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#### **HIGH LIGHTS:**

- MSI occurred in Arabidopsis seedlings only under Cd stress of 8.0 mg·L<sup>-1</sup> for 15 d.
- Aberrations in DNA methylation were induced by Cd of 0.25-5.0 mg·L<sup>-1</sup> 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.

1 Cadmium-induced genomic instability in Arabidopsis:

# 2 molecular toxicological biomarkers for early diagnosis of

#### 3 cadmium stress

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28 ABSTRACT: Microsatellite instability (MSI) analysis, random-amplified 29 polymorphic DNA (RAPD), and methylation-sensitive arbitrarily primed PCR 30 (MSAP-PCR) are methods to evaluate the toxicity of environmental pollutants 31 in stress-treated plants and human cancer cells. Here, we evaluate these 32 techniques to screen for genetic and epigenetic alterations of Arabidopsis plantlets exposed to 0-5.0 mg·L<sup>-1</sup> cadmium (Cd) for 15 d. There was a 33 34 substantial increase in RAPD polymorphism of 24.5, and in genomic 35 methylation polymorphism of 30.5-34.5 at CpG and of 14.5-20 at CHG sites under Cd stress of 5.0 mg  $L^{-1}$  by RAPD and of 0.25-5.0 mg  $L^{-1}$  by 36 37 MSAP-PCR, respectively. However, only a tiny increase of 1.5 loci by RAPD occurred under Cd stress of 4.0 mg  $L^{-1}$ , and an additional high dose (8.0 38 39 mg L<sup>1</sup>) resulted in one repeat by MSI analysis. MSAP-PCR has detected the 40 most significant epigenetic modifications in plantlets exposed to Cd stress, and 41 the patterns of hypermethylation and polymorphisms were consistent 42 witheffectsmanifest the inverted U-shaped dose responses. The presence of 43 genomic methylation polymorphism in Cd-treated seedlings, prior to the onset 44 of RAPD polymorphism, MSI and obvious growth effects, suggests that these 45 altered DNA methylation loci are the most sensitive biomarkers for early 46 diagnosis and risk assessment of genotoxic effects of Cd pollution in 47 ecotoxicology.

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

# 52 1. Introduction

53

54 Environmental contamination with heavy metals has drastically-increased

substantially over the past decades, which has aroused attention from 55 56 numerous researchers responsible for contamination diagnosis, environmental protection and ecological remediation. Cd, as one of the most highly toxic and 57 58 accumulative heavy metals, has contaminated soils and water through 59 atmospheric deposition, urban-industrial activities and agricultural practices 60 (Sun et al., 2008; Liu et al., 2011). Thus, the exploration of Cd toxicity, 61 especially its genotoxic effects of Cd, has become a focus in ecotoxicology 62 research (Herbette et al., 2006; Azevedo et al., 2007; Greco et al., 2012; 63 Pierron et al., 2014).

64 Several classic assays have been applied to examine the genotoxic 65 effects of heavy metals on organisms, e.g. the micronucleus assay and the 66 comet assay. However, neither is sensitive enough to determine the Cd 67 contamination level or able to provide information at the DNA level (Cambier et 68 al., 2010). With the rapid development of molecular biology, several 69 PCR-based techniques have been used to evaluate DNA damage in toxicology 70 studies. Random-amplified polymorphic DNA (RAPD) comprises involves the 71 use of a single "arbitrary" primer in a PCR reaction and results in the 72 amplification of several discrete DNA products (Welsh and McClelland, 1990; 73 Williams et al., 1990). RAPD has been used extensively in eco-toxicology 74 studies on animals and humans (Misra et al., 2001; Singh and Roy, 2001,2004; 75 Garnis et al., 2005). Recently, RAPD technique has been used in plant 76 eco-toxicology studies, detecting genetic variation and mutation induced by 77 stresses (Miki et al., 2001; Liu et al., 2005; 2007; Chao et al., 2008; 2009; 2012; 78 Gjorgieva et al., 2013; Malar et al., 2014). RAPD analysis is extremely efficient 79 for DNA analysis in complex genomes. Its use in investigating genomic DNA to 80 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 81 damage and mutations (Lopez-Moreno et al., 2010; Korpe and Aras, 2011). 82

83 Regarding DNA repair, Cd suppresses both eukaryotic MutS homolog 84 (MSH)2-MSH6- and MSH2-MSH3-dependent mismatch repair (MMR) activity through a nonspecific mechanism whereby involving binding of Cd binds to 85 86 multiple sites in MSH6 (Banerjee and Flores-Rozas, 2005; Wieland et al., 2009; 87 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 88 89 repeats (SSRs), leading to microsatellite instability (MSI). Previously, MSI was 90 found at two "hotspots" of microsatellite mutation in mice by analysis of a panel 91 of microsatellite markers under Cd stress (Oliveira et al., 2012; Du et al., 2014). 92 Also, there were some reports that SSRs mutations canould be promoted by 93 heavy metals, e.g. Lead, Chromium and Cd (Rodriguez et al., 2013a; 94 Rodriguez et al., 2013b; Du et al., 2014). Monteiro et al. (Monteiro et al., 2009) 95 demonstrated that SSR analyses could be used to evaluate plant genomic 96 DNA instability for deciphering DNA damage induced by Cd in a genotoxicity 97 test. Thus, MSI can be considered as a biomarker of high risk for human 98 cancer and for the assessment of different genotoxic effects of pollutants on 99 plants, respectively (Kovalchuk et al., 2000; Soreide et al., 2006). However, 100 little information is available about microsatellite mutations in Arabidopsis 101 under Cd stress.

102 Higher plants can rapidly adapt in response to stresses, but this can lead 103 to genome instability and changes in DNA methylation patterns throughout the 104 genome and at specific loci of DNA (Boyko et al., 2010; Chatterjee and Vinson, 105 2012). For example, plants employ important and sophisticated epigenetic 106 regulatory strategies, such as DNA methylation, to maintain genomic plasticity. 107 This, allowsing the simultaneous regulation of many genes involved in plant 108 defense and in facilitating relatively rapid adaptation to new internal and 109 environmental stress conditions without changesing in the DNA sequence 110 (Steward et al., 2002; Santoyo et al., 2011). The genomic DNA

111 hypermethylation pattern iswas found to be correlated with water deficit in pea 112 (Pisum sativum L.), pathogen stress in tobacco (Nicotiana tabacum), 113 radioactive contamination in Pinus silvestris, salt, UVC, cold, heat and flood 114 stresses in Arabidopsis, and chromium stress in Brassica napus, respectively 115 (Labra et al., 2002; Kovalchuk et al., 2003; Labra et al., 2004; Boyko et al., 116 2007; Mason et al., 2008). Also, DNA hypomethylation at several genomic loci 117 occurred in response to heavy metal stress in hemp and clover, to pathogen 118 stress in tobacco (Nicotiana tabacum), and to cold treatment in nucleosome 119 core regions of maize (Zea mays) seedlings (Steward et al., 2002; Aina et al., 120 2004; Boyko et al., 2007). Potentially, global DNA hypomethylation is often 121 viewed as a sign of genomic instability, which manifests as gross chromosomal 122 aberrations, genome rearrangement, MSI, deletions/insertions, and point 123 mutations, thus facilitating gene divergence and evolution of novel gene 124 functions (Boyko et al., 2007; Duan et al., 2013; Harris et al., 2013).

125 Huang et al. (Huang et al., 2012) reported that tumor cells usually exhibit 126 diverse patterns of DNA methylation, and that the CpG dinucleotide at any 127 given site of genomic DNA might differ in methylation status among 128 subpopulations of tumor cells, reflecting the heterogeneity and decreased 129 epigenetic fidelity of cancer cells. Thus, CpG dinucleotides are also called 130 methylation variable positions (MVPs) in epigenetic studies. Analysis of MVP 131 profiles might reveal stress-specific methylation patterns, and the methylation 132 frequency at any given MVPs could serve as a novel epigenetic biomarker for 133 physiological and pathologic status (Huang et al., 2012; Kit et al., 2012). 134 Moreover, minimal traces/differences of aberrant methylation profiles in blood 135 DNA could serve as early surrogate biomarkers for cancer diagnosis and for 136 risk assessment of PAHs exposure, respectively (Yang et al., 2012; Shin et al., 137 2013). Similarly, Santoyo et al (2011) reported that actual status of global DNA 138 methylation in earthworms is apparently affected by the overall effect of

metals/metalloids undergoing methylation and <u>that</u> 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).

143 Arabidopsis, with its vast resource of mutants, has long been used as a 144 model dicotyledonous higher plant for studies of plant physiology and plant 145 molecular biology. However, little information is available about potential 146 associations between the status of global DNA methylation and genomic 147 instability such as MSI and RAPD DNA polymorphisms in Arabidopsis 148 seedlings under Cd stress. Therefore, the principal aims of this work were to (1) 149 evaluate the extent of genomic instability in Arabidopsis seedlings using a 150 panel of 36 microsatellite markers and 3 RAPD primers; (2) determine the 151 status of global DNA methylation by MSAP-PCR in the Arabidopsis genome 152 under Cd stress; (3) explore potential associations between the actual status 153 of global DNA methylation and genomic instability such as MSI and RAPD 154 DNA polymorphisms in Arabidopsis under Cd stress. In-so-doing, we were 155 able to evaluate the aforementioned parameters as candidate biomarkers for 156 early diagnosis of genetic and epigenetic risks related to the presence of Cd in 157 the soil and water environment.

158 2. Materials and methods

#### 159 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.

165 Seeds were sown in sterile flasks supplemented with 150 mL  $0.5 \times$  liquid 166 MS medium (SIGMA, USA) including 0.5% (w/v) sucrose (pH5.8),

167 supplemented with Cd at a final concentration of 0 (the control), 0.25, 1.0, 4.0, 5.0, and 8.0 mg  $L^{-1}$  (only used in MSI analysis) in the form of CdCl<sub>2</sub>·2H<sub>2</sub>O of 168 169 analytical grade with purity 99.5%, PR China. Each flask with about 20 170 plantlets was placed on a rotary shaker at 50 rpm in an incubator (12 h light of 171 approximately 3000 lx and 12 h dark at 21  $\pm$  0.5 °C) for 15 d. All treatments 172 and analyses performed below were repeated in three independent replicates. 173 The seed germination rate and growth of the plantlets were measured at 174 15 d following germination, and then pooled to measure DNA content in 175 Arabidopsis shoots described in a previous study (Liu et al., 2009).

176 For MSI, RAPD, and DNA methylation assays, all DNA extractions and 177 downstream applications were performed on mixed groups using the same 178 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 179 180 storage at -70 °C. Genomic DNA from approximately 200-400 mg of fresh 181 Arabidopsis shoots was extracted respectively using Plant Genomic DNA 182 Extraction Kit. DNA concentration was examined by Eppendorf Biophotometor 183 Plus, and then verified by DL2000 DNA marker in a 0.8% agarose gel. All 184 experimental chemicals were ordered from TaKaRa Biotechnology Ltd. (Dalian, 185 PR China) unless otherwise mentioned.

# 186 2.2 MSI Analysis

187 46 pairs of SSR primers (Table A.1) were screened in this test for finding 188 genomic DNA MSI induced by Cd. PCRs were performed in a final volume of 189 25  $\mu$ L consisting of 25 ng DNA template, 1 U of Taq polymerase, 1× Taq 190 reaction buffer, 0.2 mM dNTPs, and 0.25  $\mu$ M of each primer. The touchdown 191 PCR conditions used for amplification were as follows: initial denaturation at

192 94°C for 5 min; followed by 9 cycles of denaturation at 94°C for 30 s, annealing 193 at 57°C for 30 s and primer extension for 30 s; the annealing temperature was 194 subsequently decreased by 0.5°C every second cycle until a 'touchdown' at 195 53°C, at which temperature 26 additional cycles followedwere carried out (35) 196 cycles in total). After the PCR reaction, amplification products were examined 197 using 8% (w/v) polyacrylamide gel electrophoresis (PAGE) with 7 M urea, 198 visualized by silver staining method using the Bio Image Analyzer System 199 (Bio-Rad, Vendor, Italy). To assess MSI, we compared the band pattern 200 produced after gel electrophoresis of paired PCR reactions containing the 201 control and Cd-treated DNA. If the control and Cd-treated PCR amplification 202 products displayed different electrophoretic motilities, the case was scored as 203 positive for MSI.

#### 204 2.3 RAPD genetic damage screening

205 RAPD analysis was performed using 3 primers (Primers 1, 2 and 4) 206 screened from 12 random primers (Liu et al., 2005). PCRs were performed in 207 reaction mixtures of 25 µL containing approximately 40 ng of DNA template, 208 0.5 µM primer, 0.2 mM dNTPs, 1x reaction buffer and 1 U of Taq DNA 209 polymerase. The RAPD protocol is consisted of an initial denaturing step of 5 210 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing 211 at 38 °C for 60 s and extension at 72 °C for 90 s, with an additional extension 212 period of 10 min at 72 °C. PCR products were separated by electrophoresis in 213 a 5% PAGE (50% urea). Gels were stained using silver nitrate. Images were 214 digitally captured and the intensity of bands on the gel quantified using Image 215 Lab (Bio-Rad).

# 216 2.4 Global DNA methylation assay using MSAP-PCR

217 Global DNA methylation was evaluated by methylation-sensitive arbitrarily 218 primed PCR (MSAP-PCR). Genomic DNA (1 µg) was digested in CutSmart 219 buffer with 1µL of Hpall or Mspl (NEB, USA) in a 50-µL reaction volume for 1.5 220 h at 37°C. Digested DNA was purified and collected by Takara MiniBest DNA 221 Fragment Purification Kit, then was transferred to another 50-µL reaction 222 containing 1 x EcoRI buffer and 1µL EcoRI, digested for 1.5 h at 37°C. After 223 purification, digested DNA was used for subsequent PCR reaction performed 224 as previously published (Gonzalgo et al., 1997). Hpall does not cut DNA if the 225 internal cytosine of CCGG restriction site is methylated and therefore the 226 bands generated in the PCR of DNA digested by EcoRI + Hpall lane are 227 methylated at the internal cytosine. Mspl is unable to cut DNA when the 228 external cytosine in CCGG site is methylated and so the bands generated in 229 the PCR of DNA digested by EcoRI + Mspl are methylated at the external 230 cytosine. Three primers designed by our lab and MLG2 (Gonzalgo et al., 1997) 231 were used for methylation assay (Table A.2). And PCR products were resolved 232 on 5% PAGE with 50% urea. Methods of gel staining and image capture were 233 the same aswith the above. An increase in band intensity indicates an increase 234 in methylation. Band intensity was measured to numerically determine the 235 change in DNA methylation.

# 236 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  $\geq$  50% as compared to the control. The stable polymorphism variation was calculated for

242 data statistics exclusively if it occurred simultaneously in at least two243 replications, leading to data without variance.

# 244 3. Results

## 245 3.1 MSI assay using SSRs

246 MSI was not detected in plantlets exposed to the control and 0.25-5.0 mg·L<sup>-1</sup> Cd for 15 d by a panel of 46 SSR primers. However, under an additional 247 treatment of 8.0 mg  $L^{-1}$  Cd, replication slipping of one repeat (about 2-3 bp) 248 249 was found using SSR primers of BSAT1.001 and BSAT2.012 (Fig. 1). 250 Moreover, the above replication slipping of three duplicates did appear in shoots under Cd stress of 8 mg·L<sup>-1</sup>, suggesting that MSI occurs exclusively 251 under such a strong stress that remarkably inhibited Arabidopsis growth (Fig. 252 253 A.1).



254

Fig. 1 MSI in Arabidopsis seedlings exposed to 0-8.0 mg·L<sup>-1</sup> 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<sup>-1</sup> 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.

261

#### 262 3.2 Genetic damage assay using RAPDs

Fig. 2 shows RAPD profiles in Arabidopsis seedlings grown with<u>out or and</u> with<u>out</u> Cd treatments after 15 d of exposure. In Fig. 2, each lane contains at least 10 bands ranged <u>fromin molecular size of</u> 300 to 3000 bp, in which bands of variable intensity detected in at least two replicates are calculated in Fig. 3. 267 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<sup>-1</sup> Cd 268 treatment, for different primers. 4.0 mg·L<sup>-1</sup> Cd treatment induced low 269 polymorphism whereas high polymorphism occurred at the 5.0 mg L<sup>-1</sup> Cd 270 treatment. Numbers of polymorphism loci were 1.5 and 24.5 for 4.0 and 5.0 271 mg L<sup>-1</sup> Cd, respectively (Fig. 3). In these cases, RAPD polymorphisms were 272 273 due to the loss and/or gain of amplified bands in the treated samples 274 compared with the control.





275

Fig. 2 RAPD fingerprints of Arabidopsis seedlings exposed to 0-5.0 mg·L<sup>-1</sup> 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<sup>-1</sup> 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).

282 Red arrows illustrated polymorphism between plantlets exposed to 5.0 mg·L<sup>-1</sup>

283 Cd and the control.



284

Fig. 3 Polymorphism variations detected by RAPD from Arabidopsis plantlets exposed to 0 - 5.0 mg·L<sup>-1</sup> Cd for 15 d.

287

# 288 3.3 Methylation damage assay using MSAP-PCR

289 Aberrations of global DNA methylation in Arabidopsis induced by Cd was 290 evaluated by MSAP-PCR. For all treatments, reproducible bands or cytosine 291 residues of variable intensity were evaluated (Fig. 4) and calculated in Fig. 5 292 for methylation analysis at both internal (Hpall) and external (Mspl) cytosines 293 of CCGG sites. At the external cytosine, Cd stress resulted in global 294 hypermethylation in the shoot tissues relative to the controls (Fig. 5A). This 295 hypermethylation more closely follows an inverted U-shaped dose-response to 296 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 297

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

299 At the internal cytosine, a similar trend for hypermethylation and 300 hypomethylation under Cd stress was observed as compared to the untreated 301 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·L<sup>-1</sup> Cd doses, respectively, which were 302 303 prominently more than that at the external cytosine. Moreover, 304 hypomethylation observed arose linearly albeit in a low proportion of increase 305 (Fig. 5B).

Different methylation polymorphic bands were detected at 0.25-5.0 mg·L<sup>-1</sup> 306 307 concentration of Cd for different cytosines in the shoots (Fig. 5). Also, these 308 polymorphisms indicate similar inverted U-shaped concentration response 309 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, 310 311 respectively. In all cases, methylation polymorphisms were due to the loss and 312 gain of variable intensity of reproducible amplified bands in the Cd-treated 313 seedlings as compared to the control (Fig. 4).



314

Fig. 4 The representative MSAP-PCR profiles of Arabidopsis seedlings exposed to  $0 - 5.0 \text{ mg} \cdot \text{L}^{-1}$  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 \cdot \text{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<sup>-1</sup> Cd for 15 d. (A) Frequency of hypermethylation, hypomethylation and methylation polymorphism at external cytosine (CHG) loci and (B) internal cytosine (C<sub>P</sub>G) loci.

# 330 4. Discussion

# 331 **4.1 MSI <del>is sensibility-insufficient</del> in vivo <u>is not <del>as</del> a sensitive</u>**

#### 332 biomarker for Cd stress

333 Cd has been responsible for proofreading and MMR deficiency that could 334 cause genomic MSI (Jin et al., 2003; Edelbrock et al., 2013). As far as we 335 know, SSRs was first adopted by Kovalchuk et al. (2000) as tools to assess 336 genetic instability in the offspring of wheat plants exposed to radiation near the 337 Chernobyl nuclear power plant. More recently, positive results of MSI have 338 been reported in transgenic plants consisting of a modified GUS reporter whose reading frame is disrupted by microsatellite repeat sequence (Yao and 339 340 Kovalchuk, 2011). Thus, MSI analysis has been considered as a biomarker to 341 detect genetic damages in ecotoxicology.

342 In the present study, MSI was not observed for the Arabidopsis seedlings exposed to Cd stress of 0.25-5.0 mg·L<sup>-1</sup> for 15 d, although shoots and roots in 343 344 the seedlings manifested significant inhibitory symptoms in response to Cd at 5.0 mg L<sup>-1</sup> (Fig. A.1). However, MSI was observed exclusively in seedlings 345 exposed to 8.0 mg L<sup>-1</sup>Cd, with the replication slipping of one repeat detected 346 347 by two SSRs (Fig. 2). According to a previous study (Monteiro et al., 2009), 348 MSI was plant tissue- and exposure time-dependent under Cd stress. In our 349 work, only shoots were studied because of their ease of handling and being 350 representative for the toxicity of the whole plant in contamination diagnosis. 351 Moreover, to further increase the toxicity, seedlings were exposed to Cd of additional low doses of  $(0.25 \text{ and } 1.0 \text{ mg} \cdot \text{L}^{-1} \text{ for } 21 \text{ d}$ . They were assayed by 352 353 two SSRs of BSAT1.001 and BSAT2.012, however, MSI remained negative 354 (Fig. 1).

355 Ir

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

356 be biomarkers of various cancers (Sardi et al., 2001; Vilar et al., 2014), 357 because cancers and tumors attach to severe cellular damage-strong 358 impairment of cells, eventually leading to cell deathfor animals, causing the death eventually. However in our work, Arabidopsis could still survive under 359 Cd stress of 0.25-5.0 mg  $L^{-1}$  (Fig. A.1), suggesting that repair of 360 insertion/deletion loops induced by Cd has been performed by DNA MMR 361 362 proteins MLH1, MSH2, MSH3, MSH6, PMS1 and PMS2. More importantly, 363 according to recent work (Yao and Kovalchuk, 2011), positive results of MSI 364 were induced by relatively severe stress in transgenic plants which might be 365 more sensitive in MSI because of its exogenous SSR. All the above results 366 imply that MSI is not a sufficiently sensitive biomarker in ecotoxicology studies.

# 367 4.2 DNA damage assayed by RAPD analysis is more 368 sensitive than by MSI

369 In ecotoxicology, genomic DNA damage such as DNA adducts, breakage, 370 insertion/deletion and base substitution can be detected by RAPD technique 371 as described previously, and is widely used in genotoxicity research 372 recently(Liu et al., 2009; Liu et al., 2012; Ladhar et al., 2014). In our study, 373 alterations in Arabidopsis genomic DNA induced by Cd were assayed by 374 RAPD profiles through three from 12 arbitrary primers screened. Prominent increase was observed at 5.0 mg·L<sup>-1</sup> Cd treatment and slight elevation at 4.0 375 mg L<sup>1</sup> Cd treatment for 15 d (Fig. 3). Also, note that Besides, treatment time 376 377 seems a big issue as seedlings exposed to Cd of additional low doses of 0.25 and 1.0 mg L<sup>-1</sup> for 21 d revealed a significant increase of RAPD 378 379 polymorphisms (Fig. A.2), where MSI remained negative. This suggests that 380 RAPD polymorphism is more sensitive to Cd stress than MSI as a biomarker.

381 MSI, one of genomic instability, is a consequence of MMR deficiency and
382 can<u>-no</u>t be induced by stresses straightforwardly (Clark and Kunkel, 2004;

383 Wieland et al., 2009). To our knowledge, MMR contains several proteins 384 involved in recognition and repair of DNA damage, and can regulate G2/M 385 checkpoints during the cell cycle, which can allow cells to overcome replication 386 errors before entering mitosis (Campregher et al., 2008). Thus, MSI induced 387 by stresses would be repaired by DNA MMR proteins before cell proliferation 388 and would not be predicted to occur in Arabidopsis seedlings exposed to 0.25-5.0 mg·L<sup>-1</sup> Cd. However, replication slipping errors can't be repaired when 389 390 the entire MMR system is fatally paralyzed, indeed, MSI did appeared in Arabidopsis seedlings exposed to 8.0 mg  $L^{-1}$  Cd (Fig. 1). 391

392 However, DNA adducts can be replicated and transmitted to next generation of cells through translesion replication, escaping surveillance and 393 394 repair systems (Zang et al., 2005). Under the stresses, DNA polymerases 395 during the translesion synthesis processes are forced to encounter multiple 396 types of damaged DNA, and can proceed to replicate modified DNA during the 397 cell cycle. Also, cells evolve the translesion synthesis processes to tolerate 398 genomic lesions by error-free or error-prone repair. Moreover, error-prone 399 repair could pass the errors to the next generation of cells by cell proliferation. 400 This is a type of emergency system used to maintain cells survived, while 401 severe DNA damage blocks the cell cycle, leading to cell cycle arrest or 402 apoptosis/death [58]. Additionally, RAPD technique can detect temporary DNA 403 damage and mutations caused by DNA adducts that could override the normal 404 DNA replication and concurrently pass through translesion replication (Liu et 405 al., 2005). This might explainaccount for the reasons why RAPD polymorphism 406 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  $L^{-1}$  Cd (Fig.s 1 and 3). 407

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# 409 4.3 Aberrations in DNA methylation by MSAP-PCR are the 410 optimum biomarkers in ecotoxicology.

411 Plants employ epigenetic regulatory strategies, such as DNA methylation, 412 to maintain genomic plasticity, allowing the simultaneous regulation of 413 expression of many genes involved in plant defense and in facilitating rapid 414 adaptation to new stress conditions without changing the DNA sequence 415 (Steward et al., 2002; Santoyo et al., 2011). Herein, our results indicate that Cd 416 stress does affect patterns of genomic DNA methylation in Arabidopsis shoots 417 (Fig. 5). Although the accurate timing and role of the internal and external 418 cytosines in the CCGG restriction sites remains unresolvedhas not been 419 clearly elucidated, data in Fig. 5 indicate that differential methylation patterns 420 of different cytosines in the CCGG sites in the genomic DNA are affected by Cd atof 0.25-5.0 mg·L<sup>-1</sup>-levels. The hypermethylation more closely follows an 421 422 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 423 424 external cytosines of CCGG sites. respectively. Moreover, the 425 hypermethylations were more prominently more at the internal cytosine than 426 that at the external cytosine. Also, hypomethylation was elevated albeit in a 427 low proportion of increase at both external and internal cytosines (Fig. 5). A 428 similar trend was reported foron other plant and animal species (Aypar et al., 429 2011; Fulnecek and Kovarik, 2014). Hypomethylation may increase the 430 susceptibility of chromosomes to breakage, and hypermethylaiton may also 431 cause chromosomal instability by inhibiting cell cycle-dependent checkpoint 432 controls, with deleterious effects on the cells (Kovalchuk et al., 2001). 433 Importantly, stress-induced change itself might be more important than the 434 direction of change, since any aberrations in DNA methylation is considered 435 detrimental to the cells (Aypar et al., 2011). Thereby, modified patterns of

436 global DNA methylation probably can be applied as a potential biomarker for437 biomonitoring Cd exposure.

438 Global DNA methylation modifications are immediately initiated and 439 proceed rapidly in plant cells in response to stress through signal transduction, 440 leading to fast epigenetic changes (Steward et al., 2002; Santoyo et al., 2011). 441 The mechanisms for DNA methylation effects by Cd have not been fully 442 elucidated. Nonetheless, emerging evidence shows that the major cause for 443 altered methylation is due to changes in methyltransferase activities and in 444 coordinated expression of the chromatin-related genes known to be 445 responsible for faithful maintenance of the various methylation patterns in 446 plants (Ou et al., 2012). For example, Greco et al. (Greco et al., 2012) reported 447 that Cd increased levels of chromomethylase (CMT) activities and elicited 448 concomitant increases in global genomic DNA methylation, together with an 449 heterochromatic nuclear fraction in Posidonia enhanced oceanica. 450 Alternatively, Cd can interfere with the activity of DNA methyltransferases 451 through overproduction of reactive oxygen species (ROS), and oxidative DNA 452 damage could subsequently lead to an altered DNA methylation profile and 453 subsequent alterations in gene expression, and/or siRNAs/RdDM and 454 ROS1/DME (Mirbahai and Chipman, 2014). In the current study (Fig. 5), the 455 aberrant changes of global DNA methylation we detected in Cd-treated 456 seedlings could be the consequences of siRNAs/RdDM and CMT/DNMTs 457 aberrant expression resulting from exposure to Cd for 15 d.

The utilization of biomarkers to assess <u>biotic</u>effects of exposure of the organisms to stresses has received increasing attention, and biomarkers act <u>Increasingly, biomarkers are used</u> 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 <u>E</u>epigenetic biomarkers may be indicators for early diagnosis or risk assessment of human diseases,

464 pollutant exposure and impact (Huang et al., 2012; Kit et al., 2012). Recently, 465 DNA methylation-based biomarkers have been proven to be more specific and 466 sensitive than commonly used protein biomarkers, which could clearly justify 467 their use in clinics (Kit et al., 2012). For example, emerging evidence shows 468 that minimal traces of aberrant methylation profiles in blood DNA could serve 469 as early surrogate biomarkers for cancer diagnosis and for risk assessment of 470 PAHs exposure, respectively (Yang et al., 2012; Shin et al., 2013), supporting 471 the notion that these methylation changes are the initial step that triggers 472 various genetic events in these tumors (Kit et al., 2012). In another study, DNA 473 methylation was previously introduced as an interface between the genome 474 and environment, providing partial mechanisticm explanations for the 475 sensitivity of organisms to environmental stresses. Moreover, DNA 476 methylation's persistence can usefully reflect the status of the environment in 477 which organisms reside (Mirbahai and Chipman, 2014). In our study, DNA 478 methylation polymorphism is much higher than RAPD loci and MSI in seedlings exposed to Cd stress of 0.25-5.0 mg·L<sup>-1</sup> and the effects are 479 480 dose-related, whereas DNA polymorphism using RAPD and MSI analyses is observed exclusively at doses of 4.0-5.0 mg  $L^{-1}$  and 8.0 mg  $L^{-1}$  Cd, 481 482 respectively (Fig.s 1-5). These results provide evidence that epigenetic 483 biomarker, such as DNA methylation polymorphism in seedlings, could be a 484 more sensitive biomarker for Cd exposure than RAPD polymorphism and MSI. 485 In the field of ecotoxicology, advances in DNA methylation analysis have 486 led to the recognition that aberrant DNA methylation status is potentially an 487 early, rapid and sensitive molecular biomarker indicative of pollutant exposure 488 and impact. MSAP-PCR is the most widely used method for screening 489 aberrant DNA methylation in cells, and this approach has several advantages:

490 more precision/accuracy, sensitivity, and reproducibility, and easier to carry out 491 than traditional MSAP technique in studies (Aypar et al., 2011; Fulnecek and

Kovarik, 2014; Pierron et al., 2014). <u>Moreover, In addition, today</u> MSAP-PCR is
<u>becoming</u> 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.

497 5. Conclusions

498 The present report defines modifications in the genomic DNA methylation 499 in correlation with the induction of DNA RAPD polymorphism and MSI 500 measured in Arabidopsis seedlings exposed to Cd of 0.25-5.0 mg  $L^{-1}$  for 15 d. 501 We observed that MSI and RAPD polymorphism appeared exclusively under Cd stress of 8.0 mg·L<sup>-1</sup> and of 4.0-5.0 mg·L<sup>-1</sup>, respectively. However, wethe 502 503 present study shows that prominent DNA methylation polymorphism occurred under Cd stress of 0.25-5.0 mg·L<sup>-1</sup> by MSAP-PCR. Furthermore, the inverted 504 505 U-shaped dose-response effects of Cd stress on DNA methylation 506 polymorphism were observed in Arabidopsis seedlings. Given that The ability 507 of MSAP-PCR to detects substantial epigenetic alterations in Arabidopsis 508 seedlings at Cd exposure, prior to the onset of RAPD polymorphism, MSI and 509 significant growth effects, it wshould be useful for greatly improve 510 environmental risk assessment. With further development, the MSAP-PCR 511 method may also provide a more sensitive and reliable means in identification 512 and analysis of DNA methylation modifications in the other Cd-treated plants, 513 and becomeit may be a powerful tool for early detection of genotoxic effects of 514 other heavy-metal stresses in ecotoxicology.

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1 Cadmium-induced genomic instability in Arabidopsis:

# 2 molecular toxicological biomarkers for early diagnosis of

#### 3 cadmium stress

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28 ABSTRACT: Microsatellite instability (MSI) analysis, random-amplified 29 polymorphic DNA (RAPD), and methylation-sensitive arbitrarily primed PCR 30 (MSAP-PCR) are methods to evaluate the toxicity of environmental pollutants 31 in stress-treated plants and human cancer cells. Here, we evaluate these 32 techniques to screen for genetic and epigenetic alterations of Arabidopsis plantlets exposed to 0-5.0 mg·L<sup>-1</sup> cadmium (Cd) for 15 d. There was a 33 34 substantial increase in RAPD polymorphism of 24.5, and in genomic 35 methylation polymorphism of 30.5-34.5 at CpG and of 14.5-20 at CHG sites under Cd stress of 5.0 mg  $L^{-1}$  by RAPD and of 0.25-5.0 mg  $L^{-1}$  by 36 37 MSAP-PCR, respectively. However, only a tiny increase of 1.5 loci by RAPD occurred under Cd stress of 4.0 mg  $L^{-1}$ , and an additional high dose (8.0 38 39 mg L<sup>-1</sup>) resulted in one repeat by MSI analysis. MSAP-PCR detected the most 40 significant epigenetic modifications in plantlets exposed to Cd stress, and the 41 patterns of hypermethylation and polymorphisms were consistent with inverted 42 responses. The presence of genomic methylation U-shaped dose 43 polymorphism in Cd-treated seedlings, prior to the onset of RAPD 44 polymorphism, MSI and obvious growth effects, suggests that these altered 45 DNA methylation loci are the most sensitive biomarkers for early diagnosis and 46 risk assessment of genotoxic effects of Cd pollution in ecotoxicology.

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

# 51 1. Introduction

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53 Environmental contamination with heavy metals has increased 54 substantially over the past decades, which has aroused attention from

55 numerous researchers responsible for contamination diagnosis, environmental 56 protection and ecological remediation. Cd, as one of the most highly toxic and accumulative heavy metals, has contaminated soils and water through 57 58 atmospheric deposition, urban-industrial activities and agricultural practices 59 (Sun et al., 2008; Liu et al., 2011). Thus, the exploration of Cd toxicity, 60 especially its genotoxic effects, has become a focus in ecotoxicology research 61 (Herbette et al., 2006; Azevedo et al., 2007; Greco et al., 2012; Pierron et al., 62 2014).

63 Several classic assays have been applied to examine the genotoxic 64 effects of heavy metals on organisms, e.g. the micronucleus assay and the 65 comet assay. However, neither is sensitive enough to determine the Cd 66 contamination level or able to provide information at the DNA level (Cambier et 67 al., 2010). With the rapid development of molecular biology, several 68 PCR-based techniques have been used to evaluate DNA damage in toxicology 69 studies. Random-amplified polymorphic DNA (RAPD) comprises a single 70 "arbitrary" primer in a PCR reaction and results in the amplification of several 71 discrete DNA products (Welsh and McClelland, 1990; Williams et al., 1990). 72 RAPD has been used extensively in eco-toxicology studies on animals and 73 humans (Misra et al., 2001; Singh and Roy, 2001,2004; Garnis et al., 2005). 74 Recently, RAPD has been used in plant eco-toxicology studies, detecting 75 genetic variation and mutation induced by stresses (Miki et al., 2001; Liu et al., 76 2005; 2007; Chao et al., 2008; 2009; 2012; Gjorgieva et al., 2013; Malar et al., 77 2014). RAPD analysis is extremely efficient for DNA analysis in complex 78 genomes. Its use in investigating genomic DNA to detect different kinds of DNA 79 damage and mutations suggests that RAPD can potentially form the basis of 80 novel biomarker assays for the detection of DNA damage and mutations 81 (Lopez-Moreno et al., 2010; Korpe and Aras, 2011).

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Regarding DNA repair, Cd suppresses both eukaryotic MutS homolog

83 MSH2-MSH6- and MSH2-MSH3-dependent mismatch repair (MMR) activity 84 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 85 86 or low activity of MMR proteins results in losses and gains of dinucleotide 87 repeats in microsatellites, also known as simple sequence repeats (SSRs), 88 leading to microsatellite instability (MSI). Previously, MSI was found at two 89 "hotspots" of microsatellite mutation in mice by analysis of a panel of 90 microsatellite markers under Cd stress (Oliveira et al., 2012; Du et al., 2014). 91 Also, SSRs mutations can be promoted by heavy metals, e.g. Lead, Chromium 92 and Cd (Rodriguez et al., 2013a; Rodriguez et al., 2013b; Du et al., 2014). 93 Monteiro et al. (Monteiro et al., 2009) demonstrated that SSR analyses could 94 be used to evaluate plant genomic DNA instability for deciphering DNA 95 damage induced by Cd in a genotoxicity test. Thus, MSI can be considered as 96 a biomarker of high risk for human cancer and for the assessment of different 97 genotoxic effects of pollutants on plants (Kovalchuk et al., 2000; Soreide et al., 98 2006). However, little information is available about microsatellite mutations in 99 Arabidopsis under Cd stress.

100 Higher plants can rapidly adapt in response to stress, but this can lead to 101 genome instability and changes in DNA methylation patterns throughout the 102 genome and at specific loci of DNA (Boyko et al., 2010; Chatterjee and Vinson, 103 2012). For example, plants employ important and sophisticated epigenetic 104 regulatory strategies, such as DNA methylation, to maintain genomic plasticity. 105 This allows the simultaneous regulation of many genes involved in plant 106 defense and in facilitating relatively rapid adaptation to new internal and 107 environmental stress conditions without changes in DNA sequence (Steward et 108 al., 2002; Santoyo et al., 2011). The genomic DNA hypermethylation pattern is correlated with water deficit in pea (Pisum sativum L.), pathogen stress in 109 110 tobacco (Nicotiana tabacum), radioactive contamination in Pinus silvestris, salt,

111 UVC, cold, heat and flood stresses in Arabidopsis, and chromium stress in 112 Brassica napus (Labra et al., 2002; Kovalchuk et al., 2003; Labra et al., 2004; 113 Boyko et al., 2007; Mason et al., 2008). Also, DNA hypomethylation at several 114 genomic loci occurred in response to heavy metal stress in hemp and clover, to 115 pathogen stress in tobacco (Nicotiana tabacum), and to cold treatment in 116 nucleosome core regions of maize (Zea mays) seedlings (Steward et al., 2002; 117 Aina et al., 2004; Boyko et al., 2007). Potentially, global DNA hypomethylation 118 is often viewed as a sign of genomic instability, which manifests as gross 119 chromosomal aberrations, genome rearrangement, MSI, deletions/insertions, 120 and point mutations, thus facilitating gene divergence and evolution of novel 121 gene functions (Boyko et al., 2007; Duan et al., 2013; Harris et al., 2013).

122 Huang et al. (Huang et al., 2012) reported that tumor cells usually exhibit 123 diverse patterns of DNA methylation, and that the CpG dinucleotide at any 124 given site of genomic DNA might differ in methylation status among subpopulations of tumor cells, reflecting the heterogeneity and decreased 125 126 epigenetic fidelity of cancer cells. Thus, CpG dinucleotides are also called 127 methylation variable positions (MVPs) in epigenetic studies. Analysis of MVP 128 profiles might reveal stress-specific methylation patterns, and the methylation 129 frequency at any given MVPs could serve as a novel epigenetic biomarker for 130 physiological and pathologic status (Huang et al., 2012; Kit et al., 2012). 131 Moreover, minimal traces/differences of aberrant methylation profiles in blood 132 DNA could serve as early surrogate biomarkers for cancer diagnosis and for 133 risk assessment of PAHs exposure, respectively (Yang et al., 2012; Shin et al., 134 2013). Similarly, global DNA methylation in earthworms is apparently affected 135 by the overall effect of metals/metalloids undergoing methylation and this 136 parameter might be a candidate biomarker of epigenetic risks related to the 137 presence of the metal elements in terrestrial environments (Santoyo et al., 138 2011).

139 Arabidopsis, with its vast resource of mutants, has long been used as a 140 model dicotyledonous higher plant for studies of plant physiology and plant 141 molecular biology. However, little information is available about potential 142 associations between the status of global DNA methylation and genomic 143 instability such as MSI and RAPD DNA polymorphisms in Arabidopsis 144 seedlings under Cd stress. Therefore, the principal aims of this work were to (1) 145 evaluate the extent of genomic instability using a panel of 36 microsatellite 146 markers and 3 RAPD primers; (2) determine the status of global DNA 147 methylation by MSAP-PCR under Cd stress; (3) explore potential associations 148 between the actual status of global DNA methylation and genomic instability 149 such as MSI and RAPD DNA polymorphisms in Arabidopsis under Cd stress. 150 In-so-doing, we were able to evaluate the aforementioned parameters as 151 candidate biomarkers for early diagnosis of genetic and epigenetic risks 152 related to the presence of Cd in the soil and water environment.

## 153 2. Materials and methods

#### 154 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.

160 Seeds were sown in sterile flasks supplemented with 150 mL  $0.5 \times$  liquid 161 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,

163 5.0, and 8.0 mg·L<sup>-1</sup> (only used in MSI analysis) in the form of CdCl<sub>2</sub>·2H<sub>2</sub>O 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  $\pm$ 0.5 °C) for 15 d. All treatments and analyses were repeated in three independent replicates.

168 The seed germination rate and growth of the plantlets were measured at 169 15 d following germination, and then pooled to measure DNA content in 170 Arabidopsis shoots described in a previous study (Liu et al., 2009).

171 For MSI, RAPD, and DNA methylation assays, all DNA extractions and 172 downstream applications were performed on mixed groups using the same 173 batches of reagents. In addition, the control and Cd-exposed samples were 174 collected at 15 d of incubation, and snap frozen in liquid nitrogen prior to 175 storage at -70 °C. Genomic DNA from approximately 200-400 mg of fresh 176 Arabidopsis shoots was extracted respectively using Plant Genomic DNA 177 Extraction Kit. DNA concentration was examined by Eppendorf Biophotometor 178 Plus, and then verified by DL2000 DNA marker in a 0.8% agarose gel. All 179 experimental chemicals were ordered from TaKaRa Biotechnology Ltd. (Dalian, 180 PR China) unless otherwise mentioned.

#### 181 2.2 MSI Analysis

182 46 pairs of SSR primers (Table A.1) were screened in this test for finding 183 genomic DNA MSI induced by Cd. PCRs were performed in a final volume of 184 25 µL consisting of 25 ng DNA template, 1 U of Taq polymerase, 1x Taq reaction buffer, 0.2 mM dNTPs, and 0.25 µM of each primer. The touchdown 185 186 PCR conditions used for amplification were as follows: initial denaturation at 187 94°C for 5 min; 9 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 188 30 s and primer extension for 30 s; the annealing temperature was 189 subsequently decreased by 0.5°C every second cycle until a 'touchdown' at 190 53°C, at which temperature 26 additional cycles followed (35 cycles in total). 191 After the PCR reaction, amplification products were examined using 8% (w/v)

192 polyacrylamide gel electrophoresis (PAGE) with 7 M urea, visualized by silver 193 staining method using the Bio Image Analyzer System (Bio-Rad, Vendor, Italy). 194 To assess MSI, we compared the band pattern produced after gel 195 electrophoresis of paired PCR reactions containing the control and Cd-treated 196 DNA. If the control and Cd-treated PCR amplification products displayed 197 different electrophoretic motilities, the case was scored as positive for MSI.

#### 198 2.3 RAPD genetic damage screening

199 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 200 201 reaction mixtures of 25 µL containing approximately 40 ng of DNA template, 202 0.5 µM primer, 0.2 mM dNTPs, 1x reaction buffer and 1 U of Tag DNA 203 polymerase. The RAPD protocol consisted of an initial denaturing step of 5 min 204 at 94 °C, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 205 38 °C for 60 s and extension at 72 °C for 90 s, with an additional extension 206 period of 10 min at 72 °C. PCR products were separated by electrophoresis in 207 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 208 209 Lab (Bio-Rad).

#### 210 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  $\mu$ g) was digested in CutSmart buffer with 1 $\mu$ L of Hpall or Mspl (NEB, USA) in a 50- $\mu$ L 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- $\mu$ L reaction containing 1 × EcoRI buffer and 1 $\mu$ L EcoRI, digested for 1.5 h at 37°C. After

217 purification, digested DNA was used for subsequent PCR reaction performed 218 as previously published (Gonzalgo et al., 1997). Hpall does not cut DNA if the 219 internal cytosine of CCGG restriction site is methylated and therefore the 220 bands generated in the PCR of DNA digested by EcoRI + Hpall lane are 221 methylated at the internal cytosine. Mspl is unable to cut DNA when the 222 external cytosine in CCGG site is methylated and so the bands generated in 223 the PCR of DNA digested by EcoRI + MspI are methylated at the external 224 cytosine. Three primers designed by our lab and MLG2 (Gonzalgo et al., 1997) 225 were used for methylation assay (Table A.2). PCR products were resolved on 226 5% PAGE with 50% urea. Methods of gel staining and image capture were the 227 same as the above. An increase in band intensity indicates an increase in 228 methylation. Band intensity was measured to numerically determine the 229 change in DNA methylation.

#### 230 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  $\geq$  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.

#### 238 3. Results

#### 239 3.1 MSI assay using SSRs

MSI was not detected in plantlets exposed to the control and 0.25-5.0 mg·L<sup>-1</sup> Cd for 15 d by a panel of 46 SSR primers. However, under an additional

treatment of 8.0 mg·L<sup>-1</sup> 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<sup>-1</sup>, 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<sup>-1</sup> 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<sup>-1</sup> 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.

# 255 3.2 Genetic damage assay using RAPDs

Fig. 2 shows RAPD profiles in Arabidopsis seedlings grown with or without 256 257 Cd treatments after 15 d of exposure. In Fig. 2, each lane contains at least 10 258 bands ranged from 300 to 3000 bp, in which bands of variable intensity 259 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 1.0 260  $mg \cdot L^{-1}$  Cd treatment, for different primers. 4.0  $mg \cdot L^{-1}$  Cd induced low 261 polymorphism whereas high polymorphism occurred at the 5.0 mg·L<sup>-1</sup> Cd 262 263 treatment. Numbers of polymorphism loci were 1.5 and 24.5 for 4.0 and 5.0 mg·L<sup>-1</sup> Cd, respectively (Fig. 3). In these cases, RAPD polymorphisms were 264 265 due to the loss and/or gain of amplified bands in the treated samples 266 compared with the control.



Fig. 2 RAPD fingerprints of Arabidopsis seedlings exposed to 0-5.0 268 269 mg·L<sup>-1</sup> Cd for 15 d. (A) PAGE profiles amplified by primer 1, (B) by primer 2 270 and (C) by primer 4, in which lanes (1) - (5) are PCR fragments amplified from 271 the DNA templates of Arabidopsis shoots exposed to 0, 0.25, 1.0, 4.0 and 5.0 272  $mg L^{-1}$  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). 273 Red arrows illustrated polymorphism between plantlets exposed to 5.0 mg  $L^{-1}$ 274 275 Cd and the control.



276

Fig. 3 Polymorphism variations detected by RAPD from Arabidopsis plantlets exposed to 0 - 5.0 mg·L<sup>-1</sup> Cd for 15 d.

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## 280 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<sup>-1</sup> Cd, respectively. Notably, the hypomethylation 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 and 26 at 0.25, 1.0, 4.0 and 5.0 mg·L<sup>-1</sup> Cd doses, respectively, which were 294 295 than prominently more that at the external cytosine. Moreover, 296 hypomethylation arose linearly albeit in a low proportion of increase (Fig. 5B).

Different methylation polymorphic bands were detected at 0.25-5.0 mg  $L^{-1}$ 297 298 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 mg L<sup>-1</sup> (Fig. 5) for the internal and external cytosines, respectively. In all cases, 301 302 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<sup>-1</sup> 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<sup>-1</sup> 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  $0 - 5.0 \text{ mg} \cdot \text{L}^{-1}$  Cd for 15 d. (A) Frequency of hypermethylation, hypomethylation and methylation polymorphism at external cytosine (CHG) loci and (B) internal cytosine (C<sub>P</sub>G) loci.

# 321 4. Discussion

#### 322 4.1 MSI in vivo is not a sensitive biomarker for Cd stress

323 Cd has been responsible for proofreading and MMR deficiency that could 324 cause genomic MSI (Jin et al., 2003; Edelbrock et al., 2013). As far as we 325 know, SSRs was first adopted by Kovalchuk et al. (2000) as tools to assess 326 genetic instability in the offspring of wheat plants exposed to radiation near the 327 Chernobyl nuclear power plant. More recently, positive results of MSI have 328 been reported in transgenic plants consisting of a modified GUS reporter whose reading frame is disrupted by microsatellite repeat sequence (Yao and 329 330 Kovalchuk, 2011). Thus, MSI analysis has been considered as a biomarker to 331 detect genetic damages in ecotoxicology.

332 In the present study, MSI was not observed for the Arabidopsis seedlings 333 exposed to Cd stress of 0.25-5.0 mg L<sup>-1</sup> for 15 d, although shoots and roots in 334 the seedlings manifested significant inhibitory symptoms in response to Cd at 335 5.0 mg·L<sup>-1</sup> (Fig. A.1). However, MSI was observed exclusively in seedlings exposed to 8.0 mg L<sup>-1</sup>Cd, with the replication slipping of one repeat detected 336 337 by two SSRs (Fig. 2). According to a previous study (Monteiro et al., 2009), 338 MSI was plant tissue- and exposure time-dependent under Cd stress. In our 339 work, only shoots were studied because of their ease of handling and being 340 representative for the toxicity of the whole plant in contamination diagnosis. 341 Moreover, to further increase the toxicity, seedlings were exposed to Cd of additional low doses of 0.25 and 1.0 mg  $L^{-1}$  for 21 d. They were assayed by 342 two SSRs of BSAT1.001 and BSAT2.012, however, MSI remained negative 343 344 (Fig. 1).

345 In oncology studies, MSI due to MMR deficiency is a biomarker of various 346 cancers (Sardi et al., 2001; Vilar et al., 2014), because cancers and tumors

347 attach to severe cellular damage, eventually leading to cell death. However in 348 our work, Arabidopsis could still survive under Cd stress of 0.25-5.0 mg·L<sup>-1</sup> 349 (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 350 351 and PMS2. More importantly, according to recent work (Yao and Kovalchuk, 352 2011), positive results of MSI were induced by relatively severe stress in 353 transgenic plants which might be more sensitive in MSI because of its 354 exogenous SSR. All the above results imply that MSI is not a sufficiently 355 sensitive biomarker in ecotoxicology studies.

# 356 4.2 DNA damage assayed by RAPD analysis is more 357 sensitive than by MSI

358 In ecotoxicology, genomic DNA damage such as DNA adducts, breakage, 359 insertion/deletion and base substitution can be detected by RAPD technique, 360 and is widely used in genotoxicity research (Liu et al., 2009; Liu et al., 2012; 361 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 362 primers screened. Prominent increase was observed at 5.0 mg·L<sup>-1</sup> Cd and 363 slight elevation at 4.0 mg $\cdot$ L<sup>-1</sup> Cd treatment for 15 d (Fig. 3). Also, note that 364 seedlings exposed to Cd of additional low doses of 0.25 and 1.0 mg·L<sup>-1</sup> for 21 365 366 d revealed a significant increase of RAPD polymorphisms (Fig. A.2), where 367 MSI remained negative. This suggests that RAPD polymorphism is more 368 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·L<sup>-1</sup> 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·L<sup>-1</sup> Cd (Fig. 1).

380 DNA adducts can be replicated and transmitted to next generation of cells 381 through translesion replication, escaping surveillance and repair systems (Zang et al., 2005). Under the stresses, DNA polymerases during the 382 383 translesion synthesis processes are forced to encounter multiple types of damaged DNA, and can proceed to replicate modified DNA during the cell 384 385 cycle. Also, cells evolve the translesion synthesis processes to tolerate 386 genomic lesions by error-free or error-prone repair. Moreover, error-prone 387 repair could pass the errors to the next generation of cells by cell proliferation. 388 This is a type of emergency system used to maintain cells, while severe DNA 389 damage blocks the cell cycle, leading to cell cycle arrest or apoptosis/death 390 [58]. Additionally, RAPD technique can detect temporary DNA damage and 391 mutations caused by DNA adducts that could override normal DNA replication 392 and concurrently pass through translesion replication (Liu et al., 2005). This 393 might explain why RAPD polymorphism is much more sensitive than MSI in 394 the present study, and MSI in Arabidopsis was detected only at the very high dose of 8.0 mg $\cdot$ L<sup>-1</sup> Cd (Fig.s 1 and 3). 395

396

# 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 CCGG sites in the genomic DNA are affected by Cd at 0.25-5.0 mg L<sup>-1</sup>. The 408 409 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 L<sup>-1</sup> (Fig.s 4-5) for the internal and external cytosines of CCGG sites, 411 412 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.

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

429 elucidated. Nonetheless, emerging evidence shows that the major cause for 430 altered methylation is due to changes in methyltransferase activities and in 431 coordinated expression of the chromatin-related genes known to be 432 responsible for faithful maintenance of the various methylation patterns in 433 plants (Ou et al., 2012). For example, Greco et al. (Greco et al., 2012) reported 434 that Cd increased levels of chromomethylase (CMT) activities and elicited 435 concomitant increases in global genomic DNA methylation, together with an 436 nuclear fraction Posidonia enhanced heterochromatic in oceanica. 437 Alternatively, Cd can interfere with the activity of DNA methyltransferases 438 through overproduction of reactive oxygen species (ROS), and oxidative DNA 439 damage could subsequently lead to an altered DNA methylation profile and 440 subsequent alterations in gene expression, and/or siRNAs/RdDM and 441 ROS1/DME (Mirbahai and Chipman, 2014). In the current study (Fig. 5), the 442 aberrant changes of global DNA methylation we detected in Cd-treated 443 seedlings could be the consequences of siRNAs/RdDM and CMT/DNMTs 444 aberrant expression resulting from exposure to Cd for 15 d.

445 Increasingly, biomarkers are used as an integrative measurement at the 446 sub-organism level to indicate stress status before damage to the biota arises 447 (Kit et al., 2012). Epigenetic biomarkers may be indicators for early diagnosis 448 or risk assessment of human diseases, pollutant exposure and impact (Huang 449 et al., 2012; Kit et al., 2012). Recently, DNA methylation-based biomarkers 450 have been proven to be more specific and sensitive than commonly used 451 protein biomarkers, which could clearly justify their use in clinics (Kit et al., 452 2012). For example, emerging evidence shows that minimal traces of aberrant 453 methylation profiles in blood DNA could serve as early surrogate biomarkers 454 for cancer diagnosis and for risk assessment of PAHs exposure, respectively 455 (Yang et al., 2012; Shin et al., 2013), supporting the notion that these 456 methylation changes are the initial step that triggers various genetic events in

these tumors (Kit et al., 2012). In another study, DNA methylation was 457 previously introduced as an interface between the genome and environment, 458 459 providing partial mechanistic explanations for the sensitivity of organisms to 460 environmental stresses. Moreover, DNA methylation's persistence can usefully 461 reflect the status of the environment in which organisms reside (Mirbahai and 462 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 L<sup>-1</sup> 463 464 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  $L^{-1}$  and 8.0 465 ma L<sup>-1</sup> Cd, respectively (Fig.s 1-5). These results provide evidence that 466 467 epigenetic biomarker, such as DNA methylation polymorphism in seedlings, 468 could be a more sensitive biomarker for Cd exposure than RAPD 469 polymorphism and MSI.

470 In the field of ecotoxicology, advances in DNA methylation analysis have 471 led to the recognition that aberrant DNA methylation status is potentially an 472 early, rapid and sensitive molecular biomarker indicative of pollutant exposure 473 and impact. MSAP-PCR is the most widely used method for screening 474 aberrant DNA methylation in cells, and this approach has several advantages: 475 more precision/accuracy, sensitivity, and reproducibility, and easier to carry out 476 than traditional MSAP (Aypar et al., 2011; Fulnecek and Kovarik, 2014; Pierron 477 et al., 2014). Moreover, MSAP-PCR is becoming a routine procedure in 478 laboratories. We anticipate a broad application of the described technology in 479 future studies on aberrations of DNA methylation for more accurate 480 assessment of Cd genotoxicity in other plants and for genotoxicity of other 481 heavy metal stresses in ecotoxicology.

## 482 5. Conclusions

483 The present report defines modifications in the genomic DNA methylation

in correlation with the induction of DNA RAPD polymorphism and MSI 484 measured in Arabidopsis seedlings exposed to Cd of 0.25-5.0 mg·L<sup>-1</sup> for 15 d. 485 MSI and RAPD polymorphism appeared exclusively under Cd stress of 8.0 486  $mg \cdot L^{-1}$  and of 4.0-5.0  $mg \cdot L^{-1}$ , respectively. However, we show that prominent 487 DNA methylation polymorphism occurred under Cd stress of 0.25-5.0 mg·L<sup>-1</sup> 488 by MSAP-PCR. Furthermore, the inverted U-shaped dose-response effects of 489 490 Cd stress on DNA methylation polymorphism were observed in Arabidopsis 491 seedlings. Given that MSAP-PCR detects substantial epigenetic alterations in Arabidopsis seedlings at Cd exposure, prior to the onset of RAPD 492 493 polymorphism, MSI and significant growth effects, it should be useful for 494 environmental risk assessment. With further development, the MSAP-PCR 495 method may also provide a more sensitive and reliable means in identification 496 and analysis of DNA methylation modifications in the other Cd-treated plants, 497 and become a powerful tool for early detection of genotoxic effects of other 498 heavy-metal stresses in ecotoxicology.

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**Fig. 1 MSI in Arabidopsis seedlings exposed to 0-8.0 mg·L**<sup>-1</sup> **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<sup>-1</sup> 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**<sup>-1</sup> **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<sup>-1</sup> 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 $\cdot$ L<sup>-1</sup> Cd for 15 d.



Fig. 4 The representative MSAP-PCR profiles of Arabidopsis seedlings exposed to 0-5.0 mg·L<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup> Cd for 15 d. (A) Frequency of hypermethylation, hypomethylation and methylation polymorphism at external cytosine (CHG) loci and (B) internal cytosine ( $C_PG$ ) loci.

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