Cell cycle arrest mediated by Cd-induced DNA damage in Arabidopsis root tips

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Abstract:

Accumulating evidence demonstrates that the aberrant expression of cell cycle regulation and DNA repair genes can result in abnormal cell proliferation and genomic instability in eukaryotic cells under different stresses. Herein, *Arabidopsis thaliana* (Arabidopsis) seedlings were grown hydroponically on 0.5×MS media containing cadmium (Cd) at 0–2.5 mg•L^{-1} for 5 d of treatment. Real time quantitative reverse chain reaction (qRT-PCR) analysis revealed that expression of DNA damage repair and cell cycle regulation genes, including *BRCA*1, *MRE*11, *WEE*1, *CDKA*;1 and *PCNA*1, showed an inverted U-shaped dose-response. In contrast, notably reduced expression was observed for G1-to-S transition-ralated genes, *Histone H*4, *E2Fa and PCNA*2; DSB end processing, *GR*1; and DNA mismatch repair, *MSH*2, *MSH*6 and *MLH*1 genes in root tips exposed to 0.125-2.5 mg L^{-1} Cd for 5 d. Flow cytometry (FCM) analysis revealed significant increases of cells with a ^{2}C nuclear content and with a ^{4}C and ^{8}C nuclear content under Cd stresses of 0.125 and 1-2.5 mg•L^{-1}, respectively. Our results suggest that 0.125 mg•L^{-1} Cd-induced DNA damage induced the marked G1/S arrest,

leading to accelerated growth in root tips, while 1.0-2.5 mg•L^{-1} Cd-induced DNA damage caused a notable G2/M arrest in root tips, leading to reduced growth in root tips. This may be a protective mechanism that prevents cells with damaged DNA from dividing under Cd stress.

Key words: Arabidopsis; Cd stress; DNA damage marker genes; cell cycle regulation genes; Gene expression; Cell cycle arrest

Abbreviations:

CDKs	Cyclin-dependent kinases
*CYCB*1;1	Cyclin B1;1
qRT-PCR	Real time quantitative reverse chain reaction
*BRCA*1	Breast cancer susceptibility1
PCNA	Proliferation cell nuclear antigen
DSB	double strand break
*GR*1	Gamma response1
*MSH*2	MutS homologue 2
*MLH*1	MutL homologue 1
FCM	Flow cytometry
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3-related
ROS	Reactive oxygen species

Introduction

Cadmium (Cd) is a highly persistent and accumulative heavy metals, and has been listed as one among the top ten hazardous substances by the Agency for Toxic Substances and Disease Registry (http://www.atsdr.cdc/gov/cercla/07list. html) and by the National Toxicology Program (NTP 2004). Cd is ubiquitously present in the environment mostly by derived from anthropogenic activities such as industrial processes and urban traffic, and then transferred to the food chain (Pierron et al., 2014). Numerous studies have shown that Cd stress leads to a wide variety of DNA damage

processes such as base-base mismatches, methylation, insertion/deletion loops, and DNA chain crosslinking/breaks, which can result in genotoxicity or/and cytotoxicity to cells (Filipic, 2012). Therefore, the study of the molecular mechanisms of Cd stress has become a focus in ecotoxicology research (Wang et al., 2016).

Cell proliferation is a highly concerted and tightly regulated process controlled by the cell cycle. This involves a highly conserved protein complex consisting of cyclin dependent kinases (CDKs) and cyclins, which act as multiple regulating proteins (Jia et al., 2016). Such CDK/cyclin complexes are required at cell cycle checkpoints, and activation of cell cycle checkpoints is a major mechanism in preventing genetic instability caused by threats originating from either exogenous environmental factors (such as UV-B and heavy metals) or endogenous metabolic processes (such as replication errors and metabolic byproducts) (Adachi et al., 2011; Cools and De Veylder, 2009; Hu et al., 2016). Schutter et al. (2007) demonstrated that Arabidopsis checkpoint activation upon cessation of DNA replication/DNA damage is controlled by WEE1 kinase that operates in an ATM/ ATR-dependent manner. To maintain genome integrity, signaling cascades initiated by the phosphatidylinositol-3-OH kinase–like kinases ATM and ATR control the activity of DNA repair complexes, halt cell cycle progression, and in some cases, initiate cell death programs in plants and mammals (Hu et al., 2016; Jia et al., 2016). In plants, the role of ATM/ATR-dependent signaling in the expression of several DNA damage response and DNA repair genes, such as *GR*1, *MRE*11, *RAD*51 and *BRCA*1, has been demonstrated (Jia et al., 2016; Yoshiyama, 2016; Garcia et al., 2003). Furthermore, in Arabidopsis jing he sheng 1 (jhs1) and other seedlings, many cell cycle-related genes such as *WEE*1, *CYCB*1;1, *CDKA*;1, *CDKB*1;1, *CYCD*4;1, *H*3.1, and *CYCA*2;1 were strongly induced upon DNA damage (i.e. endogenous DNA stress and /or DNA double strand breaks (DSBs)-causing treatments), and the checkpoint response is considered to be essential to inhibit transfer of damaged genetic information to daughter cells, supporting genetic stability in the cells of organisms (Cools and De Veylder, 2009; Culligan et al., 2006; Jia et al., 2016). Inhibition of *CYCB*1 and *CDKA* expression also occurred in response to Cd stresses in soybean

suspension culture cells, respectively (Burssens et al. 2000; Sobkowiak and Deckert, 2004). Furthermore, Jiang et al. (2011) reported that UV-B-induced DNA damage down-regulated expression of cell cycle related genes of *Histone H*4 and *E2F*a involved in the G1/S transition in Arabidopsis root tips. However, little information is available about the checkpoint response of cell cycle-related genes in Arabidopsis seedlings under Cd stress (Pena et al., 2012).

DNA stress either changes or perturbs the duration of different stages of cell cycle in plant cells, although the observed effects are dependent on plant species and tissue tested as well as on the type and dose of the stress (Cools and De Veylder, 2009; Hu et al., 2016;Jia et al., 2016). For example, endogenous replication stress caused by mutation of replisome factor *E2F* TARGET GENE 1 (*ETG*1) induced a prolonged cell cycle, accompanied with a high number of G2-phase cells in Arabidopsis (Cools and De Veylder, 2009). G2-phase arrest also occurred in root cells of onion (*Allium cepa* L.) reacting to X-ray-induced DSBs or hydroxyurea treatment (Pelayo et al., 2001; Carballo et al., 2006). Recently, flow cytometry analysis indicated that the DNA damage response may delay cell cycle progression and cause endoreduplication in Arabidopsis *jhs1* mutant seedlings (Jia et al., 2016). In a variety of eukaryotic cells, signals induced by Cd stress act at prereplication (G1/S) and/or premitosis (G2/M) checkpoints to inhibit the cell cycle progression, and G2/M phase cells are more sensitive to the challenge of several agents (Bakshi et al., 2008; Francis, 2011; Pena et al. 2012; Sobkowiak and Deckert, 2004; Xie and Shaikh, 2006; Yang et al., 2004). However, little information is known about cell cycle progression in response to Cd stress in Arabidopsis.

Therefore, the principal aims of this study were to (1) evaluate cell cycle progression in response to Cd in Arabidopsis seedlings; (2) determine the expression levels of cell cycle-related genes, including *CYCB*1;1, *CDKA*;1, *WEE*1, *E2F*a and *Histone H*4, by real-time, quantitative reverse transcription-PCR (qRT-PCR) analysis in Arabidopsis under Cd stress; (3) explore potential associations between the cell cycle-related indexes and expression of DNA damage marker genes in Arabidopsis under Cd stress.

2. Materials and methods

2.1 Plant material, growth and treatment conditions

Arabidopsis thaliana seeds (Arabidopsis, Columbia ecotype) were surface-sterilized in bleach solution (1:10 dilution of hypochlorite) and ethanol mix (ethanol: water: bleach 7:2:1) at about 20 ℃ for 5 min, respectively. Seeds were rinsed in sterile distilled water five times and imbibed in sterile-water for 2-4 days at 4 ℃ to obtain homogeneous germination (Pedroza-Garcia et al., 2016). The seeds were then sown in sterile flasks containing 150 mL of commercially available 0.5×Murashige and Skoog (MS) liquid medium (Basalt Salt Mixure, Caisson, USA) with 0.5% (w/v) sucrose (pH 5.8), and supplemented with Cd at a final concentration of 0 (the control), 0.125, 0.25, 1.0, and 2.5 $mg \cdot L^{-1}$ in the form of $CdCl_2 \cdot 2H_2O$ of analytical grade with purity 99.5%, PR China. Each flask with 20-30 plantlets was placed on a rotary shaker at about 50 rpm in an incubator (12 h light of approximately 3000 lx and 12 h dark at 21 ± 0.5 ℃) for 5 d. All treatments and analyses were repeated in three independent replicates.

2.2 RNA extraction and real-time, quantitative reverse transcription-PCR (qRT-PCR) analysis

For both the control and Cd treated plantlets, fresh root tip (about 0.5 cm) tissues were collected after 5 d of growth as described above, and flash frozen in liquid nitrogen prior to storage at −80 ℃. Total RNA was isolated and purified using RNA isolation and clean up kits (EZ-10 DNAaway RNA Mini-prep Kit, Sagon). First-strand cDNA was synthesized from 2μg of total RNA using a PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. qRT-PCR analysis was done using 20μL reaction mixtures containing 20 ng of template cDNA, 0.5μM of corresponding forward and reverse primers and 10μL of 2×SYBR Mix (SYBR ® Premix Ex Taq™ Ⅱ (Tli RNaseH Plus, TaKaRa). Reactions were run and analyzed on the iCycler iQ (Bio-Rad) according to the manufacturer's instructions. The specificity of amplification products was determined by melting curves. ACT2 was used for signals normalization. IQ5 relative quantification software (Bio-Rad) automatically calculates

relative expression level of the selected genes with algorithms based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data were from triplicates and are representative of at least three biological replicates. The sequence of primers used in this study is provided in Supplementary Table S1.

2.3 Flow Cytometry analysis of cell cycle progression in root tips of Arabidopsis

Nuclei were extracted by chopping approximately 0.1g of fresh root tips (about 0.5 cm) in ice cold Galbraiths Chopping buffer (45 mM $MgCl_2$; 30 mM sodium citrate; 20 mM MOPS; 0.1% (w/v) TritonX-100; pH7.0) supplemented with 10 mM DTT in a Petri dish with a razor blade (Hefner et al., 2006). After chopping, the tissue and buffer were strained through 30 μm nylon mesh, and then 15 $\mu g \cdot mL^{-1}$ RNase A were added and incubated in a water bath of 37℃ for 30 min. The suspension was stained with 50 $\mu g \cdot mL^{-1}$ propidium iodide (PI, Molecular Probes, Beyotime, PR China) at 4℃ for 30 min. The control and Cd-treated samples were analyzed within 24 h by flow cytometry on a FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with a 488 nm laser. Detector settings were determined empirically. Fluorescence intensity was analyzed in the FL2 channel with no less than 10000 nuclei measured for each sample.

Quantitation was carried out by appropriately gating the raw data and comparing the gated events for each peak and comparing that to the total number of gated nuclei. Gates of Sup-Fig. 1 were determined empirically on nuclei isolated from the root tips of the 5-day-old seedlings with FlowJo V10 software (BD Biosciences, San Jose, CA). According to data in Sup-Fig. 1, analyses of cell cycle only calculated 2N,S and 4N (i.e. 100% in total for each treatment), and analyses of ploidy distribution only calculated 2N, 4N and 8N (i.e. 100% in total for each treatment).

2.4. Statistical analysis

SPSS for Windows (version 19.0) was used for the statistical evaluation of the results. Values are expressed as mean ± standard deviation of the mean. Differences among the control and treatments were analyzed by 1-way analysis of variance (ANOVA),

taking $P< 0.05$ as significant according to the least significant differences (LSDs) tests corrected for the number of comparisons.

3. Experimental results

3.1 Cd stress decreased root growth of Arabidopsis seedlings

There were no statistically significant differences for fresh weight of shoots between the control and Cd-treated seedlings (Table 1, $P < 0.05$) although shoots treated with 2.5 mg•L^{-1} of Cd indicated a slight decrease of fresh weight. Likewise, exposure to Cd of 0.125-2.5 mg•L^{-1} for 5 d had no obvious effect on the germination rate and chlorophyll content of Arabidopsis seedlings compared to the control after 5 d of treatment (Table1, $P< 0.05$). However, the differences between the root length of the control plantlets and the plantlets treated with 1.0 and 2.5 mg•L^{-1} Cd were found to be statistically significant ($P < 0.05$, Table 1). Indeed, in plantlets exposed to 0.125-2.5 mg•L^{-1} Cd, a significant inverted U-shaped relationship was seen between the root length and Cd level (Table 1).

3.2 Cd stress triggered cell cycle arrest in root tips of Arabidopsis

To analyze cell cycle progression in Arabidopsis plantlets of 5-d-old seedlings under Cd stress, the effects of Cd stress on cell cycle arrest were examined by flow cytometry. As shown in Fig. 1, the proportion of cells with a ^{2}C nuclear content (G0/G1 phase) was 45.04% in the control plantlets whereas Cd stress significantly altered this proportion, which was 50.33, 49.54, 39.71 and 29.86% under 0.125-2.5 mg•L^{-1} Cd stresses, respectively. This alteration in the ^{2}C nuclear content was accompanied by changes in the proportion of cells with a ^{4}C and ^{8}C nuclear content, which was 48.9, 47.0, 45.6, 57.5 and 74.6% in root tips of 0-2.5 mg•L^{-1} Cd-treatment, respectively (Table 2, Sup-Fig. 1). There was no significant effect of Cd on cells in the S phase of the cell cycle (Fig. 1). This result suggests that the G1/S phase of the cell cycle is significantly delayed in the 0.125 mg•L^{-1} Cd-treated plantlets and that the G2/M phase of the cell cycle is delayed in the 1.0-2.5 mg•L^{-1} Cd-treated plantlets, respectively.

3.3 Cd stress induced the changes in expression of cell cycle-regulatory genes in root tips of Arabidopsis seedlings

The effect of Cd on the cell cycle-regulatory genes was further determined by measuring the expression of marker genes for cell proliferation (*PCNA*1 and *PCNA*2), G1/S transition (*Histone H*4 and *E2F*a), and G2/M transition (*WEE*1, *CDKA*;1 and *CYCB*1;1) in root tips under Cd stress for 5 d by qRT-PCR analysis. An increase in the gene expression of *PCNA*1, *CDKA*;1, and *WEE*1 was observed in root tips exposed to the lowest concentration (0.125 mg•L^{-1}) of Cd (Fig. 2), whereas a dose-dependent decrease was seen in expression of these genes with 0.25-2.5 mg•L^{-1} Cd, and in *CYCB*1;1, *PCNA*2, *Histone H*4 and *E2F*a with 0.125-2.5 mg•L^{-1} Cd, respectively (Fig. 2). Amongst all the Cd levels used for plantlet treatment, 0.125 mg•L^{-1} Cd caused a increase of 1.1- to 1.4-fold in gene expression of *PCNA*1 and *WEE*1, while 2.5 mg•L^{-1} Cd resulted in a maximum decrease of 1.4- to 2.5-fold in the expression of all of the cell cycle-related genes (Fig. 2), respectively. These findings support the hypothesis that Cd stress can modulate the expression of cell cycle regulatory genes involved in G1/S and G2/M transitions in Arabidopsis root tips.

3.4 Cd stress induced the changes in expression of DNA damage response genes in root tips of Arabidopsis seedlings

To examine DNA damage-response in the Cd-treated root tips of Arabidopsis seedlings, we analyzed the expression of several marker genes for the DNA damage response using qRT-PCR analysis. As shown in Fig. 2, the expression levels of *MRE*11 and BRCA1 were increased approximately 1.1- to 1.8-fold in the 0.125-1.0 mg•L^{-1}Cd-treated Arabidopsis, and decreased in the 2.5 mg•L^{-1}-Cd-treated Arabidopsis. However, a concentration-dependent reduction in the expression of *GR*1 with a minimum decrease of 1.1-fold at 0.125 mg•L^{-1} Cd and a maximum decrease of 3.3-fold at 2.5 mg•L^{-1} Cd exposure was observed. Also, a dose-dependent decrease in the expression of DNA mismatch repair genes, *MLH*1, *MSH*2 and *MSH*6, by 1.2- to 4.2-fold was observed at 0.125-2.5 mg•L^{-1} Cd exposure, respectively, in the root tips of Arabidopsis seedlings in comparison to the control. This result suggests that significant DNA damage occurred

242 in the root tips of Cd-treated plantlets for 5 d.

243

244 **4. Discussion**

245 Root tips are the most active region of plant roots for Cd influx, and Cd stress has
246 deleterious effects on plant growth and development (Filipic et al., 2012). Our results
247 indicated, however, that a significant reduction in root length appeared only in plants
248 exposed to Cd at 1.0 and 2.5 mg•L^{-1} while a significant increase in root growth was
249 observed at lower (0.125 mg•L^{-1}) Cd after 5 d of treatment. However, exposure to Cd of
250 across the concentration range tested did not significantly affect fresh weight or
251 chlorophyll content of shoots (Table 1). A similar trend of a low dose of Cd stimulating
252 cell proliferation was reported in mouse testicular Leydig cells (Singh et al., 2009).
253 Upon salt stress, the root meristematic zone was decreased in Arabidopsis root tips
254 (West et al., 2004). Furthermore, in the aluminum (Al)-sensitive variety of maize, Al
255 exposure completely blocked the entrance of cells into the S-phase in the central part
256 of the root meristematic zone (250–800 µm from the apex) (Doncheva et al., 2005).
257 Therefore, this study suggests that Al stress first rapidly blocks cell cycle progression,
258 presumably to prevent the entrance into stages when the cells are particularly
259 vulnerable to DNA damage, and to allow the cellular defense system to be activated.

260

261 DNA can be impaired in a variety of manners under various stresses, originating from
262 either exogenous (such as UV-B and heavy metal stresses) or endogenous (such as
263 replication errors and ROS) sources. To maintain genome integrity, signaling cascades
264 initiated by ATM and ATR control the activity of DNA repair complexes, halt cell cycle
265 progression, and in some cases, initiate cell death programs in plants and mammals
266 (Hu et al., 2016; Jia et al., 2016). In plants, the role of ATM/ATR-dependent signaling in
267 the expression of several DNA damage response and DNA repair genes, such as *GR*1,
268 *MRE*11, *RAD*51 and *BRCA*1, has been demonstrated (Jia et al., 2016; Yoshiyama,
269 2016; Garcia et al., 2003). However, we know very little about molecular players in
270 DNA damage response in Arabidopsis under Cd stress. Herein, we analyzed the
271 expression levels of key genes in DNA damage responses (Fig. 2). The expression of

*MLH*1, *MSH*2 and *MSH*6 was significantly reduced in the Cd-treated Arabidopsis in comparison to the control. These genes play important roles in the recognition and correction of damaged DNA bases, pyrimidine dimers and mismatches such as mispaired or unpaired bases, in the activation of cell cycle checkpoints, and in maintaining the stability of genomic DNA and fidelity of DNA replication etc (Lario et al., 2011); the decrease in their expression under Cd stress is likely therefore to result in DNA damage in these plantlets. The expression of *BRCA*1, which functions in genome surveillance and DNA damage repair (Jia et al., 2016), was enhanced more than 1.1- to 1.6-fold in the 0.125-1.0 mg•L^{-1}-Cd-treated Arabidopsis and decreased in the 2.5 mg•L^{-1}-Cd-treated Arabidopsis, respectively (Fig. 2), suggesting that DNA damage appears in these seedlings exposed to Cd stress. Similarly, the *MRE*11 nuclease, which is involved in DSB end processing (Roth et al., 2012), was significantly induced more than 1.2- to 1.8-fold in the 0.125-1.0 mg•L^{-1}-Cd-treated Arabidopsis, and decreased in the 2.5 mg•L^{-1}-Cd-treated seedlings, respectively. Also, the *GR*1 nuclease, which are involved in DSB end processing (Roth et al., 2012), was significantly decreased in the seedlings of 0.125-0.25 mg•L^{-1} Cd-treatment, respectively (Fig.2). The above results suggest that at low levels of Cd, the increased expression of the genes involved in DNA damage repair likely decreases the amount of damaged DNA in the Cd-treated cells perhaps enhancing cell proliferation and hence root extension. However at higher Cd concentrations the DNA damage is extensive, resulting in decreased expression of these genes. This would indicate a dysfunctional repair system further increasing the DNA damage (Sup-Fig. 2; Wang et al., 2016).

The mechanisms underlying a DNA damage response–dependent cell cycle arrest have been well characterized in mammals, and relatively little has been known in plant cells (Adachi et al., 2011; Cools and De Veylder, 2009; Filipic, 2012; Hu et al., 2016). In the current experiment, the results indicate that Cd stress does affect expression patterns of cell cycle regulatory genes involved in G1/S transition and G2/M transition in root tips of Arabidopsis seedlings (Fig. 2). This suggests that Cd may have an adverse effect on the regulatory process of the checkpoints of G1/S and G2/M checkpoint

transitions in the Arabidopsis seedlings. Although the accurate timing and role of various gene products at specific stages of the cell cycle has not been clearly elucidated under Cd stress, we show here that the expression of several genes involved in cell cycle regulation is affected differentially by the Cd exposure levels. For example, expression of three genes involved in the G2/M transition (i.e. *WEE*1, *CDKA*;1 and *PCNA*1) was significantly induced by exposure to 0.125 m•L^{-1} of Cd for 5 d, and therein WEE1 and CDKA;1 were a critical regulatory factor and the composition of MPF (Maturation Promoting Factor) engaged in G2/M transition, respectively (O'Connell et al., 1997). However, substantially down-regulated expression occurred at 0.25, 1.0 and 2.5 mg•L^{-1} Cd for the above three genes and at 0.125-2.5 mg•L^{-1} Cd for four genes (*CYCB*1;1, *Histone H*4, *PCNA*2 and *E2F*a which is crucial for G1/S or G2/M transition) tested, respectively (Fig. 2). However, a increase of 3.1- to 4.7-fold in gene expression of CYCB1;1 and *WEE*1 occurred in Arabidopsis plantlets exposed to 0.125-0.25 mg•L^{-1} Cd for 24 h respectively, when Arabidopsis grew under the untreated control condition for about 5 d at 21℃ after germination (data not given). Similar trend was reported on mouse testicular Leydig cells, soybean suspension-cultured cells, parsley, maize, wheat and Arabidopsis species under stresses of fungal elicitor, low temperature, UV irradiation, salt and Cd, respectively (Pena et al., 2012; Singh et al., 2009; Sobkowiak et al., 2003; Rymen et al., 2007; Xie and Shaikh, 2006). Alternatively, Pena et al. (2012) reported that Cd stress down-regulated expression of *PCNA* in wheat root apical meristems, which is cell cycle marker gene related to G1/S transition through the E2F/retinoblastoma-related (RBR) pathway. Moreover, expression of *PCNA* gene in rice seedlings was induced by exposure to a DNA-damage agent, such as UV of 25 J•m^{-2} and H_2O_2 of 1 mM treatment, indicating that the biomarker responses could be used to differentiate stress effect (Yamamoto et al., 2005). Therefore, modified expression of the cell cycle regulatory genes involved in G1/S transition and G2/M transition probably supports the assumption that Cd stress would be responsible for the decrease/decrease in cell proliferation through G1/S or/and G2/M checkpoint arrest in Arabidopsis root tips in the current research.

Cd, as a redox inactive metal, changed expression of DNA damage response and cell cycle regulatory genes in the root tips of Arabidopsis seedlings by qRT-PCR analysis, and dose-dependent manners between Cd levels applied and expression of cell cycle regulatory genes are notably reduced or inverted U-shaped curves with the maximum effect at 0.125-1.0 $mg \cdot L^{-1}$ Cd, respectively, (Fig. 2), which is in agreement with the findings of previous reports (Singh et al., 2009; Liu et al., 2009; De Schutter et al., 2007). The major mechanistic explanations for the induced expression of the above genes observed by low levels of Cd are a likely modulation of cellular signal transduction pathways by activation of transcription factors or/and modification of protein phosphorylation status as a result of the interplay among ATM, ATR, SOG1, WEE1 kinases, CDC25 phosphatases and CDKA;1 (Cools and De Veylder, 2009; Hu et al., 2016). Emerging study has demonstrated that upon different types of DNA stress from the Arabidopsis plants, the transcriptional activation of DNA repair and cell cycle checkpoint genes totally depends on ATM and/or ATR, suggesting that ATM and ATR could play a pivotal role in the DNA-damage checkpoint response in plants (De Schutter et al., 2007; Cools and De Veylder, 2009; Hu et al., 2016). Similarly, mutations in ATM or ATR render organisms hypersensitive to DNA damage-inflicting agents in plants, and the ATM mutants show growth defects when treated with γ-rays or methyl methanesulfonate (MMS), causing DSBs (Cools and De Veylder, 2009). Also, low level of Cd can interfere with antioxidant defense systems and stimulate the production of highly reactive free radicals in cells (Filipic, 2012). Thus, these reactive free radicals in cells may act as signaling molecules and induce expression of cell cycle regulatory genes in the Arabidopsis seedlings in this experiment (Fig. 2). Cools and De Veylder (2009) demonstrated that the unique behavior of increased CYCB1;1 expression hints at a specific function for this particular cyclin in DNA-stress response, but the role is unknown, which seems that increased CYCB 1;1 levels maintain the stressed cell's competence for cell division. Alternatively, Cd has a high affinity to cysteine in three dimensional protein structures and can promote specific binding of Cd to the above protein components (Filipic, 2012), which can inhibit expression of DNA repair and cell cycle regulatory genes tested under Cd stress (Fig. 2). Decreases in mRNA stability

and increased mRNA turnover rates are other possible explanations for the observed changes in expression for DNA repair and cell cycle regulatory genes (Fig. 2). Since the ubiquitin-proteasome system (UPS) is particularly important for the turnover of many cyclins-like critical proteins participating in cell proliferation process, and cyclin D and CDKA proteins conjugated with highly conserved 76-aminoacid protein ubiquitin (Ub) were specifically decreased in wheat root tips under Cd stress (Pena et al., 2012), reduction of the cell cycle regulatory genes could then represent a protective response to Cd stress in this research (Fig. 2). All the Cd effects mentioned above undoubtedly would severely affect modifications in expression of the genes observed of Arabidopsis seedlings exposed to Cd in the current study.

It is well known that when cells suffer different kinds of DNA stresses, G1/S and G2/M checkpoints can be activated that transiently inhibit cell proliferation so that DNA lesions can be repaired before the cell cycle continues, respectively, and the above effect could be performed via SOG1 transcription factor activated by ATM/ATR in plants (Cools and De Veylder, 2009; Fulcher and Sablowski, 2009; Hu et al., 2016; Furukawa et al., 2010; Yoshiyama, 2016). In the current study, the reduced growth of the root tips in the 1.0-2.5 $mg \cdot L^{-1}$ -Cd-treated seedlings suggests that a cell cycle delay was triggered (Tables 1 and 2, Fig. 1). Cd stress markedly delayed progression of G1/S transition at 0.125 $mg \cdot L^{-1}$ Cd and of G2/M transition at 1.0-2.5 $mg \cdot L^{-1}$ Cd in Arabidopsis root tips, concomitantly with enhanced DNA damage levels in Arabidopsis root tip cells (Figs. 1 and 2; Sup-Fig. 2), which illustrates that DNA damage checkpoints occurred in the Cd-treated plantlets. In immortalized human normal prostate epithelial cell line (NPrEC), Bakshi et al. (2008) observed the G1/S arrest after 8 h of exposure to Cd, whereas 32 h exposure caused the G2/M arrest. Jiang et al. (2011) showed that UV-B-induced DNA damage delayed G1/S transition in Arabidopsis root tips at least partially through changes in the regulation of the expression of cell cycle-related genes *Histone H*4 and *E2F*a. It was reported that signals induced by Cd stress act at G1/S or/and G2/M checkpoints to inhibit the cell cycle progression in a variety of eukaryotic cells (Choi et al., 2011; Pena et al. 2012; Sobkowiak and Deckert, 2004; Xie and Shaikh,

2006; Yang et al., 2004). Moreover, the aberrant expression level of several genes related to G1/ S transition and G2/M transition occurred in the Cd-treated plantlets (Fig.2), and DNA damage can affect cell cycle progression partially through changes in the mediation of the expression of cell cycle-related genes (Jia et al., 2016). Thus, all these data support the notion that the DNA damage response sensed by *BRCA*1 and MMR genes can delay G1/S transition by inhibiting E2F transcription factor which further suppresses expression of the above genes (i.e. *MSH*6, *PCNA*1 and *PCNA*2), leading to delay G2/M transition during the cell cycle progression in Arabidopsis under Cd stress (Lario et al., 2011; Pena et al., 2012). In addition, 0.125 mg•L^{-1} Cd-induced DNA damage induced the marked G1/S arrest but shorted G2/M phase, leading to accelerating growth in root tips, while 1.0-2.5 mg•L^{-1} Cd-induced DNA damage caused the notable G2/M arrest in root tips, causing reducing growth in root tips (Tables.1-2, Figs.1-2, Sup-Fig. 1). As cell cycle progression is directly related to the cell division, proliferation, growth and development (Gutierrez et al. 2002), our results suggests that Cd-induced G1/S or/and G2/M arrest can be a protective mechanism that alleviates/prevents cells with damaged DNA from dividing and may provide more explanation for the reduction in crop growth and productivity under Cd stress. Also, MMR genes with their most sensitivity and lability could be a brilliant biomarker for Cd stress.

Conclusions

The present report defines modifications in cell cycle progression in correlation with the alteration of expression of cell cycle regulatory genes and DNA damage response genes measured in root tips of Arabidopsis seedlings exposed to Cd of 0.125-2.5 mg•L^{-1} for 5 d. We observed the prominently inverted U-shaped dose-response effects of Cd stress on gene expression of *BRCA*1, *MRE*11, *WEE*1, *CDKA*;1 and *PCNA*1 in root tips of Arabidopsis seedlings at 0.125-2.5 mg•L^{-1} Cd. Also, substantially decreased expression of genes was observed for *CYCB*1;1, *Histone H*4, *E2F*a, *PCNA*2, *GR*1, *MSH*2, *MSH*6 and *MLH*1 in root tips exposed to 0.125-2.5 mg•L^{-1} Cd for 5 d, respectively. Furthermore, Cd-induced DNA damage results in the significant delay of

G1/S transition and G2/M transition at 0.125 and 1.0-2.5 mg•L^{-1} Cd in Arabidopsis root tips, respectively. Cd-induced G1/S or/and G2/M arrest may be a protective mechanism that prevents cells with damaged DNA from dividing and may explain the plant growth inhibition under Cd stress.

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532

Figure

Table 1. Effect of Cd stress on germination, total chlorophyll level, fresh weight and root growth of Arabidopsis seedlings for 5 d.

Cd level /mg·L^{-1}	Germination percentage/ %	Total chlorophyll /μg·g^{-1} FW	Fresh weight /mg·shoot^{-1}	Root growth	
				Root length/ cm	Inhibitory rate/ %
0	96.1±2.1	328.4±23.2	10.03±0.85	1.29±0.03	0
0.125	96.2±1.3	330.8±26.1	11.56±1.72	1.48±0.02a	-14.73
0.25	95.6±1.5	326.7±24.5	11.01±1.90	1.35±0.03	-0.51
1.0	95.2±2.7	331.3±21.9	10.04±1.26	1.07±0.04a	17.05
2.5	94.6±3.2	312.5±20.6	9.03±0.51a	0.76±0.01a	41.09

[a]Significantly different from the control ($P < 0.05$). Data are means ± SE (n = 3).

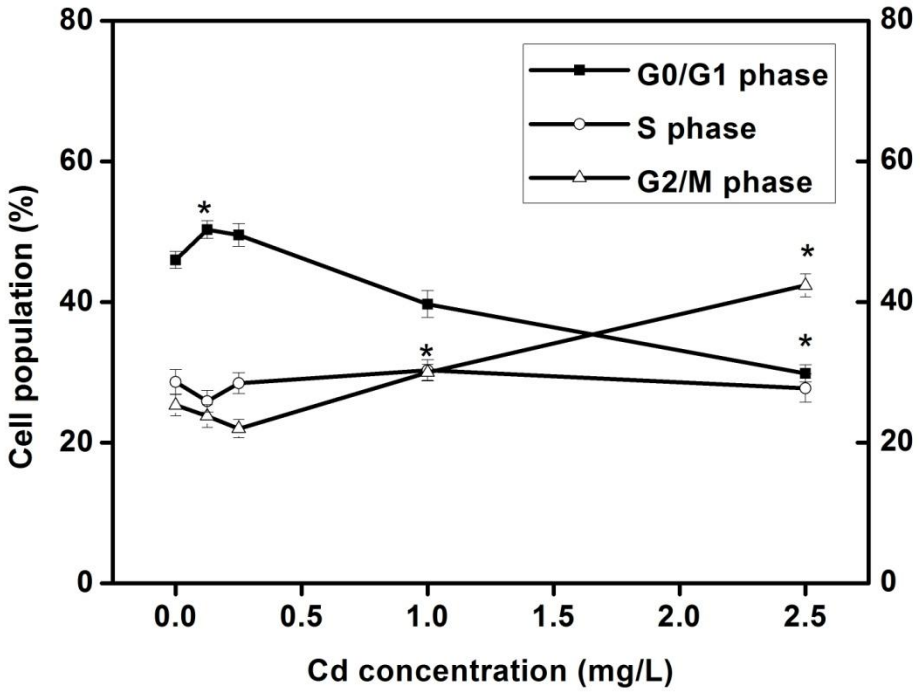

Fig.1. Effect of Cd on the cell cycle in root tips of Arabidopsis for 5 d. The percent distribution of cells in G0/G1, S, and G2/M phases was calculated and compared with the control. Each point represents the mean ± S.D. of three independent experiments. *Significantly different from the control cells ($P < 0.05$), the same below.

Table 2. Effects of Cd stress on the distribution of DNA content in root tips of Arabidopsis for 5 d.

DNA content(%)	Cd concentration(mg/L)				
	0	0.125	0.25	1.0	2.5
2C	51.1	53.0	54.4	42.5	25.4
4C	28.2	25.1	24.1	32.1	35.9
8C	20.7	21.9	21.5	25.4	38.7*

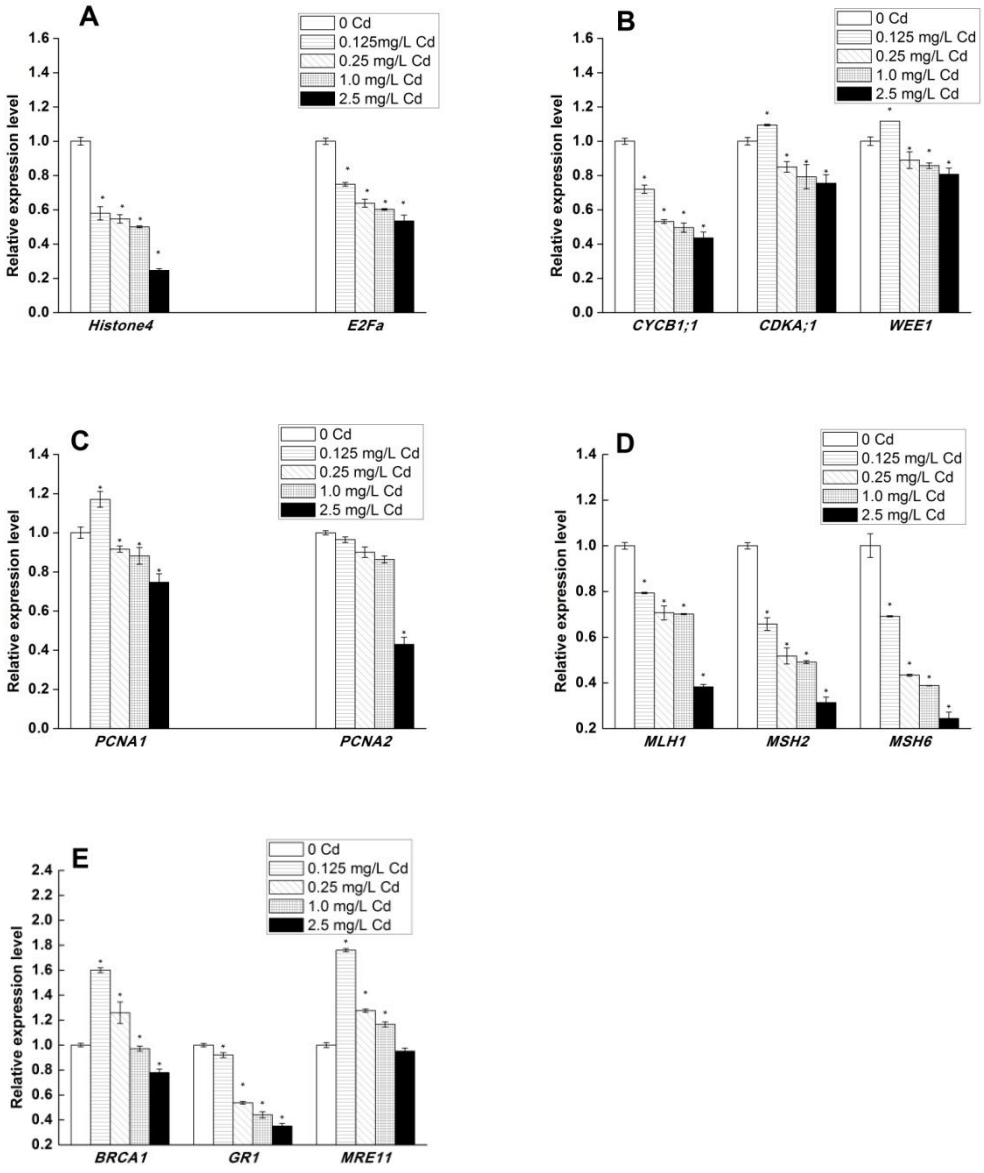

Fig.2. Effects of Cd stress on gene expression in root tips of Arabidopsis for 5 d.
(A) G1/S marker genes *Histone H*4 and *E2F*a; (B) G2/M marker genes *CYCB*1;1, *CDKA;*1 and *WEE*1; (C) Cell proloferation marker genes *PCNA*1 and *PCNA*2; (D) DNA mismatch repair genes *MLH*1, *MSH*2 and *MSH*6; (E) DNA damage repair genes *BRCA*1, *GR*1 and *MRE*11. Data are shown as mean ± SD by qRT-PCR. Data presented are average of three replicates. House-keeping gene AtACT2 was used as an internal control.

Supplementary Material

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Supplementary material

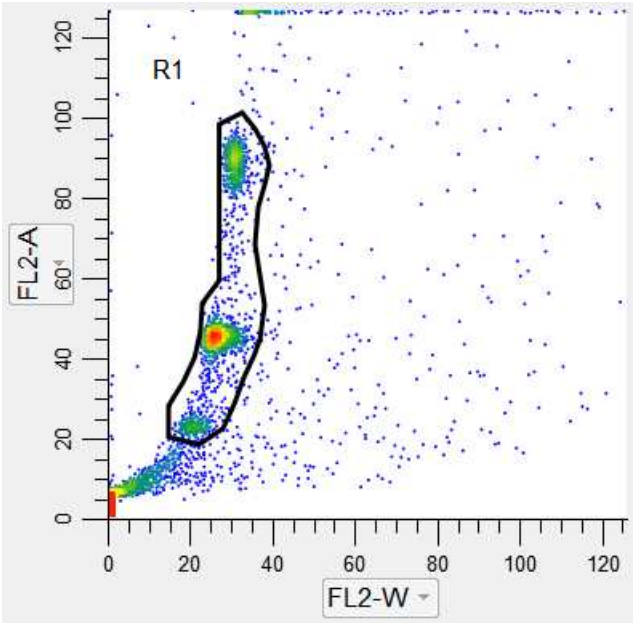
Sup--Table 1. Primer sequences used

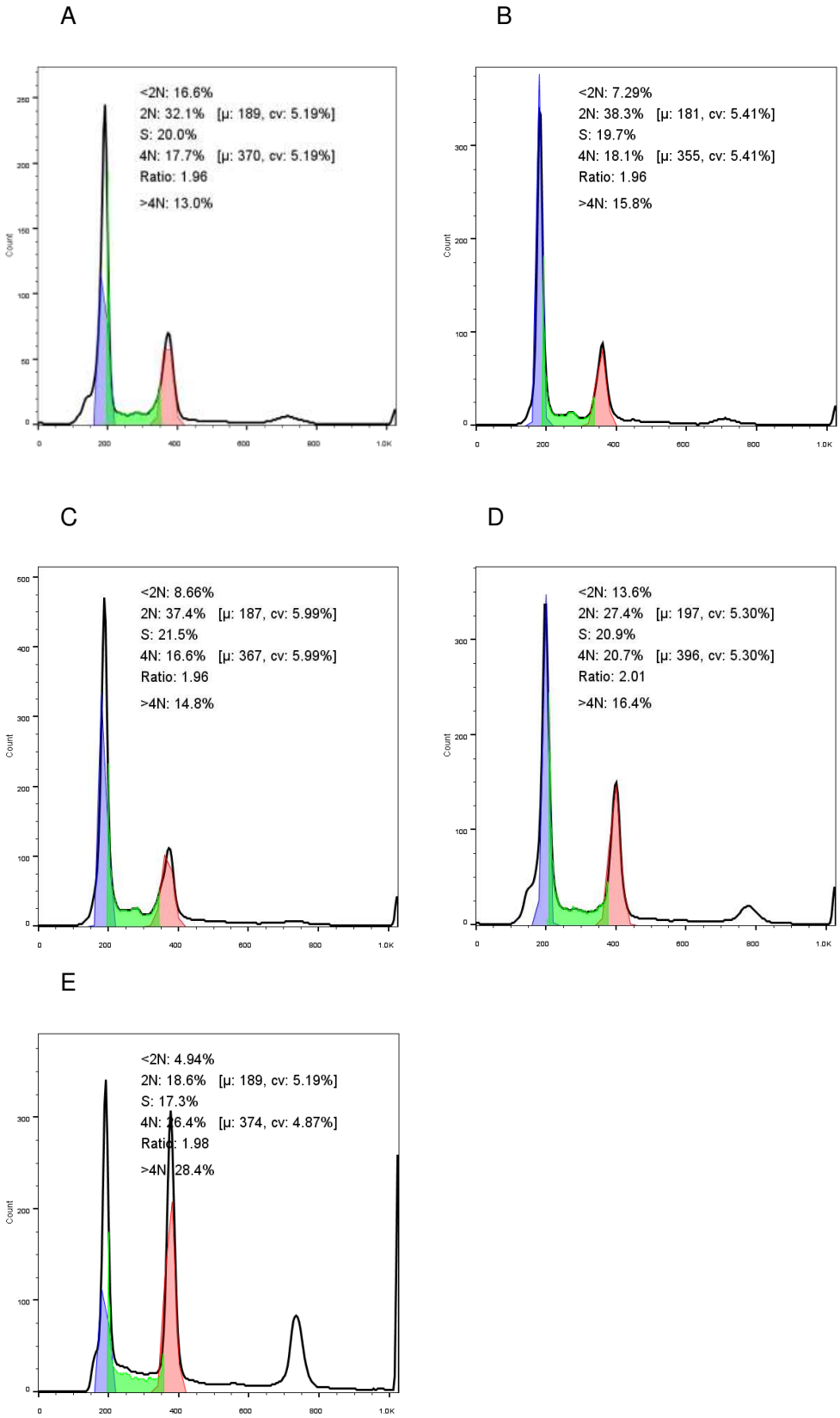
Gene name	Forward primer（5′—3′）	Reverse primer（5′—3′）	PCR product size (bp)
ACT2[a]	TCGTGGATTCCAGCAGCTTCC	CCGATGGGCAAGTCATCACG	100
MLH1[b]	GTAGTAAGGTCTTCTGCAAGGCA	TGCCATTCCAACATATGTGC	147
MSH2[b]	TCTGACTAGGCGAGTTCTT	CACCTCTCCAGGGAATCA	162
MSH6[b]	ATTAGTTAGAAAGGGCTATCGGG	AACAACTGCACATACTTCGC	127
Histone4[a]	GATTCGTCGTCTTGCTCGTAG	CAGTCACCGTCTTCCTCCTC	149
E2Fa[a]	ACCATCCACCGTCATCTC	GCTCCTGTCGTTATTATTACTG	158
CYCB1;1[c]	CTCAAAATCCCACGCTTCTTGTGG	CACGTCTACTACCTTTGGTTTCCC	110
CDKA;1[c]	CCTGTCAGGACATTTACTCATGAG	GCTTTTGGCTGATCATCTCAGC	139
WEE1[d]	TGGTGCTGGACATTTCAGTCGG	CAAGAGCTTGCACTTCCATCATAG	137
PCNA1[b]	GTGACACAGTTGTGATCTCTG	ATCACAATTGCATCTTCCGG	127
PCNA2[b]	GATGAAGCTGATGGATATCGAC	GAGATCACAACTGTGTCACC	138
GR1[c]	CAGCATGAGAAATCAGCAATCTCG	GGTGAGATGGAAGTGATAGGTGTC	161
BRCA1[c]	GTAACCATGTATTTTGCAATGCGTG	GTGACGGATTATTCTGGCTAACG	192
MRE11[c]	GTGATACACTTCGAGTACTTGTTGC	CTGACTACTTGAAACTGCACTGG	256

[a] Jiang et al. 2011; [b] Liu et al. 2009; [c] Jia et al., 2016; [d] Cools and De Veylder, 2009.

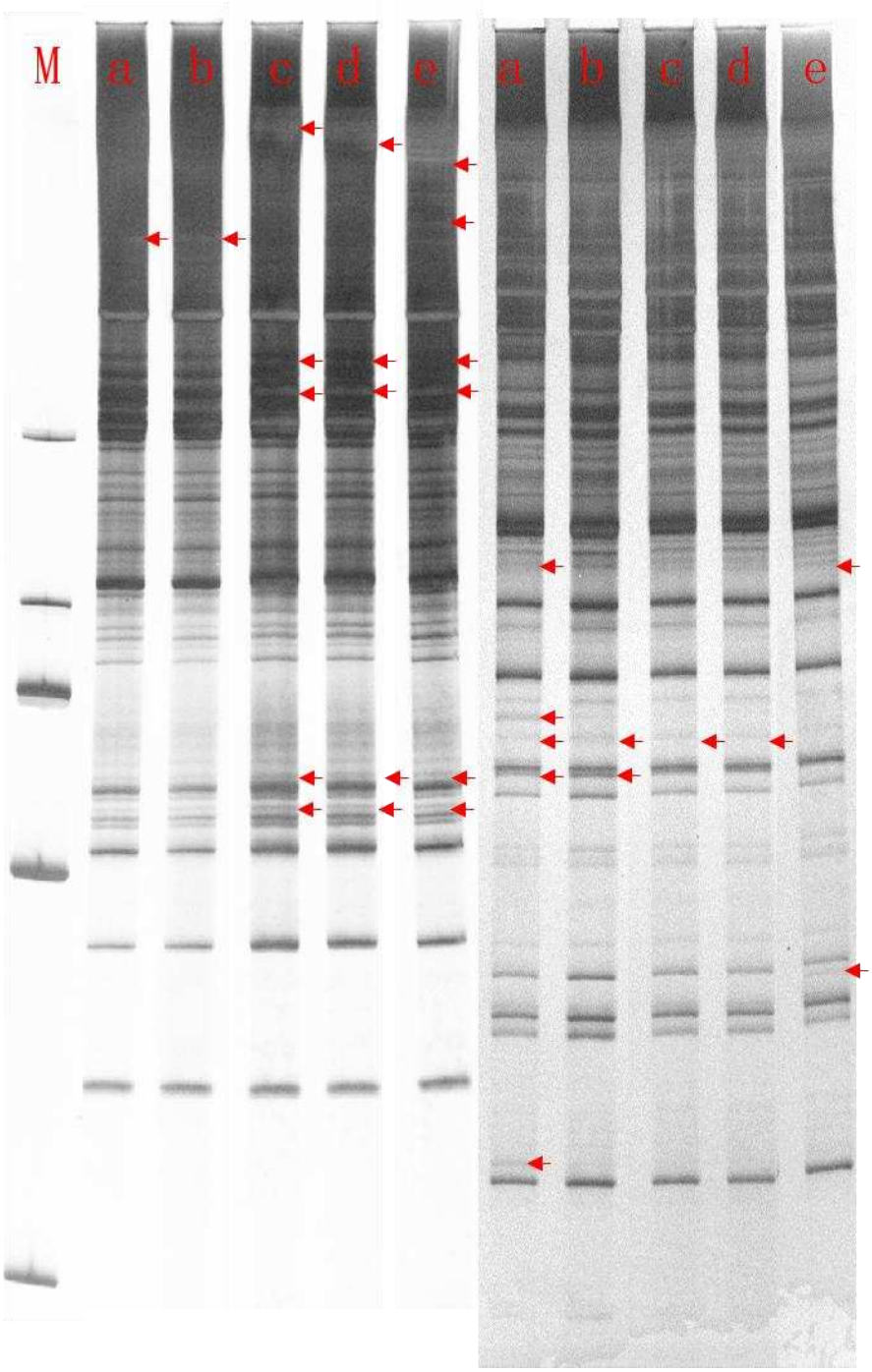
The same as the reference.

A’





Sup--Fig. 1 Effects of different concentrations of Cd on DNA distribution in Arabidopsis seedling root tip cells determined by flow cytometry analysis. Fluorescence-2 area (FL2-A) is a measure of integrated cell fluorescence signal that represents the DNA content. Data represent results from three replicates. A-E represents 0, 0.125-2.5 mg/L Cd, A', PI fluorescence signal, respectively.



Sup--Fig. 2 RAPD fingerprints of Arabidopsis seedlings exposed to 0~2.5 mg·L^{-1} Cd for 5 d. a-e represents 0, 0.125-2.5 mg/L Cd, respectively.