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ORIGINAL ARTICLE

Analysis of *Phaseolus vulgaris* gene expression related to oxidative stress response under short-term cadmium stress and relationship to cellular H_2O_2

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Abstract

In this study, we aimed to investigate the changes in the malondialdehyde (MDA) content and cellular H_2O_2 accumulation, which are the oxidative stress markers, and the expression levels of stress-related proteins such as metallothionein (*MT*) protein, catalase (*CAT*), Superoxide dismutase (*Cu/Zn-SOD*), and glutathione S transferase (*GST*) enzymes. For placing oxidative stress on plants, we used Cd in different concentrations (50,100 and 200 μM) and durations (12,24 and 48 h). The results revealed that MDA content and H_2O_2 accumulation linearly increase with exposure time and concentration. Although the *Cu–Zn/SOD* and *MT* expressions increased at 24 h exposure, they decreased at 48 h exposure. The *GST* gene is highly expressed at the beginning level of oxidative stress, whereas a decrease in the level of expression was detected with increase in exposure time. Statistical significance was detected in *CAT* gene expression at 12 h application; however, a decrease in expression level was detected in the 24 and 48 h applications. The results of this study show that transcript accumulation of stress-related genes might coincide with the oxidative levels in the cell and the H_2O_2 concentration in the signalization structure. Moreover, it could accelerate the expression of the stress genes up to a certain concentration degree, whereas a greater concentration of H_2O_2 could reduce transcript accumulation.

Keywords Oxidative signaling · ROS · Heavy metal · Real time PCR · Stress-related genes · Antioxidant enzymes

Abbreviations

MT	Metallothionein
CAT	Catalase
SOD	Superoxide dismutase
GST	glutathione S transferase
MDA	malondialdehyde
H_2O_2	hydrogen peroxide
ROS	Reactive Oxygen Species
APX	ascorbate peroxidase
GR	glutathione reductase
Cd	Cadmium
TCA	Trichloroacetic acid
TBA	thiobarbituric acid

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Introduction

Plants must cope with many negative conditions in nature because of their sessile lives. These adverse conditions are classified into two main groups: biotic and abiotic stress. Abiotic stresses such as drought, temperature, and salinity are the most frequently encountered stress types for plants; they produce considerably destructive effects including irreversible physiological and morphological changes. In addition to drought, salinity, and temperature, another frequently encountered abiotic stress factor is heavy metal stress. Domestic and industrial waste as well as the dense use of fertilizers in agriculture cause a considerable amount of heavy metals to be loaded to ground waters and agricultural areas. Consequently, heavy metal stress damages plant metabolism, disrupts ion imbalance, and affects cell permeability, thus limiting plant growth and crop yield (Schützendübel and Polle 2002; Dalvi and Bhalerao 2013).

Plants exposed to biotic and abiotic factors produce reactive oxygen species (ROS) that cause oxidative stress. ROS are radicals that cause extremely disruptive effects, such as distorting cell membrane permeability and flow and causing

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DNA damage, by attaching to cell membrane lipids (Kumar et al. 2017).

Plants contain a range of enzymes like antioxidants to relieve the harmful effects of ROS. All enzymes work in coordination to scavenge different ROS types. These enzymes can be broadly classified into two types: 1) enzymes (SOD, CAT, and POX) that directly react with ROS and decrease its level, and 2) enzymes that produce oxidized forms of antioxidants—ascorbate peroxidase (APX) and glutathione reductase (GR) (Kumar and Ganeshkhind 2017). Copper-zinc superoxide dismutase (Cu/Zn-SOD) is a 32 kDa homodimeric protein in the cytoplasm of eukaryotic and bacterial cells that catalyzes the disproportionation of superoxide into dioxygen and hydrogen peroxide (Hart et al. 1999). The typical catalase reaction is the dismutation of two molecules of H_2O_2 to water and O_2 (Mhamdi et al. 2010). Glutathione S-transferases (GSTs) catalyze the conjugation of oxidative-produced compounds to reduced glutathione, which facilitates their metabolism, sequestration, and removal (Dalton et al. 2009). Metallothionein plays a very important role in the detoxification of metals thanks to its sulfur-containing residues (Souguir et al. 2013).

The most common reactive oxygen species (ROS) are singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). The formation of ROS and a serious amount of damage, depending on the degree of destruction in the cell, are inevitable when plant cells are exposed to abiotic stress. For a long time, it has been agreed that ROS are extremely dangerous and that they have damaging effects on cells, and scientists have focused on studying ROS antioxidative enzyme scavenging activities and on the deleterious effects of ROS on the cell (Duman and Ozturk 2010; Štolfa et al. 2015, 2016). However, according to the recent definition of 'oxidative signaling', it has been emphasized that H_2O_2 is not as harmful as was predicted, and it is even necessary for opening and closing many signal pathways important for the onset of some gene expressions (Romero-puertas et al. 2004; Del Río Luis Alfonso and Puppo 2009).

Plants expend about 1% of their total O_2 on ROS synthesis. ROS are the products of aerobic metabolism, and they include superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen. ROS types are distinguished from other signaling molecules by their high reactivity, half-life, and lipid solubility (D'Autreaux and Toledano 2007). In the plant cell, depending on ROS production concentration, these molecules are considered both harmful and advantageous. At low or moderate concentrations, ROS acts as a second messenger in the intracellular signaling mechanism that interferes with numerous cellular responses in plants, whereas it may cause oxidative damage at high concentrations (Vanderauwera et al. 2009).

In this study, we used Cadmium (Cd) to place oxidative stress on plants. Cd is a very toxic heavy metal that is commonly found in nature. Because of its high solubility in water, plants may accumulate Cd, which causes severe environmental pollution as a result of anthropogenic and agricultural activities, even though it is not essential for the plant. The Cd accumulated by plants may enter the food chain indirectly, causing harmful effects on humans and organisms. Cd causes effects such as blocking signaling receptors in plants, impairing membrane permeability, affecting ATP synthesis, and most importantly, inducing oxidative stress, which is very dangerous for plants, and triggering reactive oxygen production (Chen et al. 2003; Souguir et al. 2013).

The responses of the common bean (*Phaseolus vulgaris*) to the exposure of Cd were observed. Lipid peroxidation and H_2O_2 concentrations were found to determine the oxidative level of the cell. Especially, the relationship between the changes in mRNA expression that transcripts antioxidant stress enzymes and the cellular H_2O_2 concentration in the common bean plant has been identified for the first time.

Materials and methods

Plant material and cultivation conditions

Phaseolus vulgaris seeds were purchased from Nevşehir Directorate of Provincial Agriculture and Forestry and germinated until the first leaves and roots emerged in moistened cotton. Then, seedlings were placed in a hydroponic environment containing Hoagland solution to elongate the roots. After the roots reached 10–15 cm in length, they were placed into 400 ml beakers containing 50, 100, and 200 μM $CdCl_2$ solution. They were exposed to Cd for 12, 24 and 48 h. The light/dark photoperiod on the cultivation cycle was set to 16:8. All applications were repeated three times.

Lipid peroxidation

Lipid peroxidation was determined by malondialdehyde (MDA) analysis to show the effect of stress on plants (Hodges et al. 1999). One hundred mg of root sample was homogenized in 100 ml 80% cold ethanol. Homogenates were centrifuged at 3000 g + 4 °C for 10 min. Different aliquots of the supernatant were mixed either with 20% TCA or with a mixture of 20% TCA and 0.5% TBA. Both mixtures were incubated in a water bath at 90 ◦C for 1 h. Afterwards, samples were cooled in an ice bath and centrifuged at 2000 g for 5 min at 4 ◦C. The absorbance at 440, 534, and 600 nm of the supernatant was read against a blank.

H_2O_2 determination

The method of Junglee et al. was used with modification for the determination of H_2O_2 (Junglee et al. 2014). One hundred

mg of root sample was ground in liquid nitrogen and then homogenized in 1 ml solution that contained 0.25 ml TCA (0.1%, w / v), 0.5 ml KI (1 mM), and 0.25 ml potassium phosphate at +4 °C for 10 min. Afterwards, the homogenate was centrifuged at 10000 g for 15 min at +4 degrees. The supernatant was incubated in the dark for 20 min at 20–22 °C. The H_2O_2 content was measured with a quartz cuvette at 280 nm, and the concentration of H_2O_2 in the cell was determined using the gradual concentrations of H_2O_2 (Junglee et al. 2014). The formula is $Y = 0.1922x + 0.0372$, where Y is absorbance and X is cellular H_2O_2 concentration (nanomole).

RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis

Total RNA extraction was performed using Trizol protocol. RNA quality was measured with a Donowix nanodrop spectrophotometer.

Complementary DNA (cDNA) synthesis was performed with 2 μg of RNA and with a RevertAid cDNA synthesis kit (thermo) containing 100 μM oligo (dT)18, 250 mM Tris-HCl, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT, and 10 mM dNTP Mix. The following program was applied: 60 min at 42 °C, terminating the reaction 5 min at 72 °C.

Quantitative real-time PCR (qRT-PCR) was performed using the Bioneer Exicycler Tm 96 FaST device. The sequences of primers used in the study are shown in Table 1. The housekeeping gene actin was used as the control primer. List of genes and primer used for PCR amplification. Amplifications of PCR product were monitored via SYBR Green I dye.

Livak and Schmittgen's method was used: the fold expression of target mRNAs over the reference values was performed by the comparative threshold cycle (Livak and Schmittgen 2001). Analysis of variance (ANOVA) was used for statistical analyses, and a post hoc Tukey-HSD test was used to determine the differences between the groups ($p < 0.05$) using SPSS software version 22.

Results

Lipid peroxidation

MDA concentration is a very important parameter in terms of information about the oxidative status of the cell. MDA content was calculated to determine the damage caused by cadmium exposure to the cell membrane (Fig. 1A). As a result, it was determined that Cd exposure significantly increased MDA accumulation ($p < 0.05$). It was also observed that there was an increase in MDA content with time. In the post hoc analysis, it was determined that MDA accumulations at all concentrations were statistically different from each other and that the largest amount of MDA was obtained at 48 h exposure to 200 μM concentration of Cd.

H_2O_2 concentration

The concentration of H_2O_2, formed as a result of oxidative stress in the root cells is given in Fig. 2B. H_2O_2 concentrations were determined to be steadily increasing over the control group as the duration of application and concentration increased. No statistical difference was found between the amounts of H_2O_2 occurring at 50 and 100 μM concentrations of Cd in the 24 h application, whereas the highest H_2O_2 accumulation was detected at 48 h exposure to 200 μM concentration ($p < 0.05$) (Fig. 1B).

Differential expressions of stress-related genes in *Phaseolus vulgaris*

Responses of the common bean plant, which was grown in an aquatic environment, to Cd exposure were molecularly evaluated. Transcript accumulations of *CAT, Cu-Zn/SOD, GST*, and *MT2* genes coping with oxidative stress were detected by real-time PCR method. The changes occurring in stress-related gene expression levels were assessed according to the expression of actin, which was the housekeeping gene.

When *MT* gene expression levels were evaluated, the level of gene expression as a result of 12 h Cd application showed a

Table 1 List of genes and primer used for PCR amplification and the resulting product sizes

Gene	Primer Pair	Sequence (5′ – 3′)	Reference
Metallothionein-like gene	*MT2 F*	ACGGCTGCTCAGGCTGCAAG	(Barrera-Figueroa et al. 2007)
	MT2 R	ACAGGGGTCGCATTGGCAGT	
Superoxide dismutase	*Cu-ZnSOD F*	CAAGAGTCCCAATGCTGTGAACC CACTGCA	(Nanjareddy et al. 2016)
	Cu-ZnSOD R	TCCCAGGAAACAAG	
Catalase	*Cat F*	CACATCCAGGAGAATTGGAGG	(Nanjareddy et al. 2016)
	Cat R	CCAGCTTTGCTGATGAGGGTG	
Glutatione S-transferase	*GST F*	AGCTCTTCAAGGACACTGAGCCAA	(Oliveira et al. 2015)
	GST R	AGCCTTGGGGTTAAGAGGAG	
Actin 11	*Actin F*	TGCATACGTTGGTGATGAGG	(Oliveira et al. 2015)
	Actin R	AGCCTTGGGGTTAAGAGGAG	

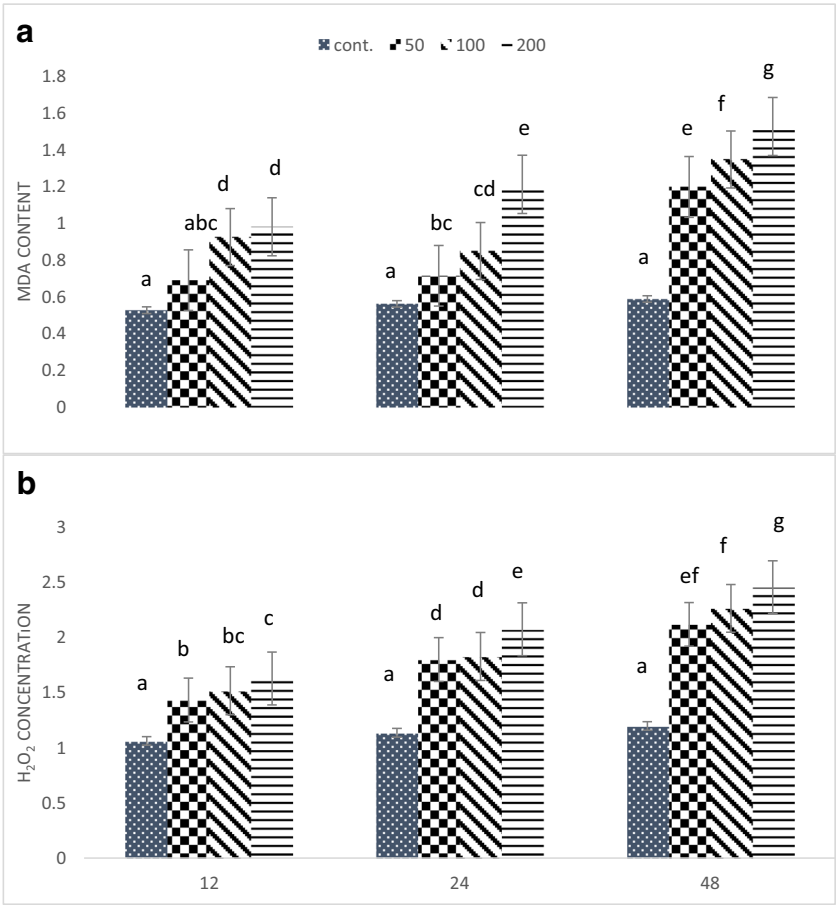

Fig. 1 Changes of MDA content nmol g^{-1}(A) and cellular H_2O_2 concentration nmol g^{-1} (B). Different letter indicate significant differences at $p < 0.05$ (ANOVA). Vertical bars indicate standard error.

statistically significant increase at 200 μM. When the 24 h application was analyzed, it was found that there was a dramatic increase in the expression levels: the highest expression level was observed at 100 μM concentration of Cd. The level of expression determined during 48 h Cd application was lower than the level of expression detected during 24 h application (Fig. 2A).

When the *GST* gene expression was observed, this substance was found to be expressed approximately four fold more than actin, the control gene, in a 12 h 50 μM application. At the same time, this level of expression is the highest among the treatments. In a 24 h application, the highest statistically significant expression was observed at a concentration of 50 μM. Yet, it was found that this gene was replicated at a lower level than that of the 12 h application. In the 48 h application, no statistically significant difference was found between concentrations except at 200 μM, and its expression level was statistically lower than the control group's (Fig. 2B).

The expression level of the *Cu/Zn SOD* enzyme did not show a significant increase in 12 h of Cd exposure except at 200 μM concentration. At 24 h exposure, the highest level of expression was found at a concentration of 200 μM. The SOD gene expression in a 24 h, 200 μM concentration was approximately sixfold greater than that of the control gene's expression level. In a 48 h application, the levels of expression were reduced compared to a 24 h application; however, the highest expression was detected in the 50 μM concentration of Cd ($p < 0.05$) (Fig. 2C).

The *CAT* gene exhibited behavior quite different from other stress gene expressions. While the highest expression levels were determined at 50 and 100 μM concentrations in the 12 h application, the *CAT* gene expression was lower than that of the control gene expression at 24 h and 48 h exposure, in which the other stress genes showed a significant increase in expression (Fig. 2D).

Discussion

In this study, cellular accumulation of H_2O_2, which is an oxidative stress indicator and an important reactive oxygen species, was detected in bean plants exposed to 50, 100, and 200 μM concentration of Cd for 12, 24, and 48 h. In addition, the amount of MDA indicative of damage caused by oxidative

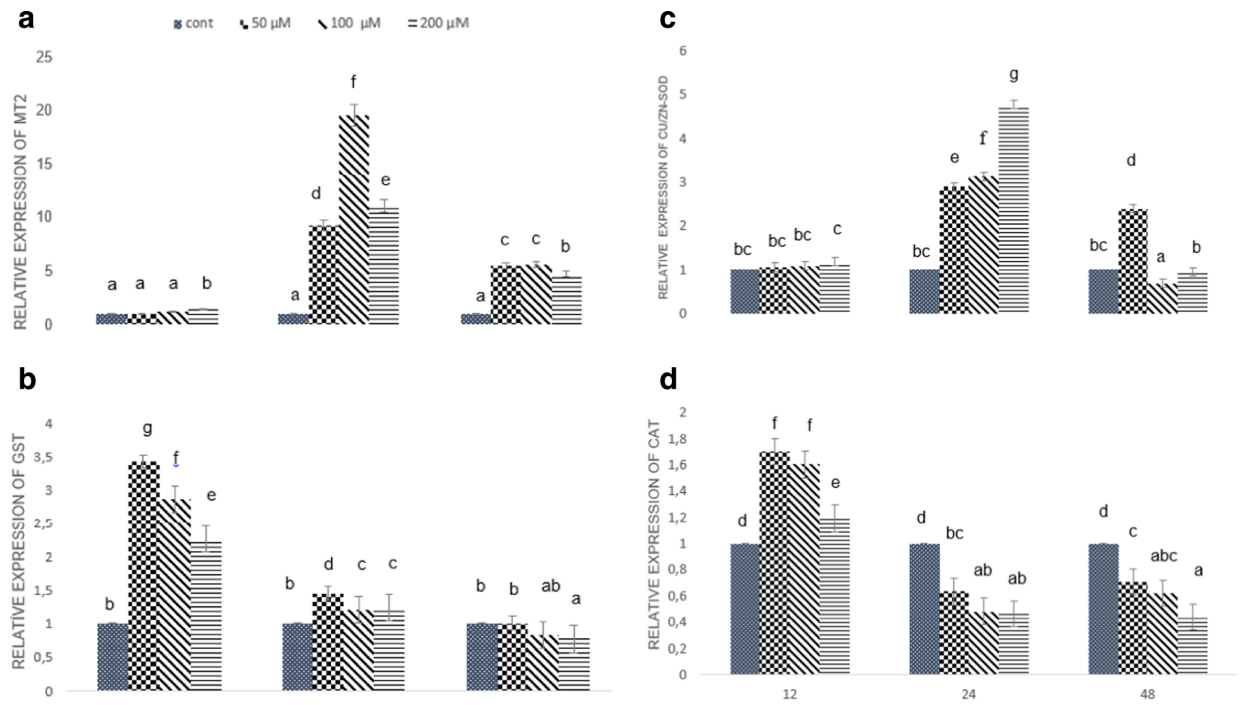

Fig. 2 Changes of transcript accumulations of *MT2* (A), *GST* (B), *Cu-Zn/SOD* (C) and *CAT* (D) different exposure and duration. To normalize the relative expressions of selected genes, *actine* was used as house-keeping gene. Different letter indicates significant differences ($p < 0.05$). Vertical bars indicate standard error

stress was calculated. Changes in the expression levels of *MT2, GST, Cu/Zn SOD*, and *CAT,* known as antioxidant stress genes, were determined based on the expression of the house-keeping gene, actin. The relationship between these expression levels and H_2O_2 has been explained for the first time.

As cell membrane lipids are very sensitive to ROS, oxidative stress first results in a radical chain reaction that breaks down membrane lipids by peroxidation. The amount of MDA may reflect the degree of damage that ROS has done to the cell membrane because this is the final degradation product of lipid peroxidation (Polle et al. 1997). In addition, MDA activity was calculated to demonstrate the effect of oxidative stress on the activity of many different antioxidant enzyme activities, and it was found that the concentration of MDA, which is the result of peroxidation in the cell membrane, increased in parallel with increased stress conditions (Singh et al. 2006; Razinger et al. 2008; Duman et al. 2010). In this study, MDA concentration also showed a steady increase in response to the duration of exposure and Cd concentration, in support of other studies.

Metallothioneins are stress response proteins that have low molecular weight and a high aversion against heavy metals because they are rich in cysteine (Kısa et al. 2017). Several gene expression studies have been conducted to identify mRNA levels in different tissues, in different developmental stages, and under heavy metal stresses. *MT* gene expression levels also change in response to tissue-specific or developmental stress, as well as to the intensity of heavy metal stress (Hossain et al. 2012). Ahn et al. exposed the *Brassica napans* plant to Fe heavy metal and investigated different MT gene expressions. They found that the *MT2* gene was down-regulated in 3 h application (Ahn et al. 2012). Souguir et al. applied Cd to *Vicia faba* roots and found the highest MT expression level at a concentration of 200 µM for 12 h and that the level of expression decreased at 24 and 48 h exposure (Souguir et al. 2013). Similarly, our study found the highest *MT2* expression at 24 h exposure and that the level of expression decreased at 48 h exposure. The results of this study also parallel the study of Tombuloglu et al. (2012).

GST allows the binding of hydrophobic and electrophilic components—which are capable of damaging the cell—with glutathione (GSH). The components connected to GSH are subject to cellular detoxification pathways (Daniel 1993). A number of factors such as growth process, pathogens, herbicides, hormones, and cellular stress substances cause the synthesis of *GST* genes. The signal by which electrophilic compounds regulate GST gene expression is believed to be a pro-oxidant state in the cells, probably resulting from reduced GSH content (Chen et al. 1996). In line with this view, in this study the highest GST expression level was found at a concentration of 50 µM for 12 h, while there was no statistically significant difference between the concentrations at 48 h of application. Furthermore, a review article emphasizes that when H_2O_2 production is inhibited, genes encoding *GST* are identified (Apel and Hirt 2004). In this study, we supported the view that the H_2O_2 concentration was the same as the

control level; the level of expression was high at 12 h exposure to 50 μM concentration, and there was no statistical difference between concentrations in the 48 h application.

SOD, a family of antioxidant enzymes, is a major defense system against oxidative stress in plants and is ubiquitous in every cell of all plant types. As the first line of defense against oxidative damage, SOD catalyzes the conversion or dismutation of toxic O_2^- radicals to H_2O_2 and molecular oxygen (O_2). In this study in particular, the increase at 24 h exposure in the expression level of the *Cu–Zn/SOD* enzyme supports Tombuloğlu et al.'s study (Tombuloglu et al. 2012). Goupil et al. found that the highest level of *SOD* expression is in arsenic application at a concentration of 160 μM. They also found that the expression at a concentration of 640 μM was lower (Goupil et al. 2009). Our work supports these studies. As Cd exposure and concentration increased, there was a decrease in the expression level of *SOD*. As noted in previous studies, SOD enzyme activity is increased in abiotic stress conditions (Faralli et al. 2015). However, Sougir reported that *SOD* expression increased in the first 12 h of exposure and decreased in other concentrations (Souguir et al. 2013). Similarly, in our findings the highest expression of SOD enzyme activity was achieved in 24 h application, and the level of expression decreased in 48 h application.

The catalase enzyme is important in the detoxification of H_2O_2 as it catalyzes toxic H_2O_2 to H_2O and O_2. Soydam-Aydın et al. examined *CAT* enzyme activity and expression in the tomato plant and reported that enzyme activity increased while expression level decreased (Soydam Aydin et al. 2016). Similarly, Saenen et al. exposed the *Arabidopsis thaliana* plant to uranium and reported that *CAT* expression decreased (Saenen et al. 2015). Paralleling these studies, in our study CAT enzyme expression increased and was statistically significant compared to the control group in 12 h exposure, and CAT enzyme expression was lower than in the control gene in 24 and 48 h exposures. Souguir et al. found that the expression of the CAT enzyme constantly increased at decreasing concentrations of antioxidant-related stress genes such as *SOD, GR1*, and *MT2* (Souguir et al. 2013). In contrast, in our study the fact that the expression of the *CAT* enzyme decreased in the concentration at which the expression of other stress genes showed a significant increase could be interpreted as an inducing effect of H_2O_2.

Cellular ROS production occurs as a response to many stimuli; on the other hand, the balance of ROS—its production and reduction driven by environmental stresses such as drought, salinity, and pathogenic attack—must be maintained. Additionally, scientists have recently focused more on the effects of ROS on growth, development, programmed cell death, and environmental stresses than on the destructive effects of ROS produced by plants (Gill and Tuteja 2010; Baxter et al. 2014; Del Rio and Lopez-Huertas 2016). In the cell, there are many different signaling pathways that the signaling molecules turn on and off as needed. For this reason, the production of signal molecules should be strictly regulated to perform this function and be synthesized in the desired amount, and subsequently, this material should be removed shortly after it has served its purpose in the cell (Kumar et al. 2017). Interestingly, it is estimated that 2% of the O_2 taken in by plants is used for ROS production (Bhattacharjee 2005). The effective antioxidant mechanism is activated when it detects ROS in the cell, and this results in ROS becoming an effective signaling molecule. Stress conditions induce cellular imbalance, which is converted into a redox signal that activates specific responses at multiple levels (Scheibe ve Dietc 2012).

In our current study, expression levels of the *MT* gene and *SOD* genes were highest at 24 h exposure to concentrations and decreased at 48 h exposure. On the other hand, the concentrations of H_2O_2 steadily increased. The continuous increase in H_2O_2 and the decrease in maximum SOD and MT2 expression levels at the 48 h exposure support studies that emphasize H_2O_2 as a signaling molecule (Farnese et al. 2016).

GST enzyme expression reached its maximum level at 12 h exposure to concentration, at which time oxidative stress began. Although this value tended to decrease at other concentrations, no statistically significant difference was found between the expressions at 48 h exposure. This demonstrates that the GST enzyme is more active in pre-oxidative states and that increased H_2O_2 concentration may be a repressor component for GST expression (Khan 2017).

When evaluated in terms of *CAT* enzymes in a previous study, no significant relationship was found between expression levels and their activity (Soydam Aydin et al. 2016). In this study, there was no evidence that H_2O_2 increased expression levels; however, the fact that H_2O_2 down-regulates the CAT expression level in 24 h applications, which is the concentration at which other enzyme expressions are increased, may be a clue that H_2O_2 is involved in signaling.

Conclusion

In the *Phaselious vulgare* plant exposed to Cd heavy metal, the amount of MDA and the concentration of H_2O_2, which are oxidative stress indicators, showed a steady increase. When the expression of oxidative stress-related enzymes are investigated, it can be stated that while H_2O_2 upregulates the expression of *MT* and *Cu/Zn-SOD* enzymes in 24 h exposure to concentrations defined as medium level, it down-regulates them in 48 h application. In addition, while the *GST* enzyme was highly expressed in low-concentration H_2O_2, it was reduced in a medium H_2O_2 concentration, and the expression level decreased to the same level as the

control at high H_2O_2 concentration. In the *CAT* enzyme, the expression level with medium H_2O_2 concentration was down-regulated, yet it did not change at other concentrations. The findings obtained in the study will contribute to work to be carried out in the subject of 'oxidative signaling'.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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