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Physio-Biochemical and Molecular Responses in Transgenic Cotton under Drought Stress

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ABSTRACT

Drought decreases the growth and productivity in cotton. Heat shock proteins accumulate in plants under water stress to protect the biochemical and physiological processes at the molecular level. In this study, plants of T_2 segregating generation of transgenic cotton, containing small heat shock protein gene (*GHSP26*) was compared with wild type plants for biochemical, physiological and molecular responses under different periods of drought stress. Transgenic plants accumulated 30% higher proline content than the wild type. Lipid peroxidation activity was reduced in transgenic plants which showed that the drought tolerance efficiency has been improved. Leaf relative water content was 69% and 45% in transgenic and wild-type plants, respectively at 10-day drought stress. Similarly, transgenic plants showed better performance for photosynthesis, stomatal conductance, transpiration and osmotic potential as compared to wild type. Real-time quantitative PCR of *GHSP26* and some other drought responsive genes such as *Gh-POD*, *Gh*-RuBisCO, *Gh-LHCP PSII*, *Gh-PIP*, *Gh-TPS* and *Gh-LEA* have supported the higher expression and proved drought tolerance in transgenic plants. The overexpression of *GHSP26* in transgenic plants improved the biochemical such as proline content and lipid peroxidation activity and physiological parameters like photosynthesis, osmotic potential and water related attributes. Hence, this study may be extended for selection of homozygous lines and breeding to improve the drought tolerance activity in plants.

Keywords: Gene expression; *Gossypium hirsutum*; Physiological analysis; Genetically modified cotton; Water stress; Biochemical analysis

Transgenik Pamuk Bitkisinin Kuraklık Stresine Fizyo-Biyokimyasal ve Moleküler Tepkisi

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ÖZET

Kuraklık, pamuk bitkisinin gelişimini ve verimini azaltmaktadır. Su stresi koşullarında biyokimyasal ve fizyolojik prosesleri moleküler düzeyde korumak için bitkide ısı şok proteinleri birikmektedir. Bu çalışmada, küçük ısı şok proteinleri (*GHSP26*) içeren transgenik pamuk bitkisinin T_2 neslinin farklı kuraklık stresi altında biyokimyasal, fizyolojik ve moleküler düzeyde tepkileri yabani-tip bitki ile karşılaştırılmıştır. Transgenik bitkiler yabani-tip bitkilere gore % 30 daha fazla prolin biriktirmişlerdir. Kuraklık tolerasyon etkinliğinin arttığını gösteren lipid peroksidasyon aktivitesi transgenik bitkilerde azalmıştır. Kuraklığın onuncu gününde, transgenik ve yabani-tip bitkilerde oransal yaprak su içeriği sırasıyla % 69 ve % 45 olmuştur. Benzer şekilde yabani-tip bitkilerle karşılaştırıldığında, transgenik bitkiler fotosentez, stoma iletkenliği, transpirasyon ve ozmotik potansiyel açısından daha iyi performance göstermiştir. *GHSP26* ve *Gh-POD*, *Gh*-RuBisCO, *Gh-LHCP PSII*, *Gh-PIP*, *Gh-TPS* ve *Gh*-LEA gibi kimi kuraklığa tepki genlerinin gerçek zaman PCR sonuçları yüksek düzeyde gen ekspresyonu olduğunu ve transgenik bitkilerin kuraklık toleranslarının iyi olduğunu göstermiştir. Transgenik bitkilerde *GHSP26*'nın yüksek ekspresyonu prolin ve lipid peroksidasyonu gibi biyokimyasal, fotosentez, ozmotik potansiyel ve su durumuna bağlı fizyolojik özellikleri iyileştirmiştir. Bu yüzden, bu çalışma bitkilerde kuraklık toleransını artırmak üzere ıslah ve homozigot hatların seçiminde kullanılabilecektir.

Anahtar Kelimeler: Gen ekspresyonu; *Gossypium hirsutum*; Fizyolojik analiz; Genetik modifiye pamuk; Su stresi; Biyokimyasal analiz

1. Introduction

Drought stress is seen as loss of water from plants and altering its various structural, physiological, and biochemical processes. These losses would result as a reduction in leaf water content (Hadiarto & Tran 2011), interruption to enzymatic reactions (Mohamed et al 2015), leaf turgor loss (Yue et al 2012), water transpiration efficiency (Ashraf & Harris 2004), cessation of photosynthesis (Chaves et al 2009), nutrient imbalance and a reduction in plants' growth and yield. Plants adapt various ways to maintain cellular activities and integrity to survive under water scarcity (Mao et al 2010). Several genes including heat-shock proteins are involved in cellular protection by re-establishing normal protein conformation and hence normalize the cell homeostasis during stress (Al-Whaibi 2011).

Cotton is a major fiber crop, and *Gossypium hirsutum* is the most widely grown species around the world. *Gossypium arboreum* adjusts well to arid lands and needs low input for agricultural practices, therefore, it is considered as a pool of vital resistant genes that may lead to improving modern cotton cultivars (Liu et al 2006). Less availability of irrigation water is one of the major limiting factors for flower development, fiber production, and lint quality. Under drought stress, plants generally display many physiological and biochemical reactions which result in the observation of certain differentially expressed genes. Therefore, it is important to understand the genes, expressed during drought stress to develop the resistant varieties (Shamim et al 2013).

Conventional breeding has produced better cotton cultivars (Ashokkumar et al 2014), but the cotton genome research has made progress to develop genomic resources and tools for basic and applied genetics, genomics and breeding such as EST's data, DNA markers, QTL's and genes for special traits (Zhang et al 2008). Applications of the biotechnology and genomics approaches have led the studies related to the introduction of transgenic plants with the hope that the development of abiotic stress-tolerant cultivars would be appropriate and comparatively efficient (Rashid et al 2014; Ünlükara et al 2015). We have previously identified, isolated and characterized, small Heat Shock Protein gene (*GHSP26*) from a local variety of *G. arboreum* (Maqbool et al 2007), transformed to local variety of *G. hirsutum* (Maqbool et al 2010) and tested its potential role in segregating generations (Shamim et al 2013; Sarwar et al 2014). Thus, objective of the present research was to elucidate the prospective role

of *GHSP26* in plants of T_2 segregating generation under 5 and 10 days drought (d) stress phase.

2. Material and Methods

2.1. Planting material, growth conditions and application of drought stress

Seeds of T_2 segregating population of *Gossypium hirsutum* previously transformed with *GHSP26* gene, which was isolated from the local variety of *G. arboreum* (Maqbool et al 2007; Maqbool et al 2010) and studied T_1 generation of the same (Shamim et al 2013) were used. Lint was removed with concentrated H_2SO_4 and washed with tap water. Sterilization was done with 0.5% $HgCl_2$ and 1% SDS for 10 min followed by washings with autoclaved water. Seeds were sown in pots (25x30 cm) containing soil, sand and peat moss (1:1:1) and kept in the greenhouse at 30±2 ^oC and 250-300 µmol m^{-2} s^{-1} light intensity. There were three replicates with five plants in each replicate. Forty-day-old plants were subjected to drought stress by stopping irrigation for 5 and 10 d (day stress), as described by (Yue et al 2012). Physiological, biochemical and molecular parameters of the transgenic and wild-type plants were observed under normal and drought stress condition. Transgenic and wild-type (WT) plants at 0 d were considered as control. So the treatments denoted as 0DS, 5DS and 10 DS.

2.2. Estimation of proline content and lipid peroxidation

The proline content in leaf was extracted as described by (Bates et al 1973). A standard curve with known concentration of proline was also obtained and proline content was calculated as µg g^{-1} of fresh leaf tissue. Malondialdehyde (MDA) was analyzed as described by (Quan et al 2004). The absorbance of the supernatant was taken at 450, 532 and 600 nm with the spectrophotometer (spectra Max plus: molecular devices, USA). The MDA content was calculated using the Equation 1.

$$C\ (\mu mol\ g^{-1}) = 6.45x(OD_{532}-OD_{600})-0.56x(OD_{450}) \quad (1)$$

Where; *C*, MDA concentration; *OD*, optical density at given wavelength.

2.3. Leaf relative water content (LRWC)

Leaf relative water content (LRWC) was determined by using the (De Ronde et al 2004) protocol with little modification. About 1 g of leaf sample was cut into smaller pieces and determined the fresh weight (FW). Then samples were immersed in double distilled H_2O for 24 h and turgor weight (TW) was determined. Samples were then oven dried at 80 ˚C for 24 h and the dry weight (DW) was obtained. The LRWC was calculated using the Equation 2.

$$\text{Leaf relative water content (LRWC)} = [(FW-DW)/(TW-DW)]x100 \quad (2)$$

Where; *FW*, fresh weight; *DW*, dry weight; *TW*, turgid weight.

2.4. Gas exchange parameters and osmotic potential (OP)

Gas exchange attributes, such as stomatal conductance (C), transpiration (E) and photosynthetic rate (PN) were calculated using an open system LCA-4 ADC portable infrared gas analyzer in the mid-day in sun shine (Analytical Development Company, United Kingdom) as described by Akram et al (2011). The Osmotic potential was measured with a Micro-Osmometer (Fiske Model 210, Fiske Associates) as mentioned by Mao et al (2010).

2.5. RNA extraction, cDNA synthesis and quantitative real time RT-PCR

Total RNA was extracted from leaves of transgenic and WT plants as described by Muoki et al (2012) with some modifications. Quantity and quality were analyzed with NanodropND-1000 spectrophotometer. RNA integrity was confirmed in 1.2% agarose gel and cDNA synthesis was done by using the kit (Fermentas cat# k1642). Gene-specific primers for *GHSP26* were designed as F-AGGCCTAAACGGTTGGCTAT, R-CCATCTTTGATGTCCCAAGG by using the primer-3 software. Expression of some drought linked genes (*Gh-POD*, *LHCP-PII*, *RuBisCO*, *Gh-TPS, LEA-5,* and *Gh-PIP*) were analyzed in transgenic and WT plants by quantitative real-time PCR. Data normalization was done by using

GAPDH as internal control. SYBER green PCR master mix (Fermentas: cat#k221) was used for reaction mix to run the cycle on Real-time PCR ABI 7500 device. The thermal profile was 3 min at 95 °C, followed by 35 cycles each at 95 °C for 30 sec, 60 °C for 40 sec and 72 °C for 30 sec. The Assay was performed in triplicate and relative gene expression analysis was done by REST 2009 V2.0.13 software provided by QIAGEN.

2.6. Statistical analysis

Experimental data are the means of at least three independent replicates, and results were determined using analysis of variance (ANOVA) Statistix software. Variation among treatment means were compared using least significant difference (LSD) ($P \leq 0.05$).

3. Results and Discussion

3.1. Proline content and lipid peroxidation

In this study, accumulation of proline was 30% higher in transgenic plants as compared to WT. So this indicates a positive correlation between the proline content and expression of the transgene against drought stress period (Figure 1A). Proline accumulation in plants in response to osmotic stress is vital for adaptation (Yue et al 2011). Proline accumulation helps the plants to minimize the dehydration damage to the cell membrane. It accumulates in a larger amount than any other amino acid in higher plants under drought and salt stress (Ashraf & Harris 2004). Statistical analysis showed that there is a significant difference for proline accumulation in transgenic and wild type cotton lines (F-test, **P<0.01) (Table 1). Similarly (Liu et al 2009) reported higher proline content in transgenic rice plants than the wild types under drought and salt stress conditions. Therefore, these reports confirm the positive function of proline in plants under abiotic stresses.

Drought stress causes oxidative damage which results in cell membrane degradation which is a sign of membrane lipid peroxidation. The value of MDA production without drought stress was 2.0

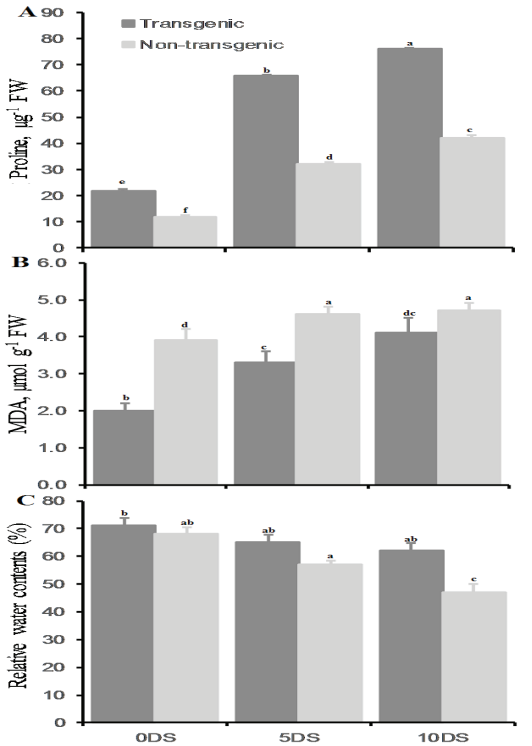

Figure 1- Biochemical and physiological indicators of transgenic and WT plants under drought stress; A, proline; B, MDA; C, leaf relative water content; each value represents the mean±SD of three replicates; values with different letters are statistically different (LSD, P≤0.05)

Şekil 1- Transgenik ve yabani tip (WT) bitkilerin kuraklık stresi altında fizyolojik ve biyokimyasal özelliklerindeki değişimler; A, prolin; B, MDA; C, yaprak relative su içeriği; değerler 3 tekerrür ortalaması±SD; farklı harflerle gösterilen ortalamalar arası fark önemli (LSD, P≤0.05)

and 4.0 µmol g^{1} FW in transgenic and WT plants respectively. Its production was raised to 3.45, and 4.1 µmol g^{1} FW in transgenic plants under 5 and 10 d respectively. However, MDA level was 4.6 and 4.9 µmol g^{1} FW in WT plants under same period of drought (Figure 1B). It also indicates the generation of ROS, superoxide radicals and hydrogen peroxide (Ashraf & Harris 2004). It protects the membranes and macromolecules that result in lowering the ion

leakage and transpiration losses and increasing water holding capacity. Statistical analysis showed that there is a significant difference for MDA accumulation F-test, **P<0.01 (Table 1). These results implied that the elevated level of small heat shock protein *in vivo* helps in efficient scavenging of ROS in transgenic plants which may be contributing to enhanceing the drought tolerance.

3.2. Over expression of GHSP26 improved the leaf relative water content (LRWC)

Leaf relative water content is directly linked to total cell size and balance between water availability to leaves and transpiration rate (Mao et al 2010). The Value of LRWC without drought stress was 72.9% and 69.7% for transgenic and WT plants respectively. Transgenic plants held 69% LRWC while WT held only 45% at 10 d drought stress (Figure 1C). With the application of 5 d drought, LRWC decreased significantly in WT plants (F-test, **P<0.01). ANOVA and testing of means for significant differences indicates that the transgenic plants were less damaged as compared to WT under drought stress (F-test, *P<0.05) (Table 1). Variation in LRWC directly influences the cell turgidity, opening, and closing of stomata and photosynthetic rate. Hadiarto & Tran (2011) suggested that drought responsive genes have a positive role to maintain the leaf relative water content in the rice plants. Verslues & Bray (2006) discussed the correlation between hormonal level and the accumulation of osmolytes which in turn affects the water potential and relative water content within plants under dehydration stress.

3.3. Photosynthesis, stomatal conductance and transpiration rate in transgenic plants

Drought stress leads to a substantial reduction in net photosynthesis, due to stomatal closure, which restricts the diffusion of CO_2 into the leaf or non-stomatal factors, such as inhibition of *RuBisCO* or ATP synthesis (Stepien & Johnson 2009). In our study, photosynthesis rate under irrigated phase was 9.04 µmol m^{-2} s^{-1} in transgenic and 5.32 µmol m^{-2} s^{-1} in WT plants. This was reduced to 5.82 and 3.08 µmol m^{-2} s^{-1} in transgenic plants and was 4.03 and 2.96 µmol m^{-2} s^{-1} in WT at 5 and 10 d drought stress, respectively (Figure 2A). This indicates that overexpressing *GHSP26* is maintaining the photosynthesis activity efficiently in transgenic plants under drought stress. ANOVA of the drought and the genotype variables and their interactions showed significant values F-test, **P<0.01 (Table 1).

Table 1- Analysis of variance (ANOVA) for physiological and biochemical parameters of transgenic and wild type plants under drought stress

Çizelge 1- Transgenik ve yabani tip (WT) bitkilerin kuraklık stresi altında fizyolojik ve biyokimyasal özelliklerindeki değişimlere ait varyans analizi sonuçları

Dependent variable	*Independent variable*								
	Drought stress			*Genotype*			*G×D*		
	SS	*MS*	*F*	*SS*	*MS*	*F*	*SS*	*MS*	*F*
Proline	5862	2931	2218**	2451	2451	1855**	541.95	270.98	205.0**
MDA	7.18	3.59	47.8**	7.70	7.70	102.4**	1.06	0.53	7.10**
LRWC	1488.7	744.33	10.3**	1093.1	1093.1	15.12**	1002.8	501.42	6.94*
PN	51.57	25.78	45.8**	15.99	15.99	28.40**	9.75	4.87	8.65**
g	10.64	5.32	79.9**	6.61	6.61	99.36**	2.52	1.26	19**
E	11.53	5.76	70.2**	3.87	3.87	47.22**	3.99	1.99	24.3**
OP	10.35	5.17	51**	0.05	0.05	0.53 ns	0.31	0.15	1.6ns
WP	0.47	0.23	23.2**	0.44	0.44	43.92**	0.02	0.01	1.3ns
F_V/F_M	55975	27987	40.2**	16272	16272	23.4**	17286	8643	12.4**

*, significant at P≤0.05; **, significant at P≤0.05; ns, non-significant; D, drought stress; G, genotype; G×D, interaction; MDA, malondialdehyde; LRWC, leaf relative water content; E, transpiration; OP, osmotic potential; WP, water potential

Small heat shock proteins play a critical role in cellular protection under drought stress and maintain water use efficiency (Gallé et al 2007). This may be a result of stomatal closure or degradation of the photosynthetic apparatus under stress. This study reports the positive response of the transgene for stomatal conductance which is enhanced under drought stress (Figure 2B). Different variables and their interaction showed significance for stomatal conductance (Table 1). Transpiration rate was 3.74 mmol m^{-2} s^{-1} in transgenic plants without drought stress and decreased to 2.0 and 1.0 mmol m^{-2} s^{-1} as the plants were subjected to 5 and 10 d of drought, respectively. Likewise in WT plants, the transpiration rate was 2.5 mmol m^{-2} s^{-1} at 0 d and decreased to 0.8 and 0.93 mmol m^{-2} s^{-1} at 5 d and 10 d of stress, respectively (Figure 2C). A significant difference in the transpiration rate was not observed under 10 d drought stresses. This may be due to the fact that negative water potential in the root generates a signal to shoot e.g. abscisic acid which has been suggested to be the operating mechanism for transpiration activity (Ashraf & Harris 2004). ANOVA determined a highly significant difference among the variables and their interactions F-test, **P<0.01 (Table 1). Transpiration activity may also be regulated by the waxy layer on the leaf surface which ultimately helps to monitor the stomatal aperture for opening and closing under environmental stresses.

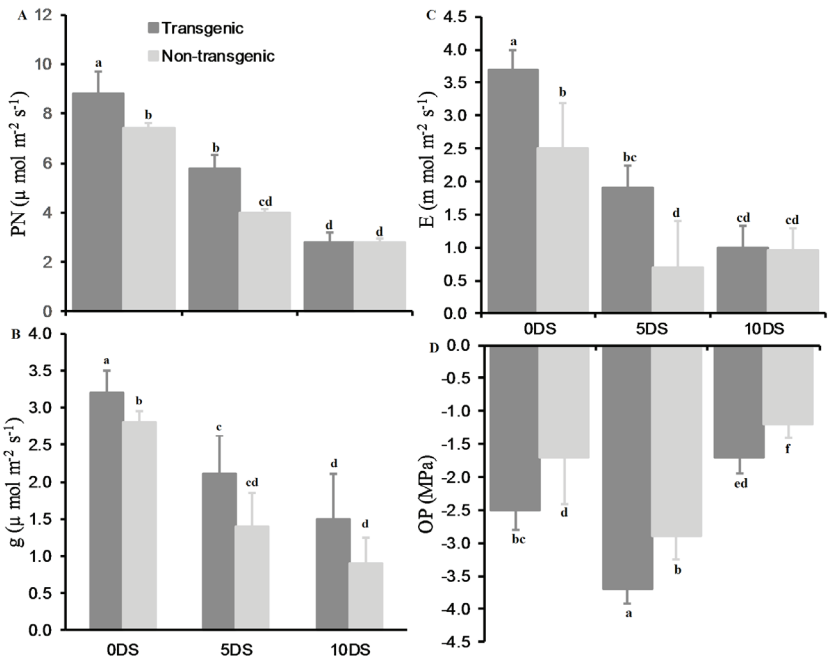

Figure 2- Physiological measurements of transgenic and WT plants under control and drought stress; A, photosynthetic rate; B, stomatal conductance; C, transpiration rate; D, osmotic potential; each value represents the mean±SD of three replicates; values with the different letter are significantly different according to LSD tests (P<0.05)

Şekil 2- Transgenik ve yabani tip (WT) bitkilerin kuraklık stresi altında fizyolojik özelliklerindeki değişimler; A, fotosentez oranı; B, stoma iletkenliği; C, terleme oranı; D, ozmotik potansiyel; değerler 3 tekerrür ortalaması±SD; farklı harflerle gösterilen ortalamalar arası fark önemli (LSD, P≤0.05)

The consequence of water stress on the stability of photosynthetic apparatus determines the chlorophyll fluorescence ratio (F_v/F_m) which is an indicator of the photochemical efficiency of Photosystem II (Efeoglu & Terzioglu 2009; Stepien & Johnson 2009). Photochemical efficiency (F_v/F_m ratio) of transgenic and WT plants differ significantly (F-test, *P<0.05) (Table 1). These results, in correlation with previous reports, confirmed that the drought stress induces the reduction in LRWC, photosynthesis, stomatal conductance and transpiration rate, but in the present study, these parameters were not reduced due to the fact that the transgene has positive impact. Reduction in water flow due to drought may cause the decline of LRWC that would result in stomatal closure to maintain water status which regulates the physiological parameters (Chaves et al 2009).

3.4. Transgenic plants over expressing GHSP26 improved the osmotic and water potential

Osmotic stress generates adverse modifications in cellular apparatus (Bartels & Sunkar 2005). The addition of an osmoprotectant to the cellular machinery is a helpful approach to improving the plants tolerance to osmotic stress (Yue et al 2012). The OP was reported as 2.5 and 1.9 (-MPa) in transgenic and WT plants under 0 d stress. But as the plants were subjected to 5 and 10 d stress, the OP was 3.75 and 1.75 (-MPa) in transgenic and 2.9 and 1.1 (-MPa) in WT plants respectively (Figure 2D). Our results strongly indicate that the improved OP and WP in transgenic plants may be due to over-expression of the transgene. This improvement usually indicates higher water retention capacity and a lower rate of water loss with higher water use efficiency (Mao et al 2010). This is in correlation to the report supported by Flexas & Medrano (2002) that leaf turgor maintains the osmotic and water potential under salt and dehydration stress, which will regulate the carbon dioxide within the cellular aperture. The photosynthetic activity is then regulated by the turgor effects, which are correlated with plant species, an age of plants and duration or nature of stress.

3.5. Over expression of GHSP26 and drought stress linked genes in transgenic cotton

The drought-related genes *LHCP-PSII, Gh-POD, Gh-PIP and Gh-RuBisCO* showed similar expression under 0 d in transgenic and WT plants, but their relative fold expressions were significantly higher under water deficiency in transgenic as compared to WT plants (Figure 3A-C and F). The light-harvesting chlorophyll a/b-binding (*LHCB*) and *Gh*-RuBisCO proteins are solely positioned in the light harvesting pigmented protein complexes of PSI and PSII and are involved in the first major step of carbon fixation of Calvin cycle (de Montaigu et al 2010; Pruneda-Paz & Kay 2010). Existing studies indicate that the down-regulation of these genes reduced the effectiveness of plant tolerance to environmental stresses resulting in inferior productivity (Xu et al 2012). Hence, elevated expression shows the comparative stability and proper regulation of the chloroplast and other membrane-bound organelles which directly play vital roles in photosynthesis. Plants use the antioxidant mechanism as a protective strategy to overcome the oxidative stress damage by up-regulation of downstream antioxidant enzymes.

The present study indicates a significant increase in POD activity in transgenic plants under drought stress. Some of the previous studies have also reported similar results in other drought tolerant crops, like *Helianthus annuus* L. (Gunes et al 2008) and Brassica *campestris* L. (Jahangir et al 2009). Expression of *LEA5 and Gh-TPS,* showed interesting results as the expression of these genes increased during stress, which is an indication of osmotic tension (Kosmas et al 2006). However, the expression level of these genes decreased in transgenic plants under drought stress (Figure 3D-E), which may show that they are sensitive to water deficient conditions and relatively lower expression under increasing drought shows reduction in tolerance against stress. Relative fold expression of transgene *GHSP26* was increased under drought stress and maximum expression was observed at 10 d (33 fold expression) (Figure 3G). Elevated gene expression is a characteristic feature of small heat shock protein due to its chaperone

activity under abiotic stress. The relative fold expression of *GHSP26* was increased in transgenic plants in under different developmental stages in T_1 generation under drought stress conditions (Shamim et al 2013). Waters et al (2008) reported the high level (100-400 fold) of small heat shock protein expression under abiotic stress which was very mild in the unstressed plants in *Arabidopsis thaliana.* Under stress conditions, the chaperone function of small heat shock protein increased to prevent irreversible aggregation and to re-solubilize proteins that have already aggregated. Our previous report also confirmed the integration and expression of the transgene in transgenic plants (Sarwar et al 2014). Analysis or the parameters presented in this study proved that the over expression of *GHSP26* improved the drought tolerance efficiency of transgenic plants.

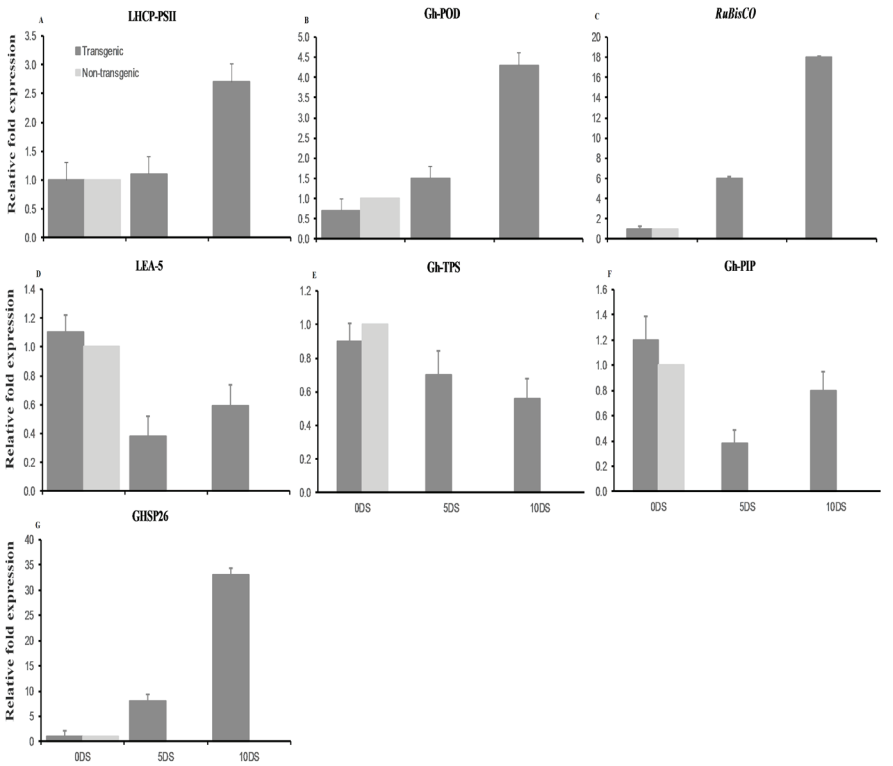

Figure 3- Quantitative real time RT-PCR expression analyses of transgene and drought linked genes; A, photosynthesis-II chlorophyll A/B-binding protein (*LHCP PS-ll*); B, peroxidase (*Gh-POD*); C, ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene (rubisco); D, late embryogenesis-abundant protein (*Gh-Lea5 D*); E, trehalose-6 phosphate (*Gh-TPS*); F, plasma intrinsic protein (Gh-PIP2); G, heat shock protein gene *GHSP26*; means were generated from three independent measurements; line on the bars indicate standard errors

Şekil 3- Transgen ve kuraklık bağlantılı genlerin kantitatif RT-PCR ekspresyon analizleri; A, fotosentez-II klorofil A/B-bağlı protein (LHCP PS-ll); B, peroksidaz (Gh-POD); C, ribuloz-1,5-bisfosfat karboksilaz/oksijenaz küçük altünite geni (rubisco); D, geç embiryogenesis-yoğun protein (Gh-Lea5 D); E, trehaloz-6 fosfat (Gh-TPS); F, plazma içi protein (Gh-PIP2); G, ısı şoku protein geni GHSP26; değerler üç tekerrür ortalaması olup çubuklar üzerindeki çizgiler standart hatadır

4. Conclusions

The results of this study support the higher expression of the heat shock protein gene in transgenic cotton plants under drought stress. The transgenic plants maintained an efficient photosynthesis rate, higher osmotic and water potential, lower ion leakage and malondialdehyde activity and increased accumulation of proline content. In this way, the transgenic plants had the better performance at the physiological, biochemical and molecular levels.

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