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Effect of nickel chloride on *Arabidopsis* genomic DNA and methylation of 18S rDNA

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ABSTRACT

Background: In recent years, nickel (Ni) has been widely applied in industrial and agricultural production and has become a kind of environmental pollution. In this study, the effect of nickel chloride ($NiCl_2$) with different concentrations on *Arabidopsis* genomic stability and DNA methylation has been demonstrated. The nucleolus variation and 18S rDNA methylation after $NiCl_2$ treatment have been analyzed.
Results: The results are as follows: (1) The $NiCl_2$ could result in heritable genomic methylation variations. The genomic DNA methylation variations have been detected by methylation-sensitive amplified polymorphism (MSAP) molecular markers, and the result showed that after $NiCl_2$ treatment, there was methylation variation in T_0 generation seedlings, and partial site changes maintained in T_1 generation, which suggested that the effects of $NiCl_2$ on DNA methylation could be heritable in offspring. (2) $NiCl_2$ brought deformity and damage to nucleolar structure in *Arabidopsis* root tip cells, and the damage was positively correlated with the $NiCl_2$ concentration. 3. In the nucleolus, there was an increased cytosine methylation in 18S rDNA. The plant nucleolus variation and 18S rDNA methylation may be used as an examination indicator for Ni pollution in soil or plant.
Conclusions: $NiCl_2$ application caused variation of DNA methylation of the *Arabidopsis* genomic and offspring's. $NiCl_2$ also resulted in nucleolar injury and deformity of root tip cells. The methylation rate of 18S rDNA also changed by adding $NiCl_2$.

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1. Introduction

Since the 20th century, the productive industry has been developing rapidly, and the heavy metals have begun to become a major source of environmental pollutants. The heavy metals can influence plant growth and greatly impact human health [1,2]. The heavy metals affect not only the growth & development, physiological & biochemical processes of plants, but also the genetic information in plant cells [3]. During the plant growth and development processes, the heavy metal stress could influence on the plant root growth, improve the cell membrane permeability, destroy the antioxidant enzymes or photosynthetic systems [4,5] in plant and poison the genes. The previous studies showed that lead and zinc damaged the roots of plant while, copper, lead, zinc, and cadmium had a destructive effect on chlorophyll and influenced photosynthesis [6,7]. The plant respiration system could be significantly inhibited by mercury, lead, and cadmium [2,3,8]. As for genetic material, heavy metal stress may influence the plant DNA replication directly or indirectly, inhibit the repair system or affect the DNA methylation or demethylation. Cadmium would enhance the DNA demethylation, and result in genomic variation of red seaweed *Gracilaria dura* [9]. Cobalt compounds would change both the methylation and demethylation state of *Vicia faba* seeds [10].

Nickel (Ni) is a kind of necessary trace elements in plant growth and development, which constituted the active site of urease as cofactor [8]. Excessive amount of Ni will influence the growth of plants as well as other heavy metal. For example, Ni could inhibit the cell division in wheat root [11]. The studies on epidemiology showed that nickel compounds were carcinogenic to animal and cell culture [12,13,14], and the insoluble Ni (Ni_3S_2) could be more carcinogenic. Ni compound was able to induce DNA methylation and chromatin variation, and result in gene silencing [15,16,17]. In addition, the histone modification of H2A, H2B, H3, and H4 may be missing, the methylation of H3K9 would increase and the ubiquitination of H2A, H2B would also improve [18,19,20,21,22]. A large number of studies have shown that the chromatin structure variation and epigenetics change may be a vital pathway which response to the carcinogenesis of Ni compounds.

In recent years, Ni has been widely applied in coins, jewelry, stainless steel, batteries, medical equipment, carbon particles in oil refining, electroplating and welding. The Ni content uptake by organism living through various pathways has been increasing year by year. As one of the most serious environmental issues, Ni has

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Table 1
Sequences of adapters and primers for AFLP and MSAP.

Primers/adapters	Sequences
Adapter	
Mse I–adapter I	5′-GACGATGAGTCCTGAG-3′
Mse I–adapter II	5′-TACTCAGGACTCAT-3′
EcoR I–adapter I	5′-CTCGTAGACTGCGTACC-3′
EcoR I–adapter II	5′-AATTGGTACGCAGTC-3′
Hpa II–Msp I–adapter I	5′-GATCATGAGTCCTGCT-3′
Hpa II–Msp I–adapter II	5′-TCATGATCCTGCTCG-3′
Preselective primers	
E–A	5′-GACTGCGTACCAATTCA-3′
M–C	5′-GATGAGTCCTGAGTAAC-3′
Hpa II–Msp I	5′-ATCATGAGTCCTGCTCGG-3′
Selective primers	
EcoR–AAC	5′-GACTGCGTACCAATTCAAC-3′
EcoR–AAG	5′-GACTGCGTACCAATTCAAG-3′
EcoR–ACA	5′-GACTGCGTACCAATTCACA-3′
EcoR–ACT	5′-GACTGCGTACCAATTCACT-3′
EcoR–AGG	5′-GACTGCGTACCAATTCAGG-3′
EcoR–ACC	5′-GACTGCGTACCAATTCACC-3′
EcoR–ACG	5′-GACTGCGTACCAATTCACG-3′
EcoR–AGC	5′-GACTGCGTACCAATTCAGC-3′
Mse–CAA	5′-GATGAGTCCTGAGTAACAA-3′
Mse–CAC	5′-GATGAGTCCTGAGTAACAC-3′
Mse–CAG	5′-GATGAGTCCTGAGTAACAG-3′
Mse–CAT	5′-GATGAGTCCTGAGTAACAT-3′
Mse–CCA	5′-GATGAGTCCTGAGTAACCA-3′
Hpa II–Msp I–TCCA	5′-ATCATGAGTCCTGCTCGGTCCA-3′
Hpa II–Msp I–TCAA	5′-ATCATGAGTCCTGCTCGGTCAA-3′

attracted widespread concerns. Recently, the studies about the influence of heavy metals on human or animal carcinogenesis and epigenetics have already made great progress; and the plant physiological ecology and biochemical processes affected by heavy metals have also been widely explored, however, there still has rarely reports on the heavy metals to the plant genomes and epigenetics.

In this study, *Arabidopsis* has been employed to explore the influence of nickel chloride ($NiCl_2$) on *Arabidopsis* genome stability and DNA methylation. The results have provided a new experimental basis for epigenetic toxicology studies of heavy metals.

2. Materials and methods

2.1. Materials

The plant material involved in this study was *Arabidopsis thaliana* Columbia wild type (*A. thaliana* ecotype the Columbia, col-0), kept in our laboratory.

2.2. Methods

2.2.1. Arabidopsis cultivation and DNA extraction

$NiCl_2$ solution with different concentrations was sterile filtered and added to the MS medium, and the final concentrations of Ni^{2+} were 50 μM, 100 μM, 150 μM, 250 μM, and 350 μM. The *Arabidopsis* seedlings were treated with $NiCl_2$ for 14 d, and the DNA in leaf cell was extracted for subsequent analysis.

Arabidopsis DNA was extracted with Universal Genomic DNA Extraction Kit Ver. 3.0 (purchased from TaKaRa Company). The DNA mass was detected with UV spectrophotometer and 0.8% agarose gel.

2.2.2. Genome information and DNA methylation analysis

The genome information was analyzed by amplified fragment length polymorphism (AFLP) molecular markers. The experimental details referred to the method from Hao et al. [23]. The DNA methylation was

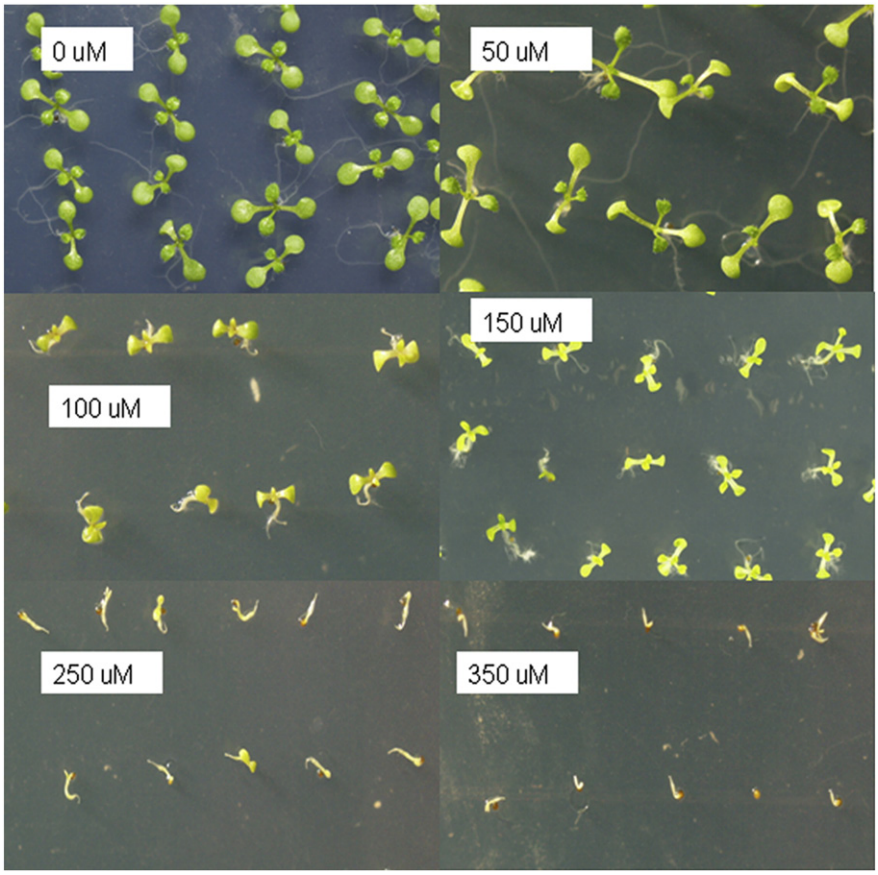

Fig. 1. Effect of $NiCl_2$ on growth of *Arabidopsis thaliana*.

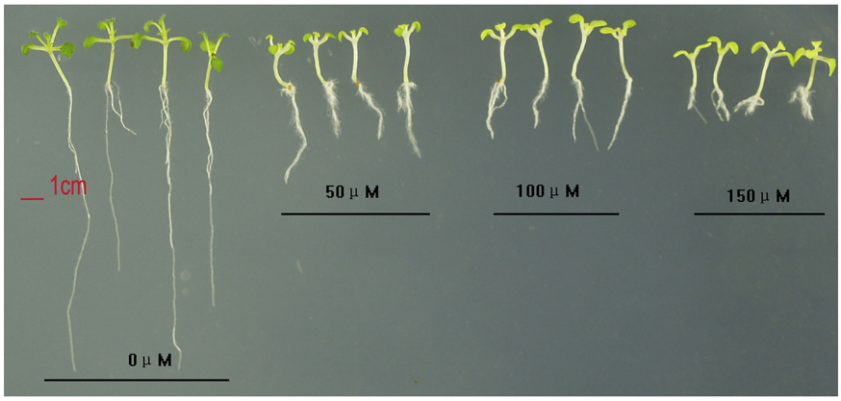

Fig. 2. Effect of $NiCl_2$ on the length of *Arabidopsis thaliana* roots.

analyzed with methylation sensitive amplification polymorphism (MSAP) molecular markers based on the method in previous study, with minor changes [24]. (All the enzymes were purchased from TaKaRa Company).

The adapters and primers involved in AFLP and MSAP molecular markers were synthesized by Shanghai Sangon Company (Table 1).

2.2.3. Other relevant experiments

Recovery and re-amplification of polymorphic fragments: the polymorphic DNA fragments were recovered from polyacrylamide gel. The target band was cut with clean blade and put into centrifuge tube. 50 μL deionized water was added to wash the ion and other impurities adsorbed on the gel surface. After washing, the water was sucked out, and 20 μL deionized water was re-added and then boiled for 10 min to release DNA. After cooling, the sample was centrifuged for 5 min at 12,000 rpm, and the target fragment in supernatant was re-amplified after centrifugation. The reaction system and thermal cycling program of secondary amplification have been the same as MSAP pre-amplification conditions. (The conditions and reaction systems of restriction, adapter-ligation, pre-amplification and selective amplification refer to Supplementary material 1).

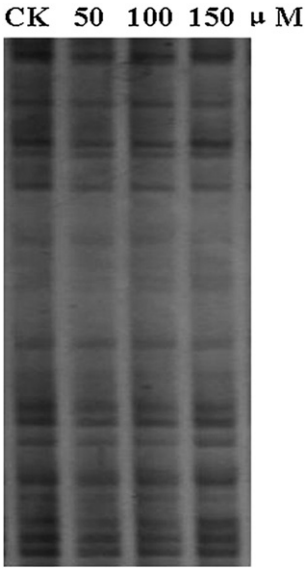

Fig. 3. AFLP mapping of *Arabidopsis* genomic DNA after $NiCl_2$ treatment. The bandings of the *Arabidopsis* genomic DNA AFLP after $NiCl_2$ treatments had no change according to control. CK represent control, 50, 100, and 150 μM represent the three kinds of $NiCl_2$ concentration.

The 18S rRNA was analyzed with EZ DNA methylation Gold kit (ZYMO RESEARCH, America).

The DNA target fragments in agarose gel were recovered and purified with TaKaRa Agarose Gel DNA Purification Kit Ver.2.0. (TaKaRa, China).

The competent cells were prepared and DNA fragments were cloned. The plasmid was extracted with TaKaRa MiniBEST Plasmid Purification Kit Ver. 2.0. (TaKaRa, China).

2.3. Statistics and analysis software

The data analyzed with SPSS 11.5. Primers were designed with Primer Premier 5. The homology was analyzed with ClustalX1.83, NCBI blast and the Contig 1 was involved for sequence assembly.

3. Results and analysis

3.1. The effects of $NiCl_2$ on the Arabidopsis growth and morphology

All the treatment of $NiCl_2$ inhibited *Arabidopsis* seed germination. The seed germination potential and germination rate had decreased obviously with the increase of the $NiCl_2$ concentration (data not show). The growth of seedlings was restrained and the seedlings showed loss of green with high concentration of $NiCl_2$ treatment (Fig. 1). The root growth also was inhibited significantly (Fig. 2).

3.2. Genome stability analysis

The 635 countable bands could be amplified by AFLP from 24 pairs of primers, and no polymorphism loci were found in these bands (Fig. 3). The result showed that the $NiCl_2$ treatment did not induce any detectable variations in DNA sequence.

3.3. The detection of genomic DNA methylation with MSAP molecular markers

MSAP molecular marker technique is a DNA methylation detection method based on isoschizomer Hpa II and Msp I, which enjoy different

Table 2
Hpa II and Msp I methylation sensitivity and restriction bands.

Methylation state	Activity		Restriction bands		Classification
	Hpa II	Msp I	H	M	
CCGGGGCC	Active	Active	+	+	I
CmCGGGGmCC	Inactive	Active	-	+	II
mCmCGGGGmCmC	Active	Inactive	+	-	III
mCCGGGGCC	Inactive	Inactive	-	-	IV

Note: "+" indicates that the site can be amplified a band; "-" means the opposite.

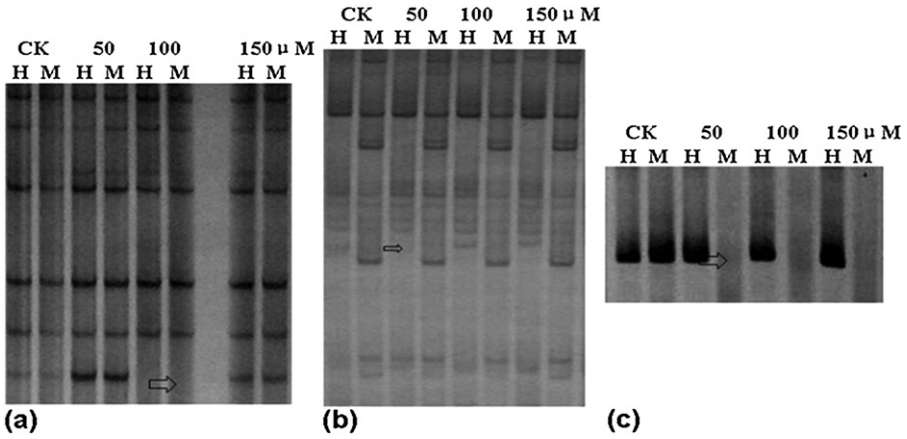

Fig. 4. MSAP mapping of *Arabidopsis* genomic DNA after $NiCl_2$ treatment. M indicated EcoR I/Msp I double digestion, H denoted the EcoR I/Hap II double digestion. Arrows point the difference bandings compare with control.

methylation sensitivities (Table 2). As for polymorphism band profile, II–I, III–I, IV–II, IV–I and IV–III demonstrated a decreased methylation of CCGG site; III–IV, I–II, I–III, I–IV, II–IV reflected increased methylation of CCGG site in genomic DNA. II–III, III–II suggested one chain with re-methylation and the other chain with demethylation and the overall level for methylation did not change (Fig. 4).

MSAP molecular marker analysis showed that, after $NiCl_2$ treatment, there was a simultaneously lowered and increased methylation in T_0 generation seedlings (Table 3). After the treatment of $NiCl_2$ with a concentration of 50 μM, 100 μM, and the increased methylation sites accounted for 4.95% and 5.94% of the total testing sites respectively, while after treatment with 150 μM $NiCl_2$, the methylation ratio reached up to 9.90%. After the treatment of these three concentrations, all the decreased methylation sites were 2.48% of the total proportion. All these data indicated that $NiCl_2$ treatment greatly influenced the methylation of *Arabidopsis* genome. The T_1 generation seedlings could be obtained by sowing T_0 generation seedling seeds in normal MS medium. In seedlings of T_1 generation (Table 4), the increased methylation sites were 1.47%, 1.49% and 1.49%, while the decreased methylation sites were 0.46%, 1.96% and 2.45%. The results suggested that the genome DNA methylation of offspring also impact by parent $NiCl_2$ treatment, which means that the $NiCl_2$ induced methylation could be heritable.

3.4. The analysis of MSAP specific band

After recycling and sequencing of the polymorphic bands in MSAP, four sequences could be obtained, which might be the specific sensitive sites for those increased methylation. The sequences analysis by NCBI Blast demonstrated that (Table 5) only the homologous sequences of S3 could be searched in GenBank (Supplementary material 2), but its function still needs to be further explored. The S1, S2 and S4 were unknown sequences because their homologous sequences could not be found in GenBank. The results were showed in Supplementary material 3.

3.5. The influence of $NiCl_2$ treatment on Arabidopsis nucleus

The previous studies suggest that Ni is an essential element for plant growth. There have been experiments showing that the low level of Ni could stimulate the plant growth, while most studies suggest that Ni with high concentration would be harmful to physiological and biochemical processes during plant growth and development. This study demonstrated that after $NiCl_2$ treatment, the nucleus structure

Table 3
Band profile variation of T_0 generation seedlings after $NiCl_2$ treatment for 10 d.

Band profile variation		$NiCl_2$ concentration (μM)			
		0 (control)	50	100	150
Bands with increased Methylation	I–III	0	0	2	8
	I–II	0	1	2	2
	I–IV	0	9	7	9
	II–IV	0	0	1	1
	III–II	0	0	0	0
	III–IV	0	0	0	0
Bands with decreased Methylation	II–I	0	0	0	0
	II–III	0	0	0	0
	III–I	0	1	1	0
	IV–I	0	4	2	1
	IV–II	0	0	2	3
	IV–III	0	0	0	1
Testing sites		202	202	202	202
Sites with increased methylation (%)		0^a	4.95^b	5.94^c	9.9^d
T-score (by T-test, $^{**}P < 0.05$)			-171.473**	-171.473**	-171.473**
Sites with decreased methylation (%)		0^a	2.48^b	2.48^b	2.48^b
T-score (by T-test, $^{**}P < 0.05$)			-170.456**	-170.456**	-170.456**

The data were analysis by one-way ANOVA statistical methods; different letters representing the difference between the two groups was significant ($P < 0.05$). The same letters indicate differences between the two groups did not reach significant levels. The ratios shown in the table are the mean.

Table 4
Band profile variation of *Arabidopsis* T1 generation after $NiCl_2$ treatment.

Band profile variation		$NiCl_2$ concentration (μM)			
		0	50	100	150
Bands with increased methylation	I–III	0	0	0	0
	I–II	0	0	0	0
	I–IV	0	0	0	0
	II–IV	0	2	3	3
	III–II	0	0	0	0
	III–IV	0	1	1	1
Bands with decreased methylation	II–I	0	0	0	1
	II–III	0	0	0	0
	III–I	0	0	0	0
	IV–I	0	1	1	2
	IV–II	0	0	1	0
	IV–III	0	0	2	2
Testing sites		204	204	204	204
Sites with increased methylation (%)		0^a	1.47^b	1.96^c	1.96^c
T-score (by T-test, $^{**}P < 0.05$)			-110.500**	-98.000**	-98.000**
Sites with decreased methylation (%)		0^a	0.49^b	1.96^c	2.45^d
T-score (by T-test, $^{**}P < 0.05$)			-148.000**	-98.000**	-91.750**

The data were analysis by one-way ANOVA statistical methods; different letters representing the difference between the two groups was significant ($P < 0.05$). The same letters indicate differences between the two groups did not reach significant levels. The ratios shown in the table are the mean.

Table 5
The analysis of polymorphic sequences.

Sequences	Band profile variation	Gene mapping
1	I–IV	No
2	50 μM, 150 μM treatment I–IV	No
3	50 μM, 150 μM treatment I–IV	EZ286504.1, 571–713 bp
4	I–II	No

Table 6
The influence of $NiCl_2$ treatment on *Arabidopsis* nucleus.

Concentration (μM)	The number of cell with abnormal nucleoli (%)		Percentage of cell with abnormal nucleoli (%)
	2 nucleoli	3 nucleoli	
0	0.098	0	0.098
50	0.413	0.048	0.461**
100	0.571	0.092	0.663**
150	0.719	0.269	0.988**

T-test: $^{**}P < 0.05$.

of *Arabidopsis* apical cell would be deformed and damaged, resulting in the formation of multiple micro-nucleoli (Fig. 5). The nucleolus injury would be intensified with increased $NiCl_2$ concentration (Table 6). From statistical analysis, the treatment of 50 μM, 100 μM and 150 μM $NiCl_2$ had a significant effect on the variation of *Arabidopsis* nucleolus. In addition, the probability of multiple nucleoli would increase with the increased $NiCl_2$ concentration, means more serious damage of nucleolus.

3.6. The methylation analysis of 18S rDNA

After treating genomic DNA with sulfite, the 18 s rDNA locus was amplified by PCR. The cytosine with methylation would be retained, while the cytosine without methylation would be directly converted to thymine. The PCR products were recovered and sequenced. The sequence analysis showed that, compared with control, the T_0 generation seedlings after treating with three concentrations of $NiCl_2$ enjoyed increased methylation sites of 11.22%, 18.88% and 12.24% respectively, while the decreased methylation sites were 6.12%, 5.61% and 3.06% respectively. The increased sites were 2–3 times more than the decreased sites, indicating that there was a trend of increased methylation (Table 7). The sequence alignment file was shown in Supplementary material 4.

4. Discussions

In general, the heavy metals in environment would result in epigenetic variation of animals and plants, which included histone modification and DNA methylation. A large number of studies have shown that the treatment of heavy metal may take an important role in the carcinogenesis of heavy metals (nickel, cadmium, arsenic, chromium, etc.). Chen et al. [17] found that nickel ions would increase the dimethylation level in H3K4; Zhou et al. [21] explored that metal compounds including nickel, chromate, arsenious acid affected the methylation of H3K4 gene, leading to the modification of histones [19,21,22]; Klein et al. [15] found that nickel compounds could induce DNA methylation and chromatin variation, resulting in gene silencing [15,24]. In addition, nickel could lead to modification missing in histone H2A, H2B, H3, and H4, and bring about increased demethylation of H3K9 and generally improved ubiquitination level of H2A and H2B [16,17,18,19,20,21,22,23,24]. The plant genomic DNA changed and rapidly responded to the environmental stress such as chilling, salts, heavy metal and pollutants [25,26,27]. The heritable methylation and phenotypic variations in these responses have been much vital for the research of plant breeding. Boyko et al. [28] studied the genome stability under chlorine ions stress, and showed that chlorine ions would influence on somatic cell and change the recombination rate. Researchers also believed that the variation of epigenetic information was an important adaptation mechanism to adversity of environment [29,30,31,32,33].

In this study, the genomic DNA methylation detected by MSAP molecular markers demonstrated that the lowered and increased methylation both existed in T_0 generation seedlings after $NiCl_2$ treatment. The proportion of methylation was significantly higher than demethylation. Part of de novo methylation and demethylation sites would maintain in T_1 generation seedlings, which mean that $NiCl_2$ could induce heritable methylation variations. A lot of previous studies also explored that the methylation of *Arabidopsis* induced by adversities such as salt, UV light, cold, heat and floods could be maintained in the offspring [34,35,36,37,38,39,40]. The genome test analyzed by AFLP molecular markers demonstrated that the genome sequence of *Arabidopsis* did not mutate, which meant that the influence of $NiCl_2$ treatment on plants may be mainly epigenetic modification. This result was consistent with that from animals. The study on animals also suggested that Ni would affect the activity of histone demethylase (JMJD1A), DNA repair enzyme (ABH2) [41], iron-and 2-oxoglutarate dependent dioxygenase [38] and histone deubiquitinating enzyme [39]. Thus, it is still necessary to study the pathways through which the Ni and related compounds change the epigenetics of plant, and whether this variation was one kind of plant adaptation to heavy metals.

The previous studies demonstrated that plant nucleus may be damaged or mutated after treatment with heavy metal. After inducing by the combination of heavy metal and calcium, the number, size and nucleolus activity of epidermal cell in pea roots could be influenced [42]. The toxic effect of heavy metals on plant nucleolus was also found in maize [43], turnip [44] and soybean [45]. After processing of copper or lead compounds with a certain concentration and processing time, it would result in disintegration of nucleolus, formation of micro-nucleolar particles and outflow of nucleolus into the cytoplasm. The similar results have also been found in this study.

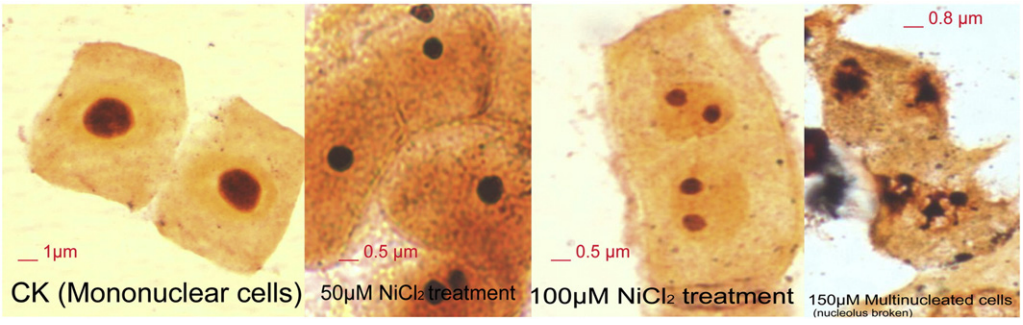

Fig. 5. The nucleolus of *Arabidopsis* root tip cell were damaged after $NiCl_2$ added.

Table 7
Analysis of the methylation of 18S rDNA.

	50 μM	100 μM	150 μM
The rate of cytosine methylation	11.22%**	18.88%**	12.24%**
The rate of cytosine demethylation	6.12%**	5.61%**	3.06%**

** Indicate differences between treatment group and control ($P < 0.05$).

The treatment of $NiCl_2$ with different concentrations damaged and deformed the nucleolar structure in root tip cells of *Arabidopsis*. There was a positive correlation between nucleolar damage and $NiCl_2$ concentration. After analyzing the cytosine methylation of nucleolar 18S rDNA, the obvious upward trend can be found. The damage of nucleolar structure and the methylation variation of rDNA would be bound to affect its normal function, which also brought genetic influence on cell physiological and biochemical processes. We assume that there may be a direct or indirect relationship between the nucleolus variation and rDNA methylation, and it is possible that the plant nucleolus variation and 18S rDNA methylation becomes an examination indicator for Ni pollution in soil or plant.

Epigenetic changes have been reported as stable, but they are also controlled by varying factors which include environmental, physiological and pathological factors. Epigenetic changes also represent a potentially rich source of novel biomarkers, from potential uses in early diagnosis and screening to multiple studies showing links to prognosis or prediction of therapeutic responses. The goal of biomarker research is to identify genes, proteins, lipids and metabolites that have diagnostic or prognostic value in managing human disease [46,47,48,49]. DNA methylation has emerged as a highly promising biomarker and aberrant DNA methylation patterns have been associated with multiple cancers and metabolic disorders such as obesity [50]. The analysis of DNA methylation-based biomarkers is rapidly advancing, and a large number of potential biomarkers have been identified [51]. Someone had suggested that DNA methylation also could be used as a biomarker to assess soil heavy metals [52]. Epigenetic changes can occur at a high frequency in plants and might generate phenotypic variation that is not correlated with genetic variation, but few studies have assessed the association between the epigenetic changes and biomarker in plants. DNA mismatch repair related gene expression was used as potential biomarkers to assess cadmium exposure in *Arabidopsis* seedlings [53]. The effects of nickel compounds on DNA methylation and gene expression in plants remain to be elucidated. In this study, nickel chloride treatment could significantly increase DNA methylation in plants in a dose-dependent and transient manner independent of sequence context, this pattern of DNA methylation could be stably inherited from one generation to the next, and DNA methylation changes were also observed in 18S rDNA. Methylation is connected with transcriptional reactivation and partial decondensation of heterochromatin [54]. Nickel chloride represents a promising new and versatile epigenetic tool for investigating the role of DNA methylation in heavy metal pollution of plants with regard to transcriptional control, maintenance and formation of (hetero-) chromatin. Nickel chloride treatment could induce nucleolus injury and increase DNA methylation, which was similar to the effects of other heavy metals on epigenetic changes [55,56]. It is possible that the epigenetic changes induced by nickel chloride could be used as a useful epigenetic marker in polluted plants and would improve environmental risk assessment.

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