Cell cycle regulation in different leaves of **Arabidopsis thaliana** plants grown under control and cadmium-exposed conditions

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HIGHLIGHTS

- Leaf position can be used as an alternative for leaf age in cell cycle analysis
- Concentrations of nuclei in leaf extracts are a proxy for effects on cell division
- Cadmium inhibits cell division and endoreduplication in *Arabidopsis thaliana* leaves
- Cadmium induces SIAMESE-RELATED gene expression in *Arabidopsis thaliana* leaves

Abstract

Cadmium (Cd) is well known to inhibit vegetative plant growth. However, knowledge regarding its influence on the cell cycle is scarce and mainly limited to cell cultures and root tissue. Therefore, the main aim of this study was to investigate the effects of Cd exposure on cell division and endoreduplication in *Arabidopsis thaliana* leaves. In order to do so, we first investigated whether different leaves of the same rosette harvested at one time point (*i.e.* leaf position) could be used as an alternative for leaf age when investigating the cell cycle. To this end, wild-type *A. thaliana* plants were grown hydroponically with or without the addition of 5 µM $CdSO_4$. Leaf growth and development, cell division, endoreduplication and the expression of cell cycle-related genes were investigated in separate leaves. The results show that different leaf positions constitute a developmental series that can be used to deduce the development of a single leaf over time. Furthermore, our data indicate that the concentration of nuclei in leaf extracts measured via flow cytometry can be used as a proxy to determine the effects of stress factors on the extent of cell division in *A. thaliana* leaves, reducing the need to perform time consuming microscopic analyses. Finally, we show that Cd exposure significantly reduces cell number, cell size and nuclear DNA content, implying an inhibition of both cell division and endoreduplication. These effects accumulate over time and contribute to the Cd-induced disturbance of leaf growth and development. At the molecular and cellular level, Cd increases hydrogen peroxide levels and induces the expression of marker genes for oxidative stress and DNA damage and genes encoding CDK inhibitors of the SIAMESE-RELATED family, suggesting that the Cd-induced inhibition of cell cycle progression is intertwined with oxidative stress and subsequent DNA damage.

Key words: *Arabidopsis thaliana*, cadmium, cell cycle, DNA damage, leaf age, oxidative stress.

1. Introduction

The cell cycle plays a key role in plant growth and development (Beemster *et al.*, 2005b; Inzé and De Veylder, 2006). In plants, the standard cell cycle consists of four phases: gap 1 (G_1), synthesis (S), gap 2 (G_2) and mitosis (M). The most important events occurring during the cell cycle are the replication of the nuclear genome (in the S phase) and the division of the cell (in the M phase). The cell cycle is regulated by cyclin-dependent kinases (CDKs), which form heterodimers with cyclins in order to become activated (Dewitte and Murray, 2003). Different cyclin-CDK complexes drive cell cycle progression by phosphorylating a broad range of substrates promoting the G_1/S and G_2/M transitions (Inzé and De Veylder, 2006). For the cell cycle to proceed, CDK activity should reach two threshold levels: one for DNA replication (at the G_1/S transition) and one for cell division (at the G_2/M transition) (De Veylder *et al.*, 2011).

In plants, an alternative version of the cell cycle termed endoreduplication exists. In contrast to the standard cell cycle, an endoreduplication cycle or endocycle only consists of a G_1 and S phase. As a consequence, nuclear DNA is replicated without intervening mitosis, resulting in endopolyploidy (Lee *et al.*, 2009; Gutierrez, 2016). In *Arabidopsis* leaves, endoreduplication occurs after cells stop dividing and coincides with their cell expansion (Beemster *et al.*, 2005a). The transition from cell proliferation to expansion takes place from the leaf tip towards the base (Donnelly *et al.*, 1999; Adriankaja *et al.*, 2012; Blomme *et al.*, 2014; Rodriguez *et al.*, 2014). The extent of endoreduplication strongly depends on the plant species and cell type studied (Sugimoto-Shirasu and Roberts, 2003). Furthermore, nuclear ploidy levels often correlate with cell size, thereby implying a role for endoreduplication in cell growth (Melaragno *et al.*, 1993;

Lee *et al.*, 2009). Similar to the standard cell cycle, endoreduplication is regulated by cyclin-CDK complexes. During an endocycle, however, CDK activity only reaches the threshold level for DNA replication and is kept below the mitosis threshold. This is achieved via (1) transcriptional downregulation of mitotic cyclins and CDKs, (2) proteolytic degradation of mitotic cyclins and (3) inhibition of CDK activity by CDK inhibitors such as the plant-specific SIAMESE-RELATED (SMR) proteins (De Veylder *et al.*, 2011). Whereas endoreduplication is involved in plant growth and development under physiological conditions, it is often affected by environmental (stress) conditions such as UV-B exposure, temperature and soil quality (Scholes and Paige, 2015).

A stress factor known to inhibit plant growth and development is cadmium (Cd). Cadmium pollution is a problem affecting many regions worldwide, as industrial and agricultural activities have caused substantial Cd releases into the environment (Vangronsveld and Clijsters, 1994; Jarup and Akesson, 2009). Although Cd is a non-essential element, it is readily taken up by plants through transport mechanisms for essential elements including calcium, iron and zinc (Benavides *et al.*, 2005; DalCorso *et al.*, 2008). Once present in cells, Cd indirectly induces oxidative stress, which is an imbalance between pro- and antioxidants in favor of the former (Cuypers *et al.*, 2010). As the levels of reactive oxygen species (ROS) increase, this results in a trade-off between signaling and damage to cellular macromolecules depending on the stress intensity (Foyer and Noctor, 2005; Cuypers *et al.*, 2016). When the stress is severe, ROS accumulation can lead to oxidative DNA damage (Gill and Tuteja, 2010), possibly affecting cell cycle regulation. Indeed, cell cycle checkpoints exist at the G_1/S and G_2/M transitions, delaying or arresting cell cycle progression and activating DNA repair systems in response to DNA damage (Yoshiyama *et al.*, 2013; Hu *et al.*, 2016). In order to prevent oxidative damage as a consequence of increased ROS

levels, plants have developed an extensive antioxidative defense system (Mittler, 2004; Cuypers *et al.*, 2012; Das and Roychoudhury, 2014).

Despite the fact that Cd exposure is well known to impair plant growth and development (Gallego *et al.*, 2012), its effects on the cell cycle are not well documented and have only been described in synchronized cell suspension cultures and root cells, in which Cd exposure negatively affects cell cycle progression (Sobkowiak and Deckert, 2004; Pena *et al.*, 2012; Zhao *et al.*, 2014). However, Fusconi *et al.* (2006) and Repetto *et al.* (2007) demonstrated that the DNA content in roots of Cd-exposed *Pisum sativum* was higher as compared to that of control plants. Similar results were reported by Cui *et al.* (2017) and Cao *et al.* (2018) in roots of Cd-exposed *A. thaliana* seedlings.

The current study aims to increase our insight into the effects of Cd exposure on growth and development, cell division and endoreduplication in developing leaves of *Arabidopsis thaliana* plants. Therefore, this study investigates (1) whether different leaf positions of the same leaf rosette harvested at one time point (*i.e.* leaf position), can be used as an alternative for leaf age when analyzing the effects of stress factors on cell cycle regulation and (2) to what extent and how Cd affects leaf growth and development.

2. Materials and methods

2.1. Plant material, growth conditions and Cd exposure

Wild-type (WT) *A. thaliana* seeds (ecotype Columbia) were surface-sterilized and grown in hydroponics using a modified Hoagland solution as described by Keunen *et al.* (2011). Growth conditions were set at a 12 h photoperiod, 22/18 °C day/night temperatures and 65 % relative

humidity. Light was provided by a combination of blue, red and far red Philips Green-Power LED-modules, simulating the photosynthetic active radiation (PAR) of sunlight. The PAR at the rosette level was 170 µmol m^{-2} s^{-1}. After three weeks, plants were either exposed to 5 µM $CdSO_4$ via the roots or further grown under control (= non-exposed) conditions. Rosette diameter and leaf number were kinetically monitored for approximately three weeks, starting from the day of exposure. Leaves 1 to 8 (with leaf 1 being the oldest leaf) or complete leaf rosettes were harvested after 72 h and 12 days of exposure. Unless otherwise stated, samples were snap-frozen in liquid nitrogen and stored at -80 °C until further analyses.

2.2. Quantification of Cd content

For the determination of leaf Cd concentrations, complete leaf rosettes were harvested and rinsed using distilled water. Subsequently, samples were oven-dried at 80 °C and digested in HNO_3 (70-71 %) in a heat block. Cadmium concentrations in the leaf extracts were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES 710, Agilent Technologies, Australia).

2.3. Flow cytometric analysis of nuclear ploidy levels

To determine the extent of endoreduplication, nuclear ploidy levels were determined in separate leaves. Leaf samples were chopped in 500 µL nuclei extraction buffer using a sharp razor blade and incubated for 1 min. The extract was filtered through a 50 µm nylon filter (CellTrics®, Sysmex Partec, Görlitz, Germany) and stained using 2 mL staining solution containing staining buffer, propidium iodide (PI) and RNase A. All products were part of the CyStain® PI Absolute

P kit (Sysmex Partec). Samples were incubated in darkness at 4 °C for at least 1 h. Subsequently, ploidy levels (2C, 4C, 8C, 16C and 32C, with C representing the haploid DNA content) of at least 5000 nuclei per sample were determined using a CyFlow® Cube 8 flow cytometer (Sysmex Partec). Nuclei were excited using a 488 nm laser and the forward scatter and PI fluorescence intensity (FL-2 channel; 580/30 nm) were measured. Data analysis was performed using FCS Express 4 software (De Novo Software, Glendale, CA, USA). The endoreduplication factor indicating the average number of endocycles per cell was calculated as follows: [(0 x % 2C) + (1 x % 4C) + (2 x % 8C) + (3 x % 16C) + (4 x % 32C)] / 100 (Cookson *et al.*, 2006; Boudolf *et al.*, 2009).

2.4. Microscopic analysis of cell surface area and number

To gain more insight into the extent of endoreduplication and cell division, a microscopic analysis was performed. Separate leaves were harvested and immediately scanned using a conventional flat-bed scanner. Subsequently, they were incubated overnight in 99 % ethanol to remove the chlorophyll. They were further cleared and stored for microscopic analysis in 90 % lactic acid. Abaxial epidermal cells were visualized using differential interference contrast (DIC) optics on a Leica DM2500 microscope equipped with a DFC 450C camera (Leica Microsystems, Wetzlar, Germany). Per leaf, two images were acquired at 25 % and 75 % from the leaf base, halfway between the midrib and the leaf margin (Fig. 1), which yields a reliable representation of the leaf as a whole (De Veylder *et al.*, 2001). The leaf surface area was determined based on the leaf contour using ImageJ software (version 1.48v, U.S. National Institutes of Health, Bethesda, MD, USA). The number of cells per image was determined using the ImageJ cell counter plugin. Subsequently, the average abaxial epidermal cell area was estimated via the ratio between the

image area and the number of cells in the image. The average abaxial epidermal cell area per leaf was calculated by taking the average of the values obtained in both images (Fig. 1). To estimate the number of abaxial epidermal cells per leaf, the ratio between leaf surface area and average cell surface area was determined.

2.5.Gene expression analysis

Leaf 1 (old), leaf 5 (young) and complete rosettes were used for the determination of gene expression levels. Frozen samples were ground using two stainless steel beads in the Retsch Mixer Mill MM 400 (Retsch, Haan, Germany). Subsequently, RNA was extracted using the RNaqueous™ Total RNA Isolation Kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Concentration and purity of the RNA samples were assessed using the NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific), while RNA integrity was confirmed via the Experion™ Automated Electrophoresis Station and the Experion™ RNA StdSens Analysis Kit (Bio-Rad, Hercules, CA, USA). Prior to cDNA synthesis, any residual genomic DNA was removed using the TURBO DNA-*free*™ Kit (Ambion, Thermo Fisher Scientific). An equal RNA input of 1 µg was used for each sample. After gDNA removal, reverse transcription was carried out using the PrimeScript™ RT Reagent Kit (Perfect Real Time, Takara Bio Inc., Kusatsu, Japan). The obtained cDNA was diluted tenfold in 1/10 TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20 °C.

Quantitative real-time PCR (qPCR) was performed using the 7500 Fast Real-Time PCR System and the Fast SYBR® Green Master Mix (Applied Biosystems, Thermo Fisher Scientific). Reactions contained 2 µL diluted cDNA sample, 5 µL 2x Fast SYBR® Green Master Mix, 2.4 µL RNase-free H_2O and forward and reverse primers (300 nM each, unless stated otherwise in

Supplemental Table S1) in a total reaction volume of 10 µL. Amplification occurred at universal cycling conditions (20 s at 95 °C, 40 cycles of 3 s at 95 °C and 30 s at 60 °C). A dissociation curve was generated to verify product specificity. Relative gene expression levels were determined via the $2^{-\Delta Cq}$ method and normalized against the expression of *RGS1-HXK1 INTERACTING PROTEIN 1* (*RHIP1*), *UBIQUITIN-CONJUGATING ENZYME 21* (*UBC21*), *ACTIN 2* (*ACT2*), *PENTATRICOPEPTIDE REPEAT* (*PPR*) and *MONESIN SENSITIVITY 1* (*MON1*) for individual leaves and *ACT2, MON1* and *YELLOW-LEAF-SPECIFIC GENE 8* (*YLS8*) for complete rosettes. Reference genes were selected based on the GrayNorm algorithm (Remans *et al.*, 2014). Forward and reverse primers (Supplemental Table S1) were designed in Primer3 and their specificity was verified *in silico* using BLAST (http://www.arabidopsis.org/Blast/index.jsp). Primer efficiencies were determined using a standard curve consisting of a two-fold dilution series of a pooled sample. Only primers with an efficiency between 90 and 110 % were used for qPCR analysis. Supplemental Table S2 shows the qPCR parameters according to the Minimum Information for publication of qPCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009) (Supplemental Table S2).

2.6. Hydrogen peroxide measurements

Hydrogen peroxide (H_2O_2) concentrations were determined in complete leaf rosettes using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Thermo Fisher Scientific). Frozen samples were ground using two stainless steel beads in the Retsch Mixer Mill MM 400 (Retsch). Subsequently, 500 µL 1X Reaction Buffer was added and samples were shaken continuously at room temperature for 30 min and then centrifuged at 12,000 x *g* for 5 min. In a 96-well plate, 95 µL of a working solution consisting of 100 µM Amplex™ Red and 0.2 U/mL

horseradish peroxidase was added to 5 µL supernatant. After 30 min incubation in the dark at 30 °C, samples were excited at 560 nm and resorufin fluorescence was measured at 590 nm in a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). A standard curve was generated with H_2O_2 concentrations ranging from 0 to 100 µM.

2.7. Statistical analysis

All statistical analyses were performed in R version 3.3.1 (R Foundation for Statistical Computing, 2016, Vienna, Austria). Normal distribution and homoscedasticity of the data were verified using the Shapiro-Wilk and Bartlett's test, respectively. In case these assumptions were not met, data were transformed (square root, inverse, exponent, logarithm). Gene expression data were standardly log-transformed. Depending on the number of variables, data were statistically analyzed using a student's t-test or two-way ANOVA followed by a post-hoc Tukey-Kramer test to correct for multiple comparisons. If the (transformed) data did not meet the normality assumption, a non-parametric Kruskall-Wallis test was used, followed by the Wilcoxon rank sum test. Outliers were determined using the Extreme Studentized Deviate method (GraphPad Software, La Jolla, CA, USA) at significance level 0.05.

3. Results and discussion

3.1. Leaf position as an alternative for leaf age in cell cycle studies

As the cell cycle is an important process involved in leaf growth and development (Beemster *et al.*, 2005a; 2006), cell cycle-related parameters (*e.g.* cell size and number, nuclear DNA content and expression of cell cycle genes) strongly depend on the developmental stage of a leaf. Therefore, leaf age should be taken into account when investigating cell division,

endoreduplication and their underlying molecular mechanisms. To this end, analyses can be performed in leaves at a specific position within the rosette (*e.g.* the first true leaf pair), harvested at different time points during leaf development (Beemster *et al.*, 2005a; 2006). However, when evaluating the effects of exposure to stress factors, this can be challenging and time consuming. As a single rosette is composed of young and old leaves, an alternative strategy to investigate leaf age-dependent effects of stress factors is to sample leaves at different positions within one rosette at one time point. The goal of the first part of this study was to investigate whether leaves at different positions harvested at the same time point (*i.e.* leaf position) could be used as a developmental series to deduce the development of a single leaf over time (*i.e.* leaf age) when investigating cell cycle-related parameters. To this end, *A. thaliana* plants were hydroponically grown under control conditions. The extent of endoreduplication was determined in (1) the first true leaf pair harvested daily between 11 and 21 DAS (Fig. 2A-B) and (2) the eight oldest leaves of one rosette harvested at 21 DAS (Fig. 2C-D).

At 11 DAS, leaves 1 and 2 almost exclusively consisted of cells with 2C and 4C DNA contents, characteristic for actively dividing cells. As a consequence of endoreduplication, the percentage of nuclei with a 2C DNA content in the first true leaf pair decreased from day 11 to day 21 after sowing (Fig. 2A – light blue bars). The relative number of 4C and 8C nuclei reached a maximum at 12 and 16 DAS respectively, and subsequently decreased over time (Fig. 2A – orange and yellow bars). Nuclei with a 16C and 32C DNA content only appeared at 12 and 16 DAS respectively, after which their levels steeply increased over time (Fig. 2A – green and dark blue bars). Graphs showing the percentage of nuclei corresponding to each ploidy level separately are provided in the Supplemental information (Supplemental Fig. S1 and Fig. S2). In general, a decrease of the percentage of nuclei with a specific DNA content coincided with an increase in

the percentage of nuclei with a double DNA content as compared to the former, indicating the progression of cells through consecutive endocycles (Galbraith *et al.*, 1991; Beemster *et al.*, 2005a; Blomme *et al.*, 2014). As a consequence, the endoreduplication factor – representing the average number of endocycles that cells have undergone – increased over time ($P < 2.2 \times 10^{-16}$) from 0.5 at 11 DAS to approximately 1.6 at 21 DAS (Fig. 2B). Similar results showing a correlation between nuclear DNA content and leaf age were observed when comparing leaves 1 to 8 of the same rosette after 21 days of growth ($P = 0.001$; Fig. 2C). The endoreduplication factor increased from approximately 0.4 in leaf 8 (the youngest leaf analyzed) to slightly over 1.6 in the first true leaf pair (Fig. 2D). These values are highly similar to those obtained in the first true leaf pair harvested daily between 11 and 21 DAS, providing a first indication that leaf position can be used as a proxy for leaf age when analyzing the cell cycle.

Subsequently, this hypothesis was further investigated at the molecular level. To this end, the expression of several genes involved in cell cycle regulation was compared between a young (leaf 5) and an old leaf (leaf 1) of the same rosette harvested at 21 DAS. The expression of cyclins and CDKs regulating the G_1/S and G_2/M transitions (Menges *et al.*, 2005) was significantly lower in leaf 1 as compared to leaf 5 (Table 1). However, the expression of G_2/M-related genes differed more strongly between both leaf positions than those of genes involved in G_1/S phase regulation (Table 1), possibly related to the occurrence of endoreduplication observed in older leaves (Fig. 2). Transcript levels of *CDKA;1* and *E2F TRANSCRIPTION FACTOR3 (E2Fa)*, expressed during all cell cycle phases (Menges *et al.*, 2005) were not affected by leaf position (Table 1). Interestingly, these results strongly correspond to those reported by Beemster *et al.* (2005a), who kinetically monitored the expression of cell cycle genes in the first true leaf pair of *A. thaliana* plants. Indeed, the authors demonstrated that the expression of *CYCA3;1*,

CYCA3;1, *CYCA2;3*, *CYCB1;1*, *CYCD3;1* and *CDKB2;1* decreased throughout leaf development, whereas *CDKA;1* and *E2Fa* were constitutively expressed (Beemster *et al.*, 2005a).

Taken together, these results indicate that leaf position can be used as an alternative for leaf age when analyzing the cell cycle at both the cellular and molecular level. The use of different leaf positions has been frequently described in experimental set-ups for the analysis of leaf senescence (Zentgraf *et al.*, 2004; Li *et al.*, 2017; Bresson *et al.*, 2018). This method offers several advantages, especially when investigating the effects of exposure to external stress factors. Firstly, it eliminates the need to daily expose plants to the stress factor of interest in experiments investigating the effects of specific exposure durations. Furthermore, it reduces the number of plants needed for analyses and minimizes experimental variation, as all leaf positions are obtained from the same plant. Therefore, the experiments described in the next paragraphs, investigating Cd-induced effects on the cell cycle, were performed in leaves at different positions within the rosette, harvested at one time point.

3.2. Cadmium exposure inhibits leaf growth and development

Although Cd exposure is well recognized to affect vegetative growth of *A. thaliana* plants (Wojcik and Tukiendorf, 2004; Weber *et al.*, 2006; Van Belleghem *et al.*, 2007; Keunen *et al.*, 2011), data regarding its effect on the cell cycle in leaves are scarce. Therefore, the second part of this study aimed to improve insight into the effects of short- and long-term Cd exposure on cell division, endoreduplication and cell cycle regulation in leaves of *A. thaliana* plants. To this end, plants were exposed to 5 µM $CdSO_4$ after three weeks of growth under control conditions. Analyses were performed in leaves at different positions within one rosette, harvested after 72 h

or 12 days of Cd exposure. Representative pictures of leaf rosettes of control and Cd-exposed plants are provided in the Supplemental information (Supplemental Fig. S3).

As shown in Fig. 3A, Cd significantly inhibited rosette growth. The rosette diameter of non-exposed control plants increased steeply over time, approaching a maximal value of approximately 80 mm at about 42 DAS. In contrast, the rosette diameter of Cd-exposed plants did not increase anymore after day 39, reaching a maximum of only 45 mm (Fig. 3A). The number of leaves was similar for control and Cd-exposed plants at all time points studied (Fig. 3B). These results are in accordance to those of Keunen *et al.* (2011), who also reported a significant Cd-induced reduction of rosette diameter and a much smaller effect on leaf number. As shown in Fig. 3C, leaf Cd concentrations did not differ between plants exposed to Cd for 72 h or 12 days, suggesting that the Cd concentration in leaves reaches a maximum shortly after start of the exposure.

3.3. Short-term cadmium exposure disturbs cell cycle progression

The inhibition of rosette growth after 72 h of Cd exposure was due to a significant negative effect on the surface area of all rosette leaves analyzed (Fig. 4A). The final size of a leaf is determined by both cell number and size (Beemster *et al.*, 2006; Cookson *et al.*, 2006; Braidwood *et al.*, 2014; Orr-Weaver, 2015). Therefore, the effects of Cd on both parameters were investigated by DIC microscopic analyses of the abaxial epidermis. The abaxial pavement cell number in leaves 1 to 5 was significantly lower after 72 h of Cd exposure, demonstrating that Cd inhibited cell division. The number of cells within leaves of non-exposed control and Cd-exposed plants differed more for leaves 4 and 5 as compared to older leaves (Fig. 4B). This is likely related to the higher cell division activity in younger leaves at the start of Cd exposure (Rodriguez *et al.*,

2014). Furthermore, the abaxial pavement cell area of leaf 5 was significantly lower after 72 h of Cd exposure, indicating that cell expansion was also inhibited (Fig. 4C). A similar trend was observed for the other leaf positions analyzed. These results show that relatively small differences in cell surface area and cell number between control and Cd-exposed leaves together result in a significantly smaller leaf surface area of Cd-exposed plants. As *Arabidopsis* epidermal cell size is often correlated to the extent of endoreduplication (Melaragno *et al.*, 1993; Sugimoto-Shirasu and Roberts, 2003; Breuer *et al.*, 2010), nuclear ploidy levels were determined using flow cytometry and compared between leaves of control and Cd-exposed plants. The nuclear DNA content of the first true leaf pair was significantly lower after 72 h of Cd exposure, as indicated by a lower endoreduplication factor, but this effect was not observed in younger leaves (Fig. 5A). This suggests that Cd primarily affects the exit from endoreduplication and not the endoreduplication rate. When comparing data from microscopic analysis with flow cytometry data, it should be kept in mind that the microscopic analysis only focuses on two regions in the abaxial epidermis, whereas the data obtained using flow cytometry are representative for the entire leaf. Therefore, it is essential to evaluate whether the effect of Cd exposure on the nuclear DNA content of the entire leaf is correlated to the microscopic effects on the abaxial epidermal cells only.

3.4. Concentration of nuclei measured via flow cytometry: a proxy for effects on cell division

In addition to the nuclear DNA content, the concentration of nuclei present in the leaf extracts was also determined using flow cytometry. This parameter was significantly lower after 72 h of Cd exposure (Fig. 5B). Furthermore, it very strongly correlated to the abaxial epidermal cell numbers determined using DIC microscopy (Fig. 5C). To the best of our knowledge, such linear

relationship has not been described before. The strong correlation between both parameters indicates that the nuclear isolation efficiency is highly similar between different leaf positions, despite differences in their size and structure. We therefore propose that the concentration of nuclei determined via flow cytometry can be used as a proxy to assess the effects of different stress factors on the extent of cell division in *A. thaliana* leaves. The use of this parameter has two important advantages as compared to the determination of cell numbers via microscopic analysis. Firstly, it is much faster, as microscopic cell measurements are generally time consuming. Furthermore, it is representative for the entire leaf, in contrast to microscopic analyses, which typically focus on two regions in one specific cell layer within the leaf. For the proxy to be reliable, care should be taken to ensure that all samples are handled in exactly the same way when performing flow cytometric analyses. This means that the volumes of extraction buffer and staining solution, the extent of chopping and the incubation time should be identical for all samples.

3.5. Effects of cadmium exposure on cell cycle progression increase over time

As Cd-induced effects on the rosette diameter increased over time (Fig. 3A), analyses were also performed after an extended exposure duration of 12 days. Again, the surface area of leaves 3 to 6 was significantly lower in Cd-exposed as compared to non-exposed control plants (Fig. 6A). The surface area of the first true leaf pair could not be determined anymore, as they had senesced. Whereas the effects of short-term Cd exposure on the cellular parameters analyzed were rather limited, the differences between control and Cd-exposed leaves became more pronounced after long-term exposure. Although Cd did not change the nuclear DNA content of most leaves after 72 h of exposure (Fig. 5A), the endoreduplication factor of all leaves was significantly lower after

12 days of exposure (Fig. 6B), indicating a time-dependent effect of Cd on the nuclear DNA content of leaves. Whereas the endoreduplication factor augmented with increasing leaf age in the plants analyzed after 72 h of exposure (Fig. 6A), it was highly similar in all leaves of plants examined after 12 days of exposure (Fig. 6B). These data indicate that most of the leaves studied were fully expanded at this stage. As mentioned before, the maximal endoreduplication factor reached in leaves of non-exposed control plants was 1.6 (Fig. 2). Leaves of Cd-exposed plants only reached an endoreduplication factor of 1.4 (Fig. 6B), suggesting a Cd-mediated reduction of the maximal ploidy level that can be reached.

To estimate the effect of prolonged Cd exposure on the number of cells, the concentration of nuclei measured using flow cytometry was compared between leaves of non-exposed control and Cd-exposed plants. This parameter was significantly reduced upon 12 days of Cd exposure in the three youngest leaves analyzed (Fig. 6B), indicating a decreased extent of cell division.

Taken together, our results indicate a negative impact of Cd exposure on the cell cycle, inhibiting or delaying both cell division and endoreduplication at different leaf positions of *A. thaliana*. These data correspond to those reported in literature in cell suspension cultures and root tissue. Sobkowiak and Deckert (2004) reported that exposure of *Glycine max* cell suspension cultures to 10 µM Cd caused an earlier entry of cells into the S phase and decreased the rate of DNA synthesis. Furthermore, transcript levels of the mitotic cyclin *CYCB1* were lower in Cd-exposed cells as compared to control cells, whereas *CDKA* expression was not affected (Sobkowiak and Deckert, 2004), consistent with the view that *CDKA* expression is constitutive and its activity is post-translationally regulated (Inzé and De Veylder, 2006). In addition, exposure to 10 µM Cd inhibited root growth of *Triticum aestivum* seedlings, reducing the number of cells in the proliferation zone of the root apex. It has been suggested that Cd-induced ROS production causes

oxidative post-translational modifications of cell cycle-related proteins, thereby affecting G_1/S transitions and blocking cell cycle progression (Pena *et al.*, 2012). Similarly, Cd significantly reduced cell length and number in the elongation zone of primary root tips of *Oryza sativa* seedlings. Furthermore, the expression of several genes encoding cyclins and CDKs was affected by Cd as well (Zhao *et al.*, 2014). These data illustrate that cell cycle proteins are regulated at the transcriptional as well as the post-translational level, indicating that studies at both regulatory levels can provide insight into Cd-induced effects on cell cycle progression.

3.6. Are cadmium-induced effects on the cell cycle modulated by ROS-induced DNA damage?

To gain more insight into the molecular mechanisms underlying Cd-induced effects on cell cycle regulation, gene expression analysis was performed on young (leaf 5) and old (leaf 1) leaves of plants exposed to 5 µM Cd for 72 h and compared to non-exposed control plants (Table 2). Cadmium exposure did not affect the expression of *CDKA;1*. In contrast, transcript levels of *HISTONE H4* (*HIS4*) and *CYCA3;1*, known to accumulate during the G_1 and S phases (Menges *et al.*, 2005), were significantly increased upon Cd exposure in leaf 1 and showed a similar trend in leaf 5. However, expression of *CYCA3;2*, also considered to be a G_1/S marker (Menges *et al.*, 2005), was not affected by Cd (Table 2). Exposure to Cd significantly increased the expression of the mitotic cyclin *CYCB1;1* in both young and old leaves, with the induction being more pronounced in the latter (Table 2). These results seem to contradict the observed cell cycle inhibition in leaves of Cd-exposed plants (Fig. 5 and 6). Therefore, we hypothesize that the transcription of cell cycle-related genes is increased in an attempt to compensate for the decreased cell cycle activity in Cd-exposed plants.

In addition to genes encoding cyclins and CDKs, the expression of other cell cycle regulators was also assessed. Expression of the transcription factor *DP-E2F-LIKE1 (DEL1)*, repressing the expression of several cell cycle regulators (De Veylder *et al.*, 2011), showed an increasing trend in both young and old leaves of Cd-exposed plants (Table 2). Since DEL1 is considered as a negative regulator of endoreduplication, the increased *DEL1* transcript levels can possibly explain the lower nuclear DNA content observed in leaves of Cd-exposed as compared to non-exposed control plants. Furthermore, expression levels of the CDK inhibitors *SMR4*, *SMR5* and *SMR7* were higher in leaves of Cd-exposed plants, with *SMR7* inductions of approximately 30-fold in both young and old leaves (Table 2). Interestingly, Yi *et al.* (2014) reported that expression of these genes increased in response to ROS-induced DNA damage. When increased ROS levels result in the production of double-strand DNA breaks (DSBs), the cell cycle checkpoint kinase ATAXIA-TELANGIECTASIA MUTATED (ATM) is activated, subsequently phosphorylating and activating the transcription factor SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1). This protein is often referred to as "the plant p53", as it regulates the expression of a plethora of genes involved in DNA repair, apoptosis and cell cycle regulation including *SMR5* and *SMR7* (Yi *et al.*, 2014; Yoshiyama, 2015).

As proteins of the SMR family are known inhibitors of cell cycle progression, we hypothesized that the observed effects of Cd exposure on the cell cycle were mediated by a ROS-induced activation of the above-mentioned ATM-SOG1 pathway. To investigate this hypothesis, the Cd-induced upregulation of *SMR4*, *SMR5* and *SMR7* observed in individual leaves (Table 2) was first confirmed in complete leaf rosettes in an independent experiment (Table 3). Subsequently, the extent of Cd-induced oxidative stress was assessed by analyzing transcript levels of five oxidative stress hallmark genes (Gadjev *et al.*, 2006). As shown in Table 3, their expression was

significantly increased upon Cd exposure. Similar results were reported by Keunen *et al.* (2015) in leaves of *A. thaliana* plants exposed to 5 µM Cd for 24 and 72 h. In addition, Cd exposure significantly induced the expression of three *RESPIRATORY BURST OXIDASE HOMOLOG* (*RBOH*) isoforms (Table 3), encoding NADPH oxidases involved in superoxide production (Remans *et al.*, 2010). These results strongly suggest that Cd exposure increases ROS production in *A. thaliana* rosettes. To further confirm this Cd-induced ROS production, H_2O_2 levels were compared between leaf rosettes of Cd-exposed and control plants. The results show that leaf H_2O_2 concentrations were increased almost 3-fold after 72 h of Cd exposure (Fig. 7). Taken together, these results indicate that Cd exposure significantly increases ROS production in *A. thaliana* leaves, thereby possibly inducing DNA damage and activating the ATM-SOG1 pathway.

To investigate whether Cd evoked DNA damage in the experimental set-up used, the expression of several DNA damage markers and DNA repair genes was compared between leaf rosettes of Cd-exposed and control plants after 72 h of exposure. Our results show that Cd exposure significantly increased the expression of *POLY(ADP-RIBOSE) POLYMERASE1 (PARP1)*, *PARP2* and *BREAST CANCER SUSCEPTIBILITY1* (*BRCA1)* (Table 3), all of which are considered as marker genes for DNA damage (Mannuss *et al.*, 2012). Furthermore, Cd induced the expression of *HOMOLOG OF X-RAY REPAIR COMPLEMENTING 1 (XRCC1)* – involved in the base excision repair (BER) pathway – and *LIGASE IV* (*LIG4*) and *DNA REPAIR PROTEIN RAD51 HOMOLOG 1* (*RAD51*), both involved in DSB repair (Manova and Gruszka, 2015; Spampinato, 2017). These data suggest that Cd exposure causes DNA damage in *A. thaliana* leaves, thereby possibly activating the ATM-SOG1 pathway, resulting in the observed cell cycle arrest. Cadmium-induced DNA damage was also observed in other studies. Monteiro *et al.*

(2012) reported that exposure of *Lactuca sativa* to 1 and 10 µM Cd significantly increased the % tail DNA as measured by the comet assay in roots and leaves. In addition, exposure to 25 µM Cd induced chromosome alterations and DNA damage in meristematic cells of *Allium cepa* and *L. sativa* root tips (Silveira *et al.*, 2017). Similarly, exposure of *Vicia faba* plants to 5 and 10 µM Cd increased the percentage of DNA damage in leaves in a concentration-dependent manner. Moreover, this coincided with an elevated level of oxidative stress as indicated by increased lipid peroxidation and decreased catalase and peroxidase activities after Cd exposure (Lin *et al.*, 2007). Recently, Cui *et al.* (2017) reported that exposure to different Cd concentrations induced cell cycle arrest in *A. thaliana* root tips. This response was also accompanied by the induction of DNA damage, as indicated by changes in the expression of genes involved in the DNA damage response (Cui *et al.*, 2017).

Taken together, our data lead to the hypothesis that the inhibitory effects of Cd exposure on the cell cycle in *A. thaliana* leaves are (at least partially) due to the induction of oxidative stress and subsequent DNA damage. Presumably via the ATM-SOG1 pathway, this DNA damage causes a strong upregulation of the CDK inhibitors *SMR5* and *SMR7*. They can subsequently reduce CDKA activity, thereby inhibiting cell cycle progression to prevent the replication of damaged DNA or its transmission by cell division. Blocking cell cycle progression also allows cells to repair their damaged DNA by specialized DNA repair mechanisms (Yoshiyama, 2015; Hu *et al.*, 2016). Using a reverse genetics approach, the involvement of SOG1 and SMRs in the Cd-induced inhibition of cell cycle progression will be unraveled in future studies.

4. Conclusion

Three main conclusions can be drawn from this study. Firstly, our results show that leaf position can be used as an alternative for leaf age when investigating the effects of stress factors on the cell cycle in *A. thaliana*. Secondly, we have shown that the concentration of nuclei measured using flow cytometry can be used as a proxy to investigate the effects of stress factors on the extent of cell division in leaves of *A. thaliana*. Finally, the results obtained in this study demonstrate that Cd exposure significantly affects cell cycle progression, inhibiting both cell division and endoreduplication in different leaf positions of *A. thaliana* plants. This response is already present after short-term exposure (72 h) and becomes more pronounced after extended exposure durations (12 days). We hypothesize that the observed inhibition of cell cycle progression is a consequence of Cd-induced oxidative stress and subsequent DNA damage, triggering the ATM-SOG1 pathway, which induces the expression of CDK inhibitors of the SMR family.

Appendix A. Supplementary data

Supplementary data consist of the following. Fig. S1: Kinetic analysis of the nuclear DNA content (%) in the first true leaf pair. Fig. S2: Nuclear DNA content (%) in the six oldest rosette leaves of three-weeks-old *Arabidopsis thaliana* plants. Fig. S3: Representative pictures of leaf rosettes of control and Cd-exposed plants. Table S1: List of primers used for reverse transcription-quantitative PCR. Table S2: Reverse transcription-quantitative PCR parameters according to the MIQE guidelines.

Author contributions

All authors participated in the conception and design of the study. SH performed the experiments and wrote the manuscript. AM assisted in conducting the experiments. EK, GB, JV and AC assisted in writing the manuscript. All authors read and approved the final manuscript after critical revision.

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Declarations of interest

None

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Figure captions

Fig. 1. For differential interference contrast (DIC) microscopic analysis of cell surface area and cell number, two images of the abaxial epidermis were acquired at 25 % and 75 % from the leaf base, halfway between the midrib and the leaf margin.

Fig. 2. Kinetic analysis of (A) nuclear DNA content (%) and (B) endoreduplication factor in the first true leaf pair of *Arabidopsis thaliana* plants grown in hydroponics under control conditions. (C) Nuclear DNA content (%) and (D) endoreduplication factor in the eight oldest rosette leaves of three-weeks-old *Arabidopsis thaliana* plants grown in hydroponics under control conditions. Values represent the average ± S.E. of four biological replicates.

Fig. 3. Leaf growth parameters of hydroponically grown *Arabidopsis thaliana* plants exposed to 0 (black circles) or 5 µM $CdSO_4$ (grey circles). Kinetic analysis of (A) rosette diameter (mm) and (B) number of rosette leaves. Values represent the average ± S.E. of 15 biological replicates. In Fig. A, values obtained from 23 DAS on significantly differed between both exposure groups (indicated with an arrow and asterisk) (Student's t-test; $P < 0.05$). (C) Cadmium concentrations (mg kg^{-1} dry weight) in leaf rosettes of hydroponically grown *Arabidopsis thaliana* plants exposed to 0 or 5 µM $CdSO_4$ for 72 h and 12 days. Values represent the average ± S.E. of 4 biological replicates.

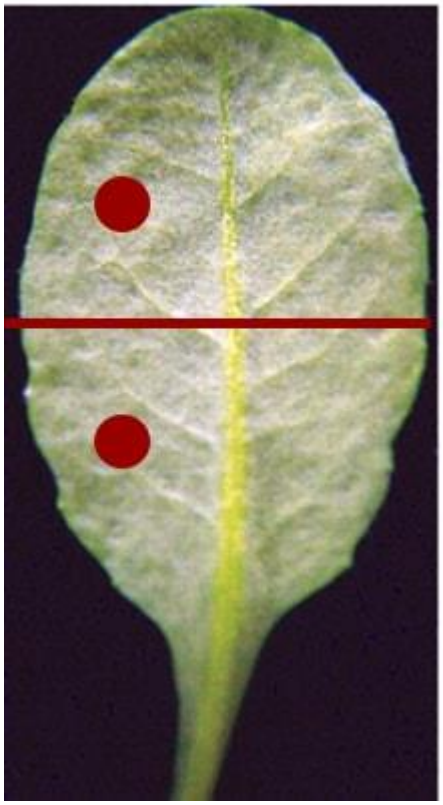
Fig. 4. (A) Leaf surface area (mm^2); (B) abaxial pavement cell number and (C) abaxial pavement cell surface area (μm^2) of the six oldest rosette leaves of *Arabidopsis thaliana* plants grown in hydroponics and exposed to 0 (white bars) or 5 µM $CdSO_4$ (grey bars) for 72 h. Values represent

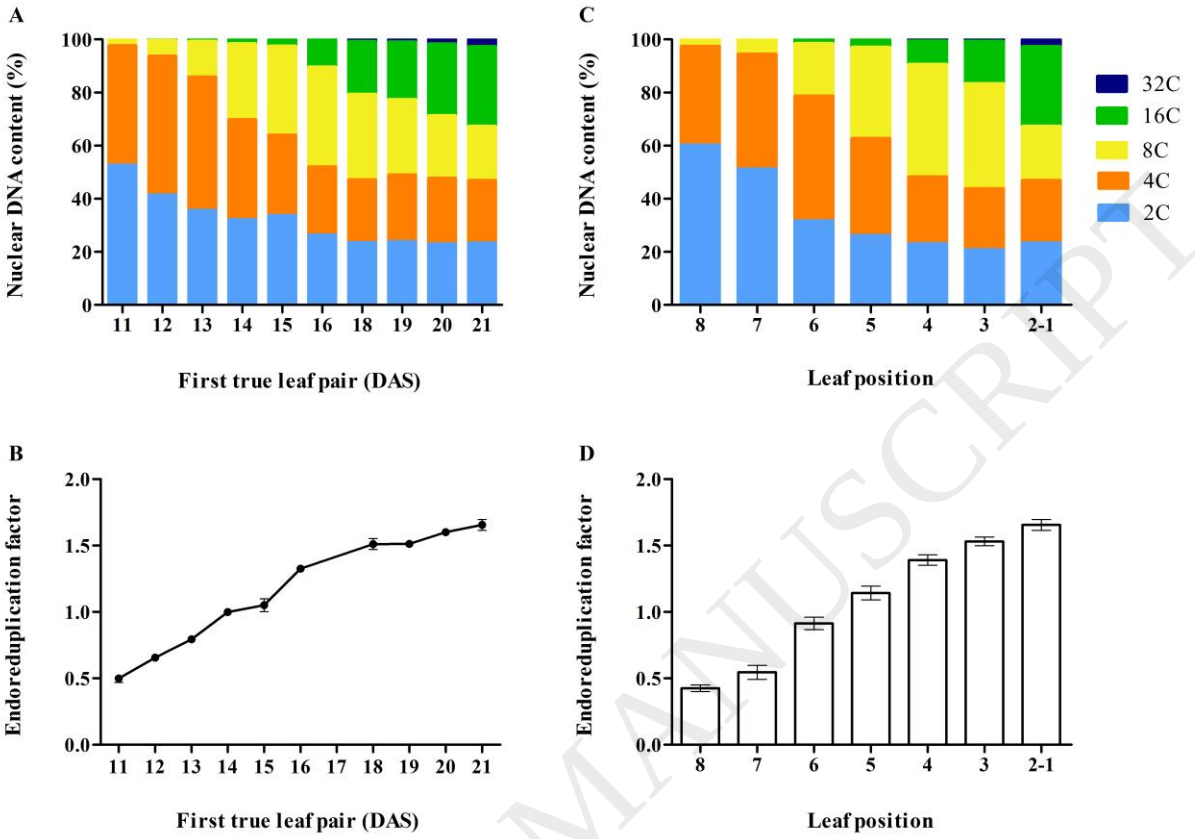
the average of eight biological replicates. Asterisks (*) indicate significant differences between the same leaf position sampled from control and Cd-exposed plants (Student's t-test; $P < 0.05$).

Fig. 5. (A) Endoreduplication factor and (B) concentration of nuclei measured using flow cytometry of the six oldest rosette leaves of *Arabidopsis thaliana* plants grown in hydroponics and exposed to 0 (white bars) or 5 µM $CdSO_4$ (grey bars) for 72 hours. Values represent the average of eight biological replicates. Asterisks (*) indicate significant differences between the same leaf position sampled from control and Cd-exposed plants (Student's t-test; $P < 0.05$). (C) Correlation between the abaxial pavement cell number determined using microscopy (on the x axis) and the concentration of nuclei measured using flow cytometry (on the y axis). Each data point represents the average value of one experimental group (leaf position and exposure condition).

Fig. 6. (A) Leaf surface area (mm^2); (B) endoreduplication factor and (C) concentration of nuclei measured using flow cytometry of the sixth to third oldest rosette leaves of *Arabidopsis thaliana* plants grown in hydroponics and exposed to 0 (white bars) or 5 µM $CdSO_4$ (grey bars) for 12 days. Values represent the average of four biological replicates. Asterisks (*) indicate significant differences between the same leaf position sampled from control and Cd-exposed plants (Student's t-test; $P < 0.05$).

Fig. 7. Relative hydrogen peroxide (H_2O_2) concentrations in leaf rosettes of hydroponically grown *Arabidopsis thaliana* plants exposed to 0 or 5 µM $CdSO_4$ for 72 h. Values represent the average ± S.E. of five biological replicates. The asterisk (*) indicates a significant difference between rosettes of control and Cd-exposed plants (Student's t-test; $P < 0.05$).





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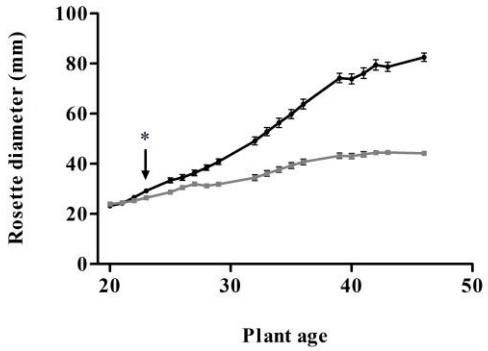

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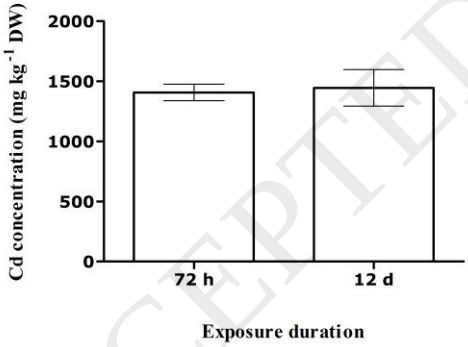

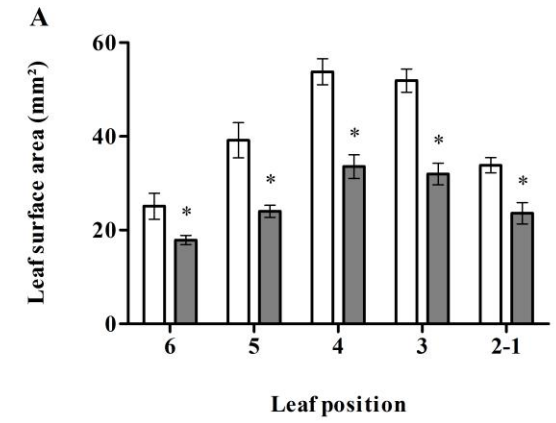

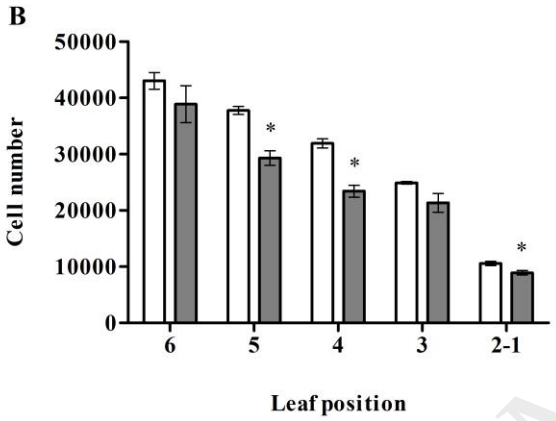

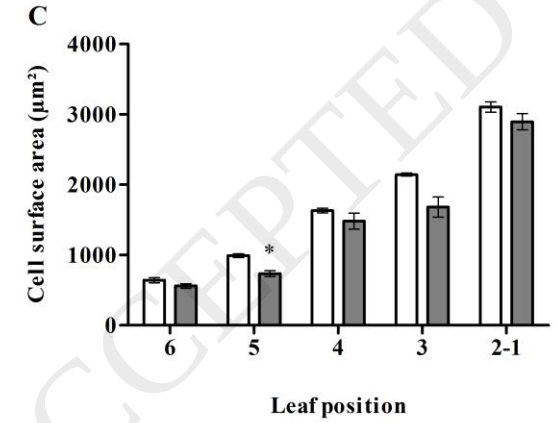

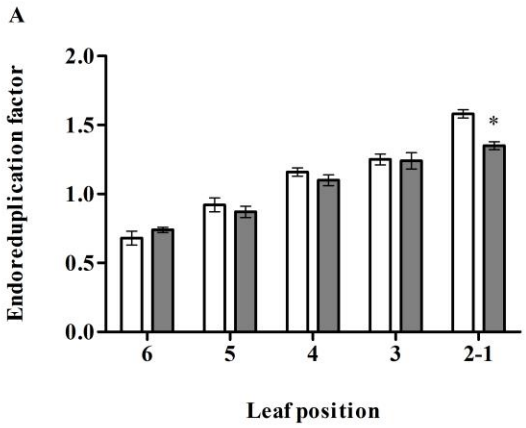

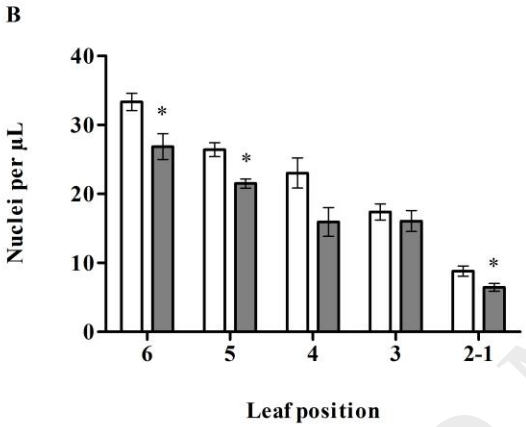

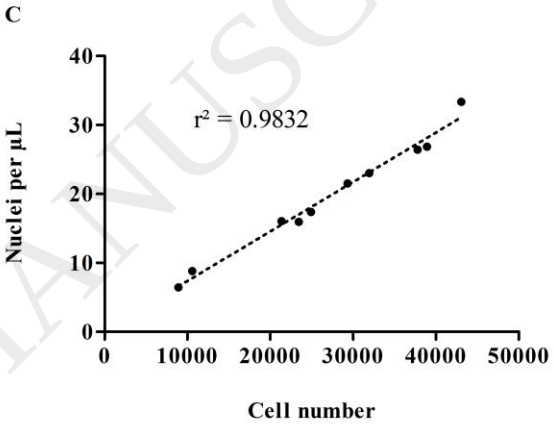

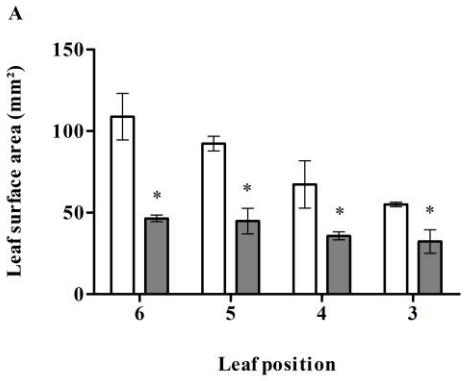

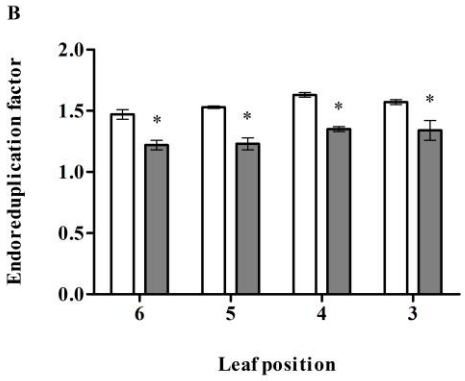

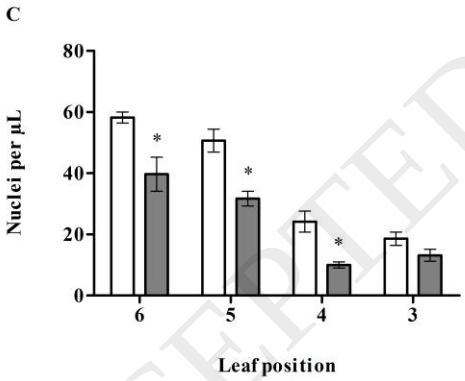

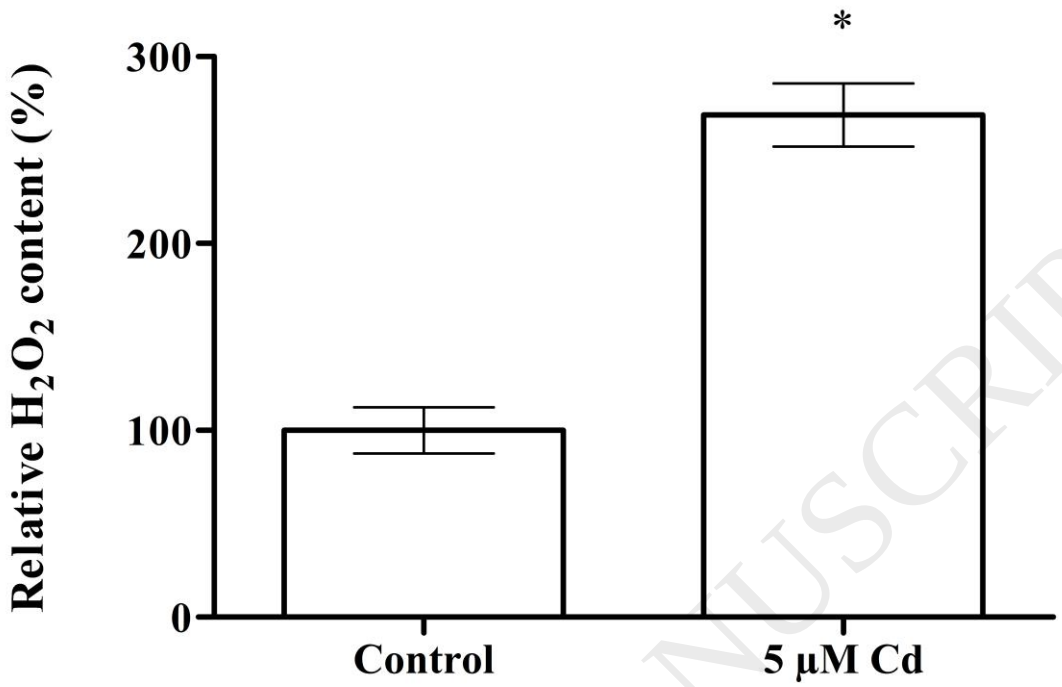

Tables

Table 1. Expression levels of cell cycle-related genes in young (leaf 5) and old (leaf 1) leaves of *Arabidopsis thaliana* plants grown in hydroponics under control conditions. Values represent the average ± S.E. of four biological replicates. Red colors indicate a significantly lower expression in the older leaf as compared to the young leaf for both normalized and non-normalized data (Student's t-test; $P < 0.05$). *CDK*: cyclin-dependent kinase; *CYC*: cyclin; *E2Fa*: E2F transcription factor 3; *HIS4*: histone H4.

	GENE	LEAF 5	LEAF 1	RATIO 1/5
General	*CKDA;1*	0.95 ± 0.03	1.07 ± 0.12	1.13
	E2Fa	1.37 ± 0.15	1.22 ± 0.10	0.89
G_1/S phase	*HIS4*	0.96 ± 0.04	0.36 ± 0.03	0.37
	CYCA3;1	1.05 ± 0.05	0.55 ± 0.05	0.52
	CYCA3;2	1.32 ± 0.08	0.72 ± 0.06	0.54
G_2/M phase	*CDKB1;1*	0.45 ± 0.04	0.11 ± 0.03	0.25
	CDKB2;1	0.53 ± 0.02	0.18 ± 0.03	0.34
	CYCA2;3	1.04 ± 0.11	0.05 ± 0.01	0.05
	CYCB1;1	0.30 ± 0.04	0.07 ± 0.00	0.24
	CYCD3;1	0.70 ± 0.05	0.10 ± 0.02	0.15

Table 2. Gene expression levels in young (leaf 5) and old (leaf 1) leaves of *Arabidopsis thaliana* plants grown in hydroponics and exposed to 5 µM $CdSO_4$ for 72 h. Values represent the average ± S.E. of four biological replicates and are expressed relative to the average of the same leaf under control conditions (set at 1.00). Significant Cd-induced upregulations for both normalized and non-normalized data are highlighted in green (2-way ANOVA; $P < 0.05$). Asterisks (*) indicate a significantly different Cd-induced response between young and old leaves for both normalized and non-normalized data (2-way ANOVA; $P < 0.05$). *CDK*: cyclin-dependent kinase; *CYC*: cyclin; *DEL1*: DP-E2F-like; *E2Fa*: E2F transcription factor 3; HIS4: histone H4; *SMR*: SIAMESE-related.

	GENE	LEAF 5	LEAF 1
Cell cycle - General	*CDKA;1*	1.25 ± 0.03	1.00 ± 0.02
	E2Fa	1.04 ± 0.06	0.70 ± 0.09
Cell cycle - G_1/S phase	*HIS4*	1.61 ± 0.12	1.94 ± 0.32
	CYCA3;1	1.54 ± 0.09	1.54 ± 0.16
	CYCA3;2	1.20 ± 0.10	1.22 ± 0.12
Cell cycle - G_2/M phase	*CDKB1;1*	1.30 ± 0.11	1.44 ± 0.32
	CDKB2;1	1.14 ± 0.02	1.20 ± 0.06
	CYCA2;3	0.97 ± 0.10	1.10 ± 0.17
	CYCB1;1	1.81 ± 0.22	2.87 ± 0.32 *
	CYCD3;1	1.40 ± 0.09	1.01 ± 0.20
Cell cycle - Other regulators	*SMR4*	3.04 ± 0.31	2.34 ± 0.76
	SMR5	6.15 ± 1.41	1.91 ± 0.30 *
	SMR7	31.48 ± 12.14	27.97 ± 7.16
	DEL1	1.64 ± 0.04	2.04 ± 0.24

Table 3. Gene expression levels in leaf rosettes of *Arabidopsis thaliana* plants grown in hydroponics and exposed to 0 or 5 µM $CdSO_4$ for 72 h. Values represent the average ± S.E. of five biological replicates. Significant Cd-induced upregulations for both normalized and non-normalized data are highlighted in green (Student's t-test; $P < 0.05$). *BRCA1*: breast cancer susceptibility 1; *LIG4*: DNA ligase IV; PARP: poly(ADP-ribose) polymerase; *RAD51*: DNA repair protein RAD51 homolog 1; *RBOH*: respiratory burst oxidase homolog; *SMR*: SIAMESE-related; *TI1*: trypsin inhibitor 1; *TIR*: toll/interleukin receptor 1; *UPOX*: upregulated by oxidative stress; *XRCC1*: homolog of X-ray repair cross complementing 1.

	GENE	**CONTROL**	**5 µM Cd**
Cell cycle regulators	*SMR4*	1.00 ± 0.09	2.74 ± 0.37
	SMR5	1.00 ± 0.05	8.38 ± 2.11
	SMR7	1.00 ± 0.09	45.78 ± 12.23
Oxidative stress	*UPOX*	1.00 ± 0.04	6.57 ± 1.76
	Defensin-like	1.00 ± 0.17	5.61 ± 0.53
	AT1G19020	1.00 ± 0.46	2.63 ± 0.29
	AT1G05340	1.00 ± 0.24	9.95 ± 1.91
	TIR-class	1.00 ± 0.46	2.32 ± 0.28
	RBOHC	1.00 ± 0.37	8.53 ± 2.77
	RBOHD	1.00 ± 0.10	2.25 ± 0.13
	RBOHF	1.00 ± 0.08	3.19 ± 0.37
DNA damage	*PARP1*	1.00 ± 0.03	1.77 ± 0.20
	PARP2	1.00 ± 0.02	11.29 ± 2.35
	BRCA1	1.00 ± 0.06	4.73 ± 0.78
	XRCC1	1.00 ± 0.09	1.65 ± 0.06
	LIG4	1.00 ± 0.05	1.71 ± 0.04
	RAD51	1.00 ± 0.08	3.75 ± 0.63