Effects of Zinc, Copper and Iron Oxide Nanoparticles on Induced DNA Methylation, Genomic Instability and LTR Retrotransposon Polymorphism in Wheat (Triticum aestivum L.)

Kamil Haliloglu 1,2, Aras Turkoıglu 3,*, Özge Balpinar 4, Hayrunnisa Nadaroşlu 5,6, Azize Alayli 7 and Peter Poczai 8,9,*

1 Department of Field Crops, Faculty of Agriculture, Ataturk University, Erzurum 25240, Turkey  
2 Department of Biology, Faculty of Science, Cankiri Karatekin University, Cankiri 18200, Turkey  
3 Department of Field Crops, Faculty of Agriculture, Necmettin Erbakan University, Konya 42310, Turkey  
4 Hemp Research Institute, Ondokuz Mayis University, Samsun 55200, Turkey  
5 Department of Food Technology, Vocational College of Technical Sciences, Ataturk University, Erzurum 25240, Turkey  
6 Department of Nano-Science and Nano-Engineering, Institute of Science, Ataturk University, Erzurum 25240, Turkey  
7 Department of Nursing, Faculty of Health Sciences, Sakarya University of Applied Sciences, Sakarya 54187, Turkey  
8 Botany Unit, Finnish Museum of Natural History, University of Helsinki, P.O. Box 7, FI-00014 Helsinki, Finland  
9 Institute of Advanced Studies Kőszeg (iASK), P.O. Box 4, H-9731 Kőszeg, Hungary  
* Correspondence: aras.turkoglu@erbakan.edu.tr (A.T.); peter.poczai@helsinki.fi (P.P.)

Abstract: Nanomaterials with unique and diverse physico-chemical properties are used in plant science since they improve plant growth and development and offer protection against biotic and abiotic stressors. Previous studies have explored the effects of such nanomaterials on different plant mechanisms, but information about the effects of nanomaterials on induced DNA methylation, genomic instability and LTR retrotransposon polymorphism in wheat is lacking. Therefore, the present study highlights the key role of nanoparticles in DNA methylation and polymorphism in wheat by investigating the effects of ZnO, CuO and γ-Fe₃O₄ nanoparticles (NPs) on mature embryo cultures of wheat (Triticum aestivum L.). Nanoparticles were supplemented with Murashige and Skoog (MS) basal medium at normal (1X), double (2X) and triple (3X) concentrations. The findings revealed different responses to the polymorphism rate depending on the nanoparticle type and concentration. Genomic template stability (GTS) values were used to compare the changes encountered in iPBS profiles. ZnO, CuO and γ-Fe₃O₄ NPs increased the polymorphism rate and cytosine methylation compared to the positive control while reducing GTS values. Moreover, non-γ-Fe₃O₄ NP treatments and 2X ZnO and CuO NP treatments yielded higher polymorphism percentages in both MspI- and HpaII-digested CRED-iPBS assays and were thus classified as hypermethylation when the average polymorphism percentage for MspI digestion was considered. On the other hand, the 3X concentrations of all nanoparticles decreased HpaII and MspI polymorphism percentages and were thus classified as hypomethylation. The findings revealed that MS medium supplemented with nanoparticles had epigenetic and genotoxic effects.

Keywords: DNA methylation; genomic instability; in vitro; nanoparticles; retrotransposons; wheat

1. Introduction

Materials with sizes of 1–100 nanometers are known as nanomaterials. The supplementation of such nanomaterials into different substances such as fertilizers and plant nutrients may improve the quality-related traits of these substances. Indeed, studies have revealed the effects of nanoparticle-containing nutrients on plant growth and development [1,2].
Since nano-sized structures are more effective than bulk materials, nutrients containing nanomaterials may have much more toxic or beneficial effects on plants [3]. For example, Zhang et al. [4] investigated the effects of many nanoparticle structures, including gold (Au), copper (Cu), zinc (Zn), aluminum (Al), titanium oxide (TiO$_2$), zinc oxide (ZnO), copper oxide (CuO) and silver (Ag), on plants. However, previous studies mainly focused on product development, protection and fertilization.

Wheat (	extit{Triticum aestivum} L.) has an essential role in world nutrition. Notably, stress factors significantly influence wheat plants; serious quality and yield losses are encountered under stress conditions. As such, biotic and abiotic stressors result in changes in wheat DNA [5,6]. Nanotechnological applications may have either stress-related negative effects or very effective beneficial effects on wheat yield. Therefore, it is thought that nanotechnology has tremendous potential to positively affect yield factors [7]. Accordingly, the effects of nanoparticles on plants have been the primary focus of many research projects in recent years. According to [8,9], nanoparticles enter plant cells and leaves and can also transport DNA and chemicals into plant cells. The majority of studies on NPs to date concern toxicity. Recently, several studies have shown that heavy metals, such as Cd, Pb, Co, Ni and Zn, cause changes in DNA methylation [10]. However, less is known about the effects of their respective nanoparticles on the induction of specific gene mutations in plants and DNA methylation changes. It is crucial to determine the effect of nanomaterials on plant genetic stability, especially in this period when the use of nanomaterials in plant fertilizers is becoming more and more widespread [11]. Hence, the current study is among the first to explore the alteration of DNA methylation caused by nanoparticles in plants.

By expressing stress-related genes, plants express their responses to environmental stimuli and stress factors. Another parameter as effective as transcription factors in regulating gene expression is epigenetics, which refers to “heritable and reversible changes in gene expression without alterations of underlying DNA sequence” [12]. Gene expression is regulated by three epigenetic mechanisms: DNA methylation, histone modification and miRNA. Observing epigenetic changes can control many vital processes in plants, from growth development to flowering. Therefore, DNA methylation has become one of the most popular epigenetic phenomena [13,14].

DNA hyper/hypomethylation is largely correlated with gene expression, cell differentiation and phylogenetic development. DNA methylation is also correlated with several biological processes, including the transcriptional silencing of genes and transposable inactivation of transposons [15]. DNA methylation, microRNA (miRNA) and retrotransposon activities may also alter gene expression profiles and ultimately result in genomic instability [10]. Various techniques, including DArTseqMet [16], semi-quantitative MSAP [17], methylRAD [18], methyl-seq [19] and a variant of MSAP [19], have recently been used to detect DNA methylation. Methylation-sensitive amplified fragment length polymorphism (metAFLP) is also used to analyze the changes in DNA methylation [20]. Several other techniques or methods, including HPLC, the bisulfites method, methylation-specific PCR (MSP), the sequencing of specific genes, AFLP, MSAP, coupled restriction enzyme digestion–random amplification (CRED-RA) [21] and coupled restriction enzyme digestion–inter-primer binding site (CRED-iPBS) [22], have also been used to detect DNA methylation changes. The CRED-iPBS method employs the methylation-sensitive enzymes 

There are limited studies on nanomaterial-induced epigenetic changes in plant DNA. This study aimed to determine whether there were epigenetic changes in the DNA of wheat that were previously developed in a medium containing nanoparticles [24]. It is thought that this study will contribute to the literature in determining the effects of nanoparticles on DNA methylation changes in wheat. The study basically measured the genomic instability of wheat against nanomaterials. The main purpose of this research was to observe how wheat would respond genetically to the nanotechnological effects that it may be exposed to in the future, due to the increasing use of nanofertilizers. Therefore, the aim of this study was to determine the effects of nanoparticles on methylation and epigenetic changes
encountered in wheat DNA using the CRED-iPBS method. It was hypothesized that the high reactivity of nanoparticles could lead to epigenetic changes in wheat DNA.

2. Results

2.1. Characterization of Nanoparticles

ZnO, CuO and γ-Fe₃O₄ nanoparticles synthesized by green synthesis were characterized by SEM, XRD and FTIR. ZnO, CuO and γ-Fe₃O₄ nanoparticles were synthesized at 30 °C, 25 °C and 20 °C, respectively. The analyses revealed that ZnO, CuO and γ-Fe₃O₄ nanoparticles had varying sizes of 60–80 nm, 5–120 nm and 30–80 nm, respectively. The reason for the broad size ranges of nanoparticles is the adhesion of tiny metallic nanomaterials synthesized by green synthesis. This situation is why small nanoparticles may appear larger [25]. According to the XRD analysis findings, it was determined that CuO nanoparticles were in face-centered cubic form, γ-Fe₃O₄ nanoparticles were spherical and ZnO nanoparticles were in hexagonal crystal from Nadaroglu et al. [26]. The article presents data associated with the characterization (SEM, XRD and FT-IR) of relevant nanomaterials by Nalci et al. [24].

2.2. iPBS Analysis

In total, 20 oligonucleotide primers with approximately 50–70% GC content were used to analyze the PCR products of the T. aestivum Kirik genome, but only 10 provided specific and stable results. Compared to the PCR products obtained from the control DNA, nanoparticle treatments resulted in apparent changes in the iPBS patterns.

As shown in Table 1, a total of 67 bands was seen in the control treatment; the highest number of bands was seen in iPBS-2382 (nine bands), while the lowest number of bands was observed in iPBS-2387 and iPBS-2392 (six bands). The molecular sizes of polymorphic bands ranged from 100 (iPBS-2385) to 850 (iPBS-2382) bp. These changes were characterized by a variation in band intensity, the loss of regular bands or the appearance of new bands (as shown in Table 1 and Figure 1). There were significant differences in the iPBS profiles of the control and nanoparticle treatments. Compared to the control, 115 new bands appeared, while 106 bands were not present in the experimental groups.

Each nanoparticle with different concentrations yielded a different response to the polymorphism rate, and decreasing polymorphism rates were seen with increasing nanoparticle concentrations of the MS medium. Polymorphism rates were respectively measured as 25.37%, 28.35%, 34.32% and 25.37% for 0, 1X, 2X and 3X ZnO treatments; as 26.86%, 23.88%, 29.85% and 22.38% for 0, 1X, 2X and 3X CuO treatments and as 37.31%, 25.37%, 28.34% and 2.89% for 0, 1X, 2X and 3X γ-Fe₃O₄ treatments.

GTS was used to compare the changes in iPBS profiles. The present findings revealed that ZnO, CuO and γ-Fe₃O₄ nanoparticle treatments at different concentrations yielded different responses to GTS values. GTS values were determined respectively as 74.62%, 71.64%, 65.67% and 74.62% for 0, 1X, 2X and 3X ZnO treatments; as 73.13%, 76.11%, 70.14% and 77.61% for 0, 1X, 2X and 3X CuO treatments and as 60.68%, 74.62%, 71.64% and 79.10% for 0, 1X, 2X and 3X γ-Fe₃O₄ treatments. The results strongly showed that the application of all nanoparticles with 3X concentration (particularly γ-Fe₃O₄ nanoparticle) enhanced GTS values under the tissue culture approach (Table 1).
Table 1. Molecular sizes (bp) of present/absent bands in iPBS profiles under different nanoparticle treatments.

| Primer | 0 \(^{a}\) | 1X \(^{a}\) | 2X \(^{b}\) | 3X \(^{c}\) | 0 \(^{b}\) | 1X \(^{b}\) | 2X \(^{b}\) | 3X \(^{b}\) | 0 \(^{c}\) | 1X \(^{c}\) | 2X \(^{c}\) | 3X \(^{c}\) |
|--------|----------|----------|----------|---------|----------|----------|----------|---------|----------|----------|----------|---------|
| iPBS 2382 | + | 9 | 627; 166 | 239 | 850; 520 | - | 458 | - | 560; 265 | - | 625; 300 | 580 | 750; 536 | 625; 245 |
| - | 605 | 287 | 485 | 698 | 308 | 146 | 335 | 420 | 352 | - | 485; 146 | 335 |
| iPBS 2383 | + | 6 | 430; 307 | 440; 207 | 520 | 330; 207 | - | - | 610 | 327 | 188 | 350 | - | 218 |
| - | 350 | 400; 258 | 350 | 515; 285 | 400 | 258 | 515; 400 | 651 | 285 | 651 | 400 |
| iPBS 2384 | + | 7 | 608 | 670; 505 | 682; 426 | 285 | 300 | 300 | 483; 322 | 584 | 480; 265 | 185 | 465 | - |
| - | 359 | 315; 105 | - | 655; 250 | - | - | 359 | 450 | 550; 450 | 315; 105 | 655 |
| iPBS 2385 | + | 8 | 397 | 305; 158 | 100 | 274 | 350 | - | - | 428; 125 | 385 | - | 505 |
| - | 350 | 307; 409 | - | - | - | 450; 158 | 158 | - | - |
| iPBS 2386 | + | 6 | 524 | 440 | 350; 205 | 450 | 287 | 405 | 620 | 355 | - | 450; 366 | - |
| - | 352 | - | 600 | - | - | 750 | 450 | - | 450; 305 | 205 | 600 |
| iPBS 2387 | + | 5 | 650 | 392 | - | 450; 205 | 650; 258 | 715 | - | 115 | 320 | - | 452 | 502 |
| - | 367 | 367; 239 | 600 | 239 | - | - | - | 367 | 560; 153 | 239 | 285 | 153 |
| iPBS 2388 | + | 6 | 507 | 467 | 565; 295 | 487 | 378 | 350; 258 | 405 | - | 482 | 489; 326 | - |
| - | 221 | 285 | 362 | 160 | 160 | - | 221; 160 | - | 450 | 650; 362 | 285 | 362 |
| iPBS 2389 | + | 8 | 118 | - | 404 | - | 858; 465 | 157 | 248 | - | 500 | 571 | 535; 228 | 776 |
| - | 450 | - | 285 | 550 | 360 | - | 517 | 600; 350; 140 | - | - |
| Total band | 67 | 17 | 19 | 23 | 17 | 18 | 17 | 16 | 305; 127 | 422 | - | 115 |
| Polymorphism (%) | 25.37 | 28.35 | 34.32 | 25.37 | 25.37 | 28.35 | 20.89 |

* ** and ***, appearance of a new band, disappearance of a normal band and without hormone, respectively; 0 \(^{a}\), MS medium containing 0X ZnO NPs; 1X \(^{a}\), MS medium containing 1X ZnO NPs; 2X \(^{a}\), MS medium containing 2X ZnO NPs; 3X \(^{a}\), MS medium containing 3X ZnO NPs; 0 \(^{b}\), MS medium containing 0X CuO NPs; 1X \(^{b}\), MS medium containing 1X CuO NPs; 2X \(^{b}\), MS medium containing 2X CuO NPs; 3X \(^{b}\), MS medium containing 3X CuO NPs; 0 \(^{c}\), MS medium containing 0X γ-Fe\(_3\)O\(_4\) NPs; 1X \(^{c}\), MS medium containing 1X γ-Fe\(_3\)O\(_4\) NPs; 2X \(^{c}\), MS medium containing 2X γ-Fe\(_3\)O\(_4\) NPs; 3X \(^{c}\), MS medium containing 3X γ-Fe\(_3\)O\(_4\) NPs.
polymorphism percentages were respectively determined as 30.58%, 39.94%, 42.59% and 37.94% for 0, 1X, 2X and 3X CuO NPs treatments and as 57.96%, 43.01%, 32.77% and 24.84% for 0, 1X, 2X and 3X ZnO NPs treatments, as 35.60%, 43.01%, 32.77% and 24.84% for 0, 1X, 2X and 3X γ-Fe3O4 NPs treatments (Table 2). Among the 20 iPBS primers, 10 produced specific and stable bands and were used in CRED-iPBS analysis (Table 2). CRED-iPBS analysis enabled the observation of any possible cytosine methylations caused by non-γ-Fe3O4 treatments. Enhancement was also seen in cytosine methylation with CuO treatments. The results of the CRED-iPBS analysis as the average polymorphism percentages in terms of HpaII and MspI digestions are provided in Table 2. In total, 69 and 67 bands were observed in the MspI- and HpaII-digested control treatments, respectively. According to the results of the CRED-iPBS pattern, the MspI polymorphism percentage was higher than the HpaII polymorphism percentage in all nanoparticle treatments. There were apparent differences in the MspI polymorphism percentages of the experimental groups. Significantly higher polymorphism rates were observed in the control treatments. The present findings revealed that ZnO, CuO and γ-Fe3O4 nanoparticle treatments at different concentrations yielded different responses for the polymorphism percentages of MspI and HpaII digestion.

For MspI digestion, polymorphism percentages were respectively determined as 32.77%, 41.34%, 50.88% and 31.50% for 0, 1X, 2X and 3X ZnO treatments, as 35.60%, 29.50%, 43.01% and 24.84% for 0, 1X, 2X and 3X CuO NPs treatments and as 57.96%, 32.28%, 37.94% and 23.85% for 0, 1X, 2X and 3X γ-Fe3O4 NPs treatments. There was a clear increase in polymorphism percentage from 0X to 2X in ZnO NPs treatments but a decrease from 2X to 3X in ZnO NPs treatments. With CuO NPs treatments, a decrease was seen in polymorphism percentage from 0X to 1X, an increase from 1X to 2X and a decrease from 2X to 3X. With γ-Fe3O4 NPs, a decrease was seen in polymorphism percentage from 0X to 1X, an increase from 1X to 2X and again a decrease from 2X to 3X (Table 2). For HpaII digestion, polymorphism percentages were respectively determined as 30.58%, 39.94%, 42.59% and 30.06% for 0, 1X, 2X and 3X ZnO NPs treatments, as 34.39%, 26.61%, 33.83% and 22.97% for 0, 1X, 2X and 3X CuO treatments and as 49.81%, 30.19%, 35.92% and 21.31% for 0, 1X, 2X and 3X γ-Fe3O4 NPs treatments (Table 2).
Table 2. Results of CRED-iPBS analysis; the molecular size of bands and polymorphism percentages.

| Primer | M */H ** | Control | Experimental Groups |
|--------|-----------|---------|---------------------|
|        | a/****|   | ZnO NPs | CuO NPs | γ-Fe₃O₄ NPs |
|        | 0        | 1X      | 2X b     | 3X c      | 0      | 1X c      | 2X c     | 3X c     |
| M –    | 8        |         | 658; 434 | 420; 156 | 590; 660 | 645     | 450       | 660; 450 | 305      | 785     | 475; 325 | 120      | 650      | 450      | -        |
| H +    | 6        | 415     | 305      | -         | 180     | -         | 350       | 254; 128 | -        | 128      | -        | -        | -        | -        | -        |
| iPBS 2382 |       |         | -        | 600       | 250; 165 | -         | 250       | 485; 375 | 298      | 205      | 165      | 345      | -        | -        | -        |
| M +    | 5        | 754; 515| 555; 450 | 550; 362; 125 | 538     | 360       | 780       | 785; 650 | 375      | -        | 605; 515 | 785      | 645; 360 | -        | -        |
| H +    | 4        | 405     | -        | 405       | -        | 395       | 540       | 305      | 155      | 305      | 155      | 305      | -        | -        | -        |
| iPBS 2383 |       |         | -        | 245       | 405; 350 | -         | -         | -        | -        | 165      | -        | 525      | -        | -        | -        |
| M +    | 7        | 390; 240| 450     | 350       | -        | 625; 120  | 390       | 350; 205 | 450; 120 | 760; 605 | -        | -        | -        | -        | -        |
| H +    | 6        | 285     | -        | 125       | 465     | 495       | -         | 275      | 375      | 260      | 580      | 262      | -        | -        | -        |
| iPBS 2384 |       |         | -        | 350       | 425; 350 | -         | 250       | 165      | 450      | 450      | 165      | 568; 425 | 425; 350 | -        | -        |
| M +    | 11       | 450     | 808; 745 | 625       | 350; 128 | 825; 486  | 760       | 456      | 785; 345 | 625      | 458; 325 | 775; 550 | 469      | 745      | 710; 650 | 330; 280 | 355      |
| H +    | 10       | 695     | 374; 125 | 560; 393  | 260     | -         | -         | 275      | 375      | 260      | 580      | 262      | -        | -        | -        | -        |
| iPBS 2385 |       |         | -        | 350       | 425; 350 | -         | 250       | 165      | 450      | 450      | 165      | 568; 425 | 425; 350 | -        | -        | -        |
| M +    | 4        | 340     | 115      | -         | 405; 300 | 807; 485  | 300; 220  | -        | 615; 430 | 405      | 345; 262 | 600      | 565; 450 | 475      | -        | -        |
| H +    | 8        | 525     | 400; 225 | 150       | 605; 150 | 360; 320  | -         | 225; 150 | 375; 225 | 400; 320 | -        | -        | -        | -        | -        | -        |
| iPBS 2386 |       |         | -        | 255; 458  | 654     | -         | 450; 355  | 355      | -        | 255      | -        | -        | -        | -        | -        | 475      |
| M +    | 3        | 285     | 185      | -         | 605; 580 | 580      | 650; 185  | -         | 580; 185 | 680; 510 | 670; 466 | 485      | 689      | -        | -        | -        |
| H +    | 9        | 850; 405| 365     | 405; 160  | 715     | 125       | 365; 125  | 160      | 345; 205 | 125      | -        | 550; 205 | -        | -        | -        | -        | -        |
| iPBS 2387 |       |         | -        | 420; 285  | 605; 225 | 450     | 500      | -         | 550; 780 | 450; 245; 125 | 600 | - | 885; 745; 600; - | 689 | 689 | - | - | - | - | - | - | - |
| Primer | M+/H- | + + +/− + + + | Control | Experimental Groups |
|--------|--------|----------------|---------|---------------------|
|        |        |                |         | **ZnO NPs**         | **CuO NPs** | **γ-Fe3O4 NPs** |
|        |        |                |         | 0*                 | 1X*        | 2X*       | 3X*       | 0*      | 1X*     | 2X*     | 3X*     |
|        |        |                |         | 1X*                | 2X*        | 3X*       | 0*      | 1X*     | 2X*     | 3X*     |
| iPBS 2388 |        | 7               |         | 450; 226          | 250; 115  | 510; 258  | 388; 520| 450; 487 | 650; 255| 125; 425| 242; 360| 220; 650| 505; 650|
| H      |        | 8               |         | 250; 397; 325     | 285; 112  | 440; 338  | 746; 380| 350; 380 | 580; 375| 120; 498| 98; 624| 404; 348|
|        |        | 15                |         | 535; 453          | 308; -     | -         | -        | 405; 355| 305; 105| 500; 462| -         | -        | 500     |
| iPBS 2389 |        | 11              |         | 375; 198          | 785; 625  | 320; 625  | 405; 550| 345; 465| 640; 460| 355; -   | 605; 485| 652; 496| 320; 128|
| H      |        | 8               |         | 285; 159          | 153; 750  | 418; 785  | 560; 245| 335; 424| 335; -   | 450; 335| 600; 505| 525; -   | -        | -        | 520     |
|        |        | 5               |         | 490; 350          | 485; 309  | 365; 263  | 785; 460| 340; -   | 750; 464| 128; -   | 560; 258| 365; 775| -         | -        | -        | 775     |
| iPBS 2390 |        | 4               |         | 365; -             | 145; 525  | 410; 669  | 285; 680| 490; 325| -         | 458; 635| -         | -        | 605     | -         | -        | -        | 605     |
| H      |        | 3               |         | 125; 600          | 450; 350  | 228; 125  | 450; 350| 320; 350| 255; 125| -         | 350; 320| -         | 350; -   | -        | -        | 320     |
| iPBS 2391 |        | 8               |         | 850; 300          | 758; 800  | 450; 750  | 450; 800| 285; 810| 450; 456| 378; 830| 805; 753| 385; 800| 850; 450| 750; 330|
| H      |        | 4               |         | 400; 350; 200     | 550; 360  | 245; 705  | 200; 345| 145; 345| 455; 775| 555; 390| 650; 350| -         | -        | -        | 495     |
| Polymorphism |        | 32.77          | 41.34   | 50.88              | 31.50     | 35.60     | 29.50   | 43.01   | 24.84   | 57.96   | 32.28   | 37.94   | 23.85    | -        | -        | 23.85   |

* *, **, *** and ****, M—Msp I, H—Hpa II, appearance of a new band, disappearance of a normal band and without hormone, respectively: 0*, MS medium containing 0X ZnO NPs; 1X*, MS medium containing 1X ZnO NPs; 2X*, MS medium containing 2X ZnO NPs; 3X*, MS medium containing 3X ZnO NPs; 0b, MS medium containing 0X CuO NPs; 1Xb, MS medium containing 1X CuO NPs; 2Xb, MS medium containing 2X CuO NPs; 3Xb, MS medium containing 3X CuO NPs; 0c, MS medium containing 0X γ-Fe3O4 NPs; 1Xc, MS medium containing 1X γ-Fe3O4 NPs; 2Xc, MS medium containing 2X γ-Fe3O4 NPs; 3Xc, MS medium containing 3X γ-Fe3O4 NPs.
In general, it was found that the non-γ-Fe3O4 NPs treatment yielded a higher polymorphism percentage in both MspI- and HpaII-digested CRED-iPBS assays (57.96% and 49.81%, respectively). On the contrary, the application of 3X treatments of all nanoparticles decreased both HpaII and MspI polymorphism percentages. In other words, the 2X treatments of ZnO and CuO NPs had an impact on cytosine methylation status and thus could be classified as hypermethylation when the average polymorphism percentage for MspI digestion was taken into consideration. On the other hand, 3X treatments resulted in an apparent decrease in average polymorphism percentages and impacted methylation status, thus classified as hypomethylation (Table 2 and Figure 2).

![Figure 2. CRED-iPBS profiles for various experimental groups with iPBS 2391 primers in wheat. M, 100–1000 bp DNA ladder; 1, control Msp I; 2, control Hpa II; 3, MS medium supplemented with 0X ZnO NPs Msp I; 4, MS medium supplemented with 0X ZnO NPs Hpa II; 5, MS medium supplemented with 1X ZnO NPs Msp I; 6, MS medium supplemented with 1X ZnO NPs Hpa II; 7, MS medium supplemented with 2X ZnO NPs Msp I; 8, MS medium supplemented with 2X ZnO NPs Hpa II; 9, MS medium supplemented with 3X ZnO NPs Msp I; 10, MS medium supplemented with 3X ZnO NPs Hpa II; 11, MS medium supplemented with 0X CuO NPs Msp I; 12, MS medium supplemented with 0X CuO NPs Hpa II; 13, MS medium supplemented with 1X CuO NPs Msp I; 14, MS medium supplemented with 1X CuO NPs Hpa II; 15, MS medium supplemented with 2X CuO NPs Msp I; 16, MS medium supplemented with 2X CuO NPs Hpa II; 17, MS medium supplemented with 3X CuO NPs Msp I; 18, MS medium supplemented with 3X CuO NPs Hpa II; 19, MS medium supplemented with 0X γ-Fe3O4 NPs Msp I; 20, MS medium supplemented with 0X γ-Fe3O4 NPs Hpa II; 21, MS medium supplemented with 1X γ-Fe3O4 NPs Msp I; 22, MS medium supplemented with 1X γ-Fe3O4 NPs Hpa II; 23, MS medium supplemented with 2X γ-Fe3O4 NPs Msp I; 24, MS medium supplemented with 2X γ-Fe3O4 NPs Hpa II; 25, MS medium supplemented with 3X γ-Fe3O4 NPs Msp I; 26, MS medium supplemented with 3X γ-Fe3O4 NPs Hpa II.]

3. Discussion

Nanotechnology has improved plant genetics through special nanoparticles (materials with dimensions between 1 and 100 nm), thus enhancing agricultural productivity [27] as growth-promoting nanoparticles may increase yield and quality traits [28]. Compared to bulk materials, nanoparticles of the same material have novel and improved physico-chemical and biological characteristics [29]. According to Nadaroglu et al. [29], nanoparticles at appropriate doses could improve seed germination and plant growth and development, increase yield levels, reduce soil pollution and protect plants against biotic and abiotic stressors. However, there are a limited number of studies on the effects of nanoparticles on induced-DNA methylation, genomic instability and LTR retrotransposon polymorphism. The present findings revealed that different concentrations (0, 1X, 2X and 3X) of ZnO, CuO and γ-Fe3O4 NPs had a significant role in wheat’s DNA methylation and genomic instability. Micronutrients are essential for plant growth and development; many...
are involved in catalytic redox reactions. In this study, inter-primer binding site (iPBS) retrotransposon and CRED-iPBS (coupled restriction enzyme digestion-iPBS) techniques were used to define the DNA damage levels and changes in DNA methylation status. As compared to the control treatments, ZnO, CuO and γ-Fe₃O₄ NPs treatments increased polymorphism rates and cytosine methylation and decreased GTS values. While 1X and 2X ZnO treatments increased polymorphism rate and cytosine methylation, 3X treatments reduced these values compared to the negative control (without Zn). Such opposite effects of ZnO NPs treatments were seen on GTS values. It can be thought that the epigenetic modifications that occur in wheat endosperms exposed to ZnO doses may be related to changes in the expression level of the PI-II gene, which may be a key mechanism responsible for developing plant immunity against stress conditions [30]. A previous study revealed that ZnO nanoparticles similarly affect the transcription factors in wheat and cause a change in the expression level of the HSFA4A gene [31]. Another mechanism of action in the epigenetic polymorphism formed by ZnO nanoparticles in callus tissues may be due to the interaction of zinc with biomolecules and cellular organelles [30]. Zinc is an essential nutrient and plays a vital role in synthesizing chlorophyll, carbohydrates and phytohormones required for plant growth and development [32]. It was reported that zinc nanoparticles enhance the plant growth and yield levels of *Cyamopsis tetragonoloba* L., *Gossypium hirsutum*, *Lycopersicum esculentum* and *Stevia* [33,34]. It was also reported that, at proper doses, zinc nanoparticles improve shoot and root lengths, chlorophyll and protein contents and increased the yield levels of *Pennisetum americanum* L. plants [35]. Excessive quantities of nanoparticles may facilitate oxidative burst, which in turn results in the production of vast quantities of reactive oxygen species (ROS) [36]. It was reported that metal nanoparticles provoke the stress response of plants [37]. According to [38], zinc nanoparticles have significant effects on biomass, root and shoot lengths, chlorophyll and protein contents, as well as enzyme activity in *Cyamopsis tetragonoloba* L. plants. Excessive or deficient micronutrients may facilitate ROS accumulation and disrupt enzyme activity, resulting in cell cycle anomalies and impairing overall biomass, yield and quality [39,40].

While 1X CuO treatments increased polymorphism rate and cytosine methylation, 2X treatments reduced them, while 3X treatments increased them compared to the negative control (without Cu). The opposite effects of ZnO treatments were seen on GTS values. It can be thought that the effect of CuO nanoparticles on epigenetic modifications is related to oxidative stress, as shown by studies that analyzed transcriptomic data related to the modification of oxidative-stress-related genes following CuO nanoparticle applications [41]. Copper is essential in electron transport and cell wall metabolism [42] and facilitating ethylene receptors [42]. Copper is an essential nutrient for plants, and since it is involved in various physiological processes, it is used mainly in agricultural activities [43]. For example, copper ions protect plant cells against oxidative damage [44] and facilitate the production of hydroxyl radicals [42] and biochemical pathways [45]. CuO nanoparticles are more soluble and toxic at low concentrations [46]; they can affect the Krebs cycle at sublethal concentrations [42]. It was reported that CuO nanoparticle toxicity negatively affects the seed germination and plant growth of lettuce, mung bean, kidney bean, alfalfa, wheat, chickpea and several other crops [47].

The present findings revealed that γ-Fe₃O₄ treatments at 1X, 2X and 3X concentrations all reduced polymorphism rate and cytosine methylation compared to the negative control (without Fe). However, an opposite effect was seen on GTS values. It has been reported that iron ions (Fe²⁺) reduce copper absorbance remarkably; therefore, malfunctions may be seen in functions related to the minor copper element in plants. For this reason, it can be thought that iron nanoparticles applied at high doses can regulate many physiological processes that require minor copper elements with epigenetic modifications [48]. Iron (Fe) is an essential nutrient for all organisms. It plays a crucial role in several physiological processes of plants, including chlorophyll biosynthesis, respiration and redox reaction [49]. It was reported that Fe nanoparticles decrease chlorophyll content and root hydraulic conductivity, thus influencing nutrient transport within the plant [30]. U Sami and U Rehman [51] indicated
that Fe nanoparticles significantly affect the growth and development of *Citrus maxima*, *Lycopersicum esculentum* and *Triticum aestivum* plants. FeO nanoparticles promote plant growth and development in soybean [52], wheat [53] and peanuts [54]. Fe nanoparticles improve FRO$_2$ gene expression levels, increase ferric reductase activity, enhance iron transformation and improve plant tolerance to iron deficiency [51].

Overall, the present findings revealed that epigenetic changes occurred using nanoparticles instead of the main element in the MS medium. Nanoparticles enhance plant growth and yield when used at proper concentrations. They interact with plant cells and change the biochemical pathway by affecting the regulation of gene expression, which enhances plant growth and development, but they also have inhibitory effects on plant growth and production when used above the optimum levels. Such negative effects could be attributed to nanoparticle type, structure, concentration and application period. Epigenetics encompasses heritable changes in gene functions without directly altering the DNA sequence [55]. Epigenetic mechanisms include DNA methylation and histone modifications [56]. In the present study, non-$\gamma$-Fe$_3$O$_4$ NPs treatment and 2X ZnO and CuO treatments yielded higher polymorphism percentages in both MspI- and HpaII-digested CRED-ipBS assays and thus could be classified as hypermethylation when the average polymorphism percentage for MspI digestion was taken into consideration.

On the contrary, the 3X concentration of all nanoparticles decreased both HpaII and MspI polymorphisms’ percentages and thus could be classified as hypomethylation. Nanoparticles can influence DNA methylation through two primary mechanisms—the reduced availability of methyl donors and altered activity of DNA methyl-transferases enzymes. The pro-oxidative characteristics of nanoparticles may alter DNA methylation patterns [57]. In addition to oxidative damage, ROS could also alter gene expression levels and thus DNA methylation status [58]. Accordingly, it was previously reported that nanoparticles induce ROS production [59]. Nanoparticles may also result in cytotoxicity, cell death, oxidative stress, genotoxicity and immunotoxicity [60], thus inducing abnormal epigenetic processes [61] and changes in the proteome [62]. Exposure to nanoparticles induces oxidative stress, lipid peroxidation and membrane damage, ultimately resulting in hypomethylation, accompanied mainly by hypermethylation. Environmental factors could also trigger epigenetic changes [46]. In general, it has been observed that nanoparticle structures have an effect on the modification of transcription factors in plants; hence, they are effective both in changing the expression profile and in signal transduction [31]. However, it is known that many factors, such as the size, shape and synthesis method of nanoparticles, can influence epigenetic modifications [63]. In addition, many factors, such as the method of application of nanoparticles on the plant, the dose and the duration of application, can determine the quality and quantity of the effect. Since this process is affected by the changes in many parameters, more studies are needed to clarify the effects of nanoparticles on plants.

### 4. Materials and Methods

The nanoparticles used in the experiment were obtained using the biological reduction method, and their nano size was confirmed as described by Nalci et al [23]. CuO, ZnO and $\gamma$-Fe$_3$O$_4$ nanoparticles were synthesized by green synthesis using a peroxidase enzyme obtained from the Euphorbia amygdaloides plant. Nanoparticles were characterized by SEM, XRD and FTIR analysis. As a result of the characterization findings, it was determined that $\gamma$-Fe$_3$O$_4$ NPs are in the size range of 30–80 nm, ZnO NPs are in the range of 60–80 nm and CuO NPs are in the range of 5–120 nm.

Briefly, iron, copper and zinc elements were removed from the Murashige and Skoog (MS) medium and replaced with a nanoparticle version of these elements at 1X, 2X and 3X concentrations [24]. The embryogenic callus is a green formation with a total plant-forming capacity that develops on callus tissue. After the embryogenic callus developed on the callus, they were transferred to different nutrient media under aseptic conditions to form a whole plant. Genomic DNA was isolated from plantlet leaves obtained from embryogenic
callus using the method specified by Hosseinpour et al. [64]. The concentration and quality of genomic DNA were measured with a Nanodrop spectrophotometer (Qiagen Qiaxpert, Qiagen, Hilden, Germany) (Thermo Fisher Scientific, Waltham, MA, USA) and run on 1.5% (w/v) agarose gel. Twenty primers were tested for iPBS-PCR amplification [65]. For iPBS analysis, a PCR reaction was carried out by Hosseinpour et al. [22]. Out of 40 iPBS oligonucleotide primers, only 10 resulted in specific and stable DNA profiles in all experimental groups (Table 3).

Table 3. Reactive primers used in iPBS PCR and their annealing (Tm) temperatures.

| No | Primer Name | Sequence (5′ to 3′) | Tm (°C) | CG (%) |
|----|-------------|---------------------|---------|--------|
| 1  | iPBS 2382   | TGTTGGCTTCCA        | 44.9    | 50     |
| 2  | iPBS 2383   | GCATGGGCTTCCA       | 50.5    | 66.7   |
| 3  | iPBS 2384   | GTAATGGGTCCA        | 40.9    | 50     |
| 4  | iPBS 2385   | CCATTGGGTCCA        | 45.7    | 58.3   |
| 5  | iPBS 2386   | CTGATCAACCCA        | 41.4    | 50     |
| 6  | iPBS 2387   | CGGCAATACCCA        | 47.3    | 58.3   |
| 7  | iPBS 2388   | TTGGAAGACCCA        | 43.4    | 50     |
| 8  | iPBS 2389   | ACATCCTTCCCA        | 43      | 50     |
| 9  | iPBS 2390   | GCAACAACCCA         | 47.6    | 58.3   |
| 10 | iPBS 2391   | ATCTGTCAGCCA        | 43.6    | 50     |

CRED-iPBS analysis was conducted as specified by Hosseinpour et al. [64], using the primers listed in Table 3 for amplification. PCR steps were the same as for iPBS analysis described by Hosseinpour et al. [22]. iPBS 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. CRED-iPBS 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. The iPBSs and CRED-iPBS PCR products were separated in a 1.5% agarose gel containing 0.05 µg/mL ethidium bromide, using 1X SB buffer in 100 V for 90 min for electrophoresis. The 100–1000 bp (Sigma Aldrich, MO, USA, No: P1473-1VL) DNA ladder was used to estimate the molecular weight of the fragments. The gels were photographed under UV light in a Universal Hood II (Bio-Rad, Hercules, CA, USA).

The iPBS and CRED-iPBS banding patterns were analyzed using TotalLab TL120 software (Nonlinear Dynamics Ltd. Newcastle, UK). Polymorphism and genomic template stability (GTS %) were analyzed as described by Hosseinpour et al. [64]. For CRED-iPBS analysis, the average values of polymorphism (%) were calculated for each concentration [23].

5. Conclusions

In this study, the effects of different nanoparticles (ZnO, CuO and γ-Fe2O3) at different concentrations (1X, 2X and 3X) on methylation and epigenetic changes encountered in wheat DNA were investigated. Based on the present findings, it was concluded that nanoparticles play an essential role in DNA methylation and genomic instability. Current findings indicated that epigenetic modification via cytosine methylation could be an essential regulatory mechanism in plants. It was determined that the three metallic nanoparticles, especially at high doses, caused changes in genomic instability. It was concluded that the excess and the absence of minor elements in the wheat plant revealed changes at the nanoscale DNA level. Exposure to nanoparticles can induce epigenetic changes, but the consequences of these changes have not been fully elucidated. Further research is recom-
mended for the high-throughput analysis of genetic and metabolic responses triggered by nanoparticles and to shed light on various aspects of nanoparticle phytotoxicity in plants.

**Author Contributions:** Concept—K.H. and A.T.; Design—K.H. and A.T.; Resource—K.H.; Materials—K.H., H.N., A.A. and Ö.B.; Data Collection and/or Processing—A.T.; Analysis and/or Interpretation—A.T.; Literature Search—A.T. and Ö.B.; Writing A.T.; Critical Reviews—K.H., H.N., A.A. and P.P. All authors have read and agreed to the published version of the manuscript.

**Funding:** Peter Poczai expresses his gratitude for the support of the iASK Research Grant. The authors thank the Helsinki University Library for supporting open access publication.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data are contained within the article.

**Acknowledgments:** Open access funding provided by University of Helsinki.

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

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