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Varied Expression Pattern of the Small Heat Shock Protein Gene Encoding HSP17.7 against UVA, UVB, Cu^{2+} and Zn^{2+} Stresses in Sunflower

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Abstract

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Today, one of the main objectives of agricultural biotechnology area is to find the responsible genes involved in stress response and engineering these genes to improve the plant response mechanisms. Therefore the current study was conducted to gain an insight on the role of HSP17.7 gene, which is a member of sHsps family, in defence mechanism of sunflower (*Helianthus annuus* L. cv. Confeta –Turkish cultivar) treated with different doses of UVA and UVB (4, 8, 12 and 20 kJ/m^2) and concentrations of copper (Cu^{2+}) and zinc (Zn^{2+}) (80, 160, 320, 640, and 1280 μM) heavy metals. Based on our data, it was observed that different doses of UVA and UVB irradiation resulted in increased levels of HSP17.7 mRNA in sunflower plants. The highest levels of these increases (8 and 12 kJ/m^2 of UVA) were seen under UVA stress. In contrast to UV stress, only the Cu^{2+}concentration of 1280 μM led to higher expression levels of HSP17.7 gene compared to the control. Besides this, the 1280 μM concentration of Zn^{2+} treatment was the peak point of increased HSP17.7 mRNA levels for all stress conditions with nearly 8 times more than in the control sample. Negative correlations were found between malondialdehyde (MDA) levels and expression levels of HSP17.7 gene in sunflower plants subjected to current abiotic stress conditions. This correlation might indicate that an effective defence mechanism was in action and it might be concluded that the HSP17.7 gene can be used for identification of cultivars tolerant to UV and high doses of Cu^{2+} and Zn^{2+} for molecular breeding studies in the near future. These findings provide evidence of the HSP17.7 gene contribution to abiotic stress response in sunflower and will be helpful for the next studies about stress tolerance improvement in sunflower plants.

Keywords: sHsps; HSP17.7 gene; qRT-PCR; abiotic stress; *Helianthus annuus* L.

All living organisms respond to environmental stress conditions that might be biotic or abiotic by the synthesis of several proteins which are referred to as 'Stress Proteins'. These proteins protect cells against harmful effects of stress conditions. Heat shock proteins (HSPs), also known as molecular chaperones, are known to belong among these stress proteins while they are present in all living organisms (Kim & Hwang 2015). Besides their role in protecting cells against environmental stress conditions, HSPs are essential components of plant cells and they play an important role in protein stabilisation and cellular functions related with plant growth (Pratt *et al.* 2001; Koo *et al.* 2015).

HSPs were first identified in the fruit fly (*Drosophila melanogaster*) and determined as highly conserved in all living organisms including plants. In plants, they are classified into five evolutionarily conserved families: HSP100, 90, 70, 60 (or chaperonins), and the small HSPs (sHsps) which have molecular weights of 100, 90, 70, 60, and 12–40 kDa, respectively (Al-Whaibi 2011; Koo *et al.* 2015). Higher plants are known to have at least 20 types of sHsps which have an α-crystallin domain consisting of 80–100 amino

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acid residues in the C-terminal region (Seo *et al*. 2006). The amount of expressed sHsps and their types change according to the plant species. The sHsps family plays an important role in the degradation of misfolded proteins and this characteristic distinguishes the family from other HSPs classes (Ferguson *et al*. 1990; Al-Whaibi 2011). Although they have no function in the refolding of non-native proteins, there are few indications that they bind to partially folded proteins to prevent their irreversible unfolding and aggregation of non-native proteins (Sun *et al*. 2002). These characteristics of sHsps make them valuable in maintaining the functional conformation of proteins under stress conditions which is crucial for a cell to survive (Al-Whaibi 2011).

Significantly increased amounts of sHsps were observed in plants subjected to abiotic stress conditions such as high temperatures (Ahn & Zimmerman 2006; Volkov *et al*. 2006), drought and salt stress (Sato & Yokoya 2008; Zahur *et al*. 2009). These previous studies suggest that sHsps might play an important role in plant resistance to environmental stress conditions. In plants, they have been well characterised in some species such as maize, soybean (Ding *et al*. 2009; Wu *et al*. 2010), and cotton (Ronde *et al*. 1993; Cottee *et al*. 2014) and it was reported that their distribution differs within tissue, organ and plant species.

Today, one of the main objectives of agricultural biotechnology is to find the responsible genes involved in stress response and engineering these genes to improve the plant response mechanisms (Pareek *et al*. 2010). Some of these genes have been well-characterised while some are suspicious in some plant species such as sunflower. Nevertheless, the analysis of these genes in many different plant species will increase the overall knowledge of their exact roles in the plant defence mechanism.

In this regard, the present study was conducted to gain an insight into the role of HSP17.7 gene, which is a member of the sHsps family, in the defence mechanism of sunflower (*Helianthus annuus* L. cv. Confeta – Turkish cultivar) treated with different doses of UVA and UVB (4, 8, 12, and 20 kJ/m^2) and concentrations of Cu^{2+} and Zn^{2+} (80, 160, 320, 640, and 1280 μM) heavy metals. For this aim, the steady-state mRNA levels of HSP17.7 gene were determined by qRT-PCR in stressed sunflowers. All results were evaluated statistically and a probable correlation between stress conditions and HSP17.7 gene expression levels was demonstrated.

MATERIAL AND METHODS

Growth of plants and stress applications. Sunflower (*Helianthus annuus* L.) cultivar Confeta seeds were kindly obtained from MAY Seed Company (Bursa, Turkey) and were germinated and grown hydroponically in pots containing 0.2 l of modified 1/10 Hoagland's solution. Hoagland's solution included macronutrients [K_2SO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $Ca(NO_3)_2 \cdot 4H_2O$, and KCl] and micronutrients (H_3BO_3, $MnSO_4$, $CuSO_4 \cdot 5H_2O$, NH_4Mo, and $ZnSO_4 \cdot 7H_2O$) with the following final concentrations of ions: 2 mM Ca, 1 μM Mn, 4 mM NO_3, 0.2 μM Cu, 1 mM Mg, 10 mM NH_4, 2 mM K, 1 μM Zn, 0.2 mM P, 0.1 mM Fe, and 1 μM B. Six plants were grown in each pot in a controlled environmental growth chamber, under light, with photosynthetic photon flux of 250 mmol/m^2·s at 25°C and 70% relative humidity. Twenty-five-days-old plants grown in controlled media were used for the stress treatments. For the heavy metal application, Cu^{2+} and Zn^{2+} were added separately to the hydroponic solution for 24 h at concentrations of 0 (control), 80, 160, 320, 640, and 1280 μM. For the UV application, twenty-five-days-old plants were irradiated with UVB and UVA using a BS-03 irradiation chamber (Dr. Gobel UV-Elektronik GmbH, Ettlingen, Germany) and UVB and UVA doses were determined as 4, 8, 12 and 20 kJ/m^2 for the plants.

Estimation of lipid peroxidation. Malondialdehyde (MDA) is a marker of oxidative lipid injury which changes in response to environmental factors that lead to stress in plants. Thiobarbituric acid-malondialdehyde (TBA-MDA) content was determined as described by Hodges *et al*. (1999). The experiment included three independent biological replicates and three technical triplicates, each for both stressed and non-stressed plant samples. Plant leaf samples were homogenised with liquid nitrogen and with 80 : 20 (v/v) ethanol–water mixture, followed by centrifugation at 3000 *g* for 10 minutes. A 1-ml aliquot of appropriately diluted sample was added to a test tube with 1 ml of either (*i*) TBA solution composed of 20.0% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene, or (*ii*) +TBA solution containing the above plus 0.65% TBA. Samples were then mixed vigorously, heated at 95°C on a hot plate (neoBlock1, 2-2503) for 25 min, cooled, and centrifuged at 3000 *g* for 10 minutes. Absorbance values were measured at 440, 532, and 600 nm by ELISA microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, USA). The equivalents of malondialdehyde were calculated by the following equations:

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$$[(\text{Abs } 532_{+TBA}) - (\text{Abs } 600_{+TBA}) - (\text{Abs } 532_{-TBA} - \text{Abs } 600_{-TBA})] = A \quad (1)$$

$$[(\text{Abs } 440_{+TBA} - \text{Abs } 600_{+TBA})\ 0.0571] = B \quad (2)$$

$$\text{MDA equivalents (nmol/ml)} = (A - B/157\,000) \times 10^6 \quad (3)$$

RNA extraction, reverse transcription and Quantitative Real-Time PCR. Total RNA extraction was performed using the Trizol protocol followed by the clean-up protocol using RNeasy mini clean-up kit (Cat No: 74104; Qiagen, Hilden, Germany) . RNA quantity/quality was measured with a NanoDrop 1000 ND-Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The quality of RNA was also confirmed by gel electrophoresis which contained 1.5% agarose. cDNA synthesis which was based on reverse transcription reactions was performed with 2 µg of RNA and high fidelity cDNA synthesis kit (Roche) which contained 2.5 µM anchored-oligo $(dT)_{18}$, 1X transcriptor high fidelity reverse transcriptase reaction buffer, 20U protector Rnase inhibitor (Roche, Mannheim, Germany), 1 mM deoxynucleotide mix, 5 mM DTT, 10U transcriptor high fidelity reverse transcriptase at final concentration. The following incubation conditions were applied; 10 min at 65°C, 30 min at 55°C, and 5 min at 85°C. cDNAs were also measured with a NanoDrop 1000 ND-Spectrophotometer.

Quantitative Real-Time PCR (qRT-PCR) was performed with LightCycler® Nano System (Roche, Mannheim, Germany), thermal cycler. The primer sequences of the target gene HSP17.7 (GenBank: U46545.1) and also housekeeping gene actin (GenBank: AF282624.1) which is used for normalization were designed with Primer3 program based on the sequences of sunflower genes available in the GenBank (http://www.ncbi.nlm.nih.gov/) (Table 1).

Table 1. Primer sequences of HSP17.7 and ACT genes

Primer name		Sequences
HSP 17.7	forward	ATAAGCGGAGAGAGGAGCAGA
	reverse	GCATTCTCCGGCAACCTAAAC
ACT	forward	TGAGCAAGGAAATCACGGCT
	reverse	TCCTCCGATCCAGACACTGT

All qRT-PCR reactions were performed in three independent biological and technical triplicates with a template free control to check any contaminations. Amplifications of PCR product were monitored using an intercalator-based method including SYBR Green I dye. After pre-denaturation, followed by 10 min at 95°C, 45 cycles of 10 s at 95°C, 30 s at 60°C, and 15 s at 72°C were applied. Melting curve analysis was performed to confirm the presence of a single product and absence of primer-dimers (Figure 1). Data collection for quantification was done during the annealing period.

Statistical analysis. The abundance of target gene transcripts was normalised to *ACT* and set relative to control plants according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). Changes in relative expression levels (REL) of the gene were checked for statistical significance according to one-way ANOVA. Fisher's least significant difference test at 0.05 significance levels was performed.

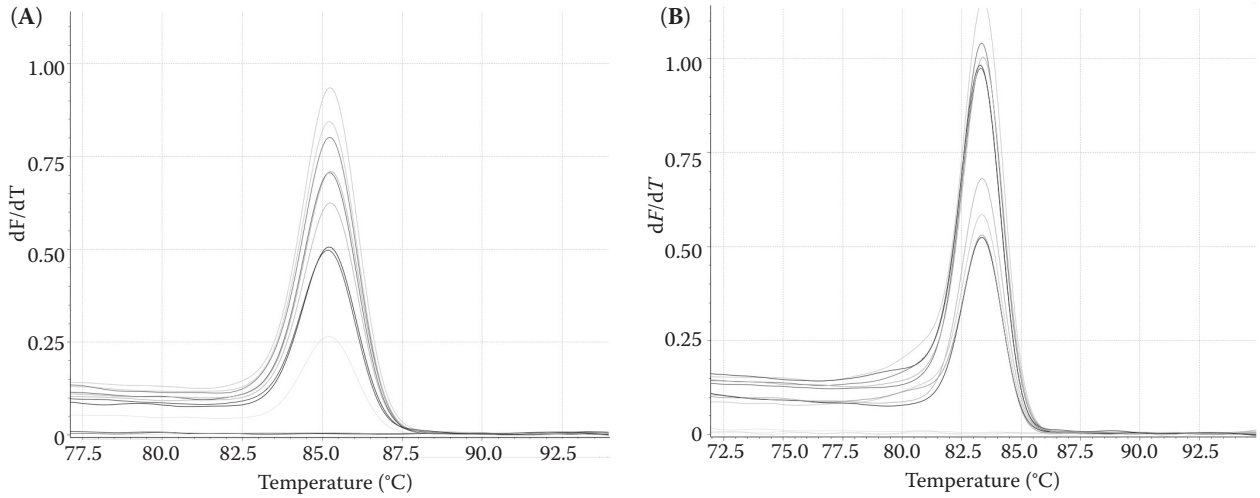

Figure 1. Melting curve analysis of qRT-PCR reactions: (**A**) Single melting curve in each RT-PCR assay for *ACT* gene; (**B**) Single melting curve in each RT-PCR assay for HSP17.7 gene

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Table 2. Malondialdehyde (MDA) content in sunflower plants subjected to different doses of UV and different concentrations of heavy metal treatments

UV doses (kJ/m^2)	UVB-MDA (nM/g)	UVA-MDA (nM/g)	Heavy metal concentrations (μM)	Cu^{+2}-MDA (nM/g)	Zn^{+2}-MDA (nM/g)
Control	0.834^a	0.834^a	Control	0.75^a	0.75^a
4	1.53^b	2.112^b	80	2.04^b	0.92^a
8	1.545^b	0.43^c	160	3.12^c	0.89^a
12	2.099^c	0.64^d	320	3.5^c	1.197^b
20	1.873^c	3.72^e	640	3.8^c	0.8^{ac}
			1280	0.6^c	0.185^d

$^{a–d}$means within each column followed by the same letter are not significantly different at the $P = 0.05$ level

RESULTS

Lipid peroxidation analysis. In the present study, primarily we evaluated malondialdehyde (MDA) levels as lipid peroxidation can be considered as a marker under stress conditions. MDA contents of sunflower plants were changed at almost all concentrations and doses of all stress conditions and these changes seemed consistent with mRNA levels of HSP17.7 gene (Figure 2). According to the analysis, all doses of UVA and UVB stresses led to statistically significantly higher levels of MDA compared to the non-stressed control sample. As documented in Table 2, the highest MDA level was seen in sunflower plants subjected to 20 kJ/m^2 UVA stress among UVA and UVB stressed plants while the lowest MDA level was found out in 8 kJ/m^2 UVA stressed plants ($P < 0.05$). Statistically non-significant increases were observed between 4–8 kJ/m^2 and 12–20 kJ/m^2 of UVA stress treatment (Table 2).

Table 2 shows that all concentrations of Cu^{2+} contamination except for 1280 μM led to statistically significantly higher levels of MDA compared to the non-stressed control plants (Table 2). When the effects of Zn^{2+} stress on MDA levels were evaluated, it was observed that statistically significantly higher levels of MDA compared to the non-stressed control only were seen at 320 μM of stress treatment. After 320 μM concentration of Zn^{2+} treatment, gradual decreases were observed in MDA levels compared to this concentration (Table 2).

Gene expression analysis. Although the expression profile of many genes of sHSPs have been studied in different plants, there are no available data on the effect of different doses and concentrations of UVA, UVB, Cu^{2+}, and Zn^{2+} stresses on HSP17.7 gene expression in the sunflower species. Therefore, qRT-PCR analysis of this gene in the sunflower species is one of the important issues of the present study.

The expression level of HSPS17.7 gene was altered in sunflower plants subjected to almost all concentrations of heavy metals and doses of UV stress conditions. Higher mRNA levels of HSP17.7 compared to the non-stressed control were seen in sunflower plants subjected to all doses of UVA and UVB stress treatments except for 20 kJ/m^2 of UVA (Figure 3A and Table 3). Based on the qRT-PCR results, it can be correctly concluded that 8 kJ/m^2 of both UVA and UVB treatments led to

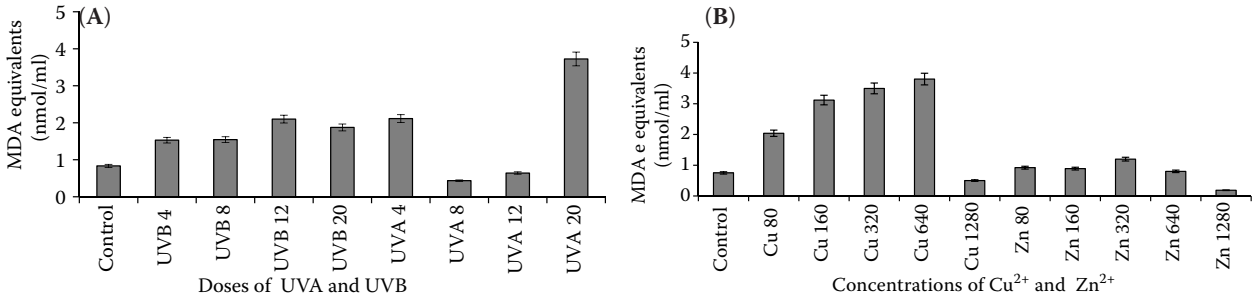

Figure 2. Effects of (A) UVA and UVB (4, 8, 12, and 20 kJ/m^2) and (B) Cu^{2+} and Zn^{2+} (80, 160, 320, 640, and 1280 μM) treatment on malondialdehyd (MDA) (lipid peroxidation) levels in sunflower plants

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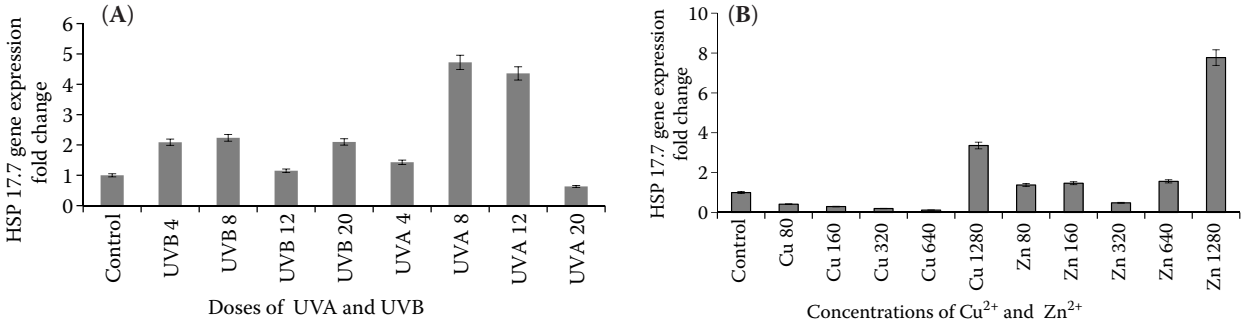

Figure 3. Effects of (A) UVA and UVB (4, 8, 12, and 20 kJ/m^2) and (B) Cu^{2+} and Zn^{2+} (80, 160, 320, 640, and 1280 μM) treatment on mRNA levels of HSP17.7 in sunflower

the highest HSP17.7 mRNA expression compared to their own control. In addition, comparative evaluation of UVA and UVB effects on sunflower plants revealed that UVA stress in that dose increased the mRNA levels of HSP17.7 nearly 5 times more than in the control sample and twice more than in UVB affected sunflower plants (Figure3A and Table 3).

The heavy metal contamination also resulted in altered mRNA levels of HSP17.7 gene as it was observed in UV stressed sunflowers. In contrast to UV stress, Cu^{2+} contaminations at concentrations of 80, 160, 320, and 640 μM reduced HSP17.7 mRNA expression. On the other hand, the gene in sunflower plants subjected to 1280 μM Cu^{2+} was expressed nearly 3 times more than in the control sample (Table 3B).

According to the results illustrated in Figure 3B, it is obvious that all concentrations of Zn^{2+} contamination except for 320 μM led to increased mRNA levels of HSP17.7 compared to the control sample (Figure 3B and Table 3). Zn^{2+} contamination at 1280 μM revealed a statistically significant increase and this was the peak point of increased HSP17.7 mRNA levels among all stress conditions with nearly 8 times more than in the control sample (Figure 3B and Table 3).

DISCUSSION

Plants are often subjected to many abiotic stresses such as salinity, drought, freezing, heavy metals, and ultraviolet radiation which affect yields of some important crops like sunflower in the world. Due to their sessile nature, plants have some adaptation and defence strategies to survive under rapidly changing environmental conditions (Boyer 1982; Soydam *et al*. 2013). In addition, the stress specific regulation of transcription is seen in plants under environmental stress conditions and some of these regulated genes and their proteins play an important role in general stress response (Rizhsky *et al*. 2004; Larkindale & Vierling 2008). Although in the past few decades, a large number of candidate genes have been identified by several researchers, several stress related molecular mechanisms still seem veiled.

Heat shock proteins which are known as chaperons are key components for cellular homeostasis in plant cells by assisting protein refolding under environmental stress conditions (Sairam & Tyagi 2004). Heat shock proteins which are also called stress proteins exist in all living organisms and the synthesis of these

Table 3. Transcription changes of HSP17.7 gene in sunflower plants subjected to different doses of UV and different concentrations of heavy metal treatments

UV doses (kJ/m^2)	UVB-HSP17.7 (nM/g)	UVA-HSP17.7 (nM/g)	Heavy metal concentrations (μM)	Cu^{+2}-HSP17.7 (nM/g)	Zn^{+2}-HSP17.7 (nM/g)
Control	1^{a}	1^{a}	Control	1^{a}	1^{a}
4	2.09^{b}	1.42^{b}	80	0.417^{b}	1.37^{b}
8	2.23^{b}	4.72^{c}	160	0.3^{c}	1.46^{bc}
12	1.151^{ac}	4.35^{c}	320	0.2^{cd}	0.48^{d}
20	2.099^{bd}	0.63^{d}	640	0.122^{e}	1.56^{bce}
			1280	3.36^{f}	7.77^{f}

$^{a–f}$means within each column followed by the same letter are not significantly different at the $P = 0.05$ level

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proteins increases under some acute environmental stress factors such as high temperature, cold, and oxygen deficiency (Henle *et al*. 1998; Wang *et al*. 2004). sHsps are among the important HSP classes and their exact roles have been investigated recently. Hence, in the present study, we aimed to investigate the possible role of HSP17.7 gene which encodes a sHSP in a sunflower plant against UVA, UVB, and heavy metal stress response.

Ultraviolet (UV) radiation is an environmental factor which has important roles in the regulation of many physiological and ecological processes in plant ecosystems and which is spectrally divided into three types: UV-A, UV-B, and UV-C (Müller-Xing *et al*. 2014). Among these UV types, UV-C (200–280 nm) is completely absorbed by the ozone layer and thus cannot reach the Earth's surface; while UV-A (320–400 nm) and UV-B (280–320 nm) can reach it and are involved in cellular processes. Hence, the present study was conducted with UVA and UVB stresses which can pose environmental adverse effects in excess quantities (Müller-Xing *et al*. 2014).

To our knowledge no studies have been published revealing the interactions between sHSPs and UV stress in a sunflower plant. There are some studies in other crop plants including maize (Ferreyra *et al*. 2010; Casati *et al*. 2011), barley (Kravets *et al*. 2012), cucumber (Shinkle *et al*. 2010), and grapevine (Pontin *et al*. 2010; Martinez-Luscher *et al*. 2013).

In the present study, different doses of UVA and UVB irradiation resulted in increased levels of HSP17.7 mRNA in sunflower plants. The highest levels of these increases (8 and 12 kJ/m^2 of UVA) were found out in UVA stressed sunflower plants for the two types of UV treatment. However, the MDA level, which gives information about damage to the cell membranes caused by stress conditions, was lower than in the non-stressed control sample at 8 and 12 kJ/m^2 of UVA treatment, indicating that an effective defence mechanism was in action (Rao *et al*. 2005). The negative correlation between MDA levels and expression levels of stress responsive genes in many organisms subjected to abiotic stress conditions was also shown by previous researches (Cho & Seo 2005; Li *et al*. 2012). All these results showed that HSP17.7 may play an important role in the defence system against UV stress in sunflower plants.

Copper and zinc are important micronutrients and they participate in many enzyme systems and structure of photosynthetic proteins. They induce toxicity above their optimal levels in plants as well as in nearly all living organisms and cause a problem of both agricultural and environmental significance (Chamseddine *et al*. 2009; Millaleo *et al*. 2010). They are accumulated until the toxic levels in plants are reached, which leads to adverse effects on plant growth and morphology (Marschner 1995). In addition, excess concentrations of these metals are also hazardous to genetic stability by leading to genotoxicity in many plants (Conte *et al*. 1998; Savva 1998; Piraino *et al*. 2006; Liu *et al*. 2007).

To identify the possible role of HSP17.7 gene in defence against stress caused by different concentrations of Cu^{2+} and Zn^{2+}, qRT-PCR and MDA data were also evaluated together. The data showed that only 1280 μM of Cu^{2+} treatment resulted in increased levels of HSP17.7 mRNA in sunflower plants compared to the control sample and the MDA level was lower than in the non-stressed control sample at this concentration of Cu^{2+}, indicating that an effective defence mechanism was in action. At the other concentrations of Cu^{2+} treatment, the MDA level was found higher compared to the non-stressed control sample while HSP17.7 expression levels were lower than in the control (Tables 2 and 3).

Zn^{2+} treatment led to statistically significant increases in the mRNA levels of HSP17.7 gene except for the application of 320 μM. Above this concentration, the expression of HSP17.7 gene was gradually increased and reached the peak level at 1280 μM and a negative correlation which reflects the possible role of this gene in defence against Zn^{2+} stress was again observed between MDA levels and HSP17.7 gene expression levels (Table 3). In previous studies, HSP18.1 and MsHsp18.2 genes in *Medicago sativa* and AtHsp17.7 gene in *Arabidopsis thaliana* were identified as the key components whose role in the protection of plants against heavy metal stresses is similar to the results of the present study (Sun *et al*. 2002).

As a result, changes in the mRNA levels of HSP17.7 gene seem more sensitive to UV stress rather than to heavy metal stress except for the Zn^{2+} treatment of 1280 μM. The results obtained in the present study about the more pronounced gene expression levels induced by UVA irradiation are in accordance with the previous studies suggesting that 8 and 12 kJ/m^2 of UVA are more efficient in triggering the genes involved in stress defence in plants. In addition, the present study showed that the HSP17.7 gene might be used for identification of cultivars tolerant to UV and high doses of Cu^{2+} and Zn^{2+} for molecular breeding

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studies in the near future. At the same time, these findings can be used for improving stress tolerance in sunflower plants by cloning and functional analyses.

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