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Estimation of genomic instability and DNA methylation due to aluminum (Al) stress in wheat (*Triticum aestivum* L.) using iPBS and CRED-iPBS analyses

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Abstract: Aluminum (Al) toxicity is a serious factor restricting crop productivity in acid soil, and Al is the major cause of phytotoxicity. However, the role of Al toxicity in interprimer binding site (iPBS) polymorphism, genomic instability, and DNA methylation has not been fully investigated. In the current study, the effects of different Al concentrations on iPBS polymorphism, genomic instability, and DNA methylation were investigated in seedlings of three wheat cultivars: Haymana 79, Kılçıksız, and Bezostaja 1. A higher aluminum concentration increased the polymorphism rate of the iPBS profile, but decreased genomic template stability in all cultivars. A higher Al concentration was found to cause DNA methylation. Furthermore, the coupled restriction enzyme digestion-iPBS technique was used to detect DNA cytosine methylation level, which could help in understanding the epigenetic mechanism. The occurrence of hypermethylation and hypomethylation was observed with respect to Al stress treatment, and Al was found to cause DNA methylation. Polymorphism in the CRED-iPBS profile and DNA methylation can be correlated to evaluate epigenetic changes under stress.

Key words: Aluminum stress, DNA methylation, iPBS profile, genomic instability, wheat

1. Introduction

Aluminum (Al) toxicity affects 15% of soils on earth (Bot et al., 2000) and is a primary stress factor in acidic arable land (Kochian, 1995). High acidification encourages the dissolution of Al minerals in ubiquitous soil, thus increasing the availability of phytotoxic Al ions (Singer and Munns, 2006). Many crop species, including wheat, are sensitive to Al, and acidic soil with Al toxicity is usually the cause of dramatic yield decrease (Mossor-Pietraszewska, 2001). Al binds to the root cell walls, and is thought to prevent the elongation of meristematic cells in sensitive species (Ma et al., 2004; Doncheva et al., 2005), resulting in root stunting, which lowers crop performance in acidic soils. Al toxicity is an important agricultural problem and has been substantially investigated in plant systems (Mossor-Pietraszewska, 2001). To cope with metal toxicity, plants have developed a constructional process (seen in many phenotypes) and an adaptive process (seen in tolerant phenotypes), both of which have been considered to be controlled genetically. More than 20 genes induced by Al stress have been isolated from a series of plant species, including wheat (Anioł, 1995; Delhaize et al., 1999), rice (Nguyen et al., 2001), soybean (Bianchi-Hall et al., 2000), and tobacco (Ezaki et al., 1997). Many of these genes appear to be common stress-associated genes induced by a series of dissimilar stresses. It has been suggested that there are several processes for gene induction under Al and oxidative stress (Mossor-Pietraszewska, 2001). Abiotic stress, such as Al toxicity, causes excessive production of reactive oxygen species (ROSs), which affect the structure and function of biological molecules in the cell (Kumar et al., 2017a). DNA damage due to oxidative stress leads to alkylation (Sharma et al., 2014), methylation and oxidation (Meriga et al., 2004), single- and double-strand breakage (Mehta and Haber, 2014), and cross-linkage to proteins (Cadet et al., 2015). Ultimately, the aggregations of these impairments result in genetic and epigenetic inequality in plants (Sharma et al., 2012). Molecular marker systems, such as random amplified polymorphic DNA, amplified fragment length polymorphism, coupled restriction enzyme digestion (CRED)-random amplification, and methylation-sensitive amplified polymorphism, have been used to detect the genetic and epigenetic modifications by induced stress (Nardemir et al., 2015; Ince and Karaca, 2016). Polymerase chain reaction (PCR)-based interprimer binding site (iPBS) amplification is based on the essential presence of a tRNA complement as a reverse transcriptase primer-binding site (PBS) in long terminal region (LTR) retrotransposons. In particular, the iPBS amplification technique has been demonstrated to be a notable DNA

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fingerprinting technology not requiring sequence data. The use of the iPBS marker is an easy and rapid method for monitoring changes in the DNA profile of plants. This technique has been successfully employed in barley, wheat, apples, maize, apricot, and guava (Nemli et al., 2015).

CRED involving the profiling of DNA with molecular markers is used to determine the changes in DNA methylation in plant genome. This technique has been effective in detecting changes in cytosine methylation due to various abiotic stresses, such as chromium nitrate, zinc, arsenic, and lead sulfate stress/toxicity in maize (Erturk et al., 2014a; Erturk et al., 2015a,b).

However, the role of Al stress in DNA methylation has not been well documented in wheat. In this study, the CRED-iPBS method was used to detect DNA methylation status using iPBS markers. To the best of our knowledge, this is the first study that combined the use of CRED with iPBS markers in the detection of genetic and epigenetic modifications under Al stress in a wheat plant.

2. Materials and methods

2.1. Plant materials and culture conditions

Three wheat (*Triticum aestivum* L.) cultivars, namely cv. Haymana 79, Kılçıksız, and Bezostaja 1, were used to evaluate Al stress tolerance. A factorial experiment was carried out in the laboratory at Atatürk University in Turkey, using a completely randomized design with four replications. The factors consisted of three wheat cultivars and five Al concentrations (7.5, 15, 22.5, 30 mM, and distilled water as control) at pH 4.5. The wheat seeds were surface-sterilized in 70% (v/v) ethanol for 3 min, rinsed twice with sterile distilled water, kept in commercial bleach (5% sodium hypochlorite) for 25 min, and rinsed twice again with sterile distilled water. Twenty-five seeds of each cultivar were germinated on two layers of filter paper in 9-cm Petri dishes, and 10 mL of $AlCl_3.6H_2O$ solution of varying concentrations were applied onto the filter paper as per the treatment. The Petri dishes were covered to prevent moisture loss and kept in 16:8-h light:dark photoperiod at 25 ± 1 °C for 10 days. Then, the seedlings were collected and stored at –80 °C for molecular studies.

2.2. Genomic DNA isolation

Young leaf tissues were collected from the control and Al-stressed wheat seedlings. Genomic DNA was isolated following the method described by Zeinalzadehtabrizi et al. (2015), and stored at –20 °C for further use. The concentration and quality of genomic DNA were determined using a spectrophotometer and electrophoresis in 0.8% (w/v) agarose gel, respectively.

2.3. iPBS-PCR amplification

Twenty eight primers were tested for iPBS-PCR amplification (Kalendar et al., 2010). PCR was performed using the master mix that consisted of 10X buffer, 2 mM $MgCl_2$, 0.25 mM of each dNTPs, 2 µM (20 pmol) primer, 0.5 U Taq polymerase, and 1 µL of 50 ng/µL template DNA in a 20-µL reaction. The amplification conditions were: initial denaturation for 3 min at 95 °C, 38 cycles of 15 s at 95 °C, 60 s at 51–56 °C and 60 s at 72 °C, and a final extension of 5 min at 72 °C. Amplification products were resolved on 1% agarose gel in 1X sodium borate (SB) buffer at 100 V/cm for 120 min, stained with ethidium bromide (1.3 mM), and visualized under UV light. Band size was estimated with the help of 100 bp DNA ladder (Vivantis product No: NM2421) loaded on the gel along with the samples. Out of 28, only 15 iPBS oligonucleotide primers resulted in specific and stable DNA profiles in all three wheat cultivars (Table 1).

2.4. CRED-iPBS amplification

For CRED-iPBS, 1000 ng of genomic DNA sample was restricted with 1 U of *Hpa*II or *Msp*I following the manufacturer's (Thermo Scientific) instructions to be used as template DNA. The primers listed in Table 1 were used for amplification. Except for template DNA, the CRED-iPBS mixture was the same as the one used for iPBS-PCR. Amplification conditions were: an initial denaturation step of 5 min at 95 °C, 42 cycles of 60 s at 94 °C, 60 s at 51–56 °C and 120 s at 72 °C, and a final extension step of 15 min at 72 °C. CRED-iPBS PCR products were run on 1% agarose gel in 1X SB buffer at 100 V/cm, stained with ethidium bromide (0.2 µg/mL), and visualized under a UV light.

2.4.1. iPBS and CRED-iPBS analyses

The iPBS and CRED-iPBS banding patterns were analyzed using TotalLab TL120 software (Nonlinear Dynamics Ltd^R). The genomic template stability (GTS %) was calculated using the following formula:

$$GTS = \left(1 - \frac{a}{n}\right) x100,$$

where a is the average number of polymorphic bands found in each treated template and n is the number of total bands in the control (Sigmaz et al., 2015).

Polymorphism in iPBS profiles was expressed as the disappearance of a normal band and the appearance of a new band compared to the control. The average was calculated for each experimental group and changes in these values were calculated as a percentage of their value in the control (set to 100%). For CRED-iPBS analysis, the average values of polymorphism (%) were calculated for each concentration using the formula, 100 × a/n.

3. Results

In this study, three wheat cultivars and five Al concentrations were used to assess genetic and epigenetic (DNA cytosine methylation) variations due to Al stress in wheat seedlings using iPBS and CRED-iPBS techniques. The iPBS profiles showed significant differences between

Table 1. Reactive primers used in iPBS-PCR and their annealing (Ta) temperature.

No.	Primer name	Sequence (5' to 3')	Tm (°C)	CG (%)	Optimal annealing, Ta (°C)
1	2077	CTCACGATGCCA	46.1	58.3	55.1
2	2095	GCTCGGATACCA	44.8	58.3	53.7
3	2375	TCGCATCAACCA	45.1	50.0	52.5
4	2377	ACGAAGGGACCA	47.2	58.3	53.0
5	2378	GGTCCTCATCCA	44.2	58.3	53.0
6	2380	CAACCTGATCCA	41.4	50.0	50.5
7	2381	GTCCATCTTCCA	40.9	50.0	50.0
8	2384	GTAATGGGTCCA	40.9	50.0	50.0
9	2387	GCGCAATACCCA	47.3	58.3	51.5
10	2388	TTGGAAGACCCA	43.4	50.0	51.0
11	2390	GCAACAACCCCA	47.6	58.3	56.4
12	2392	TAGATGGTGCCA	43.1	50.0	52.2
13	2393	TACGGTACGCCA	47.1	58.3	51.0
14	2276	ACCTCTGATACCA	42.7	46.2	51.7
15	2278	GCTCATGATACCA	42.3	46.2	51.0

the cultivars and Al concentrations. These differences were identified by variation in disappearance of normal bands seen in control (0 mM), and appearance of new bands. For the 15 reactive primers used in the study, the total bands, polymorphic bands (loss and/or gain of bands), and the GTS value were determined and compared between the Al-treated and control samples (Table 2). The results revealed that the 15 selected iPBS primers produced a total of 206, 195, and 180 bands in Haymana, Kılçıksız, and Bezostaja 1 wheat cultivars, respectively, with each primer generating 5–10, 2–14, and 3–14 bands with an average of 7.26, 7.20, and 6.80 bands per primer, respectively (Table 2).

iPBS profiles of the control and Al-treated samples varied. Depending on Al concentration, the total bands for the iPBS profiles ranged from 16 to 31, 13 to 27, and 12 to 27 in Haymana, Kılçıksız, and Bezostaja 1 cultivars, respectively. After the Al treatment, a total of 109, 108, and 102 normal iPBS bands were lost in Haymana, Kılçıksız, and Bezostaja 1 cultivars, respectively. Additionally, the changes that occurred after treatment with 4 different concentrations of Al can be summarized as the appearance of 40, 48, and 36 new bands and disappearance of 51, 42, and 39 existing bands compared to the control samples in Haymana, Kılçıksız, and Bezostaja 1 cultivars, respectively. The cultivars gave different responses to different Al levels for the total band number. There was a clear increase in the total band number with the increasing concentration of Al in all three cultivars (Table 2).

The number of polymorphic bands varied with the concentration of Al treatment. Each cultivar gave different responses to Al concentrations with respect to the polymorphism rate, showing an increase in polymorphism with increasing Al concentration in all cultivars. The highest polymorphism (28.44%) was observed at 30 mM Al in Haymana, whereas the lowest polymorphism (11.76%) was observed at 7.5 mM Al in Bezostaja 1 (Table 2).

The changes in iPBS profiles were also measured as GTS percentage. GTS is a qualitative measurement reflecting the changes in iPBS patterns. GTS calculation was performed for 15 primers, and the results are presented in Table 1. A negative relationship between the GTS value and Al concentration was observed. The response of different cultivars to Al stress varied in terms of the GTS value. The highest GTS (88.24) was observed in Bezostaja 1 at 7.5 mM Al treatment, whereas the lowest value (71.56) was observed in Haymana 79 at 30 mM Al treatment (Table 2).

A CRED-iPBS analysis was undertaken to determine the effects of Al treatment on methylation in the three cultivars. *Hpa*II polymorphism was found to be higher than *Msp*I. For *Msp*I, the mean polymorphism rate per primer ranged from 11.4% to 42.61%, 24.52% to 44.43%, and 14.77% to 44.44% for Haymana 79, Kılçıksız, and Bezostaja 1, respectively. DNA hypermethylation was observed at 30 mM Al stress, which was 44.44%, 44.43%, and 42.61% for Haymana 79, Kılçıksız, and Bezostaja 1, respectively. Hypomethylation was detected at 7.5 mM Al stress with 11.4%, 14.77%, and 24.52%, respectively (Table 3).

Table 2. The number of bands in control and disappearance (-), and/or appearance (+) of DNA bands with molecular sizes (base pair, bp), total band, polymorphism, and the average GTS value for all the primers in the shoots of three Al treated wheat cultivars.

Cultivar	Primers	Control	+/-	Al concentration			
				7.5 mM	15 mM	22.5 mM	30 mM
Haymana79	2077	10	+	585	776	1025, 776, 748, 573	1029, 779, 745
			-	--	--	--	--
	2095	7	+	706	699, 627	623	823, 680, 595
			-	--	--	794, 734	--
	2276	7	+	--	--	--	--
			-	--	--	--	--
	2278	7	+	--	--	--	--
			-	725, 401	1003, 401	1003	1003, 725, 401
	2375	5	+	--	--	457	470
			-	--	--	--	--
	2377	6	+	--	--	1011, 871	790
			-	--	--	--	--
	2378	6	+	--	--	--	--
			-	--	--	881	--
	2380	7	+	645	766, 671	761, 671	834, 676
			-	--	--	--	--
	2381	7	+	--	513	990	984, 506
			-	714	753	753	753
	2384	10	+	--	916, 481	--	--
			-	--	1199, 1015	1299, 1199, 796	1299, 1199, 796
	2387	8	+	598	589	--	855, 592
			-	988, 697	697	1038, 697	1039, 697
	2388	8	+	--	--	--	774
			-	881	881	881	881, 562
	2390	5	+	--	--	--	--
			-	849, 802	849, 802	849, 802	849, 802
	2392	7	+	--	--	--	--
			-	675, 640	473	599, 473	473
	2393	9	+	476	--	--	--
			-	643	878, 643	878, 643, 618	643, 618
Total band		**109**		**16**	**21**	**29**	**31**
Polymorphism				**14.67**	**19.26**	**26.60**	**28.44**
GTS value				**85.33**	**80.74**	**73.40**	**71.56**

Table 2. (Continued).

Cultivar	Primers	Control	+/-	Al concentration			
				7.5 mM	**15 mM**	**22.5 mM**	**30 mM**
Kılçıksız	**2077**	14	+	--	579, 488	--	597
			-	--	--	--	--
	2095	7	+	576	--	974, 628, 578	926, 803
			-	--	926	--	--
	2276	5	+	454	789, 451, 310	456	449, 302
			-	--	--	--	--
	2278	5	+	--	--	--	--
			-	--	--	--	1036
	2375	6	+	--	--	--	--
			-	671	813, 642	671	671
	2377	9	+	--	--	870, 489	--
			-	--	--	--	870, 720
	2378	8	+	--	--	590	--
			-	926	926	926	926
	2380	8	+	--	--	--	614
			-	--	--	825	757
	2381	8	+	407	768, 414	1001, 699, 407	994, 407
			-	--	--	--	--
	2384	12	+	--	--	--	--
			-	775	1249, 1205, 775	1249, 1205, 1078, 775	1249, 1078, 775
	2387	8	+	959	--	--	--
			-	873, 845	1124, 1037, 873, 845	873, 845	1037, 873, 845
	2388	6	+	751, 587	923, 575	1037, 592	582
			-	--	--	--	964
	2390	2	+	806, 711	718	808, 720	811, 715
			-	--	--	--	--
	2392	5	+	--	--	--	637, 509
			-	--	--	589	--
	2393	5	+	--	--	--	--
			-	--	880	883	886
Total band		**108**		**13**	**22**	**25**	**27**
Polymorphism				**12.03**	**20.37**	**23.14**	**25**
GTS value				**87.97**	**79.63**	**76.86**	**75**

Table 2. (Continued).

Cultivar	Primers	Control	+/-	Al concentration			
				7.5 mM	**15 mM**	**22.5 mM**	**30 mM**
Bezostaja 1	**2077**	14	+	--	516	--	721
			-	823	823, 574	823	983, 823, 646, 574,
	2095	9	+	978	833, 583	849, 774	--
			-	--	978	--	978, 932, 615
	2276	6	+	--	--	427	472, 431
			-	--	773, 504	--	--
	2278	5	+	--	691	714	694
			-	--	823	823	956
	2375	5	+	--	--	--	--
			-	--	--	--	--
	2377	7	+	--	--	--	--
			-	737	737	737, 519	737
	2378	7	+	--	--	--	759
			-	--	--	--	665
	2380	5	+	761	832	614	606
			-	--	--	--	--
	2381	7	+	--	--	979	615
			-	689	--	739	--
	2384	8	+	--	--	--	924
			-	786	786	786	813
	2387	7	+	847	--	821	878
			-	--	1048	--	1121
	2388	9	+	846	--	--	826
			-	543	779, 543	779, 543	543
	2390	3	+	--	--	--	818
			-	--	--	713	--
	2392	5	+	--	638, 477	635	643, 493
			-	595	--	--	--
	2393	5	+	867	--	997, 861	864
			-	1010	1010	1010	--
Total band		**102**		**12**	**19**	**20**	**27**
Polymorphism				**11.76**	**18.62**	**19.60**	**26.47**
GTS value				**88.24**	**81.38**	**80.4**	**73.53**

Table 3. The changes in methylation status (CRED-iPBS) of wheat seedlings exposed to different Al concentrations.

Cultivar	Primers	H/M	Al concentration											
			The number of total bands				Total polymorphic bands				Polymorphism%			
			7.5 mM	15 mM	22.5 mM	30 mM	7.5 mM	15 mM	22.5 mM	30 mM	7.5 mM	15 mM	22.5 mM	30 mM
Haymana79	2077	H	7	7	8	8	0	0	1	1	0	0	14.28	14.28
		M	7	7	8	9	0	0	1	2	0	0	14.28	28.57
	2095	H	7	7	8	9	0	0	1	2	0	0	14.28	28.57
		M	7	8	8	9	0	1	1	2	0	14.28	14.28	28.57
	2276	H	7	8	8	8	0	1	1	1	0	14.28	14.28	14.28
		M	7	6	6	6	1	0	0	0	16.67	0	0	0
	2278	H	10	9	10	10	1	0	1	1	11.11	0	11.11	11.11
		M	9	9	10	11	0	0	1	2	0	0	11.11	22.22
	2375	H	6	6	6	8	1	1	1	3	20	20	20	60
		M	4	6	6	6	0	2	2	2	0	50	50	50
	2377	H	15	14	14	16	2	1	1	3	15.38	7.69	7.69	23.08
		M	17	17	16	18	3	3	2	4	21.43	21.43	14.29	21.43
	2378	H	14	15	14	15	2	3	2	3	16.67	25	16.67	25
		M	11	10	11	13	1	0	1	3	10	0	10	30
	2380	H	10	11	12	13	0	1	2	3	0	10	20	30
		M	11	12	13	13	0	1	2	2	0	9.1	18.18	18.18
	2381	H	12	11	12	12	1	0	1	1	9.1	0	9.1	9.1
		M	11	11	11	14	1	1	1	4	10	10	10	40
	2384	H	10	10	12	12	1	1	3	3	11.11	11.11	33.33	33.33
		M	12	12	14	16	1	1	3	5	9.10	9.10	27.7	45.45
	2387	H	10	10	10	10	0	0	0	0	15.38	7.69	7.69	23.08
		M	10	10	10	10	0	0	0	0	21.43	21.43	14.29	21.43
	2388	H	8	9	8	10	0	1	0	2	0	12.5	0	25
		M	8	8	10	10	0	0	2	2	0	0	25	25
	2390	H	6	5	6	5	1	0	1	0	20	0	20	0
		M	8	9	10	9	1	2	3	2	14.29	28.57	42.86	28.57
	2392	H	11	11	11	12	2	2	2	3	22.22	22.22	22.22	33.33
		M	10	11	11	12	1	2	2	3	11.11	22.22	22.22	33.33
	2393	H	6	7	7	7	0	1	1	1	0	16.67	16.67	16.67
		M	6	6	8	8	0	0	2	2	0	0	33.33	33.33
	Average	H	9.3	9.3	9.7	10.3	0.7	0.8	1.2	1.8	14.10	14.71	22.73	34.68
		M	**9.3**	**9.5**	**10.2**	**10.9**	**0.6**	**0.9**	**1.5**	**2.3**	**11.4**	**18.61**	**28.21**	**42.61**

*H- Hpa*II, *M- Msp*I

Table 3. (Continued).

Cultivar	Primers	H/M	Al concentration											
			The number of total bands				Total polymorphic bands				Polymorphisim %			
			7.5 mM	15 mM	22.5 mM	30 mM	7.5 mM	15 mM	22.5 mM	30 mM	7.5 mM	15 mM	22.5 mM	30 mM
Kılçıksız	2077	H	9	9	10	10	0	0	1	1	0	0	11.11	11.11
		M	9	10	10	11	0	1	1	2	0	11.11	11.11	22.22
	2095	H	5	6	6	6	0	1	1	1	0	20	20	20
		M	6	5	6	7	1	0	1	2	20	0	20	40
	2276	H	9	8	8	9	3	2	2	3	50	33.33	33.33	50
		M	10	9	8	9	3	2	1	2	42.86	28.57	16.67	28.57
	2278	H	7	7	8	8	0	0	1	1	0	0	14.28	14.28
		M	8	9	8	9	1	2	1	2	14.28	28.57	14.28	28.57
	2375	H	7	6	6	7	2	1	1	2	40	20	20	40
		M	6	6	6	8	1	1	1	3	20	20	20	60
	2377	H	15	17	16	16	2	4	3	3	15.38	30.77	23.08	23.08
		M	13	12	12	13	3	2	2	3	30	20	20	33
	2378	H	16	17	16	19	3	4	3	6	23.07	30.77	23.07	30.77
		M	11	13	12	14	1	3	2	4	10	30	20	40
	2380	H	15	14	15	16	3	2	3	4	25	16.67	25	33.33
		M	11	11	12	13	1	1	2	3	10	10	20	30
	2381	H	10	10	10	12	0	0	0	2	0	0	0	20
		M	11	10	11	12	1	0	1	2	10	0	10	20
	2384	H	9	10	10	11	0	1	1	2	0	11.11	11.11	22.22
		M	10	11	11	13	1	2	2	4	11.11	22.22	22.22	44.44
	2387	H	9	10	10	9	0	1	1	0	0	16.67	8.33	25
		M	9	10	9	10	0	1	0	1	8.33	16.67	25	25
	2388	H	8	8	7	8	1	1	0	1	14.29	14.29	0	14.29
		M	8	8	8	8	0	0	0	0	0	0	0	0
	2390	H	10	11	12	13	0	1	2	3	0	10	20	30
		M	10	9	8	9	3	2	1	2	42.86	25.57	14.29	28.57
	2392	H	10	11	11	12	1	2	2	3	11.11	22.22	22.22	33.33
		M	12	12	13	14	1	1	2	3	9.10	9.10	18.18	27.27
	2393	H	6	7	7	8	0	1	1	2	0	16.67	16.67	33.33
		M	7	7	7	7	1	1	1	1	16.67	16.67	16.67	16.67
	Average	H	9.7	10.1	10.1	10.9	1	1.5	1.5	2.3	17.88	24.25	24.82	40.07
		M	**9.4**	**9.5**	**9.4**	**10.4**	**1.2**	**1.3**	**1.2**	**2.3**	**24.52**	**23.85**	**24.84**	**44.43**

*H- Hpa*II, *M- Msp*I

Table 3. (Continued).

Cultivar	Primers	H/M	Al concentration											
			The number of total bands				Total polymorphic bands				Polymorphisim %			
			7.5 mM	15 mM	22.5 mM	30 mM	7.5 mM	15 mM	22.5 mM	30 mM	7.5 mM	15 mM	22.5 mM	30 mM
Bezostaja 1	2077	H	9	9	11	12	0	0	2	3	0	0	22.22	33.33
		M	9	8	10	11	1	0	2	3	12.5	0	25	37.5
	2095	H	8	9	9	9	1	2	2	2	14.28	28.57	28.57	28.57
		M	6	7	7	8	0	1	1	2	0	16.67	16.67	33.33
	2276	H	6	7	6	7	0	1	0	1	0	16.67	0	16.67
		M	6	6	7	6	1	1	2	1	20	20	40	20
	2278	H	8	7	9	9	1	0	2	2	14.28	0	28.57	28.57
		M	9	7	8	7	2	0	1	0	28.57	0	14.28	0
	2375	H	5	5	5	5	0	0	0	0	0	0	0	0
		M	5	5	5	7	0	0	0	2	0	0	0	40
	2377	H	14	13	13	15	2	1	1	3	16.67	8.33	8.33	25
		M	13	14	15	15	1	2	3	3	8.33	16.67	25	25
	2378	H	11	10	10	11	1	0	0	1	10	0	0	10
		M	10	11	11	12	0	1	1	2	0	10	10	20
	2380	H	10	11	13	13	1	2	4	4	11.11	22.22	44.44	44.44
		M	9	9	11	13	0	0	2	4	0	0	22.22	44.44
	2381	H	8	7	8	9	1	0	1	2	14.28	0	14.28	28.57
		M	7	7	7	9	1	1	1	3	16.67	14.28	16.67	50
	2384	H	8	9	9	10	3	4	4	5	60	80	80	100
		M	6	7	8	9	0	1	2	3	0	16.67	33.33	50
	2387	H	11	12	12	12	1	2	2	2	16.67	8.33	8.33	25
		M	11	11	11	10	1	1	1	0	8.33	16.67	25	25
	2388	H	10	11	11	10	1	2	2	1	11.11	22.22	22.22	11.11
		M	8	8	10	9	0	0	2	1	0	0	25	12.1
	2390	H	10	11	10	10	3	4	3	3	42.86	57.14	42.86	42.86
		M	8	8	8	8	2	2	2	2	33.33	33.33	33.33	33.33
	2392	H	11	11	11	12	1	1	1	2	10	10	10	20
		M	12	10	11	12	2	0	1	2	20	0	10	20
	2393	H	5	5	5	5	0	0	0	0	0	0	0	0
		M	6	7	7	8	0	1	1	2	0	16.67	16.67	33.33
	Average	H	8.9	9.1	9.5	9.9	1.1	1.3	1.6	2.1	22.13	25.35	30.98	41.41
		M	8.4	8.3	9.1	9.6	0.7	0.7	1.5	2	14.77	16.10	31.32	44.44
H- HpaII, M- MspI														

4. Discussion

Environmental stresses are recognized as the cause of genetic and epigenetic variability in plants (Laird, 2010; Kumar et al., 2017a). One of the epigenetic modifications is DNA methylation, which plays a crucial role in epigenetic control by adjusting developmental and physiological mechanisms through differentially regulating gene expression at both posttranscriptional and transcriptional levels when plants are exposed to environmental stress (Gavery and Roberts, 2010; Kumar et al., 2017b). DNA methylation variability may serve as genetic diversity essential in breeding programs (Marfil et al., 2009; Kumar et al., 2017c). In addition, DNA methylation increases the mutation rate of affected cytosines, particularly in intronic and intergenic states (Mugal and Ellegren, 2011; Drewell et al., 2014; Karaca et al., 2016).

Heavy metal directly influences gene expression by binding to the metal responsive elements in target gene promoters (Cheng et al., 2012). Epigenetic changes due to variation in methylation status can also potentially cause phenotypic variations. Plants under stress can reprogram their gene expression through methylation and demethylation. Usually, hypermethylation is correlated with gene silencing, but hypomethylation is connected with active transcription (Steward et al., 2002; Li et al., 2018). The current study presents the first results on estimation of DNA methylation status using CRED-iPBS polymorphism in wheat grown under Al stress.

As revealed by the polymorphic bands in the iPBS profiles, decreased GTS evidences that Al has genotoxic effects (Table 2). For all the primers used in the study, the GTS value was lower in Al-treated plants compared to that in the control samples. This is the first report on using iPBS markers and CRED-iPBS methods for detecting DNA alteration and variation in DNA cytosine methylation. iPBS, a novel PCR-based method, is based on the presence of a tRNA complement as a reverse transcriptase PBS in LTR retrotransposons. Moreover, iPBS has proven to be a potent DNA fingerprinting technique that requires no previous sequence information (Kalendar et al., 2010; Andeden et al., 2013). The main reason for loosing normal PCR bands or seeing new bands is DNA methylation. Methylation enables or disables the restriction enzyme to recognize the cutting sites. This differentiates between normal plants and plants under stress.

In this experiment, DNA hypermethylation was observed at higher Al concentrations, whereas hypomethylation was detected at lower Al concentrations. Similar and supporting results have been reported by several researchers in maize under zinc stress (Erturk et al., 2015a), chromium nitrate in maize (Erturk et al., 2014a), arsenic trioxide in *Zea mays* (Erturk et al., 2015b), and lead sulfate solution in *Zea mays* (Erturk et al., 2014b). Excessive accumulation of Al can reduce the activity of methyl transferase and cause hypomethylation of certain specific gene regions.

In this study, we studied the effect of different Al doses on alterations in methylation in three wheat cultivars (Haymana, Kılçıksız, and Bezostaja 1). This variation can be used to choose the appropriate cultivars for plant breeding programs to enhance abiotic stress tolerance, including Al tolerance.

It demonstrates the association between cytosine methylation and Al tolerance. In conclusion, Al has a genotoxic potential and causes DNA methylation in wheat plants.

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