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Iron toxicity-induced DNA damage, DNA methylation changes, and LTR retrotransposon polymorphisms in *Zea mays*

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Abstract: The impact of Fe^{2+} (iron) toxicity on genomic instability, DNA methylation status, and Long Terminal Repeat Retrotransposons (LTR RTs) polymorphisms on *Zea mays* is unknown. We investigated the toxicity of Fe^{2+} using *Random Amplified Polymorphic DNA* (RAPD*), Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA) and Inter Retrotransposon Amplified Polymorphism (IRAP) assays* in *Zea mays* seedlings, respectively. The results indicated that each dose *of* $FeSO_4$ (50, 100, 200, and 300 mg/L) had a reducing effect on Genomic Template Stability (GTS) and increasing in RAPD pattern changes (DNA damage). The value of DNA methylation rised gradually depending on $FeSO_4$ doses. Moreover, five LTR RTs (Wltr2105, Nikita-N57, Sukkula, Nikita-E2647, and Stowaway) of the maize genome revealed polymorphism in all $FeSO_4$ doses. Furthermore, the present study indicated that there is a relationship between DNA methylation alterations and LTR RTs mobilization. It was concluded that iron caused DNA methylation changes as well as genotoxic damage in the maize genome. Also, considering the increase in some LTR RTs polymorphism we can say that it may be a part of the defense mechanism of the plant during stress.

Key words: Epigenetic, $FeSO_4$, genotoxic, retrotransposon

1. Introduction

Heavy metals are commonly known as toxic materials for human, animals, plants, and other living organisms. Yet, some heavy metals like iron, zinc, lead, etc. that are essential micronutrients for plants are called trace elements (Ghori et al., 2019). Iron is necessary for photosynthesis, also an enzyme cofactor in plants. It is essential for both chlorophyll biosynthesis, DNA synthesis, and hormone biosynthesis (Lee et al., 2016). There have been two forms of iron: Fe^{2+} and Fe^{3+} (Galaris et al., 2019). Only Fe^{2+} form is uptaken by plants because of its soluble feature. Iron toxicity commonly occurs in waterlogged soils. In these soils, the amount of acidity increases, and iron is converted from Fe^{3+} form to Fe^{2+} form making it available to plants in high concentration and increasing its potential toxicity (Krohling et al., 2016). Fe insolubility and toxicity may represent a major problem and all living organisms have evolved strategies to preserve Fe homeostasis against changes in the extracellular concentration of the metal (Rodrigues et al., 2020).

Many cereal crops, like maize, wheat, and rice are exposed to Fe^{2+} toxicity and this limits their growth, developmental durations such as photosynthesis, nucleic acid biosynthesis, production, and yield mostly (Fageria et al., 2008; Deka and Sarma, 2012). It is also known that the toxicity of heavy metals stimulates the formation of reactive oxygen species (ROS) and up-regulates antioxidative enzymes activities (Romero-Puertas et al., 2007). In cells, free Fe^{2+} catalyzes the decomposition of H_2O_2 to the extremely reactive hydroxyl (·OH) radical, and this radical leads to damage of macromolecules causing oxidative DNA damage and chromosomal abnormalities (Schützendübel and Polle, 2002; Ritambhara and Girjesh, 2010). Several assays have been developed to examine the genotoxicity of heavy metal stress conditions on plants. Previous studies supported that the RAPD technique is more reliable compared to other techniques (Wang et al., 2016; Aydin et al., 2021). The detection of different kinds of genetic variations and mutations in various plants and animals induced by stresses has made this technique more attractive than others.

Additionally, it is known that heavy metals lead to changes in DNA methylation patterns. DNA methylation is a process that consists of an addition of a methyl group into the DNA and plays a crucial suppressing role in the expressions of individual genes, transposable elements

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(transposons), pseudogenes, and repetitive sequences (Eichten et al., 2013). DNA methylation also contributes to the activation of plant responses in abiotic stress conditions (Mirouze and Paszkowski, 2011; Kumar et al., 2019). CRED RA is used in a successful manner to evaluate the methylation status of the genome depending on the stress (Aydin et al., 2021). In addition to epigenetic regulation, transposable element activities may be related to the regulation of heavy metal toxicity (Jones-Rhoades et al., 2006; Hobert, 2008; Ben Amor et al., 2009). Transposable elements (TEs) that can affect genome structure by inserting into or excising from loci, often proliferate during this process to reach high copy numbers and are widespread constituents of eukaryote genomes. LTR retrotransposons (the most abundant class of TEs) involve a reverse transcription process with an RNA intermediate, and the transposition results in an increase in the copy number. LTR RTs specifically methylated in plant genomes and their activities are regulated by gene silencing systems (Rabinowicz, 2003). *Tos17* a well-characterized Ty1/copia LTR RTs in rice was heavily methylated and immobilized under normal plant growth conditions (Cheng et al., 2006). It is well known that LTR RTs are a major target of epigenetic regulation in plants. Transcription of these LTR RTs sequences can lead to the formation of small interfering RNA, which interacts with DNA and histone methylation genes (Volpe et al., 2002; Lippman and Martienssen, 2004; Neumann et al., 2007).

While there is enough information about iron uptake and homeostasis in plants, the molecular mechanisms of this process on iron toxicity and genome stability have remained poor. So this study was designed to investigate the impact of Fe^{2+} on maize, in terms of genomic stability, the changes in DNA methylation, and LTR RTs mobilities by using RAPD, CRED-RA, and IRAP techniques, respectively.

2. Material and methods

2.1. Plant material and growing conditions

Zea mays cultivar RX9292 seeds were supplied from the Department of Field Crops, Faculty of Agriculture, Atatürk University, Erzurum, Turkey. The surface-sterilization was made with 70% ethanol for 1 min, 1.5% NaOCl (sodium hypochlorite) solution for 20 min, and double-distilled sterile water at least five times. The healthy looking uniform sized sterile seeds were kept in plastic boxes containing two layers of Whatman No-1 filter paper moistened with half strength Hoagland solution (pH 5.8) and were planted with a photoperiod of 16-h light/8-h dark and relative humidity of 60% for 7 days. Different concentrations of $FeSO_4$ (Sigma Aldrich, 12354) [0 (control/untreated), 50, 100, 200, and 300 mg/L] with Hoagland solution were added to the seedlings. Solutions were changed every 3 days. The leaves from treated and untreated plants were collected 7 days after treatment, and immediately stored at –80 °C until used for DNA isolation.

2.2. Genomic DNA extraction

Total genomic DNA (gDNA) from the control and stressed samples were extracted using the modified cetyl trimethylammonium bromide (CTAB) method by Taspinar et al. (2018). NanoDrop (Qiagen Qiaxpert) was used to measure DNA quantity and the quality of gDNA was tested using 1.5% agarose gel electrophoresis. Isolated DNA was diluted to 50 ng/µL, for use in RAPD and IRAP and 1 µg/µL for CRED-RA analysis, and both the stock and diluted portions were stored at –20 °C.

2.3. RAPDs, CRED-RAs, IRAPs, and PCR procedures

To evaluate RAPD, fourteen primers were used (Table 1) through thirty-two decamer primers (Operon Technologies Inc., Alameda, CA, USA). For IRAP reactions five LTR primers were used (Table 2) and eight decamer primers were selected for CRED-RA reactions (Table 3). The amplification contents of the three procedures were the same, but CRED-RA was a little different from the two that used *HpaII* and *MspI* endonucleases (which cut the sequence 5′-C/CGG-3′ with different sensitivity to cytosine methylation; *MspI* cuts if the inner C is methylated, whereas *HpaII* cannot cleave in the presence of methyl groups) for digesting the gDNA samples. The polymerase chain reaction (PCR) technique was carried out in a 20 µL reaction solution containing 50 ng/µL gDNA (1 µg/µL digested DNA for CRED-RA), 10 X PCR buffer, 10 mM dNTP, 25 mM $MgCl_2$, 10 pmol primer, 5 U/µL Taq DNA polymerase, and sterile nanopure H_2O. Positive and negative control with no DNA was tested for each of the primers. PCR was performed for each of three procedures in the same conditions that in a thermocycler (Sensoquest GmbH, Labcycler Gradient, Germany) with the following cycle profile: initial denaturation at 94 °C for 5 min, 38 cycles of 1 min of denaturation at 94 °C, 30 s annealing at variable temperature for each primer, 3 min extension at 72 °C, followed by 7 min at 72 °C for the final extension and finally holding adjust at 4 °C.

2.4. Electrophoresis

PCR products were electrophoresed on 2.0 % agarose gel for 2–3 h at 80 volts in 1 × TBE buffer (BIO-RAD, Sub-Cell Model 96 Cell, Hercules, CA, United States). Intercalating dye ethidium bromide was used for staining and amplified fragments visualization was made by a system (DNR MiniBis PRO, Israel).

2.5. Data analysis

RAPD, CRED-RA, and IRAP banding patterns were evaluated using the Total Lab TL120 computer software. The ratios of genomic template stability (GTS, %) and polymorphism (%) were calculated as follows: GTS = 100–

Table 1. Molecular sizes (bp) of appeared (+) /disappeared (-) bands in RAPD profiles of $FeSO_4$ treated *Zea mays* seedlings and value of GTS and polymorphism (%).

Primers	Control	+/-	50 mg/L $FeSO_4$	100 mg/L $FeSO_4$	200 mg/L $FeSO_4$	300 mg/L $FeSO_4$
OPA-13	7	+	371	397; 371	609; 492; 371; 273	397; 371
		-	900	900	900; 456; 302	900
OPH-17	12	+	-	-	-	-
		-	127	-	466; 153	1128; 466; 153; 127
OPA-2	8	+	388	388	388	388
		-	726	726	726	726
OPA-1	11	+	-	-	-	-
		-	140	140	140	140
OPA-6	9	+	1021; 791; 685; 503;456	1021; 791; 685; 503; 456	1021; 791; 685; 503; 456	1021; 791; 685; 503; 456
		-	611; 311; 213	611; 311; 213	611; 311;213	611;311;213
OPH-14	5	+	644; 469;397; 261	644; 469; 397; 261	644; 469; 397; 261	644; 469; 397; 261
		-	1096	1096	1096	1096
OPH-18	5	+	-	-	-	-
		-	841; 560; 398	841; 560; 398	841; 560; 398	841; 560; 398
OPY-6	10	+	381	381	381	381
		-	139	139	139	139
OPY-1	7	+	-	455	455	455
		-	940; 839; 721	940; 839; 721; 422	940; 839; 721; 422	940; 839; 721; 422
OPY-8	8	+	-	-	-	-
		-	700	700	700; 328	700; 328
OPY-15	12	+	802	802; 619; 519	802	802; 619; 519
		-	-	206	-	206
OPY-16	10	+	-	-	-	-
		-	866; 694; 634	866; 694; 634	866; 694; 634	866; 694; 634
OPW-1	11	+	-	-	642	642
		-	-	-	-	-
OPW-7	8	+	397	-	-	-
		-	755; 722; 273; 233	755; 722; 273; 233; 141	755; 722; 273; 233; 141	755; 722; 273; 233; 141
GTS %			**66.4**	**60.5**	**59.8**	**56.9**
Polymorphism %			**33.6**	**39.5**	**40.2**	**43.1**

(100 × a/n), a in the formula is the average number of polymorphic bands detected in each treated sample, and n is the number of total bands in the control. Polymorphisms in RAPD and IRAP profiles included the disappearance of a normal band and the appearance of a new band compared with the control. The average was calculated for each group. The average of polymorphisms (%) was calculated with the following formula: 100 × a/n to realize CRED-RA analysis.

3. Results

3.1. RAPD analysis

Totally, thirty-two decamer primers were tested for RAPD. Fourteen of them which had stable results were selected and 123 bands were counted in control as seen in Table 1. 1 (OPA-1 and OPW-1) – 8 (OPA-6) polymorphic bands out of control were recorded. Also, it was detected fairly large molecular size scale (from 127 (OPH-17) to 1096 (OPH-14) bp) for each primer. According to the results,

Table 2. Molecular sizes (bp) of appeared (+)/disappeared (-) bands in IRAP profiles of $FeSO_4$ treated *Zea mays* seedlings and value of GTS and polymorphism (%).

Primers	Control	+/-	50 mg/L $FeSO_4$	100 mg/L $FeSO_4$	200 mg/L $FeSO_4$	300 mg/L $FeSO_4$
WLTR 2105	5	+	2566; 1186	2879	2083; 879	1230; 879
		-	3737; 3020	3737; 3020	3737; 3020	3737; 3020
N57 (Nikita)	7	+	1572	1030; 922	1494; 1000; 896	1494; 1000
		-	2192; 724	2192	2192	2192; 1265
SUKKULA	6	+	1466; 975	1169	1664; 1052	1642; 1184; 1000
		-	1322	1580	1580	1580; 839
***Nikita*-E2647**	4	+	3151; 1242	791	877; 632; 470	1578; 1323
		-	2207; 1877	2563; 2207; 1877	2563; 2207	2563; 2207
STOWAWAY	4	+	-	808; 678	808; 678; 471	816; 414
		-	2161; 678534	2161; 997	2161; 997	2161; 997
GTS %			**30.4**	**32.8**	**22.5**	**15.8**
Polymorphism %			**69.5**	**67.2**	**77.5**	**84.2**

Table 3. Used CRED-RA primers and polymorphism (%).

Primers	50 mg/L $FeSO_4$		100 mg/L $FeSO_4$		200 mg/L $FeSO_4$		300 mg/L FeSO	
	HpaII	***MspI***	***HpaII***	***MspI***	***HpaII***	***MspI***	***HpaII***	***MspI***
OPA-1	14.2	0	0	14.2	0	28.5	0	28.5
OPA-2	0	14.2	0	0	14.2	14.2	14.2	28.5
OPW-1	22.2	22.2	22.2	22.2	22.2	22.2	22.2	0
OPY-6	0	0	11.1	11.1	11.1	22.2	11.1	22.2
OPB-8	0	9	9	9	0	9	9	9
OPY-15	16.6	16.6	16.6	0	16.6	0	16.6	33.3
OPH-17	0	14.2	0	28.4	14.2	28.4	14.2	0
OPH-18	12.5	12.5	12.5	12.5	0	0	12.5	12.5
Polymorphism (%)	**8.2**	**11.1**	**8.9**	**12.1**	**9.8**	**15.5**	**12.5**	**16.7**

there were significant banding pattern differences among the RAPD profiles of the control and $FeSO_4$ treated samples (Figure 1a). These differences have appeared as disappearance (-) or appearance (+) of the bands (+/-, Table 1). $FeSO_4$ treated samples had 32 disappearing bands and 20 appearing bands compared to nontreated samples. It was determined that there was a decrease in the GTS ratio as the $FeSO_4$ dose increased. The highest GTS value (66.4 %) was observed in 50 mg/L $FeSO_4$ treatment, while the lowest GTS value (56.9 %) was observed in 300 mg/L $FeSO_4$. Polymorphism ratios ranged from 33.6% to 43.1% in all $FeSO_4$ doses.

3.2. IRAP analysis

Five primers out of ten used for the IRAP technique gave specific and stable DNA profiles in *Zea mays* genome (Table 2). Significant differences in IRAP profiles existed between $FeSO_4$ treated and untreated plants (Figure 1b). These changes are characterized by variation in band intensity, loss of normal bands, or the appearance of new bands. Totally, 26 bands appeared in control as seen in Table 2. Eight-eleven polymorphic bands (loss and/or gain of bands) were detected in $FeSO_4$ treated plants out of control for all the primers used in the study. The molecular size of bands ranged from 414 (Stowaway) to 3737 (Wltr 2105) in treated plants. $FeSO_4$ treatments changed the GTS values of IRAP profiles. Increased $FeSO_4$ concentration caused decreasing GTS value in IRAP profiles. While the lowest polymorphism value (69.5%) was observed in 50 mg/L $FeSO_4$ treatment, the highest polymorphism value (84.2%) was seen in 300 mg/L $FeSO_4$ treatment (Table 2).

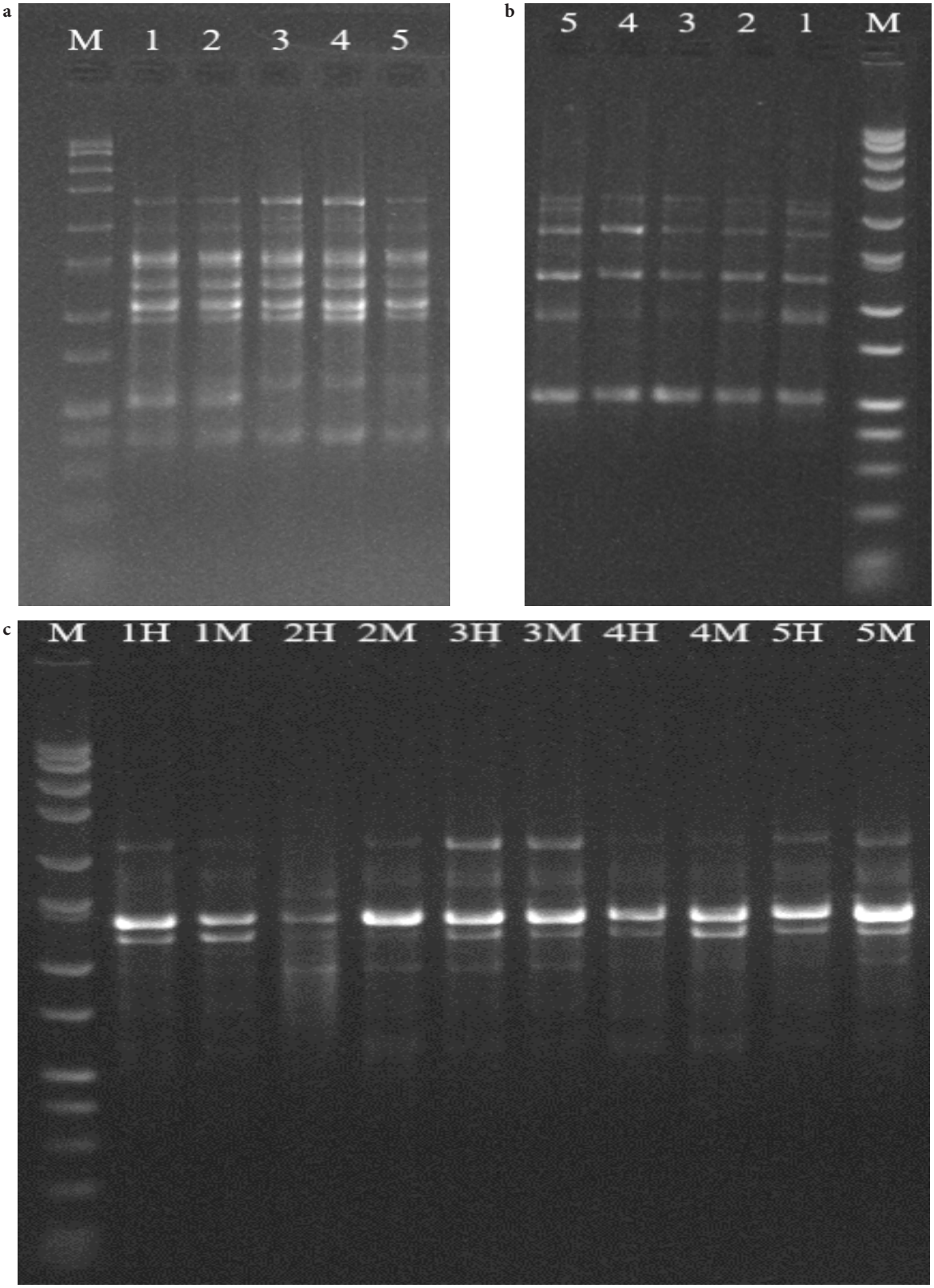

Figure 1a. RAPD banding pattern amplified with primer OPY-16. **Figure 1b.** IRAP banding pattern amplified with SUKKULA. **Figure 1c.** CRED-RA banding pattern amplified with OPY-6. (M: Marker, 1: Control, 2:50 mg/L $FeSO_4$, 3: 100 mg/L $FeSO_4$, 4: 200 mg/L $FeSO_4$, 5: 300 mg/L $FeSO_4$, H: *HpaII*, M: *MspI*).

3.3. CRED-RA analysis

Eight primers which amplified in RAPD were selected for CRED-RA technique (Table 3). The banding patterns obtained as a result of amplification with RAPD primers of cutting DNA samples with *HpaII* and *MspI* differ considerably (Figure 1c). The results of the CRED-RA assay were presented as the average polymorphism of % DNA methylation for each concentration (Table 3). When obtained the ratio of polymorphism, the *MspI* ratio was higher (11.1% to 16.7%) than the *HpaII* ratio (from 8.2% to 12.5%) and these were based on the primer and $FeSO_4$ doses (Table 3).

4. Discussion

In this research, we reported the impact of Fe^{2+} ($FeSO_4$) pollution that caused genotoxic damage, DNA methylation activities, and LTR RTs polymorphism changes in *Zea mays*. The present research effort is the first of its kind that Fe^{2+} toxicity and connection between the LTR RTs polymorphism and DNA methylation. Findings of RAPD revealed different polymorphic bands in its profiles and GTS decreasing. This indicated that Fe^{2+} had genotoxic effects on *Zea mays* seedlings. These results are in agreement with the findings of previous results on the effect of Fe^{2+} genotoxicity on different organisms such as *Pseudevernia furfuracea* (Aras et al., 2011), *Drosophila melanogaster* (Doganlar et al., 2014), etc. The amount of sufficient Fe in plants proved to be in the range of 70 to 300 mg/kg^{-1} (Wells et al., 1994). Hereby, iron deficiency or excess consists of concentrations below or above this sufficiency range. *Pseudevernia furfuracea* and *Ramalina pollinaria*, kinds of lichens that live near the iron-steel factory investigated in terms of genotoxicity by RAPD analysis and found enormous DNA damages (Aras et al., 2011; Duman et al., 2014). There is clear evidence that several environmental and genetic stimuli are known to change methylation status. Prolonged or temporary exposure to stress leads to an increase in cytosine methylation in the whole plant genome or in certain regions. (Taspinar et al., 2018; Ashapkin et al., 2020). Epigenetic marks play a role in controlling the expression of genes associated with abiotic stress. Stress signals may cause transcriptional suppression by promoting DNA methylation changes in the promoter regions of stress-related genes (Ou et al., 2012; Ueda and Seki, 2020). In this study, we used the CRED-RA technique to determine how the maize genome changes its cytosine methylation status during Fe^{2+} stress. We predicted that the expression of stress-related genes may change with increased DNA methylation due to Fe^{2+} stress. DNA methylation changes have already been detected in various stress conditions with this technique (Shams et al; 2020; Gallo-Franco et al., 2020; Hosseinpour et al., 2020; Orhan et al., 2020; Aydin et al., 2021).

Transposons are of great importance in suppressing gene expression. This can occur by methylation of a transposon located in or near a gene involved in the methylation mechanism (Galindo-González et al., 2018). In addition, the researchers reported that the transposon mobility that occurred around TEs was higher compared to other regions of the genome. The activation of TEs and adaptation to stress have been documented previously (McClintock, 1984; Boyko and Kovalchuk, 2008). Furthermore, LTR RTs might be activated by abiotic and biotic stresses in different organisms. LTR RTs can pose a threat to the integrity of the host genome because of their movement and have the potential to cause mutagenic effects through epigenetic regulation. Host genomes have developed mechanisms to control the action and possible mutagenic effects of LTR RTs. However, some LTR RTs escape from these defense mechanisms under stress conditions and continue to transpose. In our study, all of LTR RTs, which we used, have mobilized and have caused polymorphism in the maize genome against Fe^{2+} stress. Similar to our study, it was determined that the remobilization of ONSEN LTR RTs increased in heat stress in *Arabidopsis* (Ito et al., 2011), and Gypsy and Copia LTR RTs in drought, low temperature, and salinity stresses in some *Medicago* genotypes (Yin et al., 2021). In our literature research, the effect of excessive iron stress on LTR movements has not yet been clearly elucidated. Therefore, our study is unique in this respect.

5. Conclusion

In summary, it should be concluded that Fe^{2+} stress readily induced genomic instability, DNA methylation changes, and mobilization of LTR RTs. Understanding the relationship between methylation changes and LTR RTs could provide us important clues to plant adaptation in stress conditions however, the molecular functions and their interrelationships are still largely unknown. Further study is required in order to use molecular approaches in addition to genetic methods.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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