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Mammalian Sex Hormones as Steroid-Structured Compounds in Wheat Seedling: Template of the Cytosine Methylation Alteration and Retrotransposon Polymorphisms with iPBS and CRED-iBPS Techniques

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Abstract: Phytohormones are chemical compounds found naturally in plants that have a significant effect on their growth and development. The increase in research on the occurrence of mammalian sex hormones (MSHs) in plants has prompted the need to investigate the functions performed by these hormones in plant biology. In the present study, we investigated the effects of MSHs on DNA damage and DNA methylation of wheat (*Triticum aestivum* L.) during the seedling growth stage, using the CRED-iPBS (coupled restriction enzyme digestion/inter primer binding site) assay and iPBS analysis to determine DNA methylation status. Exogenous treatment with four MSHs (17-β-estradiol, estrogen, progesterone, and testosterone) was carried out at four different concentrations (0, 0.05, 0.5, and 5 µM). The highest genomic template stability (GTS) value (80%) was observed for 5 µM 17-β-estradiol, 0.5 µM testosterone, and 0.05 µM estrogen, while the lowest value (70.7%) was observed for 5 µM progesterone and 0.5 µM estrogen. The results of the CRED-iPBS analysis conducted on *Msp*I indicate that the 0.05 µM estrogen-treated group had the highest polymorphism value of 40%, while the 5 µM progesterone-treated group had the lowest value of 20%. For *Hpa*II, treatment with 0.5 µM 17-β-estradiol had the highest polymorphism value of 33.3%, while the group treated with 0.05 µM 17-β-estradiol and 0.05 µM progesterone had the lowest value of 19.4%. In conclusion, MSH treatments altered the stability of the genomic template of wheat plants and affected the cytosine methylation status at the seedling growth stage. Upon comprehensive examination of the results, it was seen that the employed methodology successfully detected alterations in cytosine methylation of genomic DNA (gDNA), as well as changes in the pattern of genomic instability.

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1. Introduction

Phytohormones, also known as plant hormones, are chemical compounds that can occur naturally in plants. These compounds have the ability to influence physiological processes even at extremely low concentrations and are responsible for coordinating activities that occur inside plant cells [1]. Phytohormones, including auxin, cytokinin, abscisic acid, salicylates, jasmonates, and gibberellin, have a significant impact on numerous phases of plant growth and development, from germination and rooting to shoot formation and flowering [2]. Steroid molecules play an important role in plant biological processes, as do phytohormones. It has been hypothesized that these structures, which are produced in plants as phytosterols, play a role in defense and signal transmission processes in plants [3].

A group of steroids known as mammalian sex hormones (MSHs) play a key role in regulating mammalian development and reproduction [4]. The ovaries of female animals and the adrenal glands of female mammals produce steroid hormones [5]. The processes of development and reproduction in organisms are regulated by these molecules, which are lipophilic and of low molecular weight. These molecules are also key substances that regulate metabolic processes involving minerals and proteins in mammals [4]. These hormones were once thought to be found only in animals; however, at the turn of the 20th century, their presence in plants was discovered [6]. As a result of advances in technology, a significant number of researchers have published results indicating that MSHs are naturally present in plants, and that their concentrations vary depending on the plant species, tissue, and stage of development [7,8].

The number of studies on the existence of MSHs in plants has increased, prompting the need to investigate the role these hormones play in plant life [9]. Recent studies have focused on evaluating the effects of exogenous application of these compounds on plant growth and development, as well as their tolerance to various abiotic and biotic stimuli and their metabolic mechanism of action [10,11].

MSHs administered exogenously affect plant development at several stages, from germination to blooming [8,9]. The effects of exogenously applied MSHs on morphologic variables such as shoot and root length, as well as biochemical components such as the activity of various proteins, nucleic acids, sugars, enzymes, and chlorophyll content, have been the subject of many studies. These researchers have shown that, even at low concentrations, MSHs significantly stimulate plant growth and development [10]. Their physiological activity, genomic DNA instability, and epigenetic effects on plants have not yet been completely described, despite the fact that the amounts, receptors, and individual binding sites of MSHs in plants have been partially revealed [12]. Mammalian sex hormones can induce molecular changes by affecting genome stability and epigenetic mechanisms [13].

Epigenetic modifications are a powerful mechanism for modulating gene expression without changing the DNA sequence. The changes involved can be characterized as modifications including, but not limited to, histone modifications such as ubiquitination, phosphorylation, and acetylation reactions, chromatin remodeling, and DNA methylation [14,15]. Modifications to the plant epigenome are necessary for them to successfully adapt to environment conditions [16]. It has been shown that plants are able to maintain their survival cycle by exploiting changes in response to conditions in their environment [17]. In particular, there is evidence of substantial connections between hormones produced by plants and epigenetic signals produced by the plants [13,18]. The study of epigenetic changes can be approached from many different angles. To detect changes in DNA, one of these methods involves the use of PCR (polymerase chain reaction) in combination with cytosine methylation-sensitive enzymes [19]. This method is used to identify methylation changes. The use of cytosine methylation-sensitive enzymes enables high-throughput studies of this epigenetic regulatory mechanism. In addition, the technique is simple and

cost-efficient [20,21]. Restriction enzyme-coupled digestion and inter-primer binding site (iPBS) methodology, also known as CRED-iPBS, is a method that is both efficient and cost-effective for studying the methylation status of plant DNA [13,22–24]. iPBS retrotransposons constitute a marker system that relies on the presence of tRNA as a binding site for reverse transcriptase primers. This system is implemented using the PCR technique [13]. After DNA restriction digestion with methylation-sensitive enzymes such as *Hpa*II and *Msp*I, the CRED-iPBS method is used to identify methylation changes that occur in different tissues or between different development stages [22–24]. Turkoglu [25] conducted a study of the DNA damage and DNA methylation of MSHs that were externally applied to the medium after callus formation on wheat grown in vitro. In contrast to the aforementioned studies, our experiment consisted of treating MSHs with different doses and observing their effects on the growth stage of seedling. This study was designed to investigate the effects of MSH (17-β-estradiol, estrogen, testosterone, and progesterone) on DNA damage and DNA methylation of wheat plants during the seedling growth stage. In addition, the CRED-iPBS method was used in combination with iPBS markers to determine the DNA methylation status of wheat plants.

2. Materials and Methods

2.1. Plant Material, Seed Germination, and Treatments

The plant material was wheat (*Triticum aestivum* L.) variety Kırik ($2n = 42$). The plant material used in this study was procured from the Agricultural Department of Field Crops at Atatürk University. The wheat seeds were subjected to a preliminary washing process using tap water to eliminate any extraneous contaminants such as dust, soil, and chemical residue. This was followed by a two-step surface sterilization procedure. At the initial stage, the seeds were subjected to immersion in 70% ethanol (EtOH) for 3 min, followed by three rinses in sterile pure water in a sterile chamber. In the second stage, the seeds were surface-sterilized by a 15-min treatment with a 10% sodium hypochlorite solution containing a small amount (0.01%) of Tween-20 (Sigma–Aldrich, St. Louis, MO, USA). After surface sterilization, the seeds were divided into four distinct groups and treated with different doses of MSHz, namely, 17-β-estradiol ($C_{18}H_{24}O_2$: 272.38 g mol^{-1}; Product Number: E2758; Sigma–Aldrich), estrogen ($C_{18}H_{22}O_2$: 270.37 g mol^{-1}; Product Number: E9750; Sigma–Aldrich), testosterone ($C_{19}H_{28}O_2$: 288.42 g mol^{-1}; Product Number: Y0002163; Sigma–Aldrich), and progesterone ($C_{21}H_{30}O_2$: 314.46 g mol^{-1}; Product Number: Y0001665; Sigma–Aldrich). The MSH groups were further divided into four distinct subgroups, each representing different concentrations. Seeds were treated with MSHs at various concentrations, including 0, 0.05, 0.5, and 5 µM. Two sterilized sheets of blotting paper in each Petri dish, and 14 mL of various prepared concentrations of MSHs were added. Then, a total of 20 wheat seeds were methodically placed at fixed intervals using forceps. After treatment, the seeds were exposed to a controlled temperature of 25 °C and a light/dark photoperiod of 16:8 h during the incubation period. After a 14-day germination period, the germinated plants were harvested for DNA isolation [13,25].

2.2. Isolation of Genomic DNA

DNA isolation was performed according to the methodology described by Zeinalzadehtabrizi et al. [26]. Following that, the DNA was kept at a temperature of −20 °C for further use. DNA genomic was assessed for concentration and purity using a Nanodrop (Qiagen Qiaxpert, Hilden, Germany) spectrophotometer and electrophoresis on a 0.8% agarose gel.

2.3. iPBS and CRED-iPBS PCR Assays

The study used a set of 10 iPBS primers to perform iPBS and CRED-iPBS analyses, which included a coupled restriction enzyme digestion and a binding site approach between the primers [27]. To perform iPBS analysis, a polymerase chain reaction (PCR) was performed using a total volume of 20 µL consisting of 10× PCR buffer, 10 mM dNTP mixture, 25 mM $MgCl_2$, ddH_2O, 10 pmol of random primer, 1 U of Taq DNA polymerase

(Thermo Fisher Scientific, Waltham, MA, USA), and 50 ng μL^{-1} DNA sample. The experiment was conducted using a PCR mixture consisting of a 10-fold PCR buffer. After the vertexing procedure, the tubes were transferred to a thermocycler apparatus (Senso-Quest Labcycler, Göttingen, Germany) to facilitate the amplification process. The initial PCR step included an initial denaturation phase, which was carried out at 95 °C for five minutes. A total of 40 cycles were then carried out, each consisting of denaturation for one minute at 95 °C, annealing for one minute (the optimal annealing temperature of the marker used), extension for two minutes at 72 °C, and final extension for ten minutes at 72 °C. To perform CRED-iPBS analysis, a quantity of 1000 ng of DNA sample from each treatment was individually digested at 37 °C for 2 h, using 1 µL of *Hpa*II and 1 µL of *Msp*I, according to the instructions provided by the manufacturer (Thermo Scientific, Waltham, USA). The digested DNA, which corresponded to each endonuclease, was used in the PCR mix as a surrogate for undigested gDNA. The amplification procedure was carried out using the primers shown in Table 1. The PCR procedural steps were analogous to those used in the previously mentioned iPBS study. The differentiation of iPBS and CRED-iPBS PCR products on the basis of their base size was achieved using the electrophoresis technique [11,13,19,22–25].

Table 1. Annealing (Ta) temperatures of primers used in iPBS PCR.

iPBS Primer Name	Sequence (5′–3′)	Tm * (°C)	CG ** (%)	Optimal Annealing Ta (°C)
2079	AGGTGGGCGCCA	56.6	75.0	65.2
2080	CAGACGGCGCCA	54.6	75.0	63.3
2217	ACTTGGATGTCGATACCA	52.5	44.4	51.4
2225	AGCATAGCTTTGATACCA	50.5	38.9	55.0
2277	GGCGATGATACCA	46.2	53.8	51.0
2231	ACTTGGATGCTGATACCA	52.9	44.4	52.0
2251	GAACAGGCGATGATACCA	54.3	50.0	53.2
2274	ATGGTGGGCGCCA	57.1	69.2	65.8
2298	AGAAGAGCTCTGATACCA	51.6	44.4	60.0
2402	TCTAAGCTCTTGATACCA	49.0	38.9	50.0

*, ** Tm: primer temperatures; CG: percentage of cytosine (C) and guanine (G) in the primary sequence, respectively.

2.4. iPBS and CRED iPBS Analysis

The iPBS and CRED-iPBS bands were both examined using the application To-talLab TL120 (Non-linear Dynamics Ltd. R brand). The genomic template stability percentage (GTS%) was calculated after band profiles using the following formula: $(1 - a/n) \times 100$, where a is the average number of polymorphic bands detected in each treated template and n is the total number of bands in the control. Polymorphisms are discovered by comparing the iPBS profile to the control, either in the form of a new band that is absent in the control or a band that is absent in the control. The means of each experimental group were calculated, and the percentage change in each group's means from the control was determined. The mean polymorphism values for the CRED-iPBS experiment were calculated using the method 100 a/n [23,24].

3. Results

3.1. iPBS Assays

The goal of the iPBS study was to determine the polymorphism effect that the simultaneous application of four MSHs at different doses would have on wheat gDNA. Only 10 of the iPBS markers out of a total of 20 were chosen for the iPBS studies. These primers were carefully selected on the basis of their ability to produce bright bands that could be easily scored (Figure 1). The experimental procedures consistently yielded stable band patterns across all samples (Table 1). The number of polymorphic bands that appeared for the primers used was assessed by comparing control plants with MSH-treated plants. The control revealed a total of 75 bands; the iPBS-2080 marker generated the highest number

of bands (14 bands), while the iPBS-2217 marker generated the lowest number of bands (1 band) (Figure 2A). The width of the polymorphic bands ranged from 206 (iPBS-2225) to 1375 (iPBS-2402) base pairs (bp). In addition, the 5 µM progesterone experimental group had the smallest molecular size (206 bp), while the 0.5 µM 17-β-estradiol experimental group had the largest molecular size (1375 bp). The MSH-treated experimental results revealed noteworthy changes in iPBS profiles after administration of different doses of various treatments. The aforementioned differences were observed in the form of reappearance of positive-labeled (+) or disappearance of negative-labeled (−) bands, as shown in Table 2. Compared to the control group, the experimental groups showed a net increase of 101 newly formed bands and a net decrease of 116 pre-existing bands.

Polymorphism rates ranged from 20% (5 µM 17-β-estradiol, 0.5 µM testosterone, and 0.05 µM estrogen) to 29.3% (5 µM progesterone and 0.5 µM estrogen) (Figure 2B). The percentage of genomic template stability (GTS) was used to measure changes in iPBS profiles. The highest GTS value (80%) was observed with treatment with 5 µM 17-β-estradiol, 0.5 µM testosterone, and 0.05 µM estrogen, while the lowest value (70.7%) was observed with treatment with 5 µM progesterone and 0.5 µM estrogen. The GTS value was lowest during treatment with 17-β-estradiol at 0.5 µM, during treatment with 5 µM progesterone, during treatment with 5 µM testosterone, and during treatment with 0.5 µM estrogen (Figure 2C). In the case of progesterone treatment, a decrease in GTS was observed with increasing concentration.

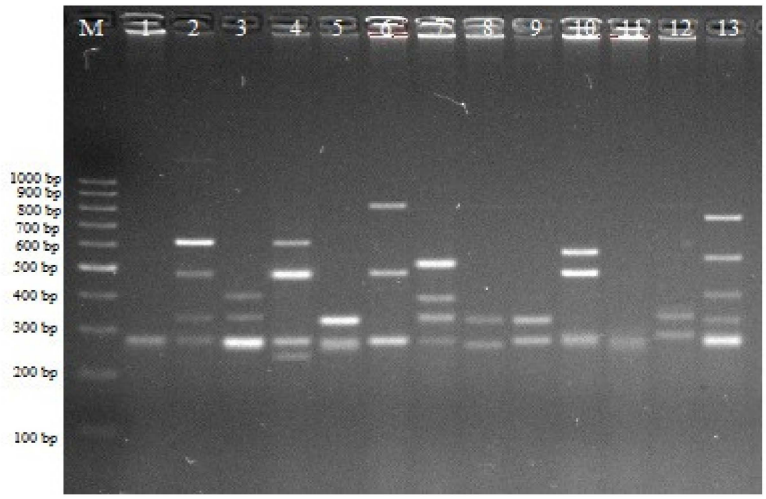

Figure 1. iPBS profiles for various MSH experimental groups with 2217 primers. M: 100–1000 bp DNA ladder; 1: control; 2: 0.05 µM 17-β-estradiol; 3: 0.5 µM 17-β-estradiol; 4: 5 µM 17-β-estradiol; 5: 0.05 µM progesterone; 6: 0.5 µM progesterone; 7: 5 µM progesterone; 8: 0.05 µM testosterone; 9: 0.5 µM testosterone; 10: 5 µM testosterone; 11: 0.05 µM estrogen; 12: 0.5 µM estrogen and 13: 5 µM estrogen.

Table 2. Molecular sizes (bp) of appearing/disappearing bands in iPBS profiles of MSH-treated wheat of varying concentration.

iPBS Primer	± [1]	Control *	Experimental Groups											
			17-β-Estradiol			Progesterone			Testosterone			Estrogen		
			0.05 µM	0.5 µM	5 µM	0.05 µM	0.5 µM	5 µM	0.05 µM	0.5 µM	5 µM	0.05 µM	0.5 µM	5 µM
2079	+	6	-	1155	853; 485	-	-	741	1142; 419	1128; 419	419	-	409	-
	−		-	-	-	-	-	-	-	-	-	-	-	612; 558; 372
2080	+	14	-	1175; 760; 655	-	671	-	-	1362; 1312	1325	987	-	648	-
	−		1000; 508; 346; 241; 400	-	508	241	508; 400; 346; 241	508; 400; 346	346	346	400; 346	508; 346; 241	1112; 1000; 900; 508	1112; 508; 241
2217	+	1	607; 473; 334	388; 334	548; 653	324	824; 493	542; 400; 344	334	339	571; 493	-	350	786; 563; 411; 334
	−		-	-	-	-	-	-	-	-	-	-	-	-
2225	+	5	-	-	385	-	469; 375; 212	206	-	700	-	-	671; 469	656
	−		-	-	-	-	-	-	-	527	527; 135	527	-	-
2231	+	4	-	1160; 1000; 884; 665	600; 530	642	-	-	-	-	-	-	1260	-
	−		315	372; 315	372	372	735; 372	735	372	735	735	735	735; 315	-
2251	+	5	-	-	485	492	-	-	-	-	-	-	-	477
	−		652	-	-	-	652	652	-	-	652	-	652	-
2277	+	12	1044	1044	400	-	1022	-	393	-	-	-	1044	1233
	−		548	708	-	774; 610; 418	610	1122; 708; 610; 548; 418	708	928; 548	708; 610	774; 708	708	-
2274	+	10	-	-	550	500	718	-	-	-	-	-	-	770
	−		1225; 1050; 900	1225	-	1225; 1050; 900	1225; 900	1225; 1050; 900	900	1050; 900	900	1225; 1050; 900	1225; 900	1050
2298	+	11	-	790	-	-	-	-	690	681	690; 531; 500	838; 700	700	848
	−		263	263	315	263	263	263	263	-	-	263	520; 315; 263	263
2402	+	7	-	1375; 1225; 482	524	1250; 518	1175	536; 482	1250; 546; 500	1275; 546	-	500	556	1250; 546; 500
	−		973	973	762	973	973	973	973	-	973	973	-	-

[1],* appearance of a new band (+), disappearance of a normal band (−), and without MSH, respectively.

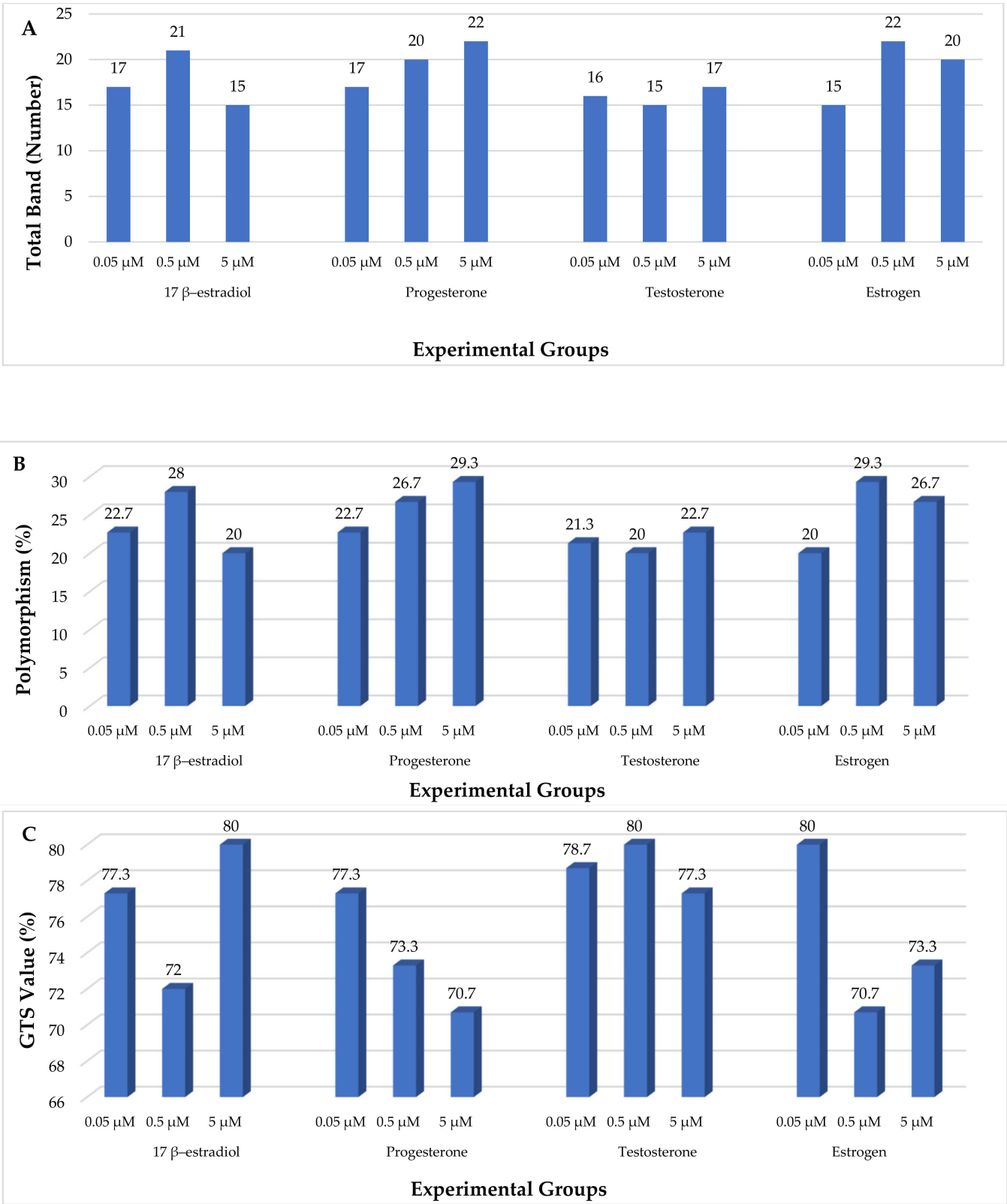

Figure 2. DNA methylation changes in the wheat exposed to MSH: (**A**) total band; (**B**) polymorphism; (**C**) GTS value as estimated using different MSH experimental groups.

3.2. CRED-iPBS Assays

The ten primers listed in Table 1 were used in CRED-iPBS analysis to detect cytosine gDNA methylation modifications. The results of the CRED-iPBS assay are indicated as the

percentage of polymorphism in CRED-iPBS assays that were digested by *Msp*I and *Hpa*II (Figure 3 and Table 3). Compared to the PCR production obtained from control DNA, the results show that whether DNA was hypermethylated or hypomethylated depended on the type and amount of MSH. The result clearly shows that the number of bands observed in the *Msp*I- and *Hpa*II-digested controls were 68 and 78 bands, respectively. The number of bands in the experimental groups digested with *Msp*I (266) was higher than in the experimental groups digested with *Hpa*II (263). The experimental groups for *Msp*I showed a net increase of 190 newly formed bands and a net decrease of 76 pre-existing bands compared to the control group. Compared to the control group for *Hpa*II, the experimental groups showed a net increase of 106 newly formed bands and a net decrease of 157 pre-existing bands.

The percentages of *Msp*I polymorphism showed a range of variation from 20% to 40%. The results of the CRED-iPBS analysis conducted on *Msp*I showed that the experimental group treated with 0.05 µM estrogen presented the highest polymorphism value of 40%, while the experimental group treated with 5 µM progesterone presented the lowest value of 20%. The results of the study indicate that there was a decrease in the polymorphism values with an increased in estrogen concentrations for *Msp*I, leading to a state of hypomethylation. In contrast, increasing doses of 17-β-estradiol, progesterone, and testosterone did not consistently decrease or increase in polymorphism level (%) in the plants.

The percentage of *Hpa*II polymorphism showed a variable range from 19.4% to 33.3%. According to the results of the CRED-iPBS analysis performed on *Hpa*II, it was observed that the experimental group treated with 0.5 µM 17-β-estradiol showed a maximum polymorphism value of 33.3%. In contrast, the experimental group treated with 0.05 µM 17-β-estradiol and 0.05 µM progesterone showed a minimum value of 19.4% (Figure 4). According to the average percentage of polymorphisms observed in *Hpa*II and *Msp*I digestion, it can be inferred that raised levels of MSHs (17-β-estradiol, progesterone, and estrogen) may confer a protective effect against hypermethylation.

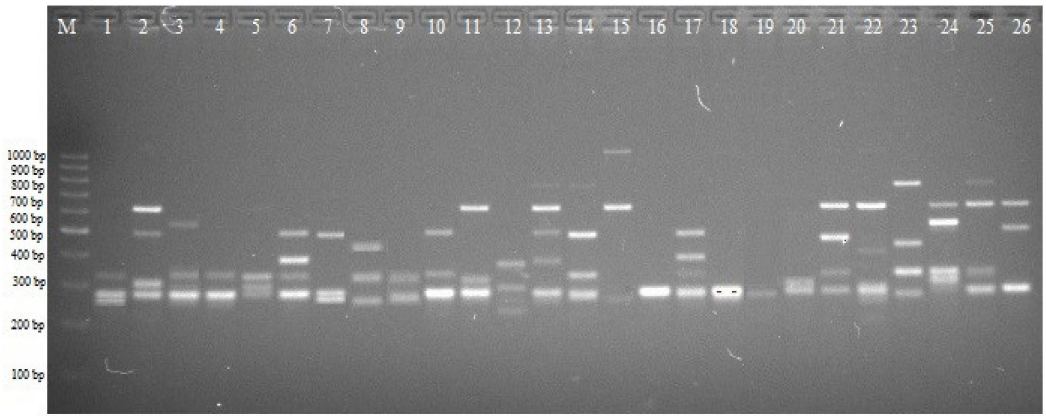

Figure 3. CRED-iPBS profiles for various MSH experimental groups with iPBS 2217 primers; 1: M, 100–1000 bp DNA ladder; 2: control *Hpa II*; 3: control *Msp I*; 4: 0.05 µM 17-β-estradiol *Hpa II*; 5: 0.05 µM 17-β-estradiol *Msp I*; 6: 0.5 µM 17-β-estradiol *Hpa II*; 7: 0.5 µM 17-β-estradiol *Msp I*; 8: 5 µM 17-β-estradiol *Hpa II*; 9: 5 µM 17-β-estradiol *Msp I*; 10: 0.05 µM progesterone *Hpa II*; 11: 0.05 µM progesterone *Msp I*; 12: 0.5 µM progesterone *Hpa II*; 13: 0.5 µM progesterone *Msp I*; 14: 5 µM progesterone *Hpa II*; 15: 5 µM progesterone *Msp I*; 16: 0.05 µM testosterone *Hpa II*;17: 0.05 µM testosterone *Msp I*; 18: 0.5 µM testosterone *Hpa II*; 19: 0.5 µM testosterone *Msp I*; 20: 5 µM testosterone *Hpa II*; 21: 5 µM testosterone *Msp I*; 22: 0.05 µM estrogen *Hpa II*; 23: 0.05 µM estrogen *Msp I*; 24: 0.5 µM estrogen *Hpa II*; 25: 0.5 µM estrogen *Msp I*; 26: 5 µM estrogen *Hpa II* and 5 µM estrogen *Msp I*.

Table 3. Results of CRED-iPBS analysis: band molecular size and proportion of polymorphism.

iPBS Primer	M/H [1]	± [2]	Control [3]	Experimental Groups											
				17-β-Estradiol			Progesterone			Testosterone			Estrogen		
				0.05 µM	0.5 µM	5 µM	0.05 µM	0.5 µM	5 µM	0.05 µM	0.5 µM	5 µM	0.05 µM	0.5 µM	5 µM
2079	M	+	6	-	-	-	-	-	-	-	-	-	-	-	-
		–		-	-	452	-	-	-	-	-	350	-	-	-
	H	+	6	-	-	360	-	-	-	415	422	532	-	-	-
		–		-	785	-	-	-	-	-	-	-	-	571	-
2080	M	+	11	810; 754	810	800	800	832; 742	810; 742	821; 754	821	820	821; 777	810	821; 777
		–		1360; 1140; 464	1140; 464	1140; 464	1140; 464	1360; 1140;	1360; 1140	1360	-	1360	1360; 1140	1360; 1140	1360
	H	+	10	405	203	520; 244	-	-	240	489; 410; 231	511; 236	-	425; 240	575	415
		–		-	1360; 810; 742	-	810	900; 373	900	-	-	810	900; 810;	-	-
2217	M	+	4	248	376	420	-	-	-	-	-	-	-	-	-
		–		610; 469	610	-	610	610; 469	-	610; 469; 308	610; 469; 308	610; 308	-	-	308
	H	+	3	522	-	456	-	620	769; 620; 484	1060; 620	477; 387	-	631; 469	779; 440	800; 642
		–		-	-	322	-	252	-	322; 252	252	322; 255	252	252	252
2225	M	+	4	651	638; 519; 439	425	651	663; 194	651	651	638	652	625	638	527
		–		-	-	231	-	-	-	-	-	-	-	231	360; 231; 127
	H	+	4	663	-	-	194	651; 200	663	651	663; 189	-	-	625	455
		–		-	-	237	-	-	-	-	-	237	-	-	237
2277	M	+	8	750	750	-	843; 750; 465	712; 677; 411	724; 472	724; 453	712; 447	-	712; 522; 465	724; 453; 340	-
		–		-	556	-	-	-	-	-	-		-	-	-
	H	+	11	-	-	364	-	800; 394	382	394; 340	358	-	364	800; 370	358
		–		1114; 573	1114; 688	-	1114; 688; 573	1114; 688;	1114	1114	1114; 688	688; 573	1114	1114; 688	1114; 688
2231	M	+	3	1020; 427; 374; 320	374; 315	421; 325	1300; 380; 346	957; 883; 711; 677; 482;442; 374	839; 427;387	1200; 937; 393	957; 435; 393; 280	-	1220; 1100; 978; 406; 380; 363	1340; 1020; 667; 427; 393	978; 450; 400; 300
		–		-	736	-	562	-	-	-	-	562	-	-	-
	H	+	6	-	-	-	-	-	-	-	-	-	1300	688	1220
		–		450; 320	450; 320	535; 320	723; 647; 562	450; 363; 320	1020; 535; 406; 320	406	1020; 835; 723;562; 406	406; 363	647; 562; 535; 363	535; 363	647; 535; 406; 320

Table 3. *Cont.*

iPBS Primer	M/H [1]	± [2]	Control [3]	Experimental Groups											
				17-β-Estradiol			Progesterone			Testosterone			Estrogen		
				0.05 µM	0.5 µM	5 µM	0.05 µM	0.5 µM	5 µM	0.05 µM	0.5 µM	5 µM	0.05 µM	0.5 µM	5 µM
2231	M	+	3	1020; 427; 374; 320	374; 315	711; 636	1300; 380; 346	957; 883; 711; 677; 482;442; 374	839; 427;387	1200; 937; 393	957; 435; 393; 280	-	1220; 1100; 978; 406; 380; 363	1340; 1020; 667; 427; 393	978; 450; 400; 300
		−		-	736	-	562	-	-	-	-	562	-	-	-
	H	+	6	-	-	769	-	-	-	-	-	-	1300	688	1220
		−		450; 320	450; 320	-	723; 647; 562	450; 363; 320	1020; 535; 406; 320	406	1020; 835; 723;562; 406	535; 320	647; 562; 535; 363	535; 363	647; 535; 406; 320
2251	M	+	3	917	917; 608; 537; 468	983	957; 689	937	978	978; 689	957; 711	632; 332	1020; 689	1000; 700; 667; 492	1000; 722
		−		-	-	-	-	-	-	-	-	-	-	-	-
	H	+	7	-		-	-	-	-	-	778	830; 980	1140	-	-
		−		500	700; 637; 500	637; 500	575	575	575	700; 575; 500	575	-	-	575; 500	575; 500
2274	M	+	6	-	-	653	987; 545	555	566	555	555	412	555	566	566
		−		-	-	-	-	-	-	-	-	-	688	-	-
	H	+	9	-	-	-	-	-	-	-	-	-	-	-	-
		−		1014; 637; 420	783; 637; 420	637; 420	637	1014; 637; 420	420	-	-	783; 637	420	1014; 420	420
2298	M	+	10	-	-	-	285	474; 318	-	-	271	-	-	285	-
		−		700	700	823	-	823	700	700	823	577	700; 577; 411; 364; 322	823	700
	H	+	9	318	346; 314; 285	550; 425	310	506	-	310	314	311	314	307; 278	442; 318
		−		-	700; 674;	-	700	778	778	700	823	-	823	823	823
2402	M	+	10	1128; 465	-	-	-	-	-	-	-	-	-	-	-
		−		-	1000	933; 534	-	933	-	1000; 933; 534	1085; 1000; 534; 408	1000; 408	1085; 1000; 933	-	933
	H	+	7	1085; 962	1085; 1014; 620;400	852; 520	1085; 416	-	1000; 392	1057; 631	1071; 948	1020; 630	1085	1200; 1085; 987	1114; 1000
		−		-	-	-	-	-	-	-	-	-	-	-	-

[1, 2, 3] M—*Msp*I, H—*Hpa*II, (+) appearance of a new band, (−) disappearance of a normal band and without MSH.

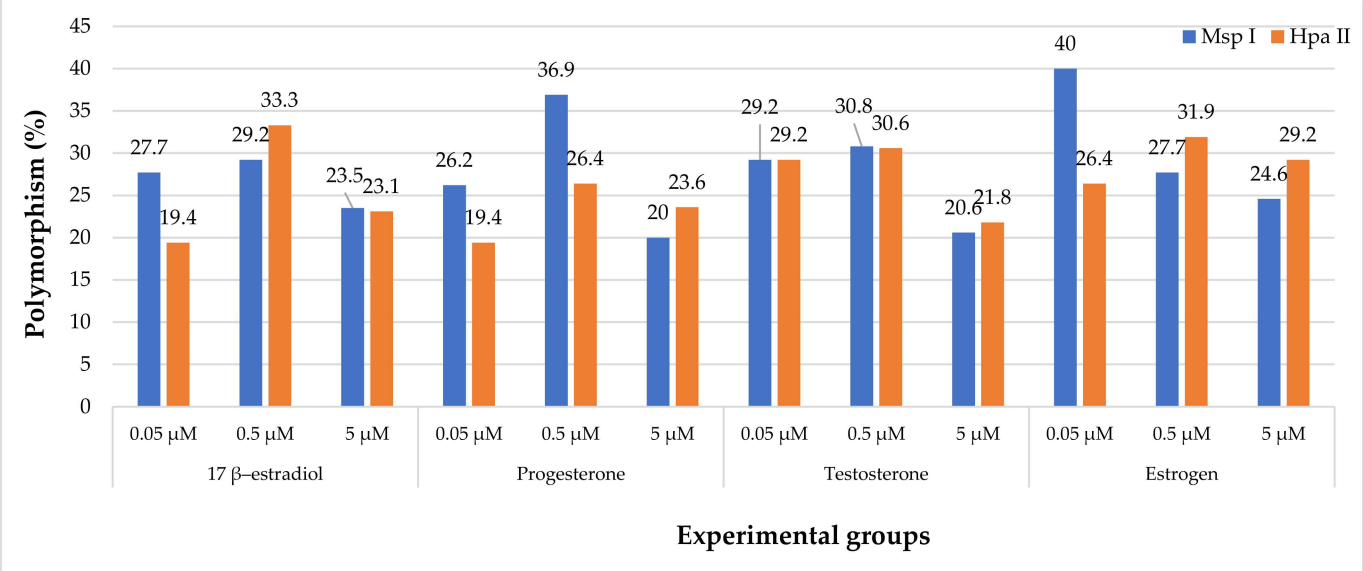

Figure 4. The effect of MSHs on polymorphism percentage in different experimental groups of wheat in seedling growth stage.

4. Discussion

The main objective of the current study was to elucidate the effects of MSHs on plant seedling growth, as well as on DNA methylation and genomic stability, of wheat plants. It has been reported that administration of exogenous MSH has a beneficial effect on plant development and growth, even under normal or stressful conditions [10,28].

A number of studies have been carried out to investigate the occurrence of MSHs in various plant species, as well as its quality and the mechanisms of action associated with their presence. According to a report by Tarkowská's [6], plants have the ability to convert sterols into steroid hormones. Dumlupinar et al. [29] conducted a study to investigate changes in the element composition of inorganic mammalian sex hormones in barley leaves. Their results indicate that inorganic element concentrations in barley leaves are significantly dependent on MSHs. In another study, Erdal [28] investigated the effect of mammalian sex hormones on the interaction between salt stress and wheat seedlings. Their results indicated that MSH treatment had a noteworthy protective effect against the detrimental consequences of salt stress in wheat seedlings. However, the effects of MSH on wheat remains largely unexplored.

In the present study, CRED-iPBS (coupled restriction enzyme digestion/inter-primer binding site) methods of retrotransposons were used to quantify levels of DNA damage and changes in DNA cytosine methylation pattern. Plant genomes contain ubiquitous, active, and abundant retrotransposons, and marker methods have generally been successful in identifying retrotransposon-derived polymorphisms [22–24]. Plants undergo molecular modifications under stress, and retrotransposons are an example of this [30]. During typical development periods, they are usually dormant. Long terminal repeat (LTR) retrotransposons are reactivated in plants by both biotic and abiotic stresses [31]. LTR retrotransposons are mobile genetic components found in plant genomes [27]. DNA methylation is a critically important enzymatic modification that also reflects a chemical change in DNA structure. It is very important for the process of regulating gene expression, as well as for protecting the genome [32]. In this study, different amounts of MSHs enhanced action of the retrotransposon, which in turn changed the rates of GTS. This was most likely the result of plants developing chemical and molecular defense mechanisms to protect themselves from the damaging effects of MSHs. According to the results of the study, it should be noted that different polymorphic bands were obtained in the IPBS profiles, and the GTS values obtained from all three testosterone doses were generally higher than those obtained from the other experimental groups. On the basis of these findings, it can be

concluded that testosterone treatment caused the least number of genotoxic effects imposed on the wheat genome. Citterio et al. [33] concluded that the iPBS method has a level of sensitivity sufficient to detect DNA damage. Observation of changes in the iPBS profile compared to that of a control sample resulted in an a change in the GTS value, either a decrease or an increase [34]. Consistent with the current study, Alzohairy et al. [35] reported that environmental stressors caused activation of LTR retrotransposons in barley plants. According to Hosseinpour et al. [24], GTS indices in wheat plants were reduced as a result of aluminum stress, which was caused by the movement of retrotransposons.

The results of this study indicate that DNA methylation levels were reduced at elevated concentrations of the hormone estrogen. Hypermethylation is commonly associated with suppression of gene expression, while hypomethylation is usually associated with the promotion of transcriptional activity [36]. On the basis of these findings, it seems that higher doses (e.g., 5 µM) of MSHs may be better than lower doses (e.g., 0.05 µM), especially when it comes to wheat genetic transformation studies, since high doses of MSHs cause hypomethylation in wheat. Epigenetic modifications at the level of DNA cytosine methylation/demethylation have a significant impact on various stress inducers in plants [24]. Although DNA sequence variation is considered a major evolutionary process leading to phenotypic changes, it is worth noting that DNA methylation modifications can also affect gene expression, potentially contributing to the inheritance of trait differences between generations [37].

Several studies have focused on the molecular effects of MSH therapy on plants. Turkoglu et al. [13] conducted a study to investigate the effect of MSHs on polymorphism and genomic instability in beans. Their study involved treatment with different concentrations of MSH to evaluate their effects on genetic or epigenetic levels, and their results indicated that iPBS polymorphism profiles showed a significant proportion (52.2%) at a concentration of 10^{-4} mM, which was the maximum dose of estrogen hormone administered. In our study, the iPBS profile of wheat showed the highest polymorphism rate treated with 0.5 µM estrogen. However, the achieved polymorphism rate was 29.3%. Furthermore, analogous to our study, their results revealed that the presence of estrogen, 17-β-estradiol, testosterone, and progesterone in bean plants affects genomic instability and induces epigenetic changes that serve as a key regulatory mechanism in gene expression. Türkoğlu [25] conducted a study to investigate the effects of MSHs on retrotransposon polymorphism and genomic instability in wheat in vitro. The researcher administered different concentrations of MSHs to Murashige and Skoog (MS) medium. It was found that treatments with 10^{-8} mM+ progesterone resulted in the highest level of genomic template stability (GTS) (80.52%), while treatments with 10^{-4} mM+ 17-β-estradiol resulted in the lowest value (68.83%). In addition, the study showed that treatment with 17-β-estradiol resulted in a higher percentage of polymorphism in CRED-iPBS assays, which were digested with *MspI* and *HpaII*, respectively. According to our results, treatment consisting of the 0.05 µM estrogen experimental group had the highest GTS value, while treatment consisting of the 5 µM progesterone experimental group and the 0.5 µM estrogen experimental group had the lowest GTS. Our iPBS and CRED-iPBS results differed from those published by Türkoğlu [25], which may be due to fact that the plant was grown in a different germination environment or because different concentrations of MSHs were used.

5. Conclusions

Since the discovery of MSHs in plants, many researchers have studied the effects of exogenous MSHs on plant development and growth. In the present study, for the first time, we investigated the effects of four different MSHs at four different concentrations on the genetic and epigenetic stability of wheat plants at the seedling growth stage using CRED-iPBS and iPBS analyses. The study identified MSH-induced retrotransposon polymorphisms, as well as methylation changes. The current results showed that MSH treatments altered the stability of the genomic template of wheat plants and affected the cytosine methylation status of wheat plants at the seedling growth stage. In the context of progesterone therapy,

an inverse relationship was found between the concentration of progesterone and the levels of GTS, indicating a reduction in GTS as the concentration of progesterone increased. On the basis of the mean proportion of polymorphisms detected in *HpaII* and *MspI* digestion, it may be deduced that elevated amounts of MSHs (17-β-estradiol, progesterone, and estrogen) would provide a safeguarding impact against hypermethylation. Nevertheless, further studies are recommended to better understand the function that MSHs play in the molecular mechanisms affecting wheat plant growth. In conclusion, our work shows that MSHs induce genetic and epigenetic changes and play an active role in maintaining genomic stability.

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References

1. Su, Y.; Xia, S.; Wang, R.; Xiao, L. Phytohormonal quantification based on biological principles. *Hormon. Met. Signal. Plants* **2017**, *13*, 431–470.
2. Gaspar, T.; Kevers, C.; Penel, C.; Greppin, H.; Reid, D.M.; Thorpe, T.A. Plant hormones and plant growth regulators in plant tissue culture. *Vitr. Cell. Dev. Biol. Plant* **1996**, *32*, 272–289. [CrossRef]
3. Milanesi, L.; Boland, R. Presence of estrogen receptor (ER)-like proteins and endogenous ligands for ER in solanaceae. *Plant Sci.* **2004**, *166*, 397–404. [CrossRef]
4. Janeczko, A.; Skoczowski, A. Mammalian sex hormones in plants. *Folia Histochem. Cytobiol.* **2005**, *43*, 71–79. [PubMed]
5. Batth, R.; Nicolle, C.; Cuciurean, I.S.; Simonsen, H.T. Biosynthesis and industrial production of androsteroids. *Plants* **2020**, *9*, 1144. [CrossRef] [PubMed]
6. Tarkowská, D. Plants are capable of synthesizing animal steroid hormones. *Molecules* **2019**, *24*, 2585. [CrossRef]
7. Iino, M.; Nomura, T.; Tamaki, Y.; Yamada, Y.; Yoneyama, K.; Takeuchi, Y.; Mori, M.; Asami, T.; Nakano, T.; Yokota, T. Progesterone: Its occurrence in plants and involvement in plant growth. *Phytochemistry* **2007**, *68*, 1664–1673. [CrossRef]
8. Janeczko, A.; Filek, W. Stimulation of generative development in partly vernalized winter wheat by animal sex hormones. *Acta Physiol. Plant.* **2002**, *24*, 291–295. [CrossRef]
9. Erdal, S.; Dumlupinar, R. Progesterone and β-estradiol stimulate seed germination in chickpea by causing important changes in biochemical parameters. *Z. Naturforsch.* **2010**, *65*, 239–244. [CrossRef]
10. Erdal, S.; Dumlupinar, R. Exogenously treated mammalian sex hormones affect inorganic constituents of plants. *Biol. Trace Element Res.* **2011**, *143*, 500–506. [CrossRef]
11. Haliloğlu, K.; Türkoğlu, A.; Balpınar, Ö.; Öztürk, H.İ.; Özkan, G.; Poczai, P. Effects of mammalian sex hormones on in vitro organogenesis of common bean (*Phaseolus vulgaris* L.). *Sci. Rep.* **2023**, *13*, 3337. [CrossRef] [PubMed]
12. Simerský, R.; Novák, O.; Morris, D.A.; Pouzar, V.; Strnad, M. Identification and quantification of several mammalian steroid hormones in plants by UPLC-MS/MS. *J. Plant Growth Regul.* **2009**, *28*, 125–136. [CrossRef]
13. Türkoğlu, A.; Haliloğlu, K.; Balpinar, Ö.; Öztürk, H.I.; Özkan, G.; Poczai, P. The effect of mammalian sex hormones on polymorphism and genomic instability in the common bean (*Phaseolus vulgaris* L.). *Plants* **2022**, *11*, 2071. [CrossRef] [PubMed]
14. Kim, J.-M.; To, T.K.; Ishida, J.; Morosawa, T.; Kawashima, M.; Matsui, A.; Toyoda, T.; Kimura, H.; Shinozaki, K.; Seki, M. Alterations of lysine modifications on the histone H3 N-tail under drought stress conditions in *Arabidopsis thaliana*. *Plant Cell Physiol.* **2008**, *49*, 1580–1588. [CrossRef]
15. Niederhuth, C.E.; Schmitz, R.J. Putting DNA methylation in context: From genomes to gene expression in plants. *Biochim. Biophys. Acta Gene Regul. Mech.* **2017**, *1860*, 149–156. [CrossRef]

16. Dowen, R.H.; Pelizzola, M.; Schmitz, R.J.; Lister, R.; Dowen, J.M.; Nery, J.R.; Dixon, J.E.; Ecker, J.R. Widespread dynamic DNA methylation in response to biotic stress. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, E2183–E2191. [CrossRef]
17. Consuegra, S.; Rodriguez Lopez, C.M. Epigenetic-induced alterations in sex-ratios in response to climate change: An epigenetic trap? *BioEssays* **2016**, *38*, 950–958. [CrossRef]
18. Yamamuro, C.; Zhu, J.-K.; Yang, Z. Epigenetic modifications and plant hormone action. *Mol. Plant* **2016**, *9*, 57–70. [CrossRef]
19. Haliloğlu, K.; Türkoğlu, A.; Balpınar, Ö.; Nadaroğlu, H.; Alaylı, A.; Poczai, P. Effects of zinc, copper and iron oxide nanoparticles on induced DNA methylation, genomic instability and LTR retrotransposon polymorphism in wheat (*Triticum aestivum* L.). *Plants* **2022**, *11*, 2193. [CrossRef]
20. Oda, M.; Glass, J.L.; Thompson, R.F.; Mo, Y.; Olivier, E.N.; Figueroa, M.E.; Selzer, R.R.; Richmond, T.A.; Zhang, X.; Dannenberg, L. High-resolution genome-wide cytosine methylation profiling with simultaneous copy number analysis and optimization for limited cell numbers. *Nucleic Acids Res.* **2009**, *37*, 3829–3839. [CrossRef]
21. Olkhov Mitsel, E.; Bapat, B. Strategies for discovery and validation of methylated and hydroxymethylated DNA biomarkers. *Cancer Med.* **2012**, *1*, 237–260. [CrossRef] [PubMed]
22. Hosseinpour, A.; Haliloglu, K.; Tolga Cinisli, K.; Ozkan, G.; Ozturk, H.I.; Pour-Aboughadareh, A.; Poczai, P. Application of zinc oxide nanoparticles and plant growth promoting bacteria reduces genetic impairment under salt stress in tomato (*Solanum lycopersicum* L. 'Linda'). *Agriculture* **2020**, *10*, 521. [CrossRef]
23. Hosseinpour, A.; Ilhan, E.; Özkan, G.; Öztürk, H.İ.; Haliloglu, K.; Cinisli, K.T. Plant growth-promoting bacteria (PGPBs) and copper (II) oxide (CuO) nanoparticle ameliorates DNA damage and DNA Methylation in wheat (*Triticum aestivum* L.) exposed to NaCl stress. *J. Plant Biochem. Biotechnol.* **2022**, *31*, 751–764. [CrossRef]
24. Hosseinpour, A.; Özkan, G.; Nalci, Ö.; Haliloğlu, K. Estimation of genomic instability and DNA methylation due to aluminum (Al) stress in wheat (*Triticum aestivum* L.) using iPBS and CRED-iPBS analyses. *Turkish J. Bot.* **2019**, *43*, 27–37. [CrossRef]
25. Turkoglu, A. Effects of mammalian sex hormones on regeneration capacity, retrotransposon polymorphism and genomic instability in wheat (*Triticum aestivum* L.). *Plant Cell Tissue Organ Cult.* **2023**, *152*, 647–659. [CrossRef]
26. Zeinalzadehtabrizi, H.; Hosseinpour, A.; Aydin, M.; Haliloglu, K. A modified genomic DNA extraction method from leaves of sunflower for PCR based analyzes. *J. Biodivers. Environ. Sci.* **2015**, *7*, 222–225.
27. Kalendar, R.; Antonius, K.; Smýkal, P.; Schulman, A.H. iPBS: A universal method for DNA fingerprinting and retrotransposon isolation. *Theor. Appl. Genet.* **2010**, *121*, 1419–1430. [CrossRef]
28. Erdal, S. Alleviation of salt stress in wheat seedlings by mammalian sex hormones. *J. Sci. Food Agric.* **2012**, *92*, 1411–1416. [CrossRef]
29. Dumlupinar, R.; Genisel, M.; Erdal, S.; Korkut, T.; Taspinar, M.S.; Taskin, M. Effects of progesterone, β-estradiol, and androsterone on the changes of inorganic element content in barley leaves. *Biol. Trace Element Res.* **2011**, *143*, 1740–1745. [CrossRef]
30. Ramakrishnan, M.; Satish, L.; Kalendar, R.; Narayanan, M.; Kandasamy, S.; Sharma, A.; Emamverdian, A.; Wei, Q.; Zhou, M. The dynamism of transposon methylation for plant development and stress adaptation. *Int. J. Mol. Sci.* **2021**, *22*, 11387. [CrossRef]
31. Mansour, A. Water deficit induction of Copia and Gypsy genomic retrotransposons. *Plant Stress* **2009**, *3*, 33–39.
32. Erturk, F.A.; Agar, G.; Arslan, E.; Nardemir, G. Analysis of genetic and epigenetic effects of maize seeds in response to heavy metal (Zn) stress. *Environ. Sci. Pollut. Res.* **2015**, *22*, 10291–10297. [CrossRef] [PubMed]
33. Citterio, S.; Aina, R.; Labra, M.; Ghiani, A.; Fumagalli, P.; Sgorbati, S.; Santagostino, A. Soil genotoxicity assessment: A new strategy based on biomolecular tools and plant bioindicators. *Environ. Sci. Technol.* **2002**, *36*, 2748–2753. [CrossRef] [PubMed]
34. Tanee, T.; Chadmuk, P.; Sudmoon, R.; Chaveerach, A.; Noikotr, K. Genetic analysis for identification, genomic template stability in hybrids and barcodes of the Vanda species (*Orchidaceae*) of Thailand. *Afr. J. Biotechnol.* **2012**, *11*, 11772–11781.
35. Alzohairy, A.; Yousef, M.; Edris, S.; Kerti, B.; Gyulai, G.; Bahieldin, A. Detection of LTR retrotransposons reactivation induced by in vitro environmental stresses in barley (*Hordeum vulgare*) via RT-qPCR. *Life Sci. J.* **2012**, *9*, 5019–5026.
36. Steward, N.; Ito, M.; Yamaguchi, Y.; Koizumi, N.; Sano, H. Periodic DNA methylation in maize nucleosomes and demethylation by environmental stress. *J. Biol. Chem.* **2002**, *277*, 37741–37746. [CrossRef] [PubMed]
37. Becker, C.; Weigel, D. Epigenetic variation: Origin and transgenerational inheritance. *Curr. Opin. Plant Biol.* **2012**, *15*, 562–567. [CrossRef] [PubMed]

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