Expression of some Genes in Response to Cadmium Stress in *Triticum aestivum*

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Abstract. Heavy metal toxicity has become a universal threat to all life forms, including plants. The main purpose of this study was to identify the gene expression profiling of MAPK, Thioredoxin, and MnSOD genes in wheat seedlings as affected by cadmium treatment. For this experiment, the quantitative Real-Time PCR on RNA isolated from shoots of wheat exposed to $CdCl_2$ at a concentration of 100 mg/L was used. Results showed that in wheat seedling that exposed to cadmium stress for six days of beginning constant cadmium stress, Thioredoxin gene expression showed a large rise compared with the control sample, MnSOD gene expression increased compared with non-treated wheat seedling at the same times, but unlike the Thioredoxin and MnSOD genes, MAPK gene expression has no significant changes. Of course, it is possible that other times of beginning treatments (instead of six days) cause a change in this gene expression.

Abbreviations: ROS: Reactive oxygen species, MAPK: Mitogen-activated protein kinase, MnSOD: Manganese Superoxide dismutase, $CdCl_2$: Cadmium chloride.

Introduction

Environmental pollution caused by heavy metals was considered a major concern around the world. The stability of these pollutants, unlike organic material, is very much and not easily decomposed by biological activity [22]. Furthermore, these materials, as a result, their undesirable accumulation in the environment, particularly due to various anthropogenic activities, will have serious consequences for health and longevity of plants, animal, and human and make it difficult food safety and the ecosystems [20; 24]. In general, heavy metals or metalloids refer to those elements with an atomic density greater than five g/cm^3 and are mostly toxic to biological systems at low concentrations. Heavy metal stress is one of the major abiotic stresses that limit crop productivity and plant growth by reason of damage to the normal metabolism, ion imbalances, water regime, etc. [8; 19]. Plants have evolved a variety of defensive mechanisms that permit them to adapt to adverse environments for continued survival, growth, and development includes the exclusion mechanism that prevents absorption by root and, internal detoxification through changes in physiological, molecular, and biochemical parameters [4; 12; 16].

Cadmium, which is highly soluble in water, is not an essential element for plants, but the plant absorbs this metal if it is present in their environment, especially in fields contaminated with fertilizers, which contain this metal. Cadmium uptake and accumulation in plants that are considered part of the food chain to create a serious health hazard to humans and living cells. Cadmium can cause a broad range of physiological and biochemical dysfunctions on plants such as blocking signaling receptors; degradation of the plasma membrane and changes in membrane permeability; the decrease of the ATP synthesis; inhibition of enzyme activity; creation of ROS. Disturbing the metabolic activities leading to leave chlorosis, growth inhibition, root tip necrosis, affecting cell membrane permeability and reducing ion uptake by roots and their transport to shoots [7; 15]. MAPK is a vital enzyme that plays an important function in signal transduction involved in the regulation of various developmental stages and responses to a large range of biotic and abiotic stress [2]. In all types of organisms, Thioredoxin is a small ubiquitous and a heat stable protein with a redox active disulfide bridge has main play in redox regulation of protein function in plant

metabolism. This regulatory protein funds in all plants [3; 13]. MnSOD is an environmental toxicology stress marker and plays a determinant role in the protection against the toxic effects of oxidative stress by scavenging superoxide radicals. MnSOD catalyzed the conversion of O_2^- to H_2O_2, which can reduce the amounts of damaging ROS produced by the adverse environments, and alleviate the harm to plants [5; 6; 21].

In this study, the molecular responses of wheat to cadmium were assessed by transcript accumulation analysis of genes (MAPK, Thioredoxin, and MnSOD) coding for products potentially involved in heavy metal tolerance.

Materials and Methods

Plant materials and growth conditions

Wheat seeds (*Triticum aestivum* L. var. Chamran) were obtained from Zarghan Agricultural Research Center, Iran. These seeds kept in a cool and dark place in the lab. Seeds surface was sterilized by soaking in 5% (w/v) sodium hypochlorite (NaOCl) for 10 minutes. They were washed three times with distilled water and air-dried on filter papers. Seeds were allowed to germinate in the dark at 25°C on moist filter papers. Five-day-old seedlings (20) were transferred into small plastic containers filled with perlite and Hoagland nutrient solution (pH 6.2). Wheat seedlings were grown in the growth chamber set at 16h/8h light-dark periods (six days). Three replicates were used for each treatment. Control and treated wheat seedlings were immediately frozen in liquid nitrogen and stored at −80 °C until nucleic acid extraction.

Cadmium chloride (CdCl$_2$) treatment

Cadmium chloride (99%) was obtained from Sigma-Aldrich (Sigma-Aldrich). The Hoagland nutrient solution was used as a control. After six days of the start of treatment, the levels of control and treated wheat were harvested and were stored at -80 ºC until RNA extraction used. Stock solution of cadmium (100 mg/L) was prepared by dissolving cadmium nitrate in double distilled water. This concentration was adapted from mentioned studies.

RNA isolation, cDNA synthesis

Total RNA was extracted using an RNA isolation kit (DENA Zist kit, Iran) according to the manufacturer's instructions. Purified RNA was kept in -80°C until DNase I treatment used. Extracted RNA was quantified using Nano-Drop ND 1000 Spectrophotometer (Wilmington, USA). The quality of total RNA was evaluated by the ratios OD_{260}/OD_{280} and OD_{260}/OD_{230}. RNA integrity was verified on a 2% agarose gel; three bands corresponding to ribosomal RNA (28S, 18S, and 5S) were apparent. DNase treatment was carried out using Fermentas (Fermentas, Hanover, MD) DNase Kit as instructed in the manufacturer's protocol and by doing confirmatory PCR, we sure that all residual of genomic DNA was removed. First strand cDNA was generated using reverse transcriptase (Fermentas Cat Num: EN0531).

Primer design

Primers used for the amplification of target cDNA were designated according to gene sequences of wheat available at the National Center for Biotechnology Information (NCBI) and used to design gene- specific Real-Time primers by Allele ID 7.8 software. Two primer pairs (whose were a relatively short sequence; approximately 100 bp and suitable for Real-Time quantitative PCR) for everyone gene, were designed. The wheat actin and 18s rRNA genes were used as housekeeping genes by the specific primers as the internal control (whose expression proved not to be influenced by heavy metal stress) for data normalization. The sequence of primers (Target and housekeeping genes) was presented in Table 1.

Table 1. Designed primers used for Real-time PCR amplification, resulting product length and Tm.

Name of genes	Primer Sequence 5' → 3'	Product length (bp)	Tm (°C)
	F: GCCACAAGAAGAACATAA	107	56
MAPK	R: AGCAACTACTCCATAACT		
Thioredoxin	F: CTGAAGTCCATTGCTGAG	108	58.8
	R: CAGTTCCTCCTTGATAGC		
MnSOD	F: TCCGCCGTCGTCCACCTC	105	63.8
	R: CCACCACCCTCGCTGATG		
Actin	F: GACATACAATTCCATCAT	153	56
	R: TTAACCTTCATACTGCTA		
18s rRNA	F: CGCTCCTACCGATTGAATGG	127	56
	R:CTTGTTACGACTTCTGCTTCC		

Real-Time quantitative PCR

Real-Time PCR reactions were performed using SYBR Green kit (Takara, Japan) according to the manufacturer's protocol. The first strand cDNAs were diluted 5X with RNase-free water. Quantitative PCR was performed in 20μl reactions using gene-specific primers, 4 μl of cDNA as a template. Gene expression was indirectly assessed using the SYBR Green dye in a lineGeneK thermal cycler (Bioer, China) with initial denaturing of 10 min at 94°C, followed by 40 cycles each consisting of 94°C for 10 sec, 57-63°C for 15 sec and 72°C for 30 sec. After 40 cycles, the specificity of the amplifications was checked based on melting curves resulted by heating the amplicons from 50 to 95°C. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis and agarose gel electrophoresis on a 2 % agarose gel. All amplification reactions were repeated twice under identical conditions, in addition to a negative control and four standard samples. The abundance of targeted gene transcripts was normalized to Actin and 18s rRNA and set relative to control plants (no heavy metal exposure) according to the 2−ΔΔCT method [14]. For quantitative Real-Time PCR data, the relative expressions for MAPK, Thioredoxin, and MnSOD were calculated based on the threshold cycle (CT) method. The mean relative levels of amplification of the target genes and standard deviations were calculated based on CT values. The CT for each sample was calculated using the Line- gene K software, where ΔCT was determined by subtracting the corresponding internal control CT value from the specific CT of the targets (MnSOD, MAPK, and Thioredoxin), and ΔΔCT was obtained by subtracting the ΔCT of each experimental sample from that of the control sample. Copy numbers of genes under stress treatments were determined by using standard curves.

Statistical analysis

The experimental designs were randomized complete block, and each value reported is the average of three repeats. The raw data was imported into Microsoft Excel 2007 and GraphPad Prism 5 programs for calculations and graphic representation. SPSS (version 16.0) software was used for analysis of variance. Quantitative changes of parameters were evaluated through analysis of variance (one-way ANOVA), with Duncan's multiple range tests at P≤0.05 to find out significant differences among treatments. All results are presented as the means ± standard deviation (SD).

Results and discussion

Quality of total RNA

Fig. 1 showed the quality of RNA extracted by Nano-Drop. The quality of total RNA was evaluated by the ratios OD260/OD280 (2.29) and OD260/OD230 (2.13). These results confirmed no organic solvent contamination and protein. Fig. 2 showed RNA electrophoresis gel photograph. In this figure, there are three wells to the right of the Ladder was before DNase treatment and Three wells to the left of the Ladder was after DNase treatment. The results clearly showed three bands corresponding to ribosomal RNA (28S, 18S, and 5S) were apparent.
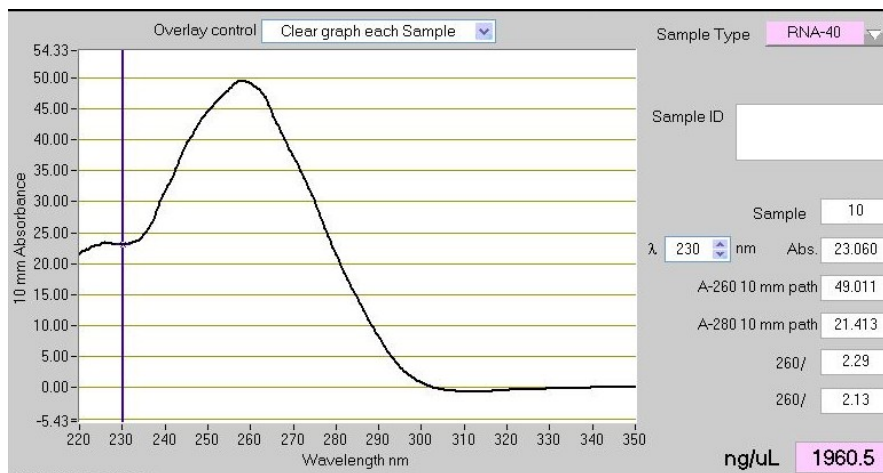

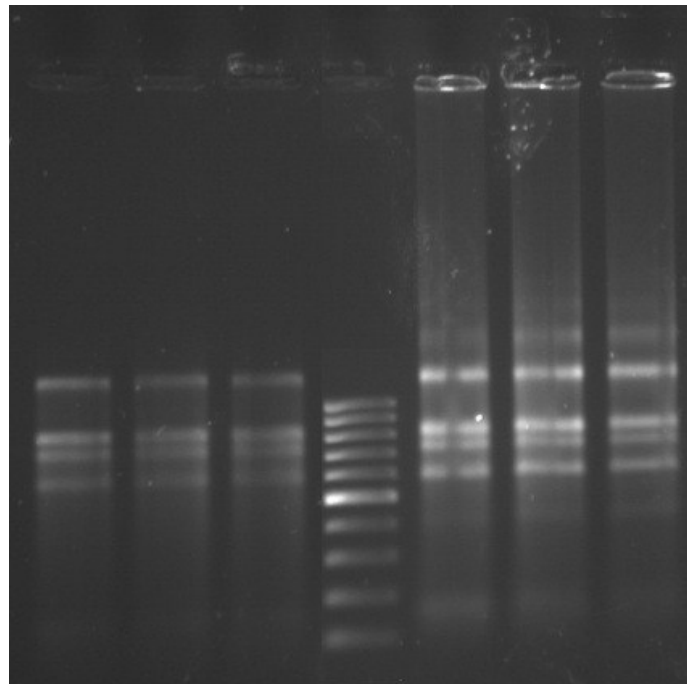**Figure 1.** Verify the quality of RNA extracted by Nano-Drop.

Figure 2. RNA electrophoresis gel photograph. Three wells to the right of the Ladder was before DNase treatment, and Three wells to the left of the Ladder was after DNase treatment (MAPK, Thioredoxin, and MnSOD RNA from left to right respectively).

PCR and sequencing

Fig. 3 showed electrophoresis gel photograph of the PCR amplification products for detection of cDNA.The identity of each Real-Time PCR product is the same target genes direct cDNA sequencing was done. PCR products after preparation were sequenced in the Macrogen Company sequencing facility using Applied Biosystems 3730 mXL automated DNA sequencer (Macrogen, Korea). MnSOD, MAPK and Thioredoxin cDNA sequences derived from wheat seedlings exposed

cadmium showed in Table 2. For identification, sequence homology searches were carried out using the BLAST search facility available through the NCBI.
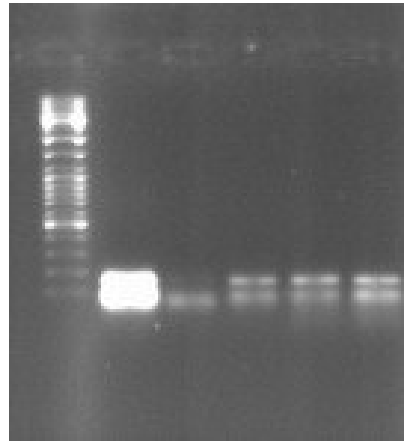

Figure 3. Electrophoresis gel photograph of the PCR amplification products for detection of cDNA (ladder, positive control, negative control, MAPK, Thioredoxin, and MnSOD cDNA from left to right respectively).

Table 2. MAPK, Thioredoxin, and MnSOD cDNA sequences derived from wheat seedlings exposed cadmium.

gene	sequence
MAPK	GGGACCTACCAGTAGAATAGTCTGTGGCAGCCAGTACCAATATC CAGGAAGTTGTTGGCAAGGGGAGTTATGGAGTAGTTGCTA
MnSOD	CCGGGGGCTACGTGTGGTGGGCATGTTAGCCATTCAATCTTCTGG AAGAACCTCAAGCCCATCAGCGAGGGTGGTGGTG
Thioredox in	CGGGTCAGCATACCACCGTTCCTGTTCATGAGGAGGAGACGTCA AGGACAGGGTTGTCGGAGCTATCAAGGAGGAACTCGA

Expression analysis of MAPK, Thioredoxin, and MnSOD genes

Real-Time PCR was performed to explore MAPK, Thioredoxin and MnSOD gene expression in response to cadmium. The results showed that the Thioredoxin and MnSOD genes were significantly up-regulated 3.65 and 2.09 fold compared with the control, respectively, but MAPK gene was not a significant change compared with the control sample (Table 3, and Fig. 4).

Table 3. MAPK, Thioredoxin, and MnSOD genes expression in the wheat seedlings exposed to exposed to cadmium (0 or control and 100 mg/L) for six days.

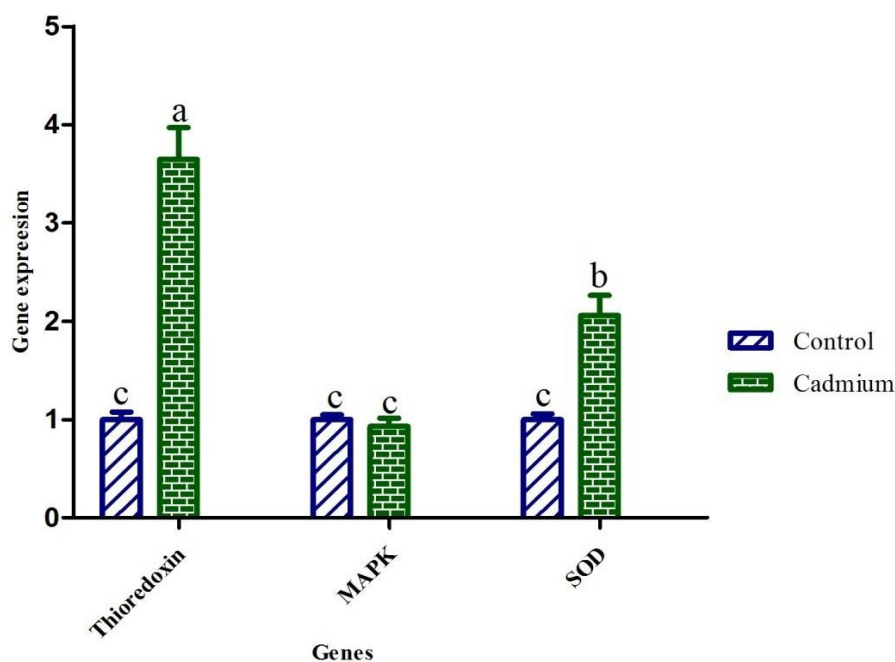| Concentration ($mg^{-1} L$)	MAPK	Thioredoxin	MnSOD
Cadmium 0 (Control)	1± 0.07c	1 ± 0.05c	1 ± 0.05c
Cadmium 100	3.68 ± 0.43a	0.92 ± 0.09c	2.18± 0.29b

Figure 4. Analyses of the expression of MAPK, Thioredoxin, and MnSOD genes in the wheat seedlings exposed to cadmium (0 or control, and 100 mg/L) for six days (Means of gene expression in the Y-axis is multiplication of expression).

After a week of beginning constant cadmium stress, Thioredoxin gene expression showed a large increase. Thioredoxin is a key ubiquitous disulfide reductase responsible for maintaining proteins in their reduced state, which is reduced by electrons from NADPH via Thioredoxin reductase. The main role of Thioredoxin proteins in heavy metal tolerance is detoxification of free radicals [3; 18].

In response to cadmium, MnSOD gene expression increased compared with non-treated wheat seedling at the same times. Heavy metals such as cadmium in plants lead to oxidative stress [17; 23]. This stress is caused by the generation of reactive oxygen species (ROS) led to being over-expression of MnSOD gene that plays an important role in the protection against the toxic effects of oxidative stress by scavenging superoxide radicals [9; 11; 25].

Unlike the Thioredoxin and MnSOD genes, MAPK gene expression in wheat exposure to six days cadmium stress has no significant change compared with the control sample. Insignificant changes in the level of MAPK gene expression may be related to since, at this time, the existing copies of MAPK gene had provided enough. As we know, as we know, MAPK is an upstream enzyme, and it involved in the signal transduction pathways associated with environmental stress responses. Some studies demonstrated expression of MAPK gene can control by light and biological clocks and heavy metals [1; 10].

Conclusion

It has been recognized that regulation of gene expression in response to heavy metals stresses is a key mechanism in protection and survival of plants. In this study, we compared the transcriptome profiles of MAPK, Thioredoxin, and MnSOD genes in wheat seedling control and exposed to cadmium. Results showed wheat seedlings that were exposed to cadmium stress for six consecutive days, had different MAPK, Thioredoxin, and MnSOD gene expression compared with the control sample. It seems extra testing at other times since the beginning of stress is necessary. These results presented here can supply benefit acknowledgments for future studies and showed that the expression of Thioredoxin and MnSOD genes significantly was induced by heavy metal stresses. We hypothesized that this gene expression can be considered as markers of heavy metals and metal nanoparticles. To better understand regarding the toxicity effects of cadmium on human, animals, and plant, additional experiments should be carried out.

Conflicts of Interest

The authors declare no conflict of interest.

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