

Evidence for a Role of *Arabidopsis* CDT1 Proteins in Gametophyte Development and Maintenance of Genome Integrity[□]

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Meristems retain the ability to divide throughout the life cycle of plants, which can last for over 1000 years in some species. Furthermore, the germline is not laid down early during embryogenesis but originates from the meristematic cells relatively late during development. Thus, accurate cell cycle regulation is of utmost importance to avoid the accumulation of mutations during vegetative growth and reproduction. The *Arabidopsis thaliana* genome encodes two homologs of the replication licensing factor CDC10 Target1 (CDT1), and overexpression of CDT1a stimulates DNA replication. Here, we have investigated the respective functions of *Arabidopsis* CDT1a and CDT1b. We show that CDT1 proteins have partially redundant functions during gametophyte development and are required for the maintenance of genome integrity. Furthermore, *CDT1-RNAi* plants show endogenous DNA stress, are more tolerant than the wild type to DNA-damaging agents, and show constitutive induction of genes involved in DNA repair. This DNA stress response may be a direct consequence of reduced CDT1 accumulation on DNA repair or may relate to the ability of CDT1 proteins to form complexes with DNA polymerase ϵ , which functions in DNA replication and in DNA stress checkpoint activation. Taken together, our results provide evidence for a crucial role of *Arabidopsis* CDT1 proteins in genome stability.

INTRODUCTION

In contrast to animal development, plant development is mostly a postembryonic process, achieved by the activity of meristems in which cells divide throughout the plant's life. In addition, the germline differentiates only late in development, implying that replication errors occurring in the shoot meristem would be transmitted to the next generation. Because of these specific features, accurate genome duplication and hence correct cell cycle regulation is of particular importance in plants. Although plants have evolved new regulators of cell cycle progression, the basic regulatory mechanisms are shared with other eukaryotes (reviewed in De Veylder et al., 2007; Costas et al., 2011). Cyclin-dependent kinase (CDK)-cyclin complexes are the core cell cycle regulators that allow the transition from one phase to another. Notably, entry into the S phase of the cell cycle requires phosphorylation of the Retinoblastoma Related (RBR) protein by CDK-cyclin D complexes. Upon phosphorylation, the inhibitory effect of RBR on E2F transcription factors is released,

allowing the expression of downstream targets such as subunits of the prereplication complex (pre-RC) (reviewed in Inz  and De Veylder, 2006). Among these subunits, CDC10 Target1 (CDT1) and Cell Division Cycle6 (CDC6) are essential factors for DNA replication licensing in all eukaryotes because they recruit the DNA helicases called Mini-Chromosome Maintenance (MCM) proteins that open the replication forks (DePamphilis, 2003). Maintenance of genome integrity requires each part of the DNA to be replicated once and only once per cell cycle; therefore, the firing of replication origins must be tightly regulated. CDT1 is considered as the key factor that determines DNA replication licensing in all eukaryotes because its overexpression is sufficient to induce rereplication (Truong and Wu, 2011). Accordingly, this protein is the target of a wealth of regulatory mechanisms, including transcriptional control by E2F transcription factors, proteolysis, and interaction with the geminin, which functions as an inhibitor of CDT1 activity (reviewed in Truong and Wu, 2011). In animals, the dynamic formation of CDT1-geminin complexes, and the modulation of the stoichiometry between the two proteins, have recently been suggested to play a major role in controlling CDT1 activity during S-phase (Lutzmann et al., 2006; De Marco et al., 2009; Kisieleska and Blow, 2012).

In *Arabidopsis thaliana*, the pre-RC consists of six Origin Recognition Complex (ORC) proteins, each of which is encoded by a single gene, except ORC1, CDT1, and CDC6, which are encoded by duplicated genes, and six canonical MCM proteins (MCM2 to MCM7) (Masuda et al., 2004), but the function of only

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[ ] Online version contains Web-only data.

www.plantcell.org/cgi/doi/10.1105/tpc.112.100156



Figure 1. *cdt1* Mutants Are Partially Sterile.

Fully developed siliques from the wild type (Col-0), *cdt1a/+*, *cdt1a/+cdt1b*, and complemented *cdt1a* mutants were opened and observed using a binocular microscope.

[See online article for color version of this figure.]

a few of these genes has been investigated. Loss-of-function approaches revealed that MCM7 is required for gametophyte development and is maternally required for embryo development (Springer et al., 2000), whereas MCM2 appears to be dispensable for gametogenesis but indispensable to embryo development (Ni et al., 2009). By contrast, overexpression of *CDC6* or *CDT1a* did not affect overall plant development but stimulated endoreduplication (Castellano et al., 2004, 2001), an atypical cell cycle during which S-phase is not followed by mitosis (De Veylder et al., 2011), resulting in increased DNA content, suggesting that these two proteins accumulate in limiting amounts for DNA replication licensing. There is evidence for proteolytic regulation of CDT1a in *Arabidopsis* (Castellano et al., 2004), but bona fide geminin homologs appear to be absent from plant genomes, although the isolation of a CDT1-interacting protein that may function analogously to geminin, at least in some cell types, has been reported (Caro et al., 2007). Therefore, it is not clear whether other posttranslational regulation mechanisms affect CDT1 activity. Another open question is the respective role of the two CDT1 homologs CDT1a and CDT1b: only the function of CDT1a was investigated via overexpression in plants (Castellano et al., 2004). Interestingly, a loss-of-function approach using an RNA interference (RNAi) construct targeting both CDT1a and CDT1b allowed us to provide evidence for a role of CDT1 proteins in the coordination of plastid division and cell cycle

progression, but again, we were not able to precisely assign distinctive roles to the two proteins (Raynaud et al., 2005).

Another crucial aspect of genome stability is the ability of cells to stop cell cycle progression upon DNA damage until repair completion. In this respect, one could expect these mechanisms to be of outstanding importance in plants. Indeed, plants are sessile organisms and therefore cannot escape from suboptimal growth conditions. Furthermore, they require light for their photosynthetic activity and thus are continuously exposed to DNA-damaging agents such as UV light or reactive oxygen species produced by chloroplasts. In all eukaryotes, including plants, genome integrity is under the surveillance of ataxia telangiectasia mutated and Rad3-related (ATR) and ataxia telangiectasia mutated (ATM) kinases: they are activated by DNA damage and simultaneously arrest cell cycle progression and promote the expression of genes encoding DNA repair proteins (Culligan et al., 2006; Bensimon et al., 2011; Nam and Cortez, 2011). As observed for the control of S-phase, regulating CDT1 activity is instrumental to the response to DNA damage in eukaryotes. First, CDT1 degradation is required to stop or delay S-phase progression until DNA is repaired, and this process involves the recruitment of CDT1 to the site of DNA damage (Roukos et al., 2011). Second, CDT1 activity is required after DNA repair is completed to re-assemble pre-RC on unfired origins (Truong and Wu, 2011). Finally, CDT1 is required for break-induced DNA repair together with MCMs and pre-RC-activating proteins, whereas ORC and CDC6 are not (Lydeard et al., 2010). How plants respond to DNA-damaging agents and how this response affects cell cycle progression are beginning to be unraveled. One key player in this process is the Suppressor Of Gamma1 (SOG1) transcription factor, which functions downstream of ATR and ATM and is required both for cell cycle arrest and the induction of DNA repair genes (Yoshiyama et al., 2009). In addition, the WEE1 protein kinase has been shown to slow down S-phase progression upon DNA stress (De Schutter et al., 2007; Cools et al., 2011) and may act downstream of SOG1 (Yoshiyama et al., 2009). Interestingly, in plants, DNA stress not only induces an arrest of proliferating cells but also cell death in stem cell niches (Fulcher and Sablowski, 2009) and endoreduplication in other cell types (Cools and De Veylder, 2009; Adachi et al., 2011). Although there is no evidence to date for a role of plant CDT1 proteins in the control of genome integrity or DNA stress response, the observation that reduced expression of *CDT1* genes both delays cell cycle progression and induces endoreduplication (Raynaud et al., 2005) suggests that a decrease of CDT1 accumulation may cause DNA stress or altered DNA stress sensing.

Table 1. Result of T-DNA Transmission in Reciprocal Crosses of *cdt1a/+*

Cross	Sulf ^R	Sulf ^S	Percentage of Sulf ^R plantlets
<i>cdt1a/+</i> × <i>cdt1a/+</i>	164	343	31%
<i>cdt1a/+</i> × Col-0	0	340	0%
Col-0 × <i>cdt1a/+</i>	140	398	25%

Seeds obtained by each cross were sown on 0.5× MS medium containing sulfadiazine, and the sulfadiazine-sensitive (Sulf^S) and sulfadiazine-resistant (Sulf^R) plantlets were counted.

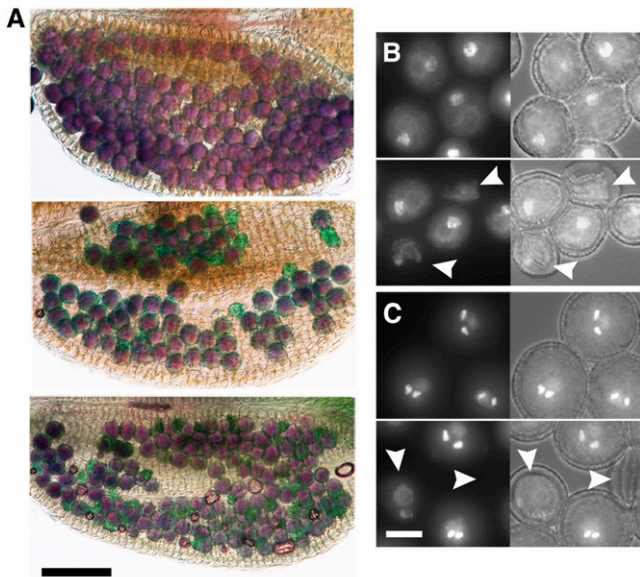


Figure 2. Pollen Development Is Affected in *cdt1a/+* Mutants.

(A) Alexander staining of mature anthers from the wild type (Col-0; top), *cdt1a/+* (middle), and *cdt1a/+cdt1b* (bottom) mutants. Viable pollen grains stain purple. Nonviable pollen grains stain blue-green and can be observed in *cdt1a/+* mutants, and their proportion is increased in the *cdt1b* background. **(B)** DAPI staining of pollen grains from wild-type (top) and *cdt1a/+* (bottom) plants at the binuclear stage. Aborting pollen grains can be detected in *cdt1a/+* plants (white arrowheads). **(C)** DAPI staining of pollen grains from wild-type (top) and *cdt1a/+* (bottom) plants at the trinuclear stage. Aborting pollen grains can be detected in *cdt1a/+* plants (white arrowheads), and some degenerating pollen grains contain two nuclei, indicating that they aborted after the first pollen mitosis. In **(B)** and **(C)**, left images are DAPI fluorescence and right images are overlays with bright-field images.

Based on our current knowledge of plant CDT1 proteins, the goal of this work was (1) to further investigate the respective functions of *Arabidopsis* CDT1a and CDT1b and (2) to determine whether these proteins are as important in plants as in other eukaryotes for the maintenance of genome integrity.

RESULTS

CDT1a and CDT1b Play Partially Redundant Roles during Gametophyte Development

We reported previously that simultaneous silencing of *CDT1a* and *CDT1b* resulted in severe developmental defects and that *cdt1b* null mutants are phenotypically identical to the wild type (Raynaud et al., 2005). *CDT1a* and *CDT1b* have very similar expression patterns, suggesting that they could be functionally redundant (Castellano et al., 2004). To elucidate the respective functions of the two genes, we searched for *cdt1a* mutants in publicly available mutant collections. We found an insertion in *CDT1a* in the GABI-Kat collection (GABI_025G08). This mutant line harbors two insertions, one in the fourth exon of *CDT1a* (see Supplemental Figure 1 online) and the second 577 bp upstream of the start codon of *HexoKinase Like3 (HKL3)* (At4g37840), which encodes a putative hexokinase. Using a segregating population, the T-DNA insertion in *CDT1a* was separated from the second T-DNA (see Methods). For three lines, no plant harboring the *HKL3* mutation was found, but all contained the *CDT1a* insertion, indicating that the parent line was mutated only for *CDT1a*. The presence of a sulfadiazine resistance maker in the GABI-Kat line allowed us to perform a segregation analysis of the T-DNA. Interestingly, the proportion of sulfadiazine-resistant plants in these lines was lower than expected (between one in four and one in three), and all *cdt1a* mutants identified in this first generation were hemizygous for the mutation ($n = 12$; hereafter referred to as *cdt1a/+*). These plants developed normally but formed short siliques containing aborted ovules (Figure 1). We counted normal and aborted seeds in siliques of seven plants (four siliques per plant) and found 49.8% aborted seeds. To confirm that this phenotype was due to the insertion in the *CDT1a* gene, we performed backcrosses. In the progeny, all sulfadiazine-resistant plants carried an insertion in *CDT1a*, and all showed a reduction in seed production. Furthermore, the mutant could be rescued by a construct encompassing a hemagglutinin (HA)-tagged version of CDT1a driven by its own promoter: *cdt1a/+* plants were transformed with the *pCDT1a*:

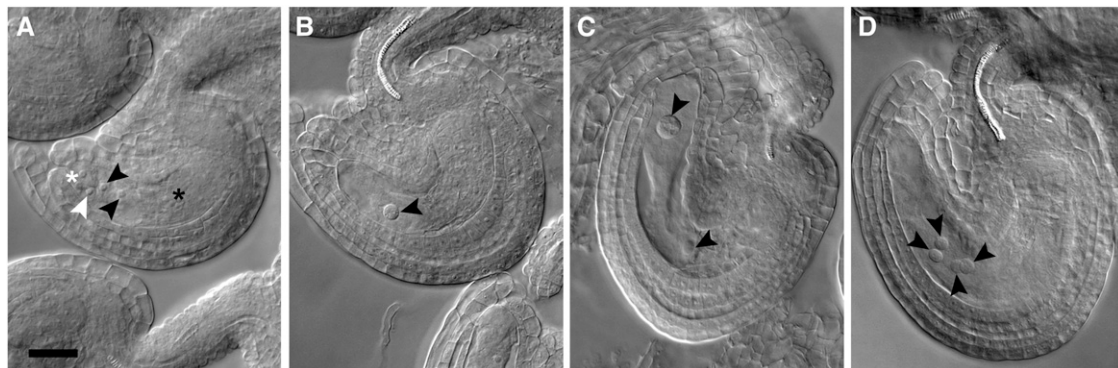


Figure 3. Embryo Sac Development Is Compromised in *cdt1a/+* Mutants.

(A) Mature embryo sac from a wild-type plant (Col-0). The positions of synergids (white star), egg cell (white arrowhead), central cell nuclei (black arrowheads), and antipodal cells (black stars) are indicated. **(B) to (D)** Abnormal embryo sacs in *cdt1a/+* mutants. Embryo sacs containing one **(B)**, two **(C)**, or four **(D)** nuclei were observed in 50% of ovules in *cdt1a/+* mutants.

CDT1a-HA construct, and phenotypically wild-type plants homozygous for the *cdt1a* mutation expressing the HA-tagged protein could be recovered (Figure 1).

To determine whether the *cdt1a* mutation was lethal during gametophyte development or during embryogenesis, we analyzed the transmission of the *cdt1a* mutation after self-pollination or reciprocal crosses. To follow the *cdt1a* mutation, we analyzed sulfadiazine resistance. Twelve plants were used for this analysis, seeds from three siliques obtained by each cross or self-fertilization were pooled, and ~40 seeds were sown on selective medium (Table 1). When *cdt1a/+* mutants were used as female, no sulfadiazine-resistant plants could be obtained, indicating that the mutation is not transmitted through the female side. When *cdt1a/+* mutants were allowed to self-fertilize or used as male, ~31 and 25% of their progeny were resistant to sulfadiazine. The difference between these two segregation values is not statistically significant (Student's *t* test; $P > 0.05$). However, these segregation values are significantly lower than the expected 50% if the mutation was transmitted normally via pollen grains ($\chi^2 = 6.48$). Hence, segregation analyses indicated that disruption of *CDT1a* affects female gametophyte development and, to a lesser extent, pollen grain development.

To confirm these observations and to determine which step of gametophyte development was affected by the *cdt1a* mutation, we tested pollen grain viability by Alexander staining and observed developing ovules from cleared siliques of *cdt1a/+* mutants. As shown in Figure 2A, *cdt1a/+* mutants show a proportion of green pollen grains after Alexander staining, indicating that they are not viable. We estimated that ~15% ($n = 200$) of pollen grains were aborted in the mutant. 4',6-diamidino-2-phenylindole (DAPI) staining on flower buds at various developmental stages revealed that meiospore formation was normal, but pollen grains stopped their development either before the first or second mitosis (Figures 2B and 2C). In developing siliques, we found that about half of the ovules contained normally developing embryos, whereas the other half appeared to have aborted. Closer examination revealed that half of the embryo sacs never reached maturity and stopped their development after one or two mitoses, giving rise to embryo sacs containing one to three nuclei (Figure 3). Consistently, *CDT1-RNAi* lines displayed similar defects during gametophyte development (data not shown). Taken together, these results suggest that *CDT1a* is strictly required for female gametophyte development and plays a role during male gametophyte development, although a majority of pollen grains harboring the *cdt1a* mutation develop normally.



Figure 4. Development of Some *cdt1a/+cdt1b* Mutants Is Affected.

(A) The progeny of a *cdt1a/+cdt1b* mutant were sown on 0.5× MS medium supplemented with sulfadiazine. After 10 d, the development of sulfadiazine-sensitive plants is arrested and they appear yellow. Some resistant plantlets show delayed growth (white arrowheads).

(B) to (E) Examples of developmental alterations observed in these smaller plantlets after transfer to soil and growth in the greenhouse. Magenta arrowheads point to abnormal plants.

Normal development of pollen grains lacking *CDT1a* may be allowed by the presence of *CDT1b*. To test this hypothesis, we obtained *cdt1a/+cdt1b*^{-/-} sesquimutants and analyzed the transmission of the *cdt1a* mutation in their progeny. We found that ~5% of plants inherited the *cdt1a* mutation ($n = 1857$).

Table 2. Summary of the Phenotypic Alterations Observed in the Progeny of *cdt1a/+cdt1b* Plants

Plant No.	Plant Height	Leaf Phenotype	Fertility
21	Normal	Normal	Reduced as shown in Figure 1
7	Reduced	Pale green	Sterile
2	Normal	Slightly pale	Sterile
1	Drastically reduced	Small, pale green, and twisted leaves (Figure 7B, right)	Entered senescence and died before flowering
1	Reduced	Round and small leaves	Fasciated stem, sterile
2	Dramatically reduced	Pale green and curly leaves (Figures 7C and 7E)	Sterile
2	Normal/slightly reduced	Slightly pale (Figures 7B, left, and 7D)	Delayed flowering, abnormal flower morphology, altered phyllotaxy, sterile

versus 30% in the *cdt1a/+* mutant ($n = 1344$). Thus, the *cdt1b* mutation significantly reduced the transmission of the *cdt1a* mutation ($\chi^2 = 27.15$). Observation of developing pollen grains revealed a higher proportion of aborted pollen in the sesqui-mutant. Flow cytometry analysis of the pollen DNA content in the wild type and mutants did not allow us to detect a difference between *cdt1a/+* and wild-type plants: pollen isolated from flowers before dehiscence distributed in a single narrow peak corresponding to three cellular pollen grains, with a minor peak toward the lower DNA contents corresponding to cell debris and empty pollen grains. However, the distribution of pollen grains from *cdt1a/+cdt1b* plants displayed a shoulder on the peak toward lower DNA content, suggesting that developing pollen grains failed to complete S-phase (see Supplemental Figures 2 and 3 online). However, we cannot rule out the possibility that the lower fluorescence observed could be due to the degeneration of nonviable pollen grains. This result indicates that *CDT1a* and *CDT1b* play partially redundant functions during pollen grain development but that pollen grains can be viable in the absence of both genes. By contrast, *CDT1a* is strictly required for female gametophyte development, suggesting that some of its functions cannot be taken over by *CDT1b*.

Downregulation of CDT1 Proteins Affects Genome Integrity

The observation that a proportion of pollen grains have a reduced DNA content in *cdt1a/+cdt1b* mutants suggests that genome integrity is compromised in the absence of *CDT1*. Indeed, upon closer examination, we noticed that ~10% ($n = 270$) of the sulfadiazine-resistant plantlets in the progeny of *cdt1a/+cdt1b* plants were smaller than their siblings (Figure 4A). When these plants were transferred to soil and allowed to grow, they displayed a variety of phenotypic alterations (summarized in Table 2; Figures 4B to 4E). Out of 36 plants, 21 grew and developed like their *cdt1a/+cdt1b* siblings, but 15 displayed a variety of developmental defects: in the most extreme cases, plants had a drastically reduced stature and deformed leaves, as shown in Figures 4B to 4E. Such a frequency of abnormal plants was never observed in the progeny of ecotype Columbia (Col-0), *cdt1a/+*, or *cdt1b* plants, indicating that these anomalies were a consequence of the loss of both *CDT1* genes in pollen grains. Most of these abnormal plants were almost completely sterile, but some produced a few seeds and gave rise to both aberrant and wild-type-looking plants in their progeny. Flow cytometry analysis of the DNA content in the second generation of aberrant plants ($n = 40$) allowed us to identify four aneuploid plants with a genome size either 10% larger or 10% smaller than the wild type. This technique did not reveal any modification in the genome size of other plants but would not allow the detection of small variations. These results indicate that the loss of both *CDT1* genes results in severe modifications of the genome during pollen grain formation.

To gain further insight into the function of *CDT1* proteins in the maintenance of genome integrity in sporophytic tissues, we investigated the consequences of reduced *CDT1* accumulation in plantlets, taking advantage of the *CDT1-RNAi* lines because homozygous *cdt1a* mutants cannot be obtained. We reported

previously that *CDT1-RNAi* plants incorporate less bromo-deoxyuridine (an analog of thymidine that incorporates into replicated DNA during S-phase) than the wild type and have smaller leaves due to a reduction of both cell expansion and cell proliferation (Raynaud et al., 2005). Furthermore, the root tip mitotic index is reduced in these plants (see Supplemental Figure 4C online). In addition to this reduction of proliferative activity, *CDT1-RNAi* lines display enhanced endoreduplication; these two features have been reported in several mutants subjected to endogenous DNA stress (reviewed in Cools and De Veylder, 2009). Many of these mutants exhibit enhanced expression of *Cyclin B1;1* (*CYCB1;1*); therefore, we monitored the expression of *CYCB1;1* in *CDT1-RNAi* plantlets. Expression of *CYCB1;1* was found to be increased in *CDT1-RNAi* plantlets and in *cdt1a/+cdt1b* but not *cdt1b* mutants (Figure 5A). This observation suggests that reduced expression of *CDT1* genes results in DNA stress. To confirm this, the expression of several genes

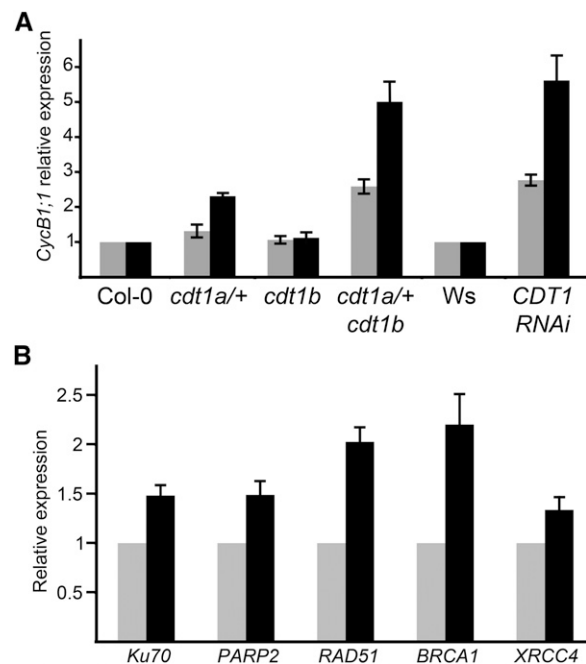


Figure 5. Downregulation of *CDT1* Genes Results in Endogenous DNA Stress.

(A) Expression of *CycB1;1* was monitored by quantitative RT-PCR. Total RNA was extracted from plantlets. Data presented here are averages of two technical replicates obtained from two biological replicates (black and gray bars). Expression of *CycB1;1* was reproducibly found to be increased in *CDT1-RNAi* and *cdt1a/+cdt1b* mutants. The increase observed in the second experiment in *cdt1a/+* mutants was not reproducible, suggesting that expression of *CycB1;1* varies a little in this background.

(B) Expression of genes involved in various DNA repair pathways was monitored by quantitative RT-PCR. All genes were found to be induced in *CDT1-RNAi* lines (black bars) compared with the wild type (Wassilewskija [Ws]; gray bars). Data presented here represent two biological replicate experiments. Each quantitative RT-PCR was repeated at least twice on each biological replicate.

For all panels, bars are averages \pm SD.

involved in various DNA repair pathways was monitored in wild-type and *CDT1-RNAi* plants. As shown in Figure 5B, the expression of *Ku70*, *PARP2*, *RAD51*, *BRCA1*, and *XRCC4* was found to be increased in *CDT1-RNAi* lines. This increase was low (between 1.3- and 2.3-fold depending on the gene) but reproducible, providing evidence for endogenous DNA stress induced by reduced CDT1 accumulation.

As an independent test for the presence of DNA stress, the DNA ploidy distribution was measured in the *cdt1a/+* and *cdt1a/+cdt1b* mutants by flow cytometry. Twelve-day-old *cdt1a/+* and *cdt1a/+cdt1b* plantlets showed a clear increase in endoreduplication in the first leaf compared with the wild-type (Figure 6A; see Supplemental Figure 5 online). This increase was also observed in whole plantlets for *cdt1a/+cdt1b* mutants but not for *cdt1a/+* mutants (data not shown) and was not observed in cauline leaves of mature plants, indicating that increased endoreduplication is transient in *cdt1a/+* and *cdt1a/+cdt1b* mutants and occurs only at early stages of development. Indeed, all viable *cdt1* mutants still possess one wild-type copy of *CDT1a*. *CDT1-RNAi* lines, therefore, are likely to accumulate lower levels of CDT1 proteins than the mutant throughout their vegetative development. Taken together, these results support the view that reduced CDT1 accumulation results in DNA stress in *Arabidopsis*.

Increased Endoreduplication in *CDT1-RNAi* Plants Is Not Dependent on WEE1 or SOG1 Activation

Genome integrity is under the control of several checkpoints during the cell cycle. In *Arabidopsis*, WEE1 activation has been proposed to govern DNA stress-dependent cell cycle arrest (De Schutter et al., 2007), and double-strand breaks have been reported to activate *CYCB1;1* expression and endoreduplication in a SOG1-dependent but WEE1-independent manner (Adachi et al., 2011). We tried to determine which of these checkpoints was activated in *CDT1-RNAi* plants. We first introduced the *CDT1-RNAi* construct in the *wee1* background. Endoreduplication

in plants displaying the *CDT1-RNAi* phenotype was measured in eight *wee1-2* homozygous mutant lines and compared with *wee1-2/+* hemizygous siblings as well as wild-type plants and *wee1-2* mutants. As described by De Schutter et al. (2007), the *wee1* mutation did not affect endoreduplication under normal growth conditions. As shown in Figure 6B and Supplemental Figure 6 online, the endoreduplication effect of the *CDT1-RNAi* construct was similar in the wild-type and the *wee1-2* mutant backgrounds, indicating that the increase in endoreduplication was not WEE1 dependent. Similarly, *CDT1-RNAi* lines still displayed increased endoreduplication in the *sog1* background, indicating that neither WEE1 nor SOG1 is responsible for the endoreduplication phenotype observed in *CDT1-RNAi* lines. Also, transmission of the *cdt1a* mutation remained unchanged in the *wee1-2* and *sog1* backgrounds, indicating that the observed developmental arrest in gametophytes does not require WEE1 or SOG1 activity. This may either suggest that the observed increase in endoreduplication is not related to the response to DNA stress or that downregulation of CDT1 occurs downstream of WEE1 and/or SOG1 activation to promote endoreduplication upon DNA damage. To gain further insight into the role of CDT1 proteins in the DNA damage response, we next investigated the sensitivity of the different lines to DNA stress.

CDT1-RNAi Plants Display Enhanced Tolerance to DNA Stress

The appearance of mutations in the progeny of *cdt1a/+cdt1b* mutants could either be due to an increase in DNA damage or to a defect in DNA stress checkpoints. To discriminate between these two hypotheses, we tested the resistance of *cdt1* mutants and *CDT1-RNAi* lines to DNA stress. We first investigated the role of CDT1 proteins in the response to replication fork stalling. To this end, plants were grown for 10 d on Murashige and Skoog (MS) medium and transferred to plates containing increasing concentrations of hydroxyurea (HU), which is an inhibitor of ribonucleoside reductase. HU treatment depletes the cellular

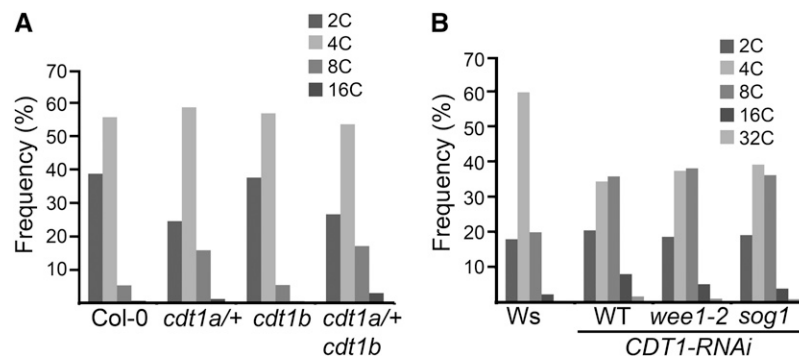


Figure 6. Endoreduplication Is Increased in Plants with Reduced CDT1 Accumulation in a WEE1- and SOG1-Independent Manner.

(A) Endoreduplication in *cdt1* mutants. Analyses were performed on the first leaf of Col-0, *cdt1a/+*, *cdt1b*, and *cdt1a/+cdt1b* plants harvested 14 d after stratification. Ten leaves were used for each sample. Representative cytometry profiles are shown in Supplemental Figure 4 online.

(B) Endoreduplication in *CDT1-RNAi* plants is independent of WEE1 and SOG1. The first cauline leaf of Wassilewskija (Ws), *CDT1-RNAi*, *wee1-2* *CDT1-RNAi*, and *sog1* *CDT1-RNAi* was used for this analysis. Increased levels of endoreduplication were observed in *CDT1-RNAi* in the wild-type (WT), *wee1-2*, and *sog1* backgrounds. Representative cytometry profiles are shown in Supplemental Figure 5 online.

content of deoxyribonucleotide and thereby induces stalling of the replication fork. Root growth was measured 2 d after transfer on HU-containing medium (Figures 7A and 7B). On MS plates, root growth of *CDT1-RNAi* plantlets was much lower than that of the wild type, and this difference was still observed on HU-containing medium. However, root length was significantly reduced by 1 mM HU treatment in the wild type but remained unchanged for *CDT1-RNAi* plants on medium containing 1 or 2 mM HU, suggesting that they show increased tolerance to stalling of the replication fork. To further confirm that growth of *CDT1-RNAi* plantlets is not as strongly inhibited as the wild type by DNA stress, we tested their tolerance to γ -irradiation. Root growth was measured 3 d after irradiation at various doses. Root growth was reduced in the wild type at a dose of 20 Gray but remained unchanged in *CDT1-RNAi* lines up to 30 Gray (Figure 7B). Furthermore, relative growth was lower in the wild type than for *CDT1-RNAi* plants in all conditions tested (Figure 7D).

Several hypotheses may account for the reduced sensitivity of *CDT1-RNAi* plantlets to DNA-damaging agents. One possibility would be that sensing of DNA damage is impaired in these plants, resulting in the absence of cell cycle arrest and DNA repair, as observed in the *sog1* mutant. Alternatively, the apparent resistance could be due to basal activation of the DNA stress checkpoint, resulting in a less severe growth inhibition after DNA damage. Because growth of *CDT1-RNAi* plantlets is further reduced after γ -irradiation compared with untreated *CDT1-RNAi* plantlets, cell cycle arrest induced by DNA damage seemed to be functional in this background. The second hypothesis, therefore, appeared the most likely. To confirm this, we tested whether the response to γ irradiation was normal in *CDT1-RNAi* plants by assessing recognition of double-strand breaks (DSB), induction of DNA repair genes, and cell cycle arrest after γ -irradiation. All steps of the DSB response were found to be functional in *CDT1-RNAi* lines (for details, see Supplemental Figure 4 and Supplemental Methods 1 online). Response to DNA damage was actually enhanced in *CDT1-RNAi* plants in terms of γ -H2AX incorporation at the site of DNA damage and *RAD51* expression, demonstrating that sensing and repair of DSB are functional in these plants and suggesting that they are “primed” by endogenous stress, allowing a faster or stronger response, at least at early steps of the pathway.

CDT1a and CDT1b Interact with DNA Polymerase ϵ

To further investigate the role of CDT1a and CDT1b in the maintenance of genome integrity, we searched for interacting partners by tandem affinity purification (TAP). We reasoned that this method may allow us to identify new protein complexes containing CDT1a and/or CDT1b together with proteins involved in DNA repair or DNA damage sensing. Protein complexes could be purified when the tag was positioned at the N-terminal end of CDT1 proteins. Few proteins were identified by this method, but the results were very reproducible (Table 3; see Supplemental Table 1 online). CDT1a and CDT1b were found to copurify with the two subunits of DNA polymerase ϵ (DPB2 and POL2A). Additionally, we found a transcription factor that appeared to copurify specifically with CDT1b.

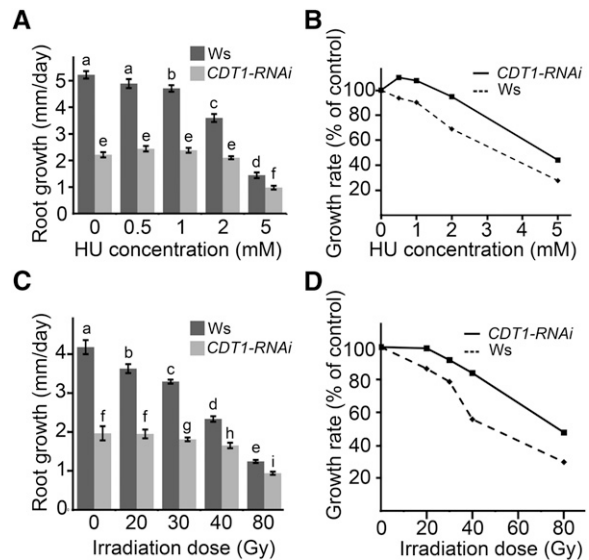


Figure 7. *CDT1-RNAi* Plants Are Tolerant to DNA Stress.

(A) Root growth of wild-type (Wassilewskija [Ws]) and *CDT1-RNAi* (RNAi) plants after HU treatment. Plantlets were germinated and grown on 0.5 \times MS medium for 10 d and transferred either to 0.5 \times MS medium or 0.5 \times MS medium supplemented with the indicated amount of HU. Root elongation was measured after 2 d. Bars are averages \pm SE. Letters indicate significantly different values (Student's *t* test; for a and b, $P < 0.05$; for all other letters, $P < 0.001$).

(B) Relative growth of roots from Ws (dashed line) and *CDT1-RNAi* (solid line) as a function of HU concentration in the growth medium. The reduction of root growth in *CDT1-RNAi* is less severe than for Ws plantlets at all concentrations tested.

(C) Root growth of wild-type (Ws) and *CDT1-RNAi* (RNAi) plants after γ -irradiation. Plantlets were germinated on 0.5 \times MS medium and irradiated at the indicated doses. Root growth was measured after 3 d. Letters indicate significantly different values (Student's *t* test; for a, b, f, and g, $P < 0.05$; for all other letters, $P < 0.001$). ns, Nonsignificantly different values ($P > 0.05$).

(D) Relative growth of roots from Ws (dashed line) and *CDT1-RNAi* (solid line) as a function of γ -ray dose (Gy). The reduction of root growth in *CDT1-RNAi* is less severe than for Ws plantlets at all doses tested.

To confirm that CDT1 proteins can interact with DNA polymerase ϵ , we used the yeast two-hybrid system. Both CDT1a and CDT1b were found to interact with the regulatory subunit DPB2 (Figure 8). The catalytic subunit is very large, and two clones encompassing \sim 500 amino acids at the C terminus of the protein (POL3 and POL5), corresponding to the region involved in DPB2 binding, were tested (Ronceret et al., 2005). We could not find an interaction between CDT1 proteins and these fragments, although the POL3 fragment could interact with DPB2 as reported (Ronceret et al., 2005) (data not shown). These results suggest that CDT1 proteins form a complex with DNA polymerase ϵ through their association with DPB2.

DISCUSSION

Although many reports have highlighted the importance of cell cycle regulation during plant development, reports regarding the relationships between cell cycle regulation and genome stability

Table 3. Proteins Identified by Tandem Affinity Purification Using CDT1a or CDT1b as Bait

Identified Proteins							
Bait Protein	Accession No.	Protein Name	No. Found/No. Expected	Protein Score	Expected	Best Ion Score	Expected
CDT1a	AT1G08260	EMB2284, POL2A, TIL1, EMB529	2/2	781	2.6E-74	65	4.20E-06
CDT1a	AT5G22110	DBP2, CYL2	2/2	452	2.10E-41	115	4.60E-11
CDT1a	AT2G31270	CDT1a	2/2	88	5.70E-05	–	–
CDT1b	AT1G08260	EMB2284, POL2A, TIL1, EMB529	2/2	911	2.60E-87	78	2.80E-07
CDT1b	AT5G22110	DBP2, CYL2	2/2	777	6.50E-74	133	7.10E-13
CDT1b	AT2G27470	CCAAT box binding transcription factor subunit HAP3 related	2/2	–	–	36	5.60E-04
CDT1b	AT3G54710	CDT1b	2/2	910	3.30E-87	115	6.60E-11

All identified proteins were found in both experiments. Values for protein score and best ion score are for two independent experiments. –, values below the threshold score. Detailed MS data can be found in Supplemental Table 1 online.

are scarce. However, it is well known in other eukaryotes and more specifically in human cells that altered regulation of DNA replication licensing due to changes in CDT1 activity results in aberrant replication, activates DNA damage checkpoints and predisposes for malignant transformation (Petropoulou et al., 2008). Therefore, regulation of pre-RC activity is likely to be crucial for the maintenance of genome integrity also in plants. Overexpression analysis suggests that accumulation of CDC6 and CDT1 is rate limiting for the initiation of S-phase, both in proliferating and in endoreduplicating cells, but that misexpression of these genes is not sufficient to alter the size of meristems (Castellano et al., 2001, 2004). We previously reported that downregulation of *Arabidopsis* *CDT1a* and *CDTb* affects cell cycle progression, plastid division, and chloroplast biogenesis, but we were not able to investigate the consequences of a complete loss of function of these genes (Raynaud et al., 2005). Our new results reveal that the two *CDT1* genes are crucial to gametophyte development and to the maintenance of genome integrity in *Arabidopsis* both in gametophytic and sporophytic tissues.

The *cdt1a* mutation prevents female gametophyte development and results in partially penetrant pollen grain development defects: ~30% of *cdt1a* pollen grains did not reach the three-nuclei stage. Although complete knockout of the *CDT1b* gene does not affect development or fertility, the penetrance of the *cdt1a* mutation was increased in this background, and only 10% of *cdt1a/+cdt1b* pollen grains were viable and allowed fertilization. These results indicate that CDT1a and CDT1b have partially redundant functions during male gametophyte development and that CDT1a, but not CDT1b, is required for female gametophyte development. Both male and female gametophyte development require accurate cell cycle regulation. After meiosis, the microspore undergoes an asymmetric division, giving birth to the vegetative cell that exits the cell cycle in the G1-phase and a generative cell. This cell divides again to produce the twin sperm cells required for double fertilization that are arrested in the S-phase of the cell cycle (Berger and Twell, 2011); hence, S-phase is initiated three times during pollen grain formation. Likewise, embryo sac development requires three mitotic events, and there is compelling evidence

that inhibition of cell cycle progression severely affects this process (Yang et al., 2010). By contrast, failure to arrest cell division because of the loss of the *RBR1* gene results in a gametophyte-lethal phenotype due to overproliferation of the embryo sac cells (Ebel et al., 2004). Intriguingly, all mutants deficient for one pre-RC subunit described so far do not display a gametophyte-lethal phenotype: *mcm2* mutants arrest development at the globular stage (Ni et al., 2009), and the *MCM7* gene is maternally required for embryo development (Springer et al., 2000) and results in leaky gametophyte lethality (Springer et al., 1995). Considering the essential function of all these proteins at the onset of S-phase, one would rather expect mutant microspores and megaspores to arrest their development before the first gametophytic mitosis. This discrepancy could result either from some functional redundancy between MCM proteins or between CDT1a and CDT1b, respectively, or from the accumulation of maternally inherited pools of the proteins, as hypothesized for the incomplete penetrance of the *cdka1;1* mutation in pollen grain development (Nowack et al., 2006). Such a hypothesis would imply that the whole pool of CDT1 proteins is not degraded after the initiation of DNA replication but inactivated in a reversible way, which hints at the existence of a yet unidentified functional homolog of geminin. The fact that CDT1b cannot compensate for the absence of CDT1a during embryo sac development and can only partially do so during male gametophyte development could mean that CDT1a accumulates at higher levels than CDT1b in these tissues or could reflect a specific function of CDT1a. Indeed, we found that CDT1a, but not CDT1b, harbored a plastid-targeting sequence and that this protein could indeed accumulate in plastids (Raynaud et al., 2005). Recently, the plastid-localized PPR2 has been reported to be essential for both male and female gametophyte development in *Arabidopsis* (Lu et al., 2011); therefore, the inability of CDT1b to fully compensate for the absence of CDT1a may relate to the function of CDT1a in plastids.

Another interesting consequence of the *cdt1a* mutation is that pollen grains arrested at the two-cell stage apparently degenerate. This is at variance with the phenotypes reported in several mutants in which CDKA;1 activity is reduced and the last pollen division is delayed (reviewed in Berger and Twell, 2011): in

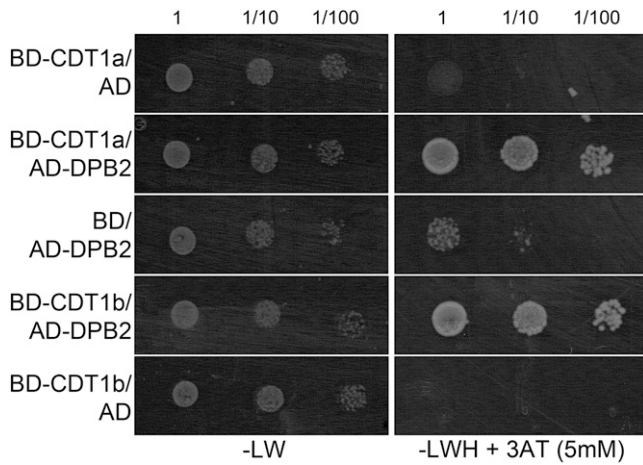


Figure 8. CDT1a and CDT1b Interact with the DPB2 Subunit of DNA Polymerase ϵ in the Yeast Two-Hybrid System.

Yeast cells were transformed with constructs encompassing fusions between CDT1a or CDT1b and the GAL4 DNA binding domain (BD-CDT1a and BD-CDT1b, respectively), between DPB2 and the GAL4 activation domain (AD-DPB2), or empty vectors (AD and BD). Transformants were resuspended in water to a final absorbance (600 nm) of 1, and serial dilutions were spotted on nonselective medium lacking only Leu and Trp (-LW) or on selective medium lacking Leu, Trp, and His and supplemented with 3-amino-1,2,4-triazole to a final concentration of 5mM (-LWH + 3AT). Restoration of His prototrophy indicates that both CDT1a and CDT1b can interact with DPB2.

this case, the two-celled pollen grains are able to fertilize ovules, resulting in the fertilization of only one cell in the embryo sac (Nowack et al., 2006). The degenerative phenotype observed in *cdt1a* and *cdt1a/+cdt1b* pollen grains could be due to incomplete genome replication during S-phase, as evidenced by flow cytometry analysis and the induction of programmed cell death. Indeed, programmed cell death has been proposed to act as a cellular surveillance mechanism to ensure the successful progression of male gametogenesis (Zhang et al., 2011). Incomplete genome replication or failure to repair some DNA damage may account for the abnormal frequency of mutant plants in the progeny of *cdt1a/+cdt1b* mutants. Indeed, plants with a modified genome size compared with the wild type were found in the progeny of *cdt1a/+cdt1b* plants. The technique used in this study would not have allowed us to identify changes in nuclear DNA content representing less than 10% of the whole genome, which corresponds to an entire chromosome. Aberrant plants with apparently normal DNA content, therefore, are likely to present genomic rearrangements, deletions, or other types of mutations that are more difficult to identify.

In addition to their implication in the onset of S-phase evidenced by the above-described gametophytic defects, our results point to a role for the CDT1 proteins in the maintenance of genome integrity. Indeed, elevated levels of *CYCB1;1* expression in *CDT1-RNAi* plants and in *cdt1a/+cdt1b* mutants together with increased endoreduplication, and constitutive induction of genes involved in DNA repair in *CDT1-RNAi* lines, also point to endogenous DNA stress when the expression of

CDT1 is low (Cools and De Veylder, 2009). Two possible models that could account for the observed DNA damage responses observed in the *CDT1-RNAi* plants are depicted in Figure 9. In both models, the reduction of CDT1 accumulation results in DNA damage, causing delayed cell cycle progression and increased expression of DNA repair-related genes, whereby the basal activation of the DNA stress response could have a priming effect on the plants, allowing them to better tolerate exogenously applied stress. Indeed, treatment with low doses of DNA-damaging agents has been shown to improve repair efficiency after exposure to higher doses of the same drug in plants (Baranczewski et al., 1997). The accompanying increase in endoreduplication observed in *CDT1-RNAi* plants does not appear to require WEE1 and SOG1 activity. In this context, two alternative hypotheses may account for the data reported here. Downregulation of CDT1 may be a component of the known pathways leading from DNA damage to endoreduplication situated downstream of WEE1 and SOG1 (Figure 9A). This is in agreement with several reports demonstrating that CDT1 proteins are degraded upon DNA stress in animal cells (Roukos et al., 2011). Such a model would imply that reduced CDT1 levels promote endoreduplication. CDT1a overexpression also stimulates endoreduplication (Castellano et al., 2004), but the similar outcome of opposite variations in CDT1 accumulation probably reflects a dual role of CDT1 in the promotion of replication licensing and in DNA damage response. Alternatively, the ability of CDT1 proteins to form complexes with DNA polymerase ϵ suggests that they could function in a new signaling cascade regulating cell cycle progression upon DNA stress occurring during S-phase (Figure 9B). Indeed, we found that both proteins interact with the regulatory subunit of DNA polymerase ϵ , DPB2. The primary function of DNA polymerase ϵ is the synthesis of the leading strand during DNA replication (Pursell et al., 2007), but it is also involved in a variety of other cellular functions, including the S-phase checkpoint (Pursell and Kunkel, 2008). The ability of plant CDT1 proteins to bind this polymerase is unexpected, as CDT1 is thought to be released

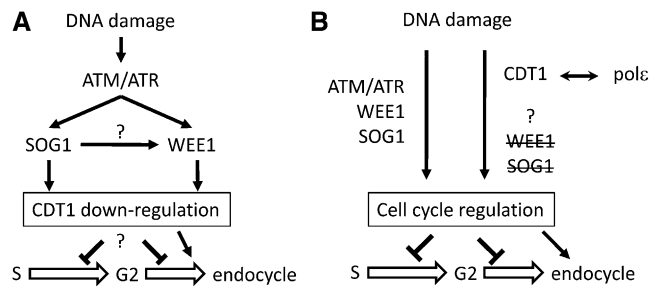


Figure 9. Two Possible Models Regarding the Role of CDT1 Proteins in Response to DNA Stress.

(A) Downregulation of CDT1 upon DNA stress may be promoted by WEE1 and/or SOG1. This would result in a delay in S-phase progression, but how this would induce endoreduplication remains unclear.

(B) CDT1 proteins, possibly via their ability to form complexes with DNA polymerase ϵ , may be at the top of a signaling pathway that could regulate cell cycle progression and endoreduplication in parallel with the WEE1- and SOG1-dependent pathway.

from chromatin upon pre-RC activation or to be maintained at replication origins (Xouri et al., 2007; Truong and Wu, 2011). However, DUP, the CDT1 homolog of *Drosophila*, has been shown to travel with the replication fork (Claycomb et al., 2002), and CDT1 has recently been proposed to participate in a surveillance mechanism that blocks nascent strand elongation upon illegitimate replication licensing in *Xenopus* (Tsuyama et al., 2009). Therefore, the polymerase ϵ /CDT1 complex may be involved in monitoring the proper progression of S-phase and in the activation of cellular responses upon events such as replication fork stalling or illegitimate licensing, which would result in the expression of DNA repair genes, inhibition of cell cycle progression, and induction of endoreduplication. This hypothesis implies that a WEE1- and SOG1-independent signaling cascade can be activated by some types of DNA stress in plant cells. Further genetic studies will be required to identify factors involved in this pathway.

Although further work is required to fully elucidate the molecular mechanisms involving CDT1 proteins in the maintenance of genome integrity, our results highlight two contrasting situations in sporophytic tissues and in gametophytes. Reduction of CDT1 accumulation in somatic cells would slow cell cycle progression and induce the activation of DNA repair genes, whereas in gametophytes lacking CDT1 proteins completely, replication errors would not systematically lead to pollen abortion: aberrant plants observed in the progeny of *cdt1a/+cdt1b* mutants likely arise from aberrant pollen grains that failed to degenerate in spite of replication defects. Taken together, this work provides strong evidence for a role of CDT1 proteins in the maintenance of genome integrity throughout the life cycle of the plant.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana seeds were surface-sterilized by treatment with Bayrochlor (Bayrol) for 20 min and washed and imbibed in sterile water for 2 to 4 d at 4°C to obtain homogeneous germination. Seeds were sown on commercially available 0.5× MS medium (Basalt Salt Mixture M0221; Duchefa) with the suitable antibiotic if needed and solidified with 0.8% agar (Phyto-Agar HP696; Kalys) and grown in a long-day (16 h of light/8 h of dark, 21°C) growth chamber. After 2 weeks, the plants were transferred to soil in a glasshouse or in a growth chamber under short-day conditions (8 h of light, 20°C/16 h of dark, 18°C) for 2 weeks before being transferred to long-day conditions.

T-DNA insertion lines from the SALK collection (SALK_001298) and from GABI (GABI_025G08) were obtained from the Nottingham Arabidopsis Stock Centre.

The *CDT1-RNAi* lines and *cdt1b* mutants have been described previously (Raynaud et al., 2005). The *cdt1a/+* mutant was obtained from the GABI-Kat collection (Scholl et al., 2000), and mutants were identified by PCR. The GABI T-DNA and CDT1a mut primers allowed us to screen for the mutated allele, and the CDT1a WT and CDT1a mut primers were used to amplify the wild-type allele. Plants harboring an insertion in the *HLK3* gene were identified using the GABI-T-DNA and HLK3mut primers. Sequences of these primers are given in Supplemental Table 2 online.

Complementation of *cdt1a/+* Mutants

For complementation of *cdt1a/+* mutants, we generated a HA-tagged version of CDT1a driven by its own promoter. To this end, a triple HA tag

was amplified by PCR using the HA-dir (containing a *KpnI* site) and HA-rev (containing a *SacI* site) primers. This fragment was sequenced and cloned between the *KpnI* and *SacI* sites of the pPZP111 vector (Hajdukiewicz et al., 1994) to generate the pPZP111-HA vector. The putative promoter sequence for CDT1a (815 bp upstream of the translation initiation codon) and the first 24 bp of the CDT1a coding sequence were amplified using the pCDT1-HindIII (containing a *HindIII* site) and pCDT1-XbaI (containing the *XbaI* site found in the CDT1a cDNA) primers, and this fragment was digested using *HindIII* and *XbaI*. The 3' end of the CDT1a coding sequence was amplified using the CDT1-Clal (situated upstream of the *Clal* site found in the CDT1a cDNA) and CDT1a-KpnI (containing a *KpnI* site) primers; after sequencing, this fragment was digested using the *Clal* and *KpnI* sites. These two fragments and the center part of the CDT1a cDNA cut at the *XbaI* and *Clal* sites were ligated into the pPZP111-HA vector to generate the *pCDT1a:CDT1a-HA* construct. The sequences of primers are indicated in Supplemental Table 2 online. The *pCDT1a:CDT1a-HA* plasmid was introduced into *Agrobacterium tumefaciens* by electroporation, and *Arabidopsis* transgenic lines were generated by the floral dip method (Clough and Bent, 1998). T1 transformants were selected on kanamycin (50 µg/mL) and sulfadiazine (5 µg/mL). Fertile *cdt1a/+* plants were identified, and their progeny were sown on selective medium. This allowed us to identify *cdt1a* homozygous mutants expressing the CDT1a-HA protein, and these plants were identical to the wild type.

Plant Treatments

For HU treatments, plants were transferred and aligned either to fresh control medium or HU-containing medium (Sigma-Aldrich) and grown vertically under long-day conditions for 2 d.

γ -irradiation assays were performed as described previously (Domenichini et al., 2006). For comparison of sensitivity to γ -rays between the wild type and *CDT1-RNAi*, plantlets irradiated as above were transferred and grown on 0.5× MS medium in a vertical position under long-day conditions for 3 d.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from seedlings with the RNeasy MiniPrep kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 µg of total RNA using ImProm-II reverse transcriptase (A3802; Promega) according to the manufacturer's instructions. One-twenty-fifth of the synthesized cDNA was mixed with 100 nM 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 the LightCycler 480 detection system. The specificity of amplification products was determined by melting curves. *PP2AA3* was used as an internal control for signal normalization. Exor4 relative quantification software (Roche Applied Science) automatically calculates the relative expression level of the selected genes with algorithms based on the $\Delta\Delta$ cycle threshold method. Data were from duplicates of at least two biological replicates. Quantitative RT-PCR analysis was performed at least twice for each biological replicate. The sequences of primers can be found in Supplemental Table 2 online.

Light and Fluorescence Microscopy

Fresh siliques were opened using a stereomicroscope (SVII; Zeiss), and images were captured with a color charge-coupled device camera (Power HAD; Sony). Anthers were stained in Alexander solution to stain pollen grains and observed by light microscopy (Alexander, 1969).

Differential interference contrast microscopy was used to observe female gametophytes that had been fixed in ethanol:acetic acid (3:1) and cleared using chloral hydrate solution (8 g of chloral hydrate, 1 mL of glycerol, and 2 mL of water). Images were captured on an Axioskop microscope (Zeiss) with a Spot RT slider camera (Diagnostic Instrument), and Z-stack projections (average of intensity) were performed using ImageJ software (rsbweb.nih.gov/ij/) and enhanced using Adobe Photoshop software.

For pollen mitosis, inflorescences were fixed as above, and for each flower, pollen grains were sorted from anthers on a polysine slide (Thermo Scientific) with a drop of Vectashield with DAPI (H-1200; Vector Laboratories). Images (Z-stacks) were captured on an epifluorescence video-microscope (DMI6000B; Leica) with an ER-Hamamatsu camera, and Z-stack projections (maximum of intensity) were performed using ImageJ software.

Flow Cytometry

For flow cytometric analysis of nuclei, tissues were chopped with a razor blade in 1 mL of Galbraith buffer (Galbraith, 1983) supplemented with 1% polyvinylpyrrolidone 10,000, 5 mM metabisulfite, and 5 mg/mL RNase from a stock solution at 50 units/mg.

Propidium iodide was added to the filtered supernatants at 50 µg/mL. Endoreduplication levels of 5,000 to 10,000 stained nuclei were determined using a Cyflow SL flow cytometer (Partec) with a 532-nm solid state laser (100 mW) for excitation, and emission data were collected after a 590-nm long-pass filter.

For quantification of genome size, a piece of tomato (*Solanum lycopersicum*) leaf was chopped with each sample to include an internal control.

TAP Experiments

CDT1a and *CDT1b* cDNAs were cloned between the *KpnI* and *EcoRI* sites of the pENTR3C vector. For C-terminal fusions to the TAP tag, we used the *CDT1a KpnI* dir and *CDT1a EcoRI* rev primers for *CDT1a* and *CDT1b KpnI* dir and *CDT1b EcoRI* rev primers for *CDT1b*. For N-terminal fusion to the TAP tag, the original stop codon of the cDNA was conserved using the *CDT1a* rev stop and *CDT1b* rev stop primers for *CDT1a* and *CDT1b*, respectively. Cloning of transgenes encoding tag fusions under the control of the constitutive cauliflower mosaic virus 35S promoter and transformation of *Arabidopsis* cell suspension cultures were then performed as described previously (Van Leene et al., 2007). Tandem affinity purification of protein complexes was done using the protein G and streptavidin binding peptide tag (Bürkstümmer et al., 2006) followed by protein precipitation and separation, according to Van Leene et al. (2008). For the protocols of proteolysis and peptide isolation, acquisition of mass spectra by a 4800 Proteomics Analyzer (Applied Biosystems), and MS-based protein homology identification based on The Arabidopsis Information Resource 8.0 genomic database, we refer to Van Leene et al. (2010). Experimental background proteins were subtracted based on ~40 TAP experiments on wild-type cultures and cultures expressing the TAP-tagged mock proteins β-glucuronidase, red fluorescent protein, and green fluorescent protein (Van Leene et al., 2010).

Yeast Two-Hybrid Experiments

Yeast two-hybrid constructs encompassing *CDT1a* and *CDT1b* were obtained by recombination using Gateway technology. Gateway-compatible yeast two-hybrid vectors were derived from the pGADT7 and pGBKT7 vectors and were a gift from Pascale Rossignol (John Innes Centre, Norwich, UK). The *DPB2* cDNA was amplified by PCR using the *Pol ε* *EcoRI* dir and *Pol ε* *XhoI* stop primers and cloned between the *EcoRI* and *XhoI* sites of pENTR1A. Yeast two-hybrid constructs were obtained by recombination. Yeast transformation and interaction assays were performed as described previously (Raynaud et al., 2005).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *CDT1a*, At2g31270; *CDT1b*, At3g54710; *DPB2*, At5g22110; *POL2A*, At1g08260; *CYCB1;1*, At4g37490; *PP2AA3*, At1g13320; *Ku70*, At1g48050; *XRCC4*, At3g23100; *PARP2*, At2g31320; *RAD51*, At5g20850; *BRCA1*, At4g21070.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Position of the T-DNA Insertion in the GABI_025G08 Line.

Supplemental Figure 2. DNA Content in Pollen Grains Isolated from a Whole Inflorescence of a Col-0 Plant.

Supplemental Figure 3. Altered DNA Content in Pollen Grains Isolated from *cdt1a/+cdt1b* Mutants.

Supplemental Figure 4. Endoreduplication Is Increased in *cdt1a/+* and *cdt1a/+cdt1b* Mutants.

Supplemental Figure 5. Endoreduplication Is Enhanced in *CDT1-RNAi* Plants in a WEE1- and SOG1-Independent Manner.

Supplemental Figure 6. Cellular Response to DNA Damage Is Normal in *CDT1-RNAi* Plants.

Supplemental Table 1. Detailed Results for TAP Analysis.

Supplemental Table 2. Primer Sequences.

Supplemental Methods 1. Immunofluorescence.

ACKNOWLEDGMENTS

We thank Martine Devic (Institut de Recherche pour le Développement, Montpellier, France) for kindly providing us yeast two-hybrid constructs encompassing fragments of *POL2A* and Pascale Rossignol (John Innes Centre, Norwich, UK) for kindly providing us Gateway-compatible yeast two-hybrid vectors. We are especially grateful to Daniel Vezon (Institut National de Recherche Agronomique, Versailles, France) for helpful advice on gametophyte development analysis and to Spencer Brown (Institut des Sciences du Végétal, Gif-sur-Yvette cedex, France) for assistance with flow cytometry experiments. We thank two anonymous reviewers for constructive remarks on the manuscript. This work was supported by the Agence Nationale de la Recherche of France (Grant ANR 2010 JCJC1207 01).

AUTHOR CONTRIBUTIONS

S.D. and M.B. designed and performed the research, G.D.J. and E.V.D.S. performed the research and analyzed the data, S.B. and M.B. performed the research, L.D.V. and C.B. wrote the article, and C.R. designed and performed the research and wrote the article.

Received May 2, 2012; revised June 7, 2012; accepted June 19, 2012; published July 6, 2012.

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