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ARTICLE

Determination by molecular methods of genetic and epigenetic changes caused by heavy metals released from thermal power plants

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Abstract The heavy metals are released into the environment due to the activities such as meeting the increasing demand for energy, industrial activities, and agricultural pesticides. In many studies, the heavy metals have been proven to have genotoxic effects. As a result of burning the lignite coal in thermal power plants, the heavy metals of Cr, Fe, Mn, Cu, Pb, Cd, Zn, and Ni are spread into the environment within the ashes. In the present study, the gene expression levels were examined on the corn and wheat plants added with 500-m interval around the thermic power plant. For this purpose, the genes from 14-3-3 protein family, expression level of which increases under abiotic stress conditions, were analyzed. For the expression levels of plants, the $2^{-\Delta\Delta C_t}$ values were calculated and then compared to $2^{-\Delta\Delta C_t}$ values of β-actin gene, that is, the housekeeping gene. The heavy metal content analyses of the samples were carried out using ICP-MS, and it was determined that there were many heavy metals at higher amounts within the structure of samples having low level of gene expression. It has been understood that heavy metal stress causes a difference in gene expression level. The change introduced by heavy metal stress into the gene expression occurs in concrete in the translation products. The level of stress-induced gene expression, which is caused by heavy metals in the environment surrounding the plant, has been successfully determined by RT-PCR.

Keywords Epigenetic · Gene expression · Genotoxic effect · Heavy metal · ICP-MS · Thermal power plant

Introduction

In proportion to the increase in world population, the intensity of vehicle traffic, the industrial production, the industrialization, and energy requirement increase [1]. The pollution occurring due to the release of heavy metals as a result of the activities conducted in order to meet the requirements became one of the most important problems in our environment [2].

The heavy metal contamination of the soil arises as a result of the factors such as agricultural pesticides, processing the underground mineral resources for the use, burning the fossil fuels such as coal, waste of batteries, and irrigation by using polluted water sources [3–6].

In previous studies, it was shown that Cu, Hg, Pb, and Cr cause the oxidative stress in organisms and this increases the DNA damage [7–9]. Although zinc has been reported to be the heavy metal species that has the lowest toxic effect, it has also been stated that excessive accumulation of zinc in plant tissues caused cytotoxic and genotoxic effects due to DNA damage [10–12]. Similarly, as a result of the increase in production of free radicals by Ni and Cr, the point mutations and chain fractures have been reported [13–16]. In previous studies, it was reported that excessive accumulation of copper in plant tissues caused formation of free oxygen radicals and its toxic

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effect for the plants was higher than other nonessential heavy metals [17, 18].

The influence of epigenetic modifications happening on the chromatin structure to the expression of genes is being controlled by the basic epigenetic mechanisms such as DNA methylation, histone modifications, and RNA [19]. The cooperation of these three mechanisms leads to inheritable differences in the gene expression. A mistake occurring in either mechanisms leads to epigenetic disorders resulting in excessive increase or suppression of gene expression [20].

The basic nutrients of living beings are the grains. Grain production in our country is equal to half of the products grown. TSI [21] our country acreage of *Triticum aestivum* L. (Wheat) and *Zea mays* L. (Corn) products in the last 5 years, according to the size and quantity of the product Fig. 1.

Our purpose in this study is to determine the plantation distance to heavy metal source by using the change in gene expression levels of staple foods wheat and corn cereals as a result of the genotoxic effects of heavy metals.

Materials and methods

Seed sowing and sampling

Selected equal-sized wheat and corn seeds were sowed at 20 distinct points so as to 500 m apart from each other in the same direction around the thermal power plant. As the control group, the seeds were sowed within the same region yet outside the effect of thermal power plant. Following a 40-day vegetation period, the sample plants were collected. Collected samples were sterilized prior to molecular investigations and heavy metal content analysis.

RNA isolation

After putting the leaves of wheat and corn samples by liquid nitrogen in dust, RNA isolation was performed by Ribospin Plant 307-150 kit in accordance with the instructions of the manufacturer.

Determination of RNA concentrations

Total data of RNA concentrations have been taken at value of A260/280 O.D by being measured using ACTGene Spectrophotometer (ACTGene UVIS-99, NJ and USA). According to these outcomes, the total RNAs of all samples were displayed on the gel, by being adjusted so as to be 0.5 μg.

cDNA synthesis

The master mix 2 μl for the first step of cDNA synthesis from the RNA obtained was prepared by being completed with ultrapure water so as to be 1 μl primer, 1 μl dNTP up to a total volume of 14 μl and left for 5 min at 65 °C. Then, the second master mix for the samples taken onto the ice was prepared in quantities as 2 μl 10× RTase reaction buffer, 2 μl 0.1 M DTT, 1 μl Hyper Script™ Reverse Transcriptase (200 μl) and 1 μl ZymAll™ RNase Inhibitor, and 6 μl hereof was added as per sample. The samples were respectively incubated for 60 min at 55 °C and 5 min at 85 °C.

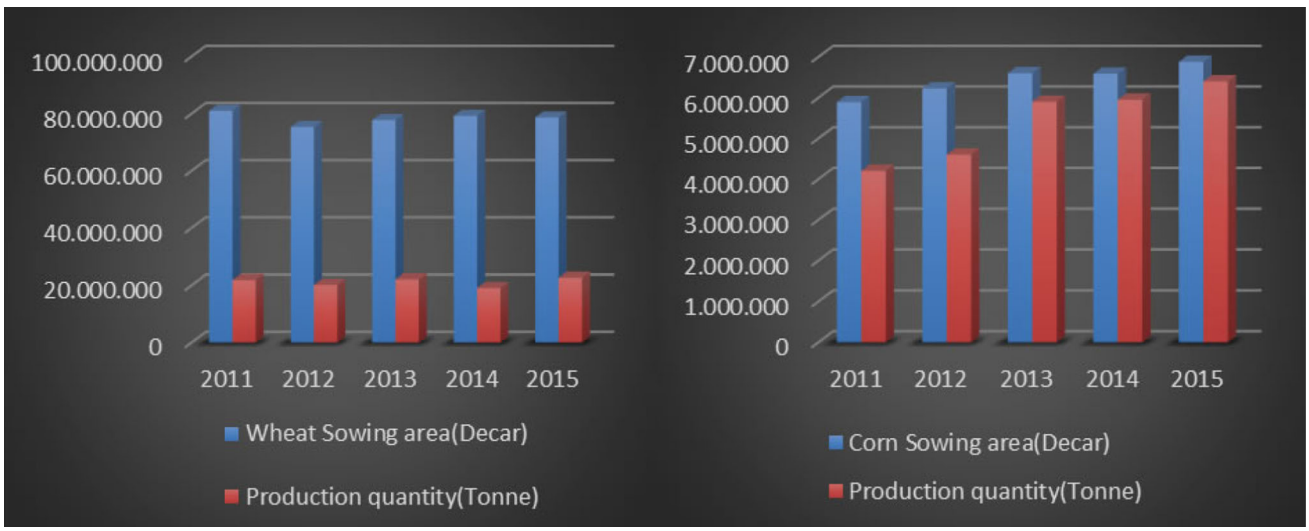

Fig. 1 Plant area and crop yield data for wheat and corn grown in our country

RT-PCR analysis

Primers Ta14S1 and Ta14S2 for wheat and ZmGF14-4 and ZmGF14-6 for corn were designed for gene expression of the 14-3-3 protein family. β-actin has been used as the housekeeping gene. The details pertaining to the primers employed are provided in Table 1. The synthesized cDNAs, primers Ta14S1, Ta14S2, ZmGF14-4, and ZmGF14-6 have been checked by using PCR analysis primer β-actin (GenBank ID: AB181991.1) has been used as reference. All real-time PCR samples have been executed in 3 repetitions. In RT-PCR analysis, cDNAs diluted at the rate of 1:200 have been used as templates. The RT-PCR reaction components and the schedule belonging to reaction temperature cycle are given below. All reactions have been performed on the Bioneer Exicycler 96® (Bioneer, South Korea). PCR components and amplification parameters are presented below.

10 µl of 2X SYBR master mix was prepared at the rates of 0.8 µl F (forward) (10 pm) primer, 0.8 µl R (reverse) (10 pm) primer, 2 µl ROX, 4 µl cDNA, and 2.4 µl ultrapure water and added to the loading wells. By loading 4 µl cDNA samples onto them, RT-PCR was carried out at 95 °C for 10 min and at 95 °C for 45 cycles in 15 s and at 60 °C for 45 cycles in 1 min.

Data analysis

The threshold cycle values were used for assessing the gene expression levels obtained from RT-PCR results. Obtained from relative or comparative qualitative quantitation results of threshold cycle value, the $2^{-\Delta\Delta C_t}$ value was used for calculating the gene expression levels [22]. For each of the samples, the mean of $2^{-\Delta\Delta C_t}$ values of 10 samples taken from the same point was calculated. Representing the gene expression level, $2^{-\Delta\Delta C_t}$ was computed using the formula below:

$$2^{-((a-b)-(c-d))},$$

where a is C_t value obtained from the gene used for each of the samples; b, C_t value obtained from the gene used for control group; c, C_t value obtained from β-actin gene of each of the samples; d, C_t value obtained from β-actin gene of control group.

Measurement of heavy metal contents in samples by ICP-MS

Upon application of combustion process as per sample in 8 ml HNO_3 + 2 ml H_2O_2 at a microwave oven branded ETHOS UP Milestone Connect, 50 ml was diluted. ICP-MS analysis was performed by taking 15 ml from the diluted solution. ICP-MS analyses were performed on a device branded Agilent Technologies 7800 (Agilent Technologies SPS 4 Autosamples).

Results

Gene expression results of wheat samples

In order to eliminate the margin of error in RT-PCR analysis results, the dimer formation was checked. In order to check the dimer formation of primers, the melting curve analysis was carried out. At the end of melting curve analysis, it was determined that primers formed no dimer.

RT-PCR was employed for Ta14S1 and β-actin genes of our study and control groups. Threshold cycle (C_t) values were obtained for using in comparative analysis of samples. $2^{-\Delta\Delta C_t}$, which is a parameter in evaluating the gene expression levels using C_t values, was calculated.

$2^{-\Delta\Delta C_t}$ values of Ta14S1 gene, which we used for determining the gene expression level of wheat, varied between 0.673 and 2.479. Since generally no change is seen in expression levels of housekeeping genes under the stress conditions, they are used for comparative analyses. The expression level of one of these genes, β-actin gene,

Table 1 Primers used in RT-PCR

Primer name	Sequence 5′ → 3′	TM (°C)	GC%
ZmGF14-4F	GAACCTCTTATCTGTTGCCT	50	45
ZmGF14-4R	GATGACTAGATGCCAGTTCC	52	50
ZmGF14-6F	GCATGCAGAAGGGTTGAGCA	56	57
ZmGF14-6R	TCAGGGCTCATCTAGCTGGTCCTG	61	58
Ta14S-F1	ACGACTCAAGCGAGGGGCA	55	63
Ta14S-R1	CGCCTGCTACGCTACAAGGAC	58	62
Ta14S-F2	GTCAATGACCGTTGCAATGTG	52	48
Ta14S-R2	GCCACCACCACCACTGTATG	56	60
β-actin-F	TTTGAAGAGTCGGTGAAGGG	52	50
β-actin-R	TTTCATACAGCAGGCAAGCA	50	45

was seen to remain low for first 5 samples closest to the heavy metal source. Mean $2^{-\Delta\Delta C_t}$ value of the products grown at closest point to heavy metal source was 0.673, that of the products grown at 2nd closest point was 0.986, that of the product grown at 3rd closest point was 0.926, that of the product grown at the 4th closest point was 0.946, and the mean $2^{-\Delta\Delta C_t}$ value of the products grown at 5th closest point to heavy metal source was 0.986. From the samples at 6th closest point to 20th closest point to heavy metal source, all of the samples were found to have higher gene expression levels than the expression level of β-actin gene. The comparison of Ta14S1 gene expression with the expression of β-actin gene is presented in Fig. 2(A).

$2^{-\Delta\Delta C_t}$ values of Ta14S2 gene, which we used for determining the gene expression level of wheat, varied between 0.176 and 2.497. Mean $2^{-\Delta\Delta C_t}$ value of the products grown at closest point to heavy metal source was 0.176, that of the products grown at 2nd closest point was 0.727, that of the product grown at 3rd closest point was 0.784, that of the product grown at the 4th closest point was 0.933, and the mean $2^{-\Delta\Delta C_t}$ value of the products grown at 5th closest point to heavy metal source was 0.840. From the samples at 6th closest point to 20th closest point to heavy metal source, all of the samples were found to have higher gene expression levels than the expression level of β-actin gene. The comparison of Ta14S2 gene expression with the expression of β-actin gene is presented in Fig. 2(B).

Gene expression results of corn samples

$2^{-\Delta\Delta C_t}$ values of ZmGF14-4 gene, which we used for determining the gene expression level of corn, varied between 0.334 and 2.531. Mean $2^{-\Delta\Delta C_t}$ value of the products grown at closest point to heavy metal source was 0.334, that of the products grown at 2nd closest point was 0.716, that of the product grown at 3rd closest point was 0.582, that of the product grown at the 4th closest point was 0.726, and the mean $2^{-\Delta\Delta C_t}$ value of the products grown at 5th closest point to heavy metal source was 0.517. From the samples at 6th closest point to 20th closest point to heavy metal source, all of the samples were found to have higher gene expression levels than the expression level of β-actin gene. The comparison of ZmGF14-4 gene expression with the expression of β-actin gene is presented in Fig. 3(A).

$2^{-\Delta\Delta C_t}$ values of ZmGF14-6 gene, which we used for determining the gene expression level of corn, varied between 0.456 and 3.837. Mean $2^{-\Delta\Delta C_t}$ value of the products grown at closest point to heavy metal source was 0.456, that of the products grown at 2nd closest point was 0.688, that of the product grown at 3rd closest point was 0.888, that of the product grown at the 4th closest point was 0.920, and the mean $2^{-\Delta\Delta C_t}$ value of the products grown at 5th closest point to heavy metal source was 0.946. From the samples at 6th closest point to 20th closest point to heavy metal source, all of the samples were found to have higher gene expression levels than the expression level of β-actin gene. The comparison of ZmGF14-6 gene expression with the expression of β-actin gene is presented in Fig. 3(B).

Gene expression level and heavy metal content combination

$2^{-\Delta\Delta C_t}$ values representing the gene expression levels of wheat and corn samples, the level of expression in comparison with β-actin gene, and the species of heavy metals found in the plants are presented in Table 2 in comparison with each other. Given these comparisons, the high

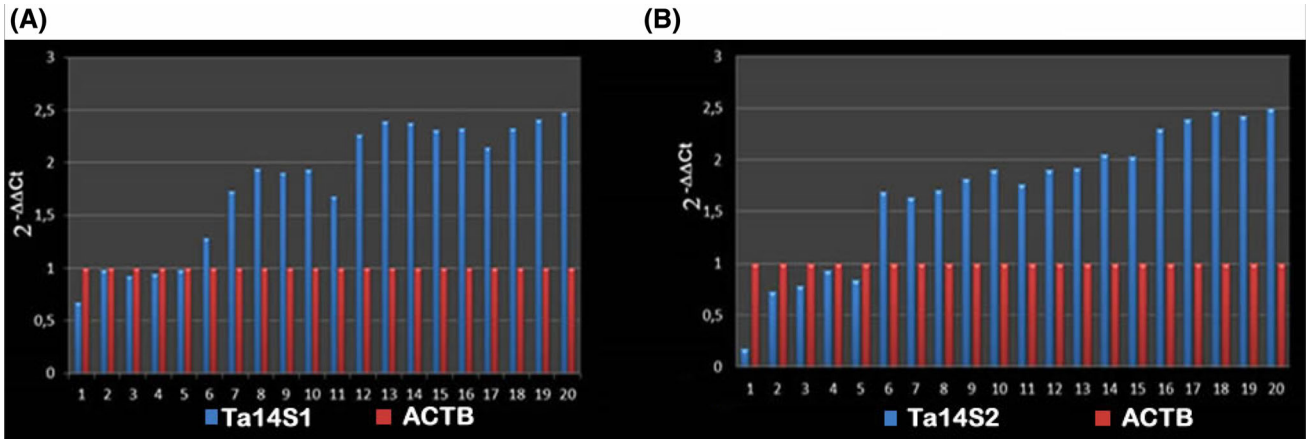

Fig. 2 (**A**) Diagram of $2^{-\Delta\Delta C_t}$ values obtained for comparing the Ta14S1 gene of wheat with β-actin gene. (**B**) Diagram of $2^{-\Delta\Delta C_t}$ values obtained for comparing the Ta14S2 gene of wheat with β-actin gene

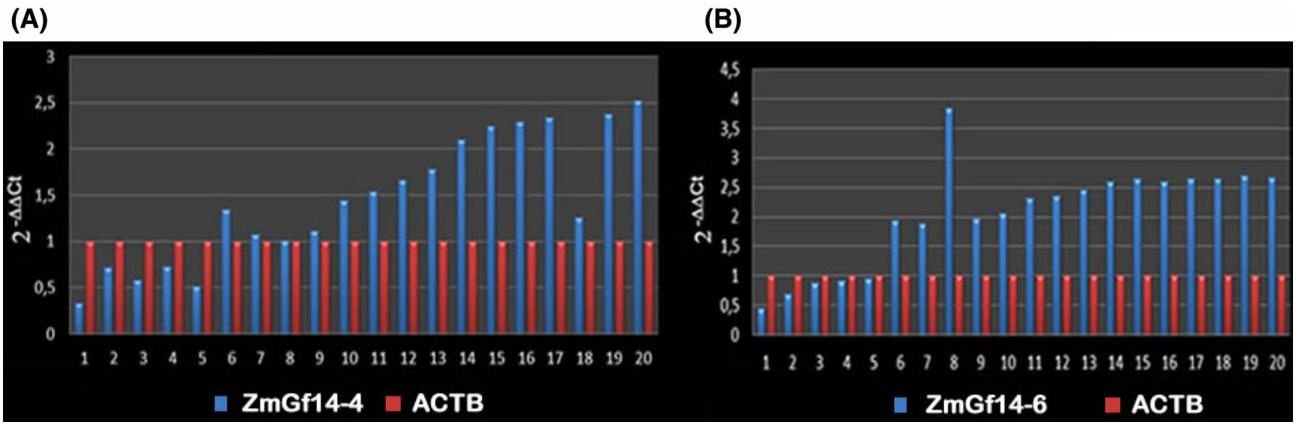

Fig. 3 (**A**) Diagram of $2^{-\Delta\Delta C_t}$ values obtained for comparing the ZmGF14-4 gene of wheat with β-actin gene. (**B**) Diagram of $2^{-\Delta\Delta C_t}$ values obtained for comparing the ZmGF14-6 gene of wheat with β-actin gene

intensity and diversity of heavy metals are remarkable in first 5 samples. The presence of such diverse and intense heavy metals suppresses the gene expression levels.

Discussion

Heavy metal contamination is an important environmental problem for living organisms in the world. Particularly, plants are directly exposed to metal-contaminated soil and water [23, 24]. In many studies carried out on the genotoxic effect of lead, it was reported to cause mutations. In addition, for *Brassica barley* seeds, lead was reported to cause DNA damage and mutations, whereas Cd was reported to have same effects on *Hordeum vulgare* and *Oryza sativa* [8, 25–28].

The gene expression regulation is a very complex process and is thought to be one of the most important mechanisms of heavy metal toxicities. The effects of heavy metals on the changes in gene expression have been reported by using plant and animal models [29–34].

The heavy metal stress is known to influence the plants through the epigenetic changes in chromatin structure such as gene expression, DNA methylation, and posttranslational modifications in histones [35].

In the present study, the gene expression levels of corn and wheat plants grown at various distances from the heavy metal source were determined. The $2^{-\Delta\Delta C_t}$ values obtained from our samples were compared to $2^{-\Delta\Delta C_t}$ values of β-actin genes, a housekeeping gene, obtained from the same samples. The results we obtained suggest that 14-3-3 protein family, the level of which is increased as a defensive reflex under the stress conditions, is suppressed when exposed to a certain concentration and/or number of heavy metals. High concentration and/or number of heavy metals suppressed the gene expression as a result of epigenetic changes. But, as the distance from heavy metal source increases, then the number and amount of heavy metals contained by the plant were observed to decrease. As a result of this decrease, the gene expression level was found to increase.

Xie et al. [36], in their study, have reported a relationship between 14-3-3 gene expression and Cd 2+, in addition to the connection between the Cd 2+ exposure and 14-3-3 transcriptional regulation. Moreover, 14-3-3s were reported to play a significant role in copper tolerance [37, 38]. Besides that, the exposure of plants to various levels of heavy metals might not always cause an increase in the expression levels of 14-3-3 proteins. For instance, in their study, Owen et al. [39] reported that the excessive accumulation of copper heavy metal suppressed the 14-3-3 gene expression and led to irrevocable DNA damage.

It has been understood that heavy metal stress causes a difference in gene expression level. The level of stress-induced gene expression, which is caused by heavy metals in the environment surrounding the plant, has been successfully determined by RT-PCR.

Suggestions

The rapid increase in the world population increases the energy requirement and consumption gradually. For this reason, the use of any available energy source is a necessity. In our country, there are lignite basins, which are very important from this aspect. The utilization of thermal energy plants is an unmissable opportunity for our country. In order to minimize the damage of heavy metals released from the thermal energy plants to the environment, these areas should be used for planting the accumulator plants absorbing the heavy metals into their structures.

Table 2 Gene expression levels of wheat samples and the determined heavy metals

Wheat												
Sample numbers	Gene expression				Heavy metals in construction							
	Ta14S1		Ta14S2									
	$2^{-\Delta\Delta C_t}$ value	Exp. level	$2^{-\Delta\Delta C_t}$ value	Exp. level	Cr	Mn	Fe	Ni	Cu	Zn	Cd	Pb
1	0.673	Low	0.176	Low	+	+	+	+	+	+	+	+
2	0.986	Low	0.726	Low	+	−	+	+	+	+	+	+
3	0.926	Low	0.784	Low	+	−	+	+	+	+	+	+
4	0.946	Low	0.933	Low	+	−	+	+	+	+	−	+
5	0.986	Low	0.840	Low	+	−	−	+	+	+	−	+
6	1.729	High	1.635	High	−	−	−	+	+	+	−	+
7	1.283	High	1.693	High	+	−	−	+	+	+	−	+
8	1.945	High	1.705	High	−	−	−	+	+	+	−	+
9	1.905	High	1.815	High	−	−	−	+	+	+	−	+
10	1.931	High	1.905	High	−	−	−	+	+	+	−	+
11	1.681	High	1.765	High	−	−	−	+	+	+	−	−
12	2.265	High	1.905	High	−	−	−	+	+	+	−	−
13	2.394	High	1.918	High	−	−	−	−	−	−	−	−
14	2.378	High	2.056	High	−	−	−	−	−	−	−	−
15	2.313	High	2.027	High	−	−	−	−	−	−	−	−
16	2.329	High	2.297	High	−	−	−	−	−	−	−	−
17	2.143	High	2.394	High	−	−	−	−	−	−	−	−
18	2.329	High	2.462	High	−	−	−	−	−	−	−	−
19	2.411	High	2.428	High	−	−	−	−	−	−	−	−
20	2.479	High	2.496	High	−	−	−	−	−	−	−	−
Corn												
Sample numbers	Gene expression				Heavy metals in construction							
	ZmGF14-4		ZmGF14-6									
	$2^{-\Delta\Delta C_t}$ value	Exp. level	$2^{-\Delta\Delta C_t}$ value	Exp. level	Cr	Mn	Fe	Ni	Cu	Zn	Cd	Pb
1	0.334	Low	0.456	Low	+	+	+	+	+	+	+	+
2	0.716	Low	0.687	Low	+	+	+	+	+	+	+	+
3	0.582	Low	0.888	Low	+	+	−	+	+	+	+	+
4	0.726	Low	0.920	Low	+	−	−	+	+	+	+	+
5	0.517	Low	0.946	Low	−	−	−	+	+	+	−	+
6	1.347	High	1.945	High	−	−	−	+	+	+	+	+
7	1.071	High	1.892	High	−	−	−	+	+	+	−	+
8	1.006	High	3.837	High	−	−	−	+	+	+	−	+
9	1.109	High	1.972	High	−	−	−	+	+	+	−	+
10	1.443	High	2.070	High	−	−	−	+	+	+	−	−
11	1.536	High	2.329	High	−	−	−	+	+	+	−	−
12	1.670	High	2.361	High	−	−	−	−	+	−	−	−
13	1.777	High	1.462	High	−	−	−	−	−	−	−	−
14	2.099	High	2.602	High	−	−	−	−	−	−	−	−
15	2.25	High	2.657	High	−	−	−	−	−	−	−	−
16	2.297	High	2.602	High	−	−	−	−	−	−	−	−
17	2.345	High	2.657	High	−	−	−	−	−	−	−	−
18	1.257	High	2.657	High	−	−	−	−	−	−	−	−

Table 2 continued

Corn												
Sample numbers	Gene expression				Heavy metals in construction							
	ZmGF14-4		ZmGF14-6									
	$2^{-\Delta\Delta C_t}$ value	Exp. level	$2^{-\Delta\Delta C_t}$ value	Exp. level	Cr	Mn	Fe	Ni	Cu	Zn	Cd	Pb
19	2.378	High	2.694	High	–	–	–	–	–	–	–	–
20	2.531	High	2.675	High	–	–	–	–	–	–	–	–

The studies on the genotoxic effects of heavy metals are based generally on the implementation of heavy metals individually and at various doses. But, given the factors causing the release of heavy metals to the nature, it can be seen that multiple heavy metals are released into the nature at the same time. From this aspect, by accurately analyzing the distribution combinations, it would be better to use these heavy metals together in studies in order to obtain more objective results.

Among the living creatures, there is a constant food chain. From this aspect, we believe that it would be better to pay attention that the nutrients to be grown will be planted at a distance from toxic sources that lead to genotoxic effects and epigenetic changes.

It has also been noted in our literature that heavy metals have a genotoxic effect on plants. Considering this situation, we believe that it would be more appropriate to determine the types of grains with high heavy metal tolerance around the facilities which cause heavy metal pollution and to plant these species.

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