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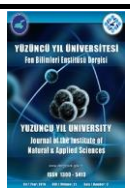

Research Article

Comparison of DNA Methylation and Changes in the Expression of Certain Cadmium-induced Genes in Bread Wheat Exposed to Cadmium (Cd) Accumulation in Soil[†]

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Abstract: As a result of natural phenomena and anthropogenic activities, cadmium (Cd) accumulation in the soil has been increasing in recent years, and plants are directly exposed to this heavy metal, which is not essential for their life. In our study, UGT-3 (UDP-Glycosyltransferase-3), LTP-4 (Lipid Transfer Protein-4), Plasma Membrane PIP-1 (Plasma Membrane Protein-1) genes were investigated in *Triticum aestivum L.* plants exposed to Cd stress. It was aimed to determine the changes in expression levels in these genes and their methylation percentages for the first time using Real-Time PCR-based quantitative DNA methylation analysis (qAMP). In this context, DNA and RNA were isolated from the roots and stems of wheat grown by exposure to control, 100, 250 and 500 µM $CdCl_2$ doses. Then, gene expression levels were determined by gene expression analysis with cDNAs obtained from RNA samples. DNA methylation percentages were determined by applying the qAMP technique. As a result, it was observed that the highest methylation percentage in the UGT-3, LTP-4 and PIP-1 genes was at 250 µM concentration in both the stem and the root. While the expression level of the UGT-3 gene was highest at a concentration of 250 µM in the stem, overexpression of the LTP-4 gene was observed at a concentration of 250 µM in the root changes in the methylation rates of UGT-3, LTP-4 and PIP-1 genes were investigated for the first time with qAMP, a new technique used in plants. A significant relationship was found out between the expression levels and methylation status of genes.

Topraktaki Kadmiyum (Cd) Birikimine Maruz Kalan Ekmeklik Buğdayda Kadmiyumun İndüklediği Bazı Genlerin DNA Metilasyonu ve İfadesindeki Değişikliklerin Karşılaştırılması

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Öz: Doğal fenomenler ve antropojenik olaylar sonucunda toprakta kadmiyum (Cd) birikimi son yıllarda giderek artmakta ve bitkiler, yaşamları için gerekli olmayan bu ağır metale doğrudan maruz kalmaktadır. Çalışmamızda Cd stresine maruz bırakılan *Triticum aestivum L.* bitkisinde UGT-3 (UDP-Glikosiltransferaz-3), LTP-4 (Lipit Transfer Proteini-4), Plazma Membran PIP-1 (Plazma Membran Protein-1) genleri çalışılmıştır. Bu genlerde meydana gelen ekspresyon seviyelerindeki değişimlerin ve Real-Time PCR temelli kantitatif DNA metilasyon analizi (qAMP) kullanılarak ilk kez metilasyon yüzdelerinin belirlenmesi amaçlanmıştır. Bu kapsamda kontrol, 100, 250 ve 500 µM $CdCl_2$ dozlarına maruz bırakılarak geliştirilen buğdayların kök ve gövdelerinden DNA ile RNA izolasyonu yapılmıştır. Daha sonra RNA örneklerinden elde edilen cDNA'lar ile gen ekspresyon analizi yapılarak gen ekspresyon düzeyleri belirlenmiştir. DNA metilasyon yüzdeleri qAMP tekniği uygulanarak saptanmıştır. Sonuç olarak UGT-3, LTP-4 ve PIP-1 genlerinde en yüksek

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metilasyon yüzdesinin hem gövdede hem de kökte 250 µM konsantrasyonda olduğu gözlenmiştir. UGT-3 geninde ekspresyon seviyesi gövdede 250 µM konsantrasyonda en yüksek oranı verirken, LTP-4 geninde kökte 250 µM konsantrasyonda aşırı ekspresyon gözlenmiştir. UGT-3, LTP-4 ve PIP-1 genlerinin metilasyon oranlarındaki değişimler bitkilerde kullanımı yeni bir teknik olan qAMP ile ilk kez araştırılmıştır. Genlerin ekspresyon seviyeleri ile metilasyon durumları arasında anlamlı bir ilişki olduğu bulunmuştur.

1. Introduction

According to the United Nations (UN) organization reports, the world population, which is currently 7 billion 924 million people, is expected to increase to 9.7 billion in 2050, and this figure is expected to rise to approximately 11 billion around 2100 (United Nations, 2022). Such a rapid increase in the world population; it also brings with it current environmental problems such as rapid industrialization, unplanned urbanization, destruction of agricultural lands, and unconscious and excessive use of natural resources. When the outcomes of these problems are reduced to specific ones, they cause air, water, radioactive and soil pollution, while these events also cause the destruction of the natural habitats of animals, plants and other microorganisms (Ramakrishnan et al., 2010; Wasi et al., 2013; Pal et al., 2015; Salbu et al., 2019; Li et al., 2021; Liu, 2021; Luo et al., 2022).

Soil is the natural living element for living things and the most basic resource that enables global food production. In addition, it is an indispensable part of the ecosystem that determines the direction and amount of water flow, regulates the climate, and ensures the continuity of biodiversity. However, according to the 2021 Global Assessment of Soil Pollution report of the United Nations Food and Agriculture Organization (FAO), 33% of our soils are degraded due to various pollutants, and heavy metals are among these pollutants (FAO, 2021). This ratio creates a rather terrible balance sheet, considering the comprehensive roles of soil.

Cadmium (Cd) is among the most important heavy metals. It occurs naturally at low concentrations, typically in soil. Wagner (1993) is reported that the Cd concentration contains 0.04-0.32 µM in uncontaminated soils, while moderately polluted soils contain 0.32–1.00 µM Cd. Cd, whose concentration has been increasing in agricultural lands in recent years, changes the structure and productivity of the soil. In addition, it seriously threatens the ecological balance of the soil and the biodiversity in the soil (Zou et al., 2021). Cd, which is not essential for plants, changes the functioning of the plant's biochemical, physiological and molecular processes after it is taken into the plant through the roots (Sun et al., 2008). In the study carried out to determine the toxicity induced by Cd in shoot and root growth, it was determined that the expression of paralog WOX genes changed in the *Arabidopsis thaliana* plant through cytokinin accumulation, causing a decrease in shoot and root size and affecting the shapes of these organs (Leonardo et al., 2021).

Cd triggers the formation of reactive oxygen species (ROS) in plants, causing changes in the activities of antioxidative enzymes and specific gene expression levels (Alshegaihi et al., 2023). Increasing the Cd concentration to which the *Vigna angularis* L. plant is exposed also increases the DNA damage levels in the plant. Moreover, increased expression levels of genes involved in DNA damage repair have been reported. Thus, it reduced the cell division rate by slowing down the G1/S phase in stem cells (Ai-Jun et al., 2007).

Being able to determine the mechanisms by which changes in the expression of genes involved in Cd stress occur contributes greatly to the explanation of stress response mechanisms. DNA methylation appears to be one of the most important of these mechanisms. In a study conducted on drm1 drm2 cmt3 mutant (ddc) and WT plants of *Arabidopsis thaliana*, which are defective in DNA methylation, the molecular and cellular mechanisms modulated by DNA methylation in response to Cd stress factor were investigated. As a result, this study determined by transcriptomic analysis that the methylation status of the plant under Cd stress plays a role in plant hormone genetic pathways (Pacenza et al., 2021). In the study conducted by Galati et al. (2023) Cd hyperaccumulator *Noccaea caerulescens* Ganges and *Arabidopsis thaliana* plants that do not accumulate Cd were used to determine the DNA damage caused by Cd and its causes. It was reported that Cd caused high DNA damage in *A. thaliana* plants, while increasing CpG DNA methylation depending on the dose and causing an increase in the expression of the MET1 gene encoding DNA-methyltransferase. Research on the effect of Cd on gene

expression and DNA methylation was conducted in *Nicotiana benthamiana*. In the Cd-stressed plant, the methylated portions of the NbMORC3, NbMUT, and NbBG genes were both CG and CHG regions, while the NbHGSNAT gene had a CHH region and this situation has been indicated that different enzymes were active in methylation. It was determined that the expression levels of the same genes were not directly proportional to the methylation levels (Xin et al., 2019).

Another study, the effects of Cd stress were investigated on DNA methylation level and chromatin reorganization in *Posidonia oceanica*. The PoMT2k gene whose significant metal tolerance gene was induced in transcription by the Cd stress itself. Apical and leaf tissue samples were analyzed by the MSAP technique and DNA methylation levels were observed to increases rapidly (Greco et al., 2012).

The increasing accumulation of Cd in soil in recent years and the fact that its effect mechanisms have not yet been fully elucidated have made Cd a focal point of scientific studies. In our study, we aimed to determine the expression levels of genes related to stress in the plant after Cd stress applied at different concentrations to bread wheat (*Triticum aestivum* L.), an important agricultural product, and to determine their methylation levels for the first time using Real-Time PCR-based quantitative DNA methylation analysis (qAMP). In this context, gene expression levels and changes in region-specific DNA methylation levels in UGT-3, PIP-1 and LTP-4 genes, which we determined to play a role in Cd stress, were determined.

2. Material and Methods

2.1. Plant material and postharvest treatments

Triticum aestivum (Gerek 79 variety, which has a wide range of adaptation ability, was used.) was obtained from the Agricultural Faculty of Ataturk University. Twenty-five seeds selected in equal size from each of them were sterilized in 1% sodium hypochlorite (NaOCl) for 3 minutes and taken for germination in a pot. They were watered with the prepared Hoagland solution (Hoagland & Arnon, 1950) and sterilized water. These were kept in pots at 25°C and 16/8 hours per day/night for 10 days. At the end of this period, each group of Cd (100, 250 and 500 µM $CdCl_2$) was performed and left at rest for an additional 10 days. After a total of 20 days, both roots and stems of each experimental group were harvested separately for the next studies.

2.2. RNA isolation and gene expression analysis

Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's instructions (Billet et al., 2018). The concentration and qualities of the obtained RNAs were determined using the Qiaxpert Nanodrop (Qiagen, Germany) and the concentrations of all samples were adjusted to be 5 µg/µl. Primers for the UGT-3, PIP-1 and LTP-4 genes (Table 1) were developed using the Primer 3 program available at http://www.primer3.ut.ee. The cDNA was synthesized using the Thermo Scientific RevertAid First Strand (Thermo Scientific) and the protocol was established according to the manufacturer's instructions. It has been prepared in a final 20µl volume. The cDNA synthesis blend contained a 4 µl Reaction buffer (5X), 1 µl RiboLock RNase Inhibitor, 1 µl Random Hexamer, 4 µl dNTP mix (20 U/µl), 1 µl RevertAid M-MuLV RT (200 U/µl), and 5 µg RNA for a total volume of 20 µl. The reverse transcription reaction took place in a thermocycler programmed to 5 min at 25°C, 60 min at 42°C, 5 min at 70°C. Subsequently, qRT-PCR was carried out using the Thermo Scientific Maxima SYBR Green/ROX qPCR kit. For each primer, the mixture was prepared as per the protocol proposed by the manufacturer's instructions in all groups. This mix is subjected to 95°C for 10 min, then 95°C for 15 sec, at a primer specific temperature of 30 sec, 72°C for 30 sec for 40 cycles.

Table 1. Base sequences of primers used in the gene expression and qAMP analysis

Gene Region	Forward Primer	Reverse Primer
UGT-3 (1st Primer)	5'-CCTATCAAAGGGTGGGGTGG-3'	5'-TAAAAACTTACGTGGCGCAGT-3'
UGT-3 (2nd Primer)	5'-GATGGCTACGACGAAGGAGG-3'	5'-AGCATCTTACGAGACTGTTTGT-3'
LTP-4 (1st Primer)	5'-ACTGCGCCACGTAAGTTTTT-3'	5'-AGTGCGTTGGCGGAAAGATA-3'
LTP-4 (2nd Primer)	5'-CCTATCAAAGGGTGGGGTGG-3'	5'-TAAAAACTTACGTGGCGCAGT-3'
Aquaporin PIP-1 (1st Primer)	5'-TGTTCTTAGCCCGGAAGCTG-3'	5'-GGGAAAAATGCTCGAAACCGA-3'
Aquaporin PIP-1 (2nd Primer)	5'-GGATGACCACGTGAGTTTCCA-3'	5'-GGACAAGGCCAGAAACCAGA-3'
UGT-3	5'-TGAATGGCTCTGGTGTTCCC-3'	5'-TTCGGATTCCCGTACAACCC-3'
LTP-4	5'-GCGGTGTTAGCATCCCCTAT-3'	5'-TATGAGTGCGTTGGCGGAA-3'
Aquaporin PIP-1	5'-TCTGTTCTTAGCCCGGAAGC-3'	5'-TTGGGATGGTTGCCAAGTGA-3'
β-actin	5'-GTGTCGCACCAGAGGATCAT-3'	5'-CGCTGGCATACAAGGACAGA-3'
GAPDH	5'-GAGGTTGCTGTGTTTGGCTG-3'	5'-GGAGCAAGGCAGTTAGTGGT-3'

2.3. DNA isolation and quantification of DNA methylation ratio by qAMP

Both roots and shoots were randomly collected from 10 plants per treatment. Isolation and purification of genomic DNA were conducted as described by Erturk et al. (2014). The concentration and qualities of the obtained DNA were determined through Qiaxpert Nanodrop. Quantitative analysis of DNA methylation using the real-time PCR (qAMP) has been used to define methylation rates in the identified gene regions. Methylation regions of these genomic DNA were determined using Methylation-Sensitive Restriction Enzymes (MSREs) with a Methylation-Dependent Restriction Enzyme (MDRE). MSRE enzymes (Hpa II, Not I, Hha I) cleaved unmethylated regions whereas the MDRE enzyme (McrBC) cleaved only when methylation occurred. The primer was then designed to detect flanked sites within these restriction regions for each gene (LTP-4, PIP-1 and UGT-3) (Table 1). The design of the primer was developed through the Primer 3 program. The quantitative real-time PCR (qRT-PCR) was done using the 2X Magic SYBR kit (Procomcure, Austria). For each primer, the mixture was prepared in the manner suggested by the manufacturer's instructions in all groups. This mix is subjected to 95°C for 5 min followed by 95°C for 10 sec, primer specific temperature 15 sec, 72°C for 20 sec for 40 cycles and 72°C for 2 min. The qPCR cycle threshold values were calculated using the data analysis spreadsheet supplied by the manufacturer (Qiagen, Rotor-gene Q).

2.4. Statistical analysis

The thresholds for all samples were determined based on gene expression analysis. The samples are evaluated over three replicates. The β-actin and GAPDH genes served as reference genes. The gene expression rate for wheat varieties was determined by the proportional calculation algorithm ΔΔCt according to Livak & Schmittgen (2001). The RT-qPCR threshold values have been analyzed in the Qiagen GeneGlobe Data Analysis Center.

The methylation rates obtained as a result of the qAMP technique were calculated using formula 100 ($e^{-0.7}$(ΔCt)) for MSREs and formula 100 (1- $e^{-0.7}$(ΔCt)) for MDRE. Welch's and Brown-Forsythe's test statistics were used to verify the homogeneity of the variances (Brown & Forsythe, 1974). Tamhane's post hoc T2 tests were used for analysis of variance and showed a significant difference between the groups. ($p<0.05$). The analysis of these data was performed by the IBM SPSS Statistics 26 program.

3. Results

3.1. Expression variations of target genes in shoot and root

Changes in expression levels of the UGT-3, LTP-4 and PIP-1 genes associated with $CdCl_2$ were applied to *T. aestivum* at four concentrations (0, 100, 250 and 500 µM). These genes were compared with the β-actin and GAPDH housekeeping genes to establish expression levels. Changes in expression of the UGT-3 gene were determined to be 0.77, 31.05, 16.64 in the shoot and 0.04, 2.85, 6.33 in the root at doses of 100, 250, 500 µM Cd, respectively. This gene was observed to increase as a function of dose, but its expression decreased excessively at a concentration of 500 µM Cd in the shoot (Table 2). The plant root had no significant increase, though the expression of the UGT-3 gene increased with dose (Table 3). The LTP-4 gene expression changes were determined to be 60.69, 21.81, 3.7 in shoot and 0.03, 5928, 14.77 in root at doses of 100, 250, 500 µM Cd, respectively. It was reported that the expression level of the LTP-4 gene decreased with increased Cd stress in the shoot. In addition, the greatest expression of this gene was ascertained at a concentration of 250 µM Cd in the plant root, and this ratio was significant. Expression changes for the PIP-1 gene were determined to be 0.04, 0.48, 0.2 in the shoot and 0.03, 6.29, 14.91 in the root at 100, 250, 500 µM Cd doses, respectively (Table 2 and Table 3.). As a consequence, plant tissues exposure, expression rates of this gene had occurred an increase of the root as a dose-dependent way. Furthermore, the level of expression increased in proportion to the increase in doses of Cd in the shoot, but no significant increase was noted.

Table 2. The variation rates in expression level of UGT-3, LTP-4, and PIP-1 genes in the shoot

	100 mM $CdCl_2$			250 mM $CdCl_2$			500 mM $CdCl_2$		
	Fold Change	p-Value	Comments	Fold Change	p-Value	Comments	Fold Change	p-Value	Comments
PIP	0.04	0.005229		0.48	0.052987		0.20	0.009885	
GAPDH	1.72	0.011854	A	19.61	0.000280	A	29.11	0.001933	A
UGT	0.77	0.342313	A	31.05	0.000111	A	16.64	0.005445	A
LPT	60.69	0.000003	A	21.81	0.000356	A	3.70	0.001490	A
ACT	0.58	0.010356		0.05	0.000209		0.03	0.000196	

Table 3. The variation rates in expression level of UGT-3, LTP-4, and PIP-1 genes in the root

	100 mM $CdCl_2$			250 mM $CdCl_2$			500 mM $CdCl_2$		
	Fold Change	p-Value	Comments	Fold Change	p-Value	Comments	Fold Change	p-Value	Comments
PIP	0.03	0.001148	C	6.29	0.002014	A	14.91	0.000008	A
GAPDH	0.03	0.019834		0.44	0.086393		0.33	0.055005	
UGT	0.04	0.013726		2.85	0.002158	A	6.33	0.001157	A
LPT	0.03	0.001148		5928.01	0.000000	A	14.77	0.000004	A
ACT	29.82	0.006092	C	2.25	0.011900		3.04	0.002321	

3.2. Methylation rates of candidate genes in wheat tissue

The results of a One-Way Analysis of Variance (ANOVA) analysis applied to the comparison of the related genes at defined doses are presented in Table 4. As well, methylation rates were calculated at all concentrations of Cd (0, 100, 250, 500 µM) for root and shoot tissues. This statistical analysis carried out with the ANOVA test is paralleled in the graphs created using the GraphPad Prism 9 application. It was determined that the dose-related differences in the MSREs of the UGT-3 gene ($F(3,47)=10,698$; $p<0.05$) (Table 4 and Figure 1). The percent methylation was significantly higher at 250 µM $CdCl_2$ dose than at 500 µM Cd dose for stem and root. Moreover, methylation rates were determined to be significantly higher than the 250 µM Cd concentration compared with the control group (Figure 1). Taking into account the percentages of MDRE in the UGT-3 gene, no statistically

significant differences were observed between Cd concentrations (p>.05). The methylation percentage of the UGT-3 gene is greater than 100% at a 250 µM dose of Cd in the root, resulting in MSRE rates (Figure 1). Analysis of the LTP-4 gene determined that the MSREs data were significantly different between applied doses Cd (F(3,47)= 10.752; p<.05). The percent methylation was found to be above 250 µM Cd concentration than 100 µM Cd concentration. The MDRE data for the LTP-4 gene were also showed dose-dependent differences (F (3,47) = 4.572; p<.05). However, the methylation percentage in the plant root was decreased to 60% at a dose of 250 µM Cd. The MSREs data of the PIP-1 gene was significantly figured out differences (F(3,47)=25.678; p<.05). The methylation rate was ascertained to be highest at the 250 µM Cd dose (Table 4 and Figure 1). Additionally, there was no statistically significant difference between doses of MDRE in the PIP-1 gene (p>.05). When MDRE methylation levels were examined, they were demonstrated to decrease at a dose of 250 µM Cd in the shoot and were increased significantly at a 250 µM concentration in the root (Table 4 and Figure 2).

Table 4. The DNA methylation rates of UGT-3, LTP-4, and PIP-1 genes

	Doses	N	Mean	Standard Deviation	F	p	Significant Difference
UGT-3 MSRE	Control	12	0.37	0.39	10.698	**0.000***	3>1
	100 µM $CdCl_2$	12	26.58	35.39			3>4
	250 µM $CdCl_2$	12	88.33	67.48			
	500 µM $CdCl_2$	12	21.87	24.54			
UGT-3 MDRE	Control	12	99.95	0.03	2.178	0.104	-
	100 µM $CdCl_2$	12	92.47	11.49			
	250 µM $CdCl_2$	12	89.38	11.92			
	500 µM $CdCl_2$	12	77.63	40.31			
LTP-4 MSRE	Control	12	0.48	0.55	10.752	**0.000***	3>1
	100 µM $CdCl_2$	12	2.12	2.75			3>2
	250 µM $CdCl_2$	12	24.58	23.29			
	500 µM $CdCl_2$	12	3.35	5.05			
LTP-4 MDRE	Control	12	99.91	0.06	4.572	**0.007***	
	100 µM $CdCl_2$	12	99.21	1.34			
	250 µM $CdCl_2$	12	78.85	33.41			
	500 µM $CdCl_2$	12	99.36	0.91			
PIP-1 MSRE	Control	12	3.44	6.05	25.678	**0.000***	3>1
	100 µM $CdCl_2$	12	16.47	22.86			3>2
	250 µM $CdCl_2$	12	65.86	15.46			3>4
	500 µM $CdCl_2$	12	17.09	24.79			
PIP-1 MDRE	Control	12	98.85	2.11	1.783	0.164	-
	100 µM $CdCl_2$	12	83.44	27.67			
	250 µM $CdCl_2$	12	84.86	23.64			
	500 µM $CdCl_2$	12	92.60	7.50			

*p< .05 One-Way ANOVA
1: Control; 2: 100 µM $CdCl_2$; 3: 250 µM $CdCl_2$; 4: 500 µM $CdCl_2$

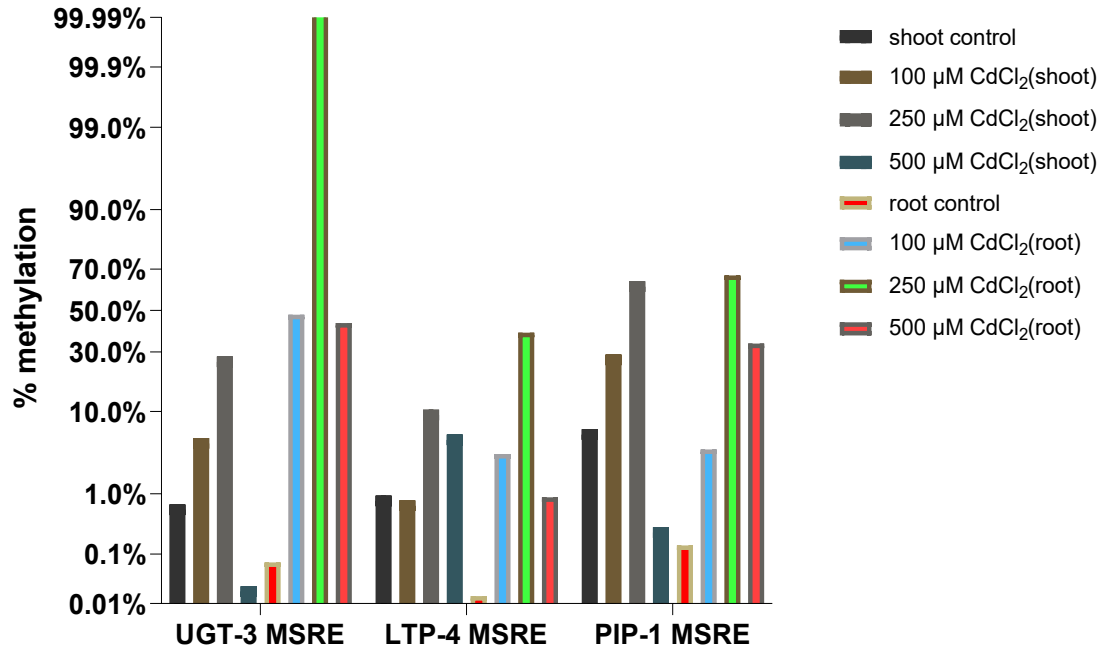

Figure 1. The methylation rates in MSREs regions of UGT-3, LTP-4, and PIP-1 genes.

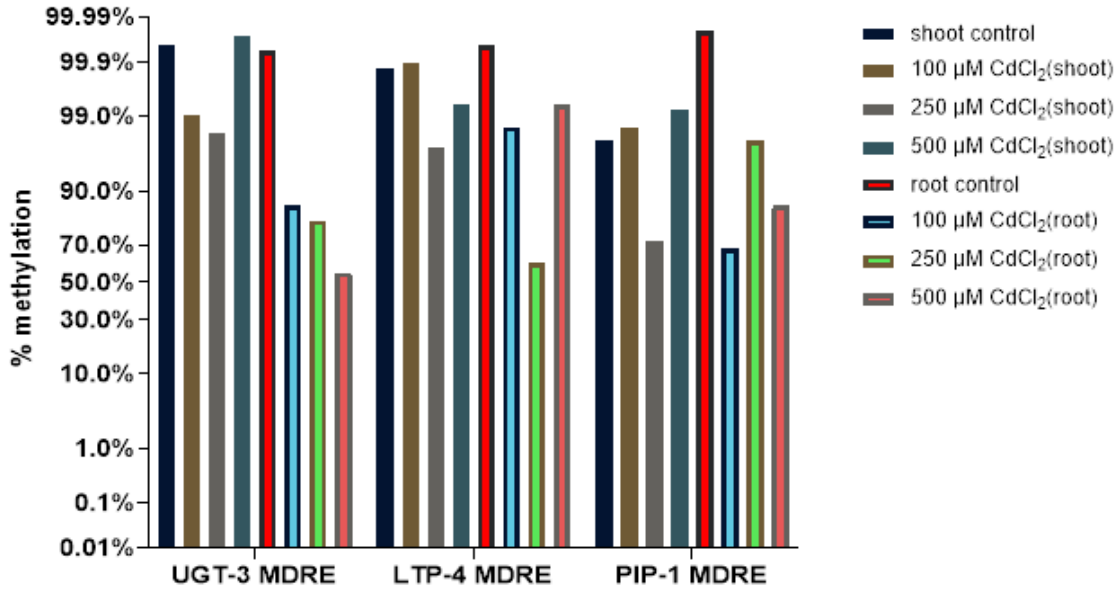

Figure 2. The methylation rates in MDRE regions of UGT-3, LTP-4, and PIP-1 genes.

4. Discussion and Conclusion

Rapid urbanization and industrialization caused by the industrial revolution along with the increasing population have significantly triggered environmental pollution, which is one of the environmental problems (Briffa et al., 2020; Fu & Xi, 2020; Mishra et al., 2021). Nowadays, heavy metals are among the main factors that cause environmental pollution. In recent years, the worldwide increase in pollution caused by the accumulation of Cd, which is among the heavy metals, poses a potential threat to food safety and human health (Zhou et al., 2018; Vareda et al., 2019). In order to elucidate the changes caused by Cd in plants, it is very important to elucidate the molecular mechanisms of the changes it causes in their vital functions and defense mechanisms. In our study, bread wheat plants grown at 100, 250 and 500 µM concentrations of Cd stress were observed to shorten of root and stem tissues and grow weaker when compared to the control group.

Plants sense changes in their environment and create responses to prevent damage (Kosová et al., 2007). Thus, the appearance of changes in gene expression, which plays a role in this complex mechanism, has been shown to be induced by stressors (Sofia Duque et al., 2013). One of the most important regulatory mechanisms for gene expression is epigenetic mechanisms. DNA methylation is an important mechanism in plant epigenetics, particularly in the regulation of gene expression. Specific methylation of DNA leads to the regulation of gene expression with activities such as the occurrence, preservation and removal of its region. The DNA methylation associated with the gene may occur in the promoter or region of the gene copied into plants. It was reported that DNA methylation in the promoter region typically inhibits gene transcription. However, it has sometimes been noted that *A. thaliana* can promote gene transcription, as in the ROS1 gene and hundreds of genes that inhibit fruit ripening in tomatoes (Zhang & Zhu, 2012; Lei et al., 2015; Williams et al., 2015). In this study, it was determined that the expression levels of the PIP-1, UGT-3 and LTP-4 genes changed both in the shoot and in the root (Table 2 and Table 3), while the methylation levels thought to be related to the expression were shown in Table 4.

Nonspecific plant lipid transfer proteins (nsLTPs), which are small and basic proteins, are known to facilitate the in vitro transfer of lipid molecules between membranes (Wang et al., 2008). LTPs in plants are thought to play a key role in many biological processes such as cuticle formation, defensive response to pathogen attacks, and plant signaling (Gomès et al., 2003).

Our study found that LTP-4 gene expression decreased in the plant stem as Cd stress concentrations increased, and it was upregulated with a sudden rise in the concentration of 250 µM in the root. The over-upregulation of this gene has been thought to occur in roots whose tissues directly exposed to Cd stress because of defensive proteins are more active (Baetz & Martinoia, 2014; Khan et al., 2021). When the methylation levels were investigated at 250 µM dose, the highest levels of methylation were observed in MSRE regions. However, these rates were reported during the onset of hypomethylation. Hence, the expression rate was highest at a concentration of 100 µM, where the lowest methylation occurred in the shoot. In addition, an excessive reduction of upregulation at a dose of 500 µM Cd was interpreted as abiotic stress that overexpressed defense genes up to the limit, and decreased the expression of defensive genes to the boundaries where the plant is approaching death (Sorrentino et al., 2017). It was also observed that this gene was methylated at a rate of 98% at a dose of 500 µM Cd. Expression and methylation rates of genes related to the vegetative growth of parental lines and the newly obtained generation were studied in the *Brassica oleracea* plant. Research using the MSAP technique, the regions increased methylation rates were determined in new generations and sequence analysis of these regions was conducted. It was observed while increasing the methylation rate of LTP-1 gene one of the genes identified and the percent expression decreased its (Salmon et al., 2009). Safi et al. (2015) applied various abiotic stress to tolerant and susceptible genotypes under abiotic stress conditions and were examined at LTP-4 expression levels. The LTP-4 gene was declared to be expressed in the more tolerant genotype than the susceptible genotype in the available data. Over-expression of the LTP-4 gene has also been reported in support of plant growth (Safi et al., 2015). This situation showed its effectiveness in the abiotic stress tolerance mechanisms of the LTP-4 gene.

Glycosyltransferases, including the UGT-3 gene also, are defined as enzymes that act as catalysts for the addition of various sugars found in prokaryotes and eukaryotes. Plant UGTs catalyze a wide range of substrates for glycosylation reactions involving plant hormones and secondary plant metabolites. In this study, the UGT-3 gene upregulated at both root and shoot. The concentration of 250 µM Cd in the shoot was the highest expression for the UGT-3 gene. Given the levels of methylation, MSREs were determined at the highest 250 µM concentration in the stem and the root. The methylation rate for the MSRE areas of the shoot was observed to be the highest (27%). When the methylation percentages of MDREs were examined, it was observed that there was no significant change between doses in the shoot, and hypermethylation took place in the regions where the enzyme severs. In addition, the expression level increased in direct proportion to the increase in Cd stress in the root of bread wheat. The highest methylation percentage (85%) of MDREs in the root was shown at 100 µM concentration. The percentage of methylation was observed to decrease to 55% at a concentration of 500 µM. Therefore, the expression of the UGT-3 gene was determined to decrease with the increase in methylation. Moreover, the higher the methylation ratio of the root compared to that the shoot, the lower the level of expression. The expression rate which was 31-fold in the plant shoot decreased to 6-fold in the root. In the study conducted, the effects of UGTs which play an important role in plant defense were

studied on *Chinese cauliflower*. This study revealed that the expression level of UGTJB1 was increased two-fold in plants with high glycosylate accumulation (Zheng et al., 2021). Additionally, the upregulation of UGT-3 has reported that salicylic acid (SA) and jasmonic acid (JA) have increased in the apex regions of the root and have positively regulated to defense responses (Xing et al., 2018). In another study on the UGT gene family, cold stress (+4℃ and -4℃) was applied to the *Chorispora bungeana* plant. Methylation levels and gene expression ratios of the genes CbADH1, CbUGT and CbPGIP were compared to these plants. It has been reported that the methylation levels of these genes diminish in +4℃ and -4℃. It was stated that there was a small change in the methylation level of CbUGT, but an increase of approximately 8-fold the level of expression (Song et al., 2017).

Transmembrane transport of water occurs through diffusion across the double lipid stratum, with increased membrane permeability as a result of water channel proteins called aquaporins (Preston et al., 1992). One of the most important plants aquaporins is PIP. They have been proven in different plants to participate in abiotic stress response mechanisms like salt, drought, cold, water stress, light and phytohormones (Yang et al., 2003). Based on the results of our study, while the PIP-1 gene was downregulated in a non-significantly from the control group in the wheat plant roots, it has been upregulated approximately 6 times because of the increased stress of Cd in the shoot. In the research carried out, it was published that if the PIP-1 and PIP-2 genes were active in the roots, only PIP-2 was expressed in the shoot. Consequently, it has been reported that PIP-2 is the most important gene that ensures the absorption and transport of water in the roots (Guo et al., 2006). The highest methylation rate of the MSRE regions of the PIP-1 gene was detected in both the shoot and root at 250 µM dose, and we determined that hypermethylation had occurred at this dose. On the other hand, the level of expression was observed to be downregulated in the shoots of the plant and that the results of methylation and expression of the gene were consistent with each other. The upregulation in the plant root had recorded at 250 and 500 µM concentrations, 6 and 14-fold, respectively. When this event was investigated, methylation levels were declining the MDRE region. Research in the literature, the PIP genes were investigated *T. aestivum* plant exposed to salt stress. This study was revealed that the methylation rate of the PIP-2 gene increased under the effect of salinity stress (Xu et al., 2013). Furthermore, it has been reported that this gene is downregulated with the rise in salt stress doses. The results from our study seem to be supported by this research.

Consequently, we examined the effect of DNA methylation on the expression of genes associated with Cd stress (UGT-3, LTP-4 and PIP-1) in bread wheat plants. Stress response mechanisms were found to be activated following the accumulation of Cd heavy metal in the plant, and there are increases and/or decreases in the expression levels of the UGT-3, LTP-4 and PIP-1 genes, which play a major role in these mechanisms. In addition, it was revealed that changes are also occurring in the DNA methylation rates of related genes due to Cd stress.

Furthermore, our study was first studied on changes in the methylation rates of the genes UGT-3, LTP-4 and PIP-1 under Cd stress. Additionally, the qAMP technique, which is generally used in mammalian cells, was used for the first time in the application of Cd stress in plant cells and has been successfully concluded. As expected, a significant correlation was observed between gene expression levels and their methylation status. On the other hand, the fact that the rates of gene expression and DNA methylation observed at certain levels of the genes are not inversely proportional, it has revealed the need to explain the results further by studying other mechanisms involved in the regulation of gene function.

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Conflict of interest

All authors declare that there is no conflict of interest to disclose.

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