Pak. J. Agri. Sci., Vol. 51(4), 935-941; 2014
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

INTEGRATION AND EXPRESSION OF HEAT SHOCK PROTEIN GENE IN SEGREGATING POPULATION OF TRANSGENIC COTTON FOR DROUGHT TOLERANCE

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Drought is one of the major abiotic stresses to restrict cotton growth and productivity. Heat shock proteins are involved in cellular shielding of plant cells and tissues affected by dehydration. In this study, T_2 segregating population of transgenic cotton containing small heat shock protein gene was compared with wild types under five and ten days drought stress. Seed germination was not affected significantly in both the lines. Amplification of 260bp *GHSP26* gene through PCR and transgene integration was observed through Southern blot and fluorescence *in-situ* hybridization, confirmed the integration of 3 copies of transgene in the transgenic plants. Total leaf area and transpirational water loss in transgenic plants were significantly different from wild type. Ten days drought stress caused 58% ion outflow from wild type and only 40% from transgenic lines and biomass was also significantly reduced in wild type plants. Plants of both the lines were not affected for root shoot ratio under drought stress. Statistical analysis proved that chlorophyll content was maintained in transgenic plants, but it was reduced in wild type. These results showed that transgenic plants sustained growth under drought stress, so may be used for classical breeding to improve crops.
Keywords: Heat shock protein gene, *GHSP26*, transgenic plants, cotton, drought tolerance

INTRODUCTION

Cotton (*Gossypium*), a key cash and main fiber crop that has been estimated to put 15–20 billion US$ to the world's agricultural financial system. Tetraploid cotton (*Gossypium hirsutum* L.)is the most widely grown species around the world; however, diploid *Gossypium arboreum* is not only the reservoir of important and resistant genes, but it is well adapted to dry land and need low inputs. These characteristics have marked it an invaluable gene pool to improve modern cotton cultivars (Liu *et al.,* 2006).

The drought is considered as moderate to extensive loss of water from different plant parts affecting various morphological, physiological and biochemical processes (Peng *et al.,* 2014). These alterations would result as stomatal closure, disruption of metabolism and cell structure, cessation of enzyme catalyzed reactions, diminished leaf water potential and turgor loss, arrest of photosynthesis and yield losses (Farooq *et al.,* 2010). Plants have evolved a series of enzymatic and non-enzymatic systems at molecular level to retain the cellular activities normal and to maintain the whole plant integrity (Mao *et al.,* 2010). Heat-shock proteins *(HSPs)* are among stress responsive proteins, which protect the cellular activities during drought stress (Wang *et al.,* 2004). Breeders need an efficient gene system in order to develop the tolerant cultivars (Ahmad *et al.,* 2009; Nawaz *et al.,* 2013). Production of transgenic plants overexpressing the genes which are able to tolerate this environmental threat is the potential technology to overcome this problem (Ai-Ke *et al.,* 2009). These studies entail the stable integration and expression of transgene to the next generations. The integration and expression level of pattern of inheritance of transgene into the segregating populations generally show extensive variation between transgenic plants carrying same genes and constructs (Tizaoui and Kchouk, 2012).

The present study was conducted to verify the potential role of transgenic *G. hirsutum* L. T_2 progeny containing small Heat Shock Protein gene *GHSP26*. Physiological, biochemical and molecular attributes were determined under 5 and 10 day drought stress (DS).

MATERIALS AND METHODS

***Planting material and growth conditions*:** Seeds of transgenic T_2 progeny of *G. hirsutum* L. were obtained from seed bank of Centre of Excellence in Molecular Biology (CEMB) previously transformed with *GHSP26* gene, which has been isolated from *G. arboreum* (Maqbool *et al.,* 2007 and 2010). Seed delinting was done with concentrated H_2SO_4 and sterilized with $HgCl_2$ for 5 minutes, followed by washings with autoclaved water. Sowing was done in pots containing a soil mixture as prepared by Rashid *et al.* (2008). Pots were kept in green house where temperature and light intensity were maintained at 30±2°C and (250-300 µmol m^-

$^{2}s^{-1}$), respectively. Germination percentage was recorded and seedlings were thinned to single plant per pot. There were 5 plants in each replicate and three replicates for each genotype were maintained. A total of 30 transgenic plants were maintained in the pots.

Amplification and Integration of Transgene in T_2 Progeny:

***Polymerase chain reaction (PCR)*:** Transgene amplification in T_2 progeny was analyzed with PCR by using gene specific primers F-AGGCCTAAACGGTTGGCTAT, R-CCATCTTTGATGTCCCAAGG. Sampling was done by randomly selecting the leaves of 5 transgenic plants and genomic DNA was isolated by using GeneJET plant genomic DNA Purification kit (Fermentas). PCR was performed in a volume of 25µl by using a 2X Fermentas master mix according to manufacturer protocol. Amplification steps included denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 30 seconds and a final extension of 10 min at 72°C and then at 4°C. The products were electrophoresed in 1.5% agarose gel and visualized under UV light by ethidium bromide staining.

***Southern blot hybridization*:** PCR positive plants were confirmed for transgene integration through Southern blot as described by Southern (1975). Genomic DNA (10µg) of 5 transgenic and Wilde Type (WT) plants was digested with enzymes NcoI, BglII, separated on 0.8% agarose gel, blotted onto nylon membrane (Hybond N, Amersham) and UV cross-linked. The amplified DNA fragment of *GHSP26* was eluted using DNA Extraction Kit and probe was labelled with Biotin DecaLabel™ DNA Labeling Kit (Fermentas). Hybridization and subsequent luminescent detection were performed using the Biotin Chromogenic Detection Kit (Fermentas) according to the manufacturer's instructions.

***Fluorescence in-situ hybridization (FISH)*:** Transgenic plants positive for PCR and Southern blot were subjected to fluorescence *in-situ* hybridization (FISH) to determine the copy number of transgene at the cellular level. Probe preparation was done by using DIG DNA Labeling and Detection Kit *(*Roche*)* according to manufacturer's instructions. Chromosomes were spread on a microscopic glass slide with a drop of fixative and then air-dried. Slides were observed under phase contrast microscope (Carl Zeiss AXIO 100), dehydrated in 70%, 95% and 100% ethanol respectively, (each for 5 minutes) and then stored at room temperature. The fluorescent signals were detected by Fluorescent microscope (Carl Zeiss AXIO 100), captured by CCD camera and analyzed by Cytovision Applied Imaging Systems software *Genus 3.7*.

Application and study of drought stress:

Forty days old Wild type (WT) and transgenic plants (30 transgenic and 15 WT plants) were subjected to drought stress by stopping the irrigation for 5 and 10 days (5DS, 10DS) as suggested by Yue *et al.* (2012). Samples taken under normal irrigation conditions (0DS) were considered as control. Growth, physiological, molecular and biochemical responses were observed and compared.

***Total leaf area*:** Total leaf area of both WT and transgenic plants was measured. Digital photographs (canon, USA) were taken with default parameters for control and drought stressed leaf and processed with ImageJ Software to calculate the total leaf area (cm^2) as suggested by Igathinathane *et al.* (2008).

***Plant height, transpirational water loss, fresh and dry biomass and root shoot ratio*:** Plants were harvested randomly under normal (control) condition and 5 and 10DS. Unsoiled the roots and length (cm) and fresh weight (FW g) of whole plant were recorded. Then plants were placed on filter paper to dry out the water content at room temperature and weighed after 24 hours. Transpirational water loss was calculated on the basis of difference in plant's fresh and wilted weight after 24 hours. Then the plants were wrapped in brown paper bags and dried for 48 hours at 80°C and dry weight was recorded (DW g). The Percent reduction in biomass was calculated as:

% Reduction in biomass =

(Fresh biomass – Dry biomass x 100)/Fresh Biomass

Root and shoot of dried plants were separated and weighed to measure the root shoot ratio as

$$Root/Shoot\ Ratio = (DW\ of\ Roots/DW\ of\ top\ of\ Plant\ Biomass)$$

Cell membrane stability (ion leakage %): Cell membrane stability (CMS) was calculated as described by Sullivan (1972). Young leaves from WT and transgenic plants were cut into 1 cm^2 discs and 0.5 g of each sample was placed into test tubes containing 20 ml of autoclaved distilled water Tubes were vortexed for 3 sec and electric conductivity (EC_0) was recorded. Then the tubes were incubated at 4°C for 24 hrs and electric conductivity (EC_1) was noticed. Samples were then autoclaved at 120°C for 15 min and electric conductivity (EC_2) was measured at room temperature. CMS was calculated as:

$$CMS(\%) = (EC1 - EC0)/(EC2 - EC0) \times 100$$

***Chlorophyll content*:** Photosynthetic pigments were determined according to Arnon and Whatley (1949). Extract was prepared by grinding 100 mg of fresh leaves (WT and transgenic) with 10 ml of 80% acetone. Homogenate was kept in dark for overnight at room temperature and then centrifuged at 800 rpm for 15 min. Absorbance of the extract was read at 663 and 645 nm using a UV-Visible spectrophotometer (spectra Max plus: molecular devices, USA). Concentration of a, b and total chlorophyll (mg g^{-1} FW) was calculated using Arnon's equations.

***Statistical analysis*:** All experimental data are the mean of three independent replicates, and results were determined using analysis of variance (ANOVA) via *Statistix* software.

Variations among treatment mean were compared using least significant difference (LSD) test ($P < 0.05$).

RESULTS AND DISCUSSION

***Seed germination*:** To estimate the applicability of transgenic breeding, an efficient system of plant regeneration is required (Karatas *et al.,* 2013). In this study, seeds from transgenic plants showed 36-91%, while WT non-transgenic seeds (CIM-496) showed 40-80% germination. Germination rate of transgenic seeds was higher than WT, but the difference was not significant (F-test, P >0.05) (Data not shown). Low germination might be due to dormancy or quality of seeds.

***Amplification and integration of transgene in T_2 progeny*:** PCR is a reliable system to determine the presence or absence of a specific DNA portion in plant genomic DNA (Nemanja *et al.,* 2013). With gene specific primers, genomic DNA of 5 transgenic plants amplified the product of 260 bp while WT showed no amplification (Fig. 1A). Transgenic plants displayed hybridization signals with probe via Southern blot analysis (Fig. 1B) and FISH analysis also confirmed the 3 copies of *GHSP26* transgene in transgenic plants during metaphase stage at the cellular level (Fig. 1C). No signals were observed in the samples taken from the wild type (WT) (Fig. 1D). These analyses confirmed the amplification and integration of transgene into the genome of the T_2 progeny of *G. hirsutum* L. Jiang *et al*. (2012) also confirmed the integration and over expression of *OSRIP18* gene through PCR, RT-PCR, Southern and Northern blot in transgenic rice plants for drought and salinity tolerance. Moreover, molecular mechanisms have extensively been studied to understand the systems that ameliorate the impact of drought stress on plants productivity (Osakabe *et al.,* 2014).

***Total leaf area and transpirational water loss*:** The transgenic plants containing *GHSP26* occupied larger leaf area as put side by side to WT as analyzed by image J software (Fig. 2A). Statistical study suggested the significant difference (*$P < 0.05$) between transgenic and wild type (non-transgenic) plants under the drought stress (Table 1).

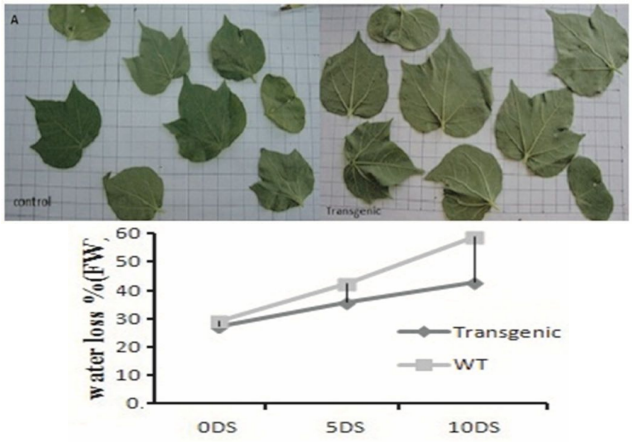

Figure 2. Comparison of total leaf area and transpirational water loss of transgenic and WT plants under drought stress. (A) Total leaf area of detached leaves on a 1cm^2 blocks (B) Transpirational water loss. Each value represents the mean ± SD of three replicate, Values with the same letter were not significantly different according to LSD tests ($P<0.05$).

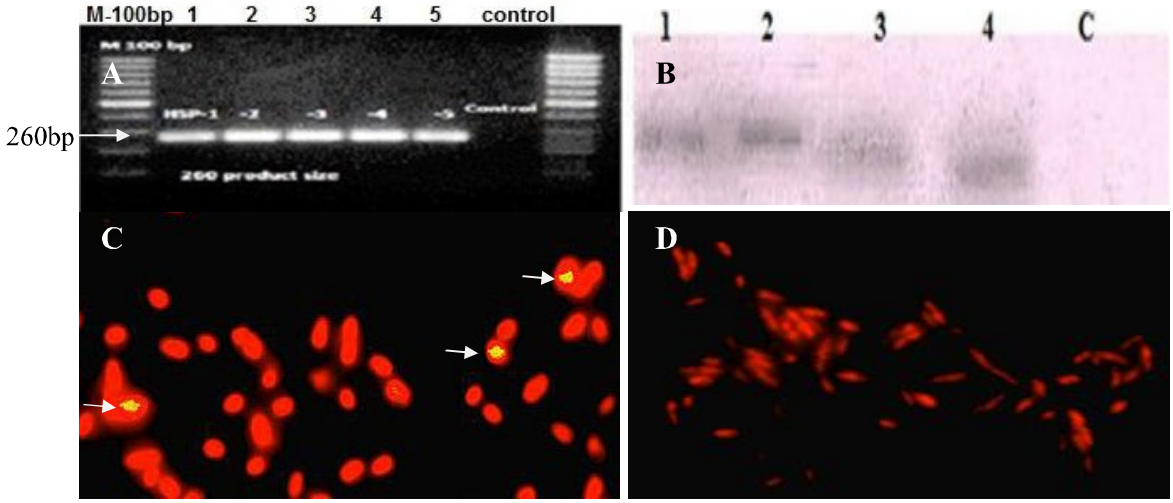

Figure 1. PCR, Southern blot and FISH analyses of *GHSP26* gene in progeny of transgenic Plants. (A) PCR with gene specific primers. Lane1-5: transgenic plants, lane control: WT, LaneM: DNA marker. (B) Southern blot hybridization. Lane1-4: transgenic plants, LaneC: controls (WT), (C) FISH analysis. Arrows indicates the transgene at Metaphase, (D): WT in FISH analyses.

Table 1. Analysis of variance (ANOVA) for growth, physiological and biochemical parameters under drought stress

Dependent Variables	Independent Variables									
	Drought Stress			Genotype			G×D			CV%
	SS	MS	F	SS	MS	F	SS	MS	F	
TLA	1670.10	835.06	6.76*	2090.89	2090.89	16.92 *	294.78	147.39	1.19 ^{NS}	13.36
PH	6.11	3.05	13.57**	3.60	3.60	15.98**	0.35	0.17	0.78*	5.10
FB	4.11	2.05	3.92*	31.52	31.52	60.09*	5.97	2.98	5.70**	15.11
DB	2.33	1.16	20.60**	2.21	2.21	39.14**	1.55	0.77	13.71**	15.27
RSR	0.15	0.07	22.88**	0.04	0.00	0.16 *NS*	0.03	0.01	4.93*	14.77
CMS	2525.50	1262.8	19.36**	449.19	449.19	6.89*	160.09	80.05	1.23 *	24.73
Total Chl	55.22	27.61	35.72**	12.21	12.21	15.80**	1.33	0.66	0.86 ^{NS}	10.36

*denotes significant differences at 5% probability level (P≤0.05).

**denotes significant differences at 1% probability level (P≤0.01), NS= non-significant.

Drought stress (D), genotype (G) and their interaction (G×D) for PH (plant height), FB (fresh biomass), DB (dry biomass), RSR (root shoot ratio), TLA (total leaf area), CMS (cell membrane stability) and total chlorophyll.

Total leaf area of transgenic and non-transgenic plants under control condition also showed the significant difference, that indicates the variable vegetative growth of both the genotypes. Means comparison analysis under drought stress pointed out that after 5 day drought stress, the difference in the total leaf area was significant at *P <0.05 at 5 and 10 days' drought stress (Table 2). Fiorani *et al*. (2005) tested *AtAOX1a*-transgenic Arabidopsis plants at low temperature and observed 30% increased leaf area for over expressing line compared with WT plants. Similarly Brini *et al*. (2007) also reported the increase in leaf area in transgenic plants over expressing *Dhn-5* gene in Arabidopsis.

With the implication of drought stress, transpirational water losses from detached leaves indicate that transgenic plants over expressing *GHSP26* gene vanished water slower when compared to WT and showed different degrees of wilting. Non-transgenic (WT) plants wilted severely while transgenic plants wilted only partially. On the basis of fresh weight, 42% water was lost through transpiration in WT plants while that was 38% in transgenic plants after 5 day stress. This indicator was raised from 42 to 58% in WT and 38 to 40% in transgenic plants after 10 days of stress treatment. So the WT plants were transpiring more when subjected to drought stress (Fig. 2B). The results implied that the elevated level of small heat shock protein may help in efficient scavenging of ROS in transgenic plants that protect the membranes and macromolecules resulting in lowering the ion leakage and transpirational losses and increasing water holding capacity which may possibly be contributed to enhance drought tolerance (Masle *et al*., 2005).

***Plant height, fresh and dry biomass and root shoot ratio*:** Plant growth including height of the plants is affected by the drought stress. A significant effect of drought stress was observed on the plants' height in both the genotypes, F-test p≤0.01 (Table 1). Interaction study between drought stress and genotypes is also significant at *P <0.05 and there was a significant statistics under drought stress and normal conditions between transgenic and WT plants (Table 2). Transgenic plants were also significantly differ from the WT when mean performance was compared at *P <0.05 under drought stress (Table 3). Trujillo *et al*. (2008) reported a number of improved morphological factors in sugarcane transgenic plants over-expressing *SodERF3* under different drought and salt stress conditions.

Table 2. Mean performance comparison for growth, physiological and biochemical parameters under different drought conditions

Drought Stress	Genotype	TLA (cm^2)	PH (cm)	FB (g)	DB (g)	RSR	CMI (%)	Total chl ($mg\ g^{-1}$)
0DS	Transgenic	100.30a	8.75bc	6.1ab	1.10cd	0.30b	18.37d	11.74a
	WT	96.67ab	8.30c	4.7c	0.99d	0.26b	19.95d	9.33b
5DS	Transgenic	77.33b	10.20a	5.4bc	1.90b	0.36b	24.01cd	9.28b
	WT	85.67ab	9.10bc	3.1d	1.50bc	0.49a	37.68bc	8.03bc
10DS	Transgenic	85.00ab	10.40a	6.8a	2.70a	0.53a	40.63b	6.89cd
	WT	54.33c	9.30b	2.6d	1.20cd	0.48a	55.38a	5.62d

Means followed by different alphabet are different at 5% level of significance based on least significant difference (LSD), while those followed by same letters are statistically non-significant.

Table 3. Mean performance comparison between transgenic and wild type plants for growth, physiological and biochemical parameters under drought stress.

Traits	TLA (cm^2)	PH (cm)	FB (g)	DB (g)	Biomass Reduction (%)	RSR	CMI (%)	Total chl ($mg\ g^{-1}$)
Transgenic	94.02a	9.76a	6.12a	1.91a	60a	0.39a	27.7b	9.30a
Wild Type	72.44b	8.88b	3.47b	1.21b	56a	0.40a	37.7a	7.66b

Means followed by different alphabet are different at 5% level of significance based on least significant difference test (LSD), while those followed by same letters are statistically non-significant.

Biomass is one of the indicators for drought tolerance in plants. Fresh biomass was measured next to the harvest of the plants. Barely significant difference was observed for FW and DW of transgenic and WT plants under normal conditions (means differ). However, as the stress prevailed, variable behavior was observed such as, statistically significant difference was found between both the genotypes under 5 and 10DS stress level in means for FW *(*P≤0.05)* and the similar behavior was found for DW *(**P≤0.01).* Interaction study (G×D) between the cotton genotypes and the drought stress are highly significant at *(**P≤0.01)* for fresh and dry biomass, that indicates the sensitivity of the biomass toward the harsh environmental condition irrespective of genotype (Table 1). Peng *et al.* (2014) observed the expression of *Hrip1* improving a number of bioassays including plant biomass in the transgenic plants under salt and drought stress.

Means performance comparison of different drought stress periods (5DS, 10DS) also showed the significant FB and DB (p≤0.05) (Table 2). Means performance between the transgenic and WT plants for FB and DB was significant to each other at (p≤0.05) but the % decrease from FB to DB in both the transgenic and WT plants is not significant (Table 3). One of the other similar studies in cotton, performed by Pasapula *et al*. (2011) showed that transgenic plants over expressing *AVP1* produced higher fresh and dry biomass than the WT .

Root to shoot ratio was measured to find out the interaction between drought stress and growth of transgenic and WT plants. Statistical analysis of data showed that drought stress had no significant *(F-test, ^{NS}P <0.05)* effect on the root shoot ratio of transgenic plants over-expressing *GHSP26* and WT; however, difference was observed in mean values (Table 1).

***Cell membrane stability*:** The degree of CMS is the measurements of electrolyte outflow from cells and it is widely used to differentiate stress tolerant and susceptible cultivars (Farooq and Azam, 2006). A mild cell membrane injury was observed in transgenic plants after drought stress but that was significantly lower (F-test, **P <0.01) than in the WT (Table 1). Drought stress at 5DS showed significant results (**P <0.05*) for the performance of transgenic plants whereas 10DS drought stress caused 55% ion outflow from WT, whereas the transgenic lines caused only 40% (Table 2). Higher ion leakage in wild plants may be due to excessive accumulation of ROS and peroxidation of lipids membrane. Lipid peroxidation product (MDA) was produced to a higher level when plants are exposed to a highly osmotic environment. Its accumulation also indicates the ROS generation, superoxide radicals and hydrogen peroxide. Ability of transgenic plants to maintain physiological behavior under stress could be related to maintain cell turgidity and water retention (Table 3). The mean comparison of replication and all other means differ significantly from each other, firmly indicated that drought stress damages were lower in transgenic plants as compared to WT (F-test, *P <0.05). The stable expression of small heat shock protein in vivo helps in efficient scavenging of ROS in transgenic cotton plants protects the membranes and macromolecules that results in lower ion leakage, higher water holding capacity and possibly be contributed to enhance the drought tolerance. Lal *et al.* (2008) reported the over expression of *HVA1* in mulberry under drought stress with less ion flow and even membrane stability. It must be well-known that humidity and hydration condition of plant are the factors that affect the physiological performance.

***Chlorophyll content*:** Chlorophyll, the green pigment common to all photosynthetic cells, absorbs wavelength of visible light except the green. There is strong evidence that drought stress affects the chlorophyll and carotenoid (Mafakheri *et al.,* 2010). In this study drought stress imposed the significant decrease in total chlorophyll content. ANOVA confirmed the higher significance of the drought application and the response of transgenic and WT genotypes at F-test p≤0.01 (Table 1). However, the G×D interaction is non-significant meaning that the two variables are working independently. The enhanced expression of the transgene at biochemical level regulates the stomatal closure and protects the photosystem to acclimatize the plants under drought and other abiotic stresses (Hozain *et al.,* 2012). Mean performance comparison for chlorophyll content and drought stress showed the significant results at p≤0.05 (Table 2). Mean performance comparison of transgenic plants with WT showed the significant difference between the chlorophyll content in both the lines at p≤0.05 (Table 3). The means comparison of replication and all other means differ significantly from each other, firmly indicate that the reduction of chlorophyll was lower in transgenic plants (F-test, **P <0.05*). Transgenic plants showed better performance in this study, which is according to the reports in other species such as Brassica (Hyoshin and Jinki, 2004)

and cotton (Shamim *et al.,* 2013). Similarly Zhu *et al.* (2013) analyzed the transcriptome of cotton (*G. hirsutum* L.) and observed a number of transcription factors associated to different functional groups for multiple abiotic stress tolerance.

***Conclusion*:** Physiological, biochemical and molecular responses of transgenic cotton plants under drought stress, confirmed the higher expression of heat shock protein gene *GHSP26* in segregating population and the transgenic plants are tolerating drought stress as compared to the wild type. Further study in field conditions for evaluation of performance and selection of homozygous lines would be suitable for future segregating generations.

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