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Maastricht University

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Master of Biomedical Sciences

Masterthesis

The involvement of SIAMESE-RELATED proteins and SUPPRESSOR OF GAMMA RESPONSE 1 in cadmium-induced stress responses in Arabidopsis thaliana leaves

Verena Iven

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :

Prof. dr. Ann CUYPERS

MENTOR :

Mevrouw Sophie HENDRIX

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LIST OF ABBREVIATIONS

A. thaliana	*Arabidopsis thaliana*
Cd	Cadmium
CDK	Cyclin-dependent kinase
$CdSO_4$	Cadmium sulphate
DAS	Days after sowing
DW	Dry weight
EF	Endoreduplication factor
FW	Fresh weight
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HU	Hyroxyurea
$O_2^{\bullet -}$	Superoxide
ROS	Reactive oxygen species
SOG1	SUPPRESSOR OF GAMMA RESPONSE 1
WT	Wildtype/wild-type

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ABSTRACT

Cadmium (Cd) present in polluted soils can be taken up by plants and hereby impair their growth. At a cellular level, Cd induces an oxidative challenge and influences cell division and endoreduplication, two important processes in plant growth that are coordinated by cyclin-dependent kinase (CDK) activity. This activity is regulated by CDK inhibitors such as SIAMESE-RELATED (SMR) proteins. Expression of these *SMRs*, in turn, can be induced by reactive oxygen species (ROS)-induced DNA damage via SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1). As preliminary results showed that Cd exposure inhibits cell division and endoreduplication and increases expression of *SMR4*, *SMR5* and *SMR7*, this study aimed to unravel whether SOG1, SMR4, SMR5 and SMR7 mediate Cd-induced effects on oxidative stress, DNA damage and cell cycle progression in leaves of *Arabidopsis thaliana*. To this end, the extent of cell division and endoreduplication, the oxidative stress response and DNA damage-related parameters were determined in leaves of wild-type (WT), *smr4/5/7* and *sog1-7 A. thaliana* plants grown in hydroponics and exposed to 0 or 5 µM $CdSO_4$ in a short-term, long-term and chronic set-up.

Assessment of leaf growth parameters and flow cytometry measurements suggested that SOG1 is involved in the Cd-induced inhibition of leaf growth, cell division and endoreduplication. These effects are presumably not regulated via SMR4, SMR5 and SMR7, as the *smr4/5/7* mutant displayed highly similar Cd-induced responses as compared to WT plants. However, it cannot be excluded that a bypass mechanism is activated in this mutant. Furthermore, the assessment of ROS-induced DNA damage using gene expression analysis of oxidative stress and DNA damage-related genes, suggested that SOG1 is involved in the Cd-induced oxidative stress and DNA damage response. Additionally, Cd-induced effects on the expression of GSH biosynthesis genes and GSH levels indicated that SOG1 is most likely involved in the regulation of GSH biosynthesis upon Cd exposure. Finally, chronic phenotypic monitoring suggested that SOG1 is mainly involved in the early response of *A. thaliana* to Cd exposure and that a lack of functional SOG1 impairs reproductive growth.

These findings contribute to the knowledge regarding Cd toxicity in plants and can be used for the optimization of plant growth on Cd-contaminated soils.

SAMENVATTING

Cadmium (Cd) is aanwezig in vervuilde bodems en kan opgenomen worden door planten, waardoor hun groei belemmerd wordt. Op cellulair niveau induceert Cd oxidatieve stress en beïnvloedt het celdeling en endoreduplicatie, twee belangrijke processen voor plantengroei die gecoördineerd worden door de activiteit van cycline-afhankelijke kinases (CDKs). Deze activiteit wordt gereguleerd door CDK-inhibitoren, zoals eiwitten van de SIAMESE-RELATED (SMR) familie. De expressie van deze *SMRs* kan op zijn beurt via SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1) geïnduceerd worden door DNA-schade veroorzaakt door reactieve zuurstof soorten (ROS). Preliminaire resultaten tonen aan dat Cd-blootstelling celdeling en endoreduplicatie inhibeert en de expressie van *SMR4*, *SMR5* en *SMR7* verhoogt. Bijgevolg is het doel van deze studie om te achterhalen of SOG1, SMR4, SMR5 en SMR7 Cd-geïnduceerde oxidative stress, DNA-schade en celcyclusinhibitie mediëren in bladeren van *Arabidopsis thaliana*. Om dit te achterhalen werden de mate van celdeling en endoreduplicatie, de oxidatieve stressrespons en DNA-schade-gerelateerde parameters bepaald in bladeren van WT, *smr4/5/7* en *sog1-7* mutante *A. thaliana* planten gegroeid in hydrocultuur en blootgesteld aan 0 of 5 µM $CdSO_4$ in een korte termijn, lange termijn en chronisch proefopzet.

Analyse van bladgroei parameters en flowcytometrische analyses gaven aan dat SOG1 betrokken is in de Cd-geïnduceerde inhibitie van bladgroei, celdeling en endoreduplicatie. Deze effecten worden waarschijnlijk niet gereguleerd via SMR4, SMR5 en SMR7, aangezien de *smr4/5/7* mutant heel gelijkaardige Cd-geïnduceerde responsen vertoonde in vergelijking met WT planten. Daarenboven toonde het onderzoek van ROS-geïnduceerde DNA schade via genexpressie-analyse van oxidatieve stress- en DNA-schade-gerelateerde genen, aan dat SOG1 betrokken is in de Cd-geïnduceerde oxidatieve stress en DNA-schade-respons. Bijkomend gaven de Cd-geïnduceerde effecten op genexpressie van GSH-biosynthese genen en GSH-niveaus aan dat SOG1 hoogstwaarschijnlijk betrokken is in de regulatie van GSH biosynthese na Cd-blootstelling. Tenslotte toonde de chronische fenotypische opvolging aan dat SOG1 vooral betrokken is bij de vroege respons van *A. thaliana* op Cd-blootstelling en dat een gebrek aan functioneel SOG1 de reproductieve groei belemmert.

Deze bevindingen dragen bij aan de kennis omtrent Cd-toxiciteit in planten en kunnen gebruikt worden voor de optimalisatie van plantengroei op Cd vervuilde bodems.

1 INTRODUCTION

Many regions worldwide, including the Campine region located in Belgium and the Netherlands, are affected by metal pollution of industrial and agricultural origin. One of the non-biodegradable metals that contribute to this persistent pollution is cadmium (Cd) (1,2). For humans, Cd is classified as a group I carcinogen and is known to affect kidney and bone function. Additionally, environmental exposure to Cd is linked to an increased risk of death (3,4). For plants, Cd is a non-essential element. Nevertheless, it can be taken up via transmembrane carriers for essential elements such as calcium, magnesium, iron, copper and zinc (5,6). Once Cd is taken up by plants it can enter and accumulate in the food chain, thereby causing increased health risks for humans (1,6,7).

Once taken up by plants, Cd indirectly causes an increase in the production of reactive oxygen species (ROS) via various mechanisms (2,6). Firstly, it increases the activity of superoxide ($O_2^{\bullet -}$) producing NADPH oxidases. This $O_2^{\bullet -}$ is then converted to hydrogen peroxide (H_2O_2), either via a spontaneous reaction or through the action of superoxide dismutase (SOD). Secondly, Cd can disturb the function of enzymes by either binding to their thiol groups or by replacing essential elements in their active sites. As a consequence the cellular redox state is disturbed (8). Finally, Cd can cause a depletion of cellular GSH levels either as a result of increased phytochelatin synthesis or as a result of thiol binding (8,9).

Reactive oxygen species have a dual role within plants. They can have beneficial roles at low concentrations, acting as signaling molecules. In this context, H_2O_2 is particularly suited as it is relatively stable, has relatively low toxicity, and is capable of crossing cellular membranes. Reactive oxygen species signaling is involved in physiological processes and also allows plants to respond to stress by initiating repair mechanisms (2,6). Excess amounts of ROS, on the other hand, can result in oxidative stress, which is a cellular redox imbalance in which pro-oxidants, such as ROS, exceed the activity of antioxidants. A state of oxidative stress can result in damage to cellular macromolecules such as DNA, proteins, and lipids (2).

Although Cd does not bind to DNA, it indirectly causes DNA damage by inducing oxidative stress (4). The ROS that are produced cause the formation of oxidized bases, such as 8-oxoadenine and 8-oxoguanine, which can subsequently result in mutations. Additionally, the deoxyribose constituent of nucleotides can be attacked by ROS and hereby oxidized (10,11). Through these mechanisms, Cd can cause different types of DNA damage, such as single strand breaks (SSBs), double strand breaks (DSBs) and chromosomal aberrations (4). Plants possess different DNA repair mechanisms, specific for each type of DNA damage. In *A. thaliana,* a number of marker genes involved in these repair mechanisms have been identified. These genes include *POLY(ADP-RIBOSE) POLYMERASES* (*PARP1* and *PARP2*), both

involved in single strand breaks (SSBs) repair, *HOMOLOGUE OF X-RAY REPAIR CROSS COMPLEMENTING 1* (*XRCC1*) involved in base excision repair (BER) and *BREAST CANCER SUSCEPTIBILITY 1* (*BRCA1*), *DNA LIGASE IV* (*LIG4*) and *DNA REPAIR PROTEIN RAD51 HOMOLOG 1* (*RAD51*), participating in double strand breaks (DSBs) repair (4,11).

In order to prevent ROS-induced damage, plants have developed an elaborate antioxidative defense system, consisting of both metabolites and enzymes (5,6,8). Antioxidative enzymes, such as SOD, ascorbate peroxidase (APX), iron superoxide dismutase (FSD), cupper/zinc superoxide dismutase (CSD) and catalase (CAT), are responsible for the conversion of ROS to less damaging molecules, eventually resulting in the formation of water and oxygen. Additionally, antioxidative metabolites, such as ascorbate (AsA) and glutathione (GSH), are also responsible for the conversion or ROS to less harmful molecules (6,12).

Glutathione is an important antioxidant metabolite, consisting of cysteine (Cys), glutamate (Glu) and glycine (Gly). Its biosynthesis occurs via a two-step reaction (Fig. 1). First Cys and γ-Glu are combined by γ-glutamylcysteine synthetase (GSH1) to form γ-glutamylcysteine (γ-EC). In a second step GSH synthetase (GSH2) adds Gly, producing GSH (Fig. 1) (13). The resulting GSH molecule can exist in a reduced (GSH) and an oxidized form, glutathione disulfide (GSSG). In its reduced form, GSH is able to directly donate an electron to unstable molecules such as H_2O_2, reducing it to H_2O. By donating its electron, however, the GSH molecules themselves become reactive and combine, forming GSSG. In order to maintain their antioxidative capacity, oxidized GSSG molecules are converted to GSH by GSH reductase (GR), using NADPH as an electron donor (Fig. 1). A second mechanism through which GSH exerts its antioxidant function is the AsA-GSH cycle (Fig. 1). In this cycle, ascorbate peroxidase (APX) reduces H_2O_2, using an electron from AsA, resulting in the formation of dehydroascorbate (DHA) and H_2O. Hereafter DHA reductase (DHAR) uses an electron from GSH to reduce DHA to AsA. The resulting GSSG molecule is converted back to its reduced state by GR (13,14).

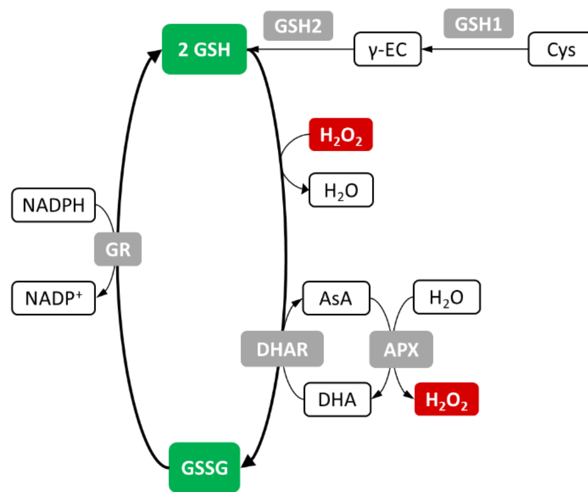

Figure 1 Glutathione synthesis and antioxidant defense mechanism. Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; Cys, cysteine; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSH1, γ-glutamylcysteine synthetase; GSH2, GSH synthetase; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; γ-EC, γ-glutamylcysteine.

In addition to its role in antioxidative defense, GSH is also involved in plant growth and development processes (15), in which the (classical) cell cycle has an important role. This cycle consists of four different phases. The DNA synthesis (S) phase in which DNA replication occurs and the mitosis (M) phase in which cells divide, these phases are separated by a gap 1 (G_1) and gap 2 (G_2) phase in which cells prepare for DNA replication and chromosome segregation and distribution respectively. Progression of cells throughout the cell cycle is controlled by the combined action of cyclins and cyclin-dependent kinases (CDKs). Two important thresholds for CDK activity need to be reached in order for the cell cycle to proceed: one for DNA replication and one for mitosis (16,17) (Fig 2A). Additionally, the cell cycle can be arrested when, for example, DNA damage is detected, hereby providing time for DNA repair (10).

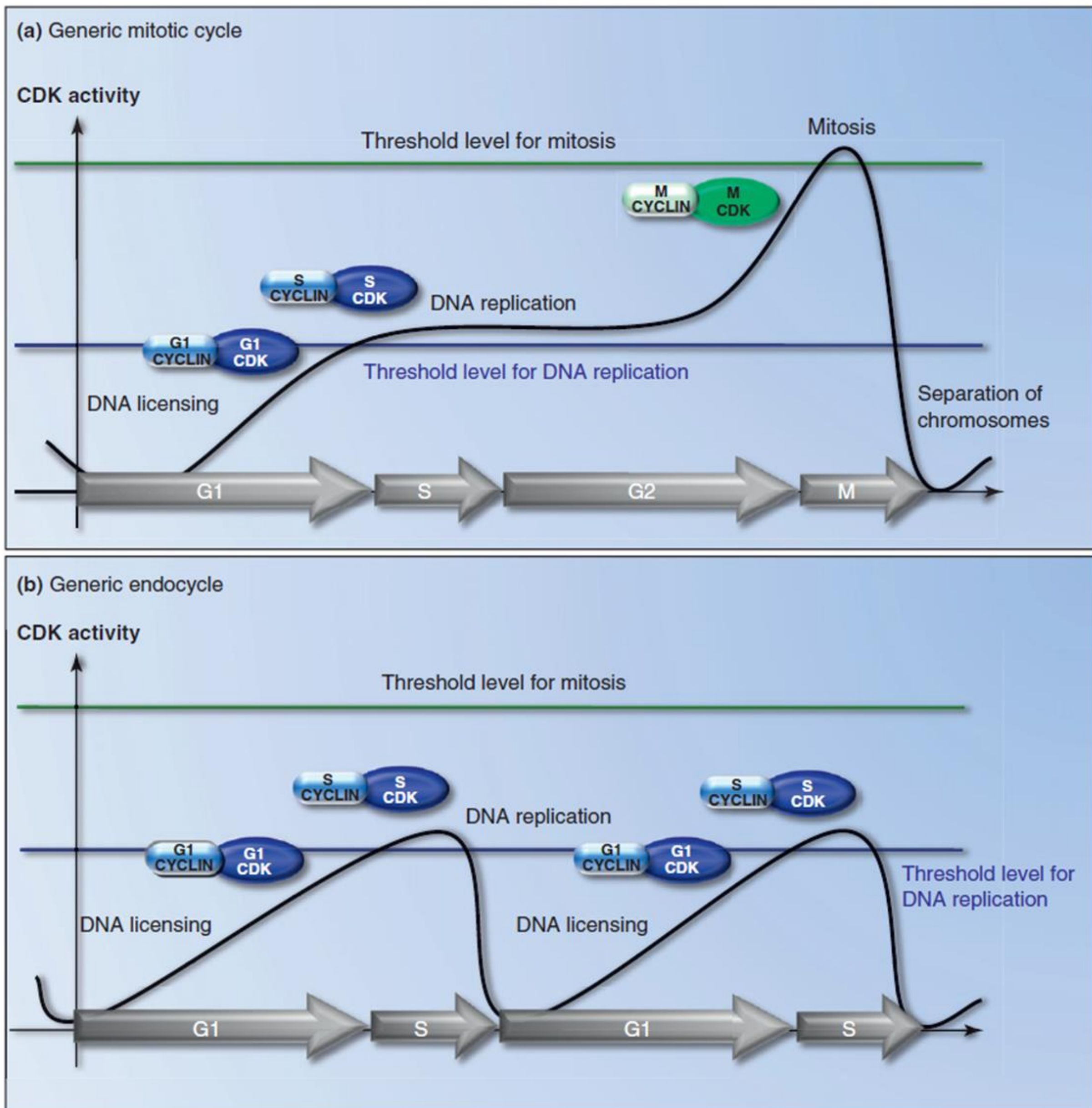

Figure 2 CDK activity during (**A**) the generic mitotic cycle or classical cell cycle and (**B**) the endocycle. De Veylder *et al.* 2011.

Besides the classical cell cycle, plants possess a second, alternative version of the cell cycle, called endoreduplication. An endoreduplication cycle or endocycle consists of a G_1 and S phase, without the intervening G_2 and M phase. As a consequence, nuclear DNA is replicated without cell division, resulting in endopolyploidy. Endoreduplication is an important process in plants as it is involved in normal plant growth and development, but can additionally be affected by environmental stress conditions such as soil quality and temperature. The extent of endoreduplication can vary between different cell types and plant species (16,18). Like the classical cell cycle, the endoreduplication cycle is regulated by CDK activity. Whereas the CDK activity reaches two thresholds for the classical cell cycle to proceed, in the endoreduplication cycle only the threshold for DNA replication is reached (Fig. 2B). The mitosis threshold is not reached due to proteolytic degradation of mitotic cyclins, transcriptional

downregulation of mitotic cyclins and CDKs, and inhibition of CDK activity by CDK inhibitors such as the SIAMESE-RELATED (SMR) proteins (18).

It is known that ROS-induced DNA damage can promote expression of two *SMR* genes: *SMR5* and *SMR7*. The pathway responsible for this response is the ATM-SOG1-SMR signaling pathway (Fig. 3) which was discovered in *A. thaliana* plants exposed to the replication inhibitory drug hydroxyurea (HU) (19). In this pathway, the formation of H_2O_2 is triggered by HU exposure. As a consequence, DNA damage occurs, causing the recruitment of ATAXIA TELANGIECTASIA MUTATED (ATM). This kinase is responsible for the phosphorylation and hence the activation of the transcription factor SUPPRESSOR OF GAMMA RESPONSE1 (SOG1). Once SOG1 is activated, it binds to the promotor regions of the genes encoding two CDK inhibitors, *SMR5* and *SMR7*, and is thereby hypothesized to cause cell cycle arrest (19,20).

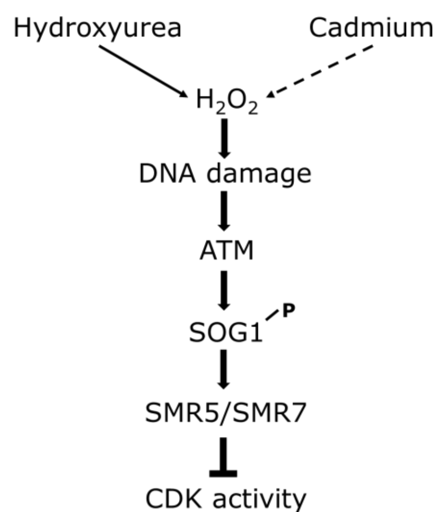

Figure 3 ATM-SOG1-SMR signaling pathway. Abbreviations: ATM, ATAXIA TELANGIECTASIA MUTATED; CDK, cyclin-dependent kinase; H_2O_2, hydrogen peroxide; SMR, SIAMESE-RELATED; SOG1, SUPPRESSOR OF GAMMA RESPONSE1. Figure adapted from Yi *et al.* 2013.

This pathway is potentially important in the response to DNA damage as a result of various stressors. Transcript levels of multiple genes involved in cell cycle control, DNA repair and apoptosis are known to be regulated by SOG1. It is therefore often referred to as the "plant p53" and is important in plant responses to stress (11,20). The SOG1-induced cell cycle arrest has a dual function. Firstly, it inhibits mitotic cell division, thereby preventing the spread of DNA damage. Secondly, it provides time for DNA repair (21).

Preliminary data of our research group, demonstrated a negative effect of Cd exposure on the extent of cell division and endoreduplication in *A. thaliana* leaves. In addition, Cd exposure induced the expression of *SMR4*, *SMR5* and *SMR7*. This response was up to thirty times stronger in leaves as compared to roots. As the expression of *SMR5* and *SMR7* can be induced upon ROS-induced DNA

damage via the ATM-SOG1-SMR signaling pathway (19) and as Cd exposure is known to generate ROS in *A. thaliana* (2,6), this mechanism could be responsible for the inhibition of cell division and endoreduplication. Therefore the hypothesis of this project is that SOG1, SMR4, SMR5 and SMR7 mediate Cd-induced effects on oxidative stress, DNA damage and cell cycle progression in leaves of *Arabidopsis thaliana.*

In order to verify this hypothesis, Cd-induced responses on oxidative stress, DNA damage, and cell cycle progression are compared between leaves of wild-type (WT) *A. thaliana* plants and *smr4/5/7* or *sog1-7* mutants. The first is a triple knockout mutant lacking functional SMR4, SMR5 and SMR7. Using this mutant allows to examine the involvement of SMR5 and SMR7 in Cd-induced responses, without any potential compensatory effects of SMR4, which has a similar function in *A. thaliana*. The second genotype is a knockout mutant lacking functional SOG1, which can provide information about the involvement of SOG1 in Cd-induced responses. Three different research questions were assessed:

1. Does the effect of Cd exposure on the amount of cell division and endoreduplication differ between WT *Arabidopsis thaliana* and *smr4/5/7* or *sog1-7* knockout mutants?
2. Does the Cd-induced oxidative stress response differ between WT *Arabidopsis thaliana* and *smr4/5/7* and *sog1-7* knockout mutants?
3. Does Cd exposure have a different effect on DNA damage-related parameters in WT *Arabidopsis thaliana* as compared to *smr4/5/7* and *sog1-7* knockout mutants?

To assess these questions WT, *smr4/5/7* and *sog1-7 A. thaliana* plants were grown in hydroponics and were exposed to either 0 or 5 µM $CdSO_4$ in multiple setups. A first setup assessed short-term effects of Cd exposure, by exposing 19 day-old plants and evaluating their response after 24 or 72 h of Cd exposure. In a second setup, plants were exposed to Cd for 7 days, starting from day 14 after sowing, to assess long-term Cd effects. Cadmium-induced effects on nuclear ploidy levels, glutathione levels and metal content, and transcription levels of genes involved in cell cycle regulation, oxidative stress, and DNA damage and repair were compared between WT and mutant plants. Additionally, vegetative and reproductive growth and survival of the different genotypes were monitored in a chronic exposure setup.

2 MATERIALS AND METHODS

2.1 Plant material, growth conditions and cadmium exposure

Seeds of WT, *smr4/5/7* and *sog1-7 A. thaliana* (ecotype Col-0) were used after their homozygosity of the double mutation was verified using PCR. Seeds were surface-sterilized in 0.1 % NaOCl solution for 1 min and washed four times with distilled water. Hereafter, seeds were incubated for two days at 4 °C in the dark to ensure equal germination. Next, plants were grown in hydroponics utilizing a modified Hoagland solution and controlled growth conditions consisting of a 12 h photoperiod, 22/18 °C day/night temperatures and 65 % relative humidity, as previously described by Keunen *et al.* (2011) (22). The photosynthetic active radiation (PAR) of sunlight was simulated by a combination of blue, red and far red Philips Green-Power LED-modules. Plants were exposed to 5 µM $CdSO_4$ via their roots at different time-points during their development. In the short-term experiment, plants were exposed to Cd after 19 days of growth under control conditions. Subsequently, rosettes and roots were weighed and harvested after 24 and 72 h of exposure. For the long-term experiment, plants were exposed to Cd after 14 days of growth under control conditions and separate leaves, rosettes and roots were weighed and harvested after one week of exposure. During the chronic experiment, plants were exposed to Cd after 19 days of growth under control conditions and exposure lasted throughout their lifetime. At the end of the experiment, root samples and seeds were collected. All samples, except those for element determinations, were snap-frozen in liquid nitrogen and stored at -80 °C until further analysis.

2.2 Analysis of nuclear ploidy levels

The CyStain® PI Absolute P kit (Sysmex Partec, Görlitz, Germany) was used to determine nuclear ploidy levels in separate leaves, in order to determine the extent of endoreduplication. Leaf samples were chopped in 500 µL of nuclei extraction buffer using sharp razor blades and incubated for approximately 1 minute. The extract was filtered through a 50 µM nylon filter (CellTrics®, Sysmex Partec, Görlitz, Germany) and hereafter 2 mL of staining solution consisting of propidium iodide (PI), staining buffer and RNase A was added. Finally, samples were incubated in the dark at 4 °C for at least 1 h. Using a CyFlow® Cube 8 flow cytometer (Sysmex Partec, Görlitz, Germany) ploidy levels (2C, 4C, 8C, 16C, and 32C, with C being the haploid DNA content) of at least 10,000 nuclei per sample were determined. After exciting nuclei with a 488 nm laser, forward scatter and PI fluorescence intensity (FL-2 channel; 580/30 nm) were determined. Data were analyzed using FCS Express 4 software (De Novo Software, Glendale, CA, USA). To indicate the average number of endocycles per cell, the endoreduplication factor (EF) was calculated using the following formula: [(0 x % 2C) + (1 x % 4C) + (3 x % 16C) + (4 x %

32C)] / 100 (23,24). Additionally, rosettes and separate leaves that were scanned during harvest, were analyzed using ImageJ (NIH, Bethesda, MD, USA) to determine respectively rosette diameter and leaf surface area.

2.3 Quantification of element content

After harvest, leaf rosettes were rinsed twice with distilled water. Roots were incubated in ice-cold 10 mM $(PbNO_3)_2$ for 15 min in order to exchange surface-bound elements and were then rinsed with distilled water. Hereafter, all samples were oven-dried at 80 °C for four weeks. Next, samples were digested in HNO_3 (70 %) and HCl (37 %) in a heating block. Dried samples were then dissolved in a 2 % HCl solution and element (Cd, Cu, Zn, Ca, K, Na, Mg, Mn, P and S) concentrations in the extracts were determined using inductively coupled plasma-atom emission spectrometry (ICP-AES, PerkinElmer, 1100B, USA). As references three blanks (containing only HNO_3 and HCl) and three replicates of a certified standard sample (Virginia Tobacco Leaves (CTA-VTL-2)) were analyzed.

2.4 Gene expression analysis

Frozen rosette samples were pulverized using two stainless steel beads in the Retsch Mixer Mill MM 400 (Retsch, Haan, Germany). Hereafter, the RNaqueous™ Total RNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) was used to extract RNA according to the manufacturer's instructions. Purity and concentration of the obtained RNA samples were determined using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To confirm RNA integrity, the Aligent 2100 Bioanalyzer was used in combination with the Aligent RNA 6000 Nano Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Any residual genomic DNA was removed using the TURBO DNA-free™ Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For each sample, an equal RNA input of 1 µg was used. Subsequently, reverse transcription was performed using the PrimeScript™ RT Reagent Kit (Perfect Real Time, Takara Bio Inc., Kusatsu, Japan), in accordance with the manufacturer's instructions. The resulting cDNA was diluted tenfold in 1/10 TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0; Sigma-Aldrich, Belgium) and stored at -20 °C.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed using the 7500 Fast Real-Time PCR System in combination with the Quantinova SYBR® Green Master Mix (Qiagen, Hilden, Germany). Each reaction consisted of 2 µL diluted cDNA and 5 µL of Quantinova SYBR® Green Master Mix, 0.05 µL of QuantiNova ROX reference dye, 2.35 µL RNase-free H_2O and forward an reverse primers (300 nM each, unless stated otherwise) with a total reaction volume of 10 µL for each sample. The following cycling conditions were used: 2 min at 95 °C, 40 cycles of 5 s at 95 °C and 10 s at 60 °C.

After amplification, a dissociation curve was created to validate product specificity. To determine relative gene expression levels, the $2^{-\Delta Cq}$ method was used. Gene expression levels were normalized against the expression of *ACTIN 2 (ATC2), MONESIN SENSITIVITY 1 (MON1) and YELLOW-LEAF-SPECIFIC GENE 8 (YLS8)*. To select reference genes, the GrayNorm algorithm was used (25). Forward and reverse primers (Table 1) were designed using Primer3 software. To confirm their specificity *in silico*, BLAST was used (http://www.arabidopsis.org/Blast/index.jsp).

Table 1 Primer sequences for RT-qPCR (in 5' to 3' direction). *APX:* ascorbate peroxidase; *BRCA1*: breast cancer susceptibility 1; *CAT*: catalase; *CSD*: copper/zinc superoxide dismutase; *Defensin*: defensin-like protein; *FSD1:* iron superoxide dismutase; *GSH1:* γ-glutamylcysteine synthetase; *GSH2*: glutathione synthetase; *LIG4*: DNA ligase IV; *PARP*: poly(ADP-ribose) polymerase; *RAD51*: DNA repair protein RAD51 homolog 1; *SMR*: SIAMESE-related; *SOG1:* SUPPRESSOR OF GAMMA RESPONSE 1; *TIR*: toll/interleukin receptor 1; *UPOX:* upregulated by oxidative stress; *XRCC1*: homologue of X-ray repair cross complementing 1.

Gene	Forward primer	Reverse primer
SMR4	TGATGGTGGTGAGAAAACGAGA	TCTCTTCGAGGCTGTGCGTAG
SMR5	CTGCTACCACCGAGAAGAACAAGT	CTGCTACCACCGAGAAGAACAAGT
SMR7	ACATCGATTCGGGCTTCACTAA	CCGTGGGAGTGATACAAATTCC
SOG1	AGTGGTGTGGAAGAGCAACC	GCAATCCTGGCCAATCATCAA
AT1G05340	TCGGTAGCTCAGGGTAAAGTGG	CCAGGGCACAACAGCAACA
AT1G19020	GAAAATGGGACAAGGGTTAGACAAA	CCCAACGAAAACCAATAGCAGA
Defensin	ATGGCAAAGGCTATCGTTTCC	CGTTACCTTGCGCTTCTATCTCC
TIR	ACTCAAACAGGCGATCAAAGGA	CACCAATTCGTCAAGACAACACC
UPOX	GACTTGTTTCAAAAACACCATGGAC	CACTTCCTTAGCCTCAATTTGCTTC
APX1	TGCCACAAGGATAGGTCTGG	CCTTCCTTCTCTCCGCTCAA
APX2	TTGCTGTTGAGATCACTGGAGGA	TGAGGCAGACGACCTTCAGG
CAT1	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA
CAT2	AACTCCTCCATGACCGTTGGA	TCCGTTCCCTGTCGAAATTG
CAT3	TCTCCAACAACATCTCTTCCCTCA	GTGAAATTAGCAACCTTCTCGATCA
CSD1	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC
CSD2	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC
FSD1	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC
GSH1	CCCTGGTGAACTGCCTTCA	CATCAGCACCTCTCATCTCCA
GSH2	GGACTCGTCGTTGGTGACAA	TCTGGGAATGCAGTTGGTAGC
BRCA1	GTGAACCTGTCTCTGCGGAT	TCCGGCTTCTTGTCAACTCC
LIG4	TGATGTATCGGATATCAAGGGCA	GAATGGGACCGAGGCACG
PARP1	TGCATTGGGAGAAATACATGAGC	CCGAGCCCTTTGGTCGAG
PARP2	ATCGGAGGTGATTGATCGGTATG	AAATCATGAGGTATCACTGTGTAGAACTCT
RAD51	GTCCAACAACAAGACGATGAAGAA	AACAGAAGCAATACCTGCTGCC
XRCC1	TGGGCCAGGGATGACCTAAG	CCGCAGCTATTCGCTTGATTT

2.5 Determination of glutathione content

Frozen leaf samples of approximately 100 mg were grounded in 200 mM HCl in a liquid nitrogen-cooled mortar. Unless stated otherwise, samples were kept at 4 °C throughout all procedures. After 10 min of centrifugation (16000 x g, 4 °C), 350 µL of supernatant was transferred to a new eppendorf and 35 µL NaH_2PO_4 (pH 5.6) was added. Subsequently, samples were brought to pH 4.5 by gradually adding 200 mM NaOH. Samples were spectrophotometrically measured at 412 nm for 5 min in a total reaction mixture of 200 µL containing 10 µL of sample, combined with 100 µL of 200 mM NaH_2PO_4 + 10 mM EDTA (pH 7.5), 10 µL of 10 mM NADPH, 10 µL of DTNB in DMSO, 10 µL of glutathione reductase, and 60 µL of H_2O. Total GSH concentrations were calculated by means of a standard curve, pipetted in duplicate, ranging from 0 to 1000 pmol GSH. Samples were measured in triplicate.

The concentration of oxidized GSSG was measured by incubating samples with 2-vinyl-pyridine (2-VP) for 30 min at 20 °C in order to complex the reduced GSH in the sample. A standard curve ranging from 0 to 400 pmol GSSG was also incubated with 2-VP. After the incubation, samples were centrifuged twice (10 min, 16000 x g, 4 °C) to precipitate the 2-VP complexes. A reaction mixture containing 40 µL of sample, 80 µL of 200 mM NaH_2PO_4 + 10 mM EDTA (pH 7.5), 10 µL of 10 mM NADPH, 10 µL of 12 mM DTNB in DMSO, 10 µL of glutathione reductase, and 50 µL of H_2O was prepared. These mixtures were spectrophotometrically measured at 412 nm for 5 min. Standards were measured in duplicate, whereas samples were measured in triplicate. Finally, the concentration of oxidized GSSG (in GSH equivalents) was subtracted from the total glutathione concentration to calculate the amount of reduced GSH present in the samples.

2.6 Chronic phenotypic analysis

The measurements assessing vegetative and reproductive growth and development of unexposed and Cd-exposed plants were started at day 19 after sowing, when $CdSO_4$ exposure started. Rosette growth was kinetically monitored by measuring the diameter at the widest point of the rosette. This parameter was followed until a plateau was reached. As soon as inflorescences started emerging, their height was monitored until a plateau was reached. As soon as siliques opened, they were harvested in order to determine the average seed weight. At the end of the experiment, roots were harvested and both fresh and dry weight (DW) was determined.

2.7 Statistical analysis

Statistical analyses were performed in R version 3.3.1 (R Foundation for Statistical Computing, 2016, Vienna, Austria). To verify the normal distribution and homoscedasticity of the data, the Shapiro-Wilk

and Bartlett's test were used, respectively. Data were transformed (square root, inverse, exponent or logarithm) if necessary. For gene expression, all data were log transformed. Hereafter, data were statistically analyzed using a two-way ANOVA in combination with a *post-hoc* Tukey-Kramer test to correct for multiple comparisons. In case data did not meet the normality or homoscedasticity assumptions, a non-parametric Kruskal-Wallis test was performed combined with a *post-hoc* Wilcoxon Rank Sum test to account for multiple comparisons. Outliers were determined using the Extreme Studentized Deviate method (GraphPad Software, La Jolla, CA, USA) at significance level 0.05.

3 RESULTS

3.1 Influence of cadmium exposure on leaf growth and cell cycle regulation after long-term exposure in wild-type, *smr4/5/7* and *sog1-7 Arabidopsis thaliana* plants

In a first part of the project, the effects of long-term Cd exposure on leaf growth and the cell cycle were assessed. To determine Cd-induced effects on leaf growth, rosette fresh weight, rosette diameter and the surface area of individual rosette leaves were assessed in plants exposed to 0 or 5 µM $CdSO_4$ for 8 days, starting from 14 days after sowing (DAS). Furthermore, the extent of cell division and endoreduplication were assessed by measuring the concentration of nuclei in flow cytometry extracts and the endoreduplication factor in individual leaves.

3.1.1 Influence of cadmium exposure on leaf growth of wild-type, smr4/5/7 and sog1-7 Arabidopsis thaliana plants after long-term exposure

To determine potential Cd-induced effects on leaf growth of different *A. thaliana* genotypes, rosette fresh weight and diameter and the surface area of individual rosette leaves were assessed. Rosette fresh weight was negatively affected by Cd exposure in both WT and *smr4/5/7* plants, but not *sog1-7* mutants (Fig. 4A). However, the *sog1*-7 mutants had a significantly lower rosette fresh weight as compared to the other genotypes under control conditions (Fig. 4A). Furthermore, Cd reduced the rosette diameter in all genotypes. It should be noted, however, that the Cd-induced effect on rosette diameter was less pronounced in *sog1-7* plants as compared to *smr4/5/7* plants and that *sog1-7* plants had a smaller rosette diameter under control conditions (Fig. 4B).

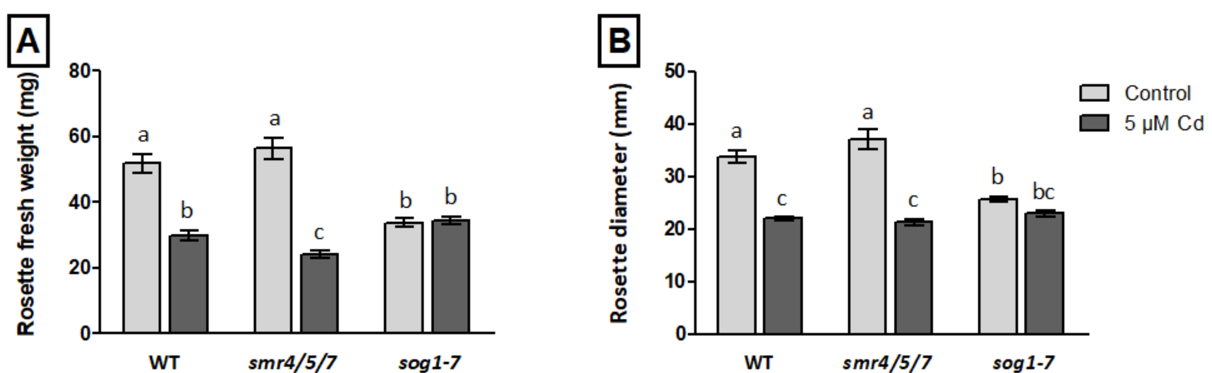

Figure 4 (**A**) Rosette fresh weight (mg) and (**B**) rosette diameter (mm) of *A. thaliana* plants grown in hydroponics and exposed to 0 or 5 µM $CdSO_4$ for 8 days, starting from day 14 after sowing. Data represent the average ± S.E. of (**A**) at least 22 and (**B**) 8 biological replicates. Significant differences ($p < 0.05$; 2-way ANOVA) are indicated using a different letter.

The leaf surface area of leaves 1, 3 and 6 was assessed. Leaf 1, the oldest leaf, was chosen because it was already present before Cd exposure was initiated. Leaf 3, which is of intermediate age, was

developing when Cd exposure started, whereas leaf 6 was not present at the onset of Cd exposure and therefore emerged during the exposure. The surface area of leaves 1 and 6 was significantly lower in Cd-exposed WT and *smr4/5/7* plants as compared to control plants of the same genotypes. In contrast, this effect was not observed in *sog1-7* plants (Fig. 5A and C). Although leaf 3 of Cd-exposed plants was significantly smaller than that of control plants for all genotypes, this response was much less pronounced in *sog1-7* plants as compared to WT and *smr4/5/7* plants (Fig. 5B).

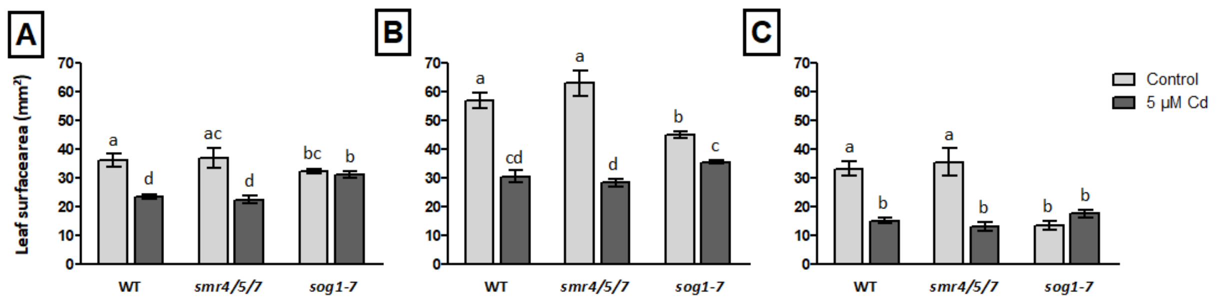

Figure 5 Leaf surface area (mm^2) of (**A**) leaf 1, (**B**) leaf 3 and (**C**) leaf 6 of *A. thaliana* plants grown in hydroponics and exposed to 0 or 5 µM $CdSO_4$ for 8 days, starting from day 14 after sowing. Data represent the average of ± S.E. of 8 biological replicates. Significant differences ($p < 0.05$; 2-way ANOVA) are indicated using a different letter.

3.1.2 Influence of cadmium exposure on cell division and endoreduplication in leaves of wild-type, smr4/5/7 and sog1-7 Arabidopsis thaliana plants

As the cell cycle is an important process contributing to leaf growth and development, the amount of cell division and endoreduplication was assessed by measuring the number of nuclei in flow cytometry extracts and the endoreduplication factor of leaves 1, 3 and 6 of WT, *smr4/5/7* and *sog1-7 A. thaliana* grown under control or Cd-exposed conditions for 8 days, starting from 14 DAS.

The number of nuclei per µL measured via flow cytometry extracts in the oldest leaf, was lower in Cd-exposed WT and *smr4/5/7* plants as compared to their control counterparts, whereas this response was absent in the *sog1-7* mutant (Fig. 6A). A similar effect of Cd exposure was observed in leaf 6, but the reduction was not significant in WT plants (Fig. 6C). Leaf 3 showed a lower concentration of nuclei in all three Cd-exposed genotypes as compared to controls (Fig. 6B). The concentration of nuclei under control conditions increased with decreasing leaf age (Fig. 6).

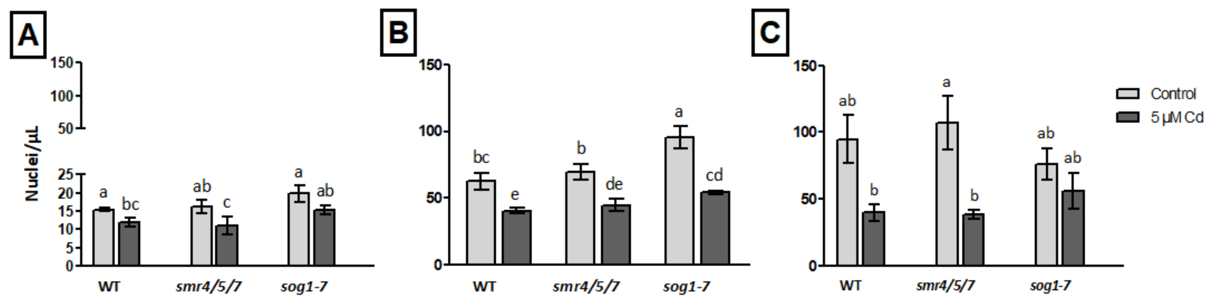

Figure 6 Concentration of nuclei measured using flow cytometry in (**A**) leaf 1, (**B**) leaf 3 and (**C**) leaf 6 of *A. thaliana* plants grown in hydroponics and exposed to 0 or 5 µM $CdSO_4$ for 8 days, starting from day 14 after sowing. Data represent the average ± S.E. of 8 biological replicates. Significant differences ($p < 0.05$; 2-way ANOVA) are indicated using a different letter.

The endoreduplication factor of leaves 1 and 3 was lower in Cd-exposed WT and *smr4/5/7* plants as compared to control plants of the same genotypes, whereas this effect was not observed in leaves of *sog1-7* plants (Fig. 7A and B). In addition, the oldest leaf of *sog1-7* plants had a higher endoreduplication factor under control conditions as compared to other genotypes (Fig. 7A). Leaf 6 did not display a Cd-induced change in endoreduplication factor in the WT, whereas *smr4/5/7* and *sog1-7* plants showed opposite effects, with respectively a Cd-induced decrease and increase in EF (Fig. 7C). It is noteworthy that under control conditions, the endoreduplication factor of leaf 6 was lower in *sog1-7* plants as compared to other genotypes (Fig. 7C).

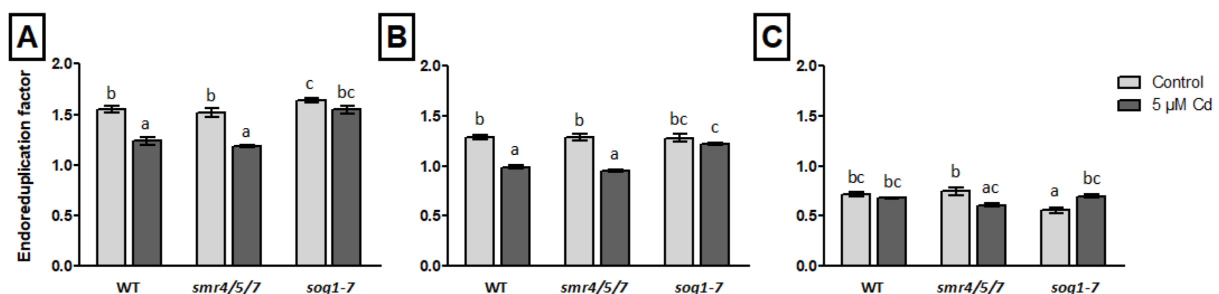

Figure 7 Endoreduplication factor of (**A**) leaf 1, (**B**) leaf 3 and (**C**) leaf 6 of *A. thaliana* plants grown in hydroponics and exposed to 0 or 5 µM $CdSO_4$ for 8 days, starting from day 14 after sowing. Data represent the average ± S.E. of 8 biological replicates. Significant differences ($p < 0.05$; 2-way ANOVA) are indicated using a different letter.

3.2 Cadmium-induced effects on oxidative stress and the DNA damage response in leaves of wild-type and *sog1-7 Arabidopsis thaliana* plants

In a second part of the project, 19-days-old WT and *sog1-7 A. thaliana* plants were exposed to Cd for 24 and 72 h to further unravel the mechanisms underlying the reduced Cd sensitivity of the *sog1-7* mutant. The *smr4/5/7* mutant was not included in this part of the project, as Cd-induced effects on leaf growth and cell cycle regulation in this mutant did not differ from those in WT plants.

3.2.1 Effects of short-term cadmium exposure on rosette weight and cadmium content in leaves of wild-type and sog1-7 Arabidopsis thaliana plants

Rosette weight was determined to verify whether *sog1-7* plants also displayed a reduced Cd sensitivity after 24 and 72h of Cd exposure. In addition, rosette dry weight and rosette Cd content were determined in WT and *sog1-7 A. thaliana* plants after 24 and 72 h of Cd exposure, starting 19 DAS.

Rosette fresh weight of WT and *sog1-7* plants was not affected by Cd exposure for both 24 and 72 h of exposure (Fig. 8A and B). However, fresh weight of Cd-exposed WT plants appeared lower as compared to their control counterparts, albeit not significantly. This trend was absent in the *sog1*-7 plants. The fresh weight of *sog1-7* rosettes was, however, lower as compared to that of the WT plants under both control and Cd-exposed conditions.

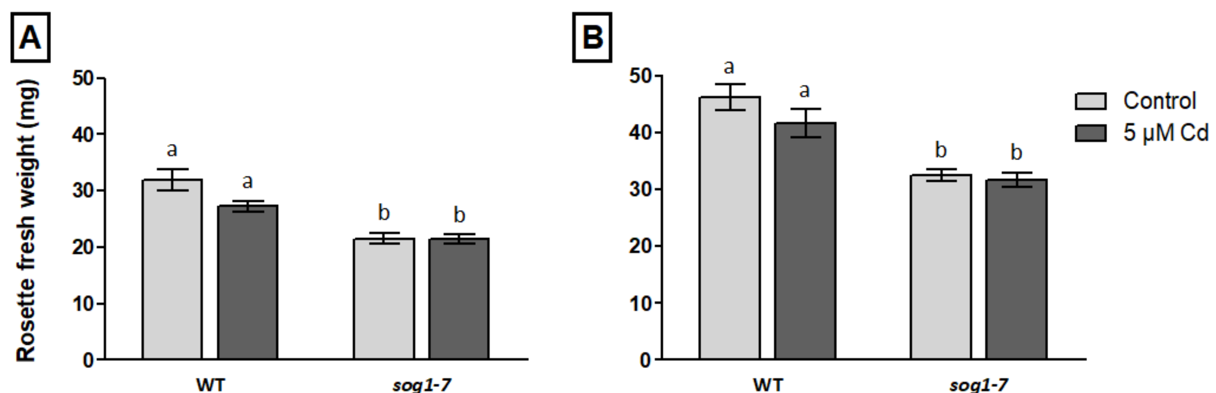

Figure 8 Rosette fresh weight of *A. thaliana* plants grown in hydroponics and exposed to 0 of 5 µM $CdSO_4$ for (**A**) 24 h or (**B**) 72 h, starting from day 19 after sowing. Data represent the average ± S.E. of at least 9 biological replicates. Significant differences ($p < 0.05$; 2-way ANOVA) are indicated using a different letter.

The percentage of rosette dry weight was not significantly influenced by Cd exposure after 24 h of exposure (Fig. 9A). However, an increasing trend could be observed, which was less pronounced in *sog1-7* plants as compared to WT plants. After 72 h of Cd exposure, in contrast, both WT and *sog1-7* had a significantly higher percentage of rosette dry weight as compared to control conditions (Fig. 9B).

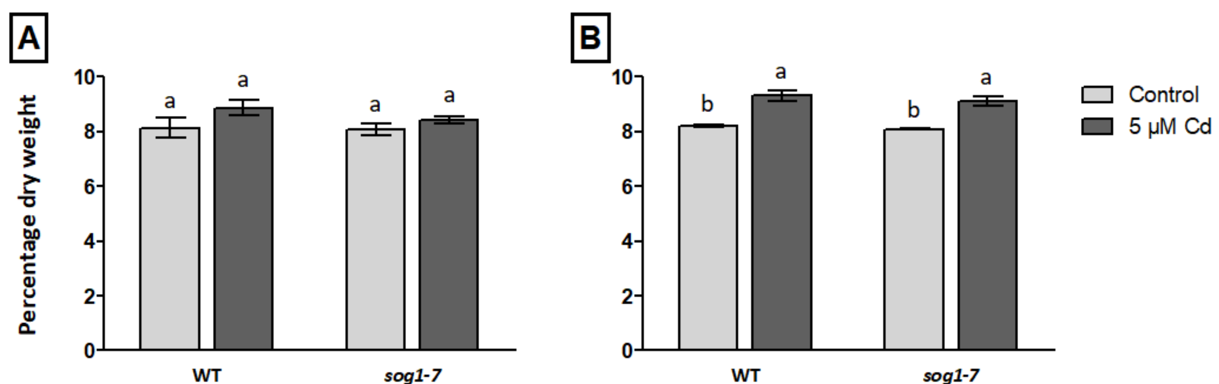

Figure 9 Percentage dry weight of rosettes of *A. thaliana* plants grown in hydroponics and exposed to 0 or 5 µM $CdSO_4$ for (**A**) 24 h or (**B**) 72 h, starting from day 19 after sowing. Data represent the average ± S.E. of 3 biological replicates. Significant differences ($p < 0.05$; 2-way ANOVA) are indicated using a different letter.

After both 24 and 72 h of Cd exposure, Cd concentrations did not significantly differ between leaves of WT and *sog1*-7 plants (Fig. 10A and B). The Cd concentration in rosettes of Cd-exposed plants of both genotypes increased over time (Fig.10).

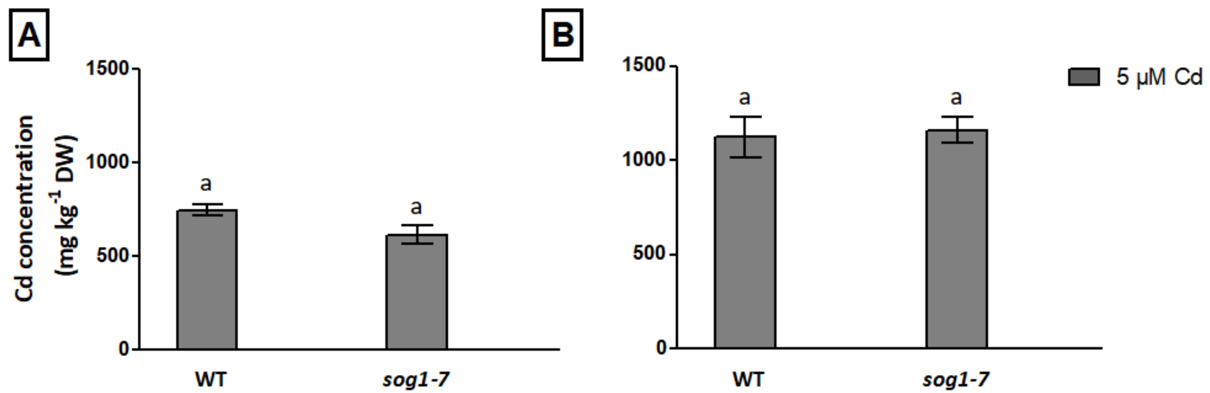

Figure 10 Cadmium concentration (mg kg^{-1} DW) in rosettes of *A. thaliana* plants grown in hydroponics and exposed to 5 µM $CdSO_4$ for (**A**) 24 h or (**B**) 72 h, starting from day 19 after sowing. Data represent the average ± S.E. of 3 biological replicates. Significant differences ($p < 0.05$; 2-way ANOVA) are indicated using a different letter.

3.2.2 Effects of short-term cadmium exposure on expression of SIAMESE-RELATED genes and SOG1 in leaves of wild-type and sog1-7 Arabidopsis thaliana plants

Gene expression analysis was performed in order to determine whether transcriptions levels of *SOG1* changed during Cd stress and whether SOG1 was responsible for the induction of *SMR4*, *SMR5* and *SMR7* in response to Cd stress.

After 24 h of exposure, both *SIAMESE-RELATED 5* (*SMR5*) and *SUPPRESSOR OF GAMMA RESPONSE 1* (*SOG1*) were upregulated in leaves of Cd-exposed WT plants (Table 2). The *sog1*-7 mutants, in contrast, showed an upregulation of *SIAMESE-RELATED 4* (*SMR4*) and *SOG1* (Table 2). After 72 h, however, all the assessed genes showed a Cd-induced upregulation in WT plants (Table 2). In *sog1-7* plants, on the other hand, none of the analyzed genes were significantly affected upon 72 h of Cd exposure (Table 2). Furthermore, it should be noted that *SMR4* expression was higher in leaves of *sog1-7* as compared to WT plants under control conditions after 24 and 72 h (Supplementary Table S2).

Table 2 Gene expression in leaves of WT and *sog1-7 A. thaliana* plants grown in hydroponics and exposed to 5 µM $CdSO_4$ for 24 or 72 h, starting from day 19 after sowing. Data are expressed relative to the control of the same genotype set at 1. A green color indicates a significant Cd-induced upregulation ($p < 0.05$; 2-way ANOVA within each time point). Asterisks (*) indicate a significantly different Cd-induced fold change in gene expression between both genotypes. Data represent the average ± S.E. of 5 biological replicates and were normalized based on the expression of *ACT2*, *MON1* and *YLS8*. *SMR*: SIAMESE-related*; SOG1:* SUPPRESSOR OF GAMMA RESPONSE 1.

	24 h		72 h	
Gene	WT	*sog1-7*	WT	*sog1-7*
SMR4	1.35 ± 0.16	2.22 ± 0.22	2.74 ± 0.37	1.08 ± 0.06*
SMR5	2.85 ± 0.20	1.33 ± 0.07*	8.38 ± 2.11	2.42 ± 0.36*
SMR7	1.72 ± 0.44	0.92 ± 0.05	45.78 ± 12.23	2.46 ± 0.63*
SOG1	1.75 ± 0.10	1.86 ± 0.11	1.66 ± 0.07	1.22 ± 0.04

3.2.3 *Effects of short-term cadmium exposure on the oxidative balance in leaves of wild-type and sog1-7 Arabidopsis thaliana plants*

As SOG1 is known to induce cell cycle arrest in response to ROS-induced DNA damage, the level of oxidative stress was assessed. To this end, expression levels of oxidative stress marker genes and genes encoding antioxidative enzymes were assessed using RT-qPCR in leaves of WT and *sog1-7 A. thaliana* plants exposed to 0 or 5 µM $CdSO_4$ for 24 and 72 h, starting 19 DAS.

After 24 h of Cd exposure, all oxidative stress marker genes were significantly upregulated in the WT, whereas this response was absent or less pronounced in the *sog1-7* mutant (Table 3). After 72 h, three of the oxidative stress marker genes were significantly upregulated in WT leaves, whereas only two were upregulated in *sog1-7* leaves. The fold change by Cd exposure is, however, smaller in the *sog1-7* mutant (Table 3). Under control conditions, *UPREGULATED BY OXIDATIVE STRESS* (*UPOX*) expression was higher in *sog1-7* as compared to WT plants, the same trend was observed for *TOLL/INTERLEUKIN RECEPTOR 1* (*TIR*), albeit not significant (Supplementary Table S2).

Most genes encoding antioxidative enzymes were significantly upregulated in WT leaves after 24 h of Cd exposure, whereas *CATALASE 2* (*CAT2*) was downregulated. In leaves of *sog1-7* plants, only *ASCORBATE PEROXIDASE 2* (*APX2*) and *COPPER/ZINC SUPEROXIDE DISMUTASE 2* (*CSD2*) displayed a significant Cd-induced change in expression levels, being up- and downregulated respectively (Table 3). After 72 h of exposure, the expression levels of *APX2* and *GLUTATHIONE SYNTHETASE* (*GSH2*) remained upregulated, whereas *CAT2* and *COPPER/ZINC SUPEROXIDE DISMUTASE 1* (*CSD1*) were downregulated in leaves of WT plants (Table 3). Leaves of *sog1-7* plants, on the other hand, showed significant Cd-induced downregulations of *ASCORBATE PEROXIDASE 1* (*APX1*), *CATALASE 1* (*CAT1*), *CSD1* and *CSD2* after 72 h of exposure (Table 3). Additionally, expression levels of *APX2* were higher in *sog1-7* mutants under control conditions (Supplementary Table S2).

Table 3 Expression of genes involved in the oxidative stress response in leaves of WT and *sog1-7 A. thaliana* plants grown in hydroponics and exposed to 5 µM $CdSO_4$ for 24 or 72 h, starting from day 19 after sowing. Data are expressed relative to the control of the same genotype set at 1. Green and red colors indicate a significant Cd-induced up- and downregulations, respectively ($p < 0.05$; 2-way ANOVA within each time point). Asterisks (*) indicate a significantly different Cd-induced fold change in gene expression between both genotypes. Data represent the average ± S.E. of 5 biological replicates and were normalized based on the expression of *ACT2*, *MON1* and *YLS8*. *APX:* ascorbate peroxidase; *CAT*: catalase; *CSD*: copper/zinc superoxide dismutase; *Defensin*: defensin-like protein*; FSD1:* iron superoxide dismutase; *GSH1:* γ-glutamylcysteine synthetase; *GSH2*: glutathione synthetase*; TIR*: toll/interleukin receptor 1*; UPOX:* upregulated by oxidative stress.

		24 h		72 h	
	Gene	WT	*sog1-7*	WT	*sog1-7*
Oxidative stress markers	*AT1G05340*	17.90 ± 3.33	1.49 ± 0.59*	9.65 ± 1.91	2.00 ± 0.84*
	AT1G19020	20.44 ± 3.07	5.79 ± 1.29*	2.63 ± 0.29	0.98 ± 0.08
	Defensin	14.92 ± 2.15	2.12 ± 0.35*	5.61 ± 0.53	2.59 ± 0.23*
	TIR	16.22 ± 2.53	2.01 ± 0.46*	2.32 ± 0.28	0.22 ± 0.01*
	UPOX	9.81 ± 1.81	2.70 ± 0.21*	6.57 ± 1.76	2.72 ± 0.36
Antioxidants	*APX1*	1.75 ± 0.20	1.07 ± 0.07*	0.83 ± 0.14	0.50 ± 0.10
	APX2	6.50 ± 1.19	3.34 ± 0.38	3.36 ± 0.45	1.38 ± 0.25*
	CAT1	2.78 ± 0.26	1.36 ± 0.19*	1.23 ± 0.12	0.56 ± 0.06*
	CAT2	0.50 ± 0.06	0.82 ± 0.03	0.73 ± 0.04	0.85 ± 0.05
	CAT3	1.88 ± 0.05	1.22 ± 0.11*	1.73 ± 0.39	1.04 ± 0.18
	CSD1	2.09 ± 0.28	0.62 ± 0.11*	0.66 ± 0.05	0.20 ± 0.02*
	CSD2	0.45 ± 0.08	0.42 ± 0.14	0.22 ± 0.02	0.18 ± 0.01
	FSD1	0.74 ± 0.25	3.35 ± 0.44	0.63 ± 0.23	1.93 ± 0.49
	GSH1	1.42 ± 0.03	1.10 ± 0.06	1.11 ± 0.05	1.08 ± 0.04
	GSH2	2.69 ± 0.07	1.29 ± 0.06*	1.43 ± 0.03	1.16 ± 0.02*

This gene expression analysis demonstrated that *sog1-7* leaves lack the induction of Cd-induced upregulation of γ-*GLUTAMYLCYSTEINE SYNTHETASE* (*GSH1*) and *GSH2* that was observed in WT leaves. As GSH plays an important role in Cd-induced responses in *A. thaliana*, GSH levels were determined in leaves of WT and *sog1-7 A. thaliana* after 24 and 72 h of Cd exposure.

After 24 h of Cd exposure, a small decrease in total GSH content was observed in leaves of *sog1-7*, but not WT plants, whereas reduced GSH concentrations did not display any significant Cd-induced changes (Fig. 11A and B). After 72 h of Cd exposure, however, both total and reduced GSH concentrations increased in leaves of WT plants. In contrast, this response was absent in the *sog1-7* mutant (C and D). It should be noted that leaves of *sog1-7* plants contained higher total and reduced GSH concentrations as compared to leaves of WT plants under control conditions (Fig. 11C and D).

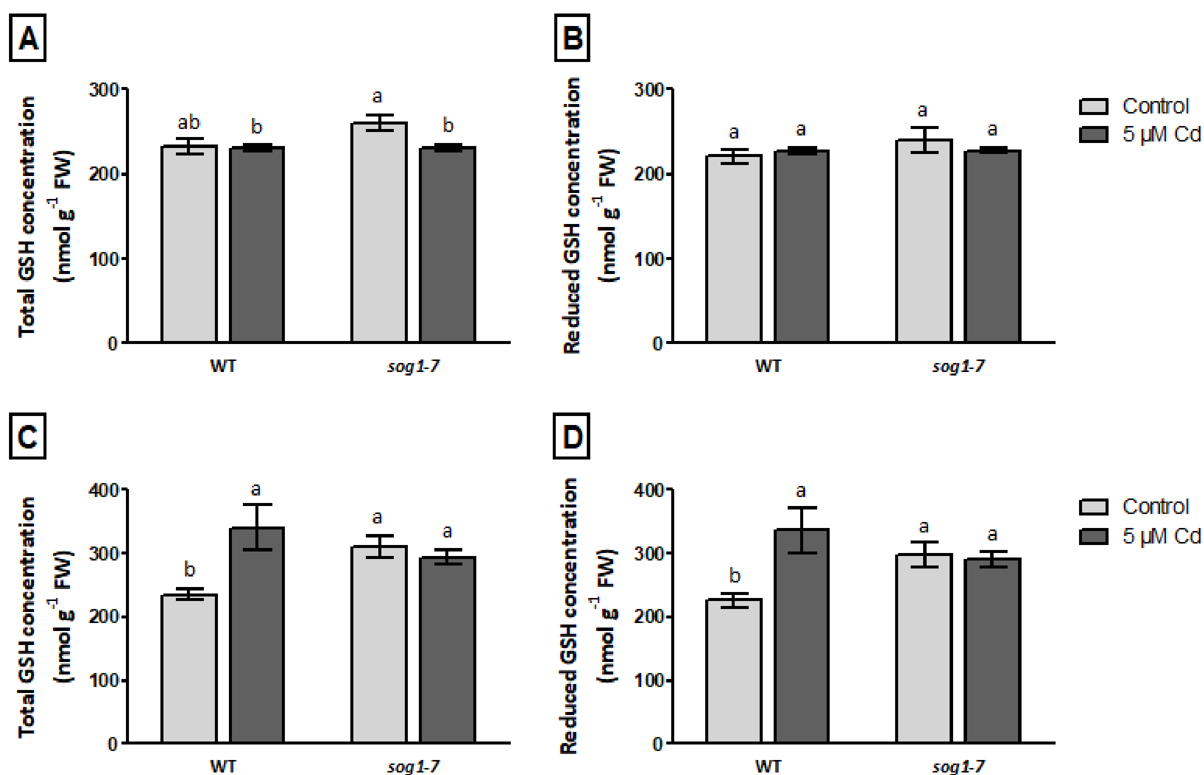

Figure 11 Concentrations (nmol g^{-1} FW) of (**A, C**) total glutathione (GSH + GSSG) and (**B, D**) reduced glutathione in leaves of WT and *sog1-7 A. thaliana* plants grown in hydroponics and exposed to 0 or 5 µM $CdSO_4$ for (**A**, **B**) 24 h or (**C**, **D**) 72 h, starting from day 19 after sowing. Data represent the average ± S.E. of 6 biological replicates. Significant differences ($p < 0.05$; 2-way ANOVA) are indicated using a different letter.

3.2.4 Influence of short-term cadmium exposure on the expression of DNA damage-related genes in leaves of wild-type and sog1-7 plants

As SOG1 causes cell cycle arrest in response to ROS-induced DNA damage, the extent of DNA damage was assessed by determining expression levels of DNA repair genes in leaves of Cd-exposed WT and *sog1-7* plants after 24 h and 72 h of exposure.

After 24 h of Cd exposure, gene expression of *DNA LIGASE IV* (*LIG4*), *POLY(ADP-RIBOSE) POLYMERASE 2* (*PARP2*) and *HOMOLOGUE OF X-RAY REPAIR CROSS COMPLEMENTING 1* (*XRCC1*) increased in leaves of WT plants (Table 4). In contrast, *POLY(ADP-RIBOSE) POLYMERASE 1* (*PARP1*) displayed a downregulation in WT plants. The *sog1-7* plants only displayed a significant Cd-induced upregulation of *LIG4* and *PARP2.* The fold change of the *LIG4* upregulation was however smaller in the *sog1-7* mutant (Table 4). In contrast, all of the analyzed DNA repair genes were upregulated in WT plants after 72 h of Cd exposure, whereas *sog1-7* plants only showed a significant upregulation of *PARP2* (Table 4). It should be noted that this induction was significantly smaller in leaves of *sog1-7* as compared to WT plants (Table 4).

Table 4 Expression of genes involved in DNA repair in leaves of WT and *sog1-7 A. thaliana* plants grown in hydroponics and exposed to 5 µM $CdSO_4$ for 24 or 72 h, starting from day 19 after sowing. Data are expressed relative to the control of the same genotype set at 1. Green and red colors indicate a significant Cd-induced up- and downregulations, respectively ($p < 0.05$; 2-way ANOVA within each time point). Asterisks (*) indicate a significantly different Cd-induced fold change in gene expression between both genotypes. Data represent the average ± S.E. of 5 biological replicates and were normalized based on the expression of *ACT2*, *MON1* and *YLS8*. *BRCA1*: breast cancer susceptibility 1; *LIG4*: DNA ligase IV; *PARP*: poly(ADP-ribose) polymerase; *RAD51*: DNA repair protein RAD51 homolog 1; *XRCC1*: homologue of X-ray repair cross complementing 1.

	24 h		72 h	
Gene	WT	*sog1-7*	WT	*sog1-7*
BRCA1	0.75 ± 0.10	1.29 ± 0.08	4.73 ± 0.78	1.72 ± 0.10*
LIG4	2.50 ± 0.14	1.63 ± 0.25*	1.71 ± 0.04	1.13 ± 0.08*
PARP1	0.61 ± 0.03	1.14 ± 0.08*	1.77 ± 0.20	1.18 ± 0.02*
PARP2	2.01 ± 0.03	1.84 ± 0.15	11.29 ± 2.35	2.39 ± 0.17*
RAD51	0.61 ± 0.06	1.42 ± 0.15*	3.75 ± 0.63	1.46 ± 0.07*
XRCC1	3.12 ± 0.20	1.43 ± 0.18*	1.65 ± 0.06	0.95 ± 0.02*

3.3 Effects of chronic cadmium exposure on growth of wild-type and *sog1-7 Arabidopsis thaliana*

As data from the previous two parts of the project indicate that the *sog1-7* mutant is less sensitive to Cd exposure, the response of this mutant after chronic exposure was examined. In order to do so, WT and *sog1-7 A. thaliana* plants were exposed to 0 or 5 µM $CdSO_4$, starting 19 DAS. From the day of exposure onwards, plants were monitored phenotypically throughout their lifetime by assessing vegetative and reproductive growth.

3.3.1 Influence of cadmium exposure on vegetative growth of wild-type and sog1-7 Arabidopsis thaliana plants

In order to determine the effects of Cd exposure on the vegetative growth of *A. thaliana* plants, rosette diameter was kinetically monitored throughout their lifetime and root weight was determined at the end of the experiment. Results display a clear Cd-induced effect on the rosette diameter of both genotypes (Fig. 12). The rosette diameter of control plants reached a plateau at 46 DAS, whereas rosettes of Cd-exposed plants stopped increasing in diameter shortly after the start of exposure. Under control conditions *sog1*-7 plants appeared to grow faster as compared to the WT, however, their final rosette diameter was similar resulting in a similar Cd-induced reduction of final rosette diameter in both genotypes (Fig. 12).

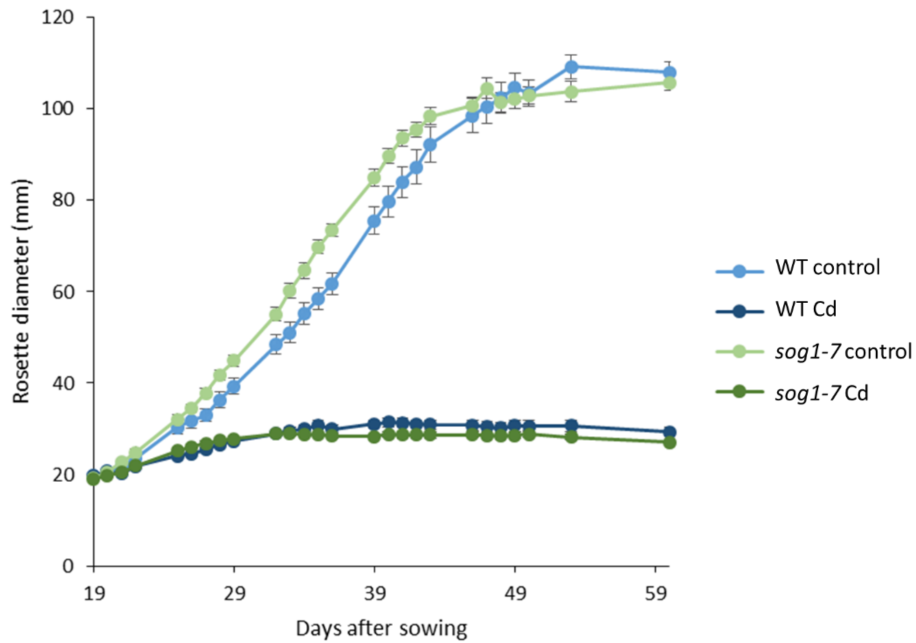

Figure 12 Rosette diameter (mm) of WT and *sog1-7 Arabidopsis thaliana* plants grown in hydroponics and continuously exposed to 0 or 5 µM $CdSO_4$, starting from day 19 after sowing. The diameter was measured at the widest point of the rosette. Data represent the average ± S.E. of 15 biological replicates.

Root weight was similarly affected by Cd exposure in both genotypes, with roots of Cd-exposed plants having a significantly lower fresh and dry weight as compared to their control counterparts (Fig. 13).

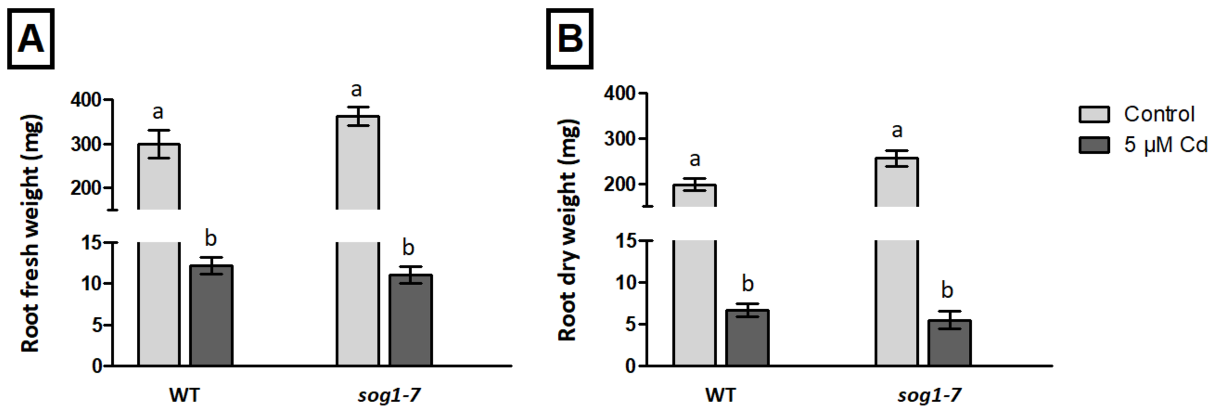

Figure 13 (**A**) Root fresh weight and (**B**) root dry weight WT and *sog1-7 Arabidopsis thaliana* plants grown in hydroponics and continuously exposed to 0 or 5 µM $CdSO_4$, starting from day 19 after sowing. The roots were harvested 95 days after sowing. Data represent the average ± S.E. of 15 biological replicates. Significant differences ($p < 0.05$; 2-way ANOVA) are indicated using a different letter.

3.3.2 Influence of cadmium on reproductive growth of wild-type and sog1-7 Arabidopsis thaliana plants

Reproductive growth was assessed by kinetically monitoring inflorescence height in control and Cd-exposed plants. Furthermore, the average seed weight was determined at the end of the experiment. The height of the inflorescence was clearly influenced by Cd exposure, as Cd-exposed plants of both genotypes had a smaller final inflorescence heights as compared to control plants of the same

genotype (Fig. 14). Under both control and Cd exposure conditions, the inflorescence of *sog1-7* mutants emerged earlier than that of WT plants. In control plants, this resulted in a larger final inflorescence height of *sog1-7* as compared to WT plants. When exposed to Cd, however, WT plants finally reached a larger inflorescence height than *sog1-7* mutants (Fig. 14). The inhibitory effect of Cd on inflorescence height was therefore more pronounced in *sog1-7* as compared to WT plants.

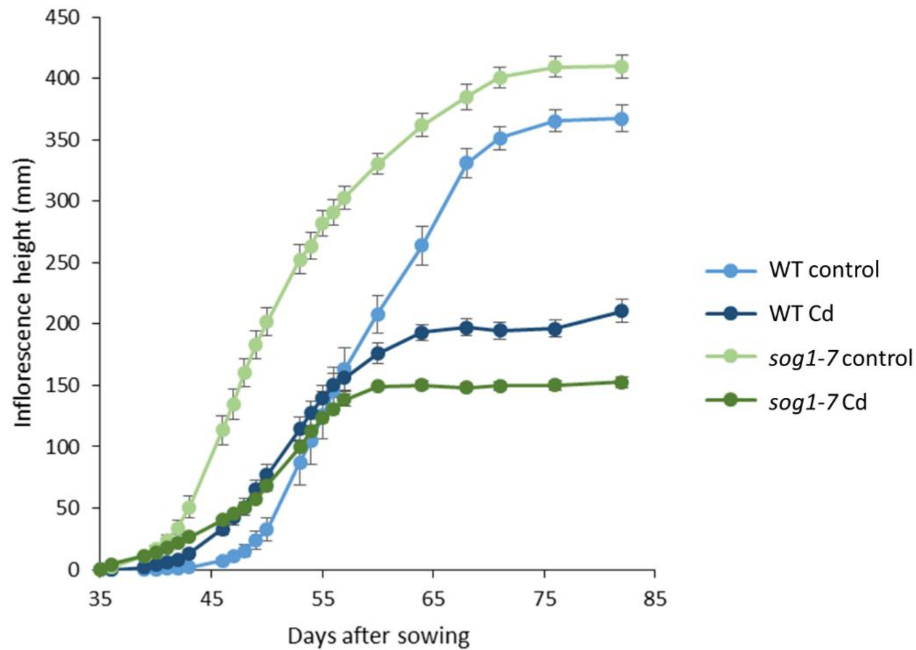

Figure 14 Inflorescence height of WT and *sog1-7 Arabidopsis thaliana* plants grown in hydroponics and continuously exposed to 0 or 5 µM $CdSO_4$, starting from day 19 after sowing. Data represent the average ± S.E. of 15 biological replicates.

Furthermore, seeds were collected to determine potential Cd- and genotype-induced effects on average seed weight. Results showed that neither Cd exposure nor genotype significantly affected seed weight (data not shown).

4 DISCUSSION

The aim of this study was to identify the involvement of SOG1, SMR4, SMR5 and SMR7 in Cd-induced cell cycle inhibition, oxidative stress and DNA damage in leaves of WT and *sog1-7 A. thaliana* plants. To this end, the effect of Cd exposure on the cell cycle in both genotypes was assessed using flow cytometry. Hereafter, oxidative stress and DNA damage, two underlying mechanisms, were assessed using gene expression analysis and glutathione determinations. Finally, the effects of chronic Cd exposure on vegetative and reproductive growth of WT and *sog1-7 A. thaliana* plants were assessed.

4.1 Cadmium-induced effects on leaf growth in wild-type, *smr4/5/7* and *sog1-7 Arabidopsis thaliana* plants

In the first part of the project, the effect of Cd exposure on leaf growth was assessed. This was done by addressing both macroscopic and microscopic effects. At the macroscopic level, rosette fresh weight, rosette diameter and surface area of individual rosette leaves were measured, whereas at the microscopic level, the concentration of nuclei in flow cytometry extracts and the endoreduplication factor were assessed. These parameters were investigated in WT, *smr4/5/7* and *sog1-7 A. thaliana* plants.

4.1.1 SOG1 is involved in the cadmium-induced inhibition of leaf growth in Arabidopsis thaliana plants after long-term exposure

Rosette fresh weight, rosette diameter and leaf surface area of control and Cd-exposed WT and *sog1-7* plants were determined in order to assess Cd-induced effects on leaf growth. Rosette fresh weight was lower in Cd-exposed WT and *smr4/5/7* plants as compared to their control counterparts. This effect was linked to the rosette diameter, which was smaller in Cd-exposed WT and *smr4/5/7* plants. Previous results from Keunen *et al.* (2011) indicate a similar Cd-induced reduction in rosette diameter of WT plants (22). These responses were, however, absent in the *sog1-7* mutant, with both rosette weight and diameter being unaffected by Cd exposure. These data indicate that the *sog1-7* mutant is less sensitive to Cd.

The effects of Cd exposure on leaf surface area displayed the same trend, with leaves of Cd-exposed WT and *smr4/5/7* plants having smaller surface areas as compared to their control counterparts. In contrast, this response was absent or less pronounced in the *sog1-7* mutant, hereby confirming the reduced Cd sensitivity of this mutant. A similar reduction in the size of the first leaves of WT, *smr5* and/or *smr7* knockout mutants as a consequence of exposure to the replication inhibitory drug HU was previously demonstrated (19).

Cadmium-exposed WT and *smr4/5/7* have a lighter rosette weight, smaller rosette diameter and leaf surface area. The absence of this response in the *sog1-7* mutant indicates that SOG1 is involved in the Cd-induced reduction of leaf growth. The similar response in *smr4/5/7* and WT plants, however, suggests that the SMR proteins are not involved in this process. As the cell cycle is an important process in leaf growth and development, it is important to address it in the different genotypes after Cd exposure to gain more insight in the molecular mechanisms underlying the Cd-induced growth inhibition.

4.1.2 SOG1 is involved in cadmium-induced effects on cell division and endoreduplication in leaves of Arabidopsis thaliana

To determine the microscopic effects of Cd exposure on leaf growth, the concentration of nuclei in flow cytometry extracts and the endoreduplication factor were determined in leaves of Cd-exposed WT, *smr4/5/7* and *sog1*-7 mutant plants. In general, the concentration of nuclei was lower in Cd-exposed WT and *smr4/5/7* plants as compared to their control counterparts, whereas for the *sog1-7* mutant this was only the case in leaf 3, further confirming the reduced sensitivity of this mutant to Cd exposure. These results are in agreement with a previously demonstrated reduction in epidermal cell number in WT, *smr5* and/or *smr7* knockout *A. thaliana* plants following HU exposure (19).

In addition, the extent of endoreduplication was determined based on the endoreduplication factor, which reflects the average number of endocycles per cell. In leaves 1 and 3, the endoreduplication factor decreased after Cd exposure in WT and *smr4/5/7* plants, but not in the *sog1-7* mutant. In leaf 6, however, the endoreduplication factor remained unaltered after Cd exposure in WT plants and displayed a decrease and increase in *smr4/5/7* and *sog1-7* mutants, respectively. As Cd exposure had little effect on the extent of endoreduplication in the *sog1-7* mutant as compared to the other genotypes, the reduced Cd sensitivity of this mutant was again confirmed. The observed Cd-induced inhibition of the cell cycle and endocycle are in accordance with preliminary data of our research group.

From these results, it can be concluded that the effects of Cd on leaf growth, cell division and endoreduplication are likely mediated by SOG1. However, these effects are presumably not regulated via SMR4, 5 and 7, as Cd-exposed *smr4/5/7* plants generally showed similar Cd-induced responses as compared to the WT. However, it cannot be excluded that a bypass mechanism is activated in the *smr4/5/7* mutant, with other CDK inhibitors from the INHIBITOR/INTERACTOR OF CYCLIN-DEPENDENT KINASES/KIP-RELATED PROTEIN (ICK/KRP) or SMR family compensating for the loss of functional SMR4, 5 and 7 (26,27). This should be further investigated in future experiments. The *smr4/5/7* mutant was not included in the following parts of the project.

4.2 Cadmium-induced effects on oxidative stress and DNA damage repair in leaves of wild-type and *sog1-7 Arabidopsis thaliana* plants

Previous research demonstrated the activation of SOG1 by ROS-induced DNA damage during HU stress and its impact on the cell cycle (19). Therefore, the second part of the project focused on oxidative stress and DNA damage as possible underlying mechanisms for the observed effects on the cell cycle. To do so, the expression of oxidative stress-related and DNA repair genes was determined in leaves of WT and *sog1-7 A. thaliana* plants after 24 or 72 h of Cd exposure. This shorter exposure duration was used as gene expression is known to transiently react to Cd exposure after short-term exposure (28,29). Additionally, preliminary data demonstrated little to no difference in the expression of multiple genes, analyzed in this project, between control and Cd-exposed plants after 8 days of exposure.

4.2.1 SOG1 does not affect leaf cadmium concentrations of Arabidopsis thaliana plants after short-term exposure

Prior to investigating Cd-induced effects on the expression of oxidative stress-related and DNA repair genes, leaf growth was determined in the short-term exposure setup to confirm the reduced Cd sensitivity of the *sog1-7* mutant. To do so, rosette fresh and dry weight were determined.

In contrast to the observations after 8 days of exposure, rosette fresh weight of WT and *sog1*-7 plants was not influenced after 24 or 72 h of Cd exposure. The absence of a significant Cd-induced effect on rosette fresh weight after short-term exposure was reported in other studies (28,30). A possible explanation is that the Cd-induced effects on leaf growth accumulate over time, resulting in more pronounced effects after longer exposure. This is in accordance with previous findings, demonstrating that Cd only significantly affected vegetative growth from 72 h of exposure onward (22,29,30). It is interesting to note, however, that a Cd-induced decreasing trend in rosette fresh weight was observed in WT plants, whereas this trend was absent in *sog1-7* plants. These data again confirm the reduced Cd sensitivity of *sog1-7* plants.

Furthermore, the percentage of dry weight in Cd-exposed WT and *sog1-7* plants was determined after 24 and 72 h of exposure. This parameter did not significantly differ between WT and *sog1-7* plants exposed to Cd. After 72 h of exposure, however, the percentage of dry weight increased in both genotypes. This is in accordance with findings of Keunen *et al.* (2016), who demonstrated a similar increase in the percentage of dry weight in leaves of *A. thaliana* exposed to 5 or 10 µM Cd for 72 h (31). The combination of a reduced rosette fresh weight and increased percentage of dry weight after Cd exposure indicate a potential Cd-induced change in the plant's water status. This could be either due to a reduced water uptake or an increased water loss caused by Cd exposure. Alternatively, a Cd-

induced increase in production of organic matter could be responsible for the observed changes. In order to determine the cause of these changes, additional experiments should be performed.

In order to ascertain that differences in Cd-induced responses between WT plants and *sog1-7* mutants were due to a reduced Cd sensitivity of the *sog1-7* mutant and not due to a reduced Cd uptake, the Cd concentration in leaves of WT and *sog1-7* plants was assessed. No significant differences in leaf Cd concentrations were detected between both genotypes after 24 or 72 h of exposure.

Taken together, these data indicate that the reduced Cd sensitivity of the *sog1-7* mutant is also present after short-term exposure and that the reduced Cd sensitivity of the mutant is not due to lower leaf Cd concentrations as compared to WT plants.

4.2.2 SOG1 regulates the expression of SIAMESE-RELATED genes in Arabidopsis thaliana leaves after cadmium exposure

In order to determine whether transcript levels of *SOG1* changed during Cd stress and whether or not SOG1 was responsible for the induction of *SMR4, SMR5* and *SMR7*, gene expression analysis was performed in leaves of WT and *sog1-7 A. thaliana* plants exposed to Cd for 24 or 72 h.

After 24 h of Cd exposure, both *SMR5* and *SOG1* were upregulated in leaves of WT plants. After 72 h of exposure, *SOG1* and all *SMR* genes analyzed were upregulated in WT plants. In the *sog1-7* mutant, only *SMR4* and *SOG1* displayed an upregulation after 24 h of exposure, whereas expression of none of the genes was significantly changed after 72 h. The upregulation of *SOG1* and *SMR* genes in Cd-exposed WT plants and the absence of this response in the *sog1-7* mutant, indicate that SOG1 is involved in the regulation of *SMR4*, *SMR5* and *SMR7* expression during Cd stress. This is in accordance with previous findings identifying all three *SMR* genes as target genes of SOG1 (32). Additionally, Yi *et al.* (2014) demonstrated that SMR5 and SMR7 are controlled by SOG1 after DNA damage induced by HU exposure. Furthermore, they also demonstrated that SMR5 and SMR7 were involved in cell cycle regulation in response to HU-induced ROS. In contrast, flow cytometry measurements from this project, indicated that this was not the case for Cd-induced ROS, as the *smr4/5/7* mutant generally showed similar Cd-induced responses as compared to the WT. These differences can originate from the different ROS-producing agents or growth conditions. Finally, it is remarkable that *SOG1* expression is upregulated after Cd exposure, as SOG1 is reported to be regulated at the post-translational level by phosphorylation (19,32).

4.2.3 SOG1 is involved in the cadmium-induced oxidative stress response in Arabidopsis thaliana leaves

In order to investigate the involvement of SOG1 in the Cd-induced oxidative stress response, the expression of genes encoding antioxidative enzymes and levels of the antioxidative metabolite GSH were determined in leaves of Cd-exposed WT and *sog1-7 A. thaliana* plants.

The extent of Cd-induced oxidative stress was assessed by measuring five oxidative stress hallmark genes, characterized by a more than 5-fold upregulation in response to different types of oxidative-stress inducing agents (11). These genes were significantly upregulated by Cd exposure in WT plants, as previously demonstrated (29), but not or to a lesser extent in *sog1-7* plants. The smaller Cd-induced upregulation of the oxidative stress hallmark genes in the *sog1-7* mutant as compared to the WT, indicates that SOG1 is most likely involved in the oxidative stress response induced by Cd exposure.

As plants respond to oxidative stress by stimulating their antioxidative defense mechanisms, the expression of genes encoding antioxidative enzymes was also determined. Transcript levels of three genes encoding superoxide dismutase (SOD) enzymes (*CSD1*, *CSD2* and *IRON SUPEROXIDE DISMUTASE* (*FSD1)*), responsible for the conversion of $O_2^{\bullet-}$ to H_2O_2 were measured (8,12). The expression of these genes was mostly unaffected by Cd exposure in WT plants and was downregulated in the *sog1-7* mutant. These results are in accordance with previous findings of Smeets *et al.* (2008), who demonstrated that the main cause of oxidative stress in leaves of *A. thaliana* exposed to 5, 10 or 20 µM Cd was not $O_2^{\bullet-}$, but H_2O_2 (33).

A second type of antioxidative genes assessed were AsA peroxidases (*APX1* and *APX2*) and catalases (*CAT1*, *CAT2* and *CAT3*), which are responsible for H_2O_2 scavenging (12). The stress-inducible *APX2* was upregulated in WT plants after 24 and 72 h of Cd exposure, indicating that the plants defend themselves against Cd-induced oxidative stress. The more pronounced effect of Cd on *APX2* as compared to *APX1* has previously been demonstrated by Jozekczak *et al.* (2014) (34). This response was less pronounced in the *sog1-7* mutant, which is in accordance with the reduced expression of the oxidative stress marker genes. After 24 h, the expression of *CAT1* and *CAT3* was upregulated in leaves of WT plants, whereas *CAT2* was downregulated. After 72 h of exposure, only *CAT2* was still significantly downregulated. This response was mostly absent in the *sog1-7* mutant, which is again in agreement with the reduced expression of the oxidative stress markers genes, indicating that SOG1 is involved in the Cd-induced oxidative challenge. The upregulations of peroxidases and catalases in the WT plants indicate that H_2O_2 is likely a key player in the Cd-induced oxidative stress response in leaves, as previously demonstrated (33,35). In order to confirm this hypothesis, H_2O_2 levels should be measured in Cd-exposed WT and *sog1-7* plants in future experiments.

Furthermore, the expression of *GSH1* and *GSH2*, both involved in the biosynthesis of GSH, was measured (13). After 24 h of Cd exposure both *GSH1* and *GSH2* were upregulated in WT plants, whereas only *GSH2* remained upregulated after 72 h of exposure. These results are in accordance with previous findings from Schellingen *et al.* (2015) (29). This response was also absent in the *sog1-7* mutant, confirming that SOG1 is likely involved in the plant response to Cd-induced oxidative stress. As alterations in gene expression levels do not necessarily correspond to differences in protein levels and enzyme activities, it is important to measure the activity of these enzymes in Cd-exposed WT and *sog1-7* plants in a future experiment.

In order to determine whether the absence of a Cd-induced upregulation of genes involved in GSH biosynthesis in the *sog1-7* mutant led to differences at the metabolite level, GSH concentrations were determined in leaves of WT and *sog1-7 A. thaliana* after 24 and 72 h of Cd exposure. A small decrease in total GSH content was observed in *sog1-7* plants, but not in the WT, after 24 h of exposure. After 72 h of Cd exposure, however, both total GSH and reduced GSH concentrations were increased by Cd exposure in WT plants. This is in accordance with results for Jozefczak *et al.* (2015), who observed similar changes in GSH concentrations in *A. thaliana* leaves after 72 h of exposure to 1 and 5 µM Cd (14). The increased concentration of total GSH in Cd-exposed WT plants after 72 h of exposure is most likely a result of the upregulated *GSH1* and *GSH2* expression after 24 h of exposure. As it takes some time for the increased gene expression to be translated to increased enzyme levels and subsequently increased GSH biosynthesis, it is possible that an increase in total GSH in only observed after 72 h of exposure. The increased levels of reduced GSH could also be due to an increased GR activity in response to Cd exposure. In order to confirm this, GR expression and/or activity levels should be measured. The absence of a Cd-induced increase in GSH concentrations in the *sog1-7* mutant suggests that SOG1 is involved in regulating GSH biosynthesis and antioxidative defense in leaves upon Cd exposure. Under control conditions, the *sog1-7* mutant had higher concentrations of total and reduced GSH as compared to the WT plants. However, this was not caused by differences in the expression of *GSH1* and *GSH2* between leaves of both genotypes. A possible explanation is that GSH1 and/or GSH2 activity in the *sog1-7* mutant are increased by regulation at the posttranscriptional level. Additionally, it is also possible that GSH degradation is reduced in the *sog1-7* mutant. In order to confirm this, however, additional experiments should be performed.

4.2.4 SOG1 is involved in the DNA damage response in Arabidopsis thaliana leaves after cadmium exposure

As Cd-induced oxidative stress can result in DNA damage, the expression of genes involved in DNA repair, known to be markers of DNA damage (11), was determined in leaves of Cd-exposed WT and *sog1-7* plants.

After 24 h of Cd exposure, *LIG4*, *PARP2* and *XRCC1* displayed an upregulated expression. In contrast, the *sog1-7* mutant only displayed an upregulation of *LIG4* and *PARP2*, which was less pronounced as compared to that in the WT. After 72 h of Cd exposure, however, all DNA repair genes analyzed were upregulated in leaves of WT plants. In the *sog1-7* mutant, only *PARP2* was upregulated and the induction of all DNA repair genes was significantly smaller in *sog1-7* as compared to WT leaves. These results suggest that Cd exposure causes DNA damage, as previously demonstrated (36,37). The overall absence of this response in the *sog1-7* mutant indicates that SOG1 is involved in the Cd-induced DNA damage response. This is in accordance with previous findings where *BREAST CANCER SUSCEPTIBILITY 1* (*BRCA1*), *PARP2* and *DNA REPAIR PROTEIN RAD51 HOMOLOG 1* (*RAD51*) expression increased in WT plants, but not in a mutant lacking functional SOG1, after exposure to different DNA-damaging agents (32,38). In addition, Ogita *et al.* (2018) identified *BRCA1*, *PARP1*, *PARP2* and *RAD51* as SOG1 target genes, which explains the fact that these genes were not upregulated by Cd exposure in the *sog1-7* mutant. The expression of DNA repair genes is, however, is an indirect measure of the extent of DNA damage caused by Cd exposure. In order to further investigate the involvement of SOG1 in Cd-induced DNA damage and repair, direct measurements of DNA damage, for example using the comet assay, should be performed in Cd-exposed WT and *sog1-7* plants in future experiments.

4.3 Chronic cadmium-induced effects on growth and development of wild-type and *sog1-7 Arabidopsis thaliana* plants

In the third part of this project, the increased Cd sensitivity of the *sog1-7* mutant was assessed during chronic Cd exposure. To this end, 19-days-old WT and *sog1-7* seedlings were exposed to 0 or 5 µM $CdSO_4$ and monitored phenotypically throughout their lifetime by assessing vegetative and reproductive growth.

4.3.1 SOG1 is involved in the early response to cadmium exposure in Arabidopsis thaliana

In order to assess the effects of Cd on the vegetative growth of WT and *sog1-7* plants, rosette diameter was kinetically monitored throughout their lifetime. Additionally, root weight was determined at the end of the experiment.

The rosette diameter of the *sog1-7* mutant displayed a Cd-induced reduction, but not to a different extent as compared to the WT. Under control conditions, however, the *sog1-7* mutant appeared to grow faster as compared to the WT, but their final rosette diameter was similar. The results obtained in WT plants are in accordance with previous findings of Keunen *et al.* (2011) and Schellingen *et al.* (2015), who described a similar Cd-induced reduction in final rosette diameter after chronic exposure to 5 µM Cd (22,39). The reduced sensitivity of the *sog1-7* mutant observed after short- and long-term Cd exposure, disappeared during chronic exposure. This suggests that SOG1 has a role in the early responses of *A. thaliana* to Cd exposure. These results are, again, confirmed by the similar Cd-induced reductions in root fresh and dry weight of chronically exposed WT and *sog1-7* plants.

4.3.2 Reproductive growth of the sog1-7 mutant is more sensitive to cadmium

The influence of Cd exposure on the reproductive growth of WT and *sog1-7* plants was addressed by kinetically monitoring inflorescence height in both control and Cd-exposed plants.

Inflorescence height was clearly influenced by Cd exposure in both genotypes as Cd-exposed plants had smaller final inflorescence heights as compared to their control counterparts. These results are in accordance with findings of Keunen *et al.* (2011), demonstrating a similar Cd-induced reduction in final inflorescence height in WT plants after exposure to 5 µM Cd (22). Under both control and Cd exposure conditions, the inflorescence of the *sog1-7* mutant emerged earlier than that of WT plants. Under control conditions, this resulted in a larger final inflorescence height of *sog1-7* as compared to WT plants. In contrast, Cd-exposed *sog1-7* plants reached a smaller final inflorescence height than WT plants. Consequently, the inhibitory effect of Cd on inflorescence height was more pronounced in the *sog1-7* mutant. A possible explanation for these results can be found in the importance of SOG1 in the regulation of the DNA damage response. After DNA damage, SOG1 responds by arresting the cell cycle and inducing DNA repair, in order to prevent the replication and transmission of damaged DNA to daughter cells (19–21). As the *sog1-7* mutant lacks functional SOG1, it is likely that this response is absent in this genotype, which is confirmed by the *sog1-7* mutant lacking a Cd-induced induction of DNA repair genes. If *sog1-7* plants are chronically exposed to Cd, known to cause ROS-induced DNA damage, DNA damage and mutations could continuously accumulate without being repaired, eventually impairing plant reproduction. This is, however, a hypothesis that needs to be further investigated in future experiments.

CONCLUSION

The aim of this study was to identify the involvement of SOG1, SMR4, SMR5 and SMR7 in Cd-induced oxidative stress, DNA damage and cell cycle inhibition in leaves of *A. thaliana* plants. In order to do so, WT, *smr4/5/7* and *sog1-7 A. thaliana* plants were exposed to 5 µM $CdSO_4$ in a short-term, long-term and chronic setup.

In a first part of the project leaf growth parameters and the extent of cell division and endoreduplication were assessed. To this end, rosette fresh weight, rosette diameter, leaf surface area, the concentration of nuclei in flow cytometry extracts and the endoreduplication factor were determined in leaves of WT, *smr4/5/7* and *sog1-7* plants exposed to 5 µM $CdSO_4$ for 8 days, starting from day 14 after sowing. Results indicated that the *sog1-7* mutant had a reduced Cd sensitivity as compared to the other genotypes, as both growth and cell cycle-related parameters displayed little to no responses to Cd exposure. Therefore, it is likely that SOG1 is involved in the Cd-induced inhibition of leaf growth, cell division and endoreduplication. However, these effects are presumably not regulated via SMR4, SMR5 and SMR7, as the Cd-induced responses of the *smr4/5/7* mutant were highly similar to those of the WT. However, it cannot be excluded that a bypass mechanism is activated in the *smr4/5/7* mutant, with other CDK inhibitors from the ICK/KRP or SMR family compensating for the loss of functional SMR4, 5 and 7. In order to confirm this hypothesis, further experiments are needed. The *smr4/5/7* mutant was not used in the subsequent parts of the project.

The second part of the project aimed to identify the molecular mechanisms underlying the reduced Cd sensitivity of the *sog1-7* mutant. To this end, ROS-induced DNA damage was assessed in leaves of Cd-exposed WT and *sog1-7* plants by determining the expression of oxidative stress and DNA damage-related genes and measuring GSH levels. Prior to investigating any of these parameters, Cd concentrations in leaves of both genotypes were determined. Results showed that there were no significant differences in leaf Cd concentrations between WT and *sog1-7* plants, indicating that the reduced Cd sensitivity of the *sog1*-7 mutant was not due to a reduced Cd uptake. In addition, the expression of *SOG1*, *SMR4*, *SMR5* and *SMR7* was assessed in Cd-exposed WT and *sog1-7* plants. The results indicated that SOG1 is involved in the regulation of SMR4, SMR5 and SMR7 during Cd stress.

Gene expression analysis of oxidative stress marker genes, genes encoding antioxidative enzymes and genes involved in DNA repair indicated that SOG1 is most likely involved in the Cd-induced oxidative challenge and DNA damage response. Additionally, expression levels of GSH biosynthesis genes and GSH levels indicated that SOG1 is involved in regulating GSH biosynthesis and antioxidative defense upon Cd exposure. However, as alterations in gene expression levels do not necessarily correlate to

changes in protein levels and enzyme activities, it is necessary to measure the activity of these enzymes in Cd-exposed WT and *sog1-7* plants in future experiments.

The final part of the project investigated whether the reduced Cd sensitivity of the *sog1-7* mutant could also be observed during chronic Cd exposure. To this end, Cd-exposed WT and *sog1-7* plants were phenotypically monitored throughout their lifetime. The effects on vegetative growth were assessed by determining root weight and rosette diameter. Neither of these parameters demonstrated a clear difference between WT and *sog1-7* plants, suggesting that SOG1 is mainly involved in the early response of *A. thaliana* to Cd exposure, as confirmed at the molecular level in the first two parts of the project. In addition, the Cd-induced effects on reproductive growth were assessed by determining the inflorescence height. The Cd-induced reduction in final inflorescence height was more pronounced in the *sog1-7* mutant as compared to the WT, indicating that it is more sensitive to Cd. This could be due to an accumulation of DNA damage in the *sog1-7* mutant, as gene expression analysis demonstrated that DNA repair is not activated upon Cd exposure in this mutant. However, this hypothesis needs to be further investigated in future experiments, for example by directly determining the extent of DNA damage by the comet assay.

The initial hypothesis of the project stating that SOG1, SMR4, SMR5 and SMR7 mediate Cd-induced effects on oxidative stress, DNA damage and cell cycle progression in leaves of *A. thaliana* cannot fully be confirmed. It is obvious from that SOG1 plays an important role in the early response of *A. thaliana* leaves to Cd, as results indicate that it is involved in the Cd-induced inhibition of leaf growth, cell division and endoreduplication, the Cd-induced oxidative stress and DNA damage response and GSH biosynthesis upon Cd exposure. Furthermore, lack of functional SOG1 impairs reproductive growth during chronic Cd exposure. The role of the SMR proteins, on the other hand, is less clear as the effects of Cd exposure on leaf growth, cell division and endoreduplication displayed little to no differences between Cd-exposed *smr4/5/7* and WT plants. These data suggest that the SMR proteins are not involved in regulating Cd-induced effects on leaf growth and the cell cycle. Nevertheless, it is possible that a bypass mechanism is activated in this mutant, thereby masking the importance of these molecules in Cd-induced stress responses. Therefore, the involvement of the SMR proteins should be further investigated in future studies.

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SUPPLEMENTAL DATA

Table S1: Element content of WT and *sog1-7 A. thaliana* leaves after 24 and 72 h of Cd exposure

Table S1 Elemental concentrations (mg kg^{-1} DW) in leaves of hydroponically grown WT and *sog1-7 A. thaliana* plants exposed to 0 or 5 µM $CdSO_4$ for 24 or 72 h, starting from day 19 after sowing. Data represent the average ± S.E. of 3 biological replicates. Significant differences ($p < 0.05$; 2-way ANOVA) are indicated using a different letter.

24 h	WT		*sog1-7*	
	Control	5 µM Cd	Control	5 µM Cd
Na	323.46 ± 33.86[a]	409.76 ± 66.77[a]	543.64 ± 21.95[a]	422.73 ± 21.62[a]
Mg	7754.75 ± 379.16[a]	7520.03 ± 199.20[ab]	6485.95 ± 258.41[bc]	5775.19 ± 159.31[c]
P	11991.80 ± 121.51[c]	12198.29 ± 57.91[bc]	13771.16 ± 357.76[a]	12899.91 ± 107.75[ab]
S	10434.10 ± 90.73[a]	10558.78 ± 172.79[a]	9407.48 ± 169.28[b]	9817.85 ± 22.14[b]
K	30094.77 ± 2096.12[b]	31170.18 ± 937.01[ab]	35019.82 ± 494.78[ab]	35878.79 ± 670.67[a]
Ca	37501.06 ± 1340.99[a]	35928.50 ± 109.76[a]	34625.01 ± 1180.84[ab]	30468.19 ± 480.37[b]
Mn	275.46 ± 14.63[a]	235.34 ± 3.34[ab]	241.46 ± 15.38[ab]	206.37 ± 2.21[b]
Cu	8.31 ± 1.33[a]	6.11 ± 0.12[a]	7.24 ± 1.30[a]	4.15 ± 0.38[a]
Zn	80.97 ± 8.14[a]	70.99 ± 2.79[a]	93.09 ± 7.33[a]	76.91 ± 4.72[a]
72 h	**WT**		***sog1-7***	
	Control	**5 µM Cd**	**Control**	**5 µM Cd**
Na	771.00 ± 217.02[a]	552.22 ± 41.83[a]	544.25 ± 41.29[a]	510.68 ± 28.04[a]
Mg	8002.61 ± 271.03[a]	8035.88 ± 148.69[ac]	6594.54 ± 103.70[bc]	6794.45 ± 432.95[b]
P	13656.32 ± 295.31[ab]	12013.99 ± 405.15[b]	14803.21 ± 407.04[a]	13464.64 ± 658.92[ab]
S	10815.96 ± 204.03[bc]	13030.81 ± 597.74[ac]	9588.68 ± 292.12[b]	13350.33 ± 711.90[a]
K	31770.77 ± 692.11[a]	32623.05 ± 1372.71[a]	33509.96 ± 1529.39[a]	34918.44 ± 1403.72[a]
Ca	38924.24 ± 1338.83[a]	33201.51 ± 778.16[b]	34280.57 ± 1300.09[ab]	29984.86 ± 1465.92[b]
Mn	260.09 ± 31.85[a]	219.24 ± 10.58[a]	222.93 ± 21.41[a]	190.41 ± 11.91[a]
Cu	6.94 ± 1.20[a]	4.75 ± 0.32[a]	6.23 ± 0.84[a]	3.78 ±0.47[a]
Zn	99.85 ± 11.26[a]	56.19 ± 4.32[b]	89.29 ± 11.86[ab]	70.23 ± 1.17[ab]

Table S2: Gene expression of WT and *sog1-7* plants under control conditions

Table S2 Gene expression in leaves of WT and *sog1-7 A. thaliana* plants grown in hydroponics under control conditions for comparison to plants exposed to 5 µM $CdSO_4$ for 24 or 72 h, starting from day 19 after sowing. Data of *sog1-7* mutants are expressed relative to WT plants set at 1. A green color indicates a significant genotype-induced upregulation ($p < 0.05$; 2-way ANOVA within each time point). Data represent the average ± S.E. of 5 biological replicates and were normalized based on the expression of *ACT2*, *MON1* and *YLS8*. *APX:* ascorbate peroxidase; *BRCA1*: breast cancer susceptibility 1; *CAT*: catalase; *CSD*: copper/zinc superoxide dismutase; *Defensin*: defensin-like protein; *FSD1:* iron superoxide dismutase; *GSH1:* γ-glutamylcysteine synthetase; *GSH2*: glutathione synthetase; *LIG4*: DNA ligase IV; *PARP*: poly(ADP-ribose) polymerase; *RAD51*: DNA repair protein RAD51 homolog 1; *SMR*: SIAMESE-related; *SOG1:* SUPPRESSOR OF GAMMA RESPONSE 1; *TIR*: toll/interleukin receptor 1; *UPOX:* upregulated by oxidative stress; *XRCC1*: homologue of X-ray repair cross complementing 1.

		24 h		72 h	
	Gene	WT	*sog1-7*	WT	*sog1-7*
Project involved genes	*SMR4*	1.00 ± 0.12	1.89 ± 0.30	1.00 ± 0.09	3.62 ± 0.70
	SMR5	1.00 ± 0.09	1.28 ± 0.02	1.00 ± 0.05	1.01 ± 0.03
	SMR7	1.00 ± 0.06	0.87 ± 0.02	1.00 ± 0.09	0.92 ± 0.09
	SOG1	1.00 ± 0.08	1.13 ± 0.04	1.00 ± 0.11	1.49 ± 0.14
Oxidative stress	*AT1G05340*	1.00 ± 0.25	2.43 ± 0.85	1.00 ± 0.24	1.11 ± 0.41
	AT1G19020	1.00 ± 0.24	0.93 ± 0.17	1.00 ± 0.46	1.09 ± 0.36
	Defensin	1.00 ± 0.21	1.09 ± 0.18	1.00 ± 0.17	1.10 ± 0.20
	TIR	1.00 ± 0.35	2.59 ± 0.95	1.00 ± 0.46	3.24 ± 1.57
	UPOX	1.00 ± 0.07	1.48 ± 0.14	1.00 ± 0.04	2.72 ± 0.05
Antioxidants	*APX1*	1.00 ± 0.09	1.08 ± 0.09	1.00 ± 0.06	1.33 ± 0.20
	APX2	1.00 ± 0.10	3.43 ± 0.74	1.00 ± 0.09	3.95 ± 0.47
	CAT1	1.00 ± 0.06	1.30 ± 0.13	1.00 ± 0.10	1.65 ± 0.28
	CAT2	1.00 ± 0.09	1.01 ± 0.07	1.00 ± 0.04	0.94 ± 0.07
	CAT3	1.00 ± 0.03	0.96 ± 0.07	1.00 ± 0.09	0.93 ± 0.00
	CSD1	1.00 ± 0.15	1.02 ± 0.15	1.00 ± 0.21	1.66 ± 0.14
	CSD2	1.00 ± 0.04	0.85 ± 0.16	1.00 ± 0.19	0.92 ± 0.07
	FSD1	1.00 ± 0.04	0.74 ± 0.28	1.00 ± 0.32	0.33 ± 0.10
	GSH1	1.00 ± 0.04	0.92 ± 0.04	1.00 ± 0.04	0.98 ± 0.03
	GSH2	1.00 ± 0.06	0.92 ± 0.03	1.00 ± 0.03	1.10 ± 0.02
DNA repair	*BRCA1*	1.00 ± 0.01	1.10 ± 0.07	1.00 ± 0.06	1.47 ± 0.06
	LIG4	1.00 ± 0.05	1.04 ± 0.04	1.00 ± 0.05	1.26 ± 0.12
	PARP1	1.00 ± 0.02	0.96 ± 0.04	1.00 ± 0.03	1.39 ± 0.08
	PARP2	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.02	1.07 ± 0.08
	RAD51	1.00 ± 0.03	1.19 ± 0.13	1.00 ± 0.08	1.58 ± 0.03
	XRCC1	1.00 ± 0.09	1.11 ± 0.09	1.00 ± 0.09	1.24 ± 0.26

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Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
The involvement of SIAMESE-RELATED proteins and SUPPRESSOR OF GAMMA RESPONSE 1 in cadmium-induced stress responses in <i>Arabidopsis thaliana </i>leaves

Richting: **Master of Biomedical Sciences-Environmental Health Sciences**
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