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Arabidopsis WEE1 Kinase Controls Cell Cycle Arrest in Response to Activation of the DNA Integrity Checkpoint [W] [OA]

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Upon the incidence of DNA stress, the ataxia telangiectasia–mutated (ATM) and Rad3-related (ATR) signaling kinases activate a transient cell cycle arrest that allows cells to repair DNA before proceeding into mitosis. Although the ATM-ATR pathway is highly conserved over species, the mechanisms by which plant cells stop their cell cycle in response to the loss of genome integrity are unclear. We demonstrate that the cell cycle regulatory *WEE1* kinase gene of *Arabidopsis thaliana* is transcriptionally activated upon the cessation of DNA replication or DNA damage in an ATR- or ATM-dependent manner, respectively. In accordance with a role for WEE1 in DNA stress signaling, *WEE1*-deficient plants showed no obvious cell division or endoreduplication phenotype when grown under nonstress conditions but were hypersensitive to agents that impair DNA replication. Induced *WEE1* expression inhibited plant growth by arresting dividing cells in the G2-phase of the cell cycle. We conclude that the plant *WEE1* gene is not rate-limiting for cycle progression under normal growth conditions but is a critical target of the ATR-ATM signaling cascades that inhibit the cell cycle upon activation of the DNA integrity checkpoints, coupling mitosis to DNA repair in cells that suffer DNA damage.

INTRODUCTION

Genome integrity of cells is threatened by DNA damage that is the consequence of environmental stresses and endogenous causes. To cope with these stress conditions, cells have developed a set of surveillance mechanisms to monitor the status and structure of DNA during cell cycle progression. In *Schizosaccharomyces pombe* (fission yeast) and mammals, DNA damage activates the ataxia telangiectasia–mutated (ATM) and Rad3-related (ATR) signaling cascades that simultaneously turn on DNA repair complexes and arrest cell division; this mechanism allows cells to repair damaged DNA before proceeding into mitosis (Zhou and Elledge, 2000; Abraham, 2001; Bartek and Lukas, 2001; Kurz and Lees-Miller, 2004). ATM responds specifically to double-stranded breaks, whereas ATR primarily senses replication stress caused by a persistent block of replication fork progression. The ATM and ATR kinases transduce the DNA stress signal to the checkpoint kinases CHK1 and CHK2, which, in turn, arrest the cell cycle by directly modulating the activity of the effectors that control cell cycle progression (Chen and Sanchez, 2004; Sancar et al., 2004), the cyclin-dependent kinase (CDK) complexes.

CDK complexes consist of a catalytic kinase subunit and a regulatory cyclin. The sequential activation of different CDK/cyclin complexes drives the cell cycle through the phosphorylation of many different target substrates. CDK/cyclin activity is highly regulated at multiple levels. Control mechanisms include the regulated synthesis and destruction of the cyclin subunits (Peters, 1998; Murray, 2004), which are thought to target the CDKs to the substrates (Ohi and Gould, 1999), and the association of CDKs with inhibitory proteins and docking factors (Lees, 1995). Moreover, CDK activity is positively regulated by phosphorylation of a conserved residue (Thr-161 or equivalent) within the T loop and negatively regulated through phosphorylation of Tyr-15 and Thr-14 by WEE1 family kinases (Berry and Gould, 1996). Phosphorylation of Tyr-15 and Thr-14 residues of the CDK subunit inhibits ATP binding and blocks substrate recognition.

In fission yeast and mammals, rapid activation of the CDK/cyclin activity at the G2-M boundary is mediated by a dual-specificity phosphatase CDC25. Maintenance of the inhibition of CDK activity by Tyr-15 phosphorylation is the ultimate target of DNA damage checkpoint signaling. By activation of CHK1 and CHK2, CDC25 is phosphorylated and targeted for ubiquitin-dependent destruction or association with a 14-3-3 protein, resulting in nuclear export and exclusion of CDC25 from the nuclear pool of CDK/cyclin complexes (Boutros et al., 2006). Both WEE1 and the functionally related kinase MIK1 have been implicated as targets of the DNA damage and replication checkpoints as well. In *Xenopus laevis* (African frog) egg extracts,

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activation of the DNA replication checkpoint stabilizes exogenously added WEE1 (Michael and Newport, 1998), whereas in fission yeast, MIK1 is a target for both the DNA damage and DNA replication checkpoints (Rhind and Russell, 2001). In response to the DNA replication checkpoint, *MIK1* mRNA levels accumulate to high levels and, simultaneously, the MIK1 protein is stabilized, leading to dramatic increases in protein levels (Boddy et al., 1998; Baber-Furnari et al., 2000; Christensen et al., 2000).

The basic machinery that controls cell cycle progression in plants is similar to that of yeast and mammals (De Veylder et al., 2003; Dewitte and Murray, 2003; Inzé and De Veylder, 2006). Multiple CDKs and cyclins are encoded by the genomes of *Arabidopsis thaliana* and *Oryza sativa* (rice) (Vandepoele et al., 2002; Wang et al., 2004; La et al., 2006). In addition, a WEE1-related kinase has been described for maize (*Zea mays*), tomato (*Solanum lycopersicum*), and *Arabidopsis* (Sun et al., 1999; Sorrell et al., 2002; Gonzalez et al., 2004). Although the plant *WEE1* gene is unable to complement mutations in its yeast homolog, its overexpression inhibits cell division in fission yeast. Additionally, recombinant purified WEE1 protein from maize is capable of inhibiting the kinase activity of biochemically purified CDKs (Sun et al., 1999). However, the in vivo role of WEE1 in plant cell cycle progression and growth is not well defined.

Our first insights into the role of DNA replication and damage checkpoints in plants came with the identification and characterization of *Arabidopsis* mutants in genes encoding orthologous ATM and ATR kinases (Garcia et al., 2003; Culligan et al., 2004). A defective DNA damage checkpoint is the reason why ATM-deficient plants are primarily hypersensitive to DNA-damaging agents, such as γ-irradiation, but rather insensitive to replication-blocking agents, such as hydroxyurea or aphidicolin (Garcia et al., 2003). In contrast, ATR mutants are hypersensitive to replication-blocking agents but also mildly sensitive toward γ-irradiation (Culligan et al., 2004). These results strongly indicate that the DNA checkpoint signaling pathways are conserved in plants. However, it is still unclear how activation of these signaling cascades leads to the arrest of the cell cycle in response to DNA damage. Here, we identify *WEE1* as an important target of the DNA replication and DNA damage checkpoints. *WEE1*-deficient plants grow normally under optimal growth conditions but are hypersensitive to DNA-damaging agents. In accordance with a role for WEE1 in arresting the cell cycle in response to replication stress, *WEE1* transcripts are found to be strongly upregulated by replication-inhibiting drugs in an ATR-dependent manner. Analogously, γ-irradiation and radiomimetic drugs induce *WEE1* transcription in an ATM-dependent manner. The cell cycle arrest observed upon induction of *WEE1* expression indicates that WEE1 is part of the mechanism that couples the onset of mitosis with the completion of DNA repair in cells that have suffered DNA damage.

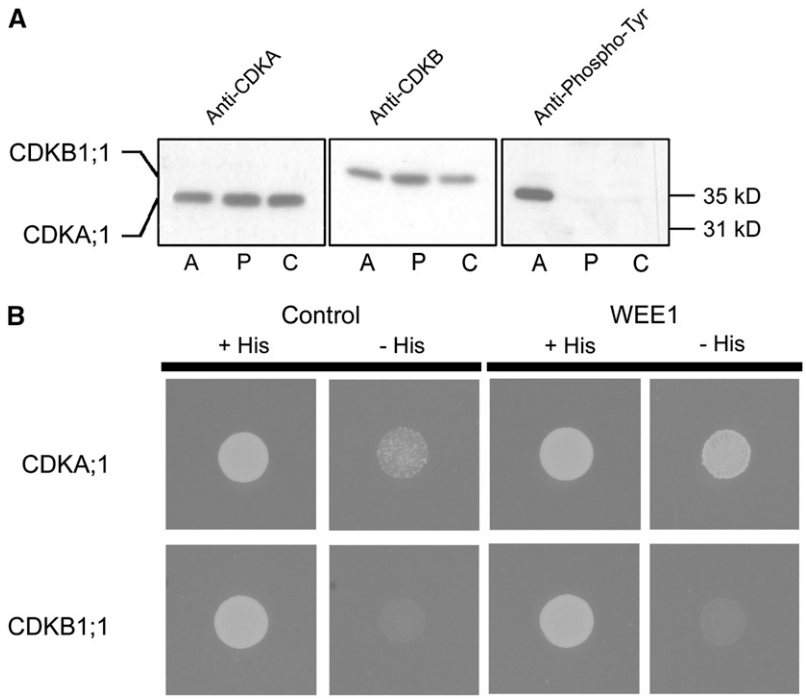

Figure 1. CDKA;1 Target for Tyr Phosphorylation and Binding WEE1.

(A) CDK phosphorylation in response to checkpoint activation. *Arabidopsis* cell cultures were treated with 10 μg/mL aphidicolin (A), with 3 μM propyzamide (P), or mock-treated in controls (C). CDKs were purified from total protein extracts (300 μg/sample) with a $p10^{CKS1At}$-Sepharose matrix, resolved by SDS-PAGE, and immunoblotted with the indicated antisera.

(B) Interaction of CDKA;1 with WEE1 in the yeast two-hybrid system. Yeast PJ69-4 cells containing a CDKA;1 or CDKB1;1 bait plasmid in combination with an empty control or WEE1 prey plasmid were spotted on plates with (+) or without (−) His. Only when the two proteins interact do cells grow on −His medium.

RESULTS

Tyr Phosphorylation of *Arabidopsis* CDKA;1 upon Activation of the DNA Replication Checkpoint

Tyr phosphorylation of CDKs has been shown to take place upon cytokinin deprivation in tobacco (*Nicotiana tabacum*) cells, application of water stress to wheat (*Triticum aestivum*) leaves, and stimulation of the DNA replication checkpoint in zygotes of the brown alga *Fucus* (Zhang et al., 1996; Schuppler et al., 1998; Corellou et al., 2000). To test whether Tyr phosphorylation occurs upon the activation of the DNA replication checkpoint in higher plants, cultured suspension cells of *Arabidopsis* were treated with aphidicolin, which inhibits all replicative DNA polymerases. Propyzamide was used to block cell cycle progression into mitosis by depolymerizing the mitotic spindle (Planchais et al., 2000). The efficiency of the drugs to stop cell cycle progression was confirmed by flow cytometric analysis (data not shown). After the drugs had been applied for 24 h, the CDK complexes were purified and analyzed on protein gel blots with specific antibodies against CDKA;1 and CDKB1;1 (Hemerly et al., 1995; Porceddu et al., 2001). CDKA;1 belongs to the archetypical CDKs, characterized by the presence of a PSTAIRE amino acid sequence motif in the cyclin binding protein domain, and CDKB1;1 belongs to the group of plant-specific CDKs (De Veylder et al., 2003; Boudolf et al., 2004a, 2004b). Neither drug treatment had an effect on the abundance of CDKA;1 protein compared with that of control cells (Figure 1A). In contrast, CDKB1;1 levels increased slightly in the propyzamide-treated cells, probably because of the preferential expression of the *CDKB1;1* gene during M-phase (Porceddu et al., 2001; Boudolf et al., 2004b). Next, the protein blots were probed with an anti-phosphotyrosine antibody. Whereas no antibody binding was detected in protein samples from control or propyzamide-treated cells (Figure 1A), a polypeptide band with the same electrophoretic mobility as that of CDKA;1 cross-reacted with the antibody in extracts prepared from the aphidicolin-treated cells. This analysis strongly indicates that CDKA;1 is the target for Tyr phosphorylation upon activation of the DNA replication checkpoint.

To analyze whether the *Arabidopsis* WEE1 kinase might be responsible for the observed Tyr phosphorylation of CDKA;1, both proteins were tested for their interaction using the yeast two-hybrid system. CDKA;1 and CDKB1;1 in fusion with the GAL4 DNA binding domain were cotransformed in an appropriate yeast reporter strain with an empty control vector or a vector encoding a fusion protein between the GAL4 transactivation domain and WEE1. Transformants were streaked on medium with or without His. Cells expressing *CDKA;1* and *WEE1* grew in the absence of His, indicating that both gene products interacted. No association was observed between CDKB1;1 and WEE1 (Figure 1B).

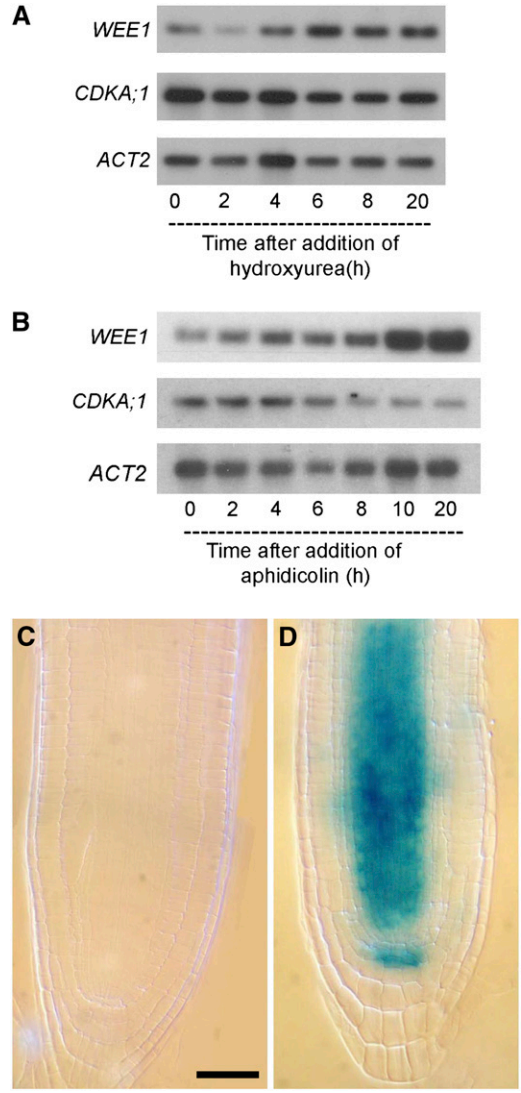

Figure 2. Transcriptional Response of the *WEE1* Gene after Activation of the DNA Replication Checkpoint.

(A) and **(B)** Transcript levels of *WEE1* and *CDKA;1* in *Arabidopsis* cells treated with 40 mM HU **(A)** and 10 μL/mL aphidicolin **(B)**. Samples were harvested at the indicated time points after addition of the drugs. Gene expression was analyzed by semiquantitative RT-PCR. The *ACT2* gene was used as a loading control.

(C) and **(D)** Transgenic *Arabidopsis* roots harboring the *WEE1* promoter fused to the *GUS* gene grown in the absence **(C)** or presence **(D)** of 10 mM HU. Plants were stained for GUS activity 20 h after drug application. Both images are at the same magnification. Bar = 50 μm.

WEE1 Gene Expression Is Induced in Response to Activation of the DNA Replication Checkpoint in Cultured *Arabidopsis* Cells and Seedlings

The observed phosphorylation of CDKA;1 upon DNA replication blockage and its association with WEE1 suggested an involvement of the WEE1 kinase in the checkpoint pathway. Therefore, we analyzed the transcriptional response of the *WEE1* gene in cell suspensions treated with either the ribonucleotide reductase inhibitor hydroxyurea (HU) or aphidicolin (Figures 2A and 2B). Drugs were added to exponentially growing cells and samples were collected at different time points for the next 20 h, after which *WEE1* and *CDKA;1* transcript levels were analyzed by semiquantitative RT-PCR. The *actin 2* (*ACT2*) gene was used as a loading control. *CDKA;1* transcript levels remained relatively constant upon HU treatment or decreased slightly after aphidicolin addition.

By contrast, *WEE1* transcript levels increased dramatically within 4 h after drug treatment, reaching maximum levels at 6 and 10 h after addition of HU and aphidicolin, respectively, indicating that replication inhibition transcriptionally activated the *WEE1* gene.

To investigate the transcriptional induction of the *WEE1* gene in response to DNA replication stress at the tissue level, transgenic *Arabidopsis* lines were generated that expressed the β-glucuronidase (*GUS*) reporter gene under the control of the *WEE1* promoter. A reproducible expression pattern was found in three independent reporter lines. Under standard growth conditions, promoter activity was detected locally in the shoot apex of seedlings as well as in the vasculature of the cotyledons and roots (Figures 3A and 3B). Also in older seedlings, expression was confined to apex and vascular tissues of roots and leaves (Figure 3C). Surprisingly, only occasionally, a faint GUS signal was detected in the apex of both main and lateral roots (Figures 3D and 3E). By contrast, GUS staining was strong in developing flowers, particularly in the anthers and gynoecia (Figure 3F), but not in mature flowers (Figure 3G).

To characterize the transcriptional induction of the *WEE1* gene in response to HU treatment, seeds of plants harboring the *WEE1:GUS* reporter were germinated on control medium. After 2 weeks, seedlings were transferred onto fresh control medium or a medium supplemented with HU. After 20 h, plants were harvested and assayed for GUS activity. No GUS staining was observed in the primary and lateral root meristems of plants transferred to the control medium (Figure 2C). In contrast, roots of the plants treated with HU for 20 h showed strong GUS staining in the root apical meristem, mostly confined to the cells of the central cylinder (Figure 2D). Similarly, *WEE1:GUS* reporter expression in the shoot apical meristem and vascular tissues was clearly induced by the HU treatment (data not shown). From these observations, we conclude that transcriptional control seems to play a major role in the regulation of WEE1 kinase activity during DNA replication stress.

WEE1 Activity Is Not Required for Cell Division or Endoreduplication

To study the role of WEE1 during normal plant development in more detail, plants were analyzed phenotypically that carried a mutation in the *WEE1* gene as a result of the insertion of T-DNA. Three different T-DNA insertion lines were used (Figure 4A). The *wee1-2* mutant allele harbored a T-DNA insertion in the first exon of the *WEE1* gene, deleting most of the WEE1 protein, whereas the *wee1-1* insert located between exons 7 and 8 resulted in a deletion of the last 197 (of 500) amino acids. Likewise, the *wee1-3* T-DNA insertion was localized in the kinase domain, with a deletion of 112

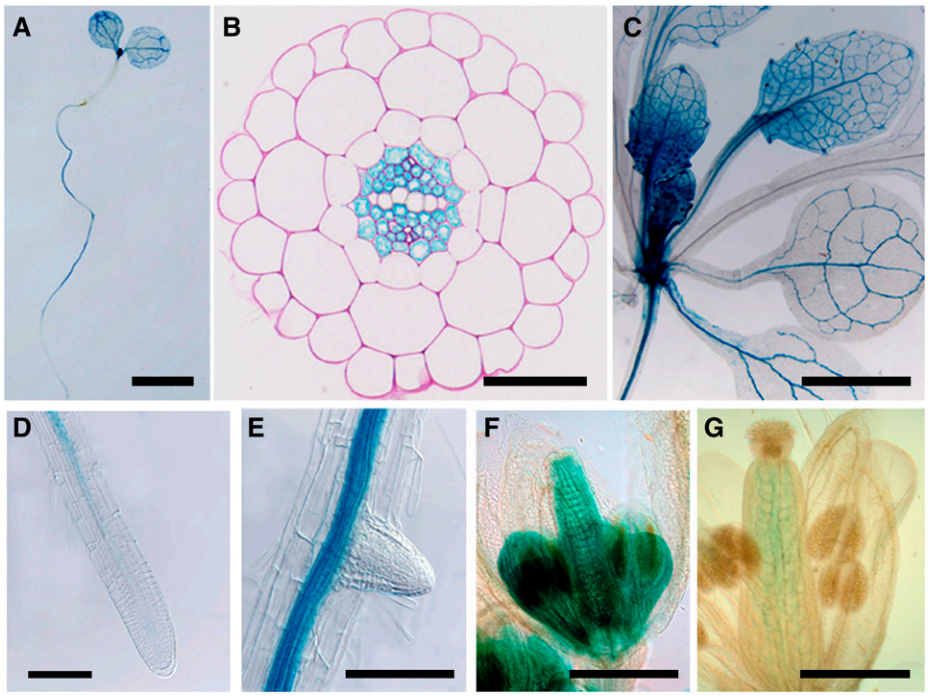

Figure 3. Spatial Expression Pattern of *WEE1*.

Promoter activity was visualized through histochemical GUS staining.
(A) Young seedling with strong GUS staining in the shoot apical meristem and vascular tissues of the root and cotyledon.
(B) Transverse section of the root with GUS staining in the vascular bundle and pericycle.
(C) Older seedling with GUS staining in the shoot apical meristem and vascular tissues.
(D) and **(E)** Primary and lateral root apex without staining or with only weak GUS staining, respectively.
(F) and **(G)** Young developing flower bud and mature flower, showing strong and weak GUS staining, respectively.
Bars = 2 mm in **(A)** and **(C)**, 50 μm in **(B)**, and 200 μm **(D)** to **(G)**.

amino acids as a consequence. Because the WEE1 kinase domain was located at the extreme C terminus of the WEE1 protein (amino acids 249 to 495), all mutants probably corresponded to null alleles. To test this hypothesis, both the full-length and the *wee1* alleles with a truncated kinase domain were cloned under the control of the *no message in thiamine* (*nmt1*) promoter, which is repressed in the presence of thiamine and can be induced by growing cells in thiamine-free medium (Maundrell, 1990). The obtained constructs were used to transform fission yeast cells. No significant difference in cell size was observed for the different constructs under noninducing conditions. In agreement with previously published results, in the absence of thiamine, expression of the full-length *WEE1* gene clearly interfered with the yeast cell cycle, resulting in an elongated cell phenotype (Figure 4B) (Sun et al., 1999; Sorrell et al., 2002). By contrast, expression of the truncated *wee1* alleles did not arrest the yeast cell cycle, because no difference in cell size was observed between cells grown in the presence or absence of thiamine. These data show that a complete kinase domain is essential for WEE1 functioning.

In none of the insertion lines was any full-length *WEE1* transcript detected (Figure 4C). When cultivated under standard growth conditions, the *WEE1* T-DNA insertion plants had no obvious morphological deviation from wild-type plants. For all plants, the mature first leaves were of similar size and contained the same number of abaxial epidermal pavement cells (Table 1). Also, the primary root length and number of lateral roots per millimeter were similar for wild-type and mutant plants (see Supplemental Figure 1 online).

In both maize and tomato, *WEE1* transcript levels have been found to peak in cell types entering the endoreduplication cycle, an alternative cell cycle during which DNA replication is not automatically followed by cytokinesis (Sun et al., 1999; Gonzalez et al., 2004). These data suggested a role for WEE1 as an important regulator of the mitosis-to-endocycle transition. However, no such role could be deduced from the *Arabidopsis WEE1* knockout plants, because the DNA ploidy distribution profile of wild-type and mutant plants was found to be identical in all tissues tested (see Supplemental Figure 2 online).

WEE1 Loss-of-Function Plants Are Hypersensitive to Replication-Inhibitory Drugs

Because of the observed induction of *WEE1* in response to HU and aphidicolin, the growth of *wee1* mutant plants was tested in the presence of drugs that block DNA replication. Wild-type and *WEE1*-deficient plants were germinated and grown on control medium for 5 d and subsequently transferred to control medium or medium containing either HU or aphidicolin at a dose that had mild, but perceptible, effects on the growth of the wild-type root (Culligan et al., 2004). In the presence of 1 mM HU or 12 μg/mL aphidicolin, the length of wild-type roots was reduced by 32 and 72%, respectively, compared with that of untreated plants (Figures 5A, 5B, 5D, and 5F). In contrast, *WEE1*-deficient root growth was reduced by >70% in the presence of HU (Figures 5A and 5E) and root growth was totally arrested in the presence of aphidicolin (Figures 5A and 5G). The growth inhibition phenotype was even more severe for plants germinated directly on aphidicolin-containing medium. Aphidicolin treatment clearly inhibited the growth of both wild-type and *wee1-1* mutant plants, although much more severely in the latter

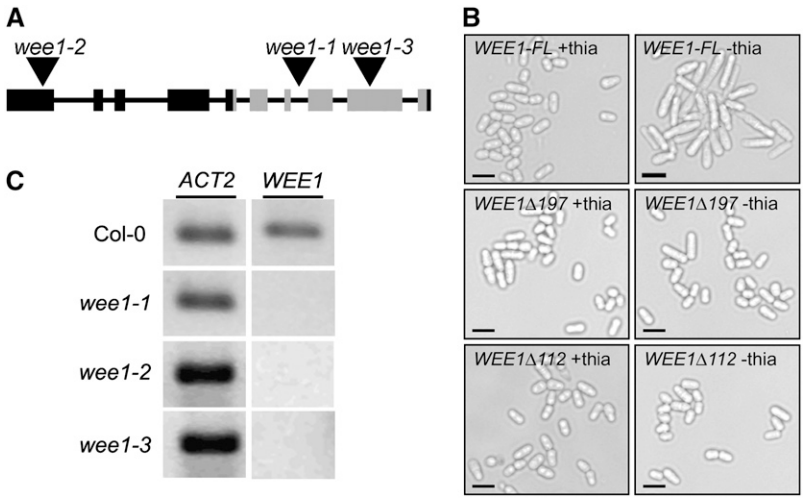

Figure 4. Molecular Analysis of *WEE1*-Deficient Plants.

(A) Intron–exon organization of the *WEE1* gene. Black and gray boxes represent exons, and lines indicate introns. The coding regions corresponding to the kinase domain are indicated in gray. The triangles correspond to the insertion sites of the different mutant alleles.

(B) Yeast cells harboring the full-length (*WEE1-FL*) or truncated (*WEE1Δ197* and *WEE1Δ112*) alleles of *WEE1*, grown in the presence (+thia) or absence (−thia) of thiamine. Bars = 10 μm.

(C) Two-step RT-PCR analysis performed on equal amounts of total RNA prepared from 8-d-old wild-type (Columbia [Col-0]) and mutant (*wee1-1*, *wee1-2*, and *wee1-3*) seedlings with primers that specifically amplify the *WEE1*-coding sequence flanking the T-DNA insertion site. The *ACT2* gene was used as a loading control.

Table 1. Size and Number of Abaxial Pavement Cells in Leaves of *WEE1*-Deficient and Control Plants

Line	Leaf Size (mm^2)	Abaxial Pavement Cell Size (μm^2)	Estimated Number
Columbia	22.4 ± 0.6	2129 ± 90	10.746 ± 298
wee1-1	22.1 ± 1.0	2183 ± 140	10.334 ± 373
wee1-2	23.0 ± 1.5	2090 ± 92	10.981 ± 519
wee1-3	20.0 ± 1.3	1946 ± 76	10.250 ± 452

All measurements were performed on leaves harvested 21 d after sowing. The indicated values are means ± SE ($n = 14$ to 30).

(Figures 5H and 5I). All three mutant lines had the same recessive phenotype that segregated with the respective insertions in *WEE1*.

We reasoned that the root growth arrest observed for the *WEE1*-deficient plants was attributable to a failure to block their cell cycle in response to DNA stress. To test this hypothesis, we compared the number of dividing cells in the root tips of the wild-type and *WEE1*-deficient plants. Under control growth conditions, the number of cells in mitosis was similar in both genotypes (Figure 6A). When control plants were treated with HU, they experienced a dramatic decrease in the number of mitotic cells. This decrease correlated with the appearance of a Tyr-phosphorylated CDK that migrated with the same electrophoretic mobility as that of CDKA;1 (Figure 6B). Moreover, a decrease in CDK activity was observed within 5 h after transfer to HU-containing medium (Figure 6C). By contrast, in the *WEE1*-deficient plants, the number of mitotic cells decreased only slightly upon HU treatment (Figure 6A). In addition, neither CDK Tyr phosphorylation nor a decrease in CDK activity was seen (Figures 6B and 6C). These data suggest that the *WEE1*-deficient plants failed to activate a G2 arrest and progressed with a not fully replicated genome into mitosis.

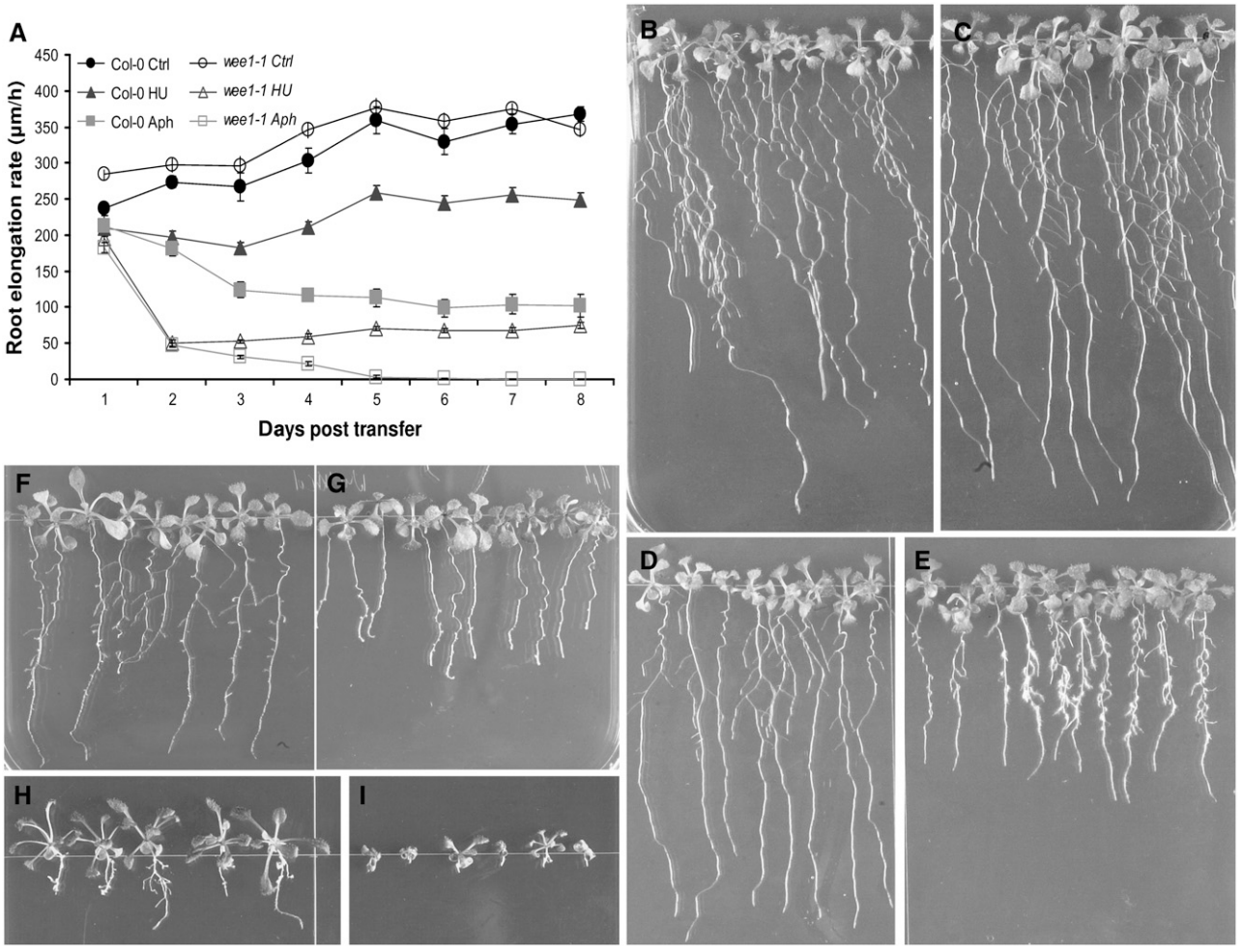

Figure 5. Phenotypic Analysis of *WEE1*-Deficient Plants under Replication Stress.

(A) Root elongation rates of plants shown in **(B)** to **(G)**. Error bars indicate SE ($n = 14$ to 20).
(B) to **(G)** Wild-type (**[B]**, **[D]**, and **[F]**) and *wee1-1* (**[C]**, **[E]**, and **[G]**) plants grown for 5 d and then transferred for 5 d to control medium (**[B]** and **[C]**), medium supplemented with 1 mM HU (**[D]** and **[E]**), or medium supplemented with 12 μg/mL aphidicolin (**[F]** and **[G]**).
(H) and **(I)** Wild-type and *wee1-1* seeds germinated on 12 μg/mL aphidicolin, respectively.

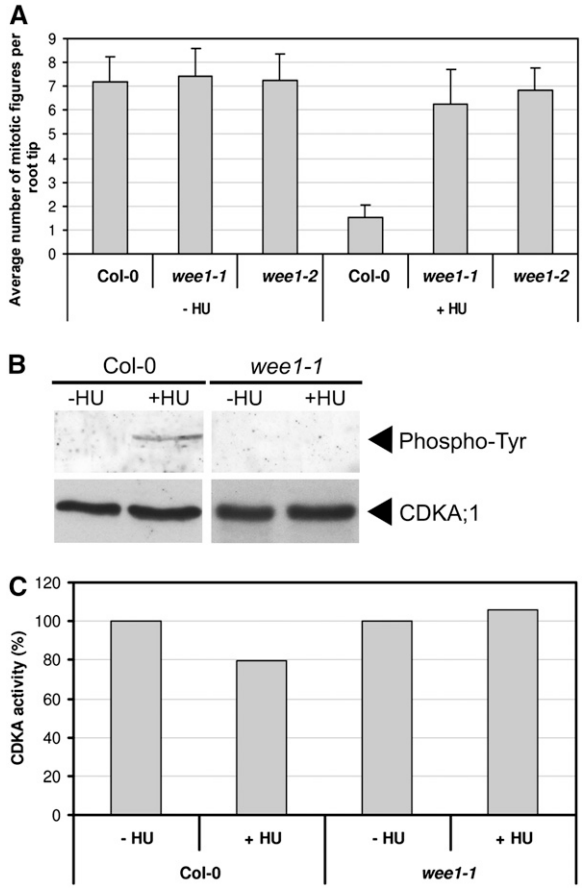

Figure 6. Root Apical Meristems of Stressed *WEE1*-Deficient Seedlings Failing to Arrest the Cell Cycle.

(A) Number of mitotic cells per root tip of 5-d-old seedlings transferred to control plates (−HU) or plates containing 1 mM HU (+HU). After 2 d, root tips (most distal 2 to 3 mm) were harvested, stained with orcein, and squashed. Error bars indicate SE ($n = 4$ to 10).

(B) Immunoblot analysis of 5-d-old wild-type (Columbia [Col-0]) and *wee1-1* plants treated with HU for 5 h (+HU) or mock treated (−HU). CDKs were purified from total protein extracts (900 μg/sample) with a $p9^{CKS1Hs}$-Sepharose matrix, resolved by SDS-PAGE, and immunoblotted with an anti-phosphotyrosine or anti-CDKA;1 antibody.

(C) Relative quantification of $p9^{CKS1Hs}$-associated CDK activity of the samples described for **(B)** using histone H1 as a substrate. The non-treated samples (−HU) were arbitrarily set at 100%. Values represent means of two independent experiments.

Transcriptional Upregulation of *WEE1* upon Replication Stress Depends on the ATR Kinase

The root phenotype of the *WEE1*-deficient plants seen upon replication stress mimicked that described for the *atr-2* mutant (Culligan et al., 2004), showing densely clustered hairs at the root tip and outgrowth of lateral roots. These observations suggest that WEE1 and ATR operate in the same pathway. To test whether *WEE1* activation upon replication stress depended on ATR, the *WEE1* expression level in wild-type and *atr-2* mutant plants was compared under control and HU stress conditions. Wild-type and *atr-2* plants were germinated and grown on control medium for 5 d and subsequently transferred to control medium or medium containing HU. Plants were grown for 24 h, after which *WEE1* transcript level was analyzed by real-time quantitative PCR. Under control growth conditions, only a low basal *WEE1* expression level was detected in both genotypes (Figure 7). When grown on HU-containing medium, the *WEE1* expression levels increased by twofold in wild-type plants. By contrast, in the *atr-2* plants, no induction of *WEE1* upon HU treatment was observed. Therefore, we conclude that the transcriptional activation of *WEE1* in response to replication-arresting drugs depends on ATR kinase function.

ATM Kinase Is Required for the Activation of the *WEE1* Gene in Response to DNA Damage

To test whether the transcriptional induction of *WEE1* is specifically linked to DNA replication stress or correlated with a general loss of DNA integrity, we analyzed whether the *WEE1* gene is modulated in response to DNA damage. To induce the in vivo formation of double-stranded breaks in plant cell DNA, we used the radiomimetic drug zeocin, belonging to the family of bleomycin/phleomycin antibiotics that are known to bind and cleave DNA. As a positive control, we used plants that carried as transgene the poly(ADP-ribose)polymerase2 (*PARP2*) gene promoter fused to *GUS* (Babiychuk et al., 1998), as ionizing radiation and radiomimetic drugs are known to induce the *Arabidopsis PARP2* gene (Doucet-Chabeaud et al., 2001; Chen et al., 2003). One-week-old *WEE1:GUS* reporter seedlings were transferred from standard germination medium to liquid medium supplemented with zeocin in serial dilutions, ranging from 1 to 100 μg/mL. Treatment of the *PARP2:GUS* reporter lines with zeocin induced a strong dose-dependent induction of GUS activity, demonstrating the efficiency of zeocin as a genotoxic drug (Figure 8).

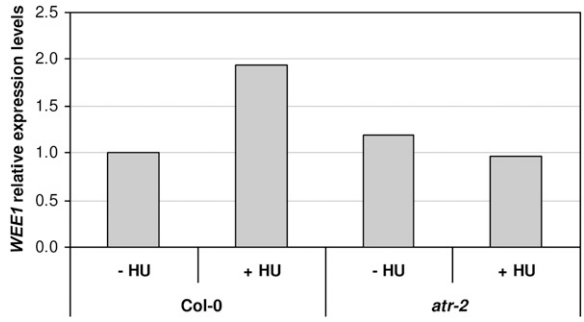

Figure 7. ATR-Dependent Induction of *WEE1* in Response to Replication Stress.

Quantification of *WEE1* transcripts in wild-type (Columbia [Col-0]) and *atr-2* mutant *Arabidopsis* plants. Five-day-old seedlings were transferred to control plates or plates containing 1 mM HU and harvested after 24 h for RNA preparation. Transcript levels were measured by real-time PCR. All values were normalized to the expression level of the *ACT2* housekeeping gene. The *ACT2*-to-*WEE1* transcript ratio of the wild-type sample under control conditions was arbitrarily set to 1. Values represent means of two independent experiments.

Similarly, *WEE1* promoter activity was induced upon zeocin treatment in both root (Figure 8) and shoot (see Supplemental Figure 3 online).

We also tested the effects of ionizing radiation on expression of the *WEE1* gene. Two-week-old plantlets exposed to 20 Gray of γ-rays were sampled for RNA preparation immediately after treatment (0 h) and after 30 min, 1, 3, 5, and 8 h. Only a low basal *WEE1* transcript level was detected just after treatment. At 1 h after irradiation, the *WEE1* mRNA abundance increased by fivefold (Figure 9A). The kinetics of *WEE1* gene induction correlated with that of the *RAD51* gene (Figure 9B), encoding an eukaryotic homolog of *RecA*, involved in double-stranded break repair, and demonstrated previously to be transcriptionally induced by γ-rays (Garcia et al., 2003). A very similar γ-irradiation–induced expression was observed for the *PARP2* gene (see Supplemental Figure 4 online). This transcriptional activation of *WEE1*, *RAD51*, and *PARP2* was only transient, although mRNA levels decreased with different kinetics.

Induction of *RAD51* by γ-irradiation was shown to depend on *ATM* (Garcia et al., 2003). To test the *ATM* dependence of the *WEE1* induction, *atm-1* plants were treated with γ-rays as described above and transcript levels were measured. As can be observed in Figure 9A, *WEE1* transcript levels did not increase in the mutant background, clearly illustrating that transcriptional activation of *WEE1* in response of DNA damage is regulated through ATM. Also, the *RAD51* and *PARP2* genes were not induced in the *atm-1* mutant (Figure 9B; see Supplemental Figure 3 online), confirming previous data (Garcia et al., 2003).

Induced Expression of *WEE1* Induces a G2 Cell Cycle Arrest

To analyze the effects of induced *WEE1* expression on plant growth, we attempted to generate transgenic *Arabidopsis* plants that constitutively overexpressed the *WEE1* gene under the control of the cauliflower mosaic virus 35S promoter but failed to do so, indicating that high WEE1 levels severely impaired growth and interfered with the regeneration of transgenic plants. To overcome this difficulty, we decided to use a switch-on constitutive overexpression approach, which relies on CRE-mediated recombination at *lox* sites (Joubès et al., 2004) (see Supplemental Figure 5 online). In this design, *CRE* recombinase expression is controlled by the promoter of the heat-shock protein gene *hsp90* and, hence, can be induced by heat treatment. CRE recombinase is able to switch on the expression of *WEE1* by catalyzing the excision of the enhanced green fluorescent protein (*EGFP*) gene, which was flanked by two colinearly orientated *lox* recombination sites and separated the open reading frame encoding WEE1 from the *CDKA;1* promoter. An additional advantage of the developed system is that the transcriptional activity of the transgene at a given chromosomal integration locus and the success of recombinational excision can be monitored visually by scoring the fluorescent tissues. All transgenic lines behaved identically upon *WEE1* expression induced by heat-shock treatment. All analyses were performed on root tissues, because heat shock–inducible CRE-dependent recombination was found to be most efficient and homogeneous.

To induce *WEE1* expression, transgenic plants were treated for 2 h at 37°C and subsequently returned to normal growth conditions. Two days after applying the heat shock, the *WEE1* transcript level had clearly increased (Figure 10A). As expected, induction of *WEE1* expression was accompanied by the loss of EGFP fluorescence in the root tissues, where the *CDKA;1* promoter is normally active (Figure 10B, panels ii and iv), and by the outgrowth of root hairs close to the root tip (Figure 10B, panel iii), probably because of shrinkage of the root meristem (see below). Root growth was completely arrested by 3 d after

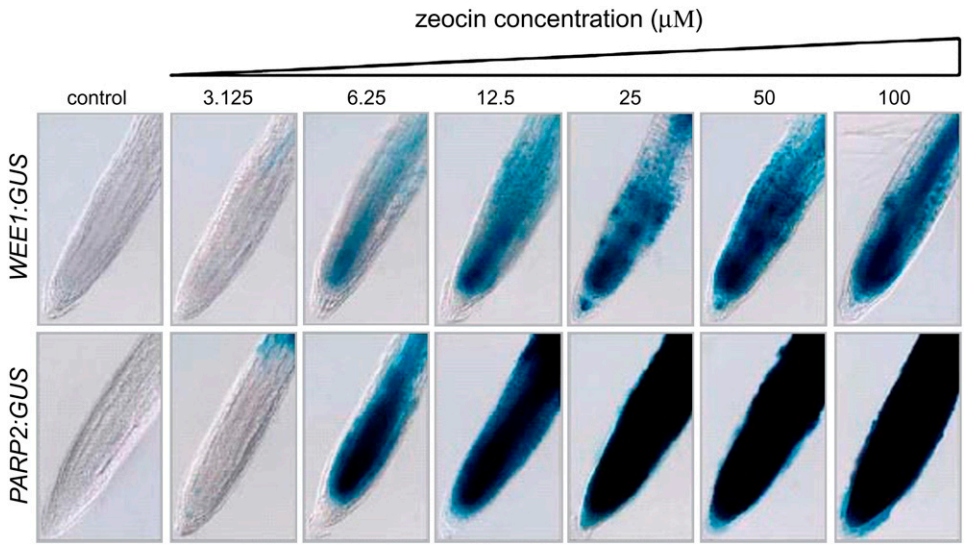

Figure 8. Transcriptional Activation of *WEE1* in Response to DNA Damage Checkpoint.

Seedlings harboring the *WEE1* or *PARP2* promoter fused to the *GUS* reporter gene (*WEE1:GUS* and *PARP2:GUS*) were treated with increasing concentrations of zeocin for 24 h. Activation of promoter activity was visualized through histochemical GUS staining.

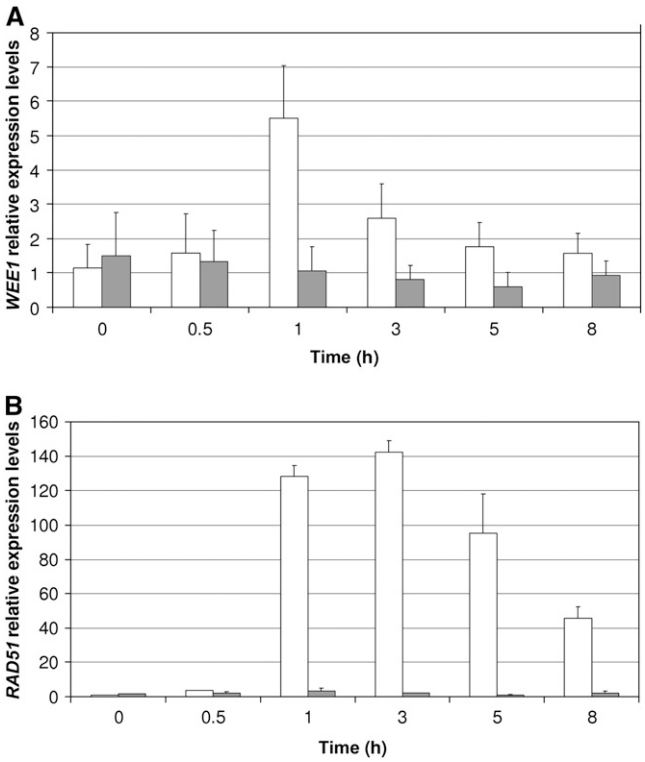

Figure 9. ATM-Dependent Induction of *WEE1* in Response to γ-Irradiation.

(A) Quantification of *WEE1* transcripts in wild-type (white bars) and *atm-1* (gray bars) *Arabidopsis* plants.

(B) Quantification of *RAD51* transcripts in wild-type (white bars) and *atm-1* (gray bars) *Arabidopsis* plants.

Plants were treated with 20 Gray of γ-rays and harvested at the indicated time points for RNA preparation. Transcript levels were measured by real-time PCR. All values were normalized against the expression level of the *ACT2* housekeeping gene. The *ACT2*-to-*WEE1* and *ACT2*-to-*RAD51* transcript ratios at time point 0 were set arbitrarily to 1. Data represent averages ± SD ($n = 3$).

applying the heat shock (Figure 10C). Longitudinal root sections of plants harvested at 7 d after the heat-shock treatment clearly showed that the pool of actively dividing meristematic cells was reduced, which are usually small and cytoplasm-rich and have a centrally positioned nucleus. By contrast, *WEE1*-overexpressing root tip cells were enlarged and highly vacuolated, resembling differentiated cells (Figure 10D). No effect of heat-shock treatment was seen for control plants harboring an inducible *GUS* gene (Figure 10C). These data clearly show that induced *WEE1* expression causes a cell cycle arrest that is accompanied by cell differentiation.

To test whether overexpression of *WEE1* blocks cells at a preferential position in the plant cell cycle, the expression levels of a number of cell cycle markers were analyzed by semiquantitative RT-PCR. No significant change in the level of the S-phase marker gene *histone H4* was observed in the root tips of control versus *WEE1*-overexpressing plants. By contrast, a substantial increase in the expression level of the G2-to-M-phase–specific cyclin *CYCB1;1* gene was seen upon *WEE1* overexpression (Figure 11). By 4′,6-diamidino-2-phenylindole and orcein staining of the genomic DNA, no increase in M-phase cells was observed, suggesting that the increase in *CYCB1;1* was attributable to a block of the cell cycle during G2 rather than to a post-G2 mitotic arrest. No effect of the heat-shock treatment on the expression of the reporter genes was observed in a control line harboring an inducible *GUS* gene (Figure 11).

DISCUSSION

DNA can be damaged in a variety of manners. To maintain genome integrity, signaling cascades initiated by the phosphatidylinositol-3-OH kinase–like kinases ATM and ATR control the activity of DNA repair complexes, halt cell cycle progression, and, in some cases, initiate cell death programs, at least in mammals. In plants, the role of ATM/ATR-dependent signaling in the expression of several DNA repair genes, such as *RAD51* and *PARP1*, has been demonstrated (Garcia et al., 2003). However, we know very little about molecular players in DNA damage response that modulate plant cell cycle progression. Here, we show that the *Arabidopsis WEE1* gene is transcriptionally activated in response to treatments that induce either DNA damage or DNA replication stress, and this induction depends on the activity of the ATR and ATM kinases, marking *WEE1* as a downstream target gene of the ATR-ATM signaling cascades. Because upregulated *WEE1* transcription blocked cell cycle progression, we propose a model in which ATR and ATM sense genotoxic stress and enforce a G2 cell cycle phase arrest by activating *WEE1* expression, allowing cells to complete the replication of their genome or to repair damaged DNA before proceeding into mitosis (Figure 12). In plants lacking WEE1, cells probably proceed into mitosis prematurely, resulting in loss of genome integrity, eventually triggering cell cycle and growth arrest. This model is corroborated by the observation that in *WEE1*-deficient lines the mitotic index in the root meristem did not decrease upon HU treatment, as can be observed in control plants (Figure 6A).

Our data indicate that CDKA;1 is a major WEE1 target. First, upon activation of the DNA replication checkpoint, a Tyr-phosphorylated CDK migrated with the same electrophoretic mobility as CDKA;1. Second, CDKA;1 associated directly with WEE1 in the yeast two-hybrid system. In addition, recently, CDKA;1 was shown to be directly phosphorylated by WEE1 in vitro in a Tyr-15–dependent manner (Shimotohno et al., 2006). Similarly, in a cell suspension culture of alfalfa (*Medicago sativa*), PSTAIRE-holding CDKs have been found to undergo Tyr phosphorylation (Mészáros et al., 2000). In mammalian cells, the drug caffeine cancels the DNA replication checkpoint through inhibition of the ATM kinase (Schlegel and Pardee, 1986; Andreassen and Margolis, 1992; Blasina et al., 1999), but in plants, it overrides the replication checkpoint only in the presence of the mitotic cyclin CYCB2 (Weingartner et al., 2003). Therefore, we postulate that among many other partnerships, CDKA;1 in complex with the B2-type cyclin might be the major target for inhibition by the activated checkpoint control pathways.

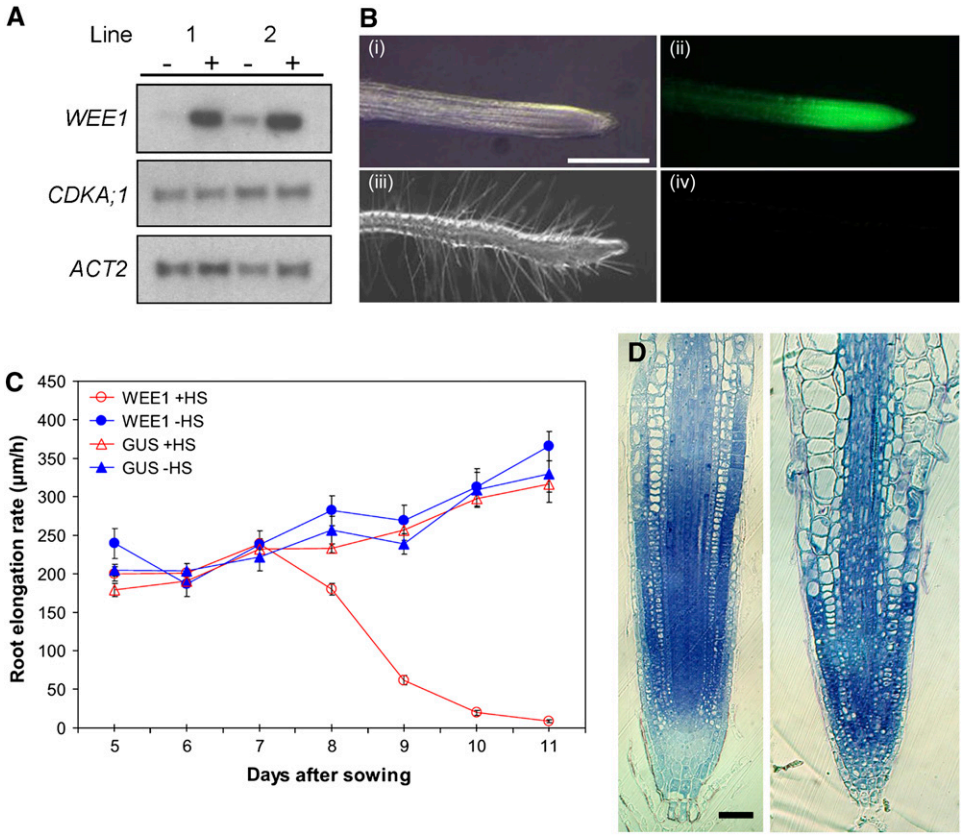

Figure 10. Molecular and Phenotypic Analysis of *Arabidopsis* Plants after Induction of *WEE1* Expression.

(A) *WEE1* expression levels as determined by semiquantitative RT-PCR analysis in two independent transgenic lines harboring the inducible *WEE1* gene expression cassette. RNA was isolated from noninduced (−) and induced (+) plants. *CDKA;1* and *ACT2* expression levels were measured as controls.
(B) Phenotype (panels i and iii) and EGFP fluorescence (panels ii and iv) of transgenic root tips before (panels i and ii) and 3 d after (panels iii and iv) induction of *WEE1* expression. All images are at the same magnification. Bar = 500 μm.
(C) Quantification of root growth rates in inducible *WEE1* and *GUS* (control) lines. Heat shock (+HS) was applied 7 d after sowing. Error bars indicate SE ($n = 10$ to 30).
(D) Median longitudinal section of untreated (left) and heat shock–treated (right) *WEE1* transgenic root tips harvested 7 d after heat-shock treatment. Both images are at the same magnification. Bar = 50 μm.

The severe growth phenotype of *WEE1*-deficient plants upon activation of the replication checkpoint suggests that WEE1 is part of the prevailing pathway that blocks the cell cycle under DNA stress, because in the presence of a redundant mechanism, no mutant phenotype would have been observed. By contrast, in cells of animals and fission yeast, the CDC25 phosphatase rather than WEE1 is the main target of the DNA damage and replication checkpoint cascades. Through its phosphorylation by the CHK1 and CHK2 kinases at specific amino acids in the N-terminal regulatory domain, CDC25 is targeted for destruction or exported from the nucleus (Boutros et al., 2006). Recently, an *Arabidopsis* gene coding for a *CDC25*-like phosphatase was identified (Landrieu et al., 2004). However, although the plant CDC25-like phosphatase displays structural homology with the mammalian CDC25 proteins within its catalytic domain and can activate CDKs in vitro, it lacks the complete N-terminal regulatory domain. Moreover, a role for the *Arabidopsis* CDC25-like protein in cell cycle control is debated, because no clear effects on cell cycle progression can be seen upon either overexpression or knockout under normal growth conditions or under stress (Bleeker et al., 2006; Dhankher et al., 2006; our unpublished data). Therefore, the *Arabidopsis* CDC25 protein very likely is not a target of the DNA damage signaling cascades.

In the absence of DNA stress, *WEE1* was expressed in the shoot apical meristem and the vascular tissues of roots and leaves, suggesting that it plays a role besides its involvement in checkpoint control. Because *WEE1* is expressed during S-phase (Gonzalez et al., 2004; Menges et al., 2005) and CDK Tyr phosphorylation occurs predominantly during DNA replication (Mészáros et al., 2000), we hypothesize that WEE1 might prevent replicating cells from entering mitosis through inhibition of the complexes required for the G2-to-M transition. Upon the incidence of DNA stress, *WEE1* expression might be maintained, arresting cells in the G2-phase until DNA synthesis or repair is completed. Such a regulation mechanism would establish a

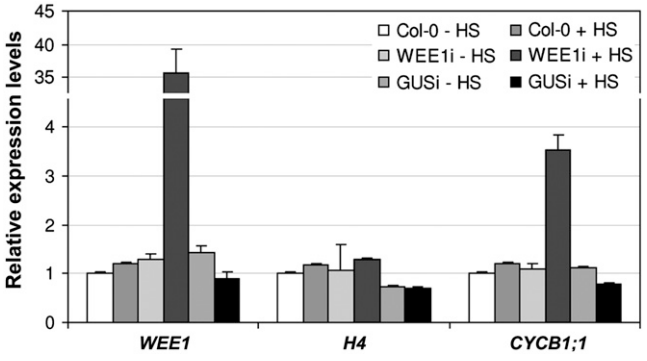

Figure 11. Expression Levels of Cell Cycle Markers upon *WEE1* Overexpression.

Seven days after sowing, seedlings were mock-treated (−HS) or heat-shocked (+HS) for 2 h at 37°C and returned to standard growing conditions; 24 h later, root tips (most distal 2 to 3 mm) were harvested for RNA preparation. *WEE1*, *histone H4*, and *CYCB1;1* transcript levels were measured by real-time PCR. All values were normalized to the expression level of the *ACT2* housekeeping gene. The normalized value of the untreated wild-type sample was arbitrarily set to 1.

situation in which cells in S-phase are intrinsically coupled to mitosis. However, no obvious cell division phenotype has been observed for *WEE1*-deficient plants under nonstressed growth conditions, indicating that CDK Tyr phosphorylation and WEE1 are not rate-limiting for the onset of mitosis. In accordance, the overexpression of a mutant *cdka;1* allele in which the putative phosphorylated Tyr residue is mutated also did not result in an obvious cell division phenotype (Hemerly et al., 1995). These data suggest that a redundant control mechanism couples DNA replication with mitosis. A putative candidate to coordinate this event together with WEE1 is CDKB1;1, whose activity has been demonstrated to limit the G2-to-M onset (Porceddu et al., 2001; Boudolf et al., 2004b, 2006).

Alternatively, the discrepancy between the observed high *WEE1* expression in dividing tissues and the lack of a cell division phenotype for *WEE1* knockout plants might be explained by posttranscriptional control of WEE1 levels. In fission yeast and African frog, WEE1 is activated posttranslationally and the protein becomes phosphorylated and stabilizes in response to either DNA damage or replication arrest (O'Connell et al., 1997; Boddy et al., 1998; Michael and Newport, 1998; Lee et al., 2001). Compared with transcriptional activation, posttranslational control of WEE1 activity might offer a more rapid mechanism to block the cell cycle in response to loss of genome integrity.

A strong expression of *WEE1* was observed in flowers, particularly during gynoecium and anther development, indicating a role for WEE1 during gametogenesis. Here again, *WEE1* might be induced in response to the double-stranded breaks that arise during meiotic recombination events. Mutant *atm* plants are partially sterile, because of aberrant meiosis accompanied by chromosomal fragmentation (Garcia et al., 2003). By contrast, *WEE1*-deficient mutants are normally fertile, illustrating that WEE1 activity is not essential for a putative meiotic checkpoint and suggesting that, if ATM controls a meiotic checkpoint, it must do so through a mechanism that does not involve WEE1 activation.

Previously, WEE1 had been attributed a role in plant genome endoreduplication, based on its expression in endoreduplicating maize and tomato tissues (Sun et al., 1999; Gonzalez et al., 2004). However, the *Arabidopsis WEE1* T-DNA insertion lines had a wild-type DNA ploidy distribution profile in all tissues tested, suggesting that if WEE1 controlled the endocycle, it would do so in a species-dependent manner. Nevertheless, at this stage, we cannot exclude the possibility that WEE1 controls the timing of differentiation events other than endoreduplication, although no significant differences in the timing of leaf development have been observed.

Our analysis of transgenic plants demonstrates that *cis* elements that are necessary and sufficient for *WEE1* gene activation are contained within the 591-bp DNA sequence upstream from the translation start. It would be of interest to identify the transcriptional cascade by which the *WEE1* promoter is activated upon DNA stress. In mammals, the tumor suppressor gene product p53 is an important transcriptional activator whose protein levels increase in response to DNA damage. Activated p53 induces genes coding for CDK inhibitor proteins, such as $p27^{Kip1}$, resulting in cell cycle arrest. However, *WEE1* is not among reported p53 gene targets. Moreover, no clear p53 homolog can be found in the *Arabidopsis* genome, suggesting that other pathways must regulate the activation of *WEE1* transcription in response to DNA stress. In fission yeast, transcriptional induction of *WEE1* in response to stress was found to rely on the mitogen-activated protein kinase (MAPK)–dependent pathway (Suda et al., 2000) that is activated by a range of stress-inducing stimuli, including DNA damage (Degols and Russell, 1997). Interestingly, *Arabidopsis* MAPK orthologs are activated in response to DNA damage, and the MAPK phosphatase mutant *mkp1* is hypersensitive to genotoxic stress treatments (Ulm et al., 2001). Therefore, it would be worthwhile to test whether MKP1 operates upstream of WEE1.

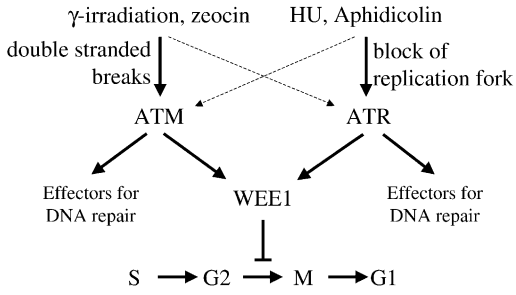

Figure 12. Model for WEE1 in the Control of the DNA Integrity Checkpoint.

DNA stress induced by double-stranded DNA breaks (as induced by γ-irradiation and zeocin) or by blockage of the replication fork (induced by HU and aphidicolin) is sensed mainly by the ATM or ATR signaling cascade, respectively (Garcia et al., 2003; Culligan et al., 2004). ATM and ATR simultaneously induce the expression of DNA repair genes and *WEE1*. WEE1 arrests cells in the G2-phase of the cell cycle, allowing cells to repair DNA before proceeding into mitosis.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana (ecotype Columbia) plants were grown under long-day conditions (16 h of light, 8 h of darkness) at 22°C on germination medium (Valvekens et al., 1988). The *wee1-1* allele (GABI_270E05) was obtained from the GABI-Kat T-DNA mutant collection (http://www.mpiz-koeln.mpg.de/GABI-Kat/GABI-Kat_homepage.html) (Li et al., 2003), whereas the *wee1-2* (SALK_147968) and *wee1-3* (SALK_039890) alleles were found in the Salk Institute T-DNA Express database (http://signal.salk.edu). The seeds were acquired from the ABRC. To screen for homozygous insertion alleles, primers were used according to GABI-kat and SALK; to screen for the presence of the full-length *WEE1* transcript, the following primer pairs were designed: 5′-TGGTGCTGGACATTTCAGTCGG-3′ and 5′-GGATATTACTCCTCGTGGTTTGAAAATG-3′ for *wee1-1*, 5′-ATGTTCGAGAAGAACGGAAGAACAC-3′ and 5′-CTATGATGGAAGTGCAAGCTCTTG-3′ for *wee1-2*, and 5′-TGGTGCTGGACATTTCAGTCGG-3′ and 5′-TGATGGATCGTGATCCGAAGCG-3′ for *wee1-3*. The *WEE1*-inducible gene construct was generated by cloning all required DNA fragments generated by PCR into pCR4Blunt-TOPO (Invitrogen). Fragments corresponding to the promoter of the *Arabidopsis HSP18.2* gene, the *CRE* recombinase–coding sequence, and the octopine synthase terminator (*OCS3′*) sequence were assembled into the pZErO-2 vector and subsequently inserted into the pCAMBIA2200 vector, resulting in the pJCRE vector. The Arath;*CDKA;1* promoter, the *EGFP*-coding sequence flanked by a 5′ end *loxP*, the OCS3′ sequences (one of them flanked by a 3′ end *loxP* site), and the GATEWAY recombination site were assembled into the pZErO-2 vector. The cassette containing all of the elements was subsequently cloned into the pCAMBIA1200 vector, resulting in the pJLOX vector. Cloning details are available upon request.

The *WEE1* gene was introduced into the pJLOX vector by the GATEWAY technology. The full-length open reading frame of *WEE1*, amplified by PCR using 5′-AAAAAGCAGGCTTCACAATGTTCGAGAAGAACGG-3′ and 5′-AGAAAGCTGGGTTCAACCTCGAATCC-3′ primers and *Arabidopsis* seedling cDNA as template, was first cloned into pDONR207 ENTRY vector by an *att*B × *att*P (BP) recombination reaction according to the manufacturer's instructions (Invitrogen). The fragment was recombined into pJLOX by an *att*L × *att*R (LR) recombination reaction at the GATEWAY recombination site. The *WEE1* promoter sequence was amplified from *Arabidopsis* genomic DNA with the 5′-AAAAAGCAGGCTTGTTTTATATCCCACATTTTAGAATTAATC-3′ and 5′-AAAAAGCAGGCTTCACAATGGAAGGGACATTGG-3′ primers. The corresponding PCR fragment was cloned into pDONR207 entry vector by BP recombination cloning and subsequently transferred into the pKGWFS7 destination vector (Karimi et al., 2002) by LR cloning, resulting in a transcriptional fusion between the *WEE1* promoter and the *EGFP-GUS* fusion gene. All constructs were transferred into the *Agrobacterium tumefaciens* C58C1Rif^R strain harboring the plasmid pMP90. The obtained *Agrobacterium* strains were used to generate stably transformed *Arabidopsis* with the floral dip transformation method (Clough and Bent, 1998). Transgenic plants were obtained on kanamycin- or hygromycin-containing medium and later transferred to soil for optimal seed production. The *atm-1* and *atr-2* mutants have been described previously (Garcia et al., 2003; Culligan et al., 2004). Suspension-cultured *Arabidopsis* ecotype Landsberg *erecta* cells were grown under long-day conditions (16 h of light, 8 h of darkness) at 25°C on a rotary shaker (120 rpm) in a modified Murashige and Skoog medium (Menges et al., 2002).

Yeast Two-Hybrid Interactions

Yeast two-hybrid bait and prey vectors were obtained through recombinational GATEWAY cloning (Invitrogen). The CDKA;1, CDKB1;1, and WEE1 open reading frames were recombined into the pDEST22 and pDEST32 vectors (Invitrogen) by an LR reaction, resulting in translational fusions between the open reading frames and the GAL4 transcriptional activation and GAL4 DNA binding domains, respectively. Plasmids encoding the baits and preys were cotransformed into the yeast reporter strain PJ69-4a (*MAT*a, *trp1-901*, *leu2-3*,*112*, *ura3-52*, *his3-200*, *gal4*D, *gal80*D, *LYS2*::*GAL1-HIS3*, *GAL2-ADE2*, *met2*::*GAL7-lacZ*) by the lithium acetate method (Gietz et al., 1992) and plated on SD plates without Leu and Trp. After 2 d of growth at 30°C, yeast was transferred to SD plates without Leu and Trp (as a control) and to SD plates without Leu, Trp, and His. Plates were incubated at 30°C and scored for growth of yeast and, hence, protein–protein interaction after 2 d.

Plant Treatments

Arabidopsis plants and cell cultures were treated with aphidicolin or HU as described by Culligan et al. (2004) and Nagata et al. (1992), respectively. For zeocin treatments, seeds were germinated under aseptic conditions. One-week-old seedlings were transferred onto 12-well plates containing 1 mL of water supplemented with different amounts of zeocin starting from a commercially available zeocin stock solution of 100 mg/mL (Invitrogen). After 24 h of treatment, plants were used for histochemical GUS staining. For γ-irradiation treatments, 2-week-old plantlets grown in vitro were irradiated with γ-rays at a dose of 20 Gray from a ^{60}Co source (Faculty of Agriculture, Ghent University). Plant material was harvested and immediately frozen in liquid nitrogen according to a time course as described.

DNA and RNA Manipulation

Genomic DNA was extracted from *Arabidopsis* leaves with the DNeasy Plant kit (Qiagen). RNA was extracted from *Arabidopsis* tissues and cultured cells with the TriZol reagent (Invitrogen). First-strand cDNA was prepared from 500 ng of total RNA with the Superscript RT II kit (Invitrogen) and oligo(dT)$_{18}$ according to the manufacturer's instructions. A 0.2-μL aliquot of the total RT reaction volume (20 μL) was used as a template in a semiquantitative RT-mediated PCR amplification, ensuring that the amount of amplified product remained in linear proportion to the initial template present in the reaction. Ten microliters from the PCR was separated on a 1.2% agarose gel and transferred onto Hybond N$^+$ membranes (GE-Healthcare). The membranes were hybridized at 65°C with fluorescein-labeled probes (Gene Images random prime module; GE-Healthcare), and the hybridized bands were detected with the CDP Star detection module (GE-Healthcare). The following primers were used: 5′-GCCAAGACTTCTTCTGCACACCTGAC-3′ and 5′-GGGAGAAAACTTGCACATTTTGATC-3′ for the *WEE1* gene (At1g02970), 5′-ATGGATCAGTACGAGAAAGTTGAG-3′ and 5′-CATTGGCATGCCTCCAAGATCC-3′ for the *CDKA;1* gene (At3g48750), and 5′-GTGCCAATCTACGAGGGTTTC-3′ and 5′-CAATGGGACTAAAACGAAAA-3′ for the *ACT2* gene (At3g18780). Quantitative PCR was performed with the SYBR Green kit (Eurogentec) with 100 nM primers and 0.125 μL of RT reaction product in a total of 25 μL per reaction. Reactions were run and analyzed on the iCycler iQ (Bio-Rad) according to the manufacturer's instructions. For each reaction, the threshold cycle was determined by setting the threshold within the logarithmic amplification phase. All quantifications were normalized to *ACT2* cDNA fragments amplified under the same conditions. Quantitative reactions were done in triplicate and averaged. Primers used were 5′-GGCTCCTCTTAACCCAAAGGC-3′ and 5′-CACACCATCACCAGAATCCAGC-3′ for *ACT2* (At3g18780), 5′-TGGTGCTGGACATTTCAGTCGG-3′ and 5′-CAAGAGCTTGCACTTCCATCATAG-3′ for *WEE1* (At1g02970), 5′-CGAGGAAGGATCTCTTGCAG-3′ and 5′-GCACTAGTGAACCCCAGAGG-3′ for *RAD51* (At5g20850), 5′-CTCAAAATCCCACGCTTCTTGTGG-3′ and 5′-CACGTCTACTACCTTTGGTTTCCC-3′ for *CYCB1;1* (At4g37490), and 5′-ATGTCTGGTCGTGGAAAGGGAG-3′ and 5′-ACCAAATTGCGTGTTTCCATTG-3′ for *H4* (At5g59970).

Antibodies and Protein Gel Blot Analysis

Protein extracts were prepared by grinding material in homogenization buffer (De Veylder et al., 1997). Protein concentrations were determined with the protein assay kit (Bio-Rad). CDKs were purified by affinity chromatography with either $p10^{CKS1At}$- or $p9^{CKS1Hs}$-Sepharose beads as described (De Veylder et al., 1997). Protein gel blotting was performed according to standard procedures with primary anti-CDKA;1 and anti-CDKB1;1 antibodies (Porceddu et al., 2001) diluted 1:5000 and 1:2500, respectively, and a secondary horseradish peroxidase–conjugated sheep anti-rabbit antibody (GE-Healthcare) diluted 1:5000 or with the mouse monoclonal horseradish peroxidase–conjugated anti-phosphotyrosine p-Tyr antibody (PY99; Santa Cruz Biotechnology) diluted 1:5000. Proteins were detected by a chemiluminescence procedure (NEN Life Science Products). Kinase assays were performed as described previously (De Veylder et al., 1997).

Histochemical GUS Assays

Complete seedlings or tissue cuttings were stained on multiwell plates (Falcon 3043; Becton Dickinson). GUS assays were performed as described by Beeckman and Engler (1994). Samples mounted in lactic acid were observed and photographed with a stereomicroscope (Stemi SV11; Zeiss) or with a differential interference contrast microscope (Leica).

Microscopy and Flow Cytometric Analyses

Leaves were harvested at 21 d after sowing, cleared overnight in ethanol, stored in lactic acid for microscopy, and observed with a microscope fitted with differential interference contrast optics (Leica). The total (blade) area was determined from images digitized directly with a digital camera (Axiocam; Zeiss) mounted on a binocular (Stemi SV11; Zeiss). From scanned drawing-tube images of outlines of at least 30 cells of the abaxial epidermis located 25 and 75% from the distance between the tip and the base of the leaf, halfway between the midrib and the leaf margin, the following parameters were determined: total area of all cells in the drawing and total numbers of pavement and guard cells, from which the average cell area was calculated. The total number of cells per cotyledon was estimated by dividing the leaf area by the average cell area. To visualize mitotic cells, seeds were germinated and grown for 5 d as described above and then transferred to control plates or plates containing HU. Root tips were harvested 2 d after transfer and fixed in three parts ethanol and one part acetic acid, macerated in 0.1 N HCl, and squashed in orcein in 45% acetic acid. The number of mitotic cells was determined by observing mitotic figures through a Leica microscope with a 63× oil lens. For flow cytometric analysis, tissues were chopped with a razor blade in 300 μL of 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, pH 7, and 0.1% Triton X-100 (Galbraith et al., 1991). One microliter of 4,6-diamidino-2-phenylindole from a stock of 1 mg/mL was added to the filtered supernatants. The nuclei were analyzed with the CyFlow flow cytometer using FloMax (Partec) software. Sections of root tips for histological analysis were prepared according to Beeckman and Viane (2000).

WEE1 Analysis in Yeast

WEE1 alleles were amplified by adapter PCR with 5′-GGGCATATGTTC-GAGAAGAACGGAAGAACAC-3′ as the universal forward primer and 5′-GGGGGATCCTTATCAACCTCGAATCCTATCAAACATG-3′, 5′-GGG-GGATCCTTAGCAAGAGCTTGCACTTCCATC-3′, and 5′-GGGGGATCC-TTAACCAAGCTTGCAAACACCGTTC-3′ as reverse primers for the full-length, *WEE1Δ197*, and *WEE1Δ112* alleles, respectively. The obtained PCR fragments were cut with *Nde*I and *Bam*HI and cloned under the control of the attenuated *nmt1* promoter in the pREP41 HA-N vector (Craven et al., 1998). All constructs were transferred into the *Schizosaccharomyces pombe h⁻ leu1-32* strain with the frozen-EZ yeast transformation kit (Zymo Research). For analysis, yeast cells were grown overnight in rich YPB medium (10 g of yeast extract, 10 g of peptone, 10 g of beef extract, and 2.5 g of NaCl per liter of water at pH 7.2), then diluted in Edinburgh minimal medium 2 (Moreno et al., 1991) with or without thiamine (5 μg/mL).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *WEE1* (At1g02970), *CDKA;1* (At3g48750), *CDKB1;1* (At3g54180), *ATM* (At3g48190), and *ATR* (At5g40820).

Supplemental Data

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

Supplemental Figure 1. Root Growth Analysis of *WEE1*-Deficient Plants.

Supplemental Figure 2. DNA Ploidy Level Distribution of Wild-Type and *WEE1*-Deficient Plants in Different Tissues.

Supplemental Figure 3. Transcriptional Activation of *WEE1* in Seedlings in Response to Zeocin Treatment.

Supplemental Figure 4. ATM-Dependent Transcriptional Induction of *PARP2* in Response to γ-Irradiation.

Supplemental Figure 5. Scheme Depicting the CRE-Inducible Transcriptional Activation of the *WEE1* Transgene.

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