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Ethyl Methanesulfonate (EMS) Mutagen Toxicity-Induced DNA Damage, Cytosine Methylation Alteration, and iPBS-Retrotransposon Polymorphisms in Wheat (*Triticum aestivum* L.)

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Abstract: The use of mutagens in plant breeding is used to create new germplasm, increase agricultural yield, quality, and resistance to diseases and pests. Mutagens are physical or chemical factors that can alter the DNA or RNA structure of an organism, causing mutations above the expected level. One of the most common and potent chemical mutagens is EMS (ethyl-methane sulfonate), which produces point mutations in plants, but to a lesser degree can also cause the loss or deletion of a chromosomal region. This study used inter-primer binding site (iPBS) and coupled restriction enzyme digestion inter-primer binding site (CRED-iPBS) technique analysis to determine the effect of EMS mutagens on methylation rates in wheat genotypes at seedling growth stage. Treatments with five different EMS concentrations (0%; control, 0.1%, 0.2%, 0.3%, and 0.4%) at four different times (0; control, 3, 6, and 9 h) were used. Inter-primer binding site (iPBS) markers were employed to assess genomic instability and cytosine methylation in treated wheat. In seeds treated with EMS at different concentrations and times, the disappearance of regular bands and the formation of new bands due to the effects of the EMS mutagen revealed that genetic diversity exists. The CRED-iPBS analysis revealed that the 3 h + 0.1% EMS treatment produced the highest *MspI* polymorphism value (19.60%), while the 9 h + 0.1% EMS treatment produced the lowest value (10.90%). The mutagenic effects of EMS treatments had considerable polymorphism on a variety of impacts on the cytosine methylation and genomic instability of wheat. According to the current research, EMS mutagenesis may be a practical method for accelerating breeding programs to produce enough genetic diversity in wheat populations. Mutation-assisted breeding and the subsequent selection of desirable mutants using genetic markers may also be carried out in wheat utilizing an integrated strategy.

Keywords: genomic template stability; DNA methylation; mutagen; iPBS

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1. Introduction

Mutagens are physical or chemical factors that change living organisms' DNA or RNA structure, naturally causing the organism to mutate above the expected level. Genetic diversity has been developed through natural methods that have been applied consciously or unconsciously in many plant groups for centuries. However, evaluating such mutations will help to fulfill the current requirements. The revelation that physical and chemical mutagens may generate mutations is one of the most significant discoveries in the history of genetics. Recently, mutagenesis has gained popularity as an efficient means of enhancing current germplasm for cultivar creation in crop improvement and breeding operations [1]. Using mutagens makes it possible to create mutant plants with specific growth patterns while retaining the required agronomic traits [2]. Traditionally, conventional mutation methods have increased agricultural output, quality, and resistance to disease and pests. The most useful chemical mutagens used to induce mutations in plants mostly belong to the class of alkylating agents such as ethyl-methane sulfonate (EMS), diethyl sulfate (DES), ethyleneimine (EI), N-nitroso-N-methyl-urethane (NEU) and methyl nitrosourea (MNH). Compared to physical mutagens, chemical mutagens cause more gene mutations but fewer chromosomal mutations [3].

Chemical mutagens are one of the mutagen methods used to enhance significant agronomic features. EMS is one of the most prevalent and potent chemical mutagens since it is exceptionally straightforward to administer and monitor the effects of mutations. In plants, EMS often produces point mutations; however, the loss or deletion of a chromosomal region may also occur to a lesser degree [4]. Consequently, EMS can modify loci or a candidate gene of interest without causing massive deletions. This provides an advantage for plant breeders seeking helpful alleles over employing exotic or wild germplasms that may include a connected collection of lethal alleles [5]. Before large-scale production for plant breeders, the first investigations of induced mutations are often conducted to obtain the best results and determine the optimal combination of these characteristics and dosage. Physical and chemical mutagens were studied on a variety of plant species, including wheat, barley, rice, and maize [6]. It has been shown that administering chemical mutagens to plants results in substantial changes in their characteristics (i.e., earliness, adaptability, endurance, yield, and quality lodging resistance; thousand-grain weight and ear-grain weight in cultivated plants; and mutants with improved agricultural performance) can be created in bread wheat [7].

Under environmental stress conditions, plants exhibit a significant decrease in yield. Mutagen breeding aims to produce plant variants that are resistant to stress. The regulation of genes in response to stress challenges largely depends on the dynamic nature of epigenetic coding [8]. The degree of quantitative phenotypic variation among species is influenced by DNA sequence variation, which is a major contributor to natural variation. Changes in chromatin structure caused by DNA methylation are known to affect stress-responsive genes [9]. In addition, some epigenetic changes such as DNA methylation play an important role in stress memory, and this memory can be transmitted to offspring [10]. Numerous recent studies have shown that epigenetic changes can cause observable phenotypic differences, making epigenetic variation a crucial factor in the study of phenotypic variation. Therefore, it is thought that EMS will cause epigenetic modifications that will be advantageous in wheat plants. Several assays have been designed to investigate the genotoxicity of plant stress situations. Previous research has shown that the iPBS approach is more dependable than other procedures [11–14]. The identification of many types of genetic abnormalities and mutations in plants caused by stressors has made this technology more appealing than others.

Epigenetic variation, such as DNA methylation, has recently been linked to both short-term and long-term evolutionary adaptation, according to certain research [15,16]. Even while DNA sequence variation is the fundamental evolutionary process causing phenotypic variations, DNA methylation modifications may affect gene expression and therefore may possibly contribute to trait differences that may be handed down to future generations [17].

DNA methylation is essential for normal development and cellular differentiation as it plays a critical role in regulating gene expression [18]. DNA methylation is an important epigenetic mechanism vital for gene expression, development, and healing [19]. DNA methylation (the addition of methyl to bases), histone modifications, the changes that occur through the incorporation of different modifications and the different properties they bring to living organisms are all included in this research study. DNA methylation occurs by the addition of a methyl group (-CH3) to the 5-carbon position of a cytosine base, usually in the context of a CpG dinucleotide. 5-Methyl cytosine is synthesized from cytosine as a result of this methylation [20]. Methylation of CpG islets in gene regulatory regions can block the binding of transcription factors and other regulatory proteins, resulting in gene silencing [18,21]. The relationship between DNA methylation and CRED (coupled restriction enzyme digestion) analysis aims to determine the effect of DNA methylation on gene expression. CRED-iPBS is successfully used to assess the methylation state of the genome in relation to stress [11–14]. Additionally, epigenetic regulation and transposable element activities may be related to the regulation of chemical mutagen toxicity.

DNA molecular markers have long been helpful tools for gaining a better understanding of the nature of genetic instability. There are several types of molecular markers, such as single nucleotide polymorphisms (SNPs), simple sequence repeats (SSRs), and amplified fragment length polymorphisms (AFLPs). Apart from these, iPBS has been used to assess polymorphism and DNA methylations and to clarify the effects of various plant treatments [22,23]. iPBS provides a universal DNA fingerprinting technique. Moreover, the iPBS marker belongs to the retrotransposon markers system according to the amplification of the zone covered by binding sites of the reverse transcriptase primer for two-joined retrotransposons that are in opposite direction [24]. It is used to identify variations in stress-induced cytosine methylation toxicity in plant genomes [22,25]. Based on the latest literature reviews, Hosseinpour et al. [26] conducted a study on the effect of EMS on germination and seedling characteristics. However, researchers have not conducted any studies on epigenetic alterations. Therefore, this is the first study on the epigenetic effects of EMS on the wheat genome using iPBS and CRED-iPBS methods. The main objective of this study was to determine the effects of EMS in different concentrations and durations in the seedling growth stage in wheat plant in terms of genomic instability and DNA methylation by using iPBS and CRED-iPBS technique analyses, respectively.

2. Materials and Methods

2.1. Plant Material, Plant Culture, and EMS Treatment

Seeds of Kirik bread wheat (*Triticum aestivum* L.) variety were used as material in the study. Seeds were washed in tap water, stirred in 70% ethyl alcohol (EtOH) for 3 min and washed 3 times with pure, sterile water in a sterile cabinet. Surface sterilization was performed in 20% sodium hypochlorite containing a few drops of Tween-20 (Sigma) for 25 min. Surface-sterilized seeds were washed with sterile distilled water and kept in aerated water for 6 h. After surface sterilization, the seeds were watered and swollen and kept at five different EMS concentrations (0%; control, 0.1%, 0.2%, 0.3%, and 0.4%) and four different times (0; control, 3, 6, and 9 h). The seeds were then immersed in EMS solution (0.1 M phosphate buffer, pH = 7.0) and contained in 50 mL Eppendorf tubes (100 seeds per tube; 0.5 mL/seed). After treatment, the seeds were washed in tap water for 4 h to remove the mutagen. In this study, 25 seeds from each treatment were germinated on two layers of filter paper in 9 cm Petri dishes for 4 replicates. Each Petri dish was filled with 14 mL of distilled water. The germination process was carried out in a germination cabinet with the temperature adjusted to 25 °C for 14 days under the conditions of 16 h light and 8 h dark period. Germination and seedling characteristics of each treatment group used in the research were previously presented in [26]. To determine the genomic DNA isolation, all the leaves for each treatment were mixed and used.

2.2. Extraction of Genomic DNA

The technique described by Zeinalzadehtabrizi et al. [27] was modified slightly to separate genomic DNA from leaves explants that had been exposed to different doses and durations of EMS mutagen for four weeks. In order to be used later, the DNA was then kept at −20 °C. NanoDrop® ND-1000 UV/V spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) (Qiagen Qiaxpert) was used to measure the quantity of DNA, and 1.5% agarose gel electrophoresis was used to check the DNA's quality.

2.3. iPBS-PCR Amplification

Twenty-five primers were evaluated for iPBS-PCR amplification; however, only 10 of them generated distinct and polymorphic banding patterns across all treatments (Table 1). The components of the PCR master mix were 20 µL of 50 ng/µL template DNA, 1 µL of 10 X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP, 10 pmol primer, and 1 U Taq polymerase. It was amplified under the following conditions: initial denaturation at 95 °C for 3 min, 38 cycles of 15 s at 95 °C, 60 s at 42.1–56 °C, and 60 s at 72 °C, then 5 min at 72 °C. An electrophoresis technique was used to distinguish iPBS and CRED-iPBS PCR products according to their base size [14].

Table 1. Sequence information of 10 iPBS primers and their annealing (Ta) temperatures.

Primer Name	Sequence (5′–3′)	Tm (°C)	CG (%)
iPBS-2075	CTCATGATGCCA	42.1	50.0
iPBS-2079	AGGTGGGCGCCA	56.6	75.0
iPBS-2080	CAGACGGCGCCA	54.6	75.0
iPBS-2217	ACTTGGA TGTCGA T ACCA	52.5	44.4
iPBS-2270	ACCTGGCGTGCCA	56.9	69.2
iPBS-2274	ATGGTGGGCGCCA	57.1	69.2
iPBS-2377	ACGAAGGGACCA	47.2	58.3
iPBS-2380	CAACCTGATCCA	41.4	50.0
iPBS-2381	GTCCATCTTCCA	49.9	50.0
iPBS-2382	TGTTGGCTTCCA	44.9	50.0

2.4. CRED-iPBS Amplification

Using 1 U of *HpaII* or *MspI* enzymes, 1000 ng of total DNA was cut. According to the manufacturer's instructions (Thermo Scientific), this procedure sought to acquire template DNA. The 10 iPBS primers mentioned above were used to amplify the fragmented DNA afterward. The PCR master mixture included 10× PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP, 10 pmol primer, 1 U Taq polymerase, and 50 ng/µL template DNA. The amplification procedure included an initial denaturation at 95 °C for 3 min, 38 cycles of 15 s at 95 °C, 60 s at 42.1–57.1 °C, 60 s at 72 °C, and a final 5 min at 72 °C. A 3% agarose gel was used to display the results of CRED-iPBS PCR samples [22,23,25].

2.5. Coupled Restriction Enzyme Digestion-iPBS Analysis

Using the program TotalLab TL120, the iPBS and CRED-iPBS bands were both examined (Non-linear Dynamics Ltd. R brand). Following band profiles, the genomic template stability percentage (GTS%) was computed as follows: the formula for the GTS is $(1 - a/n) \times 100$, 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. By comparing the iPBS profile to the control, polymorphisms are found, either in the form of a new band that is absent in the control or a band that is absent in the control. Each experimental group's mean was computed, and changes in each group's means relative to the control was calculated as a percentage. The formula 100 a/n was used to determine the mean polymorphism values for the CRED-iPBS investigation [22,23,25].

3. Results

3.1. iPBS Procedure

In this study, genetic and epigenetic effects of EMS mutagens at different times and doses were determined in wheat plants using iPBS and CRED-iPBS methods (Figure 1). Using the iPBS technique, ten selected primers generated sufficient polymorphism as well as specific and stable band profiles. According to the data obtained, significant changes were determined in wheat at different durations and concentrations of EMS mutagen (Table 2).

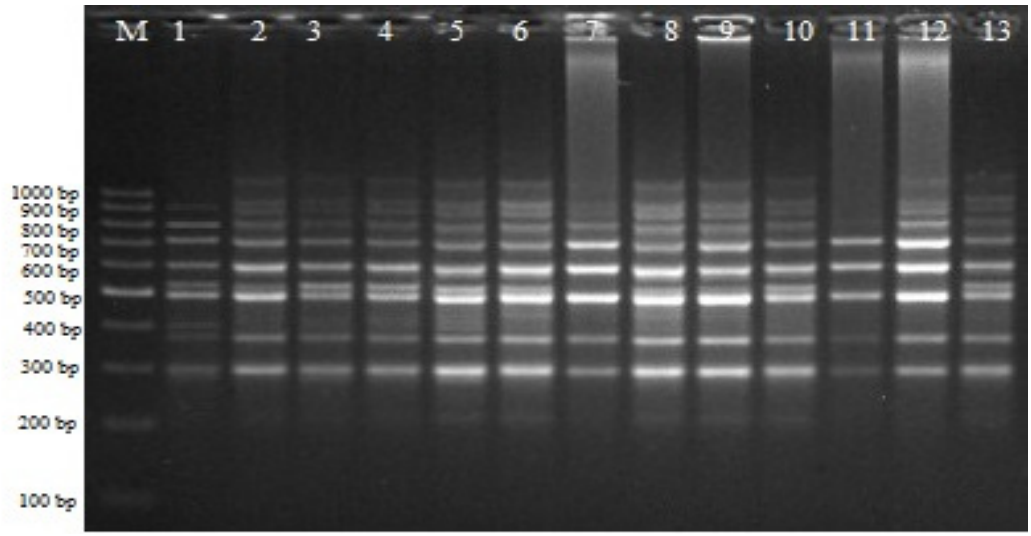


Figure 1. iPBS profiles for various EMS experimental groups with 2075 primers. 1: M 100–1.000 bp DNA ladder; 2: control; 3: 3 h + 0.1% EMS treatment; 4: 3 h + 0.2% EMS treatment; 5: 3 h + 0.3% EMS treatment; 6: 3 h + 0.4% EMS treatment; 7: 6 h + 0.1% EMS treatment; 8: 6 h + 0.2% EMS treatment; 9: 6 h + 0.3% EMS treatment; 10: 6 h + 0.4% EMS treatment; 11: 9 h + 0.1% EMS treatment; 12: 9 h + 0.2% EMS treatment; 13: 9 h + 0.3% EMS treatment; and 14: 9 h + 0.4% EMS treatment.

The total number of polymorphic bands in control iPBS group was 85. iPBS-2079 and iPBS-2270 (12 numbers) generated the most bands, whereas iPBS-2217 (3 numbers) generated the least. On the molecular scale, the widths of polymorphic bands ranged from 200 (iPBS-2075) to 1514 (iPBS-2377) base pairs. The results of the EMS mutagenesis experiment revealed that the iPBS profiles generated by administering the same treatment at different concentrations and at different times exhibited significant differences. Bands reappeared (+) or disappeared (-) to reflect these variations. In the non-experimental groups, 190 new bands formed and 93 old bands departed, as compared to the control group (Table 2).

In addition, the number of polymorphic bands at EMS application on different durations and concentrations varied between 16 and 22 numbers at 3 h, 16 and 31 numbers at 6 h, and 22 and 27 numbers at 9 h, respectively (Figure 2A). Furthermore, polymorphism rates varied depending on EMS via different concentrations and durations. The highest polymorphism rate was 36.5% at 0.1% and 32.5% at 0.3% in the 6 h treatment (Table 2). The third highest polymorphism rate was 31.8% at 0.3% dose in the 9 h treatment (Figure 2B). In addition, genomic stability (GTS) percentages, which qualitatively determine the iPBS profiles of the genotypes, were calculated (Table 2). The highest GTS value was 81.2% in 3 h and 6 h treatments (0.3 and 0.2 concentrations, respectively). The lowest GTS value was 63.5% at 0.1% concentration in the 6 h treatment (Figure 3C).

Table 2. Molecular sizes (bp) of present/absent bands in iPBS profiles after application of different EMS and different concentrations of wheat genotypes during seedling growth stage.

Primer	±	Control *	Experimental Groups											
			3 h				6 h				9 h			
			0.1%	0.2%	0.3%	0.4%	0.1%	0.2%	0.3%	0.4%	0.1%	0.2%	0.3%	0.4%
iPBS 2075	+	9	1050; 939	1062; 976; 213	1062; 939	1050; 951; 274; 210	1037; 926; 274; 210	1037; 951	1037; 939; 348; 261; 203	1037; 939; 210	1050; 265; 200	-	1050; 951	1062; 964
	-		400	-	-	-	-	531	-	-	400	900; 531; 400	531; 400	400
iPBS 2079	+	12	528	521	514	360	386; 366	-	-	948; 392	-	379	379	379
	-		-	600	748	-	-	748; 492	748	-	826; 492	-	492	600
iPBS 2080	+	10	1250	-	1300	-	785	-	1275	-	-	-	-	1250
	-		-	-	-	-	306	-	306	-	-	587; 306	-	-
iPBS 2217	+	3	845; 576	622	727	-	1442; 786	-	1028; 835; 676	622	615	1285; 940; 757; 551	1157; 1000; 800; 630; 576	1042; 630; 576
	-		443; 337	443; 337	443	475; 443	475; 443; 337	443	443	443; 337	475; 443; 337	443; 337	443; 337	443; 337
iPBS 2270	+	12	-	-	-	-	-	-	-	-	-	-	-	-
	-		1080	1080; 883	1080; 883	1160; 883	1300; 1080; 883	883	1300; 1080; 883	1300; 1080; 883	1300; 1080; 883	1300; 1080; 883	978; 810	1300; 1080; 768
iPBS 2274	+	8	-	549	-	827; 513	827; 549; 513; 486	827; 557; 527	-	549	445	-	-	513
	-		-	-	787	-	-	-	573	-	670; 573	-	476	-
iPBS 2377	+	7	1442; 1371; 1228; 900; 813; 747; 393	440; 365	1228; 856; 400; 365	1514; 1414; 1228; 856; 800; 723; 476	1228; 856; 735; 420; 365	-	1457; 1314; 1228; 856; 476; 400; 359	1228; 365	1214; 426	1228; 735; 433	1428; 1228; 786; 420	885; 760; 386
	-		-	-	-	-	-	985	-	-	-	985	-	-

Table 2. *Cont.*

Primer	±	Control *	Experimental Groups											
			3 h				6 h				9 h			
			0.1%	**0.2%**	**0.3%**	**0.4%**	**0.1%**	**0.2%**	**0.3%**	**0.4%**	**0.1%**	**0.2%**	**0.3%**	**0.4%**
iPBS 2380	+	6	886; 758; 534; 475	768; 719; 670; 516; 460	-	778	758	-	768; 516	768; 525	525	-	543	1100; 534; 483
	-		-	-	-	-	-	350	-	-	-	-	-	-
iPBS 2381	+	9	-	-	816	831	1118; 719	1118	1118	-	761	1127	1118; 700	1136; 700
	-		-	-	-	441	484	-	-	1009; 934; 622; 538; 484	-	-	441	-
iPBS 2382	+	9	871	871	1114	856	1114; 871	885	1100; 871	827	871	885; 842	1071; 871; 492	1042; 871
	-		-	-	-	-	-	1028; 638;	-	-	1028	476	-	-

+, -, and *; appearance of a new band, disappearance of a normal band, and EMS mutagen, respectively.

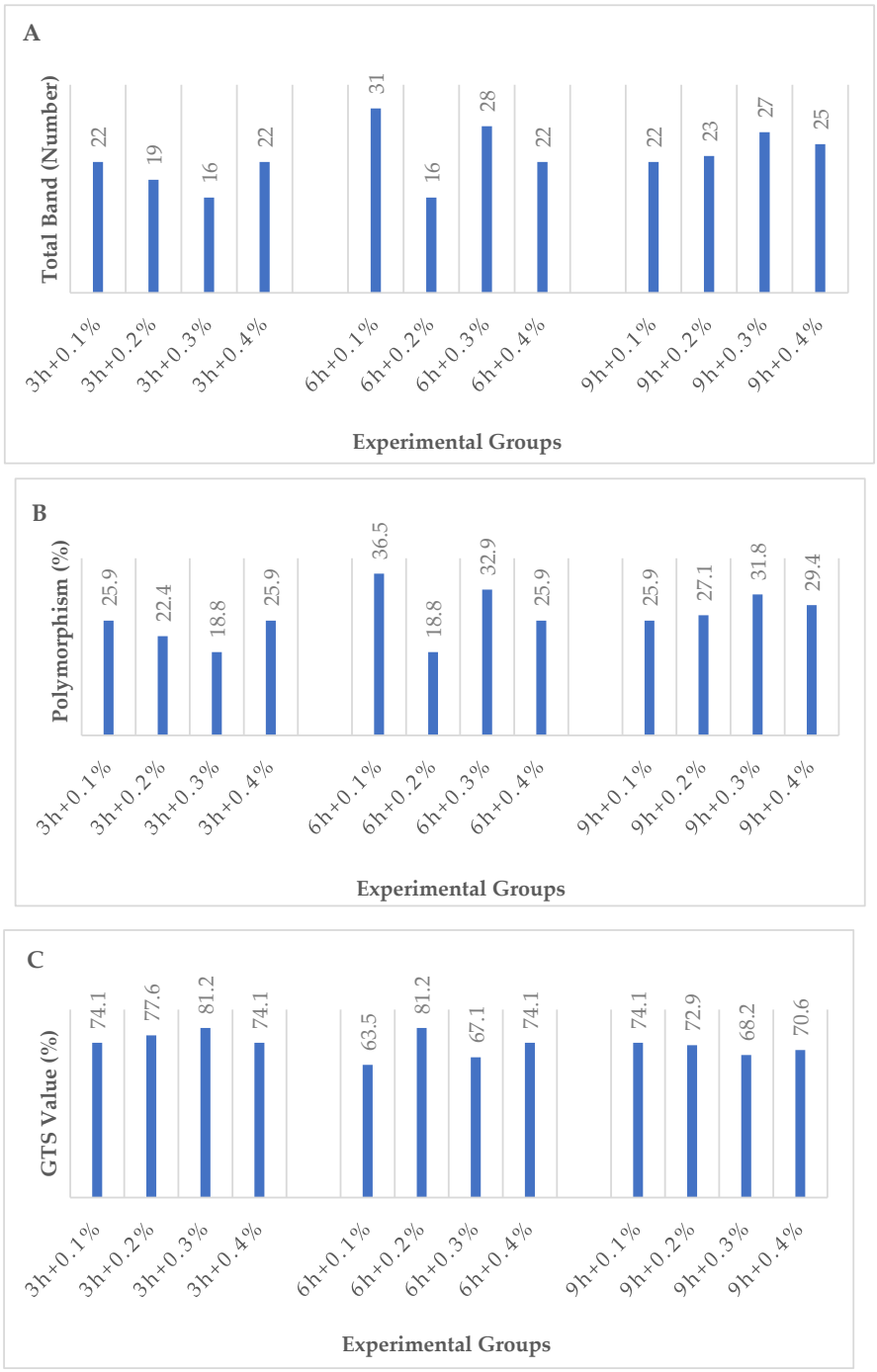


Figure 2. DNA methylation changes in the wheat exposed to EMS. (**A**) Total band, (**B**) polymorphism, (**C**) GTS value as estimated using different NaN_3 experimental groups.

3.2. CRED-iPBS Procedure

CRED-iPBS analysis was used to determine the effect of different durations and concentrations of EMS mutagen on methylation rates in wheat (Figure 3). DNA hypermethylation/hypomethylation depends on EMS duration and concentration treatments compared to a control group. Polymorphism rates were determined in CRED-iPBS assays digested with MspI and HpaII enzymes (Table 3). The rate of polymorphism due to the HpaII enzyme was higher than MspI. Hypermethylation values were determined as 19.6% (0.1% for 3 h), 17.4% (0.3% for 3 h and 0.2% for 6 h), and 16.3% (0.3% for 6 h and 0.2% for 9 h), respectively. Hypomethylation rates were 17.9%, 15.5%, and 20.2%, respectively (Table 3).

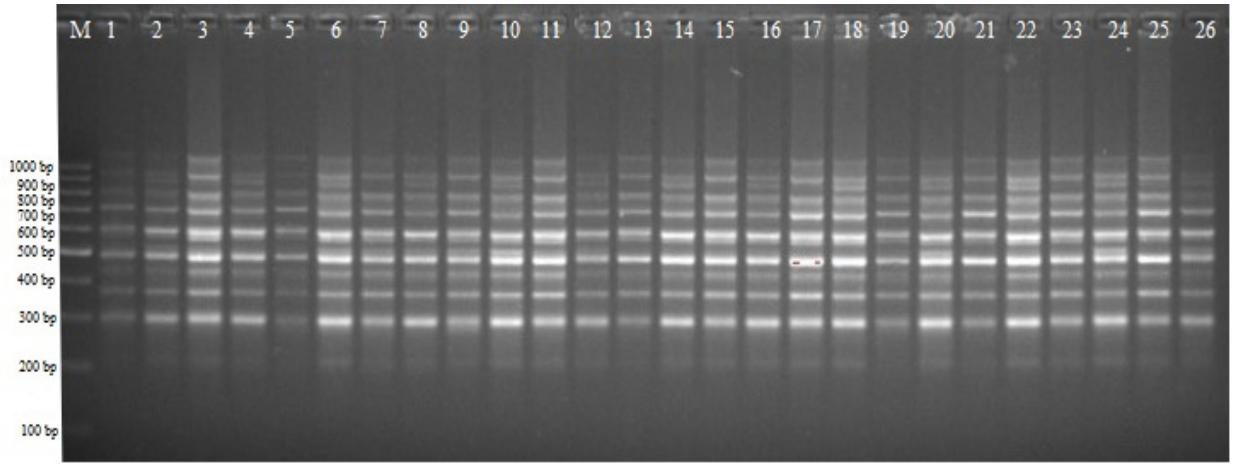


Figure 3. CRED-iPBS profiles for various experimental groups with iPBS 2075 primer. 1: M, 100–1.000 bp DNA ladder; 2: control Hpa II; 3: control Msp I; 4: 3 h + 0.1% EMS treatment Hpa II; 5: 3 h + 0.1% EMS treatment Msp I; 6: 3 h + 0.2% EMS treatment Hpa II; 7: 3 h + 0.2% EMS treatment Msp I; 8: 3 h + 0.3% EMS treatment Hpa II; 9: 3 h + 0.3% EMS treatment Msp I; 10: 3 h + 0.4% EMS treatment Hpa II; 11: 3 h + 0.4% EMS treatment Msp I; 12: 6 h + 0.1% EMS treatment Hpa II; 13: 6 h + 0.1% EMS treatment Msp I; 14: 6 h + 0.2% EMS treatment Hpa II; 15: 6 h + 0.2% EMS treatment Msp I; 16: 6 h + 0.3% EMS treatment Hpa II; 17: 6 h + 0.3% EMS treatment Msp I; 18: 6 h + 0.4% EMS treatment Hpa II; 19: 6 h + 0.4% EMS treatment Msp I; 20: 9 h + 0.1% EMS treatment Hpa II; 21: 9 h + 0.1% EMS treatment Msp I; 22: 9 h + 0.2% EMS treatment Hpa II; 23: 9 h + 0.2% EMS treatment Msp I; 24: 9 h + 0.3% EMS treatment Hpa II; 25: 9 h + 0.3% EMS treatment Msp I; 26: 9 h + 0.4% EMS treatment Hpa II; 27: 9 h + 0.4% EMS treatment Msp I.

The CRED-iPBS analysis revealed that the 3 h + 0.1% EMS treatment produced the highest MspI polymorphism value (19.6%), while the 9 h + 0.1% EMS treatment produced the lowest value (10.9%). In the experimental groups, 83 new bands appeared and 84 old bands disappeared for MspI, compared to the control group. The 6 h + 0.4% and 9 h + 0.4% EMS treatments yielded the highest polymorphism value for HpaII (23.8%), whereas the 6 h + 0.2% EMS treatment yielded the lowest value (15.5%). When looking at HpaII, the experimental groups showed a total of 140 additional bands compared to the control group, whereas 87 of the previously detected bands vanished (Table 3 and Figure 4).

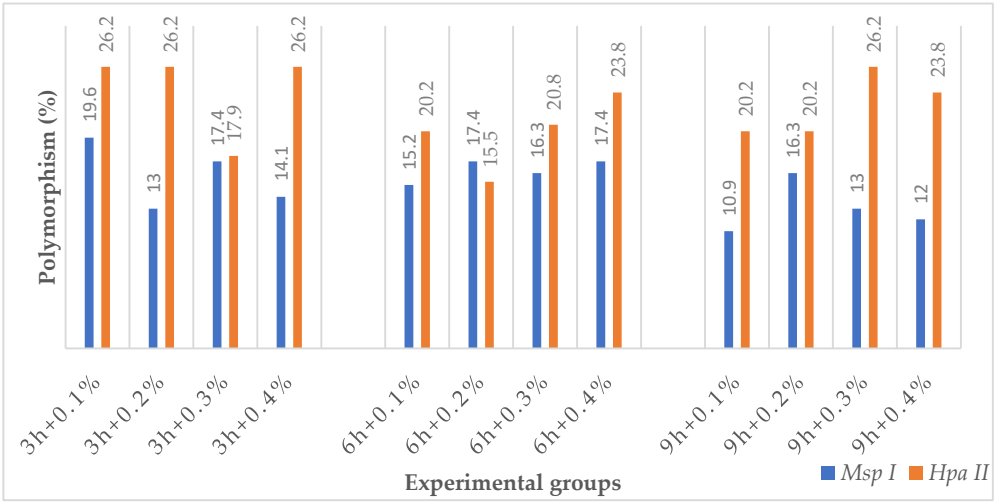


Figure 4. The effect of EMS on polymorphism percentage in different experimental groups of wheat in seedling growth stage.

Table 3. Band molecular size and polymorphism frequency according to CRED-iPBS analysis.

Primer	M/H [1]	± [2]	Control [3]	Experimental Groups											
				3 h				6 h				9 h			
				0.1%	0.2%	0.3%	0.4%	0.1%	0.2%	0.3%	0.4%	0.1%	0.2%	0.3%	0.4%
iPBS 2075	M	+	13	-	-	664	-	-	-	-	-	-	-	-	-
		-		507	-	865; 507; 210	-	865; 564; 507	507	507	507	-	507	-	564; 210
	H	+	10	213	-	477; 206	463; 203	206	-	203	200	-	-	206	815; 210
		-		-	-	-	-	-	-	-	-	-	618	-	-
iPBS 2079	M	+	12	932	-	-	-	-	-	-	-	-	-	-	-
		-		-	484; 392	392	-	484	-	800; 542; 484; 392	748; 453; 392	800; 453; 392	748; 392	748; 542; 453; 392	748; 600; 392
	H	+	11	-	800	-	-	-	-	366	-	-	-	-	-
		-		724; 492; 379	-	724; 379	724	379	724	724	724; 379	379	724; 379	724	724; 453; 379
iPBS 2080	M	+	13	1233	1200	1216	-	1233	1233; 1150	1316; 608	-	-	-	-	-
		-		288	-	-	-	-	272	-	272	-	272	868; 272	272
	H	+	11	868	868; 669; 500	868; 490	473	836	1233; 852; 683; 481	509	481	852	738; 669; 500	1083; 509	490
		-		-	300	-	-	-	-	300	-	-	-	283	-
iPBS 2217	M	+	1	1416; 1200; 1016; 750; 620	724	289	476; 372	400; 322	1400; 982	414	311	-	1050; 872	-	-
		-		-	278	278	278	-	278	-	-	-	278	-	-
	H	+	1	1250; 1050; 847; 407	555; 328	609	823; 581; 515; 379	484;300	620	600	1000; 400	823	590	982; 787; 468	1416; 1300; 492; 407
		-		273	273	-	273	273	273	273	273	-	-	273	-
iPBS 2270	M	+	10	-	943	943	943	1000; 855	1385	1014; 870	1385	943	1414; 957	1428; 971	985
		-		-	-	-	328	-	-	-	-	-	-	-	-
	H	+	9	1242	1242; 1100	-	1242; 1085	1214; 800	1200; 1071	1214; 1085	1214; 1085; 943	1228	1228; 1100; 870	1271; 1128	1271
		-		-	-	-	-		-	-		-	-	-	-
iPBS 2274	M	+	8	727	727	-	-	-	-	-	-	-	619	600	-
		-		-	-	438	-	438	438	438	-	-	-	-	-
	H	+	9	-	-	-	-	-	-	-	-	-	-	-	-
		-		451	727; 451	451	451	451	451	727; 451	-	727	451	451	660
iPBS 2377	M	+	9	1775; 1650; 1525; 576; 455; 415; 352	472; 415	447; 384	564; 439	407	463; 415	447; 400	400	463	423	439; 415	-
		-		-	-	-	1375; 921; 833; 766;	-	833	-	833; 766	-	600	-	833; 600
	H	+	6	1375; 1150; 946; 842; 778	1375; 1150; 946; 833; 776; 500; 376	1125; 946; 825	1325; 1125; 921; 833; 753; 336	1175; 921; 842; 789; 740; 368	1175	1375; 1150; 921; 842; 766; 509	1400; 1200; 500	1375; 1175; 946; 866; 740	1400; 1200	1400; 1200; 1000; 866; 789	1450; 1250
		-		-	-	-	-	-	-	-	-	-	-	463; 423	463

Table 3. *Cont.*

Primer	M/H [1]	± [2]	Control [3]	Experimental Groups											
				3 h				6 h				9 h			
				0.1%	0.2%	0.3%	0.4%	0.1%	0.2%	0.3%	0.4%	0.1%	0.2%	0.3%	0.4%
iPBS 2380	M	+	7	419	-	700; 632	700; 659	-	641	-	516	549	-	-	668
		-		-	964; 351	-	-	-	-	-	-	-	461	-	-
	H	+	7	600	-	641	-	650	659	-	641	-	-	-	-
		-		-	440	-	616; 440	-	-	-	-	440	-	-	440
iPBS 2381	M	+	11	-	-	-	-	-	-	-	-	-	-	-	-
		-		-	247	242	-	788;334	529; 438	-	1142; 378	334	-	-	-
	H	+	12	-	-	969	-	-	-	-	-	-	-	-	-
		-		392	-	-	-	-	-	392; 334	454; 392; 334	1128; 810; 392;	810; 334	1128; 810	888
iPBS 2382	M	+	8	-	-	-	-	1116	479	-	-	-	-	-	-
		-		-	-	948	-	-	-	1050; 871	1050; 871; 643	871; 643	1050; 871	643	1050
	H	+	8	-	-	-	-	-	-	493	580	-	-	-	-
		-		1116; 858; 631	858; 631	1116; 858	777; 631	858	1116	-	1116; 858	1116; 858; 631	1116; 858	858	965; 777; 631

[1, 2, 3]: M—Msp I, H—Hpa II; (+) appearance of a new band, (-) disappearance of a normal band and without EMS mutagen, respectively.

4. Discussion

Mutation breeding has become a relatively faster and effective breeding method for crop improvement [28]. Hundreds of beneficial mutants for various plant characters have been generated by treatment with physical and chemical mutagens in various crops, including wheat [5]. The basic principle of mutation breeding can be expressed as the determination of genotypes suitable for expectations among the positive and negative variations that may occur in plants at different mutagen doses [29]. Mutations cause changes in plant genome, chromosome, and gene sequences. Therefore, determination of mutations will be useful in determining the mutation-related genes. As a result of studies using mutation techniques, high mutation frequencies and low physiological damages are expected [30].

Mutagenesis using EMS is a very effective method to create variation in plants [31]. EMS is a widely used, potent, and very effective chemical mutagen, and its use is highly recommended due to its ease of application and the ability to monitor the results of mutations. Furthermore, it is one of the preferred mutagens because it causes a high frequency of mutations at random sites, and the frequency of chromosomal abnormalities is low [32]. Additionally, EMS usually causes GC ↔ AT transition-type mutations [33]. With mutation breeding, the exposure of plant DNA to mutagens can add genetic variations to a plant's genome, increasing genetic diversity within a population.

We evaluated the effects of EMS application on polymorphisms, DNA damage, and genomic instability in this work. The iPBS technique was used for investigating DNA damage caused by EMS. Our results evidently showed the effects of EMS mutagens, such as the disappearance of regular bands and the appearance of new bands, as well as explained the significant genetic diversity seen in EMS-treated seed during germination at different times and at different concentrations. According to the results, control and EMS experimental groups indicated significant differences in iPBS profile. For EMS treatment, a total of 93 normal iPBS bands disappeared and 190 new bands appeared, as compared to control. In reality, the appearance of new bands may be associated with mutation, while the loss of bands may be attributed to DNA damage, DNA methylation or chromosomal damage, all of which contribute to the production of DNA polymorphism. As the GTS is a quantitative value, the calculated changes in iPBS patterns decreased with a rise in concentration, based on the manner of EMS mutagen. The lowest GTS value (63.50%) was observed in 6 h + 0.1% EMS treatment, while the highest GTS value (81.20%) was observed in 3 h + 0.3% EMS treatment. The highest polymorphism rate was 36.5% at 0.1% and 32.5% at 0.3% in the 6 h treatment. The third highest polymorphism rate was 31.8% at 0.3% dose in the 9 h treatment. According to our results, we should point out that the variously acquired polymorphic bands in iPBS profiles and the decrease in GTS value indicated that EMS had genotoxic effects on wheat genomes. Indeed, we have recently discovered that chemical mutagens such as NaN3 induces genetic harm to wheat genomes [34]. These alterations might have been caused by point mutations, chromosomal abnormalities, or mitotic mistakes. Türkolu et al. [14] investigated the effects of ethyl-methane sulfonate on polymorphism and genomic instability in wheat under in vitro conditions. Their results showed that EMS affected the polymorphism; the genomic instability of the mature wheat embryo was more evident; and the iPBS marker could be utilized to determine this.

Epigenetics, on the other hand, refers to changes in gene expression that are not caused by changes in the underlying DNA sequence. These changes can be caused by a variety of factors, including environmental factors such as temperature, light, and nutrition, as well as developmental cues. Epigenetic changes can have a significant impact on plant growth, development, and response to stress, and are increasingly being studied in the context of plant breeding. For example, researchers are exploring ways to use epigenetic modifications to improve crop yield and quality traits [35]. In some cases, mutation breeding and epigenetic studies can be combined to create new and improved crop varieties. Researchers can use mutagenic agents to induce mutations in specific genes or regulatory regions of the genome known to be involved in epigenetic regulation. This

could lead to the development of crop varieties with altered epigenetic profiles, which could confer desirable traits such as an increased drought tolerance or disease resistance.

In reaction to chemical mutagens, DNA demethylation is also predicted to activate specific genes or restore the epigenetic mood of the genome. There have been a few studies that reveal varying DNA methylation statuses in wheat when exposed to different chemical mutagens. In this study, we used the CRED-iPBS assay. This approach was provided as the average percentage of polymorphism induced by DNA methylation for each of the EMS experimental groups. Our investigation found that following EMS treatment, alterations in DNA methylation generally increased dose-dependently. The CRED-iPBS test findings were provided as the average polymorphism of % DNA methylation at each experimental group. According to the CRED-iPBS study, the 3 h + 0.1% EMS treatment generated the highest *MspI* polymorphism value at 19.6%, whereas the 9 h + 0.1% EMS treatment produced the lowest value at 10.9%. In addition, the 6 h + 0.4% and 9 h + 0.4% EMS treatments produced the greatest polymorphism value for *HpaII* at 23.8%, whereas the 6 h + 0.2% EMS treatment produced the lowest value at 15.5%. It was also revealed that cytosine methylation changes in response to the different dosages and timings of EMS mutagens. Chemical mutagens can also alter DNA methylation and histone alterations, resulting in epigenetic silencing or the stimulation of gene expression. In the current study, our findings showed that EMS affected the iPBS band profile and cytosine methylation and that the iPBS marker was able to identify these changes along with the hyper/hypomethylations that occurred at higher concentrations. Our data showed that the EMS treatment might generate naturally occurring hypermethylation in each treatment group. DNA methylation is the primary cause of abnormal PCR banding or the appearance of new PCR bands. Similar results to the changes in epigenetic profile as a result of EMS applications have also been reported by Karaca et al. [14]. EMS is a alkylating agent that may produce base alterations, DNA backbone breaking, and mispairing due to the chemical modification of nucleotides [35]. Till et al. [36] and Maluszynski et al. [37] found that EMS acted as an alkylating agent by inducing GC>AT transitions. DNA damage is caused by EMS because it adds a methyl (-CH3) group to the oxygen and nitrogen of nucleotide bases, which is a reactive chemical. Lesions may be acquired throughout a wide range, from those with no biological consequence to those that cause cell death. As O6-meG mispairs with T, a G/C to A/T transition occurs, following DNA replication [38], and methylation at the O6 position of guanine is demonstrated to have the highest mutagenic property. When guanine on the non-transcribed strand of DNA is alkylated, it mispairs with thymine and is subsequently replaced by adenine (G>A transition) during replication. The C>T transition occurs when guanine on the transcribed strand is alkylated. According to the findings, the tested EMS at various concentrations and durations had an effect on methylation status. This condition manifested as hypermethylation. Significant data have recently been given in the literature demonstrating that stress may cause an increase or decrease in cytosine methylation across the genome. Similar findings have been reported in previous investigations, which established that chemical mutagens may change the methylation state of DNA [34].

5. Conclusions

The use of mutations in plant breeding has long been a research area of interest and induced mutagenesis is a widely used method to increase the frequency of mutations. Chemical and physical mutagens are used to increase the occurrence of mutations in plants because spontaneous mutations occur at low rates. Mutagen selection, concentration, and duration of application are factors that need to be considered when conducting mutagenesis studies. The duration and concentration of mutagen application play a crucial role in chemical mutagenesis. Higher doses of mutagens can cause more seedling damage and death, making it important to determine the optimum value for the selected mutagen. DNA methylation plays a vital role in the regulation of gene expression and differentiation of

cells and organisms. The use of iPBS polymorphisms can help identify genetic variation among wheat regenerates, which can be used to develop new varieties.

Current research focuses on the use of iPBS markers to analyze EMS-induced polymorphism in wheat at the seedling growth stage. Chemical mutagen stimulation might influence epigenetic processes such as DNA methylation. Epigenetic factors were found to be partly responsible for morphological and physiological differences in plants. The study revealed that EMS treatments have an impact on cytosine methylation level and genomic stability in wheat plants. The use of methylation pattern diversity, following recovery and sequencing, may help to identify genomic domains involved in the response to EMS treatments. In conclusion, induced mutagenesis is a useful tool to generate genetic variation in plant populations. Understanding the role of DNA methylation in plant development can help determine the effects of epigenetic factors on cell differentiation and the occurrence of diseases.

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