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Research Article
Heat Shock Proteins Expression Is Regulated by Promoter CpG Methylation/demethylation under Heat Stress in Wheat Varieties

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Abstract

Background and Objective: Heat shock proteins are induced by high temperature and other environmental stimuli to protect cellular proteins. Despite extensive research on the molecular response to heat stress, the effect of high temperatures on genes and pathways remains unclear. This study investigated the expression of the *HSP17* gene in nine Egyptian wheat varieties and the role of *HSP17* promoter CpG methylation in the regulation of *HSP17* under high temperature. **Materials and Methods:** The *HSP17* expression was investigated by using semi-quantitative PCR analysis. Methylation at the *HSP17* promoter proximal region was analyzed using bisulphite sequencing and CpG viewer software. **Results:** Under normal conditions, *HSP17* and methyltransferase 3 (*MET3*) exhibited similar expression levels in the 9 studied varieties. After exposure to high temperature, the expression level of *HSP17* in Giza155 was barely detected. Among the nine varieties, the expression level of *HSP17* was highest in Giza168 (11.3 folds of Giza155). Analysis of methylation of 14 CpG islands at the *HSP17* proximal promoter sequence showed that methylation of 10 CpG islands differed only by 10-20%, whereas methylation at the other 4 CpGs differed by 56.7-60%. The high expression of *HSP17* in Giza168 in response to high temperature was associated with low methylation of four CpGs and low *MET3* expression, whereas low expression of *HSP17* in Giza155 was associated with high methylation and high *MET3* expression. **Conclusion:** The results can aid the development of next-generation approaches to the evaluation of commercial wheat varieties and the development of next-generation approaches to plant breeding employing epiallele integration.

Key words: Wheat, high temperature, methylation, proteins, DNA-methyltransferases, epiallele

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Elevated temperature represents a major abiotic challenge for plants, especially in association with drought by negatively affecting plant growth, development and production[1-3]. One approach by which plants respond to elevated temperature is through modulation of Heat Shock Proteins (HSPs) expression[4]. The HSPs are organized in a gene family, which is induced by elevated temperature and other stresses. The HSPs are found in all living forms and help proteins to maintain their functional structure in the presence of increased temperatures[5,6]. The chaperone activities of recombinant HSPs in wheat (*Triticum aestivum*) differ. For example, the activity of TaHsp16.9C-I, which maintains the functional structure of malate dehydrogenase (prevent aggregation) is higher under heat stress than that of TaHsp17.8C-II pointing to substrate-chaperone specificity[7]. In addition to maintaining protein functional structure, HSPs have other functions during plant development[8,9] and fruit ripening in *Arabidopsis* and tomato[10-12].

The HSPs are classified into 5 types based in their size: HSP100, HSP90, HSP70, HSP60 and small HSPs[13]. The HSPs are encoded by a multigene family in plants. The tomato HSP family contains 33 genes[14,15]. Although HSPs have high sequence similarity, their gene expression patterns differ providing evidence of their complex function[16-18]. The induction of *HSP* gene expression (HSP90 and HSP70) under heat stress has been reported in several plants such as; *Salix suchowensis*[19], *T. aestivum*[3], *Panicum virgatum*[20] and *Camellia sinensis*[21]. In *Arabidopsis*, a high level of HSP90 expression was detected in response to salinity, high/low temperatures and heavy metals[22,23]. Transfer of alfalfa HSP70 and its overexpression improved transgenic plant tolerance to salinity, drought and heat stress[19,24]. The expression of various proteins including S-adenosylmethionine synthetase, glutamine synthetase and polyphenol oxidase, in thermotolerant tomato varieties was higher than that in thermosensitive tomato varieties[25].

DNA methylation, which involved the addition of a methyl group at the cytosine C5 position is a well-studied epigenetic mechanism that affects plant growth and development[26]. In plants, methylation occurs at CpG, CpNpG and CpNpN nucleotide sequences (N = A, T or C)[27]. CpG, CpNpG and CpNpN are methylated by DNA methyltransferase I (MET1)[28,29], chromomethyltransferase 3[30] and domains rearranged methyltransferase 2, respectively[31]. DNA methylation regulated a wide range of plant biological functions from plant growth, differentiation, development and metabolism[32,33] to senescence and apoptosis [34]. DNA methylation also regulated the adaptation of plants to variations in environmental conditions through epigenetic memory[35] and plant's responses to abiotic factors[36]. Methylation had been reported not only at promoters, but also at coding (gene body) DNA sequences, where it contributed to the regulation of gene expression[37].

Hypermethylation of promoters, heterochromatin, repetitive DNA and non-coding RNA have all been correlated with gene silencing[27,29,38]. Gene silencing caused by promoter DNA methylation was discovered in tobacco plants containing kanamycin and hygromycin resistance marker genes. These two transgenes were suppressed by promoter methylation[39,40]. Gene suppression by promoter methylation at the transcription level is known as transcriptional gene silencing. Previous research reported that repression of the NOS promoter was correlated with the level of DNA methylation. In tobacco mosaic virus, gene silencing resulted from promoter hypermethylation at CpG, CpNpG and CpNpN sites[40,41]. In potato, promoter of granule bound starch synthase I gene hypermethylation at CpG and CpNpN sites was correlated with transcriptional gene silencing[42]. In *Arabidopsis*, in an *in vitro* study using callus and liquid culture, the expression of many genes was affected by promoter methylation. MAPK12, BXL1 and GSTU10 were repressed by hypermethylation of CpG islands that was carried out by MET1 and domains re-arranged methyltransferase 2 (DRM-2)[43].

Abiotic stresses have a complex impact on DNA methylation causing hypo or hypermethylation at different genomic sequences. They can cause hyper or hypomethylation of promoter regions and gene body sequences. In addition, abiotic stresses may change the methylation status and activity of transposable elements[44]. In *Arabidopsis*, a genome-wide study of the effect of polyethylene glycol (PEG)-induced drought on global gene expression showed that PEG led to pronounced hypermethylation of drought-responsive genes close to the Transcription Start Site (TSS)[45]. In another study, that methylation of DNA improved adaptation to stress[46]. In the study, mangrove plants grown in salt conditions were smaller and exhibited global hypomethylation of DNA when compared to plants grown under freshwater conditions. In rice, differential DNA methylation were associated with a faster response of the rice genome to drought with drought-tolerant genotypes showing a more rapid response to drought stress due to methylation differences correlated with changes in gene expression levels[47]. Some of these drought-induced methylation marks remained after removal of the stress[47]. Low humidity, another form of drought affected

de novo methylation associated with repression of genes controlling stomata development and culminated in a lower stomata number in *Arabidopsis*[48,49].

Recently, whole-genome methylation (methylome) studies have investigated the role of methylation in regulating plant responses to changes including abiotic stresses, in their environment[33,36]. Genome-wide methylation studies do not provide precise information about site-specific methylation. In contrast, whole-genome methylation studies provide accurate information about site-specific methylation of gene promoters in response to environmental or growth changes.

Despite extensive research on the molecular response to heat stress, the effect of high temperatures on genes and pathways remains unclear[50]. A previous study on *HSP* gene expression in response to high temperature in nine Egyptian wheat varieties revealed marked differences in *HSP17* gene expression among the nine varieties[51]. Promoter methylation enables plants to regulate their genes at the transcription level. This study investigated the expression of the *HSP17* gene in nine Egyptian wheat varieties and the role of promoter methylation at CpG islands in the regulation of *HSP17* under high temperature.

MATERIALS AND METHODS

Study area: The study was carried out at Biotechnology Department, from May, 2019-January, 2020.

Plant materials: Seeds of nine Egyptian wheat (*T. aestivum*) varieties (Table 1) were surface sterilized. The sterilized seeds were germinated on 1% agar water medium in plastic vessels (100 cm^2) at 100 seeds per vessel. Two vessels per variety were germinated. All vessels were kept at normal temperature (25°C) for 1 week. One set of vessels (one vessel for each variety) served as control and the other set was treated with 40°C for 1 h^3. After germination, 20 shoots were randomly collected from both sets of plantlets and directly lyophilized for 36 h at -58°C. Subsequently, the lyophilized samples were ground in a TissueLyser II (Qiagen, Germany) and used for DNA or RNA isolation.

Primers: Primer 3 plus software was used to design DNA primers. The primers were synthesized by Macrogen (Korea). The main features of the primers are summarized in Table 2.

DNA extraction: DNA was isolated from the lyophilized leaf tissues using the CTAB protocol[52,53], with some modifications[54]. The lyophilized tissues were used as 5 mg per 0.5 mL of warm (65°C) CTAB extraction buffer in DNA extraction. Precipitated DNA was washed with 70% ethanol, air dried and dissolved in TE buffer. DNA was treated with 1 µL of ribonuclease A to remove RNA. Aliquots of DNA were run in 1% agarose to check the DNA quality.

RNA extraction: Total RNA was isolated from 5 mg of lyophilized leaf powder using QiaZol (Qiagen, Germany) according to the manufacturer's instructions. The lyophilized leaf tissue (5 mg) was added to a microfuge tube containing 1 mL of QiaZol (Qiagen). The mixture was mixed and kept at Room Temperature (RT) for 5 min. Chloroform (200 µL per 1 mL of QiaZol Qiagen) was added, mixed completely and kept at RT for 2-3 min. Phases were separated by centrifugation at 12,000 rpm for 15 min at 4°C. The top aqueous phase was transferred to a 1.5 mL tube. RNA was precipitated with an equal volume of isopropanol and the RNA pellet was recovered by centrifugation at 12,000 rpm for 15 min at 4°C. RNA was washed in 0.5 mL of 70% ethanol, dried at RT and reconstituted in DEPC water. The RNA concentration and purity were estimated by measuring its absorbance at A260 and the A260/A280 ratio.

Synthesis of cDNA and semi-quantitative PCR: Total RNA (2 µg) was used for the synthesis of cDNA using polyT primer and MMuLV reverse transcriptase (Promega, USA)[55]. The level of gene expression was estimated using a semi-quantitative PCR according to the method of Ahmed *et al.*[55]. The PCR reactions (25 µL) contained 10 pmol of primers (forward, reverse) (Table 2), 1 µL of cDNA and 1×PCR master mix (Promega, USA). The PCR program was as follows: Denaturation cycle at 94°C for 5 min, 25 amplification cycles (denaturation at 94°C for 1 min, annealing at 50-65°C for 1 min and extension at 72°C for 1 min) and a single extension cycle at 72°C. Tubulin gene expression was estimated using its unique primers (Table 1) as a control. The PCR products were separated in agarose gel (1%) containing ethidium bromide for 2 h at 70 V. Gel photographs were used for densitometric measurements of the gene expression level using the ImageJ program (http://rsb.info.nih.gov/nih-imageJ).

Table 1: Wheat (*Triticum aestivum*) varieties used in this study

Variety	Tolerance to biotic/abiotic stresses
Misr1	Stem rust
Giza168	High temperature, 3 rusts, drought
Sakha94	Three rust diseases
Gemmiza7	Stem and yellow rust
Gemmiza11	Three rust diseases
Sids1	Salinity, high temperature, yellow rust, susceptible to leaf rust
Shandawel1	Three rust diseases
Giza155	Susceptible to the 3 rusts diseases
Giza165	High temperature, moderate resistant to the 3 rusts diseases

Table 2: Primer information used in this study

Gene	AC# or reference	Primer	Primer sequence 5' 3'	Tm (°C)	PCR product
PCR primers					
HSP17	KF208539	TAHSP17p-F	CGCTCCTGAGGCATCTCGAGTTCT	69	407
		TAHSP17p-R	TGTCGATGTTGCTTTGGGTTGTAGC	68	
HSP17	JN572711	TAHSPm1F	GAGAAGGAGGACGCCAAGTA	59	246
		TAHSPm1R	CACAGGAAACTCGCTACTCTCT	57	
TaMET3	52	TAMET3m-F	CTCCACATCAACTCAATGTTT	55	530
		TAMET3m-R	CCAAGTCGATACTGTCGCTT	58	
Tubulin	U76558.1	TATub-2F	AGTGTCCTGTCCACCCACTC	60	244
		TATub-2R	TGAAGTGGATCCTCGGGTAG	60	
Bisulphite primers					
HSP17	KF208539	TAHSP17pB-F	CGCTYYTGAGGYATYTYGAGTTYT	58	407
		TAHSP17pB-R	TGTCGATRTTRCTTTRRRTTRTARC	55	

Bisulphite treatment: There were wide differences in *HSP17* expression between the control (growing at 25°C) and high temperature-treated plants in the investigated varieties, especially Giaz168 and Giza155. Therefore, methylation of the *HSP17* promoter region was investigated in these 2 varieties. The bisulphite reaction was achieved in a final volume of 18 µL containing 1-10 µg of DNA as described previously[56, 57].

PCR amplification: Bisulphite-treated DNA was amplified by using the same PCR conditions and primers (Table 2). PCR amplification of 407 bp of the Transcription Start Site (TSS) proximal region was performed using *HSP17*-specific primers. The PCR products were purified using a Wizard clean-up kit (Promega, USA).

Cloning and sequencing: Purified DNA was cloned into a pGMT-Easy vector (Promega, USA) according to the manufacturer's instructions. Thirty clones were sequenced at Macrogen (Korea) for each wheat variety to detect methylation in a single molecule. The sequences were cleaned and aligned by using Clustal Omega (EBI, https://www.ebi.ac.uk). Methylation of the CpGs islands in the target sequence was detected using a CpG viewer[58,59]. The detection of methylation is based on the fact that in the final sequence, cytosine (C) was methylated cytosine and thymine (T) was unmethylated cytosine.

Statistical analysis: Data were statistically analyzed operating one-way ANOVA variance test (SPSS version 13.0, IBM, Chicago, IL, USA) with $p<0.05$.

RESULTS

The expression level of *HSP17* in 9 Egyptian commercial wheat varieties in response to high temperature was compared with that under normal growth conditions at 25°C.

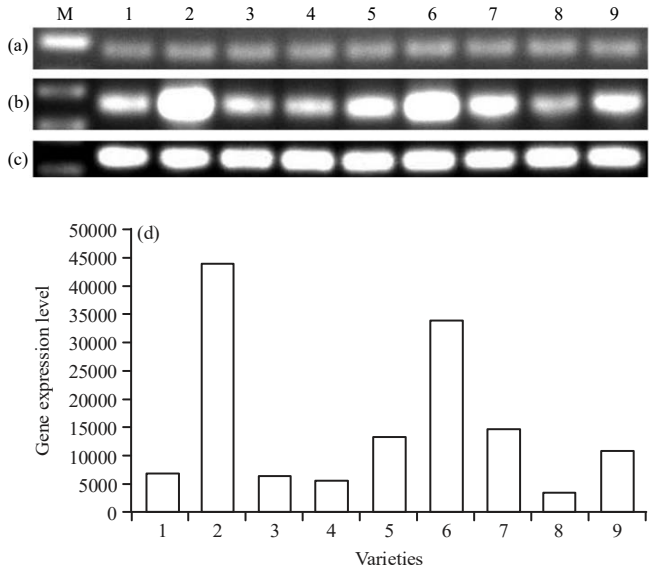

Fig. 1(a-d): *HSP17* gene expression in 9 commercial Egyptian wheat varieties under (a) Normal, (b) High temperature conditions, (c) Wheat tubulin gene expression as a reference and (d) Densitometric estimation of *HSP17* expression level (expressed as difference between b-a

1: Misr1, 2: Giza168, 3: Sakha94, 4: Gemmiza7, 5: Gemmiza11, 6: Sids1, 7: Shandawel1, 8: Giza155 and 9: Giza165

The expression level of *HSP17* was determined as the difference between expression under heat stress and expression under normal growth conditions. The expression level of wheat tubulin was employed as a reference in all gene expression experiments.

Expression of *HSP17* in wheat varieties: The *HSP17* is a low-molecular weight HSP which is induced by elevated temperature and various biotic and abiotic stresses. Under normal growth conditions, the expression level of *HSP17* was barely detected in all 9 tested wheat varieties (Fig. 1a).

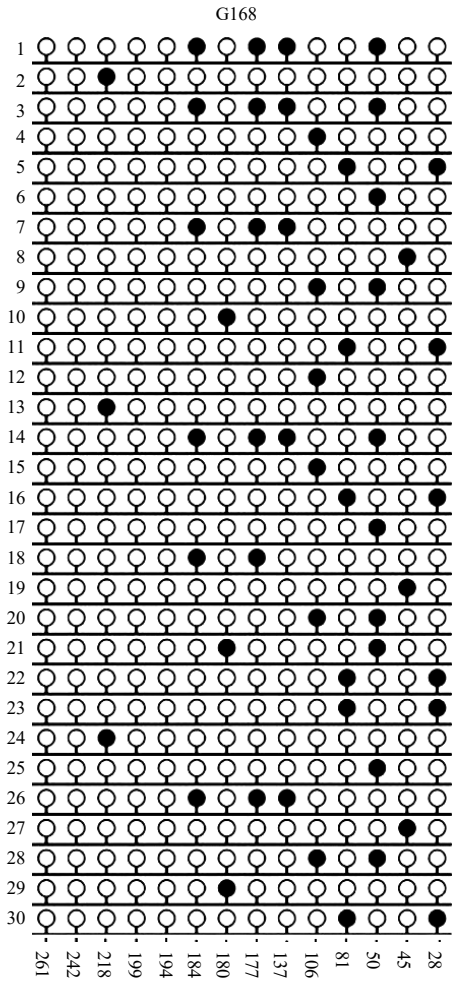

Fig. 2: Lollipop diagram of HSP17 promoter sequence methylation of Giza168 (high *HSP17* expresser)

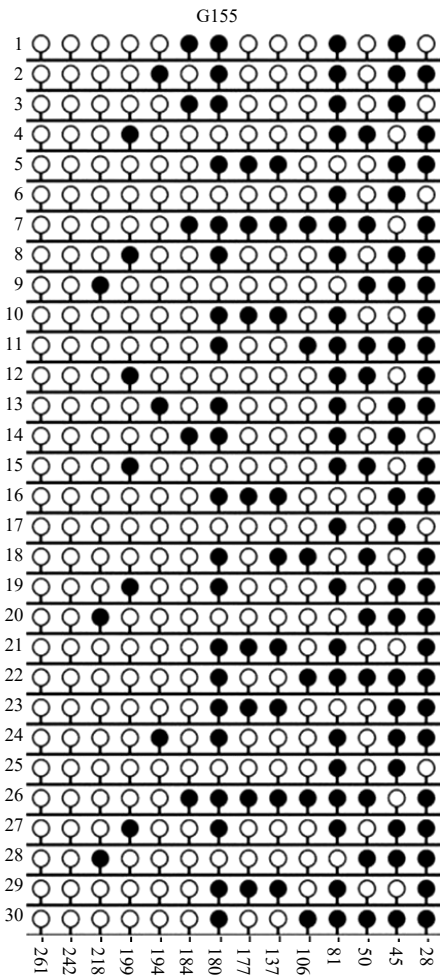

Fig. 3: Lollipop diagram of HSP17 promoter sequence methylation of Giza155 (low *HSP17* expresser)

However, after exposure to high temperature (40°C for 1 h), the expression level of *HASP17* differed markedly in the nine varieties (Fig. 1b). The lowest level of *HSP17* expression was in Giza155. Thus, the expression level of *HSP17* in the other eight varieties was compared with that (basal level) in Giza155 (Fig. 1c). The highest level of expression of *HSP17* in response to high temperature was in Giza168, with about 11.3 folds of *HSP17* expression in Giza155 (Fig. 1b, d). Sids1 also showed 8.6 folds of Giza155 expression. The expression level of HSP17 in the remaining six varieties was as follows: Misr1 (1.8 fold), Sakha94 (1.7 folds), Gemmiza7 (1.5 folds), Gemmiza11 (3.4 folds), Shandawel1 (3.7 folds), Giza165 (2.8 folds) relative to Giza155 (Fig. 1b, d).

Methylation of the *HSP17* promoter proximal region: The *HSP17* promoter proximal sequence upstream of TSS contained 14 CpG islands from -28 to -261 (Table 3). To investigate the correlation between *HSP17* expression and promoter methylation in response to high temperature, methylation in this region was compared in the wheat varieties with high *HSP17* expression (Giza168) and low *HSP17* expression (Giza155). Different levels of methylation were observed in the CpG sites in Giza168 and Giza155, with a methylation average of 13.8% in Giza168 and 36.2% in Giza155 (Table 3, Fig. 2, 3). The level of methylation in CpG sites -106, -218, -242 and -261 was 20, 10, 0 and 0%, respectively. The methylation percentage in CpG sites -50, -137, -177, -184, -194 and -199 was similar with a difference of 20% or less between Giza168 and Giza155. There was a substantial difference among the remaining four CpG sites (-28, -45, -81 and -180) in terms of the methylation percentages in Giza168 and Giza155, with low methylation in Giza168 as compared with that in Giza155. The methylation percentages were 20, 10, 20 and 10% in Giza168 as compared with 80, 70, 76.7 and 70% in

Table 3: CpG islands methylation (%) in the HSP17 promoter proximal sequence

	Wheat variety	
CpG site	Giza168	Giza155
-261	0	0
-242	0	0
-218	10	10
-199	0	20
-194	0	10
-184	20	16.7
-180	10	70
-177	20	26.7
-137	16.7	30
-106	20	20
-81	20	76.7
-50	33.3	40
-45	10	70
-28	20	80
UM	336	249
M	54	141
M (%)	13.8	36.2

UM: Unmethylated CpG, M: Methylated CpGs, M (%): Methylation (%) in HSP17 in wheat varieties

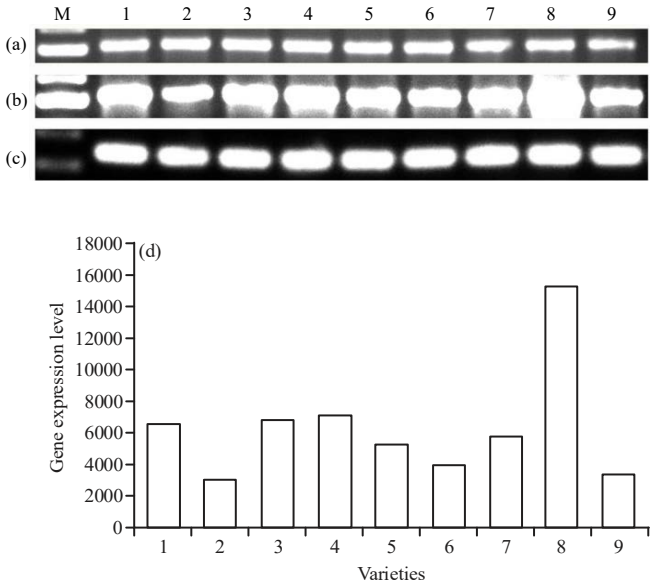

Fig. 4(a-d): Expression of *MET3* gene in commercial Egyptian wheat varieties under (a) Normal, (b) High temperature conditions, (c) Wheat tubulin gene expression as a reference and (d) Densitometric estimation of *MET3* expression (expressed as difference between b-a)

1: Misr1, 2: Giza168, 3: Sakha94, 4: Gemmiza7, 5: Gemmiza11, 6: Sids1, 7: Shandawel1, 8: Giza155 and 9: Giza165

Giza155 at -28, -45. -81 and -180 CpGs consecutively (Table 3, Fig. 2, 3). These findings indicated that the differences in CpG methylation at its promoter proximal sequence could partially explain the difference in *HSP17* expression in the Giza168 and Giza155 varieties under high temperature. This explanation was highly supported by the difference in methylation at the -28, -45, -81 ad -180 CpG sites.

Wheat DNA methyltransferase 3 (MET3) expression: DNA METs contain a methyl group at the 5th position of cytosine to maintain DNA methylation after cell division or to modulate gene expression in response to biotic/abiotic stresses.

MET3 activity was used to estimate methylation at the *HSP17* proximal promoter sequence after exposure to high temperature. Specific MET3 primers were designed to amplify a DNA fragment of about 530 bp. Under normal growth conditions, the expression level of MET3 in the nine wheat varieties was similar (Fig. 4a), whereas, there were wide differences in expression levels under high temperature. The lowest MET3 expression level was detected n Giza168 and the highest expression level was found in Giza155 (5 folds of Giza168). The other seven wheat varieties showed various levels of MET3 expression: 2.1, 2.2, 2.3, 1.7, 1.3, 1.9 and 1.1 folds for Misr1, Sakha94, Gemmiza7, Gemmiza11, Sids1, Shandawel1 and Giza165, respectively (Fig. 4b, d). Wheat tubulin gene expression as a reference (Fig. 4c). From the results, the expression level of MET3 showed a negative correlation with *HSP17* expression and a positive correlation with the methylation intensity at the promoter proximal region. Methylation of the CpG islands at the promoter proximal sequence could partially explain the response of *HSP17* to high temperature.

DISCUSSION

The expression of *HSP17* in the nine wheat varieties differed after exposure to 40°C for 1 h, with the expression in Giza168 and Sids1 markedly higher (11.3 and 8.6 folds, respectively) as compared with that in Giza155. Other varieties showed 1.5 to 3.7 folds of *HSP17* expression relative to Giza155. The high expression level of *HSP17* was associated with a low methylation level at the promoter proximal sequence in Giza168. In contrast, low *HSP17* expression in Giza155 was associated with a high methylation level at the *HSP17* promoter proximal sequence. This negative correlation of *HSP17* expression with methylation level was clearly observed at CpG sites -28, -45, -81 and -180, whereas, it was absent at the other 12 CpG sites. The findings indicated that methylation/demethylation at these four CpG sites played an indispensable role in the response of wheat to high temperature by the regulation of *HSP17* expression.

The negative correlation between the *HSP17* expression level and methylation intensity at -28, -45, -81 and -180 in Giza168 and the lack of this correlation pattern in Giza155 confirmed that methylation at these 4 CpG sites were involved in the regulation of the response of *HSP17* to high temperature. The absence of a marked difference in methylation in the other 12 CpG sites supported this conclusion. Surprisingly, these 4 CpGs were not the closest CpG sites to the TSS. For example, the methylation level of the -50 CpG site was similar in both varieties, although, it was closer to the TSS than the -81 CpG site. The methylation levels of the -106, -137 and -177 sites were all similar, all of which were closer to the TSS than -180. These findings supported the idea CpG sites closest to the TSS do not always have the greatest impact on gene expression. In our previous study, at the -77 CpG site, which lies between -56 and -88 showed a methylation difference of only 10% in wheat T0 transgenic plants[57]. In the current study, 4 sites (-50, -106, -137 and -177) represented 10% or less of a methylation difference between Giza168 and Giza155.

Several previous studies reported increased HSP expression in response to high temperatures[20,21]. Plants differ in their response to high temperature by regulating a different number of genes. The number of induced genes upon exposure to high temperature was 3-6 folds in wheat[3], rice[60] and maize[61]. The expression of 4,811 genes including 2,002 induced genes and 2,809 suppressed genes, differed in a *Panicum virgatum* transcriptome analysis after exposure to high temperature[20]. High temperature induced 50 *HSP* genes and other chaperons in switchgrass upon high-temperature exposure. One of the induced genes was upregulated up to 66 folds[20] and its rice orthologue was upregulated up to 102 folds in rice panicles after exposure to high temperature[61].

Wheat exposure to 40°C for 1h changed the expression of level of 2,618 genes. Among these genes, 1,347 genes were upregulated and 1,271 genes were downregulated. In total, 117 HSPs were upregulated up to 11.8 folds. Surprisingly, in the same study, HSP expression markedly increased after 1 h of exposure to high temperature as compared with that after 24 h of exposure[3]. Tea (*Camellia sinensis*) contains 47 *HSP* genes in its genome. These genes are organized in a gene family, which includes 7 HSP90, 22 small HSPs and 18 HSP70. All *HSP90* genes, 12 *HSP70* genes and 18 *HSP* genes were induced at a high level after exposure to high temperature[21]. Similarly, in rice, exposure to high temperature induced 2,449 genes including 72 *HSPs* (6 *HSP*90 and 19 small *HSPs*) most of them were induced after heat stress[60].

The high expression level of *HSP17* in Giza168 (11.3, 9.6 folds) and the low expression level in the other varieties (1.5 -3.7 folds) relative to that in Giza155 could be linked to its resistance to environmental stresses (biotic/abiotic). Giza168 is tolerant to rust diseases, high temperature and drought. Sids1 is tolerant to high temperature, salinity and yellow rust (Table 1). Resistance to more than one biotic/abiotic stress appears to contributed to the induction level of *HSP17* in response to high temperature. This idea is supported by the low *HSP17* expression in wheat varieties, including Misr1, Sakha94, Gemmiza7, Gemmiza11, Shandawel1 and Giza155, tolerant to only one type of stress (Table 1). It is also supported by the very low expression level in Gizza155, which is not tolerant to any type of biotic/abiotic stress (Table 1).

DNA methylation as a regulator of gene expression was previously reported in several studies reported by Bucherna *et al*.[62] and Tolley *et al*.[63]. Light-induced expression of the *PEPC* gene was regulated by methylation of promoter CpG sites. Hypermethylation of four CpG sites in the *PEPC* gene promoter in maize roots was correlated with low expression, whereas light-induced high expression in leaves was correlated with demethylation of the same four CpG sites. The authors concluded that light-induced *PEPC* expression in maize mesophyll cells was regulated by methylation/demethylation of the four CpG sites in the *PEPC* promoter region[63]. Other studies also provided evidence that promoter methylation caused gene repression[27,37-39,64]. For example, PEG-induced drought in *Arabidopsis* caused hypermethylation near the TSS of drought-induced genes[45].

In the present study, MET3 expression varied among the nine wheat varieties. The expression level of MET3 was negatively correlated with the *HSP17* expression level and positively correlated with CpGs methylation at the *HSP17* promoter proximal region as clearly observed in Giza168 and Giza155. In wheat, MET3 carries out *de novo* DNA methylation in response to environmental stimuli and growth signals. Dai *et al*.[52] studied the expression pattern of METs in wheat (*T. aestivum*) investigated variations in their expression at various developmental stages and different tissues. They reported that METs were highly expressed in embryos and dry seeds, with trace expression levels in leaves, plantules, seedlings and heading stages[52]. Three METs (MET1, chromomethyltransferase 3 and domains rearranged methyltransferase 1/2) were responsible for *de novo* methylation and maintenance of transgeneration methylation in *A. thaliana*[65].

DNA methylation and methylation marks are expected to contribute to next-generation plant breeding through epiallele application. There are several examples in this promising field. For example, allotetraploid cotton showed differential methylation at 12 million cytosines compared to its tetraploid and diploid progenitors. This involved different

methylation of 519 genes including genes controlling flowering time and dormancy of seeds. The same study showed that COL2 epialleles were responsible for photoperiodicity in allotetraploid cotton. Hypermethylation caused silencing of COL2 and led to delayed flowering. On the other hand, COL2 demethylation restored its activation and induced photoperiodic flowering in cultivated cotton[66]. In rice, hypermethylation of the OsAK1 promoter caused silencing of OsAK1 in plants carrying the Epi-ak1epiallele. This epiallele caused suppression of photosynthesis, pointing to methylation/demethylation and epiallele involvement in rice growth and development[67].

Induced hypomethylation using methylation inhibitors (5-azacytidine and 5-azadeoxycytidine) proved that the DNA methylation pattern was transferred to the next generation. Using this approach, inherited hypomethylation in different plant species such as; rice, brassica and tobacco were linked to a detectable change in the phenotype[27]. Priming represents another type of methylation pattern inheritance. Priming refers to the ability of a plant to recognize and adapt rapidly to specific environmental signals. Priming allows a plant to respond rapidly when it is exposed to the same signal or a similar signal[68]. Several reports confirmed that methylation-based priming passed to the next generation as methylation marks, which were induced by environmental stimuli. For example, infection with tobacco mosaic virus resulted in hypomethylation of R-like genes, which conferred increased resistance to various pathogens[69]. In addition, epialleles generated by drought induction were stable for many generations[49]. These findings provided evidence that changes in methylation in response to environmental changes were not random over generations[70]. Generating epialleles by global DNA methylation through methylation inhibitors or priming could lead to the development of specific phenotypes[71] as shown by the production of lines of *Brassica napus* with an increased protein content[72]. Modern molecular tools together with the technology of next-generation need to be implemented to evaluate commercial new wheat varieties under different abiotic stress toward improving yield and food security. This would be beneficial for developing new molecular tools to develop new plant varieties that could adapt well under the condition of high temperatures that are highly predicted in the future.

CONCLUSION

In the present study, variations in *HSP17* expression in Egyptian commercial wheat varieties were regulated by methylation of 4 CpG sites at the proximal promoter sequence. This was negatively associated with MET3 expression. The response of *HSP17* to high temperature through promoter methylation was linked to the genetic background of the wheat varieties. The findings can contribute to the development of next-generation approaches to evaluate the response of commercial wheat varieties to environmental conditions. In addition, they can shed light on new approaches to utilize wheat genetic resources to develop new varieties using epialleles and next-generation plant breeding.

SIGNIFICANCE STATEMENT

This study discovers the role of HSP17 promoter CpG methylation in the regulation of HSP17 under high temperature that can be beneficial for protecting cellular proteins and yield quality. This study will help the researcher to uncover the critical areas of epigenetic and next-generation approaches to evaluate commercial wheat varieties that many researchers were not able to explore. Thus, a new theory on the development of next-generation approaches to plant breeding employing epiallele integration may be arrived at.

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