characterize, and genetically transform the GUSP1 gene in local cotton and to observe its expression in transgenic plants under drought stress.

Materials and Methods: Universal Stress Protein (GUSP1) gene from *Gossypium arboreum* was cloned in pCEMBIA (-) 1301 plant expression vector by replacing Hygromycin and GUS exon with GUSP1-GFP fusion fragment. The construct was transformed into *Agrobacterium tumefaciens* and transient expression assay was confirmed by agro-infiltration of *Nicotiana benthamiana* leaves and green fluorescence under a confocal microscope. Gene integration and expression in transgenic plants was observed through Southern blot and real-time PCR analyses. Cellular localization was observed through a confocal microscope and the copy number of the transgene was observed in progeny plants.

Results: Transformation efficiency was 1.9%. Developmental and spatial expression of GUSP1 was observed through Real-time PCR in stem, root, leaf, inflorescence, and seeds of transgenic plants at the vegetative and flowering stage. Integration of GUSP1 revealed a fragment of approximately 500 bp in Southern Blot analyses. Localization of GUSP1 was detected in the intact leaf of transgenic plants through GFP fluorescence in midrib, guard cells of stomata, and trichomes. Single gene copy was detected in the chromosome of transgenic seeds.

Conclusion: GUSP1 has cloned from native species of local cotton and its integration and expression in transgenic plants confirmed that the role of GUSP1 will provide direction to breed economically important cotton varieties.

Keywords: Cotton; drought; Gene cloning; Genetic transformation; Universal stress protein

1. Background

Cotton is amongst the valuable cash crop worldwide and a basic input as fiber to the textile industry. One of the factors for crop performance and productivity is the timely availability of irrigation water. Cotton is vulnerable to drought stress during reproductive phase; plant development slows down thus causing small bolls and squares to shed, and consequently yield losses occur (1). Cotton belongs to Malvaceae family and genus *Gossypium* including approximately 50 species. *G. hirsutum* is grown worldwide because of the quality fiber that can be spun into fine yarn. *G. arboreum* is well documented as tolerant to abiotic stresses, resistant to diseases and insect pests, which makes it a valuable source of the gene pool for improvement of modern cotton cultivars (2). So, such cotton varieties that tolerate drought condition are

needed for the growing population (3).

Drought stress changes the water balance leading to alteration in plant cellular structures: that leads to cell death by series of events such as abnormal metabolic function by accumulation of reactive oxygen species (ROS), damages to cell membrane, breakdown of cellular pigments and leakage of cellular contents occurs etc. (4, 5). The universal stress protein (USP) aid in acclimatization with environmental stresses as the majority of these proteins are water soluble and hydrate the plant's structural components and serve as osmoprotectant during dehydration phase (6). They have been localized in different regions of the cells in different organisms (7). Green fluorescent proteins (GFPs) produce the green light when excited with a light of lower wavelength without the use of a substrate or an enzyme (8, 9). Necessary modifications to agricultural crops are

required to fulfill the future demands of supply to meet the requirements of increasing population. Although, progress has been done in traditional breeding and new cultivars have been produced but that process has limitations. Thus, genetic transformation is an effective method to introduce the desirable traits in crops.

2. Objectives

GUSP1 was identified from *G. arboreum* under drought stress and expressed in all plant tissues. Since GUSP1 is a regulatory protein, there is a need to understand its role against drought. So, its efficiency can also be increased by manipulating its interactions (10). This study has the aim of GUSP1 expression in *G. hirsutum* transgenic plants and GFP reporter marker will aid the localization of gene in transgenic cotton.

3. Materials and Methods

3.1. Cloning of GUSP1cDNA and GFP and Fusion in Plant Expression Vector

Cotton (*G. arboreum*) variety FDH-171 was grown in green house as routine agronomic practices and two-month-old plants were drought stressed as the water was withheld for ten days till the wilting symptoms appeared. Total RNA was isolated from leaf (11) and cDNA was synthesized by using the RevertAid™ H- first strand synthesis kit (Fermentas). GUSP1 cDNA was amplified by using Primer F: 5'-CCATGGGTACAAAAGATAGG-3' having *NcoI* restriction site and R: 5'-GCGCGCAAATCCATGAGC-3' having *PauII* restriction site. Reaction mixture (20 µL) contained: 2 µL cDNA, 2 µL 10X PCR buffer, 2 µL each primer (10 µM), 2 µL dNTPs (1 mM), 1.5 µL MgCl₂ (25 mM), 0.2 µL 5 Unit Taq DNA polymerase and 10.3 µL sterile water. Thermocycling conditions were: initial denaturation at 95 °C for 3 min followed by 25 cycles of 94 °C for the 30s, 57 °C for 30 s, and 72 °C for 30 s, then a final extension at 72 °C for 7 min and held at 25 °C.

PCR product was resolved on 1% agarose gel in 0.5× TAE and examined by UV. GUSP1 ligation was done in PCR 2.1 Vector (Invitrogen # K2020-20) as insert 25 nG, PCR 2.1 vector 50 nG, T4 DNA ligase 1 µL, 10X ligation buffer 1 µL and water was added to make volume up to 10 µL. Ligation was followed by transformation to the TOP10 *E. coli* cells by using the heat shock method. The TA clones were confirmed by PCR and restriction digestion with *NcoI* and *BamHI*. Sequencing PCR was conducted by using BigDye™ Terminator v3.0 sequencing kit (Applied Biosystem) and forward/reverse M13 universal primer to confirm the clones with reported sequence (#EU107766).

The GFP(765bp) was TA cloned after PCR amplification from the pGWB5 vector (containing an internal *NcoI* site) by using FGFP: 5-GCGCGCGGTTTCAGCTTTC-3 and RGFP: 5-GGTCACCCTTGTACAGCTCGT-3 primers. The 20 µL reaction mixture contained: 2 µL pGWB5 vector (200 ng), 2 µL 10× PCR buffer, 2 µL each primer (10 µM), 2 µL dNTPs (1 mM), 1.5 µL MgCl₂ (25 mM), 0.2 µL 5 Unit Taq DNA polymerase (Fermentas), and 10.3 µL sterile water. Thermocycling conditions were an initial denaturation at 95 °C for 3 min followed by 25 cycles of 94 °C for the 30 s, 61 °C for 30 s, and 72 °C for 50 s, then a final extension at 72 °C for 7 min and held at 25 °C.

Then cloned GUSP1cDNA was fused with GFP in plant expression vector pCAMBIA1301. For this purpose, Hygromycin fragment (1 kb) was excised from the vector by using *XhoI* site. Subsequently, the vector size was reduced from 11.8kb to 10.8kb and named pCEMBIA(-)1301. The GUSP1 TA was double digested with *NcoI* and *PauII* (Thermo Fisher Scientific, Germany). Similarly, GFP TA was also double digested with *PauII* and *BstEII*. Then vector pCEMBIA(-)1301 restricted digested with *NcoI* and *BstEII* and three-fragment ligation was conducted for GUSP1 cDNA-GFP fusion in pCEMBIA(-)1301 vector. Then cloning of GUSP1 cDNA-GFP fusion was confirmed by PCR/restriction digestion and the resultant GFP reporter construct was named pGP1. A GFP control vector was also constructed, namely pGFP. Then both these constructs were transformed in *Agrobacterium tumefaciens* strain LBA 4404 for transient expression assay and genetic transformation in a local variety of cotton.

3.2. Transient Expression Assay by Agro-Infiltration of Tobacco Leaves

The transient expression of pGP1 construct; was established through Agro-infiltration of *Nicotiana benthamiana* leaves (12) with some modifications. A single colony of *Agrobacterium* strain LBA 4404 harboring pGP1 construct was inoculated in 5 mL YEP broth supplemented with 50 µg.mL⁻¹ Kanamycin and 12.5 µg.mL⁻¹ Rifampicin followed by incubation at 28 °C for 24 h. The culture was centrifuged at 3,000 ×g for 5 min. The supernatant was discarded and the pellet was resuspended initially in 1mL induction medium and then the volume was made up to 4 mL supplemented with 100 µM Acetosyringone. *Agrobacterium* suspension grown at 28 °C for 5 to 6 h was collected by centrifugation (3000 ×g for 5 min) and resuspended in infiltration medium to OD₆₀₀=0.4. A needleless syringe was used to infiltrate the bacterial suspension to the abaxial side of the intact leaves of *N.*

benthamiana and infiltrated areas were marked with a fine-tip marker. Leaves were collected after two days and GFP fluorescence was observed. Argon laser was used for excitation of GFP at 488 nm and emission at 505 nm as a digital image by confocal laser-scanning microscope (Zeiss LSM 510).

3.3. Genetic Transformation of *Gossypium hirsutum*

A single colony of *A. tumefaciens* strain LBA 4404 harboring pGP1 & pGFP construct was inoculated in 10mL YEP broth supplemented with 50 $\mu\text{g.mL}^{-1}$ Kanamycin and incubated for 24 h at 28 °C and then pellet down for 5 min at 3000 $\times g$ and resuspended in 10 mL of MS broth. Seed delinting, surface sterilization and germination were carried out as previously described (13). Mature embryos of *G. hirsutum* local cultivar were isolated by exposing out the cotyledonary leaves from the germinating seeds were used as explant for shoot apex cut method (14). The explants were incubated with *Agrobacterium* inoculum suspension for 1h on a rotary shaker at a slow speed. Then, the embryos were implanted on Petri plates containing MS medium (15) and kept for 72 h at 28 °C and photoperiod 16 h (100-120 $\mu\text{mm}^{-2}.\text{s}^{-1}$). Then, seedlings were sub-cultured in test tubes containing MS medium supplemented with 125 $\mu\text{g.mL}^{-1}$ Cefotaxime and 100 $\mu\text{g.mL}^{-1}$ Kanamycin for selection. Plantlets were sub-cultured on fresh medium after every 2 weeks and that continued to 8 weeks. Afterward, plants were shifted to MS selection free root induction medium containing IBA 1 mg.mL^{-1} and continued to subculture for another 8 weeks for rooting. Plants were hardened off by shifting to pots containing soil mixture (16).

3.4. Confirmation of Transgenic Plants

Putative transgenic plants were preliminarily screened out on the basis of GFP fluorescence (Supplementary Fig 1) Leaf sections from the transgenic plants harboring pGP1 and pGFP constructs were observed under a confocal microscope as described in transient expression section.

Southern blotting was conducted according to the standard procedure (17) to confirm the integration of transgene in the transgenic plants. GUSP1 TA was used for probe synthesis via PCR; approximately 500 bp amplified fragment was quantified and labeled through DIG DNA Labeling Kit. Genomic DNA was isolated from leaves of transgenic and non-transgenic plants. The response of GUSP1 in transgenic plants was done by employing different molecular and biological parameters after inducing drought stress to the four-month-old plants by water withdrawal for 12 days till they showed wilting symptoms. Control plants were watered regularly.

3.5. Real-time RT-PCR Analysis of Transgenic Plants

Total RNA was isolated from three transgenic plant's tissues (stem, root, leaf, inflorescence and seeds) under drought-stress and well-watered conditions at vegetative and flowering stage (11). Their cDNA was synthesized by using RevertAidTM H- first strand synthesis kit (Fermentas) to measure the developmental and spatial relative fold expression of GUSP1 transgene through real-time RT-PCR on ABI 7500 real-time PCR machine (Applied Biosystems, USA). SYBR green PCR master mix (Fermentas) and Gene-specific primers for GUSP1: F: 5'GGTTGATATTGCTTCCGGGC 3' and R: 5' ACCCTTTGAATGGTGCCAAG 3' were used in three technical replicates. The cycling conditions were as: denaturation at 95 °C for the 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s and repeated for 40 cycles and last extension at 72 °C for 7 min. Normalization of the data was done by GAPDH reference gene. Relative fold expression was calculated by $\Delta\Delta\text{Ct}$ using SDS 3.1 software provided by ABI.

3.6. Morphological and Physiological Response of Transgenic Plants In-Vitro

Transgenic and non-transgenic plants were given stress of Polyethylene glycol (PEG) at 5% and 10% level for two weeks in the MS media. Leaf relative water content (LRWC) was measured as mentioned by (18, 19). To determine the % reduction in biomass, plants were taken out of the test tubes and washed with double distilled water (ddH_2O) to remove the media completely, and excess water was removed on filter paper. Fresh weight (FW) of the whole plant was measured with an electronic weighing balance. Then plants were wrapped in brown paper to dry out at 80 °C for 24 h and dry weight (DW) was measured and % reduction of fresh to dry biomass was calculated as

$$\% \text{ reduction in biomass} = (\text{FW} - \text{DW}) / (\text{DW}) \times 100$$

Stomatal characteristics of transgenic and non-transgenic plants under PEG stress were also studied as leaf was detached and carefully peeled off at the lower side. Transparent epidermis was placed on the glass slide with a drop of water and stomata were observed under the Olympus microscope (Olympus BX61).

3.7. Cellular Localization of GUSP1 Through Online Software

A program that predicts the subcellular localization of proteins (<http://www.bioinfo.tsinghua.edu.cn/SubLoc/>) was used to determine the GUSP1 localization (Supplementary Fig 2).

3.8. Fluorescence In-situ Hybridization (FISH) Analysis of T1 Progeny

FISH was conducted to identify the copy number of GUSP1 in seeds of T1 progeny of transgenic plants. The GUSP1 specific probe was labeled through PCR by using DIG DNA Labeling and Detection Kit (Roche#11093657910) as per manufacturer instructions. Chromosome preparation on glass slides was done as mentioned by (18). Hybridization was done by adding 35 μL of hybridization solution containing probe (20 $\text{ng}\cdot\mu\text{L}^{-1}$) at 37 °C for 18 h in a wet chamber. Fluorescent signals were detected by a fluorescent microscope (Olympus Model BX51) on the blue filter for DAPI and a red filter for PI and captured by CCD camera attached with a microscope and analyzed by Cytovision Applied Imaging Systems Software Genus 3.7.

3.9. Statistical Analysis

STATISTIX V 8.1 (Statistix. 2006. Statistix 8.1 user guide, version 1.0. Analytical Software, PO Box 12185, Tallahassee FL 32317 USA. Copyright 2006 by Analytical Software), was used for statistical analysis by subjecting the data for analysis of variance (ANOVA) <http://www.statisticssolutions.com/academic-solutions/resources/directory-of-statistical-analyses/anova/> for a factorial complete randomized design to find out the significant difference in the mean.

4. Results

4.1. Cloning and Confirmation of GUSP1 and GFP Fusion in Plant Expression Vector

Gene-specific primers amplified the 492 bp fragment of GUSP1 cDNA which further ligated in PCR 2.1 Vector (Invitrogen) and transformed in TOP10 *E. coli* cells (**Fig. 1a**). Positive clones were screened out with restriction digestion with *Nco*I and *Bam*HI. The clone #3 generated 2.5 kb, 1.5 kb and 400 bp fragments on double digestion (**Fig. 1b**), that further confirmed by Sanger sequencing for frame confirmation with already reported corresponding GUSP1 sequence, accession #EU107766.

A fragment of 765 bp of GFP from the pGWb plasmid was amplified and fused at 3' in frame with GUSP1 cDNA in pCEMBIA (-) 1301 plant expression vector by replacing GUS (**Fig. 1c**). The cloning was confirmed through PCR amplification by using gene-specific and full length primers that produced the 492 bp and 1265 bp fragments respectively (**Fig. 1d**). Restriction digestion with *Bgl*II and *Bst*EII further confirmed the fused product as it generated a 10 kb fragment for vector and another 1265 bp fusion

fragment of the GUSP1-GFP. The resultant construct was named as pGP1 (**Fig. 1e**). The right and left borders of pCAMBIA 1301 and cloning strategy of the GUSP1-GFP fusion construct, pGP1, is schematically represented in **Figures 1f and 1g** respectively.

4.2. Transient Expression Assay by Agroinfiltration of Tobacco Leaves

Transient expression of the pGP1 construct was detected in the agro-infiltrated tobacco leaves by confocal microscopy. Control leaf showed the red auto-fluorescence of chlorophyll (**Fig. 2a**) while green fluorescence was detected in the leaves infiltrated with the pGP1 construct (**Fig. 2b**).

4.3. Genetic Transformation in Cotton

A total of 2000 embryos were co-cultivated with *Agrobacterium* harboring pGP1 construct, 155 seedlings were selected on basis of fluorescence of GFP in leaves. These plants were continued to grow on the same medium for a period of two months. Finally, 38 plants survived with well-developed roots on root induction media and shifted to soil pots. The transformation efficiency of this experiment was 1.9%.

4.4. Cellular Localization of GUSP1 Through GFP via Confocal Microscopy

Localization of GUSP1 transgene via GFP reporter was carried out in real time in the intact leaf as well as in leaf tissue by using confocal microscopy. The GFP fluorescence confirmed the localization of afore mentioned transgene in different plant tissue as in midrib (M), Guard cells of stomata (S), trichome (T), globular trichome (G), pelisade (P) and spongy mesophyll (SM) (**Fig. 2c & 2d**) respectively. However, the green fluorescence was scattered throughout the leaf tissue in transgenic plants developed by pGFP construct (**Fig. 2e**). This data further strengthens the stable integration, expression, and compartmentalization of GUSP.

4.5. Real-time PCR Analysis of GUSP1 in Different Tissues of Transgenic Plants at Different Developmental Stages

Real-time PCR analyses quantified the mRNA spatial expression pattern of GUSP1 in different tissues of transgenic plants at vegetative and flowering stage under drought stress. At vegetative stage, GUSP1 cDNA relative fold expression was 5.6 fold in leaf, 2.6 fold in stem, and 1.8 fold in the roots as compared to well-watered transgenic plants (**Fig. 3a**). At flowering stage, the expression was 0.89 fold in roots of the drought-stressed transgenic plants while that was 4.1, 2.2, 1.44 fold in leaf, stem and inflorescence respectively as

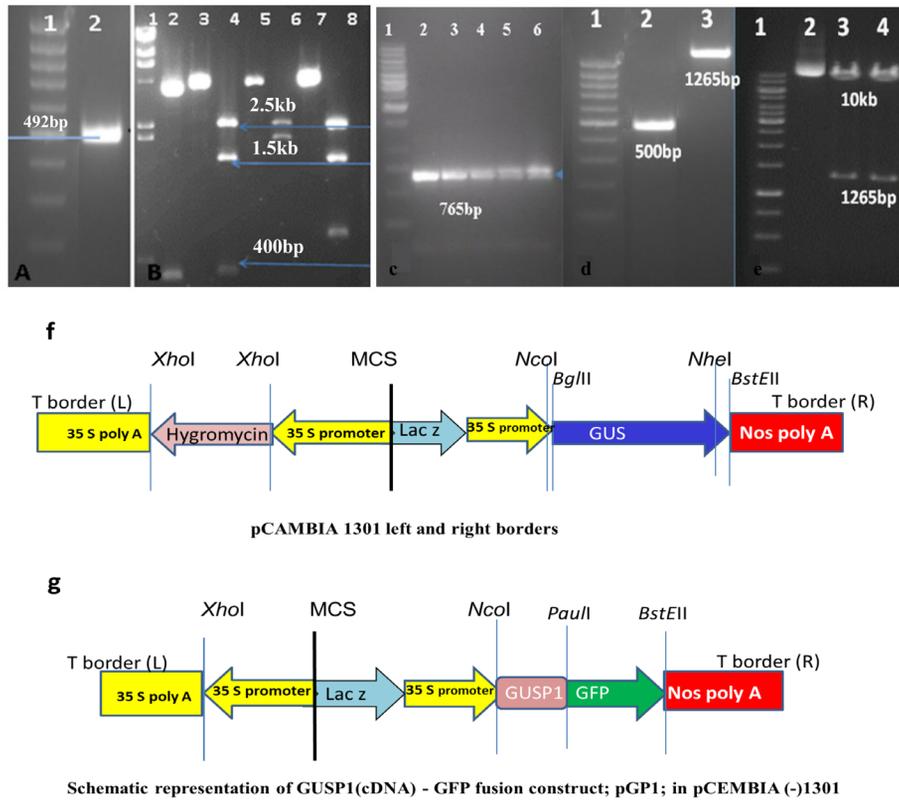


Figure 1. Cloning of GUSP1 cDNA in plant expression vector. **(A)** PCR amplification of GUSP1 (Lane1:100bp ladder; Lane 2: GUSP1. **(B)** Restriction Digestion (Lane 1: λ HindIII marker; Lane2: clone 3 *NcoI* digested; Lane3: clone 3 *BamHI* digested; Lane4: clone3 *NcoI/BamHI* double digested; Lane5: clone 4 *NcoI* digested; Lane6: clone4 *NcoI/BamHI* double digested; Lane7: Clone 5 *NcoI* digested; Lane8: clone 5 *NcoI/BamHI* double digested. **(C)** PCR amplification of GFP: Lane1: 1kb ladder; Lanes 2-6: GFP from pGWb. **(D)** GUSP1 Cloning in pCEMBIA (-) 1301; Lane 1: 100bp ladder; Lane 2: PCR amplification of GUSP1; Lane 3: PCR amplification of GUSP1-GFP fusion. **(E)** Restriction digestion confirmation of GUSP1 in pCEMBIA (-) 1301; Lane1: 1kb ladder; Lane 2: Undigested clone; Lanes 3-4: pGP1 clones digested with *BglIII* and *BstEII*. **(F)** pCambia 1301 left and right borders and **(G)** GUSP1 (cDNA) -GFP fusion clone, pGP1, in pCEMBIA (-) 1301. R: right border; L: Left Border; NOS Poly A: nopaline synthase terminator; 35S promoter: 35S cauliflower mosaic virus (CaMV).

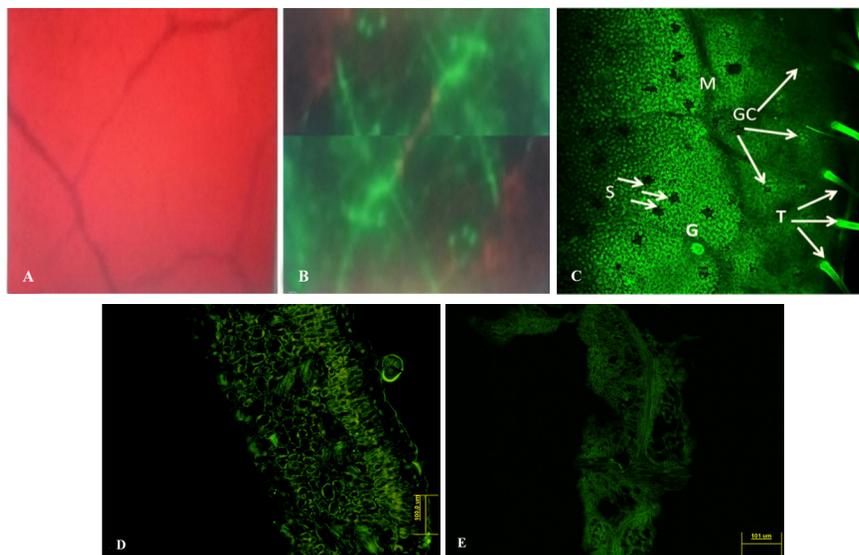


Figure 2. Transient expression assay and localization of GUSP1 in the leaf tissues through GFP Fluorescence. **(A)** Tobacco control leaf showing auto- fluorescence (red) of chlorophyll; **(B)** Tobacco leaf showing green fluorescence derived from pGP1 construct after 2 days of Agro-infiltration. **(C)** Green fluorescence in intact leaf of transgenic cotton plant containing pGP1 construct; **(D)** Green fluorescence in different regions in leaf of transgenic cotton plant containing pGP1 construct; **(E)** Green fluorescence scattered throughout the leaf of transgenic cotton plant developed by pGFP construct. M: Midrib; S: Stomata (Closed); GC: Guard Cells of stomata G: Globular Trichome; T: Trichome; P: Pelisade & SM: Spongy Mesophyll.

compared to well-watered transgenic plants (**Fig. 3b**). Overall, the highest relative fold expression of GUSP1 is observed in the leaf of drought-stressed transgenic plants at the vegetative and flowering stage in comparison to well-watered transgenic plants.

4.6. Biomass Reduction, Leaf Relative Water Content and Stomatal Characteristics of Transgenic Plants *In-vitro*

The average fresh weight of transgenic plants was 70.56% and non-transgenic plants were 71.04% without PEG stress. Reduction in biomass of transgenic and non-transgenic plants growing *in-vitro* under 5% PEG stress was increased to 72.71% and 78.54%, respectively. However, at 10% PEG stress the reduction in biomass increased to 74.74% in transgenic plants and 81.91% in non-transgenic plants. Overall the biomass reduction was observed under PEG stress. However, biomass reduction in transgenic plants is at a lower rate in comparison to non-transgenic plants (**Fig. 4a**). Similarly, a reduction in the LRWC of plants was observed. The transgenic and non-transgenic plants showed LRWC of 38.6% and

33.3% respectively under 5% PEG. At 10% PEG, it was further reduced to 36.4% and 28.6% in transgenic and non-transgenic plants respectively. The LRWC without PEG stress in transgenic and non-transgenic control plants was 56.7% and 56.5%. Overall LRWC reduction in transgenic plants was less as compared to non-transgenic plants (**Fig. 4b**).

Leaf epidermis of transgenic plant shows closed stomata which were abundant in the number represented by black arrows as compared to opened stomata as shown by blue arrows. Whereas for non-transgenic plant reverse is true; where opened stomata were numerously indicated by the blue arrows (**Fig. 4c**). Figure 4d shows the graphical overview of the number of stomata in leaf epidermis. In the transgenic plants as an average 300 stomata were counted to be closed while the same in non-transgenic plants were 72 under PEG stress. However, in the transgenic plant 60 stomata were opened while in non-transgenic plant 320 stomata were opened under PEG stress. This implies the direct role of GUSP1 in maintaining the osmotic potential for homeostasis under stress conditions.

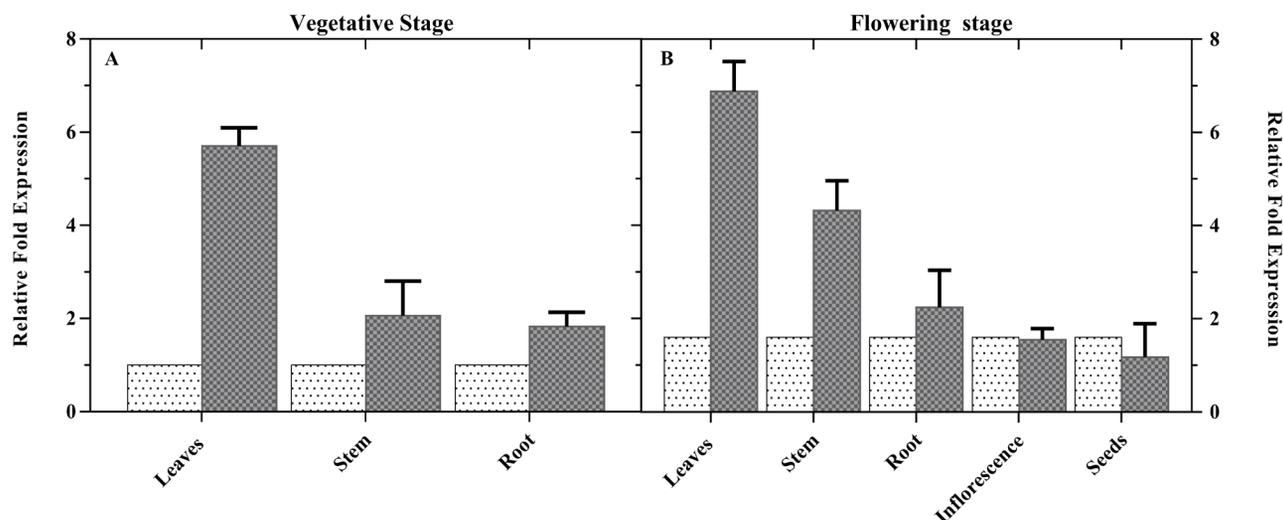


Figure 3. Spatial Relative Fold Expression of GUSP1 in different tissues of transgenic plants at different developmental stages. (A) Vegetative Stage 5.6-fold in leaf, 2.6 fold in stem and 1.8 fold in the roots as compared to well-watered transgenic plants. (B) Flowering Stage 0.89-fold in roots, 4.1 fold in leaf, 2.2 fold in stem and 1.44 fold in inflorescence as compared to well-watered transgenic plants. Each bar indicates the mean+ standard deviation (SD) of three replicates. The asterisks indicate significant differences ($P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) ns= non-significant

4.7. Southern Blot and Fluorescence *In-situ* Hybridization (FISH)

Southern blot analysis confirmed the stable integration of GUSP1cDNA in the genomic DNA of transgenic plants. Thus **Fig. 5a** shows the agarose gel run for the digested genomic DNA of the transgenic plants (lanes 2 & 4) and non-transgenic plant in lane 8. Lane 6 is positive control i.e. 500 bp PCR amplified fragment from pGP1 construct by using GUSP1 specific primers, while lanes 1, 3, 5 and 7 are empty. **Figure 5b** shows the respective blot after hybridization and exposed an

approximately 500 bp fragment. As GUSP1-GFP fusion cloned fragment (1,265 bp) contained an internal *NcoI* site thus *NcoI*-*BstEII* digestion revealed approximately 500 bp rather than 1265 bp fusion fragment in the transgenic plants genomic DNA. Genomic DNA from control plant did not show any integration (Lane 8).

The FISH analysis showed the integration of GUSP1 in the growing seeds of T1 progeny during metaphase stage at the location of chromosome number 5, indicating single copy number (**Fig. 5c**).

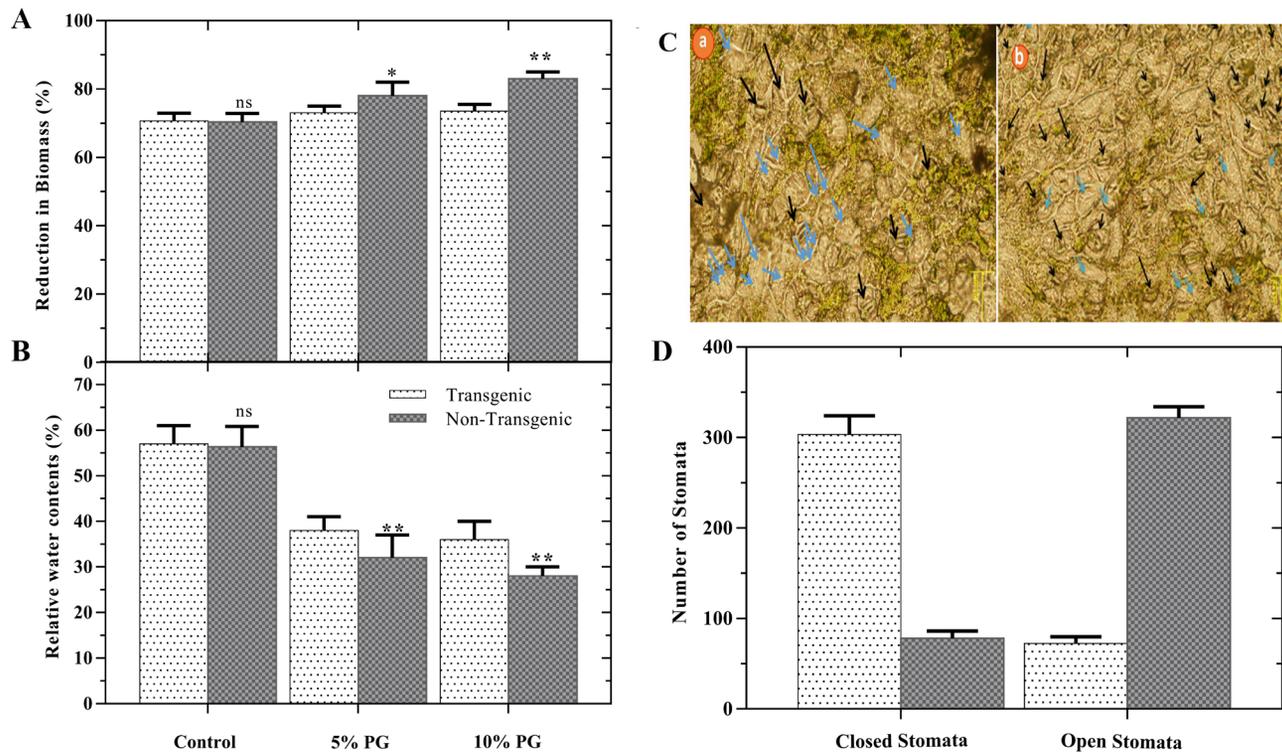


Figure 4. Physiological and stomatal characteristics of transgenic cotton plants *in vitro* under PEG stress. (A) Plant biomass % reduction of transgenic and non-transgenic cotton plants under PEG stress in comparison to control plants; (B) LRWC of transgenic and non-transgenic cotton plants under PEG stress in comparison to control plants; (C) Leaf epidermis to study the stomata black arrows showing closed stomata and blue arrows representing opened stomata; (D) Comparative analysis of opened/closed Stomata in transgenic and non-transgenic plants under PEG stress.

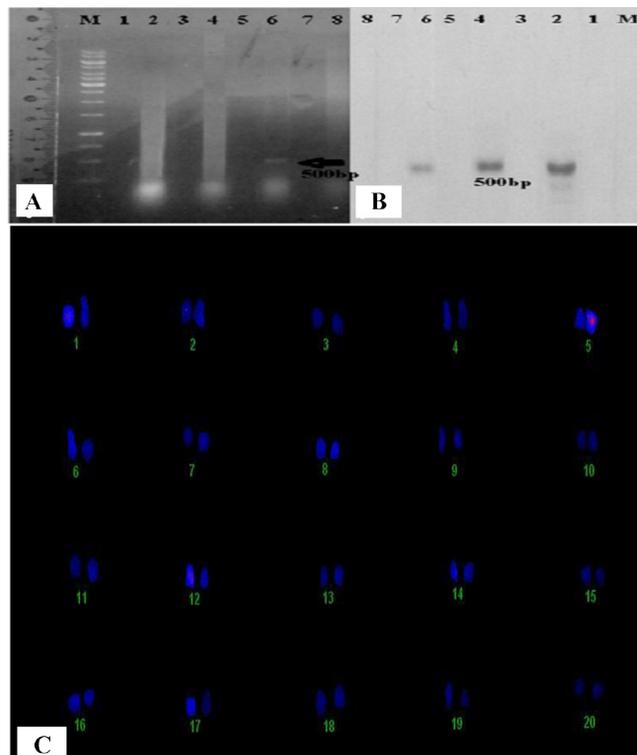


Figure 5. Southern blot and FISH analyses (A) Southern agarose gel; (B) Southern Membrane Blot. M: 1kb Ladder; Lanes 1, 3, 5 & 7: Empty. Lanes 2&4: Genomic DNA of transgenic plants double digested with *NcoI* and *BstEII*. Lane 6: positive control (500bp GUSP1 PCR product); Lane 8: negative Control. (C) FISH analysis showing integration of single copy of GUSP1 at chromosome #5 in T1 transgenic seedling during metaphase stage at the cellular level.

5. Discussion

Universal stress proteins (USPs) are prevalent and involved in a broad range of activities in plants in response to various external stimuli including drought. Present investigation involves the cloning of GUSP1 in fusion with GFP as a marker in plant expression vector, genetic transformation, cellular localization and expression in transgenic cotton plants. The GUSP1 (492 bp) fragment was cloned in pCAMBIA1301 plant expression vector after replacing GUS with GFP fused at the C terminus of GUSP1 under the control of a 35S promoter. The first USP-related gene, LeER6, was isolated and characterized in tomato and high-level expression was detected at the ripening while ethylene treatment up-regulated the expression of transcript (20). GFP as a tag has been used as visual selection marker and does not require any substrate (21), therefore, transient expression of GFP-protein fusion is considered as a time-saving and feasible approach for production of stable transformants (22). Transient expression of the GUSP1 fusion constructs pGP1 through *Agrobacterium* infiltration in leaves of *N. benthamiana* was observed. The transgenic plants of local *G. hirsutum* cultivar CIM 496 containing pGP1 construct by using *Agrobacterium*-mediated transformation system with an efficiency of 1.9% were obtained. A similar study showed that 550 bp *Solanum pennellii* SpUSP gene was cloned from the tomato species into the plant binary vector pMV, replacing the GUS reporter gene which exhibited significant expression response under salt, drought, heat and cold stress (23).

Spatial expression of GUSP1 through real-time PCR analysis indicated that GUSP1 transgene showed the highest expression in leaf under drought stress at vegetative as well as at flowering stage.

Increase in transcript expression pattern of SbUSP gene was studied under the influence of various stress treatments using real-time quantitative PCR (24). So, it is obvious that GUSP1 can also be an important genetic resource for the drought stress tolerance in cotton (25). Integration of GUSP1 was revealed by visualizing the fragment of approximately 500 bp through Southern blot. A stable insertion of AtNHX1 was observed by Southern blot and its expression was confirmed by RT-PCR (26). Protein localization is largely done by tagging proteins with fluorescent GFP marker. In chimeric proteins, GFP can stand N and C-terminal fusion, thus enabling GFP to be transcribed under the same regulatory sequence as of target gene (27). Cellular localization of CAX2 was identified in maize after generating the polyclonal antibodies against protein sequence

from the non-membranal central loop of the gene (28). In addition to this, Loctree program (29) (<http://www.bioinfo.tsinghua.edu.cn/SubLoc>) showed the cytoplasmic location of GUSP1 with an accuracy of 83%. Moreover, the transgenic plants with pGFP construct showed a mass of green fluorescence spread evenly throughout the leaf tissue section. As regulatory proteins are located at specific sites and their location is an indirect way of determining their function. Thus the cytoplasmic localization of GUSP1 emphasizes its role in signal transduction of stress signals. Cellular localization of GhMPK1 was also reported through the use of this program (30). Subcellular localization of ZmERD3 protein has also been confirmed in maize seedlings under different abiotic stresses (31).

Performance of transgenic plants in terms of physiological characteristics was better than the non-transgenic plants. The leaf water status in control and PEG stressed plants was determined by measuring the LRWC and % biomass reduction. A slight reduction in plant biomass and LRWC under drought stress conditions are desirable characteristics for plant growth. Higher reduction in plant biomass and LRWC is common phenomena in crop plants under drought stress (32). In this study, the plant biomass and LRWC in transgenic plants are reduced at a lower rate than in non-transgenic plants under *in-vitro* conditions when PEG was applied at 5 and 10% in MS media. This shows that transgenic plants are capable to survive under stressful conditions.

Stomatal responses are often more closely linked to soil moisture content than to leaf water status. Thus, partial or complete stomatal closure allows the plants to maintain a favorable water balance under drought stress (33). Transgenic plants' leaf showed a number of stomata are closed under drought stress which shows the maintenance of moisture inside the plant tissues. As stomatal opening and closing are dependent on abscisic acid (ABA), thus our data infers the direct role of GUSP1 in signal transduction for maintaining the osmotic potential in homeostasis under stress conditions.

The physical location of the site of GUSP1 transgene insertion was observed by FISH. This technique allows transgenes to be located in specific chromosome regions and is sensitive enough to detect the single copy number of the transgene (34).

In conclusion, this study deduced the cloning of GUSP1 cDNA to plant expression vector and genetic transformation to local cotton variety. The better performance of transgenic plants *in-vitro* under drought stress showed its direct role in signal transduction in homeostasis under the control of ABA. Moreover,

GUSP1 cDNA can provide direction for future engineering of drought-stress tolerance in other important crops. Further studies under field conditions may be useful for sustainability of plants under drought conditions (35).

Conflict of interests: The authors declare that there is no conflict of interests

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References

1. Abbas HG, Mahmood A, Ali Q. Genetic Variability and Correlation Analysis for Various Yield Traits of Cotton (*Gossypium hirsutum* L.). *J Agric Res.* 2015;53(4):481-491.
2. Liu D, Guo X, Lin Z, Nie Y, Zhang X. Genetic Diversity of Asian Cotton (*Gossypium arboreum* L.) in China Evaluated by Microsatellite Analysis. *Genet Resour Crop Evol.* 2006;53(6):1145-1152. doi: <https://doi.org/10.1007/s10722-005-1304-y>.
3. Yildiz Aktas L, Dagnon S, Gurel A, Gesheva E, Edreva A. Drought Tolerance in Cotton: Involvement of Non Enzymatic ROS Scavenging Compounds. *J Agron Crop Sci.* 2009;195(4):247-253. doi: <https://doi.org/10.1111/j.1439-037x.2009.00366.x>.
4. Mahdiah M, Mostajeran A, Horie T, Katsuhara M. Drought Stress Alters Water Relations and Expression of PIP-Type Aquaporin Genes in *Nicotiana tabacum* Plants. *Plant Cell Physiol.* 2008;49(5):801-813. doi: <https://doi.org/10.1093/pcp/pcn054>.
5. Mohamed BB, Aftab B, Sarwar MB, Rashid B, Ahmad Z, Hassan S, et al. Identification and Characterization of the Diverse Stress-Responsive R2R3-RMYB Transcription Factor from *Hibiscus sabdariffa* L. *Int J Genom.* 2017;2017:2763259. doi: <https://doi.org/10.1155/2017/2763259>.
6. Kosma DK, Bourdenx B, Bernard A, Parsons EP, Lü S, Joubès J, et al. The Impact of Water Deficiency on Leaf Cuticle Lipids of Arabidopsis. *Plant Physiol.* 2009;151(4):1918-1929. doi: <https://doi.org/10.1104/pp.109.141911>.
7. Sousa MC, McKay DB. Structure of the Universal Stress Protein of *Haemophilus influenzae*. *Structure.* 2001;9(12):1135-1141. doi: [https://doi.org/10.1016/s0969-2126\(01\)00680-3](https://doi.org/10.1016/s0969-2126(01)00680-3).
8. Ei-Shemy HA, Khalafalla MM, Ishimoto M. The role of Green Fluorescent Protein (GFP) in Transgenic Plants to Reduce Gene Silencing Phenomena. *Curr Issues Mol Biol.* 2009;11(1):I21. doi: <https://doi.org/10.21775/cimb.011.i21>.
9. Zhang J, Campbell RE, Ting AY, Tsien RY. Creating New Fluorescent Probes for Cell Biology. *Nat Rev Mol Cell Biol.* 2002;3(12):906. doi: <https://doi.org/10.1038/nrm976>.
10. Maqbool A, Zahur M, Husnain T, Riazuddin S. GUSP1 and GUSP2, Two Drought-Responsive Genes in *Gossypium arboreum* have Homology to Universal Stress Proteins. *Plant Mol Bio Rep.* 2009;27(1):109-114. doi: <https://doi.org/10.1007/s11105-008-0049-0>.
11. Jaakola L, Pirttila AM, Halonen M, Hohtola A. Isolation of High Quality RNA from Bilberry (*Vaccinium myrtillus* L.) Fruit. *Mol Biotechnol.* 2001;19(2):201-203. doi: <https://doi.org/10.1385/mb:19:2:201>.
12. Lee MW, Yang Y. Transient Expression Assay by Agroinfiltration of Leaves. *Arabidopsis Protocols: Springer;* 2006. p. 225-229. doi: <https://doi.org/10.1385/1-59745-003-0:225>
13. Rashid B, Saleem Z, Husnain T, Riazuddin S. Transformation and Inheritance of Bt Genes in *Gossypium hirsutum*. *J Plant Biol.* 2008;51(4):248-254. doi: <https://doi.org/10.1007/bf03036123>.
14. Gould J, Banister S, Hasegawa O, Fahima M, Smith RH. Regeneration of *Gossypium hirsutum* and *G. barbadense* from Shoot Apex Tissues for Transformation. *Plant Cell Rep.* 1991;10(1):12-16. doi: <https://doi.org/10.1007/bf00233024>.
15. Murashige T, Skoog F. A Revised Medium for Rapid Growth and Bio-Assays with Tobacco Tissue Cultures. *Physiol Plant.* 1962;15(3):473-497. doi: <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>.
16. Rashid B, Husnain T, Riazuddin S. In Vitro Shoot Tip Culture of Cotton (*Gossypium hirsutum*). *Pak J Bot.* 2004;36(4):817-823. doi: <https://doi.org/10.11.625.3552>
17. Southern EM. Detection Of Specific Sequences among DNA Fragments Separated by Gel Electrophoresis. *J Mol Biol.* 1975;98(3):503-517. doi: <https://doi.org/10.1016/b978-0-12-131200-8.50041-1>.
18. Sarwar MB, Batool F, Rashid B, Aftab B, Hassan S, Husnain T. Integration and Expression of Heat Shock Protein Gene in Segregating Population of Transgenic Cotton for Drought Tolerance. *Pak J Agri Sci.* 2014;51(4):935-941. doi: <https://doi.org/10.2135/cropsci2007.03.0120>.
19. Sarwar MB, Sadique S, Hassan S, Sania R, Rashid B, Mohamed BB, et al. Physio-Biochemical and Molecular Responses in Transgenic Cotton under Drought Stress. *Tarim Bilim Derg.* 2017;23(2):157-166.
20. Zegzouti H, Jones B, Frasse P, Marty C, Maitre B, Latch A, et al. Ethylene-Regulated Gene Expression in Tomato Fruit: Characterization of Novel Ethylene-Responsive and Ripening-Related Genes Isolated by Differential Display. *Plant J.* 1999;18(6):589-600. doi: <https://doi.org/10.1046/j.1365-313x.1999.00483.x>
21. Leckie BM, Stewart CN. Agroinfiltration as a Technique for Rapid Assays for Evaluating Candidate Insect Resistance Transgenes in Plants. *Plant Cell Rep.* 2011;30(3):325-334. doi: <https://doi.org/10.1007/s00299-010-0961-2>.
22. Hanson MR, Kohler RH. GFP Imaging: Methodology and Application to Investigate Cellular Compartmentation in Plants. *J Exp Bot.* 2001;52(356):529-539. doi: <https://doi.org/10.1093/jexbot/52.356.529>
23. Loukehaich R, Wang T, Ouyang B, Ziaf K, Li H, Zhang J, et al. SpUSP, An Annexin-Interacting Universal Stress Protein, Enhances Drought Tolerance in Tomato. *J Exp Bot.* 2012;63(15):5593-5606. doi: <https://doi.org/10.1093/jxb/ers220>.
24. Udawat P, Mishra A, Jha B. Heterologous Expression of an Uncharacterized Universal Stress Protein Gene (SbUSP) from the Extreme Halophyte, *Salicornia brachiata*, which Confers Salt and Osmotic Tolerance to *E. coli*. *Gene.* 2014;536(1):163-170. doi: <https://doi.org/10.1016/j.gene.2013.11.020>.
25. Hassan S, Rashid B, Hafeez MN, Sarwar MB, Husnain T. Genetic Modification of *Gossypium arboreum* Universal Stress Protein (GUSP1) Gene to Improve Drought Tolerance in Transgenic *Gossypium hirsutum*. *Mol Biol Rep.* (Submitted). 2019: .
26. Fan W, Deng G, Wang H, Zhang H, Zhang P. Elevated Compartmentalization of Na⁺ into Vacuoles Improves

- Salt and Cold Stress Tolerance in Sweet Potato (*Ipomoea batatas*). *Physiol Plant*. 2015;154(4):560-571. doi: <https://doi.org/10.1111/ppl.12301>
27. Cutler SR, Ehrhardt DW, Griffiths JS, Somerville CR. Random GFP:: cDNA Fusions Enable Visualization of Subcellular Structures in Cells of Arabidopsis at a High Frequency. *Proc Natl Acad Sci*. 2000;97(7):3718-3723. doi: <https://doi.org/10.1073/pnas.97.7.3718>.
28. Chen H, Zhang X. Subcellular localization of CAX Proteins in Plants. *Mol Soil Bio*. 2015;6:1-5. doi: <https://doi.org/10.5376/msb.2015.06.0003>
29. Goldberg T, Hecht M, Hamp T, Karl T, Yachdav G, Ahmed N, et al. LocTree3 Prediction of Localization. *Nucleic Acids Res*. 2014;42(Web Server issue):W350-355. doi: <https://doi.org/10.1093/nar/gku396>.
30. Shi J, Zhang L, An H, Wu C, Guo X. GhMPK16, A Novel Stress-Responsive Group D MAPK Gene from Cotton, is Involved in Disease Resistance and Drought Sensitivity. *BMC Mol Biol*. 2011;12(1):22. doi: <https://doi.org/10.1186/1471-2199-12-22>.
31. Song X, Weng Q, Zhao Y, Ma H, Song J, Su L, et al. Cloning and Expression Analysis of ZmERD3 Gene from *Zea mays*. *Iran J Biotechnol*. 2018;16(2):140-147. doi: <https://doi.org/10.21859/ijb.1593>.
32. Jaleel CA, Manivannan P, Wahid A, Farooq M, Al-Juburi HJ, Somasundaram R, et al. Drought Stress in Plants: a Review on Morphological Characteristics and Pigments Composition. *Int J Agric Biol*. 2009;11(1):100-105.
33. Franks PJ. Passive and Active Stomatal Control: Either or Both? *New Phytol*. 2013;198(2):325-327. doi: <https://doi.org/10.1111/nph.12228>
34. Harwood WA, Bilham LJ, Travella S, Salvo-Garrido H, Snape JW. Fluorescence *In Situ* Hybridization to Localize Transgenes in Plant Chromosomes. *Transgenic Plants: Methods and Protocols*: Springer; 2005. p. 327-339. doi: <https://doi.org/10.1385/1-59259-827-7:327>
35. Hassan S, Samiullah TR, Rashid B, Husnain T. Cloning, Antibody Production, Expression and Cellular Localization of Universal Stress Protein Gene (USP1-GFP) in Transgenic Cotton. *J Plant Biochem Biotechnol*. 2017:1-11. doi: <https://doi.org/10.1007/s13562-017-0427-5>.