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HIGH LIGHTS:

- MSI occurred *in Arabidopsis* seedlings only under Cd stress of 8.0 $mg \cdot L^{-1}$ for 15 d.
- Aberrations in DNA methylation were induced by Cd of 0.25-5.0 $mg \cdot L^{-1}$ using MSAP-PCR.
- Sensitivity of biomarkers for Cd followed: DNA methylation loci >> RAPD > MSI.
- MSAP-PCR emerged as *a powerful tool for* early diagnosis *of Cd stress.*

Cadmium-induced genomic instability in Arabidopsis: molecular toxicological biomarkers for early diagnosis of cadmium stress

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ABSTRACT: Microsatellite instability (MSI) analysis, random-amplified polymorphic DNA (RAPD), and methylation-sensitive arbitrarily primed PCR (MSAP-PCR) are methods to evaluate the toxicity of environmental pollutants in stress-treated plants and human cancer cells. Here, we evaluate these techniques to screen for genetic and epigenetic alterations of Arabidopsis plantlets exposed to 0-5.0 $mg \cdot L^{-1}$ cadmium (Cd) for 15 d. There was a substantial increase in RAPD polymorphism of 24.5, and in genomic methylation polymorphism of 30.5-34.5 at CpG and of 14.5-20 at CHG sites under Cd stress of 5.0 $mg \cdot L^{-1}$ by RAPD and of 0.25-5.0 $mg \cdot L^{-1}$ by MSAP-PCR, respectively. However, only a tiny increase of 1.5 loci by RAPD occurred under Cd stress of 4.0 $mg \cdot L^{-1}$, and an additional high dose (8.0 $mg \cdot L^{-1}$) resulted in one repeat by MSI analysis. MSAP-PCR ~~has~~ detected the most significant epigenetic modifications in plantlets exposed to Cd stress, and the patterns of hypermethylation and polymorphisms were consistent with~~effectsmanifest the~~ inverted U-shaped dose responses. The presence of genomic methylation polymorphism in Cd-treated seedlings, prior to the onset of RAPD polymorphism, MSI and obvious growth effects, suggests that these altered DNA methylation loci are the most sensitive biomarkers for early diagnosis and risk assessment of genotoxic effects of Cd pollution in ecotoxicology.

Keywords: Microsatellite instability, Random-amplified polymorphic DNA, Methylation-sensitive arbitrarily primed PCR, Cadmium, *Arabidopsis thaliana*

1. Introduction

Environmental contamination with heavy metals has ~~drastically~~ increased

substantially over the past decades, which has aroused attention from numerous researchers responsible for contamination diagnosis, environmental protection and ecological remediation. Cd, as one of the most highly toxic and accumulative heavy metals, has contaminated soils and water through atmospheric deposition, urban-industrial activities and agricultural practices (Sun et al., 2008; Liu et al., 2011). Thus, the exploration of Cd toxicity, especially its genotoxic effects ~~of Cd~~, has become a focus in ecotoxicology research (Herbette et al., 2006; Azevedo et al., 2007; Greco et al., 2012; Pierron et al., 2014).

Several classic assays have been applied to examine the genotoxic effects of heavy metals on organisms, e.g. the micronucleus assay and the comet assay. However, neither is sensitive enough to determine the Cd contamination level or able to provide information at the DNA level (Cambier et al., 2010). With the rapid development of molecular biology, several PCR-based techniques have been used to evaluate DNA damage in toxicology studies. Random-amplified polymorphic DNA (RAPD) comprises~~involves the use of~~ a single “arbitrary” primer in a PCR reaction and results in the amplification of several discrete DNA products (Welsh and McClelland, 1990; Williams et al., 1990). RAPD has been used extensively in eco-toxicology studies on animals and humans (Misra et al., 2001; Singh and Roy, 2001,2004; Garnis et al., 2005). Recently, RAPD ~~technique~~ has been used in plant eco-toxicology studies, detecting genetic variation and mutation induced by stresses (Miki et al., 2001; Liu et al., 2005; 2007; Chao et al., 2008; 2009; 2012; Gjorgieva et al., 2013; Malar et al., 2014). RAPD analysis is extremely efficient for DNA analysis in complex genomes. Its use in investigating genomic DNA to detect different kinds of DNA damage and mutations suggests that RAPD can potentially form the basis of novel biomarker assays for the detection of DNA damage and mutations (Lopez-Moreno et al., 2010; Korpe and Aras, 2011).

Regarding DNA repair, Cd suppresses both eukaryotic MutS homolog ~~(~~MSH~~)~~2-MSH6- and MSH2-MSH3-dependent mismatch repair (MMR) activity through a nonspecific mechanism whereby~~involving binding of~~ Cd binds to multiple sites in MSH6 (Banerjee and Flores-Rozas, 2005; Wieland et al., 2009; Li et al., 2013). Loss or low activity of MMR proteins results in losses and gains of dinucleotide repeats in microsatellites, also known as simple sequence repeats (SSRs), leading to microsatellite instability (MSI). Previously, MSI was found at two “hotspots” of microsatellite mutation in mice by analysis of a panel of microsatellite markers under Cd stress (Oliveira et al., 2012; Du et al., 2014). Also, ~~there were some reports that~~ SSRs mutations can~~ould~~ be promoted by heavy metals, e.g. Lead, Chromium and Cd (Rodriguez et al., 2013a; Rodriguez et al., 2013b; Du et al., 2014). Monteiro et al. (Monteiro et al., 2009) demonstrated that SSR analyses could be used to evaluate plant genomic DNA instability for deciphering DNA damage induced by Cd in a genotoxicity test. Thus, MSI can be considered as a biomarker of high risk for human cancer and for the assessment of different genotoxic effects of pollutants on plants~~, respectively~~ (Kovalchuk et al., 2000; Soreide et al., 2006). However, little information is available about microsatellite mutations in Arabidopsis under Cd stress.

Higher plants can rapidly adapt in response to stress~~es~~, but this can lead to genome instability and changes in DNA methylation patterns throughout the genome and at specific loci of DNA (Boyko et al., 2010; Chatterjee and Vinson, 2012). For example, plants employ important and sophisticated epigenetic regulatory strategies, such as DNA methylation, to maintain genomic plasticity. This~~,~~ allow~~sing~~ the simultaneous regulation of many genes involved in plant defense and in facilitating relatively rapid adaptation to new internal and environmental stress conditions without change~~sing~~ in ~~the~~ DNA sequence (Steward et al., 2002; Santoyo et al., 2011). The genomic DNA

hypermethylation pattern is~~was found to be~~ correlated with water deficit in pea (*Pisum sativum* L.), pathogen stress in tobacco (*Nicotiana tabacum*), radioactive contamination in *Pinus silvestris*, salt, UVC, cold, heat and flood stresses in Arabidopsis, and chromium stress in *Brassica napus*~~, respectively~~ (Labra et al., 2002; Kovalchuk et al., 2003; Labra et al., 2004; Boyko et al., 2007; Mason et al., 2008). Also, DNA hypomethylation at several genomic loci occurred in response to heavy metal stress in hemp and clover, to pathogen stress in tobacco (*Nicotiana tabacum*), and to cold treatment in nucleosome core regions of maize (*Zea mays*) seedlings (Steward et al., 2002; Aina et al., 2004; Boyko et al., 2007). Potentially, global DNA hypomethylation is often viewed as a sign of genomic instability, which manifests as gross chromosomal aberrations, genome rearrangement, MSI, deletions/insertions, and point mutations, thus facilitating gene divergence and evolution of novel gene functions (Boyko et al., 2007; Duan et al., 2013; Harris et al., 2013).

Huang et al. (Huang et al., 2012) reported that tumor cells usually exhibit diverse patterns of DNA methylation, and that the CpG dinucleotide at any given site of genomic DNA might differ in methylation status among subpopulations of tumor cells, reflecting the heterogeneity and decreased epigenetic fidelity of cancer cells. Thus, CpG dinucleotides are also called methylation variable positions (MVPs) in epigenetic studies. Analysis of MVP profiles might reveal stress-specific methylation patterns, and the methylation frequency at any given MVPs could serve as a novel epigenetic biomarker for physiological and pathologic status (Huang et al., 2012; Kit et al., 2012). Moreover, minimal traces/differences of aberrant methylation profiles in blood DNA could serve as early surrogate biomarkers for cancer diagnosis and for risk assessment of PAHs exposure, respectively (Yang et al., 2012; Shin et al., 2013). Similarly, ~~Santoyo et al (2011) reported that actual status of~~ global DNA methylation in earthworms is apparently affected by the overall effect of

metals/metalloids undergoing methylation and ~~that~~ this parameter might be ~~considered as~~ a candidate biomarker of epigenetic risks related to the presence of the metal elements in terrestrial environments (Santoyo et al., 2011).

Arabidopsis, with its vast resource of mutants, has long been used as a model dicotyledonous higher plant for studies of plant physiology and plant molecular biology. However, little information is available about potential associations between the status of global DNA methylation and genomic instability such as MSI and RAPD DNA polymorphisms in Arabidopsis seedlings under Cd stress. Therefore, the principal aims of this work were to (1) evaluate the extent of genomic instability ~~in Arabidopsis seedlings~~ using a panel of 36 microsatellite markers and 3 RAPD primers; (2) determine the status of global DNA methylation by MSAP-PCR ~~in the Arabidopsis genome~~ under Cd stress; (3) explore potential associations between the actual status of global DNA methylation and genomic instability such as MSI and RAPD DNA polymorphisms in Arabidopsis under Cd stress. In-so-doing, we were able to evaluate the aforementioned parameters as candidate biomarkers for early diagnosis of genetic and epigenetic risks related to the presence of Cd in the soil and water environment.

2. Materials and methods

2.1 Plant materials, treatment conditions and DNA isolation

Arabidopsis thaliana seeds (Arabidopsis, Columbia ecotype) were sterilized in dilute bleach solution (bleach with surfactant diluted 1:10 with water) for 5 min, washed in sterile water and placed in an ethanol mix (ethanol : water : bleach 7:2:1) for 5 min. Seeds were then rinsed in sterile distilled water five times and placed in 4 °C for 24-36 h.

Seeds were sown in sterile flasks supplemented with 150 mL 0.5× liquid MS medium (SIGMA, USA) including 0.5% (w/v) sucrose (pH5.8), supplemented with Cd at a final concentration of 0 (the control), 0.25, 1.0, 4.0, 5.0, and 8.0 $mg \cdot L^{-1}$ (only used in MSI analysis) in the form of $CdCl_2 \cdot 2H_2O$ of analytical grade with purity 99.5%, PR China. Each flask with about 20 plantlets was placed on a rotary shaker at 50 rpm in an incubator (12 h light of approximately 3000 lx and 12 h dark at 21 ±0.5 °C) for 15 d. All treatments and analyses ~~performed below~~ were repeated in three independent replicates.

The seed germination rate and growth of the plantlets were measured at 15 d following germination, and then pooled to measure DNA content in Arabidopsis shoots described in a previous study (Liu et al., 2009).

For MSI, RAPD, and DNA methylation assays, all DNA extractions and downstream applications were performed on mixed groups using the same batches of reagents. In addition, the control and Cd-exposed samples were collected at 15 d of incubation, and snap frozen in liquid nitrogen prior to storage at -70 ˚C. Genomic DNA from approximately 200-400 mg of fresh Arabidopsis shoots was extracted respectively using Plant Genomic DNA Extraction Kit. DNA concentration was examined by Eppendorf Biophotometor Plus, and then verified by DL2000 DNA marker in a 0.8% agarose gel. All experimental chemicals were ordered from TaKaRa Biotechnology Ltd. (Dalian, PR China) unless otherwise mentioned.

2.2 MSI Analysis

46 pairs of SSR primers (Table A.1) were screened in this test for finding genomic DNA MSI induced by Cd. PCRs were performed in a final volume of 25 μL consisting of 25 ng DNA template, 1 U of Taq polymerase, 1× Taq reaction buffer, 0.2 mM dNTPs, and 0.25 μM of each primer. The touchdown PCR conditions used for amplification were as follows: initial denaturation at

94°C for 5 min; ~~followed by~~ 9 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and primer extension for 30 s; the annealing temperature was subsequently decreased by 0.5°C every second cycle until a 'touchdown' at 53°C, at which temperature 26 additional cycles followed~~were carried out~~ (35 cycles in total). After the PCR reaction, amplification products were examined using 8% (w/v) polyacrylamide gel electrophoresis (PAGE) with 7 M urea, visualized by silver staining method using the Bio Image Analyzer System (Bio-Rad, Vendor, Italy). To assess MSI, we compared the band pattern produced after gel electrophoresis of paired PCR reactions containing the control and Cd-treated DNA. If the control and Cd-treated PCR amplification products displayed different electrophoretic motilities, the case was scored as positive for MSI.

2.3 RAPD genetic damage screening

RAPD analysis was performed using 3 primers (Primers 1, 2 and 4) screened from 12 random primers (Liu et al., 2005). PCRs were performed in reaction mixtures of 25 µL containing approximately 40 ng of DNA template, 0.5 µM primer, 0.2 mM dNTPs, 1× reaction buffer and 1 U of Taq DNA polymerase. The RAPD protocol ~~is~~ consisted of an initial denaturing step of 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 38 °C for 60 s and extension at 72 °C for 90 s, with an additional extension period of 10 min at 72 °C. PCR products were separated by electrophoresis in a 5% PAGE (50% urea). Gels were stained using silver nitrate. Images were digitally captured and the intensity of bands on the gel quantified using Image Lab (Bio-Rad).

2.4 Global DNA methylation assay using MSAP-PCR

Global DNA methylation was evaluated by methylation-sensitive arbitrarily primed PCR (MSAP-PCR). Genomic DNA (1 μg) was digested in CutSmart buffer with 1μL of HpaII or MspI (NEB, USA) in a 50-μL reaction volume for 1.5 h at 37°C. Digested DNA was purified and collected by Takara MiniBest DNA Fragment Purification Kit, then was transferred to another 50-μL reaction containing 1 × EcoRI buffer and 1μL EcoRI, digested for 1.5 h at 37°C. After purification, digested DNA was used for subsequent PCR reaction performed as previously published (Gonzalgo et al., 1997). HpaII does not cut DNA if the internal cytosine of CCGG restriction site is methylated and therefore the bands generated in the PCR of DNA digested by EcoRI + HpaII lane are methylated at the internal cytosine. MspI is unable to cut DNA when the external cytosine in CCGG site is methylated and so the bands generated in the PCR of DNA digested by EcoRI + MspI are methylated at the external cytosine. Three primers designed by our lab and MLG2 (Gonzalgo et al., 1997) were used for methylation assay (Table A.2). ~~And~~ PCR products were resolved on 5% PAGE with 50% urea. Methods of gel staining and image capture were the same as~~with~~ the above. An increase in band intensity indicates an increase in methylation. Band intensity was measured to numerically determine the change in DNA methylation.

2.5 Statistical analyses

Polymorphism frequency of RAPD and MSAP-PCR was calculated from PAGE results and analyzed in linear graphs using Microsoft Excel. Presence or absence of a band was counted as one locus in polymorphism frequency, while a band was calculated as 0.5 locus if its intensity changed $\geqslant$ 50% ~~as~~ compared to the control. The stable polymorphism variation was calculated for

data statistics exclusively if it occurred simultaneously in at least two replications, leading to data without variance.

3. Results

3.1 MSI assay using SSRs

MSI was not detected in plantlets exposed to the control and 0.25-5.0 $mg \cdot L^{-1}$ Cd for 15 d by a panel of 46 SSR primers. However, under an additional treatment of 8.0 $mg \cdot L^{-1}$ Cd, replication slipping of one repeat (about 2-3 bp) was found using SSR primers of BSAT1.001 and BSAT2.012 (Fig. 1). Moreover, the above replication slipping of three duplicates did appear in shoots under Cd stress of 8 $mg \cdot L^{-1}$, suggesting that MSI occurs exclusively under such a strong stress that ~~remarkably~~ inhibited Arabidopsis growth (Fig. A.1).

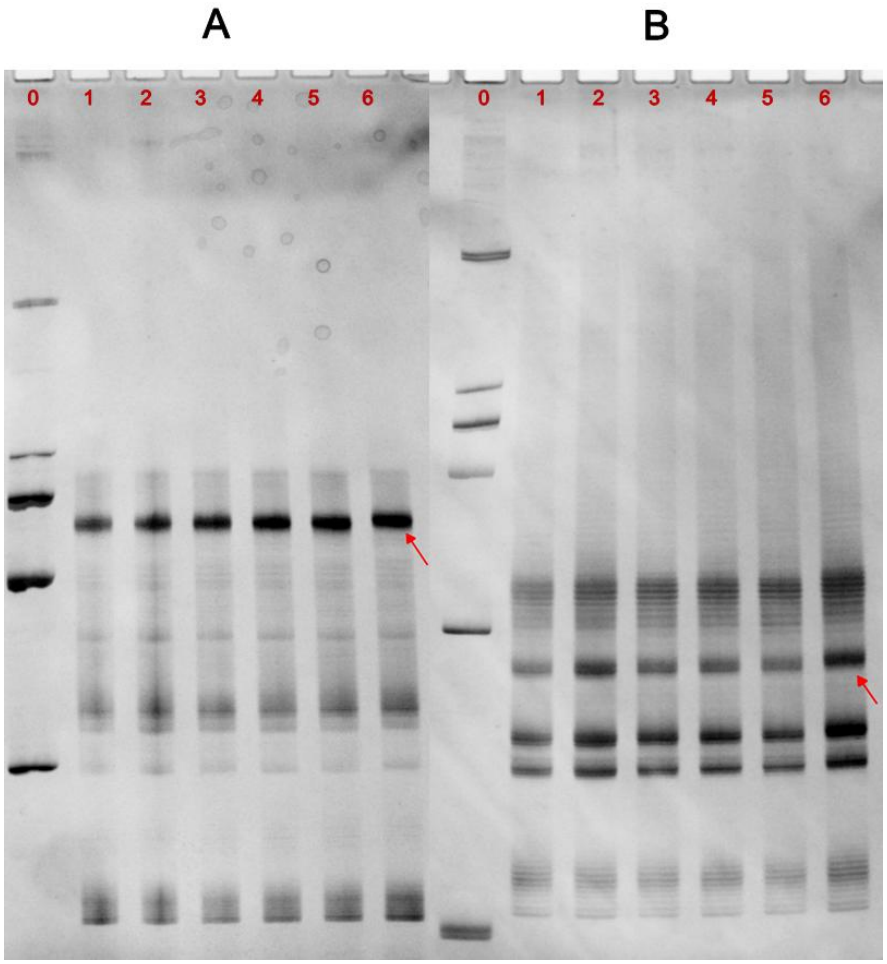


Fig. 1 MSI in Arabidopsis seedlings exposed to 0-8.0 mg·L^{-1} Cd for 15 d. (A) PAGE profiles amplified by primer BSAT1.001, and (B) by BSAT2.012, in which lanes (1) - (6) are PCR fragments amplified from the DNA templates of Arabidopsis shoots exposed to 0, 0.25, 1.0, 4.0, 5.0 and 8.0 mg·L^{-1} Cd for 15 d, respectively. Lane (0) is Takara DNA Marker 2000 (2000, 1000, 750, 500, 250 and 100 bp from top to bottom), and the same is below.

3.2 Genetic damage assay using RAPDs

Fig. 2 shows RAPD profiles in Arabidopsis seedlings grown with~~out~~ or ~~and~~ without Cd treatments after 15 d of exposure. In Fig. 2, each lane contains at least 10 bands ranged from~~in molecular size of~~ 300 to 3000 bp, in which bands of variable intensity detected in at least two replicates are calculated in Fig. 3.

Compared with the controls, pPolymorphic bands were not detected between in the unexposed and exposed plantlets of in the 0.25 and 1.0 mg·L^{-1} Cd treatment, for different primers. 4.0 mg·L^{-1} Cd treatment induced low polymorphism whereas high polymorphism occurred at the 5.0 mg·L^{-1} Cd treatment. Numbers of polymorphism loci were 1.5 and 24.5 for 4.0 and 5.0 mg·L^{-1} Cd, respectively (Fig. 3). In these cases, RAPD polymorphisms were due to the loss and/or gain of amplified bands in the treated samples compared with the control.

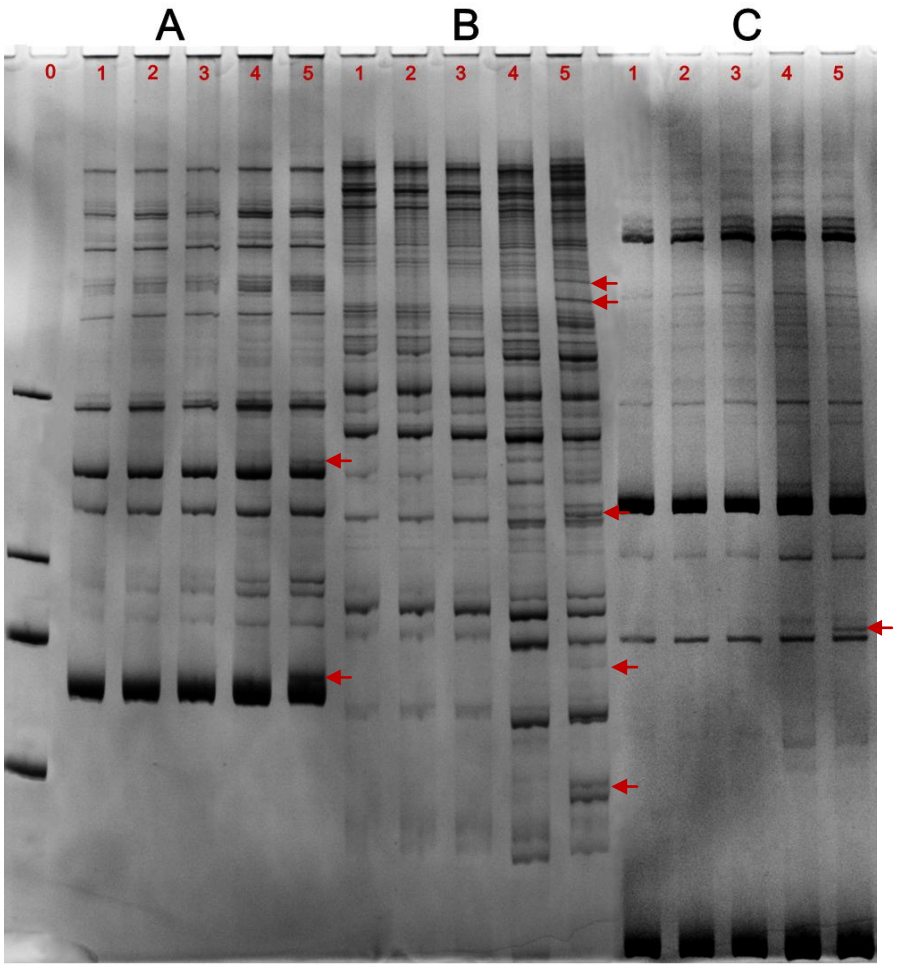


Fig. 2 RAPD fingerprints of Arabidopsis seedlings exposed to 0-5.0 mg·L^{-1} Cd for 15 d. (A) PAGE profiles amplified by primer 1, (B) by primer 2 and (C) by primer 4, in which lanes (1) - (5) are PCR fragments amplified from the DNA templates of Arabidopsis shoots exposed to 0, 0.25, 1.0, 4.0 and 5.0 mg·L^{-1} Cd for 15 d, respectively, and lane (0) is Takara DNA Marker 2000 (100

and 250 bp bands of DNA marker migrated out of the gel for high resolution). Red arrows illustrated polymorphism between plantlets exposed to 5.0 mg·L^{-1} Cd and the control.

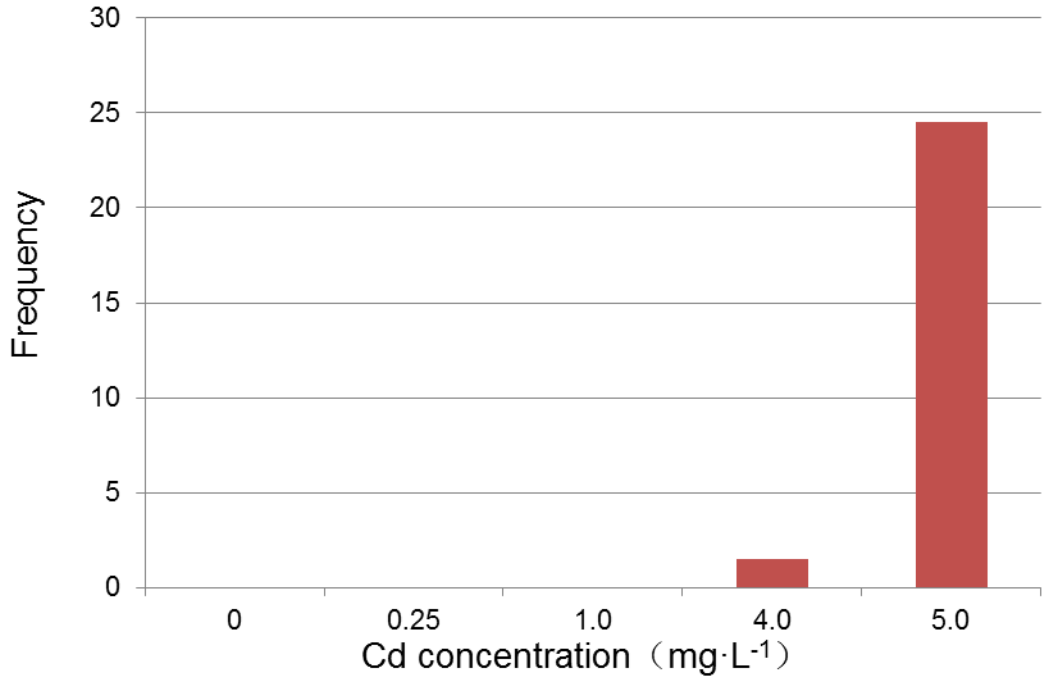


Fig. 3 Polymorphism variations detected by RAPD from Arabidopsis plantlets exposed to 0 - 5.0 mg·L^{-1} Cd for 15 d.

3.3 Methylation damage assay using MSAP-PCR

Aberrations of global DNA methylation in Arabidopsis induced by Cd was evaluated by MSAP-PCR. For all treatments, reproducible bands or cytosine residues of variable intensity were evaluated (Fig. 4) and calculated in Fig. 5 for methylation analysis at both internal (HpaII) and external (MspI) cytosines of CCGG sites. At the external cytosine, Cd stress resulted in global hypermethylation in the shoot tissues relative to the controls (Fig. 5A). This hypermethylation more closely follows an inverted U-shaped dose-response to Cd. The increase was 9.5, 9.5, 14.5 and 9 loci in plantlets exposed to Cd of 0.25, 1.0, 4.0 and 5.0 mg·L^{-1} Cd, respectively. Notably~~Yet~~, the hypomethylation

manifested an invariable status under all Cd stresses tested (Fig. 5A).

At the internal cytosine, a similar trend for hypermethylation and hypomethylation under Cd stress was observed as compared to the untreated control (Fig. 5B). The numbers of hypermethylation loci were 24.5, 27.5, 26 and 26 at 0.25, 1.0, 4.0 and 5.0 $mg \cdot L^{-1}$ Cd doses, respectively, which were prominently more than that at the external cytosine. Moreover, hypomethylation ~~observed~~ arose linearly albeit in a low proportion of increase (Fig. 5B).

Different methylation polymorphic bands were detected at 0.25-5.0 $mg \cdot L^{-1}$ ~~concentration of~~ Cd for different cytosines in the shoots (Fig. 5). Also, these polymorphisms indicate similar inverted U-shaped concentration response curves under Cd stress, and the inverted U-shaped curves were maximum at Cd of 1.0 and 4.0 mg L^{-1} (Fig. 5) for the internal and external cytosines, respectively. In all cases, methylation polymorphisms were due to the loss and gain of variable intensity of reproducible amplified bands in the Cd-treated seedlings as compared to the control (Fig. 4).

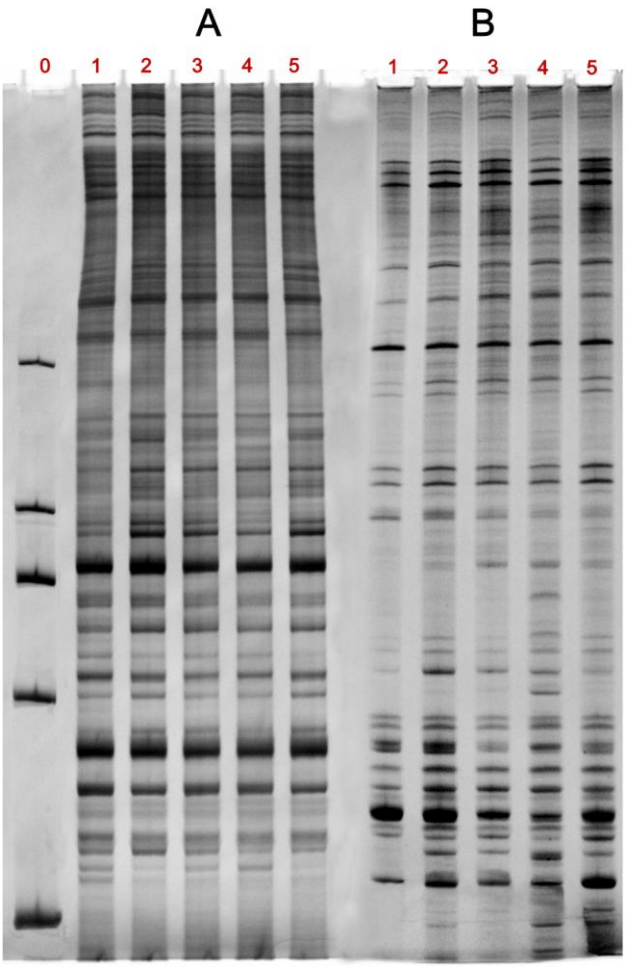
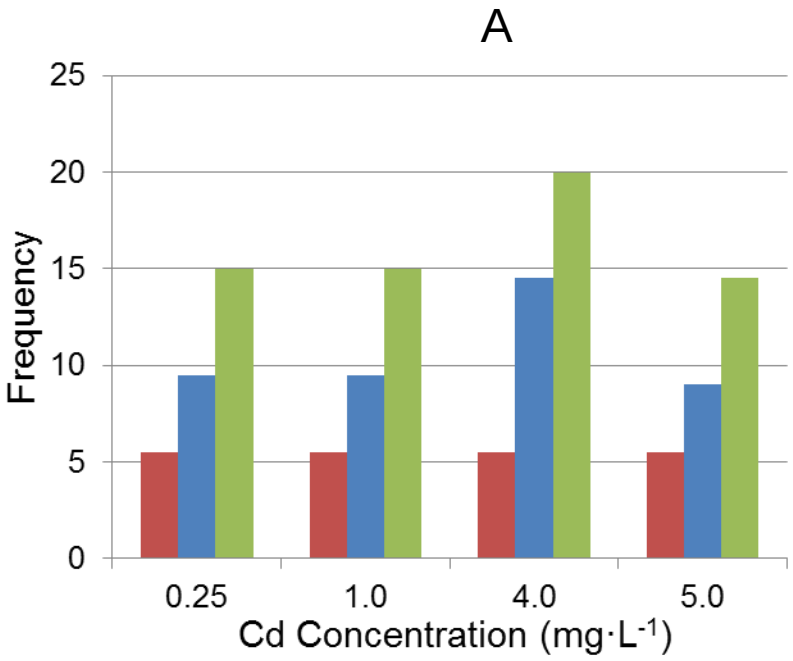


Fig. 4 The representative MSAP-PCR profiles of Arabidopsis seedlings exposed to 0 - 5.0 mg·L^{-1} Cd for 15 d. (A) PAGE profiles amplified by primer MLG2 from DNA templates digested by EcoRI+MspI and (B) EcoRI+HpaII, in which lanes (1) - (5) are PCR fragments amplified from the DNA templates of Arabidopsis shoots exposed to 0, 0.25, 1.0, 4.0 and 5.0 mg·L^{-1} Cd for 15 d, respectively, and lane (0) is Takara DNA Marker 2000 (100 bp band of DNA marker migrated out of the gel for high resolution).



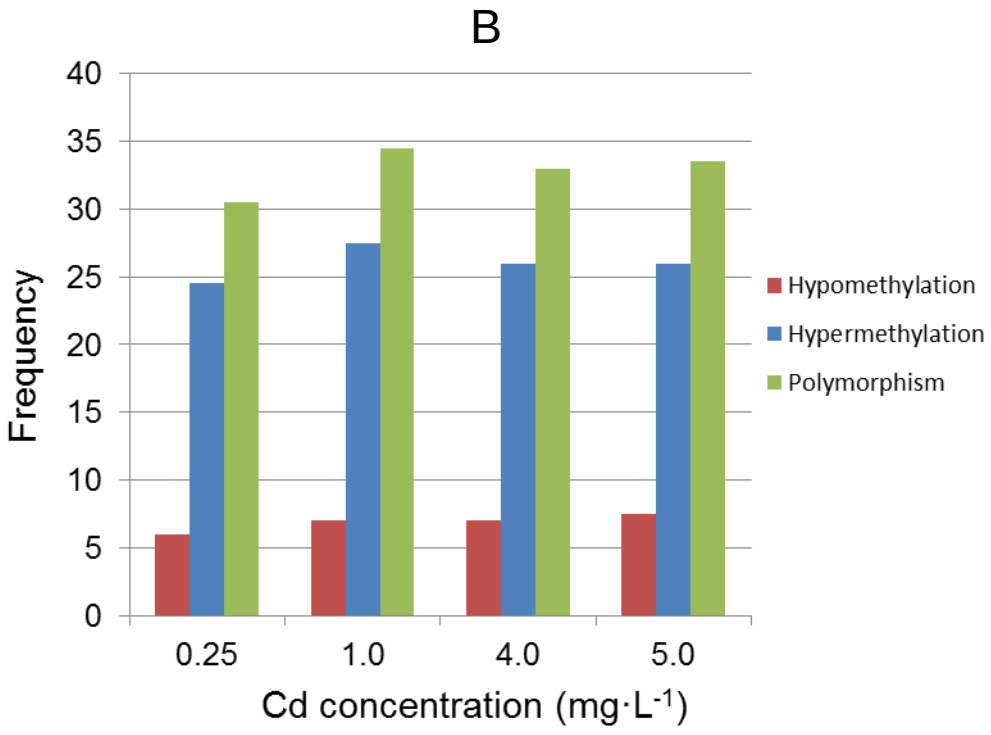


Fig. 5 DNA methylation variations detected by MSAP-PCR from Arabidopsis plantlets exposed to 0 - 5.0 mg·L^{-1} Cd for 15 d. (A) Frequency of hypermethylation, hypomethylation and methylation polymorphism at external cytosine (CHG) loci and (B) internal cytosine (C_PG) loci.

4. Discussion

4.1 MSI ~~is sensibility-insufficient~~ in vivo is not ~~as~~ a sensitive biomarker for Cd stress

Cd has been responsible for proofreading and MMR deficiency that could cause genomic MSI (Jin et al., 2003; Edelbrock et al., 2013). As far as we know, SSRs was first adopted by Kovalchuk et al. (2000) as tools to assess genetic instability in the offspring of wheat plants exposed to radiation near the Chernobyl nuclear power plant. More recently, positive results of MSI have been reported in transgenic plants consisting of a modified *GUS* reporter whose reading frame is disrupted by microsatellite repeat sequence (Yao and Kovalchuk, 2011). Thus, MSI analysis has been considered as a biomarker to detect genetic damages in ecotoxicology.

In the present study, MSI was not observed for the Arabidopsis seedlings exposed to Cd stress of 0.25-5.0 $mg \cdot L^{-1}$ for 15 d, although shoots and roots in the seedlings manifested significant inhibitory symptoms in response to Cd at 5.0 $mg \cdot L^{-1}$ (Fig. A.1). However, MSI was observed exclusively in seedlings exposed to 8.0 $mg \cdot L^{-1}$ Cd, with the replication slipping of one repeat detected by two SSRs (Fig. 2). According to a previous study (Monteiro et al., 2009), MSI was plant tissue- and exposure time-dependent under Cd stress. In our work, only shoots were studied because of their ease of handling and being representative for the toxicity of the whole plant in contamination diagnosis. Moreover, to further increase the toxicity, seedlings were exposed to Cd of additional low doses of~~(~~ 0.25 and 1.0 $mg \cdot L^{-1}$ for 21 d. They were assayed by two SSRs of BSAT1.001 and BSAT2.012, however, MSI remained negative (Fig. 1).

In oncology studies, MSI due to MMR deficiency is a~~have been reported to~~

~~be~~ biomarker~~s~~ of various cancers (Sardi et al., 2001; Vilar et al., 2014), because cancers and tumors attach to severe cellular damage ~~strong impairment of cells~~, eventually leading to cell death~~for animals, causing the death eventually~~. However in our work, Arabidopsis could still survive under Cd stress of 0.25-5.0 $mg \cdot L^{-1}$ (Fig. A.1), suggesting that repair of insertion/deletion loops induced by Cd has been performed by DNA MMR proteins MLH1, MSH2, MSH3, MSH6, PMS1 and PMS2. More importantly, according to recent work (Yao and Kovalchuk, 2011), positive results of MSI were induced by relatively severe stress in transgenic plants which might be more sensitive in MSI because of its exogenous SSR. All the above results imply that MSI is not a sufficiently sensitive biomarker in ecotoxicology studies.

4.2 DNA damage assayed by RAPD analysis is more sensitive than by MSI

In ecotoxicology, genomic DNA damage such as DNA adducts, breakage, insertion/deletion and base substitution can be detected by RAPD technique ~~as described previously~~, and is widely used in genotoxicity research ~~recently~~(Liu et al., 2009; Liu et al., 2012; Ladhar et al., 2014). In our study, alterations in Arabidopsis genomic DNA induced by Cd were assayed by RAPD profiles through three from *12* arbitrary primers screened. Prominent increase was observed at 5.0 $mg \cdot L^{-1}$ Cd ~~treatment~~ and slight elevation at 4.0 $mg \cdot L^{-1}$ Cd treatment for 15 d (Fig. 3). Also, note that~~Besides, treatment time seems a big issue as~~ seedlings exposed to Cd of additional low doses of 0.25 and 1.0 $mg \cdot L^{-1}$ for 21 d revealed a significant increase of RAPD polymorphisms (Fig. A.2), where MSI remained negative. This suggests that RAPD polymorphism is more sensitive to Cd stress than MSI as a biomarker.

MSI~~, one of genomic instability,~~ is a consequence of MMR deficiency and can 'not be induced by stresses straightforwardly (Clark and Kunkel, 2004;

Wieland et al., 2009). To our knowledge, MMR contains several proteins involved in recognition and repair of DNA damage, and can regulate G2/M checkpoints during the cell cycle, which can allow cells to overcome replication errors before entering mitosis (Campregher et al., 2008). Thus, MSI induced by stresses would be repaired by DNA MMR proteins before cell proliferation and would not be predicted to occur in Arabidopsis seedlings exposed to 0.25-5.0 $mg \cdot L^{-1}$ Cd. However, replication slipping errors can't be repaired when the entire MMR system is fatally paralyzed, indeed, MSI did appeared in Arabidopsis seedlings exposed to 8.0 $mg \cdot L^{-1}$ Cd (Fig. 1).

~~However,~~ DNA adducts can be replicated and transmitted to next generation of cells through translesion replication, escaping surveillance and repair systems (Zang et al., 2005). Under the stresses, DNA polymerases during the translesion synthesis processes are forced to encounter multiple types of damaged DNA, and can proceed to replicate modified DNA during the cell cycle. Also, cells evolve the translesion synthesis processes to tolerate genomic lesions by error-free or error-prone repair. Moreover, error-prone repair could pass the errors to the next generation of cells by cell proliferation. This is a type of emergency system used to maintain cells ~~survived~~, while severe DNA damage blocks the cell cycle, leading to cell cycle arrest or apoptosis/death [58]. Additionally, RAPD technique can detect temporary DNA damage and mutations caused by DNA adducts that could override ~~the~~ normal DNA replication and concurrently pass through translesion replication (Liu et al., 2005). This might explain~~account for the reasons~~ why RAPD polymorphism is much more sensitive than MSI in the present study, and MSI in Arabidopsis was detected only at the very high dose of 8.0 $mg \cdot L^{-1}$ Cd (Fig.s 1 and 3).

4.3 Aberrations in DNA methylation by MSAP-PCR are the optimum biomarkers in ecotoxicology.

Plants employ epigenetic regulatory strategies, such as DNA methylation, to maintain genomic plasticity, allowing the simultaneous regulation of expression of many genes involved in plant defense and in facilitating rapid adaptation to new stress conditions without changing the DNA sequence (Steward et al., 2002; Santoyo et al., 2011). Herein, our results indicate that Cd stress does affect patterns of genomic DNA methylation in Arabidopsis shoots (Fig. 5). Although the accurate timing and role of the internal and external cytosines in the CCGG restriction sites remains unresolved~~has not been clearly elucidated~~, data in Fig. 5 indicate that differential methylation patterns of different cytosines in the CCGG sites in the genomic DNA are affected by Cd at~~of~~ 0.25-5.0 mg·L^{-1} ~~levels~~. The hypermethylation more closely follows an inverted U-shaped dose-response to Cd, and the inverted U-shaped curves were maximum at Cd of 1.0 and 4.0 mg L^{-1} (Fig.s 4-5) for the internal and external cytosines of CCGG sites, respectively. Moreover, the hypermethylations were more prominent~~ly more~~ at the internal cytosine than that at the external cytosine. Also, hypomethylation was elevated albeit in a low proportion of increase at both external and internal cytosines (Fig. 5). A similar trend was reported for~~on~~ other plant and animal species (Aypar et al., 2011; Fulnecek and Kovarik, 2014). Hypomethylation may increase the susceptibility of chromosomes to breakage, and hypermethylaiton may also cause chromosomal instability by inhibiting cell cycle-dependent checkpoint controls, with deleterious effects on the cells (Kovalchuk et al., 2001). Importantly, stress-induced change itself might be more important than the direction of change, since any aberrations in DNA methylation is considered detrimental to the cells (Aypar et al., 2011). Thereby, modified patterns of

global DNA methylation probably can be applied as a potential biomarker for biomonitoring Cd exposure.

Global DNA methylation modifications are immediately initiated and proceed rapidly in plant cells in response to stress through signal transduction, leading to fast epigenetic changes (Steward et al., 2002; Santoyo et al., 2011). The mechanisms for DNA methylation effects by Cd have not been fully elucidated. Nonetheless, emerging evidence shows that the major cause for altered methylation is due to changes in methyltransferase activities and in coordinated expression of the chromatin-related genes known to be responsible for faithful maintenance of the various methylation patterns in plants (Ou et al., 2012). For example, Greco et al. (Greco et al., 2012) reported that Cd increased levels of chromomethylase (CMT) activities and elicited concomitant increases in global genomic DNA methylation, together with an enhanced heterochromatic nuclear fraction in *Posidonia oceanica*. Alternatively, Cd can interfere with the activity of DNA methyltransferases through overproduction of reactive oxygen species (ROS), and oxidative DNA damage could subsequently lead to an altered DNA methylation profile and subsequent alterations in gene expression, and/or siRNAs/RdDM and ROS1/DME (Mirbahai and Chipman, 2014). In the current study (Fig. 5), the aberrant changes of global DNA methylation we detected in Cd-treated seedlings could be the consequences of siRNAs/RdDM and CMT/DNMTs aberrant expression resulting from exposure to Cd for 15 d.

~~The utilization of biomarkers to assess biotic~~effects ~~of exposure of the organisms to stresses has received increasing attention, and biomarkers act~~ Increasingly, biomarkers are used as an integrative measurement at the sub-organism level to indicate stress status before damage to the biota arises (Kit et al., 2012). ~~In the field of epigenomics, the~~ Epigenetic biomarkers may be indicators for early diagnosis or risk assessment of human diseases,

pollutant exposure and impact (Huang et al., 2012; Kit et al., 2012). Recently, DNA methylation-based biomarkers have been proven to be more specific and sensitive than commonly used protein biomarkers, which could clearly justify their use in clinics (Kit et al., 2012). For example, emerging evidence shows that minimal traces of aberrant methylation profiles in blood DNA could serve as early surrogate biomarkers for cancer diagnosis and for risk assessment of PAHs exposure, respectively (Yang et al., 2012; Shin et al., 2013), supporting the notion that these methylation changes are the initial step that triggers various genetic events in these tumors (Kit et al., 2012). In another study, DNA methylation was previously introduced as an interface between the genome and environment, providing partial mechanistic~~m~~ explanations for the sensitivity of organisms to environmental stresses. Moreover, DNA methylation's persistence can usefully reflect the status of the environment in which organisms reside (Mirbahai and Chipman, 2014). In our study, DNA methylation polymorphism is much higher than RAPD loci and MSI in seedlings exposed to Cd stress of 0.25-5.0 $mg \cdot L^{-1}$ and the effects are dose-related, whereas DNA polymorphism using RAPD and MSI analyses is observed exclusively at doses of 4.0-5.0 $mg \cdot L^{-1}$ and 8.0 $mg \cdot L^{-1}$ Cd, respectively (Fig.s 1-5). These results provide evidence that epigenetic biomarker, such as DNA methylation polymorphism in seedlings, could be a more sensitive biomarker for Cd exposure than RAPD polymorphism and MSI.

In the field of ecotoxicology, advances in DNA methylation analysis have led to the recognition that aberrant DNA methylation status is potentially an early, rapid and sensitive molecular biomarker indicative of pollutant exposure and impact. MSAP-PCR is the most widely used method for screening aberrant DNA methylation in cells, and this approach has several advantages: more precision/accuracy, sensitivity, and reproducibility, and easier to carry out than traditional MSAP ~~technique in studies~~ (Aypar et al., 2011; Fulnecek and

Kovarik, 2014; Pierron et al., 2014). Moreover,~~In addition, today~~ MSAP-PCR is becoming a routine procedure in laboratories. We anticipate a broad application of the described technology in future studies on aberrations of DNA methylation for more accurate assessment of Cd genotoxicity in other plants and for genotoxicity of other heavy metal stresses in ecotoxicology.

5. Conclusions

The present report defines modifications in the genomic DNA methylation in correlation with the induction of DNA RAPD polymorphism and MSI measured in Arabidopsis seedlings exposed to Cd of 0.25-5.0 mg·L^{-1} for 15 d. ~~We observed that~~ MSI and RAPD polymorphism appeared exclusively under Cd stress of 8.0 mg·L^{-1} and of 4.0-5.0 mg·L^{-1}, respectively. However, we~~the present study~~ show~~s~~ that prominent DNA methylation polymorphism occurred under Cd stress of 0.25-5.0 mg·L^{-1} by MSAP-PCR. Furthermore, the inverted U-shaped dose-response effects of Cd stress on DNA methylation polymorphism were observed in Arabidopsis seedlings. Given that~~The ability of~~ *MSAP-PCR* ~~to~~ detect~~s~~ substantial epigenetic alterations in Arabidopsis seedlings at Cd exposure, prior to the onset of RAPD polymorphism, MSI and significant growth effects, it ~~w~~should be useful for ~~greatly improve~~ environmental risk assessment. With further development, the MSAP-PCR method may also provide a more sensitive and reliable means in identification and analysis of DNA methylation modifications in the other Cd-treated plants, and become~~it may be~~ a powerful tool for early detection of genotoxic effects of other heavy-metal stresses in ecotoxicology.

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Cadmium-induced genomic instability in Arabidopsis: molecular toxicological biomarkers for early diagnosis of cadmium stress

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ABSTRACT: Microsatellite instability (MSI) analysis, random-amplified polymorphic DNA (RAPD), and methylation-sensitive arbitrarily primed PCR (MSAP-PCR) are methods to evaluate the toxicity of environmental pollutants in stress-treated plants and human cancer cells. Here, we evaluate these techniques to screen for genetic and epigenetic alterations of Arabidopsis plantlets exposed to 0-5.0 $mg \cdot L^{-1}$ cadmium (Cd) for 15 d. There was a substantial increase in RAPD polymorphism of 24.5, and in genomic methylation polymorphism of 30.5-34.5 at CpG and of 14.5-20 at CHG sites under Cd stress of 5.0 $mg \cdot L^{-1}$ by RAPD and of 0.25-5.0 $mg \cdot L^{-1}$ by MSAP-PCR, respectively. However, only a tiny increase of 1.5 loci by RAPD occurred under Cd stress of 4.0 $mg \cdot L^{-1}$, and an additional high dose (8.0 $mg \cdot L^{-1}$) resulted in one repeat by MSI analysis. MSAP-PCR detected the most significant epigenetic modifications in plantlets exposed to Cd stress, and the patterns of hypermethylation and polymorphisms were consistent with inverted U-shaped dose responses. The presence of genomic methylation polymorphism in Cd-treated seedlings, prior to the onset of RAPD polymorphism, MSI and obvious growth effects, suggests that these altered DNA methylation loci are the most sensitive biomarkers for early diagnosis and risk assessment of genotoxic effects of Cd pollution in ecotoxicology.

Keywords: Microsatellite instability, Random-amplified polymorphic DNA, Methylation-sensitive arbitrarily primed PCR, Cadmium, *Arabidopsis thaliana*

1. Introduction

Environmental contamination with heavy metals has increased substantially over the past decades, which has aroused attention from

numerous researchers responsible for contamination diagnosis, environmental protection and ecological remediation. Cd, as one of the most highly toxic and accumulative heavy metals, has contaminated soils and water through atmospheric deposition, urban-industrial activities and agricultural practices (Sun et al., 2008; Liu et al., 2011). Thus, the exploration of Cd toxicity, especially its genotoxic effects, has become a focus in ecotoxicology research (Herbette et al., 2006; Azevedo et al., 2007; Greco et al., 2012; Pierron et al., 2014).

Several classic assays have been applied to examine the genotoxic effects of heavy metals on organisms, e.g. the micronucleus assay and the comet assay. However, neither is sensitive enough to determine the Cd contamination level or able to provide information at the DNA level (Cambier et al., 2010). With the rapid development of molecular biology, several PCR-based techniques have been used to evaluate DNA damage in toxicology studies. Random-amplified polymorphic DNA (RAPD) comprises a single “arbitrary” primer in a PCR reaction and results in the amplification of several discrete DNA products (Welsh and McClelland, 1990; Williams et al., 1990). RAPD has been used extensively in eco-toxicology studies on animals and humans (Misra et al., 2001; Singh and Roy, 2001,2004; Garnis et al., 2005). Recently, RAPD has been used in plant eco-toxicology studies, detecting genetic variation and mutation induced by stresses (Miki et al., 2001; Liu et al., 2005; 2007; Chao et al., 2008; 2009; 2012; Gjorgieva et al., 2013; Malar et al., 2014). RAPD analysis is extremely efficient for DNA analysis in complex genomes. Its use in investigating genomic DNA to detect different kinds of DNA damage and mutations suggests that RAPD can potentially form the basis of novel biomarker assays for the detection of DNA damage and mutations (Lopez-Moreno et al., 2010; Korpe and Aras, 2011).

Regarding DNA repair, Cd suppresses both eukaryotic MutS homolog

MSH2-MSH6- and MSH2-MSH3-dependent mismatch repair (MMR) activity through a nonspecific mechanism whereby Cd binds to multiple sites in MSH6 (Banerjee and Flores-Rozas, 2005; Wieland et al., 2009; Li et al., 2013). Loss or low activity of MMR proteins results in losses and gains of dinucleotide repeats in microsatellites, also known as simple sequence repeats (SSRs), leading to microsatellite instability (MSI). Previously, MSI was found at two “hotspots” of microsatellite mutation in mice by analysis of a panel of microsatellite markers under Cd stress (Oliveira et al., 2012; Du et al., 2014). Also, SSRs mutations canbe promoted by heavy metals, e.g. Lead, Chromium and Cd (Rodriguez et al., 2013a; Rodriguez et al., 2013b; Du et al., 2014). Monteiro et al. (Monteiro et al., 2009) demonstrated that SSR analyses could be used to evaluate plant genomic DNA instability for deciphering DNA damage induced by Cd in a genotoxicity test. Thus, MSI can be considered as a biomarker of high risk for human cancer and for the assessment of different genotoxic effects of pollutants on plants (Kovalchuk et al., 2000; Soreide et al., 2006). However, little information is available about microsatellite mutations in Arabidopsis under Cd stress.

Higher plants can rapidly adapt in response to stress, but this can lead to genome instability and changes in DNA methylation patterns throughout the genome and at specific loci of DNA (Boyko et al., 2010; Chatterjee and Vinson, 2012). For example, plants employ important and sophisticated epigenetic regulatory strategies, such as DNA methylation, to maintain genomic plasticity. This allows the simultaneous regulation of many genes involved in plant defense and in facilitating relatively rapid adaptation to new internal and environmental stress conditions without changes in DNA sequence (Steward et al., 2002; Santoyo et al., 2011). The genomic DNA hypermethylation pattern is correlated with water deficit in pea (*Pisum sativum* L.), pathogen stress in tobacco (*Nicotiana tabacum*), radioactive contamination in *Pinus silvestris*, salt,

UVC, cold, heat and flood stresses in Arabidopsis, and chromium stress in *Brassica napus* (Labra et al., 2002; Kovalchuk et al., 2003; Labra et al., 2004; Boyko et al., 2007; Mason et al., 2008). Also, DNA hypomethylation at several genomic loci occurred in response to heavy metal stress in hemp and clover, to pathogen stress in tobacco (*Nicotiana tabacum*), and to cold treatment in nucleosome core regions of maize (*Zea mays*) seedlings (Steward et al., 2002; Aina et al., 2004; Boyko et al., 2007). Potentially, global DNA hypomethylation is often viewed as a sign of genomic instability, which manifests as gross chromosomal aberrations, genome rearrangement, MSI, deletions/insertions, and point mutations, thus facilitating gene divergence and evolution of novel gene functions (Boyko et al., 2007; Duan et al., 2013; Harris et al., 2013).

Huang et al. (Huang et al., 2012) reported that tumor cells usually exhibit diverse patterns of DNA methylation, and that the CpG dinucleotide at any given site of genomic DNA might differ in methylation status among subpopulations of tumor cells, reflecting the heterogeneity and decreased epigenetic fidelity of cancer cells. Thus, CpG dinucleotides are also called methylation variable positions (MVPs) in epigenetic studies. Analysis of MVP profiles might reveal stress-specific methylation patterns, and the methylation frequency at any given MVPs could serve as a novel epigenetic biomarker for physiological and pathologic status (Huang et al., 2012; Kit et al., 2012). Moreover, minimal traces/differences of aberrant methylation profiles in blood DNA could serve as early surrogate biomarkers for cancer diagnosis and for risk assessment of PAHs exposure, respectively (Yang et al., 2012; Shin et al., 2013). Similarly, global DNA methylation in earthworms is apparently affected by the overall effect of metals/metalloids undergoing methylation and this parameter might be a candidate biomarker of epigenetic risks related to the presence of the metal elements in terrestrial environments (Santoyo et al., 2011).

Arabidopsis, with its vast resource of mutants, has long been used as a model dicotyledonous higher plant for studies of plant physiology and plant molecular biology. However, little information is available about potential associations between the status of global DNA methylation and genomic instability such as MSI and RAPD DNA polymorphisms in Arabidopsis seedlings under Cd stress. Therefore, the principal aims of this work were to (1) evaluate the extent of genomic instability using a panel of 36 microsatellite markers and 3 RAPD primers; (2) determine the status of global DNA methylation by MSAP-PCR under Cd stress; (3) explore potential associations between the actual status of global DNA methylation and genomic instability such as MSI and RAPD DNA polymorphisms in Arabidopsis under Cd stress. In-so-doing, we were able to evaluate the aforementioned parameters as candidate biomarkers for early diagnosis of genetic and epigenetic risks related to the presence of Cd in the soil and water environment.

2. Materials and methods

2.1 Plant materials, treatment conditions and DNA isolation

Arabidopsis thaliana seeds (Arabidopsis, Columbia ecotype) were sterilized in dilute bleach solution (bleach with surfactant diluted 1:10 with water) for 5 min, washed in sterile water and placed in an ethanol mix (ethanol : water : bleach 7:2:1) for 5 min. Seeds were then rinsed in sterile distilled water five times and placed in 4 °C for 24-36 h.

Seeds were sown in sterile flasks supplemented with 150 mL 0.5× liquid MS medium (SIGMA, USA) including 0.5% (w/v) sucrose (pH5.8), supplemented with Cd at a final concentration of 0 (the control), 0.25, 1.0, 4.0, 5.0, and 8.0 $mg \cdot L^{-1}$ (only used in MSI analysis) in the form of $CdCl_2 \cdot 2H_2O$ of analytical grade with purity 99.5%, PR China. Each flask with about 20

plantlets was placed on a rotary shaker at 50 rpm in an incubator (12 h light of approximately 3000 lx and 12 h dark at 21 ±0.5 °C) for 15 d. All treatments and analyses were repeated in three independent replicates.

The seed germination rate and growth of the plantlets were measured at 15 d following germination, and then pooled to measure DNA content in Arabidopsis shoots described in a previous study (Liu et al., 2009).

For MSI, RAPD, and DNA methylation assays, all DNA extractions and downstream applications were performed on mixed groups using the same batches of reagents. In addition, the control and Cd-exposed samples were collected at 15 d of incubation, and snap frozen in liquid nitrogen prior to storage at -70 ˚C. Genomic DNA from approximately 200-400 mg of fresh Arabidopsis shoots was extracted respectively using Plant Genomic DNA Extraction Kit. DNA concentration was examined by Eppendorf Biophotometor Plus, and then verified by DL2000 DNA marker in a 0.8% agarose gel. All experimental chemicals were ordered from TaKaRa Biotechnology Ltd. (Dalian, PR China) unless otherwise mentioned.

2.2 MSI Analysis

46 pairs of SSR primers (Table A.1) were screened in this test for finding genomic DNA MSI induced by Cd. PCRs were performed in a final volume of 25 μL consisting of 25 ng DNA template, 1 U of Taq polymerase, 1× Taq reaction buffer, 0.2 mM dNTPs, and 0.25 μM of each primer. The touchdown PCR conditions used for amplification were as follows: initial denaturation at 94°C for 5 min; 9 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and primer extension for 30 s; the annealing temperature was subsequently decreased by 0.5°C every second cycle until a ‘touchdown’ at 53°C, at which temperature 26 additional cycles followed (35 cycles in total). After the PCR reaction, amplification products were examined using 8% (w/v)

polyacrylamide gel electrophoresis (PAGE) with 7 M urea, visualized by silver staining method using the Bio Image Analyzer System (Bio-Rad, Vendor, Italy). To assess MSI, we compared the band pattern produced after gel electrophoresis of paired PCR reactions containing the control and Cd-treated DNA. If the control and Cd-treated PCR amplification products displayed different electrophoretic motilities, the case was scored as positive for MSI.

2.3 RAPD genetic damage screening

RAPD analysis was performed using 3 primers (Primers 1, 2 and 4) screened from 12 random primers (Liu et al., 2005). PCRs were performed in reaction mixtures of 25 μL containing approximately 40 ng of DNA template, 0.5 μM primer, 0.2 mM dNTPs, 1× reaction buffer and 1 U of Taq DNA polymerase. The RAPD protocol consisted of an initial denaturing step of 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 38 °C for 60 s and extension at 72 °C for 90 s, with an additional extension period of 10 min at 72 °C. PCR products were separated by electrophoresis in a 5% PAGE (50% urea). Gels were stained using silver nitrate. Images were digitally captured and the intensity of bands on the gel quantified using Image Lab (Bio-Rad).

2.4 Global DNA methylation assay using MSAP-PCR

Global DNA methylation was evaluated by methylation-sensitive arbitrarily primed PCR (MSAP-PCR). Genomic DNA (1 μg) was digested in CutSmart buffer with 1μL of HpaII or MspI (NEB, USA) in a 50-μL reaction volume for 1.5 h at 37°C. Digested DNA was purified and collected by Takara MiniBest DNA Fragment Purification Kit, then was transferred to another 50-μL reaction containing 1 × EcoRI buffer and 1μL EcoRI, digested for 1.5 h at 37°C. After

purification, digested DNA was used for subsequent PCR reaction performed as previously published (Gonzalgo et al., 1997). HpaII does not cut DNA if the internal cytosine of CCGG restriction site is methylated and therefore the bands generated in the PCR of DNA digested by EcoRI + HpaII lane are methylated at the internal cytosine. MspI is unable to cut DNA when the external cytosine in CCGG site is methylated and so the bands generated in the PCR of DNA digested by EcoRI + MspI are methylated at the external cytosine. Three primers designed by our lab and MLG2 (Gonzalgo et al., 1997) were used for methylation assay (Table A.2). PCR products were resolved on 5% PAGE with 50% urea. Methods of gel staining and image capture were the same as the above. An increase in band intensity indicates an increase in methylation. Band intensity was measured to numerically determine the change in DNA methylation.

2.5 Statistical analyses

Polymorphism frequency of RAPD and MSAP-PCR was calculated from PAGE results and analyzed in linear graphs using Microsoft Excel. Presence or absence of a band was counted as one locus in polymorphism frequency, while a band was calculated as 0.5 locus if its intensity changed $\geqslant$ 50% compared to the control. The stable polymorphism variation was calculated for data statistics exclusively if it occurred simultaneously in at least two replications, leading to data without variance.

3. Results

3.1 MSI assay using SSRs

MSI was not detected in plantlets exposed to the control and 0.25-5.0 $mg \cdot L^{-1}$ Cd for 15 d by a panel of 46 SSR primers. However, under an additional

treatment of 8.0 mg·L^{-1} Cd, replication slipping of one repeat (about 2-3 bp) was found using SSR primers of BSAT1.001 and BSAT2.012 (Fig. 1). Moreover, the above replication slipping of three duplicates did appear in shoots under Cd stress of 8 mg·L^{-1}, suggesting that MSI occurs exclusively under such a strong stress that inhibited Arabidopsis growth (Fig. A.1).

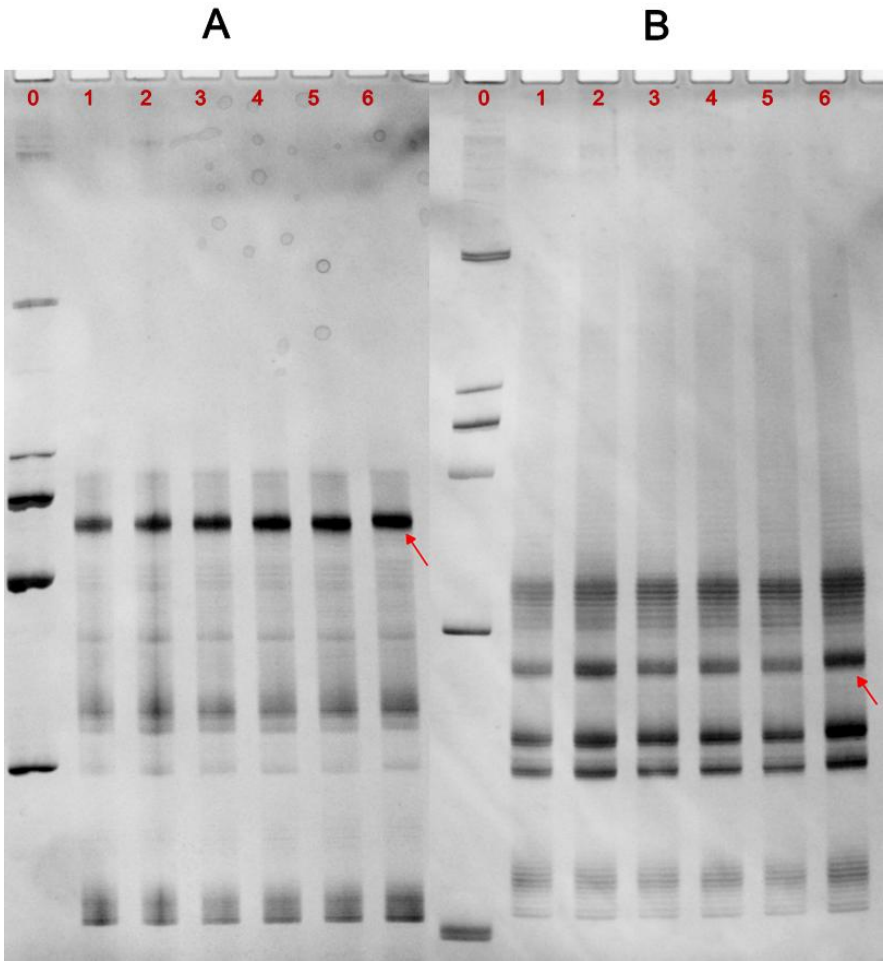

Fig. 1 MSI in Arabidopsis seedlings exposed to 0-8.0 mg·L^{-1} Cd for 15 d. (A) PAGE profiles amplified by primer BSAT1.001, and (B) by BSAT2.012, in which lanes (1) - (6) are PCR fragments amplified from the DNA templates of Arabidopsis shoots exposed to 0, 0.25, 1.0, 4.0, 5.0 and 8.0 mg·L^{-1} Cd for 15 d, respectively. Lane (0) is Takara DNA Marker 2000 (2000, 1000, 750, 500, 250 and 100 bp from top to bottom), and the same is below.

3.2 Genetic damage assay using RAPDs

Fig. 2 shows RAPD profiles in Arabidopsis seedlings grown with or without Cd treatments after 15 d of exposure. In Fig. 2, each lane contains at least 10 bands ranged from 300 to 3000 bp, in which bands of variable intensity detected in at least two replicates are calculated in Fig. 3. Compared with the controls, polymorphic bands were not detected in the in the 0.25 and 1.0 $mg \cdot L^{-1}$ Cd treatment, for different primers. 4.0 $mg \cdot L^{-1}$ Cd induced low polymorphism whereas high polymorphism occurred at the 5.0 $mg \cdot L^{-1}$ Cd treatment. Numbers of polymorphism loci were 1.5 and 24.5 for 4.0 and 5.0 $mg \cdot L^{-1}$ Cd, respectively (Fig. 3). In these cases, RAPD polymorphisms were due to the loss and/or gain of amplified bands in the treated samples compared with the control.

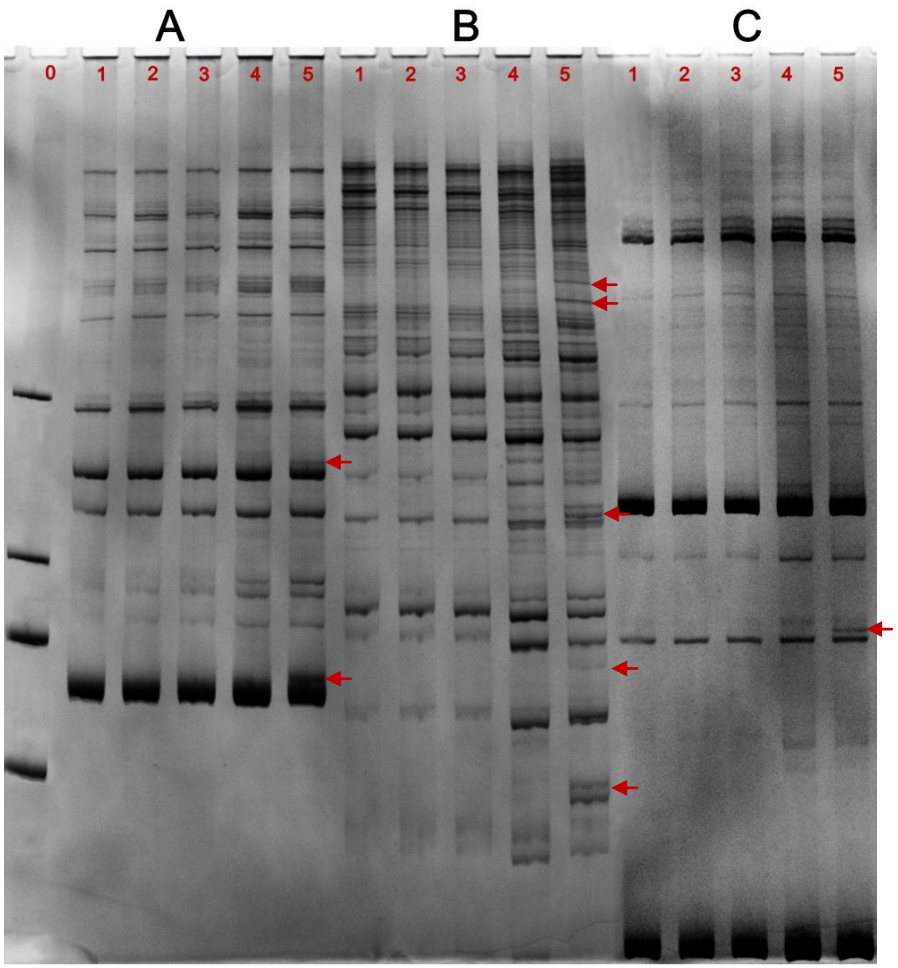

Fig. 2 RAPD fingerprints of Arabidopsis seedlings exposed to 0-5.0 mg·L^{-1} Cd for 15 d. (A) PAGE profiles amplified by primer 1, (B) by primer 2 and (C) by primer 4, in which lanes (1) - (5) are PCR fragments amplified from the DNA templates of Arabidopsis shoots exposed to 0, 0.25, 1.0, 4.0 and 5.0 mg·L^{-1} Cd for 15 d, respectively, and lane (0) is Takara DNA Marker 2000 (100 and 250 bp bands of DNA marker migrated out of the gel for high resolution). Red arrows illustrated polymorphism between plantlets exposed to 5.0 mg·L^{-1} Cd and the control.

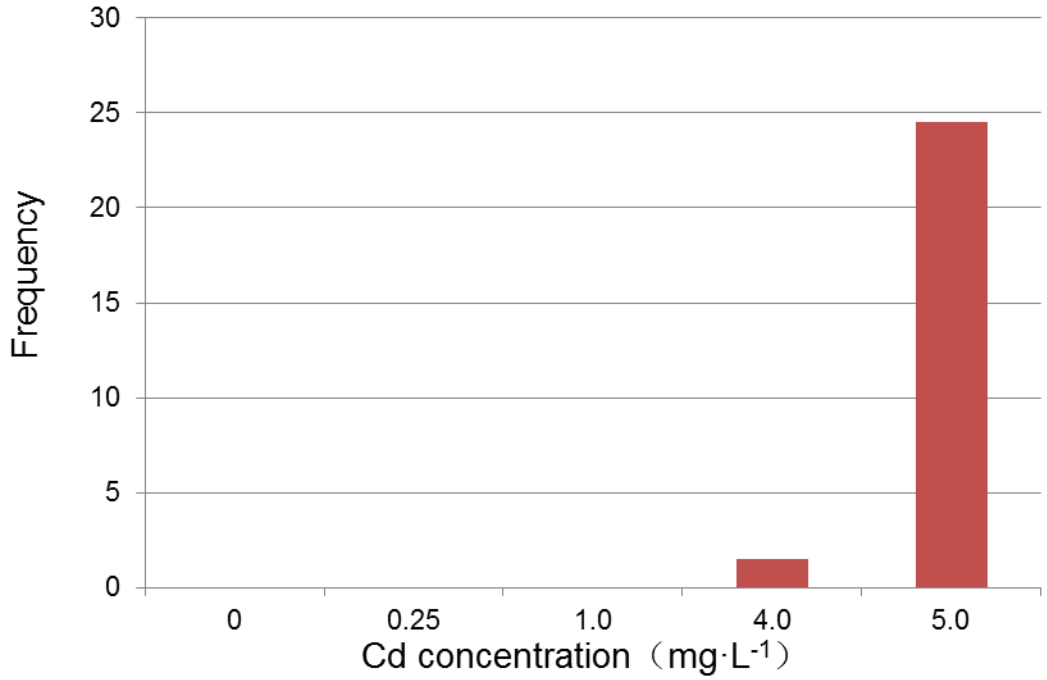

Fig. 3 Polymorphism variations detected by RAPD from Arabidopsis plantlets exposed to 0 - 5.0 mg·L^{-1} Cd for 15 d.

3.3 Methylation damage assay using MSAP-PCR

Aberrations of global DNA methylation in Arabidopsis induced by Cd was evaluated by MSAP-PCR. For all treatments, reproducible bands or cytosine residues of variable intensity were evaluated (Fig. 4) and calculated in Fig. 5 for methylation analysis at both internal (HpaII) and external (MspI) cytosines

285 of CCGG sites. At the external cytosine, Cd stress resulted in global
286 hypermethylation in the shoot tissues relative to the controls (Fig. 5A). This
287 hypermethylation more closely follows an inverted U-shaped dose-response to
288 Cd. The increase was 9.5, 9.5, 14.5 and 9 loci in plantlets exposed to Cd of
289 0.25, 1.0, 4.0 and 5.0 $mg \cdot L^{-1}$ Cd, respectively. Notably, the hypomethylation
290 manifested an invariable status under all Cd stresses tested (Fig. 5A).
291 At the internal cytosine, a similar trend for hypermethylation and
292 hypomethylation under Cd stress was observed as compared to the untreated
293 control (Fig. 5B). The numbers of hypermethylation loci were 24.5, 27.5, 26
294 and 26 at 0.25, 1.0, 4.0 and 5.0 $mg \cdot L^{-1}$ Cd doses, respectively, which were
295 prominently more than that at the external cytosine. Moreover,
296 hypomethylation arose linearly albeit in a low proportion of increase (Fig. 5B).
297 Different methylation polymorphic bands were detected at 0.25-5.0 $mg \cdot L^{-1}$
298 Cd for different cytosines in the shoots (Fig. 5). Also, these polymorphisms
299 indicate similar inverted U-shaped concentration response curves under Cd
300 stress, and the inverted U-shaped curves were maximum at Cd of 1.0 and 4.0
301 mg L^{-1} (Fig. 5) for the internal and external cytosines, respectively. In all cases,
302 methylation polymorphisms were due to the loss and gain of variable intensity
303 of reproducible amplified bands in the Cd-treated seedlings as compared to
304 the control (Fig. 4).

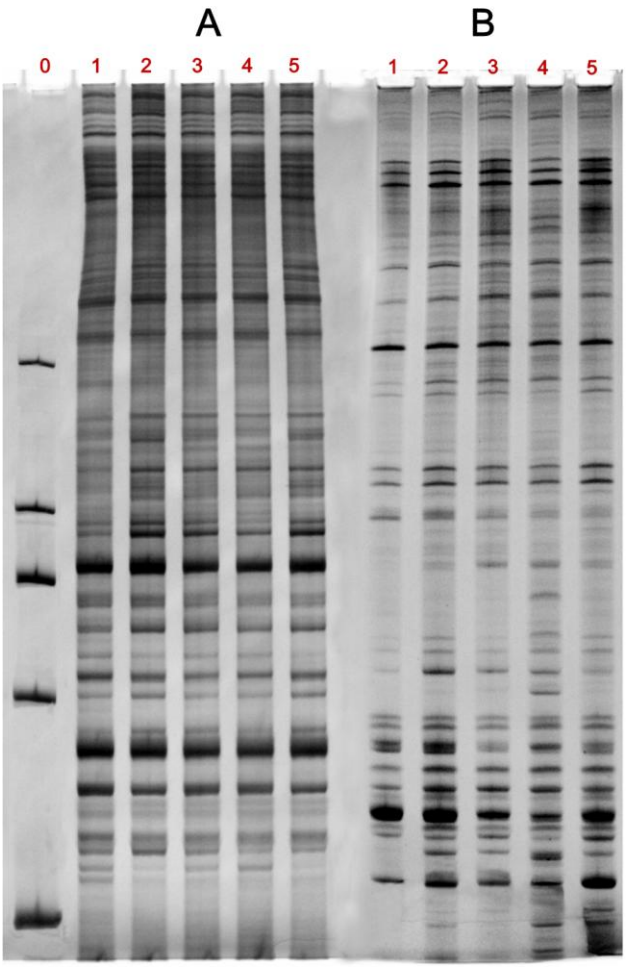

Fig. 4 The representative MSAP-PCR profiles of Arabidopsis seedlings exposed to 0 - 5.0 mg·L^{-1} Cd for 15 d. (A) PAGE profiles amplified by primer MLG2 from DNA templates digested by EcoRI+MspI and (B) EcoRI+HpaII, in which lanes (1) - (5) are PCR fragments amplified from the DNA templates of Arabidopsis shoots exposed to 0, 0.25, 1.0, 4.0 and 5.0 mg·L^{-1} Cd for 15 d, respectively, and lane (0) is Takara DNA Marker 2000 (100 bp band of DNA marker migrated out of the gel for high resolution).

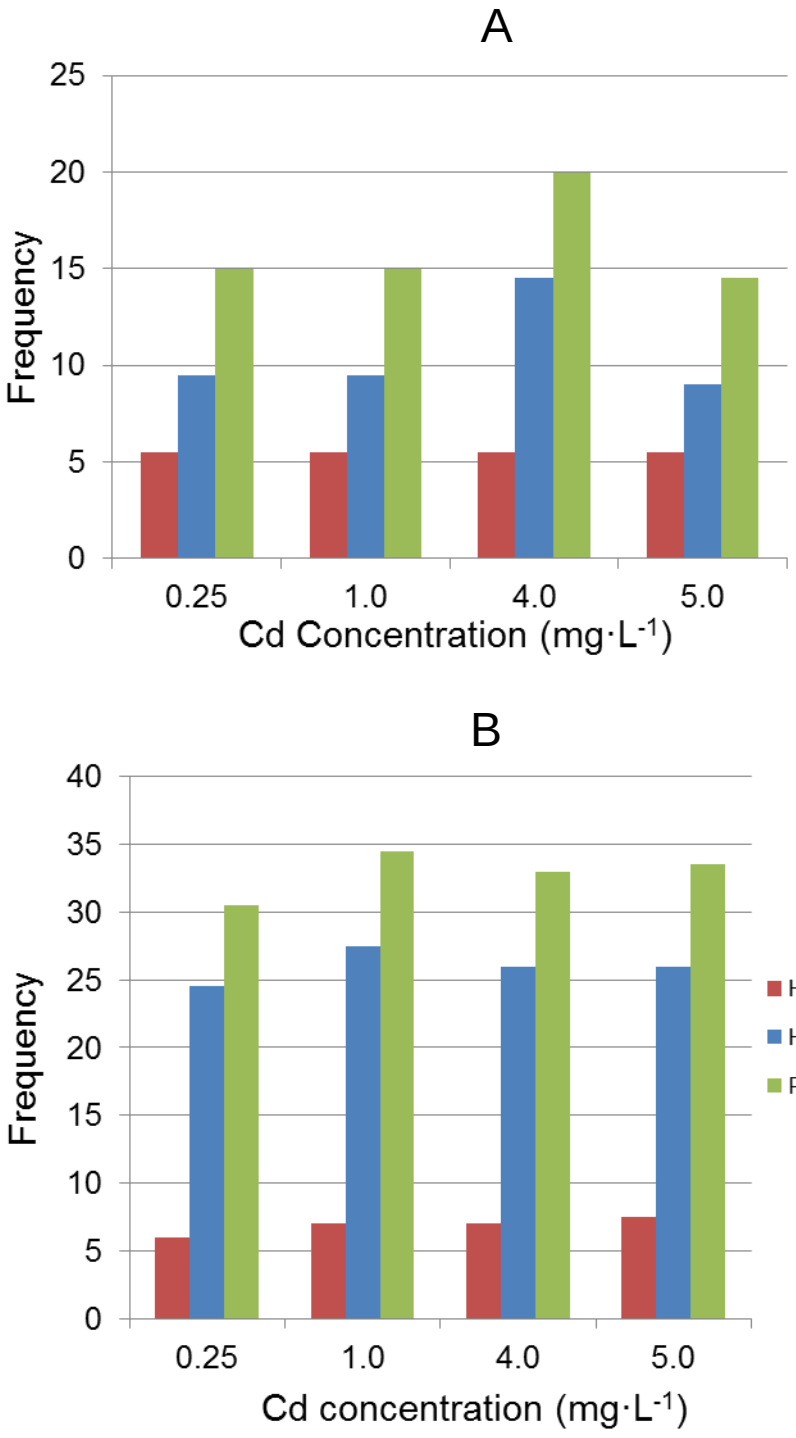

Fig. 5 DNA methylation variations detected by MSAP-PCR from Arabidopsis plantlets exposed to 0 - 5.0 mg·L^{-1} Cd for 15 d. (A) Frequency of hypermethylation, hypomethylation and methylation polymorphism at external cytosine (CHG) loci and (B) internal cytosine (C_PG) loci.

4. Discussion

4.1 MSI in vivo is not a sensitive biomarker for Cd stress

Cd has been responsible for proofreading and MMR deficiency that could cause genomic MSI (Jin et al., 2003; Edelbrock et al., 2013). As far as we know, SSRs was first adopted by Kovalchuk et al. (2000) as tools to assess genetic instability in the offspring of wheat plants exposed to radiation near the Chernobyl nuclear power plant. More recently, positive results of MSI have been reported in transgenic plants consisting of a modified *GUS* reporter whose reading frame is disrupted by microsatellite repeat sequence (Yao and Kovalchuk, 2011). Thus, MSI analysis has been considered as a biomarker to detect genetic damages in ecotoxicology.

In the present study, MSI was not observed for the Arabidopsis seedlings exposed to Cd stress of 0.25-5.0 $mg \cdot L^{-1}$ for 15 d, although shoots and roots in the seedlings manifested significant inhibitory symptoms in response to Cd at 5.0 $mg \cdot L^{-1}$ (Fig. A.1). However, MSI was observed exclusively in seedlings exposed to 8.0 $mg \cdot L^{-1}$ Cd, with the replication slipping of one repeat detected by two SSRs (Fig. 2). According to a previous study (Monteiro et al., 2009), MSI was plant tissue- and exposure time-dependent under Cd stress. In our work, only shoots were studied because of their ease of handling and being representative for the toxicity of the whole plant in contamination diagnosis. Moreover, to further increase the toxicity, seedlings were exposed to Cd of additional low doses of 0.25 and 1.0 $mg \cdot L^{-1}$ for 21 d. They were assayed by two SSRs of BSAT1.001 and BSAT2.012, however, MSI remained negative (Fig. 1).

In oncology studies, MSI due to MMR deficiency is a biomarker of various cancers (Sardi et al., 2001; Vilar et al., 2014), because cancers and tumors

attach to severe cellular damage, eventually leading to cell death. However in our work, Arabidopsis could still survive under Cd stress of 0.25-5.0 $mg \cdot L^{-1}$ (Fig. A.1), suggesting that repair of insertion/deletion loops induced by Cd has been performed by DNA MMR proteins MLH1, MSH2, MSH3, MSH6, PMS1 and PMS2. More importantly, according to recent work (Yao and Kovalchuk, 2011), positive results of MSI were induced by relatively severe stress in transgenic plants which might be more sensitive in MSI because of its exogenous SSR. All the above results imply that MSI is not a sufficiently sensitive biomarker in ecotoxicology studies.

4.2 DNA damage assayed by RAPD analysis is more sensitive than by MSI

In ecotoxicology, genomic DNA damage such as DNA adducts, breakage, insertion/deletion and base substitution can be detected by RAPD technique, and is widely used in genotoxicity research (Liu et al., 2009; Liu et al., 2012; Ladhar et al., 2014). In our study, alterations in Arabidopsis genomic DNA induced by Cd were assayed by RAPD profiles through three from *12* arbitrary primers screened. Prominent increase was observed at 5.0 $mg \cdot L^{-1}$ Cd and slight elevation at 4.0 $mg \cdot L^{-1}$ Cd treatment for 15 d (Fig. 3). Also, note that seedlings exposed to Cd of additional low doses of 0.25 and 1.0 $mg \cdot L^{-1}$ for 21 d revealed a significant increase of RAPD polymorphisms (Fig. A.2), where MSI remained negative. This suggests that RAPD polymorphism is more sensitive to Cd stress than MSI as a biomarker.

MSI is a consequence of MMR deficiency and can not be induced by stresses straightforwardly (Clark and Kunkel, 2004; Wieland et al., 2009). To our knowledge, MMR contains several proteins involved in recognition and repair of DNA damage, and can regulate G2/M checkpoints during the cell cycle, which can allow cells to overcome replication errors before entering

mitosis (Campregher et al., 2008). Thus, MSI induced by stresses would be repaired by DNA MMR proteins before cell proliferation and would not be predicted to occur in Arabidopsis seedlings exposed to 0.25-5.0 $mg \cdot L^{-1}$ Cd. However, replication slipping errors can't be repaired when the entire MMR system is fatally paralyzed, indeed, MSI did appear in Arabidopsis seedlings exposed to 8.0 $mg \cdot L^{-1}$ Cd (Fig. 1).

DNA adducts can be replicated and transmitted to next generation of cells through translesion replication, escaping surveillance and repair systems (Zang et al., 2005). Under the stresses, DNA polymerases during the translesion synthesis processes are forced to encounter multiple types of damaged DNA, and can proceed to replicate modified DNA during the cell cycle. Also, cells evolve the translesion synthesis processes to tolerate genomic lesions by error-free or error-prone repair. Moreover, error-prone repair could pass the errors to the next generation of cells by cell proliferation. This is a type of emergency system used to maintain cells, while severe DNA damage blocks the cell cycle, leading to cell cycle arrest or apoptosis/death [58]. Additionally, RAPD technique can detect temporary DNA damage and mutations caused by DNA adducts that could override normal DNA replication and concurrently pass through translesion replication (Liu et al., 2005). This might explain why RAPD polymorphism is much more sensitive than MSI in the present study, and MSI in Arabidopsis was detected only at the very high dose of 8.0 $mg \cdot L^{-1}$ Cd (Fig.s 1 and 3).

4.3 Aberrations in DNA methylation by MSAP-PCR are the optimum biomarkers in ecotoxicology.

Plants employ epigenetic regulatory strategies, such as DNA methylation, to maintain genomic plasticity, allowing the simultaneous regulation of

401 expression of many genes involved in plant defense and in facilitating rapid
402 adaptation to new stress conditions without changing the DNA sequence
403 (Steward et al., 2002; Santoyo et al., 2011). Herein, our results indicate that Cd
404 stress does affect patterns of genomic DNA methylation in Arabidopsis shoots
405 (Fig. 5). Although the accurate timing and role of the internal and external
406 cytosines in the CCGG restriction sites remains unresolved, data in Fig. 5
407 indicate that differential methylation patterns of different cytosines in the
408 CCGG sites in the genomic DNA are affected by Cd at 0.25-5.0 $mg \cdot L^{-1}$. The
409 hypermethylation more closely follows an inverted U-shaped dose-response to
410 Cd, and the inverted U-shaped curves were maximum at Cd of 1.0 and 4.0 mg
411 L^{-1} (Fig.s 4-5) for the internal and external cytosines of CCGG sites,
412 respectively. Moreover, the hypermethylations were more prominent at the
413 internal cytosine than that at the external cytosine. Also, hypomethylation was
414 elevated albeit in a low proportion of increase at both external and internal
415 cytosines (Fig. 5). A similar trend was reported for other plant and animal
416 species (Aypar et al., 2011; Fulnecek and Kovarik, 2014). Hypomethylation
417 may increase the susceptibility of chromosomes to breakage, and
418 hypermethylaiton may also cause chromosomal instability by inhibiting cell
419 cycle-dependent checkpoint controls, with deleterious effects on the cells
420 (Kovalchuk et al., 2001). Importantly, stress-induced change itself might be
421 more important than the direction of change, since any aberrations in DNA
422 methylation is considered detrimental to the cells (Aypar et al., 2011). Thereby,
423 modified patterns of global DNA methylation probably can be applied as a
424 potential biomarker for biomonitoring Cd exposure.

425 Global DNA methylation modifications are immediately initiated and
426 proceed rapidly in plant cells in response to stress through signal transduction,
427 leading to fast epigenetic changes (Steward et al., 2002; Santoyo et al., 2011).
428 The mechanisms for DNA methylation effects by Cd have not been fully

elucidated. Nonetheless, emerging evidence shows that the major cause for altered methylation is due to changes in methyltransferase activities and in coordinated expression of the chromatin-related genes known to be responsible for faithful maintenance of the various methylation patterns in plants (Ou et al., 2012). For example, Greco et al. (Greco et al., 2012) reported that Cd increased levels of chromomethylase (CMT) activities and elicited concomitant increases in global genomic DNA methylation, together with an enhanced heterochromatic nuclear fraction in *Posidonia oceanica*. Alternatively, Cd can interfere with the activity of DNA methyltransferases through overproduction of reactive oxygen species (ROS), and oxidative DNA damage could subsequently lead to an altered DNA methylation profile and subsequent alterations in gene expression, and/or siRNAs/RdDM and ROS1/DME (Mirbahai and Chipman, 2014). In the current study (Fig. 5), the aberrant changes of global DNA methylation we detected in Cd-treated seedlings could be the consequences of siRNAs/RdDM and CMT/DNMTs aberrant expression resulting from exposure to Cd for 15 d.

Increasingly, biomarkers are used as an integrative measurement at the sub-organism level to indicate stress status before damage to the biota arises (Kit et al., 2012). Epigenetic biomarkers may be indicators for early diagnosis or risk assessment of human diseases, pollutant exposure and impact (Huang et al., 2012; Kit et al., 2012). Recently, DNA methylation-based biomarkers have been proven to be more specific and sensitive than commonly used protein biomarkers, which could clearly justify their use in clinics (Kit et al., 2012). For example, emerging evidence shows that minimal traces of aberrant methylation profiles in blood DNA could serve as early surrogate biomarkers for cancer diagnosis and for risk assessment of PAHs exposure, respectively (Yang et al., 2012; Shin et al., 2013), supporting the notion that these methylation changes are the initial step that triggers various genetic events in

these tumors (Kit et al., 2012). In another study, DNA methylation was previously introduced as an interface between the genome and environment, providing partial mechanistic explanations for the sensitivity of organisms to environmental stresses. Moreover, DNA methylation's persistence can usefully reflect the status of the environment in which organisms reside (Mirbahai and Chipman, 2014). In our study, DNA methylation polymorphism is much higher than RAPD loci and MSI in seedlings exposed to Cd stress of 0.25-5.0 $mg \cdot L^{-1}$ and the effects are dose-related, whereas DNA polymorphism using RAPD and MSI analyses is observed exclusively at doses of 4.0-5.0 $mg \cdot L^{-1}$ and 8.0 $mg \cdot L^{-1}$ Cd, respectively (Fig.s 1-5). These results provide evidence that epigenetic biomarker, such as DNA methylation polymorphism in seedlings, could be a more sensitive biomarker for Cd exposure than RAPD polymorphism and MSI.

In the field of ecotoxicology, advances in DNA methylation analysis have led to the recognition that aberrant DNA methylation status is potentially an early, rapid and sensitive molecular biomarker indicative of pollutant exposure and impact. MSAP-PCR is the most widely used method for screening aberrant DNA methylation in cells, and this approach has several advantages: more precision/accuracy, sensitivity, and reproducibility, and easier to carry out than traditional MSAP (Aypar et al., 2011; Fulnecek and Kovarik, 2014; Pierron et al., 2014). Moreover, MSAP-PCR is becoming a routine procedure in laboratories. We anticipate a broad application of the described technology in future studies on aberrations of DNA methylation for more accurate assessment of Cd genotoxicity in other plants and for genotoxicity of other heavy metal stresses in ecotoxicology.

5. Conclusions

The present report defines modifications in the genomic DNA methylation

in correlation with the induction of DNA RAPD polymorphism and MSI measured in Arabidopsis seedlings exposed to Cd of 0.25-5.0 $mg \cdot L^{-1}$ for 15 d. MSI and RAPD polymorphism appeared exclusively under Cd stress of 8.0 $mg \cdot L^{-1}$ and of 4.0-5.0 $mg \cdot L^{-1}$, respectively. However, we show that prominent DNA methylation polymorphism occurred under Cd stress of 0.25-5.0 $mg \cdot L^{-1}$ by MSAP-PCR. Furthermore, the inverted U-shaped dose-response effects of Cd stress on DNA methylation polymorphism were observed in Arabidopsis seedlings. Given that *MSAP-PCR* detects substantial epigenetic alterations in Arabidopsis seedlings at Cd exposure, prior to the onset of RAPD polymorphism, MSI and significant growth effects, it should be useful for environmental risk assessment. With further development, the MSAP-PCR method may also provide a more sensitive and reliable means in identification and analysis of DNA methylation modifications in the other Cd-treated plants, and become a powerful tool for early detection of genotoxic effects of other heavy-metal stresses in ecotoxicology.

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Figure

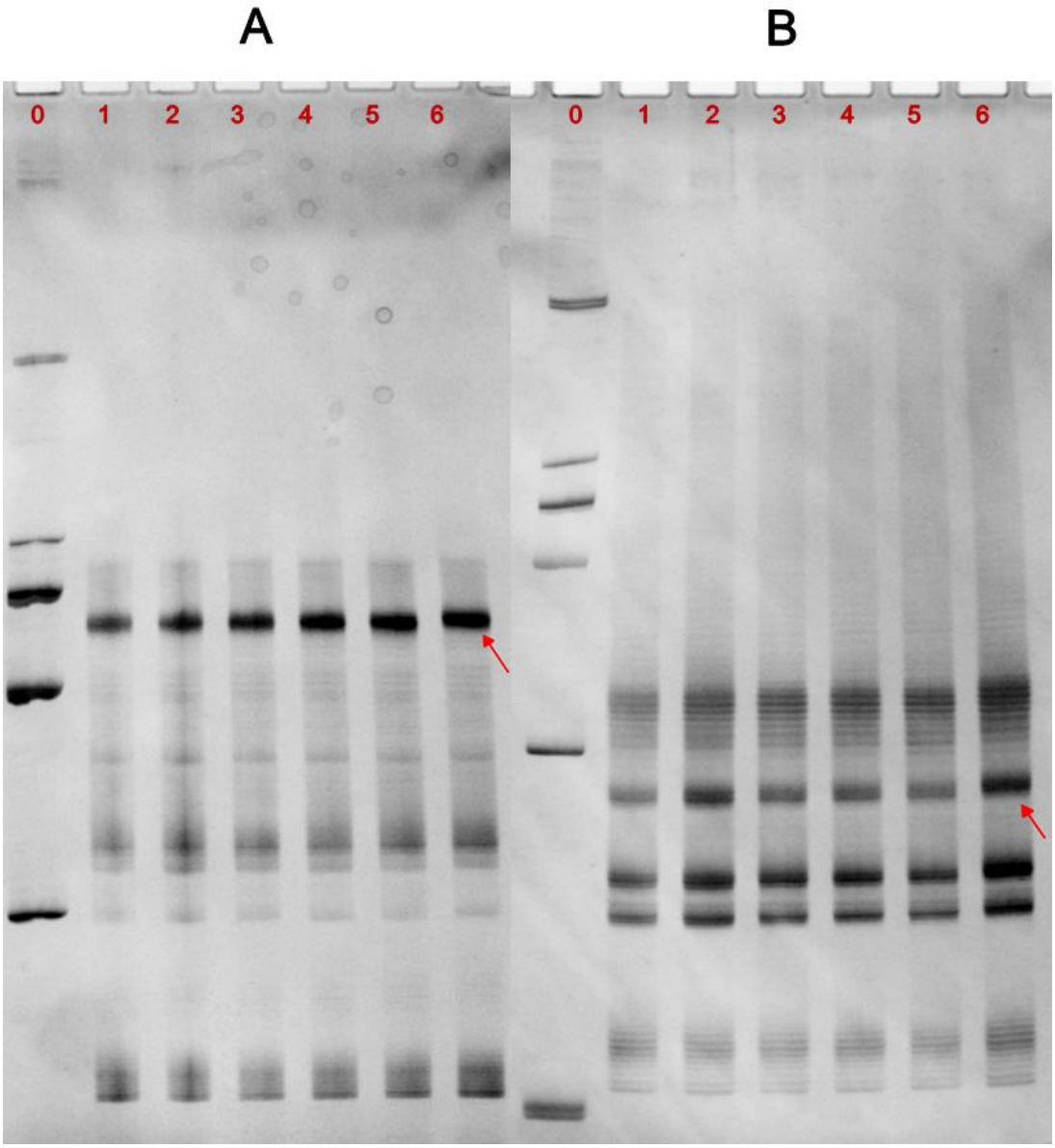

Fig. 1 MSI in Arabidopsis seedlings exposed to 0-8.0 mg·L^{-1} Cd for 15 d. (A) PAGE profiles amplified by primer BSAT1.001 and (B) BSAT2.012, in which lanes (1) - (6) are PCR fragments amplified from the DNA templates of Arabidopsis shoots exposed to 0, 0.25, 1.0, 4.0, 5.0 and 8.0 mg·L^{-1} Cd for 15 d, respectively, and lane (0) is Takara DNA Marker 2000.

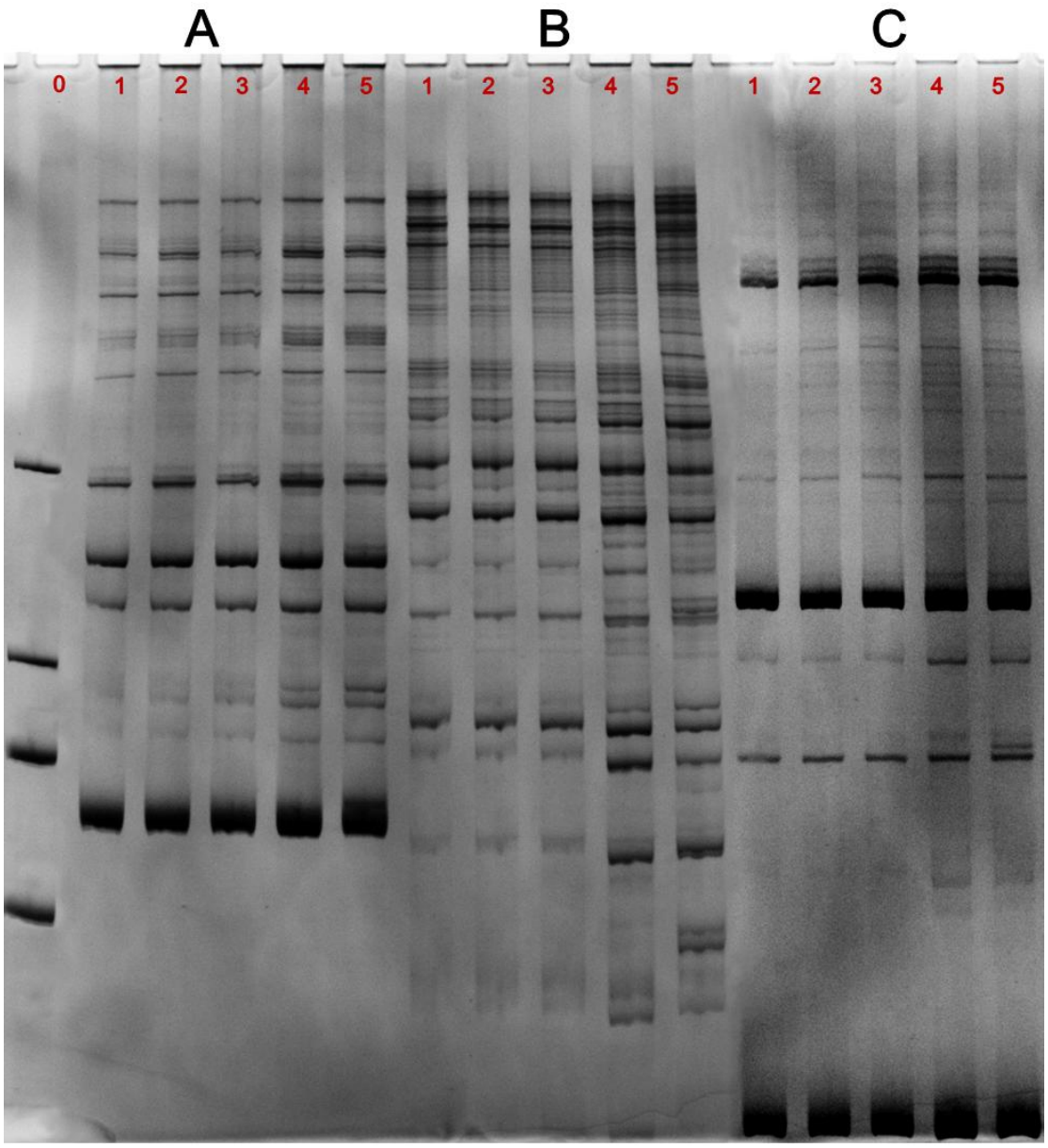

Fig. 2 RAPD fingerprints of Arabidopsis seedlings exposed to 0-5.0 mg·L^{-1} Cd for 15 d. (A) PAGE profiles amplified by primer 1, (B) by primer 2 and (C) by primer 4, in which lanes (1) - (5) are PCR fragments amplified from the DNA templates of Arabidopsis shoots exposed to 0, 0.25, 1.0, 4.0 and 5.0 mg·L^{-1} Cd for 15 d, respectively, and lane (0) is Takara DNA Marker 2000 (100 and 250 bp bands of DNA marker migrated out of the gel for high resolution).

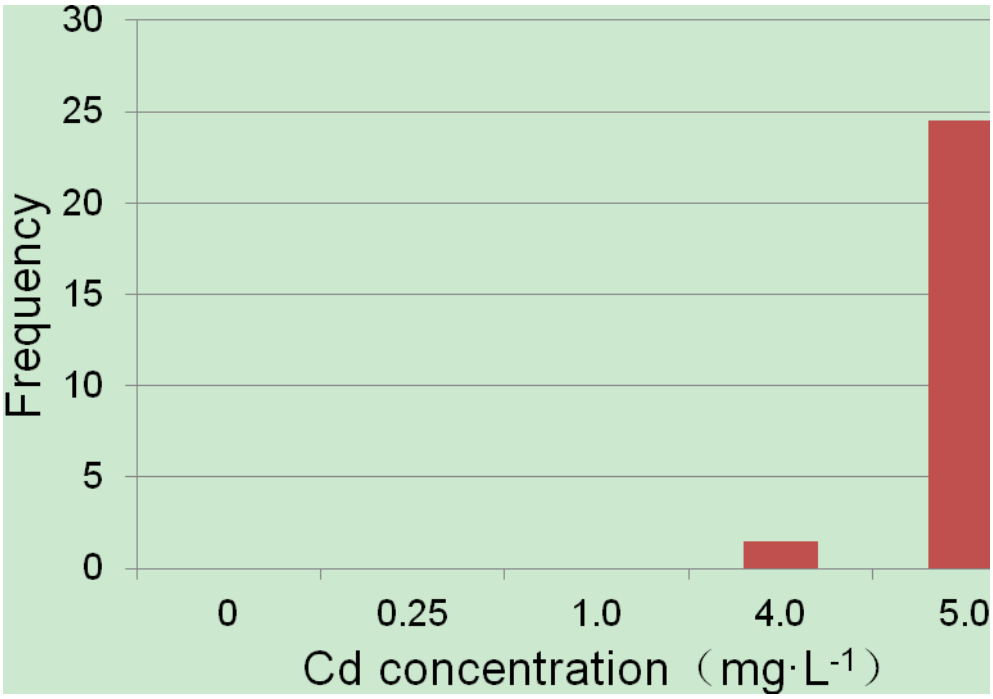

Fig. 3 Polymorphism variations detected by RAPD from Arabidopsis plantlets exposed to 0-5.0 mg·L^{-1} Cd for 15 d.

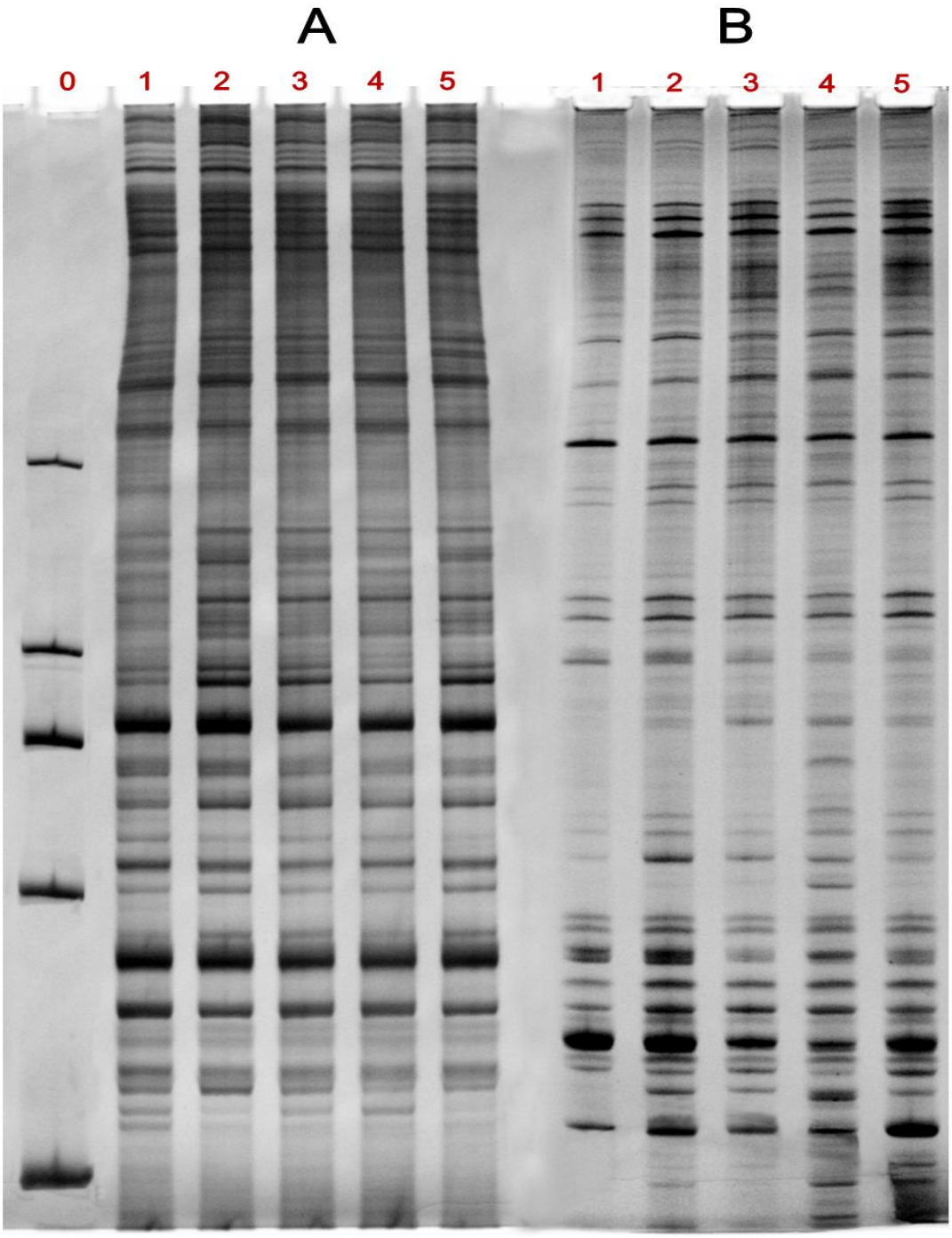

Fig. 4 The representative MSAP-PCR profiles of Arabidopsis seedlings exposed to 0-5.0 mg·L^{-1} Cd for 15 d. (A) PAGE profiles amplified by primer MLG2 from DNA templates digested by EcoRI+MspI and (B) EcoRI+HpaII, in which lanes (1) - (5) are PCR fragments amplified from the DNA templates of Arabidopsis shoots exposed to 0, 0.25, 1.0, 4.0 and 5.0 mg·L^{-1} Cd for 15 d, respectively, and lane (0) is Takara DNA Marker 2000 (100 bp band of DNA marker migrated out of the gel for high resolution).

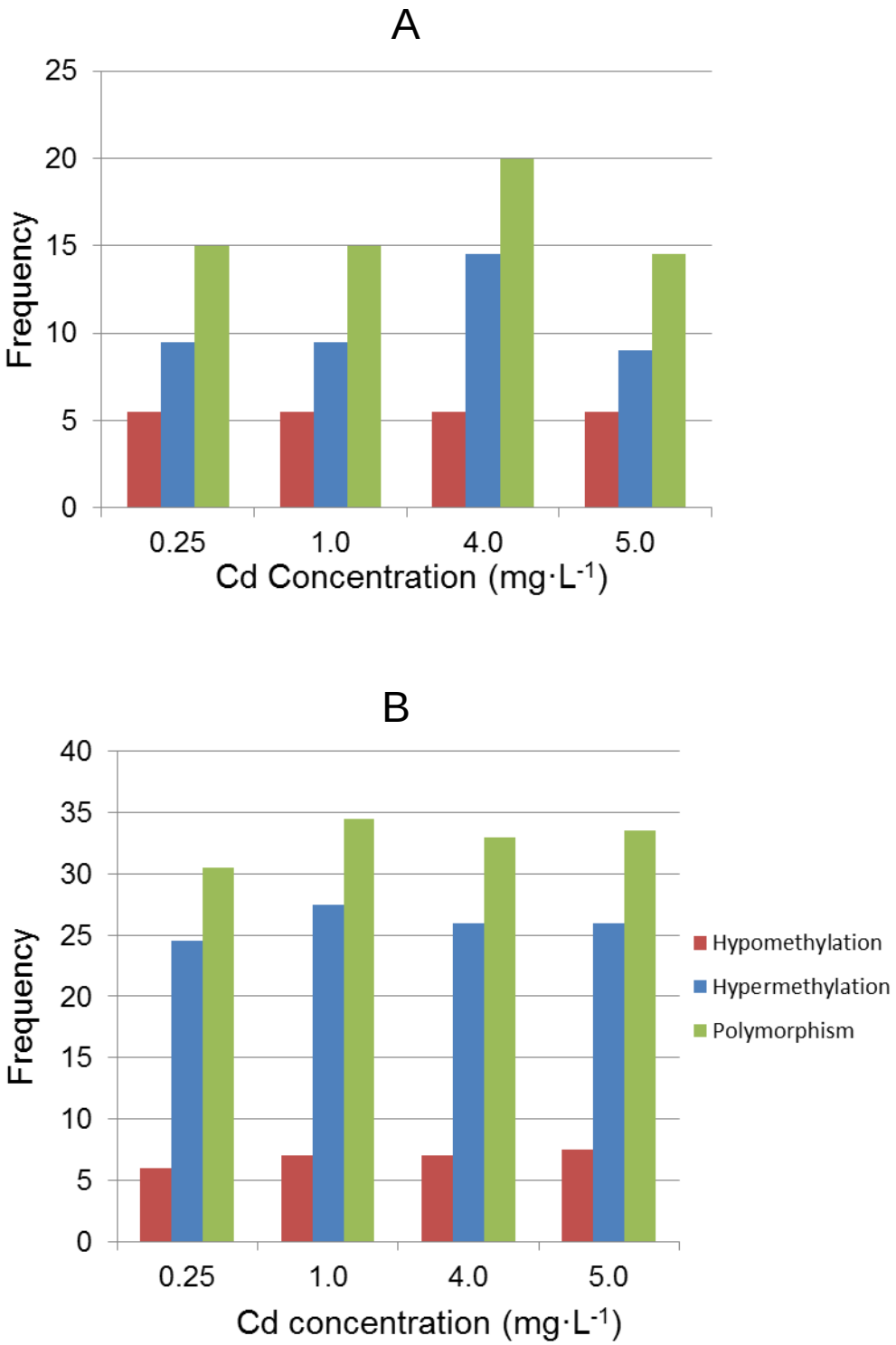

Fig. 5 DNA methylation variations detected by MSAP-PCR from Arabidopsis plantlets exposed to 0 - 5.0 mg·L^{-1} Cd for 15 d. (A) Frequency of hypermethylation, hypomethylation and methylation polymorphism at external cytosine (CHG) loci and (B) internal cytosine (C_PG) loci.

Supplementary Material

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