



Functional characterization of the DNA Polymerase epsilon and its involvement in the maintenance of genome integrity in Arabidopsis

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Par

M. José Antonio Pedroza-Garcia

**Functional characterization of the DNA Polymerase epsilon and its
involvement in the maintenance of genome integrity in *Arabidopsis***

Thèse présentée et soutenue à Orsay, le 22 septembre 2016 :

Composition du Jury :

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Résumé de la thèse en Français :

Contrairement aux animaux, les plantes ont un développement largement post-embryonnaire et forment continuellement de nouveaux organes et tissus grâce à l'activité de leurs méristèmes. Ces massifs de cellules indifférenciées conservent la capacité à se diviser tout au long de la vie de la plante, et c'est également à partir du méristème caulinaire que se forment les gamètes. Chaque cycle de division peut être la source de mutations, suite par exemple à des erreurs de réplication. De plus, les méristèmes sont relativement exposés aux stress environnementaux qui peuvent également endommager l'ADN des cellules. Les mécanismes impliqués dans la détection des lésions de l'ADN ou des défauts de réplication et l'arrêt de la prolifération cellulaire en réponse à ces dommages jouent donc un rôle fondamental dans le maintien de la stabilité du génome, aussi bien au cours du développement végétatif que lors de la reproduction sexuée.

Le développement des plantes repose sur l'activité de cellules souches présentes au sein des méristèmes qui conservent leur capacité proliférative tout au long de leur vie. De plus, contrairement à ce qui est observé chez les Animaux, la lignée germinale n'est pas individualisée à une étape précoce du développement mais se forme à partir des méristèmes de manière relativement tardive. Ce mode de développement pose la question des mécanismes particuliers qui assurent le maintien de l'intégrité du génome au fil des divisions cellulaires chez les plantes. En effet, les différentes étapes du cycle cellulaire sont la source de lésions de l'ADN, par exemple lors d'erreur de réplication, ou de défaut de ségrégation des chromatides sœurs pendant la mitose. L'objectif de ce travail de thèse était donc de mieux comprendre ces processus en utilisant la plante modèle *Arabidopsis thaliana*, et en étudiant plus particulièrement les mécanismes mis en jeu pendant la phase S (au cours de laquelle a lieu la réplication de l'ADN) pour assurer la duplication fidèle de l'information génétique.

L'une des protéines clés impliquées dans la réplication de l'ADN nucléaire chez tous les eucaryotes est l'ADN Polymérase ϵ (Pol ϵ) : elle assure la synthèse du brin précoce pendant la réplication, mais est également impliquée dans la perception du stress réplicatif et l'activation de la réponse cellulaire. Elle est constituée de quatre sous-unités conservées chez tous les eucaryotes: une sous-unité catalytique (Pol2A) et trois sous-unités accessoires (DPB2, 3 et 4). L'étude détaillée de sa fonction est cependant rendue difficile chez beaucoup d'organismes par le fait que son inactivation est létale. Dans ce travail, nous avons utilisé des approches de génétique pour étudier le rôle de l'ADN Pol ϵ d'*Arabidopsis* au cours de la progression du cycle cellulaire et dans la réponse au stress réplicatif et aux lésions de l'ADN

Au cours de ce travail de thèse nous avons caractérisé la fonction des sous-unités POL2A and DPB2 et leur interaction avec les voies de réponses aux lésions de l'ADN. Les gènes *POL2A* et *DPB2* sont des

gènes essentiels, mais des mutants faibles pour la sous-unité POL2A existent. Afin de caractériser ces protéines, nous avons généré des sur-expresssurs de la protéine DPB2 et comparé leurs phénotypes à celui des mutants partiellement déficients pour la sous-unité catalytique.

Les sur-expresssurs de DPB2 présentent une forte réduction de croissance, un cycle cellulaire et notamment une phase S très allongée et une activation constitutive des gènes de réponse aux lésions de l'ADN. Des analyses génétiques nous ont permis d'établir que cette activation est largement dépendante de la protéine kinase ATR qui est connue pour son rôle dans signalisation activée par le stress répliatif. Cependant, les sur-expresssurs de DPB2 présentent également une formation spontanée de cassures double-brin dans les cellules des méristèmes, ce qui active l'activation de la kinase ATM, spécialisée dans la détection de ce type de lésions. L'activation de cette voie confère aux plantes une tolérance accrue aux dommages de l'ADN. Notre modèle de travail est que la suraccumulation de DPB2 déstabilise le réplisome (structure multiprotéique qui assure la répliation de l'ADN), ce qui conduirait à des défauts de progression de la fourche de répliation et à une activation de la réponse au stress répliatif.

Les mutants partiellement déficients pour la sous-unité catalytique POL2A présentent des défauts similaires aux sur-expresssurs de DPB2 (croissance réduite, activation de la réponse aux lésions de l'ADN, tolérance au stress répliatif). La viabilité de ces mutants dépend strictement de la kinase ATR. Cependant ces plantes sont hypersensibles aux agents induisant des cassures double-brin de l'ADN, et notre analyse génétique révèle que ce phénotype est probablement induit par une interférence entre les voies de signalisation ATR et ATM dépendantes. Enfin, en utilisant une approche de RNAi, nous avons pu montrer que la sous-unité POL2A elle-même est nécessaire à la perception du stress répliatif.

Nous avons ainsi pu montrer que la sous-unité catalytique du complexe Pol ϵ ainsi que sa principale sous-unité accessoire DPB2 sont essentielles à la détection des défauts de répliation, et fonctionnent en amont de la kinase ATR pour induire l'arrêt du cycle cellulaire et activer les voies de réparation au cours du développement végétatif. En outre, nous avons découvert un nouveau point de contrôle activé lors de la phase de répliation pré-méiotique qui permet l'activation d'une mort cellulaire programmée en réponse à des défauts survenus pendant cette phase, grâce au facteur de transcription SOG1.

Enfin, ces travaux ont mis en évidence l'existence d'un nouveau point de contrôle activé par le stress répliatif, dépendant de la Pol ϵ et médié par le facteur de transcription SOG1, un intégrateur central de la réponse aux lésions de l'ADN. L'ensemble de ces résultats fait l'objet d'une publication parue dans la revue *Nucleic Acids Research*, et d'une seconde soumise pour publication.

Tous les stress biotiques ou abiotiques auxquels la plante est soumise pouvant conduire à la formation de lésions au niveau de l'ADN, nos résultats ouvrent des perspectives de recherche pour comprendre la réponse des plantes aux stress environnementaux. En outre, la disponibilité de mutants viables pour différents facteurs impliqués dans la réplication ou la réponse aux lésions de l'ADN nous a permis d'explorer chez un eucaryote pluricellulaire des mécanismes qui sont pour l'instant essentiellement décrits chez la levure, et ainsi d'acquérir des connaissances qui pourront être transférées aux systèmes animaux et notamment à l'Homme.

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ABBREVIATIONS

ABA	Absciscic Acid
APC	Anaphase Promoting Complex/Cyclosome
ATM	Ataxia Telangiectasia Mutated
ATR	ATM and Rad3 related
BER	Base Excision Repair
BIR	Break-Induced Replication
CDK	Cyclin Dependent Kinase
CDK/cyclin	Cyclin Dependent Kinase/cyclin complexes
CKI	CDK inhibitors
ChIP	Chromatin Immunoprecipitation
CHAC	CHRoatin Accessibility Complex
CMG complex	(CDC45, MCM, GINS)
C-terminal	Carboxyl-terminal
CYC	Cyclin
KDa	KiloDalton
DDK	DBF4-dependent kinase

DNA	Deoxyribonucleic acid
DDR	DNA damage Response
DSB	Double-Strand Break
dNTP	deoxynucleoside triphosphate
FPC	Fork Protection Complex
G1 and G2	Gap Phases
G2/M	G2/M transition
γ H2AX	Histone Variant H2X
HR	Homologous Recombination
HU	Hydroxy-urea
KRP	KIP-related Proteins)
MAPK	Mitogen-Activated Protein Kinase
MMR	Mismatch Repair
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End-Joining
M-Phase	Mitosis-Phase
N-terminal	Amino Terminal
ORC	Origin Replication Complex
Pol	Polymerase

PCNA	Proliferating Cell Nuclear Antigen
pre-LC	pre-Loading Complex
Pre-RC	pre-Replication Complex
QC	Quiescent Centre
RBR	RetinoBlastoma Related
Rb	Retinoblastoma
RFC	Replication factor C
RNA	Ribonucleic Acid
RNR	Ribonucleotide Reductase
RPA	Replication Protein A
ROS	Reactive Oxygen Species
S-Phase	Synthesis-Phase
SSBs	Single-Strand DNA Breaks
ssDNA	single-stranded DNA
TAP	Tandem Affinity Purification
TLS	Translesion Synthesis

INTRODUCTION

At variance with other eukaryotes, plant development is largely post-embryonic, and relies on the proliferative activity of meristematic cells that can form new organs and tissues throughout the life cycle of the plant. Because each round of division can lead to the generation of mutations due to replication errors or to the transmission of mutations caused by exogenous stress, this developmental programme raises intriguing questions regarding the mechanisms that allow safeguarding the genetic information. This question is relevant not only in developing organs, but also in the context of reproduction. Indeed, germ cells form relatively late in the life cycle of the plant, from meristematic cells that have undergone many rounds of cell division. Both the shoot meristem and the root meristem contain a pool of slowly dividing cells at their centre: these cells undergo much fewer rounds of division than the rapidly dividing cells that surround them and form new organs or tissues; they therefore have a reduced probability of accumulating replication errors or mutations. Quiescent centre cells may thus function as a reservoir of genetic information (Heyman et al., 2014). However, specific mechanisms are also at work in proliferating cells to safeguard genome integrity.

During my PhD, I studied the role of the replicative DNA Polymerase ϵ in the maintenance of genome integrity in *Arabidopsis*. In the introduction, I will therefore describe our current knowledge on plant cell cycle regulation, with a particular emphasis on the control of DNA replication as well as the mechanisms involved in DNA Damage Response. Because the cellular processes described here are largely conserved between eukaryotes, some sections will be dedicated to the state of the art in yeast or animal systems, and I will discuss the common features and specificities of mechanisms at work in plant cells.

I-Cell cycle regulation in plants

Even though the pace of cell division varies between regions of the meristem and in developing organs, the basic mechanisms that govern its progression are largely similar in all dividing plant cells, and more generally conserved in all eukaryotes. Here we will focus mainly on our current knowledge on plant cell cycle regulation. This section of the introduction is part of a book chapter entitled “Plant Cell Cycle Transitions” (Molecular Cell Biology of the Growth and Differentiation of Plant Cells Edited by Ray J. Rose CRC Press 2016). The full version of this book chapter is available in the Appendix section of the manuscript.

The cell cycle is divided in four phases. The two main phases are replication (S-phase; for synthesis) and the segregation of replicated DNA between the two daughter cells (M-phase). These two phases are separated by the so-called gap phases (G1 and G2 respectively) during which the cell prepares for the next step, and checkpoints can be activated, for instance if DNA damage occurs, in order to delay cell cycle progression until lesions are repaired. Over 40 years ago, Cyclin Dependent Kinase (CDK)-cyclin complexes were identified as the universal motors of cell cycle regulation in all eukaryotes. CDKs are protein kinases that phosphorylate various substrates to promote transitions from one phase to the next. Their activity is modulated by their association with the regulatory sub-units called cyclins that take their name from the fact that their accumulation fluctuates during the cell cycle (Cools and De Veylder, 2009).

Basic mechanisms regulating cell cycle progression, DNA replication and mitosis are conserved in all eukaryotes including plants. This high degree of conservation allowed fast improvement of our understanding of cell cycle regulation. For example, the first plant CDK was isolated by functional complementation of a yeast mutant with an Alfalfa cDNA (Hirt et al., 1991), and considerable progress has been made in the last 35 years in our understanding of plant cell cycle transitions.

Even though core mechanisms regulating the cell cycle are highly conserved, the plant cell cycle has a number of specificities. One obvious difference concerns plant mitosis that is characterized by

the absence of centrosomes and mechanisms governing cytokinesis. Another hallmark of the plant cell cycle is the relatively frequent occurrence of endoreduplication, a particular type of cell cycle consisting of several rounds of DNA replication without mitosis, and leading to an increase in cell ploidy. Although this process can be found in animals, it is generally restricted to relatively specific cell types such as the salivary glands in *Drosophila* and hepatocytes in mammalian (Fox and Duronio, 2013). By contrast in plants, it is widely distributed in various organs such as fruits, , endosperm in cereals or even leaves in plants such as *Arabidopsis* (Fox and Duronio, 2013).

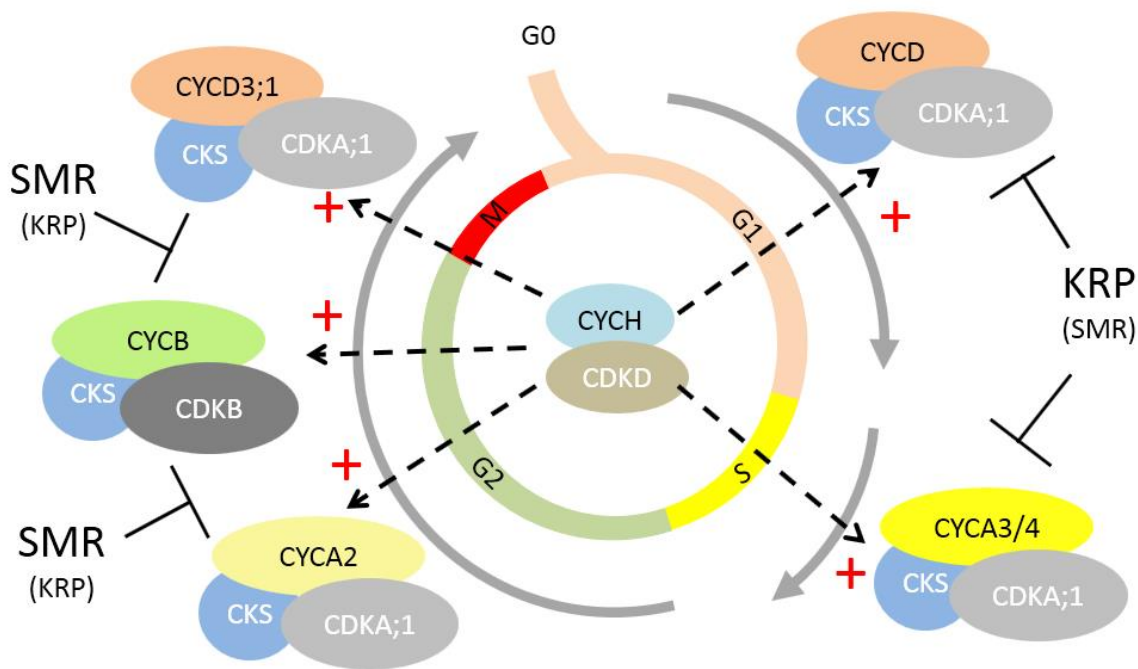


Figure 1. Succession of CDK/Cyclin complexes during the cell cycle (adapted from Van Leene et al, 2010). CYCD/CDKA, CYCA/CDKA and CYCB/CDKB sequentially accumulate and are activated to allow progression through the various phases of the cell cycle. CKS sub-units are scaffolding proteins associated with all complexes. Likewise, all CDK/Cyclin complexes are activated by the CYCH/CDKD kinase

A-Plant CDKs and Cyclins, motors of cell cycle progression with an intriguing diversity

One feature of plants is the surprisingly high diversity of core cell cycle regulators encompassed by their genomes. Indeed, the *Arabidopsis* genome encodes 5 CDKs distributed in two sub-classes (a single A-type CDK and four B-type CDKs) and 31 Cyclins belonging to three families (10 CycA, 11 CycB and 10 CycD), whereas *Saccharomyces cerevisiae* has a single CDK and 9 Cyclins, and *Homo sapiens* has 4 CDKs and 9 Cyclins (Van Leene et al., 2010). The number of putative CDK/Cyclin pairs is thus very large in plants, making the elucidation of their role problematic. One important step forward in the understanding of how plant CDK/Cyclin complexes control cell cycle transitions has been the comprehensive analysis of their expression in synchronized cell suspensions (Menges et al., 2005).

These results led to a global picture of CDK/cyclin complexes around the cell cycle (Figure 1). According to these study, CDKA;1 is expressed throughout the cell cycle and stably associates with D-type cyclins and S-phase expressed A-type cyclins as well as with CYCD3;1 in G2/M, suggesting it could be involved in the control of the G1/S as well as the G2/M transition. Consistently, expression of a dominant negative form of CDKA;1 drastically inhibits cell proliferation (Gaamouche et al., 2010). Likewise, CDKB2s are required for normal cell cycle progression and meristem organisation (Andersen et al., 2008). More recently, analysis of *cdka* and *cdkb* knock-out mutants revealed that CDKA;1 is required for S-phase entry, while it redundantly controls the G2/M transition with B-type CDKs (Nowack et al., 2012).

Very schematically, D-type Cyclins are thought to control cell cycle onset whereas A-type cyclins would be involved at later stages during the S and G2-phases in complex with CDKA1;1 or CDKBs and B-type cyclins bound to CDKBs would control the G2 and M phases (Figure 1, (Van Leene et al., 2010)). However, Cyclin D3;1 has the particularity of peaking both at the G1/S and at the G2/M transition (Menges et al., 2005).

Globally, results available so far suggest that a lot of redundancy exists between closely related cyclins. However, the potential role of specific cyclins in response to stress or changes in external conditions have to date little been explored, and could shed light on the physiological role of such a diversity of CDK/cyclin complexes.

Multiple mechanisms acting at the post-translational level modulate CDK/Cyclin activity. The WEE1 protein kinase can inhibit CDKs by phosphorylating them on Tyr15 and Thr14 (Berry and Gould, 1996). This phosphorylation plays an important role in the control of the G2/M transition in eukaryotes and functions to avoid premature division of cells that have not sufficiently expanded as well as to delay mitosis after DNA damage. However, in Arabidopsis, the WEE1 kinase seems to be predominantly involved in DNA stress response and not in growth regulation under normal conditions (De Schutter et al., 2007; Cools et al., 2011). Finally, CDK/cyclin complexes can be inhibited by binding of small proteins called CDK inhibitors (or CKI). In plants they are distributed between two unrelated families: the KRP (for KIP-related Proteins) that share homology with the human cell cycle inhibitor p27 and the SMR (for SMR related) (Van Leene et al., 2010). Like CDKs and cyclins, these inhibitors are extremely diverse: the Arabidopsis genome encompasses 7 KRPs and 14 SMRs. KRPs (also called ICKs for Inhibitors of Cyclin-dependant Kinases) were the first identified plant cell cycle inhibitors (Wang et al., 1998). They associate preferentially with CYCD or CYCA/CDKA;1 complexes (Van Leene et al., 2010). The respective roles of the various KRPs remain to be elucidated. SIAMESE (SIM), the founding member of the SMR family, also appears to positively regulate endoreduplication: *sim* mutants display multicellular trichomes, indicating that the SIM protein is required not only to promote endoreduplication but also to inhibit cell proliferation (Churchman et al., 2006). SIM-RELATED proteins (SMRs) have been proposed to play a role in cell cycle arrest during stress response (Peres et al., 2007). Consistently, SMR5 and SMR7 are involved in cell cycle arrest caused by reactive oxygen species, for example during high light stress (Yi et al., 2014), and contribute to the growth reduction caused by chloroplasts

dysfunction (Hudik et al., 2014). SMR5 and SMR7 are involved in cell cycle arrest in the G2/M interphase (Figure 1).

B-Control of the G1/S transition: the E2F/RBR pathway

As previously described, CYCD/CDKA complexes the first CDK/Cyclin complexes activated for cell cycle onset. Consistently, expression of a number of CYCD responds to external cues (see below). In all eukaryotes, CYCD/CDKA complexes promote the G1/S transition by phosphorylating the Retinoblastoma (Rb) protein and alleviating its inhibitory action on E2F transcription factors that can in turn activate genes involved in DNA replication (Berckmans and De Veylder, 2009) (Figure 2). This pathway is conserved in plants, and the Arabidopsis genome encompasses a single Rb homologue (RBR, RetinoBlastoma Related) and six E2Fs (Lammens et al., 2009).

Plant E2F transcription factors can be divided in two sub-groups: canonical E2Fs (E2Fa, b and c) require a Dimerization Partner (DP) to efficiently bind DNA, whereas atypical E2Fs (E2Fd, e and f) function as monomers. Plant E2Fs also differ by their function in cell cycle regulation, E2Fa and b being activators of the cell cycle whereas E2Fc behaves as a negative regulator (Berckmans and De Veylder, 2009).

Upon RBR release, activating E2Fs stimulate the expression of genes required for DNA replication, including the ones encoding the pre-replication complex (pre-RC). Assembly of the pre-RC on replication origin and DNA replication licensing are key steps to the regulation on the G1/S transition. ORC (origin replication complex) proteins bind to replication origins and recruits CDC6 and CDT1 that in turn allow binding of MCM proteins that function as helicases to open the replication fork (DePamphilis, 2003). All these factors are conserved in Arabidopsis, and interactions between the various constituents of the pre-RC have been observed in the yeast two-hybrid system (Shultz et al., 2007).

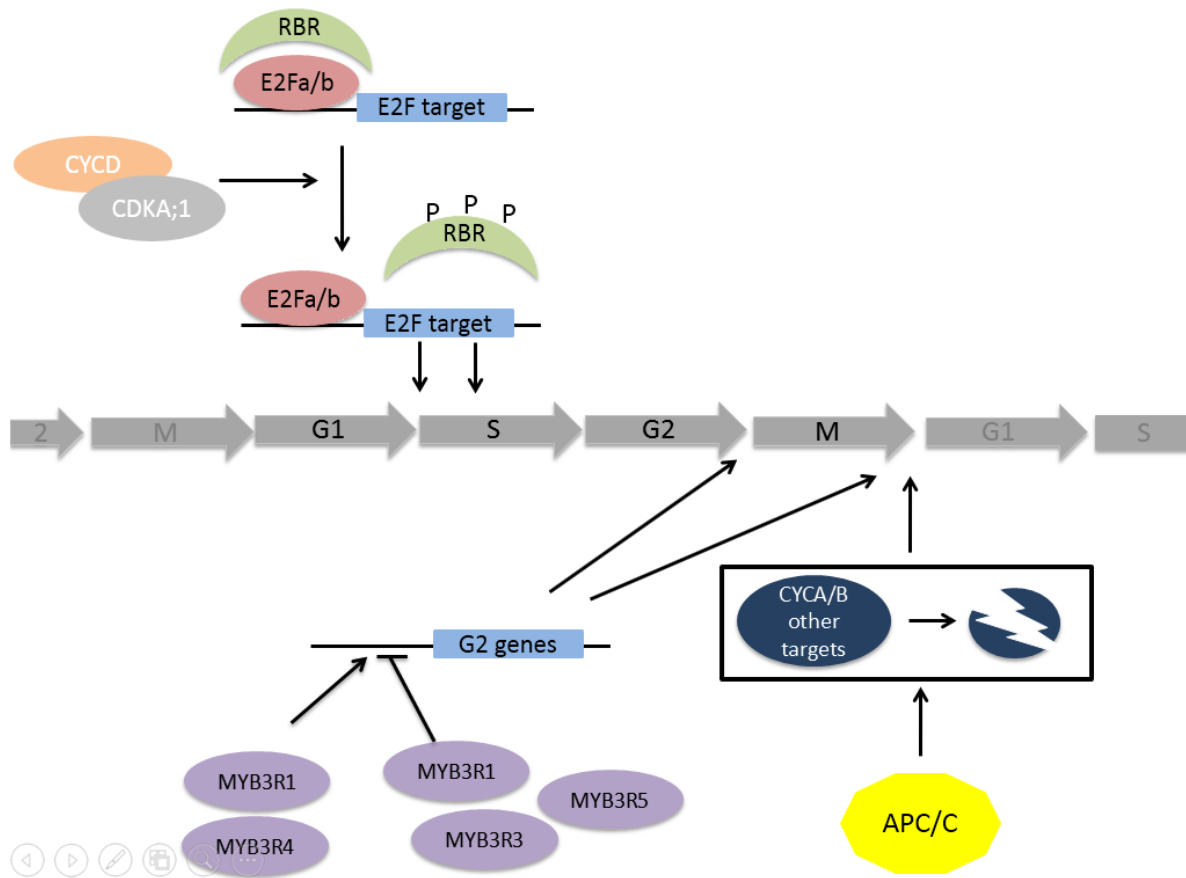


Figure 2. Regulation of cell cycle transitions. Activation of CYCD/CDKA complexes leads to phosphorylation of RBR and release of its inhibitory action on E2F factors thereby allowing expression of S-phase genes. G2 and M genes are under the control of MYB3R transcription factors. Activation of the APC/C is required to degrade various targets and allow exit from mitosis

C-Regulation of G2 and mitosis

Many genes expressed during the G2 and M phases harbour a specific regulatory sequence in their promoter called MSA (mitosis-specific activator) (Ito et al., 1998; Menges et al., 2005) that is recognized by MYB3R transcription factors (Haga et al., 2011). Mutants deficient for MYB3R1 and 4 display a drastically reduced stature due to aberrant cytokinesis activate the expression of G2/M specific genes such as *KNOLLE* to allow proper cytokinesis (Haga et al., 2011).

In addition to the transcriptional regulation of G2/M gene expression, targeted protein degradation plays a pivotal role for progression through mitosis. The Anaphase Promoting Complex/Cyclosome is a highly conserved E3-ubiquitin ligase that specifically targets cell cycle regulators towards proteolysis (Heyman and De Veylder, 2012), that was named for its role in the degradation of the mitosis inhibitor securin (Vodermaier, 2004). This complex comprises 11 sub-units (APC1-11, (Van Leene et al., 2010)), some of which are constitutively expressed while others accumulate specifically during G2 and M (Heyman and De Veylder, 2012) (Figure 2).

D-Cell Cycle regulation in response to stress

In addition to the programmed changes in cell proliferation associated with normal plant development, the ability to modulate the cell cycle in response to stress is a key parameter for the ability to cope with changing environmental conditions and to adjust their body plan accordingly. As a general rule, stress induces cell differentiation, possibly to avoid the transmission of induced mutations to the progeny of the cells (Cools and De Veylder, 2009). However, *CYCB1;1* has the particularity of being induced by genotoxic stress, and has been proposed to function to block some cells in G2, thereby allowing to preserve some proliferative potential until conditions become favourable again (Cools and De Veylder, 2009). In the root of *Arabidopsis*, replenishment of the meristem after initial cell death is achieved by stimulating the division of quiescent centre (QC) cells that are probably less vulnerable to stress because of their low division rate: when plants are transferred from a medium containing DNA damaging agents back to normal growth medium, the

ERF115 transcription factor that is a positive regulator of QC cell division is activating, thereby allowing the replacement of cells that have undergone programmed cell death (Heyman et al., 2013). Yet another mechanism has been described in rice where the RSS1 protein is required to maintain the proliferative capacity of meristematic cells during salt stress (Ogawa et al., 2011), but this factor is not conserved in eudicots. In parallel, stress also induces premature cell differentiation in growing organs. In leaves, drought activates gibberellin signalling and thus stabilization of DELLA proteins that in turn activate the atypical E2F factor E2Fe thereby stimulating the expression of CCS52A and triggering early endoreduplication (Claeys et al., 2012). High light stress also promotes early cell differentiation by activating the expression of the cell cycle inhibitors SMR5 and SMR7 (Hudik et al., 2014; Yi et al., 2014). Likewise, DNA damage causes early differentiation of root meristematic cells (Cools et al., 2011). The analysis of cell cycle progression in response to stress is still in its infancy, but there is also accumulating evidence that biotic stresses also impinge on cell cycle regulation (Reitz et al., 2015), opening exciting new research prospects.

As described above, initiation of DNA replication is one of the key steps of cell cycle regulation because it commits one cell towards division, but can also be the initial step of a differentiation programme. In the next section, I will therefore summarize the key regulatory steps in the control of DNA replication in yeast and animals. Indeed, most of the knowledge on eukaryotic DNA replication has been acquired in yeast and animal cells, and the described mechanisms are generally assumed to function in a similar fashion in plants, even though little biochemical evidence is available to fully support this view.

II-DNA replication

DNA replication during S-phase results in the duplication of the entire genome, which needs to be faithful to avoid problems in gene expression, chromatid cohesion and maintenance of epigenetic features (Costas et al., 2011). DNA replication is a mechanism that involves three steps: initiation, elongation, and termination: the initial steps of DNA replication are depicted on Figure 3. The sequence of events allowing the initiation of DNA replication is so well described now that it has recently been reconstructed *in vitro* (Yeeles et al., 2015). In this section and throughout the introduction, we will use the appropriate nomenclature for the different eukaryotic organisms we refer to: yeast protein names are spelled in lowercase letters starting with a capital letter (ex: Dpb2), whereas names used in animals are spelled in capital letters (ex: DPB2). For the sake of clarity, when the same protein has a specific name in one model, the name of its homolog in other organisms will be systematically added in brackets.

A-Regulation of replication initiation

Genome duplication in dividing plant cells has the same requirements and constraints as in animal cells (Sanchez Mde et al., 2012). Thus, the initiation step is strongly regulated because it must occur once and only once per cell cycle. Replication initiation can be divided in two temporally separated steps: the origins first need to be licensed and subsequently to be activated. Thus, initiation of DNA replication in eukaryotes depends on the assembly of pre-replication complex (pre-RC) on many sites of the genome known as replication origins. Pre-RC formation occurs during the late G1-phase of the cell cycle and is called origin licensing; its further activation to initiate DNA replication marks the onset of S-phase and is known as the origin firing process (Hills and Diffley, 2014).

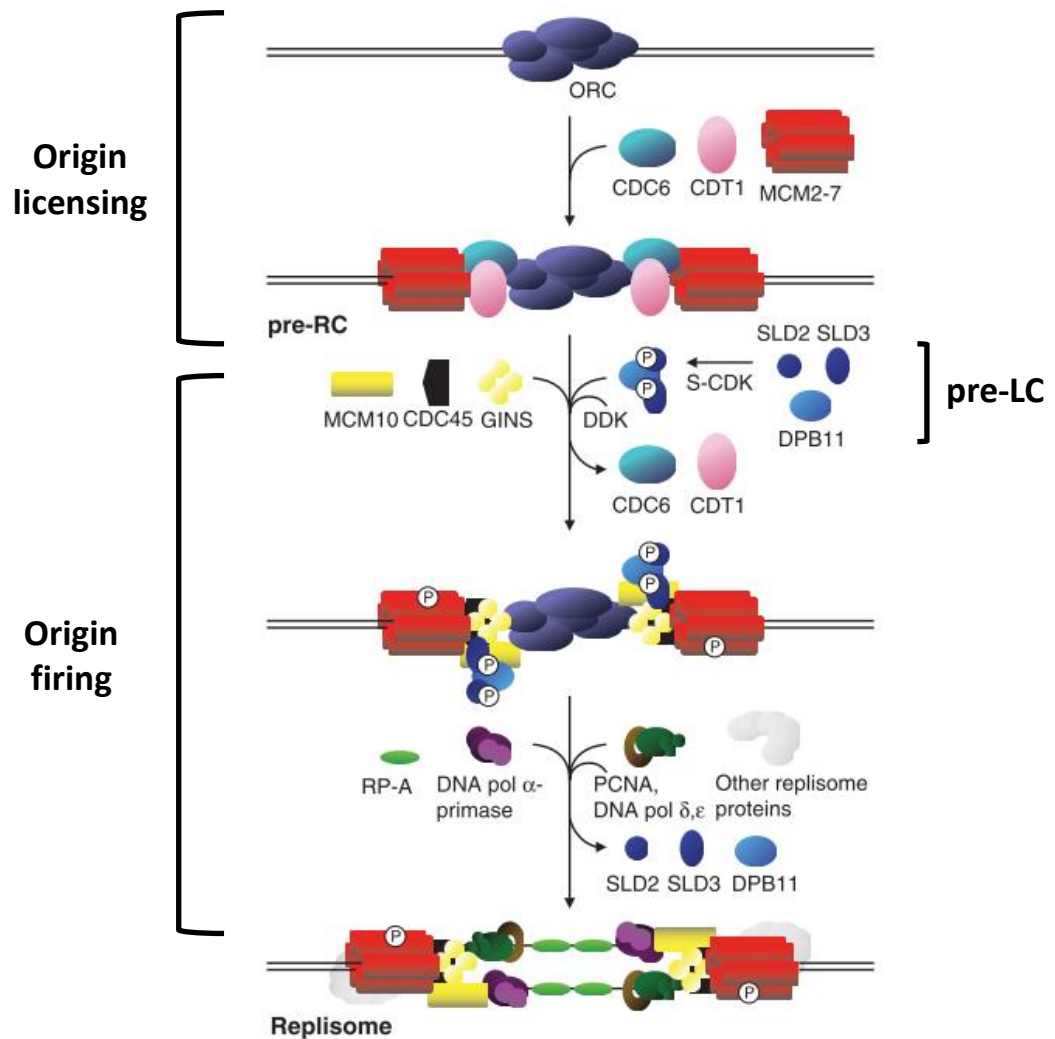


Figure 3. Initiation of DNA replication in eukaryotes (Taken and adapted from Bryant and Aves, 2011). Replication origins are marked by the ORC complex. During the licensing process, ORCs recruit CDT1 and CDC6 that in turn allow chromatin loading of MCM hexamers. Origin firing next involves MCM10, CDC45 and GINS loading, as well as association with the pre-LC complex (that contains SLD2, SLD3 and DPB11) that depends on CDK and DDK kinase activities. CDC6 and CDT1 dissociate from chromatin at this step. Finally, other components of the replisome such as DNA Polymerases or RPA and loading, and replication is initiated bi-directionally.

Origin licensing

In eukaryotic cells, replication initiates at multiple origins, each one of which needs to assemble a replication apparatus that will completely replicate its portion of the chromosome with high fidelity (Karnani and Dutta, 2011). In *S. cerevisiae*, the Origin Recognition Complex (ORC) recognizes a consensus sequence of 11 nucleotides. However, *S. cerevisiae* appears to be an exception among eukaryotes because no DNA sequence specifying replication origins could be identified in other organisms. Nevertheless, in *Schizosaccharomyces pombe*, replication origins are associated with enriched sequences in adenine and thymine, suggesting that enrichment in these bases allows an easy unwinding of DNA favourable for replication initiation (Costas et al., 2011).

The number of replication origins varies between organisms, cell types and/or the physiological state. In *Arabidopsis thaliana*, replication origins were recently identified using massive sequencing of short-pulse BrdU-labelled DNA and complementary studies of ORC1 and CDC6 binding regions, by chromatin immunoprecipitation and microarray experiments (ChIP-chip). These two proteins are part of the machinery involved in the recognition of replication origins (see below). The analysis of these data led the identification of ~1500 putative replication origins across the *Arabidopsis* genome (Costas et al., 2011).

In all eukaryotes, the Origin Recognition Complex (ORC) that allows assembly of the pre-replication complex (pre-RC) at the end of the G1 phase, marks replication origins. As described above, expression of pre-RC components is cell cycle regulated via the E2F/Rb pathway. The licensing process specifies which replication origins may be used during the S-phase; it takes place only at the end of mitosis and during the G1 phase and involves the sequential assembly of pre-RC members onto the replication origins (Xouri et al., 2007). The first pre-RC component that associates with the potential origins of replication is ORC that acts as a scaffold for the recruitment of CDC6 and CDT1 that in turn allows binding of MCM proteins that function as helicases to open the replication fork (Sanchez Mde et al., 2012; DePamphilis, 2003). This is the final step in order to

lead to a licensed state of the replication origins. As soon as MCM proteins are loaded onto the replication origins, CDC6 and CDT1 are no longer required to maintain MCMs at these origins at least in *in vitro* assays (Yeeles 2015).

Licensing of replication origins must be tightly controlled so that it occurs once and only once per cell cycle in order to avoid incomplete DNA replication or re-replication of fractions of the genome (Xouri et al., 2007). Therefore, the cell has developed different strategies to control licensing. First, the assembly of the pre-RC is cell cycle regulated not only at the transcriptional, but also at the post-transcriptional level: it depends on activation and inactivation of CDKs, and assembly of the pre-RC can only occur in short time window during the low CDK activity period from late mitosis through G1 phase. Thus, inappropriate re-assembly is suppressed during S, G2, and M phases (Fujita, 2006). In addition, CDKs inhibit origin licensing by phosphorylating several members of the pre-RC. Phosphorylation of CDC6 may cause its nuclear export, while phosphorylation of ORC2 inhibits its chromatin binding and phosphorylation of ORC1 and CDT1 targets them for ubiquitin-mediated degradation (Hills and Diffley, 2014). Additionally, CDT1 is degraded through replication-coupled PCNA-mediated ubiquitination. Moreover, in Animals, from S-phase to mitosis, an inhibitor of CDT1 called geminin is present: CDT1 is sequestered by geminin, preventing its chromatin binding, and thus restraining its activity to the G1 phase (Xouri et al., 2007). Finally, origin licensing is also differentially regulated between early and late-firing origins: early replicating regions corresponding mainly to euchromatin while heterochromatin is replicated at the end of the S-phase (Hayashi et al., 2013; Bass et al., 2014).

All the factors of pre-RC are conserved in Arabidopsis, and interactions between the various constituents of the pre-RC have been observed in the yeast two-hybrid system (Shultz et al., 2007). In addition, there is genetic evidence that the function of CDC6, CDT1, MCM2 and MCM7 in DNA replication is conserved in plants (Springer et al., 2000; Castellano et al., 2001, 2004; Ni et al., 2009; Domenichini et al., 2012). Although these regulatory mechanisms are very well described

in Animals, it is much less clear how they function in plants. CDT1 that is the target of many regulatory pathways in animals also appears to be regulated by proteolysis in plants (Castellano et al., 2004), but whether *bona fide* homologs of Geminin exist in plants remains to be clarified (Caro et al., 2007; Caro and Gutierrez, 2007).

During pre-RC assembly, the MCM2-7 replicative helicase is loaded onto double stranded DNA as an inactive double hexamer. Subsequent helicase activation is required to initiate DNA replication in S-phase, this mechanism is also known as origin firing (Zegerman, 2013). This process is tightly controlled to prevent multiple firing from a single origin that would lead to duplication of portions of the genome.

Origin firing

In human cells, hundreds of thousands of MCM2-7 complexes are loaded onto DNA in form of head-to-head double hexamers and 30-50 thousand of these are activated per human cell during DNA replication. Thus, only a small proportion of chromatin loaded MCM2-7 complexes are activated during S-phase, while the remaining serve as dormant origins, which are activated only in the case of replication stress to bypass replication defects occurring at a neighbouring fork (Blow et al., 2011).

At the G1/S transition, origin firing begins by the action of two kinase activities; G1/S-phase cyclin-dependent kinase (CDK) and DDK (DBF4-dependent kinase; CDC7 kinase). Both kinases stimulate the phosphorylation of MCM2-7. To be activated, the MCM complex also needs to associate with the GINS (consisting of four proteins (Sld5-Psf1-Psf2-Psf3) named for the Japanese 'go-ichi-ni-san', which means 5-1-2-3) and CDC45. All together these sub-units form the CMG complex (CDC45, MCM, GINS), that is instrumental to the stabilization of the replication fork (Friedel et al., 2009). CDKs act, at least in yeasts, by phosphorylating the Sld2 and Sld3 proteins, causing them to form a complex with Dpb11 (TopBP1 in Mammals). Dpb11–Sld2–Sld3 trimers then associate with origins and are required for Cdc45 and DNA polymerase loading but, unlike Mcm10 and Cdc45, do

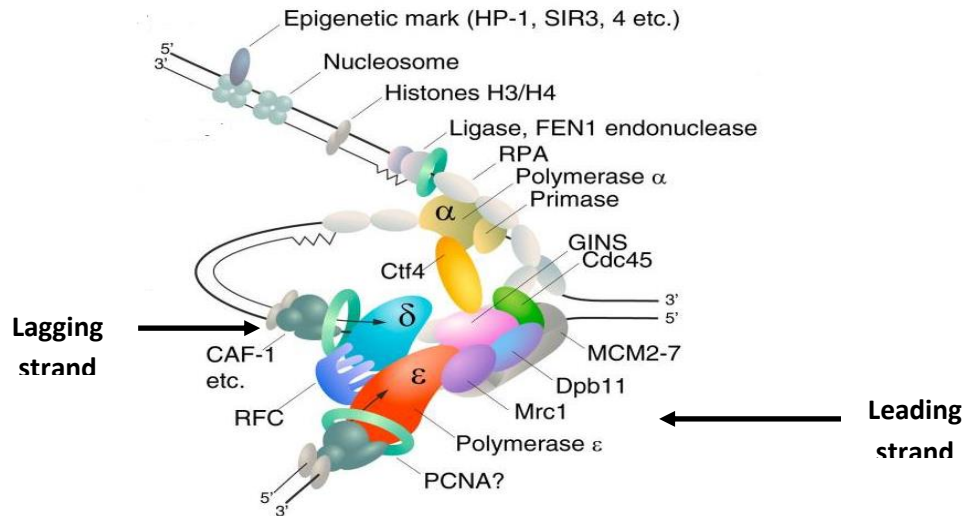
not progress with the replication fork. Recently, a complex called the pre-loading complex (pre-LC) was identified, which forms in a CDK-dependent manner before replication initiation in budding yeast. The pre-loading complex (pre-LC) contains the essential CDK target Sld2 and its binding partner Dpb11 (TopBP1 in Mammals); it is likely an important regulatory complex that specifically targets DNA Polymerases and GINS to load MCM at origins in a CDK-dependent manner (Muramatsu et al., 2010). Thus, the conversion of an inactive double hexamer into two functional helicases involves several firing factors, including Sld2, Sld3, Sld7, Mcm10, Dpb11 and the replicative DNA Polymerase ϵ , which together aid the recruitment of Cdc45 and GINS to form the CMG complex, thereby stimulating the helicase activity of the Mcm2-7 complex (Hills and Diffley, 2014) and connecting it to the replicative polymerases. Data regarding the function of these factors in plants is scarce but down-regulation of CDC45 in meiocytes results in DNA fragmentation independently of programmed double-strand breaks that form during meiosis, suggesting that CDC45 is required for DNA replication to proceed normally (Stevens et al., 2004). In addition, plant genomes encode homologues of the CDC7/Dbf4 kinase involved in replication licensing (Shultz et al., 2007), but their function has never been studied.

B-Organisation and function of the replisome (elongation and termination of replication)

DNA replication is a mechanism that leads to the production of two identical sister chromatids that contain one strand from the parental DNA duplex and one new antiparallel strand. This mechanism is conserved from prokaryotes to eukaryotes and is known as semiconservative DNA replication (Leman and Noguchi, 2013). In all living organisms, the DNA replication machinery is a complex and dynamic structure called the replisome. The eukaryotic replisome comprises 48 polypeptides, many of which are absent from the prokaryotic replisome, which reflects the complexity of eukaryotic replication. Replisome assembly occurs only upon entry into S-phase (Kurth and O'Donnel, 2013), and individual subunits are highly regulated by post-translational modifications in

a cell cycle dependent manner (Kurth and O'Donnel, 2013) to achieve faithful duplication of the genome.

A



B

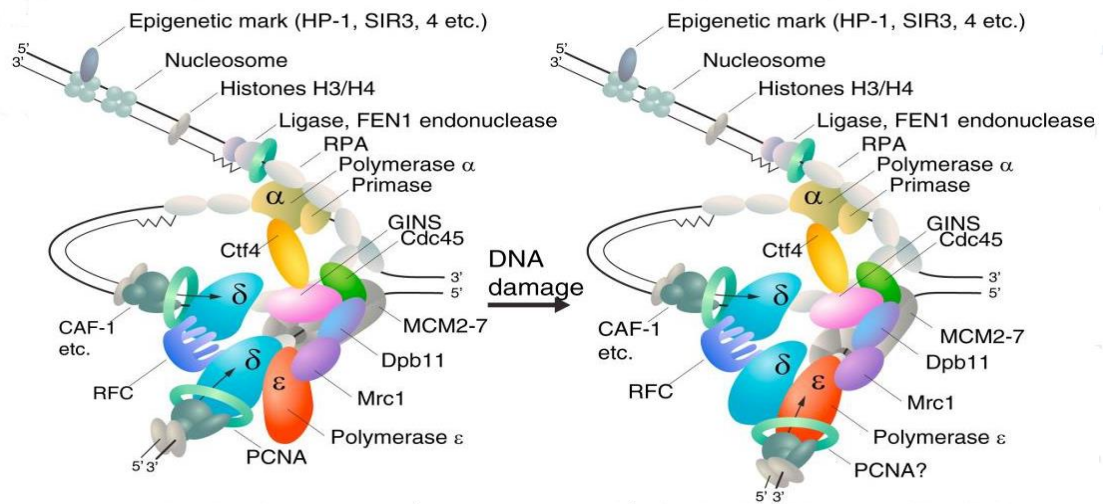


Figure 4. DNA polymerases at the eukaryotic DNA replication fork (Taken and adapted from Stillman, 2015).

A: according to the generally accepted view DNA polymerase δ synthesizes the lagging strand and polymerase ε the leading strand.

B: in a new model, DNA polymerase δ normally replicates both strands and, upon DNA damage in the leading strand template, a switch to polymerase ε occurs, linking DNA-damage detection to the essential role for polymerase ε and associated checkpoint proteins. In all cases, DNA polymerase α is coupled with primase to synthesize a RNA-DNA primer on the lagging strand that is recognized by RFC and PCNA to switch to the replicative polymerase couples other events at the replication fork, such as nucleosome assembly.

During replication DNA polymerases synthesize a DNA strand complementary to the original template strand. To this end, the double-stranded DNA is unwound by DNA helicases ahead of polymerases, forming a replication fork containing two single-stranded templates. As a consequence of the antiparallel nature of DNA, DNA replication occurs in opposite directions between the two new strands at the replication fork. However, all DNA polymerases synthesize DNA in the 5' to 3' direction. Leading-strand synthesis thus proceeds continuously, whereas lagging-strand synthesis occurs in a discontinuous manner. The discontinuous stretches of DNA replication products on the lagging strand are known as Okazaki fragments and are about 100 to 200 bases.

Once the replication fork is opened by the CMG, a RNA/DNA primer produced by the DNA polymerase α /primase complex initiates leading-strand synthesis and every Okazaki fragment on the lagging strand (Garg and Burgers, 2005). Polymerases δ and ϵ are required to elongate these primers (Kurth and O'Donnel, 2013), but their respective roles at the fork are debated. For the past few years, the generally accepted view has been that Pol δ synthesizes the lagging strand (Garg and Burgers, 2005), (Figure 4A) while Pol ϵ is responsible for the synthesis of the leading strand (Pursell et al., 2007). However, recent work suggests that polymerase δ may replicate both strands, while Pol ϵ would be involved in the removal of replication errors generated by Pol δ (Johnson et al., 2015), and would play an important scaffolding role at the fork (Figure 4B).

Replication forks are fragile structures, that are prone to recombination or DNA break formation, and tight coordination is thus required during DNA replication to avoid genomic instability. The different strategies have consequences for the machineries that copy the strands, including which DNA polymerases are involved and how DNA damage can be repaired if it occurs (Stillman, 2015). The lagging strand generally contains a longer stretch of ssDNA (single-stranded DNA) that is coated by the heterotrimeric complex RPA (Replication Protein A), which stabilizes ssDNA templates by preventing secondary structure formation or other transactions at the exposed ssDNA.

Each Okazaki fragment is preceded by an RNA primer, which is displaced by the procession of the next Okazaki fragment during synthesis. In eukaryotic cells, a small amount of the DNA segment immediately upstream of the RNA primer is also displaced, creating a flap structure. This flap is then cleaved by endonucleases (such as Fen1). At the replication fork, the gap in DNA after removal of the flap is sealed by DNA ligase I (Leman and Noguchi, 2013).

In addition, the replication fork includes other factors to support DNA replication *in vivo*. The Proliferating Cell Nuclear Antigen (PCNA) acts as a sliding clamps, that forms ring structure interacting with DNA polymerases, and especially with Pol δ (Garg and Burgers, 2005). This interaction allows secure tethering of DNA polymerases to DNA. PCNA-dependent stabilization of DNA polymerases has a significant effect on DNA replication because it enhances polymerase processivity up 1000-fold. In addition, PCNA interacts with several constituents of the replisome to regulate diverse processes (Leman and Noguchi, 2013). Recently, the ternary structure of Pol ϵ catalytic domain was reported, showing that Pol ϵ can tightly clamp onto DNA even without PCNA, making it an excellent candidate for the leading-strand polymerase (Hogg et al., 2014). However, PCNA may still be required on the leading strand to enable coupling with nucleosome dynamics and other PCNA-associated functions (Stillman, 2015). Indeed, during replication, chromatin is disassembled ahead of the fork to allow its progression and reassembled behind the fork. Histones are removed from chromatin ahead of the replication fork probably by the FACT chaperone, whereas CAF-1 loads newly synthesized histones to re-establish chromatin after replication. These two complexes are associated with replisome proteins such as PCNA (Leman and Noguchi, 2013).

Additionally, other accessory proteins are required for adequate progression of the replisome, including the FPC (Fork Protection Complex), Mrc1/Claspin, and RFC (the Replication Factor C clamp loader). These factors are regulators of polymerase functions and control DNA synthesis: notably, Mrc1/Claspin and RFC are involved in replicative stress checkpoint activation (see below, (Leman and Noguchi, 2013)).

Because DNA replication is a bidirectional process, each replication fork terminates when it encounters a fork moving in the opposite direction, leading to the displacement of the MCM proteins from DNA (Xouri et al., 2007). Termination involves at least four processes, not necessarily in the following order. First, the last stretch of parental DNA between forks is unwound (“dissolution”) and replisomes come into contact; second, any remaining gaps in the daughter strands are filled in and nascent strands are ligated (“ligation”); third, double-stranded DNA are removed (“decatenation”); fourth, the replisome is disassembled (Dewar et al., 2015).

Different protein homologues required for the different steps of replication have been identified in plants, specially *Arabidopsis* and rice (*Oryza sativa*). However experimental evidence about their functions is scarce (Sanchez Mde et al., 2012). In plants, the study of protein-protein interactions and biochemical analysis of replisome components is still in its infancy but, given the conservation of the essential coding sequences, it is supposed that these processes in plants do not differ significantly from other eukaryotes (Bryant and Aves, 2011).

Among the proteins of the replication fork that have been partially characterized are the three replicative polymerases: α , δ , and ϵ (Barrero et al., 2007; Yin et al., 2009; Iglesias et al., 2015). Data regarding these three polymerases in plants will be described in the next section. Other replisome proteins that have been studied in plants include PCNA; RFC, RPA, endonuclease 1 (FEN1), and Ligase 1 (Amoroso et al., 2011; Xia et al., 2007; Aklilu et al., 2014; Zhang et al., 2016b). Intriguingly, plant genomes contain multiple copies of most of these genes, which contrasts with genomes of other eukaryotes (Sanchez Mde et al., 2012). An example that may reflect the complexity of the plant replisome regulation is the copy number for RPA in *Arabidopsis*: five paralogs of RPA1, two of RPA2 and two of RPA3 have been identified, in contrast to the single RPA1, RPA2 and RPA3 subunits found in yeasts and mammals. Genetic analysis of the five paralogs of RPA1 from *Arabidopsis* revealed shared and unique functions for each gene. One group appears responsible for promoting genomic replication, and another group appears devoted to DNA

repair and recombination. Furthermore, within the repair/recombination group individual RPA1 subunits display unique functions in response to DNA damage (Aklilu et al., 2014).

The above-described example illustrates that although the global scheme for DNA replication is largely conserved, some specificities may exist in plants. Interestingly, our group recently found an interaction between the plant CDT1 protein and the DNA Pol ϵ (Domenichini et al., 2012). This interaction has never been described in other organisms and drew our interest to analyze the function of plant Pol ϵ into more details. In the next section, we will summarize the current knowledge on replicative polymerases, with a particular emphasis on Pol ϵ .

III-Polymerase epsilon (DNA polymerases)

DNA polymerases (Pol) are enzymes that carry out DNA synthesis. In mammalian genomes, fifteen different DNA polymerases have been identified, which are specialized for distinct cellular mechanisms, including DNA replication, DNA repair, recombination, and translesion synthesis (TLS, a process that allows DNA replication to proceed past DNA lesions; (Lange et al., 2011)). Despite the availability of several DNA polymerases, only three of them are responsible for genome duplication. Pol δ and Pol ϵ are the main eukaryotic DNA replicases, and together perform the bulk of DNA replication, following priming by Pol α (Rayner et al., 2016).

Studies of mutant Pol ϵ and Pol δ polymerases with particular error signatures in *S. cerevisiae* and *S. pombe* have suggested a model of DNA replication in which Pol δ replicates the lagging strand, whereas Pol ϵ replicates the leading strand. This division of labours is broadly accepted (Rayner et al., 2016), but this model has been recently questioned. Indeed a very recent publication reported that Pol δ normally replicates both strands of the DNA, but that occasionally a switch to Pol ϵ on the leading strand can be induced by replication errors, thereby coupling checkpoint signaling to repair of the DNA damage (Johnson et al., 2015), (Figure 4B), thus Pol ϵ would preferentially ensure leading-strand fidelity. This model may explain why mutations in the Pol ϵ catalytic residues have a

dominant negative effect, suggesting that this inactive polymerase gums up replication (Dua et al., 1999). Consistent with the notion that DNA polymerases play non-overlapping roles at the fork, combination of a collection of mutations with hypomorphic alleles of the three replicative polymerases revealed specialized genetic networks interacting with each polymerase, and corroborated the central role of Pol ϵ at the pre-initiation steps of DNA replication (Sengupta et al., 2013). In addition, several independent findings may support a model in which Pol δ is the main replicative polymerase for both strands. For instance, in yeast, the catalytic domain of Pol ϵ is not required for survival; the essential activity actually lies within the C-terminal domain that is involved in the intra-S phase detection of DNA damage and induction of checkpoint signaling to repair damage and maintain fork stability (Dua et al., 1999). In addition, replication of the simian virus 40 genome only requires Pol α and Pol δ activities. These data suggest that strand-specific variations in the error rate that were attributed to replication errors may have rather been a result of differential mismatch repair (MMR; (Stillman, 2015)).

However, all of the genetic studies dealing with this issue have used mutant strains in which Pol ϵ or δ activity is modified. Therefore, the results must be taken with caution because mutations may lead to errors in the interpretation of the wild-type situation (Stillman, 2015). For instance, in yeast, DNA synthesis of leading and lagging strands is carried out by DNA polymerase δ after homologous recombination-dependent fork restart when fork inactivation is persistent (Miyabe et al., 2015). Such a mechanism may well be activated in mutants with impaired Pol ϵ activity, which would lead to the erroneous conclusion that Pol δ is the main actor of DNA replication in normal conditions.

Although Pol ϵ may not be responsible for DNA synthesis *per se*, it is of particular interest because it stands at the interface between DNA replication, DNA repair, cell cycle regulation upon DNA damage and chromatin remodelling (Henninger and Pursell, 2014; Pursell and Kunkel, 2008). In all eukaryotes, it is a four sub-unit complex comprising a catalytic sub-unit (POL2A) and three

accessory sub-units DPB2, 3 and 4, that are not required for the DNA polymerase activity. The largest accessory sub-unit, DPB2, is essential for cell viability and could be involved in the stabilization of the Pol ϵ complex (Pursell and Kunkel, 2008). In addition, Dpb2 interacts with a sub-unit of the helicase complex, thereby inserting Pol ϵ in the replisome on the leading strand (Sengupta et al., 2013). The other two smaller subunits are dispensable for cell viability, but their inactivation leads to genetic instability, suggesting that they affect Pol ϵ fidelity (Aksenova et al., 2010). In addition, they have also been implicated in chromatin remodelling (Pursell and Kunkel, 2008). In yeast, Pol ϵ is also required for the activation of the S-phase checkpoint upon replication defects such as replication fork stalling, collapse or DNA damage. Figure 5 summarizes the functions associated to subunits of Pol ϵ complex.

Investigating the functions of the POL2 and DPB2 subunits in multicellular organisms has been complicated due to the lethality of mutations, thus most studies have been performed in yeast and cell lines. In plants, these proteins have been poorly characterized (Pursell and Kunkel, 2008).

In the following sections of this manuscript, the cell mechanisms where Pol ϵ is involved will be described briefly, with emphasis on the respective roles of the two essential Pol ϵ subunits, POL2A and DPB2.

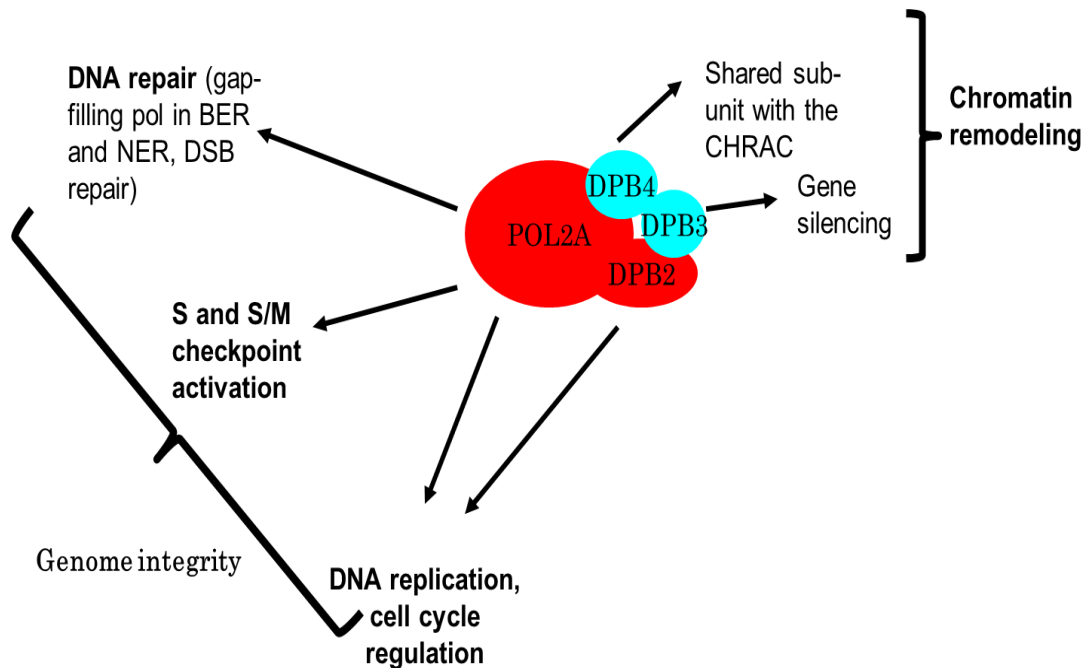


Figure 5. Pol ϵ at the intersection between diverse cellular mechanisms (Adapted from Pursell & Kunkel, 2008).

Pol ϵ is involved in several cellular processes. The involvement of each subunit is depicted with arrows. In addition to its role in DNA replication, Pol ϵ subunits also participate in cell cycle checkpoint regulation, in different DNA repair mechanisms as base excision repair (BER), nucleotide excision repair (NER), and double strand break (DSB) repair. Pol ϵ has also been implicated in the propagation of chromatin modification states. This last function could be associated with the DPB3 and DPB4 subunits. Indeed, DPB4 is also part of CHRomatin Accessibility Complex (CHRAC)

A-Structure and properties of DNA polymerases

Pol ϵ catalyzes DNA template-dependent DNA synthesis by a phosphoryl transfer reaction involving nucleophilic attack by the 3' hydroxyl of the primer terminus on the α -phosphate of the incoming deoxynucleoside triphosphate (dNTP). The products of this reaction are pyrophosphate and a DNA chain increased in length by one nucleotide. The catalytic mechanism is conserved among DNA polymerases (Lange et al., 2011).

All DNA polymerases share a common polymerase fold, which has been compared to a human right hand, composed of three subdomains; fingers, palm, and thumb. The palm, a highly conserved fold composed of four antiparallel β strands and two helices. In contrast, the thumb and fingers subdomains exhibit substantially more structural diversity. The fingers undergo a conformational change upon binding the DNA template and the correct incoming nucleotide. This movement allows residues in the fingers subdomain to come in contact with the nucleotide in the nascent base pair. The thumb holds the DNA duplex during replication and contributes to processivity (Doublié and Zahn, 2014).

DNA-dependent DNA polymerases are classified into six families based on primary amino acid sequence similarity in the enzyme active site: A, B, C, D, C, D, X, and Y (Lange et al., 2011). For instance, Y-family DNA polymerases have significantly smaller finger and thumb domains than those of replicative DNA polymerases and spacious active sites that enable them to bypass bulky DNA adducts (Rayner et al., 2016). The three replicative polymerases are part of B-family.

All B family polymerases are formed of five subdomains; the fingers, thumb, and palm are the core of the polymerase activity, whereas an exonuclease domain and an N-terminal domain have independent roles (Doublié and Zahn, 2014).

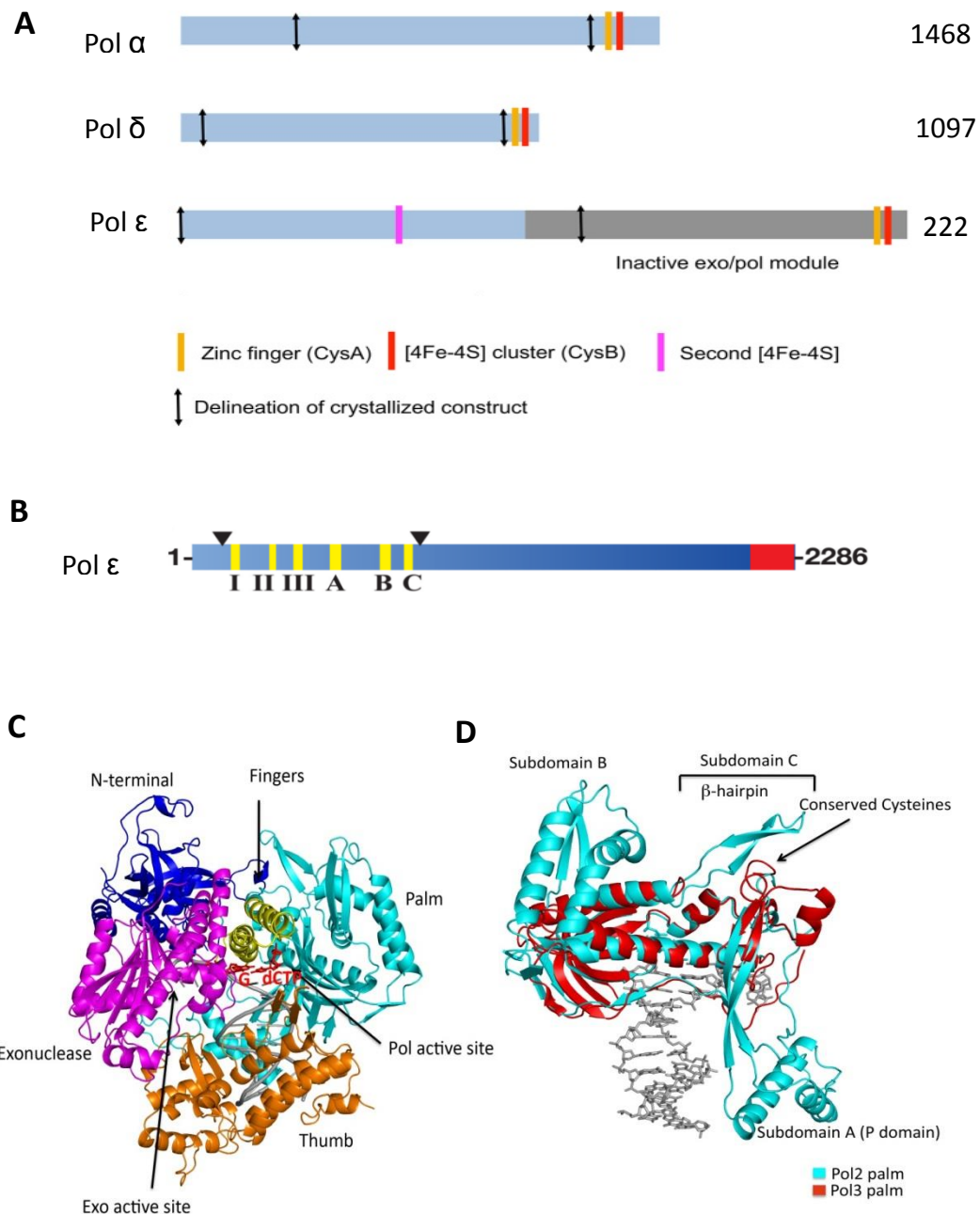


Figure 6. Structural differences amongst Pol ϵ and the other replicative polymerases; Pol δ and Pol α (Taken and Adapted from Doublié & Zahn. 2014a (A); Pursell & Kunkel, 2008 (B); Jain et al., 2014 (C-D)).

A depicts a schematic diagram of the three *S. cerevisiae* replicative DNA polymerase α , δ , and ϵ . B depicts a scheme of the Pol ϵ catalytic subunit from *H. sapiens*. Conserved motifs in the exonuclease and polymerase domains are shown in yellow, with the C-terminal protein-protein interaction region in red. DEAD-box cleavage sites in human Pol ϵ are shown as black arrows. C depicts the ternary structure of catalytic domain of POL2 from yeast. D depicts the comparison between the palm domain of Pol ϵ (Pol2) and Pol δ (Pol3)

Exonuclease proofreading refers to the action of a 3'-5' exonuclease activity that removes mis-incorporated nucleotides prior to their extension. In eukaryotes, only Pols ϵ , δ , and γ contain intrinsic 3'-5' exonuclease proofreading activities. Pol γ replicates mitochondrial DNA (and the chloroplast genome in plants, (Oldenburg and Bendich, 2015)). Multiple studies in model organisms have confirmed the essential role of DNA polymerase proofreading in the maintenance of genomic stability (Rayner et al., 2016), thus these polymerases contribute to avoid the accumulation of mutations in the genome.

Other characteristics that differ amongst DNA polymerases are the properties of fidelity and processivity. The fidelity of a DNA polymerase indicates the DNA synthesis error rate: high fidelity DNA synthesis is beneficial for maintaining genetic information from one generation to the next and for avoiding mutations. Pol ϵ and Pol δ display very high fidelity due to their 3'-5' endonuclease activity that allows them to correct replication errors. The processivity is the average number of nucleotides added by DNA polymerase per association event with the template strand; Pol α displays poor processivity, whereas Pol ϵ and Pol δ are highly processive. However, Pol δ requires to be associated to PCNA (Proliferating Cell Nuclear Antigen) to show high processivity, whereas that Pol ϵ *per se* has high processivity (Rayner et al., 2016).

B-Specificities of Pol ϵ subunits

The catalytic subunit (POL2)

The open reading frame encoding the catalytic subunit of Pol ϵ is among the longest of the many known eukaryotic polymerases. The catalytic subunits of human and yeast Pol ϵ contain 2286 and 2222 amino acids, respectively. The 140 kDa N-terminal half of the protein is fairly well conserved across different species, with 63% sequence identity shared between the yeast and human enzymes. This conservation reflects the fact that the amino terminal residues of Pol ϵ harbour the polymerase and exonuclease activities. The structural framework for catalysis of the polymerization reaction comprises highly conserved motifs A, B and C that are characteristic of the “right-handed”

polymerases (Figure 6A). In addition, amino terminus of Pol ϵ also contains three conserved motifs that contribute to exonuclease activity; called ExoI, ExoII, and III (Figure 6B, (Pursell and Kunkel, 2008)).

Pol ϵ differs from Pol δ and Pol α in that it contains an inactive domain in its C-terminal half. This is a large domain of approximately 120 KDa. This sequence is poorly conserved among homologs, but it contains two conserved cysteine-rich motifs that are shared among the B family replicative polymerases. In contrast to the catalytic domain that is dispensable for the survival, this C-terminus is essential for the viability of the cell in yeast (Kesti et al., 1999; Feng and D'Urso, 2001). The C-terminal domain is required for multiple interactions with other proteins, including the three regulatory subunits and proteins involved in cell cycle checkpoint regulation (Pursell and Kunkel, 2008).

Additionally, Pol ϵ differs from Pol δ in that it does not require the DNA sliding clamp PCNA for high processivity. This feature of Pol ϵ has been recently elucidate thanks to the elucidation of its structure by crystallization (Figure 6C, (Doublié and Zahn, 2014; Jain et al., 2014). The crystal revealed the existence of a domain absent in the corresponding Pol δ subunit (Pol3 in yeast) that could explain its enhanced processivity in the absence of PCNA. The Pol ϵ catalytic subunit contains a unique domain that projects around the DNA near its active site, thus reducing Pol ϵ dissociation from DNA. Consistently, loss of this domain or certain positively charged residues within it causes a loss of processivity (Doublié and Zahn, 2014). Two unique insertions, residues 533-555 and 682-760, encompass this novel domain, which the authors named the processivity domain (P-domain). This novel domain also could contribute to sense replication errors and thus may help facilitate active site switching (Doublié and Zahn, 2014).

Accessory subunits

In all eukaryotes studied to date, the Pol ϵ holoenzyme is formed of four subunits. The biochemical characterization of the Pol ϵ complex and analysis of the native holoenzyme purified from yeast, showed a 1:1:1:1 stoichiometry for each of the four subunits (Chilkova et al., 2003). The second largest subunit of Pol ϵ is DPB2 in budding yeast and p59 in humans, the latter name being based on its predicted molecular weight (Pursell and Kunkel, 2008). This subunit together with DBP3 and DPB4 does not have catalytic activity. These three accessory subunits interact with the C-terminal half of the POL2 catalytic subunit, possibly reducing polymerase dissociation and increasing processivity. Interestingly each subunit shows different interactions with proteins involved in mechanisms such as DNA replication, checkpoint activation, and chromatin remodelling.

DPB2 is a protein of 527 residues in yeast; it is not essential for Pol ϵ catalytic activity *in vitro*. However, disruption of DPB2 decreases the stability of Pol ϵ complex. In addition, Dpb2 is phosphorylated in S-phase, which may facilitate its interaction with Pol2 or the activity of the Pol ϵ complex (Kesti et al., 2004). However, to date, there is not clear data shedding light on the role of Dpb2 phosphorylation. In a recent study, Dpb2 was shown to regulate replication initiation. A fragile, Pol ϵ -containing complex called the pre-loading complex (pre-LC) has been identified, which forms in a phosphorylation-dependent manner before replication initiation in budding yeast (Handa et al., 2012), suggesting that the phosphorylation of Dpb2 may be required for the activation of this mechanism. In addition, Segupta and collaborators showed that Dpb2 has two critical roles during chromosome replication in budding yeast. First, it is required for initiation, because it allows loading the complex of proteins to initiate the replication. Second, during elongation, it links the leading strand DNA polymerase to the helicase complex (Sengupta et al., 2013).

Several studies have shown that alterations in Pol2-Dpb2 interaction, lead to genome instability. Mutations in the yeast Dpb2 subunit that destabilize its interaction with Pol2 caused reduced

survival and an increase in spontaneous mutagenesis rate (Jaszczur et al., 2009, 2008). However it is unclear whether the mutations affect holoenzyme stability or some other process.

Due to the lethality of *dpb2* mutations, data is scarce regarding its role in multicellular organisms. Nevertheless, the recent *in vivo* analysis of its function in *Drosophila* revealed that this protein has an important role in progression of S-phase in mitotic cell cycles, and is also required for endoreduplication (Sahashi et al., 2013), suggesting that DPB2 functions are conserved in eukaryotes.

C- Roles of Pol ϵ subunits at different steps of DNA replication

DNA Pol ϵ has multiples roles for DNA replication, which are independent of its catalytic activity. Understanding the non-catalytic functions of DNA polymerases will be key to figure out why eukaryotes have three distinct polymerases at the replication fork (Zegerman, 2013).

As briefly described above, recent studies have shed light on important roles for Pol ϵ in the early stages of the assembly of an active replisome. The role of Pol ϵ in the replication initiation in budding yeast may be regulated by the formation of the pre-LC, which consists of four subunits GINS, Pol ϵ complex, and the Dpb11 and Sld2 proteins. It is formed in a CDK-dependent manner before replication initiation (Muramatsu et al., 2010). The role of Pol ϵ in this mechanism has been associated with the ability of DPB2 interacts with Psf1, a sub-unit of GINS (Handa et al., 2012). This interaction is required to load the rest of replisome proteins to chromatin, including the catalytic subunit of Pol ϵ , and thus start the DNA replication (Sengupta et al., 2013). During replication progression, Dpb2 directly interacts with Psf1, a sub-unit of the CMG complex, allowing the connection between the DNA Pol ϵ and the helicase complex. This interaction requires the N-terminus of Dpb2: deletion of the domain prevents interaction between Pol ϵ and the replisome (Sengupta et al., 2013). Additionally, overexpression of the Dpb2 N-terminus in yeast mutants lacking Dpb2, demonstrated that this fragment of the protein is sufficient for CMG complex assembly and DNA replication initiation but not for recruitment of the other subunits of Pol ϵ (Sengupta et al., 2013). Consistent with this function, the removal of Pol2 prevents the conversion of the inactive MCM to the active form CMG helicase complex, and mutations within the non-catalytic carboxyl terminus still allow CMG assembly but prevent the progression of the helicase away from origin (Handa et al., 2012).

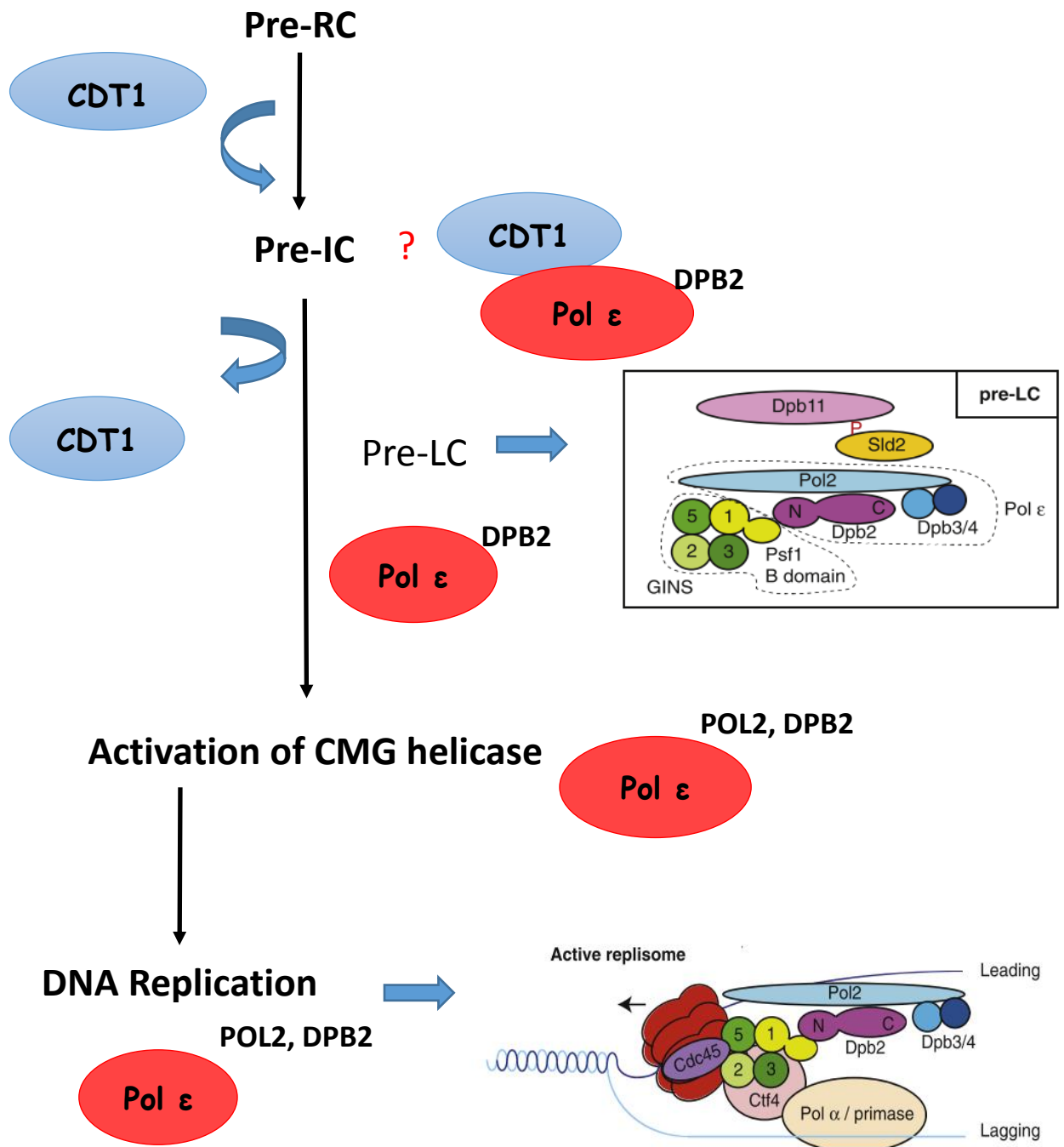


Figure 7. Multiple roles of Pol ϵ subunits in the DNA replication mechanism (Drawings of replisome were taken and adapted from Zegerman 2013). The scheme depicts the different steps of replication where Pol ϵ sub-units are required. The question mark indicates that the function has not been established.

Thus, Pol ϵ has two roles in replication initiation: first in the assembly of the CMG, probably through the pre-LC, and second in activating the CMG at origins (Figure 7). Whether these mechanisms are conserved in plants, or whether the role of DPB2 in DNA replication initiation is mediated by other interactions remains to be established. Indeed, *Arabidopsis* CDT1 homologues form complexes with DNA Pol ϵ both in yeast two-hybrid and in Tandem Affinity Purification experiments, and this interaction has never been described in other organisms, suggesting that the molecular events occurring during pre-RC formation or fork progression may differ in plants and other eukaryotes (Domenichini et al., 2012).

In the current models of DNA replication, this interaction is unexpected because CDT1 is supposed to be unloaded from chromatin before of Pol ϵ recruitment (Yeeles et al., 2015). However, these two proteins are involved in other mechanisms than DNA replication itself where this interaction may be acting. For instance, both proteins participate in the Break-Induced Replication (BIR) mechanism, which is an efficient homologous recombination (HR) pathway employed to repair a DNA double-strand break (DSB) when homology is restricted to one end (Lydeard et al., 2010). BIR is thought to restart stalled or collapsed replication forks during S phase. How it can restart replication in the absence of an origin remains to be deciphered. It was hypothesized that the primary role of Cdt1 is to load Mcm2-7 onto the BIR template DNA, and that this mechanism does not depend on either Orc or Cdc6 (Lydeard et al., 2010). In addition, BIR requires proteins of pre-LC and CMG helicase such as Dpb11-Sld3, Mcm10, Ctf4 (Lydeard et al., 2010), and subsequently the 3 replicative DNA polymerases are required together with the other sub-units of the replisome. Because Pol ϵ participates in the replication initiation as was mentioned above, it may be supposed that the interaction between DPB2 and CDT1 is required to initiate replication in special conditions. However, the existence of BIR is debated in plants (Schubert et al., 2011). Furthermore, the CDT1/Pol ϵ complex was observed in TAP experiments using both CDT1 and DPB2 as baits, but

no other sub-units of the replisome were identified in these assays (Domenichini et al 2012, our unpublished data) suggesting that these complexes might be quite stable within plant cells.

On the other hand, CDT1 is recruited at foci of DNA repair after DNA damage induction, although after a short time it is degraded: CDT1 accumulates at the site of DNA damage through interaction with PCNA, and this interaction might function as scaffold to coordinate the cell cycle and DNA damage Response (DDR) (Roukos et al., 2011). Because CDT1 is specifically expressed during the G1 phase of the cell cycle, its recruitment at the site of damage could signal to the DDR the phase of the cell cycle for the choice of the appropriate repair pathway. For example, the choice between homologous recombination (HR) and non-homologous end-joining (NHEJ) in repairing DSBs has been shown to be dependent on the cell cycle phase (Roukos et al., 2011). Thus, we may speculate that interaction between Pol ϵ and CDT1 could be involved in connecting the cell cycle to the DDR pathways. Future studies in Arabidopsis will be needed to figure out the role of interactions of Pol ϵ with other proteins, including CDT1.

Hence CDT1/Pol ϵ complexes could function either in DNA replication *per se*, or could be required to safeguard genome integrity in response to DNA damage or replicative stress. Either way, DNA Pol ϵ clearly stands at the crossroad between DNA replication and DDR. In the next section, we will therefore summarize our current knowledge on DDR in Animals and Plants.

IV- Mechanisms involved in the maintenance of genome integrity

In response to DNA damage, eukaryotic cells activate highly coordinated cellular networks, collectively termed DDR, that are critical for maintaining genome integrity. These signaling pathways lead to DNA repair, cell cycle checkpoints activation, and in some cases cell death; all these processes contribute to eliminate or tolerate lesions in the genetic material (Yoshiyama et al., 2014). When DNA damage is not severe, cell cycle progression is delayed or arrested to allow DNA repair. However, when DNA damage is too severe to be repaired, animal cells undergo apoptosis, as it is preferable to eliminate cells with unrepairable DNA than to allow them to propagate incorrect genetic information (Yoshiyama et al., 2013b). Defects in this system in Animals contribute to various disorders, including cancer and developmental defects, which highlights the crucial importance of an efficient DDR for the viability of both the cell and the organism (Yoshiyama et al., 2014). Although carcinogenesis does not occur in plants, DNA damage leads to growth inhibition and developmental defects. Endogenous DNA damage resulting from defective DNA repair machinery results in aberrant organogenesis and development (Hu et al., 2016). Thus, all eukaryotes require proper cell cycle regulation and more specifically, cell cycle checkpoints activated by DNA damage are instrumental for the maintenance of genome integrity particularly in meristems from which the germ line differentiates after many rounds of division.

A-Genotoxic Stress

Due to their sessile lifestyle, plants are particularly exposed to adverse environmental conditions present ubiquitously in the air, soil, and water, most of which can induce DNA damage via various mechanisms (Tuteja et al., 2009). The major environmental genotoxic agent is the UV-B light from sun that mainly generates thymidine dimers (also known as photoproducts). These lesions distort the DNA helix and block the replication fork (Al Khateeb and Schroeder, 2009). More generally, a wide range of biotic and abiotic stresses can lead indirectly to DNA damage because they induce oxidative stress that can alter all cellular macromolecules, including DNA. All primary components

of DNA that contribute to the genetic information such as sugar residues, phosphodiester bounds, purine and pyrimidine bases are susceptible to suffer damage (Bray and West, 2005). In general, DNA lesions are divided into two main categories: single and double-stranded. The first category comprises lesions disturbing only one DNA strand, such as oxidized or alkylated bases, base loss, DNA adducts, intra-strand cross-links, DNA photoproducts, and single-strand DNA breaks (SSBs). The second category includes lesion affecting both DNA strands, such as inter-strand cross-links and double-strand DNA breaks (DSBs), the latter being the most severe type of DNA damage that can affect the genome (Manova and Gruszka, 2015). The most common cause of endogenous DNA lesions is the intracellular metabolism, which increases the concentration of free radicals. In plants, reactive Oxygen Species (ROS) are produced by the active metabolism in the chloroplasts and mitochondria and they are the major source of SSBs through of deoxyriboses destruction and covalent modifications on bases (Bray and West, 2005). High concentrations of ROS can also generate abasic sites by spontaneous hydrolysis of the N-glycoside bond or as intermediates resulting from the repair of deaminated, alkylated or oxidized bases (Manova and Gruszka, 2015). Moreover, all DNA-associated cellular processes involved in the transmission, expression and maintenance of genetic information have the potential to cause SSB or DSB in DNA. To face all these hazards, complex networks of mechanisms for DNA damage detection and repair exist in plants, like in other eukaryotes (Manova & Gruszka, 2015).

With exceptions, plants have been shown to possess all common DNA repair mechanisms that have been described in other eukaryotes, such as yeast and mammals. Among these mechanisms are the main DNA double-strand break repair pathways- HR and NHEJ that have been shown to be essential in plants for preservation of their genetic stability (Waterworth et al., 2011). Additionally, the two classical forms of excision repair, base (BER) and nucleotide (NER), play an essential role in proliferative tissues (Kimura and Sakaguchi, 2006; Tuteja et al., 2009). Homologues of genes involved in the MMR (MisMatch Repair) mechanism, which is required for the repair of incorrectly

paired nucleotides, have also been found in plants (Manova and Gruszka, 2015). Finally, polymerases that participate in translesion synthesis and allow the replication fork to travel pass a DNA lesion have been also characterized in *Arabidopsis* (Curtis and Hays, 2011; Amoroso et al., 2011). Photoreactivation (a light-dependent reaction allowing the dissolution of pyrimidine dimers) is absent in Mammalian cells. By contrast, in plants, this mechanism is one of the main DNA-repair mechanisms: it is required to maintain the genome stability on a daily basis because of their inherent necessity for solar light exposure (Bray and West, 2005; Kimura and Sakaguchi, 2006). It is worth mentioning that photoreactivation does not require the checkpoint machinery to be activated, at variance with most other DNA repair mechanisms.

Figure 8 illustrates the DNA damaging-agents that can cause DNA lesions, the main kinds of damages and repair mechanisms. The lesion caused in the DNA depend on the type of genotoxic stress as well as on the exposure time, and the DNA repair mechanisms activated depend on the type of lesion. In addition, in plants, the choice of a repair pathway and its action is primarily dependent not only on the type of lesion and its genomic context but also on the type of the cell, its proliferation status and cell cycle stage (Britt, 1999). For instance; some of the repair mechanisms as photoreactivation are highly specialized for a particular damage, however, others, like excision or recombination pathways may deal with a variety of lesions. Moreover, NER and BER are specific mechanisms of meristematic tissue, whereas photoreactivation is active both in differentiated and proliferative tissues (Kimura and Sakaguchi, 2006).

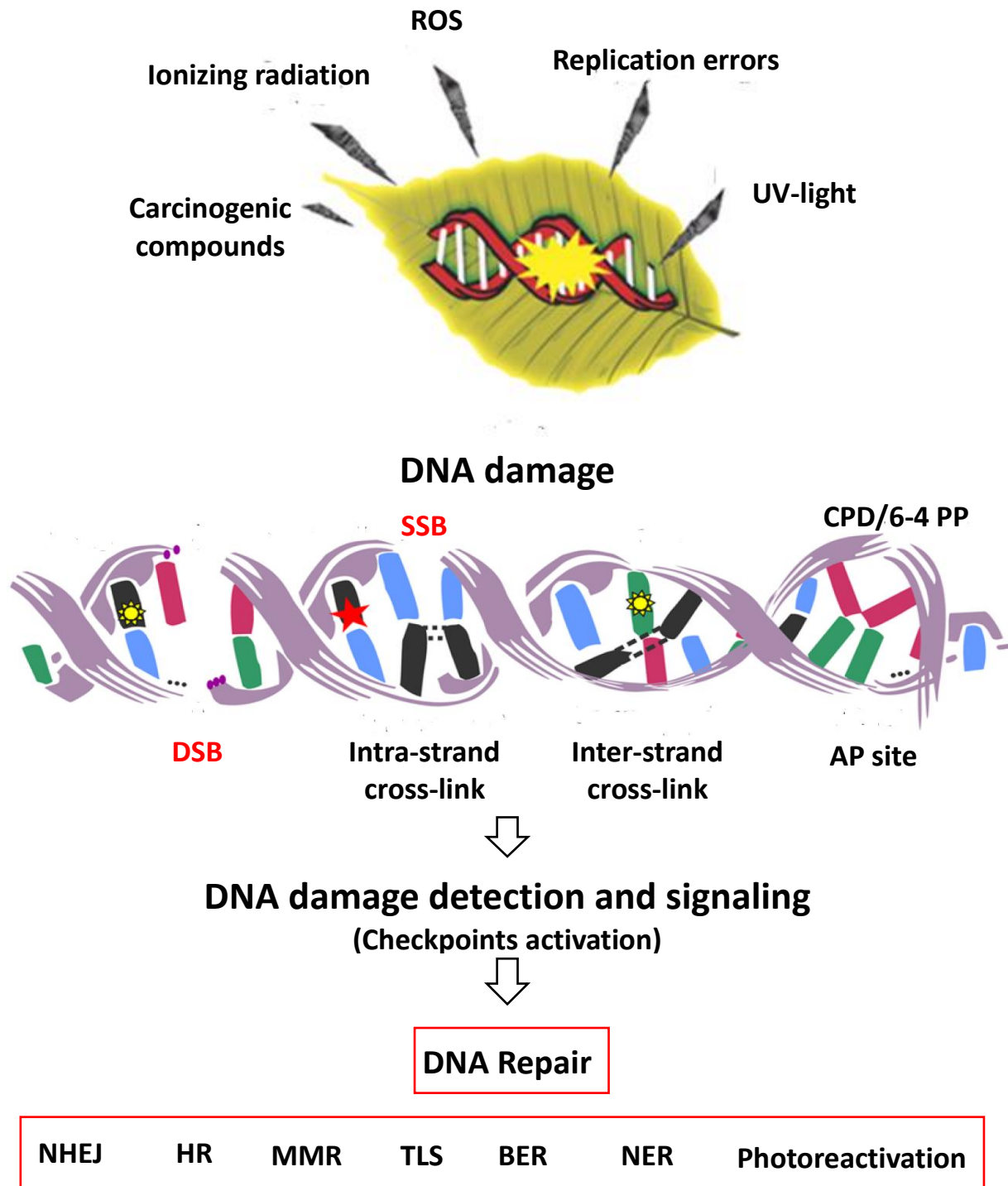


Figure 8. Schematic representation of the major DNA lesions induced by various external and endogenous factors, and the types of DNA repair mechanisms employed to remove them from the eukaryotic genome (Taken and adapted Kimura & Sakaguchi, 2006; and Manova & Gruszka, 2015). Different kinds of DNA damage can be generated in the DNA structure, and the lesion generated depends on the type of DNA-damaging agents that plant cell has been exposed to DSB: Double Strand Break, SSB: Single Strand Break, AP site: apuric/apyrimidic site also known as abasic site, CPD/6-4PP: cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (lesions induced by UV irradiation). Genotoxic stress triggers the DDR activation, resulting in the activation of DNA repair mechanisms. However, photoreactivation does not require DDR activation. NHEJ: non-homologous End Joining, HR: Homologous Recombination, MMR: mismatch repair, TLS: translesion synthesis, BER/NER: Base/Nucleotide Excision Repair

B-DDR in Mammals and Plants

Plant cells have evolved unique mechanisms to control the proliferative activity of their meristematic cells, to stop cell cycle progression in response to DNA damage, and to maintain their ability of stem cells to divide when favorable conditions return. Thus, they can avoid permanent DNA damage and transmission of mutations to the developing organs via mitosis and then to the next generation via meiosis. Because many key players are conserved in most or even all eukaryotes, it is generally assumed that plant DDR functions similarly to what has been described in Mammals and yeast. I will therefore first describe the DDR in Mammals. However, a number of recent results indicate that significant differences exist at some DDR steps (Cools and De Veylder, 2009; Yoshiyama et al., 2013b; Hu et al., 2016). In addition, relationships between plant response to various biotic and abiotic stresses and the control of DNA repair and genome stability are beginning to be revealed (Dona et al 2013). Specificities of the plant DDR will thus be detailed in the second sub-section.

DDR in Mammals

At the molecular level, the DDR pathway contains several key components: DNA damage sensors, signal transducers, mediators, and effectors. When DNA damage occurs, the first step is the activation of one of the two main protein kinases ATM (Ataxia Telangiectasia Mutated) and ATR (ATM and Rad3 related). The ATM kinase is activated by DSBs, whereas that ATR kinase is critical to respond to single stranded DNA and SSBs. The eukaryotic cells have distinct sensor protein complexes to detect different kinds of damage, which activate ATM or ATR (Yoshiyama et al., 2013b).

Recognition of DNA damage is an essential step for repair; the lesion can be detected by multiple mechanisms, which precede signaling initiation. When a DSB occurs, the ends of the DSB are recognized by the MRE11-RAD50-NBS1 (MRN) complex (Waterworth et al., 2011), which

recruits ATM at the DSB sites through the C-terminus of NBS1. This interaction triggers ATM activation by autophosphorylation and the phosphorylation of downstream target proteins involved in DDR signaling pathway. One of the earliest consequences of ATM activation at the DSB site is phosphorylation of the histone-variant H2AX producing γ H2AX. These γ H2AX foci act as a signal for DNA damage and recruit other proteins, forming foci of DDR proteins at DSB sites to trigger downstream signaling events (Roy, 2014).

On the other hand, replication protein A (RPA) carries out the sensing of single-stranded DNA (ssDNA), generated by replication stress. The hallmark of replication stress is the presence of long stretches of single-stranded DNA caused by an uncoupling of DNA polymerase activity from the unwinding of the DNA at the replication fork. This ssDNA is sensed and coated by RPA, which in turn independently recruits two checkpoint complexes: the ATR-interacting protein (ATRIP)-ATR complex and the Rad9-Hus1-Rad1 (9-1-1) complex. Whereas the (ATRIP)-ATR complex directly binds RPA, the loading of the 9-1-1 complex on DNA requires several steps. First, DNA polymerase α is bound to RPA-ssDNA and initiates the recruitment of the clamp loader complex RAD17-replication factor C (RFC). Next, the RAD17-RFC complex loads the 9-1-1 complex onto the damaged site (Waterworth et al., 2011; Yoshiyama et al., 2013b; Hu et al., 2016). Both checkpoint complexes are essential for optimal activation of ATR (Yoshiyama et al., 2013b). DNA Pol ϵ , together with other proteins, is involved in the activation the 9-1-1 complex (see below).

Once ATR or ATM is activated, it triggers phosphorylation cascades until the activation of DDR effectors. Intriguingly, although each kinase is activated by different kinds of DNA damage and sensor complexes, both share many downstream signaling players. CHK1 and CHK2 are the key transducers that receive signal from ATR and ATM: ATR predominantly phosphorylates CHK1, whereas ATM activates CHK2. Both signals converge towards p53, which is a tumor suppressor protein that plays a central role in the decision for a cell to either undergo cell cycle arrest and DNA repair or apoptosis after DNA damage. In normal conditions, the p53 protein levels are low because

of MDM2-mediated ubiquitination and degradation through the proteasome pathway. Upon DNA damage, p53 is activated by phosphorylation at several N-terminal sites in its transactivation domain by ATM, ATR, CHK1, and CHK2, and this phosphorylation inhibits the interaction of p53 with MDM2, resulting in p53 stabilization (Yoshiyama et al., 2013b). In addition, ATM directly inactivates MDM2 (Wang et al., 2012).

Two main effectors downstream of signaling are the WEE1 kinase and CDC25 phosphatase, which operate antagonistically to regulate CDK activity. When DSBs occur during S-phase, ATM signaling inactivates CDC25 and promotes activation of the WEE1 kinase, resulting in the repressive phosphorylation of the nuclear CDK-cyclin complex, triggering cell cycle arrest (Hu et al., 2016). In addition, when DSBs occur during the G1 phase, ATM activates p53 that induces the expression of several downstream targets, including the gene encoding p21, an inhibitor of CDK-cyclin complexes, triggering a cell cycle arrest at the G1/S transition (Yoshiyama et al., 2013b). In contrast to the role of the ATM pathway which predominantly regulates the G1/S and G2/M checkpoints, the ATR pathway controls DNA replication at the G1/S and intra-S checkpoints (Yoshiyama et al., 2013b). WEE1 is a key regulator of the S phase checkpoint together with ATR (Hu et al., 2016). The main players of checkpoint activation in mammals are shown on Figure 9.

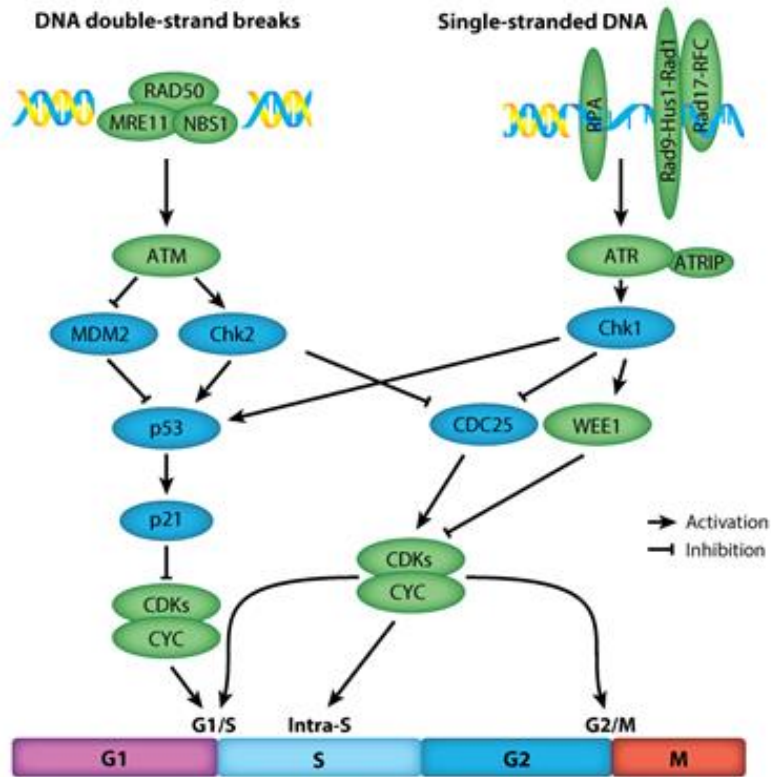


Figure 9 . Cell cycle checkpoints activated in response to DNA stress in Mammals (Taken from Hu et al., 2016). SSBs and DSBs are recognized by different complexes to trigger the activation of either ATR or ATM kinases respectively. Downstream in the signalling cascade are effectors (CHK1, CHK2 and MDM2), the p53-transcription factor, and the inhibitors of CDKs/cyclin complexes such as p21 and WEE1, resulting in the arrest of cell cycle. The role of each signalling component is detailed in the text

The Plant DDR, conserved mechanisms and unique features

Plant response to DNA damage has been studied for several years and it is beginning to be well described (Yoshiyama et al., 2013b). The mechanisms for detection of DNA damage are conserved between animals and plants, while the mechanisms for signal transduction and gene regulation have diverged (Yoshiyama et al., 2014). Plants have evolved a subset of unique DNA damage response regulators, particularly with respect to cell cycle control (Hu et al., 2016).

In plants, the homologues of genes encoding subunits of the MRN complex for sensing DSBs are conserved. In *A. thaliana*, γ H2AX accumulation did not occur in *rad50* or *mre11* mutant plants, suggesting that the MRN complex is required for H2AX phosphorylation by the ATM and ATR kinases in response to DNA damage (Amiard et al., 2010). These results indicate that the MRN complex acts as a sensor for the DDR like in animals. In the case of complexes involved in ssDNA sensing, orthologous genes have been identified in plant genomes for subunits of both complexes. The functions of some 9-1-1 complex subunits have been partially characterized by genetic studies (Takashi et al., 2009; Heitzeberg et al., 2004). *rad17* and *rad9* mutants of *A. thaliana* are sensitive to bleomycin (BLM, that induces DNA breaks) and mitomycin C (MMC, an alkylating agent that induces inter-strand crosslink) and show defects similar to those of mutants for the corresponding human genes, suggesting that the two proteins are involved in the same pathway in plants and in animals (Heitzeberg et al., 2004). Moreover, inactivation of *A. thaliana* RPA70a, which is similar to the largest subunit of human RPA, causes increased sensitivity to the replicative stress agent hydroxyurea (HU), suggesting a role for *AtRPA* in the ATR pathway (Takashi et al., 2009).

In Mammals, knockout ATR mutation results in embryonic lethality, while an ATM mutation results in pleiotropic defects such as growth defects, neurologic dysfunction, and infertility. By contrast, *atr* and *atm* Arabidopsis mutants grow normally (Culligan et al., 2004; Culligan and Britt, 2008), although *atm* plants are partially sterile (Ricaud et al., 2007), and *atm atr* double mutants are completely sterile (Culligan et al., 2004). However, the functions of ATR and ATM are conserved

in plants. Like in other eukaryotes, ATM appears to be predominantly involved in double-strand break perception whereas ATR senses replication stress and induces a G2 cell cycle arrest after DNA damage (Culligan et al., 2006, 2004).

The CHK1 and CHK2 proteins, that function downstream of ATM and ATR, are apparently missing in plants. However, substrates of CHK1 and CHK2 in animals such as BRCA1 and E2F can be found in plants (Yoshiyama et al., 2013b), suggesting that other kinases may work as functional homologs of CHK1 and CHK2. Likewise, the p53 effector that coordinates the transcriptional response after DNA damage in animals (Branzei and Foiani, 2009), appears to be missing from plant genomes. However, the SOG1-transcription factor could be the functional homolog of p53 (Yoshiyama et al., 2009), and is a substrate of ATM (Yoshiyama et al., 2013a). SOG1 was identified in a genetic screen for suppressors of the gamma irradiation-induced cell-cycle arrest of the *Arabidopsis xpf-2* mutant, which is defective for the repair endonuclease XPF (Preuss and Britt, 2003). SOG1 is the first identified plant-specific transcription factor involved in the DDR pathway. It is also the central regulator of the DDRs because it can be activated both by ATM and by ATR (Sjogren et al., 2015; Hu et al., 2015), and its activation is required for the majority of plant responses to DNA damage (Yoshiyama et al., 2009) including cell-cycle arrest, and death of stem cells (Yoshiyama et al., 2014).

In terms of cellular response, activation of ATM or ATR by DNA damage causes programmed induction of endoreduplication (several rounds of DNA replication without mitosis, (Adachi et al., 2011)), cell cycle arrest and in some instances programmed cell death (Fulcher and Sablowski, 2009). In proliferating cells, cell cycle progression must be stopped until DNA damage is repaired to avoid transmission of mutations to daughter cells. In plants, like in animals, this is achieved by activation of inhibitors of CDK (Cyclin Dependent Kinase)/cyclin complexes and of the WEE1 kinase, which phosphorylates and inhibits CDKs (De Schutter et al., 2007), although CDC25 appears to be missing from plant genomes (Boutros et al., 2006). Similarly to what is described with

the p53/p21 module, cell cycle arrest upon DNA damage seems to depend partly on a SOG1/SMR module. SOG1 induces the transcription of *SMR5* and *SMR7* in response to different kinds of DNA stress, indicating that they are potential DNA damage checkpoint regulators. Consistently, mutation of SOG1 or depletion of the ATM kinase completely abolishes *SMR5/SMR7* induction upon DNA damage inducing conditions (Hu et al., 2016). In parallel, DNA damage also triggers the transcription of the *WEE1* gene, thereby allowing inhibitory phosphorylation of CDKs/cyclin complexes to arrest the cell cycle. However in plants, WEE1 has been also associated not only with the intra-S checkpoint but also with DSBs response (De Schutter et al., 2007). Interestingly, the SOG1-dependent replication checkpoint may be controlled by mitogen-activated protein kinase (MAPK) pathway, which controls cell cycle progression upon UV-B stress and operates independently from ATR (González Besteiro and Ulm, 2013).

These cell cycle checkpoints allow not only cell cycle arrest in S or G2 phase, which can be visualized by increased expression of the G2 marker CYCLIN B1;1, but also lead to induction of endoreduplication which is thought to allow early differentiation of cells whose genome has been damaged. This response is SOG1-dependent, and likely relies on the activation of SMRs (Yi et al., 2014). It has been recently reported that genotoxic stress can also promote endoreduplication in animals, but the function of this response is not clear because endoreduplication can block mitosis but can also promote cancer progression depending on the genetic background (Lee et al., 2009). The general molecular control of the DNA damage response in plants is represented on Figure 10.

Many of the above-described mechanisms can be activated irrespective of the cell cycle phase, or even in differentiated cells. However, the S-phase of the cell cycle is a major source of DNA lesions and dedicated mechanisms have evolved to activate cell cycle checkpoints in response to replicative stress. The underlying mechanisms will therefore be more specifically detailed in the next section.

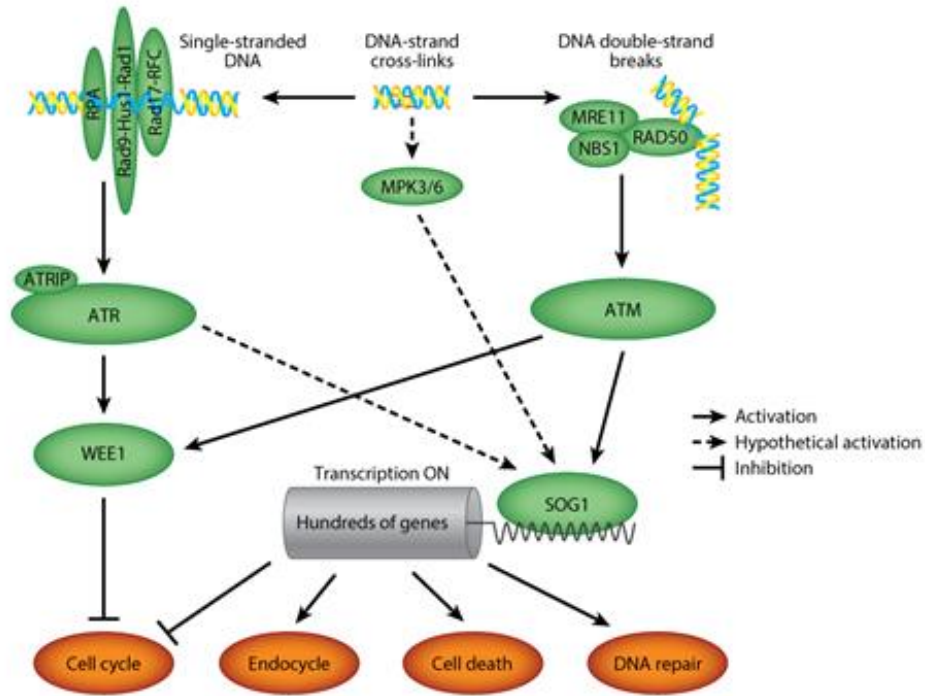


Figure 10. Molecular control of the DNA damage response in plants (Taken and adapted from Hu et al., 2016). The sensing of DNA damage is conserved with other eukaryotes. CHK1 and CHK2 as well as p53 are missing in comparison to mammals. However, the SOG1-transcription factor is the functional homolog of p53: it controls the transcription in response to DNA damage. WEE1 is mainly required to arrest the cell cycle in S-phase or G2. Strikingly, an additional mechanism to activate SOG1 may be dependent on MAPK signalling. The role of each component is detailed in the main text

C- Specific mechanisms triggered by replicative stress

Most studies aimed at elucidating molecular mechanisms involved in replication stress response have been performed in yeast (Friedel et al., 2009). Mechanisms involved avoiding the accumulation of errors during DNA replication and DDR appear to be conserved in plants, but are poorly described compared to animal or yeast models.

A number of factors such as lesions, difficult to replicate sequences, collision with the transcription machinery etc... can impede fork progression during the S-phase and trigger replicative stress. Stalled forks are fragile structures that can lead to genetic instability; cells have therefore evolved complex sensing mechanisms to activate checkpoints in response to replicative stress (Jossen and Bermejo, 2013). Experimentally, the replication stress can be induced by exposure to HU (hydroxy-urea); it depletes the dNTP pool by inhibiting the ribonucleotide reductase, resulting in fork stalling. In fact most findings on replication stress have been obtained in systems where HU was used to artificially generate the stress.

Replication checkpoint activation triggers multiple mechanisms including the inhibition of late-firing origins of DNA replication, replication fork stabilization and induction of genes required for DNA repair, nucleotide biosynthesis and cell cycle arrest (Friedel et al., 2009; Segurado & Tercero, 2009).

Roles of Pol ϵ in the S-phase checkpoint activation

The S-phase checkpoint response is activated by an increased presence of single-strand DNA (ssDNA) at replication forks. The ssDNA are sensed by RPA protein on the leading strand and by the 9-1-1 complex on the lagging strand (Branzei and Foiani, 2009). Several intermediates are required to activate ATR after recognition of the damage (Friedel et al., 2009; Segurado & Tercero, 2009); for instance in yeast, an important player in Mec1 (ATR) activation appears to be Dpb11 (TopBP1 in Human). In UV-exposed G1 cells, Dpb11 binds to the 9-1-1 complex and interacts directly with Mec1 (ATR), thereby allowing its activation (Puddu et al., 2011). Moreover, the

amplification of the replication checkpoint signal depends on Mrc1 (Claspin in Mammals). Claspin interacts with the effector kinase CHK1; the molecular mechanism allowing checkpoint signal amplification in Mammalian cells is complex, and it seems that different signals trigger the formation of alternative Claspin-CHK1 complexes (Branzei and Foiani, 2009).

Both Dpb11 (TopBP1) and Mrc1 (Claspin) proteins interact with Pol ϵ (Muramatsu et al., 2010; Lou et al., 2008), consistent with the early report of the role of Pol ϵ in DNA stress response in yeast (Navas et al., 1995). The blockage of DNA replication triggered by DNA damage (by exposure to MMS), depletion of nucleotide pools (by exposure to HU), or inactivation of DNA polymerases is sensed by the Pol ϵ catalytic subunit (Pol2). The checkpoint activation generated by Pol2 and other sensors is transduced through Rad53 (CHK1), and leads to cell cycle arrest and the inactivation of the Dun1 kinase (Figure 11A; Navas et al., 1995); this protein kinase controls several DNA damage-inducible genes such as *RNR* (RiboNucleotide Reductase). Mec1 (ATR) and Rad53 (CHK1) are also required for the activation of Dun1 (Branzei and Foiani, 2009). In this way, yeast cells regulate nucleotide biosynthesis in response to replication stress.

Several molecular mechanisms have been elucidated that could account for this role of Pol ϵ in replicative stress sensing. First, Puddu et al have reported that Mec1 (ATR) can be activated by two independent pathways during HU-exposure, one of which requires the C-terminus of Pol2 as well as the accessory sub-unit Dpb4 (Puddu et al., 2011). The activation of Mec1 (ATR) in this pathway requires Dpb11 and Sld2. Pol ϵ , Sld2 and Dpb11 thus likely work together in replication stress sensing. This hypothesis is consistent with the fact that Dpb11, Sld2, Pol ϵ and the GINS form a complex at the beginning of S-phase in yeast and that Dpb11 is recruited at the sites of replication stalling and co-localizes with Pol ϵ during initiation of DNA replication, but not during elongation (Puddu et al., 2011). Dpb4 and Sld2 probably function upstream of Dpb11 during checkpoint signaling (Figure 11B). Pol ϵ and its interacting subunits may function in sensing replication stress

on the leading strand, while the 9-1-1 complex may be more important to detect lagging strand fork arrest (Figure 11B).

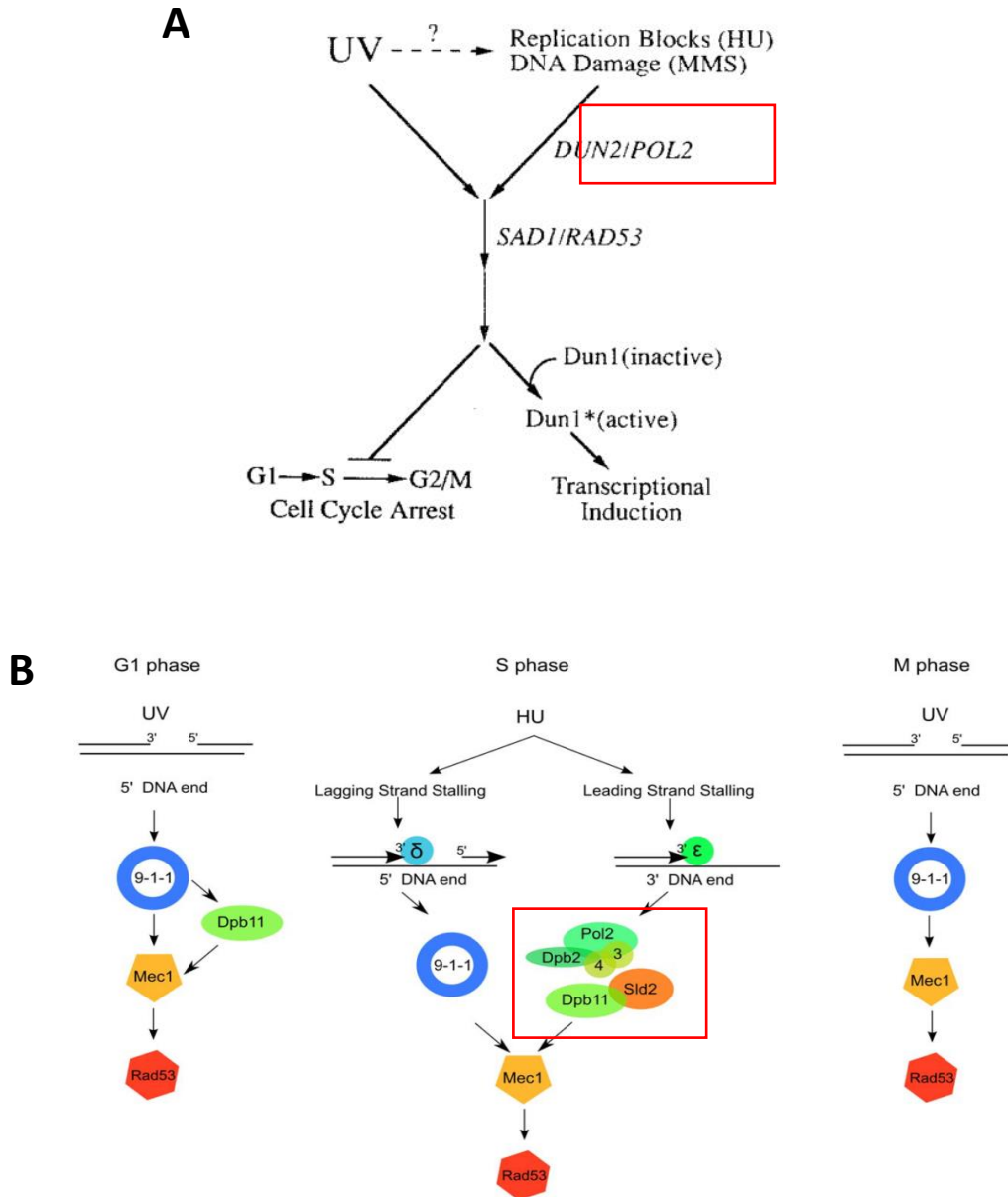


Figure 11. Models of replication stress checkpoint activation triggered by Pol ϵ during S-phase in yeast (Taken and adapted from Navas et al., 1995; Puddu et al., 2011). A: model describing how Pol2 is involved in the sensing of DNA damage generated by HU or MMS. Signal is then transduced to *SAD1/RAD53* (the CHK1 homologs), resulting in cell cycle arrest and the transcriptional activation of *RNR*. B: Model for 9-1-1 and Dpb11 function in Mec1 (ATR) activation. Pol ϵ is involved in the checkpoint activation during S-phase in response to HU, which depends on its interaction with Dpb11 and Sld2. Moreover, this model illustrates that the complexes responsible for DNA damage sensing are dependent on the cell cycle phase and the type of genotoxic agent.

Additionally, Pol ϵ has been found to interact physically with Mrc1 (Claspin) in yeast and *Xenopus* respectively, which allows full checkpoint activation (Henninger and Pursell, 2014). In response to HU, Mrc1 (Claspin) carries out the adaptor function between Mec1 (ATR) and Rad53 (CHK1). In normal conditions, Mrc1 (Claspin) interacts with the N and C-terminus of Pol ϵ catalytic subunit. Upon replicative stress, phosphorylation of Mrc1 disrupts its interaction with Pol ϵ N-terminus while its association with the C-terminus remains unchanged and could be important for the stabilization of the fork by anchoring of Pol ϵ to DNA. This mechanism has been proposed to function as a molecular switch in replicative stress sensing (Lou et al., 2008). In plants, the homologue of Claspin has not been identified, and whether such a mechanism can operate thus remains to be established (Yoshiyama et al., 2013b).

Recently in budding yeast, S-phase checkpoint activation was shown to require binding of Pol ϵ to a replication factor called Ctf18-RFC (Okimoto et al., 2016; García-Rodríguez et al., 2015); only the 200 to 300 first amino acid of the Pol2 N-terminus are required for this interaction (Figure 12A). The RFC protein contains a module of interaction with Pol2, and this sequence is conserved in different organisms including *Arabidopsis thaliana* and *Oryza sativa* (Figure 12B). Moreover, the incorporation of Pol ϵ into the replisome is required downstream of Mec1 (ATR), for activation of the S-phase checkpoint. This incorporation in the replisome requires Dpb2 (García-Rodríguez et al., 2015), using the same mechanism as replication in normal conditions (Sengupta et al 2013). However, disruption of Pol ϵ insertion of the replisome with a truncated version of Dpb2 only partially affects checkpoint activation, whereas loss Ctf18-RFC completely suppresses it, suggesting that the role of Ctf18-RFC is only partly mediated by its interaction with Pol ϵ (García-Rodríguez et al., 2015, Figure 12C-D).

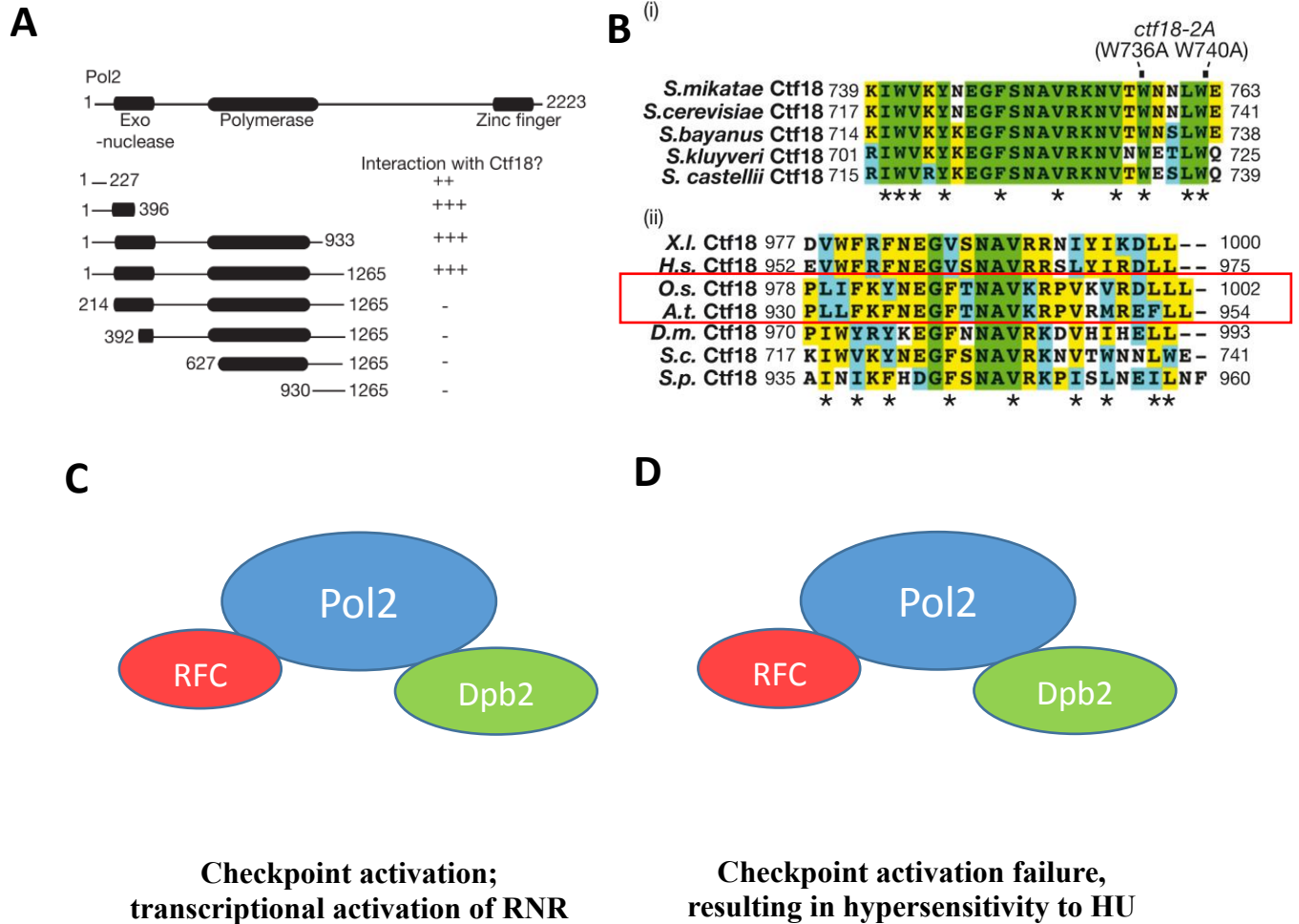


Figure 12. Pol ϵ requires the association with Ctf18-RFC for S-phase checkpoint activation (Taken and adapted from García-Rodríguez et al., 2015). A: Pol2 truncations that were tested for their ability to interact with Ctf18 in the two-hybrid assay. B: (i) Alignment of the carboxyl terminus of Ctf18 from each of the indicated yeast species, asterisks denote conserved hydrophobic residues. (ii) An analogous alignment of the end of the Ctf18 protein from diverse eukaryotic species, showing conservation of 9/10 residues that are required for interaction with Pol2 (X. l.= *xenopus laevis*; H.s.=*Homo sapiens*; O.s.=*Oryza sativa*; A.t.=*Arabidopsis thaliana*; D.m.=*Drosophila melanogaster*; S.c.=*Saccharomyces cerevisiae*; S. p.=*Schizosaccharomyces pombe*). C: scheme that represents the interactions required for replicative stress response; POL2 interacts with Ctf18-RFC to trigger checkpoint activation, and the loading of Pol ϵ in the replisome by the DPB2 subunits is partially required for checkpoint activation. D: when either of the two events described above are affected, checkpoint activation fails.

In fission yeast, deletion of the N-terminus of Pol ϵ catalytic subunit encompassing the polymerase activity is not lethal, but these cells show prolonged S-phase (Feng and D'Urso, 2001). The viability of such mutants depends on components of the DNA damage checkpoint: synthetic lethality was observed when this Pol2 truncated version was introduced in deletion mutants such as *chk1*, *rad3* (ATR), and *hus1* (9-1-1 clamp). In addition, the Pol δ homolog (Cdc6 in yeast) is essential for their survival (Feng & Urso, 2001), indicating that Pol δ catalytic activity is able to partially compensate for Pol ϵ catalytic deficiency. Consistently, mutations in the extreme C-terminus of the yeast Pol ϵ catalytic subunit lead to hypersensitivity to HU and inability to activate the ribonucleotide reductase (*RNR3*) transcriptional response. These mutants also display a catastrophic mitosis after HU treatment, indicating that they fail to activate the appropriate checkpoint (Navas et al., 1995). By contrast, catalytic deletion mutants are insensitive to HU and highly sensitive to MMS (Feng & Urso, 2001), confirming that the C-terminus is responsible for checkpoint signaling. In addition, replication stress has been shown to induce degradation of DNA Pol ϵ , which could thus function both as a sensor and a target of DNA Damage Response (DDR) (Roseaulin et al., 2013). However, the molecular mechanisms involved are not clear.

Interestingly in human cells, whether Pol ϵ promotes S-phase entry or affects S-phase progression is debated (Bermudez et al., 2011). Since Pol ϵ is involved in multiple mechanisms linked to genome integrity maintenance, mutations that affect this protein could potentially affect a wider range of cellular activities than just replication fidelity (Rayner et al, 2016). Hence, different outcomes could result from mutations affecting distinct parts of the POL2 protein, or its regulatory subunits. Consistently, many mutations in DNA Pol ϵ have been associated with different types of cancer (Henninger & Pursell, 2014).

Although the plant DDR and more specifically the replication stress response is beginning to be well described (Cools and De Veylder, 2009), the relationships between DNA replication proteins such as Pol ϵ and DDR remain to be elucidated. This type of analysis is now made possible by the

isolation in genetic screens of viable hyphomorphic alleles of Pol ϵ or Pol δ catalytic sub-units (Yin et al., 2009; del Olmo et al., 2010; Iglesias et al., 2015).

Hence, although questions remain regarding the exact molecular mechanisms underlying the contribution of Pol ϵ to replicative stress response, its involvement in the cellular response is clearly established. In the next paragraph, we will describe the sequence of events that occurs after checkpoint activation to ensure genome integrity and allow replication to resume.

Mechanisms activated by replication stress checkpoint

The stabilization of DNA replication forks seems to be the most important checkpoint function to ensure cell survival when DNA is damaged during S-phase. How the replication checkpoint prevents fork collapse has been extensively investigated, but, despite considerable progress, the precise mechanisms by which the checkpoint executes this essential function remains unclear (Segurado & Tercero, 2009).

Checkpoint proteins are required to avoid catastrophic irreversible events that cause the collapse of DNA replication forks: when *atr* and *chk1* mutants are exposed to replication stress, for instance by treatment with HU, the replisome dissociates from the stalled replication fork. This breakdown of the replisome, results in fork collapse (Branzei and Foiani, 2009), which correlates with increased cell death (Segurado & Tercero, 2009). In addition, both DNA Pol α and Pol ϵ are stably associated at forks stalled by HU exposure (Friedel et al., 2009), and this association is dependent on Mec1 (ATR). The probable function of this response is to maintain the polymerases at stalled fork to restart the replication (Segurado & Tercero, 2009). Furthermore, in the presence of HU, *mec1* (*atr*) mutants display Pol ϵ recruitment and inappropriate firing at late origins, highlighting the relationship between replicative stress signaling and activation of late origins (Pursell & Kunkel, 2008).

An alternative mechanism involved in replisome stabilization has been proposed to occur in RFC deficient cells, or when Pol ϵ activity limits DNA synthesis. The Brc1 protein binding to γ H2AX could stabilize the replisome without involving Rad17-RFC or the 9-1-1 clamp. This mechanism could be activated by limiting DNA synthesis, or by defects in Pol ϵ tethering to DNA; in the latter case, Brc1 binding to γ H2AX would suppress catastrophic formation of ssDNA (Mejia-Ramirez et al., 2015).

Another important function of the checkpoint is the induction of genes involved in the DNA damage response, but it does not seem to be essential for fork stabilization or cell viability (Branzei & Foiani, 2009). Indeed, the inhibition of protein synthesis in wild-type cells during S-phase does not render cells sensitive to HU and does not impede fork resumption after HU block, suggesting that the effect of the checkpoint on gene expression is not primordial (Friedel et al., 2009).

As mentioned above, S-phase checkpoint also prevents late origin firing. Indeed, DNA Pol α was shown to bind early but not late firing origins in wild-type cells treated with HU (Friedel et al., 2009). Importantly, replication fork stabilization also allows the resumption of DNA synthesis once the stress is removed, since *de novo* assembly of the pre-replication complex required for replication fork firing is not possible during S-phase (Friedel et al., 2009). Instead, dormant replication origins become important for ensuring the completion of DNA replication if replication forks stall or are inhibited during S-phase (McIntosh and Blow, 2012). Indeed, MCM2-7 complexes are loaded onto DNA in excess, and only around of 10 % of origins are firing in normal conditions. Most licensed origins normally remain dormant and are passively replicated by oncoming forks (McIntosh & Blow, 2012). Replication forks travel bi-directionally, meaning that if a single fork stalls, a fork coming from the opposite direction can rescue this. However, when two converging forks stall, the genome between them is left in a compromised state, and if there is no dormant origin between the stalled forks, completing DNA replication will be difficult, possibly depending on recombination-dependent fork restart. However, if a dormant origin is activated between the

stalled forks, this provides a simple mechanism for replication of all intervening DNA (McIntosh & Blow, 2012). When replication forks are arrested, it only makes sense for dormant origins to be activated near the stalled forks, and not elsewhere in the genome (Blow et al., 2011).

ATR and CHK1 inhibit replication initiation and delay progression through the replication timing program. At first sight it appears paradoxical that replication inhibition simultaneously activates dormant origins but also suppresses overall origin initiation via ATR and CHK1. When cells experience low levels of replication fork inhibition, which leads to maximal activation of dormant origins, ATR and CHK1 predominantly suppress initiation by reducing the activation of new replication origins. This means that the super-activation of origins is restricted to already active replication clusters, also termed fabrics (Figure 13). Additionally, modest changes in CDK activity preferentially alter the activation of new replication origins, leaving initiation within clusters unaffected (Blow et al., 2011).

Since checkpoint mutants are unable to resume replication following treatment with either HU or DNA-damaging agents, checkpoints are also likely to play an important role in promoting completion of chromosome replication in the presence of, or following, genotoxic stress. Consistent with the data obtained in yeast, a role for ATM/ATR in the restart of damaged replication forks was also demonstrated by studies in *Xenopus* (Branzei and Foiani, 2009).

Although S phase checkpoint activation has been studied intensively, not much is known about how cells inactivate the checkpoint once the replication stress is removed (“**recovery**”) or how they down-regulate the checkpoint when DNA repair fails (“**adaptation**”). Both processes are important for resumption of replication after damage and for completing replication in the presence of damaged DNA (Friedel et al., 2009).

All together mechanisms activated by replicative stress contribute to the maintenance of genome integrity in eukaryotes. In Mammals, deficiency in these processes triggers genomic instability resulting in cancer. In plants, these mechanisms have little been investigated until now.

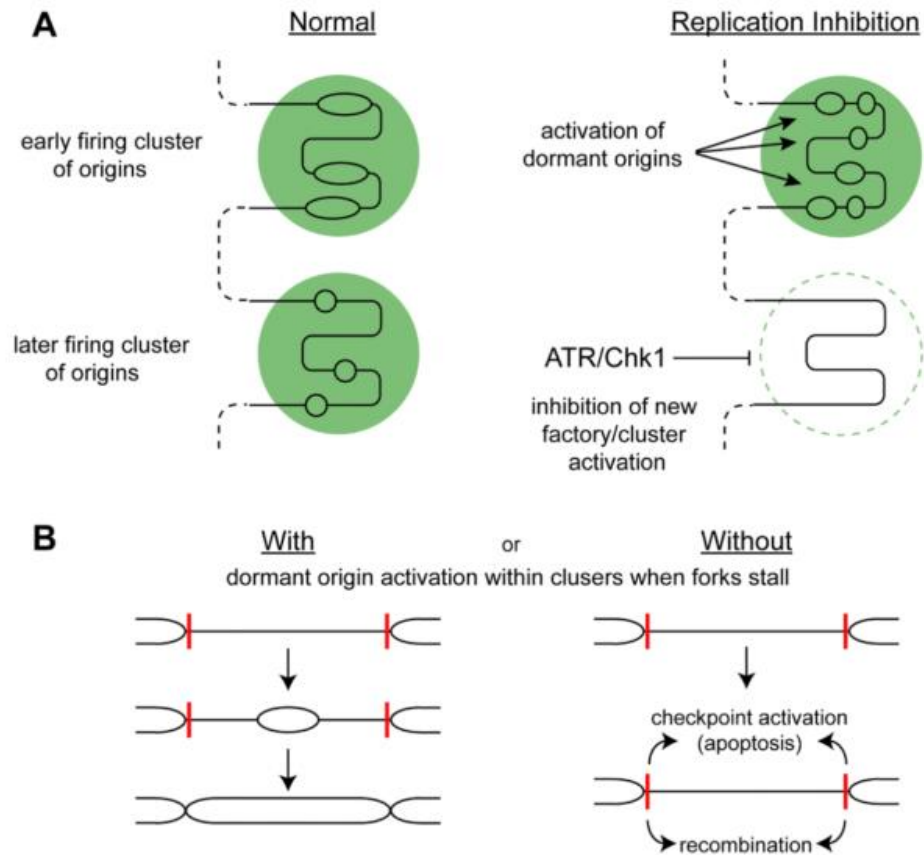


Figure 13 . Model for how dormant origins promote complete genome replication upon low levels of replicative stress, (Taken from Blow et al., 2011). A: two clusters of origins (“factories” represented as green circles) adjacent on DNA. Under normal conditions (left), the upper factory is activated slightly earlier than the factory below and each initiates three origins. Upon low levels of replicative stress (right), replication forks are inhibited in the earlier replicating cluster, which promotes the firing of dormant origins as a direct consequence of stochastic origin firing. Replicative stress activates DNA damage checkpoint kinases, which preferentially inhibit the activation of the unfired later clusters/new factories. B: A single fragment of DNA (black line) is shown with two converging forks that have stalled (red bars). If a dormant origin is activated between them, replication can be rapidly rescued (left). If there is no dormant origin firing between the stalled forks (right), the DNA damage response can lead to recombination or induction of apoptosis.

Coordination between ATM and ATR signaling

Both exposure to DNA damaging agents and fork collapse due to failure of fork stabilization can lead to the formation of DSBs. In this case, DNA repair is essential for both cell viability and maintenance of genomic integrity. Two major pathways take care of repairing DSBs, Non-Homologous End-Joining (NHEJ) and Homologous Recombination (HR). NHEJ directly ligates together the two broken ends with little or no processing and is highly efficient, but it can lead to mutations at the joining sites, as well as inversions and translocations. HR is more accurate, because it uses undamaged homologous DNA sequences (sister chromatids or homologous chromosomes). Making the right choice between NHEJ and HR is important to ensure genome stability (Gobbini et al., 2013). Interestingly the response triggered by ATR also can activate HR in order to repair ssDNA (Figure 14).

DNA damage simultaneously triggers DNA breaks and replication fork stalling, causing ATM and ATR to regularly cooperate in response to DNA stress. A challenging question is how ATM and ATR actions are coordinated at DSBs. Interestingly, while activation of both ATM and ATR depends on the ss/dsDNA junctions they are oppositely regulated by the lengthening of single-stranded overhangs. Blunt double-strand ends, as well as ends with short single-stranded tails, are the preferred substrates for ATM activation. As the single-stranded tail increases in length, it simultaneously potentiates ATR activation and attenuates ATM activation. In both Human and yeast, ATM (Tel1) activation promotes the accumulation of ssDNA at DSB ends and therefore is critical for the subsequent activation of ATR (Mec1). As generation of ssDNA ultimately leads to ATM inactivation, this mechanism ensures an efficient switch from ATM (Tel1) to ATR (Mec1) (Clerici et al., 2014). Moreover, when replication stress occurs during meiosis in *S. pombe* and *S. cerevisiae*, the replication checkpoint machinery suppresses the formation of DSBs through transcriptional repression of essential regulators of DSB formation in normal conditions

(Subramanian and Hochwagen, 2014). Thus, constitutive activation of ATR signaling may impact the ATM-dependent response.

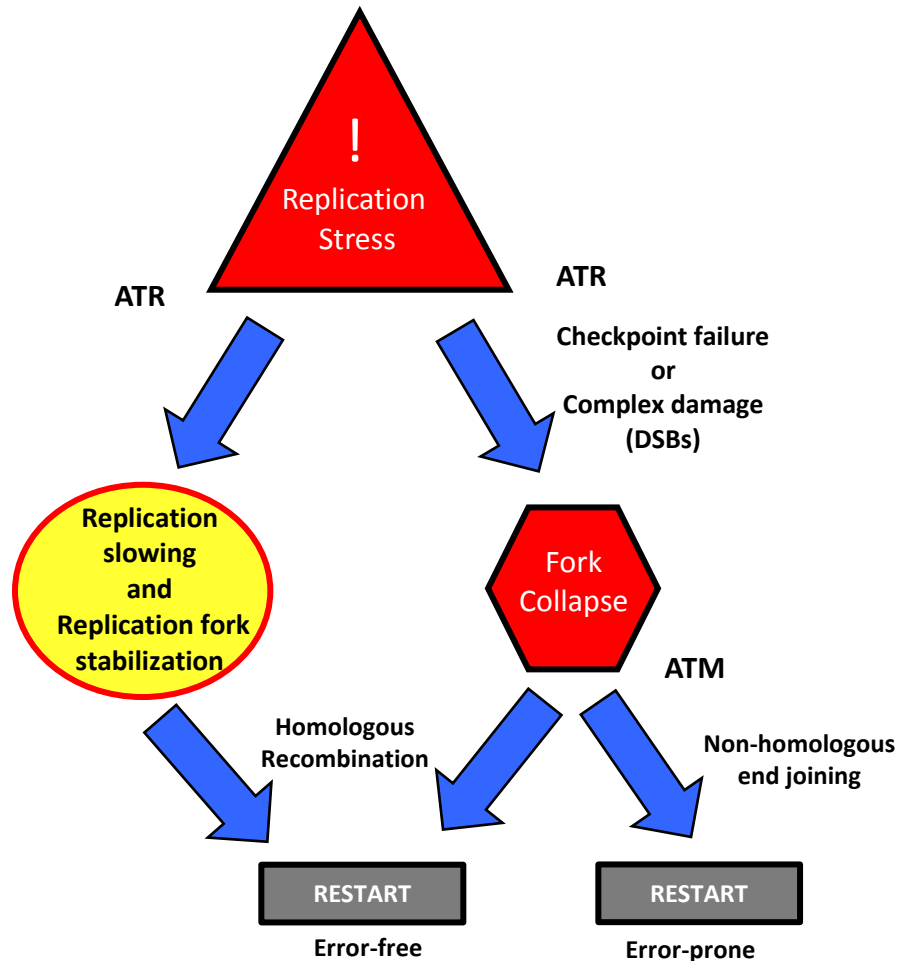


Figure 14. Fork stabilization and fork collapse. Upon replication stress, the cell primarily activates the ATR- signalling to allow fork stabilization and arrest the cell cycle. A DNA repair mechanism that can be activated by damage is homologous recombination, which can repair in an error-free manner (left). If ATR checkpoint fails to occur, fork collapse and generation of DSBs activate ATM. DSBs may promote homologous recombination or Non-homologous end joining which is an error prone mechanism (right).

The different complexes that sense ssDNA are involved in the switch of ATM to ATR signaling. Thereby mutations affecting the constituents of these complexes may compromise the coordination between the two signaling pathways. Since Pol ϵ forms part of complexes involved in ssDNA detection, it will be interesting to evaluate its role in this process.

One of the central mechanisms allowing DNA replication to proceed in the presence of DNA damage involves TLS to bypass the lesion. Interestingly, Pol ϵ has been shown to play a role in this process.

TLS and the role of Pol ϵ

During replication, DNA lesions can block fork progression. These injuries can be bypassed when a translesion DNA synthesis (TLS) polymerase is available, by two alternative mechanisms. One pathway uses a combination of replicative and translesion synthesis (TLS) polymerases to replicate across the lesion and the outcome can sometimes be error-prone. The second mechanism is known as template switching, and is essentially error-free because it is mediated by recombination, to bypass the lesion using the undamaged information of the sister duplex (Branzei & Foiani, 2009; Lange et al., 2011). In the complete absence of a TLS polymerase, no translesion bypass occurs (although there will still be some template switching) and replication forks collapse, leading to DSBs and chromosome instability. TLS DNA polymerases are thus factors that allow tolerating DNA damage (Lange et al., 2011).

The DNA damage checkpoint complex (9-1-1) was shown to facilitate the recruitment and damage bypass mediated by pol ζ (zeta). The damage and replication checkpoints have multiple connections with TLS: phosphorylation events mediated by the checkpoint kinases are likely to control the choice of the repair pathway (Branzei & Foiani, 2009).

Interestingly, Pol ϵ has been shown to have an essential role in regulating the switch of bypass polymerases. Temperature-sensitive mutants of the Dpb2 subunit of Pol ϵ that no longer bind stably

to the Pol2 catalytic subunit confer a strong mutator phenotype that is partially dependent on Pol ζ , suggesting that low-fidelity polymerases have greater access to the leading strand when the stability of Pol ϵ complex is affected. Because Pol ϵ and CMG are connected via Dpb2, these results have allowed to propose a model where the Pol ϵ –CMG connection is flexible, allowing TLS and other polymerases to bind the DNA temporarily without displacing Pol ϵ from the replisome. This complex may tether the polymerase to the replisome so that, even if it falls off the primer terminus, it can quickly rebind and continue synthesis. The proximity of Pol ϵ that is tethered to the fork by CMG serves to limit the action of the low-fidelity polymerase and allow rapid recovery of the primer terminus by the high-fidelity Pol ϵ as soon as the lesion is bypassed (Figure 15, (Langston et al., 2014)).

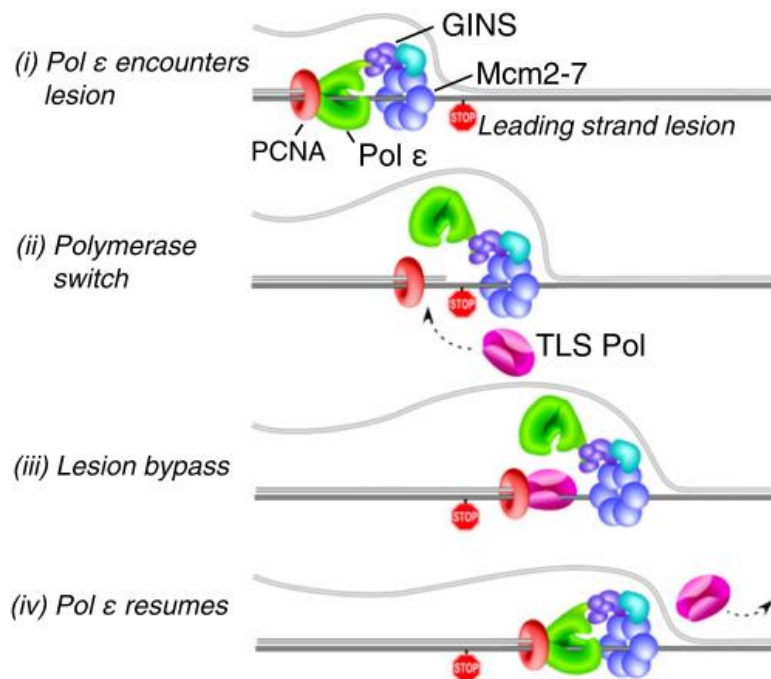


Figure 15. TLS and Pol ϵ (Taken from Langston et al., 2014). This drawing shows a model where Pol ϵ retention at a fork is mediated by binding the CMG. (i) the fork progression is stopped by a DNA lesion; (ii) Pol ϵ releases PCNA and the primer terminus upon encountering the lesion but remains bound to CMG, allowing access to a translesion synthesis (TLS) polymerase; (iii) The TLS Pol (s) bypasses the lesion; (iv) Pol ϵ rebinds the primer terminus and resumes high-fidelity replication.

In yeast, upon response to DNA damage, PCNA is ubiquitinated which might facilitate recruitment of TLS polymerases and their exchange with the replicative polymerase for lesion bypass (Branzei & Foiani, 2009). The participation of plant PCNA in DNA damage processing has been shown in Arabidopsis. Thus, POL θ (AtPol θ) and POL λ (AtPol λ), members of the Y and X families, respectively, interact with PCNA2 (Anderson et al., 2008; Amoroso et al., 2011) to catalyze TLS and restore replication fork progression when DNA is damaged.

In Arabidopsis, a study genetic between *atm*, *atr*, pol η , and *pol*, ζ mutants revealed complex pathways to tolerate DNA stress involving TLS polymerases. These pathways depend on the four proteins for response to replication fork blockage. Authors challenged double mutant lines with UV-light and followed the survival of daughter cells in stem cells. This work led to the conclusion that Pol η and Pol ζ tightly cooperate for TLS. They may usually be sufficient to complete DNA replication. TLS by Pol η is activated as a first alternative to bypass the lesion; its function does not depend on DDR kinases. By contrast, ATR appears to promote TLS by facilitating recruitment of Pol ζ and may indirectly promote damage tolerance. In this model, the role of ATM would be to promote repair of DSBs that result from failed TLS. Only when the activation of these pathways fails, leading to accumulation of DSBs, ATM and ATR trigger cell death (Figure 16). ATR and ATM seem to play multiple and seemingly contradictory roles in replication-blocked stem cells. The dynamic balances among the death-avoidance and death-promotion functions of ATR and of ATM seem to depend on the various factor (Curtis and Hays, 2011).

This summary of the current knowledge on DNA damage and replicative stress response highlights the central role of DNA polymerases in these processes. In the last section of the introduction, we will describe more specifically data available on plant replicative polymerases.

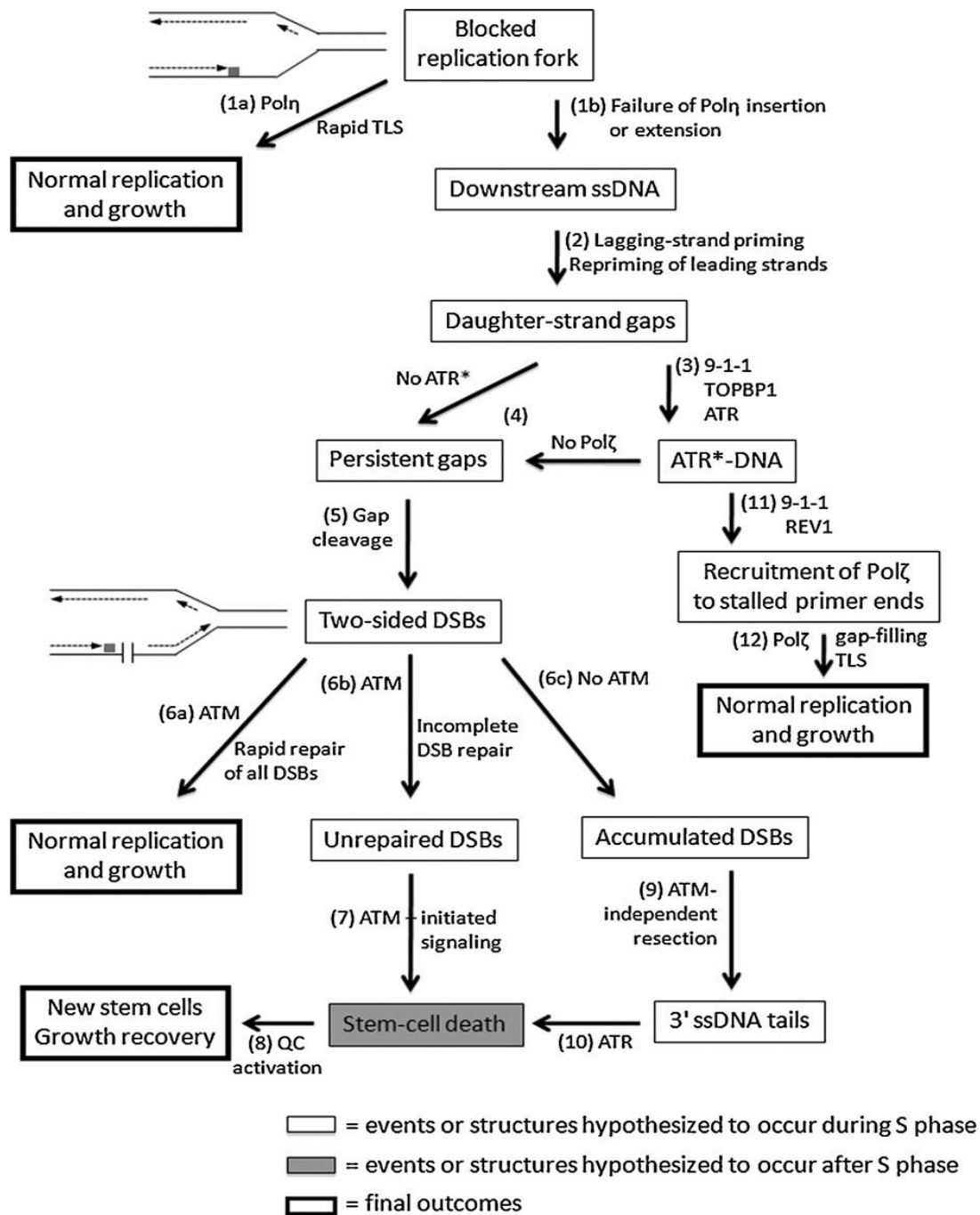


Figure 16. DDR and plant TLS polymerases (Taken and adapted from Curtis & Hays, 2011). The scheme depicts the different steps that plant cells may follow to tolerate DNA stress involving TLS polymerases *pol* η , and *pol* ζ , and their regulation by *atm* and *atr*. In Arabidopsis, these complex pathways depend on the four proteins for response to replication fork blockage. To details of each step to see text

V-Replicative polymerases in plants

All components of the replisome are conserved in plants. The three DNA polymerases that carry out the replication are also conserved: polymerase alpha ($\text{Pol}\alpha$). Polymerase delta ($\text{Pol}\delta$) and polymerase epsilon ($\text{Pol}\epsilon$) (Shultz et al. 2007; Sanchez et al 2012).

A-Polymerase α

DNA polymerase α (POLA) activity in plants has been shown biochemically in maize, wheat, pea, and cauliflower. The genome sequence analysis of Arabidopsis and rice allowed the identification of the four putative subunits of POLA complex (POLA1 to 4). The largest subunit (POLA1) contains the DNA polymerase activity whereas the two smallest subunits have the DNA primase activity (POLA3 and POLA4, also known as PRI1 and PRI2, respectively). The plant POLA1 sequence is conserved compared to its yeast and animal homologues, with the exception of a YYRRLFP motif of unknown function present in POLA4 subunits (Shultz et al. 2007). Conservation of the essential motifs suggests a similar function of POLA subunits in plants (Shultz et al. 2007; Sanchez et al 2012).

In Arabidopsis, the first hypomorphic mutant for POLA1 was isolated in a genetic screen for abnormal leaf morphology (Barrero et al., 2007). This mutant, also known as *incurvata 2* (*icu2*), harbours a point mutation and displays early flowering, leaf incurvature, and homeotic transformations of floral organs. The *icu2-1* mutation also influences the development of the shoot apical meristem, and delays the G2/M phase with high expression of a G2/M marker gene *CYCBI;1:GUS*. Furthermore, the frequency of homologous recombination is enhanced compared to wild-type plants (Liu et al., 2010).

The early flowering phenotype was associated with increased FT expression which led to upregulation of genes involved in the control of flowering time, floral meristem and floral organ identity. In addition, ICU2 interacts with genes involved in chromatin-mediated cellular memory

such as TLF2 (Barrero et al., 2007). Consistent with the first study about of *icu2-1* plant, it was reported that *icu2-1* mutants displayed modifications of histone marks compared to the wild-type, but no changes in DNA methylation (Liu et al., 2010; Hyun et al., 2013). Analysis of *icu2-1*, demonstrated that Pol α is involved in mediating epigenetic regulation; the *icu2-1* mutation strongly impairs Pol α function in the maintenance of repressive epigenetic marks but does not seem to affect ICU2 polymerase activity.

Recently, microarray analysis of *icu2-1* mutant plants revealed that up-regulated genes include genes encoding transcription factors and targets of the Polycomb Repressive Complexes. The down-regulated genes included many known players in salicylic acid (SA) biosynthesis and accumulation, ABA signaling and ABA-mediated responses. The *icu2-1* seedlings showed hypersensitivity to ABA and salt stress. Interestingly, *icu2-1* seedlings accumulated more ABA than the wild-type plants in response to salt stress. These results indicate a role for ICU2 in the regulation of genes involved in ABA signaling as well as in SA biosynthesis and accumulation (Micol-Ponce et al., 2015), but how this can be connected to the molecular function of Pol α as a replicative polymerase remains to be fully established.

B- Polymerase δ

Plant Pol δ has been described in rice, wheat, maize, and Arabidopsis. The polymerase activity of plant DNA Pol δ has been assessed in wheat. Pol δ also contains an associated 30–50 exonuclease activity, which confers a proofreading ability, and is highly stimulated by PCNA. The Arabidopsis POLD complex contains four subunits (POLD1 to D4) whereas in rice it contains five subunits. The essential motif that mediates the interaction of POLD2 and POLD3 subunits with PCNA is conserved as well. Rice POLD1 and POLD2 genes are regulated by sucrose and UV treatment, indicating a function in environmental stresses response (Sanchez et al., 2012).

In Arabidopsis as in other eukaryotes, the deletion of the catalytic subunit from Pol δ (POL δ 1) gene is lethal. The study of *pol δ 1* hemizygous mutants (T-DNA insertions) and *POL δ 1-RNAi* lines

revealed that reduced expression levels of *POL δ1* lead to increased HR in differentiated cells which occurred during endoreduplication, and was thus unlinked from the essential function in meristematic cells. Additionally transcriptome analysis of *polδ1* hemizygous lines showed almost no changes in gene expression with only three genes that were significantly mis-regulated compared to wild-type plants. Furthermore these mutant plants were not found to be hypersensitive to genotoxic agents. The increased HR in somatic cells and the lack of up-regulation of genes involved in DNA repair, suggest that reduction of *POLD1* may interfere with replication in a DNA structure-specific manner: fork stalling would be particularly likely at repeats, leading to the preferential occurrence of HR in these regions of the genome (Schuermann et al., 2009).

Recently, a viable thermosensitive mutation in Arabidopsis *POLD1* has been described. This mutant was isolated in a genetic screen for the identification of flowering time regulators by the isolation of suppressors of *Gigantea*. The *gis5* (*gigantea* suppressor 5) flowers early and displays curly leaves. It has a point mutation in the catalytic subunit of the Arabidopsis POLδ1. This mutant is thermosensitive: under restrictive temperatures, the *gis5* allele leads to early flowering and curly leaf phenotypes, which are dependent on the *FT* gene. These plants are unable to complete development at 28° while at 18°C they are identical to the wild-type. However at 24°C *gis5* plants display increased expression of DNA replication stress marker genes, enhanced HR and ectopic expression of *SEPALLATA* (*SEP3*) due to epigenetic changes at this locus. These data suggest that Pol δ is required for proper establishment of transcriptionally active epigenetic marks during replication (Iglesias et al., 2015). By contrast, in lines with lowered *POLδ1* expression, no epigenetic changes were observed (Schuermann et al 2009). One likely explanation would be that the reduction of *POL δ1* expression in these lines is very mild, and the phenotypic consequences are therefore not as drastic as in the hypomorphic mutant. Together, these results suggest that *gis5* effects on epigenetic inheritance might result from a specific change in Pol δ activity rather than decreased levels of the protein (Iglesias et al., 2015).

Very recently, a mutation in POLD2 (*pold2-1* mutant) was isolated in a genetic screen aimed at identifying components of the Transcriptional Gene Silencing (TGS) machinery. POLD2 is an accessory subunit of DNA polymerase δ , and had not been characterized in plants because it is an essential gene. *POLD2* is highly expressed in the shoot meristem, cotyledons, and older true leaves. The *pold2-1* plants display high frequency of homologous recombination and short telomere length. They also show hypersensitivity to genotoxins such as hydroxy-urea, MMS, and cisplatin. Whole-genome bisulfite sequencing indicated that POLD2 is not involved in the regulation of DNA methylation, although its inactivation induces defects in histone mark deposition. These results suggest that POLD2 is required for maintaining genome integrity and properly establishing some chromatin marks during DNA replication (Zhang et al., 2016a).

POLD2 genetically interacts with ATR and DNA polymerase α . The *pold2-1atr* double mutant show severe defects in leaf development and fertility, whereas that the *pold2 atm* double mutant showed growth phenotypes similar to those of *pold2-1*. These results suggest that POLD2 and ATR have additive roles in controlling plant development. On the other hand, *pold2-1 pola* double mutant plants were smaller and exhibited more severe growth phenotypes than *pola* or *pold2-1* single mutants, suggesting that the two genes have additive effects on plant growth and development. In contrast, *pola* and *pol2a* do not have additive effects on plant growth, suggesting that both work in the same pathway. Finally, the *pold2-1pol2a* double mutant was similar to *pold2-1*, indicating that POLD2 has an epistasis effect on Pol ϵ for controlling plant development (Zhang et al., 2016a).

C-Polymerase ϵ

Pol ϵ has been studied in Arabidopsis and rice, including genetic analyses that have not been performed in other multicellular organisms (Sanchez et al., 2012). The Arabidopsis genome encodes two isoforms of the catalytic sub-unit POL2A and POL2B, one DPB2 sub-unit (Jenik et al., 2005; Ronceret et al., 2005), two putative homologues of DPB3 and one DPB4 (Petroni et al., 2012).

Catalytic subunit (POL2A)

The catalytic subunit is highly conserved among eukaryotic species and possesses a DNA polymerase domain and proofreading exonuclease domain in the amino-terminal half of the protein. The carboxyl-terminal half of the protein is less conserved, with the exception of the two zinc fingers motifs, and is thought to be involved in cell cycle regulation during S-phase (Jenik et al 2005).

The *AtPOL2A* gene is annotated to be 15949 bp, with 48 exons, accounting for an open reading frame of 6818 bp. It encodes a protein of 2271 amino acids, with a predicted molecular mass of 261 KDa. The POL2B protein sequence is 79% identical (84% similar) to POL2A, but only POL2A is an essential gene (Jenik et al., 2005; Del Olmo et al., 2010).

POL2A is highly expressed in synchronized Arabidopsis cells and in the apical region and inflorescence while *AtPOL2B* is expressed at detectable level mostly under stress conditions (Ronceret et al., 2005; Jenik et al., 2005). Both Arabidopsis POL2 proteins possess each of the motifs necessary for a functional DNA Pol ϵ catalytic subunit, however only *POL2A* is an essential gene, suggesting that POL2A has the most prominent role during DNA replication, at least under normal growth conditions (Ronceret et al., 2005). However, analysis of double mutants revealed that POL2B function is partially redundant with POL2A at least during embryogenesis, floral transition and in meristems (Jenik et al., 2005; Ronceret et al 2005; Del Olmo 2010).

Genetic analysis revealed that the *emb529* mutants lacking POL2A are arrested at a very early stage during embryo development, which precluded detailed analysis (Ronceret et al., 2005; Jenik et al., 2005). More recently, the isolation of hypomorphic alleles of POL2A has shed more light on the biological function of Pol ϵ in plants. So date, four hypomorphic alleles for POL2A have been isolated; three of them have been found in genetic screens for phenotypes that are not directly related to DNA replication, such as embryo development (*till-4*; Jenik et al., 2005), hormonal signaling (*abo4-1*; Yin et al., 2009) and flowering (*esd7-1*; Del Olmo et al., 2010): they contain point mutation. Another viable mutant allele is a T-DNA insertion mutant (*abo4-2*; Yin et al., 2009).

The characterization of new pathways involved in embryo patterning, led to the isolation of *till-4* (*tilted*) mutant. The *till-4* plants contain two G-to-A mutations: one at position 3927 (counting from the first ATG) in exon 12 and one at position 5005 in intron 14. The former mutation changes a conserved Gly (position 472) into Arg. It is localized between catalytic domain and endonuclease domain. These plants display slowly developing embryos in which the root pole is displaced laterally. This abnormal placement of the root pole can be phenocopied by treatment of wild-type embryos with a DNA polymerase inhibitor (aphidicolin). During embryo development *till-4* mutant displays prolonged S phase, and at vegetative stage these plants show growth defects, slightly delayed flowering, and reduced fertility (Jenik et al., 2005).

The second *POL2A* hypomorphic allele was isolated by screening for mutants with altered sensitivity to ABA, *abo4* (*abscisic acid over-sensitive*) mutants; the *abo4-1* mutant line has a point mutation near the catalytic domain of the polymerase. This mutation changes Gly (position 534) to Arg (G to A in position 4171 counting from the first putative ATG, in the 13th exon). In this study, a Salk T-DNA insertion line (SALK 0963441) was also found to be viable: in the *abo4-2* mutant, the T-DNA is inserted at position 3972 (in the 12th intron), counting from the first putative ATG of the genomic coding sequence. The *abo4-2* mutant showed ABA sensitivity like the *abo4-1* mutant.

Moreover, these mutants show reduced growth, as well as disorganized meristems and early flowering, and the flowering phenotype was controlled by epigenetic changes. Moreover, *abo4* seedlings displayed hypersensitivity to DNA-damaging agents such as: MMS and UV-B. Finally, *abo4-1* mutant show enhanced homologous recombination in somatic cells and constitutive activation of DNA repair genes (Yin et al., 2009).

The *abo4-1* mutation also leads to lengthening of the S-phase of the cell cycle with a high expression of H4B in *Arabidopsis* and probable cell cycle arrest at the G2/M phase (Yin et al., 2009). Although *abo4-1* and *abo4-2* plants share several features, only *abo4-2* plants are almost completely sterile, suggesting that the *abo4-2* may affect other processes. Together, these results provided evidence for the role of Pol ϵ in epigenetic regulation, HR, and ABA signaling in plants.

The isolation of the third point mutation for *POL2A* resulted from a genetic screen for early flowering mutants. The *esd7-1* (*early in short days7-1*) mutant displays reduced growth, as well as disorganized meristems and early flowering. The *esd7-1* mutation consists of a guanine to adenine transition in the 26th exon, which substituted Gly (G) by Arg (R) in amino acid position 992, a residue located in the region between the predicted PolV and C1 domains. Detailed genetic analysis of the *esd7-1* mutant revealed the role of Pol ϵ in the replication of the genetic and epigenetic information, notably in the repression of flowering by the deposition of repressive histone marks (Del Olmo et al 2010). The FAS2 chromatin assembly factor is a sub-unit of the CAF-1 complex and is involved in chromatin packaging and DNA replication. The *fas2* mutations suppress the *esd7-1* early flowering phenotype. Expression levels of *FT* and *SOC1* (flowering activators) in the *esd7-1fas2-1* double mutant were similar to *fas2-1*. The epistatic relationship established between *FAS2* and *ESD7* indicates that in the absence of a functional CAF-1 complex, gene de-repression could not take place in *esd7-1*, suggesting that the CAF-1 complex acts downstream of ESD7, facilitating the formation of nucleosome on newly replicated DNA (Del Olmo et al 2010). On the other hand, the *esd7-1icu2-1* double mutant showed a flowering phenotype, and *FT* and *SOC1*

expression patterns similar to *icu2-1*, suggesting that both genes may regulate *FT* and *SOC1* expression through the same regulatory system (De Olmo et al 2010). Recently POL2A was reported to interact both genetically and physically with PRC2 (POLYCOMB REPRESSIVE COMPLEX 2) components such as CURLY LEAF (CFL), the catalytic subunit, EMF (EMBRYONIC FLOWER) and MSI1 (MULTICOPY SUPPRESSOR OF IRA1). A domain of the C-terminal region of POL2A mediates the binding to the different PRC2 components and this interaction is necessary for the proper recruitment of PRC2 to *FT* and *SOC1* loci, thereby regulating flowering time through a mechanism involving the deposition of repressive histone marks (Del Olmo et al., 2016).

Finally, POL2A has also been reported to play an important role during meiosis. The analysis of meiosis progression in *till-4*, *abo4-2* and transgenic plants expressing a *POL2A-RNAi* construct driven by a meiosis-specific promoter showed that the different POL2A-deficiency leads to chromosomal fragmentation, which is dependent on SPO11-1 at least for the *abo4-2* background. SPO11 is an endonuclease responsible for the formation of DSBs required for the HR-process that drives chromosome pairing during prophase I of meiosis. This report thus suggests that Pol ϵ is required for the repair of these breaks. Consistently, the authors reported that bivalent formation and formation of crossovers were altered in Pol ϵ deficient lines (Huang et al., 2015). Strikingly, the *esd7-1* and *abo4-1* plants do not display alterations during meiosis, suggesting that the effects triggered by alterations of Pol ϵ depend on which domain of the protein is affected by mutations.

Most of mutational analysis of DNA Pol ϵ has been done in yeast. Null mutants of DNA Pol ϵ cause lethality in yeast as they do in Arabidopsis. Viable mutants of Pol ϵ sub-units have been described in few multicellular organisms; the scarcity of viable replicative DNA polymerase mutant points to the essential nature of these proteins and makes Arabidopsis *pol2a* mutants a very useful tool for the detailed analysis of its functions.

DPB2

Isolation of hypomorphic alleles of *POL2A* has shed more light on the biological function of Pol ϵ in plants. By contrast, very little is known regarding the contribution of largest accessory sub-unit of Pol ϵ DPB2.

Genetic analysis revealed that mutants *cyclops2* (*cyl2*) lacking DPB2 are arrested at a very early stage during embryo development, which precluded further analysis (Ronceret et al., 2005). Like the *POL2A* gene, *DBP2* contains consensus binding sites for E2F factors in its promoter and is regulated cell cycle-regulated. Both genes display a maximal induction in the S-phase and a low but significant induction in G2/M. Moreover, DPB2 also seems be regulated at fertilization; DPB2 was activated in the embryo sac only after fertilization (Ronceret et al., 2005).

DPB3 and DPB4

In yeast, the other accessory subunits DPB3 and 4, play a role in chromatin remodeling (Pursell & Kunkel, 2008), and also contribute to the proper functioning of the replication fork (Aksenova et al., 2010). However in plants, the function of these two accessory subunits has not been determined. According to phylogenetic analyses, two putative homologues of DPB3: DPB3-1 (NF-YC10) and DPB3-2 (NF-YC13) and one homologue of DPB4 (NF-YB11) have been identified. The three proteins are part of the Nuclear Factor Y family, which are sequence-specific transcription factors with histone like subunits, with the unique characteristic that they bind DNA at CCAAT sites as heterotrimeric complexes composed of single subunits from each of three protein families: NF-YA, NF-YB, and NF-YC. The expansion of NF-Y families in plants, combined with their heterotrimeric nature, means that many possible NF-Y complexes can theoretically form. This leads to a flexible, combinatorial system of transcription factors that may allow subtle adjustments to many different environmental conditions (Petroni et al., 2012). To date, there is no experimental data supporting the role of these Nuclear Factor Y (NF-Y) factors as sub-units of DNA Pol ϵ . Indeed, the homolog for DPB3-1 in *Arabidopsis thaliana* and *Oriza sativa* (Sato et al., 2016, 2014) have been involved

in abiotic stress response. Arabidopsis DPB3 (NF-YC10) has been reported to act as a positive regulator of Dehydration-Responsive Element Binding protein 2A (DREB2A), and the overexpression of DPB3-1 was shown to enhance heat stress tolerance without growth retardation (Sato et al., 2014). Additionally AtDPB3-1 interacts with DREB2A homologues in rice and soybean and *OsDPB3-2* (the homolog of AtDPB3-1) function is conserved (Sato et al., 2016). How these roles could relate to DNA replication is unclear and these results suggest (i) that the DPB3-1 is a *bona fide* NF-Y transcription factor rather than a sub-unit of a replicative polymerase or (ii) that it is a bifunctional protein that can participate in the two processes.

Hence, although our knowledge of the function of plant replicative polymerases remains incomplete, striking similarities emerge from the genetic analyses of their functions. As expected, hypomorphic mutants deficient for either Pol α , Pol ϵ or Pol δ show defects in DNA replication, increased HR due to endogenous DNA stress and sensitivity to genotoxic stress, reflecting the essential role of all these polymerases in the maintenance of genome integrity. Likewise, genetic interaction between these three polymerases and histone modifiers has been observed, consistent with the notion that both the DNA and chromatin marks have to be faithfully duplicated during the S-phase of the cell cycle, and that coupling of the two processes likely involve DNA Polymerases themselves. The hypersensitivity of Pol ϵ and Pol α deficient lines to ABA is more of a puzzle, although Yin and colleagues have postulated that it could relate to the formation of DSBs upon exposure to ABA (Yin et al., 2009). One question that clearly remains open is the interaction of replicative polymerases with the DDR machinery, and more specifically the role of Pol ϵ in these responses. Indeed, dissecting the dual function of this polymerase in replication itself and replication stress activation is tricky, because impeding DNA replication automatically triggers DNA stress, as observed in Pol α mutants in which loss of the ATR kinase enhances the severity of developmental defects. The aim of my PhD was thus to investigate the role of DNA Pol ϵ and more specifically of its two largest sub-units POL2A and DPB2 in the control of DNA replication and

DNA stress response activation, in order to assess the conservation of its dual role in a multicellular organism. To this end, I developed genetic approaches and took advantage of the large number of available mutants to address the function of these two proteins, and their genetic interaction with components of the DDR machinery.

RESULTS

Results

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Role of the Polymerase ϵ sub-unit DPB2 in DNA replication, cell cycle regulation and DNA damage response in Arabidopsis

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Abstract

Faithful DNA replication maintains genome stability in dividing cells and from one generation to the next. This is particularly important in plants because the whole plant body and reproductive cells originate from meristematic cells that retain their proliferative capacity throughout the life cycle of the organism. DNA replication involves large sets of proteins whose activity is strictly regulated, and is tightly linked to the DNA damage response to detect and respond to replication errors or defects. Central to this interconnection is the replicative polymerase DNA Polymerase ϵ (Pol ϵ) which participates in DNA replication *per se*, as well as replication stress response in animals and in yeast. Surprisingly, its function has to date been little explored in plants, and notably its relationship with DNA Damage Response (DDR) has not been investigated. Here we have investigated the role of the largest regulatory sub-unit of Arabidopsis DNA Pol ϵ : DPB2, using an over-expression strategy. We demonstrate that excess accumulation of the protein impairs DNA replication and causes endogenous DNA stress. Furthermore, we show that Pol ϵ dysfunction has contrasting outcomes in vegetative and reproductive cells and leads to the activation of distinct DDR pathways in the two cell types.

INTRODUCTION

In all living organisms, DNA replication is the fundamental process that faithfully duplicates the genome prior to its distribution between daughter cells during cell division. Plants continuously form new organs throughout their life cycle thanks to meristematic cells that retain their proliferative capacity and also give rise to reproductive cells relatively late in the life of the plant. One mechanism that has been proposed to avoid extensive accumulation of replication errors in meristems is the presence of slowly dividing cells in its centre that divide less frequently than their neighbours, and can thus function as a reservoir of cells in which genome integrity is preserved (1). However, it is also possible that plant-specific mechanisms exist to avoid the accumulation of errors during DNA replication.

In both prokaryotes and eukaryotes, the DNA replication machinery is a complex and dynamic structure called the replisome. The eukaryotic replisome comprises an 11 subunit helicase complex and replicative DNA polymerases (2). The helicase activity is brought by the MCM2-7 (Mini Chromosome Maintenance) heterohexamer that forms a ring unwinding unreplicated DNA. To be activated, the MCM complex needs to associate with the GINS (consisting of four proteins (Sld5-Psf1-Psf2-Psf3) named for the Japanese 'go-ichi-ni-san', which means 5-1-2-3) and CDC45. All together these sub-units form the CMG complex (CDC45, MCM, GINS). Once the replication fork is opened by the CMG, chromosomes are replicated by the DNA polymerase α /primase complex that synthesizes primers for the leading strand and Okazaki fragments, and Polymerases δ and ϵ that are thought to elongate these primers (2), but whose respective roles at the fork are debated. For the past few years, the generally accepted view has been that Pol δ synthesizes the lagging strand (3) while Pol ϵ is responsible for the synthesis of the leading strand (4). However, recent work suggests that polymerase δ replicates both strands, while Pol ϵ would be involved in the removal of replication errors generated by Pol δ (5), and would play an important scaffolding role at the fork.

Although it may not be responsible for DNA synthesis *per se*, DNA Pol ϵ is of particular interest because it stands at the interface between DNA replication, DNA repair, cell cycle regulation upon DNA damage and chromatin remodelling (6). In yeast and animals, it is a four sub-unit complex comprising a catalytic sub-unit (Pol2A) and three accessory sub-units DPB2, 3 and 4 (6), that are not required for the DNA polymerase activity. The largest accessory sub-unit, DPB2, is essential to cell viability and could be involved in the stabilization of the Pol ϵ complex (6). In addition, DPB2 interacts with Psf1, thereby inserting Pol ϵ in the replisome on the leading strand (7, 8). Another unique feature of Pol ϵ is its involvement in DNA stress response: yeast mutants in the C-terminal

region of its catalytic sub-unit are sensitive to Hydroxy-Urea (HU) induced replication stress and undergo catastrophic mitosis, indicating that they fail to activate the appropriate checkpoint (9). This function appears to be conserved in animals (6), but the underlying molecular mechanisms remain unclear. In addition, replication stress has been shown to induce degradation of DNA Pol ϵ , which could thus function both as a sensor and a target of DNA Damage Response (DDR) (10).

DDR has been studied into detail in yeast and animals. Briefly, two main protein kinases ATM (Ataxia Telangiectasia Mutated) and ATR (ATM and Rad3 related) are involved respectively in the perception of double-strand breaks and single stranded DNA. Once activated, they trigger a phosphorylation cascade leading to the activation of p53, a transcription factor which in turn stimulates the expression of DNA repair genes, checkpoint factors that delay cell cycle progression etc... (11). Because fork stalling can lead to the dissociation of the helicase complex from the polymerases and thus formation of large stretches of single stranded DNA, ATR is the main kinase involved in replication stress response (11). Its activation at replication forks involves the detection of single-stranded DNA coated with the RPA protein on the leading strand and activation by the 9-1-1 complex on the lagging strand (11). Interestingly, Puddu *et al* have reported that the yeast ATR homolog Mec1 can be activated by replication stress via two independent pathways, one of which requires the C-terminus of Pol2A as well as the accessory sub-unit DPB4 (12). Mechanisms involved in DNA replication and DDR appear to be conserved in plants, but are poorly described compared to animal or yeast models.

All components of the replisome and DNA polymerases are conserved in plants (13). The Arabidopsis genome encodes two isoforms of the catalytic sub-unit (Pol2A and Pol2B), one DPB2 sub-unit (14) (At5g22110), two putative homologues of DPB3 and one DPB4 (15). Genetic analysis revealed that the *emb529* or *tilted1* mutants lacking Pol2A or *cyclops2* (*cyl2*) lacking DPB2 are arrested at very early stage during embryo development, which precluded detailed analysis of the Pol ϵ function or of its interaction with DNA damage response (14, 16). However, identification of Pol2A, Pol2B and DPB2 as sub-units of DNA polymerase is corroborated by their expression in dividing tissues, as well as by the observation that treatment with the replication inhibitor aphidicolin induces similar embryo development defects as the ones observed in *Pol2A* or *DPB2* deficient mutants, and that DPB2 interacts with the C-terminus of Pol2A (14, 16). By contrast, evidence for a role of putative DPB3 or DPB4 homologues in DNA replication is lacking, and the Arabidopsis DPB3-1 protein appears to participate in the transcriptional regulation of heat-stress genes (17). More recently, the isolation of hypomorphic alleles of Pol2A has shed more light on the biological function of Pol ϵ in plants. Both *abo4* (*abscisic acid over-sensitive*) and *esd7* (*early in*

short days) mutants display partial loss of function of Pol2A and show reduced growth, as well as disorganized meristems and early flowering (18, 19), providing evidence for the role of Pol ϵ in the replication of the genetic and epigenetic information (20). In addition, *abo4* mutants show enhanced recombination and expression of DNA repair genes, indicating that the role of Pol ϵ in the perception of DNA stress during S-phase may be conserved in plants (18).

The main actors of the DNA damage response and S-phase checkpoint are also conserved in plants, although many intermediaries of the phosphorylation cascade are apparently missing (21). The *Arabidopsis* genome encodes one ATM and one ATR kinase; mutants deficient for these proteins are viable although double mutants are completely sterile (22). Like in other eukaryotes, ATM appears to be predominantly involved in double-strand break perception whereas ATR senses replication stress and induces G2 cell cycle arrest after DNA damage (22, 23). Both ATM and ATR can activate the SOG1 transcription factor, the functional homologue of p53, which in turn stimulates the expression of DNA repair genes (24). Activation of ATM or ATR by DNA damage also causes programmed induction of endoreduplication (several rounds of DNA replication without mitosis, (25)), cell cycle arrest via activation of the WEE1 protein kinase which inhibit CDK (Cyclin Dependent Kinase)/Cyclin complexes (26) and in some instances programmed cell death (27). The plant DDR and more specifically the replication stress response is thus beginning to be well described (28). Nevertheless, the relationships between DNA replication proteins such as Pol ϵ and DDR remain to be fully elucidated. In addition, very little is known regarding the contribution of accessory sub-units to this interconnection since null mutants are lethal and no partial loss of function mutant has been isolated.

In this work, we have generated over-expression lines to gain insight into the role of the largest accessory sub-unit of Pol ϵ DPB2 and its genetic interaction with DDR pathways.

MATERIAL AND METHODS

Cloning procedures

DPB2 cDNA was amplified using the DPB2 EcoRI and DPB2 XhoI stop primers and clones between the EcoRI and XhoI sites of the pENTRTM3C vector (Life Technologies). To generate the DPB2-CFP construct, the cDNA was subsequently transferred to the pB7CWG2 vector (<https://gateway.psb.ugent.be/search>) using the Gateway technology according to manufacturer's instructions. To generate a DPB2 over-expression construct without adding a tag to the protein, the cDNA was recombined in the pK7WG2 vector (<https://gateway.psb.ugent.be/search>). For *cyl2* mutant complementation, the 35S promoter of the pH7FWG2 (<https://gateway.psb.ugent.be/search>) was replaced by the *DPB2* promoter described in (14) amplified with primers introducing a HindIII and a SpeI site at its 5' and 3' ends respectively. The *DPB2* cDNA alone or the *CFP-DPB2* cDNA was subsequently cloned downstream of the *DPB2* promoter. To generate DPB2-RNAi inducible lines, a 500bp fragment of the DPB2 cDNA was cloned between the EcoRI and KpnI and ClaI and BamHI sites of the pKannibal vector. The RNAi cassette was then transferred to a modified pPZP111 downstream of the *alcA* promoter for inducible expression as described in (29). Sequence for primers is provided in Table S1.

Plant material and growth conditions

Seeds were surface-sterilized by treatment with bayrochlore for 20 min, washed and imbibed in sterile-water for 2–4 days at 4 °C to obtain homogeneous germination. Seeds were sown on commercially available 0.5x Murashige and Skoog (MS) medium (Basalt Salt Mixure M0221, Duchefa) with the appropriate antibiotic if needed and solidified with 0.8% agar (Phyto-Agar HP696, Kalys), and grown in a long days (16h light, 8h night, 21°C) growth chamber. After 2 weeks, the plants were transferred to soil in a glasshouse under short-day conditions (8 h light 20°C, 16h night at 18°C) for 2 weeks before being transferred to long-day conditions. For selection of *DBP2OE* lines, seeds of the T1 generation were sown on sand and watered with a solution of glufosinate (7,5mg/L). Independent lines were allowed to self-fertilize, and homozygous lines of the T3 generation were used for all subsequent experiments, unless otherwise specified.

RNA Extraction and quantitative RT-PCR

Total RNA were extracted from seedlings with the RNeasy MiniPrep kit (Qiagen, according to the manufacturer's instructions. First strand cDNA was synthesized from 2µg of total RNA using Improm-II reverse transcriptase (A3802, Promega) according to the manufacturer's instructions.

1/25th of the synthesized cDNA was mixed with 100nM of each primer and LightCycler® 480 Sybr Green I master mix (Roche Applied Science) for quantitative PCR analysis. Products were amplified and fluorescent signals acquired with a LightCycler® 480 detection system. The specificity of amplification products was determined by melting curves. *PDF2* was used as internal control for signals normalization. Exor4 relative quantification software (Roche Applied Science) automatically calculates relative expression level of the selected genes with algorithms based on $\Delta\Delta C_t$ method. Data were from triplicates and are representative of at least two biological replicates. The sequence of primers used in this study is provided in Table S1.

Transcriptome Studies

Three independent biological replicates were produced. For each biological repetition and each point, RNA samples were obtained by pooling RNAs from more than 200 plants. Whole plantlets were collected on plants at 1.04 developmental growth stages (30), cultivated *in vitro* under long-day conditions. Total RNA was extracted as described above. RNA-seq experiment was carried out at the POPS Transcriptomic Platform, Institute of Plant Sciences - Paris-Saclay (Orsay, France). PolyA RNA was purified using the Dynabeads mRNA direct micro kit (Ambion, France). The sequencing libraries were constructed with the Ion Total RNA-Seq Kit v2 and the sequencing spheres were prepared with the Ion PI™ Template OT2 200 Kit v3 before sequencing on an Ion Proton using the Ion PI™ Sequencing 200 Kit v3 and Ion PI v2 chips (Life Technologies, France) with 520 run flows.

RNA-seq bioinformatic treatment and analysis

To allow comparisons, each RNA-Seq sample followed the same pipeline from trimming to count of transcript abundance as follows. Read preprocessing criteria included trimming library adapters and performing quality control checks using the Torrent suite (Version 4.2.1) with default settings. The reads corresponding to rRNAs were identified by mapping on *A. thaliana* rRNAs using bowtie version2 (with `-local` option) (31) and removed. The same software was used to align the remaining reads against the *A. thaliana* transcriptome (33 602 mRNA from TAIR 10 (32)) without ambiguous hits (multi-hits are removed). According to these rules, around 75% of the initial reads aligned to transcripts for each sample. Genes which do not have at least 1 read after a counts-per-million (CPM) normalization in at least three samples among the six were discarded. The differential analysis has been performed by using a likelihood ratio test in a negative binomial generalized linear model where the dispersion is estimated by the method proposed in edgeR and where a biological replicate effect was taken into account. A gene was declared differentially expressed if its

raw p-value adjusted by the Benjamini-Hochberg procedure to control the FDR is lower than 0.05. Analyses were performed with the software 'R' (Version 3.1.0) and the edgeR package (version 3.6.8) of Bioconductor.

Light and fluorescence microscopy

Fresh siliques were opened under a stereo-microscope (SVII, ZEISS) and images were captured with a colour CCD camera (Power HAD, Sony).

For meiotic analyses, flower buds were fixed in EtOH : Acetic Acid (3 :1). 4',6-Diamidino-2-phenylindole staining of meiotic chromosomes was performed according to a previously described method (33). Slides were observed on an epi-fluorescence videomicroscope (SVII; Zeiss), and images were captured with a colour charge-coupled device camera (Power HAD; Sony).

For cell cycle length analysis, we used a method adapted from (34). Plants were grown on supplemented MS medium (10 g L⁻¹ sucrose, 0.1 g L⁻¹ myo-inositol, 0.5 g L⁻¹ MES, 100 µl thiamine hydrochloride (10 mg mL⁻¹), 100 µl pyridoxine (5 mg mL⁻¹), 100 µl nicotinic acid (5 mg mL⁻¹), pH 5.7, adjusted with 1 M KOH, and 10 g L⁻¹ agar) for 5 days, and transferred to the same medium supplemented with EdU (10 µM). Samples were collected after 3, 6, 9 and 12h, fixed in paraformaldehyde (4% in PME buffer: 50 mM piperazine-N,N'-bis(2-ethanesulphonic acid) (PIPES), pH 6.9; 5 mM MgSO₄; 1 mM EGTA) for 45 min and washed with PME buffer. Root apices were dissected on a glass slide and digested in a drop of enzyme mix (1% (w/v) cellulase, 0.5% (w/v) cytohelicase, 1% (w/v) pectolyase in PME) for 1h at 37°C. After three washes with PIPES, root apices were squashed gently between the slide and a coverslip, and frozen in liquid nitrogen. After removal of the coverslip and drying of the slides for 1h at room temperature, EdU revelation and Hoechst counterstaining were performed as described in (35). The percentage of EdU positive nuclei was plotted as a function of time. The percentage of EdU positive nuclei increases linearly with time, and follows an equation that can be written as $y = at + b$ where y is the percentage of EdU positive nuclei and t is time. Total cell cycle length is estimated as 100/a, and S phase length is b/a.

Detection of γ-H2AX foci by immunostaining was performed as described previously (36).

Flow cytometry

For flow cytometric nuclei analysis, tissues were chopped with a razor blade in 1 mL of Gif nuclei-isolation buffer (45 mM MgCl₂, 30 mM sodium citrate, 60 mM MOPS, 1% (w/v) polyvinylpyrrolidone 10,000, pH 7.2) containing 0.1% (w/v) Triton X-100, supplemented with 5 mM sodium metabisulphite and RNase (5 U/ml). Propidium iodide was added to the filtered

supernatants to a final concentration of 50 µg/ml. Endoreduplication levels of 5,000-10,000 stained nuclei were determined using a Cyflow SL3 flow cytometer (Partec-Sysmex) with a 532-nm solid state laser (30 mW) excitation and an emission collected after a 590-nm long-pass filter. For cell cycle analysis, we used the algorithm available in the Flomax software.

For EdU incorporation analysis, platelets were incubated on MS supplemented with EdU (10µM) for 7h. Nuclei were extracted as described above, pelleted by centrifugation (5 min at 1000g). The revelation reaction was performed as described in (35) and analysed on a Moflo Astrios flow cytometer (Beckman-Coulter).

Histochemical staining of GUS activity

After 15-min fixation in 100 % cold acetone, β-glucuronidase (GUS) activity was revealed as described previously (37). After 1 h at 37°C, samples were washed in 70% ethanol, fixed with PFA during 20 min under vacuum, and then cleared using chloral hydrate solution overnight at room temperature (8 g of chloral hydrate (Sigma), 2 ml of 50% glycerol and 1 ml of water). Images were captured on a macroscope (AZ100, NIKON) with a video camera Nikon RI1.

Statistical analysis

All statistical analysis was performed using the R software (<https://www.r-project.org/>).

RESULTS

Molecular and morphological characterization of the DPB2 over-expressing lines

Dpb2 null mutants are lethal (14), and partial loss-of-function lines are not available. To decipher the biological function of DPB2, we thus generated lines over-expressing the DPB2 protein fused to CFP under the control of a 35S promoter (hereafter referred to as *DPB2OE*). In the T1 generation, *DPB2OE* plants displayed severe developmental defects including reduced stature and partial or complete sterility depending on the severity of the phenotype (Figure 1A). Three independent over-expressor lines were selected for further analysis, and all subsequent experiments were performed on seeds of the T3 generation. These lines were selected because they were representative of the different classes of phenotype observed, *DPB2OE 1* being the most, and *DPB2OE 3* the less severely affected (Figure 1B). The degree of phenotypic alterations during development correlated with *DPB2* transcript accumulation (Figure 1C). In addition to reduced rosette size and stem height, root growth was inhibited in these lines, similarly to that observed in the *abo4-1* mutant which is deficient for the catalytic sub-unit of Pol ϵ ((18), Figure 1D). As shown on Figure S1A-B, the CFP-DPB2 fusion expressed downstream of the *DPB2* promoter could complement the *cyl2* mutant which is deficient for DPB2 (14), indicating that the tagged version of DPB2 is functional. Identical phenotypes were observed in lines over-expressing DPB2 without tag (Figure S1C), further confirming that the observed defects are due to excess accumulation of a functional DPB2 protein.

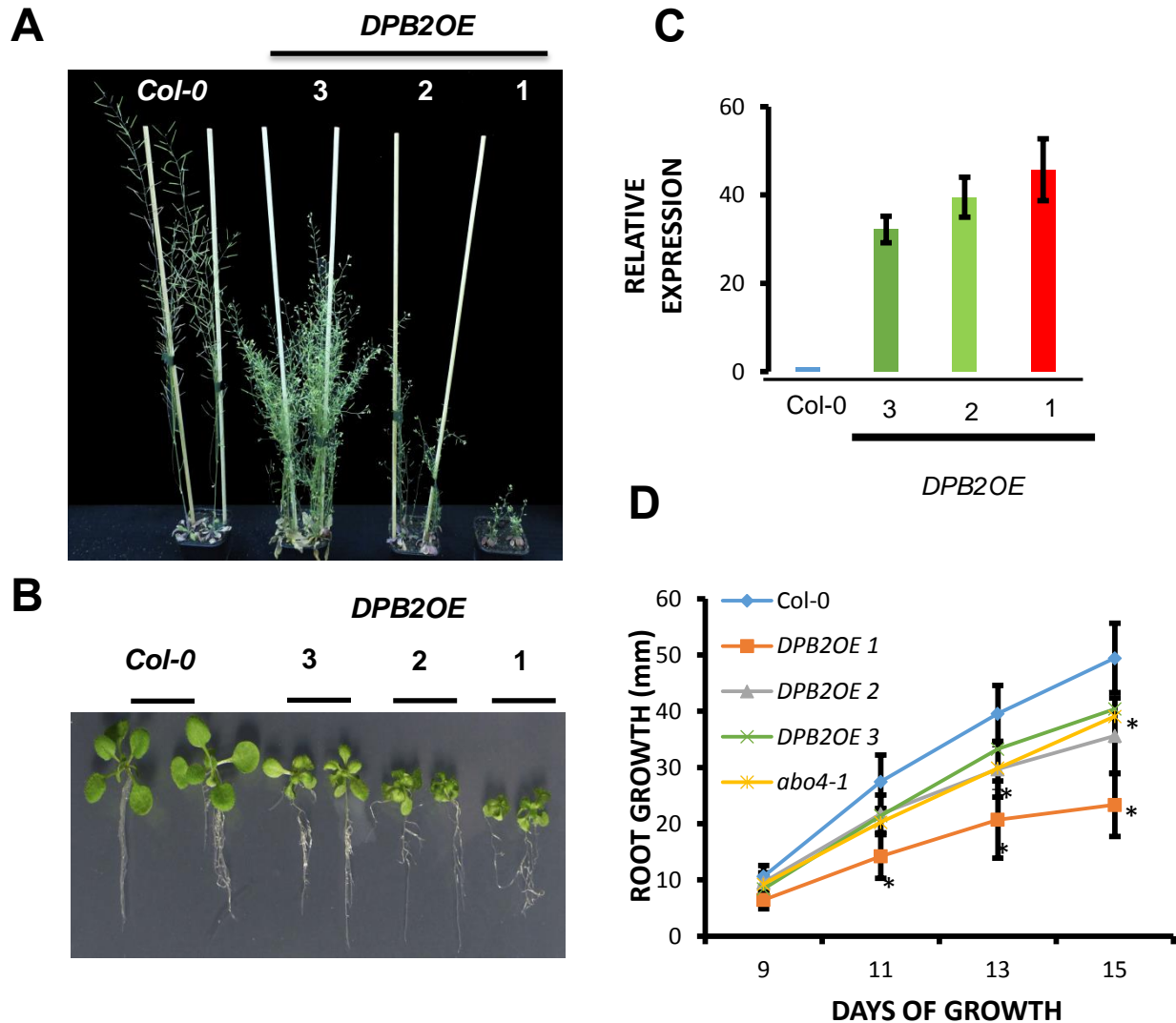


Figure 1: *DPB2* over-expression inhibits plant growth.

A: Six-week-old plants display severe dwarfism compared to the wild type.

B: Seedlings of 14 days-old of wild-type (*Col-0*) and three independent *DPB2OE* lines.

C: Quantification of *DPB2* expression in the wild-type (*Col-0*) and three independent *DPB2OE* lines by RT-qPCR. Results are average \pm standard deviation from three technical replicates and are representative of two independent experiments.

D: Root growth is reduced in *DPB2OE* lines. Wild-type (*Col0*) and *DPB2OE* lines were grown vertically for 2 weeks, and root length was measured every second day. The *abo4-1* mutant (Yin et al, 2009) that is deficient for the catalytic sub-unit of Pol e was included as a control. Growth reduction was similar in *abo4-1* and in *DPB2OE* 2 and 3, but more pronounced in *DPB2OE1*. Error bars indicate the SD between three biological replicates with 20 seedlings each. Asterisks indicate significant differences respect to wild type plants (Student t test: $P < 0.05$).

Plants displaying the most severe phenotype (*DPB2OE1*, Figure 1A) in the T1 generation displayed reduced fertility, and some were completely sterile. The latter lines displayed few pollen grains of very heterogeneous size, which prompted us to analyse meiosis progression in wild-type (Figure 2A-E) and these *DPB2OE* lines (Figure 2F-I). In the wild-type chromosomes condense during prophase I to form bivalents (Figure 2A, B). Homologous chromosomes segregate during the first division (Figure 2C), and sister chromatids segregate during the second (Figure 2D) to form tetrads (Figure 2E). Although the early steps of meiosis appeared normal in *DPB2OE* (Figure 2F), bivalents were never identified. Instead, we observed severe chromosome fragmentation both during the first and the second meiotic division (Figure 2G-H) resulting in the formation of polyads (Figure 2I) that contained unequal amounts of fragmented chromosomal material. During meiosis, the SPO11 endonuclease produces programmed double-strand breaks (DSB) to initiate homologous recombination, cross-over formation and thereby chromosome pairing (38). To determine whether this fragmentation was due to defects in the repair of these DSB we introduced the *DPB2OE* construct in the *spo11-1* mutant (38). Because this mutant does not form DSB, homologous chromosomes do not pair and segregate randomly during the first meiotic division (Figure 2 J), resulting in the formation of polyads containing random combinations of chromosomes (Figure 2L-M). Because Arabidopsis has few chromosomes, this abnormal meiosis does not result in complete sterility: 1 gamete in 32 is expected to contain exactly one chromosome of each pair. Chromosome fragmentation was identical in *spo11-1 DPB2OE* lines to that observed in *DPB2OE* lines with apparently normal early prophase (Figure 2N-Q). The failure of the *spo11-1* mutation to rescue *DPB2OE*-induced fragmentation was also reflected in the enhanced sterility of *spo11-1 DPB2OE* (Figure S2), suggesting that DNA fragmentation occurred before SPO11 activation, possibly due to defects in pre-meiotic DNA replication.

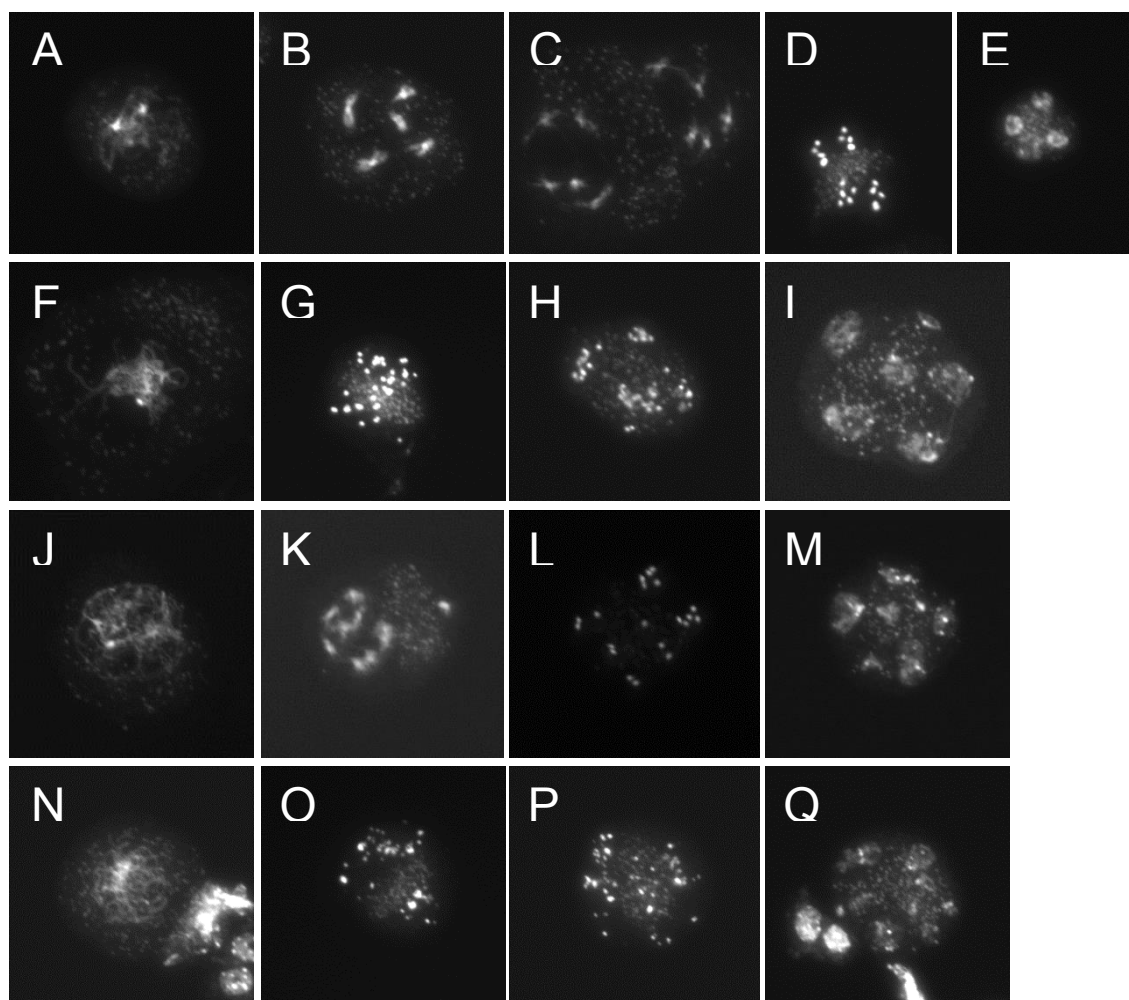


Figure 2: *DPB2* over-expression causes *SPO11*-independent DNA fragmentation during meiosis.

Meiosis was observed in WT (A-E), *DPB2OE* (F-I), *spo11* (J-M) and *spo11 DPB2OE* (N-Q) lines. In the wild-type, chromosomes begin to condense and pair during prophase I (A), until they form five bivalents displaying chiasmata (B). The first meiotic division segregates five homologue chromosome at each cellular pole (C), and sister chromatids segregate during the second meiotic division (D), resulting in the formation of tetrads (E). By contrast, in *DPB2OE*, although early steps of meiosis appeared normal (F), bivalents were never observed. Instead, severe chromosome fragmentation was observed both during the first (G) and the second meiotic division (H), resulting in the formation of polyads (I). In the *spo11* background, early steps of prophase I proceed normally (J), but chromosomes fail to pair due to the absence of double-strand breaks required to trigger the formation of crossing-overs. Instead, the first meiotic division randomly segregates 10 univalents (K). Subsequent segregation of sister chromatids during the second division (L) results in the formation of polyads (M), although a small number of balanced gametes are formed. Meiosis in *spo11 DPB2OE* lines was identical to what was observed in *DPB2OE* lines with apparently normal early prophase (N), severe chromosome fragmentation visible both during D1 and D2 (O, P) and formation of polyads (Q). Bar in A = 10 μ m for all panels

DPB2 over-expression affects DNA replication and cell cycle progression

To evaluate whether the observed growth reduction of DPB2 over-expressing plants might be caused by defects in DNA replication, we first tested the sensitivity of *DPB2OE* lines to aphidicolin, an inhibitor of the B-family polymerases comprising the three replicative polymerases: α , δ , and ϵ (39). We included the *abo4-1* mutant in this test as a control. Both genotypes were hyper-sensitive to aphidicolin compared to the wild-type: they displayed chlorotic leaves (Figure 3A, B) and reduction of root length (Figure 3C, D), indicating that *DPB2* over-expression, like the *abo4* mutation, impairs DNA replication. To corroborate this hypothesis, we generated ethanol-inducible RNAi lines targeting the *DPB2* transcript. Prolonged growth on ethanol resulted in complete growth arrest, but on low doses of ethanol plants could be maintained for a few days and, we observed a reduction of root elongation comparable to the one observed in *DPB2OE* lines (Figure S3). Furthermore, plants over-expressing DPB2 showed a clear acceleration of flowering time as described for the Pol2A mutant *esd7*, providing further evidence for partial complex inactivation caused by excess DPB2 accumulation (Figure S4).

To further analyse the DNA replication defects caused by *DPB2* over-expression, we monitored cell cycle progression in *DPB2OE* lines. We first performed flow cytometry analysis on cauline leaves of *DPB2OE* lines. Endoreduplication was increased in all *DPB2OE* lines (Figure 4A), regardless of the severity of the phenotype, indicating that *DPB2* over-expression induces extra rounds of DNA replication in developing organs. In addition, we reproducibly observed that flow-cytometry profiles obtained on lines displaying a severe phenotype showed poorly separated peaks compared to the wild-type (Figure 4B-C), suggesting that a higher proportion of nuclei contained intermediate DNA contents. To determine whether this phenomenon could be due to an increase in the proportion of S-phase nuclei, the cell cycle distribution of nuclei extracted from young flower buds was analysed. The proportion of S-phase cells was higher in *DPB2OE* lines, and the proportion of G1 cells was reduced (Figure 4D-E). This was reproducibly observed on independent samples (Figure 4F). To confirm that the proportion of nuclei in S-phase is increased in *DPB2OE* lines, we used ethynyl deoxyuridine (EdU) incorporation; EdU is a thymidine analogue that can be incorporated into genomic DNA during S-phase. The results obtained by EdU incorporation combined with flow-cytometry analysis of whole seedlings were consistent with an increased proportion of S-phase cells in *DPB2OE* lines (Figure 4G).

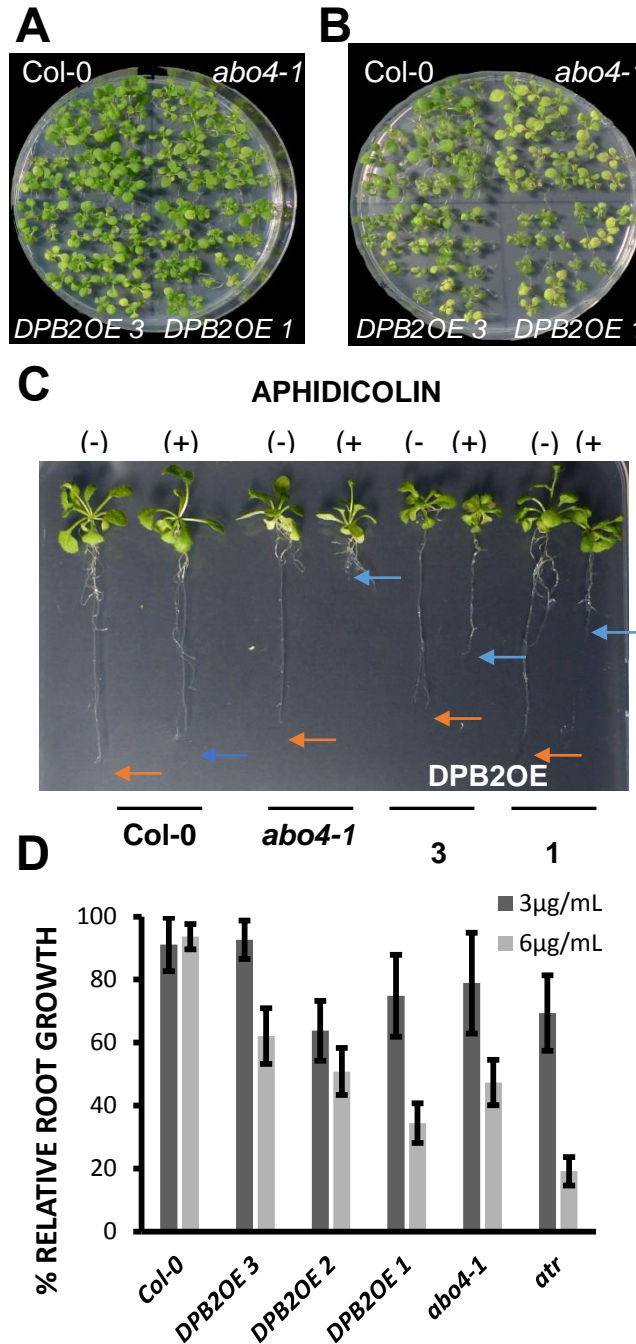


Figure 3: *DPB2OE* lines are hypersensitive to the replication inhibitor aphidicolin.

A, B: Four-day-old seedlings of the wild-type (Col-0), *DPB2OE* and *abo4-1* mutants were transferred for 7 days to half strength MS medium supplemented with aphidicolin 6μg/mL (B) or DMSO (A).

C: Growth of seedlings after of 14 days on mock (DMSO) or aphidicolin 6μg/mL.

D: Relative root growth of seedlings after a 14-day of growth on aphidicolin. The results are showed as the percentage with respect of the corresponding mock genotypes. Data represent mean ± SD of three independent experiments (n=25 for each experiment), the asterisks denote significant difference with respect to wild type plants with the same treatment (Student t test: P<0.05).

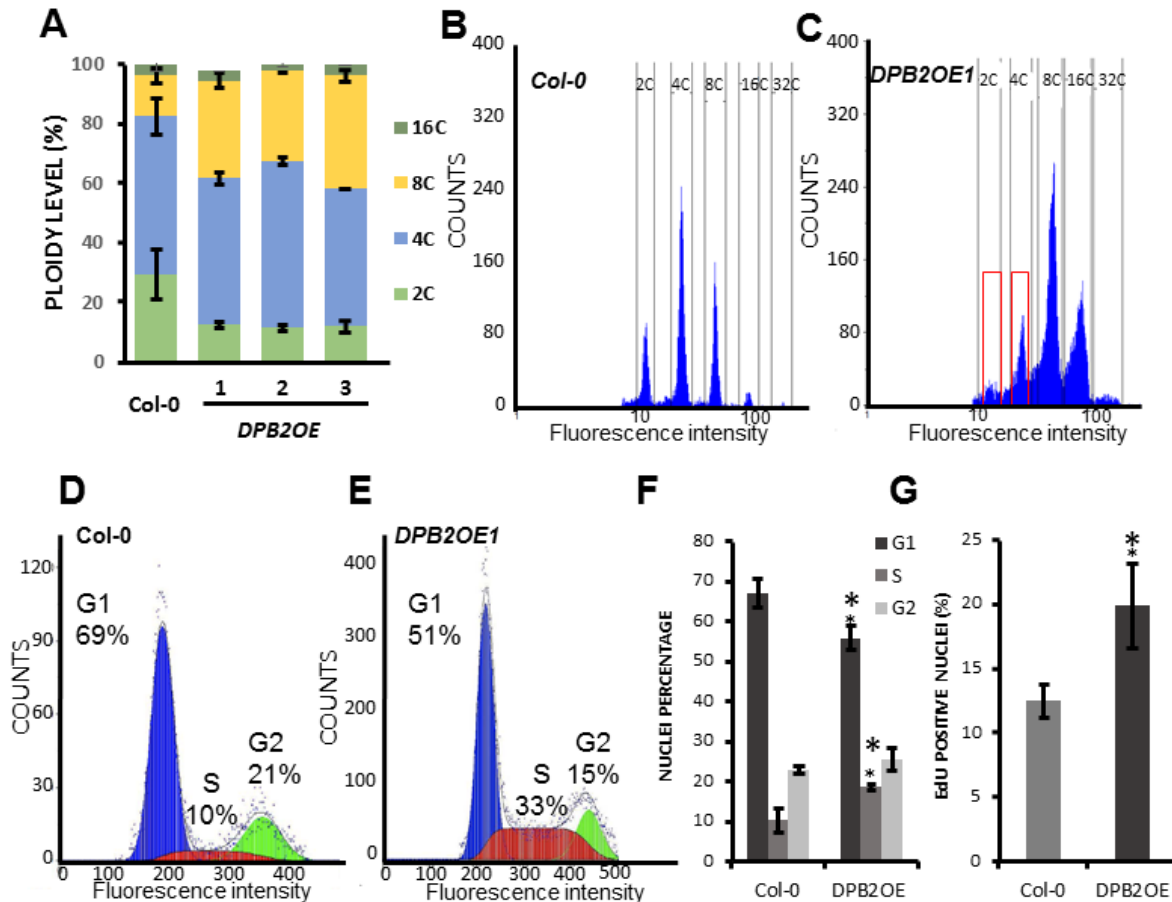


Figure 4: S-phase progression is impaired in *DPB2OE* plants.

A: Endoreduplication is increased in *DPB2OE* lines. The DNA content of nuclei extracted from the first cauline leaf of wild-type (Col-0) and three independent *DPB2OE* lines was analyzed by flow cytometry. All lines displayed an increased proportion of 8C nuclei and a decreased proportion of 2C nuclei, irrespective of the severity of the phenotype. Values are average \pm SD (n=3).

B, C: Flow-cytometry profiles obtained in Col-0 (B) and one *DPB2OE* line (C). The number of events (count) is plotted against fluorescence intensity (log scale). Peaks corresponding to different DNA contents are poorly separated in *DPB2OE* plants. Graph presented here are representative of more than 10 independent observations in three independent *DPB2OE* lines.

D, E, F: The proportion of S-phase nuclei is increased in *DPB2OE* lines. Nuclei were extracted from flower buds of the wild-type (Col-0) and *DPB2OE* lines, stained with propidium iodide and their DNA content was measured. A cell cycle was then fit on the profile, displaying G1 nuclei in blue, S nuclei in red and G2 nuclei in Green. The proportion of each type of nuclei is indicated in the graph (D-E), data are representative of 10 independent observations. F: proportion of S-phase nuclei in flower buds of wild-type Col-0 and *DPB2OE* lines, data are average \pm S.D. (n=3 independent lines).

G: EdU incorporation is increased in *DPB2OE* lines. Seven-day-old plantlets were incubated for 3h on 0.5x MS containing EdU, and the proportion of labelled nuclei was quantified by flow cytometry (n= 10000), the result is the average of three independent *DPB2OE* lines. For F and G, the asterisks denote significant differences with respect to wild type plants (Student t test; $P < 0.05$).

The observed increase in the proportion of S-phase cells could either be due to enhanced proliferative activity, or to a prolongation of S-phase duration. To discriminate between these two possibilities, we estimated cell cycle and S-phase length as described in (34) by following EdU incorporation as a function of time in root meristems of wild-type, *DPB2OE1* and *DPB2OE2* lines (Figure S5). As shown in Table I, total cell cycle length was increased in both *DPB2OE* lines. S-phase length was about twice that of the wild type for both lines. However, the increase of total cell cycle length was systematically higher than that of S-phase duration, indicating that another cell cycle phase (likely the G2 phase) was prolonged by *DPB2* over-expression. Furthermore, a similar increase in S-phase and cell cycle length was observed in *DPB2-RNAi* lines grown in the presence of ethanol for 3 days (Figure S6), confirming that this increase in S-phase length is due to partial loss of Pol ϵ function.

In summary, these results suggest that *DPB2* over-expression affects cell cycle progression leading to an increase in S-phase length, possibly due to checkpoint activation.

Table I: Cell cycle length is increased in *DPB2OE* lines.

Cell cycle and S-phase length were estimated as described in (34) by following EdU incorporation for 12h in root tip cells. Values are for two independent experiments.

Line	Cell cycle length (h)		S-phase length (h)	
Col-0	19,5	20,4	3,7	3,3
DPB2OE1	26,3	27,2	7,4	7,3
DPB2OE2	24,4	23,3	6,7	6

DPB2 over-expression induces symptoms of DNA damage accumulation.

To evaluate whether delay in the cell cycle progression observed in *DPB2OE* lines could be due to constitutive activation of the DDR, we analysed the presence of DNA damage hallmarks. Indeed, several mutants subjected to endogenous DNA stress show enhanced endoreduplication (28) which was the case in *DPB2OE* (Figure 4A). In addition, DNA stress often leads to increase expression of *CYCB1;1*. Consistently, in the absence of HU, the *CYCB1;1::DB-GUS DPB2OE* lines showed increased GUS staining in proliferating tissue compared to the wild-type (Figure S7). This observation suggests that *DPB2* over-expression delays the G2/M progression possibly due to DNA damage stress. Furthermore, a number of genes that are upregulated in response to DNA damage such as *RAD51*, *CYCB1;1* or *PARP2* (23) were up-regulated in *DPB2OE* lines (Figure 5A).

To further investigate the effects of *DPB2* over-expression, the transcriptome of *DPB2OE* lines 1 and 3 was compared to that of wild-type plants by RNA sequencing. Overall 45 genes were significantly up-regulated in line 1, and 455 in line 3 whereas numbers of down-regulated genes were 319 and 640 respectively (fold change ≥ 1.5 P value ≤ 0.01 ; Table S2). There was significant overlap between up-regulated genes in the two lines (Figure S8A) and gene ontology analysis of significantly up-regulated genes in the two lines revealed over-representation of the “DNA metabolic process” category (Figure S8B). Consistently, among a set of 61 genes identified before as DNA stress hallmark genes (23, 40), 28 were up-regulated in *DPB2OE* plants considering both RNA sequencings (Table II). Although not all genes reported were up-regulated in both cases, RT-qPCR analysis revealed for example, that *RAD51* was up-regulated in all *DPB2OE* lines analysed (Figure 5A), and that failure to detect it as being mis-regulated in one of the lines was likely due to the heterogeneity of the replicates. To further corroborate that Pol ϵ dysfunction systematically results in activation of the DDR, expression of *PARP2*, *RAD51*, *XRI-1* and *BRCA1* was also monitored in *abo4-1* mutants and in inducible *DPB2-RNAi* lines (Figure 5B-C). As expected, all these genes were up-regulated in *abo4-1*, consistent with previous results (18). All 5 genes were also induced in both RNAi lines in the presence of ethanol, indicating that these lines have similar defects to *DPB2OE* plants.

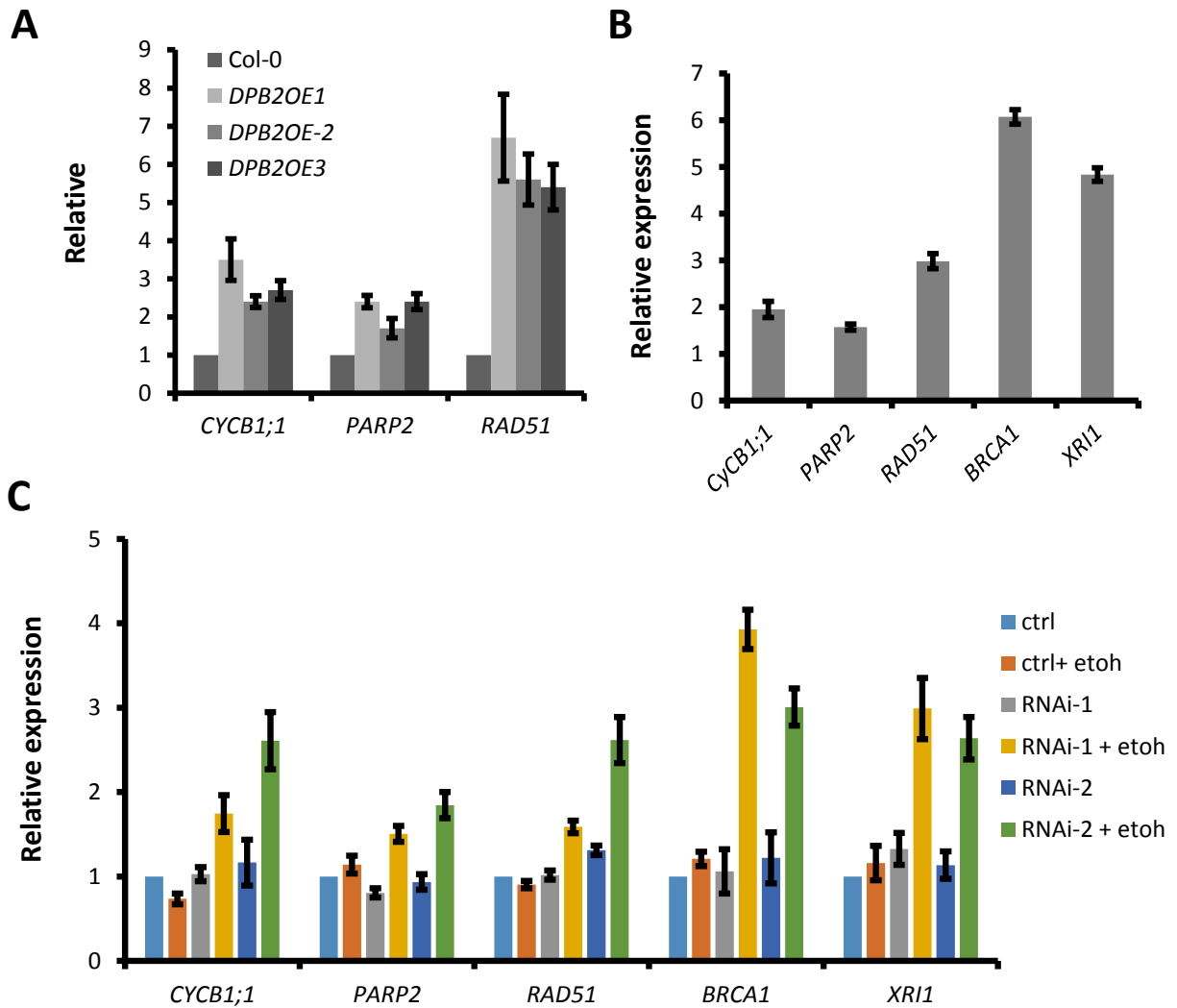


Figure 5: *DPB2OE* plants display symptoms of DNA damage accumulation.

A: Transcript levels of DNA damage-induced genes *CYCB1*, *PARP2*, and *RAD51* in *DPB2OE* flowers were measured by quantitative RT-PCR and normalized to *PDF2*. B-C: Transcript levels of DNA damage-induced genes *CYCB1*, *PARP2*, *RAD51*, *BRCA1* and *XRI-1* in *abo4-1* seedlings (B) or *DPB2-RNAi* seedlings (C) were measured by quantitative RT-PCR and normalized to *PDF2*. For all panels, values are normalized with respect to wild-type (Col-0). In C, expression was monitored in all genotypes both in absence and in the presence of ethanol. Values are average \pm SD from three technical replicates and are representative of two independent experiments

Table II: DNA stress hallmark genes induced in *DPB2OE* seedlings compared with wild type.

- : no significant difference.

Locus	Description	<i>DPB2OE 1</i> Fold change	<i>DPB2OE3</i> Fold change
AT4G29170	Mnd1 family protein	2,3	2,6
AT3G27060	TSO2	1,9	2,5
AT4G02390	PARP-2	2,4	2,0
AT5G24280	Gamma-irradiation and mitomycin c induced 1	2,4	2,3
AT5G48720	X-ray induced transcript 1 (XRI1)	2,1	2,3
AT4G21070	Breast cancer susceptibility 1 (BRCA1)	2,8	3,3
AT2G30360	SOS3-interacting protein 4	2,2	2,3
AT5G61000	Replication protein a 1D (RPA1D)	1,9	1,8
AT4G19130	Replication protein A 1E (RPA1E)	2,0	2,3
AT5G23910	Kinesin-related, ComEA domain	2,1	1,8
AT2G21790	Ribonucleotide reductase 1 (RNR1)	1,7	1,7
AT3G07800	Thymidine kinase 1a (TK1a)	2,8	3,7
AT4G22960	Protein of unknown function (DUF544)	9,9	13,0
AT3G27630	Siamese-related 7 (SMR7)	5,0	9,9
AT5G60250	zinc finger (C3HC4-type RING finger) family protein	4,2	4,6
AT1G08260	Catalytic subunit of polymerase epsilon (POL2A)	1,8	-
AT5G03780	TRF-LIKE 10	2,4	-
AT1G20750	RAD3-LIKE	10,0	-
AT4G24610	unknown protein	1,8	-
AT5G55490	SMC1-related, SbcC-related, ZipA-related	6,4	-
AT5G20850	RAD51	-	2,5
AT4G25580	stress-responsive protein-related	-	3,6
AT5G64060	NAC domain containing protein 103	-	2,9
AT1G05490	Chromatin remodeling 30	-	2,4
AT5G67460	O-Glycosyl hydrolases family 17 protein	-	2,0
AT3G45730	unknown protein	-	2,9
AT1G07500	Siamese-related 5 (SMR5)	-	17,3

To determine if the constitutive activation of DDR genes in *DPB2OE* lines was caused by accumulation of DNA damage, we performed *in situ* immuno-staining experiment in *DPB2OE* root tip nuclei using γ -H2AX antibodies directed against the Arabidopsis protein (41, 42). As expected, no foci were detected in root tips of wild type plants. In contrast *DPB2OE* roots displayed γ -H2AX foci (Figure 6). We observed about 22% of nuclei containing at least one γ -H2AX focus in *DPB2OE 1* and 6% in *DPB2OE 2*. These results suggest that *DPB2OE* plants present endogenous DNA damage leading to constitutive activation of DDR.

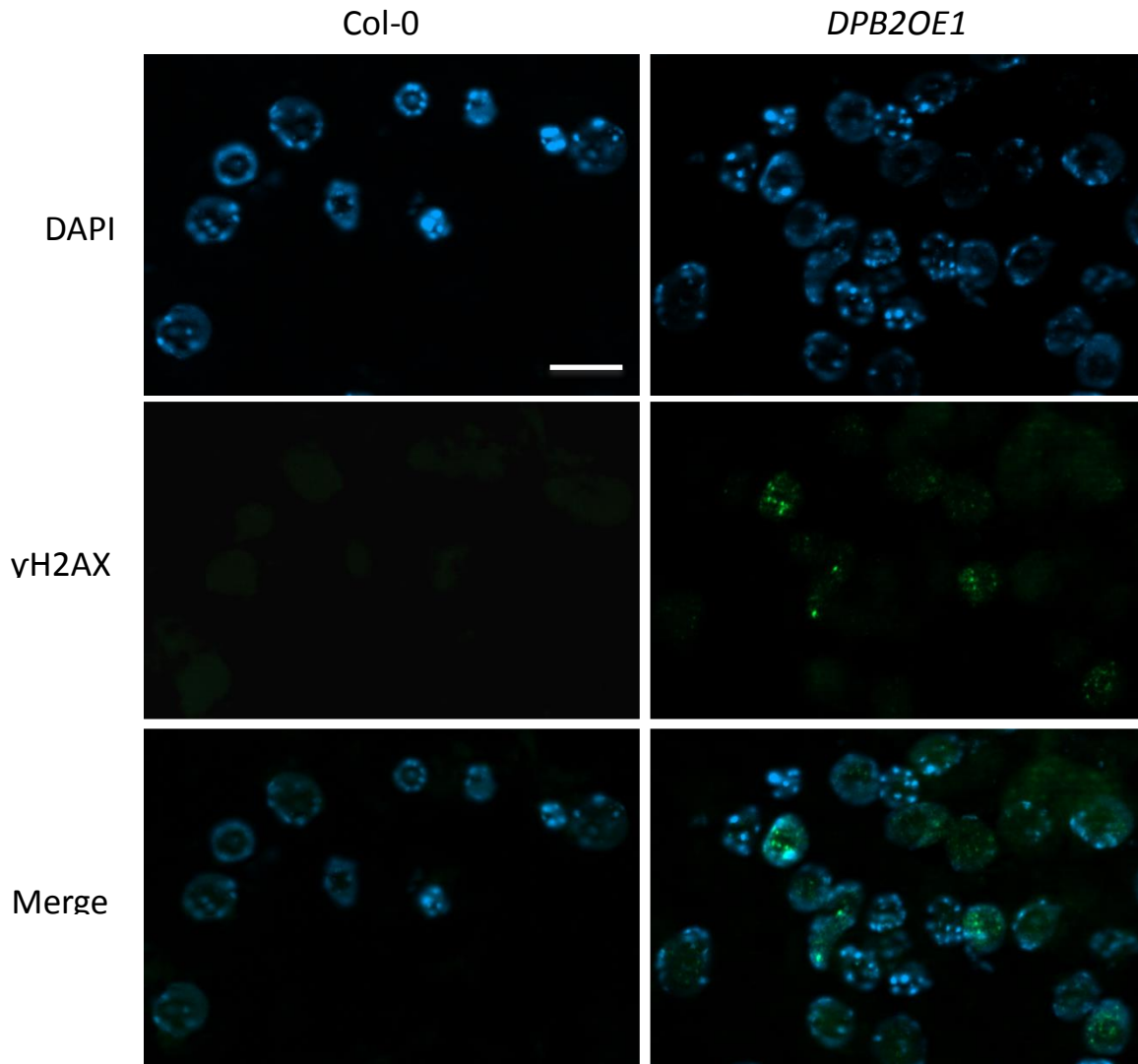


Figure 6: Detection of γ -H2AX immunofluorescence in root tip nuclei.

No foci were detected in Col-0 (left panel) plants, while *DPB2OE* nuclei displayed foci (right panel). DNA was stained with DAPI (blue), γ -H2AX foci are coloured in Green, and merged images overlay γ -H2AX foci onto nuclei. Bar = 10 μ m for all panels

Tolerance to DNA damage is increased in *DPB2OE* plants

The *abo4-1* mutant has been reported to be hypersensitive to DNA-damaging agents (18), indicating that the catalytic sub-unit of Pol ϵ is required for efficient DDR. To investigate the role of DPB2 in this process, we performed DNA damage sensitivity assays with *DPB2OE* seedlings. *atr* and *atm* mutants that are known to be sensitive to replicative stress and DSBs respectively were included as controls in our assays.

We first investigated the response to replication fork stalling. To this end, plants were exposed to different concentrations of hydroxyurea (HU), which is an inhibitor of ribonucleotide reductase. HU treatment depletes cellular deoxyribonucleotide pools, and thereby induces stalling of replication forks. The sensitivity to DNA stress was determined by assessing the proportion of plants displaying true leaves after 10 days of incubation on HU (43). This proportion was higher in all three *DPB2OE* lines than in the wild-type (Figure 7A-B), whereas the *atr* mutant showed increased sensitivity as expected. Because HU induces oxidative stress in the leaves (40), the effect of HU on root development was also monitored. Roots of *DPB2OE* displayed a lower growth-inhibition compared to the wild type (Figure 7C). Although the observed difference was relatively modest, it was statistically significant; suggesting that *DPB2OE* seedlings show increased tolerance to replication fork stalling. Since HU does not directly damage DNA, we also assessed the sensitivity of *DPB2OE* lines to a range of DNA-damaging agents. We used UV-C, the alkylating agents mitomycin C and methyl methanesulfonate (MMS) that produce both mutagenic and replication blocking lesions (44) and zeocin that intercalates DNA and directly cleaves it (45). *DPB2OE* lines were tolerant to all genotoxic stresses tested (Figure S9), although the tolerance was more pronounced in the case of UV than upon DSB-inducing treatments. Together our results indicate that at variance with deficiency in the catalytic sub-unit that results in hypersensitivity to all kinds of DNA damage, DPB2 over-expression induces a mild but statistically robust increase in DNA damage tolerance.

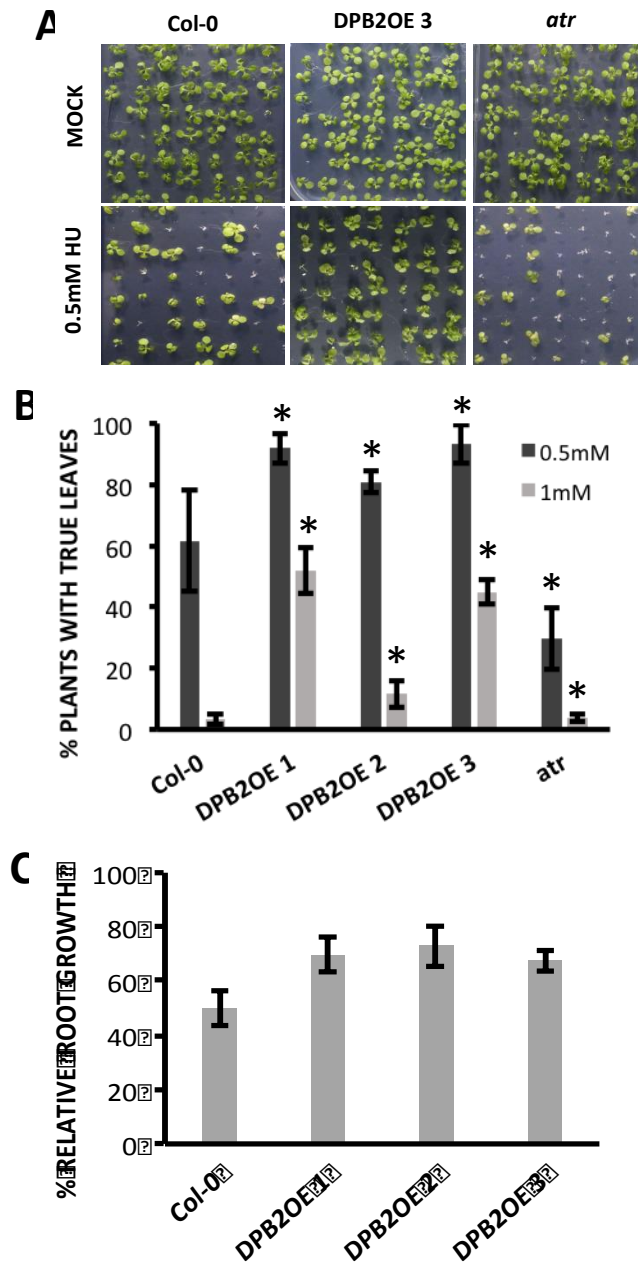


Figure 7: DPB2 over-expression enhances tolerance to replication stress.

A: Phenotype of 10-day-old of wild-type (Col-0), *DPB2OE*, and *atr* seedlings germinated with and without HU (0.5mM), B: Relative percentage of plants with true leaves in the presence of 0.5 or 1mM HU, relative to mock-treated populations. Values are mean \pm SE of at least three biological replicates with 80-100 seedlings each. C: Quantification of root length of HU-grown seedlings. Four-day-old seedlings were transferred to half-strength MS containing HU (2.0mM) and root length after was measured after 4 days. Results are expressed as relative growth with respect to the corresponding genotype without treatment. Values are mean \pm SE of three biological replicates with 20 seedlings each. For B and C, the asterisks denote significant differences with respect to wild-type plants (Student t test; $P < 0.05$).

Genetic analysis reveals complex interactions of DPB2 function with DDR

The tolerance to different types of DNA damage that trigger replicative stress or DNA breaks, may be due to constitutive activation of ATR, and/or ATM. To investigate the contribution of the DNA damage response to the growth defects caused by DPB2 over-expression, the *DPB2OE* construct was introduced in *atm*, *atr*, and *sog1* mutants. For each background, two transformation batches were analysed and at least 63 plants of each T1 generation were grown in the greenhouse. We were able to identify plants displaying a clear *DPB2OE* phenotype in the T1 generation in all backgrounds (Table III, Figure S10A-C), but the distribution of plants in the different phenotypic categories was significantly different from what was observed in the wild-type in all genotypes (χ^2 , p-value < 0.01). *DPB2* over-expression was quantified using qRT-PCR in different lines representative of the different phenotypic groups (Figure S10D). Interestingly, plants with similar phenotypes in the Col-0, *atm* or *sog1* background had similar *DPB2* expression levels. By contrast, in the *atr* background, much higher *DPB2* over-expression was required to induce severe and intermediate developmental defects, indicating that ATR activation accounts for some of the phenotypic alterations induced by *DPB2* over-expression. Root growth assays confirmed that plants had been assigned to the proper phenotypic category: root growth inhibition was similar in the severe, intermediate and mild lines of all tested backgrounds (Figure S11). Interestingly, viable plants with very severe phenotype could only be obtained in the *sog1*, and *atr* backgrounds (Figure S10A and B), but many of these plants died before flowering, and they were obtained at a lower frequency than in the Col-0 background (Table III). In the *atm* background, we only identified plants with intermediate and mild vegetative phenotype (Figure S10C). These results suggest that ATM is required for survival of severe *DPB2OE* lines. However, failure to obtain plants with severe phenotype may also be due to 35S interference that would prevent *DPB2* expression to reach sufficient levels, since the *atm* mutant allele was from the SALK collection (46). To determine whether ATM activity was essential to *DPB2OE* plants survival, we tested the effect of a specific inhibitor of ATM activity (IATM), and found that *DPB2OE* lines are hypersensitive to this drug (Figure S12) supporting the notion that ATM activity is required for survival of *DPB2OE* whereas SOG1 and ATR are not. To further analyse the genetic interaction between *SOG1* and *DPB2*, we asked whether the tolerance to DNA damage observed in *DPB2OE* lines required SOG1 activation. To this end, we assessed the sensitivity of *sog1* and *sog1DPB2OE* to HU. As previously demonstrated (47), *sog1* was hypersensitive to HU, and *sog1DPB2OE* displayed an intermediate phenotype between wild-type and *sog1* mutants (Figure 8A-B). This additivity of the *sog1* and *DPB2OE* phenotypes suggests that DPB2 and SOG1 control HU sensitivity via independent pathways. This is further corroborated by the observation that some DNA damage response genes

were up-regulated in flower buds despite of SOG1 deficiency in *sog1 DPB2OE* lines (Figure S13): induction of *XRI-1*, *PAPR2*, *BRCA1* or *SMR5* and 7 was lost or greatly reduced in the *sog1* background. Surprisingly, *WEE1*, *CYCB1;1* and *RAD51* were still up-regulated in the *sog1* mutant, indicating that DPB2 over-accumulation activates a SOG1-independent replicative stress checkpoint. Together, these results indicate that ATR and SOG1 are dispensable to the survival of *DPB2OE* lines, and that their activation only partly accounts for growth retardation observed in *DPB2OE* lines.

Table III: Distribution of *DPB2OE* T1 plants in the mild, intermediate and severe phenotypic classes in the wild-type (Col-0) and DDR mutant backgrounds. Transgenic plants were selected on sand watered with glufosinate. For each background, two independent transformations were performed, which corresponds to the two rows. The indicated number of T1 plants was transferred to the green house, and phenotypes were scored. For the “Severe” category, numbers in brackets indicate the number of plants that died before flowering.

Line	Mild	Intermediate	Severe	Total
<i>Col-0 DPB2OE</i>	40	23	25 (4)	88
	59	26	32 (3)	117
<i>atr DPB2OE</i>	54	18	11 (5)	83
	46	12	7 (3)	65
<i>atm DPB2OE</i>	56	6	1 (1)	63
	74	11	0	85
<i>sog1 DPB2OE</i>	9	35	29 (18)	73
	15	53	24 (14)	92

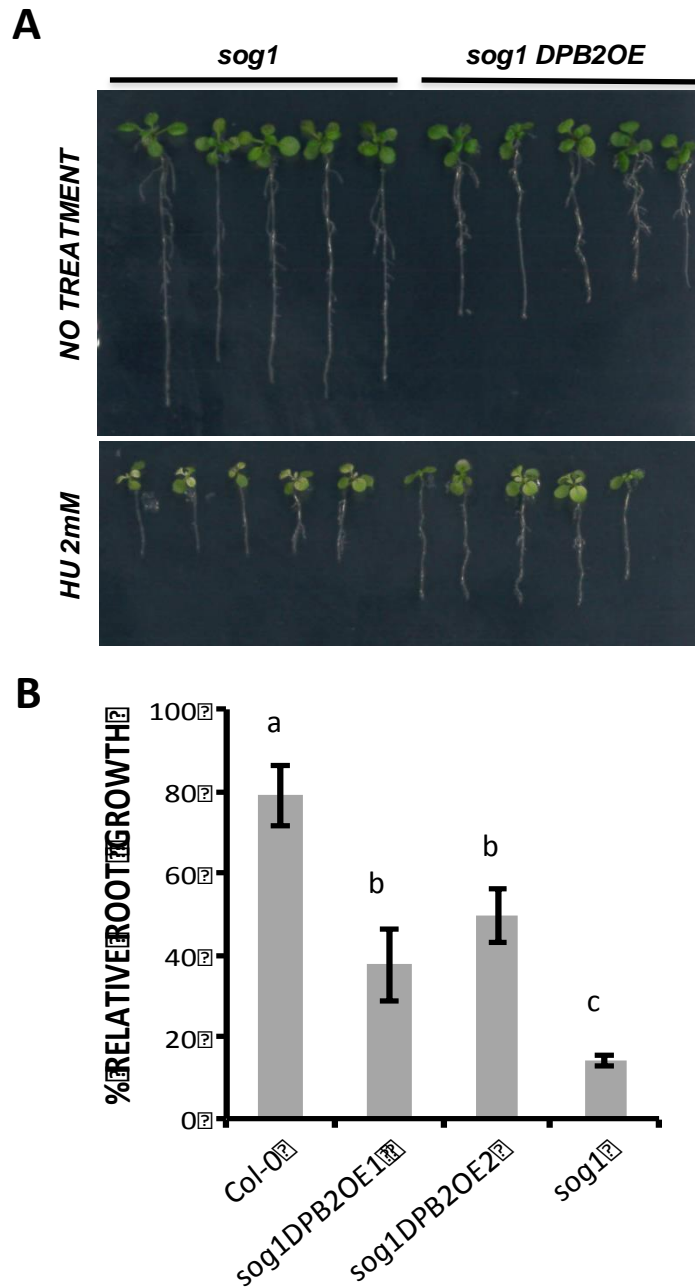


Figure 8: DPB2 over-expression partially rescues HU hypersensitivity in the *sog1* mutant.

A, B: Seedlings were germinated and grown on 0.5X MS medium for 4 days and transferred either to 0.5x MS medium or 0.5x MS medium supplemented with 2mM of HU. A: phenotype of *sog1* and *sog1DPB2OE* seedlings after 8 d with or without treatment; B: relative root growth. Results are shown as relative length with respect of the corresponding genotype without treatment, and values are mean \pm SE of three biological replicates with 20 seedlings each. Letters indicate statistically significant differences (Student t test; $P < 0.05$)

Unexpectedly, inactivation of ATR and SOG1 rescued the fertility of *DPB2OE* lines (Figure 9A-F). This effect was particularly striking in the *sog1* background: we observed that plants with identical *DPB2* mRNA levels were as severely affected as in the Col-0 background with respect to plant size, but the above-described sterility phenotype was completely lost (Figure 9F). The *sog1 DPB2OE* lines were propagated until the T5 generation, and the observed fertility rescue was conserved. Most of obtained seeds germinated although 30%-40% died at the seedling stage. This result suggests that the partial sterility of *DPB2OE* plants is dependent of SOG1 protein function. Analysis of meiosis progression in the most severe *sog1 DPB2OE* lines confirmed that DNA fragmentation was largely lost (Figure S14), indicating that the meiotic DNA-fragmentation phenotype observed in *DPB2OE* lines is an active process requiring SOG1. Together, our results indicate that DPB2 over-expression activates distinct DDR pathways during the vegetative and reproductive phases.

DISCUSSION

DNA polymerase ϵ plays a key role during DNA replication. Until recently, it was considered as the polymerase responsible for the synthesis of the leading strand, but evidence suggest that its main role may be in the progression of the CMG complex to unwind the replication fork and in the repair of replication errors (5). Here, we have investigated the role of the regulatory sub-unit DPB2 in Arabidopsis using an over-expression strategy to overcome the lethality of DPB2 deficiency (14). DPB2 over-expression impairs the replicative function of the Pol ϵ complex. Indeed, *DPB2OE* lines displayed the same aphidicolin sensitivity and the same early flowering phenotype as *abo4/esd7* mutant lines that are deficient for the catalytic sub-unit (18, 19). Because complementation experiments demonstrate that the CFP-DPB2 protein is functional, we can rule out the possibility that the phenotype of DPB2OE lines could be the result of a dominant-negative effect. Additionally, inducible inactivation of DPB2 via RNAi induced similar cell cycle delay and root growth inhibition as DPB2 over-expression. Analysis of the native holoenzyme purified from yeast suggests a 1:1:1:1 stoichiometry for the Pol ϵ sub-units (48); although the complex composition has not been investigated in plants, our results reveal the importance of stoichiometric sub-unit accumulation for complex functionality. In yeast, DPB2 is not required for Pol2A catalytic activity *in vitro*, but it improves its stability (49), and enhances the fidelity of DNA replication (50). Recently, Sengupta et al (2013) have shown that DPB2 is required to integrate DNA Pol ϵ into the replisome, and that over-expression of the N-terminus of DPB2 is sufficient to rescue the lethality of *dpb2* null mutants. These lines are capable to produce a replisome lacking Pol ϵ and are viable although they grow extremely poorly (7). Strikingly, over-expression of DPB2 N-terminus has a

dominant negative effect, and yeast cells with a replisome lacking Pol2A show delayed S-phase progression, as is the case for *DPB2OE* lines. Our results therefore suggest that plant DPB2 protein functions like its yeast counterpart to link Pol ϵ and the helicase into a functional replisome.

Detailed phenotypic analysis of *DPB2OE* lines revealed dramatic defects both in the mitotic and the meiotic cell cycle. In somatic cells, DPB2 over-expression led to a delay in S-phase progression as reported in Pol2A-deficient mutants (16, 18), further supporting the notion that defects caused by DPB2 over-expression are due to impaired Pol ϵ functionality. Interestingly, the observed increase in S-phase length did not fully account for the total increase in cell cycle length. Together with the enhanced expression of the G2/M marker *CYCBI;1*, this result points to constitutive activation of the DDR and subsequent cell cycle checkpoint activation in *DPB2OE* lines, leading to a G2 arrest. Eukaryotic cells respond to DNA replication block or DNA damage by activating checkpoints that delay the onset of mitosis until DNA replication and repair are completed (28). *DPB2OE* lines display several features observed in response to DNA stress including *CYCBI;1* over-expression, enhanced endoreduplication likely reflecting early onset of differentiation, and increased expression of DNA repair genes (28). Similar defects were reported in several mutants deficient for proteins involved in DNA replication such as *caf* (*chromatin assembly factor*), *fas1* (*fasciata*), *pol2a*, *rpa2a* (*replication protein a*), (18, 51–53), and were hypothesized to result from stalled replication forks during S-phase (18, 28). Consistently, we were able to show that *DPB2OE* lines constitutively accumulate DNA damage, as evidenced by the presence of phosphorylated γ H2A-X foci in root meristem nuclei, suggesting that altered fork progression in these lines ultimately leads to fork collapse and formation of DSB. Taken together, our results strongly suggest that control of DPB2 accumulation plays a key role at the replication fork to prevent DNA damage from accumulating during replication.

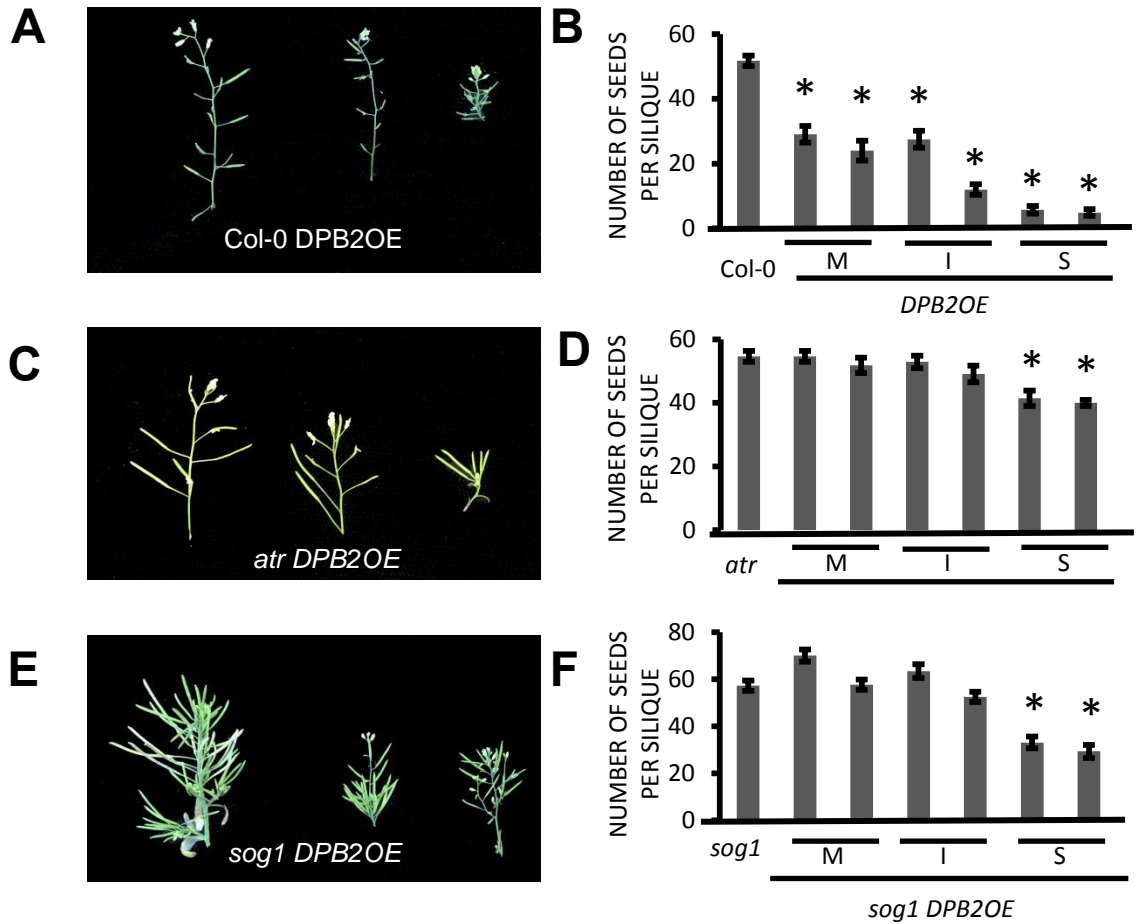


Figure 9: Sterility caused by *DPB2* over-expression largely depends on *SOG1* and *ATR* activity.

Panels A, C, and E show the representative siliques phenotype of *DPB2* over-expressing Col-0, *atr*, and *sog1* lines. B, D, and F show the average number of seeds produced per silique in independent *DPB2OE* lines displaying mild (M), intermediate (I) and severe (S) phenotypes, corresponding to the phenotypes of *DPB2OE* lines 3, 2 and 1 respectively. Values are mean \pm SE (n=30) for each line. For B, D, and E; the asterisks denote significant differences with respect to the background in which *DPB2OE* was introduced (Student t test; $P < 0.05$).

To further explore the role of DPB2 in DNA replication and repair, we tested the sensitivity of *DPB2OE* lines to replication stress and direct DNA damage. By contrast to mutations in replisome sub-units, DPB2 over-expression conferred tolerance to replicative stress induced by hydroxy-urea as well as to DNA-damaging agents. The increased tolerance to DNA damage could be due to basal activation of the DNA stress checkpoints and constitutive expression of DNA repair genes, as proposed in the case of CDT1-deficient lines (54), as well as to activation of bypass mechanisms allowing DNA replication to proceed through lesions. This hypothesis correlates with the basal increase in the transcription of DNA repair genes and the presence of phosphorylated form of H2AX histone variant (γ H2AX), which plays a key role in the recruitment and accumulation of DNA repair proteins at sites of DSBs (55). Consistently, the line displaying the highest basal accumulation of DSB is not tolerant to zeocin, suggesting that DPB2 over-accumulation confers tolerance to DNA damage up to a certain threshold above which pre-activation of DNA repair pathways is not sufficient for the plant to cope with genotoxic stress. Thus DPB2 over-expression likely triggers the pre-activation of DNA damage response, resulting in less growth inhibition after of DNA damage exposure. Intriguingly, *DPB2OE* lines shared features with the Pol ϵ catalytic subunit mutant (*abo4-1*) in the alterations of cell cycle and DNA repair genes increased transcript levels, suggesting also the pre-activation of DDR in *abo4-1*, however, this mutant is hyper-sensitive to DNA damaging agents such as MMS and UV-C (18). POL2A participates in various DNA repair pathways such as nucleotide excision repair (NER), base excision repair (BER), break-induced replication (BIR), and homologous recombination (HR) (56, 57) in many organisms. Our observation that DPB2 over-expression interferes with DNA replication but not with DNA repair suggests that POL2A functions independently of DPB2 in DNA repair, or that the relative abundance of the two sub-units plays a less prominent role in this pathway. Regarding the observed tolerance to HU, it could either reflect this improved tolerance to various kinds of DNA damage, or the impairment of Pol ϵ -dependent S-phase checkpoint activation. Since our work nevertheless indicates that ATR is activated by DPB2 over-expression, one can postulate that multiple sensing mechanisms cooperate at the fork to signal defects in replication progression to downstream components some possibly mediated by Pol ϵ itself, and others mediated via the formation of single stranded DNA upon uncoupling of the replicative machinery and the helicase complex. Indeed Pol ϵ catalytic subunit is required for the activation of the S-phase checkpoint upon replication defects such as fork stalling, collapse or DNA damage (12, 58), and this role has recently been shown to require Pol ϵ association into the replisome (59); hence, impairment of Pol ϵ association to the replisome by DPB2 over-expression may prevent checkpoint activation in these lines.

To further connect DPB2 functions with DDR, we have used genetic approaches. The frequency of lines displaying a severe phenotype in the T1 generation was reduced in both *atm* and *atr* mutants, which may suggest that the two kinases contribute to the survival *DPB2OE* plants. In the case of *atm*, this is supported by the hypersensitivity of *DPB2OE* lines to an inhibitor of ATM and the fact that we could not recover plants with a high DPB2 over-expression or a severe phenotype. By contrast, we observed very high accumulation of *DPB2* mRNA in *atr DPB2OE* lines that did not result in a severe phenotype, indicating that ATR activation is partly responsible for the growth defects in *DPB2OE* lines. It is worth noting that the few plants displaying a severe phenotype had a poor survival rate, suggesting that failure to activate ATR-dependent responses can lead to developmental arrest likely caused by extensive DNA damage. By contrast, plants with intermediate and severe phenotype were obtained at a higher frequency in the *sog1* mutant, and root growth inhibition was slightly more pronounced in the *sog1* than in the Col-0 background for lines with similar levels of *DPB2* accumulation (Figure S11), suggesting that SOG1 activation partially alleviates defects induced by DPB2 over-expression. However, the survival rate of severe lines was lower than in the wild-type, indicating that SOG1 also contributes to the viability of these plants. Meristem arrest caused by DPB2 over-expression in the *sog1* background may also account for the bushy appearance of *sog1 DPB2OE* plants, as arrest of the main meristem would favour branching. In addition, the *sog1* mutation restored only partially the sensitivity to HU of *DPB2OE* roots, and some DNA damage response genes were still up-regulated in flower buds despite of SOG1 deficiency. Recently, Hu et al have reported that replication checkpoint activation upon HU treatment relies on two parallel pathways: one involving WEE1 activation via the ATR kinase, and the other involving SOG1 activation via ATR (47) (Figure 10A, black arrows). Our results are consistent with the notion that SOG1 is not the only regulator of this checkpoint. Interestingly, they also show that *CYCB1;1*, that is activated in a SOG1-dependent manner by DNA damage (24), is activated via SOG1-independent pathways by Pol ϵ dysfunction. Together our results suggest that replication stress induced by DPB2 over-accumulation activates the ATR-SOG1 module that is required for sustained growth, possibly via its role in the regulation of DNA repair genes. In parallel, ATR regulates cell cycle progression independently of SOG1, and in the absence of ATR, plants show a less pronounced growth inhibition but lower survival. In addition, ATM appears to be required for survival of *DPB2OE* lines, indicating that DPB2 over-expression also leads to fork collapse and DSB formation, which would activate DDR in an ATR-independent manner. Indeed, proteins from the mitogen-activated protein (MAP) kinase pathway have been shown to play a role in DDR independently of ATR and could thus be activated by Pol ϵ deficiency (60). Figure 10A summarizes the proposed model for the effect of DPB2 over-expression in vegetative cells; together

our results indicate that perturbed Pol ϵ function activates the two main DNA stress response pathways previously described in plants, but also provide evidence for yet unidentified regulatory pathways. Notably, they demonstrate that SOG1-independent pathways can activate both DNA repair genes and cell cycle delay.

In addition to the defects observed in somatic cells, severe *DPB2OE* lines showed DNA fragmentation during meiosis. Similar defects have been reported in various mutants deficient for replisome sub-units such as *CDC45-RNAi* lines, *rpa*, or *abo4-2* (Stevens et al., 2004; Aklilu et al., 2014; Huang et al., 2015). The chromosome fragmentation observed in all these lines could be due to defects during pre-meiotic DNA replication, which is essential to chromosome cohesion, meiotic recombination and chromosome segregation, but could also reflect a role of the replicative machinery in DSB repair via homologous recombination. Consistently, POL2A is expressed in meiocytes in mouse (63) and in Arabidopsis (62). Furthermore, meiotic defects observed in Arabidopsis POL2A-deficient lines were SPO11-dependent, suggesting that they were due to defects in DNA repair (62). By contrast, disruption of CDC45 or MEI1, like DPB2 over-expression, results in SPO11-independent DNA fragmentation, suggesting that defects occurred during pre-meiotic replication (61, 64). Because DPB2 forms complexes with CDC45 and MEI1 in different steps of replication initiation where the catalytic subunit is not required (65), its over-expression could affect the stability of interactions required for correct DNA replication prior to meiosis, leading to delayed completion of DNA replication or replication errors. Strikingly, although SOG1 is essential to normal meiosis in the *uvh1* background (66), the fertility of *DPB2OE* lines was restored in the *sog1* background, and DNA fragmentation was largely lost. Hence, our results provide evidence for the involvement of SOG1 in an active DNA fragmentation program triggered by replication defects in meiocytes (Figure 10B), consistent with the role of SOG1 in the transcriptional activation of cell death genes (24, 67). This hypothesis is further supported by the observation that the survival of *sog1 DPB2OE* T2 seedlings was affected, suggesting that a large proportion of embryos derived from gametes with major genetic anomalies. Interestingly, in mammals, a link exists between the regulation of Pol ϵ activity and p53, the functional homologue of SOG1: MDM2 (Mouse Double Minute 2) can bind the C-terminus of Pol2A and is thought to simultaneously modulate Pol ϵ functions in response to stress and control cell cycle progression by regulating p53 degradation (68), similar mechanisms might be conserved in plants to maintain genome integrity.

Overall, our work provides evidence for the exquisite complexity of DNA damage and replication stress response in plants, and further questions the central role of SOG1 as the central integrator of

DNA damage response in plant cells. Additionally, it reveals the tissue specificity of cellular responses, defects in pre-meiotic DNA replication triggering a cell death response that does not occur in vegetative tissues in response to Pol ϵ dysfunction.

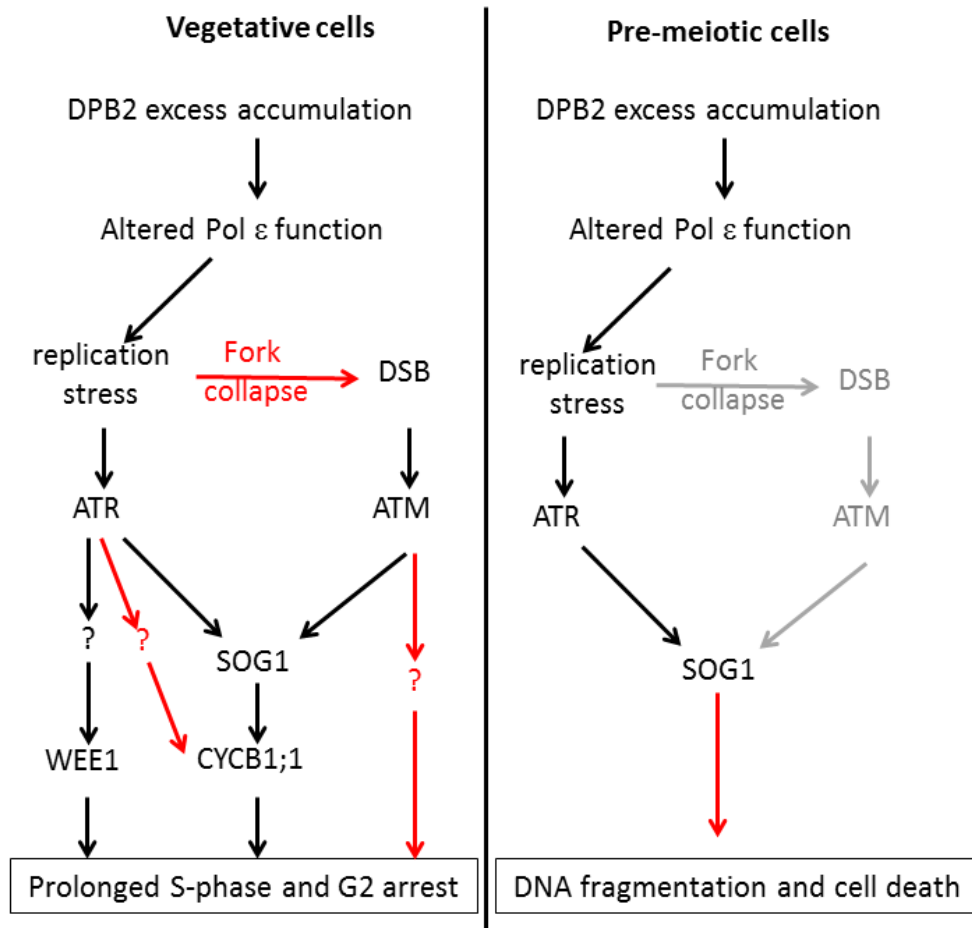


Figure 10: Involvement of DPB2 in DDR regulation in somatic and reproductive cells.

A: In vegetative cells, excess DPB2 accumulation alters Pol ϵ function either by excluding Pol2A from the replication fork or by altering the stability of protein complexes at the fork. This leads to fork stalling and replication stress, thereby activating ATR which in turn can delay cell cycle progression by activating WEE1 and CYCB1;1 and possibly other cell cycle regulators. This activation occurs independently of SOG1, even though CYCB1;1 is a known target of this transcription factor. In addition, altered fork progression leads to fork collapse and creation of DSB, which activate ATM. The observation that ATM but not SOG1 is required for survival of *DPB2OE* lines indicates that unknown SOG1-independent pathways are required to regulate cell cycle progression and DNA repair upon fork collapse.

B: In pre-meiotic cells, the above-described initial events occur similarly, but in this tissue, SOG1 activation leads to an active process of DNA fragmentation probably associated with cell death. This mechanism may represent a pre-meiotic replication specific checkpoint that would prevent transmission of replication errors through gametogenesis. Arrows in red represent regulatory pathways inferred from this work, whereas black arrows correspond to previously identified mechanisms

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *DPB2* (AT5G22110), *SMR5* (AT1G07500), *SMR7* (AT3G27630), *PARP2* (AT2G31320), *KU70* (AT1G16970), *CYCBI-1* (AT4G37490), *XRI1* (AT5G48720), *WEE1* (AT1G02970), *BRCA1* (AT4G21070).

Data Deposition

Microarray data from this article were deposited at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>), accession no. GSE71002) and at CATdb (<http://urgv.evry.inra.fr/CATdb/>; Project: NGS2014_10_Epsilon) according to the “Minimum Information About a Microarray Experiment” standards.

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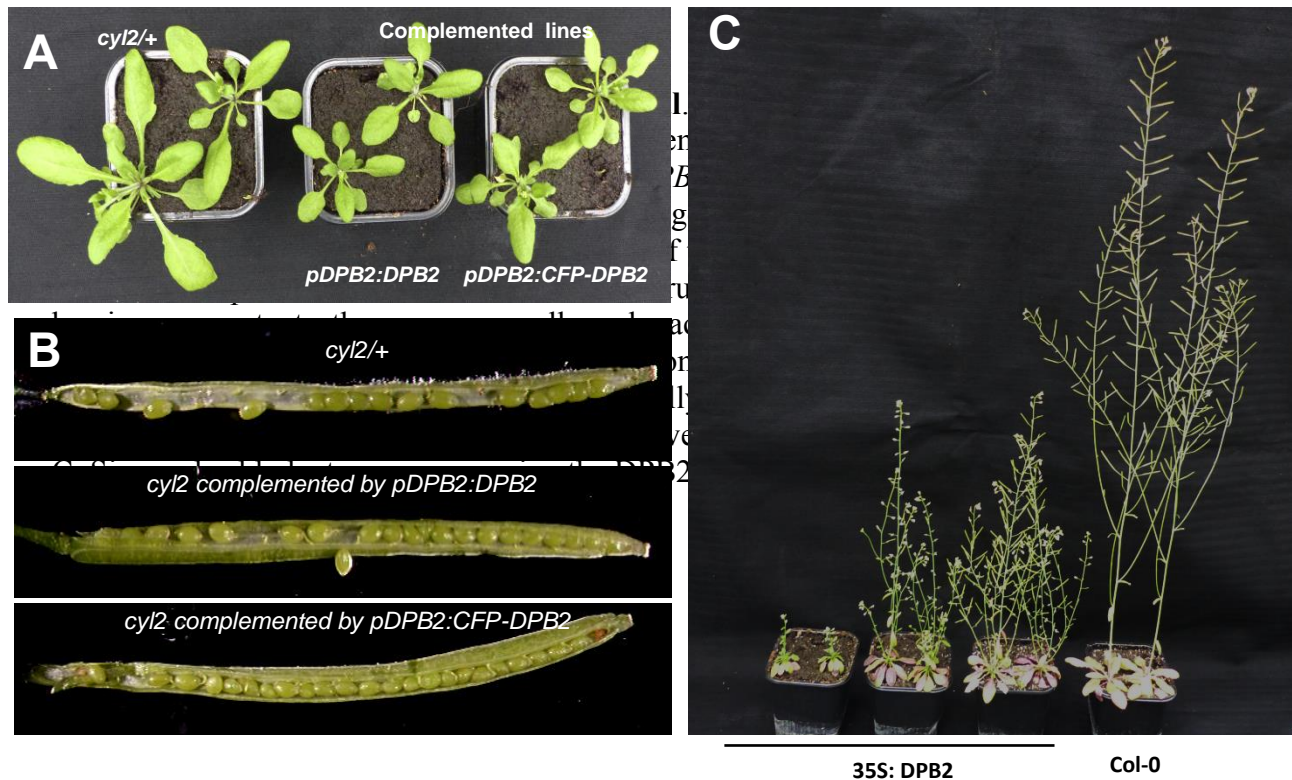


Figure S1. The CFP-DPB2 fusion is functional.

A: Rosettes of four-week-old plants of *cyl2/+* hemizygous mutants and lines complemented either with the *DPB2* cDNA alone (*pDPB2:DPB2*) or with the CFP fusion (*pDPB2:CFP-DPB2*). The *DPB2* promoter corresponds to the genomic fragment defined in Ronceret et al (2005) as sufficient to allow complementation of the *cyl2* mutant. Although 30-day-old *cyl2* mutants complemented with the two constructs were slightly smaller than *cyl2/+* hemizygous mutants, they grew normally and reached the same final size.

B: Expression of *DPB2* or the CFP-*DPB2* fusion under the control of the *DPB2* promoter fully restores the fertility of the *cyl2* mutant. Fully developed siliques from *cyl2/+* mutants and complemented lines were opened and observed using a binocular.

C: Six-week-old plants over-expressing the *DPB2* protein without tag (35S:*DPB2*).

Figure S2. Phenotype of *spo11-1* DPB2OE plants.

A: Vegetative development is similarly altered by DPB2 over-expression in the *spo11-1* background as in the wild-type.

B, C: The fertility of *spo11-1* DPB2OE lines is more severely affected than that of the *spo11-1*. Siliques phenotype of *spo11-1* DPB2OE with different range of affectation (B). Seeds produced by silique of independent *spo11-1* DPB2OE lines (C). The mean are of 50 siliques and the asterisk denotes significant difference respect to *spo11-1* mutant (Student t test; $P < 0.05$).

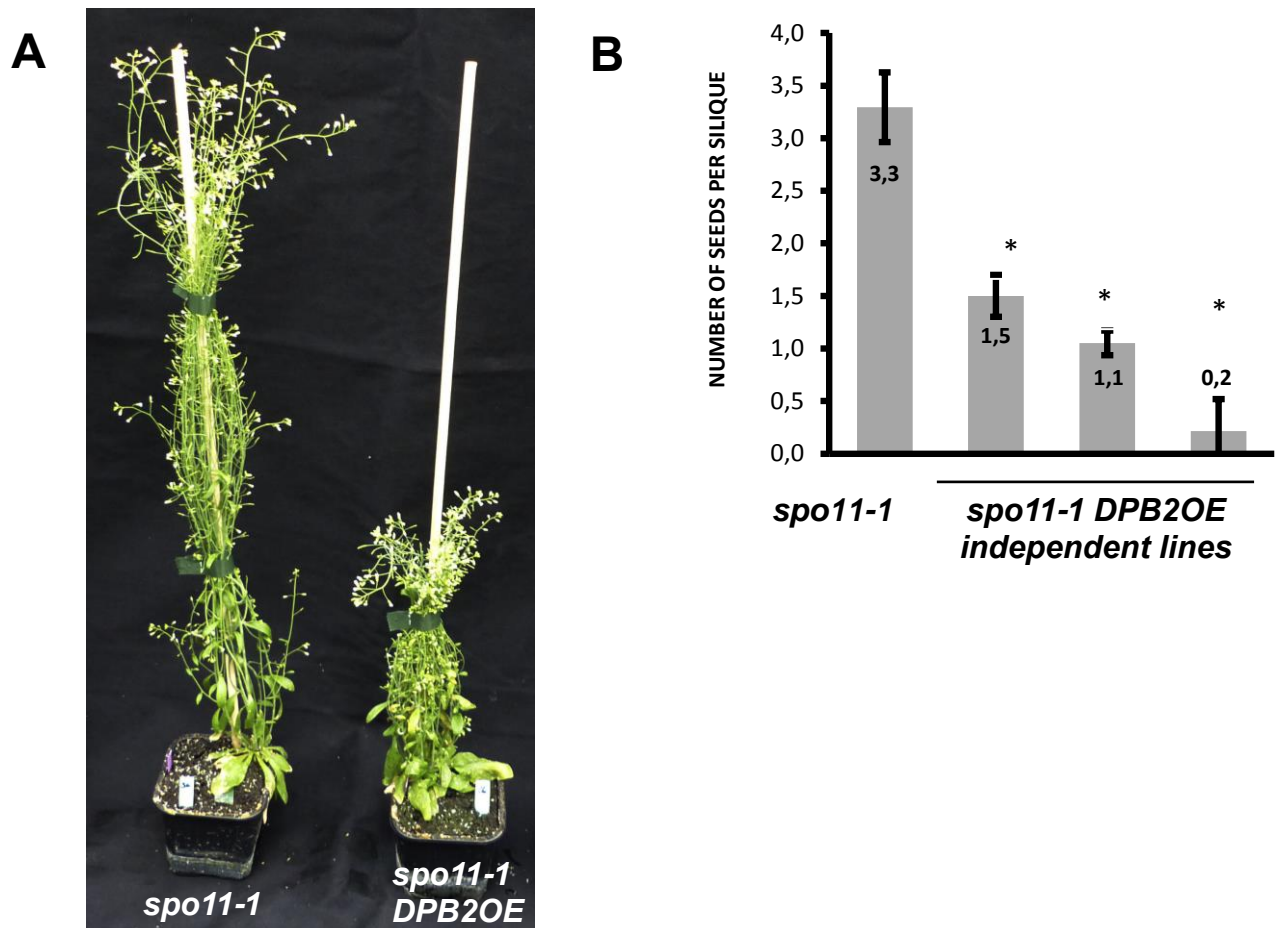


Figure S3. Phenotype of ethanol-inducible *DPB2-RNAi* lines.

A: Relative expression of *DPB2* s reduced upon ethanol addition in *DPB2-RNAi* lines compared to the control (Ctrl).

B : Phenotype of plantlets grown for 4 days on half-strength MS and transferred for 4 days on control medium (-) or medium supplemented with ethanol to the final concentration of 0.05%.

C: Quantification of the relative root growth of control and *DPB2-RNAi* plantlets. After 4 days of growth on ethanol supplemented or control medium, root length was measured with the Image J software. Data are expressed as the average percentage of control growth observed on ethanol supplemented medium +/- standard deviation (n>30).

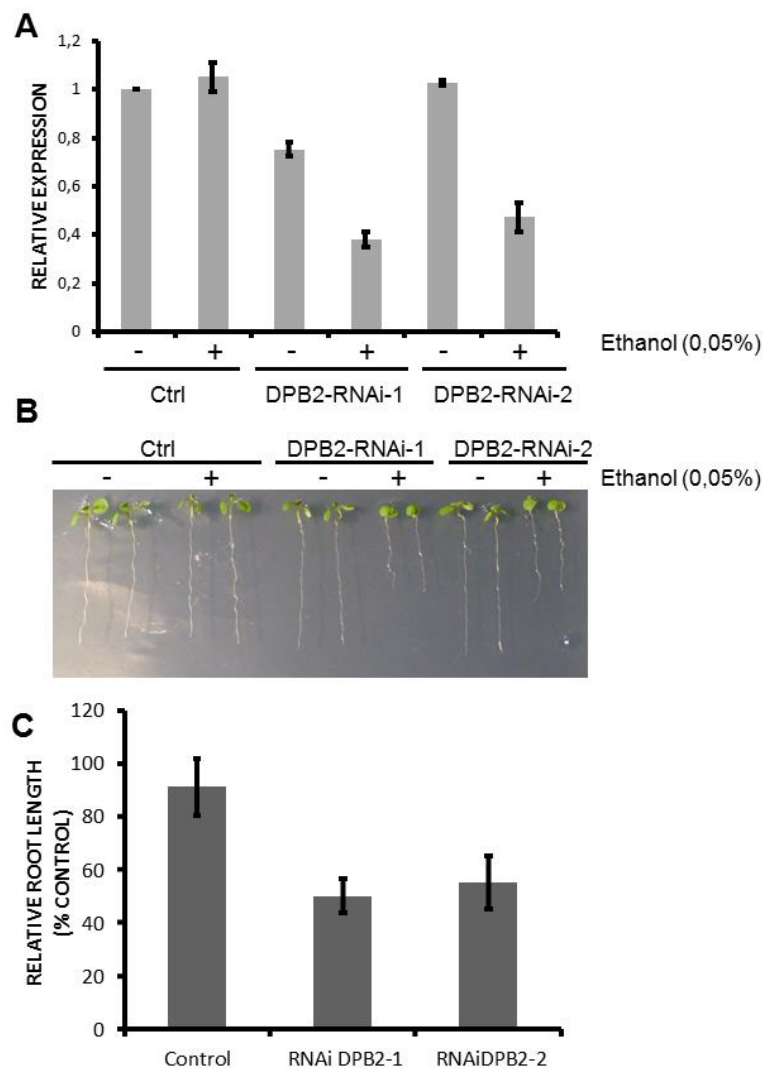
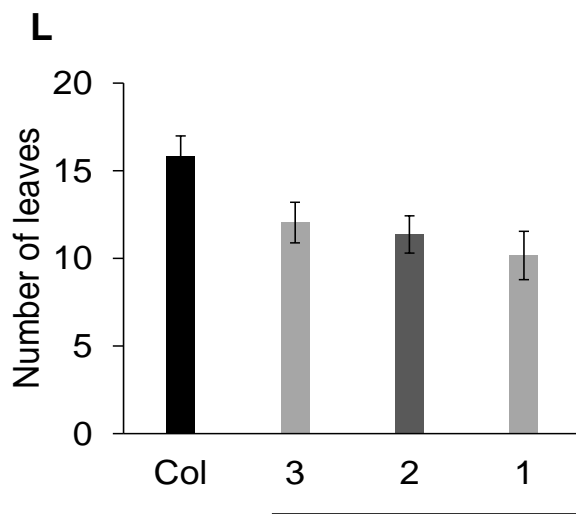
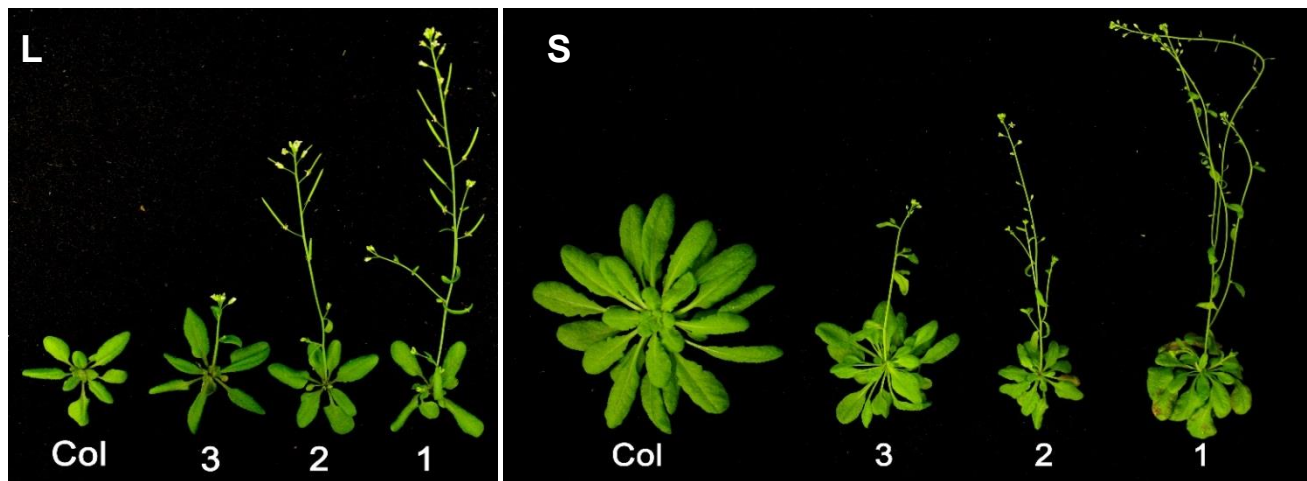
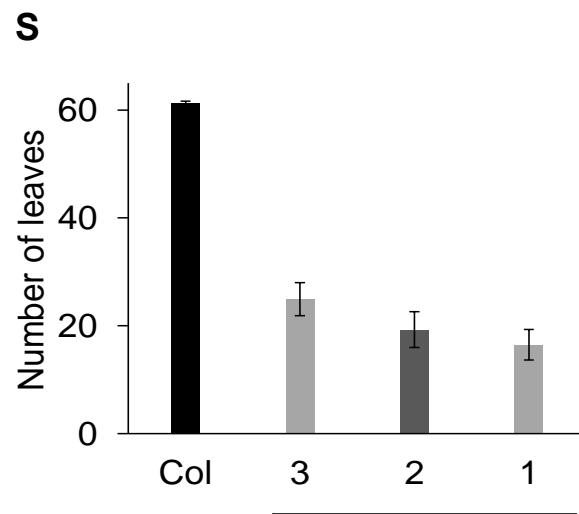


Figure S4. DPB2 over-expressors are early flowering

Plants were grown under long days (left) or short days (right) and the number of leaves at the onset of flowering was counted. Leaf number was lower in *DPB2OE* lines than in wild-type plants (Col), particularly in short days, as described in *esd7-1* mutants that harbor a point mutation in the catalytic sub-unit of Pol ϵ Pol2A (19).



DPB2OE



DPB2OE

Figure S5: Cell cycle length is increased in DPB2OE lines.

A: representative images of EdU labelling performed in squashed root tips of wild-type (Col-0) and DPB2OE1 plants incubated on EdU for the indicated time.

B: Graphs representing the percentage of EdU labelled cells as a function of incubation time for the indicated genotypes. Data are average \pm SE, n=10. The equation of the slope was calculated using the least-squares method, and used to estimate cell cycle length and S-phase length as described in (33). Data are representative of two independent experiments

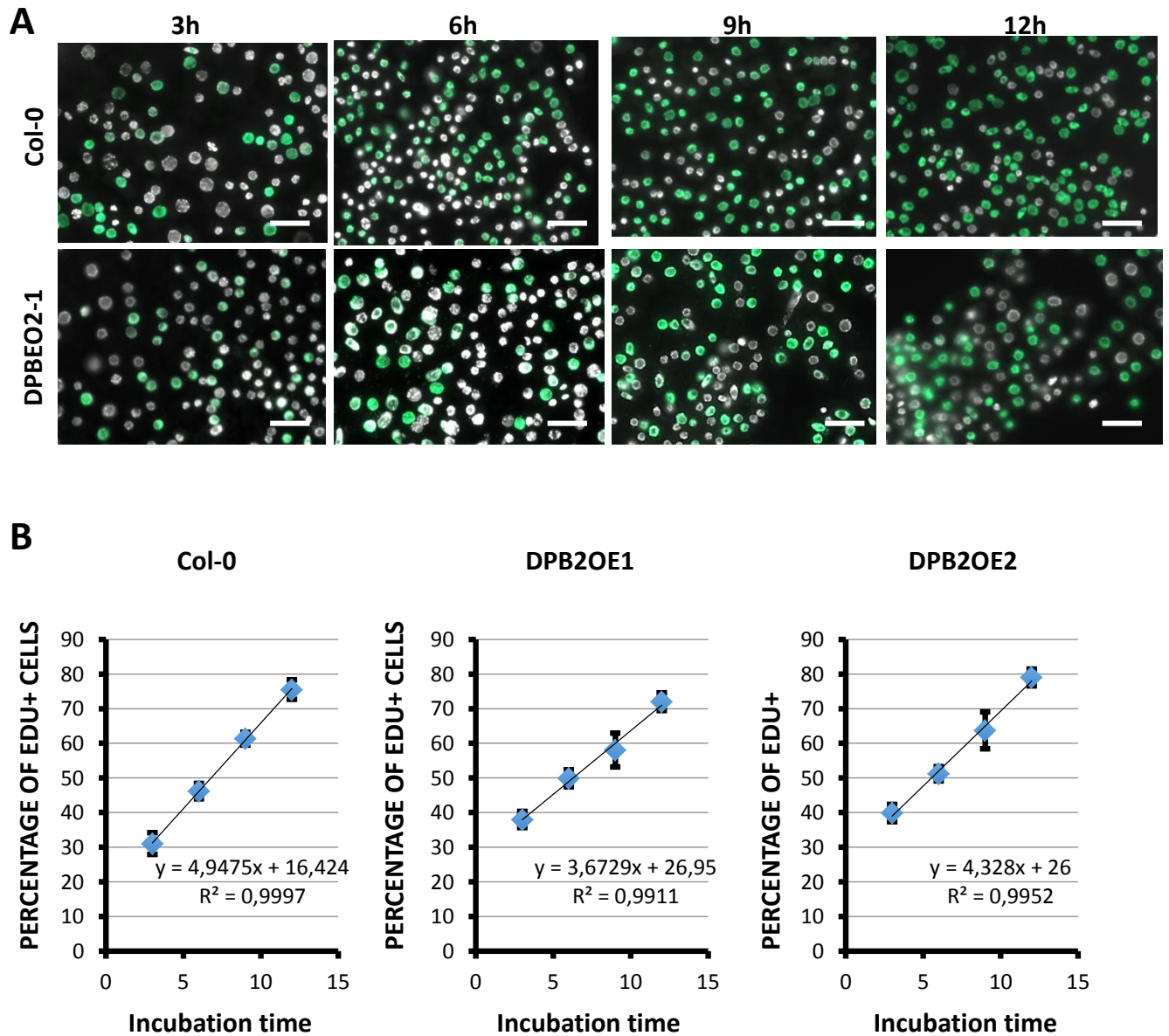


Figure S6: Cell cycle length is increased in DPB2OE-RNAi lines.

Graphs representing the percentage of EdU labelled cells as a function of incubation time for the indicated genotypes. Data are average \pm SE, n=10. The equation of the slope was calculated using the least-squares method, and used to estimate cell cycle length and S-phase length as described in (28), and obtained values are indicated below the graphs

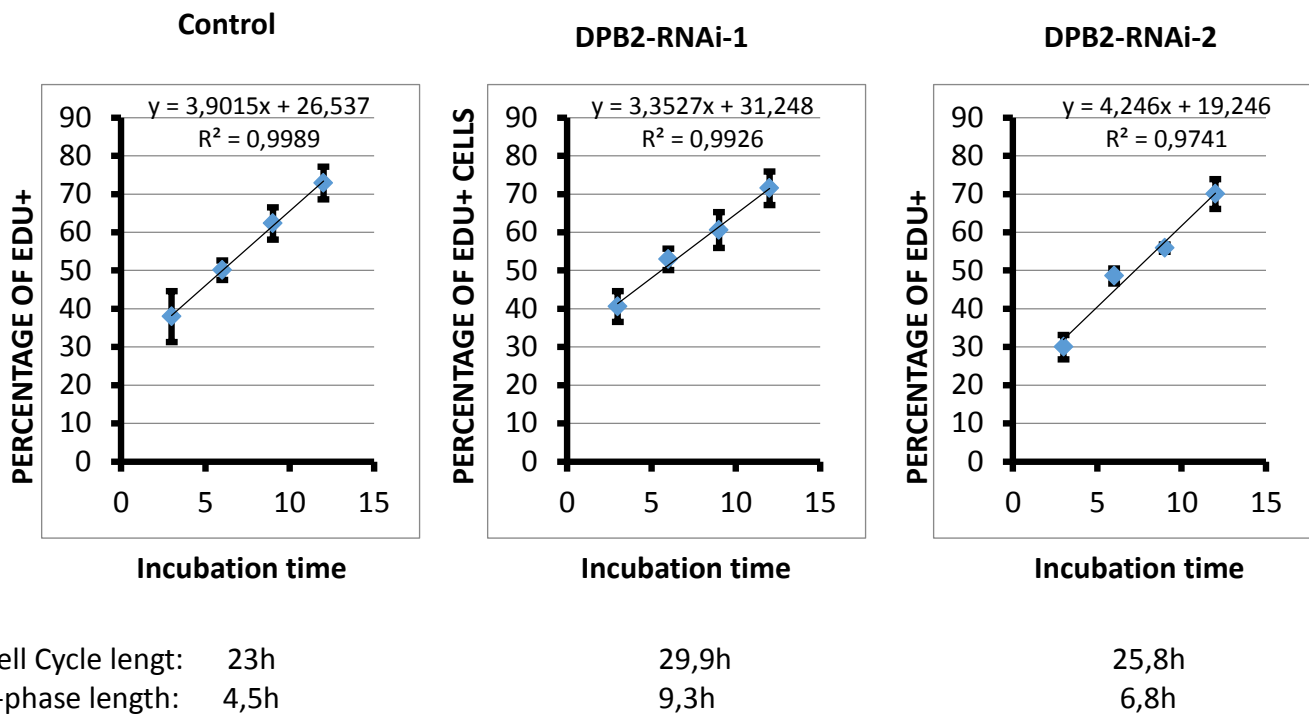


Figure S7. *DPB2OE* plants display symptoms of DNA damage accumulation.

GUS-staining was performed on inflorescences (A) and roots of 5-day-old plantlets (B) from the *CYCB1;1::DB-GUS* line and *CYCB1;1::DB-GUS DPB2OE* lines; images are representative of results obtained in 10 independent lines

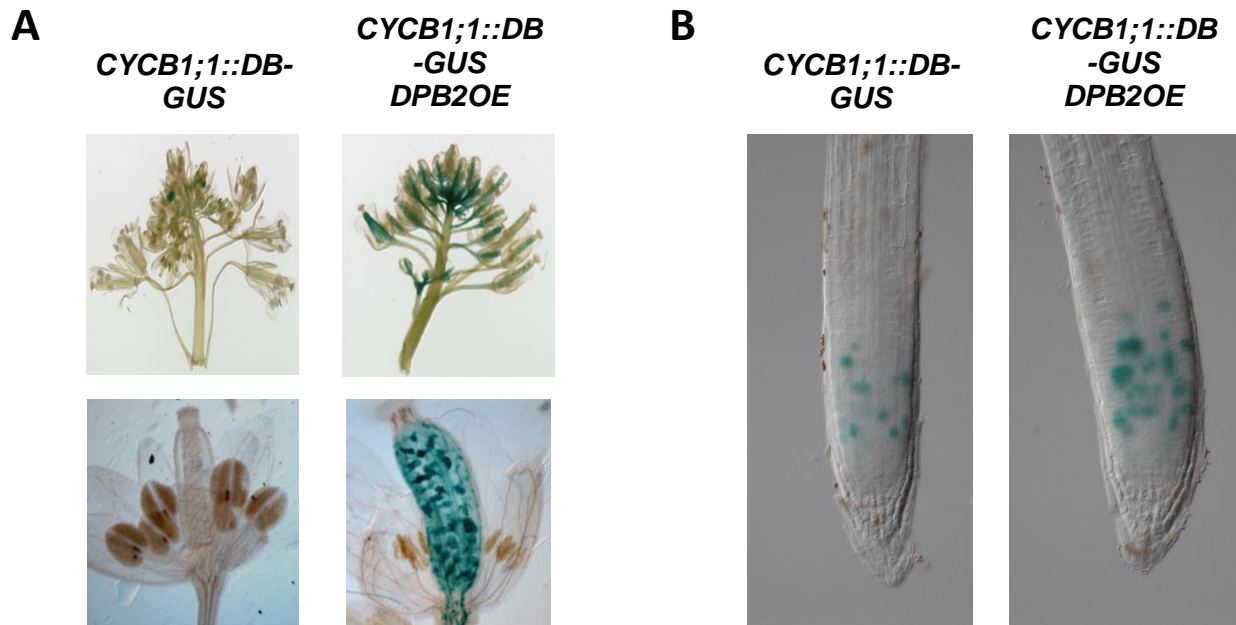
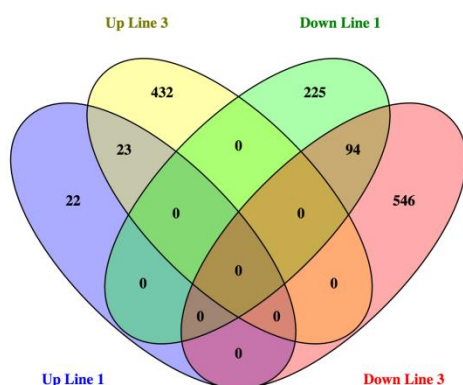


Figure S8. Transcriptome analysis of DPB2OE lines reveals mis-regulation of genes involved in DNA replication and repair.

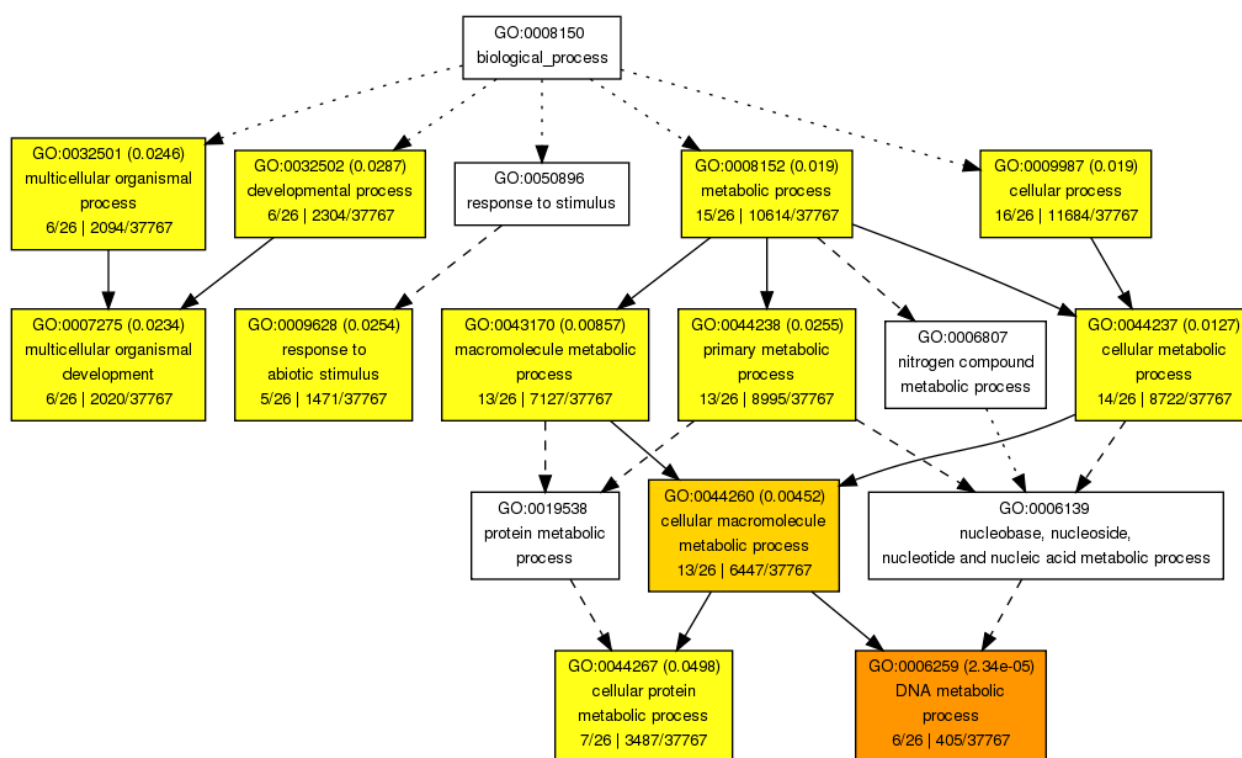
A: Venn diagram showing overlap between differentially regulated genes in the two lines.

B: GO analysis of up-regulated genes in *DPB2OE* seedlings. The yellow color represents significantly over-represented GO category (≤ 0.01). Analysis was performed using the agriGO software (<http://bioinfo.cau.edu.cn/agriGO/analysis.phd>)

A



B



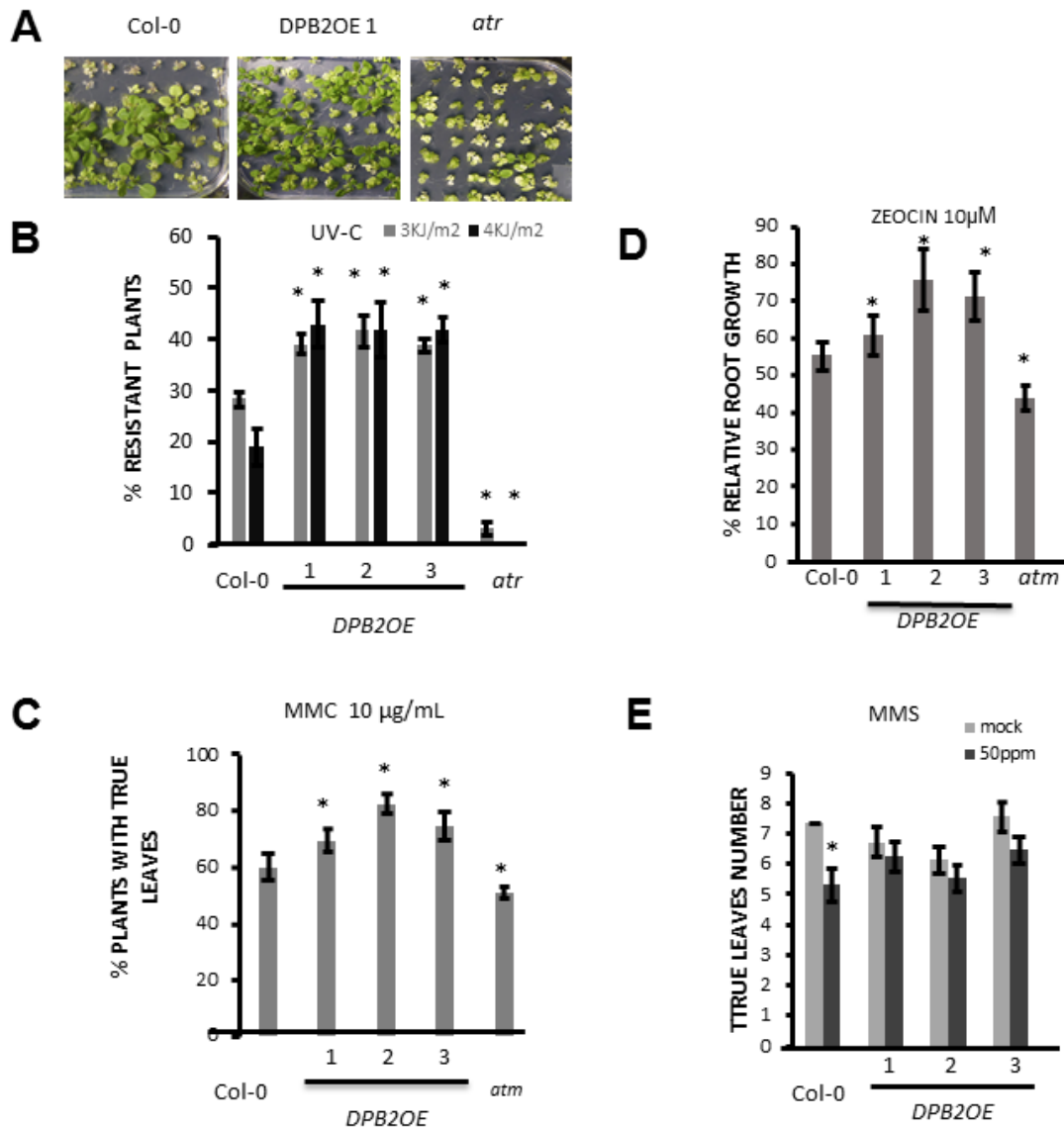


Figure S9. *DPB2OE* lines are tolerant to DNA damage.

A: Phenotype of seedlings 17 days after UV-C irradiation (3KJ/m²). B: percentage of resistant seedlings 12 days after treatment with 3 or 4KJ/m² UV-C. Resistant seedlings are the ones that remain green after treatment. C: Mitomycin C (MMC) sensitivity assay. Wild-type (Col-0), *DPB2OE* 1 and *atr* seedlings 4 days-old seedlings were transferred to liquid 0.5x MS supplemented with MMC 10µg/mL or to control medium. After 5 days of incubation the seedlings were transferred on control medium for recovery for 24h before analysis. D: Four-day-old seedlings were transferred to MS containing zeocin and root length was measured after 12 days. The results are showed as relative growth with respect of the corresponding genotype without treatment. E: Four-day-old seedlings were transferred to MS containing MMS at the indicated concentration, the number of true leaves was recorded at day 12. The DNA damage sensitive mutants *atr* and *atr* were used as control. For B and C percentage was calculated in relation to mock populations. The result showed are mean \pm SE of three or more biological replicates with at least 30 seedlings each. Asterisk denotes significant difference relative to the wild-type (Student t test; $P < 0.05$), except for panel E where they denote significant difference relative to the untreated control.

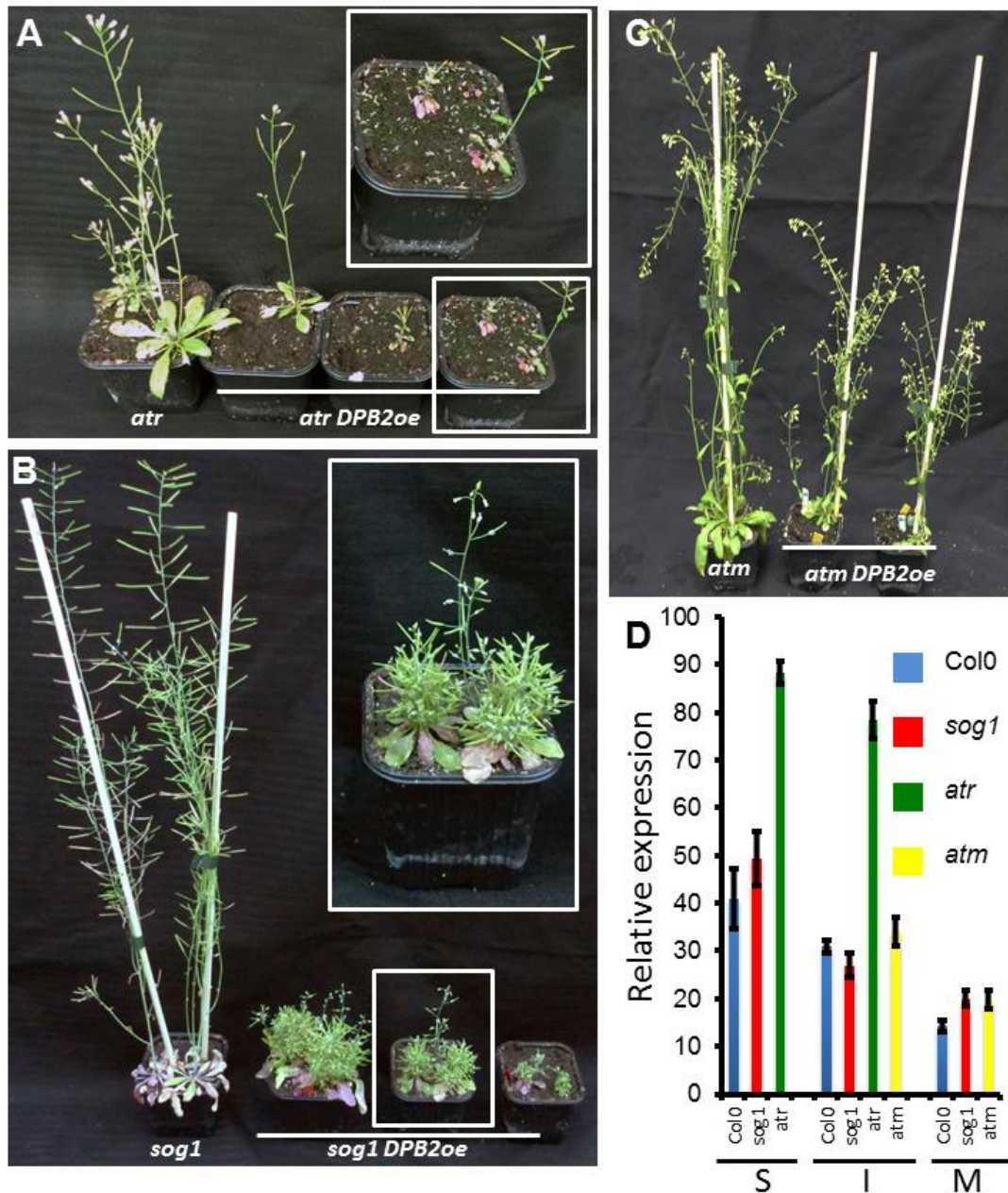


Figure S10. DDR mutants transformed with *DPB2OE* display different morphological defects.

A-C: Phenotypic appearance of *atr* DPB2OE, *sog1* DPB2OE, and *atm* DPB2OE pictures are of 6 week-old-plants. In A and B, insets are magnifications of areas in white boxes. D: qRT-PCR quantification of DPB2 over-expression in the various backgrounds. Total RNA were extracted from flower buds collected on several T1 individuals displaying the same phenotype, and DPB2 accumulation was compared to what was observed in lines 1, 2 and 3 in the Col-0 background. S = severe, I = intermediate, M = mild. Results are relative expression compared to wild-type plants. For DPB2OE lines in the Col0 background, lines S, I and M are lines 1, 2 and 3 respectively.

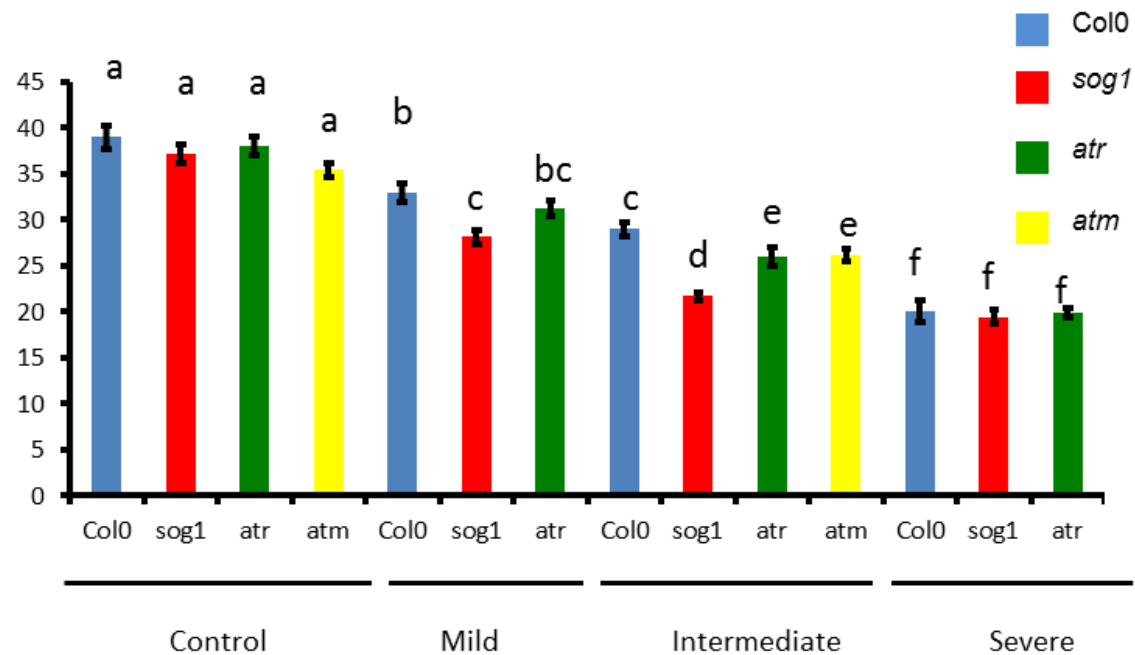


Figure S11. DPB2 over-expression alters root growth in DDR mutants.

Plantlets of the indicated genotype were grown vertically on half-strength MS medium for 14 days, and root length was measured using the Image J software. Values are average \pm SE ($n > 30$). Different letters indicate statistically relevant differences (Student t-test $p < 0.05$). Values for the Col-0 background are from graph presented on Figure 1.

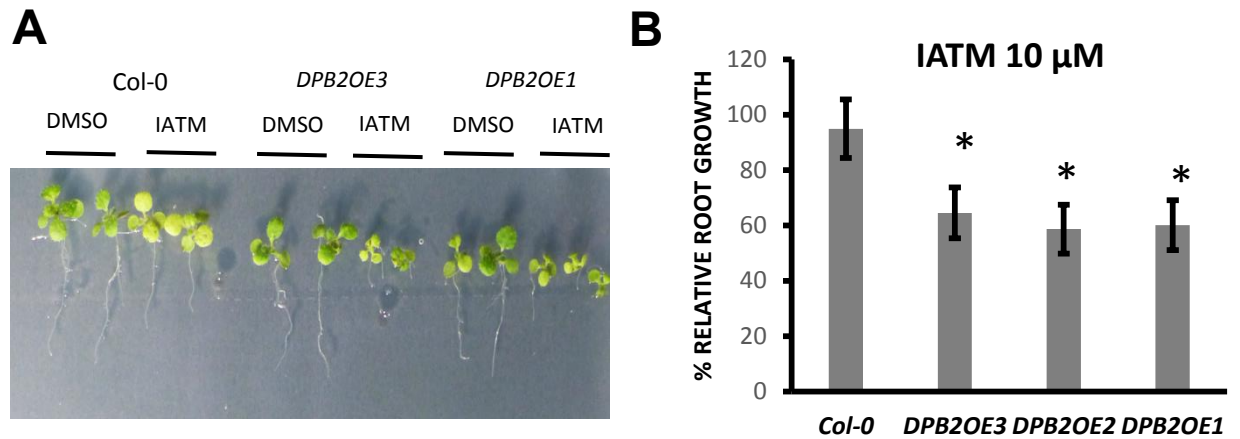


Figure S12. ATM activity is essential to the survival of *DPB2OE* lines.

Seedlings were germinated and grown on 0.5X MS medium or 0.5X MS medium supplemented with IATM for 14 days. A: Phenotype of *Col-0* and three independent *DPB2OE* lines grown in the presence of DMSO or IATM (20 μ M). At this concentration, growth of *DPB2OE* lines was completely stopped and many plantlets died. However, growth of wild-type plants was severely affected. Growth assays were therefore performed on a lower dose of IATM. B: relative root growth of the different genotypes on IATM (10 μ M) with respect to the corresponding genotype grown on mock. Values are mean \pm SE of two biological replicates with 20 seedlings each. Asterisks indicate significant differences with respect to plants with the same genotype without exposure to IATM (Student t test; $P < 0.05$).

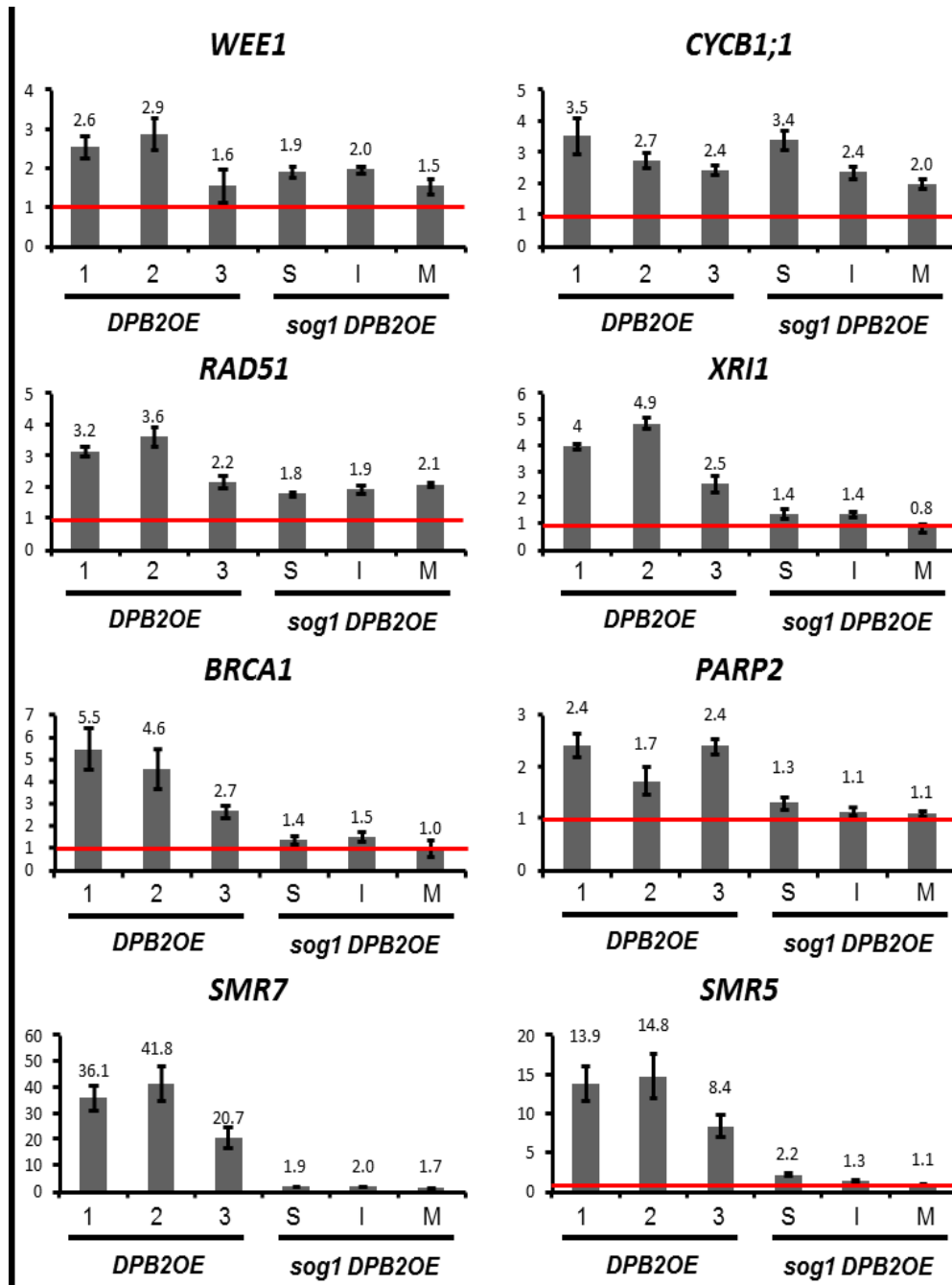


Figure S13. DPB2 over-expression activates both SOG1-dependent and SOG1-independent responses.

Total RNA were extracted from flower buds of wDPB2OE lines in the wild-type of *sog1* mutant background and expression of a set of genes was measured by RT-qPCR. In the *sog1* background lines with a severe (S), Intermediate (I) or Mild (M) phenotype were selected for comparisons with lines 1, 2 and 3 in the Col-0 background respectively. Data are expressed as fold changes compared to the Col-0 or *sog1* control and are average \pm SD from triplicates and are representative of two biological replicates.

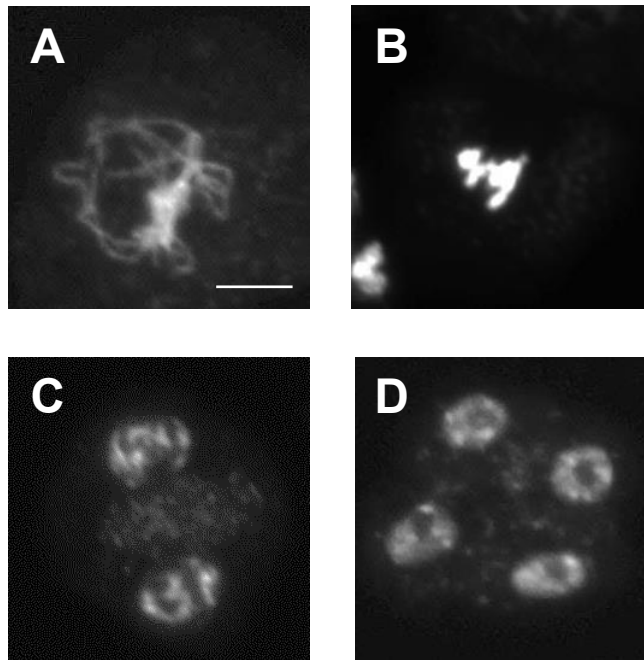


Figure S14. *SOG1* deficiency restores normal meiosis in *DPB2OE* lines.
A: Prophase I, B: Metaphase I, C: end of first division, D: tetrads. Bar = 10 μ m.

Table S1: Primers used in this study.

qPCR primers			
Gene	Accession Number		Sequence 5'→3'
DPB2	AT5G22110	Forward	TCGATAACCTCCAGGAAACG
		Reverse	AAACTTTGGCACCAGAAACG
SMR5	AT1G07500	Forward	AAACTACGACGACGGAGATACG
		Reverse	GCTACCACCGAGAAGAACAAGT
SMR7	AT3G27630	Forward	GCCAAAACATCGATTCTGGGCTTC
		Reverse	TCGCCGTGGGAGTGATACAAAT
PARP2	AT2G31320	Forward	AGCCTGAAGGCCCGGGTAACA
		Reverse	GCTGTCTCAGTTTTGGCTGCCG
KU70	AT1G16970	Forward	GGTGTAGCTGCTCCTCGCGC
		Reverse	GCATAGTGTGTCTGCAAAGCGGG
CYCB1-1	AT4G37490	Forward	GGAAGCAACAAGAAGAAGGGAG
		Reverse	AGGGATCAAAGCCACAGCG
XRI1	AT5G48720	Forward	GCTACCTGATGACTTAAACTTTGGTTC
		Reverse	CATTTGGAGAAGATCGAGTCACAG
WEE1	AT1G02970	Forward	GGCCATTCTGTTGCAGTTACA
		Reverse	TCTCGACATCTGAGAGACTC
BRCA1	AT4G21070	Forward	AGGTGAACCTGTCTCTGCGGATTT
		Reverse	TTCTCCGGCTTCTTGTCAACTCCA
Cloning primers			
DPB2 promoter	pDPB2 SacI dir		GCGAGCTCCTTTCTGTTTTTTGGATTGTAT
	pDBP2 SpeI rev		GCACTAGTTCTCGCTACTTCTCTCTTTTT
DPB2 cDNA	DPB2 EcoRI dir		GGGAATTCATGAGCAGCACCAGTCTCAGAAGAGG
	DPB2 XhoI rev		TCCTCGAGTCACAAAGCAGAGAGTTCGACTTCT
DPB2-RNAi	DPB2-RNAi BamHI EcoRI dir		CGGGATCCGAATTCGGACGCAGGTCCTTCAACAGTC
	DPB2-RNAi ClaI KpnI rev		ATATCGATGGTACCACAAAGCAGAGAGTTCGACT

Second article: Function of the plant DNA Polymerase epsilon in replicative stress sensing, a genetic analysis (to be submitted)

Function of the plant DNA Polymerase epsilon in replicative stress sensing, a genetic analysis

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ABSTRACT

Faithful transmission of the genetic information is essential in all living organisms. DNA replication is therefore a critical step of cell proliferation, because of the potential occurrence of replication errors, and of the fragile nature of replication forks. Indeed, replication stress frequently occurs in the absence of external perturbations; replication stress sensing and subsequent stabilization of the fork are therefore instrumental to the maintenance of genome integrity. More than 20 years ago, the replicative DNA polymerase ϵ (Pol ϵ) was shown to activate the S-phase checkpoint in yeast upon replicative stress. Since then, in spite of the increasing knowledge of the signaling pathways involved in DNA Damage Response (DDR), the molecular mechanisms underlying the Pol ϵ -dependent checkpoint activation have been little investigated, particularly in multicellular eukaryotes, due to the lethality of mutations affecting the involved factors. Here, we took advantage of hypomorphic *Arabidopsis* mutants partially deficient for the Pol ϵ catalytic subunit to explore the genetic interaction between Pol ϵ and the DDR. We found that Pol ϵ directly contributes to the activation of cell cycle checkpoints both in somatic and in meiotic cells, and provide a genetic dissection of the signaling pathways that function downstream of Pol ϵ .

INTRODUCTION

Faithful duplication of the genome is a key step during cell proliferation in all living-organisms. In eukaryotes, it requires the activity of three replicative polymerases (DNA Pol α , δ and ϵ) that are associated to a large protein complex called the replisome. This structure encompasses all the core activities required for DNA replication including DNA unwinding, synthesis of short RNA primers, primer elongation, tethering of the polymerases to DNA via a sliding clamp and protection of single stranded DNA (1). The helicase activity is brought by the CMG complex (CDC45, MCM, GINS) consisting of MCM (mini-chromosome maintenance) proteins that are the active helicases and their activators: CDC45 and the GINS (Psf5, 1, 2 and 3 also called Go, Ichi, Nii, San, Japanese for 5, 1, 2, 3) (2). GINS maintains association of Cdc45 with MCM in replisome progression complexes (Gambus et al., 2006).

All steps of DNA replication are under tight control to ensure that it initiates only once from each activated replication origin, and to avoid the accumulation of replication errors. The sequence of events and required factors for DNA replication are well understood, and it has recently been possible to reproduce replication initiation from purified proteins *in vitro* (3). Although it is clear that Pol α is responsible for the synthesis of RNA/DNA primers, the exact roles of Pol δ and ϵ are still a matter of debate. The most widely accepted view is that Pol δ and ϵ synthesize the lagging and leading strands respectively (4, 5), but according to an alternative model, Pol δ could be the main replicative polymerase whereas Pol ϵ would be involved in the repair of replication errors and play a scaffolding role (6). Consistent with a division of labours between Pol α , δ and ϵ , combination of a collection of mutations with hypomorphic alleles of the three replicative polymerases revealed specialized genetic networks interacting with each polymerase. This study corroborated the non-overlapping functions of the three polymerases in yeast as well as the central role of Pol ϵ at the pre-initiation steps of DNA replication (7).

In animals and fungi, DNA Pol ϵ consists of four sub-units: one large catalytic sub-unit (called Pol2 in yeast) and three accessory sub-units DPB2, 3 and 4. Pol2 and DPB2 are essential to cell viability whereas DPB3 and 4 are dispensable(8). Pol2 has two functional moieties: the highly conserved N-terminal domain encompassing the polymerase and exonuclease activities, and a C-terminal domain less well conserved. Surprisingly, only the C-terminal extension is required for cell survival and DNA replication (9), further supporting the notion that Pol ϵ has an essential scaffolding function, independently from DNA synthesis *per se*. Indeed, DPB2 was shown to directly interact with Psf1, a sub-unit of the CMG complex thereby connecting the DNA polymerase with the helicase complex

(10). Furthermore, detailed genetic analysis demonstrated that in *Schizosaccharomyces pombe*, Pol2 is required both for the chromatin loading and the progression of the CMG complex (11).

A number of factors such as DNA lesions, difficulty replicating sequences, collision with the transcription machinery etc... can impede fork progression during the S-phase and cause replicative stress. Stalled forks are fragile structures that can lead to genetic instability; cells have therefore evolved complex sensing mechanisms allowing checkpoint activation in response to replicative stress (12). Checkpoint activation triggers the expression of multiple genes required for replication fork stabilization, cell cycle arrest, and DNA repair (13, 14). In yeast, replicative stress activates the Mec1 kinase, that leads to the expression of DNA repair genes, nucleotide biosynthesis, expression of the DNA repair machinery and cell cycle arrest (12). Interestingly, Mec1 activation is mediated via two independent pathways, one triggered by single stranded DNA (ssDNA) accumulation and the other requiring the C-terminal domain of DNA Pol ϵ (15, 16). This sensor role of DNA Pol ϵ likely involves its ability to interact with the checkpoint protein Rad17 (17) and the mediator protein Mrc1 (18). In addition, in budding yeast, association of the Ctf18-RFC complex with the N-terminus of Pol ϵ was shown to be instrumental for the activation of the S-phase checkpoint, indicating that both domains of the protein can contribute to this sensor role of Pol ϵ (19).

Many mutations in DNA Pol ϵ have been associated with different types of cancer (20). Furthermore in *Xenopus laevis*, Pol ϵ interacts with Claspin (the homolog of Mrc1) (21), and the essential role of the C-terminus is conserved in *Drosophila* (22). Together, these reports suggest that the dual function of Pol ϵ in DNA replication and replicative stress response is conserved in all eukaryotes. However, most of the knowledge regarding the role of Pol ϵ in replicative stress sensing has been obtained in yeast because the lethality of Pol ϵ deficiency has precluded detailed analysis in multicellular organisms.

The *Arabidopsis* genome encompasses two genes encoding the catalytic sub-unit of Pol ϵ : *POL2A* and *POL2B*, but only *POL2A* is an essential gene (23). Over the past 10 years, a number of hypomorphic alleles of *POL2A* have been isolated: the *esd7-1* (*early in short days*7) mutant, which harbours a mutated amino acid at the junction of N- and C-terminal regions of the protein and shows early flowering as well as overall reduced growth (24), the *abo4-1* (*abscisic acid over-sensitive* 4) mutant line, which has a point mutation in the catalytic domain of the polymerase and the *abo4-2* mutant (with a T-DNA insertion in the middle of the gene), which display enhanced homologous recombination in somatic cells and constitutive activation of DNA repair genes (25); likewise, the *til1-4* (*tilted*1) mutant displays prolonged S phase during embryo development (26).

Defects observed in mutants that are partially deficient for POL2A suggest that the role of DNA Pol ϵ in replicative stress sensing is also conserved in plants. In this work, we took advantage of the viability of Arabidopsis hypomorphic mutant lines to investigate the role of Pol ϵ in replicative stress sensing and to genetically test its interaction with the main players of DNA Damage Response (DDR) that are conserved in all eukaryotes (27). Our results indicate that plant Pol ϵ functions upstream of the DNA Damage Response Kinase ATR (Ataxia Telangectasia mutated and Rad3-related) to activate replication stress response, providing evidence for the conservation of its key role in genome stability in multicellular eukaryotes.

MATERIAL AND METHODS

Cloning procedures

Transgenic *POL2ARNai* lines were generated after Agrobacterium-mediated transformation of Col plants with the plasmid CATMA1a07250, which harbours a fragment of 155 bp corresponding to the nucleotides 3368 to 3522 of the coding sequence of *ESD7/POL2A* cDNA, cloned in sense and antisense orientation in the pAgrikola vector (28), a Gateway® destination vector based closely on the Hellsgate 12 vector. Several transgenic independent plants were selected in medium containing phosphinothricin (PPT) at 10mg/ml and later were established as homozygous lines. All of them displayed lower levels of expression of *POL2A* mRNA in comparison to non-transformed control plants.

Plant material and growth conditions

Seeds were surface-sterilized by treatment with bayrochlore for 20 min, washed and imbibed in sterile-water for 2–4 days at 4 °C to obtain homogeneous germination. Seeds were sown on commercially available 0.5x Murashige and Skoog (MS) medium (Basalt Salt Mixure M0221, Duchefa) with the appropriate antibiotic if needed and solidified with 0.8% agar (Phyto-Agar HP696, Kalys), and grown in a long days (16h light, 8h night, 21°C) growth chamber. After 2 weeks, the plants were transferred to soil in a glasshouse under short-day conditions (8 h light 20°C, 16h night at 18°C) for 2 weeks before being transferred to long-day conditions.

RNA Extraction and quantitative RT-PCR

Total RNA were extracted from seedlings with the RNeasy MiniPrep kit (Qiagen, according to the manufacturer's instructions. First strand cDNA was synthesized from 2µg of total RNA using Improm-II reverse transcriptase (A3802, Promega) according to the manufacturer's instructions. 1/25th of the synthesized cDNA was mixed with 100nM of each primer and LightCycler 480 Sybr Green I master mix (Roche Applied Science) for quantitative PCR analysis. Products were amplified and fluorescent signals acquired with a LightCycler[®] 480 detection system. The specificity of amplification products was determined by melting curves. *PDF2* was used as internal control for signals normalization. Exor4 relative quantification software (Roche Applied Science) automatically calculates relative expression level of the selected genes with algorithms based on $\Delta\Delta C_t$ method. Data were from triplicates and are representative of at least two biological replicates. The sequence of primers used in this study is provided in Table S1.

Transcriptome Studies

Three independent biological replicates were produced. For each biological repetition and each point, RNA samples were obtained by pooling RNAs from more than 200 plants. Whole plantlets were collected on plants at 1.04 developmental growth stages (29), cultivated *in vitro* under long-day conditions. Total RNA was extracted as described above. RNA-seq experiment was carried out at the POPS Transcriptomic Platform, Institute of Plant Sciences - Paris-Saclay (Orsay, France). PolyA RNA was purified using the Dynabeads mRNA direct micro kit (Ambion, France). The sequencing libraries were constructed with the Ion Total RNA-Seq Kit v2 and the sequencing spheres were prepared with the Ion PI[™] Template OT2 200 Kit v3 before sequencing on an Ion Proton using the Ion PI[™] Sequencing 200 Kit v3 and Ion PI v2 chips (Life Technologies, France) with 520 run flows.

RNA-seq bioinformatic treatment and analysis

To allow comparisons, each RNA-Seq sample followed the same pipeline from trimming to count of transcript abundance as follows. Read preprocessing criteria included trimming library adapters and performing quality control checks using the Torrent suite (Version 4.2.1) with default settings. The reads corresponding to rRNAs were identified by mapping on *A. thaliana* rRNAs using bowtie

version2 (with –local option) (30) and removed. The same software was used to align the remaining reads against the *A. thaliana* transcriptome (33 602 mRNA from TAIR 10 (31)) without ambiguous hits (multi-hits are removed). According to these rules, around 75% of the initial reads aligned to transcripts for each sample. Genes which do not have at least 1 read after a counts-per-million (CPM) normalization in at least three samples among the six were discarded. The differential analysis has been performed by using a likelihood ratio test in a negative binomial generalized linear model where the dispersion is estimated by the method proposed in edgeR and where a biological replicate effect was taken into account. A gene was declared differentially expressed if its raw p-value adjusted by the Benjamini-Hochberg procedure to control the FDR is ≤ 0.01 and its absolute fold change is ≥ 1.5 . Analyses were performed with the software 'R' (Version 3.1.0) and the edgeR package (version 3.6.8) of Bioconductor.

Light and fluorescence microscopy

Fresh siliques were opened under a stereo-microscope (SVII, ZEISS) and images were captured with a colour CCD camera (Power HAD, Sony).

For meiotic analyses, flower buds were fixed in EtOH : Acetic Acid (3 :1). 4',6-Diamidino-

2-phenylindole staining of meiotic chromosomes was performed according to a previously described method (32). Slides were observed on an epi-fluorescence videomicroscope (SVII; Zeiss), and images were captured with a colour charge-coupled device camera (Power HAD; Sony).

Observations were done with a widefield fluorescence microscope (AxioImager Z.2, Carl Zeiss GmbH, Germany) fitted with a metal halide lamp and the appropriate shifted free filter sets for imaging DAPI dye (Zeiss n°49). Images were acquired with a cooled CCD camera (AxioCam 506 monochrome, Zeiss) operated using Zen Blue software (Zeiss, GmbH). Cell cycle length analysis was performed as described in (33).

Flow cytometry

For flow cytometric nuclei analysis, tissues were chopped with a razor blade in 1 mL of Gif nuclei-isolation buffer (45 mM MgCl₂, 30 mM sodium citrate, 60 mM MOPS, 1% (w/v) polyvinylpyrrolidone 10,000, pH 7.2) containing 0.1% (w/v) Triton X-100, supplemented with 5

mM sodium metabisulphite and RNase (5 U/ml). Propidium iodide was added to the filtered supernatants to a final concentration of 50 µg/ml. Endoreduplication levels of 5,000-10,000 stained nuclei were determined using a Cyflow SL3 flow cytometer (Partec-Sysmex) with a 532-nm solid state laser (30 mW) excitation and an emission collected after a 590-nm long-pass filter. For cell cycle analysis, we used the algorithm available in the Flomax software.

Statistical analysis

All statistical analysis was performed using the R software (<https://www.r-project.org/>).

RESULTS

Partial deficiency for POL2A confers tolerance to replicative stress and induces constitutive activation of the DNA damage response.

To investigate the role of the catalytic sub-unit of Pol ϵ in the activation of the DDR, we initially analysed the *abo4-1* mutant. This mutant harbours a point mutation leading to a Gly₅₃₄ to Arg change affecting a highly conserved amino acid in the catalytic domain of the protein. Flow-cytometry analysis of cauline leaves revealed broadened peaks compared to the wild-type (Figure S1A) as previously described for plants over-expressing the Pol ϵ regulatory sub-unit DPB2 (33), suggesting that this mutant accumulates cells with intermediate DNA contents due to defects in cell cycle progression. This hypothesis was further supported by flow-cytometry analysis of flower buds nuclei (Figure S1B-C) and EdU incorporation assay (Table S2) and likely results from constitutive activation of cell cycle checkpoints (25). In addition, *abo4-1* mutants are hypersensitive to a wide range of genotoxic stresses ((25) and this study Figure S2) probably because the catalytic activity of Pol ϵ is required for DNA repair. However, how they respond to replicative stress had not been investigated. To explore the role of POL2a in the replicative stress response, *abo4-1* mutants were challenged with hydroxyurea (HU), an inhibitor of ribonucleotide reductase that induces stalling of replication forks by depleting cellular deoxyribonucleotide pools. This treatment thus induces fork stalling without creating DNA damage directly. As shown on Figure 1, *abo4-1* was more tolerant to replication fork stalling than the wild type.

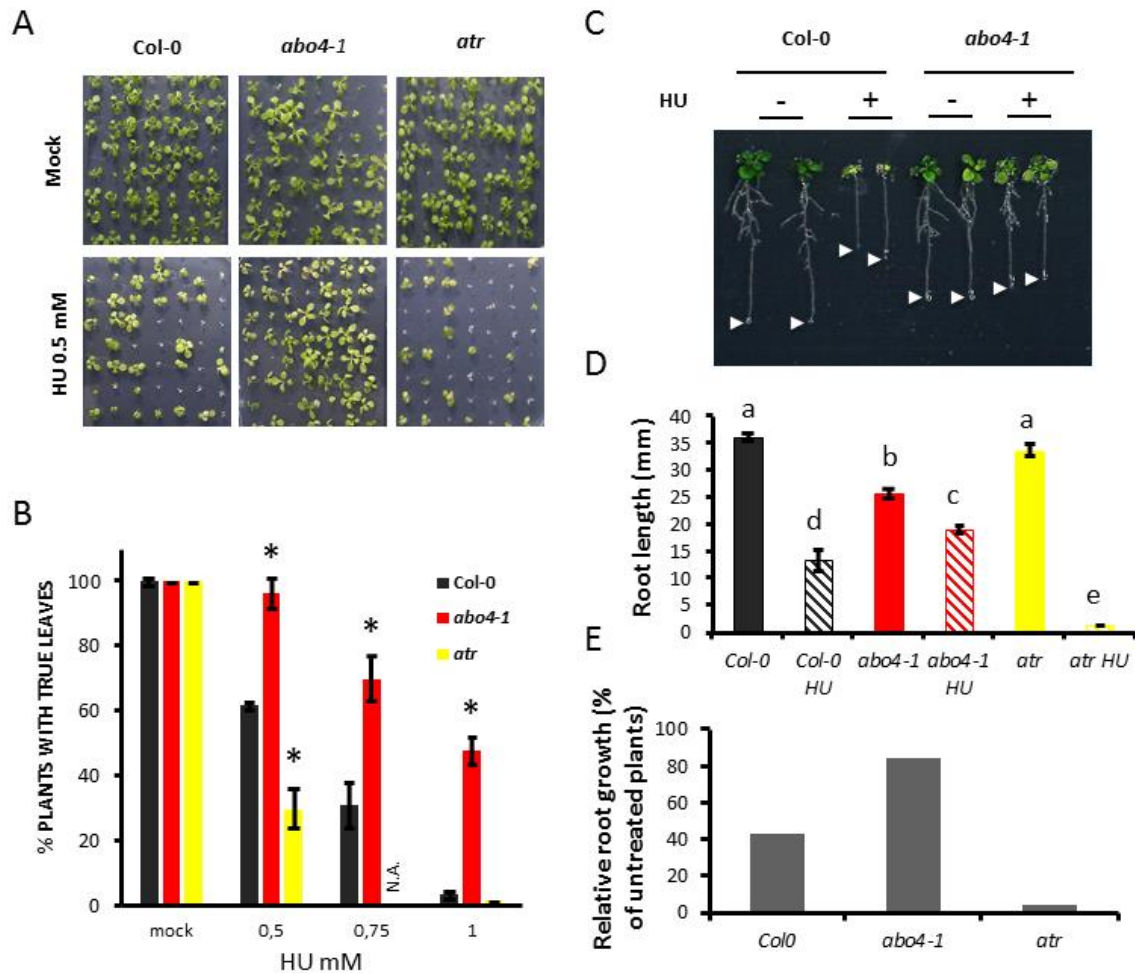


Figure 1: The *abo4-1* mutant shows increased tolerance to HU-induced replicative stress.

A-B: Wild-type (Col-0) and *abo4-1* mutant seedlings were germinated on HU-supplemented medium and plants with true leaves were counted after 12 days. The *atr* mutant was used as a hypersensitive control. In B, values are average \pm s.e. of three biological replicates. Asterisks indicate statistically relevant differences with respect to the wild-type in the same conditions (Student t-test $p < 0.05$). C-E: Wild-type (Col-0) and *abo4-1* mutant seedlings were grown for 4 days on half strength MS and transferred to HU-supplemented medium for 9 days to monitor root growth. C: by contrast with wild-type plants, root length was almost unchanged by HU exposure in the *abo4-1* mutant arrowheads mark the position of the root tip. D: average root length was measured after 9 days on HU, at least 20 plantlets were used for each treatment, values are average \pm s.e. Different letters indicate significantly different values (Student t-test $p < 0.05$). Data are representative of two independent experiments. E: the relative growth of each genotype after 9 days on HU was calculated compared to untreated plants of the same genotype

Previous studies have shown that a number of genes involved in the DDR are constitutively activated in *POL2A*-deficient mutants (25). To obtain a broader view of this response, the transcriptome of the *abo4-1* line was compared to that of wild-type plants by RNA sequencing. The *abo4-1* line showed that 218 genes were significantly induced while 153 were repressed (absolute fold change ≥ 1.5 p value ≤ 0.01 ; Table S3). Interestingly, we observed significant overlap with previous RNA-seq analysis of this mutant (34), although a large proportion of genes were identified as specifically mis-regulated in only one of the studies (Figure S3A). As expected, gene-ontology (GO) analysis of significantly up-regulated genes revealed over-representation of DNA metabolic process, response to DNA damage, and cell cycle (Figure S3B). Among these were several genes involved in DNA replication such as DNA polymerase δ subunit 4, which could reflect the ability of Pol δ to compensate for Pol ϵ deficiency. Three genes encoding the single stranded DNA (ssDNA) binding proteins RPA1C, D and E as well as RAD17 were also up-regulated, indicating that *abo4-1* mutants are subjected to constitutive replicative stress. In addition, different ATR-downstream targets such as the WEE1 kinase, that participates in the inactivation of cyclin-dependent kinases (CDKs), or SMR7, a plant-specific CDKs inhibitor, were activated together with B-type CDKs (CDKB1;1, CDKB1;2, and CDKB2;1) and B-type cyclin (CYCB1;1, CYCB1;4, CYCB2;1, and CYCB2;4), consistently with previous reports (25). Finally, expression of genes involved in DNA repair was also induced in the *abo4-1* mutant line (Table S4).

This analysis confirms that constitutive DNA replicative stress results in the activation of cell cycle checkpoints in the *abo4-1* line.

The ATR-WEE1-dependent checkpoint is required for the viability of *POL2A* mutants.

We next used a genetic approach to determine which DDR pathways are activated in *abo4-1*. In plants as in other eukaryotes, the ATM (Ataxia Telangectasia Mutated, also called Tel1 in yeast) and ATR (Mec1 in yeast) are the two main kinases involved in the response to double-strand breaks and replicative stress respectively (27). In plants, signals from these two pathways converge towards the SOG1 transcription factor that can activate cell cycle inhibitors as well as DNA repair genes (27). We first crossed *abo4-1* with the *atr* and *wee1* mutants that are deficient for replicative stress response (35). Double mutants could never be recovered, and siliques of sesquimutants

contained around ¼ of aborted seeds (Figure 2A-F). Closer observation of embryo development in *abo4-1 atr/+* and *abo4-1 wee1/+* sesquimutants, showed that ¼ of embryos stopped development at various stages and displayed aberrant division patterning (Figure 2G-N). Similar defects in embryo development have been already described in T-DNA insertion homozygous mutants for *pol2a*, and when wild-type embryos were exposed to aphidicolin, an inhibitor of replicative polymerases (26). These results thus suggest that ATR and WEE1 are required during embryo development in *POL2A*-deficient lines for cell proliferation progress in spite of replicative stress.

Part of the response to replicative stress mediated by ATR depends on the SOG1 transcription factor that acts independently of WEE1 (35). We therefore asked whether SOG1 also contributed to the checkpoint activation observed in *abo4-1* mutants. *abo4-1 sog1* mutants were viable, albeit smaller than *abo4-1* single mutants (Figure S4), indicating that SOG1 activity is required to sustain growth in *abo4-1*, but not for embryo development.

We next asked whether the tolerance to DNA damage observed in *abo4-1* plants required SOG1 activation. As previously demonstrated (35), *sog1* was hypersensitive to HU. By contrast, *abo4-1 sog1* mutants behaved like wild type plants on medium supplemented with 0.25mM or 0.5 mM of HU and thus displayed an intermediate phenotype between the two parental lines (Figure 3A-B and S5A-B). To confirm that DDR activation observed in *abo4-1* mutants was partly dependent on SOG1 activity, we performed qRT-PCR on 11 genes that were up-regulated in *abo4-1* seedlings according to the RNA-seq data (Table S3). These genes are representative of different mechanisms such as cell cycle regulation (*CYCB1;1*, *WEE1*, and *SMR7*), DNA repair genes (*RAD51*, *BCRA1*, *XR11*, and *PARP2*), and nucleotide synthesis genes (*TK1a*, *TSO2*, and *RNR1*). Consistently, up-regulation of some DDR genes was lost in *abo4-1 sog1* mutants while others were still up-regulated albeit to a lower extent than in *abo4-1* single mutants (Figure 3C).

The additivity of the *sog1* and *abo4-1* phenotypes suggests that *POL2A* deficiency triggers replicative-stress checkpoint activation via the two SOG1-dependent and independent pathways. Finally, we tested the contribution of SOG1 to the sensitivity of *abo4-1* mutants to DSB-inducing agents. As shown in Figure 3D-E and S5C, the *abo4-1 sog1* double mutant was similarly tolerant to zeocin like the *sog1* mutant, suggesting that the sensitivity of *abo4-1* mutant to DBSs requires SOG1 activity.

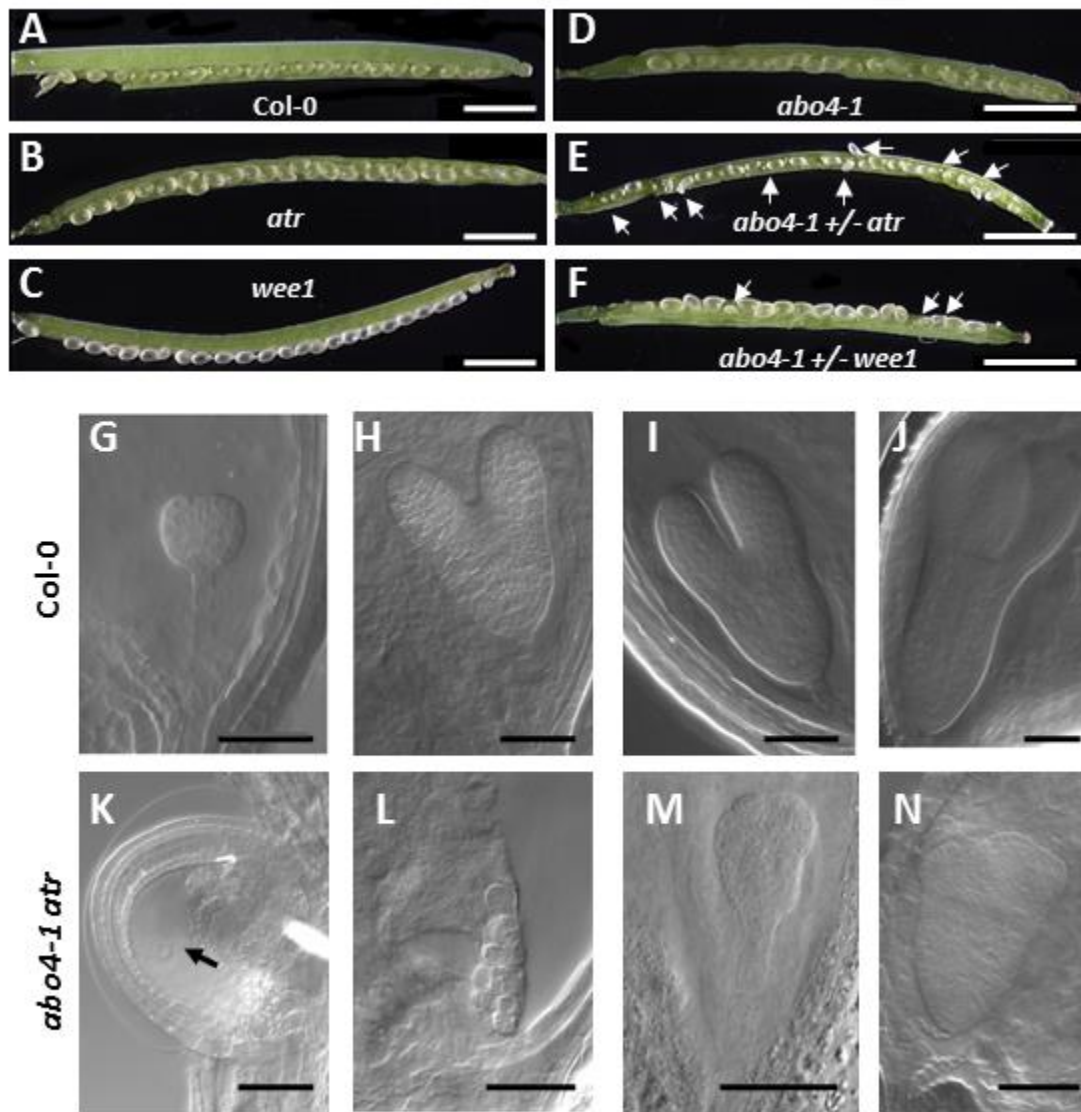


Figure 2. ATR and WEE1 are required for *abo4-1* mutant viability.

A-F: open siliques of wild-type (A), *atr* (B), *wee1* (C), *abo4-1* (D) mutants, *atr/+ abo4-1* (E) and *wee1/+ abo4-1* (F) sequimutants. Arrows point to aborted seeds. Bar= 2mm for all panels.

G-N: embryo development in wild-type (G-J) and *wee1/+ abo4-1* sequimutants. G: globular stage, H: late heart stage, I-J: early and late torpedo stage. In the siliques of *wee1/+ abo4-1* mutants, about $\frac{3}{4}$ of embryos undergo normal development as in the wild-type, *abo4-1* or *wee1* single mutants. However, $\frac{1}{4}$ of embryos stop development, at various stages and show aberrant division patterning. K: arrested embryo just after fertilization, the arrow points to the single nucleus of the endosperm, L, M: embryos at the globular stage with abnormal cell organization, N: embryo at the late torpedo stage with misshapen cotyledons. Bar = 50 μm for all panels

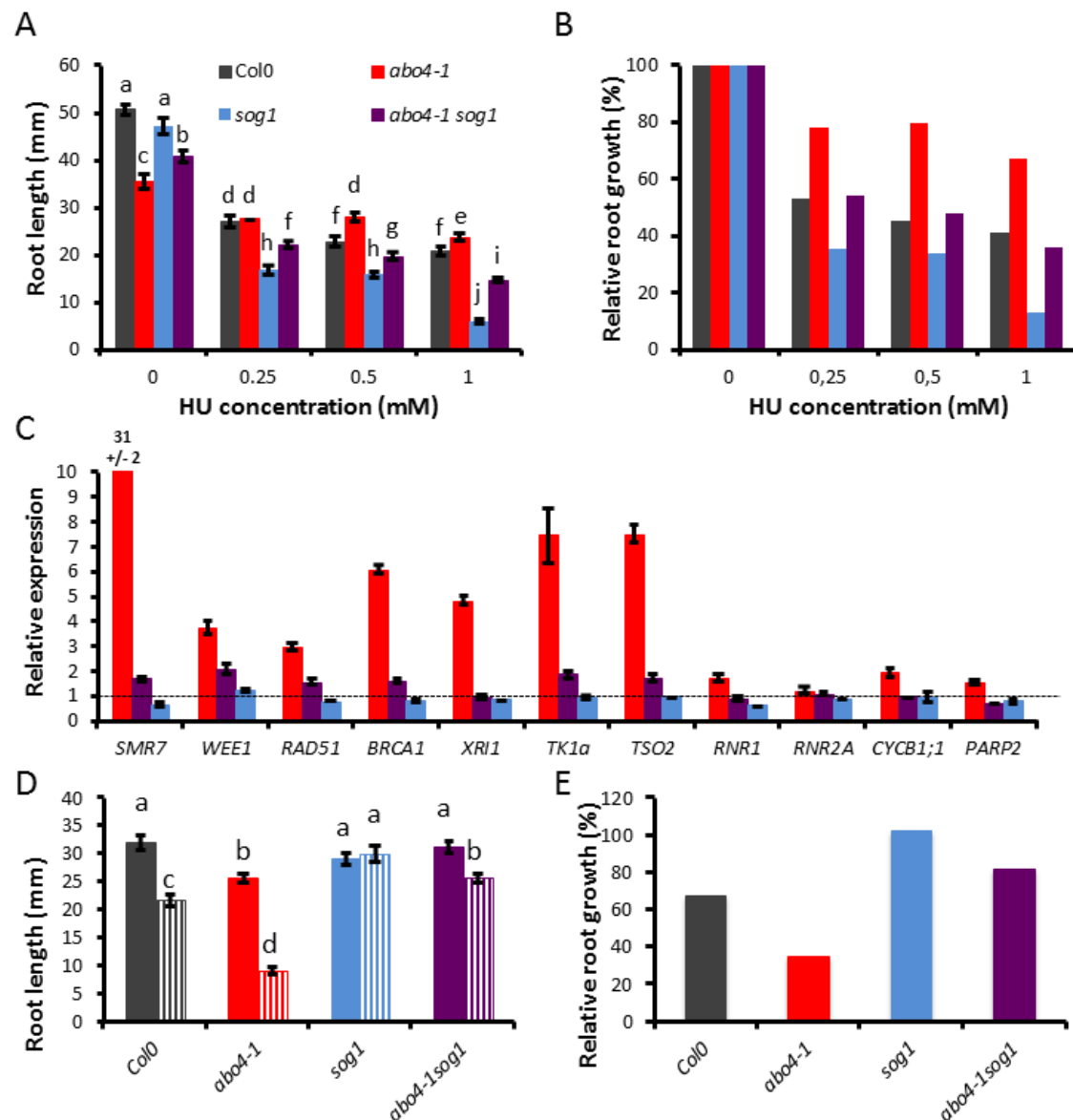


Figure 3. The checkpoint activated by the *abo4-1* mutation is partially dependent on SOG1.

A-B: HU sensitivity in wild-type (Col-0), *abo4-1*, *sog1* and *abo4-1 sog1* mutants. Plantlets were grown on half strength MS for 4 days and transferred to control medium (full bars) or HU supplemented medium (dashed bars) for 9 days. A: Root length, values are average \pm s.e. obtained on at least 15 plantlets. B: relative root growth, values are expressed as percentage of length on MS medium. C: qRT-PCR analysis of the expression of selected genes in *abo4-1*, *sog1* and *abo4-1 sog1* mutants, values are average \pm s.d. D-E: Zeocin sensitivity in wild-type (Col-0), *abo4-1*, *sog1* and *abo4-1 sog1* mutants. Plantlets were grown on half strength MS for 4 days and transferred to control medium (full bars) or zeocin supplemented medium (dashed bars) for 9 days. D: Root length, values are average \pm s.e. obtained on at least 15 plantlets. In A and D, different letters indicate significantly different values (Student t-test, $p < 0.05$). For all panels, data are representative of at least two independent experiments

The checkpoint activated by POL2A deficiency is ATM-independent

We next asked whether the ATM pathway involved in DSB response was also required for *abo4-1* viability: *abo4-1 atm* double mutants could be recovered, indicating that the ATM pathway is dispensable for *abo4-1* survival. To determine whether constitutive activation of DDR in *abo4-1* involves ATM activity, we treated *atm* and *abo4-1 atm* seedlings with HU. Intriguingly, *atm* seedlings also displayed HU tolerance compared to wild-type plants (Figure 4A-B), suggesting that HU affects root growth at least partly via ATM activation, likely due to DSB formation after fork collapse. HU tolerance was the same in *abo4-1 atm* mutants as in *abo4-1* single mutants (Figure 4A-B). Consistently, expression of DDR genes was induced to the same level in *abo4-1* and *abo4-1 atm* mutants compared to wild-type plants, indicating that DDR activation in *abo4-1* does not require ATM (Figure 4C).

These results suggest that ATM and POL2A could act in parallel in the DSBs response. To test this hypothesis, we monitored the sensitivity of single and double mutants to zeocin. Prolonged exposure to zeocin had a similar effect on root growth in *abo4-1* and *atm abo4-1* (Figure 4D). However, when the seedlings were allowed to recover for 6 days after a 6 days treatment to zeocin, *abo4-1 atm* seedlings displayed reduced growth compared to *abo4-1* single mutant, and their fresh weight was dramatically decreased (Figure 4E-F), corroborating that the *atm* and *abo4-1* mutations have additive effects on tolerance to DSBs.

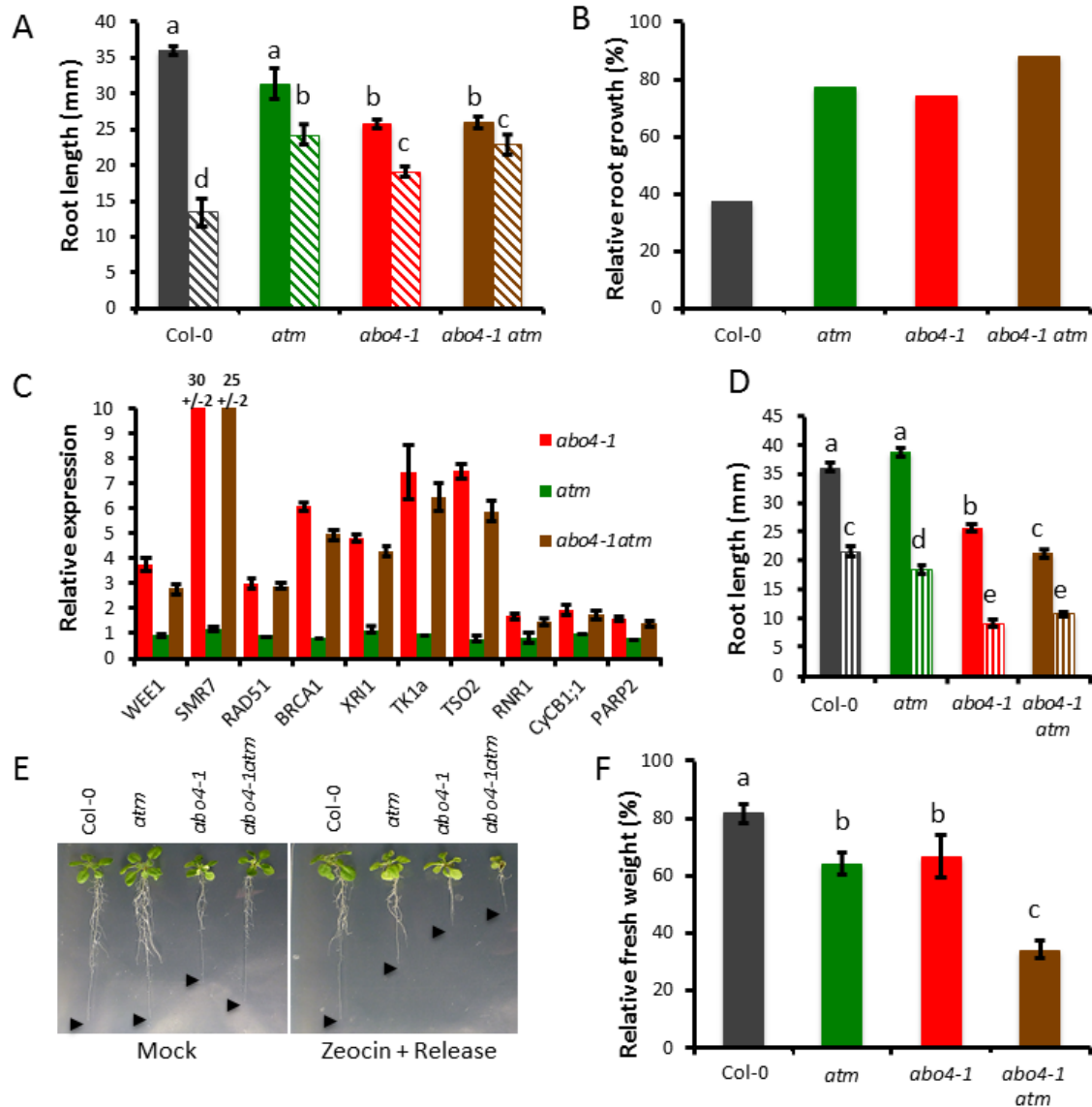


Figure 4. The checkpoint activated by the *abo4-1* mutation is ATM-independent

A-B: HU sensitivity in wild-type (Col-0), *abo4-1*, *atm* and *abo4-1 atm* mutants. Plantlets were grown on half strength MS for 4 days and transferred to control medium (full bars) or HU supplemented medium (dashed bars) for 9 days. A: Root length, values are average \pm s.e. obtained on at least 15 plantlets. B: relative root growth, values are expressed as percentage of length on MS medium. C: qRT-PCR analysis of the expression of selected genes in *abo4-1*, *atm* and *abo4-1 atm* mutants, values are average relative expression compared to the wild-type \pm s.d. D: Zeocin sensitivity in wild-type (Col-0), *abo4-1*, *atm* and *abo4-1 atm* mutants. Plantlets were grown on half strength MS for 4 days and transferred to control medium (full bars) or zeocin supplemented medium (dashed bars) for 9 days. D: Root length, values are average \pm s.e. obtained on at least 15 plantlets. E: Representative picture of plantlets grown on half strength medium (mock), or grown on MS supplemented with zeocin for 6 days and allowed to recover for 6 more days. Arrowheads mark the position of the root tip. F: Relative fresh weight of plantlets after recovery. Values are average \pm s.e. from 6 replicates. In A, D and F different letters indicate statistically relevant differences (Student t-test, $p < 0.05$).

Contrasting outcomes of distinct forms of *POL2A* deficiency on DDR.

Because mutations in different regions of the POL2 protein led to diverse defects in other eukaryotes (20, 36), we tested whether other hypomorphic alleles of *POL2A* could lead to constitutive activation of DNA stress response and tolerance to HU. The *abo4-2* mutant harbours a T-DNA insertion in the 12th intron. In this mutant, the 5' and 3' portions of the cDNA situated on each side of the T-DNA insertion accumulate at almost wild-type levels (Figure S6A). In addition, RT-PCR analysis using primers located on each side of the insertion revealed the accumulation of low levels of wild-type mRNA and additional splicing variants corresponding to the elimination of exon 12 or exons 12 and 13 (amino acids 427 to 481 or 427 to 540 respectively); these splicing variants do not generate a frameshift, and thus allow the production of a modified protein lacking conserved amino-acids of the active site (Figure S6B). This mutant thus likely accumulates different isoforms of the POL2A protein and possibly its N or C-terminal domain on its own. In addition, *abo4-2* displays defects in cell-cycle regulation like the *abo4-1* mutant (Figure S7, and Table S2).

As observed in *abo4-1*, *abo4-2* mutant also displays tolerance to HU and hypersensitivity to zeocin (Fig S8A-B) and shows constitutive expression of genes involved in DDR (Figure S8C). To determine whether the same pathways as in *abo4-1* were activated, we crossed *abo4-2* with *atr*, *atm* and *sog1* mutants: only *abo4-2 sog1* and *abo4-2 atm* double mutants were viable. As in *abo4-1*, transcriptional activation of DDR genes was largely SOG1-dependent but ATM-independent (Figure S8C). However, although the *sog1* mutation induced a further reduction of rosette growth in the *abo4-1* background, the *abo4-2 sog1* mutant displayed improved development compared to the *abo4-2* single mutant (Figure S4, Figure S8A), indicating that the *abo4-1* and *abo4-2* mutations have different consequences on DDR activation. To gain further insight into these differences, we tested the sensitivity of *abo4-2*, *abo4-2 sog1* and *abo4-2 atm* mutants to genotoxic stress. The tolerance to HU was less pronounced than in *abo4-1* but it was higher than in the wild-type (Figure S8A-B). To further establish whether altered POL2A function leads to HU tolerance but hypersensitivity to DNA damage, we also analyzed the *esd7-1* mutant that harbors a point mutation leading to a substitution of Gly by Arg at position 992, situated at the junction between the active site of the protein and its C-terminal domain (24). Like *abo4-1* and *abo4-2*, this mutant was tolerant to HU, but hypersensitive to a variety of genotoxic stresses (Figure S9), further corroborating that incorporation of modified POL2A at the fork leads to replicative stress.

As stated above, DNA Pol ϵ plays a dual role at the replication fork because it performs both a catalytic and a scaffolding function, and defects described in *abo4-1* and *abo4-2* mutants could either be direct consequences of the signalling role of DNA Pol ϵ or indirect effects of replicative stress. To discriminate between these two hypotheses, we generated *POL2A* RNA interference lines (*POL2A-RNAi*). *POL2A-RNAi* plants display a range of developmental alterations such as reduced size, early flowering, and partial sterility (Figure S10A). These features are shared with *POL2A*-deficient mutants. However, only *POL2A-RNAi* lines with a mild phenotype that reached a similar size as the wild-type showed a sufficiently stable phenotype over generations to be used for further analysis (Figure S10B). Although these lines showed a slight increase in S-phase length, flow cytometry revealed no obvious accumulation of cells in S-phase (Table S2 and Figure S7).

By contrast to *abo4-1* and *abo4-2*, *POL2-RNAi* seedlings were hypersensitive to HU (Figure 5A-B), and they were not affected by zeocin exposure (Figure 5C-D). Consistently *POL2A-RNAi* did not display constitutive up-regulation of DDR genes (Figure 5E), suggesting that adequate levels of *POL2A* are essential for transcriptional activation triggered by replication stress checkpoint.

Failure to activate suitable response upon replication stress can lead to fork collapse and thus generate DSBs that in consequence trigger ATM activation. To determine whether ATM activity is essential to *POL2-RNAi* plants survival, we tested the effect of a specific inhibitor of ATM activity (IATM), and found that *POL2A-RNAi* lines are hyper-sensitive to this drug (Figure 5F) supporting the notion that ATM pathway activation is required for plant survival when *POL2A* accumulation is decreased.

In summary, these results suggest that the presence of mutated *POL2A* in the replication fork affects cell cycle progression leading to an increase in S-phase length due to checkpoint activation that confers tolerance to HU, whereas lowered concentration of *POL2A* prevents proper checkpoint activation in response to replicative stress.

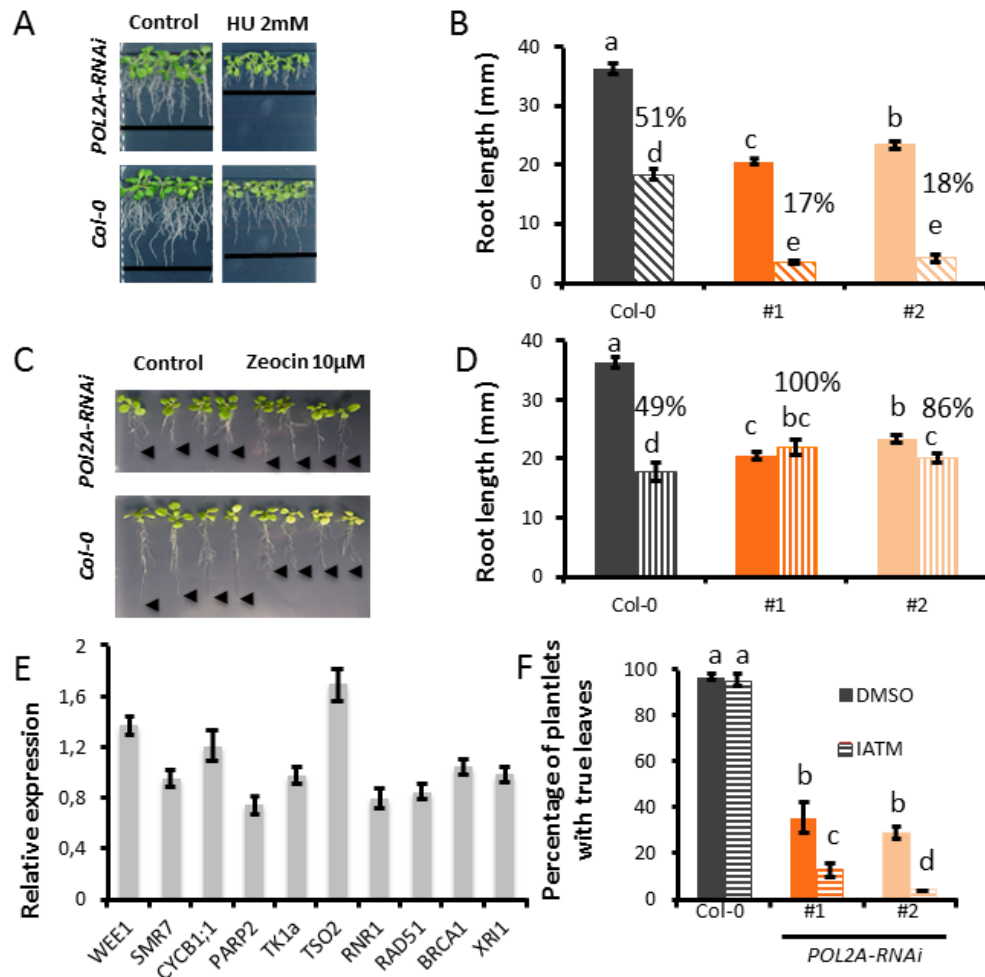


Figure 5: Proper levels of *POL2A* are required for checkpoint activation in DNA damage response.

A,B: Wild-type (*Col-0*) and *POL2A-RNAi* seedlings were grown for 4 days on half strength MS and transferred to HU-supplemented medium for 9 days. *POL2A-RNAi* lines were hypersensitive to this drug: lines indicate the extremity of roots (A). After 9 days, root length was measured on plants kept on control medium (full bars) or on HU supplemented medium (dashed bars) (B). Values above the bar indicate the relative root growth compared to the respective untreated control. C, D: Wild-type (*Col-0*) and *POL2A-RNAi* seedlings were grown for 4 days on half strength MS and transferred to zeocin-supplemented medium for 9 days. *POL2-RNAi* lines were unaffected by this drug (C). Arrowheads mark the position of the root tip. After 9 days, root length was measured on plants kept on control medium (full bars) or on zeocin-supplemented medium (dashed bars) (D). E: qRT-PCR quantification of selected genes in *POL2A-RNAi* seedlings. Values are average \pm s.d. compared to the wild-type. F: *POL2A-RNAi* plantlets are hypersensitive to IATM. Plants were germinated on MS medium containing DMSO or IATM (10 μ M). After 10 days, the percentage of plants with true leaves was monitored. Germination and development are severely affected in *POL2A-RNAi* lines, and the proportion of plants with true leaves was therefore reduced compared to the wild-type on control medium. However, this reduction was even more pronounced in the presence of IATM, whereas this compound had no effect on wild-type plants. In B, D and E, values are average \pm s.e. of data obtained on at least 15 plantlets. Different letters indicate statistically relevant differences (Student t-test $p < 0.05$). All data are representative of at least two biological replicates.

Role of Pol ϵ catalytic subunit during Arabidopsis reproductive development.

Recently, we have shown that increased accumulation of the Pol ϵ regulatory sub-unit DPB2 led to the activation of a cell cycle checkpoint during pre-meiotic DNA replication and induced SOG1-dependent DNA fragmentation (33). Furthermore, Huang and colleagues reported that the *til4-1* and *abo4-2* alleles of *POL2A* display meiotic DNA fragmentation, although they attributed these defects to impaired DSB repair (37). We observed that the fertility of *abo4-2 sog1* mutants was improved compared to *abo4-2*, suggesting that meiotic defects are at least partly due to SOG1 function. To clarify whether *POL2A* also participates in a pre-meiotic checkpoint, we analysed meiosis progression in *abo4-2* and in *abo4-2 sog1* mutants. In our hands, DNA fragmentation was observed in 89% of meiocytes (n=123) in the *abo4-2* mutant (Figure 6). This proportion was lowered to 40% in *abo4-2 sog1* mutants (n=93), confirming the hypothesis that partial *POL2A* inactivation can trigger the SOG1-dependent checkpoint in response to defects during pre-meiotic DNA replication.

Surprisingly, the fertility of *abo4-1 atm* and *abo4-2 atm* double mutants was modified compared to parental lines. Indeed, *abo4-1 atm* plants showed improved fertility compared to *atm* (Figure S11A-B), suggesting that constitutive activation of the DDR in *abo4-1* might partially rescue the meiotic defects of *atm* (38). We also observed that the fertility of *abo4-2 atm* mutants was improved compared to *abo4-2*: the number of seeds per silique was comparable to what was observed in *atm* (Figure S11C-D). Previous studies have shown that *atm* mutants are partially deficient for repair of meiotic DSBs (38), as described for *abo4-2*. However, the rescue of *abo4-2* sterility by the *atm* mutation suggests that the SOG1-dependent checkpoint triggered by pre-meiotic replication defects requires ATM activity.

Taken together, our results indicate that *POL2A* plays a role in replicative stress checkpoint activation both in somatic and in reproductive tissues, but that the signaling events differ between the different cell types.

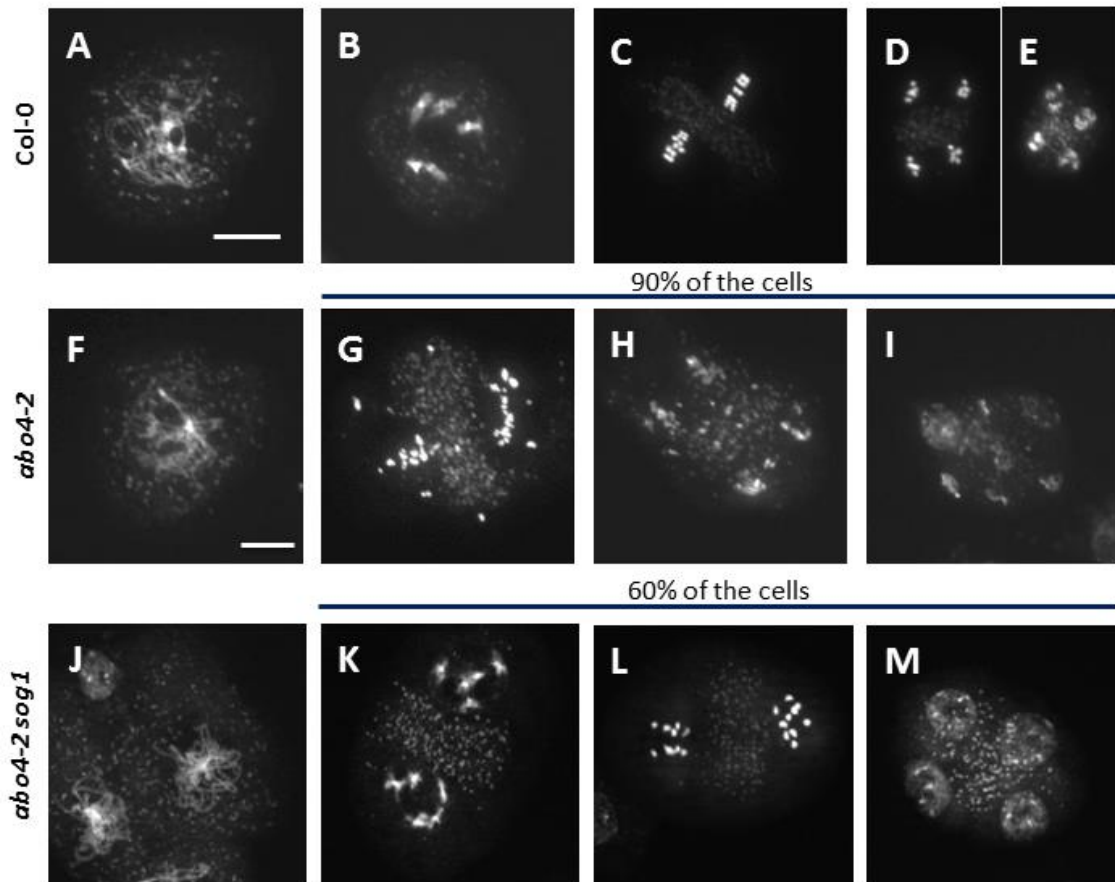


Figure 6: *abo4-2* mutants show SOG1-dependent meiotic fragmentation

Meiosis progression in the wild-type (A-E), *abo4-2* mutant (F-I) and *abo4-2 sog1* mutant. In the wild-type, after early prophase (A), bivalents were formed (B), homologous chromosomes segregated during division I, and sister chromatids segregate during division II (C, metaphase, D, anaphase) to form tetrads (E). In the *abo4-2* mutant, early prophase was normal (F), but bivalents were never observed. Instead, in 90% of the cells, extensive DNA fragmentation was observed both during the first (G) and the second division (H), leading to the formation of polyads (I). In *abo4-2 sog1* mutants, 40% of the cells still showed DNA fragmentation, but 60% of meiocytes were wild-type like (K: end of division I, L anaphase of division II, M: tetrad). Bar = 10 μ m for all pannels

DISCUSSION

Arabidopsis POL2A participates in cell cycle checkpoints activation and fork stabilization

DNA Pol ϵ is required not only for DNA synthesis *per se* during DNA replication, but also for sensing of replication stress. This dual role is well established in yeast, and likely conserved in animals, but detailed investigation has been hampered by the lethality of mutants deficient for its catalytic subunit. In this work, we took advantage of hypomorphic alleles encompassing partially defective versions of the Pol ϵ catalytic sub-unit POL2A (*abo4-1*, *abo4-2* (25) and *esd7-1* (24)) available in Arabidopsis to explore its contribution in checkpoint activation upon replicative stress. Although they are hypersensitive to DNA damaging agents ((25), this study), *abo4* and *esd7* mutant alleles display specific tolerance to HU-induced replicative stress. This phenotype is likely due to basal activation of the replication stress checkpoint, as evidenced by the prolonged S-phase and constitutive expression of DDR genes observed in the mutants. Indeed, *abo4-1* seedlings displayed up-regulation of genes encoding proteins required for replicative stress response such as the ssDNA sensors Rad 17 and RPA (39, 40) or CYCB1;1 that promotes G2 arrest (41). One of the key elements during replication stress response is the activation of nucleotide biosynthesis by the RNR that in yeast has been shown to depend on Pol ϵ (15, 42, 43). In addition to the *de novo* pathway involving the Ribonucleotide reductase (RNR), plants like all eukaryotes with the exception of yeast also rely on a salvage pathway comprising Thymidine Kinase 1 (TK1) (44), and these two pathways have redundant functions in DDR (45–48). Elements of both pathways are up-regulated in *abo4* mutants, which may account for their tolerance to HU.

To further explore the role of Pol ϵ in replicative stress response, we generated knock-down lines expressing lower levels of *POL2A* mRNA. Surprisingly, these lines were viable, and the less severe ones reached a similar size to wild-type plants, either because Pol δ can synthesize both DNA strands when Pol ϵ accumulation is reduced as has been hypothesized in other organisms (6, 8), or because expression of Pol ϵ is still sufficient to allow proper S-phase progression. By contrast with partial inactivation of POL2A, down-regulation of *POL2A* accumulation did not induce constitutive activation of the replicative stress checkpoint but resulted in enhanced sensitivity to HU, further supporting the direct involvement of plant Pol ϵ in replicative stress sensing. The hypersensitivity of *POL2A-RNAi* lines to HU may also be a consequence of defects in fork stabilization due to decreased POL2A accumulation. Indeed, Pol ϵ plays an essential scaffolding role to stabilize stalled forks (18, 20, 49), independently of checkpoint activation (50). Alternative mechanisms have been

described to stabilize the replisome when Pol ϵ is limiting (51), but whether they are conserved in plants and could be operating in *POL2A-RNAi* lines still remains to be established.

POL2A-RNAi lines and *abo4* mutants also showed contrasting responses to DSBs: *abo4* mutants are hyper-sensitive to zeocin whereas *POL2A-RNAi* lines are tolerant to this drug. The sensitivity of *abo4* mutants to zeocin may reflect the direct involvement of Pol ϵ in DNA repair, but it could also be due to defects in the transcriptional response induced by DNA damage. This tolerance to zeocin of *POL2A-RNAi* lines suggest that bypass mechanisms possibly involving translesion synthesis (TLS) polymerases compensate Pol ϵ down-regulation. The activation of TLS polymerases in response to DNA damage has been shown to require ATM (52), consistent with the hypersensitivity of *POL2A-RNAi* lines to the ATM inhibitor. Interestingly, *sog1* mutants are hypersensitive to HU and tolerant to zeocin, and the *sog1* mutation was epistatic on the *abo4* mutations for these responses. These results indicate (i) that *abo4* functions upstream of SOG1 to confer tolerance to HU and (ii) that the sensitivity of *abo4* mutants to zeocin requires SOG1 activity, suggesting that bypass mechanisms can be activated independently of SOG1 (53). In yeast, the Mec1 (ATR) pathway has been shown to attenuate Tel1 (ATM) signaling (54), and constitutive activation of SOG1 via ATR may thus prevent proper response to DSBs in Arabidopsis and account for the sensitivity of *POL2A* hypomorphic mutants to DNA damaging agents.

Signaling downstream of POL2A

Replicative stress sensing depends on the ATR kinase that activates WEE1 and SOG1 via two independent pathways (35), but does not involve ATM (55). Consistently, the viability of *abo4* mutants does not require ATM, but depends on components of the replication stress checkpoint: ATR and WEE1. In *S. pombe*, deletion of the POL2 catalytic domain led to tolerance to HU and hypersensitivity to MMS, and survival of this mutant was strictly dependent on Rad3 (ATR), and Pol δ (56), because ATR is required to stabilize the association of Pol ϵ with stalled forks (49), indicating that the mechanisms described in yeast are conserved in multicellular organisms.

Interestingly, *abo4 sog1* mutants are viable, suggesting that Pol ϵ deficiency can activate both branches of the replicative stress response as previously described (33), and demonstrating that only the ATR-WEE1 branch of the pathway is required for embryo development in the presence of replicative stress.

A puzzling result was the observation that the *sog1* mutation had opposite effects on the growth of the two *abo4* alleles studied here: *abo4-1 sog1* mutants showed further reduced growth reduction whereas *abo4-2 sog1* were larger than *abo4-2* mutants. In mammals, the consequences of POL2 mutations differ depending on the mutated protein domain (36, 49). A tempting possibility could be that the *abo4-1* mutation triggers mainly the ATR branch of the DDR, whereas the *abo4-2* mutation also activates the ATM branch of the pathways, possibly because this particular mutated allele affects not only POL2A activity but also its accumulation, leading to endogenous DNA damage as a consequence of fork collapse. Antagonistic roles of the ATR and ATM pathways could also explain this observation (54): in *abo4-2* mutants, loss of SOG1 may allow more effective activation of the ATM pathway and thus rescue growth defects. Finally, our results showed that ATM and POL2A likely function in parallel in response to DSBs: *atm abo4* double mutants were unable to resume growth after release from genotoxic stress, indicating an additive effect of the two mutations, consistent with the model according to which ATM and ATR-signaling cooperate in DDR (27).

Pol ϵ and meiosis

Recently, we reported that over-expression of the Pol ϵ accessory sub-unit DPB2 results in SPO11-independent DNA fragmentation during meiosis, and showed that this process required SOG1 activity, suggesting that the observed fragmentation is the consequence of an active process triggered by defects occurred during pre-meiotic replication (33). By contrast, Huang and colleagues recently described SPO11-dependent DNA fragmentation in *pol2a* mutants, and proposed that they were due to defects in DNA repair (37). However, *abo4-1* mutants that are also hypersensitive to genotoxic stress do not display meiotic defects, suggesting that impairment of the POL2 activity *per se* does not trigger DNA fragmentation. Furthermore, we showed that SOG1-deficiency partially rescued the meiotic defects of *abo4-2* mutants. Together, our results provide evidence for the involvement of POL2A in the pre-meiotic checkpoint previously described, although residual DNA fragmentation observed in *abo4-2 sog1* mutant likely results from defects in the repair of programmed DSBs. Furthermore, the finding that the *atm* mutation partially rescues the fertility of *abo4-2* mutants suggests that the meiotic checkpoint activated by Pol ϵ deficiency involves ATM signaling. One possible model would thus be that inactivation of Pol ϵ triggers fork collapse, thereby generating DSBs and ATM-dependent SOG1 activation. Consistently, the SOG1-dependent fragmentation phenotype was observed in *abo4-2* mutants that accumulate reduced levels

of full length POL2A but not in *abo4-1* mutants in whom production of the full length protein is unchanged.

Intriguingly, the *abo4-1* mutation also improved the fertility of the *atm* mutant. Both in budding and in fission yeast, replicative stress induced by stalled forks inhibits the formation of DSBs (57); it is therefore possible that the constitutive activation of replicative stress in the *abo4-1* mutant leads to the formation of fewer DSBs, thereby alleviating the subsequent repair defects caused by the *atm* mutations. Further investigation of meiosis progression in double mutants should help clarify this point. Together, our results further demonstrate that pre-meiotic DNA replication is a critical step for gamete formation, and that severe replicative stress occurring during this phase activates a cell death program that requires the SOG1 transcription factor.

Concluding remarks

Overall, this work has shed light on the diverse roles of plant POL2 in DDR activation, as summarized on Figure 7. We show that the role of Pol ϵ in S-phase checkpoint activation is a universal mechanism operating similarly in a multicellular organism as in yeast. Mutations affecting Pol ϵ can lead to a rare autosomal recessive disease (58), and have been associated with various types of cancer (36). Intriguingly, the consequences of Pol ϵ deficiency are much less dramatic in plants, possibly because of the plasticity of their development that allows replacement of damaged cells by neighboring ones in meristems (59). Because many mutants deficient for DDR are viable in plants but lethal in animal systems, future studies in *Arabidopsis* could reveal mechanisms that have not been elucidated in other multicellular eukaryotes and could be translated into Mammalian cells to further elucidate the association of Pol ϵ deficiency with tumorigenesis.

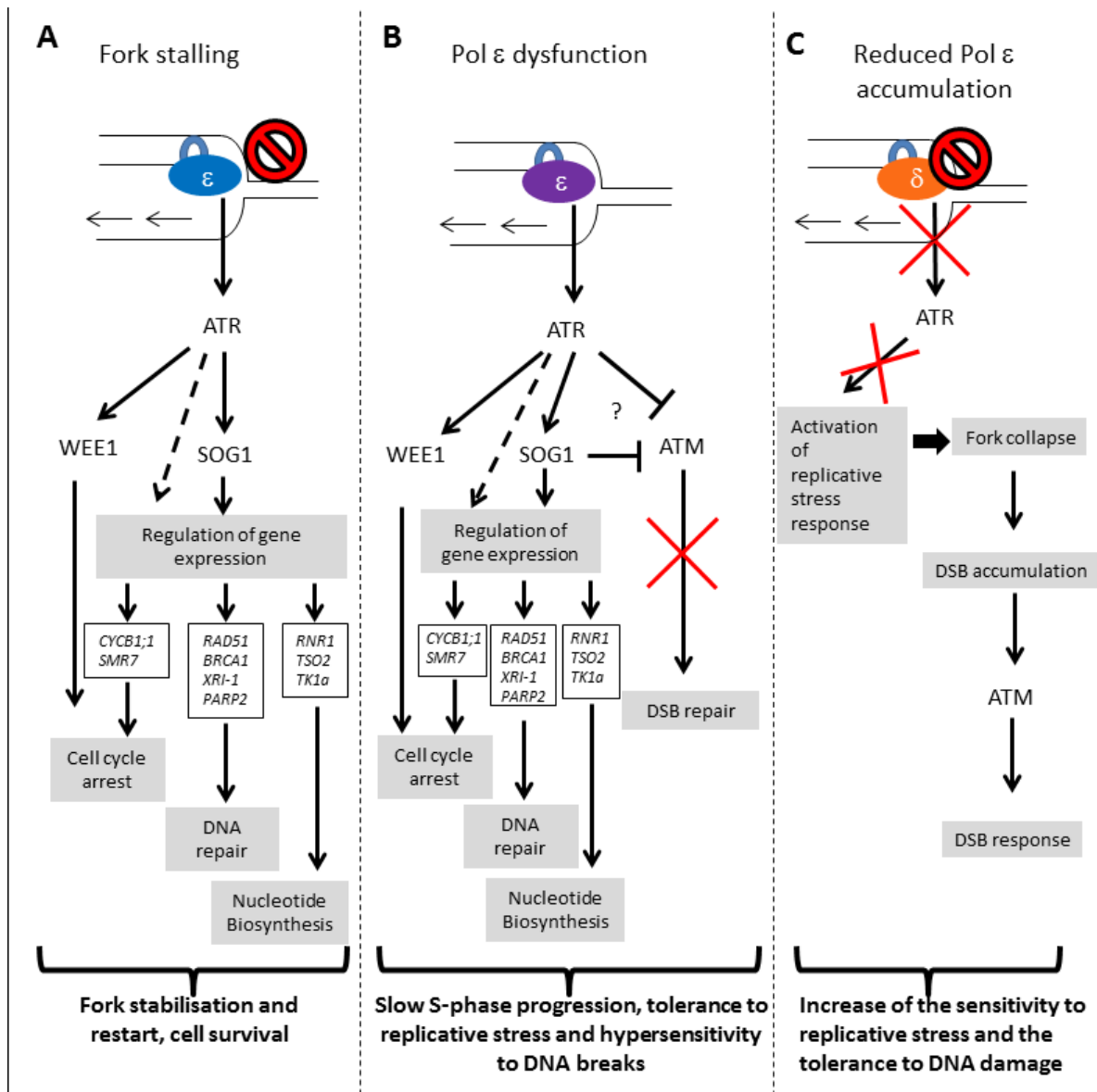


Figure 7: Model for Pol ε function in plant DDR

A: In the wild-type, Pol ε catalytic sub-unit POL2A is involved in replication stress sensing, leading to ATR-dependent activation of the WEE1 and SOG1 pathways, allowing the expression of genes involved in cell cycle arrest, DNA repair and nucleotide biosynthesis, ultimately leading to fork stabilization and completion of DNA replication and cell survival.

B: In *pol2A* mutants with point mutations affecting POL2A activity, the abnormal Pol ε sub-unit likely gums up replication, leading to constitutive replication stress and activating ATR. The WEE1 branch of the downstream pathway is essential to plant survival, whereas the SOG1 branch of the pathway is dispensable, but confers tolerance to replicative stress. SOG1 activation may also negatively regulate ATM signalling leading to enhanced sensitivity to DNA damaging agents.

C: When accumulation of POL2A is reduced, Pol δ likely replaces it and replicates both DNA strands. In the absence of Pol ε, replicative stress signalling is not properly activated, which may lead to fork collapse and DNA lesions that can in turn activate ATM signalling and promote tolerance to DSB-inducing agents.

In all panels, dashed arrows indicate putative pathways that remain to be molecularly identified

Data Deposition

RNAseq data from this article were deposited at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>), accession no. GSE71002) and at CATdb (<http://urgv.evry.inra.fr/CATdb/>; Project: NGS2014_10_Epsilon) according to the “Minimum Information About a Microarray Experiment” standards.

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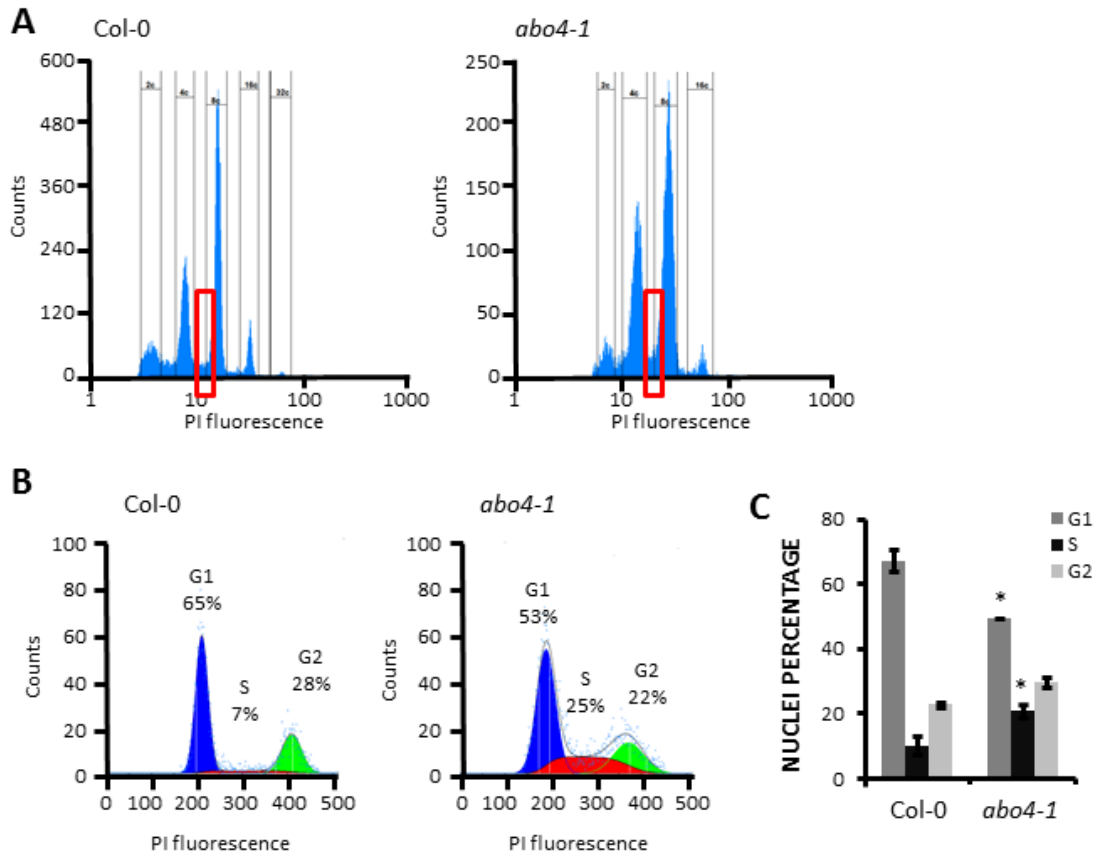


Figure S1: Cell cycle regulation is altered in *abo4-1* mutants.

A: Representative flow cytometry profiles obtained on cauline leaves of wild-type (Col-0) and *abo4-1* mutants. As highlighted by the red rectangle, peaks are broader in *abo4-1* suggesting that a higher proportion of nuclei have intermediate DNA contents. B: Representative flow cytometry profiles obtained on flower buds of wild-type (Col-0) and *abo4-1* mutants. The proportion of S-phase nuclei is increased in *abo4-1*. C: average proportion of G1, S and G2 nuclei in flower buds of wild-type (Col-0) and *abo4-1* mutants. Data are average \pm s.d. from three biological replicates

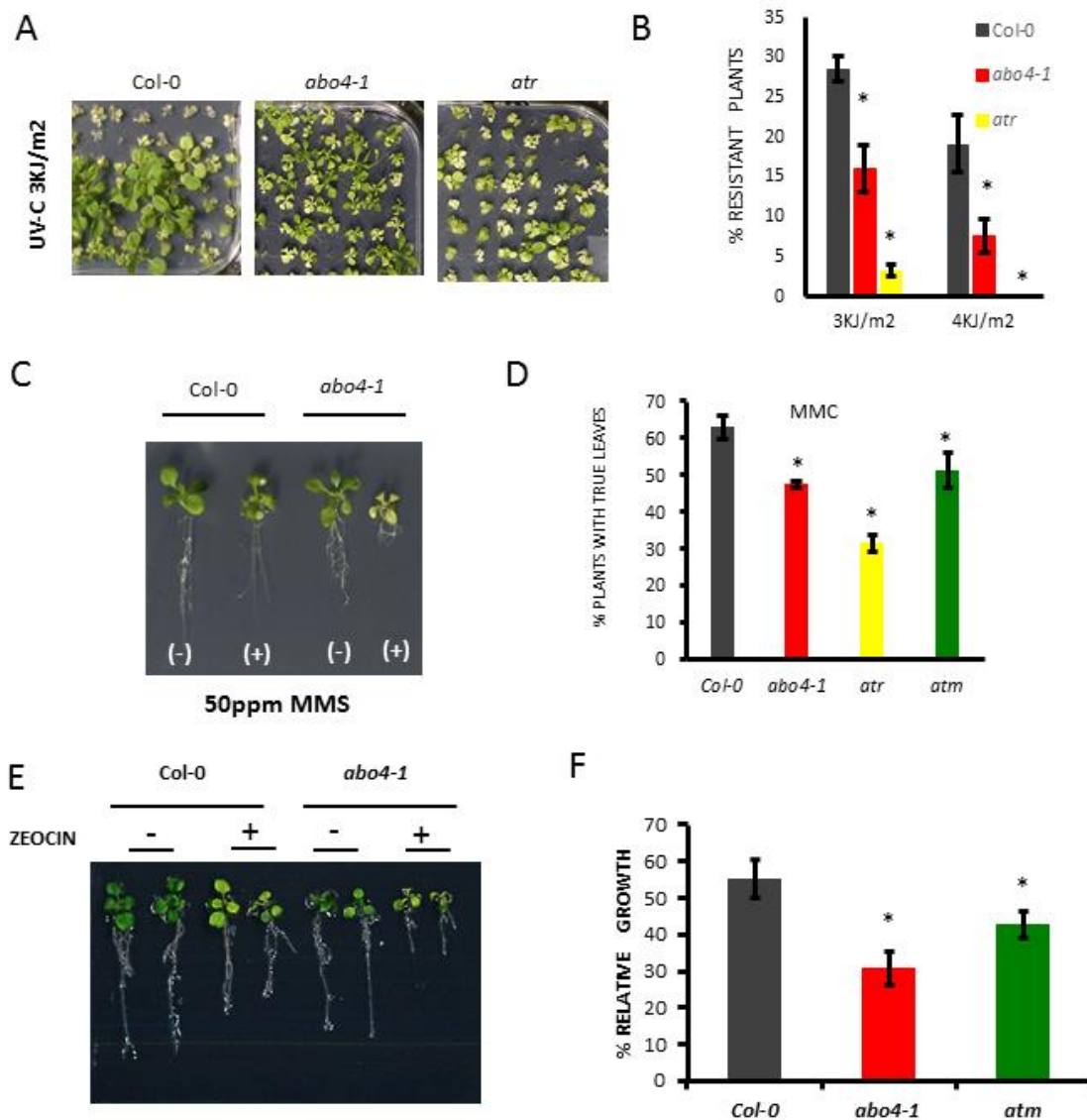


Figure S2. The *abo4-1* mutant is hypersensitive to genotoxic stress

Wild-type (Col-0) and *abo4-1* seedlings were challenged with UV-C (A, B) MMS (C) mitomycin C (D) and zeocin (E,F). All these treatments induce not only replication blocking lesions but also DNA damage. The tolerance of seedlings to these genotoxic stresses were monitored as previously described (Pedroza-Garcia et al 2016). The *atr* and *atm* mutants that are hypersensitive to these genotoxic agents were used as a positive controls. For all graphs, values are average \pm s.e. of three biological replicates and asterisks indicate statistically significant differences compared to the wild-type (student t-test p value < 0.05).

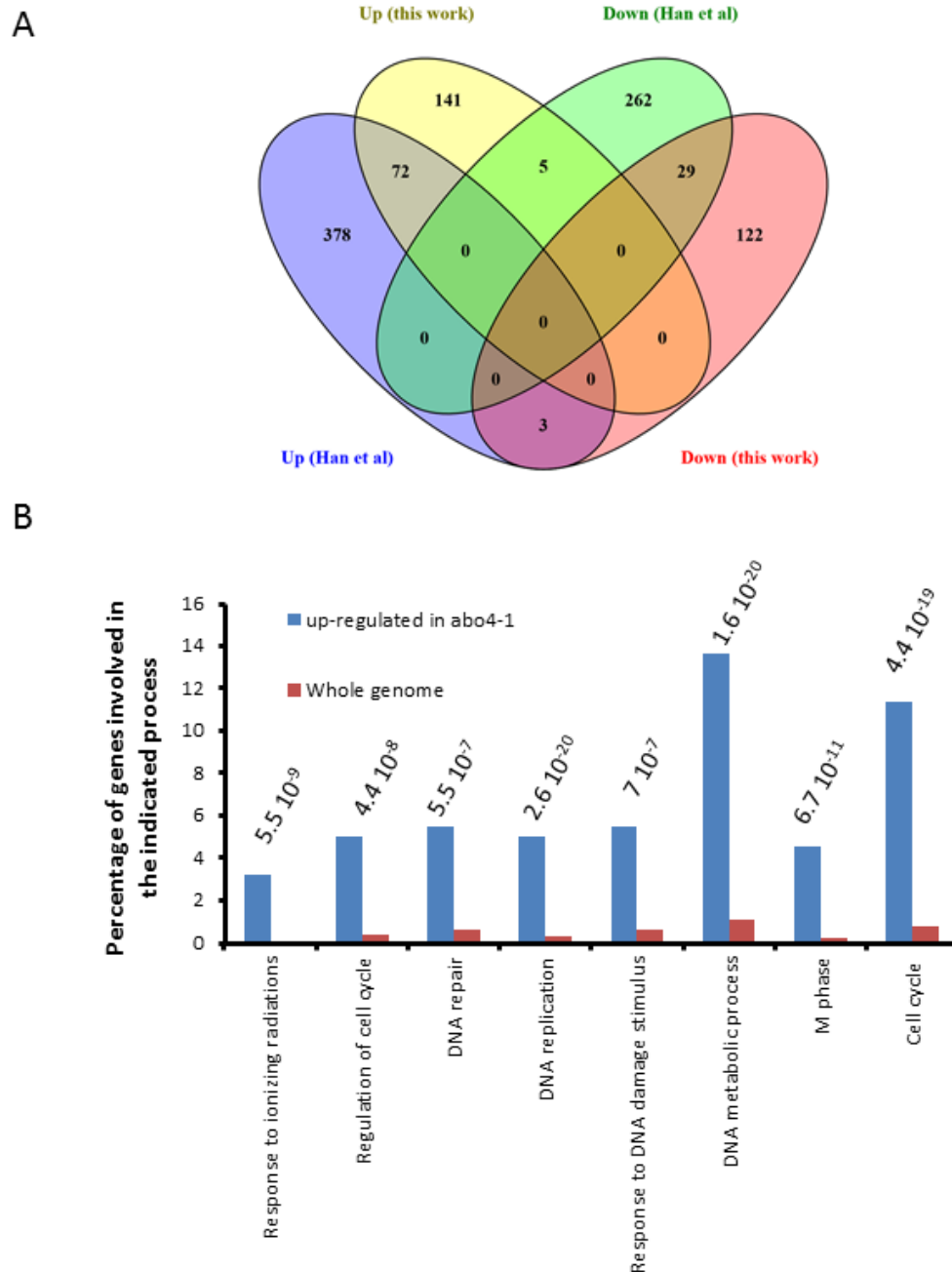


Figure S3: GO analysis of significantly induced genes in *abo4-1* seedlings.

A: GO analysis was performed using the GO enrichment analysis tool (geneontology.org/page/go-enrichment-analysis). Values above bars indicate the p value associated with the enrichment (Bonferroni corrected).

B: Comparison between our RNAseq data and results published by Han et al (2015). Overlap between the two datasets is significantly higher than expected at random, although the majority of misregulated genes are identified in only one of the datasets. Genes found to be up-regulated in both datasets are all related to DNA repair, cell cycle or chromatin organization.



Figure S4: The *sog1* mutation significantly reduces vegetative growth of the *abo4-1* mutant but partially rescues the *abo4-2* mutant.

Plants were grown in vitro for 10 days, and subsequently transferred to soil and grown in a green house for one month.

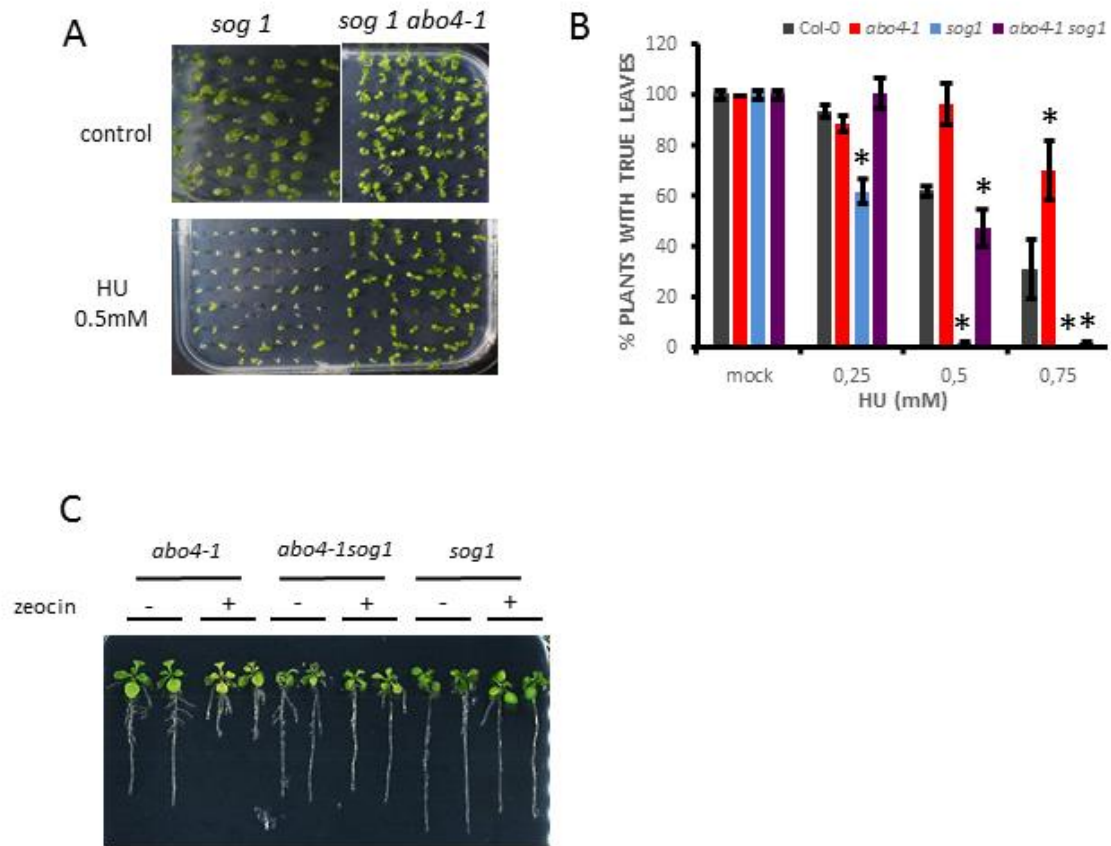


Figure S5. The SOG1 transcription factor is partly responsible for the tolerance of *abo4-1* mutants to HU and their sensitivity to zeocin

A-B: Wild-type (Col-0) and *abo4-1*, *sog1* and *abo4-1sog1* seedlings were germinated on half strength MS supplemented with HU to the indicated concentration. After 12 days, the percentage of plants with true leaves was monitored.

C: Representative phenotype of *abo4-1*, *sog1* and *abo4-1 sog1* plantlets 8 days after transfer to zeocin containing medium. The *sog1* mutant is completely resistant to zeocin, whereas the *abo4-1* mutant is highly sensitive, and the double mutant displays an intermediate phenotype.

For all graphs, values are average \pm s.e. and asterisks indicate statistically significant differences compared to the wild-type (student t-test p value < 0.05). Data are representative of at least 2 independent experiments

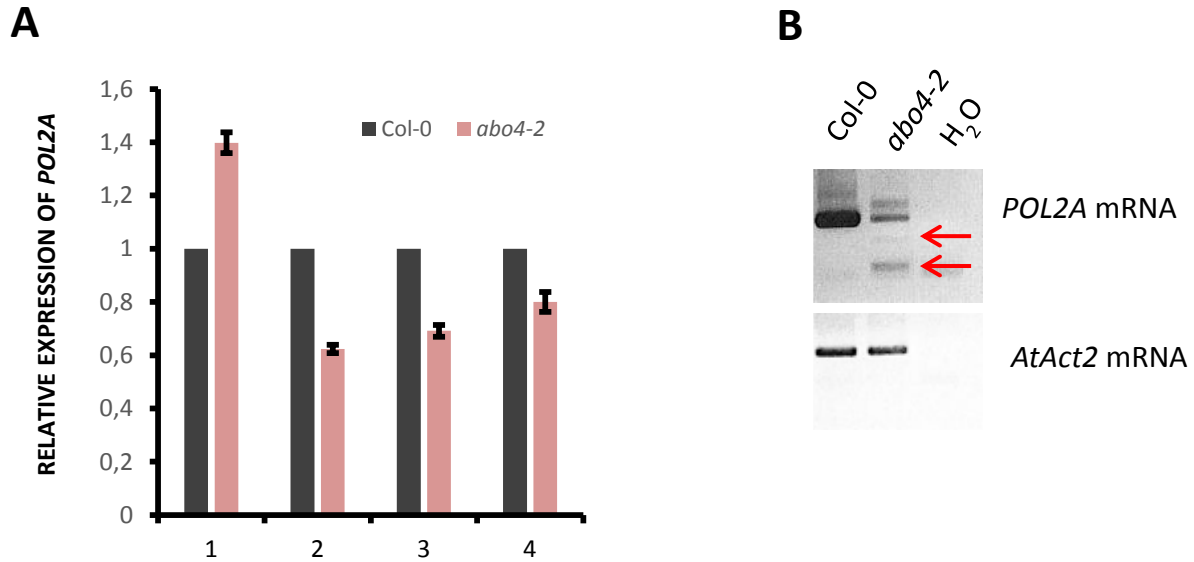


Figure S6: The T-DNA insertion in the *abo4-2* mutant leads to production of different variants of the *POL2A* mRNA

A: All regions of the *POL2A* mRNA are accumulated in the *abo4-2* mutant. qRT-PCR quantification of *POL2A* mRNA accumulation. Primer pair 1 is situated upstream of the T-DNA insertion, pairs 2, 3 and 4 are situated downstream. The mutant shows slight hyper-accumulation of the 5' moiety of the *POL2A* mRNA, and some reduction in the accumulation of its 3' part. B: RT-PCR amplification pattern obtained with primers situated on both sides of the T-DNA insertion (in exons 11 and 14). Some cleanly spliced transcript can be amplified, and its sequence was confirmed to be identical to the wild-type. Additional PCR products of lower molecular weight can also be observed (indicated by red arrows), sequence analysis revealed that they result from alternative splicing events leading to the elimination of exon 12 (corresponding to amino acids 427 to 481) or exons 12 and 13 (corresponding to amino-acids 427 to 540).

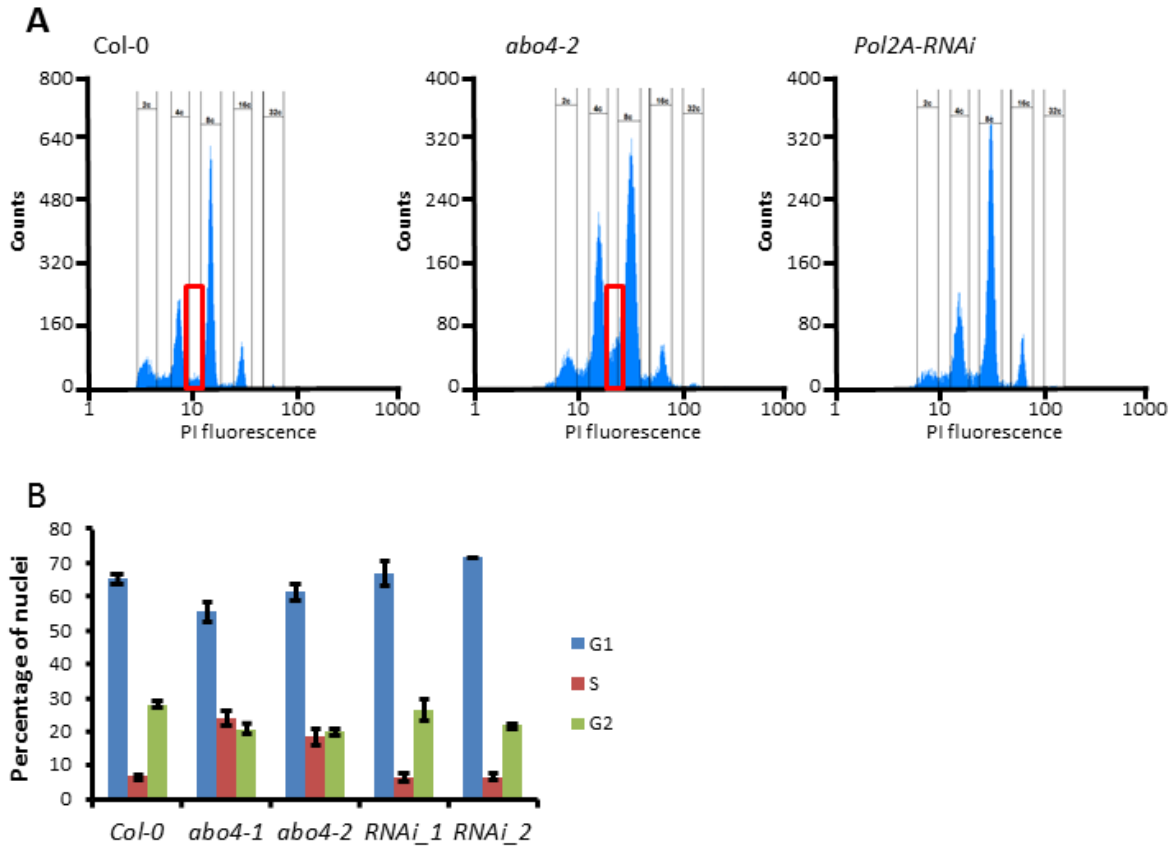


Figure S7: Partial inactivation and down-regulation of POL2A have contrasting effects on cell cycle regulation

A: Flow-cytometry analysis on cauline leaf nuclei. The *abo4-2* mutant displays the same accumulation of nuclei with intermediate DNA contents (outlined with a red rectangle between the 4C and 8C peaks) as the *abo4-1* mutant, whereas RNAi lines are comparable to the wild-type.

B: Distribution of flower buds nuclei between the G1, S and G2 phases of the cell cycle. *abo4-1* and *abo4-2* mutants show a significant increase in the number of S phase cells whereas RNAi lines are comparable to the wild-type.

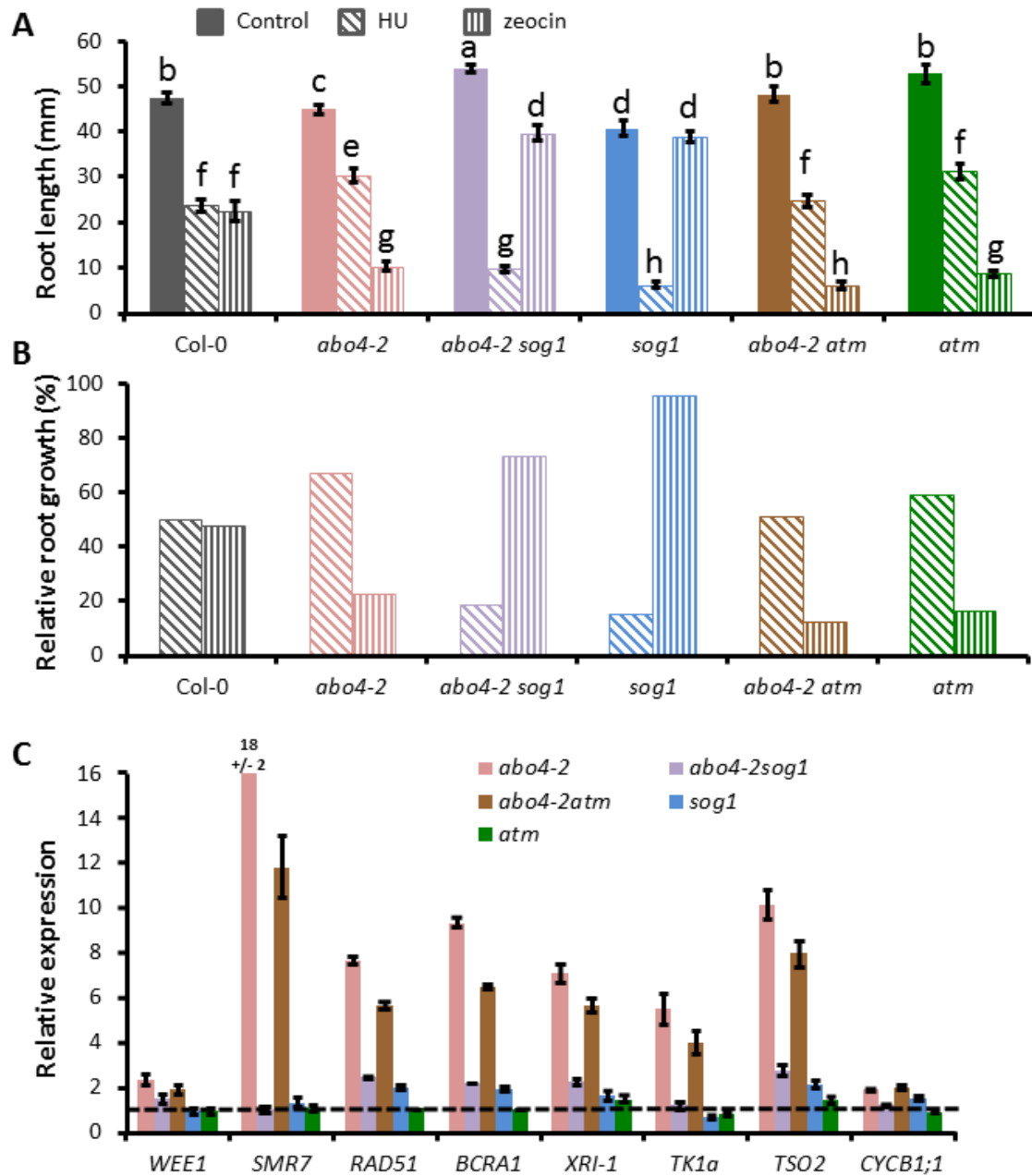


Figure S8: The *abo4-2* mutation confers HU tolerance that is partly dependent on SOG1.

A: Root length of wild-type (Col-0), *abo4-2*, *abo4-2 sog1*, *sog1*, *abo4-2 atm* and *atm* mutants after 9 days on control medium, or medium supplemented with HU or zeocin. Values are average \pm s.e. Different letters above bars indicate statistically significant differences (Student t-test $p < 0.05$). B: Relative root growth of wild-type (Col-0), *abo4-2*, *sog1*, *abo4-2 atm* and *atm* mutants after 9 days on HU or zeocin. C: qRT-PCR quantification of DDR genes expression in flower buds of the *abo4-2*, *abo4-2 sog1*, *sog1*, *abo4-2 atm* and *atm* mutants. Values are expressed relative to the wild-type (Col-0). For all panels results are representative of two independent experiments.

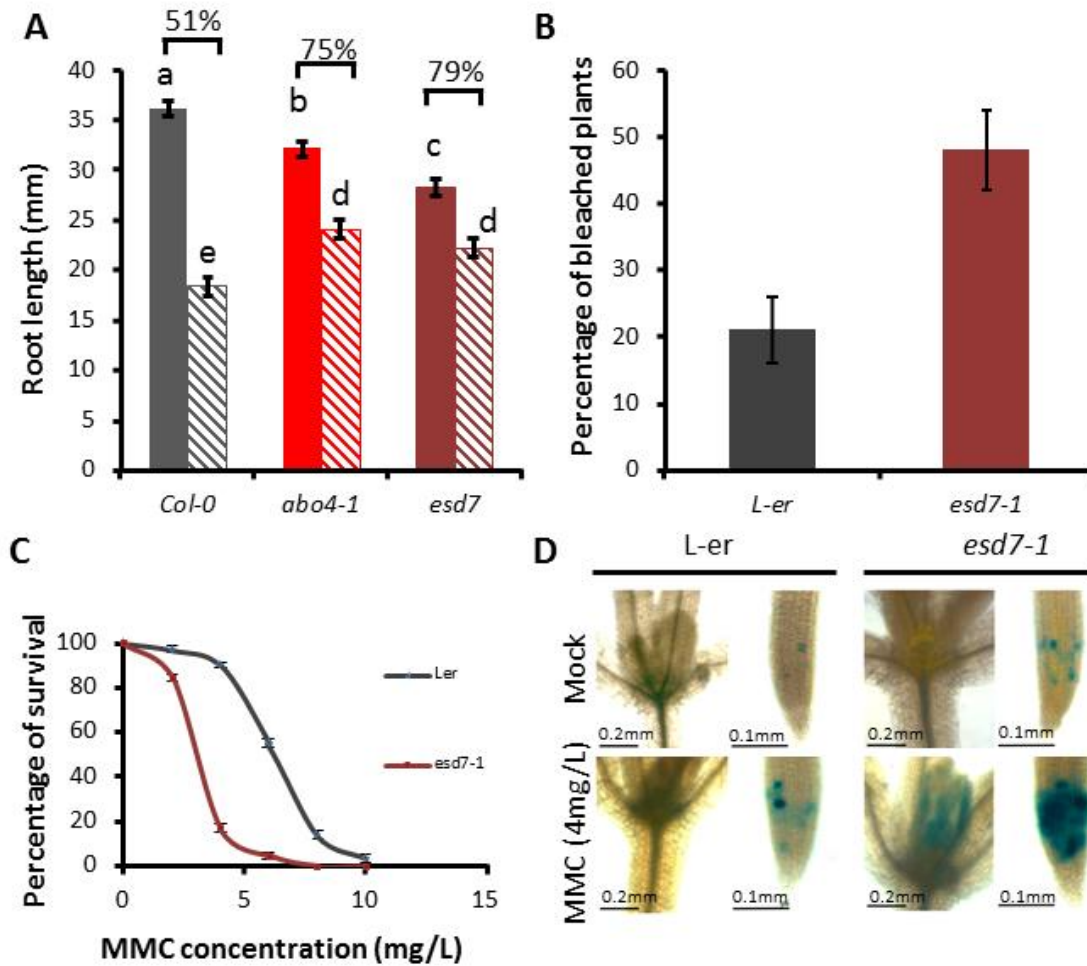


Figure S9: The *esd7* mutant is tolerant to HU but hypersensitive to DNA damaging agents.

A: HU sensitivity in wild-type (*Col-0*), *abo4-1*, and *esd7* mutants (the *esd7* allele was introgressed into the *Col-0* background for this experiment). Plantlets were grown on half strength MS for 4 days and transferred to control medium (full bars) or HU supplemented medium (dashed bars) for 9 days. Values are average \pm s.e. obtained on at least 15 plantlets; different letters indicate statistically relevant differences (Student t-test, $p < 0.05$) and values above bars indicate the relative growth observed on HU supplemented medium compared to control medium.

B: *esd7* seedlings were more sensitive to UV-C light than the wild type. Seven-day-old seedlings of the wild type and the *esd7* mutant were irradiated with UV-C (3000 J/m²) 3 times in a row every 2 days.

C: *esd7* seedlings were more sensitive to MMC than the wild type. Seven-day-old seedlings of the wild type and the *esd7* mutant were grown in liquid medium with different concentrations of MMC ranging from 0 to 10mg/ml.

D: *CYCB1;1* is hyper-induced by genotoxic stress in the *esd7* background. The Representative examples of GUS staining; *CYCB1;1* promoter-GUS was expressed higher in shoot and root meristems of *esd7* than the wild type, and its expression was clearly hyper induced by MMC treatment (4 mg/L) in *esd7* compared to the wild-type.

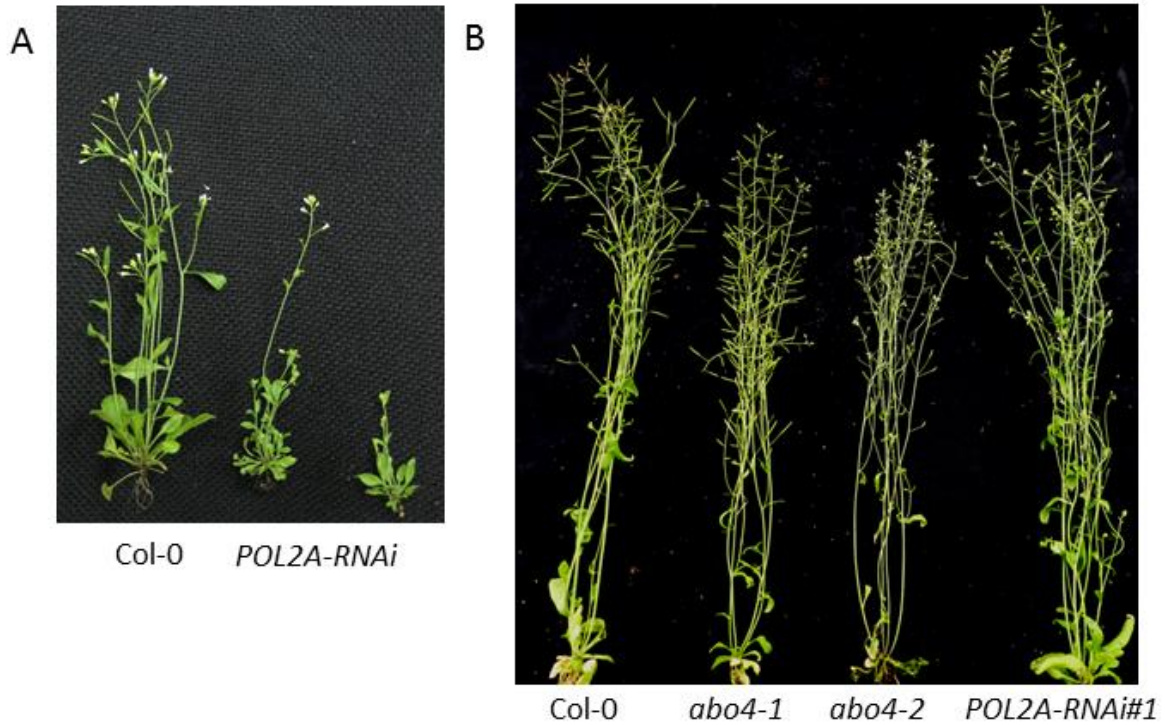


Figure S10: Down-regulation of *POL2A* affects plant growth and fertility.

A: examples of severe phenotypes obtained in several *POL2A-RNAi* lines.

B: phenotype of adult wild-type (Col-0), *abo4-1*, *abo4-2* and *POL2A-RNAi* #1. *POL2A-RNAi* lines used for further analysis reach the same size as wild-type plants but show a severe reduction in fertility.

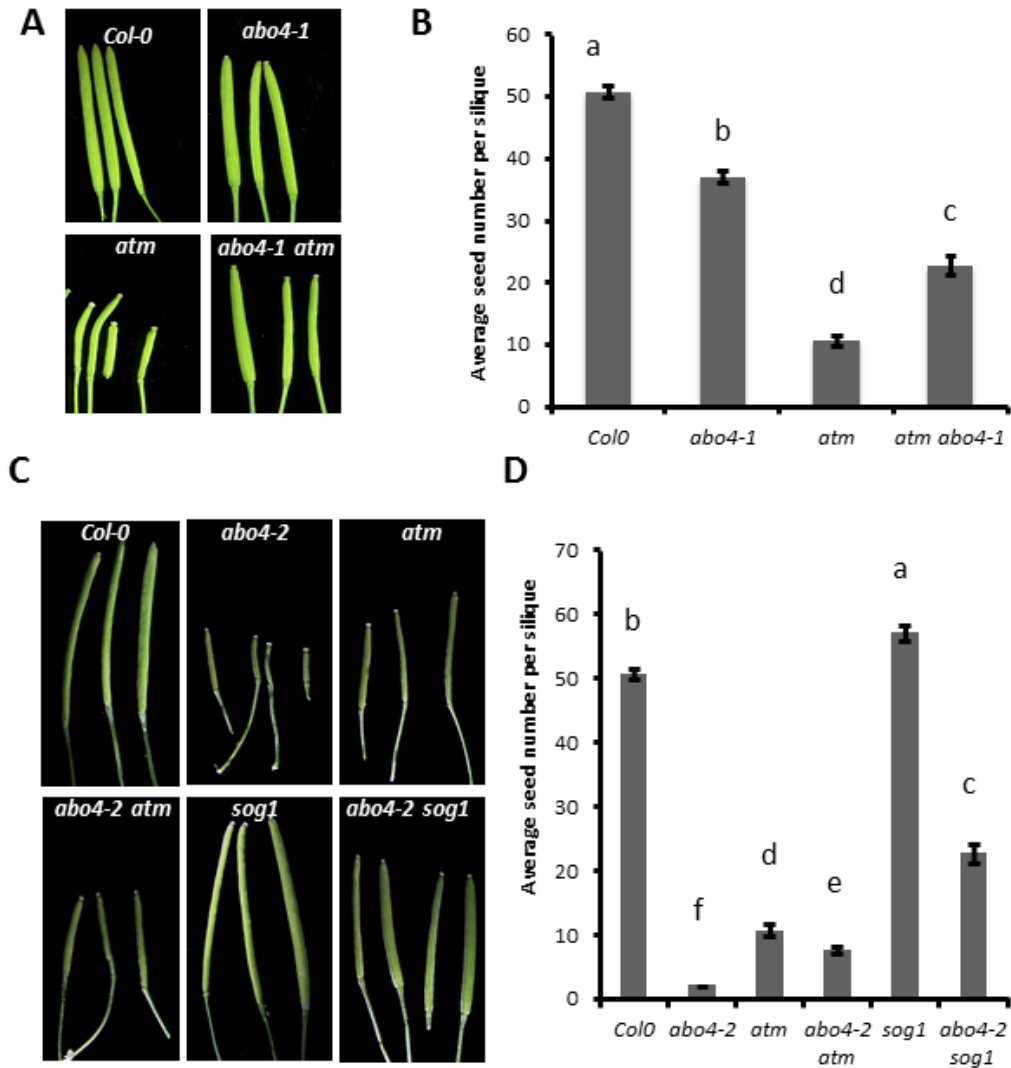


Figure S11. Genetic interactions between Pol e and DDR genes during reproductive development

A,B: The *abo4-1* mutation partially restores the fertility of *atm* mutants. A: representative pictures of siliques of wild-type (*Col0*), *abo4-1*, *atm* and *abo4-1 atm* mutants. B: Average seed number in siliques of the different genotypes. Values are average \pm s.e. of at least 20 siliques.

C,D: The *abo4-2* mutant is almost completely sterile, and these defects are partially rescued by the *sog1* mutation, and to a lesser extent by the *atm* mutation. C: representative pictures of siliques of the indicated genotypes, D: average seed number per silique in the different mutants, values are average \pm s.e. of data obtained in at least 20 siliques. In B and D, different letters indicate statistically significant differences (Student t-text $p < 0.05$).

Table S1: Primers used in this study.

qPCR primers			
Gene	Accession Number		Sequence 5'→3'
<i>RNR1</i>	AT2G21790	Forward	GCTTAGCAGTGACCATTGTG
		Reverse	TCAGCAGCCAACTCATCATCAAG
<i>TSO2</i>	AT3G27060	Forward	TCGCTTGTCTACTCTACACG
		Reverse	CCGCGTCGCAGACGATTGA
<i>SMR7</i>	AT3G27630	Forward	GCCAAAACATCGATTCGGGCTTC
		Reverse	TCGCCGTGGGAGTGATACAAAT
<i>PARP2</i>	AT2G31320	Forward	AGCCTGAAGGCCCGGTAAACA
		Reverse	GCTGTCTCAGTTTTGGCTGCCG
<i>TK1a</i>	AT3G07800	Forward	TTGGAAGATTCTGACAAGGCTA
		Reverse	CAACATTAAGGATAAACCAGACCA
<i>CYCB1-1</i>	AT4G37490	Forward	GGAAGCAACAAGAAGAAGGGAG
		Reverse	AGGGATCAAAGCCACAGCG
<i>XRI1</i>	AT5G48720	Forward	GCTACCTGATGACTTAACTTTGGTTC
		Reverse	CATTGGAGAAGATCGAGTCACAG
<i>WEE1</i>	AT1G02970	Forward	GGCCATTTCGTTGCAGTTACA
		Reverse	TCTCGACATCTGAGAGACTC
<i>BRCA1</i>	AT4G21070	Forward	AGGTGAACCTGTCTCTGCGGATTT
		Reverse	TTCTCCGGCTTCTTGTCAACTCCA
<i>RNR2A</i>	AT3G23580	Forward	TGGCTCAGAACCAGAGATTC
		Reverse	AGAAACTGGCTTCAGCCTTC

Table S2: Cell cycle length is drastically modified in *abo4* mutants but only mildly in *POL2-RNAi* lines.

Total cell cycle and S-phase length were assessed as described in Pedroza-Garcia et al (2016). In both *abo4* mutants, S-phase was three times as long as in the wild-type (Col-0), corresponding to an 8h delay, and total cell cycle length was increased by 13h, pointing to a G2/M arrest . In *POL2-RNAi* lines we observed only a 2h delay that could be attributed to a small increase in S-phase length.

	Col-0	<i>abo4-1</i>	<i>abo4-2</i>	<i>POL2A-RNAi 1</i>	<i>POL2A-RNAi 2</i>
Cell Cycle length (h)	19	32	32	21	22
S-phase length (h)	4	11	12	6	6
S-phase length (%)	21	34	37	29	27

Table S3**Up-regulated**

AT4G29170	AT3G27060	AT2G30360	AT3G16650	AT1G80760	AT5G17160
AT5G52290	AT4G02390	AT5G23910	AT1G16070	AT3G06030	AT1G69770
AT3G27730	AT5G24280	AT2G18193	AT5G40942	AT4G26660	AT3G54750
AT1G53490	AT5G48720	AT4G22960	AT3G22231	AT3G22235	AT3G02640
AT5G40840	AT4G21070	AT5G07610	AT2G47780	AT1G16520	AT1G57820
AT1G06660	AT5G61000	AT5G60250	AT1G24260	AT3G20150	AT5G63090
AT3G25100	AT2G21790	AT1G68200	AT5G12970	AT5G05160	AT3G50220
AT1G51130	AT3G07800	AT5G16080	AT1G11070	AT5G03260	AT1G52690
AT1G49910	AT1G08260	AT1G73010	AT5G60020	AT1G62500	AT5G20850
AT3G42860	AT4G03130	AT1G32560	AT3G08860	AT5G62550	AT5G16410
AT3G59550	AT1G20750	AT5G10890	AT1G36180	AT5G02490	AT1G07880
AT2G34790	AT1G02670	AT5G22794	AT4G02110	AT4G35350	AT2G26180
AT3G57860	AT5G64060	AT5G58610	AT4G11650	AT4G14770	AT3G19430
AT4G15890	AT3G52115	AT4G25580	AT1G66100	AT4G14310	AT5G03545
AT2G46980	AT5G49110	AT2G35300	AT1G65570	AT2G36200	AT1G67180
AT1G34355	AT5G03780	AT1G60460	AT1G59930	AT2G34490	AT3G58270
AT3G44050	AT2G18600	AT3G02000	AT1G53480	AT1G18250	AT4G38780
AT3G22880	AT2G42260	AT3G06630	AT3G03470	AT3G30720	AT5G46740
AT5G37630	AT3G27640	AT2G04032	AT4G31805	AT3G27620	AT3G45730
AT5G55820	AT4G19130	AT4G21090	AT5G38690	AT3G53040	AT3G51740
AT4G32830	AT5G49160	AT3G42725	AT4G13370	AT5G60910	AT1G80080
AT5G62410	AT2G07400	AT4G34510	AT2G27550	AT5G48390	AT1G08560
AT1G15660	AT3G42927	AT2G31980	AT1G52070	AT5G15800	AT3G16770
AT3G27630	AT1G49920	AT1G52270	AT1G04650	AT1G03710	AT5G42720
AT1G48605	AT1G05490	AT3G17520	AT1G54960	AT2G47770	AT3G54600
AT2G17620	AT3G24340	AT4G12540	AT5G67460	AT2G41260	
AT3G01330	AT5G61070	AT2G21770	AT1G65710	AT2G31250	
AT1G02970	AT3G09480	AT1G61450	AT1G76740	AT4G32510	
AT2G38620	AT2G19920	AT2G20110	AT1G59660	AT4G18550	
AT5G13060	AT1G01370	AT1G06100	AT5G05940	AT3G59200	
AT4G37490	AT1G67105	AT1G73220	AT3G23290	AT3G55920	
AT1G76310	AT2G07170	AT3G01600	AT5G01370	AT5G45400	
AT3G60840	AT5G67200	AT1G21120	AT4G28230	AT1G04020	
AT3G54180	<u>AT3G58650</u>	AT3G63480	AT5G05180	AT1G09815	
AT3G03130	AT5G63550	AT4G26960	AT3G20490	AT4G24610	
AT1G72670	AT5G66750	AT1G66620	AT5G54370	AT5G48020	
AT2G26760	AT5G39550	AT1G51470	AT2G05510	AT5G66130	
AT1G76540	AT1G31280	AT3G55290	AT3G57830	AT3G19210	

Down-regulated

AT1G03743	AT2G16367	AT3G59010	AT5G54490	AT4G38650
AT1G28370	AT3G01190	AT2G44370	AT4G08555	AT2G17040
AT3G27940	AT5G37770	AT5G04150	AT4G23810	AT2G22122
AT4G25490	AT3G23840	AT3G53650	AT5G19470	AT2G24600
AT2G47880	AT1G01600	AT1G68585	AT2G28960	AT1G49570
AT3G56710	AT2G43920	AT3G46280	AT5G08565	AT5G45340
AT3G56880	AT1G02300	AT3G10570	AT1G60740	AT5G59680
AT5G01870	AT2G28305	AT5G18430	AT4G36280	AT5G34800
AT3G52400	AT4G17030	AT5G38140	AT3G47790	AT5G34790
AT1G26250	AT4G14130	AT5G10140	AT1G65450	AT4G08040
AT3G04640	AT5G35935	AT5G26270	AT5G48657	AT5G24230
AT5G42800	AT1G79840	AT1G77960	AT5G15022	AT5G19170
AT3G05727	AT4G22520	AT1G72910	AT1G24577	AT1G07135
AT1G07610	AT3G25600	AT1G16960	AT5G23990	AT4G17490
AT5G25980	AT4G36500	AT2G48080	AT5G26660	AT2G43120
AT2G34430	AT5G17220	AT1G19380	AT3G58000	AT5G64110
AT3G14210	AT2G28630	AT3G42658	AT1G53635	AT2G42170
AT1G22690	AT1G73540	AT1G63710	AT1G14250	AT3G58550
AT1G02205	AT5G55930	AT5G48430	AT2G21045	AT1G57990
AT1G04800	AT1G26800	AT1G14540	AT2G07080	AT3G13610
AT4G22513	AT4G24570	AT2G38300	AT1G20390	AT1G25450
AT4G22517	AT2G28400	AT1G79910	AT1G52040	AT4G04570
AT2G41100	AT5G33370	AT2G36790	AT4G18010	
ATCG01130	ATCG00220	AT5G22250	AT2G38470	
AT1G55330	AT3G44260	AT2G42060	AT4G27280	
AT1G02930	AT2G37750	AT2G39690	AT3G50060	
AT2G33850	AT5G54585	AT5G52750	AT1G27730	
AT1G33811	AT3G03820	AT1G02065	AT4G29780	
AT2G21140	AT4G17970	AT5G25880	AT1G25400	
AT3G19030	AT2G40960	AT1G48500	AT5G03406	
AT2G37470	AT5G61600	AT1G50590	AT3G51350	
ATCG01020	AT1G10060	AT5G40330	AT2G36690	
AT5G50200	AT1G76600	AT5G35480	AT1G80840	

Table S4

Cell cycle and DNA damage response		
Locus	Description	fold change
AT1G02970	WEE1	2,4
AT3G27630	SIAMESE-RELATED	21,6
AT3G01330	DEL3 (DP-E2F-like 3)	2,2
AT1G76310	CYCLIN 1	1,7
AT4G37490	CYCB1;1	2,2
AT2G17620	CYCLIN B2;1	2,4
AT2G26760	Cyclin B1;4	1,8
AT2G38620	CDKB1;2	2,4
AT1G76540	CDKB2;1	1,9
AT3G54180	CDKB1;1	2,2
AT4G19130	REPLICATION PROTEIN A 1E, RPA1E	3,5
AT5G61000	REPLICATION PROTEIN A 1D	2,4
AT5G45400	REPLICATION PROTEIN A 1C, RPA1C	1,9
AT5G66130	RAD17	2,2
AT1G20750	RAD3-like DNA-binding helicase protein	8,1
AT5G20850	RAD51	3,7
AT3G19210	RAD54	2,5
AT4G21070	Breast cancer susceptibility1	4,8
AT1G04020	BARD1 (BREAST CANCER ASSOCIATED RING 1)	2,1
AT1G09815	"DNA polymerase delta subunit 4 family"	1,8
AT1G08260	POL2A catalytic subunit of Pol ϵ	1,5
AT3G07800	TK1a; Thymidine kinase	7,1
AT2G21790	RNR; RNR large subunit	2,1
AT3G27060	TSO2; RNR subunit	7,1
AT4G02390	Poly(ADP-Rib) polymerase	6,1
AT5G24280	γ -Irradiation and mitomycin c induced 1	5,8
AT5G48720	X-ray induced transcript 1	4,8
AT5G03780	TRF-like 10	4,0
AT4G03130	BRCT domain-containing DNA repair protein	3,0
AT3G52115	GR1; GAMMA RESPONSE 1	2,4
AT5G64060	ANAC103 ; transcription factor	4,5
AT2G42260	UVI4 (POLYCHOME, UV-B-INSENSITIVE 4)	2,1
AT2G18600	RUB1-conjugating enzyme, putative	2,5
AT3G27640	"transducin family protein / WD-40 repeat family protein"	1,9
AT1G02670	helicase-related	2,3
AT5G49110	unknown protein	2,9
AT4G24610	unknown protein;	1,8
AT5G48020	2-oxoglutarate (2OG)	1,7
AT5G13060	armadillo domain-containing protein	2,0
AT3G60840	microtubule associated protein (MAP65/ASE1)	2,0
AT3G03130	unknown protein	1,8
AT1G72670	IQD8; calmodulin binding	1,8
AT1G48605	"ATHAL3B (Hal3-like protein B); electron carrier"	2,9
Mitosis/Meiosis		
Locus	Description	fold change
AT4G29170	ATMND1	2,6
AT5G52290	XPF endonuclease-like	2,8
AT3G27730	RCK (ROCK-N-ROLLERS)	2,3
AT1G53490	HEI10: Required for class I crossover .	3,5
AT5G40840	SYN2 (Sister chromatid cohesion 1 (SCC1)	2,3
AT1G06660	JASON	1,9
AT3G25100	CDC45	2,1
AT1G51130	Nse4, Smc5/6 DNA repair complex	2,2
AT1G49910	mitotic checkpoint protein,	2,3
AT3G42860	zinc knuckle (CCHC-type) family protein	2,1
AT3G59550	SYN3 (Sister chromatid cohesion 1 protein 3	1,9
AT3G57860	UV-B-INSENSITIVE 4-LIKE	1,9
AT4G15890	mitotic chromosome condensation	2,0

AT2G46980	ASY3, that is required for normal meiosis	2,0
AT1G34355	Encodes PS1 (Parallel Spindle 1)	1,8
AT3G44050	"kinesin motor protein-related"	1,7
AT3G22880	ATDMC1 (RECA-LIKE GENE)	2,3
AT5G55820	inner centromere protein (INCENP)	1,7
AT4G32830	ATAUR1 (ATAURORA1); histone ser kinase	1,7
AT5G62410	SMC2 (STRUCTURAL MAINTENANCE OF CHROMOSOMES 2)	2,0
AT1G15660	homologue of the human centromeric protein C (CENP-C)	1,8
AT2G34790	EDA28	1,9
AT5G37630	EMB2656	1,7

GENERAL DISCUSSION

AND

PERSPECTIVES

Discussion

In eukaryotes, several lines of evidence support multiple roles of Pol ϵ in the maintenance of genome stability (Navas et al., 1995; Henninger and Pursell, 2014; Rayner et al., 2016; Puddu et al., 2011), but the underlying mechanisms remain unclear. Recently, in *Arabidopsis*, the isolation of hypomorphic alleles of *POL2A* has shed more light on the biological function of Pol ϵ in plants (Jenik et al., 2005; del Olmo et al., 2010; Yin et al., 2009). By contrast, DPB2 which is the largest regulatory subunit has not been characterized because null mutations are lethal (Ronceret et al., 2005). In this study, we have investigated the role of the two main Pol ϵ subunits from *Arabidopsis thaliana* in the processes of DNA replication, cell cycle regulation and DNA damage response. To study the regulatory subunit DPB2 we used an over-expressing strategy, while the catalytic subunit POL2A was studied through the characterization of the hypomorphic mutants termed *abo4* (“abscissic acid oversensitive”) (Yin et al., 2009).

Role of Pol ϵ sub-units during the replicative stress response in somatic cells

Alteration in the relative abundance of Pol ϵ sub-units appears to impair the replicative function of the complex. Indeed DPB2 overexpressing (*DPB2OE*) lines displayed the same aphidicolin sensitivity and the same early flowering phenotype as *abo4/esd7* mutant lines that are deficient for the catalytic subunit (del Olmo et al., 2010; Yin et al., 2009). Additionally, inducible inactivation of DPB2 via RNAi induced similar cell cycle and root growth inhibition as DPB2 overexpression. We have shown that *abo4-1* mutation severely affects cell cycle regulation: these lines display enhanced endoreduplication and slower cell cycle progression, together with constitutive activation of DNA repair and cell cycle regulation genes. All these features are shared with *DPB2OE* lines, suggesting that both lines display endogenous DNA damage and pre-activation of the DDR. Similar defects were reported in several mutants deficient for proteins involved in DNA replication such as *caf* (chromatin assembly factor), *fas1*, *rpa2a* (replication protein a) (Endo et al., 2006; Bolaños-Villegas

et al., 2013; Aklilu et al., 2014), and were hypothesized to result from stalled replication forks during S-phase (Cools and De Veylder, 2009). Consistently, *abo4-1* and *DPB2OE* plants displayed delay in S-phase progression. Interestingly, the observed increase in S-phase length did not fully account for the total increase in cell cycle length. Together with the enhanced expression of G2/M markers as *CYCB1:1*, these results indicate to constitutive activation of the DNA damage response and subsequent cell cycle checkpoint activation, leading to a G2 arrest. Additionally, both *abo4* and *DPB2OE* plants display tolerance to hydroxy-urea (HU), an analogue of thymidine that inhibits RNR (Ribonucleotide reductase), thereby depleting the nucleotide pools and triggering replication fork arrest, suggesting that in these lines, constitutive activation of the replication stress response leads to improved tolerance to this stress, possibly because the expression of genes involved in the response to this stress is already induced. A similar mechanism has been suggested to account for the tolerance of CDT1 deficient lines to HU (Domenichini et al., 2012).

All together the aforementioned characteristics suggest that mutations in *POL2A* or over-expression of *DPB2* affect the Pol ϵ activity in the same manner; leading to endogenous DNA damage, constitutive activation of the DDR and finally cell cycle arrest. However, further analysis revealed molecular and phenotypic differences between hypomorphic alleles of *POL2A* and *DPB2OE* plants. First, *abo4-1* mutant is hypersensitive to various agents that damage DNA directly such as zeocin, MMC and UV-C ((Yin et al., 2009); this study). By contrast, *DPB2OE* seedlings are tolerant to these genotoxic agents. Second, by contrast with *DPB2OE*, *abo4-1* roots tips did not display γ -H2AX foci in normal conditions (Figure 17A). Because γ -H2AX formation is a marker of the activation of DDR triggered by the formation of DSBs (Roy, 2014), this result suggests that only *DPB2OE* plants present endogenous DNA damage leading to constitutive activation of DDR. Finally, there was only a partial overlap between the transcriptomes of *abo4-1* and *DPB2OE* (Figure 17B). Most genes up-regulated in *DPB2OE* lines were also up-regulated in *abo4-1* mutants, but many genes were specifically induced in *abo4-1*. Genes up-regulated in both *abo4-1* mutants

and *DPB2OE* were mainly genes associated with DNA repair. A large degree of variability was observed between the biological replicates used to generate these RNAseq data. For this reason, we cannot rule out the possibility that the overlap between the transcriptome changes induced by *DPB2* over-expression and the *abo4-1* mutation may be underestimated. Nevertheless, the possibility that POL2A deficiency may trigger specific responses is in agreement with the notion that alterations in each subunit of Pol ϵ may affect different mechanisms (Sengupta et al., 2010).

As mentioned above, the specific tolerance of *abo4* plants to HU is likely attributable to constitutive activation of replication stress response, which triggers the activation of a specific transcriptional program and fork stabilization. Since HU leads to fork stalling without generating collapse, it is frequently used for studying replication stress (Poli et al., 2012). In yeast, POL2 is required to sense ssDNA, triggering the replication checkpoint activation (Navas et al., 1995; Puddu et al., 2011). Deletion of the POL2 N-terminus, which encompasses the catalytic domain does not compromise cell survival (Feng and D'Urso, 2001), showing that the catalytic activity is not essential for the survival, likely because Pol δ may compensate the deficiency of Pol ϵ activity. Recent studies have shown that Pol δ can perform the synthesis of both strands (Miyabe et al., 2015; Johnson et al., 2015) and that Pol ϵ catalytic activity is required for the repair of replication errors (Johnson et al., 2015). Together, our results suggest that the point mutation in POL2A present in the *abo4-1* allele might trigger constitutive checkpoint activation by endogenous replicative stress, possibly by gumming up replication. This checkpoint could stabilize single-strand breaks (SSB) at stalled fork, and thus avoid fork collapse and generation of DSBs. Consistent with the absence of γ -H2AX foci and DSBs (Yin et al., 2009) in normal conditions. This checkpoint has been described in other eukaryotes (García-Rodríguez et al., 2015; Navas et al., 1995; Puddu et al., 2011); it allows completing the DNA replication despite of the presence of replicative stress. To achieve this, homologous recombination is increased (Blow et al., 2011), which likely accounts for the enhanced HR phenotype displayed by *abo4-1* and other replicative polymerase mutants (Liu et al., 2010; Yin

et al., 2009). The tolerance to HU resulting from POL2 mutation also has been reported in yeast; as well as, the hypersensitivity to DNA-damaging agents (Pursell and Kunkel, 2008). This hypersensitivity may be a direct consequence of the need for Pol ϵ activity during DNA repair or an indirect consequence of the constitutive activation of this branch of DDR that would prevent the correct activation of DSBs response. To date, the mechanism is unclear. However, *DPB2OE* plants display tolerance to both HU and DNA-damaging agents: these characteristics indicate that DPB2 over-expression triggers constitutive replication stress activation without compromising DSBs response, supporting the former hypothesis. This finding would imply that the 1:1:1:1 stoichiometry of the complex (Chilkova et al., 2003) is less crucial for DNA repair than for DNA replication. Consistently, in yeast, Dpb2 is not required for Pol2A catalytic activity *in vitro*, although it improves its stability (Li et al., 1997) and enhances the fidelity of DNA replication (Jaszczur et al., 2008). Hence, the POL2A-DPB2 interaction may be crucial for the insertion of the polymerase in the replisome (Handa et al., 2012), as well as for the activation of the MCM helicases (Zegerman, 2013), but dispensable during DNA repair. Together, our results support the role of both subunits of Pol ϵ in the maintenance of genome integrity, although each sub-unit appears to also have specific functions.

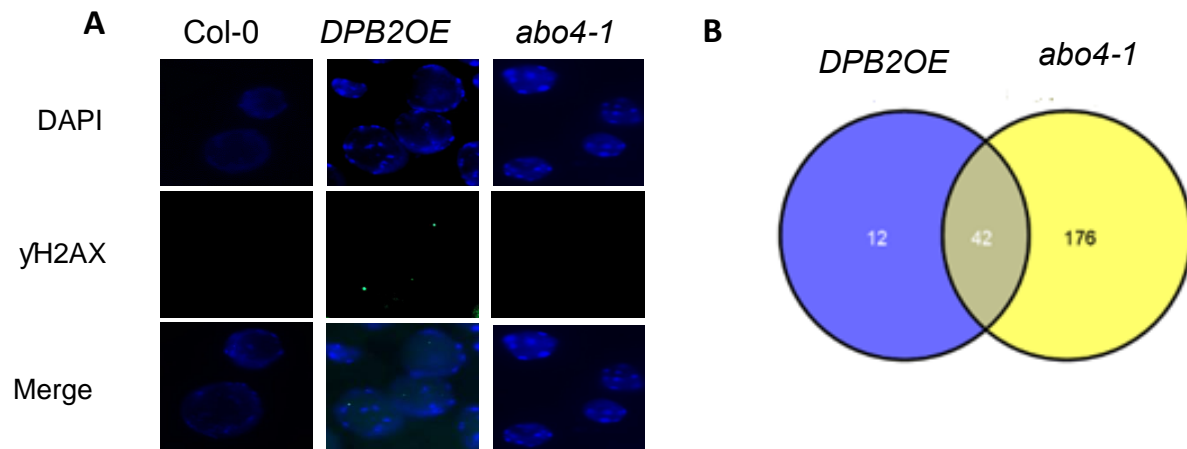


Figure 17. Molecular differences between hypomorphic allele of *POL2A* and *DPB2OE* plants.

A: Detection of γ -H2AX immunofluorescence in root tip nuclei.

B: overlap of up-regulated between RNAseqs of *DPB2OE* and *abo4-1* plants. For this analysis only genes that were up-regulated in both *DPB2OE* independent lines were included.

To figure out the DNA damage response pathways activated by *DPB2OE* and *abo4-1*, we have used genetic approaches. The *DPB2OE* construct was introduced in *atm*, *atr*, and *sog1* mutants and *abo4* plants were crossed with these mutants. The reduced frequency of *DPB2OE* lines displaying severe phenotype in both *atm* and *atr* mutants may suggest that both kinases contribute to the survival of *DPB2OE* plants. Interestingly, we observed very high accumulation of *DPB2* mRNA in *atr DPB2OE* lines that did not result in a severe phenotype, indicating that ATR activation is partially responsible for the growth defects in *DPB2OE* lines. However, it is worth noting that the few plants displaying a severe phenotype had a poor survival rate, suggesting that failure to activate ATR-dependent responses ultimately leads to developmental arrest likely caused by extensive DNA damage, reminiscent of the lethality of the *abo4 atr* double mutants. Likewise, the low survival rate of *sog1 DPB2OE* with severe phenotype indicates that SOG1 also contributes to the viability of these plants. In addition, the *sog1* mutation restored only partially the sensitivity to HU of *DPB2OE* roots, and some DNA damage response genes were still up-regulated in flower buds despite of SOG1 deficiency. Together our results suggest that replication stress induced by DPB2 over-accumulation or POL2A partial loss of function activates the ATR-SOG1 module that is required for sustained growth. Very similar results were obtained with *abo4* mutants: ATR and WEE1, but not SOG1 or ATM, were required for the viability of the mutants, and their tolerance to HU was at least partly mediated by SOG1. All together, these results gave us evidence that plant Pol ϵ might be directly involved in replicative stress sensing and acts upstream of ATR to trigger checkpoint activation via the two SOG1-dependent and independent pathways previously described (Hu et al., 2015, 2016). Importantly, our results demonstrate that SOG1-independent pathways can activate both repair genes and cell cycle delay (Figure 18).

In yeast, combination of mutation affecting Pol2 and Mec1 (ATR) also are lethal (Feng and D'Urso, 2001): in the absence of Mec1, fork stabilization upon replicative stress (or Pol2 deficiency) is compromised, which generates a massive fork collapse (Branzei and Foiani, 2009; Segurado and

Tercero, 2009). Interestingly, the *atr DPB2OE* plants are viable, suggesting that ATR-independent mechanisms are being activated to avoid these catastrophic events. The checkpoint activated by the *abo4* mutations is completely independent of ATM, demonstrating that only endogenous replication stress is present in these lines. By contrast, ATM appears to be required for survival of *DPB2OE* lines, indicating that DPB2 over-expression also leads to fork collapse and DSBs formation, which would activate DDR in an ATR-independent-manner (Figure 18). One possible model would thus be that ATM signaling is activated by DSBs as a consequence of fork collapse, which may allow the survival of *atr DPB2OE*, although we cannot rule out that other unknown mechanisms involving POL2A could be at work. Indeed, the former hypothesis does not fully explain why *abo4 atr* mutants are not viable, because ATM-dependent activation of DSB response should also function in these mutants. It is however possible that the catalytic activity of Pol ϵ itself is required in at least some of the ATM-dependent pathways. Figure 18 summarizes the proposed model for the effect of DPB2 over-expression and *abo4* mutations in vegetative cells.

The results of genetic analysis for *DPB2OE* revealed the mechanisms of DDR activated by the stress generated by the accumulation of DPB2. However, this did not allow us to clarify whether plant Pol ϵ is directly involved in replication checkpoint activation, up-stream of ATR (Pursell and Kunkel, 2008) and involved in replication stress sensing. The analysis of POL2A-RNAi lines was therefore instrumental to address this question: reduced accumulation of POL2A results in HU hypersensitivity, confirming that Pol ϵ is primordial for sensing of replication stress and activation of appropriate responses.

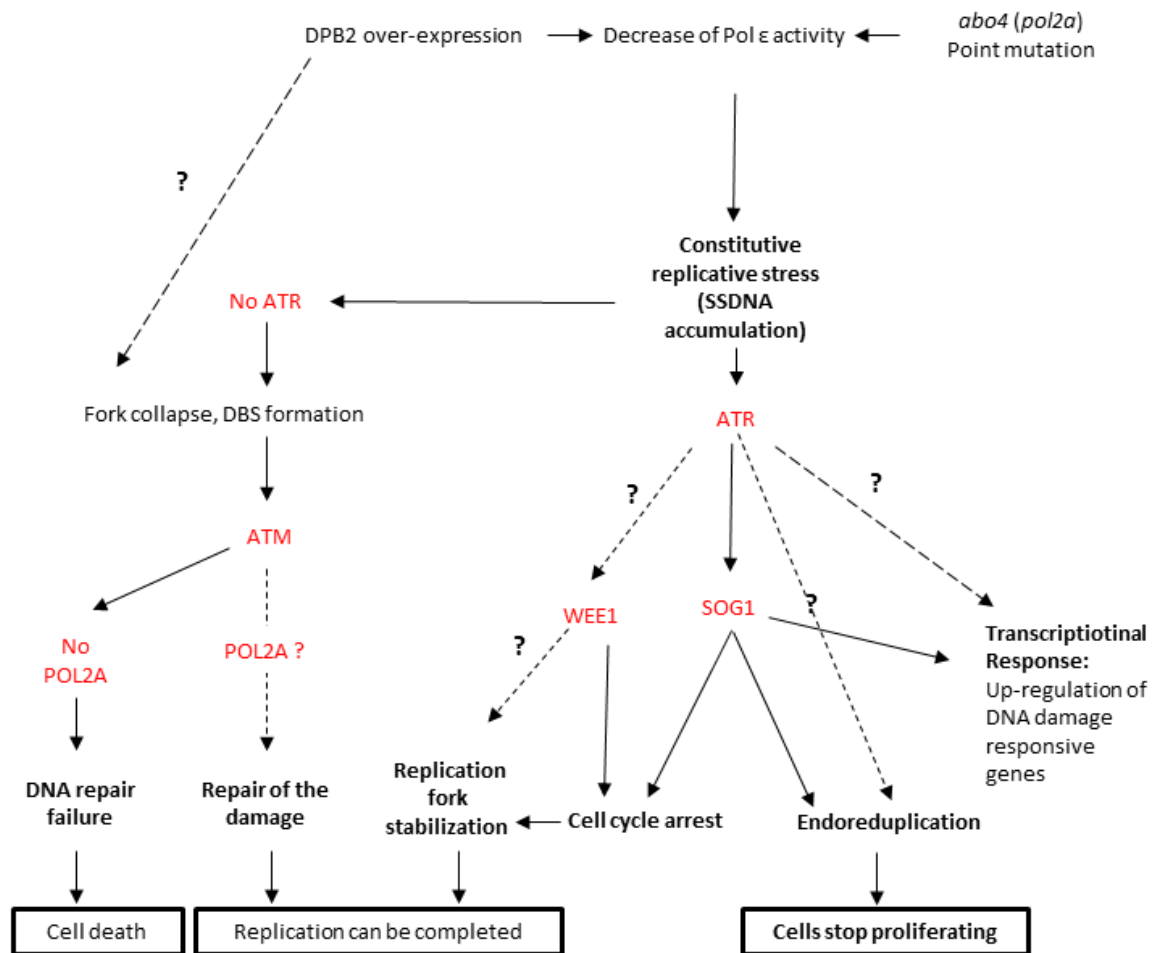


Figure 18. Model for POL2 involvement in the activation of replicative stress response in somatic cells. .

Deficiency of Pol ϵ catalytic domain or excess accumulation of DPB2 leads to constitutive activation of replication stress checkpoint. The ATR –WEE1 module is essential for embryo viability in *abo4* mutants (green arrow), and possibly for the viability of *DPB2OE* lines, although ATR activation is also partly responsible for their growth delay due to cell cycle arrest and premature induction of endoreduplication. In both cases, ATR activates SOG1-independent and –dependent mechanisms for triggering up-regulation. All these branches of DDR contribute to stabilize the replication forks to complete DNA replication upon constitutive replication stress. In the absence of ATR, or if it is not sufficiently activated, fork collapse is likely to happen, leading to the production of DSBs that can activate ATM-dependent responses. This pathway may require POL2A activity, which would explain why *atr DPB2OE* but not *atr abo4* mutants are viable. Unknown mechanisms and regulators are showed with dashed arrows, and question marks. The final outcome of each pathway is highlighted in a box. Effectors are written in red

Role of Pol ϵ in an adaptation checkpoint involved in DNA damage tolerance

In parallel of our analysis of replicative stress response, we have tested the sensitivity of Pol ϵ deficient lines to DNA damaging agents such as zeocin. To our surprise, *POL2A-RNAi* and to a lesser extend *DPB2OE* lines are resistant to zeocin. These results indicate that the hypersensitivity displayed by *abo4* plants to DNA-damaging agents might not be consequence of loss of DNA synthesis activity for repair as was previously suggested (Yin et al., 2009). In addition, *abo4-1 sog1* plants also were insensitive to zeocin like *sog1* and *POL2-RNAi* interference lines, supporting this hypothesis. In yeast, the Mec1 (ATR) signaling activation attenuates Tel1 (ATM) signaling in order to allow proper termination of the checkpoint response (Clerici et al., 2014). DNA damage often simultaneously triggers DNA breaks and stalled forks, causing that ATM and ATR regularly cooperate in response to DNA damage (Friedel et al., 2009; Branzei and Foiani, 2009). Thus, the hypersensitivity to zeocin showed by *abo4* seedlings may be caused by the constitutive activation of ATR signaling which could concomitantly inhibit ATM signaling thereby affecting the correct activation or inactivation of key players specific to this kind of response. However, this hypothesis does not account for the tolerance of *DPB2OE* lines to this treatment. It could be explained by the fact that DSBs are generated in the absence of exogenously applied stress in these lines, which would more efficiently activate ATM.

An alternative hypothesis would be that plant Pol ϵ could regulate an “adaptation checkpoint”. Indeed in Mammalian cells, upon prolonged exposure to agents that produce DSBs, cell survival requires the repression of DNA damage induced genes even if DNA damage repair has failed and lesions have accumulated (Syljuåsen, 2007). In this way, DNA replication and cell cycle can continue despite the presence of damaged DNA. This mechanism is termed “adaptation checkpoint” (Christmann and Kaina, 2013; Shaltiel et al., 2015; Syljuåsen, 2007). Indeed, constitutive activation of DNA repair is dangerous because several of these genes encode endonucleases that cut DNA, which is a step required for some DNA repair mechanisms (Syljuåsen, 2007; Christmann and

Kaina, 2013; Shaltiel et al., 2015). When DNA damage is prolonged cells thus activate the “adaptation checkpoint” to avoid accumulation of DSBs, and DNA damage is bypassed (Shaltiel et al., 2015). In yeast and mammal cells, the “adaptation checkpoint” triggers accumulation of DNA damage and genomic instability, leading to cell death after a certain time (Christmann and Kaina, 2013; Shaltiel et al., 2015). However, this mechanism appears to be very frequent in plants: Arabidopsis wild-type seedlings can grow on medium with genotoxins during prolonged time at variance with yeast and mammal cells. It therefore makes sense to postulate that an “adaptation checkpoint” is present in plants. We have tested this hypothesis: wild-type, *abo4* and *POL2A-RNAi* seedlings were exposed to zeocin and the transcriptional response for several genes associated to DDR was evaluated over time. The results shown that in wild-type plants the transcriptional response peaks after around 12h on genotoxin, and that at later time points the up-regulation of DDR genes starts to decrease, showing characteristic behavior of “adaptation checkpoint”. By contrast, when *abo4* plants were exposed to zeocin, expression of the DDR genes that was high even in the absence of treatment failed to reach the levels observed in the wild-type, at least for some genes but seemed to remain high, even at later time points. *POL2-RNAi* lines also showed some defects in the full induction of the DDR, but mRNA accumulation of the tested genes seemed to decrease after prolonged exposure as in the wild-type. Because *POL2A-RNAi* plants are tolerant to zeocin, this suggests that the sensitivity of *abo4* mutants to this drug can be attributed to the failure to reduce the transcription levels rather than to reduced activation of these genes at earlier time points. Nevertheless, this hypothesis needs to be confirmed by extending our time-course analysis at least to 48h to really confirm the tendency observed after 24h. Finally, this hypothesis is supported by the observation that *abo4-1 sog1* plants were insensitive to zeocin like *sog1*: the main role of SOG1 is to control the transcriptional regulation in response to DSBs. Thus, complete failure to activate the DDR results in apparent tolerance of the plant to the DNA damaging agent, although it is likely that close analysis of plants that have been exposed to these treatments or their progeny would reveal severe genetic anomalies. In any case, future work will be needed to clarify why

DPB2 over-expression and partial inactivation of *POL2A* by RNAi would not interfere with the “adaptation checkpoint” whereas modifications directly affecting the POL2A protein would.

Finally, one last hypothesis would be that lesion bypass could be more efficient in *POL2-RNAi* lines and possibly in *DPB2OE* lines. As described in the introduction, yeast mutants of the Dpb2 subunit of Pol ϵ that no longer bind stably to the Pol2 catalytic subunit confer a strong mutator phenotype that is partially dependent on the TLS polymerase Pol ζ , likely because these polymerases have more access to the leading strand when the stability of the Pol ϵ complex is affected (Langston et al., 2014). Thus, the accumulation of reduced levels of *POL2A* or *DPB2* over-expression may facilitate the access to TLS polymerases to carry out the replication in response to damage thereby conferring tolerance to zeocin by allowing cell proliferation to continue even in the presence of replication-blocking lesions.

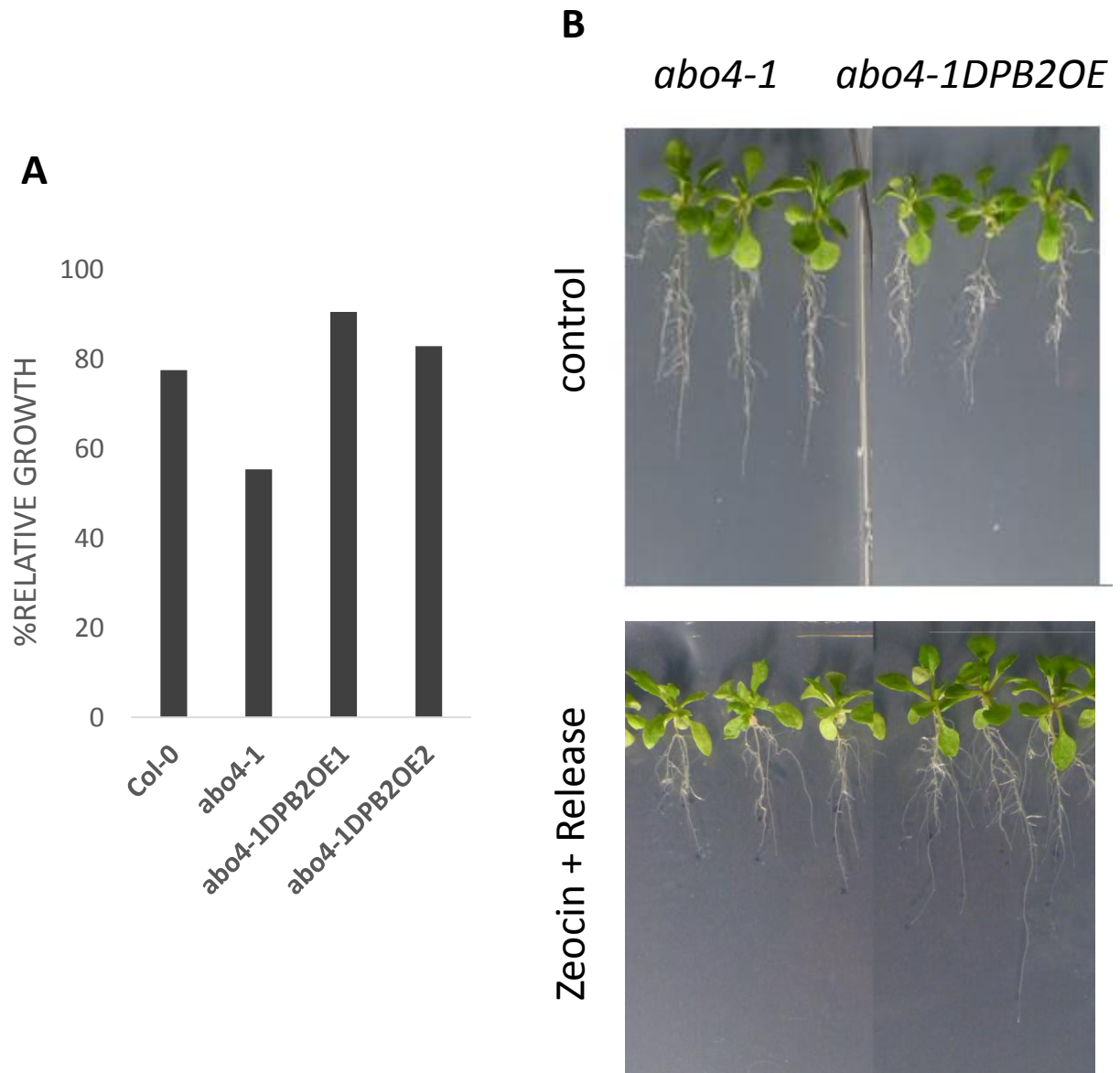


Figure 19. DPB2 over-expression leads to tolerance to zeocin in *abo4-1* plants. A, B: seedlings were germinated and grown on 0.5X medium for 4 days and transferred either to 0.5X MS medium or 0.5 X medium supplemented with 10 μ M of zeocin; A: relative root growth, after of 10 days on zeocin; B: upper panel shows plant without treatment and lower panel shows plants after release from zeocin, seedlings were exposed to zeocin for 6 days and then they were transferred to medium without genotoxin for 6 days to allow recovery

Roles of Pol ϵ sub-units during the pre-meiotic DNA replication and meiosis progression

Both *DPB2* over-expression and the *abo4-2* mutation result in extensive DNA fragmentation during meiosis, pointing to a central role of Pol ϵ during this crucial developmental step. Interestingly, these defects were at least partly rescued in the *sog1* (for both *abo4-2* and *DPB2OE*) and *atr* (for *DPB2OE*) backgrounds. This result suggest that the partial sterility of Pol ϵ deficient results from an active DNA fragmentation program triggered by replication stress in meiocytes, consistent with the role of SOG1 in the transcriptional activation of cell death genes (Yoshiyama et al., 2009, 2013b). Our results are in apparent contradiction with previous work by Huang et al, showing that the DNA fragmentation in *abo4-2* is SPO11-dependent, which would point to defects in DNA repair rather than activation of a cell death program (Huang et al., 2015). However, we propose that both mechanisms account for the meiotic phenotype of the mutant, and that previous reports overlooked the contribution of SOG1-dependent DNA fragmentation. Double *abo4-2 spo11* and triple *abo4-2 spo11 sog1* mutants are currently being produced to confirm this hypothesis.

One pending question is why the *abo4-1* mutation does not cause the same severe meiotic fragmentation as *DPB2* over-expression or the *abo4-2* mutation. This finding indicates that problems in DNA synthesis *per se* do not trigger the observed defects. It is however possible that the intensity of the replicative stress triggered by the *abo4-2* mutation is higher than in *abo4-1*. An alternative explanation may be that POL2 is required for scaffolding of other proteins for proper meiotic checkpoint activation, and that this scaffolding function is specifically compromised in *DPB2OE* and *abo4-2* lines. The molecular consequences of the *abo4-2* mutation are complex, but *abo4-2* mutants likely accumulate both low levels of normal POL2A protein and truncated variants of the protein. In this respect, this mutation could affect the insertion of Pol ϵ in the replisome in a similar way to what is postulated for *DPB2* over-expression, thereby compromising its scaffolding role at the fork. Such a defect would also be expected to occur in *POL2-RNAi lines*, although we

still need to confirm that their partly sterility results from SOG1-dependent DNA fragmentation. In general, the cell cycle regulation of meiosis is similar to that of mitosis (Lydall et al., 1996) and although some regulators are specific to each mechanism, most players are conserved (Subramanian and Hochwagen, 2014). Components of the DDR play an important role in the elimination of meiotic products with genetic anomalies. Indeed, disruption of the 9-1-1 complex component HUS1 was recently shown to bypass both pachynema arrest and apoptosis in mouse spermatocytes (Lyndaker et al., 2013), and disruption of CHK2 has similar effects in mouse oocytes (Bolcun-Filas et al., 2014), supporting a role of the meiotic checkpoint mechanism in this decision (Subramanian and Hochwagen, 2014). Similar mechanism may be activated in *abo4-2* and *POL2-RNAi* plants because POL2A interacts with 9-1-1 complex for checkpoint activation.

Interestingly, defects in various proteins involved in the onset of DNA replication led to similar meiotic phenotypes: SPO11-independent meiotic DNA fragmentation have been described in in *CDC45-RNAi* and *mei1* lines (Stevens et al., 2004; Grelon et al., 2003), as well as in lines in which the interaction between CDT1a and DPB2 is compromised (our unpublished data). Because DPB2 forms complexes with CDC45 and MEI1 in different steps of replication initiation where the catalytic subunit is not required (Handa et al., 2012), its over-expression could affect the stability of interactions required for correct DNA replication prior to meiosis, leading to delayed completion of DNA replication or replication errors.

To conclude, our results show that alteration of both the catalytic and regulatory sub-units of the Pol ϵ complex activates the DDR and cell cycle arrest but that the underlying mechanisms may be only partially overlapping, suggesting that each subunit could have different roles during DNA replication, and in the maintenance of genome integrity. To support this hypothesis, we have introduced the *DPB2OE* construct in *abo4-1* plants. Strikingly, DPB2 over-expression leads to tolerance to zeocin in *abo4-1* plants (Figure 19). Since Pol ϵ catalytic subunit is required for the activation of the S-phase checkpoint upon replication defects such as fork stalling, collapse or DNA

damage (Feng and D'Urso, 2001; Puddu et al., 2011), and this role has recently been shown to require DPB2 to achieve the Pol ϵ association into the replisome (García-Rodríguez et al., 2015); we may hypothesize that an impairment of Pol ϵ association to the replisome generated by DPB2 accumulation may interfere with checkpoint activation in these plants. Under such a hypothesis, constitutive activation of the DDR response observed in the *abo4* and *DPB2OE* lines would in fact rely on independent pathways rather than on similar mechanisms as proposed on Figure 18. This question remains to be addressed, but would be consistent with the existence of Pol2-dependent and Pol2-independent pathways for replicative stress sensing as described in the introduction.

Most studies about of role Pol ϵ have been carried out in yeast and cell lines. In this work we show that several roles of Pol ϵ subunits are conserved in plants. Thus, futures studies in *Arabidopsis* could allow elucidating mechanisms and functions that have not been found in other eukaryotes. In addition, the hypomorphic mutants of *POL2A* represent a valuable tool to study replication stress in a multicellular organism, notably to investigate cell-type specific responses.

PERSPECTIVES

Isolation of replicative polymerases mutants resulted from genetic screens aimed at isolating mutants deficient for processes as diverse as hormone signaling, epigenetic silencing, and embryo development. This fact illustrates of course the crucial importance of DNA replication for all aspects of plant development, but also points to the diversity of the functions encompassed by the Pol ϵ complex. A large number of research leads could thus be implemented following this work.

Function of Pol ϵ subunits

To better understand the role of Pol ϵ , we could characterize the *abo4-1 DPB2OE* plants into more detail. We could first determine whether transcriptional response to DNA damage induction is affected compared to *abo4-1* and *DPB2OE*, this approach we would allow us to know whether *DPB2* over-expression interferes with the checkpoint activation or inactivation.

Furthermore, we have generated constitutive *DPB2-RNAi* interference lines; these plants can be used to investigate the consequences of *DPB2* down-regulation at various developmental stages; which was not possible to analyze in inducible *DPB2-RNAi*-lines. It will be interesting to compare the obtained results with the *DPB2* over-expressing lines: do *DPB2* hyper and hypo-accumulation alter Pol ϵ function in the same way or do they lead to distinct features?

Currently the host laboratory has the four *POL2A* hypomorphic alleles and the T-DNA insertion line for *POL2B*. Three of the *POL2A* hypomorphic alleles appear to be affecting the polymerase domain because the mutation is within of domain or near it (del Olmo et al., 2010; Yin et al., 2009). We have shown that two of these mutants (*abo4-1*, *abo4-2*) display constitutive activation of the replication checkpoint and similar phenotype during the vegetative development. The third mutant is *esd7-1* (del Olmo et al., 2010); in this mutant, the mutation is situated within the catalytic domain and the mutant displays a phenotype similar to that of *abo4* plants; it is therefore likely replicative stress is also constitutively activated in these lines. The fourth hypomorphic mutant is *till-4*; this

mutation is within of endonuclease domain (Jenik et al., 2005). Interestingly, *till-4* plants display differences in the phenotype compared with rest of *pol2a* mutants: these plants do not show drastic change on flowering time compared to wild type plants and the vegetative growth is similar to wild-type line (Jenik et al., 2005), suggesting that other mechanisms may be affected by this mutation. Strikingly, *abo4-2* and *till-4* showed chromosomal fragmentation during meiosis and enhanced sterility (Huang et al., 2015), while *abo4-1* and *esd7-1* do not display problems during meiosis. In other eukaryotes, the consequences of mutations affecting POL2 depend on the region of the protein that is mutated it is thus likely that the same holds true in plants. Further analysis of *till-4* plants to explore whether they display the same features as *abo4/esd7* plants in terms of sensitivity to genotoxic agents will be interesting to further explore this possibility. Finally, the analysis of *pol2b* and *pol2bpol2a* lines will allow elucidating what is the contribution of POL2B in the observed phenotype and to further elucidate its level of redundancy with POL2A (Jenik et al., 2005; del Olmo et al., 2010).

The role of Pol ϵ during DNA replication and DNA damage response could be investigated into more detail. For instance, genetic interactions between replicative polymerases have been reported: *pol2a pola* plants do not have additive effects on plant growth, suggesting that both work in the same pathway (Zhang et al., 2016a; del Olmo et al., 2010); however, it has not been studied whether they both contribute to DDR. This would notably help determine whether the dual role in replicative stress sensing and DNA replication is unique to Pol ϵ or whether this feature is shared with other replicative polymerases. The single and double mutants are currently available in the host laboratory to address these issues.

Another crucial question will be to determine if chromatin changes in Pol ϵ deficient lines can account for defects in DNA repair or enhanced sensitivity to DNA damage, and more generally to deepen our understanding of the role of Pol ϵ in chromatin metabolism and histone marks maintenance. Indeed, Pol ϵ is involved in the replication of the genetic and epigenetic information,

notably in the context of the control of flowering time (Del Olmo et al., 2016; del Olmo et al., 2010). However, genome-wide analyses would be required to determine whether this role of Pol ϵ is confined to certain loci or whether it operates on the whole genome.

Interestingly, mutation in the FAS2 chromatin assembly factor, which is related with chromatin packaging and DNA replication, suppress the *esd7-1* phenotype in terms of flowering time (del Olmo et al., 2010). However, it has not been established whether this mutation affects DNA damage response in *pol2a* mutant. Additionally, the analysis of whole-genome bisulfite sequencing in *icu2-1* and *pold2-1*, indicated that Pol α and POLD2, the second small subunit of DNA polymerase δ , are not involved in the regulation of DNA methylation, however both showed epigenetic changes on histones (Hyun et al., 2013; Liu et al., 2010; Zhang et al., 2016a). By contrast, our preliminary results indicated that *abo4-1* and DPB2 over-expressing lines contain DNA methylation changes, several regions of DNA being hypomethylated in Pol ϵ deficient plants. However, these results need to be confirmed with a better depth of sequencing. Together these approaches will allow clarifying the role of Pol ϵ in properly establishing the chromatin marks during DNA replication to modulate gene expression and for maintaining genome integrity.

We would like to emphasize that the Arabidopsis plants with reduced or deficient Pol ϵ levels presented here offer an attractive genetic system to investigate mechanisms and proteins that are involved in safeguarding DNA replication, as well as the mechanisms activated by replication stress checkpoint, which are poorly described in plants. Notably, the depletion of DDR components or chromatin remodeling machinery has been found to be detrimental in other eukaryotes but did not impede plant growth. Thus, Pol ϵ functions and genetic interactions with factors involved in DNA replication can be more easily assessed in the context of a multicellular organism without interfering with cell viability.

Pol ϵ complex and interactions

To gain insight into the complexes that contain plant Pol ϵ , we performed Tandem Affinity Purification (TAP) using the DPB2 protein. The identified interactors are shown in Table I. Amongst the candidate partner proteins were found POL2A, CDT1a, and CDT1b, which were previously reported to interact with DPB2 in two-hybrid experiments (Ronceret et al., 2005; Domenichini et al., 2012). Interestingly, NF-YB11 and NF-YB13 were also identified as interactors of DPB2; according to phylogenetic analyses, they could be the plant DPB4 and DPB3-2 proteins (Petroni et al., 2012; Laloum et al., 2013). This is relevant because to date, there is no experimental data supporting the role of these Nuclear Factor Y (NF-Y) factors as sub-units of DNA Pol ϵ . Hence, other members of the host laboratory have begun to test the interactions through of two-hybrid and Bimolecular Fluorescence Complementation (BiFC) assays. The preliminary results showed that NF-YB11 and NF-YB13 interact physically with other Pol ϵ subunits and CDT1. Various single mutants and double mutant for these genes have been kindly provided to the host laboratory by the group of J.A. Jarillo (INIA Madrid). These plants do not display phenotypic differences compared with wild-type plants. They will be studied in response to genotoxic stress to explore whether their function is linked to specific response as was found for DPB3-1, which is involve heat stress response (Sato et al., 2016, 2014).

Additionally, MCM7 protein was found to interact with DPB2, suggesting that the complexes required for replication initiation may be conserved with other eukaryotes. Other interesting candidates include the ELONGATOR 3 (ELO3 factor) that encodes a histone acetyl-transferase required for normal cell proliferation (Skylar et al., 2013), and RNaseH-like proteins. Systematic interaction analyses will be performed using yeast two-hybrid and BiFC and confirmed interactants can then be considered for further characterization.

Table I: Proteins Identified by Tandem Affinity Purification Using DPB2 as Bait. Overview MS results. Possible background identifications are in orange font.

AT number	Name	NGSrhino-1	NGSrhino-2	Total/2 exps
AT5G22110	ATDPB2, CYL2, DPB2 DNA polymerase epsilon subunit B2	1	1	2
AT1G08260	EMB142, EMB2284, POL2A, TIL1, EMB529, ABO4, ESD7 DNA po	1	1	2
AT2G31270	ATCDT1A, CDT1A, CDT1 homolog of yeast CDT1 A	1	1	2
AT3G54710	ATCDT1B, CDT1B, CDT1 homolog of yeast CDT1 B homolog o	1	1	2
AT3G42660	transducin family protein / WD-40 repeat family protein	1	1	2
AT5G47890	NADH-ubiquinone oxidoreductase B8 subunit, putative	1	1	2
AT1G50370	Calcineurin-like metallo-phosphoesterase superfamily protein		1	1
AT2G27120	POL2B, TIL2 DNA polymerase epsilon catalytic subunit		1	1
AT2G27470	NF-YB11 nuclear factor Y, subunit B11		1	1
AT5G43250	NF-YC13 nuclear factor Y, subunit C13		1	1
AT4G02060	PRL, MCM7 Minichromosome maintenance (MCM2/3/5) family protein		1	1
AT3G55410	2-oxoglutarate dehydrogenase, E1 component	1		1
AT5G50320	ELO3, HAG3, HAC8, ELP3, AtELP3 radical SAM domain-containing protein / C		1	1
AT3G11770	Polynucleotidyl transferase, ribonuclease H-like superfamily	1		1
AT5G06450	Polynucleotidyl transferase, ribonuclease H-like superfamily	1		1

A link between DNA Damage Response and auxin signaling?

Finally, some of our results suggest that Pol ϵ may act as a possible linker between DNA replication and repair and hormone signaling. Indeed, the *abo4-1* allele of *POL2A* was originally isolated in a genetic screen for mutants affected in ABA response. Several mutants of replication fork proteins such as: RPA, CFA, and FAS1 also displayed hypersensitivity to ABA. Interestingly, we observed that *abo4-1* mutants are also hypersensitive to auxin, and that this phenotype is partly SOG1-dependent, providing evidence for a direct connection between phytohormone signaling and DNA stress. Our main obtained results will be described in the next paragraphs, together with approaches that will be implemented to investigate this putative link between phytohormone signaling and DNA stress.

The *abo4-1* seedlings display increased number of cells in S-phase in growth normal conditions (Fig 20 left), as determined via incorporation of the thymidine analogue EdU to newly synthesized DNA. We used auxin to induce the induction of lateral root formation, which increases the signal of the incorporation of BrdU (another thymidine analogue) in the wild-type. To our surprise, we obtained contrasting results between the seedlings exposed to auxin and not treated (Figure 20A), suggesting inhibits cell proliferation in *abo4-1* seedlings. A further analysis of cell cycle progression will be performed in presence of auxin.

To confirm the negative effects of auxin in *abo4-1* seedlings, we performed sensitivity assays: seedlings *abo4-1* were exposed to NAA (synthetic auxin) 0.2 μ M. *Abo4* seedlings were hypersensitive to the hormone compared with the wild-type (Figure 20B). Additionally, *abo4-1* seedlings displayed defective gravitropism: wild-type roots showed a bending angle of around 90° after 24h whereas in *abo4-1* was clearly less than 90°, indicating that auxin transport or response may be affected in Pol ϵ deficient plant.

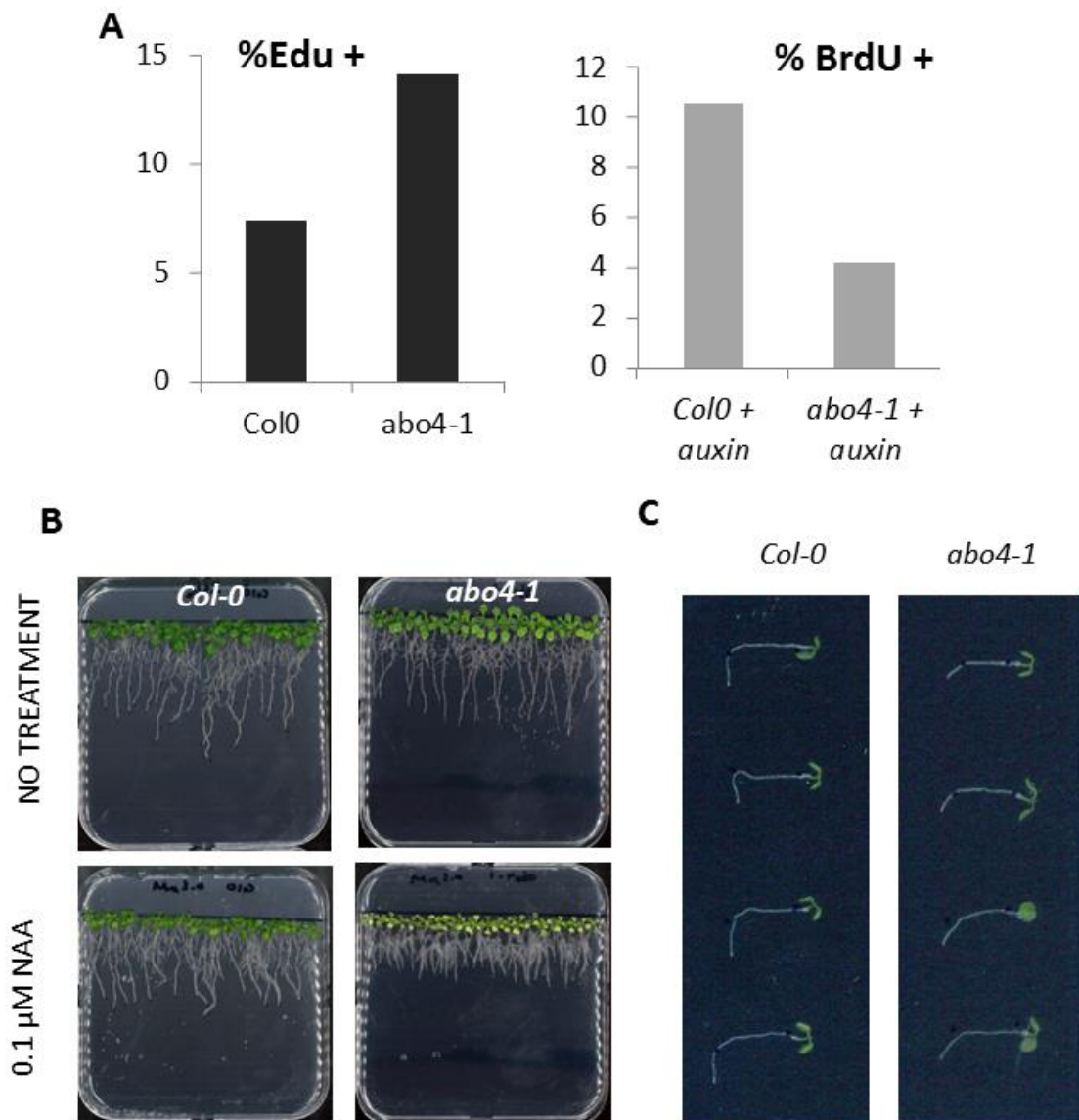


Figure 20. *abo4-1* mutants are hypersensitive to auxin and display alteration of root gravitropism. A: Proportion S-phase nuclei in untreated seedlings (left, % Edu +) and auxin-treated *abo4* seedlings (right, % BrdU +). In the presence of auxin, the proportion of cells in S-phase increases in the wild-type but decreases drastically in *abo4-1*. B: seedlings were germinated and grown on 0.5X medium for 8 days and transferred either to 0.5X MS medium or 0.5 X medium supplemented with 0.2 μ M of auxin (NAA) for 9 days. C: 5 day-old seedlings were used for a bending test, the bending of root was recorded after 24h: root bending was more efficient in the wild-type than in *abo4-1*.

In parallel, we evaluated the sensitivity to auxin of DPB2 over-expressing plant; these lines are less sensitive to auxin than wild-type plants (Figure 21), the *DPB2OE* seedlings were greener than Col-0. This result was confirmed in main root growth assays (data not shown). To explore if the sensitivity showed could relate to auxin distribution and/or response, we used the auxin reporter DR5-GUS line (Ulmasov et al., 1995), to examine indirectly the location of auxin maxima in roots from *abo4-1* and *DPB2OE* seedlings. In the wild type, *DR5* expression showed a characteristic pattern of auxin flux, while Pol ϵ deficient lines showed an abnormal pattern of auxin location (Figure 21B) indicating that auxin transport or response is affected in both lines. Surprisingly, *DR5* expression was lower in both lines, even though they showed opposite phenotypes in terms of auxin tolerance, suggesting that these lines show complex alterations in the auxin response program. Recently it was reported that prolonged exposure to low concentration of HU leads to changes in the polarity of auxin efflux in onion cells (Žabka et al., 2015). To explore the possible changes of auxin efflux in the Pol ϵ deficient lines; we will perform immunolocalization of PIN2; this is an auxin efflux carrier which is expressed in root tissues and is selectively localized to the apical (upper) side of root cap cells and epidermal cells (Dai et al., 2012). Polar localization of PIN proteins facilitates auxin flow and determines the direction of local intercellular auxin transport, and subsequently regulates plant development (Dai et al., 2012).

We next used the DR5 reporter lines to follow the response to exogenous auxin in Pol ϵ deficient lines; in the wild-type background, DR5-GUS expression increased throughout the root with exogenous auxin application (0.2 μ M NAA; Figure 22A). Notably, the tip region showed the maximum induction (Figure 22A). In the *abo4-1* line, the induction maximum was attenuated at the root tip (Figure 22A); likewise, in the rest of the root, DR5-GUS expression was greatly reduced (Figure 22A). All observed roots showed the same pattern of DR5-GUS expression. These changes in DR5-GUS activation indicate that *abo4-1* phenotypes may be caused by defects in auxin transport and/or possibly also in auxin signaling.

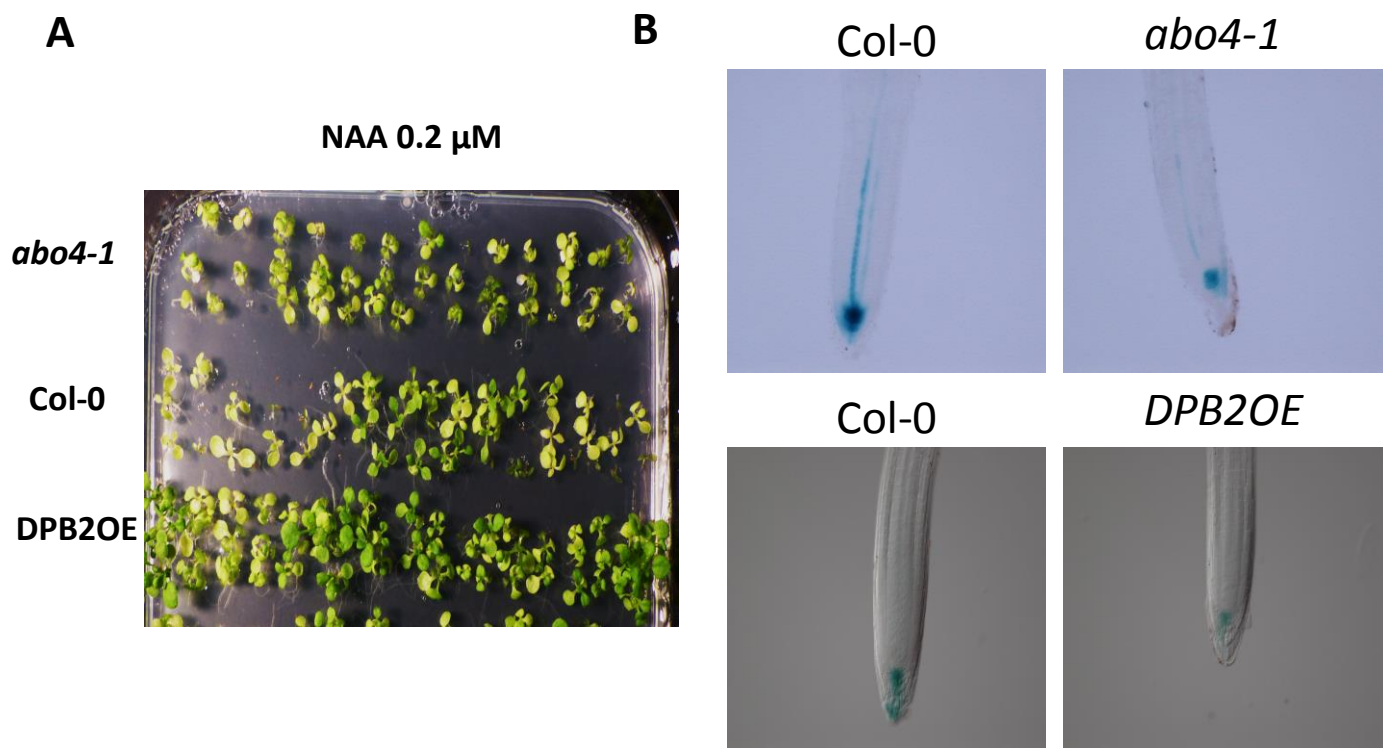


Figure 21. DPB2 over-expression affects auxin sensitivity and auxin response.

A: seedlings were germinated and grown on 0.5X medium supplemented with 0.2 μ M of auxin (NAA), phenotype was recorded at 10 days. B: GUS-staining was performed on wild-type, *abo4-1*, and *DPB2OE* seedlings expressing the DR5-GUS reporter.

To further study how auxin response is modified in *abo4-1* mutants, we evaluated the mRNA levels of auxin-responsive genes. Both *PID* (*PINOID*) and *APOLO* have been demonstrated to respond to exogenous auxin. The *APOLO* gene allows the production of a long noncoding RNA (Ariel et al., 2014); while *PINOID* (*PID*) is a Ser/Thr kinase that was reported to directly phosphorylate PIN proteins and thus to play an important role in mediating the polar targeting of PIN proteins (Zourelidou et al., 2014). Loss of *PID* function causes apical to basal shift in PIN polarity, while *PID* gain function results in the opposite basal-to-apical shift in PIN polarity. Altered *PID* activity causes changes in auxin flow, leading to defects in the development (Dai et al., 2012). In wild-type plants, both genes are up-regulated by the auxin exposure (Figure 22B). In *abo4-1* seedlings, both *APOLO* and *PID*, were up-regulated in basal conditions and auxin responsiveness was largely lost for both genes (Figure 22B).

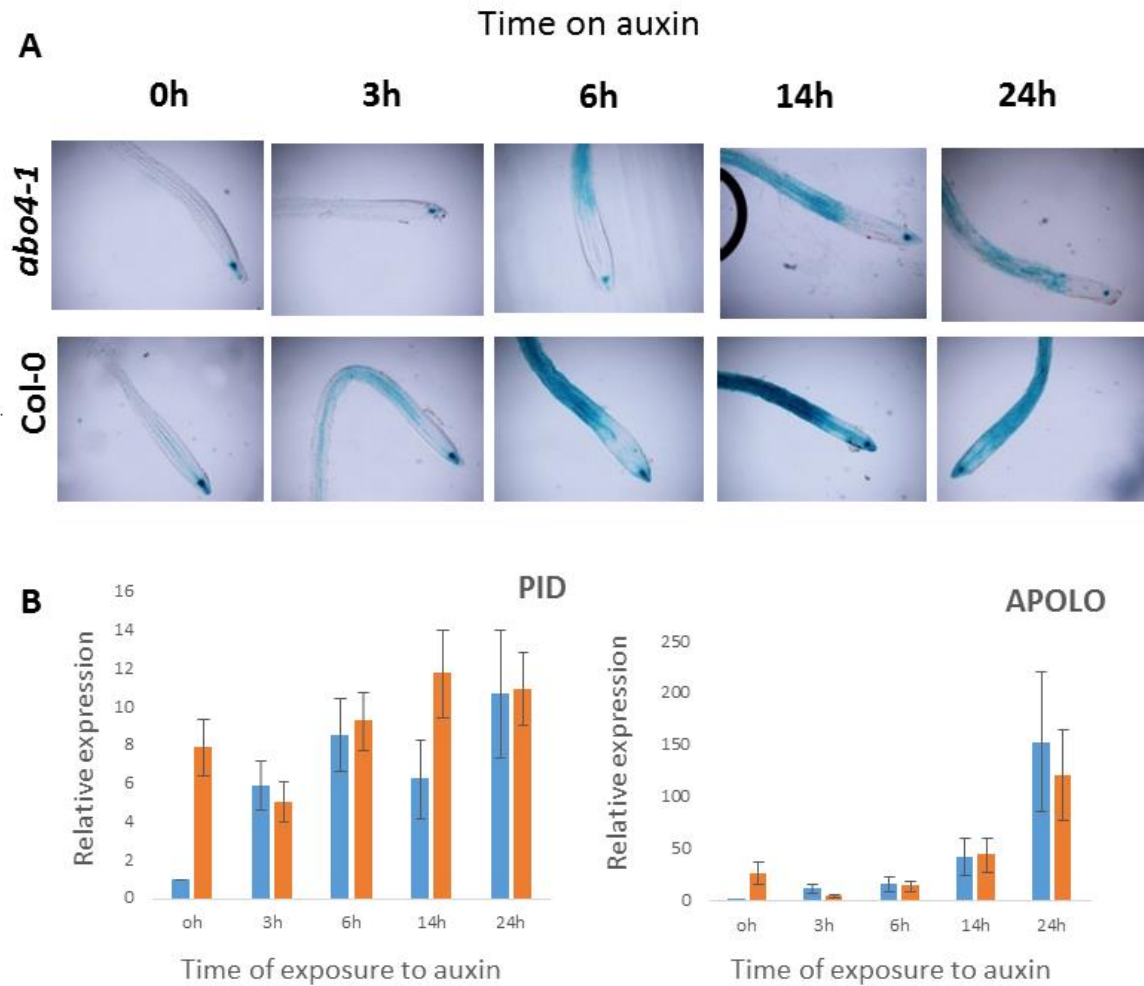


Figure 22. Response to exogenous auxin in *abo4-1* seedlings. A: GUS-staining was performed on seedlings of the DR5-GUS reporter line and *abo4-1* DR5-GUS lines at different time points after exposure to auxin (0.2 μ M NAA). B: Transcript levels of the auxin responsive genes *PID*, and *APOLO* in 8-day-old seedlings. Values are expressed relative to wild-type (Col-0) without treatment. Transcript levels were measured by quantitative RT-PCR and normalized to PDF2.

In addition, we searched for auxin-related transcriptional changes by analyzing the transcriptomes of *abo4-1* and *DPB2OE* seedlings, with the Visual Lateral Root Transcriptome Compendium tool (Parizot et al., 2010). The analysis allowed us to identify amongst the mis-regulated genes in the two lines, the genes associated with lateral root initiation (LRI) and auxin response factors (ARF). These genes represented a high proportion of the total number of mis-regulated genes in Pol ϵ deficient lines. The numbers of genes in each category for both RNAseqs that are either up or down regulated are show in Figure 23. In each categories, we found both up and downregulated genes, suggesting that the consequences of Pol ϵ deficiency on auxin signaling are complex. This analysis allowed us to identify some candidate genes for auxin signaling, which will be monitored its expression by qRT-PCR to confirm whether auxin response is pre-activated in these plants. Further genetic approaches will be required to dissect the connection between Pol ϵ deficiency and auxin response.

Recently, microarray analysis of Pol α mutant plants revealed that a large number of genes involved in ABA signaling as well as in SA biosynthesis and accumulation are down-regulated in these mutants (Micol-Ponce et al., 2015). Interestingly, we also identified genes associated to these mechanisms amongst mis-regulated genes in Pol ϵ deficient lines. Moreover, both *icu2-1* and *abo4-1* lines are hypersensitive to ABA (Micol-Ponce et al., 2015; Yin et al., 2009), suggesting that both mutations activate the same type of response, although the underlying molecular mechanisms remain elusive.

	<i>abo4-1</i>	<i>DPB2OE3</i>
Total	389	785
LRI	61	152
ARF	22	93

	Down-regulated	Up-regulated
LRI	23	38
ARF	18	4

	Down-regulated	Up-regulated
LRI	82	70
ARF	47	46

Figure 23. Analysis of the transcriptomes of *abo4-1* and *DPB2OE* seedling, using the tool called Visual Lateral Root Transcriptome Compendium (Parizot et al., 2010). The genes associated with lateral root initiation (LRI) process and auxin responsive factor (ARF) were identified and grouped in down- and up-regulated genes. Both *abo4-1* and *DPB2OE3* RNA seqs were analysed.

It has been shown that cell proliferation-promoting genes, which included seven Elongator (histone acetyltransferase complex), two CAF-1, and one POL2 subunit genes, all contribute to leaf shape formation (Xu et al., 2012). Strikingly, DPB2 over-expression rescued partially leaf shape in *abo4-1* plant (Figure 24A). Since, leaf shape is partly dependent of auxin transport, we asked if DPB2 over-expression would also rescue the sensitivity of *abo4-1* seedlings. *abo4-1 DPB2OE* seedlings were less sensitive to auxin compared to *abo4-1* single mutant (Figure 24B), indicating that excess accumulation of DPB2 has an opposite effect on auxin response compared to partial POL2A deficiency. Similar results were observed in the DNA-damaging agents sensitivity assays, which led us to ask whether the SOG1 mutation also rescues the sensitivity of *abo4-1* mutants to auxin. *sog1* seedlings display less sensitivity to auxin compared to wild-type plants (Figure 25A). In addition, the *sog1* mutation partially rescued the auxin sensitivity of *abo4-1* mutants (Figure 25B), as well as the leaf shape defects (Figure 25C). Interestingly, it was reported that the expressions of the auxin response element *IAA5* and the auxin efflux regulator *PIN3*, which are plant-specific hormone regulators, are induced and repressed by γ -irradiation, respectively, in a SOG1-dependent manner (Yoshiyama et al., 2013a). These results suggest that a connection exists between auxin signaling and the DDR. Consistently, transcript profiling revealed that DNA damage induces the up-regulation of several genes that are essential for the auxin response (Ricaud et al., 2007). Moreover, *atm* and *atr* mutants display alteration in the regulation of auxin-responsive genes after DNA damage (Culligan et al., 2006).



Figure 24. DPB2 over-expression rescues the auxin sensitivity and leaf shape defects of *abo4-1* mutants. A: *abo4-1* plants display modified leaf shape and this defects is partly rescued by *DPB2* over-expression. B: seedlings were germinated and grown on 0.5X medium for 4 days and transferred either to 0.5X MS medium or 0.5 X medium supplemented with 0.2 μ M of auxin (NAA), phenotype was recorded after 10 days on auxin.

Additionally, expression of the *KU70* gene, which is involved in DNA repair, can be down-regulated by phytohormones such as auxin and ABA in etiolated hypocotyls of *Virginia radiata* (Liu et al., 2007, 2008). Furthermore, an evident link between ABA signaling and plant response to genotoxic stress was provided by the characterization of the *abo4-1* mutant: ABA treatment increases homologous recombination in both *abo4-1* mutants and wild-type plants, however, it led to accumulation of DSBs in somatic cells only in the *abo4-1* mutant (Yin et al., 2009). Finally, a recent study has shown that treatment with SA in wild-type seedlings induces DSBs and activates the HR pathway via ATR and RAD17 (Yan et al., 2013) leading to improved plant immunity. All together, these results point to connections between hormone signaling and the DDR. The observation that plant stress hormones can induce the DDR may relate to the fact that a number of stress conditions induce oxidative stress that can lead to DNA damage. However, the connection between auxin response and DNA damage is more difficult to explain. In addition, how DDR could in turn affect hormone-signaling remains to be established.

To continue the study of the link between auxin and DDR proteins such as ATM, ATR, SOG1, and Pol ϵ , we will perform sensitivity assays to auxin in mutant lines. The assays performed to date, indicate that *POL2-RNAi* seedlings are tolerant to auxin, although we need to confirm this result. This result could further support the hypothesis that the hypersensitivity of *abo4* mutants to auxin could be due to auxin-induced accumulation of DNA lesions. Additionally, we have crossed DR5-GUS marker line with *atm*, *atr*, *sog1*, *abo4-1sog1*, *abo4-1atm* mutant lines; currently these lines are in the F1 generation. The analysis of GUS expression in these lines will give us information about how these mutations impact transport and/or response to auxin. If changes are observed, immuno-localization of PIN proteins could be performed in the various mutant lines. Finally, we tested the sensitivity to ABA of *DPB2OE* lines, these plants displayed less reduction in the relative percentage of germination rate compared with wild-type line (Figure 26). By contrast, *abo4-1* plants are hypersensitive to ABA (Yin et al., 2009). Thus, *abo4-1* and *DPB2OE* lines have contrasting

sensitivity to both auxin and ABA, indicating that each deficiency of Pol ϵ likely affects by different mechanism the transport and/or signaling of hormone.

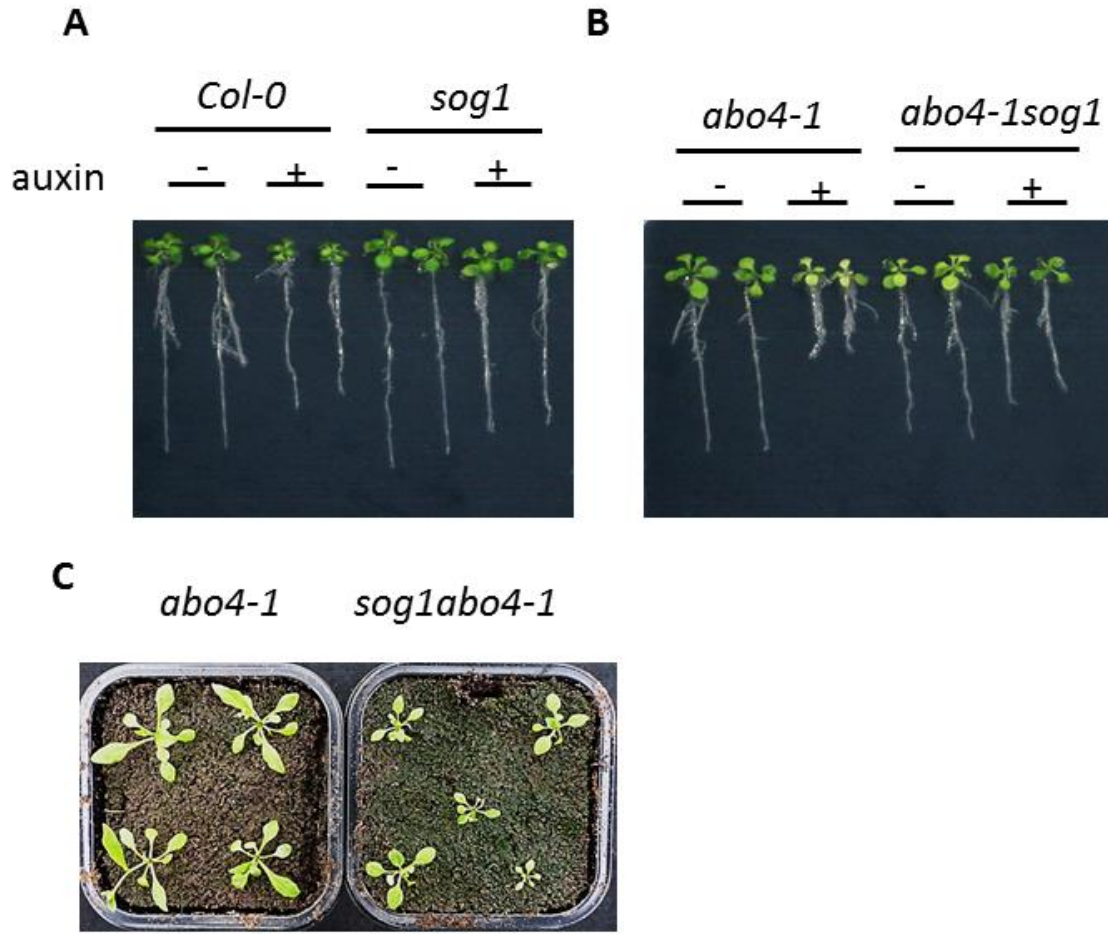


Figure 25. Hypersensitivity to auxin and leaf shape alteration displayed by *abo4-1* mutants are partly SOG1-dependent A, B: seedlings were germinated and grown on 0.5X medium for 4 days and transferred either to 0.5X MS medium or 0.5 X medium supplemented with 0.2 μ M of auxin (NAA), phenotype was recorded after of 10 days on auxin. C: *abo4-1* plants display changes in leaf shape that are partly rescued by the *sog1* mutation.

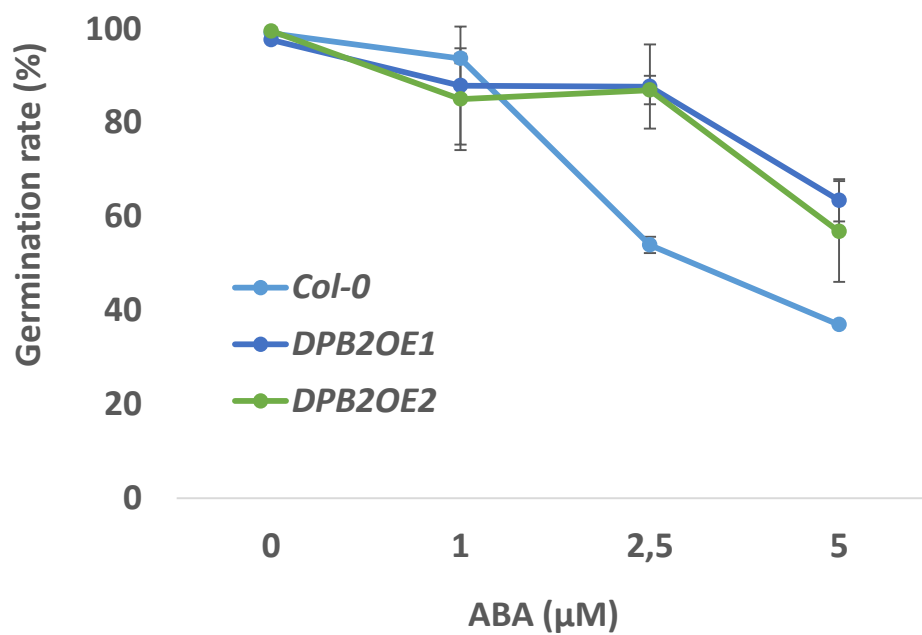


Figure 26. DPB2 over-expression confers tolerance to Abscicic acid (ABA). Seedlings were germinated and grown on 0.5X medium supplemented with different ABA concentrations. The number of germinated seeds were counted at 5 days-old.-growth

Interestingly, TAP experiments identified the PP6 phosphatase catalytic subunit one, termed FYPP1 as a putative interactor of DPB2 (AT1G50370). Two homologous genes encoding the catalytic subunits of PP6; *FYPP1* and *FYPP3* with redundant functions have been described (Dai et al., 2013a). Interestingly, in Arabidopsis, this phosphatase forms a holoenzyme complex, which interacts with a subset of PIN proteins and regulates PIN phosphorylation. Thus, PP6 acts antagonistically with PID to regulate the reversible phosphorylation of PIN and polar targeting, subsequently impacting polar auxin transport and plant development (Dai et al., 2012). Moreover, PP6 proteins physically interact with ABI5 *in vitro*; ABI5 is a transcriptional activator that binds to an ABA-responsive element (ABRE), a conserved cis-acting element found in the promoters of many ABA-induced genes (Finkelstein and Lynch, 2000). The specificity PP6 function *in vivo* is conferred by a group of regulatory subunits that are unique to their holoenzymes (Dai et al., 2013a). PP6 proteins and SnRK2 have opposite effects on ABI5 phosphorylation and ABA responses. Thus, the Arabidopsis PP6 phosphatase regulates ABA signaling through dephosphorylation and destabilization of ABI5 (Dai et al., 2013b).

Protein phosphorylation by kinases and dephosphorylation by phosphatases represent a major mechanism regulating eukaryotic cell signaling (Moorhead et al., 2007). In Mammalian cells, much of the DDR signaling transduction is relayed and amplified by Ser/Thr phosphorylation, it is intuitive that Ser/Thr phosphatases could negatively regulate these phosphorylation events and maintain the balance of phosphorylation during and after DDR. This allows cells to prevent illegitimate activation of DDR in the absence of DNA damage as well as rapid cessation of DDR once the lesion is repaired (Liu et al., 2016; Moorhead et al., 2007). Indeed, the human homologue of PP6 directly dephosphorylates sites ATM, ATR or DNA-PKcs kinases and enhances their activity (Liu et al., 2016). All these evidence suggest that PP6 could be a shared regulator between hormone and DDR signaling to allow the coordination of both pathways, and that Pol ϵ is likely a

shared target. To date, we have confirmed the interaction of PP6 with DPB2 via yeast two-hybrid experiments, but further work will be needed to clarify the role of this interaction.

Together, all these approaches will allow us to clarify the role of Pol ϵ and DDR proteins in the hormone signaling in the context of DNA damage response.

Hence, this work sets ground for diverse research lines in the field of DDR response, chromatin dynamics or plant physiology. Exploring all these roles of DNA Pol ϵ , the connections between the various pathways, and the contribution of other replicative polymerases to similar functions should allow obtaining a more comprehensive view of these seemingly independent processes.

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APPENDIX

Book chapter entitled “Plant Cell Cycle Transitions” (Molecular Cell Biology of the Growth and Differentiation of Plant Cells Edited by Ray J. Rose CRC Press 2016). The full version of this book chapter is available in the printed version

Titre : Analyse fonctionnelle de l'ADN polymérase epsilon : à l'interface entre réplication de l'ADN, régulation du cycle cellulaire et réponse aux lésions de l'ADN.

Mots clés : Polymérase epsilon, cycle cellulaire, point de contrôle, réplication de l'ADN, réparation de l'ADN

Résumé : Contrairement aux animaux, les plantes ont un développement largement post-embryonnaire et forment continuellement de nouveaux organes et tissus grâce à l'activité de leurs méristèmes. Ces massifs de cellules indifférenciées conservent la capacité à se diviser tout au long de la vie de la plante, et c'est également à partir du méristème caulinaire que se forment les gamètes. Chaque cycle de division peut être la source de mutations, suite par exemple à des erreurs de réplication. De plus, les méristèmes sont relativement exposés aux stress environnementaux qui peuvent également endommager l'ADN des cellules. Les mécanismes impliqués dans la détection des lésions de l'ADN ou des défauts de réplication et l'arrêt de la prolifération cellulaire en réponse à ces dommages jouent donc un rôle fondamental dans le maintien de la stabilité du génome, aussi bien au cours du développement végétatif que lors de la reproduction sexuée. Chez tous les eucaryotes, l'ADN Polymérase ϵ est un acteur central de ces mécanismes parce qu'elle assure non seulement la réplication fidèle de l'ADN au cours de la phase S du cycle cellulaire, mais est également directement impliquée dans la réparation de l'ADN, et dans la perception du stress réplcatif. L'étude détaillée de sa fonction est cependant rendue difficile chez beaucoup d'organismes par le fait que son inactivation est létale. Dans ce travail, nous avons utilisé des approches de génétique pour étudier le rôle de l'ADN Pol ϵ d'Arabidopsis au cours de la progression du cycle cellulaire et dans la réponse au stress réplcatif et aux lésions de l'ADN. Nous avons ainsi pu montrer que la sous-unité catalytique du complexe Pol ϵ ainsi que sa principale sous-unité accessoire DPB2 sont essentielles à la détection des défauts de réplication, et fonctionnent en amont de la kinase ATR pour induire l'arrêt du cycle cellulaire et activer les voies de réparation au cours du développement végétatif. En outre, nous avons découvert un nouveau point de contrôle activé lors de la phase de réplication pré-méiotique qui permet l'activation d'une mort cellulaire programmée en réponse à des défauts survenus pendant cette phase, grâce au facteur de transcription SOG1. Tous les stress biotiques ou abiotiques auxquels la plante est soumise pouvant conduire à la formation de lésions au niveau de l'ADN, nos résultats ouvrent des perspectives de recherche pour comprendre la réponse des plantes aux stress environnementaux. En outre, la disponibilité de mutants viables pour différents facteurs impliqués dans la réplication ou la réponse aux lésions de l'ADN nous a permis d'explorer chez un eucaryote pluricellulaire des mécanismes qui sont pour l'instant essentiellement décrits chez la levure, et ainsi d'acquérir des connaissances qui pourront être transférées aux systèmes animaux et notamment à l'Homme.

Title: Functional characterization of the DNA Polymerase epsilon and its involvement in the maintenance of genome integrity in Arabidopsis

Keywords: Pol epsilon, cell cycle, checkpoint, DNA replication, DNA repair

Abstract: Plant development is a largely post-embryonic process that depends on the activity of meristems. These pools of undifferentiated cells retain the ability to proliferate throughout the lifespan of the plant, and are at the origin of gamete formation relatively late in its life cycle. Mutations can arise at each round of cell division, for example due to replication errors. In addition, meristems are relatively exposed to all kinds of environmental stresses that can also induce DNA damage. Detection of DNA lesions or replication defects and subsequent cell cycle arrest are thus instrumental to the maintenance of genome integrity, both during vegetative and reproductive growth. In all eukaryotes, DNA Pol ϵ is a key player of these mechanisms because it is not only responsible for the faithful reproduction of the genetic information during S-phase, but also directly involved in DNA repair and replicative stress perception. Detailed analysis of its function has however been complicated by the lethality of its inactivation in most organisms. In this work, we have used genetic approaches to investigate its role during cell cycle progression and replicative stress response. We have shown that both its catalytic sub-unit and its main accessory sub-unit DPB2 are involved in replicative stress sensing and that they function upstream of the ATR kinase to induce cell cycle arrest and DNA repair during vegetative growth. In addition, we have found that a specific checkpoint exists during pre-meiotic DNA replication that activates a cell death programme via the SOG1 transcription factor upon replicative stress. Because all types of biotic and abiotic stresses can generate DNA damage, our work opens new research prospects to understand how plants cope with adverse conditions. Furthermore, the viability of Arabidopsis mutants deficient for various factors involved in DNA replication or DNA Damage Response allowed us to analyse into details in a multicellular eukaryote crucial cellular mechanisms that had until now been mainly investigated in yeast. This work thus allowed us to generate data that can be transferred to animal systems and notably to Human.