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1 Roles of MSH2 and MSH6 in Cadmium-induced G2/M checkpoint arrest in 2 Arabidopsis roots

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15

16 ABSTRACT

17

18 DNA mismatch repair (MMR) proteins have been implicated in sensing and correcting
19 DNA damage, and in governing cell cycle progression in the presence of structurally
20 anomalous nucleotide lesions induced by different stresses in mammalian cells. Here,
21 Arabidopsis seedlings were grown hydroponically on 0.5×MS media containing cadmium
22 (Cd) at 0-4.0 mg L⁻¹ for 5 d. Flow cytometry results indicated that Cd stress induced a
23 G2/M cell cycle arrest both in *MLH1*-, *MSH2*-, *MSH6*-deficient, and in WT roots,
24 associated with marked changes of G2/M regulatory genes, including *ATM*, *ATR*, *SOG1*,
25 *BRCA1*, *WEE1*, *CYCD4;1*, *MAD2*, *CDKA;1*, *CYCB1;2* and *CYCB1;1*. However, the Cd-
26 induced G2/M phase arrest was markedly diminished in the *MSH2*- and *MSH6*-deficient
27 roots, while a lack of *MLH1* had no effect on Cd-induced G2 phase arrest relative to that in
28 the wild type roots under the corresponding Cd stress. Expression of the above G2/M

29 regulatory genes was altered in *MLH1*, *MSH2* and *MSH6*-deficient roots in response to Cd
30 treatment. Furthermore, Cd elicited endoreplication in *MSH2*- and *MSH6*-deficient roots,
31 but not in *MLH1*-deficient Arabidopsis roots. Results suggest that *MSH2* and *MSH6* may
32 act as direct sensors of Cd-mediated DNA damage. Taken together, we conclude that
33 *MSH2* and *MSH6*, but not *MLH1*, components of the MMR system are involved in the G2
34 phase arrest and endoreplication induced by Cd stress in Arabidopsis roots.

35

36 **Key words:** Arabidopsis; Cd stress; Cell cycle; G2 phase arrest; DNA damage; DNA
37 Mismatch repair

38

39 **Abbreviations:**

40

41	Arabidopsis	<i>Arabidopsis thaliana</i>
42	ATM	Ataxia-telangiectasia mutated
43	ATR	ATM and Rad3-related
44	<i>BRCA1</i>	Breast cancer susceptibility1
45	CDKs	Cyclin-dependent kinases
46	<i>CYCB1;1</i>	Cyclin B1;1
47	DAPI	4,6-diamidino-2-phenylindole
48	DDR	DNA damage response
49	DSB	Double strand break DNA
50	FCM	Flow cytometry
51	<i>GR1</i>	Gamma response1
52	MAPK	Mitogen-activated protein kinase
53	MMR	DNA Mismatch repair

54	<i>MSH2</i>	Mutated S homologue 2
55	<i>MSH6</i>	Mutated S homologue 6
56	<i>MLH1</i>	Mutated L homologue 1
57	<i>mlh1</i>	T-DNA insertion line of MLH1 deficiency
58	<i>msh2</i>	T-DNA insertion line of MSH2 deficiency
59	<i>msh6</i>	T-DNA insertion line of MSH6 deficiency
60	NER	Nucleotide excision repair
61	PCNA	Proliferation cell nuclear antigen
62	qRT-PCR	Real time quantitative reverse transcript polymerase chain reaction
63	RAPD	Random amplified polymorphism DNA
64	ROS	Reactive oxygen species
65	ssDNA	Single strand DNA
66	SOG1	Suppressor of gamma response 1
67	TLS	Trans-lesion synthesis
68	WT	Wild type (Col-0) line

69

70 **1. Introduction**

71

72 Cd is considered to be a highly toxic, persistent and accumulative heavy metal element,
73 and has been listed among the top ten hazardous substances by the National Toxicology
74 Program (NTP 2004) and by the Agency for Toxic Substances and Disease Registry
75 (<http://www.atsdr.cdc.gov/cercla/07list.html>). Cd exists ubiquitously in the soil and water,
76 mainly due to anthropogenic activities such as urban traffic and industrial processes, and
77 is then transferred to the food chain, which may lead to genotoxicity or/and cytotoxicity to
78 an organism's cells (Filipic, 2012; Pierron et al., 2014; Zhou et al., 2015). Thus, research

79 into the molecular mechanisms of Cd stress has become an important topic in
80 environmental studies (Cui et al., 2017; Pena et al., 2012; Wang et al., 2016).

81

82 It is well known that Cd, even at low concentrations, can bind directly to DNA and lead
83 to a wide variety of DNA damage processes such as base-base mismatches,
84 insertion/deletion loops, DNA adducts, and DNA chain cross linking and breaks (Filipic,
85 2012). DNA stress in eukaryotic cells induces elaborate repair mechanisms and signal
86 transduction pathways that can cause transient arrest of the progression through the cell
87 cycle (Hu et al., 2016; Wang et al., 2013; Xiang et al., 2017). ATM and ATR kinases act as
88 sensors of different types of DNA stress, coordinating stress responses with cell cycle
89 checkpoint control and repair of such lesions (Yoshioka et al., 2006; Spampinato, 2017).
90 Cell cycle checkpoints provide the cells with sufficient time to either cope with the
91 damaged DNA or undergo cell death. In particular, the G2/M checkpoint allows cells to
92 repair replication errors and damage before proceeding into mitosis, thereby ensuring
93 genomic integrity. In plant cells, key components of the G2/M checkpoint comprise WEE1,
94 BRCA1, ATM, ATR, and SOG1 which is activated through phosphorylation via the MAPK
95 signalling pathway (Cools and De Veylder, 2009; Opdenakker et al., 2012;
96 Pedroza-Garcia et al., 2016; Sjogren and Larsen, 2017; Yamane et al., 2007).
97 Subsequently, active SOG1 induces hundreds of genes controlling the DDR including cell
98 cycle arrest, DNA repair, endocycle onset and programmed cell death. The induction of
99 these genes (i.e. *MAD2*, *MRE11*, *CYCB1;2*, *BRCA1*, *CDKA;1* and *RAD51*) and
100 accumulation of their encoded proteins results in inhibition of CDK activity and arrest in the
101 G2/M phase in response to various stresses tested (Carballo et al., 2006; Hu et al., 2016;
102 Jia et al., 2016; Pelayo et al., 2001; Rounds and Larsen, 2008; Weimer et al., 2016;
103 Yoshiyama et al., 2009; Yoshiyama, 2016). More recently, FCM analysis showed that the

104 DDR can delay cell cycle progression and cause endoreplication in *Arabidopsis jhs1*
105 mutant seedlings (Jia et al., 2016; Pena et al. 2012). However, little information is available
106 about the checkpoint response of G2 phase-related *ATM*, *ATR* and *SOG1* genes in
107 response to Cd stress in *Arabidopsis* seedlings.

108

109 Among the different DNA repair pathways in both animals and plants, MMR systems
110 are involved in a wide range of important cellular processes. These include: (1) sensing
111 DNA damage, signaling, reacting to and repairing DNA lesions such as mispaired bases
112 (e.g. G/T, A/G or T/C), unpaired bases, and small insertion-deletion loop-outs (IDLs; e.g.
113 TTTT/AAA) in DNA, which arise from escaping the DNA polymerase proof-reading activity
114 during DNA replication, 5-methylcytosine deamination and the action of chemical
115 mutagens, (2) inhibiting recombination between divergent DNA sequences, (3)
116 maintaining barriers against massive genetic flow, and (4) preventing productive meiosis
117 in interspecies hybrids (Hays, 2002; Emmanuel, 2006; Cadet and Davies, 2017). Thus,
118 MMR plays a crucial role in confirming fidelity of DNA replication, maintaining genomic
119 stability and governing cell cycle progression in the presence of DNA damage
120 (Campregher et al., 2008; Wu and Vasquez, 2008). Thus, a fully functional MMR
121 machinery can modulate prolonged G2/M phase arrest by up-regulation of G2/M
122 regulatory proteins (i.e. Cyclin B1, Cdc2/p-Cdc2, and Cdc25C/p-Cdc25C) and/or by
123 activating the p53, ATM and ATR signaling pathways in human cells under exogenous and
124 endogenous stresses (Wang et al., 2013; Yan et al., 2003). In contrast, MMR deficient
125 human cell lines are resistant to alkylating agents and bypass the G2/M arrest, indicating
126 that the MMR has a role in post-replication checkpoints (O'Brien and Robert Brown, 2006).
127 Pabla et al. (2011) demonstrated that MLH1, MSH6 and MSH2 are the main MMR proteins
128 in human cells, and can play differential roles in G2 phase arrest following DNA damage

129 under different stresses. For example, MNNG (N-methyl-N-nitro-N-nitrosoguanidine)- and
130 ST (Sterigmatocystin)-induced G2/M phase arrest requires hMLH1 in animal cells (O'Brien
131 and Brown 2006; Wang et al., 2013). In contrast, nitric oxide (NO) and H₂O₂ are capable of
132 arresting G2/M phase in hMLH1 mutant cells (Chang et al., 2003; Hofseth et al., 2003).
133 However, IR (ionizing radiation)- and neutrophil-induced G2 phase arrest requires the
134 MSH2 protein in primary mouse embryonic fibroblasts and colon epithelial cells
135 (Campregher et al., 2008; Cejka et al., 2003; Marquez et al., 2003). Additionally, Yamane
136 et al. (2007) showed that both MSH2 and MLH1 activate G2/M phase checkpoint via the
137 BRCA1-ATR-Chk1 signaling pathway in human HCC1937 lines under 6-thioguanine
138 (6-TG) stress. Recently, two models have been suggested to explain how the DNA
139 damage recognized by MMR proteins can lead to cell cycle checkpoint activation. Firstly,
140 the “futile repair cycle model” proposes that the MMR system plays an indirect role by
141 initiating futile cycles of DNA repair, in which DNA breaks and gaps are continuously
142 produced, ultimately causing the production of secondary lesions. In contrast, the “general
143 DNA damage sensor model” proposes that MMR proteins may trigger stress signaling
144 directly, leading to the induction of cell cycle arrest (Pabla et al., 2011; Wang et al., 2013).
145 In Arabidopsis, there is little information on whether MLH1, MSH2 and MSH6 initiate G2
146 phase arrest of cell cycle progression in response to Cd stress. Thus, it is important to
147 evaluate the putative roles of different MMR proteins in Cd-induced DNA damage and cell
148 cycle arrest in Arabidopsis cells.

149

150 The principal objectives of the current study were to (1) measure cell cycle progression
151 in response to Cd in Arabidopsis seedlings comparing WT with *mlh1*, *msh2* and *msh6*
152 mutants; (2) determine the expression levels of DNA damage and G2M-phase-related
153 genes, such as *ATR*, *ATM*, *SOG1*, *CYCB1;1*, *CDKA;1*, *WEE1*, by qRT-PCR analysis in the

154 above *Arabidopsis* seedlings under Cd stress, and (3) evaluate the potential roles of MMR
155 genes *MLH1*, *MSH2* and *MSH6* in G2/M phase arrest and endoreplication in *Arabidopsis*
156 under Cd stress.

157

158 **2. Materials and methods**

159

160 *2.1. Plant materials, growth and treatment conditions*

161

162 *Arabidopsis thaliana* (*Arabidopsis*) plants used in this study were of the Columbia
163 ecotype WT (Col-0) and of the *mlh1*, *msh2* and *msh6* mutants. T-DNA insertion mutant
164 lines of *msh2* (SALK_002708), *msh6* (SALK_089638), and *mlh1* (SALK_123174C) were
165 obtained from the *Arabidopsis* Biological Resource Center (ABRC, Columbus, OH, USA),
166 and the background of the three mutants is from Col-0. The above seeds were
167 surface-sterilized using bleach solution (1:10 dilution of hypochlorite) and ethanol mix
168 (ethanol: water: bleach 7:2:1) at about 20 °C for 5 min, respectively, and were rinsed in
169 sterile distilled water five times and imbibed in sterile-water for 2-4 days at 4 °C to obtain
170 homogeneous germination (Pedroza-Garcia et al., 2016). The seeds were then sown in
171 sterile flasks containing 150 mL of commercially available 0.5× Murashige and Skoog (MS)
172 liquid medium (Basal Salt Mixture, Caisson, USA) with 0.5% (w/v) sucrose (pH 5.8), and
173 supplemented with Cd at a final concentration of 0 (the control), 1.25, 2.5, and 4.0 mg L⁻¹
174 in the form of CdCl₂·2H₂O of analytical grade with purity 99.5% (PR China). Each flask with
175 20-30 plantlets was placed on a rotary shaker at about 50 rpm in an incubator (12 h light of
176 approximately 3000 lx and 12 h dark at 21 ± 0.5 °C) for 5 d following germination. All
177 treatments and analyses were repeated in three independent replicates.

178

179 2.2. RNA extraction and qRT-PCR analysis

180

181 For both the control and Cd treatments, about 100 mg of fresh roots were collected at 5
182 d following germination in the growth chamber, and flash frozen in liquid nitrogen prior to
183 storage at -80°C . Total RNAs were extracted and purified using RNA isolation and clean
184 up kits (EZ-10 DNAaway RNA Mini-prep Kit, Sagon). First-strand cDNA was synthesized
185 from 2 μg of total RNA using the PrimeScriptTM 1st strand cDNA Synthesis Kit (TaKaRa)
186 following the manufacturer's protocols. qRT-PCR analysis was carried out using 20 μL
187 reaction mixtures containing 0.4 μL of template cDNA, 0.5 μM of corresponding forward
188 and reverse primers and 10 μL 2 \times SYBR Mix (SYBR R Premix Ex TaqTM II (Tli RNaseH
189 Plus, TaKaRa). Reactions were run and analyzed on an iCycler iQ (Bio-Rad) according to
190 the manufacturer's instructions. PCR products were run on a 2% (w/v) agarose gel to
191 confirm the size of the amplification products and to verify the presence of a unique PCR
192 product. The specificity of amplification products was determined by melting curves, and
193 the gene expression level was normalized to that of the reference genes, ACT2 or UBQ10
194 (Konishi and Yanagisawa, 2011). IQ5 relative quantification software (Bio-Rad)
195 automatically calculates relative expression level of the selected genes with algorithms
196 based on the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001). All analyses were repeated at
197 least three times. The primer pairs used for qRT-PCR are listed in supplemental (Sup)
198 Table S1. For detailed descriptions of expression of SOG1 and the other genes, see
199 Sjogren et al. (2015) and Cui et al. (2017), respectively.

200

201 2.3. FCM analysis of cell cycle progression in roots of Arabidopsis

202

203 To study the ploidy level of the WT and three mutant plantlets, approximately 0.1 g of

204 fresh roots were excised and chopped in ice cold chopping buffer (Partec, Germany) with
205 a single-edged razor blade in a glass Petri dish (diameter, 5 cm). After 5-10 minutes, crude
206 samples, consisting of finely minced tissue fragments, were put through two nylon filters
207 (pore size, 50 and 30 μm) to remove cell debris. The nuclei in the filtrate were stained with
208 DAPI (Partec, Germany) following the manufacturer's instructions. After 15 to 30 minutes
209 at about 25 °C in the dark, the stained nuclei of the control and Cd-treated samples were
210 analyzed using a CyFlow flow cytometer (Partec, Germany) equipped with a 365 nm laser.
211 Fluorescence intensity was analyzed for ≥ 5000 nuclei, and four independent replicates
212 were performed for each sample. Gates (Sup-Fig. S2) were determined empirically on
213 nuclei extracted from the roots of the 5-day-old plantlets with Flowjo 10 win 64 software
214 (BD Biosciences, San Jose, CA). Ploidy distribution calculated proportions of 2C, 4C, 8C
215 and 16C nuclei (i.e. 100% in total for each treatment; Sup-Fig. S2).

216

217 *2.4. DNA extraction and RAPD analysis*

218

219 Fresh roots (about 100 mg) were collected as for the RNA extraction. Total genomic DNA
220 was extracted and RAPD analysis was performed using 2 primers (Primers 3 and 11)
221 screened from 12 random primers as previously described (Liu et al., 2005; Sup-Table S2).
222 PCRs were performed, and polymorphism frequency of RAPDs, assessed by PAGE gel
223 electrophoresis, was calculated according to Wang et al. (2016).

224

225 *2.5. Statistical analysis*

226

227 nnnSPSS for Windows (version 23.0) was used for statistical analysis of the results. Data
228 are expressed as the means \pm standard deviation (SD). Statistical differences among the

229 control and treatments were calculated using 1-way analysis of variance (ANOVA), taking
230 $P < 0.05$ as significantly different according to the least significant differences (LSDs) tests
231 corrected for the number of comparisons.

232

233 **3. Results**

234

235 *3.1. Cd stress affected root growth of Arabidopsis plantlets*

236

237 Exposure to Cd ($1.25 - 4.0 \text{ mg}\cdot\text{L}^{-1}$) for 5 d had no obvious effect on the germination
238 rate of WT Arabidopsis seedlings compared to the control ($P < 0.05$; Table 1). Likewise,
239 there were no statistically significant differences for fresh weight between the control and
240 Cd-treated plantlets ($P < 0.05$) with the exception that seedlings treated with $4.0 \text{ mg}\cdot\text{L}^{-1}$ Cd
241 indicated a notable decrease of fresh weight. However, a significant inverted U-shaped
242 relationship was seen between root length and Cd level, with a correlation coefficient (r^2)
243 of 0.939 using regression way (Table 1; Sup-Fig. S1).

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255

256 **Table 1**

257 Effect of Cd on germination, fresh weight and root growth of Arabidopsis seedlings for 5 d.

Lines of Arabidopsis	Cd concentration/ mg·L ⁻¹	Germination percentage/ %	Fresh weight of plantlet ⁻¹ /mg	Root growth	
				Root length/cm	Suppression rate/ %
WT	0	95.1±2.1	10.31±0.39	1.20±0.05	0.00
	1.25	95.8±1.9	11.04±0.42	1.32±0.04*	-10.00
	2.5	96.1±3.3	10.14±0.48	0.81±0.05*	32.50
	4.0	95.3±1.7	7.78±0.42*	0.52±0.09*	56.67
<i>mlh1</i>	0	95.6±3.2	10.32±0.24	1.22±0.04	0.00
	1.25	94.9±1.3	10.13±0.75	1.09±0.08	10.66
	2.5	95.2±3.5	9.08±0.78	0.88±0.02*	27.87
	4.0	95.6±1.8	7.69±0.34*	0.55±0.09*	54.92
<i>msh2</i>	0	94.2±1.6	10.29±0.31	1.23±0.03	0.00
	1.25	95.1±2.5	10.01±0.67	1.06±0.06*	13.82
	2.5	95.3±3.2	8.51±0.56	0.62±0.07*	49.59
	4.0	96.2±6.4	7.01±0.29*	0.4±0.09*	67.48
<i>msh6</i>	0	95.7±1.8	10.34±0.35	1.16±0.04	0.00
	1.25	95.2±3.3	9.75±0.49	0.87±0.05*	20.91
	2.5	96.5±7.7	8.73±0.53	0.55±0.07*	50.00
	4.0	94.8±3.1	7.13±0.45*	0.35±0.02*	68.18

258 * Significantly statistical difference from the control, respectively ($P < 0.05$).

259

260 Under the control conditions, the WT and three mutants (*mlh1*, *msh2* and *msh6*) had
261 similar root growth and fresh weight (Table 1). A significant negative relationship was
262 observed for the root length between the above mutants and Cd levels of 0 and 4.0 mg·L⁻¹
263 for 5 d, with correlation coefficients (r^2) of 0.962, 0.983 and 0.985, respectively.
264 Interestingly, seed germination percentage, fresh weight and suppression of root growth in
265 the WT seedlings was not different from that of MLH1-deficient seedlings under Cd
266 stresses of 2.5 - 4.0 mg·L⁻¹. In contrast, suppression of root growth was much greater in
267 *MSH2*- and *MSH6*-deficient seedlings with a similar reduced trend under Cd stresses of
268 1.25 - 4.0 mg·L⁻¹. These results indicate that *MSH2*- and *MSH6*-deficient seedlings were

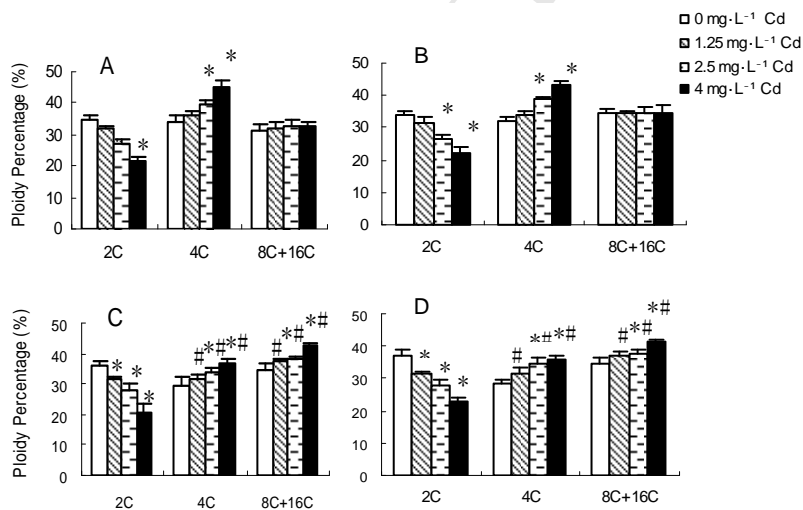
269 more sensitive to Cd toxicity than the WT and *MLH1*-deficient seedlings in this experiment
 270 (Table 1).

271

272 3.2. Cd stress induced G2 phase arrest in the WT *Arabidopsis* roots

273

274 To evaluate cell cycle progression in the WT roots under Cd stress for 5 d, effect of Cd
 275 stress on cell cycle arrest was determined using FCM analysis. As shown in Fig. 1, the
 276 proportion of cells with a 2C nuclear content (G0/G1 phase) was 35.2% in the control, but
 277 Cd stress significantly decreased this proportion at 4.0 mg·L⁻¹ Cd, which was 23.1%. This
 278 alteration in the 2C nuclear content was accompanied by a significant increase in the
 279 proportion of cells with a 4C nuclear content: which was 39.8% and 41.4% in roots at the
 280 highest two Cd concentrations of 2.5 and 4.0 mg·L⁻¹, respectively (Fig. 1A, Sup-Fig. S1).
 281 The FCM result suggests that Cd stress could induce G2/M phase arrest in roots of the
 282 WT plantlets.



283

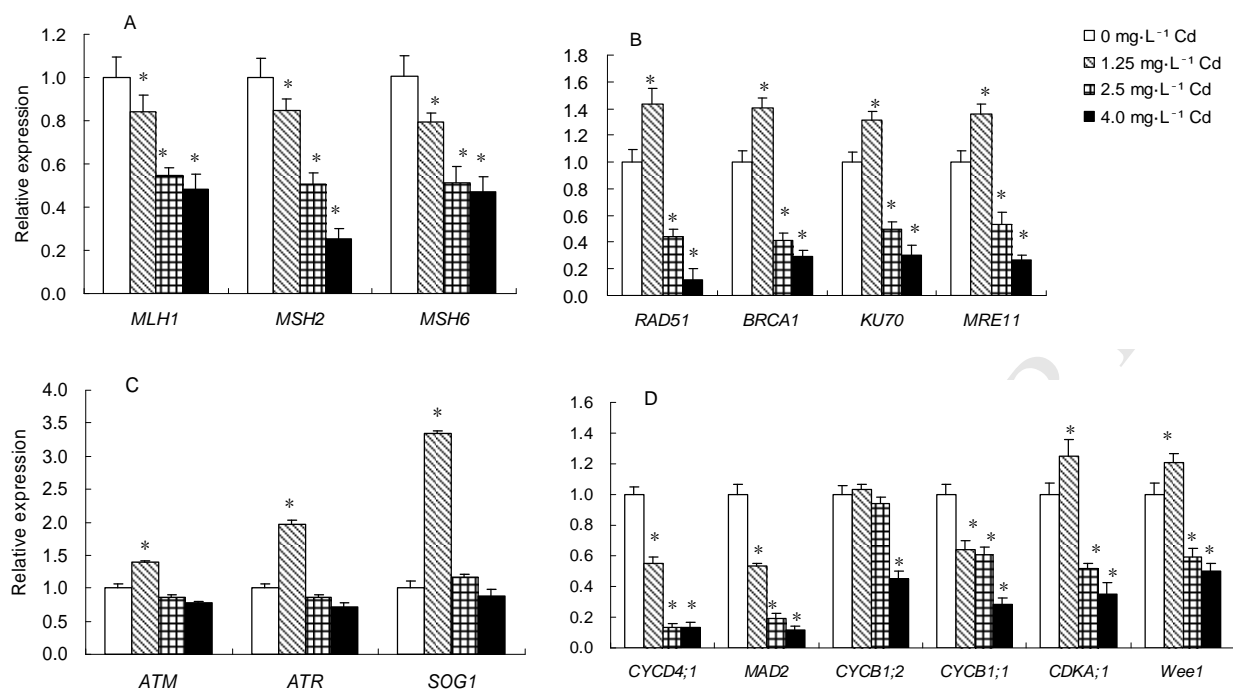
284 **Fig.1.** FCM analysis on the nuclear DNA contents of WT (A), *mlh1* (B), *msh2* (C), and
 285 *msh6* (D) in *Arabidopsis* roots exposed to 0 - 4.0 mg·L⁻¹ Cd for 5 d. The percent
 286 distribution of cells in 2C, 4C and 8C+16C was calculated and compared with the control.

287 Each point represents the mean \pm SD of three independent experiments. *Significantly
288 different from the control in A-D, respectively ($P < 0.05$), and # significantly different from
289 the WT under the corresponding Cd stress in C-D ($P < 0.05$).

290

291 The effect of Cd stress on cell cycle-regulatory genes was determined by measuring
292 the expression of marker genes for G2/M transition (*ATM* and *ATR*, *SOG1*, *WEE1*,
293 *CYCD4;1*, *MAD2*, *CDKA;1*, *CYCB1;2* and *CYCB1;1*) in the WT roots with and without Cd
294 stress by qRT-PCR analysis. Two patterns of gene expression were noted. Gene
295 expression of *ATM*, *ATR*, *SOG1*, *CDKA;1*, and *WEE1* increased by 1.21- to 3.3-fold at the
296 lowest concentration ($1.25 \text{ mg}\cdot\text{L}^{-1}$) of Cd, but a dose-dependent decrease was observed
297 in expression of *CDKA;1*, and *WEE1* with Cd concentrations above $1.25 \text{ mg}\cdot\text{L}^{-1}$ (Fig. 2C
298 and 2D). The second group of cell cycle-regulatory genes, *CYCD4;1*, *MAD2*, *CYCB1;2*
299 and *CYCB1;1* showed a dose-dependent reduction in the expression from 0 to $4.0 \text{ mg}\cdot\text{L}^{-1}$
300 Cd. For all these genes the maximum reduction in expression was with $4.0 \text{ mg}\cdot\text{L}^{-1}$ Cd with
301 a maximum decrease of 0.12- to 0.50-fold in the expression of the *CDKA;1*, *WEE1*,
302 *CYCD4;1*, *MAD2*, *CYCB1;2* and *CYCB1;1* genes relative to the control (Fig. 2C and 2D).
303 Taken together, these findings support the hypothesis that Cd stress can mediate the
304 aberrant expression of the above G2 phase cell cycle regulatory genes partially involved in
305 G2/M arrest in Arabidopsis roots.

306



307

308 **Fig. 2.** Effect of Cd stress on gene expression in Arabidopsis roots for 5 d. (A) DNA
 309 mismatch repair genes *MLH1*, *MSH2* and *MSH6*; (B) DNA damage repair genes *RAD51*,
 310 *BRCA1*, *KU70* and *MRE11*; (C) DNA damage response genes *ATM*, *ATR* and *SOG1*; (D)
 311 G2/M marker genes *CYCB1;1*, *CDKA;1*, *WEE1*, *CYCD4;1*, *MAD2* and *CYCB1;2*. The
 312 expression level of these genes was set to 1 in the control. Data are shown as mean \pm SD
 313 by qRT-PCR. Data presented are average of three replicates. * Significantly different from
 314 the control ($P < 0.05$). House-keeping gene AtUBQ10 was used as an internal control.

315

316 3.3. Cd stress caused an MSH2- and MSH6-dependent G2/M arrest in Arabidopsis roots

317

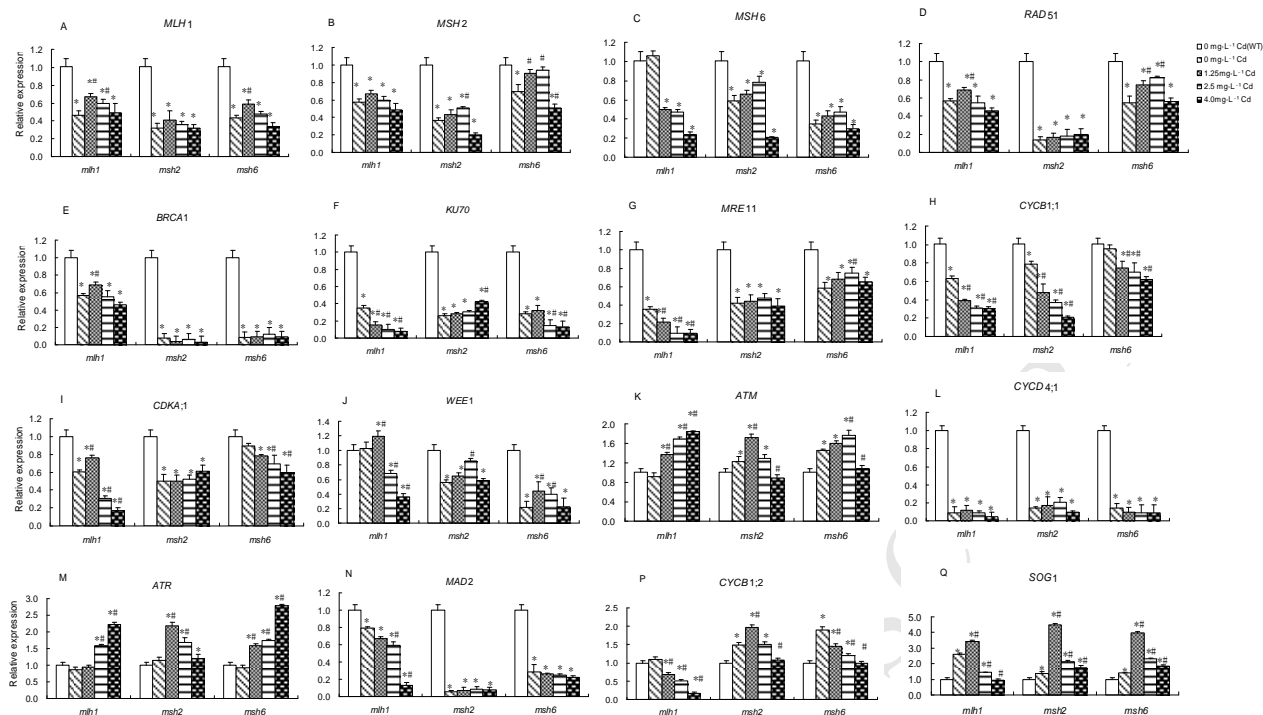
318 FCM analyses showed that the Cd-induced G2 arrest was dramatically attenuated in
 319 the *msh2* and *msh6* mutants compared with the WT under the corresponding Cd stress
 320 (Fig. 1). The attenuation was of 12.7%, 14.7% and 11.6% in the *MSH2*-deficient roots, and
 321 of 10.9%, 13.2% and 15.7% in *MSH6*-deficient roots under Cd stresses of 1.25 - 4.0 mg
 322 L⁻¹, respectively. In contrast, mutation of *MLH1* had no effect on Cd-induced G2 phase

323 arrest. Cd stress dramatically increased the proportion of cells with 8C and 16C nuclear
324 content, and the increase was of 6.7%, 11.3%, 15.2%, and 15.5% for MSH2-deficient
325 roots, and of 5.7%, 11.3%, 14.31, and 12.4% for MSH6-deficient roots under 0, 1.25, 2.5
326 and 4.0 mg L⁻¹ Cd treatments compared to WT at each Cd concentration tested,
327 respectively. However, again there was no effect of *MLH1* mutation on the proportion of
328 8C and 16C nuclear content in response to Cd treatment (Fig. 1, Sup-Fig.S1). Based on
329 the above results, we concluded that MSH2 and MSH6, but not *MLH1*, of the MMR system
330 are involved in the G2 phase arrest induced by Cd stress in Arabidopsis roots.

331

332 In *MSH2*-, *MSH6*- or *MLH1*-deficient Arabidopsis roots, exposure to Cd stress strongly
333 activated expression of *ATR*, *ATM*, *SOG1* and *CYCB1;2* genes compared with the WT
334 control, whereas expression of *CYCD4;1*, *RAD51*, *BRCA1*, and *MAD2* was sharply
335 diminished (i.e. a decrease of 0.12- to 0.23-fold) in MSH2-deficient roots under Cd stress
336 (Fig.3). Some genes (i.e. *KU70*, *CYCB1;1*, *MRE11*) tested were down-regulated in a Cd-
337 dependent manner in *MLH1*-deficient Arabidopsis roots. Notably, expression of *MAD2*,
338 *MRE11*, *CYCB1;2*, *BRCA1* and *RAD51* genes showed obvious differences between
339 *MLH1*-deficient and MSH2/MSH6-deficient roots under Cd stress. In addition, expression
340 of *MSH2*, *MSH6* and *MLH1* genes was significantly suppressed in MSH2/MSH6- and
341 *MLH1*-deficient roots in response to 1.25-4.0 mg L⁻¹ Cd treatment compared with the WT
342 control to some extent, respectively (Fig. 3). Taken together, the altered expression of the
343 above genes suggests that (1) most of the genes are down-regulated in each mutant
344 compared to the WT control, and (2) DDR is activated in Cd-stressed seedlings, which
345 may be partially implicated in the G2 phase arrest.

346



347

348 **Fig. 3.** Transcript expression levels in roots of *mlh1*, *msh2* and *msh6* mutants exposed to
 349 0- 4.0 mg·L⁻¹ Cd for 5 d. In A-Q, MMR genes *MLH1*, *MSH2* and *MSH6*; G2/M phase
 350 marker genes *CYCB1;1*, *CDKA;1*, *WEE1*, *CYCD4;1*, *MAD2* and *CYCB1;2*; DNA damage
 351 repair genes *RAD51*, *BRCA1*, *KU70* and *MRE11*; and DNA damage response genes *ATM*,
 352 *ATR* and *SOG1*. The expression levels of the WT were set to 100% in the control by
 353 qRT-PCR analysis. Data were shown mean ± SD at least three independent experiments,
 354 and house-keeping gene *AtUBQ10* was used as an internal control. * and # significantly
 355 statistical difference from the WT control and the corresponding mutant control,
 356 respectively ($P < 0.05$).

357

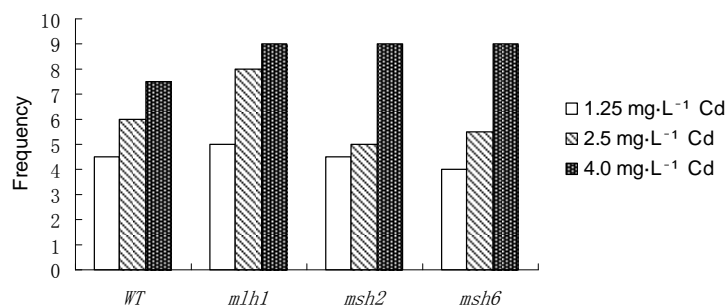
358 **3.4. *MSH2* and *MSH6* may act as direct sensors of Cd-mediated DNA damage in**

359 *Arabidopsis* roots

360

361 To assess whether MMR proteins are acting via the futile repair cycle model or the
 362 direct DNA damage sensor signaling model in response to the Cd treatment, we examined
 363 the level of DNA damage in WT, *MSH2*- and *MSH6*-deficient roots under Cd stress using a
 364 RAPD assay. The WT and the two MMR mutants tested exhibited similar frequencies of
 365 RAPD polymorphism after Cd stress of 1.25 - 4.0 mg L⁻¹ for 5 d (Fig. 4, Sup-Fig. S3).
 366 These results indicate that *MSH2* and *MSH6* did not lead to the formation of secondary

367 damage, suggesting that DNA lesions recognized by MSH2 and MSH6 could lead to G2/M
 368 cell cycle arrest through the direct signaling model in Arabidopsis roots under Cd stress.



369

370 **Fig. 4.** RAPD polymorphism variations of Arabidopsis roots exposed to 0- 4.0 mg·L⁻¹ Cd
 371 for 5 d. For all treatments, reproducible bands in at least two replicates were evaluated
 372 and calculated for polymorphism analysis.
 373

374 4. Discussion

375

376 Many studies have proved that the MMR system can sense, react and repair DNA
 377 damage, thus has an utmost important role in confirming fidelity of DNA replication, in
 378 maintaining genomic stability and in governing the cell cycle progression in the presence
 379 of DNA damage induced by different stresses in mammalian cells. Such experiments
 380 indicated that MMR deficiency can lead to tumorigenesis in response to stresses through
 381 loss of cell cycle regulation and decreased apoptosis (Tsaalbi-Shtylik, 2015; Wang et al.,
 382 2013). However, little information is known about whether *MLH1*, *MSH2* and *MSH6* initiate
 383 G2/M phase arrest of cell cycle progression in higher plants under Cd stress. Results
 384 presented here show that Cd exposure could induce DNA damage and change
 385 remarkably gene expression of G2/M-transition-related regulation and MMR system, and
 386 thus lead to G2/M phase arrest in Arabidopsis seedlings (Figs. 1 - 2). Moreover, mutation
 387 of two MMR genes, *MSH2* and *MSH6* results in a significant attenuation of G2 arrest and
 388 in a marked increase of cells with 8C and 16C nuclear content compared with the WT
 389 under the corresponding Cd stress (Fig. 1), indicating that these two genes may be

390 important in the MMR-mediated response to Cd. Furthermore, MSH2 and MSH6 may act
391 as direct sensors of Cd-mediated DNA damage and participate in the G2/M arrest and
392 endoreplication under Cd stress (Figs. 1, 4). These findings provide new insights into the
393 molecular basis of *MLH1*, *MSH2* and *MSH6* roles in the G2/M phase arrest caused by
394 DNA lesions in Arabidopsis seedlings under Cd stress.

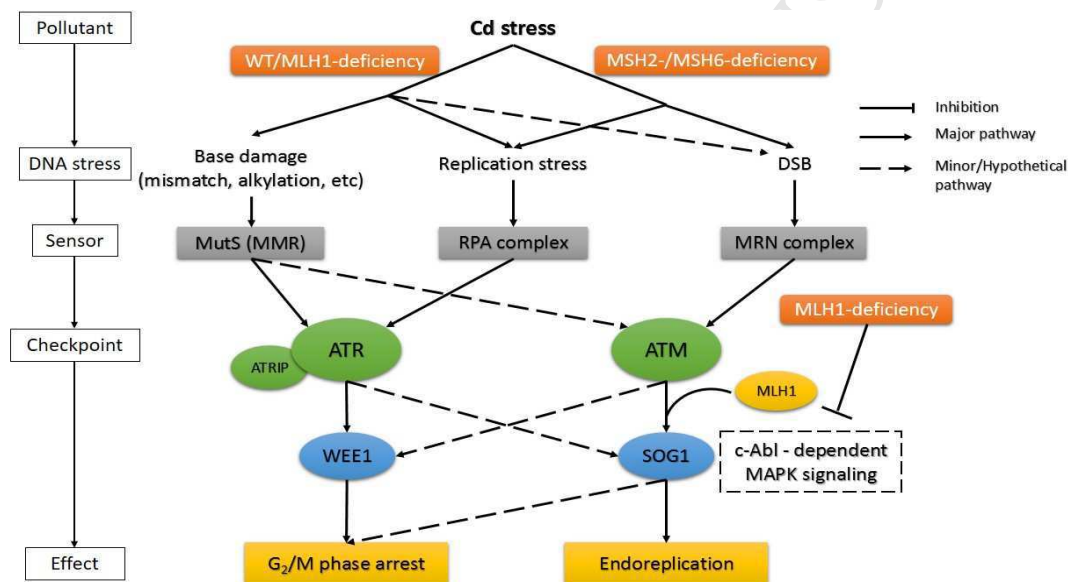
395
396 Endogenous replication stress induced by replisome factor E2F TARGET GENE1
397 mutant triggered a prolonged cell cycle, accompanied with a high number of the G2/M
398 phase cells in Arabidopsis (Cools and De Veylder, 2009), while X-ray or hydroxyurea
399 stress led to a G2/M phase arrest in root cells of onion (*Allium cepa* L.) (Pelayo et al., 2001;
400 Carballo et al., 2006). Recently, we demonstrated that Cd stress could induce G₂ phase
401 arrest in Arabidopsis seedlings (Cui et al., 2017). In the current study, FCM analysis
402 indicated that Cd stress could significantly reduce the proportion of 2C cells and induce a
403 G2/M phase arrest in the WT root cells of Arabidopsis seedlings (Fig. 1; Sup-Fig. S1).
404 Additionally, the expression of the G₂ phase marker genes confirmed that Cd stress
405 induced the abnormal expression of *ATM*, *ATR*, *SOG1*, *WEE1*, *CYCD4;1*, *MAD2*, *CDKA;1*,
406 *CYCB1;2* and *CYCB1;1* genes (Fig. 2), which may be involved in the G2 phase arrest
407 triggered by Cd stress in Arabidopsis roots. Although some signaling pathways, such as
408 *ATM*, *ATR*, *SOG1*, *WEE1*, *BRCA1*, *RAD51*, *CDKA;1*, *CYCB1;2* and *CYCB1;1*, have been
409 found to be involved in the mechanism of cell cycle arrest by Cd and other stresses (Cui et
410 al., 2017; O'Brien and Brown, 2006; Hu et al., 2016), further studies validating the exact
411 mechanism are warranted.

412
413 A number of reports have suggested that *MLH1*, *MSH2* and *MSH6* proteins can
414 recognize DNA damage and act as signaling mediators for activation of cellular DNA

415 damage responses (O'Brien and Brown, 2006; Tennen et al., 2013; Yoshioka et al., 2006).
416 Herein, we found that Cd stress caused significant DNA damage in Arabidopsis roots (as
417 shown by RAPD polymorphism, Fig. 4), indicating that Cd stress can produce a genotoxic
418 effect, including DSB and ssDNA, in Arabidopsis roots. In addition, downregulated
419 expression of *MLH1*, *MSH2* and *MSH6* genes occurred at the mRNA level by Cd stress at
420 all the concentrations tested, which was obviously different from other repair genes (i.e.
421 *KU70*, *BRCA1*, *RAD51*) (Fig. 2), showing that Cd stress was prone to impairing MMR
422 system in response to DNA damage in Arabidopsis roots. Furthermore, knockdown
423 studies highlighted that *MSH2* and *MSH6*, not *MLH1*, caused a G2/M arrest of the cell
424 cycle in Arabidopsis roots following Cd stress (Fig. 1), which was consistent with the
425 activation of a post-replication DNA-damage checkpoint (Cools and De Veylder, 2009).
426 Also, evidence for the installation of such a checkpoint, apart from the G2/M arrest (Fig. 1),
427 includes significantly changed expression of G2/M phase and its regulation-related genes
428 such as *ATM*, *ATR*, *SOG1*, *WEE1*, *CYCD4;1*, *MAD2*, *CDKA;1*, *CYCB1;2* and *CYCB1;1* in
429 *MLH1*-, *MSH2*- or *MSH6*-deficient roots under Cd stress (Figs. 2 - 3). When the cells
430 undergo diverse stresses, expression of *ATM* or/and *ATR* is significantly increased, which
431 phosphorylates *SOG1*; subsequently, activated *SOG1* and/or *WEE1* could severely affect
432 *CDKA;1* to form an active complex with Cyclin B1, leading to a G2/M arrest (Hu et al.,
433 2016; O'Brien and Brown, 2006; Weimer et al., 2016). In this study, expression of *ATM*
434 and *ATR* was enhanced by DNA stress via cell cycle checkpoints although MutSα was
435 uncoupling with them in *MSH2*- and *MSH6*-deficient roots, activating *SOG1* and *WEE1*,
436 which could cause G2/M arrest (Figs. 1 - 5). However, in the *MLH1*-deficient roots, *ATR*
437 and *ATM* received signals from MutS and activated *WEE1* and *SOG1*, leading to G2/M
438 phase arrest (Figs. 1 - 3, 5). Indeed, expression of *MAD2*, *MRE11*, *CYCB1;2*, *BRCA1* and
439 *RAD51* genes indicated obvious differences between *MLH1*-deficient and *MSH2/MSH6*-

440 deficient roots under Cd stress (Fig. 3). Herein, sharply repressed expression of *MAD2*
 441 may mediate G2-M arrest through the regulation of chromatid segregation with a dual
 442 mechanism: (i) transcriptional regulation of gene expression profiling; and/or (ii)
 443 post-transcriptional ubiquitination (Sisinni et al., 2017). Also, aberrant expression of the
 444 other genes responsible for DNA repair could affect G2/M arrest probably by altering
 445 repair efficiency (Figs. 1 - 3, 5). Taken together, the above results suggest that Cd stress
 446 induced G2/M arrest, independent of *MLH1*, but dependent on *MSH2* and *MSH6* genes in
 447 *Arabidopsis* roots.

448



449

450 **Fig. 5.** Model of Cd-induced G2/M arrest and endoreplication in *Arabidopsis* roots. Base
 451 damage is usually major form in Cd-induced DNA stress. In WT and *MLH1*-deficient roots,
 452 base damage and replication stress could be sensed by MutS and RPA complex
 453 respectively, leading to activation of ATR. Then ATR activates Wee1 through
 454 phosphorylation, which can phosphorylate the inhibiting tyrosine residue of CDK in
 455 cyclin-Cdk complex, finally causing cell cycle arrest. In *MSH2*- and *MSH6*-deficient roots
 456 because of recession of sensing function caused by MutS-deficiency, more DSB are
 457 produced and sensed by MRN complex which activate ATM. ATM, as a protein kinase, has
 458 been proved that it can cross nuclei membrane through *MLH1*- and C-abl-dependent
 459 MAPK signaling and activates transcription factor SOG1, causing endoreplication.

460

461 Two models have been proposed to account for the reason why DNA damage

462 signaling recognized by MMR proteins may cause cell cycle checkpoint activation (Pabla
463 et al., 2011). The futile cycle model emphasizes DNA repair as the single function of MMR.
464 According to this model, a futile attempt of the MMR system to repair damaged DNA leads
465 to the generation of DNA strand breaks, as damage on the template strand is repeatedly
466 processed. However, the direct signaling model proposes two distinct functions for MMR:
467 DNA repair and DNA damage signaling. In this model, MMR proteins might directly initiate
468 DNA damage signaling that permits activation of one or more cell cycle checkpoints.
469 Indeed , these two models are not mutually exclusive, and are supported or contradicted
470 by the good experimental evidence (O'Brien and Brown, 2006; Pabla et al., 2011). Our
471 results suggest that under Cd stress, DNA damage (as evidenced by the presence of
472 RAPD polymorphism) was similar in the WT and the *msh2/msh6* mutant tested after Cd
473 treatment (Fig. 4), which indicates that *MSH2* and *MSH6* did not lead to the formation of
474 secondary damage. Similarly, direct sensors of DNA damage signaling were recognized
475 by MLH1 and MSH2 proteins in human esophageal epithelial Het-1A cells and in mouse
476 embryonic fibroblasts, respectively (Pabla et al., 2011; Wang et al., 2013). Moreover, under
477 1.25 and 2.5 mg L⁻¹ Cd stress, DNA damage was different between *msh2/msh6* and *mlh1*
478 mutant (Fig. 4), suggesting functional dissociation of DNA damage repair and recognition
479 signaling. The nicks near base mismatches, O⁶MeG or IDLs loci are produced during DNA
480 mismatch repair processes after replication and sensing damages (Culligan and Hays,
481 2000; Hu et al., 2016), leading to RAPD polymorphism (Fig. 4). In *mlh1* mutant, *MLH1* and
482 many other repair genes (i.e. *KU70*, *BRCA1*, *MRE11*, *RAD51*) were prominently
483 repressed (Fig. 3) to cause low-efficient repair and long-duration of nick maintenance,
484 however, initiating MMR is significantly inhibited in *msh2/msh6* mutant, which accounts for
485 differences in DNA damage between *mlh1* and *msh2/msh6* mutant (Fig. 4). The above
486 observations support the direct signaling model, wherein MSH2 and MSH6 might act as

487 direct sensors of Cd-mediated DNA damage and be directly implicated in the initiation of
488 DNA damage signaling responses.

489

490 The recent findings have revealed that the G2/M arrest and endoreduplication in
491 response to different stresses are strictly dependent on MMR activity and the roles of
492 MMR proteins in mismatch repair can be uncoupled from the MMR-dependent damage
493 responses (Luo et al., 2004). Although MMR system repairs only DNA mismatches or
494 mispairs in cells, it is involved in checkpoint activation in response to various forms of DNA
495 damage (i.e. O⁶MeG lesions). Moreover, while the repair can function efficiently at
496 subnormal levels of hMLH1 or hMSH2, the checkpoint activation requires a full level of
497 them (Luo et al., 2004; O'Brien and Brown, 2006). In the current study, the WT roots
498 showed marked G2 phase arrest but increase growth of 10% under 1.25 mg•L⁻¹ Cd stress
499 (Table 1; Sup-Fig. S2), which suggests that the functions of MMR proteins in MMR and
500 checkpoint signaling may involve different molecular processes (Pabla et al., 2011). The
501 possible outcomes are complicated following MMR-dependent G2/M arrest and
502 endoreduplication under Cd stress as follows: (1) the prolonged G2/M arrest is associated
503 with the appearance of plantlets that display a inhibition-like phenotype such as
504 suppressed root growth observed in both WT and mutants, mainly through ATR/ATM-
505 Wee1 cascade (Table 1; Fig. 5); (2) some of them appear to escape from G2 arrest but
506 undergo endoreduplication observed only in the *msh2* and *msh6* mutants, probably
507 through TLS to bypass Cd-induced lesions in an ATM/ATR-SOG1-dependent manner
508 (Figs. 1, 5) (Adachi et al, 2011; Hirose et al., 2003; Hu et al., 2016; Reyes et al., 2015;
509 Tsaalbi-Shtylik et al., 2015). Alternatively, an interaction between MMR system (MSH2,
510 MSH6 or MHL1) and DNA damage sensors/repair proteins (i.e. ATR, ATM, SOG1, MRE11,
511 BRCA1, CYCB1;1, KU70 and MAPK) has been required for endoreduplication and for the

512 installation of cell cycle arrest as well as the co-localisation of MMR proteins, γ -H2AX foci
513 and the MRN (Mre11-Rad50- Nbs1) complex to foci of DNA damage (Hirose et al., 2003;
514 Hu et al., 2016; Manke et al., 2005; Opdenakker et al., 2012; Stojic et al., 2004; Weimer et
515 al., 2016). Herein, expression of *ATM*, *ATR* and *SOG1* genes was strongly activated,
516 accompanied with significantly increased proportion of cells with 8C+16C nuclear content
517 in *msh2* or *msh6* mutant roots under Cd stress (Figs.1-3), which accounts for
518 endoreduplication via ATM/ATR-SOG1-dependent pathway. Additionally, expression of
519 *RAD51*, *BRCA1*, and *MAD2* genes was sharply diminished in *msh2* mutant roots (Figs. 3,
520 5), suggesting that these genes' expression could be mediated through a MSH2-
521 dependent pathway of ATR-SOG1 activation (Pabla et al., 2011; Sisinni et al., 2017),
522 which could similarly promote endoreduplication. However, endoreplication was not seen
523 in MLH1-deficient Arabidopsis roots under Cd stress, and many genes tested were
524 down-regulated in a Cd-dependent manner (Figs. 1, 3, 5), probably because MLH1 in
525 Arabidopsis could play an important role in c-Abl-dependent MAPK signaling just like that
526 in human/animal and then in activating SOG1 in response to DNA lesions (Kim et al.,
527 2007). Campregher et al. (2010) demonstrated that several other proteins have been
528 involved in MMR system, including clamp PCNA, DNA polymerase delta, single-strand
529 binding protein RPA, clamp loader replication factor C (RFC), exonuclease 1 (EXO1), and
530 endonuclease FEN1, which are associated with synthesizing DNA and the replication fork.
531 Thus, we could speculate that the accelerated activation of endoreplication observed
532 exclusively in the *msh2* and *msh6* mutants, but not in the *mlh1* mutant, could be a
533 consequence of the interaction among RPA, MRN and MutS complex with DNA damage
534 checkpoint signaling such as ATR/ATM-Wee1 or/and ATM/ATR-SOG1 cascade under Cd
535 stress (Figs.1, 5). Taken together, exposure to Cd stress strongly activates the major
536 MSH2-ATR-Wee1 signaling cascade in WT and *mlh1* mutant, but the major MRN-ATM-

537 SOG1 cascade in *msh2* and *msh6* mutants (Fig. 5).

538

539 More recently, research focus has turned to elucidating the mechanisms that regulate
540 the dephosphorylation of checkpoint proteins, and to revealing phosphatases that govern
541 SOG1 and other checkpoint proteins implicated in checkpoint resolution and mitotic
542 progression (Friedhoff et al., 2016). Moreover, many results point to ATR/ATM/SOG1 as
543 master regulators of checkpoint maintenance and resolution, and subsequent mitotic exit
544 in plants (O'Brien and Brown, 2006; Sjogren et al., 2017; Yoshiyama, 2016).

545 Interestingly, SOG1 exists usually in cytoplasm, and can enter the nuclei to regulate
546 hundreds of genes' expression when SOG1 was phosphorylated via MAPK signal
547 pathway, which is dependent on c-abl and MLH1 (Kim et al., 2007; Opdenakker et al.,
548 2012). Thus, unravelling the possible roles of MMR proteins in maintenance and resolution
549 of the G2/M checkpoint and the subsequent mitosis after recovery from DNA lesions in
550 Arabidopsis and other plants under Cd and other stresses could prove interesting and
551 fruitful.

552

553 **Conclusions**

554

555 This study indicated that Cd stress induced DNA lesions and G2/M arrest in
556 Arabidopsis roots, which was mediated by *MSH2* and *MSH6* genes, but not *MLH1*, of the
557 MMR system via altered expression of G2/M regulatory genes, including *ATM*, *ATR*,
558 *SOG1*, *WEE1*, *CYCD4;1*, *MAD2*, *CDKA;1*, *CYCB1;2* and *CYCB1;1*. To our knowledge,
559 this is the first study showing that *MSH2* and *MSH6* are implicated in G2/M phase arrest
560 triggered by Cd stress in Arabidopsis roots. In addition, endoreduplication occurred mainly
561 from impairment of *MSH2* and *MSH6*, and was not seen in *MLH1*-deficient Arabidopsis

562 roots in response to Cd stress. Moreover, we observed that MSH2 and MSH6 could act as
563 direct sensors of Cd-induced DNA lesions in Arabidopsis plantlets. Because Cd pollution is
564 ubiquitously present in the soil and water, these results provide new insight into the
565 essential mechanisms of *MLH1*, *MSH2* and *MSH6* in the G2/M phase arrest induced by
566 DNA damage under Cd stress in other plant seedlings.

567

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HIGHLIGHTS

- Cd-caused endoreplication occurred mainly by impairment of MSH2 and MSH6 in roots.
- Cd-induced G2 phase arrest was markedly reduced in the MSH2- and MSH6-deficiency.
- Cd-triggered endoreplication was eliminated in MLH1-deficient Arabidopsis roots.
- MSH2-ATR/ATM is the major signaling cascade in Cd-governed DDR in Arabidopsis.
- MSH2 and MSH6 can act as direct sensors of Cd-induced DNA damage in roots.