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Expression Analysis of Some Stress-Related Genes Induced by Cadmium on Tomato (*Solanum Lycopersicum* L.) Plants

Ekrem Bolukbasi[1,2]
[1]Amasya University, Department of Environmental Protection and Technologies, Amasya,!Turkey
[2]Amasya University, Central Research Laboratory, Amasya,!Turkey

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***Correspondence to**: Ekrem Bolukbasi, Amasya University, Department of Environmental Protection and Technologies, 05100, Amasya, Turkey.*
E-Mail: ekrem.bolukbasi@amasya.edu.tr
Phone: +90 (358) 211 5043
Fax: +90 (358) 417 7794

ABSTRACT

Environmental pollution occurs in nature as air, soil and water pollution and as a result it affects whole ecosystem including human beings. Although industrialization and technological developments have made life easier than before, in recent years, they have triggered environmental pollution. Cadmium, which is a toxic pollutant for all living things, is one of the most important element in heavy metal pollutants. In this study, it was aimed to determine gene expression changes in tomato plant under Cd stress. Molecular response of tomato plants to Cd stress was examined by transcript accumulation analysis of two stress-related genes: (i) MT-2 (metallothionine-2) gene encodes metal binding protein and (ii) The GR-1 (glutathione reductase-1) gene encodes the glutathione reductase enzyme and is a marker of the ROS scavenging mechanism. Expression differences in MT-2 and GR-1 genes in tomato seedlings exposed to cadmium stress at different concentrations ranging from 20 to 1280 mg L-1 for 24 hours were determined performing quantitative real-time PCR. The results obtained from this study were showed that MT-2 and GR-1 genes play an important role in the mechanism of protection against heavy metal of Cd stress. In addition, the physiological properties of tomato have been associated with cadmium accumulation.

Keywords:
Cadmium stress; Tomato; Metallothionein; Glutathion reductase; qRT-PCR

INTRODUCTION

Tomato is an important fruit crop grown mainly as an annual and economically valuable plant for growers in the Mediterranean basin (*Solanum lycopersicum* L. formerly Lycopersicon esculentum Mill. belongs to the Solanaceae family). It is an important source of vitamins, minerals, fiber and a dietary antioxidants [1]. It is also among the anti-carcinogenic foods due to the carotenoids it contains. Consumed as fresh and dried fruit, tomatoes are also processed in industry as tomato paste. Turkey, Egypt, Italy, Spain, Greece and Morocco are among the world's largest tomato producers and exporters [2].

With the increasing population, unplanned urbanization and developing technology, heavy metal pollution has become an important environmental problem worldwide. In addition to these, as a result of industrial activities, mining, using pesticide in agriculture, metallurgy, combustion of fossil fuels, faulty waste disposal, metal-enriched some materials, automotive emissions and domestic wastes and many other factors [3-5]. Heavy metal pollution, one of the most important environmental pollutants, is reported to accumulate in soil and water at high concentration, causing genotoxicity and damage to many functional biomolecules in living things. Heavy metals, which accumulate intensely in soil and water ecosystems, can be included in the food chain, especially by means of plant-based nutrients [6,7]. Thus, the heavy metals included in the food chain, may deteriorate the structure of many biomolecules such as proteins, enzymes and especially nucleic acids [8-11].

Very few metals such as Zinc, Copper, Nickel, Manganese and Iron are required nutrients in low concentrations for plant life and normal growth. This situation is similar for humans and animals, too [12,13]. These metals act as co-factors for many enzymes in most metabolic pathways. Especially in the structural and catalytic functions of proteins. On the other hand, the presence of the same metals in high concentrations in tissues causes toxic effects [10,14]. Thus, it adversely affects many biological molecules. For example, reactive

oxygen species (ROS) such as hydrogen peroxide and singlet oxygen formed due to heavy metal toxicity, cause conformational changes in enzymes involved in important metabolic formation pathways such as protein and nucleic acid. This causes oxidative damage, impairment of cellular homeostasis and stress, in plants as well as in many living things [15-17]. Cadmium (Cd) heavy metal in particular is a highly toxic pollutant for all living things, especially plants. Cadmium, which is not an essential element especially for plants, is generally found in low amounts in the soil and adversely affects plant growth and development. It is not an essential nutrient for plants, but it quickly enters the cells. This metal has some serious effects on plants such as growth inhibition, decrease in enzyme activities, photosynthesis and nutrient intake. Cadmium accumulated in plant tissues can cause serious damage to biological molecules such as proteins, enzymes and nucleic acids [6,18-20]. For example; ROS formed via cadmium stress may cause changes such as incompatibilities in DNA bases and instability of the double helix structure [16,21]. This causes changes in the expression of some genes that play a role in dealing with stress caused by heavy metal pollution. This change in gene expression allows plants to cope with stress [3,7,22-24].

In the present work, gene expression changes due to Cd stress in tomato plant were analyzed by examining two different stress-related genes. (i) MT-2 (metallothionine-2) gene encodes metal binding protein and (ii) The GR-1 (glutathione reductase-1) gene encodes the glutathione reductase enzyme and is a marker of the ROS scavenging mechanism [9,25-28].

Metallothionein is a protein that combines with metals to form complex structures as chelators. Many studies have shown that metallothioneins are highly expressed in metallophyte tolerant plant varieties. Thus, the plants can protect themselves against metal stress [26-29]. These metallothionein proteins are able to bind heavy metals by the thiol groups in the cysteine residue. Also, metallothionein proteins are involved in scavenging of ROS. Glutathione reductase is an enzyme that reduces oxidized glutathione. Glutathione reductase, an enzyme especially active in the ascorbate-glutathione (ASH-GSH) pathway, plays a role in defense against ROS by maintaining the low status of GSH [30]. By determining the changes in gene expression levels of metallothionein and glutathione reductase in plants, the tolerance of plants to various types of stress can be determined [29-33].

The results suggest that the early molecular response of hydroponically cultivated tomato plants might develop different strategies to cope with Cd toxicity by manipulating the expression level of stress-related genes. Therefore, in this study, changes in metallothionein and glutathione reductase genes in tomato seedlings exposed to cadmium heavy metal stress at different concentrations for a certain period of time were demonstrated. The quantitative real-time PCR technique was performed to determine the change in gene expression levels. Finally, the data obtained from the study; showed that the change in the expression levels of these genes in the tomato plant could serve as an additional Cd-tolerance mechanism to deal with the toxic effect of cadmium.

MATERIALS AND METHODS

Growth of Plant Samples and Cadmium Stress Treatments

Before planting tomato seeds, their surfaces were sterilized with 70 % alcohol and 30 % sodium hypochlorite solution. The seeds were then washed three or four times with distilled water. For the germination and growth of tomato seeds, viols prepared using sterile perlite were arranged. Tomato seeds were germinated in sterile perlite by irrigating with 0.2 L modified 1/10 Hoagland solution and grown hydroponically. Tomato seeds planted in three biological replicates were grown in a controlled environmental growth chamber at 23-26 ^{0}C with 250 mmol m-2 s-1 photosynthetic photon flux and 50-60 % relative humidity. After 21 days of growing period, tomato seedlings were exposed to cadmium solution at different concentrations of 20, 40, 80, 160, 320, 640, 1280 mg L-1 in the growth chamber for 24 hours. At the end of the 24 hour period, the roots and shoots of tomato seedlings were harvested and stored at -80 ^{0}C until RNA isolation.

Root and shoot fragments (~200 mg) taken from samples which exposed to cadmium stress were powdered using liquid nitrogen. Subsequently, RNA was isolated from these samples. For RNA extracting, Trizol RNA extraction protocol was followed by RNeasy mini kit (Qiagen, Cat no: 74104) to cleanup. Quantity and quality measurement of isolated genomic RNAs were determined by Nanodrop (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific). And then it was confirmed by 1.5 % agarose (containing 0.05 µl ml-1 EtBr) gel electrophoresis.

First Strand cDNA Synthesis Assay

For the first strand cDNA synthesis assay, a two-step procedure was performed for real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The reverse transcription reaction was performed using a high quality cDNA synthesis kit (Roche). According to the protocol; 2 µg isolated RNA, 2.5 µM Anchoredoligo (dT)18, 1X Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 20 U RNase Inhibitor, 1 mM deoxynucleotide

mix and 10 U Transcriptor high fidelity reverse transcriptase were used. Quantity and quality measurements of cDNA were determined by Nanodrop (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific).

The Quantitative Real-time PCR Analysis of GR-1 and MT-2 Genes

Following cDNA synthesis, Real-Time PCR applications were performed using SYBR Green I Master dye via Light Cycler Nano (Roche) device. Sequences of the target genes identified in the study were searched/determined from the NCBI database and primers specific to these regions were designed using the Primer-3 program [36]. The designed primers were commercially synthesized. In addition, the Actin (ACT) gene was selected as the housekeeping gene to be used in the normalization process. During the Real-Time PCR reaction, Melting curve analysis was performed to determine the efficiency of PCR and to observe if there was any dimer formation, following the quantification (quantification = determination of expression) performed using SYBR Green I dye. Before starting the actual experimental work, the optimization of the reaction conditions was ensured. As a result of the experiments, the most suitable primer and cDNA concentrations were determined. The sequences and melting temperatures (Tm) of the primers used throughout the reactions are given in Table-1 and the homology analysis information of the qRT-PCR amplified transcripts in the NCBI database are given in Table 2.

Table 1. Sequences and melting temperatures of primers used in qRT-PCR

Genes/Primers name	Sequence (5'-3')	Tm (°C)
MT-2F	GCTGTGGATCTAGCTGCAAGTGCG	58-60
MT-2R	AAGGGTTGCACTTGCAGTCAGATC	
GR-1F	CGTGCTGTGATACTTGGTGG	58-60
GR-1R	TCGTGCAAGGATGCATAGTG	
ACT-F	GGGATGGAGAAGTTTGGTGGTGG	58-60
ACT-R	CTTCGACCAAGGGATGGTGTAGC	

Real-Time PCR reactions were performed in triplicate (as technical iterations) using the optimal conditions obtained as follows were initial denaturation 10 minutes at 95 °C, (40 cycles) 95 °C for 10 seconds, 58-60 °C for 20 seconds, 72 °C for 20 seconds, and increasing incrementally from 55 °C to 95 °C temperature 0.5 °C min-1.

Normalization and Statistical Analysis of Real-Time PCR Results

Gene expression results determined as Ct (Cycle Threshold) value, ACT (actin) and control conditions used in the study were normalized by considering housekeeping gene (Livak and Schmittgen 2001). The obtained data were normalized according to the 2-ΔΔCt method of Livak and Schmittgen [37].

Table 2. Homology analysis information of qRT-PCR amplified transcripts in NCBI database.

Genes	Length	Homology	Accession no.
MT-2	170 bp	*Solanum lycopersicum* type 2 metallothionein mRNA, partial cds	EU884310
GR-1	87 bp	*Solanum lycopersicum* glutathione reductase mRNA, partial cds	FJ265823
ACT	398 bp	*Solanum lycopersicum* ACT mRNA for actin, partial cds	AB199316.1

ANOVA, Tukey and Dunnett multiple comparison tests were performed to reveal differences between groups. The homogeneity of variances was determined with the statistical program (IBM SPSS Statistic-21) and Levene's test. The post-hoc Tukey HSD and Dunnett test were applied to the homogeneously distributed variables (also Dunnett's test to confirm the results) and the Dunnett T3 test was applied to the non-homogeneously distributed variables. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

It is one of the most important effects of heavy metal toxicity known to inhibit root and shoot development. The toxicity of heavy metals due to increased concentrations negatively affects the development of roots and shoots and seed germination in plants. Similarly, regression in root development of tomato plants used in the study was detected. As expected, these results are similar to previous studies [8,9,16,30].

Metallothionein binding proteins (MTs) protect plants from metal stress and toxicity. Numerous studies have shown that MTs are highly expressed in many plants exposed to heavy metal toxicity [7,8,27]. Cysteine residue has the ability to bind heavy metals both physiologically (such as zinc, copper, and selenium) and xenobiotically (such as cadmium, mercury, silver, and arsenic) via thiol groups. In addition, it has been reported that MTs have important roles in both metal chaperoning and scavenging of ROS [28]. Many studies have shown that arsenic, cadmium and copper stresses induce metallothionein expression and accumulation [26,27,29].

Glutathione reductase (GR) is an important enzyme in the ASH-GSH (Ascorbate-Glutathione) pathway in enzymatic antioxidant system in plants, as in many living things. It plays a critical role in the defense system against

ROS, which occurs as a result of stress factors, by maintaining the GSH level and acts as a substrate for glutathione-S-transferases. In many recent studies, it was stated that GR-1 expression increased against cadmium stress in various plants [7,9,31-35].

Considering the mRNA expression profiles of genes (MT-2 and GR-1) and actin (ACT) used as a housekeeping gene and control conditions of root and shoot samples of tomato plants with different concentrations of cadmium (Cd) stress applied by Real-time PCR (Light CyclerNano, Roche). Normalized according to the 2-ΔΔCt method of Livak and Schmittgen [37]. In addition, the quantification and melting curve analysis of the transcripts are given in Figure 1. Normalized gene expression data were averaged and according to the results obtained, the changes in the concentration-dependent expression level of MT-2 and GR-1 genes occurring in different tissues of each tomato samples were shown on the graphs. For accuracy of results, MT-2, GR-1 and ACT transcript levels of all samples were measured in triplicate for different concentration of cadmium stress.

In this current study, different amounts of GR-1 accumulation were observed depending on varying concentration ranges in roots and shoots samples taken from tomato seedlings exposed to cadmium heavy metal stress. When the GR-1 expression data in both root and shoot parts were evaluated; The changes in the expression levels of the GR-1 gene, depending on the concentration, in the root samples of tomato seedlings subjected to cadmium stress are given in the Figure 2. Compared to the control group, the GR-1 gene expression level was approximately 5.9 and 7.8-fold, with the highest concentrations of 160 and 320 mg L-1, and the lowest at 1280 mg L-1 concentration, approximately 1.5-fold. GR-1 gene expression change in shoots; it was detected at the highest level with 80 mg L-1 and 320 mg L-1 concentrations, 8.8 and 11.9 fold, respectively. Similarly, fold change was observed as 7.0 fold at 160 mg L-1 concentration. On the other hand, the lowest expression data is; it was detected at the concentration of 1280 mg L-1 with approximately 0.9 fold (Figure 2).

Additionally, the GR-1 expression data in root and shoot samples were examined; an abruptly decrease was observed after the cadmium concentration of 160 mg L-1 in the root and 320 mg L-1 in the shoot, especially. This result decreased almost to the control group expression level with the increase of the cadmium concentration. This also shows that; it is the ineffectiveness of the resistance mechanism against cadmium stress in both roots and shoots after the specified concentrations. It was determined that the GR pathway was inhibited due to possible cellular damage and it could not provide protection against cadmium stress by using the stress recovery pathway [7,31-34].

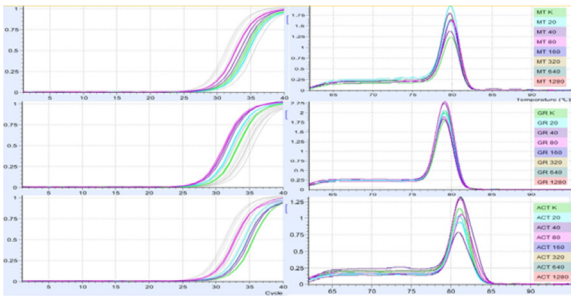

Figure 1. The quantification and melting curve analysis of the transcripts. The graphs on the left side shows transcript accumulation; right side shows the melting curve analysis of MT-2, GR-1 and Actin genes.

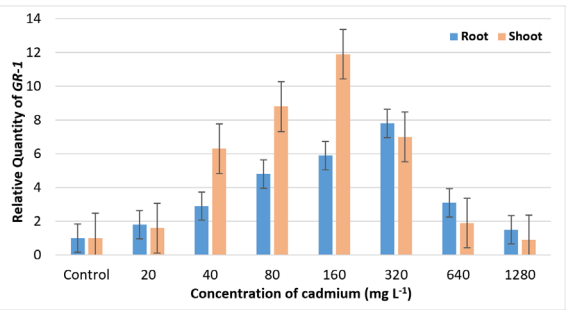

Figure 2. The changes in the expression levels of the Glutathion reductase-1 (GR-1) gene, depending on the concentration, in the root and shoot samples of tomato seedlings subjected to cadmium stress

On the other hand, metallothionein-2 (MT-2) gene expression data were examined, MT-2 results similar to GR-1 data were obtained. When the expression data of root and shoot samples of tomato seedlings under cadmium stress were evaluated; MT-2 gene expression level was found to be 3.4 and 4.4 fold in root samples at 80 and 160 mg L-1 concentrations, respectively. After the concentration of 160 mg L-1, there was a remarkable decrease in the expression of the MT-2 gene like GR-1. Similarly, 4.2 and 4.7 fold changes were detected in shoots at 80 and 160 mg L-1 concentrations, respectively (Figure 3). It is cleared from the results obtained that the change in the expression of the metallothionein binding protein gene MT-2 can induce cadmium toxicity tolerance in tomato plant [7,29,32,33].

In addition support these results, the change in GR-1 and MT-2 expressions data in both root and shoot samples was found to be statistically significant ($P < 0.05$). Detailed

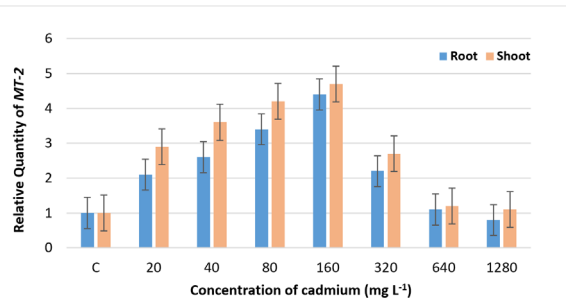

Figure 3. The changes in the expression levels of the metallothionein-2 (MT-2) gene, depending on the concentration, in the root and shoot samples of tomato seedlings subjected to cadmium stress

Table 3. Sequences and melting temperatures of primers used in qRT-PCR

		Concentrations						
	Genes	20 mg L^{-1}	40 mg L^{-1}	80 mg L^{-1}	160 mg L^{-1}	320 mg L^{-1}	640 mg L^{-1}	1280 mg L^{-1}
root	GR-1	*	*	***	**	***	*	*
	MT-2	*	Ns	*	***	*	***	*
shoot	GR-1	*	*	**	**	*	*	*
	MT-2	*	*	**	*	*	Ns	*

* p<0,05, ** p<0,01, *** p<0,001, Ns: non significant

information on the statistical significance levels of GR-1 and MT-2 expressions data in root and shoot samples of tomato seedlings exposed to cadmium stress at different concentrations compared to the control group has been given in the Table 3.

The results obtained with the GR-1 and MT-2 genes and their activities in this current study support different studies in the literature [7-9,26,27,33]. Stress related genes such as GR-1 and MT-2 are used in different applications (especially phytoremediation) against various stress factors. It has been reported that the evaluation of the expression data of these genes is effective in determining the level of damage to the living thing by the pollution in question and determining the molecular biological limits of the defense mechanism [7,26,28-35]. According to Tombuloglu et al. (2012) the expression data of GR-1 and MT-2 genes, which are stress-related genes due to boron stress, were examined in tomoto seedlings. While gene expression increased due to increasing concentration, a decrease was observed after certain concentrations [27]. Also, in our previous study (2019), tomato seedlings were exposed to varying concentrations of zinc heavy metal stress and the expression levels of the same genes were investigated. Although similar results were obtained, its toxic effect was revealed at higher concentrations (eg: 320 mg L-1 - 640 mg L-1) than cadmium toxicity, since zinc is a microelement. After these concentrations, sudden decreases were observed in the same way. The results of the present study also support these [7]. Finally, Wang et al., (2018) cloning and characterization of the glutathione reductase gene and Rono et al. (2021) showed that metallothionein-like gene groups were identified for cadmium detoxification and potential phytoremediation. Thus, it has been shown that there are different and new stress-related genes or clones as GR-1 and MT-2 [31,33].

CONCLUSIONS

In the present study, it was shown that the activation of MT-2 and GR-1 like protein transcripts under cadmium stress. These genes expression increased at first and then, the expression curve was showed a descending profile due to inhibition of stress mechanisms which regulates the cellular homeostasis under high cadmium level. Due to the increased concentration of cadmium heavy metal, the change of these genes, which are known to be stress-related in plants, such as tomato, has shown that these genes play an important role in the mechanism of protection against heavy metal stress. Also, this study results have indicated that tomato has physiological traits associated with accumulation of cadmium. The early molecular response of hydroponically cultivated tomato plants might develop different strategies to cope with Cd toxicity by manipulating the expression level of stress-related genes. Furthermore, these results showed that the resistance mechanism could not cope with cadmium stress and toxicity due to possible cellular damage using the GR pathway. Finally, the data obtained from the study; showed that the change in the expression levels of these genes in the tomato plant could serve as an additional Cd-tolerance mechanism to deal with the toxic effect of cadmium.

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CONFLICTS OF INTEREST

Author had no any financial or personal relationships with other individuals or organizations that might inappropriately influence this work during the submission process.

STATEMENT OF ETHICS

There is no need for an ethics committee decision for the studies in the article.

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