Ghent University

Faculty of Sciences

Department of Plant Biotechnology and Genetics

IDENTIFICATION OF SIGNALING COMPONENTS THAT ACTIVATE THE DNA DAMAGE CELL CYCLE CHECKPOINT IN PLANTS

Dalong Yi

Promotor: Prof. Lieven De Veylder

25th of Feb 2014

VIB, Plant Systems Biology

Cell Cycle Group

Technologiepark 927, B-9000 Ghent, Belgium

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衣大郎

This work was conducted in the Plant Systems Biology department (PSB) of the Flanders Institute for Biotechnology (VIB).

This work was supported by a PhD grant from the China Scholarship Council (CSC).

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Jury members

Promotor

Prof. Dr. Lieven De Veylder
Department of Plant Biotechnology and Genetics
Ghent University
E-mail: livey@psb.vib-ugent.be

Promotion commission

Prof. Dr. Sofie Goormachtig (chair)
Department of Plant Biotechnology and Genetics
Ghent University
E-mail: sogoo@psb.vib-ugent.be

Prof. Dr. Crisanto Gutierrez
Centro de Biologia Molecular "Severo Ochoa"
CSIC-UAM
E-mail: cgutierrez@cbm.uam.es

Prof. Dr. Geert Berx
Inflammation Research Center
Ghent University
E-mail: Geert.Berx@irc.vib-UGent.be

Prof. Dr. Tom Beeckman
Department of Plant Biotechnology and Genetics
Ghent University
E-mail: Tom.Beeckman@psb.vib-ugent.be

Prof. Dr. Alain Goossens
Department of Plant Biotechnology and Genetics
Ghent University
E-mail: algoo@psb.vib-ugent.be

Prof. Dr. Moritz Nowack
Department of Plant Biotechnology and Genetics
Ghent University
E-mail: monow@psb.ugent.be

Dr.Marie Huysman
Department of Plant Biotechnology and Genetics
Ghent University
E-mail: mahuy@psb.vib-ugent.be

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Abbreviations

53BP1	p53 binding protein 1
9-1-1	Rad9-Hus1-Rad1
Ala, A	Alanine
Asp, D	Aspartate
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3 related
ATRIP	ATR Interacting Protein
BM	Bleomycin
BRCA1	Breast Cancer 1
CHK	Checkpoint Kinase
CDC	Cell Division Cycle
CDK	Cyclin Dependent Kinase
cDNA	Complementary DNA
Cdr	Changed Divison Response
Cip	CDK-Interacting Protein
CKI	CDK Inhibitor Protein
CYC	Cyclin
DDR	DNA Damage Response
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triposphate
DP	Dimerization Partners
DSB	Double Strand Breaks
dsDNA	Double Stranded DNA
E2F	E2 Transcription Factor
ETG1	E2F Target Gene 1
FACS	Fluorescence-activated Cell Sorting
G1-phase	Gap 1-phase
G2-phase	Gap 2-phase
GFP	Green Fluorescent Protein
GO	Gene Ontology
GUS	Beta-glucuronidase
Hsl7	Histone Synthetic Letal 7
HU	Hydroxyurea

INK4a	Inhibitor Of Kinase 4
Kip	Kinase Inhibitor Protein 1
KO	Knock-Out
KRP	Kip Related Protein
M-phase	Mitosis
MCM	Minichromosome maintenance complex
MKP1	MAP Kinase Phosphatase
MMS	Methyl Methanesulfonate
Mre11	Meiotic Recombination 11
MRN	Mre11-Rad50-Nbs1
Myt1	Membrane Associated Tyrosine/Threonine Protein Kinase 1
Nbs1	Nijmegen Breakage Syndrome
ORF	Open Reading Frame
PARP2	Poly (Adenosinediphosphate-Ribose) Polymerase 2
PCD	Programmed Cell Death
PCNA	Proliferating Cell Nuclear Antigen
RB/RBR	Retinoblastoma/Retinoblastoma Related Protein
RNA	Ribonucleic Acid
RNR	Ribonucleotide Reductase
ROS	Reactive Oxygen Species
S-phase	Replication Phase
SCF	Skp, Cullin, F-box containing complex
Ser, S	Serine
SIM	Siamese
SMR	Siamese Related
SOG	Suppressor Of Gamma Response 1
SSB	Single Strand Break
ssDNA	Single-stranded DNA
TERT	Telomerase Reverse Transcriptase
TOPBP1	DNA Topoisomerase Binding Protein 1
UV	Ultraviolet
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Overview and Objectives

In the beginning of winter 2013, a gigantic haze that shrouded north and east China lasted for over a month. Particulate matter (PM) is the main constituent of air pollutant in haze. Millions of people in China breathe a cocktail of hazardous chemicals every day. These chemicals are produced by coal-fired power plants, factories and vehicles. Unsurprisingly, such air pollution events occur in many places around the world including London, Los Angeles, New Delhi and others. PM 2.5 (diameter lesser than 2.5 µm) containing genotoxic chemicals is proven to be harmful for plant and animal cells (André et al., 2011; Brito et al., 2013). There is accumulating epidemiologic evidence that exposure to air pollutants, including particulate matter (PM) and polyaromatic hydro carbons (PAHs) could induce oxidative DNA damage, eventually causing significant reductions in both crop quality and yield, or inducing cancers and other diseases in humans and animals.(Sørensen et al., 2003; Huang et al., 2012)

Pollution has been found to be present widely in the environment. Soil contamination from metal elements and xenobiotic (human-made) chemicals are dangerous to health or to the environment. The metal ion such as aluminium (Al), iron (Fe), copper (Cu), and cobalt (Co) or chemicals toxicity involves the production of superoxide radicals and hydroxyl radicals (Jomova and Valko, 2011) which cause DNA damage (Dizdaroglu et al., 2002; Roldán-Arjona and Ariza, 2009).

To counteract the risks of DNA damage, eukaryotic organism developed a complex mechanism to maintain the integrity of their genome. Generally, upon detection of DNA damage, three different responses can occur: cell cycle arrest, DNA damage repair or apoptosis if DNA can't be repaired sufficiently. Cell cycle checkpoints are the control mechanisms that ensure the fidelity of the cell cycle process. An important function of checkpoints is to assess DNA damage (Veylder et al., 2003; Cools and De Veylder, 2009). However, we know very little about the molecular players that adjust the plant cell cycle in response to DNA stress. In *Arabidopsis thaliana*, the cell cycle inhibitor WEE1 interacts with CDKA;1 upon replication stress (Cools and De Veylder, 2009; Cools et al., 2011). However, this mechanism was found to be essential only under replication stress and single strand breaks.

The objectives of this study were to understand cell cycle regulation in response to DNA damage and identify new components and mechanisms in DNA damage related stress in plants. Therefore, the first part of this work was focused on determining new CDK inhibitors (CKI) belonging to SIAMESE/SIAMESE RELATED (SIM/SMR) family (Peres et al., 2007). Three family members (*SMR4*, *SMR5*, and *SMR7*) respond specifically towards DNA damaging drugs, suggesting that they control the cell cycle checkpoint upon the occurrence of DNA stress. We focused on the function and transcriptional regulation of *SMR* genes upon DNA damage. To this end, we have developed an independent strategy to identify signal transduction components driving *SMR7* expression, We opt to use a positive selection strategy, making use of the D-amino acid oxidase (*DAO1*) selection marker (Erikson et al., 2004). We generated transgenic lines that hold the *DAO1* selector under control of the *SMR7* promoter.

E2F transcription factors act as transcriptional regulators of cell cycle, and are known to play important roles upon DNA damage response in animals (Martinez et al., 2010; Chen et al., 2012). Plants possess a set of E2F transcription factors, but there are no adequate reports that describe their role upon DNA stress (Cools and De Veylder, 2009). The second part of this work focused on investigating new elements of the DNA damage pathway in plants. Thus we investigated the $E2F^{KO}$ phenotypes and transcriptome upon DNA stress to connect E2Fs with environmental or endogenous DNA damage stress.

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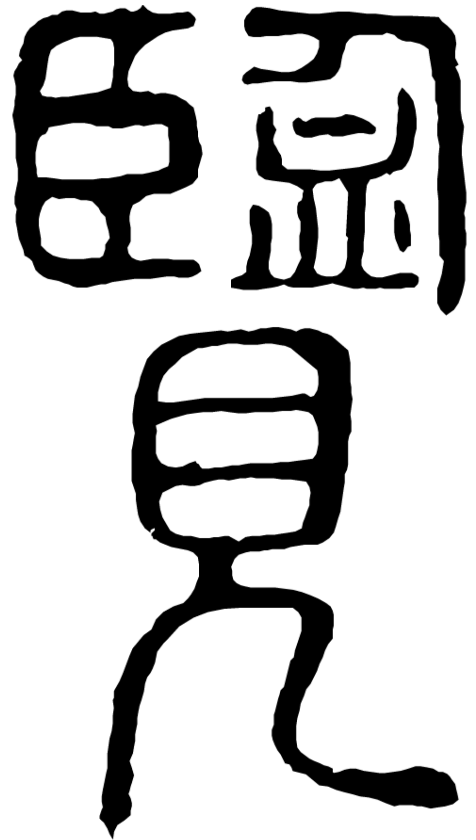

CHAPTER 1

CELL CYCLE REGULATION IN DNA DAMAGE RESPONSE

INTRODUCTION

DNA, Deoxyribonucleic acid, is the basic blueprint for all life. It provides the starting template for every new cell. In cells, DNA exists as long structured macromolecules called chromosomes. These contain the genetic instructions for all of the traits that control organism growth, development and reproduction. DNA reproduction is the primary process of cell division. During cell division, the genome is duplicated in a process called DNA replication, providing each cell with its own complete set of the genome. In eukaryotic cells, DNA replication usually occurs as part of a cell cycle process. Multiple cell cycle checkpoints have been identified, which make sure each phase of cell cycle is completed before progression into the next phase (Watson and Berry, 2009).

Cyclins and cyclin-dependent kinases (CDKs) combine to form kinase complexes that are conserved in all eukaryotes. Several distinct CDKs and cyclins have been shown to work in different stages of the cell cycle in animals (Morgan, 2007). Similar to animals, there are several types of CDKs and cyclins in plants (Veylder et al., 2003). Besides CDKs and cyclins, other key regulators like CAK (CDK-activating kinase), CKI (cyclin-dependent kinase inhibitor), and RBR/E2F control the cell cycle through interacting with CDK/Cylin complexes (van den Heuvel and Dyson, 2008) (Figure 1). In the first part of this chapter, we will focus on the functions of cell cycle regulation elements and the differences between plants and animals.

DNA can be damaged by many kinds of mutagens from exogenous and endogenous sources, which change the DNA sequence or break the DNA structure. Double stranded breaks (DSBs) and single stranded breaks (SSBs) are the two main types of DNA damage which are produced by these different types of mutagens. These mutagens include physical mutagens like ionizing radiation (IR) or ultraviolet light (UV) and DNA reactive chemicals agents such as reactive oxygen species (ROS), metal ions or intercalating agents (De Bont and van Larebeke, 2004; Harper and Elledge, 2007).

To maintain the integrity of the genome, organisms have developed a DNA damage response system that is involved in a variety of responses including cell cycle regulation, DNA repair and apoptosis. When damage is detected, the DNA damage checkpoint is activated and a cell cycle arrest is induced, allowing the cell time to repair

the damage. If the damage is too extensive, programmed cell death (PCD) is induced (Nyberg et al., 2002).

The DNA damage response pathway and cell cycle checkpoints are conserved but not exactly the same in all eukaryotes. ATM and ATR are two important conserved DNA damage sensing mechanisms in animals and plants. After ATM/ATR are activated by DNA damage, they will modulate the cell cycle through controlling the CDK/cyclin complexes. In animals, this process has been described in detail, with the identification of CHK1/CHK2, p53, CDC25 and WEE1 as downstream elements of ATM/ATR. In contrast, much less is known about this process in plants. Plant specific regulators such as SOG1 and SIM/SMRs have been investigated in recent reports. In the second part of this chapter, we will mainly describe the DNA damage response and the recent discoveries of DDR in plants (Harper and Elledge, 2007).

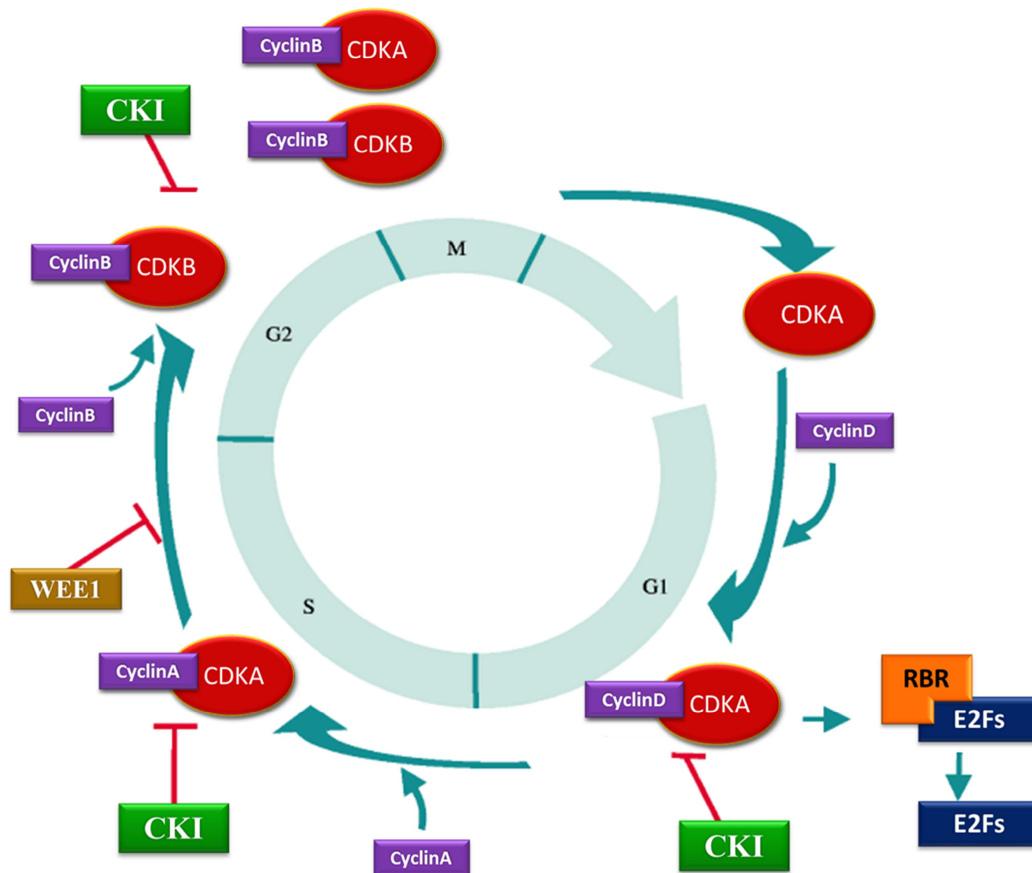

Figure 1. Schematic representation of cell cycle control in plants.

Progression through the mitotic cycle involves the successive formation, activation and subsequent inactivation of cyclin-dependent protein kinases (CDKs). The kinases bind sequentially to a series of cyclins, which are responsible for differential activation of the kinase during the cell cycle. The G1 to S transition is thought to be controlled by CDKs containing D-type cyclins that phosphorylate the retinoblastoma protein, releasing E2F transcription factors. E2F are involved in the transcription of genes needed for the G1 to S transition. The G2 to M transition is carried by CDK complexes containing CycA and CycB cyclins. CDK complexes are kept in inactive state by phosphorylation by the WEE1 kinase, and by interaction with inhibiting proteins (CKIs). At the G2 to M boundary activation of the kinase is brought about by release of the CKI protein.(base on the Mironov et al., 1999)

CELL CYCLE REGULATION

The cell cycle represents the series of events that take place in a cell leading to its duplication and division, resulting in a parent cell dividing into two daughter cells . In prokaryotes, the cell cycle is termed binary fission, which takes place without the formation of spindles. In contrast to the prokaryotic cell division, the eukaryotic cell cycle is more complex (Morgan, 2007).

There are two distinct types of eukaryotic cell division: a vegetative division, whereby each daughter cell obtains the complete genome from the parent cell (mitosis) and a reductive cell division, whereby the number of chromosomes in the daughter cells is reduced by half, to produce haploid gametes (meiosis). The vegetative cell cycle consists of four consecutive phases: G1 phase, S phase (synthesis), G2 phase and M phase (mitosis). G1, S and G2 phases are collectively referred to as interphase. For multicellular organisms, the cell-division cycle is a critical process by which a single zygote cell develops into a mature organism. Due to its importance for development and multiplication of the organism, it is crucial to understand the molecular mechanisms regulating the eukaryotic cell cycle (Inzé and De Veylder, 2006; Morgan, 2007).

CDKs and Cyclins

Cyclin-dependent kinases (CDKs) are a group of serine/threonine kinases regulating the cell cycle process together with their binding partners, the cyclin proteins. The first CDK member was identified from yeast in 1975 (Nurse, 1975). For their enzymatic activation CDKs need to associate with a cyclin regulatory subunit, which determines the temporal CDK activation and substrate specificity (Gopinathan et al., 2011).

In animals

Up to date, there are more than 20 CDK family members described in the human genome (Malumbres et al., 2009). Throughout the CDK gene family, a number of domains are conserved and essential for their function during the cell cycle. Basically, we can distinguish 3 core motives in the CDKs protein sequences: 1) an ATP-binding pocket, 2) a cyclin-binding domain and 3) an activating T-loop motif. There are inhibitory phosphorylation sites in the ATP-binding pocket, and an activating

phosphorylation site in the T-loop motif. Collectively, these domains control the temporal activation of CDKs (Lim and Kaldis, 2013).

The size of the CDK and cyclin gene families suggest that they exert many related but different roles during cell cycle regulation. For example, the M phase is controlled by CDK1/CycA and CDK1/CycD complexes. CDK4/CDK6 are mostly working on G1 and S phase and respond to DNA stress by interacting with cyclin D. (Dean et al., 2012).

In plants

CDKs in plants were found based on the homology analysis with CDKs in animals and yeasts. CDKs in arabidopsis can be classified into at least six subsets (CDKA-F) based on protein sequences (Inzé and De Veylder, 2006; Wang et al., 2008). Two classes of them, CDKAs and CDKBs, participate in core cell-cycle regulation in arabidopsis. A-type CDKs contain the conserved PSTAIRE cyclin domain pocket, which is present in animals and yeast CDKs as well (Ferreira et al., 1991; Takashi et al., 1991; Porceddua et al., 1999). CDKA activity controls both the G1-S and G2-M transitions of the cell cycle. CDKBs contain a PPTALRE or PPTTLRE motif instead of the PSTAIRE motif in their cyclin binding domain (Joubès et al., 2000; Boudolf et al., 2001). This group of CDKs responds to light and plant hormones such as brassinosteroid, gibberellic acid and jasmonic acid (Yoshizumi et al., 1999; Fabian et al., 2000; Świątek et al., 2004). There are two subgroups of CDKB. CDKB1 with the PPTALRE motif is expressed in the S, G2 and M phase, and CDKB2 with the PPTTLRE motif is expressed during the G2 to M phase. Both regulate the G2-M transition (Umeda et al., 1999; Oakenfull et al., 2002). Two other kinds of CDKs named CDKC and CDKE exist in plants, but no clear roles for them have been described in the cell cycle so far (Inzé and De Veylder, 2006). CDKD and CDKF are to classes CDK-activating kinase (CAK) which activates the cyclin-CDK complex by phosphorylation (Inzé and De Veylder, 2006)

In Arabidopsis, the cyclins constitute a large gene family. There are at least 32 members predicted to be involved in cell cycle progression (Vandepoele et al., 2005). According to their sequence similarity to animals, cyclins in Arabidopsis can be divided into 10 A-type cyclins, 11 B-type cyclins, 10 D-type cyclins and 1 H-type cyclin. Generally, cyclin A proteins are involved during S-to-M phase, whereas cyclin B proteins mainly control the G2-M and intra-M phase. D-type cyclins are thought to regulate the G1-S

transition, but there is numerous data indicating that D-type cyclins also take part in the G2-M transition (Schnittger et al., 2002; Kono et al., 2003).

CKI

CDK inhibitors (CKI) are a group of proteins that tightly interact with CDK/cyclin complexes which broadly exist in animals (Lim and Kaldis, 2013) and plants (Wang et al., 2008). They have been described as important factors for organism development and external signal response, during which they modulate the cell cycle process by influencing the CDK/cyclin complexes.

In animals

In animals, CKIs can be categorized into two gene families based on their evolutionary origins, structure, and CDK specificities, namely INK4 and Cip/Kip. The INK4 gene family contains $p16^{INK4a}$ (Cdkn2a), $p15^{INK4b}$ (Cdkn2b), $p18^{INK4c}$ (Cdkn2c) and $p19^{INK4d}$ (Cdkn2d), all of which target CDK4/CDK6 and inhibit their kinase activities by interfering with their ability to interact with D-type cyclins. Conversely, Cip/Kip family proteins, including $p21^{Cip1}$ (Cdkn1a), $p27^{Kip1}$ (Cdkn1b) and $p57^{Kip2}$ (Cdkn1c), widely interfere with both cyclin and CDK subunits and modulate the activity of cyclin D, E, A, and B/CDK complexes (Sherr and Roberts, 1999).

INK4 gene family

The INK4 family consists out of proteins that have conserved sequences containing ankyrin repeats. Expression analysis revealed distinct tissue-specificity and developmental expression of the different family members in mice (Ortega et al., 2002; Pei and Xiong, 2005). The different expression patterns of *INK4* genes imply there are various functions for each INK4 protein in the DNA damage response, cellular processes and development through regulation of the cell cycle.

Regulation of INK4 is mainly controlled at the transcription level by transcription factors such as ETS, FOXO and SP1, resulting in stable INK4 protein levels in the cell (Ohtani et al., 2001; Xue et al., 2004; Katayama et al., 2007). Besides inhibiting CDK4/CDK6 directly, INK4 proteins can interact with the p53 and E2F transcription factors to modulate the cell cycle.

Cip/Kip family

Cip/Kip family proteins are more broad regulators of CDK/Cylin complexes in cell cycle regulation process, compared to the INK4 family. The three members of the Cip/kip family, $p21^{Cip1}$ (Cdkn1a), $p27^{Kip1}$ (Cdkn1b) and $p57^{Kip2}$ (Cdkn1c), have related but different functions in cellular processes and organism development. $p21^{Cip1}$ (Cdkn1a) mostly responds to DNA damage under transcriptional control of p53 tumor suppressor. Activated p21 arrests cells in the G1 or G2 phases to allow DNA damage repair (Dulić et al., 1998; Nakayama and Nakayama, 1998). Accumulation of $p27^{Kip1}$ (Cdkn1b) causes cells to exit the cell cycle and enter a quiescent state, and it will be rapidly degraded when cells re-enter the cell cycle (Chu et al., 2008). $p57^{Kip2}$ (Cdkn1c) plays a very important role in embryonic development, because embryos lacking a functional p57 die off due to the inability to promote cell differentiation. Besides interaction with CDKs, Cip/Kip proteins also modulate cell cycle gene expression by CDK-independent functions in transcriptional regulation (Besson et al., 2008). Control of Cip/Kip protein activity occurs mainly on the protein level, such as through phosphorylation (Dash and El-Deiry, 2005; Chu et al., 2007) and proteasomal degradation through ubiquitination by E3-ubiquitin ligases (Glickman and Ciechanover, 2002). Cip/Kip inhibitors can be redistributed under the influence of INK4 proteins to repress the kinase activity of Cdk2/cyclinE complexes (Sherr and Roberts, 1999). INK4 proteins compete with Cip/Kip proteins for CDK4/cyclin D. Increased INK4 protein results in formation of INK4–CDK complexes and destabilization of cyclin D. Consequently, release of Cip/Kip proteins from the complexes inhibits cyclin E (and A)-dependent CDK2.

In plants

In contrast to animals, little is known about CKIs in plants. Three groups of CKIs have been discovered: the Inhibitor of CDK/Kip Related Protein (ICK/KRP) CDK inhibitor family (Wang et al., 1998; De Veylder et al., 2001); the SIAMESE/SIAMESE-RELATED (SIM/SMR) gene family (Churchman et al., 2006; Peres et al., 2007) and the tissue-specific inhibitors of CDK (TIC) (DePaoli et al., 2011; 2012). They are three different but related groups of CDK inhibitors.

KRPs

The most studied family of plant CDK inhibitors family is the ICK/KRP CDK inhibitor family. This CKI family was discovered through a plant yeast two-hybrid library screen using *Arabidopsis thaliana* CDKA;1 and CYCD3;1 as bait. In Arabidopsis, the ICK/KRP family consists of seven members (De Veylder et al., 2001).

All seven ICK/KRP proteins in Arabidopsis contain a CDK inhibitory region which shows amino acid sequence homology to the mammalian cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Wang et al., 1998; Lui et al., 2000; De Veylder et al., 2001; Zhou et al., 2002) This conserved C-terminal domain was proven to interact with CDKA (De Veylder et al., 2001)**.** In addition, another shorter conserved domain adjacent to the CDK inhibitory motif (Zhou et al., 2002) was shown to interact with D-type cyclin (Wang et al., 1998; Jakoby et al., 2006). Based on their evolutionary conservation, the ICK/KRP family genes in Arabidopsis can be classified into 3 groups: (i) *KRP1* and *KRP2*, (ii) *KRP6* and *KRP7* and (iii) *KRP3, KRP4* and *KRP5* (Wang et al., 2008).

The ICK/KRP family of CDK inhibitors plays an important role in cellular processes and plant development, through its interaction and regulation of the CDKA and D-type cyclin proteins. Several reports confirm that ICK/KRP proteins are cell division inhibitors, as seen by the reduction in cell number and growth inhibition upon overexpression of *ICK/KRP* genes. Overexpressing *ICK/KRP* Arabidopsis plants display all similar phenotypes including smaller plant size, serrated leaves, reduced cell number and enlarged cells.(Wang et al., 2000; De Veylder et al., 2001; Jasinski et al., 2002; Zhou et al., 2002; Barrôco et al., 2006). Interestingly, the influence of *ICK/KRP* on endoreduplication is dose-dependent. Although overexpression of *ICK/KRP* mainly inhibits endoreduplication, weak overexpression of ICK1/KRP1 or ICK2/KRP2 has the opposite effect on endoreduplication (Verkest et al., 2005; Weinl et al., 2005). In contrast to overexpression, downregulation of multiple ICK/KRPs in plants leads to enhancement of seedling dry weight and cotyledon and leaf size (Cheng et al., 2013). *ick3/krp5* mutants display a decrease in the 16C population both in etiolated seedlings and roots (Jégu et al., 2013; Wen et al., 2013). This suggests that *KRP5* promotes higher endoreduplication levels. Corresponding with the role of endoreduplication in promoting growth, *ick3/krp5* primary roots show a growth reduction compared to wild type controls (Wen et al., 2013) .

ICK/KRP genes show different expression patterns. In the shoot apex, *KRP1* and *KRP2* expression can solely be detected in the tissues that are undergoing endoreduplication, but *KRP4* and *KRP5* are mainly expressed in mitotically dividing cells. Expression of the other KRPs (*KRP3, KRP6* and *KRP7*) can be detected in both mitotically active and endoreduplicating tissues (De Veylder et al., 2001; Wen et al., 2013). This classification result is partially compatible with the evolutionary conservation analysis of KRP proteins (Wang et al., 2008). Besides transcriptional regulation, the level of ICK/KRP proteins is also regulated post-translationally. The N-terminal region of KRP1 protein controls the stability, while the C-terminal region is important for CDK inhibition activity through interacting with the kinase complex. Interestingly, the central domain of ICK1 is responsible for nuclear localization (Zhou et al., 2003). ICK/KRP proteins are degraded through the ubiquitin–proteasome pathway. Two different ubiquitin protein ligases, SCFSKP2 and the RING protein RKP, work on its degradation(Ren et al., 2008). Furthermore, similar as KRP1, KRP6 and KRP7 proteins are also degraded by RING-finger E3 ligases RHF1a and RHF2a or the SKP1–Cullin1–F-box protein FBL17, which is involved in gametocyte development. Subcellular localization studies using the GFP reporter proved that the Arabidopsis ICK/KRP family proteins are all localized in the nucleus, which is important for CKI function or to regulate their functions(Zhou et al., 2003; Bird et al., 2007). Interestingly, these seven ICK/KRP proteins show two types of nuclear localization, KRP1, KRP3, KRP4 and KRP5 present a punctate pattern distribution in nucleus, and KRP2, KRP6 and KRP7 always exist throughout the nucleoplasm. However, there is no detail on the relation between these two type sub-unclear localization patterns and the functions of the ICK/KRP proteins.

SMRs

The *SIAMESE/SIAMESE-RELATED (SIM/SMR)* gene family encodes for a new group of plant-specific CKIs. Homologs of SIM/SMR were detected both in dicots and in monocots (Churchman 2006; Peres et al 2007 Walker 2000). Based on protein analysis, SIM/SMRs have a conserved motif that resembles the cyclin-binding domain of ICK/KRP proteins. Peres et al. (2007) reported that the Arabidopsis genome encodes five SIAMESE-RELATED genes, and recent research expanded this to 13 family members (Yi et al., 2014). Besides *Arabidopsis thaliana*, SIM/SMR also exist in other plants. There are at least four conserved motifs in the *SIM/SMR* gene family. As discussed before, Motif 4 of the SIM/SMR proteins is similar to the motif of the

ICK/KRP CDK-inhibitory proteins (De Veylder et al., 2001). Motif 3, containing the Cy or zRxL motif, is predicted to interact with some CYCA and CYCD/CDK complexes (Adams et al., 1996; Wohlschlegel et al., 2001). Motifs 1 and 2 show no obvious similarity to any domain with known function.

According to subcellular localization experiments, we know that all the investigated SIM/SMR proteins (SIM, SMR1, SMR2, SMR3, SMR4, SMR5, SMR7; Orysa;EL2) are localized in the nucleus (Churchman et al., 2006; Peres et al., 2007). These results support the function of SIM/SMRs as CDK inhibitors.

In accordance to being a part of the family of CKI proteins, SIM/SMR proteins influence the cell cycle process. In the meantime, there is data showing that SIM/SMR proteins not only exist in proliferating tissues like the root and shoot apical meristem and in leaf primordia, but also in differentiated cells such as vascular cells and in the root elongation zone (Churchman et al., 2006; Peres et al., 2007; Yi et al., 2014), indicating there are different functions of SIM/SMRs in plants. This speculation has been supported by the report from Van Leene et al (2010). Based on their co-purification with CDK/Cyclin, we can discern at least two groups of SIM/SMR proteins. The first group contains the SIM, SMR1 and certain SMR family members linked with endocycle onset as CKIs function to inhibit the activity of B1-type of CDKs (Boudolf et al., 2004; 2009). The second group of SIM/SMR proteins, including SMR4, SMR5 and SMR7, only co-purified with A-type CDK and D-type cyclins (Van Leene et al., 2010).

Mutation of the *SIAMESE* gene triggers multi-cellular trichomes and a decreased in the DNA content in these cells, which indicates that the *SIM* gene can block mitosis and trigger endoreduplication. (Churchman 2006). SIAMESE RELATED 1(SMR1) gene is also named LGO (loss of giant cells from organs) (Roeder et al., 2010). In the *lgo* mutant, high ploidy epidermis cells in both leaves and sepals are reduced, but overexpressing LGO gene produces excess giant cells. We can predict that the SIM and SMR1, standing by the first group SIM/SMR protein, inhibit CDK kinase activity to trigger endocycle onset.

New research showed that the second group of *SIM/SMR* genes including *SMR4*, *SMR5* and *SMR7* respond to abiotic stress (Peres et al., 2007; Yi et al., 2014). This result can be confirmed by the functions of CYCD/CDKA complex. This complex is responsible

for control of cell cycle onset in response to intrinsic and extrinsic signals, and specifically G1-to-S phase progression (Riou-Khamlichi et al., 2000; Dewitte and Murray, 2003; Nowack et al., 2010; Nowack et al., 2012). Specifically, it was confirmed that *SMR5* and *SMR7* respond to oxidative stress together with genotoxic stress (Yi et al., 2014). In this process, expression of *SMR5* and *SMR7* is under control of ATM and SOG1, which are involved in the double-stranded DNA damage response (DDR), but not under control of ATR that responds to single-stranded DNA damage. In summary, SMR4, SMR5 and SMR7 are important factors linking abiotic stress signals to cell cycle checkpoint activation. Meanwhile, a SIM-related gene in rice (*Oryza sativa*), *Orysa;EL2* also is considered to belong to the second group. Orysa;EL2 protein interacts with CDKA1;1 and D-type cyclins, but not with B-type CDKs (Peres et al., 2007).

SCI1

Stigma/style cell cycle inhibitor 1 (SCI1) is the first and unique tissue-specific inhibitor of CDK (TIC) described in plants (DePaoli et al., 2011; 2012). The *SCI1* gene encodes a 156 amino acids protein, which is mainly expressed in the early stages of tobacco stigma/styles. SCI1 controls the development of stigma/style through modulating cell proliferation/differentiation(DePaoli et al., 2011), a process which depends on auxin signaling (DePaoli et al., 2012). In Arabidopsis, *SCI1* expression is both cell cycle- and auxin signaling-dependent through the *cis*-acting elements in its upstream regulatory region (URR) (DePaoli et al., 2012) Both *in silico* and experimental observations suggest that SCI1 protein functions as a CDK inhibitor. From protein sequence analysis, SCI1 shows no sequence similarity with ICK/KRP proteins and only very limited similarity region with the SIM/SMR family. In summary, SCI is proposed to be a new kind CDK inhibitor, giving us new insights about cell cycle regulation and tissue-specific development (DePaoli et al., 2011; DePaoli et al., 2012).

E2F transcription factors

E2F transcription factors are well-studied cell cycle regulators. In the beginning, E2F was identified as a cellular factor that promotes the expression of the adenovirus E2 promoter (Kovesdi et al., 1986). Then E2F proteins were been found to stimulate the expression of a wide variety of genes that are mostly involved cell cycle process

(Pagano et al., 1992; Ramírez-Parra et al., 1999). Retinoblastoma/Retinoblastoma related protein (RB/RBR) acts as a repressor that binds E2F/DP complexes, inhibiting their activity and therefore inhibiting cell cycle progression (Murphree and Benedict, 1984; Weinberg, 1995). Typically, E2F proteins associate with dimerization partners (DP) proteins to form a heterodimeric complex that binds to the promoter of a multitude of target genes (van den Heuvel and Dyson, 2008).

Over the last decades, the core functions of E2F/DP have been partially characterized. These transcription factors are crucial for the regulation of DNA replication, endoreduplication onset and maintenance, checkpoint control, apoptosis, and cell differentiation. Specifically, E2Fs play a crucial role in the regulation of G1-to-S-phase transition(Ren et al., 2002). In mammals, deregulation of E2F/DP activity has a big impact on health and disease by controlling transcription of a wide range of genes which are involved in cell-cycle progression and DNA synthesis, replication and repair. (Tsuge et al., 2005; DeGregori and Johnson, 2006; Hoglinger et al., 2007). In contrast, there is few report investigated their functions in DNA damage response.

In human, there are eight E2F (E2F1-8) and three DP proteins (DP1, DP2/3, and DP4) present and in Arabidopsis there are six E2F (E2Fa-f) proteins and two DP (DPa and DPb) proteins. (Mariconti et al., 2002; Attwooll et al., 2004; Christensen et al., 2005; Dimova and Dyson, 2005; Maiti et al., 2005). The E2Fs can be classified into typical (E2F1–6 in mammals and E2Fa, E2Fb, E2Fc in Arabidopsis) and atypical (E2F7, E2F8 in mammals and DEL1/E2Fe, DEL2/E2Fd, and DEL3/E2Ff in Arabidopsis) subgroups based on their structure and function (Lammens et al., 2009).

Typical E2F members

Typical E2F proteins contain a DNA binding domain, a dimerization domain and a transcriptional activation domain that includes an RBR binding domain. DP proteins. E2F and DP interact with each other as a heterodimer to regulate downstream gene expression. In mammals, six classical E2F proteins have been described (E2F1–6). According to whether E2Fs act positively or negatively on gene transcription, they are grouped into transcriptional activators (E2F1–3) or suppressors (E2F4–6). E2F transcription factors play an integral role in the coordination of DNA replication events. The activators E2F behave as sequence-specific transcriptional activators of cellular genes, including those associated with growth and proliferation, whereas the repressors

play opposing roles (Wong et al., 2011). E2Fs regulate CYC/CDK to trigger DNA damage checkpoints or apoptosis. This process can be regulated by CDK4/6 , RB or CHKs (Martinez et al., 2010; Dean et al., 2012). Furthermore, E2Fs can mediate DDR that centers on activation of the ATM kinase and p53 (Rogoff et al., 2002; E et al., 2011).

As typical E2Fs in Arabidopsis, E2Fa, E2Fb and E2Fc need to cooperate with a dimerization partner, being DPa or DPb, which assists E2F protein binding to defined DNA sequences to induce gene expression (Mariconti et al., 2002). E2Fa and E2Fb are two positive regulators that promote S-phase entry and progression. They are mostly expressed in proliferating tissues, have specific expression patterns and play similar but distinct roles during cell cycle progression (De Veylder et al., 2002; Mariconti et al., 2002; Sozzani et al., 2006). Co-overexpressing *E2Fa* and *DPa* plants show extreme cell proliferation and increased endoreduplication, resulting into severe developmental defects. A clear induction of S-phase specific gene expression could be observed in *E2Fa–DPa* co-overexpressing seedlings (Vandepoele et al., 2005). These results indicate the E2Fa-DPa complex is a key regulator controlling cell proliferation, differentiation and endoreduplication in plants. E2Fb and E2Fa recognize the same E2F consensus *cis*-regulatory elements in the promoter of target genes(Kosugi and Ohashi, 2002). Therefore it is very difficult to indicate the different specific target genes of E2Fa and E2Fb, respectively. But some evidence suggests non-overlapping regulation exists between E2Fa and E2Fb. *E2Fa* and *E2Fb* overexpression lines show a different reaction to auxin in cell suspension cultures(Magyar et al., 2005). E2Fa and E2Fb have a different function in lateral root development (Sozzani et al., 2006; Berckmans et al., 2011b). Moreover, chromatine-IP experiments shows there are E2Fa- and E2Fb-specific target genes in the Arabidopsis genome (Naouar et al., 2009).

In contrast to E2Fa and E2Fb, E2Fc is a transcriptional repressor (del Pozo et al., 2002). There is no transcription activator domain in E2Fc, but it can combine with DPb to bind DNA sequences with E2F binding sites (Mariconti et al., 2002). Thus, the reasonable conclusion is that E2Fc operates as a competitive inhibitor of E2Fa and E2Fb. As a repressor, E2Fc blocks entry into S-phase to arrest the cell cycle, and reduced E2Fc activity increases cell proliferation (del Pozo et al., 2002; del Pozo et al., 2006). Consequently, there are studies which reveal the competitive relation between E2Fb and E2Fc in regulation of the target gene expression (Berckmans et al., 2011a). Such kind of

relation also exists between E2F1 and E2F2 in animals (Frolov et al., 2001; Cayirlioglu et al., 2003). This means that interplay between positive regulators and repressors of E2F is conserved and an important way to balance the modulation in cell cycle onset.

Atypical E2F members

Atypical E2Fs are a set of novel E2F transcription factors, which includes E2Fd/DEL (DP–E2F-like) 2, E2Fe/DEL1, and E2Ff/DEL3 in Arabidopsis and E2F7 and E2F8 in mammals. However, the sequence similarity between typical E2Fs and atypical E2Fs is relatively low, showing about 20% similarity (Lammens et al., 2009). In contrast to typical E2Fs, atypical E2F proteins contain two DNA binding domains instead of a dimerization domain, which indicates that atypical E2Fs can bind to DNA containing the consensus E2F binding site without the help from a DP dimerization partner. The absence of a transcriptional activation domains ensures that atypical E2Fs can’t perform all the functions of typical E2F proteins, therefore they negatively modulate downstream gene expression, acting as transcription repressors.

E2Fe/DEL1 controls the onset of the endocycle through a direct transcriptional repression of *CCS52A2* that is involved in controlling the switch from cell division to endoreduplication by regulating APC/C activity (Lammens et al., 2008). *E2Fe/DEL1*^{KO} plants show higher UV-B tolerance compared to wild type, due to increased expression of the photolyase photoreactivating enzyme *PHR1*, which is a type-II cyclobutane pyrimidine dimer-photolyase DNA repair gene, being under control conditions repressed by *DEL1* (Radziejwoski et al., 2011). E2Fd/DEL2 is a factor that has been shown to promote cell proliferation and reduce cell size. This protein accumulates by the auxin signaling pathway at the post-transcriptional level (Sozzani et al., 2010). E2Ff/DEL3, without RB binding domain, negative regulates cell size but does not influence the DNA ploidy level distribution and cell proliferation(Ramirez-Parra et al., 2004). Similarly to E2Fe/DEL1, the mammalian atypical E2F7 and E2F8 also have crucial roles in endocycle control. Loss of *E2F7* and *E2F8* results in endocycle defects in the trophoblast giant cells and probably governing the maintenance of endocycle progression (Lammens et al., 2009; Meserve and Duronio, 2012).

Regulations of E2F

In animals, as a group of transcription factors, E2Fs can regulate each other on the transcriptional level. E2F3 acts upstream of E2F1. E2F1 in turn acts upstream of E2F2. Upon DNA damage, E2F2 responds to the DNA damage through E2F3, initializing apoptosis, making E2F3 a key regulator of DNA damage-induced apoptosis (Martinez et al., 2010). Such cross regulation also exist between typical and atypical E2Fs. Atypical members *E2F7* and *E2F8* are under the control of typical E2F1(Christensen et al., 2005). Furthermore, the increase of E2F7 and E2F8 protein level can give a feedback to reduce *E2F1* expression. In contrast, low level of E2F7/E2F8 proteins increases *E2F1* expression (Chen et al., 2012). In Arabidopsis, E2Fb accumulates in *E2Fa*^{OE} plants, both on the transcriptional and post-transcriptional level (Sozzani et al., 2006). Overexpression of *E2FD/DEL2* increases the expression of *E2Fa* (Sozzani et al., 2010). Meanwhile, *DEL1*, the homologue of E2F7 and E2F8, is a transcriptional target of the E2Fb and E2Fc, which are regulated by light signal(Berckmans et al., 2011a). Furthermore, *E2Fa* expression is regulated by the auxin-signaling pathway through the LATERAL ORGAN BOUNDARY DOMAIN18/LATERAL ORGAN BOUNDARY DOMAIN33 (LBD18/LBD33) dimer to mediate lateral root organogenesis (Berckmans et al., 2011b).

At the post-transcriptional level, two distinct mechanisms have been described. One of them is based on the RB protein. Under genotoxic conditions, the interaction between E2F and RB protein is enhanced by ATM-CHK1/2 mediated phosphorylation (Inoue et al., 2007). Through an independent mechanism, E2F1 protein can be phosphorylated by ATM and CHK2 kinases upon DNA damage (Stevens et al., 2003). So far, in plants there has been no data on transcriptional and posttranscriptional control of E2Fs in response to DNA stress. Under the stimulation of glucose, target-of-rapamycin (TOR) kinase phosphorylates E2Fa, which leads to activate E2Fa target gene expression to promote cell proliferation in the root (Xiong et al., 2013).

RBR

E2Fs can be regulated by RBs, present both in animals and plants. The Retinoblastoma protein (RB) is a tumor suppressor discovered in humans (Murphree and Benedict, 1984) which can interact with E2F proteins (Cobrinik, 2005). It belongs to the pocket protein family, containing RB, Retinoblastoma-like protein 1 (RBL1) (Ewen et al.,

1991) and Retinoblastoma-like protein 2 (RBL2) (Soprano et al., 2006). In plants, RB homologues (RBR, retinoblastoma-related) have been isolated from maize (Grafi et al., 1996), tobacco (Nakagami et al., 1999), and Arabidopsis(Kong et al., 2000; Ebel et al., 2004). Yeast-two-hybrid assays showed that RBs interacts with E2F proteins through two pocket domains (Ramírez-Parra et al., 1999). This type of interaction normally happens using the C-terminal domain of the E2F, repressing the transcriptional activation of the E2F/DP complex. RB proteins in plants and animals contain conserved pocket domains A and B. Due to the high degree of conservation of the E2F/DP/RB pathway, RB proteins from plants can inactivate the transactivation activity of human E2F (Huntley et al., 1998).

RBR is a crucial regulator of cell cycle, DNA damage response and organ development. Apparently, the main regulatory activity of RB proteins operates through the E2F/DP pathway. In animals, mutants of *p105(Rb1)* can be detected in nearly all cancerous specimens, implying its functions as tumor suppressor (Nguyen and McCance, 2005). RB proteins are phosphorylated by CDK/cyclin complexes in the G1-S phase (Harbour and Dean, 2000; Nakagami et al., 2002; Magyar et al., 2012). After phosphorylation, RB protein detaches from E2F/DP complexes. These complexes can subsequently promote downstream gene expression and thus drive the cell into S-phase. In animals, *RBR* defective mutants can form gametes, but homozygous embryos fail to develop and initialize apoptosis (Jacks et al., 1992; Du and Dyson, 1999). Actually, E2F proteins are not the unique targets of RB proteins. Besides E2F proteins, RB can also regulate several other transcription factors (Korenjak and Brehm, 2005; Nguyen and McCance, 2005; Calo et al., 2010). In animals, RB can interact with the α-globin promoter by recruiting a tissue-specific transcription factor PU.1 (Rekhtman et al., 2003). It can also cooperate with Mitf1 to regulate $p21^{CIP}$ gene expression (Carreira et al., 2005).

In Arabidopsis, RETINOBLASTOMA RELATED (RBR) is the single homologue of pRB, and the pRB-E2F pathway is largely conserved. There are 3 T-DNA insertion knockout lines available of rbr^{KO} mutant alleles (*rbr1-1*, *rbr1-2* and *rbr1-3*). There is no *rbr1-1* and *rbr1-3* homozygous line because these alleles cause lethality in gametophytes, especially in female gametophytes (Ebel et al., 2004). Recent work demonstrated that RBR genetically interact with the conserved epigenetic regulators of the Polycomb Repressive Complex 2 (PRC2) to control the development of both male and female gametophytes (Johnston et al., 2008) Homozygous *rbr1*-2 plants are viable,

though they are not fully knockout, but have a strong temperature-sensitive phenotype (Nowack et al., 2012). Therefore an RBR RNA interference system has been developed to research the function of RBR protein (Borghi et al., 2010). This report shows that decreasing the RBR expression level leads to repression of cell differentiation and disruption of stem cell niche and meristem maintenance, thus arresting root growth, leaf development and inflorescence development. RBR protein interacts with cytokinin signaling to stimulate cell differentiation in the root meristem (Perilli et al., 2013). In these processes, inhibition of RBR expression will drive cell ploidy switch from 2C to 4C, but will not increase the endoreduplication further. Similar phenotypes also are detected in other plants such as maize (Sabelli et al., 2013). In Arabidopsis, pRB is known to interact with members of the E2Fs transcription factor proteins, thus interfering with their ability to activate transcription of genes necessary for the G1-to-S transition (Magyar et al., 2012). Whereas, the report from Cruz-Ramírez et al. (2012) shows that in Arabidopsis root, RBR1 can directly interact with the SCARECROW transcription factor to modulate asymmetric stem cell division in stem cells. Together, these reports suggested that the *RBR* regulatory network can function differently depending on the developmental context. The developmental role of *RBR* during sporophytic development remains poorly understood, primarily due to the lack of genetic tools.

DNA DAMAGE AND DNA DAMAGE RESPONSE

As the genome contains all the information required for development and maintenance of an organism, it is of utmost importance that the DNA content is efficiently repaired upon the occurrence of DNA breaks or replication mistakes. These breaks and errors can arise from environmental stresses, including drought, soil contamination by heavy metals, and increasing genotoxic chemicals owing to pollution from industry (Ma et al., 2012; Xu et al., 2012). These conditions often result in the production of DNA damage in the cell, eventually causing significant reductions in both crop quality and yield. Reactive oxygen species (ROS) are mainly direct agents that inhibit DNA replication and cause DNA damage (De Bont and van Larebeke, 2004). In absence of a cell division arrest, cells would proceed with damaged DNA into mitosis, causing cell death or oncogenesis. Therefore, a functional DNA stress cell cycle checkpoint is of utter importance for cell survival. To cope with these stress conditions, cells have developed

a set of complicated mechanisms that monitor the status and structure of the DNA during cell cycle progression. DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to DNA damage.(Nyberg et al., 2002)

Mutagens

DNA damage can occur spontaneously or be induced by external mutagens which is is a physical or chemical agent like metal ions, IR, or UV to break DNA directly or through the ROS (Table 1).

ROS inducing DNA damage

Reactive oxygen species can oxidize and damage a wide range of organic macromolecules, including lipids, proteins and nucleic acids (Dizdaroglu et al., 2002; Roldán-Arjona and Ariza, 2009). In living cells, ROS are produced by exogenous and endogenous sources. Exogenous elements are mainly environmental genotoxic agents, including high-light conditions, ultraviolet light (UV) and pollutants in the air and soil, e.g. heavy metal ions and toxic chemicals. Endogenous sources are mainly chloroplasts, mitochondria and peroxisomes (Foyer and Noctor, 2003)

Aerobic respiration is crucial for many organisms, especially for eukaryotes. In this process, ROS are continuously produced from mitochondria. In humans, excessive ROS will lead to many kinds of diseases such as Parkinson's and amyotrophic lateral sclerosis(Emerit et al., 2004). In plants, besides mitochondria, ROS are also generated by photosynthesis as byproducts in chloroplasts. H_2O_2 and O2 are the products of O_2^- disproportionation (Asada, 2006). Oxidative attacks on DNA generate altered bases and damage sugar residues causing fragmentation and consequently single-strand breaks (SSB) (Roldán-Arjona and Ariza, 2009). Thus organisms will initialize the processes of base excision repair (BER) and nucleotide excision repair (NER) to remove the oxidized nucleotides (Dizdaroglu, 2005). In these processes, the poly (ADP-ribose) polymerase (PARP) superfamily proteins are considered as a group of modulating elements both in animal and plants cells (Lindahl et al., 1995; Babiychuk et al., 1998). Furthermore, oxidative DNA damage generated by ionizing radiation can also cause double-strand breaks (DSB) through the generation of clusters of radicals that affect nearby sites on both strands(Culligan et al., 2006). This leads to a DNA damage response involving

both ATM and ATR. ATM can also be activated by oxidative stress directly in the form of exposure to H_2O_2 in human cells (Guo et al., 2010b).

HU induced genotoxicity

Hydroxyurea (HU) is a ribonucleotide reductase inhibitor, which inhibits class 1 ribonucleotide reductase and limits the cellular supply of deoxyribonucleotides (Timson, 1975; Lopes et al., 2001; Sogo et al., 2002). Depletion of dNTP pools through HU leads to DNA replication fork arrest and subsequent genomic instability, most likely through substrate starvation (Foti et al., 2005).

Besides inhibition of DNA replication, HU also causes DNA damage through the formation of DSBs(Kurose et al., 2006). This means that HU can initialize two kinds of DNA damage response pathways, which are based on ATM and ATR. Furthermore, besides damaging the DNA, HU also shows more broad possibilities as a cytotoxic compound. According to recent research, HU can directly target and inhibit catalase that catalyzes the decomposition of H_2O_2 to water and oxygen (Juul et al., 2010). This inhibition leads to an increasing H_2O_2 concentration *in vivo*. Additionally, there are studies that have demonstrated that HU causes H_2O_2 induction in the presence of Cu(II) and Fe in E.*Coli* (Sakano et al., 2001; Davies et al., 2009). After H_2O_2 accumulation, ROS-derived DNA oxidation will lead to DNA damage (Vanderauwera et al., 2011).

Metal ion induced DNA damage

Metal elements like aluminium (Al), iron (Fe), copper (Cu), chromium (Cr) and cobalt (Co) are important nutrients for organisms. They play important roles in many biological processes and organism development. However, an excess of heavy metal ions is toxic for cells. Heavy metal ion toxicity involves the production of superoxide radicals and hydroxyl radicals (Jomova and Valko, 2011). Meanwhile, accumulation of these ion will repress DNA damage repair and influence cell cycle process (Hartwig et al., 2002).

Fe (II) and (III) are soluble in biological fluids and produce highly reactive hydroxyl radicals through the Fenton reaction. This leads to free radical-mediated DNA damage (Dizdaroglu et al., 2002). Cu(II) ions occur in nuclei and bind to DNA (Kamunde and MacPhail, 2011; Linder, 2012). Copper ions will damage DNA and chromatin by oxidative activity, producing DNA single and double strand breaks, crosslinks and

adducts, point mutations, and even chromosome instability (Cao and Wang, 2007; Ruiz et al., 2010; Buchtik et al., 2011). Even more, copper also can alter DNA methylation and histone acetylation to influence gene expression on an epigenetic level (Tang and Ho, 2007; Fragou et al., 2011; Ziech et al., 2011). Co(II) ions produce DNA DSBs through production of ROS that results in the activation of ATM, p53 and Rad51 (Galanis et al., 2009). The same research also describes that exposure to non-toxic doses of Co nanoparticles will result in oxidative DNA damage.

Aluminum (Al) is the most abundant metal element in the Earth's crust. Al (III) is a strong hydroxide ion, which can produce oxidative stress and DNA damage (Yamamoto et al., 2002; Panda et al., 2009). In agriculture, aluminum toxicity is a serious factor limiting crop productivity, especially in acid soil. Besides ROS, aluminum binding to DNA causes condensation of DNA molecules, blocking DNA replication and repressing gene transcription by reducing the capacity to provide a viable template (Rounds and Larsen, 2008). In *Arabidopsis*, the *als3*-mutant is hypersensitive to aluminium and a suppressor screen revealed the *alt1-1* mutant, which showed increased root growth on the heavy metal. This could later be attributed to a partial loss of function of ATR in the mutant. Further research showed that the *atr*-mutant is impaired in the detection of DNA damage caused by aluminium and subsequently fails to respond to the stress. Aluminium leads to the terminal differentiation of stem cells in the root meristem in an ATR-dependent manner (Rounds and Larsen, 2008). This could mean that the brittle root system in wild type plants is the result of a plant defense mechanism and not because of the aluminium stress itself. The modulation of this defense mechanism could therefore lead to plants that grow better on marginal soils.

Boron (B) is an essential nutrient in plants. It is involved in the cell wall and membrane structure and function, which are important for plant root growth. There is an antagonistic effect between B and Al. Supplementing boron can rescue aluminum stress in root growth and cell culture (Lukaszewski and Blevins, 1996; Koshiba et al., 2009; Horst et al., 2010). However, in high concentrations B is harmful to both plants and animals. In animals, B can cause reproductive abnormalities, such as a decrease in the X:Y sperm ratio (Robbins et al., 2008). In plants, excess B induces DNA damage by producing ROS (Cervilla et al., 2007). But this is not the only mechanism of action of boron to induce genotoxicity. In the report from Sakamoto et al. (2011), they found two knockout mutants *heb1-1* and *heb2-1*, which are hypersensitive to high concentration of

B and the genotoxic chemicals zeocin and aphidicolin, but not to ROS generating agents. *HEB1* and *HEB2* encode the CAP-G2 and CAP-H2 subunits of condensin II (Fujimoto et al., 2005), which is an important component to ameliorate DSBs. Supplemented excess B combined with defect of condensin II induces the accumulation of DSBs which lead to hypersensitive *heb* mutants. Taken together, these data indicate that B toxicity might be caused by a combination of DSBs and replication stress, which is induced by some unidentified (new) mechanism besides the production of ROS.

Table 1. Overview of genotoxic agents

Type	Agents	Action
Ionizing radiations	X-rays, Gamma rays, Alpha particles	Cause DNA breakage and other damages
Ultraviolet		Absorbed strongly by bases, producing pyrimidine dimers, which can cause error in replication if left uncorrected.
Reactive oxygen species (ROS)	Superoxide, Hydroxyl radicals, Hydrogen peroxide	Production of many base adducts, as well as DNA strand breaks and crosslinks.
Alkylating	Ethylmethylsulfone (EMS), Nitrosamines	Transfer methyl or ethyl group to bases or the backbone phosphate groups.
Intercalation	Ethidium bromide, Proflavine	Insert between bases in DNA, causing frameshift mutation, block transcription and replication.
Metals	Arsenic, Cadmium, Chromium, Nickel, Iron	Associated with the production of ROS, alter the fidelity of DNA replication, DNA hypermethylation and histone deacetylation
DNA replication inhibitor	Hydroxyurea, Aphidicolin, Actinomycin D	Block the cell cycle process
Cytotoxic antibiotics	Bleomycin, Mitomycin	DNA intercalation, generation of highly reactive free radicals that damage intercellular molecules

DNA Damage Response

After DNA damage occurred, the DNA damage response (DDR) mechanism will be activated to arrest cell division. DDR is a conserved bio-process in eukaryotes. When damage is detected, the DNA damage checkpoint is activated and a cell cycle arrest is induced, allowing the cell time to repair the damage. If the damage is too extensive, programmed cell death (PCD) is induced (Nyberg et al., 2002)But such kind conservation just a part conservation. There are lots of related but different components existing in animals and plants (Figure 2).

DDR in animals

ATM and ATR

The first important step in the DDR is initializing Ataxia Telangiectasia Mutated (ATM) and Ataxia telangiectasia mutated and Rad3-related (ATR), which are two important conserved DNA damage sensing mechanisms. Subsequently, a set of DDR genes will be promoted to rescue the damage cells. ATM and ATR are two phosphotidylinositol-3-kinase (PI3K)-related protein kinases that trigger the activation, stabilization or degradation of a number of transducer and effector proteins in the DNA damage response to arrest cell division and allow cells to repair damaged DNA before entering mitosis (Zhou and Elledge, 2000; Abraham, 2001; Bartek and Lukas, 2001; Kurz and Lees-Miller, 2004). These PI3K regulators have different but related functions in the DNA damage response. ATM mainly senses double-strand breaks (DSBs) and ATR responds to replication inhibition and single-strand breaks (SSBs) generated by processing of DSBs (Kastan and Bartek, 2004) (Figure 2A).

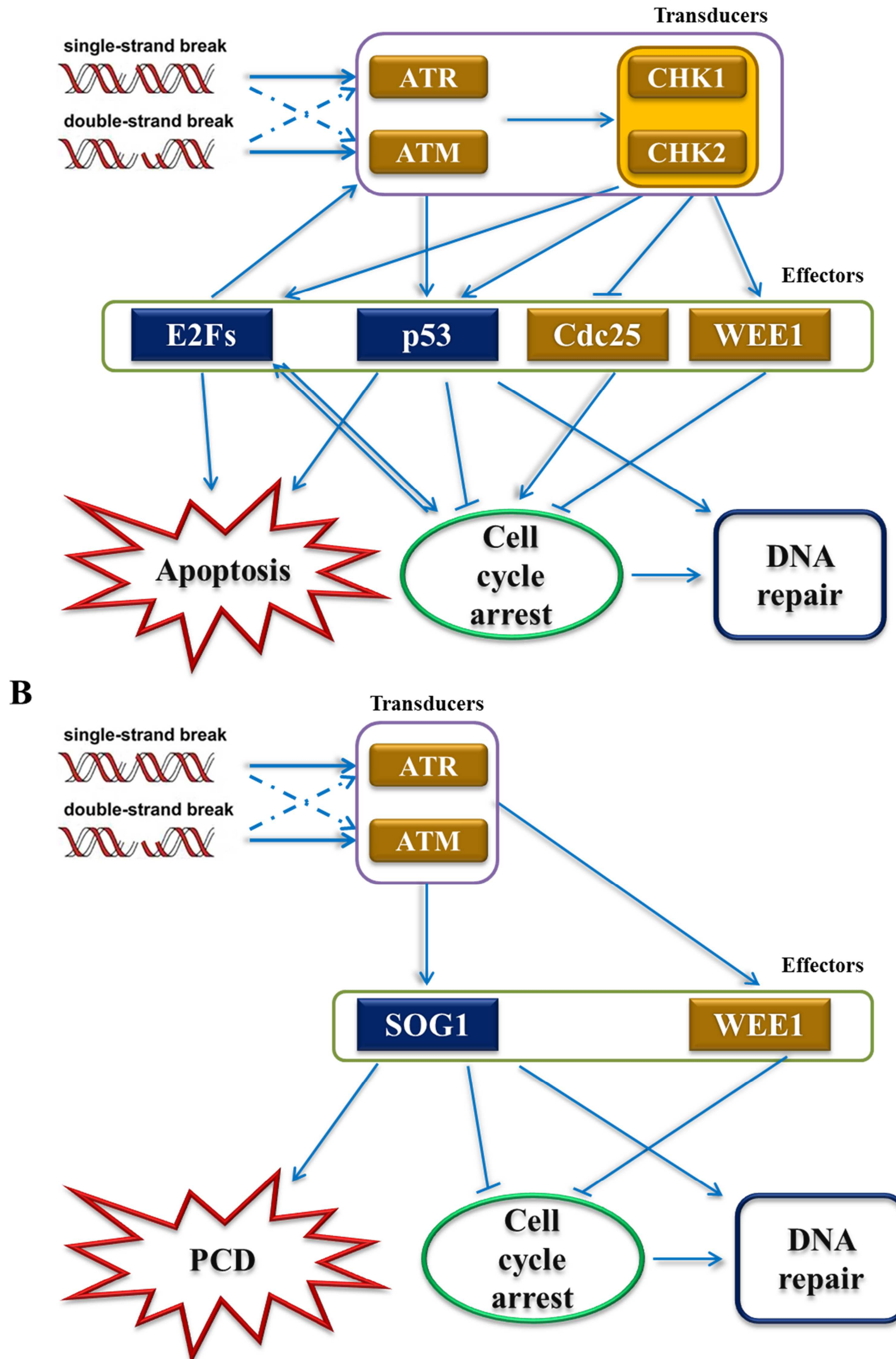

Figure 2. DNA damage response.

(A) In animals, the ATM ATR signaling pathway is activated, which leads to the phosphorylation and activation of Chk1 and Chk2 and to the subsequent modulation of Cdc25, p53 and WEE1.As cell cycle regulators, E2Fs also take part in the DDR process. **(B)** In plants, several key components of DDR existing in the animal are absent (p53, CHK1, and CHK2) or non-functional homologs (CDC25). DNA damage signals through ATM/ATR transducing kinases, SOG1 transcription factor and WEE1 leading to DNA repair, cell-cycle checkpoint or programmed cell death.

Under normal conditions, ATM is not essential for cell survival and differentiation, however, *ATM* mutations lead to predispose carriers to cancer formation by facilitating senescence and bypassing apoptosis and cellular proliferation despite the accumulation of DNA damage(Shiloh and Kastan, 2001; Luo et al., 2009). Meanwhile, *atm*-null mice show meiotic defects because of absence of ATM-dependent DSBs repair during meiotic recombination (Lange et al., 2011; Daniel et al., 2012). Beyond the DNA damage response, there are experiments that demonstrate the direct ATM activation in the presence of hydrogen peroxide (H_2O_2) independently of both DNA and MRN (Guo et al., 2010a; Guo et al., 2010b). ATM protein is constitutively expressed in cells. This means that ATM needs some kind of posttranslational activation under genotoxic stress. First step to initialize the DNA damage response is detecting the DNA damage. Reports show that the Mre11/Rad50/Nbs1 (MRN) complex detects DSBs and recruits ATM to the site of the damage (Carson et al., 2003; Lee and Paull, 2004). Upon the modulation of the MRN complex, DSBs stimulate the ATM homodimer to autophosphorylate its subunits. This leads to the dissociation into two active ATM monomers that can promote the downstream DNA damage response genes (Bakkenist and Kastan, 2003).

ATR is another PI3K which contains a motif that is conserved between both ATM and SpRad3 (Cimprich et al., 1996). ATR controls and co-ordinates DNA replication origin firing, replication fork stability, cell cycle checkpoints and DNA repair (Nam and Cortez, 2011). Unlike ATM, ATR is crucial for cell development and differentiation. Absence of ATR in mice results in early embryonic death (Brown and Baltimore, 2000) and deletion of *ATR* in adult mice has also shown to lead to stem cell loss and premature aging (Ruzankina et al., 2007) ATR exists in a form that is constitutively ready to phosphorylate substrates. Furthermore, its kinase activity doesn't increase during DNA damage stress. It appears the activation to ATR is largely controlled through its subcellular localization (Abraham, 2001; Kastan and Bartek, 2004). In human cells, there is an ATR interacting protein (ATRIP) that forms a stable complex with ATR, and this protein is considered as a potential regulatory partner for ATR (Cortez et al., 2001; Ünsal-Kaçmaz and Sancar, 2004). Replication protein A (RPA), an ssDNA-binding protein, can recruit ATR-ATRIP complexes to ssDNA produced by DNA damage (Lee et al., 2003; Zou and Elledge, 2003). Recruitment of ATR–ATRIP to DNA lesions or stalled forks is not the only reaction for checkpoint signalling. A new ATR activator TOPBP1 which contains a region termed the AAD (ATR-activating domain) binds

surfaces on both ATR and ATRIP ant it is recruited independently of ATR–ATRIP localization. (Mordes et al., 2008).

CHK1 CHK2

The ATM and ATR kinases transduce the DNA stress signal to the checkpoint kinases CHK1 (Walworth et al., 1993) and CHK2 (Murakami and Okayama, 1995), which in turn arrest the cell cycle by directly modulating the activity of the effectors that control cell cycle progression (Chen and Sanchez, 2004). CHK1 and CHK2 are regulated by ATR and ATM, respectively, in response to DNA replication blocks or DNA damage (Liu et al., 2000).

When ATR is translocated to DNA replication foci, this kinase phosphorylates CHK1 to activate it. Consequently, activated CHK1 phosphorylates downstream elements such as Cdc25-A, Cdc25-B and Cdc25-C. Apparently, phosphorylation of CHK1 will be blocked in cells that lack the ATR kinase. Like ATR, a deficiency in CHK1 will block mice embryo development, which implies an essential role of the ATR-CHK1 pathway in the cell cycle (Liu et al., 2000).

In contrast to CHK1, CHK2 is dispensable for prenatal embryo development in mice. CHK2 responds in an ATM-dependent way to DSBs (Hirao et al., 2000; Melchionna et al., 2000). Biochemical analysis indicates that activated CHK2 phosphorylates Cdc25A, Cdc25C, BRCA1 and p53 (Bartek and Lukas, 2003). The ATM–CHK2 pathway is important during the DNA damage checkpoint, resulting in cell cycle arrest and DNA repair, or induction of apoptosis (Smith et al., 2010).

WEE1 and CDC25

In mammals, CDC25 and WEE1 are two other key factors. They are a pair of analogous opposites. At the G2/M transition, Cdk1 is activated by Cdc25 through dephosphorylation of Tyr15 and inactivated by WEE1 through phosphorylation at its C-terminus. Under genotoxic conditions, they are modulated by ATM and ATR directly or through CHK1 and CHK2 (Lee et al., 2001; Harper and Elledge, 2007).

CDC25 was first described in yeast *Schizosaccharomyces pombe* as a cell cycle defective mutant (Strausfeld et al., 1991). The CDC25 proteins represent a group of phosphotyrosine phosphatases, which activates CDKs by removing inhibitory

phosphates from residues in the CDK active site to promote cell cycle. There are 3 members of CDC25 (CDC25A CDC25B CDC25C) in animals.

Under genotoxic conditions, CDC25 proteins are phosphorylated by CHK1/CHK2. The consequent binding of 14-3-3 proteins to phosphorylated CDC25 leads to downregulation of their phosphatase activity (Blasina et al., 1999; Uto et al., 2004), through their exclusion from the nucleus (Peng et al., 1997), and degradation by the ubiquitin-proteasome pathway (Falck et al., 2001).

WEE1 encodes a tyrosine kinase that through phosphorylation of CDKs inhibits their activity. Nurse (1975) described this kinase first in yeast. In yeast and animal cells, WEE1 kinase regulates CDK/cyclin activity together with CDC25 (Harper and Elledge, 2007). Knockout of WEE1 in yeast results in premature entry in mitosis. In contrast, constitutive expression blocks cells in the G2 phase (Nurse, 1975; Russell and Nurse, 1987). Under replication stress conditions, WEE1 and CDC25 will be phosphorylated by ATR-CHK1/2. After phosphorylation, CDC25 will be degraded through the ubiquitin-proteasome pathway to arrest cell entry into mitosis. In contrast, phosphorylated WEE1 can interact with 14-3-3 to bind mitotic CDK/cyclin and repress complex activity (Lee et al., 2001; Rothblum-Oviatt et al., 2001).

p53

The mammalian tumor suppressor p53 protein is a key regulator in preventing cancer (Prives and Hall, 1999; Green and Chipuk, 2006). The activity of p53 is mainly regulated by post-transcriptional modifications like phosphorylation, SUMOlylation, neddylation and acetylation (Appella and Anderson, 2001). Under normal conditions, p53 protein is degraded though the Mdm2-mediated ubiquitin-proteasomes pathway (Liang and Clarke, 2001). Upon DNA damage stress, p53 protein degradation is inhibited by its hyperphosphorylation by ATR/ATM directly and through CHK1/CHK2 (Maya et al., 2001). Activated p53 arrests cells in the G1 phase through the induction of expression of $p21^{CKI}$. Meanwhile, p53 promotes many DNA damage response genes to initialize cell cycle arrest, DNA repair or apoptosis (Wahl and Carr, 2001; Green and Chipuk, 2006) .

DDR in plants

The sedentary nature and plasticity of plants has resulted in specific adjustments of the checkpoints (Cools and De Veylder, 2009). In plants, ATM/ATR-dependent signaling pathways also control the activation of a cell cycle checkpoint upon DNA stress (Garcia et al., 2000; Garcia et al., 2003; Culligan et al., 2004; Culligan et al., 2006). Besides ATM and ATR, several key components of the animal signaling pathways appear to be absent (p53, CHK1, and CHK2) or non-functional homolog (CDC25) in plants (Figure 2B), indicating that fundamental differences exist between distantly related phyla in the recognition and signaling of DNA stress. Insight into the plant DNA damage response will be described below, with a focus on the differences with animals.

ATM

Similar as in animals, ATM plays a very important role in plants. ATM in Arabidopsis was isolated based on sequence homology cloning (Garcia et al., 2000). ATM protein in plants is highly similar to human ATM (67 and 45% similarity in the PI3K-l and rad3 domains, respectively).

As one of core DNA damage response reactors in plants, ATM is playing a similar role as in animals. ATM controls a DSB checkpoint in Arabidopsis. ATM^{KO} mutants are hypersensitive to γ-radiation and methylmethane sulfonate but not to UV-B light (Garcia et al., 2003). Activated ATM proteins induce the expression of downstream genes involved in cell cycle arrest and DNA repair (Ricaud et al., 2007b). Specifically, ATM phosphorylates the transcription factor SOG1, which is an important plant-specific transcription factor involved in the DNA damage response (Adachi et al., 2011; Yoshiyama et al., 2013). If DNA damage can’t be repaired effectively, ATM can induce a SOG1-dependent apoptosis program (Fulcher and Sablowski, 2009; Furukawa et al., 2010).

In response to DNA damage, ATM is mostly regulated at the protein level (Garcia et al., 2000). Like animals, the Mre11-Rad50-Nbs1 (MRN) complex in Arabidopsis cells is the first sensor of double-strand breaks, which will subsequently activate ATM during the DNA damage response. (Waterworth et al., 2007; Amiard et al., 2010). Different with animals, an ATM defect in Arabidopsis leads only to partial sterility (Garcia et al., 2003), indicating that ATM activity is crucial during meiosis. *ATM* deficient

Arabidopsis plants show a fragmentation of chromosomes in the early prophase I. This fragmentation is generated by SPO11, a topoisomerase-like endonuclease, to form recombinations between homologous chromosomes during the prophase of the first meiotic division. *ATM* is needed to induce the repair process of these endogenous DSBs. Without functional ATM this will lead to a semisterile phenotype (Culligan and Britt, 2008).

ATR

ATR in Arabidopsis is highly conserved with ATR proteins in other species. Besides its conserved parts, it contains motifs that are partially similar to ATM. Interestingly, different with animals, Arabidopsis ATR^{KO} lines show the same phenotype as wild type plants under normal growth conditions (Culligan et al., 2004). However, ATR^{KO} mutants show hypersensitivity to replication stress inducing agents such as HU, aphidicolin and UV-B light, and only mild sensitivity to DSB-inducing agents like γ-radiation, which implies that ATR is primarily required to respond to replication stress, and secondary to double-strand breaks. Together with ATM, ATR is involved in the maintenance of chromosomal stability (Amiard et al., 2010; 2011). ATR, cooperating with a telomere constituent CTC1/STN1/TEN1 (CST) complex, is an essential element to maintain plant telomeres (Boltz et al., 2012). Upon genotoxic stress, or lacking functional CST, ATR activates a G2 checkpoint or induces cell death (Furukawa et al., 2010; Amiard et al., 2011; Boltz et al., 2012). Similar with animals, ATR is also regulated by an ATRIP protein. The *HUS2* gene encodes an ATRIP ortholog protein in Arabidopsis. *hus2-1* mutants have been identified as mutants showing hypersensitivy to HU treatment (Sweeney et al., 2009).

WEE1

In plants, WEE1-related kinases have been described in maize (Zea mays) (Sun et al., 1999), tomato (*Solanum lycopersicum*) (Gonzalez et al., 2004) and Arabidopsis (Sorrell et al., 2002). Similar to animals and yeast, plant WEE1 kinase also plays an important role in the DNA damage response. HU increases *WEE1* expression at the transcriptional level in an ATR-dependent way (De Schutter et al., 2007). This result in an arrest of cell cycle progression during S-phase. $WEE1^{KO}$ mutant plants on the other hand are hypersensitive to HU and show a large amount of dead cells. After a prolonged and strong HU treatment, $WEE1^{KO}$ plants show disorganization in the xylem tissue (Cools et

al., 2011). Meanwhile, *WEE1* expression induced by γ-irradiation was shown to be controlled by ATM, but *WEE1*^{KO} plants do not show sensitivity to BM (Cools et al., 2011), which means that the WEE1 pathway is dispensable in response to DSBs. In other words, there should be other mechanisms to control the DSB response besides WEE1. Above all, WEE1 is regulating the S-phase process under DNA damage conditions.

SOG1

Besides WEE1, SOG1 is the other important cell cycle regulator in the DNA damage response and cell cycle regulation. *SOG1 (suppressor of gamma response)* encodes a transcription factor. It is required for the induction of a large amount of transcripts in response to gamma radiation (Yoshiyama et al., 2009). Although the amino-acid sequences of SOG1 are unrelated to those of mammalian p53, the two proteins functions are similar to each other (Yoshiyama et al., 2013). *SOG1*^{KO} plants show resistance to DNA double stranded break (DSB) inducing agents such as bleomycin/zeocin and γ-irradiation (Yoshiyama et al., 2009). Furthermore, endoreduplication under DNA damage is repressed in *SOG1*^{KO} (Adachi et al., 2011). Meanwhile there is only a low level of cell death in root stem cells in *SOG1*^{KO} during genotoxic stress (Furukawa et al., 2010). Taken together, SOG1 plays an important role in the DNA damage response, especially in the DSB response. SOG1 promotes hundreds of transcripts in response to DNA damage to arrest cell proliferation for DNA repair. On the other hand, if DNA damage can't be rescued efficiently, SOG1 will initialize programmed cell death (PCD) progress(Furukawa et al., 2010).

SOG1 is activated by ATM (Preuss and Britt, 2003; Culligan et al., 2006; Ricaud et al., 2007a). Expression of *SOG1* was significantly increased by 100 Gy γ-irradiation in wild type and *ATR*^{KO} mutants, but not in *ATM*^{KO}. Besides regulation on the transcriptional level, SOG1 is also activated post-translationally by ATM. A serine-glutamine (SQ) domain in SOG1 protein is phosphorylated in an ATM-dependent manner under genotoxic stress, including γ-irradiation, zeocin or ROS (Yoshiyama et al., 2013; Yi et al., 2014). Interestingly, there is no evidence that SOG1 is directly regulated by ATR, but several reports showed that the ATR-SOG1 pathway still exists (Yoshiyama et al., 2009; Furukawa et al., 2010). DDR-induced cell expansion occurred in *ATM*^{KO} or *ATR*^{KO} single mutants, but was significantly suppressed in the *ATM*^{KO}/*ATR*^{KO} double

mutant or in the *SOG1*^{KO} (Adachi et al., 2011). In the long-term response to irradiation, *ATR*^{KO} mutants but not *ATM*^{KO} exhibit a similar phenotype to *SOG1*^{KO} seedlings. It suggests that SOG1 is activated in response to the activation of ATR by some replication-blocking lesions (Yoshiyama et al., 2009). SOG1 also triggers stem-cell death by gamma irradiation or UVB, which is activated in both cases by either ATR or ATM (Furukawa et al., 2010). These results emphasize the importance and complexity of SOG1 during the DNA damage response.

CONCLUSION

The regulation of cell cycle process and DNA damage response is conserved in eukaryotes. Because of that, the main elements involved in cell cycle and DNA damage in plants are identified based on comparative research with animal and yeast. For example, CDKA proteins exhibit high similarity to the CDKs in animal and yeast, both on a structural and functional level. ICK/KRP proteins contain the CDK inhibitory region that is analogous to the mammalian CKI protein p27^{Kip1}. The E2F/RBR pathway, which is crucial to regulate cell cycle process both in eukaryotic growth and development or response of environmental signaling, shows resemblance from functional mechanisms to regulatory pathways.

However, in pace with recent research results, it appears that plants have evolved different specific elements and regulatory pathways involved in cell cycle regulation and DNA damage response. For instance, there is a special CDKB family in plants that regulates cell division under the control of plant specific hormones. New CKI members e.g SIM/SMR and SCI1 are found in Arabidopsis. They are involved in plant organogenesis including stigma and trichomes, or response to abiotic stress. Besides the new elements of cell cycle regulation, new components involved in the DNA damage response also are identified. In plants, SOG1 plays a core role to inhibition of cell cycle, DNA repair and initialization of programmed cell death, analogously to the role of p53 in mammals.

The discovery of new components and mechanisms of cell cycle regulation and DNA damage response in plants is just beginning. It is foreseeable that to reveal more such mechanisms should be based on the understanding of plant features. These features can include plant hormone signaling pathways, cell wall formation, heavy metal ion

accumulation from soil, or photoperiodism and photosynthesis regulation. Identifying the pathway involved in DDR and cell cycle regulation is important to understand the reaction network of plants when they face extrinsic and intrinsic stresses.

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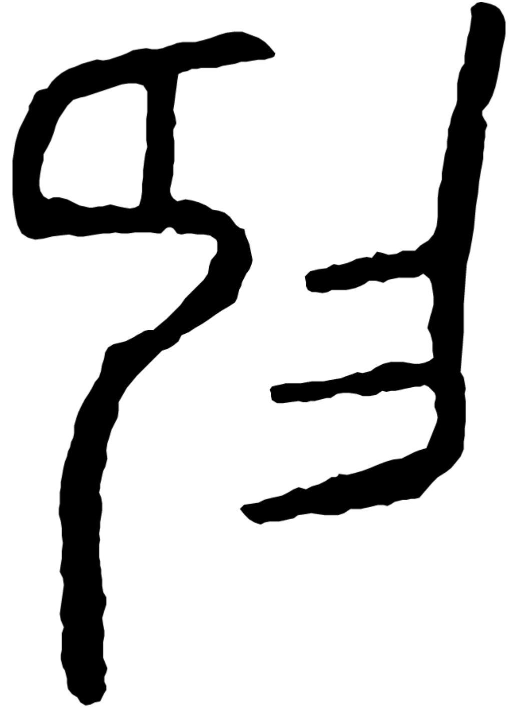


CHAPTER 2

THE ARABIDOPSIS THALIANA SIAMESE-RELATED CYCLIN-DEPENDENT KINASE INHIBITORS SMR5 AND SMR7 CONTROL THE DNA DAMAGE CHECKPOINT IN RESPONSE TO REACTIVE OXYGEN SPECIES

The *Arabidopsis thaliana* SIAMESE-RELATED cyclin-dependent kinase inhibitors SMR5 and SMR7 control the DNA damage checkpoint in response to reactive oxygen species

Dalong Yi,[a,b,1] Claire Lessa Alvim Kamei,[a,b,1,2] Toon Cools,[a,b] Sandy Vanderauwera,[a,b] Naoki Takahashi,[c] Yoko Okushima,[c] Thomas Eekhout,[a,b] Kaoru Okamoto Yoshiyama,[c] John Larkin,[d] Hilde Van den Daele,[a,b] Phillip Conklin,[e] Anne Britt,[e] Masaaki Umeda,[c,f] and Lieven De Veylder[a,b,3]

[a]Department of Plant Systems Biology, VIB, B-9052 Gent, Belgium

[b]Department of Plant Biotechnology and Bioinformatics, Ghent University, B-9052 Gent, Belgium

[c]Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0192, Japan

[d]Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

[e]Department of Plant Biology, University of California Davis, Davis, CA 95616, USA

[f]JST, CREST, Nara 630-0192, Japan

[1]These authors contributed equally to this work

[2]Present address: Wageningen UR - Plant Breeding, Wageningen University and Research Center, P.O. Box 386. 6700 AJ Wageningen. The Netherlands.

Running title: SMRs and DNA checkpoint control

AUTHOR CONTRIBUTIONS

D.Y., C.L.A.K., T.C., S.V., A.B., M.U. and L.D.V. conceived and designed the research. D.Y., C.L.A.K., T.C., S.V., N.T., Y.O., T.E., K.O.Y., H.V.d.D. and A.B. performed the experiments. D.Y., C.L.A.K., T.C., S.A., N.T., J.L., A.B., M.U. and L.D.V. analyzed the data and wrote the manuscript. All authors read, revised and approved the manuscript.

ABSTRACT

Whereas our knowledge about the diverse pathways aiding DNA repair upon genome damage is steadily increasing, little is known about the molecular players that adjust the plant cell cycle in response to DNA stress. By a meta-analysis of DNA stress microarray datasets, three family members of the *SIAMESE/SIAMESE-RELATED* (*SIM/SMR*) class of cyclin-dependent kinase inhibitors were discovered that react strongly to genotoxicity. Transcriptional reporter constructs corroborated specific and strong activation of the three *SIM/SMR* genes in the meristems upon DNA stress, whereas overexpression analysis confirmed their cell cycle inhibitory potential. In agreement with being checkpoint regulators, *SMR5* and *SMR7* knockout plants displayed an impaired checkpoint in leaf cells upon treatment with the replication inhibitory drug hydroxyurea (HU). Surprisingly, HU-induced *SMR5/SMR7* expression depends on ATAXIA TELANGIECTASIA MUTATED (ATM) and SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1), rather than on the anticipated replication stress-activated ATM AND RAD3-RELATED (ATR) kinase. This apparent discrepancy was explained by demonstrating that, in addition to its effect on replication, HU triggers the formation of reactive oxygen species (ROS). ROS-dependent transcriptional activation of the *SMR* genes was confirmed by different ROS-inducing conditions, including high-light treatment. We conclude that the identified *SMR* genes are part of a signaling cascade inducing a cell cycle checkpoint in response to ROS-induced DNA damage.

Adapted from manuscript published online before print January 2014, doi: http://dx.doi.org/10.1105/tpc.113.118943. *The Plant Cell,* 2014 tpc.113.118943.

INTRODUCTION

Being sessile, plants are continuously exposed to changing environmental conditions that can impose biotic and abiotic stresses. One of the consequences observed in plants subjected to altered growth conditions is the disruption of the reactive oxygen species (ROS) homeostasis (Mittler et al., 2004). Under steady-state conditions, ROS are efficiently scavenged by different non-enzymatic and enzymatic antioxidant systems, involving the activity of catalases, peroxidases, and glutathione reductases. However, when stress prevails, the ROS production rate can exceed the scavenging mechanisms, resulting into a cell- or tissue-specific rise in ROS. These oxygen derivatives possess a strong oxidizing potential that can damage a wide diversity of biological molecules, including the electron-rich bases of DNA, which results into single- and double-stranded breaks (Amor et al., 1998; Dizdaroglu et al., 2002; Roldán-Arjona and Ariza, 2009). Hydrogen peroxide (H_2O_2) is a major ROS compound and is able to transverse cellular membranes, migrating into different compartments. This feature grants H_2O_2 not only the potential to damage a variety of cellular structures, but also to serve as a signaling molecule, allowing the activation of pathways that modulate developmental, metabolic and defence pathways (Dizdaroglu, 2005). One of the signaling effects of H_2O_2 is the activation of a cell division arrest by cell cycle checkpoint activation (Tsukagoshi, 2012), however the molecular mechanisms involved remain unknown.

Cell cycle checkpoints adjust cellular proliferation to changing growth conditions, arresting it by the inhibition of the main cell cycle controllers: the heterodimeric complexes between the cyclin-dependent kinases (CDK) and the regulatory cyclins (Lee and Nurse, 1987; Norbury and Nurse, 1992). The activators of these checkpoints are the highly conserved ATAXIA TELANGIECTASIA MUTATED (ATM) and ATM AND RAD3-RELATED (ATR) kinases that are recruited in accordance with the type of DNA damage (Zhou and Elledge, 2000; Abraham, 2001; Bartek and Lukas, 2001; Kurz and Lees-Miller, 2004). ATM is activated by double-stranded breaks (DSBs); whereas ATR is activated by single-strand breaks or stalled replication forks, causing inhibition of DNA replication. In mammals, ATM and ATR activation result in the phosphorylation of the Chk2 and Chk1 kinases, respectively. Both kinases subsequently phosphorylate p53, a central transcription factor in the DNA damage response (Chaturvedi et al., 1999;

Shieh et al., 2000; Chen and Sanchez, 2004; Rozan and El-Deiry, 2006). Chk1, Chk2, and p53 seemingly appear to have no plant ortholog, although an analogous role for p53 is suggested for the plant-specific SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1) transcription factor that is under direct posttranscriptional control of ATM (Yoshiyama et al., 2009; Yoshiyama et al., 2013). Another distinct plant feature relates to the inactivation of CDKs in response to DNA stress. CDK activity is in part controlled by its phosphorylation status at the N-terminus, determined by the interplay of the CDC25 phosphatase and the antagonistic WEE1 kinase, acting as the "on" and "off" switches of CDK activity, respectively (Francis, 2011). Whereas in mammals and budding yeast the activation of the DNA replication checkpoint, leading to a cell cycle arrest, is predominantly achieved by the inactivation of the CDC25 phosphatase, plant cells respond to replication stress by transcriptional induction of *WEE1* (Horst et al., 2010). In absence of WEE1, *Arabidopsis thaliana* plants become hypersensitive to replication inhibitory drugs such as hydroxyurea (HU), which causes a depletion of dNTPs by inhibiting the ribonucleotide reductase (RNR) protein. However, *WEE1*-deficient plants respond similarly as control plants to other types of DNA damage (Dissmeyer et al., 2009; Horst et al., 2010). These data suggest the existence of yet to be identified pathways controlling cell cycle progression under DNA stress, operating independently of WEE1.

Potential candidates to operate in checkpoint activation upon DNA stress are CDK inhibitors (CKIs). CKI proteins are mostly low molecular weight proteins that inhibit cell division by their direct interaction with the CDK and/or cyclin subunit (Sherr and Roberts, 1995; Marnett, 2000). The first identified class of plant CKIs was the ICK/KRP (interactors of CDK/Kip-related protein) protein family comprising seven members in *A. thaliana*, all sharing a conserved C-terminal domain being similar to the CDK-binding domain of the animal CIP/KIP proteins (Dulić et al., 1998; Xue et al., 2004; Koshiba et al., 2009). The TIC (tissue-specific inhibitors of CDK) is the most recently suggested class of CKIs (DePaoli et al., 2012) and encompasses SCI1 in tobacco (DePaoli et al., 2011). SCI1 shares no apparent sequence similarity with the other classes of CKIs in plants, and has been suggested to connect cell cycle progression and auxin signaling in pistils (DePaoli et al., 2012). The third class of CKIs is the plant-specific *SIAMESE/SIAMESE-RELATED* (*SIM/SMR*) gene family. SIM has been identified as a cell cycle inhibitor with a role in trichome development and endocycle

control (Jomova and Valko, 2011). Based on sequence analysis, five additional gene family members have been identified in *A. thaliana*, and together with EL2 from rice, been suggested to act as cell cycle inhibitors modulated by biotic and abiotic stresses (Peres et al., 2007). Plants subjected to treatments inducing DSBs showed a rapid and strong induction of specific family members (Culligan et al., 2006; Buchtik et al., 2011), suggesting that SIM/SMR proteins might include interesting candidates to complement WEE1 in the global response to DNA stress.

In this work we identified three *SMR* genes (*SMR4*, *SMR5* and *SMR7*) that are transcriptionally activated by DNA damage. Cell cycle inhibitory activity was demonstrated by overexpression analysis, whereas knockout data illustrated that both *SMR5* and *SMR7* are essential for DNA cell cycle checkpoint activation in leaves of plants grown in the presence of HU. Remarkably, we found that *SMR* induction mainly depends on ATM and SOG1, rather than ATR as would be expected for a drug that triggers replication fork defects. Correspondingly, we demonstrate that the HU-dependent activation of *SMR* genes is triggered by ROS rather than replication problems, linking *SMR* genes with cell cycle checkpoint activation upon the occurrence of DNA damage-inducing oxidative stress.

RESULTS

Meta-Analysis of DNA Stress Datasets Identifies DNA Damage-Induced *SMR* Genes

When DNA damage occurs, two global cellular responses are essential for cell survival: activation of the DNA repair machinery, and delay or arrest of cell cycle progression. In recent years, gene expression inventories have been collected that focus on the transcriptional changes in response to different types of DNA stress (Culligan et al., 2006; Ricaud et al., 2007; Panda et al., 2009; Yoshiyama et al., 2009). To identify novel key signaling components that contribute to cell cycle checkpoint activation, we compared bleomycin-induced genes to those induced by HU treatment (Panda et al., 2009) and γ-radiation (Culligan et al., 2006; Yoshiyama et al., 2009). Twenty-two genes were upregulated in all DNA stress experiments and can be considered as transcriptional hallmarks of the DNA damage response (DDR), regardless of the type of DNA stress

(Figure 1; Table 1). Within this selection, genes known to be involved in DNA stress and DNA repair are predominantly present, including *PARP2*, *BRCA1* and *RAD51*. In addition, we recognized one member of the *SIM/SMR* gene family, being *SMR5* (At1g07500). When expanding the selection by considering genes induced in at least two of the three DNA stress experiments, we identified a total of 61 genes (Supplemental Table 1). Besides DDR-related genes, this expanded dataset included an additional *SMR* family member (*SMR4*; At5g02220) being expressed upon HU treatment and γ-radiation.

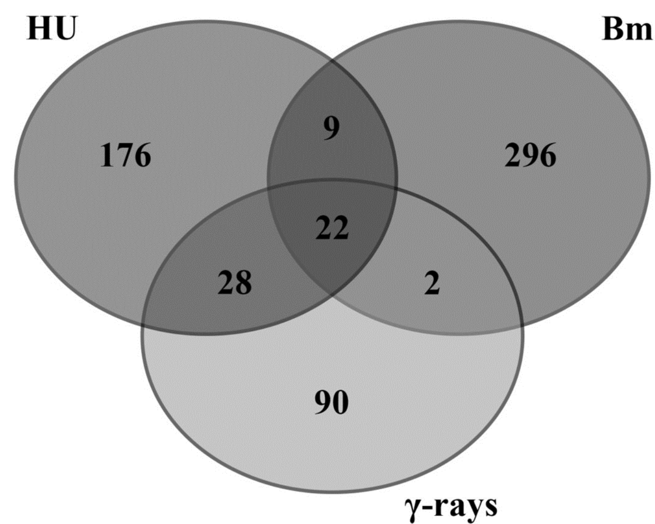


Figure 1. DNA stress meta-analysis.

Venn diagram showing the overlap between transcripts induced by hydroxyurea (HU), bleomycin (Bm), and γ-radiation (γ-rays). In total, 61 genes were positively regulated in at least two DNA stress experiments, and 22 genes accumulated in all DNA stress experiments.

Table 1: Overview of the transcriptionally induced core DNA damage genes

AGI locus	Annotation	HU 24h/0h[a]	γ-rays - 1[b]	γ-rays - 2[c]	Bleo-mycin
AT4G21070	Breast cancer susceptibility1	10.375	581.570	57.803	2.386
AT5G60250	Zinc finger (C3HC4-type RING finger) family protein	8.907	34.918	40.000	2.352
AT1G07500	Siamese-related 5	7.863	38.160	35.842	1.595
AT4G02390	Poly(ADP-ribose) polymerase	7.701	131.865	59.172	2.663
AT3G07800	Thymidine kinase	7.160	46.179	20.492	2.759
AT5G03780	TRF-like 10	7.111	108.316	23.474	1.600
AT5G64060	NAC domain containing protein 103	5.579	28.086	13.755	2.153
AT2G18600	Ubiquitin-conjugating enzyme family protein	5.521	21.462	11.481	1.972
AT4G22960	Unknown function (DUF544)	5.315	36.380	14.451	2.282
AT5G48720	X-ray induced transcript 1	5.296	285.166	65.789	2.228
AT5G24280	Gamma-irradiation and mitomycin c induced 1	4.823	108.578	42.918	2.584
AT5G20850	RAS associated with diabetes protein 51	4.643	186.456	31.250	1.765
AT3G27060	Ferritin/ribonucleotide reductase-like family protein	4.595	37.351	8.741	1.970
AT2G46610	RNA-binding (RRM/RBD/RNP motifs) family protein	3.593	19.913	7.331	1.546
AT5G40840	Rad21/Rec8-like family protein	3.375	113.919	27.473	1.692
AT1G13330	Hop2 homolog	2.949	17.349	13.495	1.580
AT5G66130	RADIATION SENSITIVE 17	2.888	30.411	10.384	1.627
AT1G17460	TRF-like 3	2.378	18.925	10.661	1.681
AT2G45460	SMAD/FHA domain-containing protein	2.378	45.673	21.053	1.575
AT5G49480	Ca2+-binding protein 1	1.952	15.106	5.851	1.580
AT3G25250	AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family protein	1.853	12.995	17.794	1.517
AT5G55490	Gamete expressed protein 1	1.670	71.489	34.722	2.407

a: According to Cools et al., 2011

b: According to Culligan et al., 2006

c: According to Yoshiyama et al., 2009

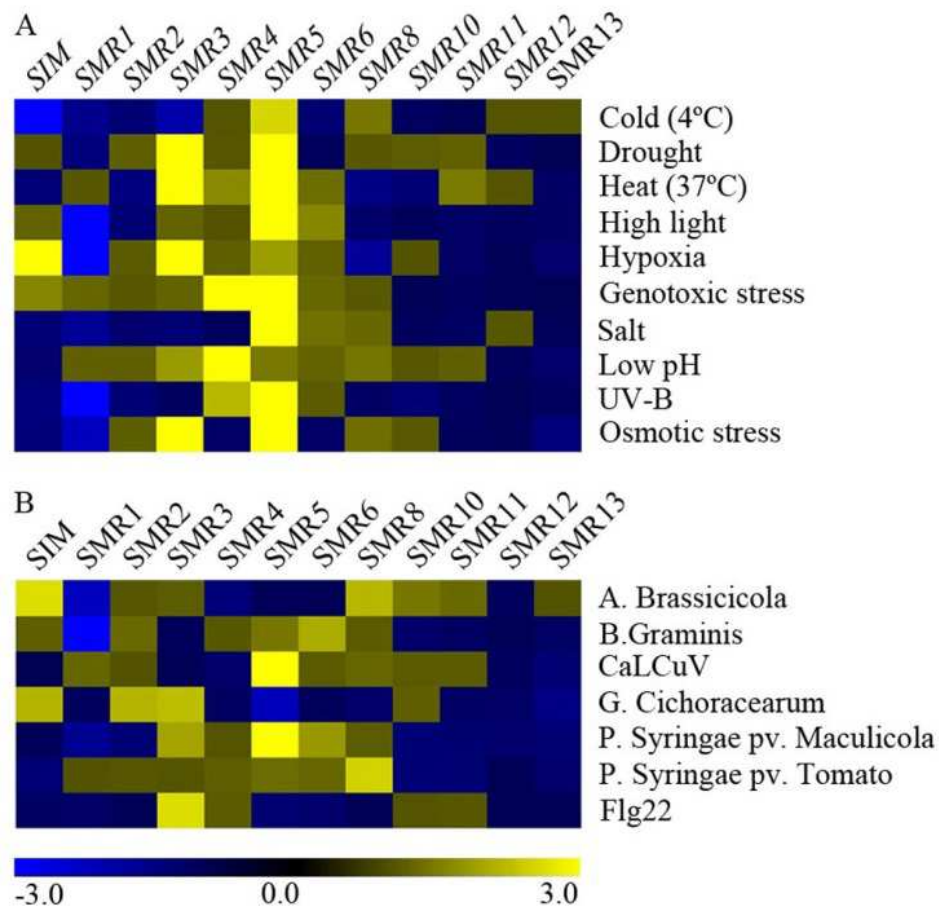


Figure 2. Hierarchical average linkage clustering of *SIM/SMR* genes induced in response to different abiotic (A) and biotic stresses (B).

Data comprise the *SIM/SMR* represented in publicly available Affymetrix ATH1 microarrays obtained with the Genevestigator toolbox. Blue and yellow indicate down- and up-regulation, respectively, whereas black indicates no change in expression.

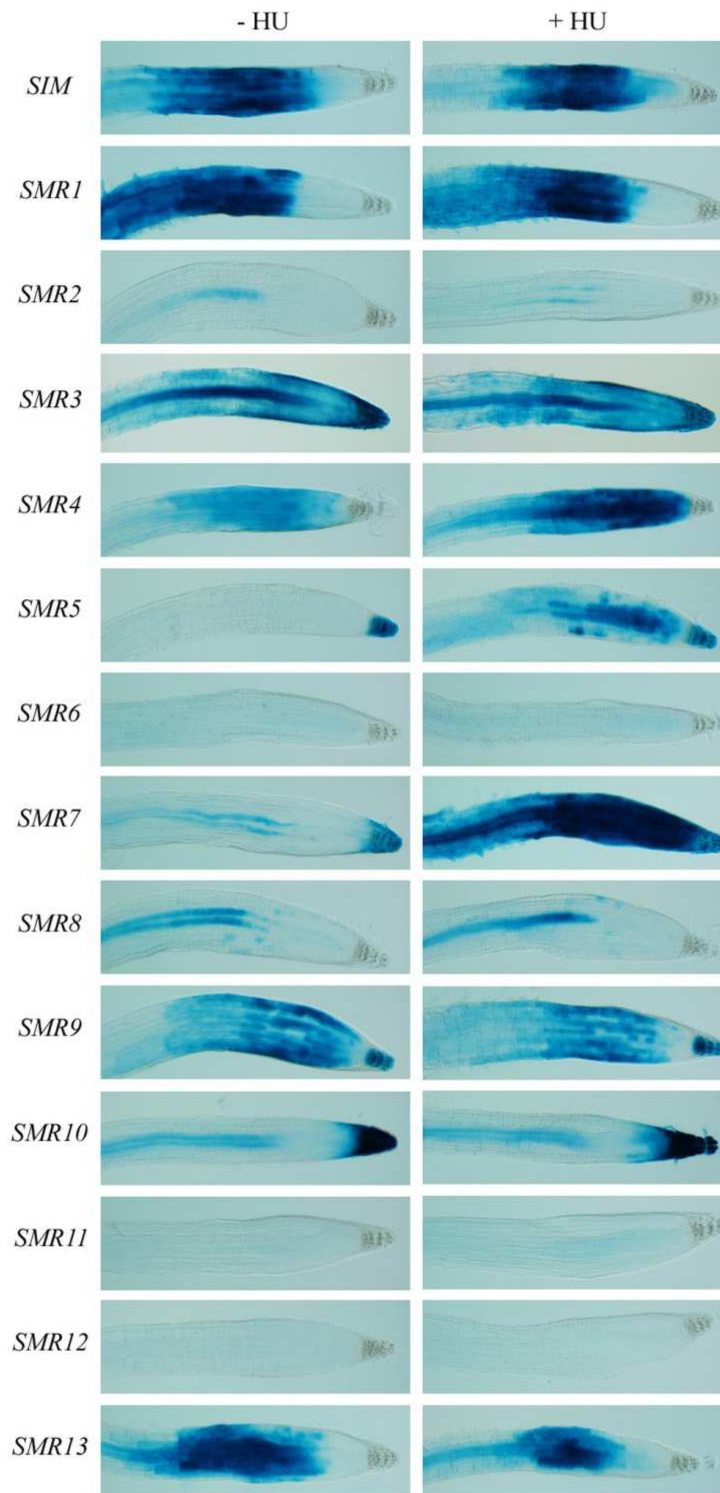


Figure 3. ***SIM/SMR*** **induction in response to HU.**

One-week-old transgenic Arabidopsis seedlings were transferred to control (-HU) medium or medium supplemented with 1 mM HU (+HU). GUS assays were performed 24 h after transfer.

The *SMR* Gene Family Comprises 14 Family Members that Respond to Different Stresses

Previously, we reported on the existence of one *SIM* and five *SMR* genes (*SMR1*-*SMR5*) in the *A. thaliana* genome (Peres et al., 2007), whereas protein purification of CDK/cyclin complexes resulted into the identification of two additional family members (*SMR6* and *SMR8*) (Ohtani et al., 2001). With the availability of newly sequenced plant genomes, we re-examined the Arabidopsis genome using iterative BLAST searches for the presence of additional *SMR* genes, resulting in the identification of six non-annotated family members, nominated *SMR7* to *SMR13* (Supplemental Table 2). With the Genevestigator toolbox (Maxwell et al., 1999), the expression pattern of the twelve *SIM/SMR* genes represented on the Affymetrix ATH1 microarray platform was analyzed in response to different biotic and abiotic stress treatments. Distinct family members were induced under various stress conditions, albeit with different specificity (Figure 2). Every *SMR* gene appeared to be transcriptionally active under at least a number of stress conditions, with *SMR5* responding to most diverse types of abiotic stresses. In response to DNA stress (genotoxic stress and UV-B treatment), two *SMR* genes responded strongly, namely *SMR4* and *SMR5*, corresponding with their presence among the DNA stress genes identified by our microarray meta-analysis.

To confirm their involvement in the genotoxic stress response, transcriptional reporter lines containing the putative upstream promoter sequences were constructed for all *SIM/SMR* genes. After selection of representative reporter lines, one-week-old seedlings were transferred to control medium, or medium supplemented with HU (resulting into stalled replication forks) or bleomycin (causing DSBs). Focusing on the root tips revealed distinct expression patterns (Figure 3; Supplemental Figure 1), with some family members being restricted to the root elongation zone (including *SIM* and *SMR1*), while others were confined to vascular tissue (e.g. *SMR2* and *SMR8*), or columella cells (e.g. *SMR5*). When plants were exposed to HU, three *SMR* genes showed transcriptional induction in the root meristem, being *SMR4*, *SMR5* and *SMR7*, with the latter two displaying the strongest response (Figure 3). In the presence of bleomycin, an additional weak cell-specific induction of *SMR6* was observed (Supplemental Figure 1). Transcriptional induction of *SMR4*, *SMR5* and *SMR7* by HU and bleomycin was

confirmed by qRT-PCR experiments (Supplemental Figure 2). These data fit the above described microarray analysis, with the lack of *SMR7* (At3g27630) being explained by its absence on the ATH1 microarray of the HU and γ-irradiation experiments, although being induced 5.68-fold in the bleomycin experiment performed using the Aragene array. Next to HU and bleomycin, we confirmed transcriptional activation of *SMR4*, *SMR5* and *SMR7* by γ-irradiation (Supplemental Figure 3).

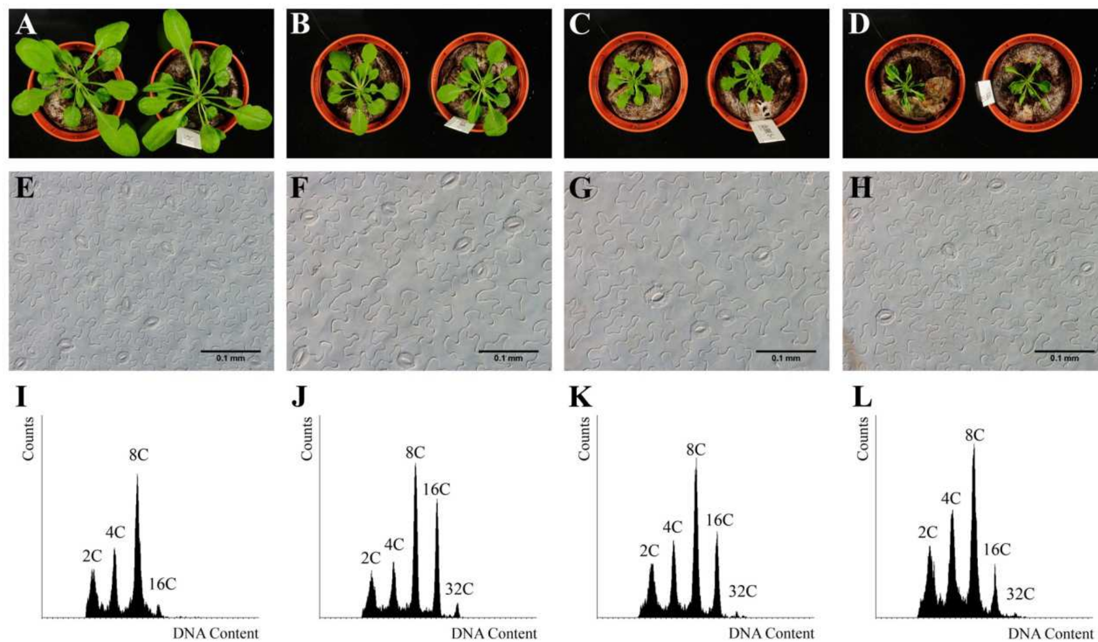


Figure 4. Ectopic *SMR4, SMR5* and *SMR7* expression inhibits cell division.

(A-D) Four-week-old rosettes of control **(A)**, *SMR4*^{OE} **(B)**, *SMR5*^{OE} **(C)** and *SMR7*^{OE} **(D)** plants. **(E-H)** Leaf abaxial epidermal cell images of *in vitro*-grown 3-week-old control **(E)**, *SMR4*^{OE} **(F)**, *SMR5*^{OE} **(G)** and *SMR7*^{OE} **(H)** plants. **(I-L)** Ploidy level distribution of the first leaves of 3-week-old *in vitro*-grown control **(I)**, *SMR4*^{OE} **(J)**, *SMR5*^{OE} **(K)** and *SMR7*^{OE} **(L)** plants.

DNA Stress-Induced *SMR* Genes Encode Potent Cell Cycle Inhibitors

SIM had been proven to encode a potent cell cycle inhibitor, since its ectopic expression results into dwarf plants holding less cells compared to control plants (Jomova and Valko, 2011). To test whether the DNA stress-induced *SMR* genes encode proteins with cell division inhibitory activity, *SMR4*-, *SMR5*- and *SMR7*-overexpressing (*SMR4*^{OE}, *SMR5*^{OE} and *SMR7*^{OE}) plants were generated. For each gene, multiple lines with high transcript levels were isolated, all showing a reduction in rosette size compared to wild-type plants (Figures 4A to 4D). This decrease in leaf size correlated with an increase in cell size (Figures 4E to H), indicative of a strong inhibition of cell division. Similar to *SIM* (Jomova and Valko, 2011), ectopic expression did not only inhibit cell division but also triggered an increase in the DNA content by stimulation of endoreplication (Figures 4I to L; Supplemental Table 3), likely representing a premature onset of cell differentiation. Together with the previously described biochemical interaction between SMR4 and SMR5, and CDKA;1 and D-type cyclins (Ohtani et al., 2001), it can be concluded that the DNA stress-induced *SMR* genes encode potent cell cycle inhibitors.

SMR5 and *SMR7* Control a HU-Dependent Checkpoint in Leaves

To address the role of the different *SMR* genes in DNA stress checkpoint control, the growth response to HU treatment of plants being knocked out for *SMR5* or *SMR7* (Supplemental Figure 4) was compared to that of control plants (Col-0). No significant difference in leaf size was observed for plants grown under standard conditions. In contrast, when comparing plants grown for 3 weeks in the presence of HU, the size of the *SMR5*^{KO} and *SMR7*^{KO} leaves was significantly bigger than that of the control plants (Figure 5A). This difference was attributed to a difference in cell number. Control plants responded to the HU treatment with a 47% reduction in epidermal cell number, reflecting an activation of a stringent cell cycle checkpoint. In contrast, in *SMR5*^{KO} and *SMR7*^{KO} plants this reduction was restricted to 29% and 30%, respectively (Figure 5B). Within the *SMR5*^{KO} *SMR7*^{KO} double mutant, the reduction in leaf size and cell number was even less (Figures 5A and 5B), suggesting that both inhibitors contribute to the cell cycle arrest observed in the control plants by checkpoint activation upon HU stress. A similar role of SMR4 could not be tested due to the lack of an available knockout.

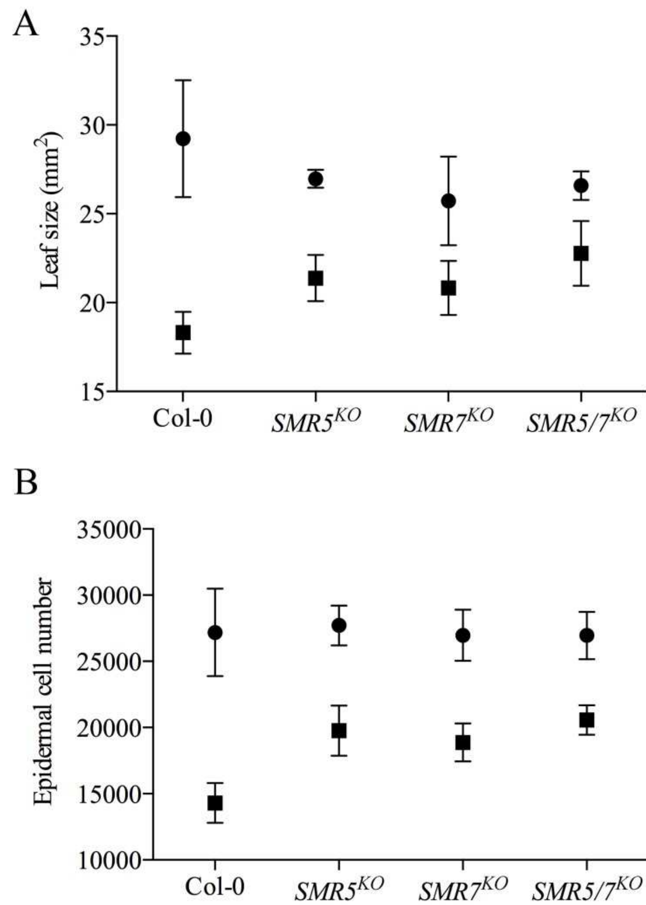


Figure 5. SMR5 and SMR7 are required for an HU-dependent cell cycle checkpoint.

(A-B) Leaf size **(A)** and abaxial epidermal cell number **(B)** of the first leaves of 3-week-old plants grown on control medium (circles) or medium supplemented with 1 mM HU (squares). Data represent mean with 95% confidence interval (2-way ANOVA, n = 10).

SMR5 and *SMR7* Expression is Triggered by Oxidative Stress

Because of the observed role of the *SMR5* and *SMR7* genes in DNA stress checkpoint control, we analyzed the dependence of their expression on the ATM and ATR signaling kinases and the SOG1 transcription factor by introducing the *SMR5* and *SMR7* GUS reporter lines into the *atr-2*, *atm-1* and *sog1-1* mutant backgrounds. Both genes were induced in the proliferating leaf upon HU and bleomycin treatment (Figure 6). Moreover, as would be expected for a DSB-inducing agent, the transcriptional activation of *SMR5* and *SMR7* by bleomycin depended on ATM and SOG1. Surprisingly, the same pattern was observed for HU, whereas one would expect that *SMR*5/*SMR7* induction after arrest of the replication fork would rely on ATR-dependent signaling. These data indicate that the HU-dependent activation of the *SMR5* and *SMR7* genes might be caused by a genotoxic effect of HU being unrelated to replication stress induced by the depletion of dNTPs. A recent study demonstrated that HU directly inhibits catalase-mediated H_2O_2 decomposition (Juul et al., 2010). Analogously, in combination with H_2O_2, HU has been demonstrated to act as a suicide inhibitor of ascorbate peroxidase (Chen and Asada, 1990). Combined, both mechanisms are likely responsible for an increase in the cellular H_2O_2 concentration, which might trigger DNA damage and consequently transcriptional induction of the *SMR5* and *SMR7* genes. Indeed, extracts of control plants treated with HU displayed a reduced H_2O_2 decomposition rate (Figure 7A). As catalase and ascorbate peroxidase activity are essential for the scavenging of H_2O_2 that is generated upon high-light exposure, we subsequently tested the effects of HU treatment on photosystem II (PSII) efficiency in one-week-old seedlings after transfer from low- to high-light conditions. As illustrated in Figure 7B, transfer for 48 h to high light resulted in a decrease of maximum quantum efficiency of PSII (Fv'/Fm'). In the presence of HU, the Fv'/Fm'decrease was even more pronounced, which again corroborates the idea that HU might interfere with H_2O_2 scavenging. Macroscopically, plants grown in the presence of HU showed visible anthocyanin pigmentation in the young leaf tissue within 48 h after transfer, whereas plants grown on control medium showed no effect of the transfer to high light (Figure 7C).

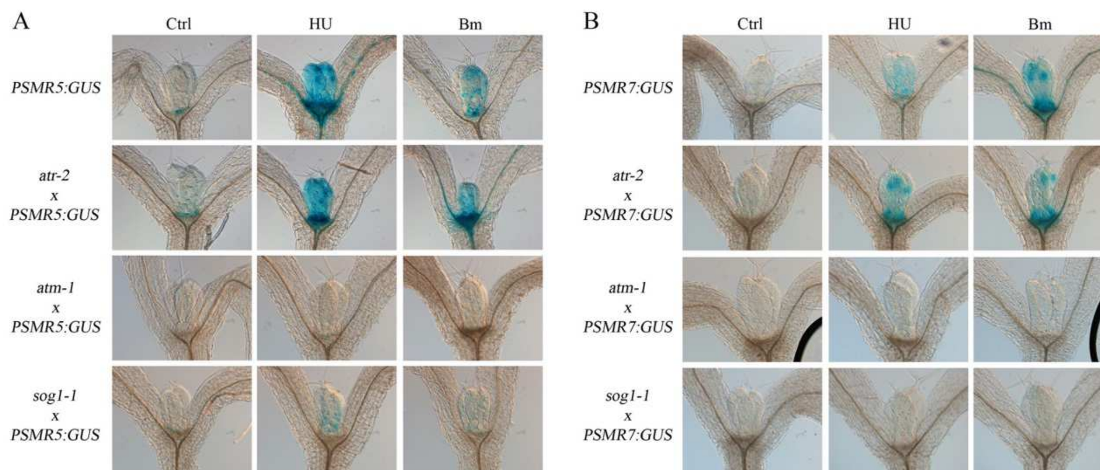


Figure 6. ***SMR5*** **and** ***SMR7*** **expression is ATM- and SOG1-dependent.**

(A-B) *PSMR5:GUS* **(A)** and *PSMR7:GUS* **(B)** reporter constructs introgressed into *atr-2*, *atm-1* and *sog-1* mutant backgrounds were control-treated (Ctrl), or treated with 2 mM HU or 0.3 µg/ml bleomycin (Bm) for 24 h.

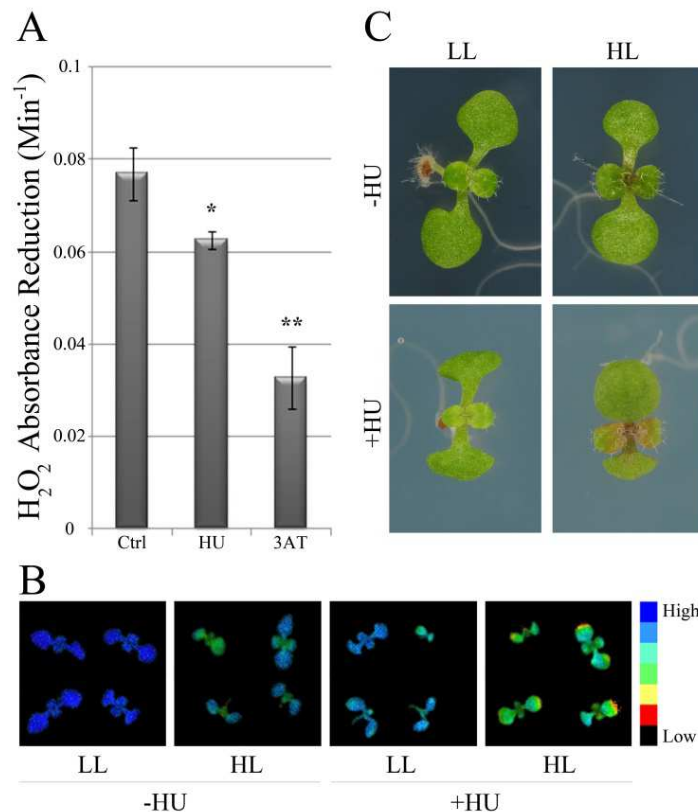


Figure 7. HU triggers oxidative stress.

(A) H_2O_2 scavenging of in extracts from one-week-old control (Ctrl), HU-treated (1 mM) and 3AT-treated (6 µM) (positive control) plants. Error bars show SE (n = 3-4). *, P-value < 0.05; **, P-value < 0.01 (two-tailed student's T-test). **(B)** Maximum quantum efficiency of PSII (Fv'/Fm') of 6-day-old seedlings grown under low (LL) and high light (HL) for 48 hrs, in absence (-HU) and presence (+HU) of 1 mM HU. **(C)** Light microscope pictures of plants shown in **(B)**.

To examine whether an increase in H_2O_2 might trigger expression of *SMR* genes, *SMR5* and *SMR7* expression levels were analyzed in plants that are knockout for *CAT2* and/or *APX1*, encoding two enzymes important for the scavenging of H_2O_2. Whereas *SMR5* transcript levels appeared to be stable over all genotypes, *SMR7* expression levels were clearly induced in the single *apx1* and *apx1 cat2* double mutant (Figure 8A). As an independent strategy to induce ROS, *SMR5* and *SMR7* GUS reporter lines were transferred from control to high light conditions for two days. Whereas *PSMR7:GUS* plants displayed little to no increase in GUS activity, *SMR5* promoter activity was strongly stimulated under high light, as confirmed by RT-PCR (Figure 8B; Supplemental Figure 5). To examine whether this transcriptional induction contributed to a high light-induced cell cycle checkpoint, we measured epidermal cell numbers in mature first leaves of control (Col-0), $SMR5^{KO}$ and $SMR7^{KO}$ plants that were transferred for 4 days to high light condition at the moment that their leaves were proliferating. This high light treatment resulted into a 34% and 38% reduction in cell number in control and $SMR7^{KO}$ plants, respectively (Figure 8C). In contrast, $SMR5^{KO}$ plants displayed only a 13% reduction in cell number, illustrating that SMR5 is essential to activate a high light-dependent cell cycle checkpoint.

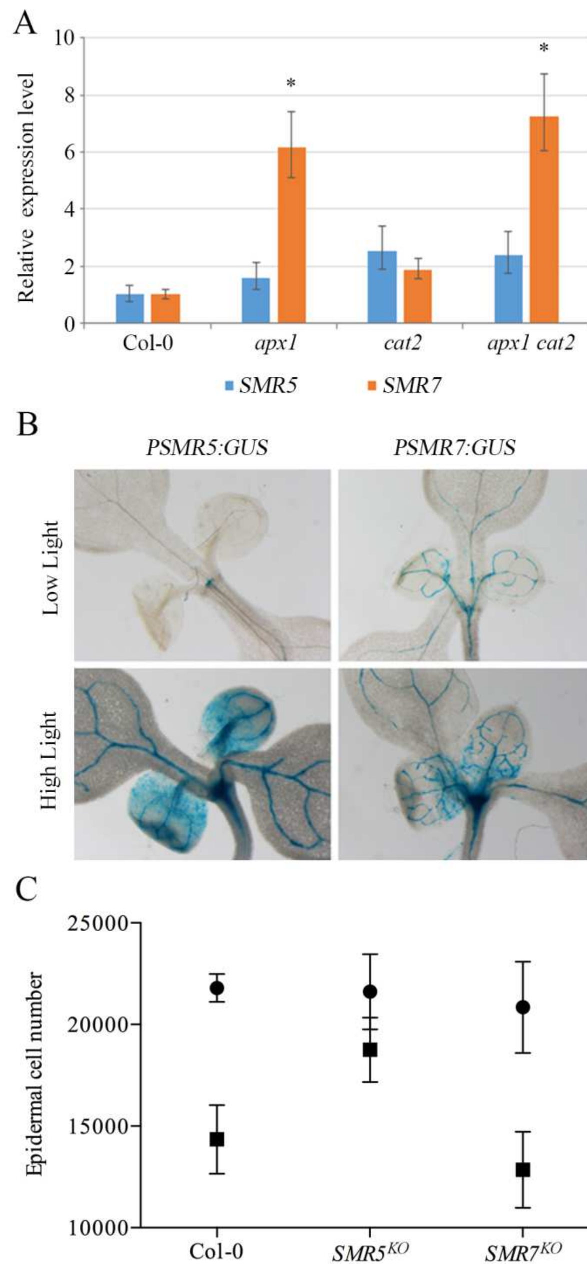


Figure 8. ***SMR5*** **and** ***SMR7*** **are induced by oxidative stress-inducing stimuli.**

(A) Relative *SMR5* and *SMR7* expression levels in shoots of 6-day-old wild-type (Col-0), *apx1*, *cat2* and *apx cat2* mutant plants. Data represent least square means ± SE, normalized to wild type levels that were arbitrary set to one (n = 3, *P-value < 0.01). **(B)** One–week-old *PSMR5:GUS* and *PSMR7:GUS* seedlings grown under low- versus high-light conditions for 48 hrs. **(C)** Abaxial epidermal cell number of the first leaves of 3-week-old plants transferred at the age of 8 days for 96 h to control (circles) or high light (squares) conditions. Data represent mean with 95% confidence interval (2-way ANOVA, n = 8).

SMR5 and *SMR7* are under Direct Control of SOG1

Recently, it was found that the SOG1 transcription factor becomes hyperphosphorylated in an ATM-dependent manner upon the occurrence of DSBs, such as induced by γ–irradiation or treatment with the radiomimetic drug zeocin, and that this phosphorylation is essential for SOG1 activity (Yoshiyama et al., 2013). As *SMR5* and *SMR7* transcription was found to depend on SOG1, and because both *SMR* genes respond to oxidative stress, we tested whether SOG1 phosphorylation occurs in response to H_2O_2 treatment. Lines expressing a Myc-tagged *SOG1* under control of its own promoter (*PSOG1:SOG1-Myc*) were either control-treated or treated with H_2O_2. As described previously, immunoblotting using anti-Myc antibody detected two bands under control conditions (Figure 9A), with the upper band corresponding to SOG1 being phosphorylated in a DNA stress-independent manner by a yet to be identified kinase (Yoshiyama et al., 2013). Upon H_2O_2 treatment, a third slowly migrating band appeared at a similar position as detected by zeocin treatment (Yoshiyama et al., 2013). This band disappeared when protein extracts were treated with the λ protein phosphatase (λPP), indicating that it corresponds to a phosphorylated form of SOG1 (Figure 9A).

Subsequently, as *SMR5* and *SMR7* transcription was found to depend on SOG1 (Figure 6), it was tested whether both genes are under direct control of SOG1. Direct binding of SOG1 to the *SMR5* and *SMR7* promoters was tested through chromatin-immunoprecipitation using *PSOG1:SOG1-Myc* seedlings being control-treated or treated for 2 h with the DSB-inducing drug zeocin. Promoter scanning revealed that SOG1 binds in a DNA stress-dependent manner to both *SMR* promoters in close proximity to their transcription start site (Figures 9B and 9C). These data illustrate that both *SMR* genes are under direct control of SOG1.

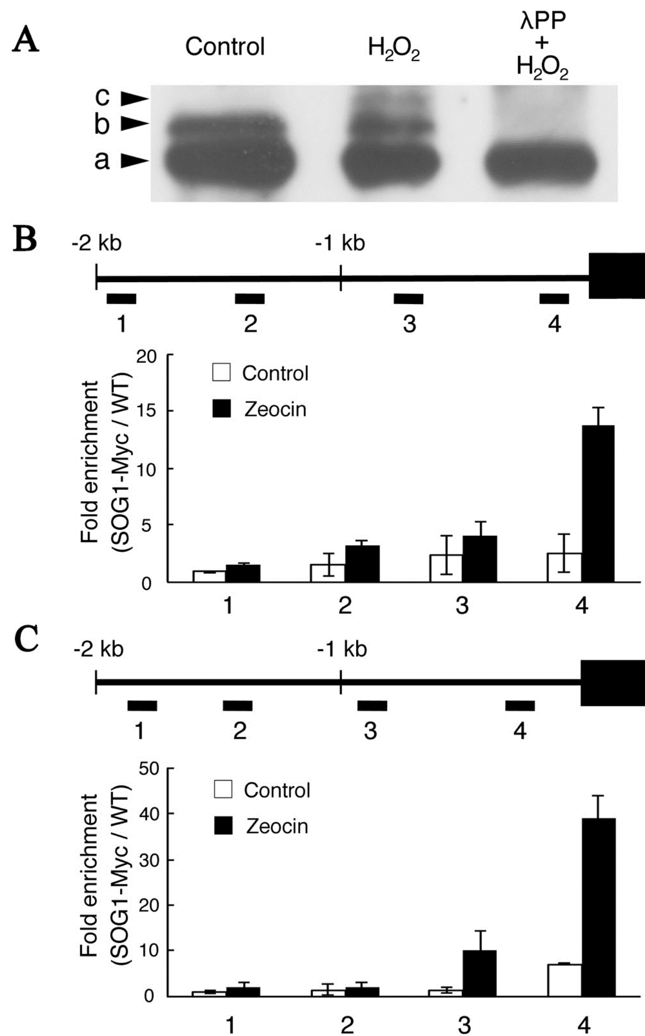


Figure 9. ***In vivo*** **phosphorylation of SOG1 by H_2O_2 and its association to the** ***SMR5*** **and** ***SMR7*** **promoters.**

(A) Total protein was immunoblotted with anti-Myc antibody. Plants harboring *PSOG1:SOG1-Myc* were treated with or without H_2O_2, and total protein was extracted. Total protein from H_2O_2-treated plants was incubated with λ protein phosphatase (λPP). The phosphorylated forms of SOG1 were separated in an SDS-PAGE gel containing Phos-tag. Non-phosphorylated, phosphorylated and hyperphosphorylated SOG1-Myc (band a, b and c, respectively) are indicated by arrowheads. **(B-C)** Chromatin bound to the promoter regions of *SMR5* **(B)** and *SMR7* **(C)** was collected by immunoprecipitation with anti-Myc antibodies from *PSOG1:SOG1-MYC* plants treated with (black bars) and without (white bars) 15 μM zeocin and subjected to qPCR analysis. Fold enrichment for each DNA fragment was determined by dividing the recovery rate with that of wild-type plants (WT=1). Bar graphs represent the average of two biological replicate ChIP experiments ± SE. Positions of PCR amplicons 1–4 are also shown.

DISCUSSION

SMRs Categorize to Minimally Two Different Functional Groups

In this work, we analyzed the SIM/SMR group of CKIs. All share mutually only a limited sequence homology, being restricted to short amino-acid (AA) regions scattered along the protein sequences, among which is a six-AA domain corresponding to a cyclin-binding motif (Peres et al., 2007). Although this poor sequence alignment does not allow a clear phylogenetic analysis, biochemically it appears that SIM/SMR proteins fall into at least two different categories. A first category includes the founding members SIM and SMR1 that both have been linked to endocycle onset (Roeder et al., 2010; Jomova and Valko, 2011), being an alternative cell cycle in which mitosis is repressed in favour of repetitive rounds of DNA replication, resulting in an increase in DNA ploidy level. Through protein purification, these two SMRs were found to co-purify with the B-type CDKB1;1 (Ohtani et al., 2001), in agreement with the observation that this particular CDK needs to be inhibited for endocycle onset (Boudolf et al., 2004; 2007). A role in endocycle onset is supported by their expression pattern in the root, showing specific transcription in the cell elongation zone, likely representing the zone where cells start the endocycle. Next to SIM and SMR1, also SMR2 exclusively co-purifies with CDKB1;1, suggesting that this particular CKI might also be an SMR family member linked with endocycle onset. As second category, other SMRs, including SMR4 and SMR5, exclusively co-purify with the A-type CDK and D-type cyclins (Ohtani et al., 2001). CDKA;1 is the main driver of S-phase progression (1998; Nowack et al., 2010), whereas the CYCD/CDKA;1 complex is responsible for control of cell cycle onset in response to intrinsic and extrinsic signals (Lukaszewski and Blevins, 1996; Riou-Khamlichi et al., 2000). Therefore, CYCD/CDKA;1 appears to be the most logical CYC/CDK complex to be targeted by those SMRs that aim to link DNA stress signals with cell cycle checkpoint activation.

HU Affects DNA Integrity in Multiple Ways

HU is known for its inhibitory effect on RNR activity, resulting into a depletion of the available dNTPs, causing impaired progression of the replication fork and activation of an ATR-dependent replication checkpoint. However, the observed ATM-dependent induction of *SMR5* and *SMR7* upon HU treatment suggests that HU affects DNA

integrity also in an RNR-independent manner. In particular, our data indicate that ROS might be the primary trigger of *SMR5* and *SMR7* expression upon HU treatment. A link between HU and oxidative stress has been observed previously in *Saccharomyces cerevisiae,* where, next to a DNA replication arrest caused by RNR inhibition, exposure to HU results in the activation of the Yap regulon that reacts to oxidative stress and encompasses genes involved in cellular redox homeostasis (Dubacq et al., 2006). In Arabidopsis, Juul et al. (2010) reported a direct interaction between HU and catalase, resulting in a stereo-inhibition of the detoxifying capabilities of the catalase protein. Analogously, HU was demonstrated to be a suicide inhibitor of ascorbate peroxidase (Chen and Asada, 1990). In agreement, we demonstrated that HU treatment results in a decrease in the H_2O_2 scavenging rate. A second source of HU-induced ROS might originate from displacement of the essential cofactor iron from the RNR catalytic site (Nyholm et al., 1993), probably resulting into an increase in the intracellular iron concentration. This increase might contribute to the raise in ROS, as iron catalyzes the production of hydroxyl radicals from H_2O_2 through the Fenton reaction. Together, the increased H_2O_2 and iron levels after HU treatment represent a potent source of oxidative stress. The HU-induced oxidative state results into an accumulation of anthocyanin pigments and the reduction in PSII efficiency. The latter is likely due to the deceleration of PSII repair, consequently resulting in further increased levels of intracellular ROS and enhanced photo-inhibition (Murata et al., 2013).

Because of its relatively long life and permeability, H_2O_2 is able to migrate into different cellular compartments. Besides PSII inhibition, H_2O_2 and hydroxyl radicals are known to affect the DNA in multiple ways, including the oxidation of bases, the creation of DNA interstrand cross-links and DSBs (Cadet et al., 2012), being different types of DNA damage that induce ATM-dependent signaling. In mammals, oxidation of ATM directly induces its activation (Guo et al., 2010), however, whether a similar mechanism is functional in plants is unknown. In agreement with H_2O_2 acting as a putative DNA stress-inducing compound, it has been reported that the lack of both catalase and cytosolic ascorbate peroxidase activity results in the transcriptional activation of DNA stress genes, including *PARP2* and *BRCA1* (Katayama et al., 2007). The fact that within these *apx1 cat2* double mutants no detectable rise in ROS levels could be measured suggests that experimentally undetectable levels of H_2O_2 can already trigger a DNA damage response. Interestingly, the resulting constitutive DNA damage

response of the *apx1 cat2* plant grants them enhanced tolerance to DNA stress inducing conditions.

SMR5 and *SMR7* Respond to ROS-Induced DNA Damage

Next to their ATM-dependent transcriptional activation upon HU treatment, expression analysis under different ROS accumulating conditions strongly indicates that the transcriptional activation of *SMR5* and *SMR7* in response to HU is primarily mediated through changes in ROS homeostasis rather than by replication stress. Interestingly, *SMR5* and *SMR7* appear to display a differential transcriptional response towards distinct sources of ROS. Under high light treatment, likely generating singlet oxygen rather than H_2O_2 (Mittler et al., 2002), it is mainly *SMR5* that is induced, in agreement with the observation a high light induced cell cycle checkpoint was only abrogated in the *SMR5*^{KO} plants. In contrast, *SMR7* is the main gene induced the *apx1* and *apx1 cat2* mutants. Similar to mature *apx1 cat2* double mutant plants, young *apx1* mutants display an activated DNA stress response, as supported by the elevated expression of DNA damage reporter genes under control conditions in 8-day-old seedlings (see Supplemental Figure 2 in Vanderauwera et al., 2011). This constitutive DNA damage response likely results from H_2O_2 leakage from the chloroplast (Davletova et al., 2005), being able to traverse to the nucleus in the absence of cytosolic scavenging by APX1. The mechanisms by which different *SMR* genes respond to different types of ROS are currently unknown.

From our data it can be concluded that HU triggers simultaneously two different cell cycle checkpoint cascades, one related to replication stress and one responding to H_2O_2, controlled by ATR and ATM, respectively (Figure 10). Roots of plants being knockout for the replication stress checkpoint activators *ATR* or *WEE1* are hypersensitive towards HU, indicating that in roots the HU-induced replication defect prevails. In contrast, despite their transcriptional induction, no outspoken root phenotype was observed for the *SMR5*^{KO} and *SMR7*^{KO} plants (Supplemental Figure S6). The restriction of a HU-sensitive phenotype to tissues with photosynthetic activity therefore suggests that the primary response of HU in the shoot tissue might be ROS accumulation (Figure 10). Remarkably, our data indicate that the signaling pathway by which oxidative stress induces *SMR5/SMR7* expression is relatively short, with ATM phosphorylating the SOG1 transcription factor that binds directly to the *SMR* promoters to activate their

transcription, as supported by the observation that no *SMR5/SMR7* expression is observed in the *sog1-1* mutant background. Because SOG1 only associates to the *SMR5* and *SMR7* promoters in the samples in which DNA stress was induced, we can speculate that phosphorylation of SOG1 is a prerequisite for binding to its target genes.

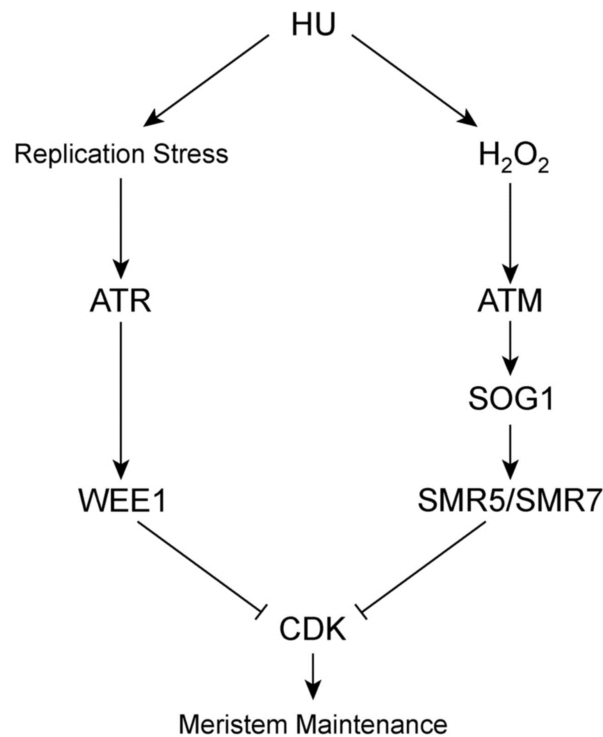


Figure 10. Model for HU-dependent cell cycle checkpoint activation.

HU treatment results in replication stress and an increase in the cellular H_2O_2 concentration, likely resulting in DNA damage sensed by the ATR and ATM signaling cascades, respectively. ATR activates a checkpoint response through transcriptional induction of *WEE1*, whereas ATM does the same through activation of *SMR5* and *SMR7*. Both pathways allow cells to adapt to the occurring DNA stress, and contribute in that way to meristem maintenance.

Next to being induced by genotoxic stress, *SMR5* displays a strong transcriptional response toward many different abiotic stress conditions that share the involvement of ROS signaling, including drought, high light and salt (Figure 2). Therefore, SMR5 might be a general integrator of ROS signaling with cell cycle progression. ROS signaling has been linked with cell cycle progression before. Treatment of tobacco cells with a ROS-inducing agent results into an impaired G1-to-S transition, retarded S-phase progression and delayed entry into M-phase, being correlated with a downregulation of CDK activity (Reichheld et al., 1999). Moreover, it has been demonstrated that the G1-to-S transition requires adequate levels of the antioxidant glutathione. Accordingly, the ROOT MERISTEMLESS1 gene, encoding a gluthatione biosynthetic enzyme, is required to establish an active meristem (Vernoux et al., 2000). Additionally, recent evidence indicates that the balance of ROS controls the transition from proliferation to differentiation: the basic helix-loop-helix transcription factor UPBEAT1 (UPB1) is expressed at the root transition zone and controls the distribution of ROS by monitoring the expression level of peroxidase genes (Lim and Kaldis, 2013). Strikingly, the same study revealed the *SIM* promoter to be bound by the UPB1 protein, fitting with the observation that *SIM* expression is restricted to the root elongation zone, which is also the site of maximum H_2O_2 concentration (Dunand et al., 2007). Likewise, ROS signaling has been implicated in pathogen response, whereas the first rice *SIM/SMR*-like gene (*EL2*) was described originally as a gene being induced within minutes after addition of the elicitator *N*-acetylchitoheptaose or purified flagellin protein of the pathogen *P. Avenae I* (Minami et al., 1996; Che et al., 2000). Moreover, H_2O_2 has also been detected in root columella cells, root cap cells and vascular cells (Dunand et al., 2007; Lim and Kaldis, 2013), to which specific *SMR* expression patterns can be linked. These data suggest that the transcriptional activation of *SIM/SMR* genes in response to ROS signals might be a general mechanism to link the oxidative status of a cell with its cell division activity.

METHODS

Plant Materials and Growth Conditions

The *smr5* (SALK_100918) and *smr7* (SALK_128496) alleles were acquired from the Arabidopsis Biological Research Center. Homozygous insertion alleles were checked

by genotyping PCR using the primers listed in Supplemental Table 2. The atm-1, atr-2 *and* sog1-1 mutants have been described previously (Garcia et al., 2003; Preuss and Britt, 2003; Culligan et al., 2004; Yoshiyama et al., 2009). Unless stated otherwise, plants of *Arabidopsis thaliana* (L.) Heyhn. (ecotype Columbia) were grown under long-day conditions (16 h of light, 8 h of darkness) at 22°C on half-strength Murashige and Skoog (MS) germination medium (Lindahl et al., 1995). Arabidopsis plants were treated with HU as described by Cools et al. (2011). For bleomycin treatments, five-day-old seedlings were transferred into liquid MS medium supplemented with 0.3 µg/mL bleomycin. For γ-irradiation treatments, five-day-old *in vitro*-grown plantlets were irradiated with γ-rays at a dose of 20 Gy. For light treatments, one-week-old seedlings were transferred to continuous high-light conditions (growth rooms kept at 22°C with 24-h day/0-h night cycles and a light intensity of 300-400 µmol m^{-2} s^{-1}) for 4 days, and subsequently retransferred to low-light conditions (70-80 µmol m^{-2} s^{-1}).

DNA and RNA Manipulation

Genomic DNA was extracted from Arabidopsis leaves with the DNeasy Plant Kit (Qiagen) and RNA was extracted from Arabidopsis tissues with the RNeasy Mini Kit (Qiagen). After DNase treatment with the RQ1 RNase-Free DNase (Promega), cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad). A quantitative RT-PCR was performed with the SYBR Green kit (ROCHE) with 100 nM primers and 0.125 µL of RT reaction product in a total of 5 µL per reaction. Reactions were run and analyzed on the LightCycler 480 (Roche) according to the manufacturer's instructions with the use of the following reference genes for normalization: ACTIN2 (At3g46520), *EMB2386* (At1g02780), *PAC1* (At3g22110) and *RPS*26C (At3g56340). Primers used for the RT-PCR are given in Supplemental Table 4. Statistical analysis was executed with the Statistical Analysis Software (SAS Enterprise Guide 5.1; SAS Institute, Inc.) using the mixed model procedure and P-values were Bonferroni adjusted for multiple measurements.

SIM/SMR promoter sequences were amplified from genomic DNA by PCR using the primers described in Supplemental Table 4. The product fragments were created with the Pfu DNA Polymerase Kit (Promega, Catalog #M7745), and were cloned into a pDONR P4-P1r entry vector by BP recombination cloning and subsequently transferred into the pMK7S*NFm14GW,0 destination vector by LR cloning, resulting in a

transcriptional fusion between the promoter of the *SMR* genes and the *nlsGFP-GUS* fusion gene (Asada, 2006). For the overexpression constructs, the *SMR* coding regions were amplified using primers described in Supplemental Table 4, and cloned into the pDONR221 vector by BP recombination cloning and subsequently transferred into the pK2GW7 destination vector (Karimi et al., 2002) by LR cloning. Based on the available annotation, the amplification of the *SMR5* coding sequence yielded in a fragment of smaller size than expected, which suggested sequence mis-annotation. Further sequencing analysis confirmed the lack of the intronic region. The corrected coding sequencing of *SMR5* is represented in Supplemental Figure S4. All constructs were transferred into the *Agrobacterium tumefaciens* C58C1RifR strain harboring the pMP90 plasmid. The obtained *Agrobacterium* strains were used to generate stably transformed Arabidopsis lines with the floral dip transformation method (Yamamoto et al., 2002). Transgenic plants were selected on kanamycin-containing medium and later transferred to soil for optimal seed production. All cloning primers are listed in Supplemental Table 4.

GUS Assays

Complete seedlings or tissue cuttings were stained in multiwell plates (Falcon 3043; Becton Dickinson). GUS assays were performed as described by Ruiz et al. (2010). Samples mounted in lactic acid were observed and photographed with a stereomicroscope (Olympus BX51 microscope) or with a differential interference contrast (DIC) microscope (Leica).

Microscopy

For leaf measurements, first leaves were harvested at 21 days after sowing on control medium or on medium supplemented with 1 mM HU. Leaves were cleared overnight in ethanol, stored in lactic acid for microscopy, and observed with a microscopy fitted with DIC optics (Leica). The total (blade) area was determined from images digitized directly with a digital camera (Olympus BX51 microscope) mounted on a binocular (Stemi SV11; Zeiss). From scanned drawing-tube images of the outlines of at least 30 cells of the abaxial epidermis located between 25% to 75% of the distance between the tip and the base of the leaf, halfway between the midrib and the leaf margin, the following parameters were determined: total area of all cells in the drawing and total

numbers of pavement and guard cells, from which the average cell area was calculated. The total number of cells per leaf was estimated by dividing the leaf area by the average cell area (De Veylder et al., 2001). Leaf sizes and epidermal cell numbers in the different lines were analyzed and compared by performing a 2-way-ANOVA (P-value < 0.05). Tukey's test was used to correct for family-wise error-rate. For confocal microscopy, root meristems were analyzed 2 days after transfer using a Zeiss LSM 510 Laser Scanning Microscope and the LSM Browser version 4.2 software (Zeiss). Plant material was incubated for 2 min in a 10 µM PI solution to stain the cell walls and was visualized with a HeNe laser through excitation at 543 nm. GFP fluorescence was detected with the 488-nm line of an Argon laser. GFP and PI were detected simultaneously by combining the settings indicated above in the sequential scanning facility of the microscope. Acquired images were quantitatively analyzed with the ImageJ v1.45s software (http://rsbweb.nih.gov/ij/) and Cell-o-Tape plug-ins (French et al., 2012). Chlorophyll *a* fluorescence parameters were measured using the IMAGING PAM M-Series Chlorofyll Fluorescence (Walz) and associated software.

Flow Cytometry Analysis

For flow cytometric analysis, root tip tissues were chopped with a razor blade in 300 µL of 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, pH 7 (Galbraith et al., 1991). One microliter of 4,6-diamidino-2-phenylindole (DAPI) from a stock of 1 mg/mL was added to the filtered supernatant. Leaf material was chopped in 200 µL of Cystain UV Precise P Nuclei extraction buffer (Partec), supplemented with 800 µL of staining buffer. The mix was filtered through a 50-µm green filter and read by the Cyflow MB flow cytometer (Partec). The nuclei were analyzed with the Cyflogic software.

Catalase Assay

Plants were germinated on either control medium, medium with 1 mM HU or 6 µM 3-AT. Leaf tissue of 10 plants was ground in 200 µL extraction buffer (60 mM Tris (pH 6.9), 1 mM phenylmethylsulfonylfluoride, 10 mM DTT) on ice. The homogenate was centrifuged at 13,000 g for 15 min at 4°C. A total of 45 µg protein extract was mixed with potassium phosphate buffer (50 mM, pH 7.0) (Galanis et al., 2009). After addition of 11.4 µL H_2O_2 (7.5%), the absorbance of the sample at 240 nm after 0 and 60 s was

measured to determine catalase activity by H_2O_2 breakdown (Nakayama and Nakayama, 1998; Galanis et al., 2009).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were carried out as described (Roldán-Arjona and Ariza, 2009) with minor modifications. Surface-sterile *PSOG1:SOG1-Myc* (Yoshiyama et al., 2013) seeds were germinated in 100 mL of 0.5X MS medium containing 1.5% sucrose (pH 5.7) and cultured under continuous light at 23°C with gentle shaking (50 rpm). After a 14-d culture period, the seedlings were treated with 15 µM zeocin (Invitrogen) or water for 2 h. Wild-type (Col-0), no-treatment seedlings were used as a negative control. Sonicated chromatin solution (corresponding to 0.3 g tissue) was used for immunoprecipitation with anti-Myc antibodies (clone 4A6, Millipore) and an antibody recognizing an invariant domain of histone H3 (AB1791, Abcam). The ChIP products were used for qPCR analysis with the primers listed in Supplemental Table 4. Quantitative PCR was performed with the LightCycler system (Roche) and Thunderbird SYBR qPCR Mix (Toyobo) according to the following reaction conditions: 95 °C for 1 min; 70 cycles at 95 °C for 10 s, at 60 °C for 10 s, and at 72 °C for 20 s. The signal obtained from ChIP with an anti-Myc antibody was normalized to that obtained from ChIP with an anti-Histone H3 antibody. Finally, each normalized ChIP value was divided by the normalized wild-type ChIP value to calculate the fold enrichment.

Microarray Analysis

Seeds were plated on sterilized membranes and grown under a 16-h/8-h light/dark regime at 21°C. After 2 days of germination and 5 days of growth, the membrane was transferred to MS medium containing 0.3 µg/mL bleomycin for 24 h. Triplicate batches of root meristem material were harvested for total RNA preparation using the RNeasy plant mini kit (Qiagen). Each of the different root tip RNA extracts were hybridized to 12 Affymetrix® Arabidopsis Gene 1.0 ST Arrays according to the manufacturer's instructions at **the Nucleomics Core Facility** (Leuven, Belgium; http://www.nucleomics.be). Raw data were processed with the RMA algorithm (Emerit et al., 2004) using the Affymetrix Power Tools and subsequently subjected to a Significance Analysis of Microarray (SAM) analysis with "MultiExperiment Viewer 4"

(MeV4) of The Institute for Genome Research (TIGR) (Tusher et al., 2001). The imputation engine was set as 10-nearest neighbor imputer and the number of permutations was 100. Expression values were obtained by log2-transforming the average value of the normalized signal intensities of the triplicate samples. Fold changes were obtained using the expression values of the treatment relative to the control samples. Genes with Q-values < 0.1 and fold change > 1.5 or < 0.666 were retained for further analysis.

Microarray Meta-Analysis

Transcripts induced by bleomycin (Q-value < 0.1 and fold change > 1.5) were compared with different published DNA stress-related data sets. For γ-irradiation, an intersect of the genes with a significant induction (P-value < 0.05, Q-value < 0.1, and fold change >1.5) in 5-day-old wild-type seedlings 1.5 h post-irradiation (100 Gy) was made of two independent experiments (Culligan et al., 2006; Yoshiyama et al., 2009). For replication stress, genes were selected that showed a significant induction (P-value (Time) < 0.05, Q-value (Time) < 0.1 and fold change >1.5) in 5-day-old wild-type root tips after 24 h of 2-mM hydroxyurea treatment (Cools et al., 2011). Meta-analysis of the *SMR* genes during various stress conditions and treatments were obtained using Genevestigator (Maxwell et al., 1999). Using the "Response Viewer" tool, the expression profiles of genes following different stimuli were analyzed. Only biotic and abiotic stress treatments with a more than 2-fold change in the transcription level (P-value < 0.01) for at least one of the *SMR* genes were taken into account. Fold-change values were hierarchically clustered for genes and experiments by average linkage in MeV from TIGR.

SOG1 Phosphorylation Assay

Plants harboring *PSOG1:SOG1-Myc* (Yoshiyama et al., 2013) were grown on MS media [1 x MS salts including vitamins, 2% (w/v) sucrose, 0.8% (w/v) gellangum (pH 6.0)] under continuous light at 23°C. Five-day-old seedlings were transferred onto a new MS medium or a medium supplemented with 5 mM H_2O_2, and incubated for 24 h. Total protein was extracted from roots and immunoblotted with anti-Myc antibody (Santa Cruz) as described by Yoshiyama et al. (2013). To detect phosphorylated SOG1 proteins, Phos-tag reagent (NARD Institute) was used for the phoshoprotein mobility

shift assay (Kinoshita et al., 2006). λ protein phosphatase (λPP) (New England Biolabs) was used to dephosphorylate the phosphorylated forms of SOG1.

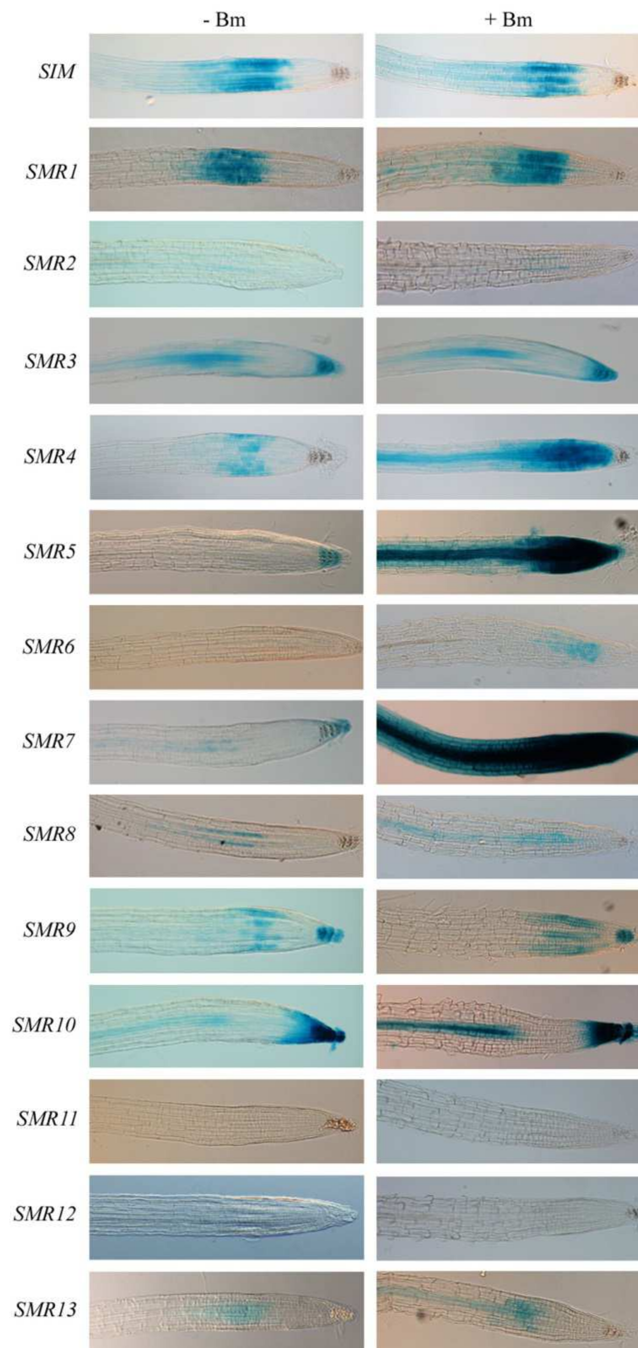
Accession Numbers

Microarray results have been submitted to MiamExpress (*www.ebi.ac.uk/miamexpress),* with accession number *E-MEXP-3977.* Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *SMR4* (At5g02220); *SMR5* (At1g07500); *SMR7* (At3g27630); *ATM* (At3g48490); *ATR* (At5g40820); *SOG1* (At1g25580).

ACKNOWLEDGMENTS

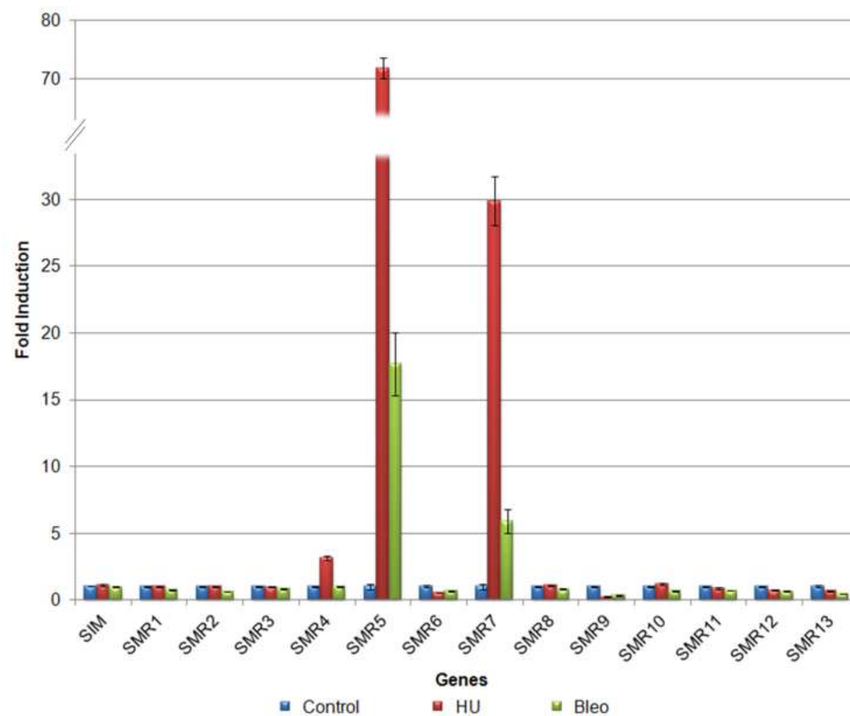
The authors thank Annick Bleys for help in preparing the manuscript, Maheshi Dassanayake for pointing out the mis-annotation of the *SMR5* transcript, and Lorin Spruyt and Frank Van Breusegem for use of the high light infrastructure. This work was supported by Ghent University (Multidisciplinary Research Partnership "Bioinformatics: from nucleotides to networks"), and the Interuniversity Attraction Poles Programme (IUAP P7/29 "MARS"), initiated by the Belgian Science Policy Office. T.C. is a Postdoctoral Fellow of the Research Foundation-Flanders. D.Y. is indebted to the China Scholarship Council (CSC File No. 2009685045) for a predoctoral scholarship. M.U. was supported by MEXT KAKENHI grant number 22119009 and JST, CREST.

Supplemental Data



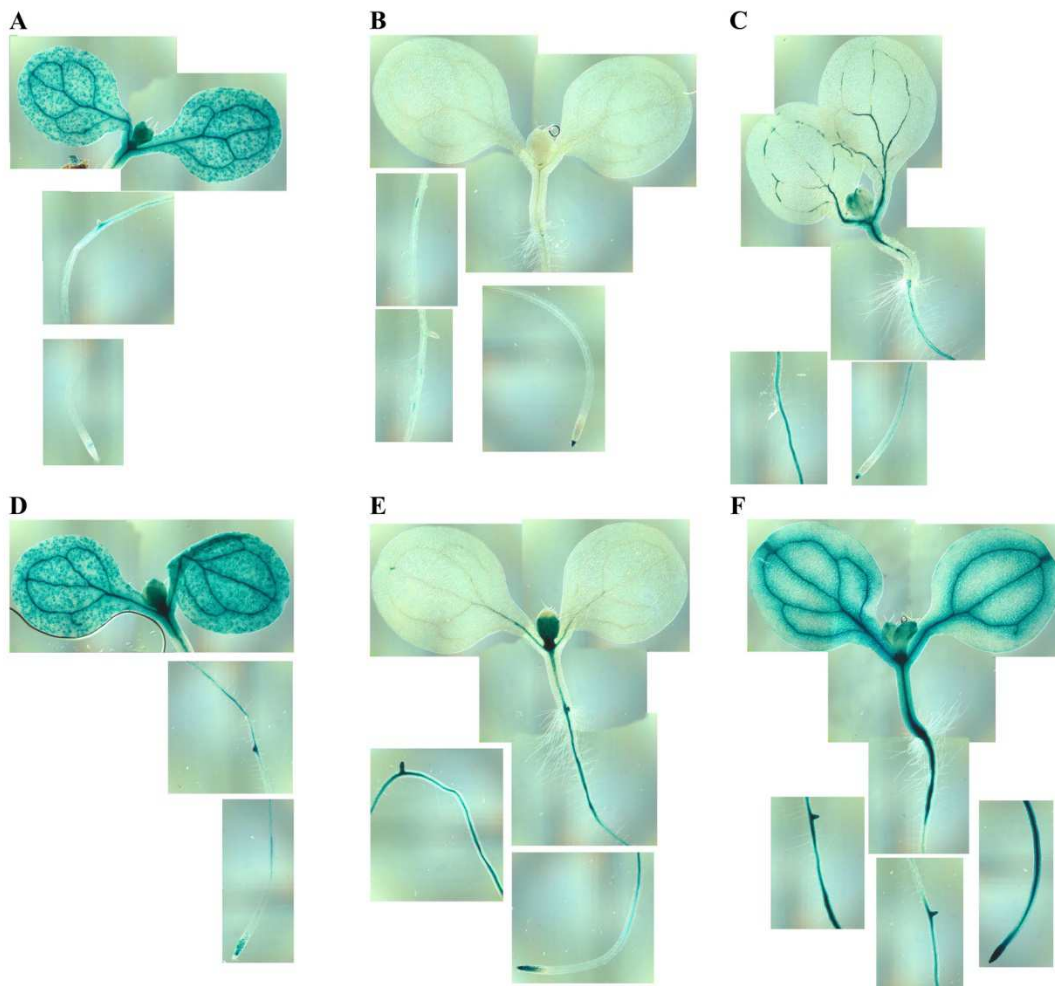
Supplemental Figure 1. *SIM/SMR* induction in response to bleomycin

One-week-old transgenic Arabidopsis seedlings were transferred to control (-Bm) medium or medium supplemented with 0.3 µg/mL bleomycin (+Bm). GUS assays were performed 24 h after transfer.



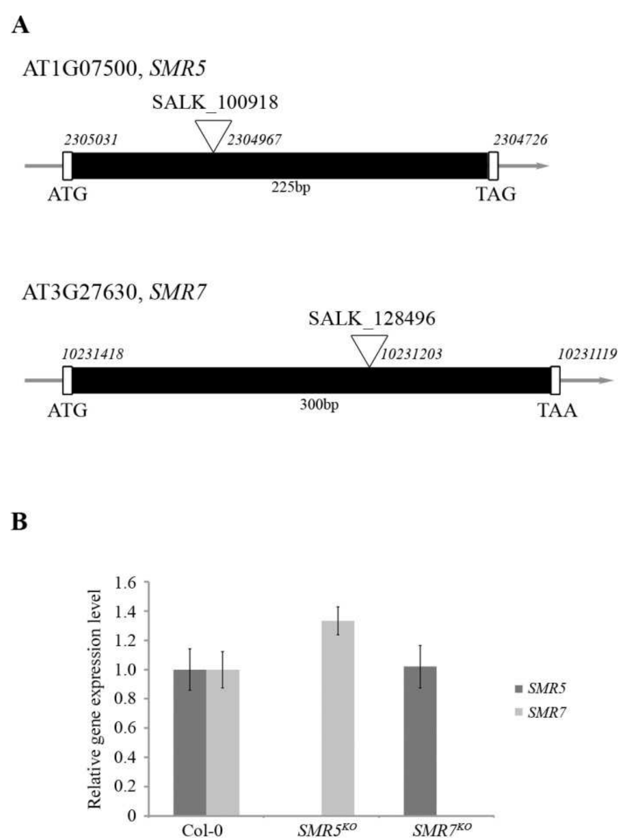
Supplemental Figure 2. Transcriptional induction of *SIM/SMR* genes upon HU and bleomycin treatment.

One-week-old wild type Arabidopsis seedlings were transferred to control medium (blue), or medium supplemented with 1 mM HU (red) or 0.3 µg/mL bleomycin (green). Root tips were harvested after 24 h for RT-PCR analysis. Expression levels in control condition were arbitrary set to one. Data represent mean ± SE (n = 3).



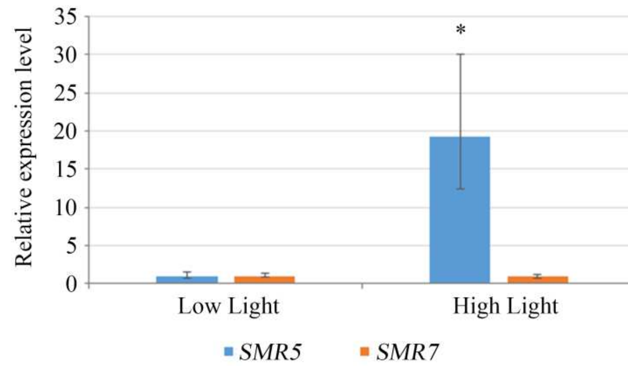
Supplemental Figure 3. Transcriptional induction of *SIM/SMR* genes upon γ-irradiation.

(A-F) *PSMR4:GUS* **(A** and **D)**, *PSMR5:GUS* **(B** and **E)** and *PSMR7:GUS* **(C and D)** either control-treated **(A-C)** or irradiated with 20 Gy of γ-rays **(D-F)**. GUS assays were performed 1.5 h after irradiation.



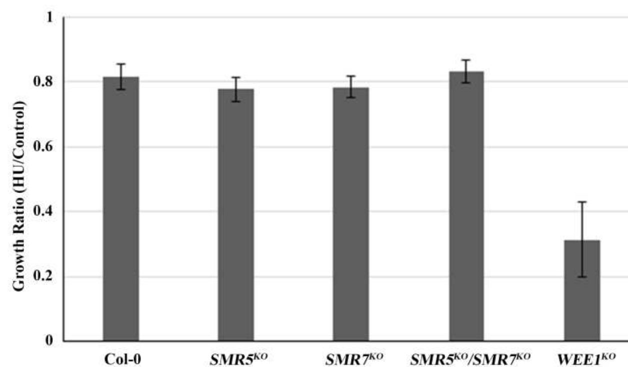
Supplemental Figure 4. Graphical representation of the *SMR5* and *SMR7* T-DNA insertion lines.

(A), Intron-exon organization of the Arabidopsis *SMR5* and *SMR7* genes. Black and white boxes represent coding and non-coding regions, respectively. The white triangles indicate the T-DNA insertion sites. **(B)**, qRT-PCR analysis on wild-type, *SMR5*^{KO}, *SMR7*^{KO}*, and SMR5*^{KO} *SMR7*^{KO} seedlings using primers specific to either *SMR5* or *SMR7*. Expression levels in wild type were arbitrary set to one. Data represent mean ± SE (n = 3).



Supplemental Figure 5. ***SMR5*** **and** ***SMR7*** **expression levels in response to high light treatment.**

One-week old wild type Col-0 plants were either control treated or exposed for 48 h to high light. Complete seedlings were harvested for RT-PCR analysis. Data represent mean ± SE (n = 3).



Supplemental Figure 6. Relative root growth of ***SMR5***^{KO}**,** ***SMR7***^{KO}**, and** ***SMR5***^{KO} ***SMR7***^{KO} **plants upon HU treatment.**

Five-day-old seedlings were transferred to control medium or medium supplemented with 1 mM HU. Data plot the root growth ratio on HU versus control plates over 4 days after transfer. HU-hypersensitive *WEE1*^{KO} plants were included as positive control. Data represent mean ± SE (n > 15).

Supplemental Table 1. Meta-analysis of genes induced in multiple DNA damage experiments.

Locus	Description	q-value (HU - Time)[a]	p-value (HU - Time)[a]	HU 24h/0h[a]	q-value (γ-rays - 1)[b]	p-value (γ-rays - 1)[b]	γ-rays - 1[b]	q-value (γ-rays - 2)[c]	p-value (γ-rays - 2)[c]	γ-rays - 2[c]	q-value Bleomycin	Bleomycin
Significantly Induced by HU, BM and gammarays												
AT4G21070	breast cancer susceptibility1	0.018	0.001	10.375	0.000	0.000	581.570	0.000	0.000	57.803	0.000	2.386
AT5G60250	zinc finger (C3HC4-type RING finger) family protein	0.000	0.000	8.907	0.001	0.000	34.918	0.000	0.000	40.000	0.000	2.352
AT1G07500	unknown protein; Has 4 Blast hits to 4 proteins in 3 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 4; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink).	0.000	0.000	7.863	0.003	0.000	38.160	0.000	0.001	35.842	0.000	1.595
AT4G02390	poly(ADP-ribose) polymerase	0.000	0.000	7.701	0.001	0.000	131.865	0.000	0.000	59.172	0.000	2.663
AT3G07800	Thymidine kinase	0.033	0.002	7.160	0.000	0.000	46.179	0.000	0.004	20.492	0.000	2.759
AT5G03780	TRF-like 10	0.018	0.001	7.111	0.005	0.000	108.316	0.000	0.003	23.474	0.036	1.600
AT5G64060	NAC domain containing protein 103	0.014	0.000	5.579	0.004	0.000	28.086	0.000	0.008	13.755	0.002	2.153
AT2G18600	Ubiquitin-conjugating enzyme family protein	0.009	0.000	5.521	0.004	0.000	21.462	0.000	0.014	11.481	0.004	1.972
AT4G22960	Protein of unknown function (DUF544)	0.012	0.000	5.315	0.009	0.000	36.380	0.000	0.009	14.451	0.000	2.282
AT5G48720	x-ray induced transcript 1	0.048	0.003	5.296	0.004	0.000	285.166	0.000	0.000	65.789	0.000	2.228
AT5G24280	gamma-irradiation and mitomycin c induced 1	0.026	0.001	4.823	0.009	0.000	108.578	0.000	0.000	42.918	0.000	2.584
AT5G20850	RAS associated with diabetes protein 51	0.031	0.002	4.643	0.002	0.000	186.456	0.000	0.001	31.250	0.000	1.765
AT3G27060	Ferritin/ribonucleotide reductase-like family protein	0.012	0.000	4.595	0.001	0.000	37.351	0.000	0.018	8.741	0.000	1.970
AT2G46610	RNA-binding (RRM/RBD/RNP motifs) family protein	0.027	0.001	3.593	0.002	0.000	19.913	0.000	0.021	7.331	0.021	1.546
AT5G40840	Rad21/Rec8-like family protein	0.052	0.004	3.375	0.005	0.000	113.919	0.000	0.002	27.473	0.002	1.692
AT1G13330	Arabidopsis Hop2 homolog	0.014	0.000	2.949	0.019	0.000	17.349	0.000	0.009	13.495	0.046	1.580
AT5G66130	RADIATION SENSITIVE 17	0.009	0.000	2.888	0.003	0.000	30.411	0.000	0.015	10.384	0.002	1.627
AT1G17460	TRF-like 3	0.052	0.004	2.378	0.000	0.000	18.925	0.000	0.015	10.661	0.007	1.681
AT2G45460	SMAD/FHA domain-containing protein	0.012	0.000	2.378	0.000	0.000	45.673	0.000	0.004	21.053	0.010	1.575
AT5G49480	Ca2+-binding protein 1	0.021	0.001	1.952	0.002	0.000	15.106	0.000	0.026	5.851	0.010	1.580
AT3G25250	AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family protein	0.014	0.000	1.853	0.003	0.000	12.995	0.000	0.004	17.794	0.035	1.517
AT5G55490	gamete expressed protein 1	0.034	0.002	1.670	0.000	0.000	71.489	0.000	0.001	34.722	0.000	2.407
Significantly induced by HU and gammarays												
AT4G28950	RHO-related protein from plants 9	0.021	0.001	9.680	0.000	0.000	36.081	0.000	0.008	13.569		
AT3G45730	unknown protein; Has 3 Blast hits to 3 proteins in 1 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 3; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink).	0.034	0.002	5.637	0.000	0.000	46.290	0.000	0.009	14.286		
AT5G11460	Protein of unknown function (DUF581)	0.006	0.000	5.483	0.003	0.000	41.596	0.000	0.005	16.863		
AT5G02220	unknown protein; Has 30201 Blast hits to 17322 proteins in 780 species: Archae - 12; Bacteria - 1396; Metazoa - 17338; Fungi - 3422; Plants - 5037; Viruses - 0; Other Eukaryotes - 2996 (source: NCBI BLink).	0.023	0.001	4.500	0.001	0.000	45.759	0.000	0.004	20.534		
AT2G47680	zinc finger (CCCH type) helicase family protein	0.031	0.002	3.422	0.022	0.000	50.849	0.000	0.004	17.513		
AT4G29170	Mnd1 family protein	0.060	0.005	2.898	0.000	0.000	40.733	0.000	0.006	16.694		
AT5G06190	unknown protein; BEST Arabidopsis thaliana protein match is: unknown protein (TAIR:AT3G58540.1); Has 30201 Blast hits to 17322 proteins in 780 species: Archae - 12; Bacteria - 1396; Metazoa - 17338; Fungi - 3422; Plants - 5037; Viruses - 0; Other Eukaryot	0.012	0.000	2.878	0.008	0.007	3.757	0.001	0.092	2.690		
AT5G67460	O-Glycosyl hydrolases family 17 protein	0.031	0.002	2.799	0.005	0.000	18.032	0.000	0.004	17.271		
AT4G35740	DEAD/DEAH box RNA helicase family protein	0.037	0.002	2.594	0.002	0.000	21.434	0.000	0.021	7.037		
AT2G21790	ribonucleotide reductase 1	0.045	0.003	2.514	0.000	0.000	13.702	0.000	0.034	4.948		
AT3G02400	SMAD/FHA domain-containing protein	0.052	0.004	2.479	0.025	0.002	9.474	0.000	0.022	6.649		
AT2G31320	poly(ADP-ribose) polymerase 2	0.020	0.001	2.445	0.001	0.000	39.238	0.000	0.015	9.970		
AT3G42860	zinc knuckle (CCHC-type) family protein	0.039	0.002	2.445	0.001	0.000	30.770	0.000	0.010	13.351		
AT1G09815	polymerase delta 4	0.026	0.001	2.354	0.000	0.000	19.771	0.000	0.021	7.310		
AT3G20490	unknown protein; Has 754 Blast hits to 165 proteins in 64 species: Archae - 0; Bacteria - 48; Metazoa - 26; Fungi - 25; Plants - 36; Viruses - 0; Other Eukaryotes - 619 (source: NCBI BLink).	0.043	0.003	2.313	0.003	0.000	17.593	0.000	0.029	5.291		
AT4G19130	Replication factor-A protein 1-related	0.093	0.010	2.305	0.010	0.000	59.037	0.000	0.010	13.089		
AT2G30360	SOS3-interacting protein 4	0.033	0.002	2.274	0.004	0.000	11.137	0.000	0.017	9.346		
AT3G12510	MADS-box family protein	0.006	0.000	2.266	0.001	0.000	17.935	0.000	0.029	5.426		
AT1G12020	unknown protein; BEST Arabidopsis thaliana protein match is: unknown protein (TAIR:AT1G62422.1); Has 89 Blast hits to 88 proteins in 16 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 87; Viruses - 0; Other Eukaryotes - 2 (source: NCBI	0.030	0.001	1.873	0.006	0.000	8.806	0.001	0.080	2.976		
AT1G31280	Argonaute family protein	0.014	0.000	1.866	0.002	0.000	24.264	0.000	0.017	9.302		
AT1G59660	Nucleoporin autopeptidase	0.033	0.002	1.860	0.014	0.000	15.946	0.000	0.013	11.933		
AT3G15240	Serine/threonine-protein kinase WNK (With No Lysine)-related	0.027	0.001	1.790	0.016	0.001	6.471	0.001	0.060	3.552		
AT1G30600	Subtilase family protein	0.093	0.010	1.711	0.013	0.000	9.920	0.001	0.066	3.299		
AT5G67360	Subtilase family protein	0.029	0.001	1.676	0.001	0.000	4.720	0.001	0.082	2.923		
AT1G76180	Dehydrin family protein	0.062	0.005	1.659	0.017	0.010	3.048	0.001	0.080	2.975		
AT4G11740	Ubiquitin-like superfamily protein	0.084	0.008	1.653	0.000	0.000	7.747	0.001	0.067	3.272		
AT2G36910	ATP binding cassette subfamily B1	0.012	0.000	1.569	0.000	0.001	3.596	0.001	0.092	2.693		
AT5G14930	senescence-associated gene 101	0.000	0.000	1.542	0.000	0.000	9.606	0.000	0.018	8.993		
Significantly inducedby HU and BM												
AT5G66985	unknown protein; Has 30201 Blast hits to 17322 proteins in 780 species: Archae - 12; Bacteria - 1396; Metazoa - 17338; Fungi - 3422; Plants - 5037; Viruses - 0; Other Eukaryotes - 2996 (source: NCBI BLink).	0.088	0.009	3.294							0.007	1.612
AT5G14920	Gibberellin-regulated family protein	0.027	0.001	2.789							0.000	2.122
AT4G15480	UDP-Glycosyltransferase superfamily protein	0.081	0.008	2.196							0.000	2.394
AT3G27620	alternative oxidase 1C	0.077	0.007	2.056							0.025	1.883
AT3G27950	GDSL-like Lipase/Acylhydrolase superfamily protein	0.045	0.003	1.641							0.000	4.012
AT4G04750	Major facilitator superfamily protein	0.082	0.008	1.625							0.011	1.689
AT5G60100	pseudo-response regulator 3	0.037	0.002	1.619							0.018	1.801
AT5G25810	Integrase-type DNA-binding superfamily protein	0.000	0.000	1.558							0.040	1.573
AT1G49030	PLAC8 family protein	0.044	0.003	1.553							0.000	2.653
Significantly induced by BM and gammarays												
AT4G05370	BCS1 AAA-type ATPase				0.014	0.000	8.214	0.000	0.050	3.949	0.007	1.807
AT5G49110	unknown protein; INVOLVED IN: biological_process unknown; LOCATED IN: cellular_component unknown; EXPRESSED IN: cultured cell; Has 30201 Blast hits to 17322 proteins in 780 species: Archae - 12; Bacteria - 1396; Metazoa - 17338; Fungi - 3422; Plants - 503				0.004	0.001	7.611	0.000	0.037	4.819	0.002	1.562

Supplemental Table 2. Annotated Arabidopsis *SIM/SMR* genes.

AGI locus	Annotation
At5g04470	*SIM*
At3g10525	*SMR1*
At1g08180	*SMR2*
At5g02420	*SMR3*
At5g02220	*SMR4*
At1g07500	*SMR5*
At5g40460	*SMR6*
At3g27630	*SMR7*
At1g10690	*SMR8*
At1g51355	*SMR9*
At2g28870	*SMR10*
At2g28330	*SMR11*
At2g37610	*SMR12*
At5g59360	*SMR13*

Supplemental Table 3. DNA ploidy level distribution in transgenic plants overexpressing *SMR4*, *SMR5*, or *SMR7*.

Ploidy (%)	Col-0	*SMR4* ^{OE}	*SMR5* ^{OE}	*SMR7* ^{OE}
2C	19.6 ± 0.2	17.1 ± 0.1	23.6 ± 0.9	24.2 ± 1.3
4C	26.3 ± 1.2	19.4 ± 0.5	21.3 ± 0.8	29.2 ± 0.7
8C	49.2 ± 0.5	34.9 ± 3.4	34.8 ± 0.5	36.1 ± 0.2
16C	4.6 ± 0.7	27.1 ± 3.1	19.6 ± 0.2	9.5 ± 0.9
32C	0.2 ± 0	1.5 ± 0.6	0.7 ± 0.1	1.1 ± 0.1

Supplemental Table 4. List of primers used for cloning, genotyping and RT-PCR.

Promoter cloning primers

SIAMESE	Fw	ATAGAAAAGTTGGTATTGTAATTATATATGAAAAAATAGTAAT
	Rev	GTACAAACTTGTTCTTTTTTGTTTATATAAATATTAAATGT
SMR1	Fw	ATAGAAAAGTTGTCACAAGTGCATTTTTAATTTGTAGGA
	Rev	GTACAAACTTGCATCTAAACTTGTGTATGTTTTTGTTTTTTGG
SMR2	Fw	ATAGAAAAGTTGGTAACTCCTTCGGCATCTTTGT
	Rev	GTACAAACTTGTGGTCACATGGATGTGAAAGTTT
SMR3	Fw	ATAGAAAAGTTGGTATTTTAAATTACGATTTCAAAATCTTGA
	Rev	GTACAAACTTGTTAGACAAGTTTTACAGAGAGAAAGAAGAG
SMR4	Fw	ATAGAAAAGTTGGTGAAACACAAAGCATCTTCG
	Rev	GTACAAACTTGTTCTTCTCTCTCGAACTCG
SMR5	Fw	ATAGAAAAGTTGGTCAGAACGAACAAAAG
	Rev	GTACAAACTTGTTTTTGTCCGCTCTCTCG
SMR6	Fw	ATAGAAAAGTTGGTCAGTGTGTCAAAACCGACG
	Rev	GTACAAACTTGTCTCTCTTTAACTAACTCAAAACCAAGA
SMR7	Fw	AGAAAAGTTGCGTTGACGCGGGAAAATTAA
	Rev	GTACAAACTTGCTTAAAACAGTTGGAGATTGAG
SMR8	Fw	ATAGAAAAGTTGGTAGATCCCACATTACTTAAGAAATTGG
	Rev	GTACAAACTTGTGACTTCTCTCGAATGTGAATGAAGA
SMR9	Fw	ATAGAAAAGTTGGTACATATAAAGGTGTTATACACACCCTT
	Rev	GTACAAACTTGTTTTTGAGACCAGAATAAGAGAGAAG
SMR10	Fw	ATAGAAAAGTTGGTTTTAAAAAACCGTTTCAAACTAGTGC
	Rev	GTACAAACTTGTCTTTGAGAAGAAACGTCGCTC
SMR11	Fw	ATAGAAAAGTTGGTTGTGGTAATCTACATGGAATTTGC
	Rev	GTACAAACTTGTTTGGATTCACGAGATCTAAGCA
SMR12	Fw	ATAGAAAAGTTGGTTCGGCTCACCTTGTTTTCC
	Rev	GTACAAACTTGTGTGCGCTTTTTTTTCTTCTCAG
SMR13	Fw	ATAGAAAAGTTGGTAAAACTCAAGACACTTCTTTTTTTGG
	Rev	GTACAAACTTGTCTTATCACAAACAGGAAAAGAGAGAGT

T-DNA genotyping primers

SMR5	SALK_100918	LB	GAACGAACAAAAGTGAGCTCG
		RB	TTTCCCAACCTGACAGAAAAC
SMR7	SALK_128496	LB	AAAATCGATAACTAAAACGAACCG
		RB	AGGCCTTCAATATAGCCCATG

Primers used for ChIP experiments

SMR5-ChIP-F1	GGAACAAAGTCATGAGAATTAACGC
SMR5-ChIP-R1	TTCCTGCTAAAGGACGTGGTG
SMR5-ChIP-F2	GTTGTCAACAATCCTACAATTGTGTG
SMR5-ChIP-R2	GATGTCGAATCCATTTGGTACTATG
SMR5-ChIP-F3	ATCACAACGAAACGAACCTTAGAAC
SMR5-ChIP-R3	TGGGTTTCTATATATTATGCGAGCTC
SMR5-ChIP-F4	ACGTGGCAGTACGTTCCTCC
SMR5-ChIP-R4	GTCCGCTCTCTCGCACTTTC
SMR7-ChIP-F1	ATCACCAGAAGCAGTCAGAAGAC
SMR7-ChIP-R1	ACATTTCTTGGATCAAGGTGTG
SMR7-ChIP-F2	TAAACCTAAATCACAAACGACCA
SMR7-ChIP-R2	GTTCTGTTGATTACTCAATGTAGCTAG
SMR7-ChIP-F3	GGTGTGGTCTCTCATTTGACGC
SMR7-ChIP-R3	GGCCATCATATATGGGCCTTAC
SMR7-ChIP-F4	TAGTCTCAAAACCATGGCGC
SMR7-ChIP-R4	GAAGCTTTCAGAGGAAGATTATTAGG

RT-PCR primers

SIAMESE	Fw	CACAAGATTCCTCCCACCACAG
	Rev	CAGAGGAGAAGAACCGCTCGAT
SMR1	Fw	CACCCACATCCCAAGAACACAAG
	Rev	GACGGAGGAGAAGAAACGGTCAA
SMR2	Fw	AGAGCAGAAACCCAGAAGCCAAG
	Rev	GAAATCTCACGCGGTCGCTTTCTT
SMR3	Fw	CGATCACAAGATTCCGGAGGTG
	Rev	CGGCTCAGATCAATCGGTATGC
SMR4	Fw	GCCGAGAAGCACGATGTATAG
	Rev	AGATCTGGTGGCTGAAAGTACC
SMR5	Fw	AAACTACGACGACGGAGATACG
	Rev	GCTACCACCGAGAAGAACAAGT
SMR6	Fw	GGGCTTCGTTGAAACCAGTCAAG
	Rev	TTTCTCGGTGCTGGTGGACATTC
SMR7	Fw	GCCAAAACATCGATTCGGGCTTC
	Rev	TCGCCGTGGGAGTGATACAAAT
SMR8	Fw	TAACCTATCTCCCGGCGTCACA
	Rev	GCACTTCAACGACGGTTTACGC
SMR9	Fw	GCCACTTCAAGAACCCATCTCC
	Rev	TCCGGAGTACAACATCCACTCTCT
SMR10	Fw	GCAAAGAAGGAGCAACCGTCAAG
	Rev	CGGTGGACAAATTCTTGGCATCG
SMR11	Fw	CTGCTTCGATCTCGGATTGTGTT
	Rev	GACGAAGGAGGCGGTGTTTTAC
SMR12	Fw	GGTATGTCGGAGACGAGCTTGA
	Rev	GAGTCGGTGTCTTGAACCCATCA
SMR13	Fw	GAACCACCAACACCGACAACAAG
	Rev	GTTCGAGTTTCTCGGCGTCTCT
Actin2	Fw	GGCTCCTCTTAACCCAAAGGC
	Rev	CACACCATCACCAGAATCCAGC
EMB2386	Fw	CTCTCGTTCCAGAGCTCGCAAAA
	Rev	AAGAACACGCATCCTACGCATCC
PAC1	Fw	TCTCTTTGCAGGATGGGACAAGC
	Rev	AGACTGAGCCGCCTGATTGTTTG
RPS26C	Fw	GACTTTCAAGCGCAGGAATGGTG
	Rev	CCTTGTCCTTGGGGCAACACTTT

ORF cloning primers

SMR4	Fw	AAAAAGCAGGCTTCATGGAGGTGG TGGAGAGGAA G
	Rev + stop co	AGAAAGCTGGGTCCTAAGCGCAAGCTTCTCTTC
	Rev - stop coc	AGAAAGCTGGGTCAGCGCAAGCTTCTCTTC
SMR5	Fw	AAAAAGCAGGCTTCATGGAGGAGAAAAACTACGACG
	Rev + stop co	AGAAAGCTGGGTCCTAGGTTGCCGCTTGGG
	Rev - stop coc	AGAAAGCTGGGTCGGTTGCCGCTTGGGA
SMR7	Fw	AAAAAGCAGGCTTCATGGGAATTTCGAAAAAATCTC
	Rev + stop co	AGAAAGCTGGGTCTTAACGGCGTTGTATAAACACC
	Rev - stop coc	AGAAAGCTGGGTCACGGCGTTGTATAAACACCA

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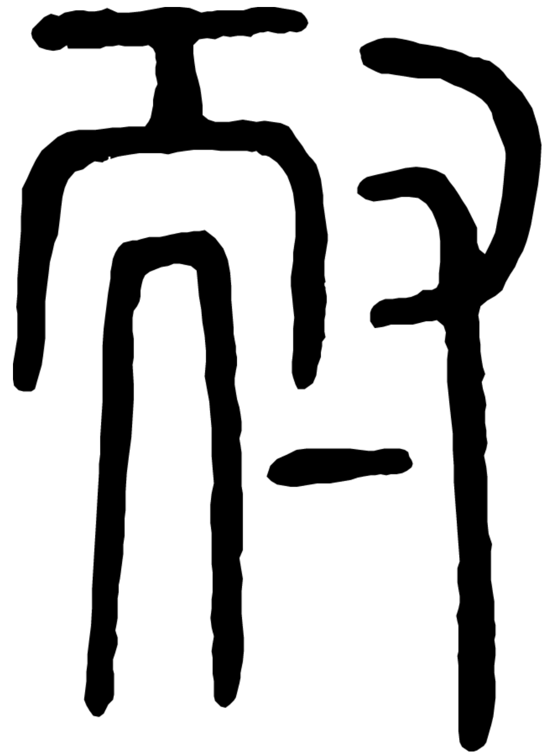


CHAPTER 3

E2F DEPLETION RENDERS DNA STRESS RESISTANCE

E2F depletion renders DNA stress resistance

Dalong Yi[a,b], Sandy Vanderauwera[a,b], Barbara Berckmans[a,b], Claire Lessa Alvim Kamei[a,b], Toon Cools[a,b], Hilde Van den Daele[a,b], Lorenzo Borghi[c], Wilhelm Gruissem[c], Lieven De Veylder[a,b]

[a]Department of Plant Systems Biology, VIB, B-9052 Gent, Belgium

[b]Department of Plant Biotechnology and Bioinformatics, Ghent University, B-9052 Gent, Belgium

[c]Department of Biology, Plant Biotechnology, ETH Zurich, Zurich, Switzerland.

Running title: E2Fs and DNA checkpoint control

AUTHOR CONTRIBUTIONS

D.Y., S.V., B.B., C.L.A.K., and L.D.V. conceived and designed the research. D.Y., S.V., C.L.A.K., B.B. T.C., H.V.d.D. and L.B. performed the experiments. D.Y., S.V., and L.D.V. analyzed the data and wrote the manuscript.

ABSTRACT

As important cell cycle transcription factors, E2F proteins play a crucial function in DNA damage response in mammals. However, little is known about E2F features upon genotoxic stress in plants. In our work, mutants of E2Fa and E2Fb were found to be resistant to DNA damage caused by the radiomimetic drug bleomycin. We illustrate that this resistance is likely due to a constitutive weak activation of the DNA damage response (DDR) pathway triggered through the depletion of MCM proteins, without inhibiting growth of plants in the absence of exogenous DNA damage inducing agents. In the *e2fa-2/e2fb* double mutant *MCM* expression falls to minimal levels, triggering a more severe DDR that results in shrinkage of the root meristem under control conditions. Our results suggest that E2F transcription factors counteract DNA damage, while E2F absence leads to an endogenous DNA damage response that at slightly increased levels allows for an adaptive response that makes the plant more tolerant to higher genotoxic levels.

Adapted from manuscript: **Dalong Yi, Sandy Vanderauwera, Barbara Berckmans, Claire Lessa Alvim Kamei, Toon Cools, Hilde Van den Daele, Lorenzo Borghi, Wilhelm Gruissem, Lieven De Veylder**. E2F depletion renders DNA stress resistance (Manuscript in preparation)

INTRODUCTION

E2F transcription factors are well-known transcription factors controlling cell division. They regulate the expression of many genes that are mostly involved in DNA replication (Pagano et al., 1992; Ramírez-Parra et al., 1999; Vandepoele et al., 2005). Dimerization partner (DP) proteins associate with E2Fs to form a heterodimeric complex that binds the promoters of genes containing a E2F *cis*-acting element (van den Heuvel and Dyson, 2008). Activity of E2F/DP complexes can be inhibited by the Retinoblastoma/Retinoblastoma related protein (RB/RBR) (Murphree and Benedict, 1984; Weinberg, 1995). Over the last decades, the core functions of plant E2F/DP transcription factors have been partially characterized. They appear to be crucial for the regulation of DNA replication, endoreplication, and cell differentiation. Specifically, E2Fs play a crucial role in the regulation of G1-to-S-phase transition (Ren et al., 2002). Six members of E2F family transcription factor family (E2Fa, E2Fb, E2Fc, DEL1/E2Fe, DEL2/E2Fd, and DEL3/E2Ff) have been identified in Arabidopsis. E2Fa and E2Fb are two positive regulators that promote S-phase entry and progression. They are mostly expressed in proliferating tissues (De Veylder et al., 2002; Mariconti et al., 2002; Sozzani et al., 2006).

In mammals, E2F/DP activity has a big impact on the DNA damage response (DDR) (Tsuge et al., 2005; DeGregori and Johnson, 2006; Hoglinger et al., 2007) by controlling transcription of a wide range of genes that are involved in cell-cycle progression and DNA synthesis, replication and repair. E2F1 protein accumulates to trigger DNA repair genes for DNA double-strand break and UV radiation–induced damage repair (Anup K. Biswas et al., 2012). E2F3 acts upstream of E2F1. E2F1 in turn acts upstream of E2F2. Upon DNA damage, E2F2 responds to the DNA damage through E2F3, initializing apoptosis, making E2F3 a key regulator of DNA damage-induced apoptosis (Martinez et al., 2010). Meanwhile, E2F1 control E2F7 and E2F8 (Christensen et al., 2005), and E2F7 and E2F8 can give a feedback E2F1 expression (Chen et al., 2012). Microarray analysis of E2F target gene expression upon DNA damage showed they are involved in DNA replication, DNA repair and mitosis. These results indicate that E2F-dependent gene activation contributes to the cellular response

to DNA damage both at S phase and during mitosis (Polager et al., 2002; Ren et al., 2002).

The minichromosome maintenance complex (MCM) is a helicase complex that plays a role in both the initiation and the elongation phases of DNA replication, specifically during the formation and elongation of the replication fork. It is a key component of the pre-replication complex to initialize DNA replication, and also a key component of replicative helicase (Tsuge et al., 2005; DeGregori and Johnson, 2006; Hoglinger et al., 2007). The first identified MCM protein in Arabidopsis is PROLIFERA (PRL, MCM7) (Springer et al., 1995). *PRL/MCM7* is expressed in dividing cells and plays a role during embryo development. Its gene product is localized in the nucleus during the G1 phase (Springer et al., 2000). MCM5 and MCM7 proteins were also reported to accumulate in G1, S and G2 phases (Shultz et al., 2009). There are reports showing that reduction of MCM levels lead to the defects in genome stability in yeast (Liang et al., 1999; Fitch et al., 2003). Analogously, depletion of the *E2F TARGET GENE 1* (ETG1) protein that interacts with MCM proteins during DNA replication triggered a DDR response (Takahashi et al., 2008; 2010).

The mechanisms by which most of the E2F family members respond to DNA damage are poorly understood in plants. Moreover, the role they play in the DNA damage response has not been extensively explored. Here, we demonstrate that the depletion of E2Fa or E2Fb confers resistance to DNA damage caused by the radiomimetic drug bleomycin (BM). We illustrate that this resistance is likely due to a drop in of *MCM* gene expression, triggering a DDR without compromising the cell cycle. Contrary, in the $E2Fa^{KO}$ $E2Fb^{KO}$ double mutant *MCM* expression fall to minimal levels, triggering a more severe DDR that results in shrinkage of the root meristem.

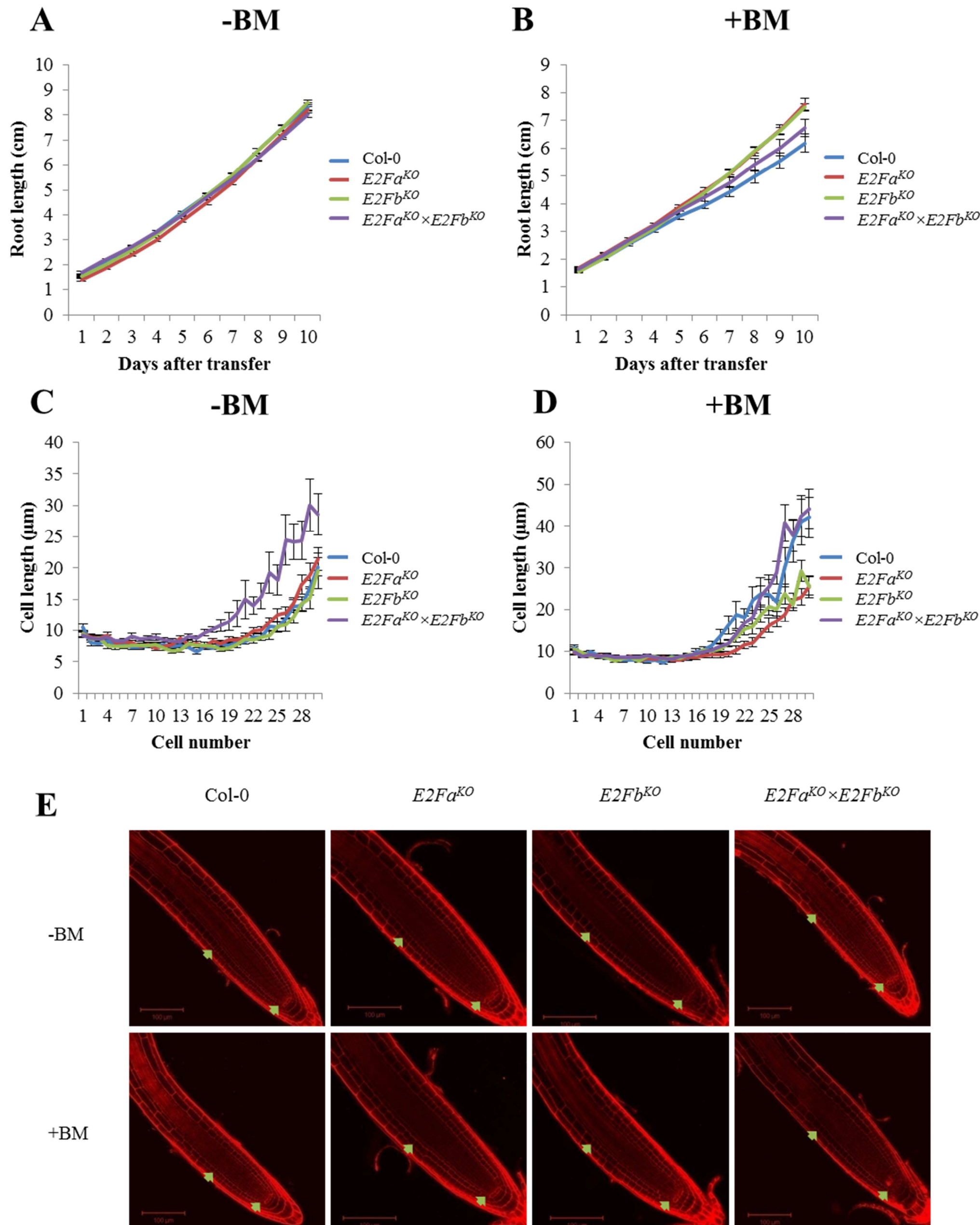


Figure 1. *E2F*^{KO} primary root growth in response to bleomycin.

(A-B) Average primary root lengths of wild-type (Col-0), *E2Fa*^{KO}, *E2Fb*^{KO} and *E2Fa*^{KO}×*E2Fb*^{KO} and seedlings transferred at 5 DAG to mock **(A)** or 0.3 µg/mL BM-containing medium **(B)**. **(C-D)** Cortex cell lengths in function of distance from the QC for wild-type (Col-0), *E2Fa*^{KO}, *E2Fb*^{KO} and *E2Fa*^{KO}×*E2Fb*^{KO} seedlings transferred at 5 DAG for 24 hrs. to mock **(C)** or 0.3 µg/mL BM-containing medium **(D)**. Error bars represent standard error of the mean ($n > 15$). **(E)** Representative examples of root meristems of wild-type (Col-0), *E2Fa*^{KO}, *E2Fb*^{KO} and *E2Fa*^{KO}×*E2Fb*^{KO} after transfer for 24 hrs. to mock (-BM) or 0.3 µg/mL BM-containing (+BM) medium. Arrows mark the size of the meristems.

RESULTS

E2F^{KO} plants show resistance towards bleomycin

E2F transcription factors are important for cell cycle regulation and cell proliferation, and have been implicated in other organisms to be part of the DNA damage stress response (De Veylder et al., 2002; Mariconti et al., 2002). To analyse a putative role for the Arabidopsis E2Fa and E2Fb transcription factors in DDR, we measured root growth of plants being exposed to BM. Wild-type (Col-0) and *E2Fa* and *E2Fb* T-DNA insertion mutant lines (*e2fa-2* and *e2fb*) (Berckmans et al., 2011) were germinated on vertical plates for 5 days and subsequently seedlings were transferred to control medium or medium supplemented 0.3 µg/mL BM. Using this concentrantion of BM, the root growth was inhibited rather than completely arrested. For 10 days root growth was measured. Root growth of the *E2F*^{KO} mutant lines was identical to control plants in the absence of BM (Figure. 1A). In the presence of BM, growth of the control plants was inhibited. In contrast, both *E2F*^{KO} lines appeared to be insensitive to BM treatment (Figure. 1B), resulting in an approximately 20% longer primary root length, compared to the control plants. A significant difference in root length between wild-type and the *E2F*^{KO} lines appeared from day 6 onwards (Student's t-test P-value < 0.05).

Table 1. Length of cortex meristem

Length of cortex meristem		Level		Mean
Bleo-	Col-0	**A**		243.10
	e2fa-2×e2fb	**A**		242.53
	e2fa-2	**A**		242.25
	e2fb	**A**	**B**	229.39
Bleo+	*e2fa-2×e2fb*	**A**		240.47
	e2fa-2	**A**	**B**	226.92
	e2fb		**B**	216.49
	Col-0		**B**	211.70

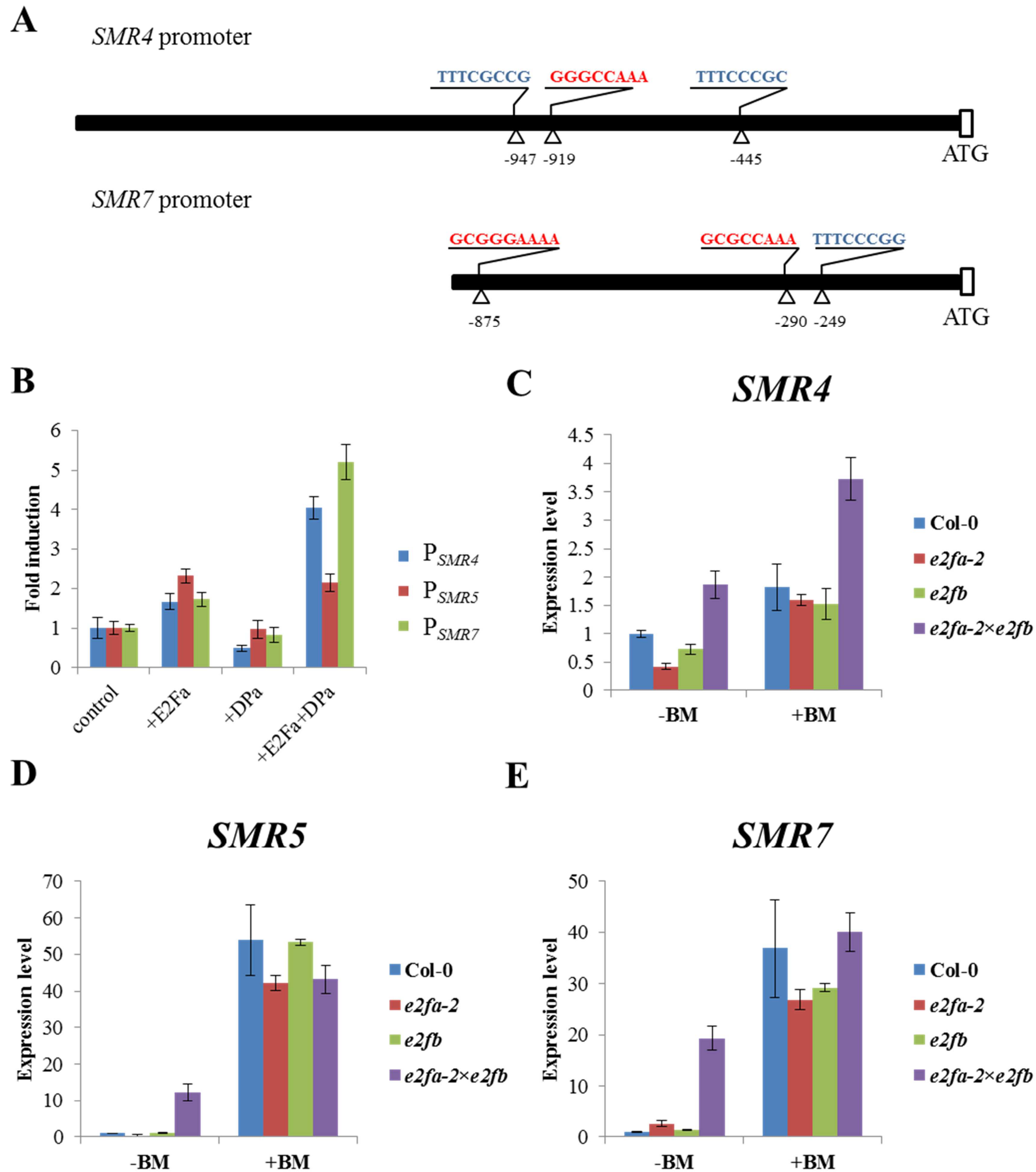


Figure 2. E2Fa stimulates expression of *SMR4* and *SMR7*.

(A) E2F *cis*-acting elements in promoters of *SMR4* and *SMR5*. Triangles denote the position of the different elements. **(B)** Transcriptional activation of *pSMRs:LUC* constructs by E2Fa and DPa. Protoplasts were either controlled transformed with *pSMRs:LUC* only (control), or in combination with *CaMV35:E2Fa* (+E2Fa), *CaMV35:DPa* (+DPa), or *CaMV35:E2Fa* and *CaMV35:DPa* (+E2Fa+DPa). Data represent mean with SEM (n = 6). (C-E) Relative *SMR4* **(C)**, *SMR5* **(D)**, and *SMR7* **(E)** expression level in wild-type (Col-0), $E2Fa^{KO}$, $E2Fb^{KO}$ and $E2Fa^{KO}\times E2Fb^{KO}$ root tips, both in control treated plants (-BM) of plants treated for 24 hrs. with 0.3 µg/mL BM (+BM). Data represent mean ± SE (n = 3).

To investigate the underlying reasons of the BM resistance phenotype, we plotted cortical cell length in function form distance from the stem cell niche. Five-day-old seedlings were transferred to 0.3 µg/mL BM-containing or mock medium for 24 h. In absence of BM no significant difference was observed between the different genotypes. Upon growth on BM, wild type cortical cells entered the cell expansion phase earlier, resulting into shrinkage of the root meristem (Figure 1E). This was not observed in both $E2F^{KO}$ lines, which maintained a meristem size being identical to that of the non-treated plants (Figure 1, Table 1). These data indicate the ability of the $E2F^{KO}$ lines to withstand the BM treatment, probably resulting from the inability to activate a cell cycle checkpoint.

E2F transcriptional factors stimulate *SMR* gene expression.

SIM/SMR proteins belong to a new family of CDK inhibitors (Yi et al., 2014), with SMR4, SMR5 and SMR7 activating a DNA stress induced cell cycle checkpoint. From promoter analysis we found E2F *cis*-acting binding site in the promoter regions of *SMR4* and *SMR7* (Figure 2A), suggesting that the impaired checkpoint activation in the $E2F^{KO}$ lines might be due the inability to activate *SMR4* and *SMR7* expression. Co-expressing the *E2Fa* and *DPa* transcription factors resulted in a dramatic induction in expression of both *SMR4* and *SMR7* in Arabidopsis protoplasts, illustrating that E2Fa/DPa indeed has the potential to activate both genes. In contrast, *SMR5* expression was not significantly induced, correlating with absence of an E2F *cis*-acting element in its promoter (Figure 2B).

The RETINOBLASTOMA RELATED 1 (RBR1) protein is a plant homolog of the tumor suppressor Retinoblastoma (pRb), which is a key regulator of the cell cycle though regulating activity of E2F/DP complex (Grafi et al., 1996; Ramírez-Parra et al., 1999; Harbour and Dean, 2000). An inducible *RBR1* RNA interference system has been developed to silence RBR1 activity in Arabidopsis (Grafi et al., 1996; Ramírez-Parra et al., 1999; Borghi et al., 2010; Gutzat et al., 2011). Three-day-old inducible *RBR1* RNA seedlings that contained either the P_{SMR4}:*GUS* or P_{SMR7}:*GUS* reporter construct were germinated in the medium supplemented with β-estradiol. Both lines displayed a dramatic increase in GUS activity upon silencing of RBR1 (Figure S1). Again, the P_{SMR5}:*GUS* reporter was not induced. These results corroborate the control of *SMR4* and *SMR7* transcription by E2F transcription factors.

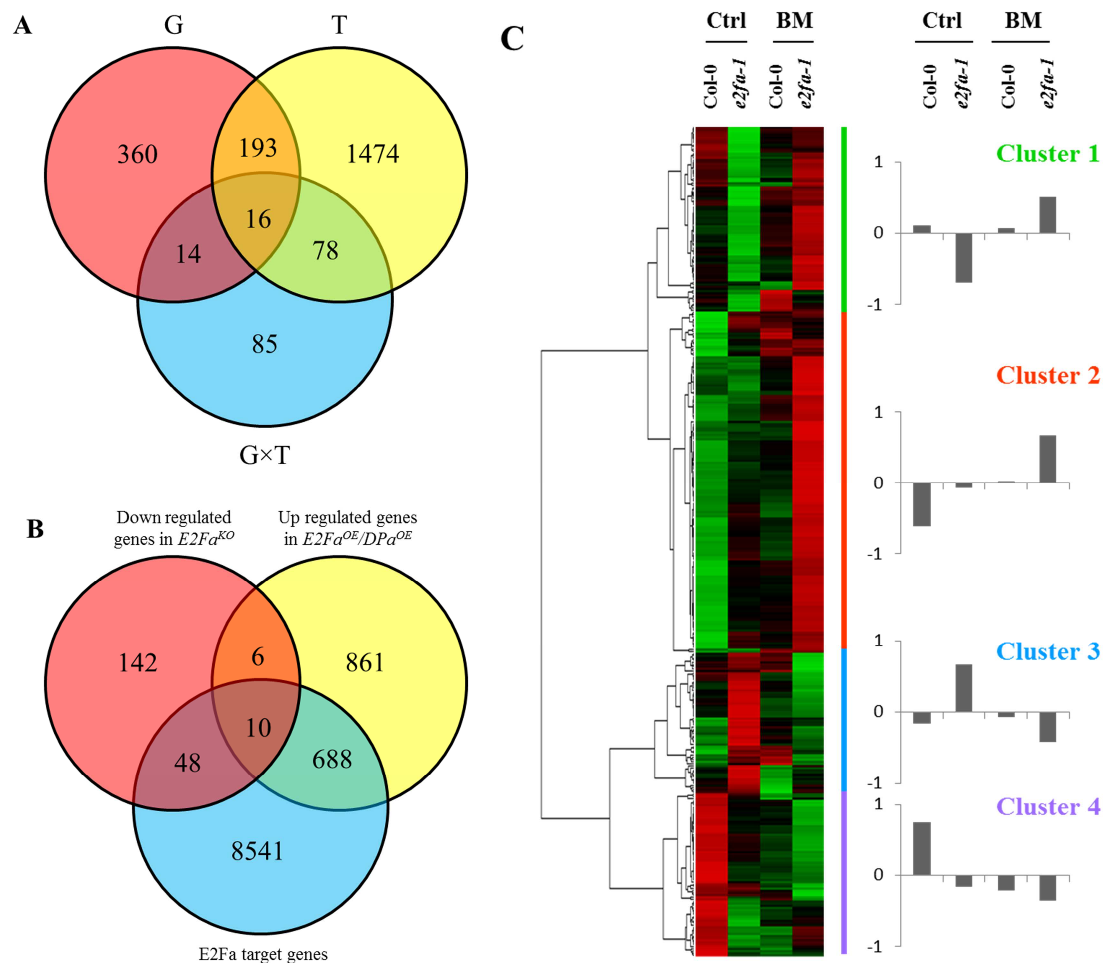


Figure 3. Meta-analysis of E2Fa controlled DNA damage response genes.

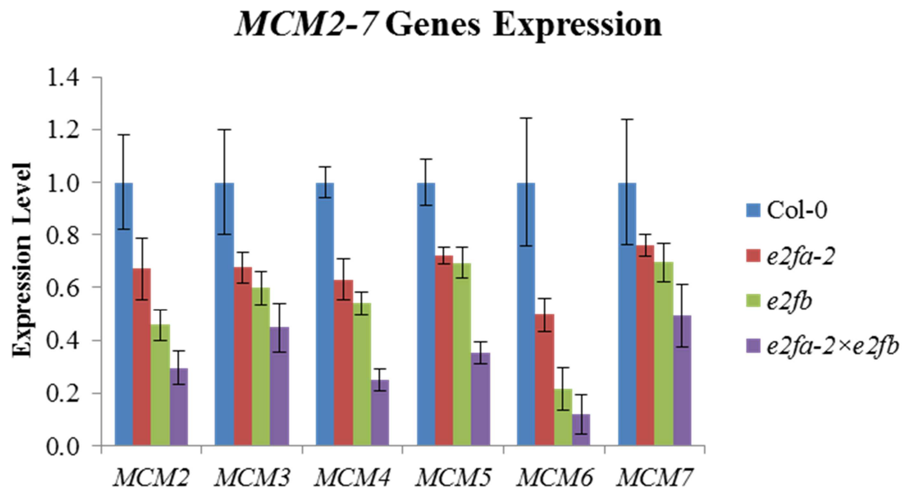
(A) Comparison of genes differentially expressed in response to *E2Fa* knockout (G), bleomycin treatment (T), or both (G×T). **(B)** Overlap between genes being down-regulated in *E2Fa*^{KO}, upregulated in *E2Fa/DPa*^{OE} plants, and bound by E2F, as defined by Naouar et al. (2009). **(C)** Hierarchical cluster analysis of G+T and G×T genes. Green and red represent down- and up-regulated gene expression, respectively.

To address whether the E2F transcription factors contribute to the DNA stress inducibility of the *SMR4*, *SMR5*, and *SMR7* genes, we measured their expression levels by RT-PCR in *E2F*^{KO} lines grown under control conditions, or in the presence of BM. Only the *SMR4* gene displayed a significant ($P<0.05$ for comparison with WT) decrease in expression level in both knockout backgrounds (Figure 2C-E). Nevertheless, its expression was still induced upon BM treatment, like observed for *SMR5* and *SMR7*. Thus, although *SMR* genes are under transcription control of E2F transcription factors, these transcription factors appear not to contribute to their DNA stress inducibility. Thus the BM resistance of the *E2F*^{KO} lines is unlikely to be due to impaired SMR-dependent checkpoint activation.

E2Fa^{KO} is affected in expression of DDR and replication genes

To uncover the mechanism by which the *E2F*^{KO} lines confer BM resistance, we performed a microarray analysis comparing 5-day-old wild-type and *E2Fa*^{KO} root tips of plants control treated or grown for 24h on medium supplemented with 0.3 µg/mL BM. Statistical analysis identified 583 genes being differentially expressed ($P < 0.001$) (G sign) between both genotypes under control conditions, of which 206 and 377 being down-regulating and up-regulated, respectively (Table S1, Figure 3A). GO analysis of the 206 downregulated genes from the *E2Fa*^{KO} microarray suggests an involvement of these genes in stress response (Figure S3C) and cell cycle regulation (Figure S3D). We compared the *E2Fa*^{KO} significant down-regulated genes from our microarray datasets with the E2F target genes that have at least one putative E2F *cis*-acting element in their 1-kb promoter and the significant upregulated genes in *E2Fa/DPa*^{OE} co-expressing plants, as identified through microarray analysis (Naouar et al., 2009) (Figure 3B). Interestingly, 58 E2Fa target genes (Table S2) were downregulated in the *E2Fa*^{KO} line, and 10 of these E2F target genes were also significantly upregulated in the *E2Fa/DPa*^{OE} (Table S2, Figure 3B). From the corresponding GO analysis it can be seen that they are mainly involved in DNA replication (Figure S3A).

A



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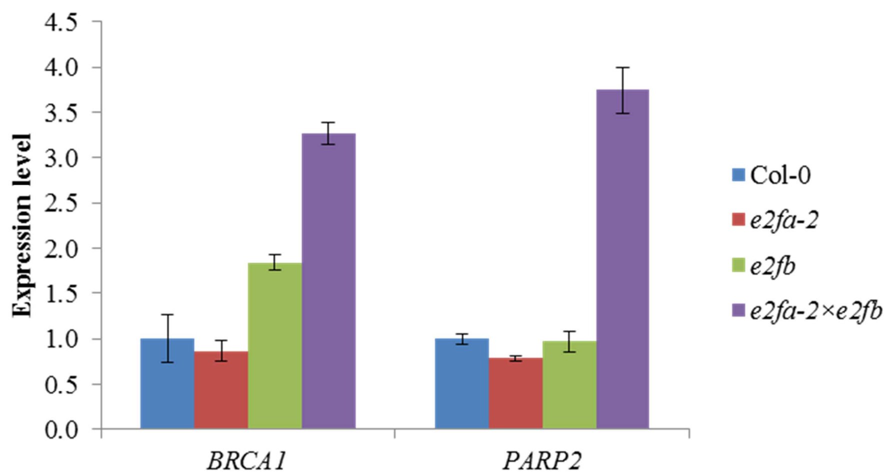


Figure 4. *MCM* family genes are under the control of E2F transcription factors.

(A) The expression level in wild-type (Col-0), *E2Fa*^{KO} (*e2fa-2*), *E2Fb*^{KO} (*e2fb*) ,and *E2Fa*^{KO}×*E2Fb*^{KO} (*e2fa-2×e2fb*) root tips of *MCM* genes being identified as differentially expressed in the microarray dataset. **(B)** Relative *BRCA1* and *PARP2* expression levels in wild-type (Col-0), *E2Fa*^{KO}, *E2Fb*^{KO} and *E2Fa*^{KO}×*E2Fb*^{KO} seedlings. Expression levels in wild type were arbitrary set to one. Data represent mean ± SE (n = 3).

Table 2. *MCM* expression in *E2Fa*^{KO} microarray

AGI code	Gene Description	E2F binding sites in pomoter	p-values (Genotype)	P<0.001 (Genotype)	p-values (Treatment)	P<0.001 (Treatment)
AT2G07690	Minichromosome maintenance *MCM5*	**3**	1,34E-07	Genotype significant	9,41E-06	Treatment significant
AT5G46280	Minichromosome maintenance *MCM3*	**2**	7,37E-06	Genotype significant	1,17E-05	Treatment significant
AT4G02060	Minichromosome maintenance *MCM7 PRL*	**1**	1,37E-04	Genotype significant	8,07E-04	Treatment significant
AT2G16440	Minichromosome maintenance *MCM4*	**1**	1,03E-05	Genotype significant	2,04E-06	Treatment significant
AT5G44635	minichromosome maintenance *MCM6*	**2**	0,624179		5,06E-05	Treatment significant
AT1G44900	minichromosome maintenance *MCM2*	**2**	0,715426		2,64E-05	Treatment significant

Several MCM family members are present in the 10 overlapping E2F target genes that are down and upregulated in the *E2Fa*^{KO} and *E2Fa/DPa*^{OE} lines respectively (Table 2). The minichromosome maintenance (MCM) complex is important for DNA replication as a heterohexamer complex composed of MCM2 to MCM7 proteins. The MCM complex is part of the helicase that unwinds DNA during replication (Tye and Sawyer, 2000; Labib and Diffley, 2001; Forsburg, 2004). The Arabidopsis *MCM* genes can be divided into 2 groups. The promoters of the first group of genes which include *MCM5*, *MCM3*, *MCM7* (*PRL*), *MCM4*, *MCM6*, *MCM2*, and *MCM8* hold an E2F *cis*-acting element. All components of the MCM2-7 complex belong to this group. The remaining *MCM* genes (*MCM9, MCM10, EMB1688* and *AT1G67460*) do not contain such binding site in their promoters (Naouar et al., 2009). Among those genes with an E2F *cis*-acting element, *MCM3, MCM4, MCM5* and *MCM7* were significantly down and upregulation in the *E2Fa*^{KO} and *E2Fa/DPa*^{OE} lines, respectively. *MCM2* and *MCM6* showed a non-significant reduction in expression level in *E2Fa*^{KO} microarray datasets. These results were confirmed by quantitative RT-PCR (Figure 4A).

The BM treatment resulted into 1761 differentially genes (Table S1), among which 963 genes were up-regulating and 798 genes were down-regulating. In total 209 genes showed a significant change both in genotype and treatment (G+T). These genes are thus induced by BM in an E2Fa independent manner. To investigate the interaction between the DDR and E2Fa regulating pathway, a two-way ANOVA analysis was

performed, identifying 193 genes for which expression is dependent both upon E2Fa and DDR (G×T). The expression of these genes upon DNA damage is affected by E2Fa. To investigate the genes that are both influenced by the E2Fa transcription factor and DNA damage response, we pooled G+T and G×T genes together for cluster analysis. Using TreeView (Page, 1996) (Figure 3C), we can divide these 387 genes to 4 clusters. Cluster 1 and Cluster 2 genes show dramatic induction upon BM treatment (Figure S4-S5). The genes belonging to Cluster 1 show low expression level in the *E2Fa*^{KO} line under control conditions. GO categorization showed that they are involved in DNA-dependent DNA replication initiation, DNA geometric change, DNA conformation change and protein folding. The genes of Cluster 2 show an elevated expression level in *E2Fa*^{KO} under both control and DNA stress conditions, and appear to be enriched for DNA and nucleic acid metabolic process, DNA replication, response to DNA damage and DNA repair. Cluster 3 and 4 hold genes that have a low expression level in the presence of BM, being enriched for genes involved iron transport (Figure S6).

Knockout of both *E2Fa* and *E2Fb* triggers endogenous DNA damage response

Next to the single *E2F*^{KO} lines, we analyzed root growth of the double mutant. Similar to the single mutants, the *E2Fa*^{KO}×*E2Fb*^{KO} mutant showed under control conditions no clear growth phenotype. Surprisingly, in the presence of BM the double mutant grew worse than both single mutants, although still better than the control plants (Figure 1B). When measuring the cortical cell length, it appeared that under both control and BM conditions cells expanded prematurely (Figure 1C,D), resulting into a significant shrinkage of the root meristem (Figure 1E, Table 1).

The decrease in meristem size indicated the activation of a cell cycle checkpoint in the *E2F*^{KO} double mutant, already in the absence of DNA damage. To confirm this hypothesis we examined *SMR4*, *SMR5* and *SMR7* expression levels in the *E2Fa*^{KO} *E2Fb*^{KO} mutant. All were strongly induced in the double mutant both under control conditions and in the presence of BM, in comparison to the control line and single mutants (Figure 2C-E). Additionally, expression of the DDR *BRCA1* and *PARP2* genes was strongly activated (Figure 4B), indicating indeed that the co-depletion of E2Fa and E2Fb triggered endogenous DNA stress. When measuring *MCM* expression levels, it

appeared that the expression values those that were found to be downregulated in the *E2Fa*^{KO} were even lower in the double mutant (Figure 4A).

DISCUSSION

The role of E2F transcription factors in DNA damage response and cell proliferation

To maintain genome integrity, DNA repair should be finished before the next cell division. When DNA damage occurs, the cell cycle will be arrested for the damage to be repaired. A cell cycle arrest is an important reaction of the DDR in multicellular organisms. In plants, the E2F transcription factors likely perform important functions during the DDR. E2Fa co-localizes with γH2AX upon genotoxic conditions, which recruitment depends on ATM (Lang et al., 2012). Ribonucleotide reductase (RNR) and RNR-like genes are under the control of E2Fa in response of DNA damage (Roa et al., 2009). Under UV-B stress, E2Fe/DEL1 controls the expression of the photolyase photoreactivating enzyme type-II cyclobutane pyrimidine dimer-photolyase DNA repair gene (*PHR1*) (Radziejwoski et al., 2011). ANTI-SILENCING FUNCTION1 (ASF1) is a key histone H3/H4 chaperone that participates in DNA repair processes in post-UV response. *ASF1A* and *ASF1B* encoding ASF1 proteins are also under control of E2Fa during cell cycle progression in Arabidopsis (Lario et al., 2013). FASCIATA1 (FAS1) encodes the CAF-1 large subunit, is another target of E2F transcription factors, and depletion of FAS1 causes hypersensitivity to both DNA replication stress and DNA damage (Kirik et al., 2006; Ramirez-Parra and Gutierrez, 2007; Hisanaga et al., 2013). In our results, we can find some genes like *RNR1* and *FAS1* being significantly upregulated among the different genotypes under control condition. In contrast, *ETG1* expression is reduced in *E2Fa*^{KO}. *PHR1* and *ASF1* did not present differences in expression in the absence of *E2Fa*^{KO}, which might be due to redundancy of different members of E2F family. These data suggest that E2Fs operates upstream regulation in DNA damage response.

Surprisingly, despite E2Fa's role as a transcriptional activator to DNA response genes, we observed that *E2Fa*^{KO} mutants display resistance to DNA induced by BM compared to control plants. A similar DNA stress resistance phenotype was observed for *E2Fb*^{KO}

plants. A likely mechanism by which E2Fs might contribute to a cell cycle checkpoint might have been the transcriptional activation of *SMR* genes, shown recently to arrest the cell cycle in an ATM dependent manner (Yi et al., 2014). However, despite the observation that the *SMR4* and *SMR7* genes holds E2F *cis*-acting bindings sites in their promoter region, and despite their transcriptional activation by E2Fa-DPa, *E2F^{KO}* plants still displayed a transcriptional activation of *SMR4/SMR7* upon administration of BM. Thus, likely a transcription factor being different to E2Fs control *SMR* activation in response to DNA damage. A likely candidate is the SOG1 (Yi et al., 2014). Rather than playing an active role in response to DNA damage, E2F-dependent transcription of *SMR4* and *SMR7* might be linked to S-phase progression. Indeed, in a *sog1-1* mutant background a patchy expression pattern of *SMR5* and *SMR7* can be observed (Figure S7), likely reflecting E2F-driven cell cycle phase dependent gene expression. The role of *SMR* expression during the S phase awaits further characterization.

E2Fa and E2Fb have partial redundant functions but they are different.

Microarray analysis of *E2Fa^{KO}* plants showed that many genes are significantly reduced in expression, even in the absence of BM. These genes are mainly involved in DNA replication (Figure S3D) and stress response (Figure S3C). In the comparison analysis with E2F target genes, we found not all the E2Fa target genes being down-regulated (Figure 3B). These data can be can explained functional redundancy between E2Fa and E2Fb. As we discussed before, E2Fa and E2Fb likely recognize the same *cis*-acting element (Naouar et al., 2009). Furthermore, E2Fa target genes like *MCMs* show a strong reduction in the E2Fa/E2Fb double knockout line in comparison with the reduction in the single knockout lines (Figure 4). On the other hand, the many genes that are differential expressed in the *E2Fa^{KO}* line indicates that E2Fa and E2Fb are not completely redundant. Similar conclusions can be made from previous reports, such as *E2Fa* and *E2Fb* present different function to auxin in cell suspension cultures (Magyar et al., 2005) and lateral root development (Sozzani et al., 2006; Berckmans et al., 2011). The double knockout line in our experiment is *e2fa-2×e2fb*. This line does not present any obvious growth defect but the other double knock line which contains another E2Fa knockout allele (*e2fa-1*) is infertial. Thus, we can suppose that the absence of phenotype difference between *e2fa-2×e2fb* and WT is due to the partial loss-of-function product of *E2Fa*. Interestingly, from these data we can conclude that the *E2Fa^{KO}* downregulated

genes can be divided in two bio-process sets The genes with reduced expression in *E2Fa*^{KO} and upregulated in *E2Fa/DPa*^{OE} can mainly be categorized into DNA replication, the remaining genes appear to correspond to stress response related processes (Figure S3).

Activation of the DNA damage response renders genotoxicity resistance

The *E2Fa*^{KO} and *E2Fb*^{KO} mutants show longer roots than wild-type plants under genotoxic stress, likely attributed to the maintenance of their meristem length upon BM treatment, contrary to the control meristems that display a clear shrinkage. Contrasting, *E2Fa*×*E2Fb* double knockout plants display a reduced meristem size under both control and DNA stress conditions. We postulate that this reduction in meristem size reflects the activation of an endogenous DNA stress checkpoint, likely due to a depletion of essential DNA replication factors. Candidate replication factors accounting for the endogenous DNA damage are subunits of the MCM2-7 complex. In yeasts, low levels of MCM2-7 complex proteins lead to accumulating genome damage caused by the abnormal DNA replication process (Liang et al., 1999). Actually, from the GO analysis of genes induced in *E2Fa*^{KO} plants in the absence of exogenous applied DNA stress, we can enrichment for DNA repair. In contrast with the induced genes in the *E2Fa/DPa*^{OE} line, these genes are activated by E2Fs depletion which caused DDR rather than transcrioptional regulators by E2F (Figure 5). We speculate that the DDR is already activated at marginal levels in the single *E2F*^{KO} plants, resulting into a basal transcriptional activation of DNA repair genes and granting *E2F*^{KO} plants the potential to deal with BM stress better than wild type plants do. Especially, in our experiments, DNA damage stress was given by low doses of BM which inhibited root growth rather than completely arrested.

In the *E2Fa*×*E2Fb* double mutant, *MCM* levels are reduced even more, probably to a level that causes more severe DNA damage, and thus already affecting plant growth in the absence of external applied DNA stress. This hypothesis is supported by the strong transcriptional activation of the *SMR*4, *SMR5, SMR7* and the DDR makers *BRCA1* and *PARP2*. A trade-off between a constitutive active DDR conferring resistance towards DNA damage inducing agents, and an already decreased cell division rate before BM treatment because of checkpoint activation, might explain why the double mutant

displays a slightly worse resistance towards DNA damage compared to the single *E2F* mutants.

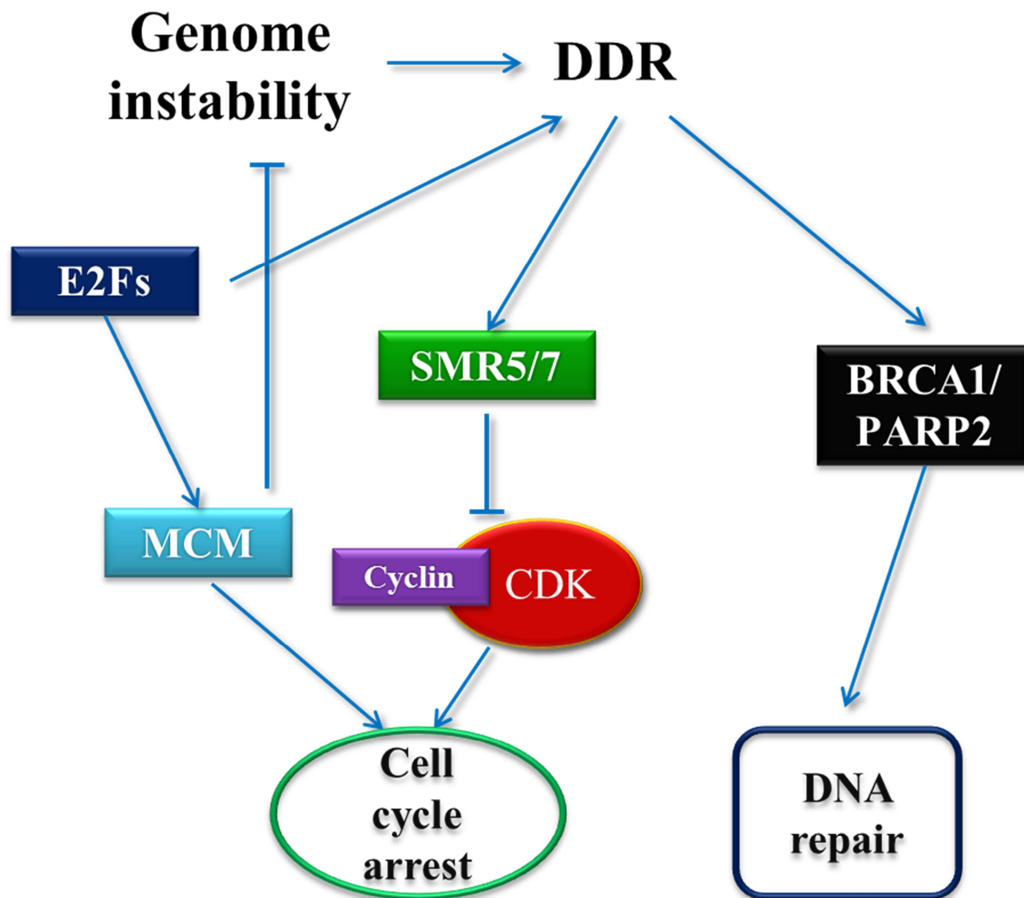


Figure 5. Model for E2Fs occurrences in DNA damage response.

The resistance for DNA damage that is given by the absence of E2Fs is likely due to a constitutive weak activation of the DNA damage response (DDR) pathway triggered through the depletion of MCM proteins, without inhibiting growth of plants in the absence of exogenous DNA damage inducing agents.

Bleomycin effects metal-related DDR genes.

In our research, BM was used as a DNA damaging agent. BM causes DNA DSBs (Favaudon, 1982) and inhibits the growth of both animal and plant cells by the accumulation of unrepaired DSBs. DNA cleavage by BM depends on oxygen and metal ions. The exact mechanism of DNA strand scission is unresolved, but it has been suggested that BM chelates metal ions (primarily iron), producing ROS that cleaves DNA (Burger et al., 1981; Favaudon, 1982). However more research is needed to describe the bio-processes of the BM-induced growth phenotype in plants. In our work, we showed that of the > 1700 differentially expressed genes upon treatment with BM, many genes being important for DNA repair, abiotic stress and cell cycle regulation. Interestingly, GO analysis showed there is a group of genes involved in iron transport (Fig). This can be explained by the function of BM (see before). Meanwhile, metal elements like iron (Fe), copper (Cu), chromium (Cr) and cobalt (Co) are toxic to DNA due to the production of superoxide radicals and hydroxyl radicals (Jomova and Valko, 2011). Accumulation of these ions will repress DNA damage repair and influence cell cycle process (Hartwig et al., 2002) . From this part, we can find a new direction to discover the DNA damage response.

METHODS

Plant Materials and Growth Conditions

The *E2Fa*^{KO} lines GABI_348E09 (*e2fa-2*) and MPIZ-244 (*e2fa-1*) alleles were acquired from the Max-Planck-Institut für Züchtungsforschung of Cologne (Rios et al., 2002) and *E2Fb*^{KO} line SALK_103138 (*e2fb*). Homozygous insertion alleles genotype them by metheod from (Berckmans et al., 2011). Primer sequences used for genotyping are given in Supplemental Table 4. Unless stated otherwise, plants of *Arabidopsis thaliana* (L.) Heyhn. (ecotype Columbia) were grown under long-day conditions (16 h of light, 8 h of darkness) at 22°C on half-strength Murashige and Skoog (MS) germination medium (Lindahl et al., 1995). For bleomycin treatments, five-day-old seedlings were transferred into MS medium supplemented with 0.3 µg/mL bleomycin.

DNA and RNA Manipulation

Genomic DNA was extracted from Arabidopsis leaves with the DNeasy Plant Kit (Qiagen) and RNA was extracted from Arabidopsis tissues with the RNeasy Mini Kit (Qiagen). After DNase treatment with the RQ1 RNase-Free DNase (Promega), cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad). A quantitative RT-PCR was performed with the SYBR Green kit (ROCHE) with 100 nM primers and 0.125 μL of RT reaction product in a total of 5 μL per reaction. Reactions were run and analyzed on the LightCycler 480 (Roche) according to the manufacturer's instructions with the use of the following reference genes for normalization: *ACTIN2* (At3g46520), *EMB2386* (At1g02780). Statistical analysis was executed with the Statistical Analysis Software (SAS Enterprise Guide 5.1; SAS Institute, Inc.) using the mixed model procedure and P-values were Bonferroni adjusted for multiple measurements. Primers used for the RT-PCR are given in Supplemental Table 3.

Transient reporter assay in Arabidopsis protoplasts

The 500- and 1500-bp promoter sequences upstream of the translational start of *SMRs*, respectively, were amplified from genomic DNA using specific primers, cloned in the pDONR^{TM} P4-P1R vector (Invitrogen) (Chapter 2) and subsequently cloned simultaneously with the fLUC sequence in the pm42GW7,3. These sequences were cloned in the pDONR^{TM} P4-P1R vector and subsequently cloned simultaneously with the pENTR-min35S(-46)promoter containing a minimal *Caulifower Mosaic virus (CaMV) 35S* promoter, and the *firefly Luciferase* (*fLUC*) sequence of the pEN-R1-L+-L2 vector in the pm42GW7 vector (Karimi et al., 2007) by multisite gateway cloning (Invitrogen). *E2Fa* and *DPa* sequences were amplified from genomic DNA by PCR using the primers described in Supplemental Table 4. The product fragments were created with the Pfu DNA Polymerase Kit (Promega, Catalog #M7745), and were cloned into a pDONR221 entry vector by BP recombination cloning. For generating the effector constructs, the full-length open reading frames of the *A. thaliana E2F* and *DP* genes were recombined in the p2GW7 vector by gateway cloning, containing the *CaMV 35S* promoter(Asada, 2006). Both reporter and effector plasmids were used to transfect protoplasts using the polyethylene glycol (PEG)/Ca^{2+} method, as described by De Sutter et al., 2005. Luciferase measurements were performed using the Dual-luciferase Reporter

1000 Assay System (Promega), according to the manufacturer's instructions and as described before (De Sutter et al., 2005). Primers used are given in Supplemental Table 3

Microscopy

For confocal microscopy, root meristems were analyzed 2 days after transfer using a Zeiss LSM 510 Laser Scanning Microscope and the LSM Browser version 4.2 software (Zeiss). Plant material was incubated for 2 min in a 10μm PI solution to stain the cell walls and was visualized with a HeNe laser through excitation at 543nm. GFP fluorescence was detected with the 488-nm line of an Argon laser. GFP and PI were detected simultaneously by combining the settings indicated above in the sequential scanning facility of the microscope. Acquired images were quantitatively analyzed with the ImageJ v1.45s software (http://rsbweb.nih.gov/ij/) and Cell-o-Tape plug-ins (French et al., 2012).

Microarray Analysis

Seeds were plated on sterilized membranes and grown under a 16-h/8-h light/dark regime at 21°C. After 2 days of germination and 5 days of growth, the membrane was transferred to MS medium containing 0.3 μg/mL bleomycin for 24 h. Triplicate batches of root meristem material were harvested for total RNA preparation using the RNeasy plant mini kit (Qiagen). Each of the different root tip RNA extracts were hybridized to 12 Affymetrix® Arabidopsis Gene 1.0 ST Arrays according to the manufacturer's instructions at the Nucleomics Core Facility (Leuven, Belgium; http://www.nucleomics.be). Raw data were processed with the RMA algorithm (Emerit et al., 2004) using the Affymetrix Power Tools and subsequently subjected to a Significance Analysis of Microarray (SAM) analysis with "MultiExperiment Viewer 4" (MeV4) of The Institute for Genome Research (TIGR) (Tusher et al., 2001). The imputation engine was set as 10-nearest neighbor imputer and the number of permutations was 100. Expression values were obtained by log2-transforming the average value of the normalized signal intensities of the triplicate samples. Fold changes were obtained using the expression values of the treatment relative to the control samples. Expression profiles of genes following different experiment setting were analyzed by Two-way ANOVA,. Only significant change in the transcription level (P-value < 0.001) genes were taken for next analysis. Fold-change values were

hierarchically clustered for genes and experiments by average linkage in MeV from TIGR. To determine significantly (P-value < 0.001) overrepresented GO categories among up- and down-regulated genes, we used the BiNGO plugin for Cytoscape (http://www.psb.ugent.be/cbd/papers/BiNGO/) (Maere et al., 2005).

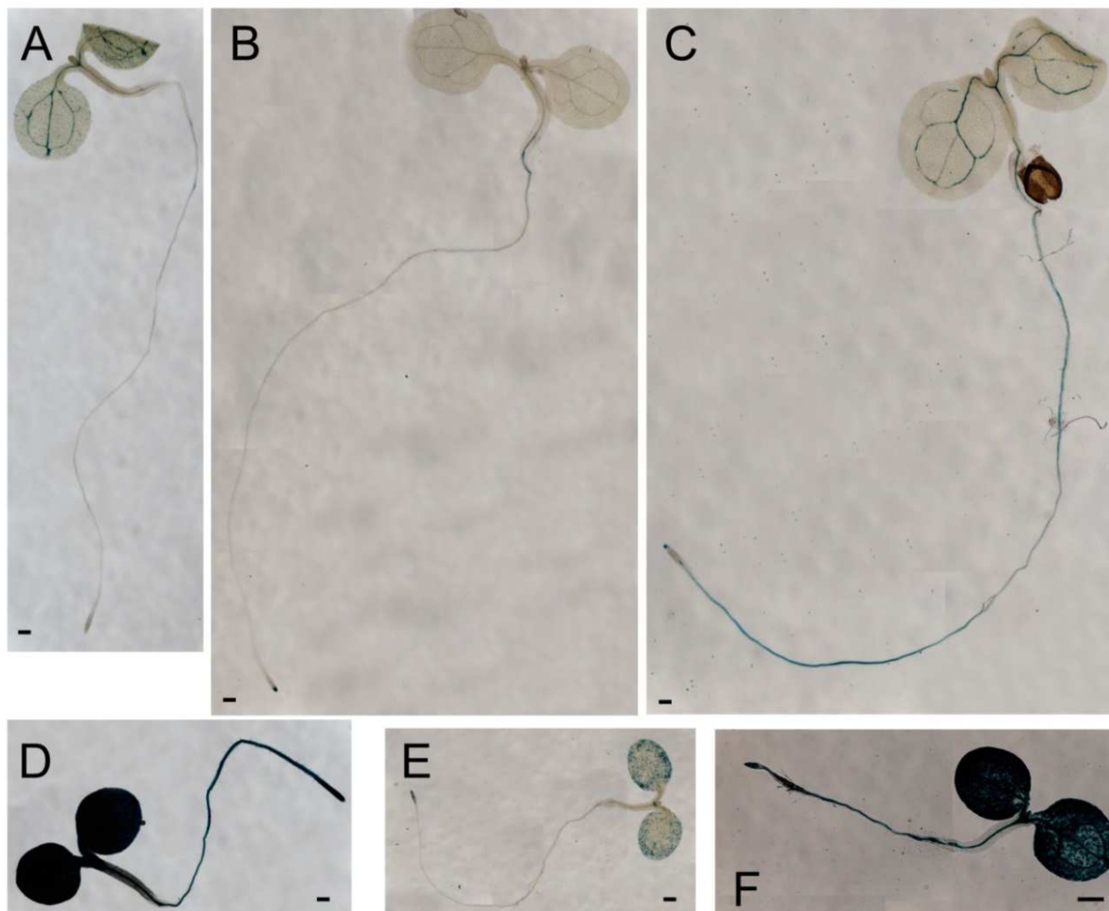
Accession Numbers

Microarray results have been submitted to MiamExpress (www.ebi.ac.uk/miamexpress), with accession number E-MEXP-3977. Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *SMR4* (At5g02220); *SMR5* (At1g07500); *SMR7* (At3g27630); AT1G67460; *MCM2* (AT1G44900); *MCM3* (AT5G46280); *MCM4* (AT2G16440) *MCM5* (AT2G07690); *MCM6* (AT5G44635); *PRL* (AT4G02060); *MCM8* (AT3G09660); *MCM9* (AT2G14050); *MCM10* (AT2G20980); *EMB1688* (AT1G67440); *E2Fa* (AT2G36010); *E2Fb* (AT5G22220).

ACKNOWLEDGMENTS

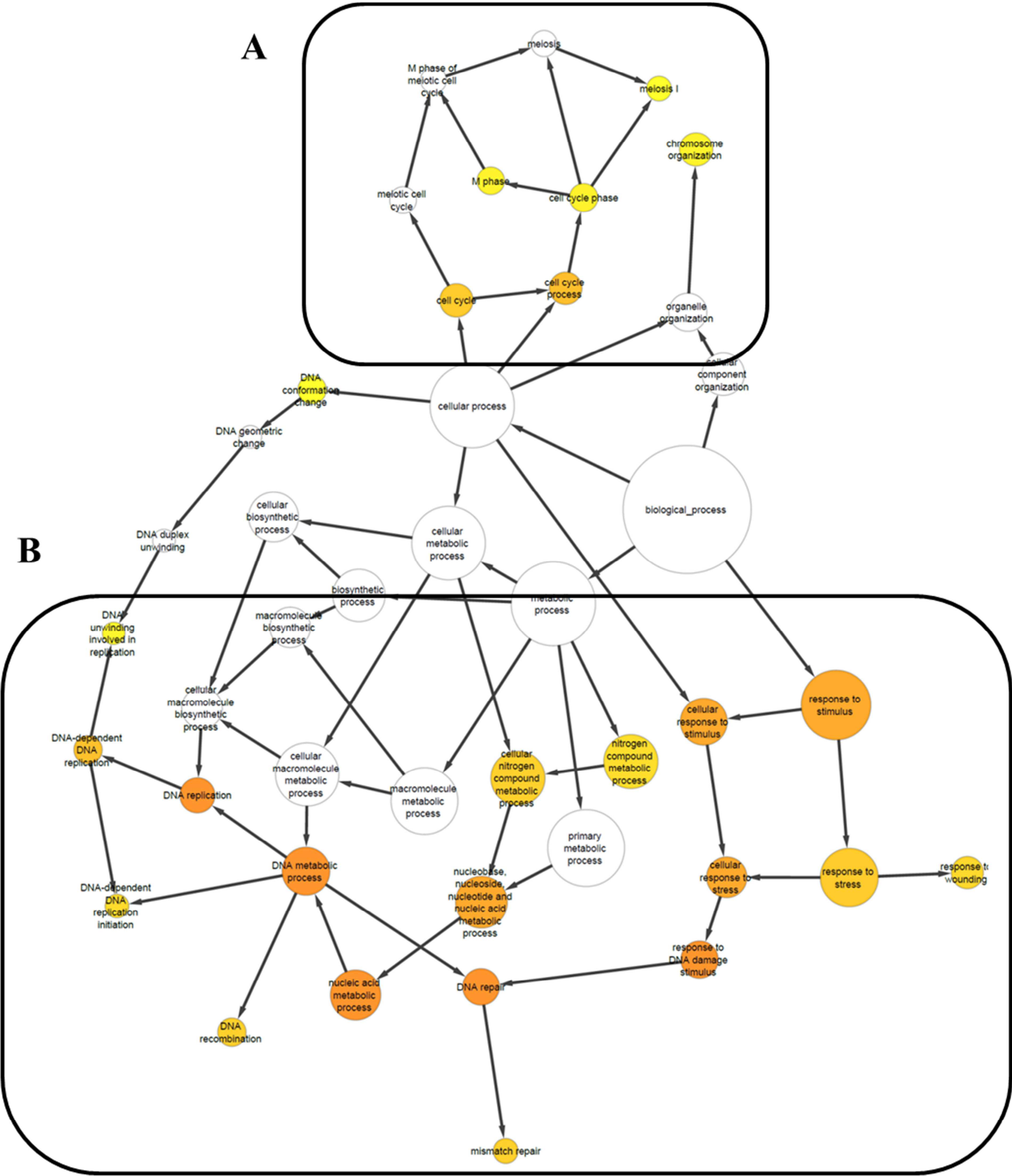
This work was supported by Ghent University (Multidisciplinary Research Partnership “Bioinformatics: from nucleotides to networks”), and the Interuniversity Attraction Poles Programme (IUAP P7/29 "MARS"), initiated by the Belgian Science Policy Office. T.C. is a Postdoctoral Fellow of the Research Foundation-Flanders. D.Y. is indebted to the China Scholarship Council (CSC File No. 2009685045) for a predoctoral scholarship.

Supplemental Data

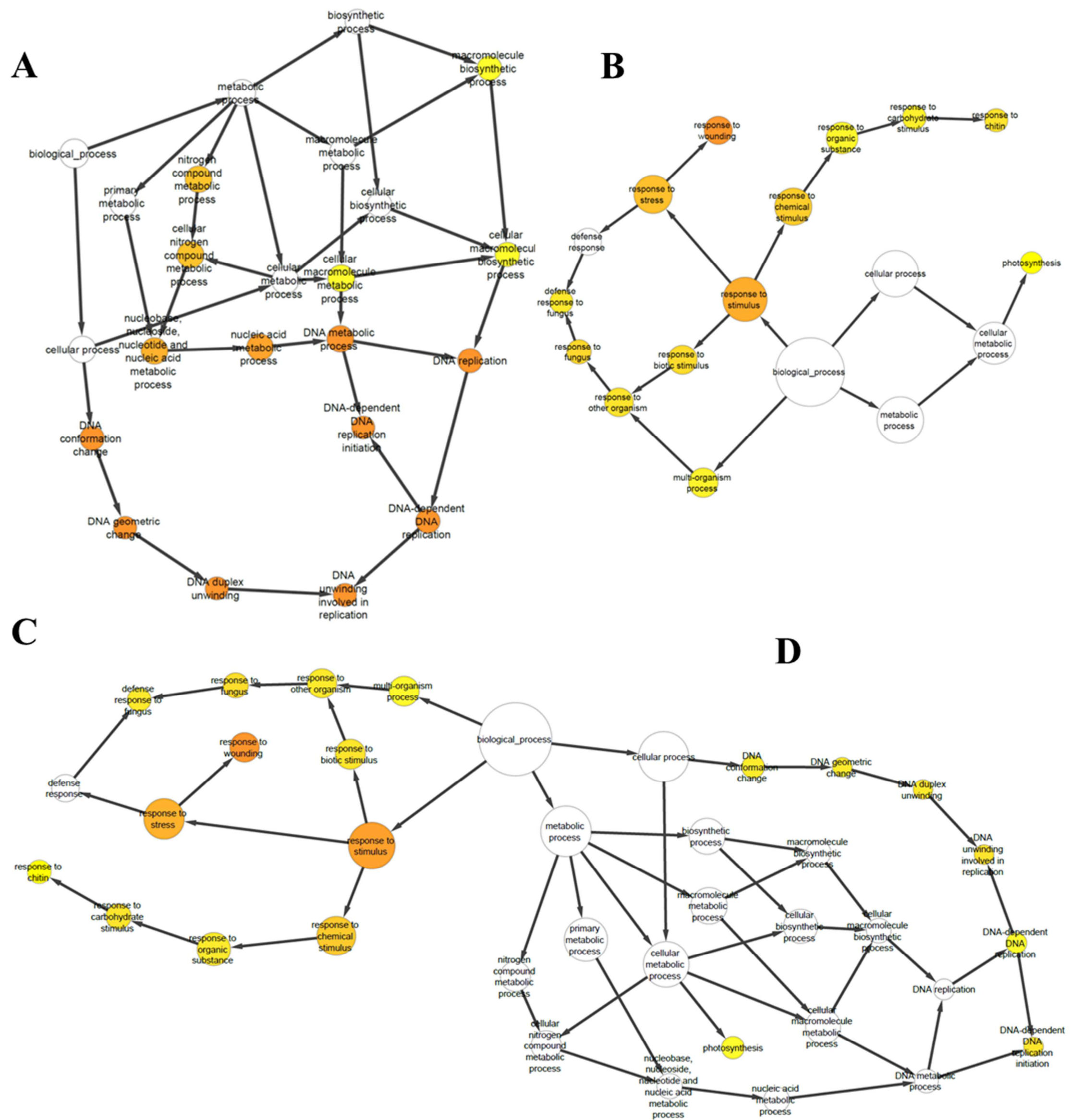


Supplemental Figure 1. *SMR5* and *SMR7* expression is RB -dependent

P_{SMR4}:*GUS* (**A,D**), P_{SMR5}:*GUS* (**B,E**), and P_{SMR7}:*GUS* (**C,F**) reporter constructs introgressed into inducible *RBR1* RNA interference backgrounds germinated on control medium (**A, B, C**) or medium supplemented with without 5 µM β-estradiol (**D, E, F**).

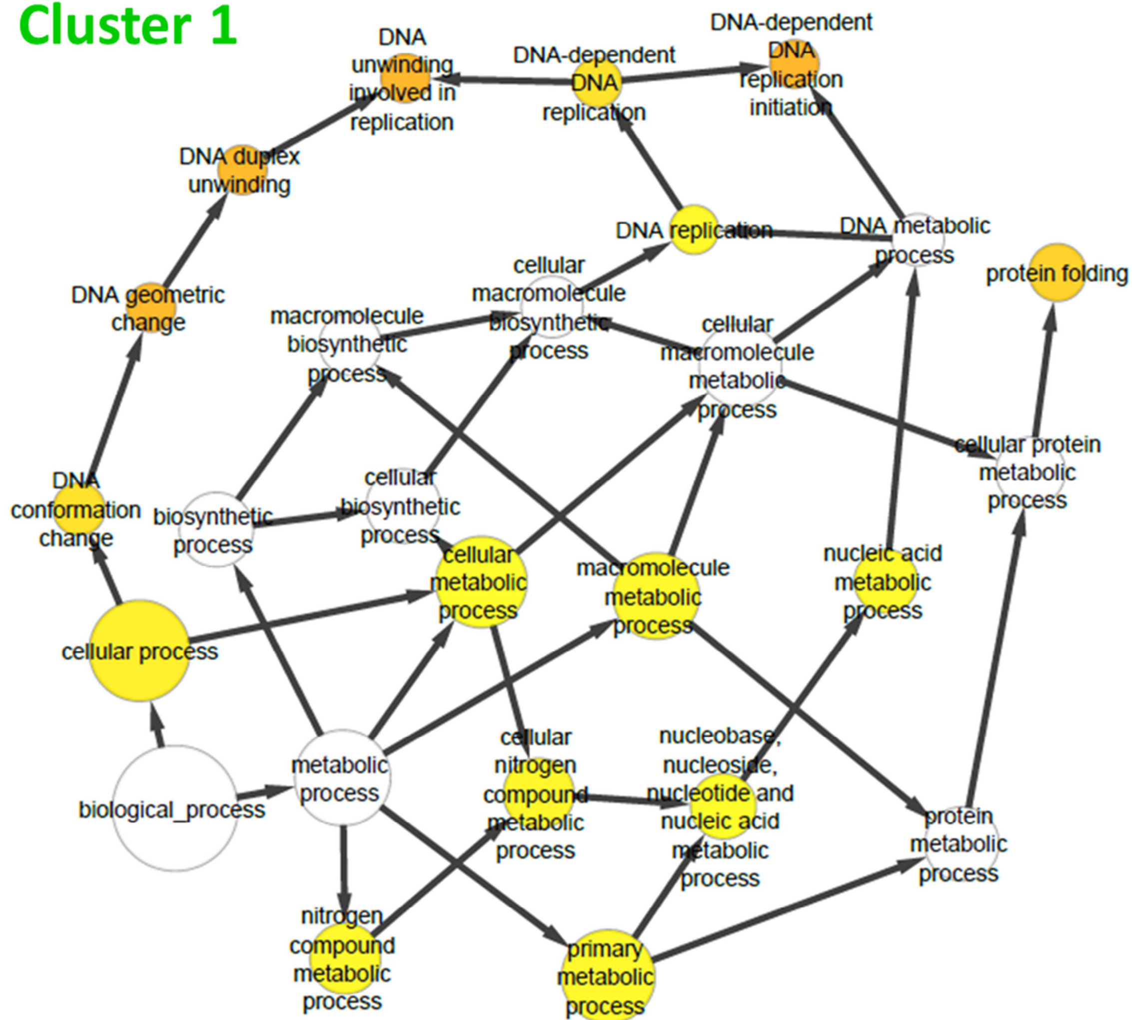


Supplemental Figure 2. GO analysis of G sign genes.

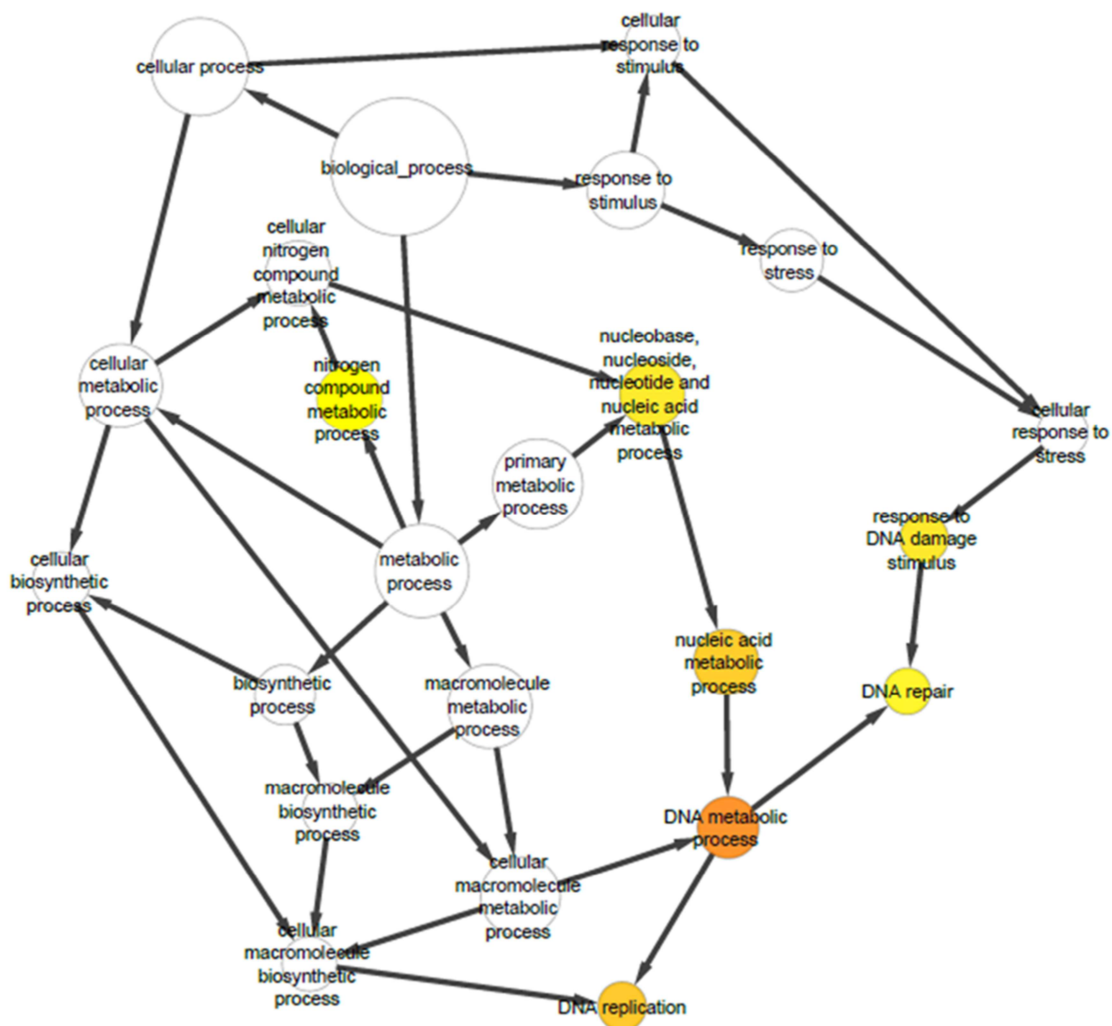


Supplemental Figure 3. GO analysis of reduced G sign genes.

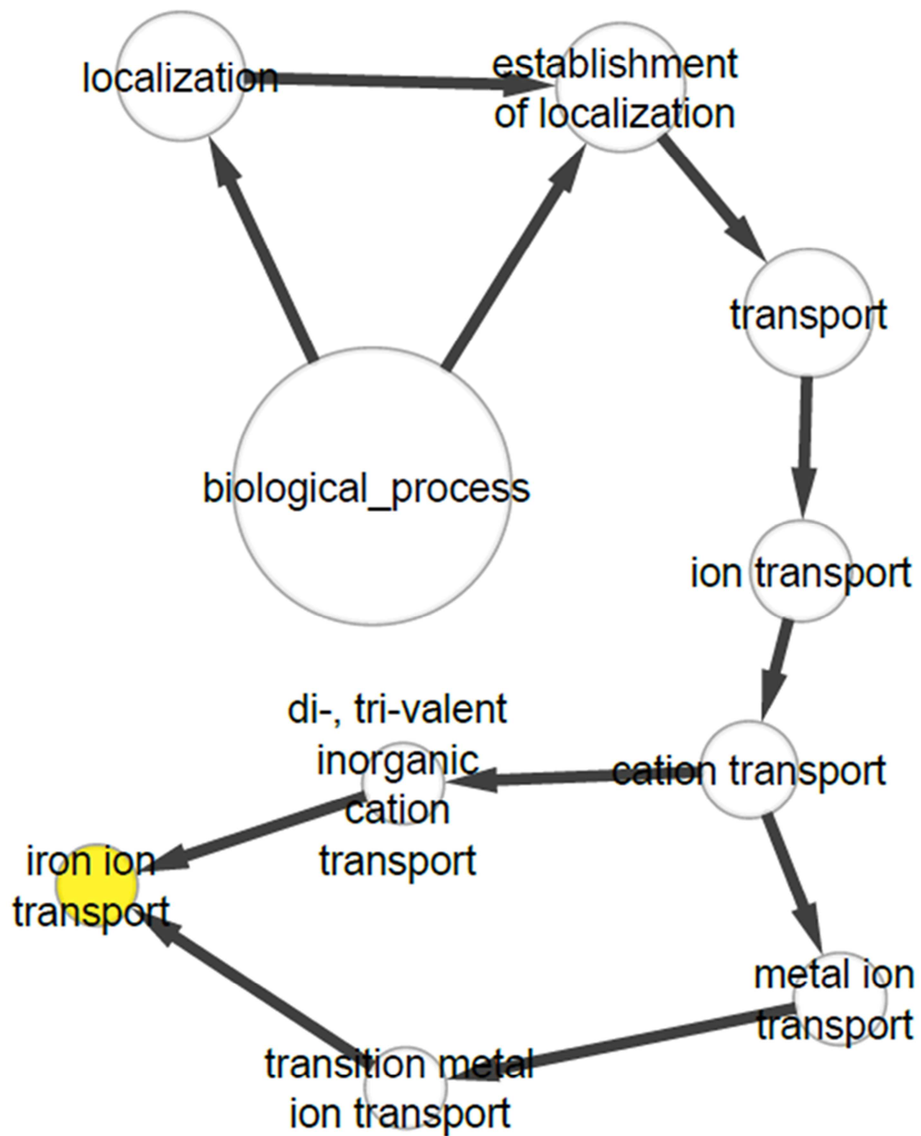
(A) GO analysis of overlapping E2F target genes that are down and upregulated in the *E2Fa*^{KO} and *E2Fa/DPa*^{OE}. **(B)** GO analysis of genes only reduced in *E2FaKO* line but not induced in *E2Fa/DPa*^{OE} . **(C-D)** GO analysis of E2Fa all reduced G sign genes.



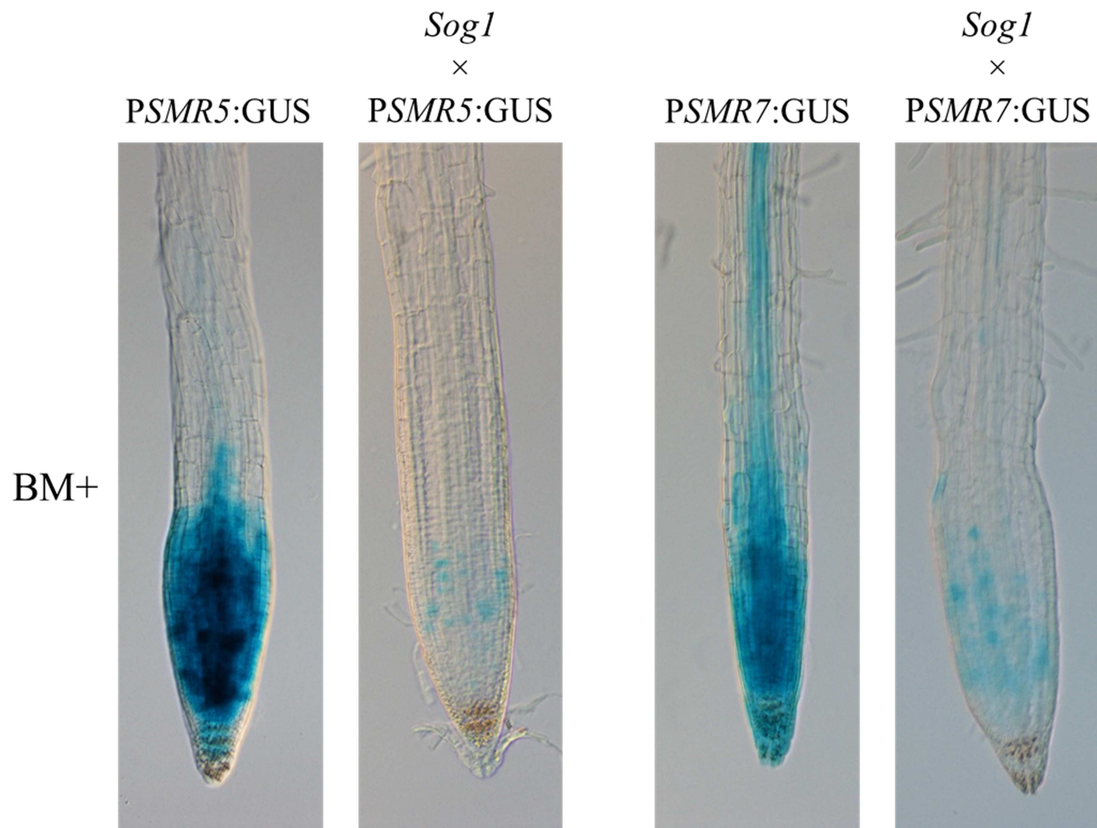
Supplemental Figure 4. GO analysis of Cluster 1 from G+T&G×T genes.



Supplemental Figure 5. GO analysis of Cluster 2 from G+T&G×T genes.



Supplemental Figure 6. GO analysis of Cluster 3+4 from G+T&G×T genes.



Supplemental Figure 7. *SMR5* and *SMR7* expressed in *sog1-1* mutant background

Supplemental Table 1. List of significant differentially expressed genes in Microarray (P < 0.001)

> G sign reducted genes

AT3G30720 AT4G04223 AT3G01345 AT5G38005 AT2G24850 AT5G13220 AT2G20520 AT5G24110 AT2G36010 AT5G64510 AT1G15040 AT1G80840 AT5G20230 AT3G27940 AT3G26570 AT1G17420 AT4G34410 AT1G70700 AT1G28480 AT1G76640 AT1G72520 AT4G14548 AT1G54870 AT5G62210 AT4G25220 AT2G44840 AT2G34600 AT1G76650 AT5G18670 AT1G74930 AT5G51190 AT5G13370 AT1G74950 AT2G26530 AT1G20340 AT5G07190 AT1G15010 AT4G10340 AT4G36040 AT5G39580 AT4G24230 AT3G32030 AT3G48540 AT1G76470 AT1G10300 AT5G36925 AT2G22860 AT5G22410 AT1G44030 AT2G32150 AT4G29780 AT3G51860 AT5G45110 AT5G47110 AT1G31800 AT1G09932 AT4G25050 AT1G06680 AT4G23180 AT1G22980 AT4G22080 AT3G63540 AT1G74470 AT3G47650 AT5G40460 AT1G03600 AT3G51450 AT2G06520 AT2G34930 AT1G23710 AT4G17490 AT3G01830 AT1G68590 AT1G18382 AT3G47470 AT5G11070 AT1G61340 AT2G32270 AT4G11280 AT3G56010 AT2G07690 AT4G02530 AT3G21670 AT1G01770 AT1G73500 AT5G44490 AT1G18460 AT5G16120 AT3G23700 AT1G20510 AT5G58650 AT4G40090 AT1G01140 AT2G38470 AT2G33460 AT1G71500 AT2G35260 AT5G57180 AT3G59080 AT3G13724 AT2G18710 AT4G18020 AT1G02074 AT4G14860 AT5G64630 AT2G23320 AT3G01480 AT1G16130 AT5G19220 AT3G12930 AT2G43330 AT2G06050 AT5G57625 AT1G05560 AT4G02330 AT4G38390 AT5G18840 AT5G46280 AT3G51870 AT3G62010 AT4G01050 AT5G18748 AT2G31070 AT3G20670 AT3G01410 AT3G02790 AT1G33700 AT2G18800 AT5G41610 AT4G20325 AT3G47490 AT3G27580 AT5G01100 AT2G36990 AT5G47910 AT4G12720 AT5G04140 AT1G69050 AT4G02060 AT5G67400 AT5G58680 AT1G78915 AT5G26740 AT1G44350 AT2G33860 AT1G30950 AT1G72680 AT5G07440 AT4G36030 AT1G06760 AT1G01720 AT3G12540 AT5G03160 AT4G28300 AT3G15518 AT1G11050 AT3G62420 AT2G16440 AT5G30510 AT5G13190 AT2G35490 AT1G32990 AT2G47540 AT4G14210 AT1G67320 AT1G02850 AT3G11340 AT4G27020 AT4G02070 AT2G39670 AT3G11420 AT5G63790 AT5G61670 AT1G65040 AT1G66760 AT3G12480 AT3G56510 AT1G13910 AT1G64280 AT4G20360 AT1G54100 AT3G55610 AT1G59910 AT4G36050 AT2G26800 AT2G28190 AT1G24267 AT1G49480 AT2G47250 AT5G13750 AT3G20560 ATMG00610 ATCG00540 AT1G31970 AT1G28250 AT3G52230 AT3G16480 ATCG00860 ATCG01280 AT3G23990 AT3G04600 AT3G50910 ATMG01220 AT4G26900 AT4G37910 ATCG00500

> G sign inducted genes

AT5G02500 AT5G13710 AT5G35180 AT1G29050 AT5G10170 AT1G25145 AT5G14550 AT2G21790 AT4G36945 AT5G15230 AT1G66270 AT5G47820 AT3G56480 AT2G13820 AT1G24793 AT2G43610 AT2G01190 AT5G12250 AT3G56370 AT1G73340 AT2G42070 AT1G20100 AT5G46740 AT3G22850 AT3G01820 AT5G47540 AT1G14210 AT1G22530 AT4G35560 AT1G25210 AT3G03380 AT2G34020 AT4G28410 AT4G28650 AT5G45510 AT2G40840 AT1G74660 AT4G39400 AT3G05990 AT2G28790 AT4G35890 AT3G09540 AT5G02190 AT5G63760 AT1G64440 AT1G72050 AT4G26670 AT1G27210 AT4G35380 AT5G35670 AT1G79060 AT2G32590 AT5G11000 AT1G06470 AT5G20960 AT1G18910 AT5G27100 AT1G33750 AT1G21880 AT5G01881 AT3G47680 AT2G39040 AT2G01420 AT2G20750 AT2G25640 AT4G22910 AT3G20150 AT2G02680 AT5G18550 AT3G59830 AT4G16970 AT5G62410 AT1G79450 AT2G39530 AT3G63430 AT1G52050 AT1G77320 AT2G31900 AT1G65470 AT5G46600 AT3G48340 AT2G24490 AT3G23510 AT1G79580 AT1G75620 AT3G16440 AT4G18300 AT2G21610 AT4G34260 AT4G13990 AT3G54750 AT2G13540 AT2G12646 AT1G79460 AT1G49030 AT2G31130 AT2G28960 AT1G09910 AT4G10640 AT2G13550 AT1G50060 AT5G40820 AT2G23410 AT1G10760 AT2G35020 AT1G71110 AT1G03840 AT5G45050 AT2G45870 AT5G38280 ATCG00590 AT1G09450 AT5G08260 AT5G38140 AT3G59320 AT1G60390 AT3G10410 AT5G60630 AT1G69526 AT5G54020 AT4G03820 AT5G20830 AT1G29630 AT4G23400 AT4G37160 AT3G62110 AT1G78520 AT5G09960 AT2G28870 AT5G44510 AT1G76730 AT3G61760 AT1G27110 AT3G49190 AT4G31920 AT3G01516 AT1G10520 AT1G57790 AT1G66730 AT5G23220 AT3G19430 AT1G05835 AT5G11540 AT1G05650 AT5G63960 AT4G20350 AT3G14890 AT5G62720 AT2G17080 AT2G17230 AT2G14095 AT3G50340 AT3G51330 AT3G24495 AT2G16230 AT2G03090 AT2G21050 AT4G18550 AT4G08770 AT4G39740 AT4G19130 AT1G69770 AT5G10280 AT5G23420 AT5G10278 AT5G04200 AT3G20490 AT1G57820 AT3G01840 AT3G04980 AT3G05480 AT3G19210 AT3G23730 AT5G38690 AT2G46570 AT4G39380 AT1G10530 AT5G65450 AT2G37700 AT5G44130 AT5G27000 AT1G79110 AT5G10390 AT1G34340 AT5G25754 AT5G62960 AT4G22214 AT4G39230 AT4G01533 AT1G06080 AT2G03420 AT4G15890 AT1G55265 AT3G29810 AT4G22212 AT3G48260 AT5G56320 AT5G61000 AT4G35520 AT1G08730 AT1G79760 AT2G32940 AT3G49380 AT4G39630 AT4G14770 AT2G21540 AT5G01370 AT3G52115 AT3G59210 AT5G61455 AT1G02740 AT2G28100 AT5G62550 AT3G01850 AT5G63920 AT1G05530 AT3G07980 AT1G53543 AT3G03500 AT5G48600

AT3G10470 AT3G19230 AT3G17998 AT2G31902 AT5G59660 AT1G03502 AT1G23160 AT1G03070 AT2G35850 AT1G27140 AT4G16770 AT5G14920 AT1G73620 AT5G02790 AT1G12420 AT1G78940 AT5G49110 AT4G25540 AT3G16300 AT3G20840 AT2G37290 AT1G05780 AT2G31920 AT1G49160 AT5G60520 AT1G66040 AT2G41190 AT5G49290 AT3G55040 AT4G39000 AT5G35740 AT3G09660 AT4G03270 AT3G63375 AT4G37950 AT1G08260 AT3G50870 AT3G55734 AT3G05740 AT3G12220 AT3G54710 AT4G21902 AT5G37800 AT3G07580 AT5G16850 AT1G32860 AT2G27970 AT5G20045 AT3G29300 AT5G51470 AT5G27610 AT3G59100 AT2G47230 AT3G29780 AT1G73780 AT5G25380 AT4G36880 AT5G43500 AT4G21070 AT5G54960 AT3G23360 AT4G16745 AT3G53680 AT5G49160 AT3G24320 AT2G16850 AT1G64910 AT5G07400 AT1G17460 AT3G18500 AT5G06590 AT3G48346 AT3G26380 AT3G21420 AT5G13060 AT5G25580 AT5G24205 AT3G46616 AT1G49952 AT1G11735 AT4G09250 AT4G21590 AT5G53380 AT3G14740 AT1G57770 AT2G43890 AT4G09200 AT4G09310 AT5G44010 AT5G10440 AT3G61810 AT2G47610 AT2G47590 AT3G18730 AT2G26910 AT4G14970 AT3G10420 AT5G26270 AT1G10810 AT2G23910 AT5G60250 AT5G48390 AT1G19940 AT4G37235 AT1G71750 AT4G35200 AT2G14050 AT4G17380 AT1G04650 AT5G01630 AT4G17760 AT2G01905 AT1G02670 AT3G05625 AT1G15550 AT1G50970 AT4G16807 AT1G67180 AT1G06460 AT2G26560 AT2G41810 AT2G47650 AT1G67370 AT3G47460 AT3G28880 AT3G44765 AT2G47580 AT1G79890 AT2G47630 AT4G22217 AT4G30650 AT2G47600 AT2G28560 AT4G15393 AT5G07660 AT4G20210 AT1G04600 AT2G46980 AT1G56500 AT2G46980 AT2G47640 AT1G79640 AT3G20475 AT5G35870 AT5G25640 AT3G47040 AT2G47620 AT3G60660 AT1G26540 AT4G09300 AT5G24206 AT1G15310 AT2G24970 AT2G20250 AT4G33870 AT1G77860 AT4G13555

>G×T

AT3G30720 AT4G04223 AT3G01345 AT5G38005 AT2G24850 AT5G13220 AT2G20520 AT5G24110 AT2G36010 AT5G64510
AT1G15040 AT1G80840 AT5G20230 AT3G27940 AT3G26570 AT1G17420 AT4G34410 AT1G70700 AT1G28480 AT1G76640
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Supplemental Table 2. E2FaKO down regulated genes & E2Fa/DPa OE up regulated genes & E2F target genes

AGI code	Gene Description	Up regulated in E2Fa/DPa ^{OE}	E2Fmotifs
AT1G01140.1	CBL-interacting protein kinase 9 CIPK9		ATACGCGC -59 +
AT1G03600.1	photosystem II family protein		GTTCCCGC -531 +
AT1G11050.1	Protein kinase superfamily protein		TTTGGCGG -85 -
AT1G15010.1			TTTAGCGC -82 - ATTCGCGG -762 +
AT1G20340.1	Cupredoxin superfamily protein DRT112		TCTCCCGG -503 - TTAGGCGC -704 -
AT1G31800.1	cytochrome P450, family 97, subfamily A, polypeptide 3 CYP97A3		TTTCGCGC -239 +
AT1G32990.1	plastid ribosomal protein l11 PRPL11		TCTCCCGC -639 -
AT1G67320.2	DNA primase, large subunit family	Yes	ATTCCCGC -97 + TTTCCCGC -74 +
AT1G71500.1	Rieske (2Fe-2S) domain-containing protein		ATGCGCGC -267 + TTTCGCGC -263 - TCGCGCGC -265 -
AT1G74930.1	Integrase-type DNA-binding superfamily protein		ACTGGCGC -83 - ATTCCCGC -132 +
AT1G74950.1	TIFY domain		TTTCCCCC -461 + TTACGCGG -746 -
AT2G06520.1	photosystem II subunit X PSBX		TTTGCCCC -991 -
AT2G07690.1	Minichromosome maintenance (MCM2 MCM5	Yes	GTTCCCGC -58 + TTTGGCGG -54 - TTTCCCGC -72 +
AT2G16440.1	Minichromosome maintenance (MCM2 MCM4	Yes	TTTGGCGC -78 -
AT2G22860.1	phytosulfokine 2 precursor PSK2		TTTCCCCC -689 +
AT2G24850.1	tyrosine aminotransferase 3 TAT3		ATACGCGC -624 +
AT2G26530.1	Protein of unknown function (DUF1645)		TTGGCCGC -486 -
AT2G28190.1	copper CSD2		GTTGCCGC -724 +
AT2G33460.1	ROP-interactive CRIB motif-containing protein 1 RIC1		TTTGCCGG -725 +
AT2G35260.1			ATTGGCGG -170 -
AT2G36990.1	RNA polymerase sigma-subunit F SIGF		ATTGCCGC -539 -
AT2G43330.1	inositol transporter 1 INT1		ACTCCCGC -141 -
AT3G01345.1	Expressed protein		TTGGGCGC -869 -
AT3G01410.1	Polynucleotidyl transferase, ribonuclease H-like superfamily protein		ATGCCCGC -971 -
AT3G01830.1	Calcium-binding EF-hand family protein		GTTCCCGC -154 - TTAGCCGC -713 -
AT3G12540.1	Protein of unknown function, DUF547		TTTCCCGC -809 -
AT3G23700.1	Nucleic acid-binding proteins superfamily		ATTGGCGG -632 - ACTCCCGC -636 + ATTCGCGG -585 - TTTCCCGC -595 +
AT3G48540.1	Cytidine	Yes	TTTCCCGC -80 +
AT3G51450.1	Calcium-dependent phosphotriesterase superfamily protein		TTAGCCGC -648 -
AT3G52230.1			GTTCGCGG -263 -
AT3G59080.1	Eukaryotic aspartyl protease family protein		TTTGGCGC -547 - TTTGGCGG -853 +
AT3G62420.1	basic region BZIP53		TTTCCCCC -683 +
AT4G01050.1	thylakoid rhodanese-like TROL		TTCGCCGC -529 -
AT4G02060.1	Minichromosome maintenance (MCM2 PRL	Yes	TTTCCCGC -21 +
AT4G02070.2	MUTS homolog 6 MSH6	Yes	ATTGGCGG -157 - TTTCCCGG -128 + TTGCGCGG -110 - ATTCCCGC -114 + TTTCCCGC -161 +
AT4G04223.1	other RNA		TTTCCCGG -225 -
AT4G12720.4	MutT		TATCCCGC -884 -
AT4G20325.1		Yes	ATGCGCGC -72 + ATTGGCGC -49 -
AT4G28300.1	Protein of unknown function (DUF1421) DUF1421		TTTCCCCC -236 +
AT4G40090.1	arabinogalactan protein 3 AGP3		TATGGCGC -519 +
AT5G07190.2	seed gene 3 ATS3		ATACCCGC -534 -
AT5G19220.1	ADP glucose pyrophosphorylase large subunit 1 APL1		TTTCCCCG -482 -
AT5G20230.1	blue-copper-binding protein BCB		TTTAGCGC -185 - TTACGCGC -189 +
AT5G22410.1	root hair specific 18 RHS18		TTTCCCCC -521 -
AT5G24110.1	WRKY DNA-binding protein 30 WRKY30		TTTGCCCC -856 +
AT5G30510.1	ribosomal protein S1 RPS1		TTTCGCGC -144 +
AT5G40460.1		Yes	ATTGGCGG -942 - TTTGGCGC -176 - TTTCCCGC -269 - TATGGCGC -180 +
AT5G44490.1	FBD, F-box, Skp2-like and Leucine Rich Repeat domains containing protein		TCTCCCGG -537 -
AT5G46280.1	Minichromosome maintenance (MCM2 MCM3	Yes	TTTGGCGC -217 - TTTGGCGG -106 -
AT5G47110.1	Chlorophyll A-B binding family protein		TTTGCCGC -513 +
AT5G51190.1	Integrase-type DNA-binding superfamily protein		TTACCCGG -905 +
AT5G58650.1	plant peptide containing sulfated tyrosine 1 PSY1		ACTCCCGC -191 +
AT5G64510.1			TCTCGCGC -112 + TTTCCCGG -694 -
AT5G64630.1	Transducin FAS2	Yes	TTTGCCGG -880 + TTTCCCCC -544 - TTTCGCGC -18 +
ATCG00860.1	Chloroplast Ycf2;ATPase, AAA type, core		TTGGGCGC -118 +
ATCG01280.1	Chloroplast Ycf2;ATPase, AAA type, core		TTGGGCGC -118 +
ATMG00610.1	Putative membrane lipoprotein		TTTCGCCC -235 + GTTGGCGC -779 +
ATMG01220.1			TTTCGCCC -370 -

Supplemental Table 3. List of primers used for cloning, genotyping, and RT-PCR

Promoter cloning primers

Gene	Primer	Sequence
SMR4	Fw	ATAGAAAAGTTGGTGAAACACAAAGCATCTTCG
	Rev	GTACAAACTTGTTCTTCTCTCTCGAACTCG
SMR5	Fw	ATAGAAAAGTTGGTCAGAACGAACAAAAG
	Rev	GTACAAACTTGTTTTTGTCCGCTCTCTCG
SMR7	Fw	AGAAAAGTTGCGTTGACGCGGGAAAATTAA
	Rev	GTACAAACTTGCTTAAAACAGTTGGAGATTGAG

ORF cloning primers

Gene	Primer	Sequence
SMR4	Fw	AAAAAGCAGGCTTCATGGAGGTGG TGGAGAGGAA G
	Rev + stop code	AGAAAGCTGGGTCCTAAGCGCAAGCTTCTCTTC
	Rev - stop code	AGAAAGCTGGGTCAGCGCAAGCTTCTCTTC
SMR5	Fw	AAAAAGCAGGCTTCATGGAGGAGAAAAACTACGACG
	Rev + stop code	AGAAAGCTGGGTCCTAGGTTGCCGCTTGGG
	Rev - stop code	AGAAAGCTGGGTCGGTTGCCGCTTGGGA
SMR7	Fw	AAAAAGCAGGCTTCATGGGAATTTCGAAAAAATCTC
	Rev + stop code	AGAAAGCTGGGTCTTAACGGCGTTGTATAAACACC
	Rev - stop code	AGAAAGCTGGGTCACGGCGTTGTATAAACACCA
E2Fa	Fw	GGCCATGGCCGGTGTCGTACGATCTTCTCCCGA
	Rev	GGGGATCCTCATCTCGGGGTTGAGT
DPa	Fw	GGCCATGGAGTTGTTTGTCACTCC
	Rev	GGAGATCTTCAGCGAGTATCAATGG

T-DNA genotyping primers

Gene	Line	Primer	Sequence
SMR5	SALK_100918	LP	GAACGAACAAAAGTGAGCTCG
		RP	TTTCCCAACCTGACAGAAAAC
SMR7	SALK_128496	LP	AAAATCGATAACTAAAACGAACCG
		RP	AGGCCTTCAATATAGCCCATG
E2Fa-1	MPIZ-244	LP	TTGTTTCCTTCACTCGCCGTCGCTT
	MPIZ-244	RP	CTCGGTGCTCCTCTACAACATCACTCTA
E2Fa-2	GABI_348E09	LP	TTCCAGGTCTGTCTTTCCTATTTC
	GABI_348E09	RP	ATTCCTCCTACTTGCTCTTGC
E2Fb	SALK_103138	LP	TGCGAACTCTGTTATGCAATG
	SALK_103138	RP	GCAAGCATAACGTTTGAGGAC
	SALK	T-DNA_I	GATAGACGGTTTTTCGCCCTTTGAC
	GABIKAT	T-DNA_I	CCCATTTGGACGTGAATGTAGACAC
	MPIZ	T-DNA_I	CTCGGGAATGGCGAAATCAAGGGCATC

RT-PCR primers

Gene	Primer	Sequence
SMR4	Fw	GCCGAGAAGCACGATGTATAG
	Rev	AGATCTGGTGGCTGAAAGTACC
SMR5	Fw	AAACTACGACGACGGAGATACG
	Rev	GCTACCACCGAGAAGAACAAGT
SMR7	Fw	GCCAAAACATCGATTCGGGCTTC
	Rev	TCGCCGTGGGAGTGATACAAAT
MCM2	Fw	GGCAATATGTCACAGAAGAGGATG
	Rev	GAGAAGAACGAGCAGCAATGAG
MCM3	Fw	GCAACCAACGCAGGAATAATGAAG
	Rev	GAGACCGTGCCGCTGAATTG
MCM4	Fw	CGAGGTGATGGAACAGCAGAC
	Rev	CTAGATAGCAAGGTTGGAGGAAGG
MCM5	Fw	CGGTGCTTGCTGCTGCTAAC
	Rev	ATGGCTGGCTATTTCCTTGTCTTG
MCM6	Fw	CAAGATGCTAATGGTGACAAC
	Rev	CTATCATATTCTTCCTCGCTAATC
MCM7	Fw	TCCAGCATCAGGCAAGAAG
	Rev	GTCGGTCATCAGCATACAATG
EMB2386	Fw	CTCTCGTTCCAGAGCTCGCAAAA
	Rev	AAGAACACGCATCCTACGCATCC
Actin2	Fw	GGCTCCTCTTAACCCAAAGGC
	Rev	CACACCATCACCAGAATCCAGC
PARP2	Fw	ATGGCGTTCTGCTCCTCTGC
	Rev	GGTGCTGTTTTCCCCACACC
BRCA1	Fw	TGTTCCCTCTTTCAGCGATTTGATG
	Rev	GGCCTCTGAGTCCATTCAAACA

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CHAPTER 4

NEW SCREENING SYSTEM FOR TRANSCRIPTIONAL REGULATORS WORKING UPSTREAM OF *SMR7*

New screening system for transcriptional regulators working upstream of *SMR7*

Dalong Yi[a,b], Toon Cools[a,b], Ilse Vercauteren[a,b], Hilde Van den Daele[a,b], Claire Lessa Alvim Kamei[a,b], Lieven De Veylder[a,b]

[a]Department of Plant Systems Biology, VIB, B-9052 Gent, Belgium

[b]Department of Plant Biotechnology and Bioinformatics, Ghent University, B-9052 Gent, Belgium

Running title: SMR7and transcriptional regulator

AUTHOR CONTRIBUTIONS

D.Y., T.C., C.L.A.K., and L.D.V. conceived and designed the research. D.Y., T.C., C.L.A.K., ., H.V.d.D. and I.V. performed the experiments. D.Y., T.C., and L.D.V. analyzed the data and wrote the manuscript.

ABSTRACT

***D-amino acid oxidase* 1 (*dao1*) gene of the yeast *Rhodotorular gracilis* . Its protein product catalyzes the conversion of the D-amino acids to its corresponding imino acid. Some of the D-amino (e.g. D-Ala and D-Ser) acids are toxic to plants unless converted and other D-amino acids are non-toxic (e.g. D-Ile and D-Val) until converted. By putting the *dao1* gene under control of the DNA damage inducible *SMR7* promoter, we are able to detect mutants in the DNA damage response. *SMR7* has a clear transcriptional induction during DNA-stress and upon treatment with D-Val together with BM, plants holding the *SMR7:Dao1* gene need to have an impaired checkpoint to prevent the conversion of D-Val to its toxic imino acid. The use of BM and HU as the trigger to induce *SMR7* allows us to detect specific elements that react to the stress in two separate pathways. The screening system is designed for the discovery of transcriptional mutants, but in contrast to the use of any other reporter gene, requires little or no researcher effort besides sowing the plants.**

INTRODUCTION

The DNA damage pathway is a conserved and essential molecular mechanism that safeguards proper propagation of genomic content during cell division and to subsequent generations (Cools and De Veylder, 2009). It involves a complex interaction network that senses DNA stress and integrates developmental signals and environmental cues to ultimately invoke the most favorable response for the cell and organism. Among eukaryotes, three major responses to DNA damage are recognizable. First of all, the activation and transcriptional induction of DNA repair proteins that are set for a swift restoration of the damaged nucleic acids (Bartek and Lukas, 2001; Harper and Elledge, 2007). This branch of the DNA damage pathway works in concert with the cell cycle checkpoints whose action will result in a cell cycle arrest or retardation. The interplay between both should allow the cell sufficient time for repair before progressing into the next cell cycle phase. To prevent that the DNA damage puts the organism in jeopardy, a third branch will induce programmed cell death (PCD)(Furukawa et al., 2010) whenever the damage is too excessive and the risk for mutations that are detrimental for the organism is simply too high. Although many elements of the pathway are conserved amongst eukaryotes, each of them is in need of distinct elements that translate and adapt the response for the unique development of the organism.

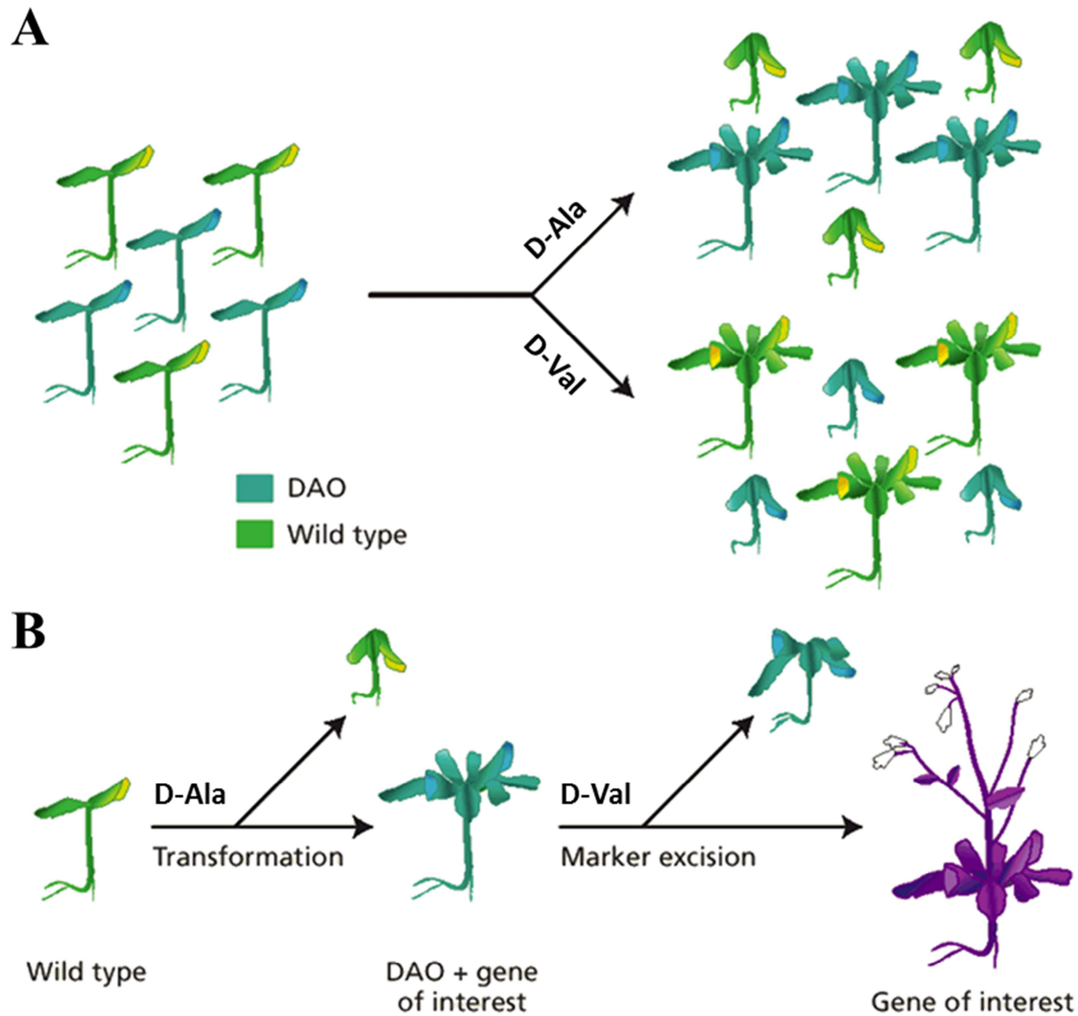

Figure 1. The potential *DAO1* –based Either/or selection markers selection system

(A) Growth of nontransgenic wild-type plants lacking DAO activity is inhibited by D-amino acids such as D-alanine (D-Ala) but is not affected by D-valine (D-Val). In contrast, plants expressing the transgenic *DAO1* gene detoxify D-Ala and survive, whereas they metabolize D-Ile to toxic compounds that kill the plants. **(B)** Hypothetical application: plants that have integrated a gene of interest together with the *DAO1* marker are first detected by positive selection. Subsequent negative selection identifies plants from which the no-longer-desirable selection marker has been removed, leaving the gene of interest as the only transgenic sequence in place (Scheid, 2004).

In plants, the search for members of the DNA damage pathway was initially mainly based on the search for mammalian/yeast orthologues. As such, the core kinases Ataxia telangiectasia mutated (ATM) (Garcia et al., 2000; Garcia et al., 2003) and ATM and Rad3-related (ATR) protein kinases(Culligan et al., 2004) and many of the sensing and signaling proteins could be found. On the other hand, for Checkpoint kinase 1 and 2 (CHK1 and CHK2) (Walworth et al., 1993; Murakami and Okayama, 1995) and Polo-like kinase (PLK) (Casaluce et al., 2013), three essential kinases in the DNA damage pathway in mammalians, no functional or sequence ortholog was found to date in plants. Similarly, Cell Division Cycle 25 (CDC25) has a sequence ortholog, but all evidence suggests that its function has shifted to other physiological processes. It is only recently that plant-specific components were discovered. The existence of Suppressor Of Gamma Response 1 (SOG1) was already shown in 2003 (Preuss and Britt, 2003), but it was only six years later that the mutation was mapped to a NAC transcription factor gene. SOG1 has a functional resemblance to the mammalian p53 oncogene, as it is a core transcription factor of the DNA damage response and *SOG1*^{KO} plants are less prone to PCD upon DNA stress(Preuss and Britt, 2003; Yoshiyama et al., 2009; Adachi et al., 2011; Yoshiyama et al., 2013). Besides its downstream targets, namely the *SIAMESE-RELATED* 5 and 7 (*SMR5* and *SMR7*) genes that inhibit CDK activity and hence the cell cycle upon DNA damage and oxidative stress (Yi et al., 2014) (Chapter 2), there is no report of any critical plant specific components in the DNA damage response (DDR). Moreover, the list of core DNA damage genes (Chapter 3) hints to the existence of many potential new members that are transcriptionally regulated upon DNA-stress that have no clear mammalian or yeast ortholog. Moreover, no inactivators of the DNA damage pathway are known to date. These data suggest that there is still uncovered ground waiting to be found.

The DDR can be experimentally activated by treatment with several drugs. Two extensively used and documented agents that are capable to do this are hydroxyurea (HU) and bleomycin (BM). The method action of BM is still under debate, but it is clear that it results in the formation of double strand breaks (DSBs) that can be sensed and transduced by ATM. In plants, treatment with BM will result in stem cell death with concomitant growth reduction of the root. On a molecular level, it will induce *SMR5* and *SMR7* via SOG1. HU will act on 2 separate fronts: at the one hand it will inhibit the small subunit (R2) of ribonucleotide reductase (RNR) causing reduced dNTP-levels and

replication stress(Roa et al., 2009). The resulting stalled replication forks are sensed by ATR. At the other hand, HU causes oxidative stress, likely through displacement of Fe^{3+} from the active site of RNR with the formation of an aminocarbonylaminooxyl radical (Chapter 2), in combination with potential inhibition of catalase activity (Chapter 2). The induction of *SMR5/7* upon HU treatment is completely dependent on the ATM-SOG1 pathway suggesting that this upregulation is solely the result of oxidative stress.

As a result of the low number of signaling elements discovered to date, new screening methods are necessary that go beyond traditional suppressor screens. Classical screening methods in plants are typically based on easy to detect phenotypes that allow high-throughput, such as screening for dwarf growth, agravitropism, reduced root growth, revertants and many others. To specifically test transcriptional induction, the discovery of reporter genes, such as those encoding for Green Fluorescent Protein (GFP) and its derivatives or firefly luciferase, allowed to screen for the molecular control without the necessity for an obvious visual phenotype. Despite their high sensitivity, staining based on the activity of beta-glucuronidase (GUS) is less suitable since it requires destruction of plant material for detection. Still, experimental procedures are needed to determine transcriptional upregulation or downregulation in the generated mutants.

We present a new screening method for the identification of novel DDR regulators based on the use of the *D-amino acid oxidase* 1 (*dao1*) gene of the yeast *Rhodotorular gracilis* (Pilone, 2000; Erikson et al., 2004). The *dao1* gene is normally not present in plants. Its protein product catalyzes the conversion of the D-amino acids to its corresponding imino acid. Since some of the D-amino (e.g. D-Ala and D-Ser) acids are toxic to plants unless converted and other D-amino acids are non-toxic (e.g. D-Ile and D-Val) until converted, the construct can be used for either positive or negative selection (Erikson et al., 2004) when introgressed under control of a strong promoter (Figure 1). However until now, the DAAO approach has only been used as a selection marker.

By putting the *dao1* gene under control of the *SMR7* promoter, we are able to detect mutants in the DDR. *SMR7* has a clear transcriptional induction during DNA-stress and upon treatment with D-Val together with BM, plants holding the *SMR7:Dao1* gene need to have an impaired checkpoint to prevent the conversion of D-Val to its toxic imino

acid. This concept was proven by crossing the *SMR7:Dao1* line in an *atm1* background, hereby impairing the DDR signaling. The use of HU and BM as the trigger to induce *SMR7* allows us to detect specific elements that react to the stress in two separate pathways. The screening system is designed for the discovery of transcriptional mutants, but in contrast to the use of any other reporter gene, requires little or no researcher effort besides sowing the plants.

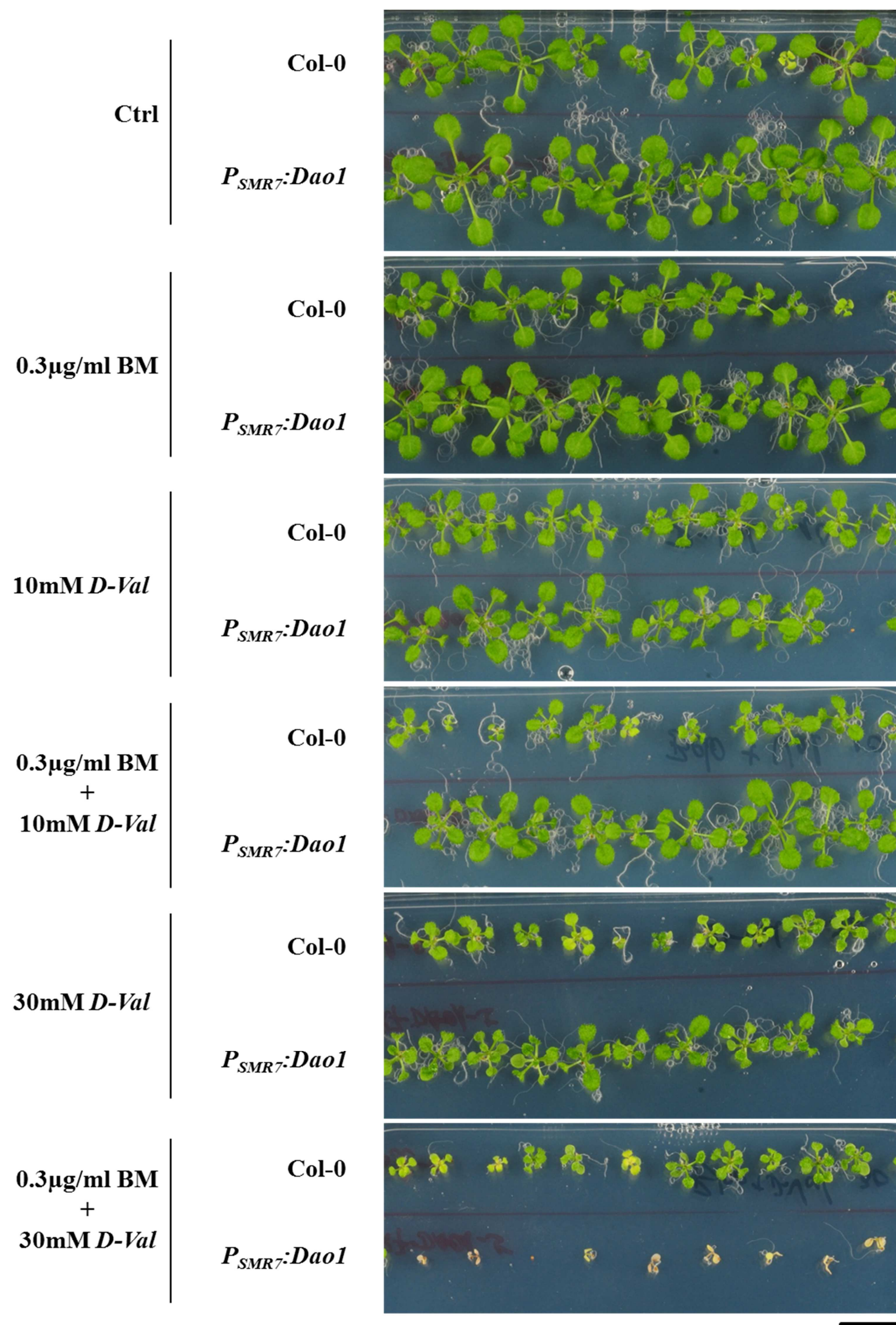

Figure 2. Optimization of *D-Val* Concentration

P_{SMR7}:*DAO1* transgenic plants were sowed on 10 mM or 30mM D-Val containing medium in the presence or absence of 0.3 μg/ml bleomycin. The scale bar is 1cm

RESULTS

Because the *dao1* gene is protected by a patent, the use of the material for research purposes was granted by the BASF Plant Science Company. Under this license agreement the vector VC-RLM208-1 and sequence was delivered to us, which allowed us to clone *dao1* gene in a pDONR221 entry vector. The full promoter sequence of *SMR7* (888bp until the next gene) was similarly cloned in a pDONR-P4P1R entry vector. By means of a multisite gateway experiment with a pK7m24GW;3 destination vector, the *dao1* was put under control of the promoter of *SMR7* and transformed in *Arabidopsis thaliana* ecotype Col-0. *SMR7* is a gene that is transcriptionally induced upon DNA damage and will consequently induce the *dao1* gene in this setting. The addition of D-Val or D-Ala will respectively kill or rescue the plant upon conversion to the corresponding imino acid.

In a prescreening experiment, growth conditions were established via a T3 generation of transformants with the construct. Based on the results shown by Erikson and colleagues(2004), two D-Val concentrations were tested in the presence or absence of 0.3 µg/ml bleomycin (Figure 2). Col-0 plants grown under the same conditions mimic plants that are unable to induce the *DAO1* gene upon DNA-stress conditions and that consequentially are potential positive screening targets. Since the growth without BM but in the presence of 30 mM D-Val gave good growth in control plants and was able to kill lines expressing *DAO1* upon BM treatment, this concentration was used as a starting point. At this moment, the best responding line was selected to use in subsequent screening experiments and for EMS mutagenesis. Hereafter, we lowered the BM concentration that could deliver a uniform response among plants, but that was able to stress them to a minimum. Ultimately, the screening conditions were selected to germinate plants on 30 mM D-Val in addition to 0.15 µg/mL BM, when screening for plants that lack the induction of *SMR7* during DNA-stress (Figure 3A). In addition, the use of HU also allows for a similar screen but one that reacts to replication stress. The ideal screening conditions were determined in an analogous manner and were set at 30 mM D-Val in the presence of 0.5 mM HU (Figure 3B). In both cases, the screening was highly dependent on the light regime, as better screening results in terms of survival and health were achieved under low light conditions. The use of HU and BM allows

discriminating whether the mutation is located in a shared part of the DNA damage pathway that reacts to both types of stress. In this way, we can catalog the different mutants and determine which lines will be further used for mapping, dependent on our interests.

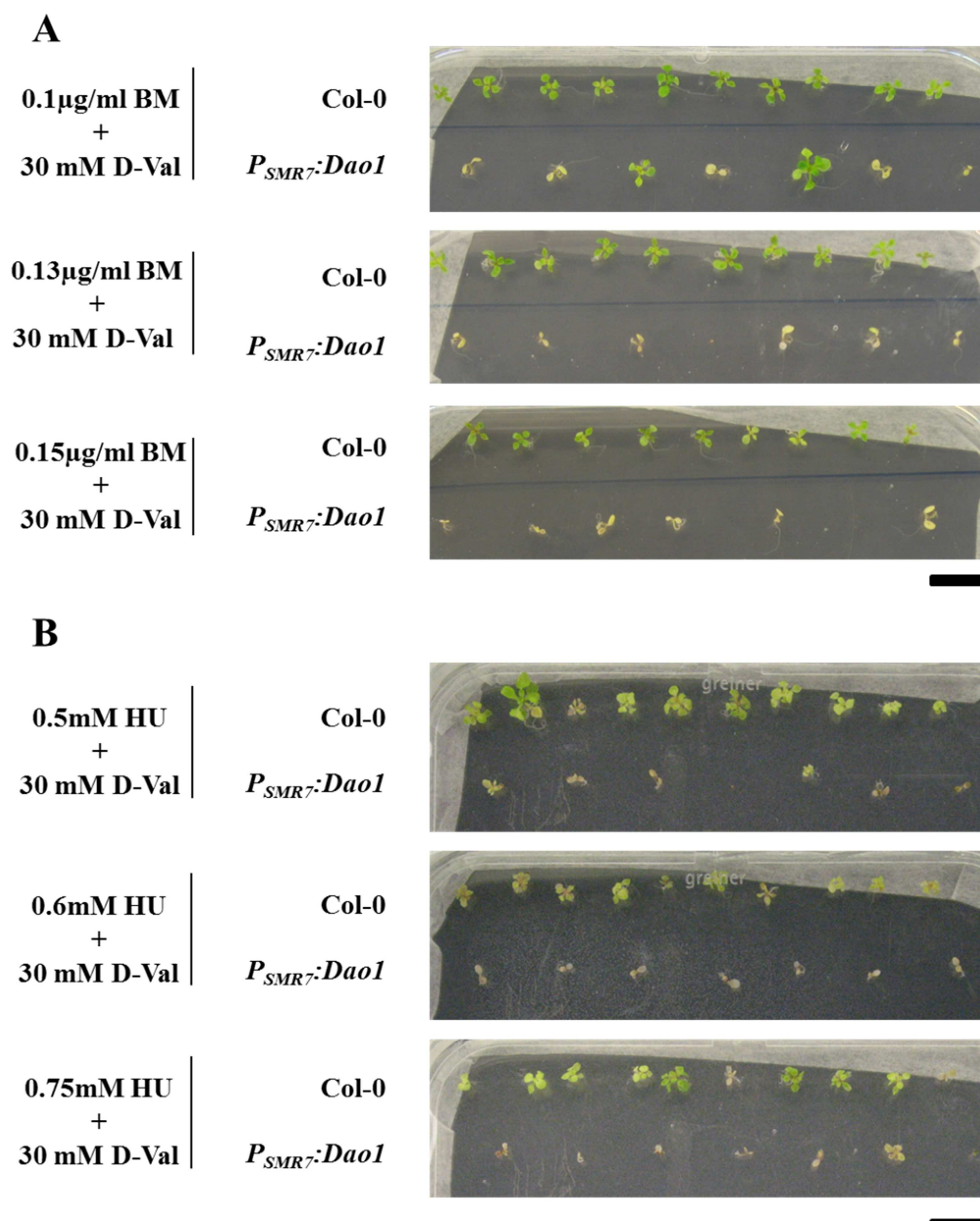

Figure 3. Optimization of BM Concentration

P_{SMR7}:DAO1 transgenic plants were sowed on 30mM D-Val medium in the presence of different concentrations of bleomycin **(A)** or HU **(B)**. The scale bar is 1cm

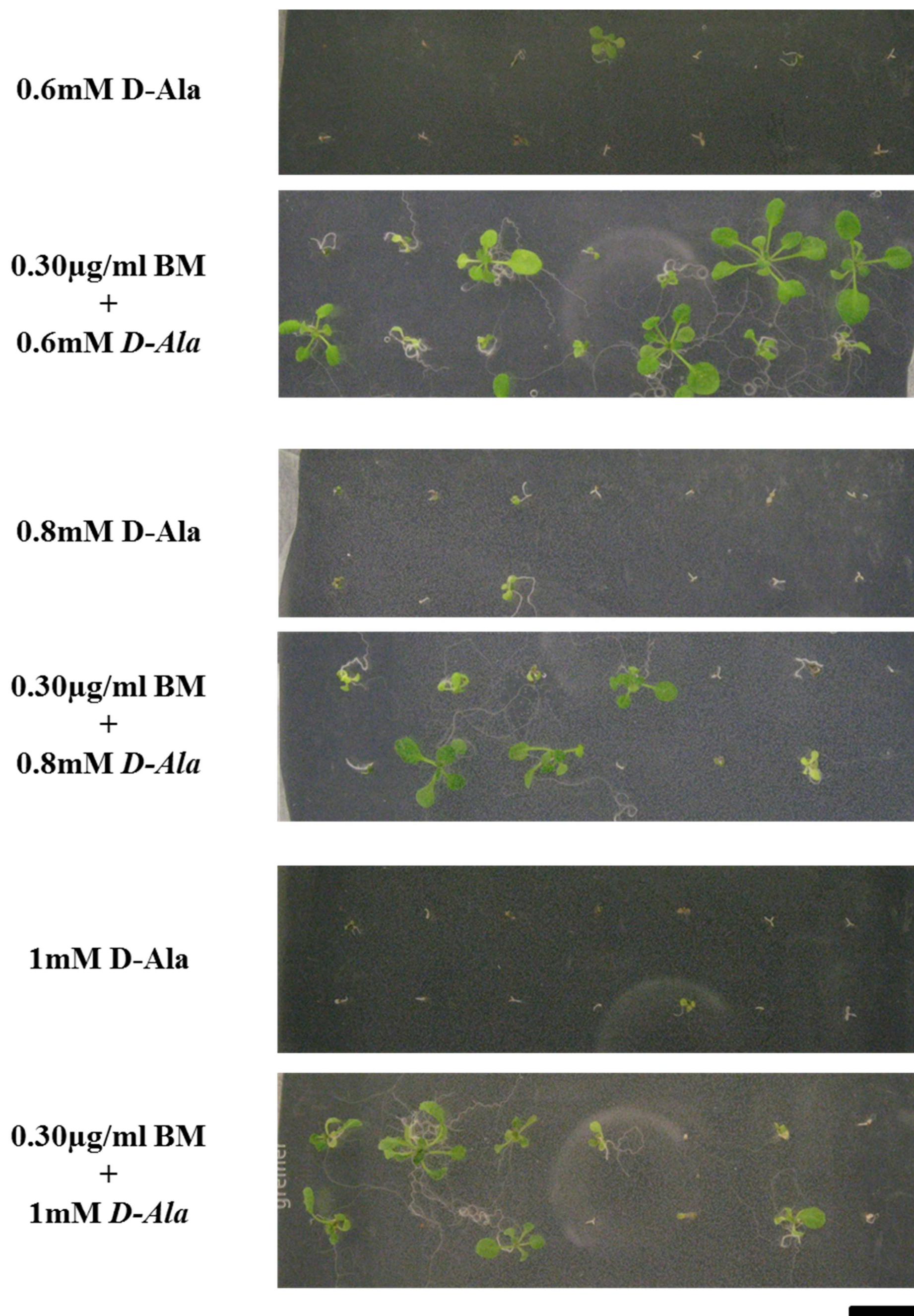

Figure 4. Transgenic line survive in *D-Ala*

WT plants as well as plants bearing the *DAO1* gene under control of the *SMR7* promoter died when grown on medium supplemented with 1mM D-Ala, In contrast, when the medium was also supplemented with 0.3μg/mL BM the transgenic plants survived since the *DAO1* gene was induced by the *SMR7* promoter. The scale bar is 1cm.

Besides screening for the lack of transcriptional induction, the use of an either/or system also allows to do the reverse and design screening conditions that will result in the detection of mutants that constitutively induce the gene of interest. Instead of using D-Val, a toxic D-type amino acid, D-Ala, was used to do the screening. Both 0.5mM and 1mM D-Ala were used to see which of them didn't gave rise to any survivors and consequently minimized the chance to pick up any false positives. Next, as 1mM D-Ala gave rise to a uniform death response of both WT plants and plants bearing the DAO1 gene under control of the SMR7 promoter, we used this concentration in combination with 0.3µg/mL BM (Figure 4). As expected, plants that contained the DAO1 gene were able to induce the gene through the *SMR7* promoter and consequently could convert D-Ala to its non-toxic imino acid, while all WT plants died. Although the response was not uniform, it shows that plants that are able to generate sufficient DAO1 transcript for whatever reason can cope with the stress brought from the toxic D-Ala. In addition, it also shows that despite continuous SMR7 induction with concomitant cell cycle inhibition, these mutant plants would still be able to grow at a reasonable pace and deliver offspring that can be used for mapping.

DISCUSSION AND PRINCIPLES

In this chapter, we presented the development of a new screening concept for transcriptional regulators of the *SMR7* gene based on the use of negative selection. Aside from a phenotypical screen, we managed to base plant survival of our mutagenized plant population on the lack or presence of transcriptional induction of the gene of interest. This appears to be a major advantage of using the *dao1* gene for screening purposes: time consumption for the researcher is drastically reduced except for selecting survivors for upscale. The use of the *SMR7* promoter shows that the method is sensitive enough to discriminate for weakly expressed genes. For stronger promoters, lower concentrations of D-Val could even be possible, since plants bearing the *dao1*-gene under control of the *CaMV 35S* promoter displayed a very clear phenotypic difference on 15mM D-Val (Erikson et al. 2004). Moreover, with the use of D-Ala we showed that a second screen is possible on the same seed stock, hereby doubling the discovery potential of the mutagenized seeds. In addition, it is likely that the two screens will target two distinct groups of the same pathway, since they will respectively look for the lack of and increased induction. This drastically reduces the overlap and consequently, finding the same elements in both screens.

When performing the *dao1*-screen, there are certain precautions that need to be taken into account. When performing a loss-of-induction screen with D-Val, contamination of the seed stock with seeds that do not bear the *dao1* gene will lead to false positives as they will not convert D-Val to its toxic imine. Therefore it is essential to confirm the transcriptional reduction of the endogenous gene by for instance RT-PCR or the presence of the construct in the subsequent generation. The latter can be done by growing the seeds on the appropriate selection marker or by PCR. Additionally, EMS mutagenesis or any other random mutagenesis method can result in the disruption of the *dao1* gene or controlling promoter. In this case, controlling by RT-PCR whether the endogenous gene is also affected by the mutation or sequencing the *dao1 gene* should allow to discriminate between a true and a false positive. A gain-of-induction screen with D-Ser/D-Ala does not suffer from these drawbacks since a functional *dao1* is the prerequisite for a successful screen. Still, growth on D-Ser/D-Ala cannot be used to

confirm the loss-of-induction screen in the next generation, since true positives are also unable to properly induce the gene.

We showed that the external application of certain types of drugs in concert with D-type amino acids is possible. However, take into account that established working concentrations are likely to be reduced, due to the toxicity of the D-type amino acids or converted forms. This is especially true when using agents that are detrimental for the organism.

In our screen based on the transcriptional control of *SMR7*, we expect to find several elements of the DNA damage pathway. Among these, new mutant alleles of *SOG1* and *ATM* are expected that might help to uncover essential residues for activity. In addition, members of the Mre11-Rad50-Nbs1 (MRN) complex that senses DSBs could be retrieved in our screen(Bakkenist and Kastan, 2003; Carson et al., 2003; Lee and Paull, 2004). However, it is likely that new elements might pop up as the large number of unknowns in DNA damage microarrays, like *SMR5* and *SMR7* until recently, hints at a more complex control of the checkpoint that is influenced by plant-specific elements.

In conclusion, we presented here a new screening method based on the use of the *dao1* gene. Since both positive and negative screens are possible with the use of the same line, we were able to drastically increase efficiency of an EMS mutagenesis screen. The coupling of a transcriptional response to the plant's fate provides a very easy-to-screen method with far exceeding possibilities.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana (L.) Heyhn. (ecotype Columbia) were grown under long-day conditions (16 h of light, 8 h of darkness) at 22°C on half-strength Murashige and Skoog (MS) germination medium(Murashige and Skoog, 1962). For bleomycin treatments, five-day-old seedlings were transferred into liquid MS medium supplemented with 0.3 µg/mL bleomycin.

Vector construction and plant transformation

SMR7 promoter sequence was amplified from genomic DNA by PCR. The product fragments were created with the Pfu DNA Polymerase Kit (Promega, Catalog #M7745), and were cloned into a pDONR P4-P1r (Chapter 2) (Karimi et al., 2002). *Dao1* gene sequence cloned from VC-RLM208-1 into pDONR221 vector by BP recombination cloning and subsequently transferred into the destination vector pm42GW7,3 by LR recombination cloning. All constructs were transferred into the *Agrobacterium tumefaciens* C58C1RifR strain harboring the pMP90 plasmid. The obtained *Agrobacterium* strains were used to generate stably transformed Arabidopsis lines with the floral dip transformation method (Yamamoto et al., 2002).

Selection analysis.

Transgenic T1 plants were selected on medium containing kanamycin (25 ug/ml). Lines containing a single T-DNA insertion locus were selected by statistical analysis of T-DNA segregation in the T2 population that germinated on kanamycin-containing medium. Plants with a single locus of inserted T-DNA were grown and self-fertilized. Homozygous T3 seed stocks were then identified by analyzing T-DNA segregation in T3 progenies. T1 seeds were surface-sterilized and sown in Petri plates that were sealed with gas-permeable tape. The growth medium was half strength Murashige and Skoog medium19 with 1% (wt/vol) sucrose and 0.8% (wt/vol) agar, plus 0.3 µg/mL BM combined with D-Alanine, or D-Val as the selective agent. Plants were grown for 14 d after germination with a 16 hrs photoperiod at 21 °C.

EMS mutagenesis

Root growth revertants were screened from an M2 population homozygous *P_{SMR7}:DAO1* transgenic line, mutagenized by ethyl metanesulfonate (EMS). For this, *PS_{MR7}:DAO1* transgenic seeds were added to 5 ml water and mutagenized by adding 50 µl EMS. Incubation in the EMS containing solution was performed for 12 hours on a rotating wheel. Seeds were washed in sodiumthiosulfate, dried and sown in 56 pools of each 250 seeds. M2 seeds were harvested from each pool and screened for restoration of root growth on vertical plates.

ACKNOWLEDGMENTS

This work was supported by Ghent University (Multidisciplinary Research Partnership “Bioinformatics: from nucleotides to networks”), and the Interuniversity Attraction Poles Programme (IUAP P7/29 "MARS"), initiated by the Belgian Science Policy Office. T.C. is a Postdoctoral Fellow of the Research Foundation-Flanders. D.Y. is indebted to the China Scholarship Council (CSC File No. 2009685045) for a predoctoral scholarship.

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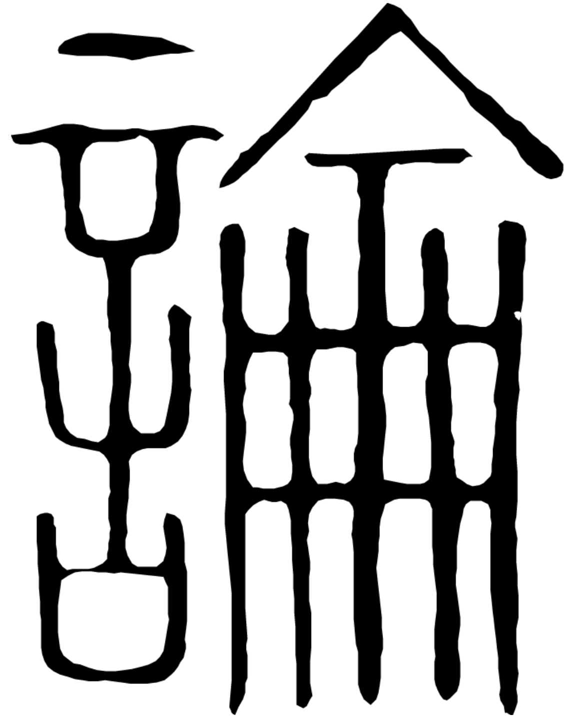

CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

DNA Damage response in plants

The DNA damage response (DDR) is of utmost importance to maintain genome integrity. ATM and ATR are two crucial components in the DNA damage response pathway. ATM and ATR respond to double strand and single strand DNA damage, respectively. In animals, these pathways have been described extensively. A crucial step in the response to DNA damage is the blocking of the cell cycle making time for the reparation of the DNA since proper DNA replication is needed to ensure the cellular functionality. However, in plants, the mechanisms that arrest the cell cycle upon genotoxic stress are still not completely understood. Up to now, only WEE1 has been well characterized as a cell cycle regulator, responding to single strand DNA breaks and replication stress (Cools and De Veylder, 2009; Cools et al., 2011). The *WEE1* gene is transcriptionally induced during the S-phase upon DNA stress. Thus the WEE1 protein specifically accumulates in S-phase cells encountering DNA-stress. This regulation is under control of both ATR and ATM (De Schutter et al., 2007). it has been seen that WEE1 also accumulates upon treatment with the double strand damage inducing agent bleomycin, but *WEE1*^{KO} does not show sensitivity towards the DSB agent bleomycin (Cools et al., 2011). These data suggests that there must be other pathways regulating cell cycle in response to DNA damage. The SOG1 protein is encoded by *SOG1 (suppressor of gamma response)* gene, which only exists in plants and is an important regulator in the DNA damage response. Up to now, SOG1 has been found to be under the control of ATM on both the transcriptional and post-transcriptional level (Ricaud et al., 2007; Yoshiyama et al., 2013). Being a transcription factor, SOG1 activates the expression of a large number of genes in response to DNA damage.

Since transcriptional regulation is an important part of DDR, the identification of other transcriptional regulators of DDR is an interesting question for future studies. We focused on the E2F transcription factors which are involved in the cell cycle regulation and synthesis of DNA in eukaryotes (Chapter 3). Subsequent studies revealed that *E2F*^{KO} line presents tolerance to DNA damage indicating that E2Fs have a role in the regulation of cellular response to DNA damage. But both E2F and SOG1 appear to be upstream regulators of DDR rather than a cell cycle regulator. It is supposed that one or several cell cycle regulators influence cell cycle process by directly interacting on

CDK/Cyclin complex under DNA damage stress. Based on this hypothesis, we tried to discover new elements and mechanisms involved in the plant DNA damage response. In our research, we chose a novel CKI family proteins SIM/SMRs as candidates of cell cycle checkpoints (Chapter 2). In the end, we clearly saw that SMR proteins were inhibiting cell division upon DNA damage agent HU and ROS conditions via strongly and rapidly transcriptional induction. And this regulation depends on the ATM-SOG1 pathway.

DNA damage and CKIs

Based on their evolutionary origin, structure and functional specificities, there are two groups of CKI in animals, namely INK4 and Cip/Kip and three groups of CKIs exist in plants, namely KRP, SIM/SMR and TIC (Chapter 1 and 2). As important components of the cell cycle checkpoints, CKI act on cell cycle onset to elicit a cell cycle arrest as part of the DDR in animals (Lim and Kaldis., 2013). Compared to CKIs in animals, little is known about their function in plants. Preceding our research, there was no report describing the mechanism of plant's CKIs in the DNA damage response pathway. ICK/KRP proteins are the first CKIs described in plants, but no obvious link between KRPs and DNA stress has been reported up to now (Cools and De Veylder, 2009). In chapter 2, we discussed SIM/SMRs functions in DNA damage response.

In our research, three SIM/SMR proteins (SMR5, SMR7 and SMR7) had been demonstrated to strongly respond to DNA damage at the transcriptional level (Chapter 2). Indeed, SMR5 participates in the control of cell division in leaf development upon DNA stress. This process is under the control of ATM-SOG1 pathway. More interestingly, SMR5 and SMR7 also respond to ROS induced DNA damage depending on SOG1. Thus, we can conclude that the transcriptional response of *SIM/SMR* genes link the oxidative stress with cell division activity upon DNA damage. From previous research, we know that SMR4, SMR5 and SMR7, only co-purified with A-type CDK and D-type cyclin, and the CYCD/CDKA;1 complex is responsible for the control of cell cycle onset in response to intrinsic and extrinsic signals (Lukaszewski and Blevins, 1996; Riou-Khamlichi et al., 2000). Which implied the mechanism of how they take part in the cell cycle regulation of DNA damage response. Thus, SIM/SMR proteins appear to be plant specific and are the first CKIs described in plant to control DDR.

To understand more about DNA damage response, we need to try to discover new components and mechanisms in the DNA damage response pathway. Ongoing mutagenesis screens could uncover these components. We constructed *pSMR7:DAO1* transgenic plants to screen the upstream regulators of SMRs. Currently, an EMS-based mutagenesis screen using these plants is in progress. The mutated genes will be identified through map-based cloning. Once a mutation is mapped, the nature of the gene cloned will help to define the pathways that control SMR abundance under genotoxic stress.

Another important aspect is to understand how SMRs proteins influences cell cycle upon DNA damage stress at the biochemical level. It is already know that SMR4, SMR5 and SMR7 interact with CDKA/CYCD complexes, but we still need additional information about the structure of SMR proteins. Identification of the interaction partners of these CDKs can reveal the mechanism on how cell cycle arrest adapted to the conditions of DNA damage stress.

DNA damage and E2Fs

The members E2F transcription factor family can be divided into typical and atypical subgroups based on sequence analysis, or classified as transcriptional activator and repressor based on their function in transcription regulation (Chapter 1). The different E2F family members exhibit redundancy but diverge in function in the cell cycle regulation (Magyar et al., 2005; Sozzani et al., 2006; Berckmans et al., 2011). Meanwhile, activity of E2Fs is under control of CDKA/CYCD complex via phosphorylation of the retinoblastoma-related (RBR) protein (de Jager et al., 2009). Whereas CDKA/CYCD complex is responsible for the control of cell cycle onset in response to stress signals. These components texture a complex regulating network (Chapter 1 and Chapter 3) that modulates cell cycle progression in plant growth or respond to changing environments.

Previous reports presented E2F3, which is the homologue of E2Fa, as a regulator of cell proliferation, cell cycle arrest and apoptosis through transcriptional control of a number of genes including other E2F family members like E2F1 in animal cells (Chen et al., 2012). Similarly, in Arabidopsis, E2Fs are controlling the expression of several target

genes involved in the DNA damage response (Christensen et al., 2005; Martinez et al., 2010),

In our results, we found that plants containing a non-functional *E2Fa* allele showed increased resistance to bleomycin (Chapter 3). This resistance was mainly observed in primary root length and cell proliferation in the root meristem. Furthermore, through transcriptome profiling experiments, we can see that E2Fa influences gene expression in response to DNA damage. These genes can be divided into two main groups: cell cycle related genes and DNA damage response genes (Chapter 3). Interestingly, from the transcirptome analysis of genes in *E2Fa*^{KO} plants, in absence of exogenously applied DNA stress, a number of DNA repair genes had an enhanced expression (Chapter 3). We supposed that the DDR is already activated at marginal levels in the *E2Fa*^{KO} plants. Subsequently, basal transcriptional activation of DNA repair genes granted these plants the potential to deal with genotoxic stress induced by BM better than wild type plants do. Moreover, the *E2Fa/E2Fb* double knockout mutants display a root meristem proliferation arrest. In the double KO mutant, expression of the genes that are under transcriptional control of E2Fs (e.g. the *MCM* family) is strongly reduced compared to the *E2Fa*^{KO}, which causes more spontaneous genotoxic and DDR, already affecting plant growth in the absence of external applied DNA stress (Chapter 2).

By combining these results, we can see how E2Fa takes part in the meristem cell proliferation regulation in different ways. On one hand, E2F transcription factors are suggested to regulate DNA damage pathway by affecting modulating the transcription of genes involved cell cycle regulation. On the other hand, changes in the expression of the cell cycle and the DNA replication genes in the plants absence of E2Fs resulted in spontaneous stimulation of checkpoint activation, which can enhance the tolerance of DNA damage and also can affect plant growth in overreaction.

Up to now, based on the conclusions we got, the main goals in the future should be to determine the E2F transcription factors influencing DNA damage response components in detail. Since E2Fs are important regulators of the cell cycle, we should focus on the cell cycle and DNA replication genes which are under control of E2F transcription factors. On the meanwhile, we already know that endogenous DNA damage response is triggered on E2F depleted plants to block the cell cycle onset. What mechanism induces this compensation will be an interesting question for future studies.

In plants, except E2Fs family, there are several important families of transcription factors involved in cell cycle regulation, such as MYB and WRKY. Interestingly, an increase in the levels of transcripts of MYB and WRKY transcription factors was also detected upon bleomycin plus mitomycin C treatment (Chen et al., 2003). For instance, the *PARP1* promoter region presents two MYB binding motif sequences (AACGG). Moreover, in Arabidopsis CycB1;1 expression is also under control of the binding of MYB protein to the *cis*-acting element of its promoter (Planchais et al., 2002). Also the *Rad51* promoter is containing W-box (TTGACc/t) of WRKY. The presence of MYB or WRKY binding motifs in the DNA damage response genes and the cell cycle regulation genes promoter regions suggests these transcription factors might have an important function in the DNA damage response.

ROS and DNA damage response

However, during organism growth and development the genome is continuously exposed to DNA damaging agents. These agents originate from the environment or biological processes. ROS is an important DNA damage agent, which can cause different kinds of DNA damage including SSB and DSB (Dizdaroglu et al., 2002; Roldán-Arjona and Ariza, 2009). Plants need to absorb nutrients from soil directly, which means they are easily stressed by heavy metal ions which are broadly existing on earth (Jomova and Valko, 2011; Rymen and Sugimoto, 2012). The metal ions including iron, copper, cobalt and aluminum induce DNA damage via the activity of ROS (Chapter 1). And as we know, antineoplastic chemical Hydroxyurea (HU) is a ribonucleotide reductase inhibitor which can block the cell cycle (Lopes et al., 2001, Sogo et al., 2002 and Timson, 1975). Besides this known function, HU can reduce catalase activity to trigger oxidative stress (Chapter 2). Bleomycin acts by induction of DNA breaks depending on oxygen and metal ions (primarily iron) (Burger et al., 1981; Favaudon, 1982). From Chapter 3, we can see the iron transport genes participating in the DDR induced by the bleomycin, and this process is regulated by E2F transcription factors. In plants, ROS are also generated by photosynthesis and aerobic respiration (Asada, 2006). From these results, we can suppose ROS is a crucial agent for DNA damage generated from different kinds of stresses.

Oxidative stress is a widespread stress in plants. ROS is already known to cause a cell cycle arrest by the ATM-SOG1 DNA damage response pathway (Chapter 2). It would be very interesting to obtain more details about the interaction between the DNA damage response and ROS signaling. and how they link to cell cycle regulation, DNA repair stimulation and programmed cell death. From our research, we suppose that SMR5 is the molecular switch of cell division under ROS induced genotoxicity (Chapter 2). However, it would be interesting to see whether cell division blockage is directly triggered by oxidative stress or if the DNA damage induced by ROS in leaf cells pushes them out of the cell cycle and consequently reduces cell proliferation.

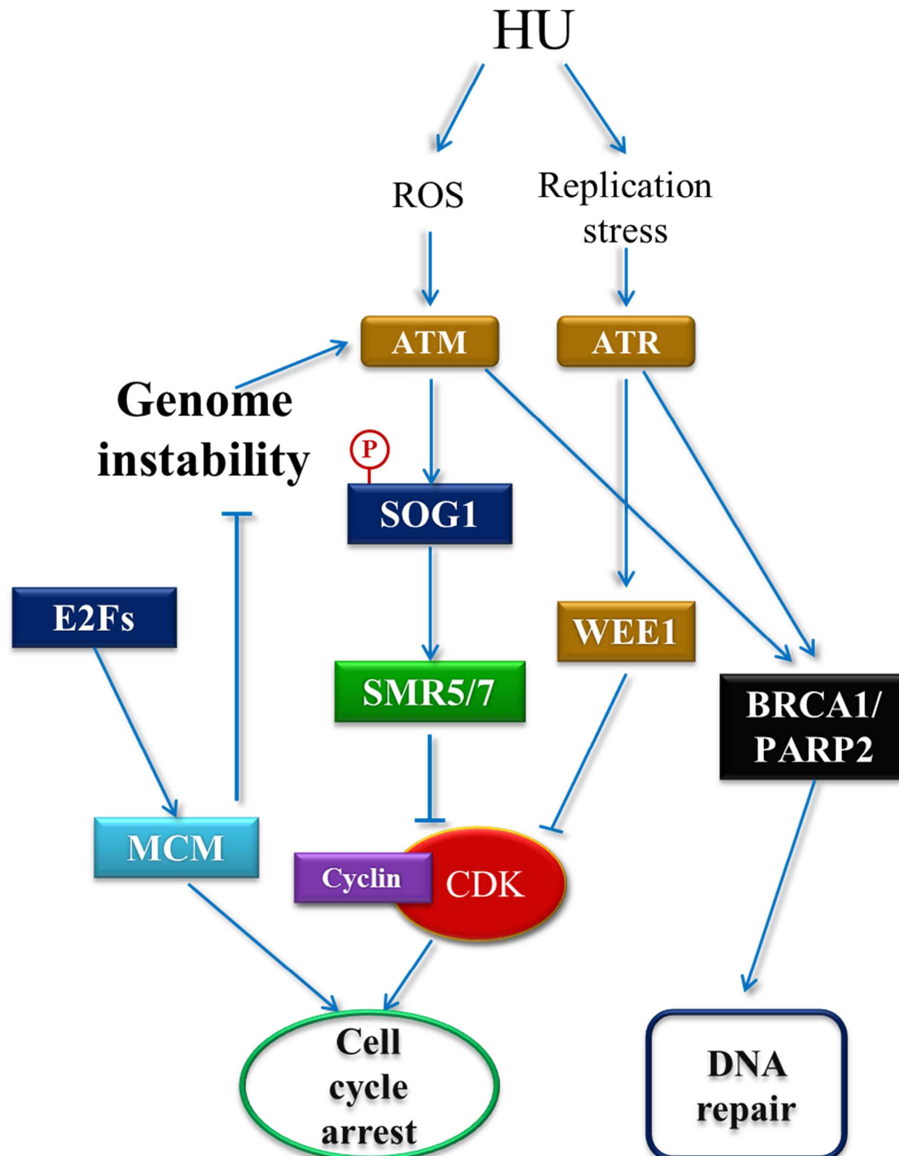

Figure 1. Current model of cell cycle regulation in DNA damage response.

CDK inhibitor protein SMR5 and SMR7 were inhibiting cell division upon DNA damage agent HU and ROS conditions via strongly and rapidly transcriptional induction. And this regulation depends on the ATM-SOG1 pathway. Meanwhile, E2F transcription factors are involved in the cell cycle regulation and synthesis of DNA in eukaryotes and $E2F^{KO}$ line presents tolerance to DNA damage due to DDR is already activated at marginal levels in the $E2Fa^{KO}$ plants via reduction of E2F target genes proteins suck as MCMs.

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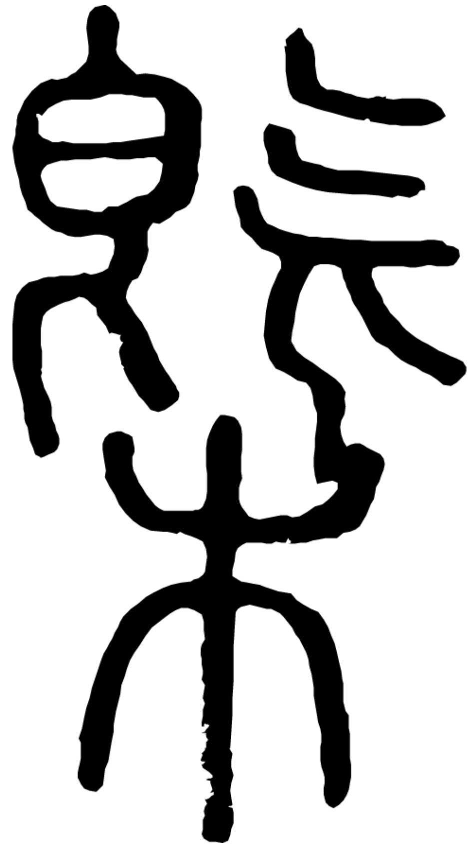


CHAPTER 6

SUMMARY

Summary

Genetic damage can be catastrophic when cells progress to the next cell cycle phase before the previous phase is properly completed. To minimize the occurrence of such mistakes during cell cycle events, the cell cycle progression is monitored at several checkpoints. Control mechanisms operating at these checkpoints ensure the intactness of chromosomal DNA and the completion of each cell cycle stage before the following stage is initiated. For instance, the DNA damage checkpoint blocks progression through the cell cycle until the damage is repaired. The existence of cell cycle checkpoints isn't apparent in normal unperturbed cell cycle **(Chapter 1**).

Recently, a new group of genes, nominated as *SIAMESE/SIAMESE-RELATED* (*SIM/SMR*), have been identified. These genes encode small proteins that inhibit the cell cycle through their direct interaction with cyclin-dependent kinases (CDK). In addition, they are strongly and rapidly transcriptionally induced by different stress conditions. In **Chapter 2**, we can see three *SIM/SMR* family members (*SMR4*, *SMR5*, and *SMR7*) respond specifically towards genotoxic stress, suggesting that they control the cell cycle checkpoint upon the occurrence of DNA stress. SMR5 and SMR7 control cell division in response to ROS-induced DNA damage. Their expression depends on ATAXIA TELANGIECTASIA MUTATED (ATM) and SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1), rather than on the anticipated replication stress-activated ATM AND RAD3-RELATED (ATR) kinase. We conclude that the identified SMR genes are part of a signaling cascade inducing a cell cycle checkpoint in response to ROS-induced DNA damage.

In **Chapter 3**, we focus on the E2F transcription factors, which are important elements to control the cell cycle. We have shown that E2F transcription factors are required for the cell proliferation arrest upon DNA damage. Under genotoxic stress, E2Fa affected expression of a number of DDR genes which are mostly involved in cell cycle regulation and stress response. We already know E2Fa and E2Fb are homologous, and they present partial overlapping/redundant functions **(Chapter 1 and 3)**. However, we detected that E2Faoccurs specific roles in the DNA damage induced cell cycle regulation. We detected that DDR genes were up regulated in *E2F*^{KO} lines. Such kind basal transcriptional activation of DNA repair genes endue *E2Fa*^{KO} plants the potential to tolerant BM stress rather than wild type plants do.

In **Chapter 4**, we develop a protocol to search for the transcriptional level regulators of the SMR7 promoter causing inducible *D-amino acid oxidase 1* (*Dao1*) gene, which was discovered from yeast . Expressing the Dao1 in the presence of D-amino (e.g. D-Ala and D-Ser) acids is toxic to plants, but save plants from the other D-amino acids (e.g. D-Ile and D-Val). We construct P_{SMR7}*:DAO1* transgenic lines. Using DNA damage agents like BM and HU to stimulate expression of Dao1 under control of *SMR7* promoter can be used as a screening system to discover transcriptional mutants in DNA damage response. This system can also be used to characterize other target genes or pathways.

Acknowledgments

Acknowledgments

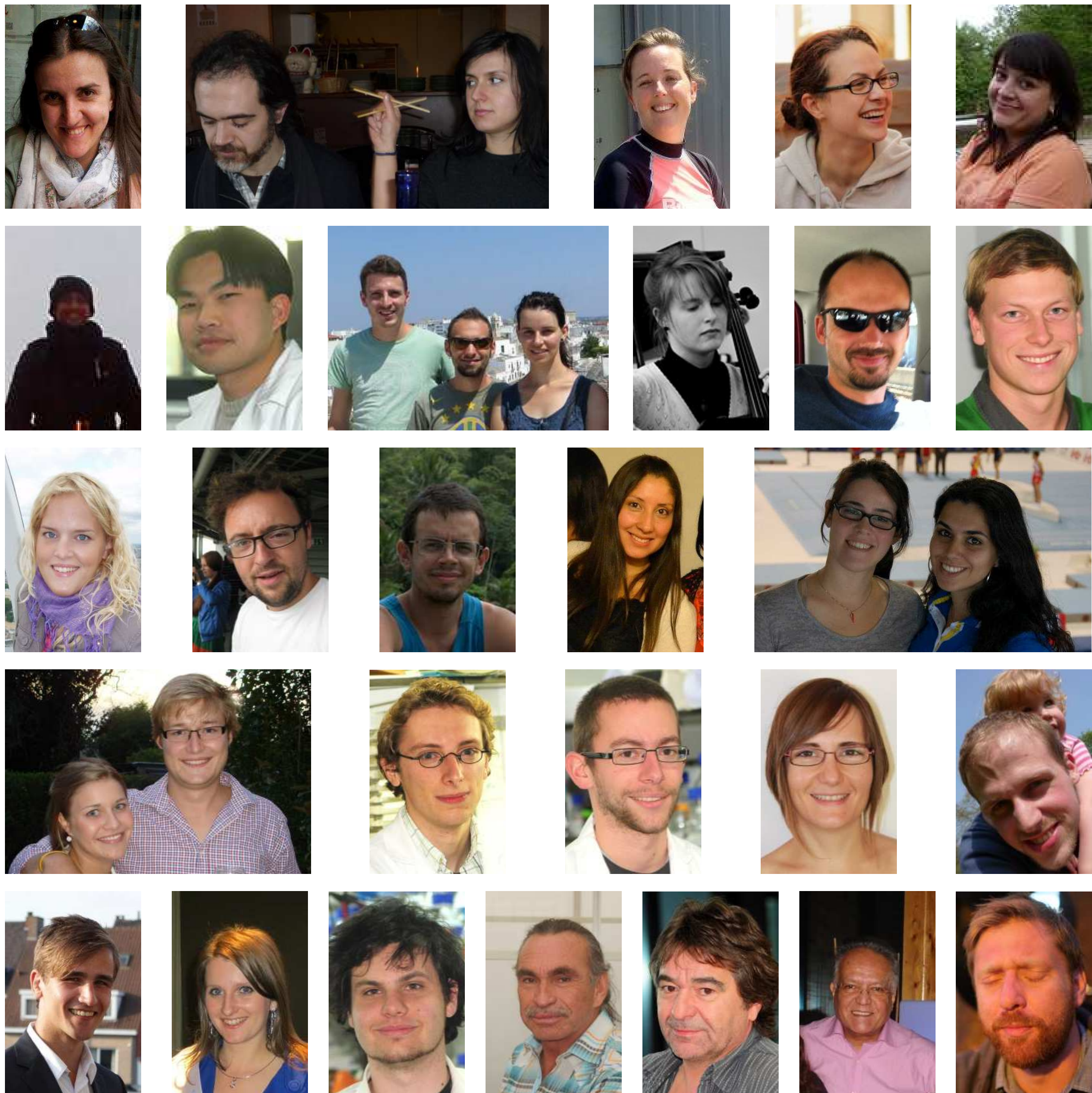
I thank the China Scholarship council for supporting my PhD grant.

Actually, this is the most difficult part to write for my dissertation, because I cannot express my gratitude fully in my poor English. During the course of my PhD there were so many people affecting the life that I went through.

Prof. Dr. **Lieven De Veylder**, promotor of my PhD, is the first I would like to thank. Lieven allowed me to start my PhD in his lab and always guided me and gave me the necessary corrections to grow as a researcher. I am so proud of working in this amazing lab. You are the best and most important promotor of my life.

Looking back these four years, I realize that time is running so fast. I came from a Far East country alone and struggled in the begining with communicating to all colleagues, butthis

condition changed quickly. I still remember **Claire, Barbara, Mauricio, Jefri, Toon, Amandine, Sandy, Pooneh, Marie, Veronique, Lieven, Sara, Hilde and Ilse** came to me and gave me a warm welcome, showed me around and guided me during the start of my PhD. They also helped me after work by going to drink amazing Belgian beers, eating spare rips and showing me this beautiful city. Most of all they were also good friends that helped me in gaining back my self confidence after some difficult first months. Furthermore, I want to thank you for the assistance and fun you gave me during my stay in the lab.

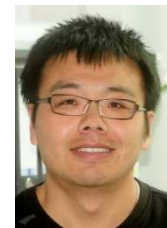


After several months I got more friends: **Mara** (Belgian and Greek)**, Aleksandra and Fernando, Mandy, Dora, Lorena, Sinta, Long, Brecht, Igor and Petra, Dorien, Geert,**

Wannes, Lisa (Swedish version)**, Ward, Paula, Pedro, Juliana, Nathália, Alexandra and Sander, Hannes** (Both of them)**, Nathalie, Gert, Nathan**, **Eveline, Lorin,** and many many more friends will stay in my memory forever. Thanks for all the fun and impressive moments during my work and life. Also thanks to **Wilson, Rudy, Gert, Nino, Kristof, Jacky, Nico, Carine, Tim, Diane, Christine and Sophie** for all technical and administrative help.

I also thank **Stefanie** (squirrel girl in our lab)**, Alex, Fabi, Thomas, Jefri, Marc, Toon, Mandy, Sinta**, who can tolerate my poorly written English and help me to perfect my manuscripts. Without you my friends, I cannot finish my amazing thesis.

During my writing phase, **Alex, Youn-Jeong, Stefanie, Luiz, Fabi, Livia, Lisa** (Belgian version), gave me so much happiness and encouragement, which I will never forget...



Our Chinese gang: **宣伟 (Xuan, We)**, **老丁 (Ding, Zhaojun)**, **田姐(Tian, Huiyu)**, **张静 (Zhang, Jing)**, **何莹 (He, Ying)**, **岳坤 (Yue, Kun)**, **王峰 (Wang, Feng)** , **陈谦 (Chen, Qian)**, **晓欢 (Sun, Xiaohuan)**, **邓磊 (Deng, Lei)**, **李臻 (Li, Zhen)**, **高震(Gao, Zhen)**, **晓光 (Yu, Xiaoguang)**, **中华 (Zhang, Zhonghua)**, **筑兵 (Hu, Zhubing)**, **罗昱 (Luo, Yu)**, **晓剑 (Xia,Xiaojian)**, **凌香 (Jiang, Lingxiang)**, **陈栩 (Chen, Xu)**, **朱强(Zhu, Qiang)**, **红姜 (Li,Hongjiang)**, **刘洋 (Liu, Yang)**, **龙哥 (Du,Yunlong)**, **晶晶 (Huang Jingjing)**, **小崔(Cui, Guangling),** all of you gave me the feeling of home, helping me not to feel alone.

Also many thanks toProf. Dr. **Dirk Inzé,** Prof. Dr. **Tom Beeckman,** Prof. Dr. **Frank Van Breusegem,** Prof. Dr. **Moritz Nowack,** Prof. Dr. **Geert De Jaeger**, and the other Pis, for helping me during my PhD research.

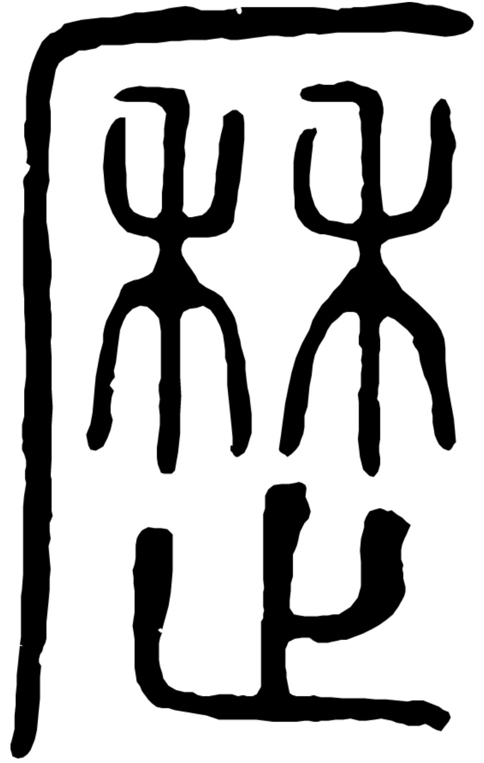
In addition I also want to thank my jury members: **Prof. Dr. Sofie Goormachtig (chair), Prof. Dr. Crisanto Gutierrez, Prof. Dr. Geert Berx, Prof. Dr. Tom Beeckman, Prof. Dr. Moritz Nowack, Prof. Dr. Alain Goossens and Dr. Marie Huysman** for giving me many useful remarks.

Thanks very much to my parents for always supporting me and giving me the opportunity to study abroad! Love you very much!

Finally I want to thank all the people that helped or affected me somehow during my PhD lifeIapologize that I cannot list all of them or maybe forgot to mention.

As last I would like to thank my papa, mama, Erlong grandma for all the support!

My dear colleages, family and friends, from my heart and soul, I thank all of you and I will never forget this amazing time.



CURRICULUM VITAE

Curriculum Vitae

Personal details

Name	**Dalong Yi**
Nationality	**CHINA**
Gender	**Male**
Date of birth	**November 19, 1982**
Place of Birth	**Chengde, Hebei Province**
Address for correspondence:	**Stalhof 49, G532** **Gent, 9000, Belgium**
Telephone:	**+32485045044**
Email:	**dalyi@psb.ugent.be**

Education

2009.12-2013.2

PhD fellowship, Doctor of Science: Biochemistry and Biotechnology, VIB-Plant Systems Biology (PSB), Universiteit Gent

Subject: IDENTIFICATION OF SIGNALING COMPONENTS THAT ACTIVATE THE DNA DAMAGE CELL CYCLE CHECKPOINT IN PLANTS

Promoter: Lieven De Veylder

2005.9-2009.5

M.S, major in Plant Biochemistry & Molecular Biology, College of Life Sciences, Nanjing Agricultural University, P.R.China,

Promoter: Zhengqiang Ma

2000.9-2004.6

B.S, major in Biotechnology, College of Life Sciences, Hebei University, P.R.China,

Research Interests

1. DNA damage response;
2. Regulation mechanism of cell cycle;

Recent Publications

Dalong Yi, Claire Lessa Alvim Kamei, Toon Cools, Sandy Vanderauwera, Naoki Takahashi, Yoko Okushima, Thomas Eekhout, Kaoru O. Yoshiyama, John Larkin, Hilde Van den Daele, Anne Britt, Masaaki Umeda, and Lieven De Veylder. (2013) The Arabidopsis thaliana SIAMESE-RELATED cyclin-dependent kinase inhibitors SMR5 and SMR7 control the DNA damage checkpoint in response to reactive oxygen species. The Plant Cell online.

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Personal Interests

I am well in Tabletennis. I also have great interests in music such as Hard Rock and play guitar well.