ATR Regulates a G2-Phase Cell-Cycle Checkpoint in Arabidopsis thaliana

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Ataxia telangiectasia-mutated and Rad3-related (ATR) plays a central role in cell-cycle regulation, transmitting DNA damage signals to downstream effectors of cell-cycle progression. In animals, ATR is an essential gene. Here, we find that Arabidopsis (Arabidopsis thaliana) atr−/− mutants were viable, fertile, and phenotypically wild-type in the absence of exogenous DNA damaging agents but exhibit altered expression of AtRNR1 (ribonucleotide reductase large subunit) and alteration of some damage-induced cell-cycle checkpoints. atr mutants were hypersensitive to hydroxyurea (HU), aphidicolin, and UV-B light but only mildly sensitive to γ-radiation. G2 arrest was observed in response to γ-irradiation in both wild-type and atr plants, albeit with slightly different kinetics, suggesting that ATR plays a secondary role in response to double-strand breaks. G2 arrest also was observed in wild-type plants in response to aphidicolin but was defective in atr mutants, resulting in compaction of nuclei and subsequent cell death. By contrast, HU-treated wild-type and atr plants arrested in G1 and showed no obvious signs of cell death. We propose that, in plants, HU invokes a novel checkpoint responsive to low levels of deoxynucleotide triphosphates. These results demonstrate the important role of cell-cycle checkpoints in the ability of plant cells to sense and cope with problems associated with DNA replication.

INTRODUCTION

The detection of DNA damage is an important aspect of damage resistance. Cells do not routinely express all DNA repair mechanisms. Instead, plants upregulate these functions as required, concomitantly slowing the cell cycle to permit time for repair before S-phase (as replication past damaged sites may be mutagenic) or before M-phase (after which point double-strand breaks and lesions in daughter strand gaps may become more problematic to repair). Damage is generally detected by protein complexes in the form of single-stranded DNA (Crowley and Courcelle, 2002; Zou and Elledge, 2003), created when the replication fork is physically blocked by DNA lesions or during the process of DNA repair (e.g., homologous recombination, nucleotide excision repair, and mismatch repair). These damage sensors then activate downstream checkpoint mechanisms to prevent progression into the next phase of the cell cycle as well as upregulate DNA repair (Nyberg et al., 2002).

Some damage sensors also are involved in the regulation of deoxynucleotide triphosphate (dNTP) pools, which may influence the mode of DNA replication. One of the primary targets for dNTP pool regulation is the enzyme ribonucleotide reductase (RNR), which catalyzes the final reduction step in the production of dNTPs. Genetic studies in Saccharomyces cerevisiae suggest that the elevation of dNTP levels (through transcriptional and/or posttranslational regulation of RNR) in response to DNA damage may result in more efficient translesion DNA synthesis, enabling the cell to complete replication in spite of the presence of persistent lesions (Tanaka et al., 2000; Chabes et al., 2003; Yao et al., 2003).

The proteins that sense damaged or single-stranded DNAs and trigger these elaborate responses have been extensively characterized in organisms ranging from bacteria to human (Crowley and Courcelle, 2002; Melo and Toczyski, 2002). In plants, however, the regulation of DNA replication and repair and the cell cycle in response to DNA damage is only beginning to be understood.

The Schizosaccharomyces pombe Rad3 homologs (ataxia telangiectasia-mutated and Rad3-related [ATR] and ataxia telangiectasia-mutated [ATM]) are essential regulators of cell-cycle checkpoints, sensing DNA damage and/or single-stranded DNA and activating downstream effectors of cell-cycle progression and DNA repair. ATM is activated primarily by DNA double-strand breaks. ATM-deficient plants and animals are hypersensitive to γ-irradiation but not to replication-blocking agents such as UV-B light, hydroxyurea (HU), or aphidicolin (Abraham, 2001; Garcia et al., 2003; K. Culligan and A. Britt, unpublished data). Furthermore, the Arabidopsis (Arabidopsis thaliana) ATM homolog is required for the transcriptional induction of repair genes in response to γ-irradiation and for efficient progression through meiosis (Garcia et al., 2003), though its role in plant cell-cycle responses to DNA damage has not yet been determined. In animals, activated ATM phosphorylates downstream components of checkpoint pathways that include p53, BRCA1, NBS1, and CHK2, initiating G1-, S-, or G2-phase arrest and/or apoptosis.

By contrast, ATR is activated primarily by agents that block the progression of replication forks. ATR dominant-negative
and conditional knockout mammalian cell lines display hypersensitivity to UV-B light, HU, and aphidicolin but are also sensitive to γ-radiation. Thus, in animal cells, ATR is thought to play a more generalized role in response to DNA damage than ATM. Activated ATR phosphorylates CHK1 to initiate G2-phase arrest but may also act at other points of the cell cycle, as evidenced by ATR-dependent phosphorylation of BRCA1 (Tibbets et al., 2000; Melo and Toczyski, 2002). However, the determination of the full extent of ATR’s role in genome maintenance has been complicated by the fact that ATR homozygous knockouts die early in embryogenesis (Brown and Baltimore, 2000; de Klein et al., 2000); cre/lox-induced nulls survive only a few rounds of cell division, and the dominant-negative reduction-of-function cell lines have yielded conflicting results (Cliby et al., 1998; Wright et al., 1998). Thus, a tractable genetic system in which a null allele of ATR could be tolerated throughout the development of a higher organism would be particularly desirable.

Here, we investigate the effects of an ATR T-DNA insertion allele in the plant Arabidopsis. The allele has a 6-kb insertion within the kinase domain and is presumably null. We find that the mutant plant (atr) is viable and developmentally normal in the absence of exogenous DNA-damaging treatments but is hypersensitive to the replication-blocking agents UV-B light, HU, and aphidicolin. Thus, AtATR does appear to act as a functional homolog of its mammalian counterpart. However, the Arabidopsis atr mutants differ from mammalian mutants not only in terms of their viability but also in their degree of sensitivity to γ-radiation.

In the course of these experiments, we also observed a differential response to HU versus aphidicolin in wild-type cells, with HU primarily inducing a G1 arrest, whereas aphidicolin induced arrest in G2. This stands in contrast with the G2 arrest induced by both agents in yeast and mammals (at concentrations that allow S-phase progression) and suggests that plants may possess a novel G1 checkpoint response to low dNTP levels. The G2 arrest in response to aphidicolin was abrogated in atr mutants, suggesting that ATR regulates the G2 checkpoint response to replication blocks, as it does in mammals. Moreover, programmed cell death was observed in atr mutants in response to aphidicolin, but not HU, under our experimental conditions. We propose a model that suggests that the HU and aphidicolin hypersensitivities of atr mutants are the result of its defect in the G2 checkpoint response to blocked replication forks.

RESULTS

Identification of the ATR Ortholog in Arabidopsis

ATR orthologs, including Mec1 of S. cerevisiae and Rad3 of S. pombe, are members of a large gene family in eukaryotes that encode Ser-Thr kinases, whose C-terminal catalytic domains share similarity to yeast and mammalian phosphoinositide 3-kinases (Tibbets and Abraham, 2000). We searched the Arabidopsis genome for homologs of ATR, using the S. pombe Rad3 C-terminal domain as a query sequence, and found several candidate genes. Phylogenetic analysis of the corresponding predicted full-length protein sequences indicated that one was an ortholog of Target of Rapamycin (TOR), one an ortholog of ATM, and one was an ortholog of ATR (Figure 1A). No other candidate genes fell into these broad categories. The ATR-orthologous sequence (previously reported in GenBank as mRNA AtRad3 [AB040133], which we designate here as AtATR) revealed high similarity throughout its entire length to other ATR proteins, but its similarity to ATM and TOR sequences was limited only to the highly conserved middle and C-terminal regions. We confirmed the GenBank sequence and intron/exon boundaries by isolating and sequencing cDNAs of AtATR (~8.1 kb, ecotype Wassilewskija [Ws]) using RT-PCR, and the resulting sequence predicted 17 exons encoding a protein of 2702 amino acids (Figure 1B).

Identification of T-DNA Insertion-Mutation Alleles in AtATR

To study the function of ATR in Arabidopsis, we searched for lines with T-DNA insertions within ATR. Three independent lines were identified from different collections and were termed atr-1 (Arabidopsis Knockout Facility, ecotype Ws), atr-2 (SIGnAL database, ecotype Columbia), and atr-3 (FLAG database, ecotype Ws). atr-1 and atr-2 contain insertions in the middle of ATR, in exons 7 and 10, respectively (Figure 1B). atr-3 contains a single, ~6-kb T-DNA insertion within the highly conserved C-terminal kinase (PI3Kc) domain (Figures 1B to 1D), which causes a 30-bp deletion adjacent to the catalytic Asp required for kinase function. RT-PCR analysis of the C-terminal region of ATR confirms that the transcript is absent in atr-3 (Figure 7). Thus, we believe atr-3 is likely to be a null mutation and have focused the majority of our characterization on this line (termed atr below). Nonetheless, all three lines exhibit a similar recessive phenotype (see below) that cosegregates with their respective T-DNA insertions in ATR.

Neither AtATR nor AtATM Are Required during Normal Somatic Development

All three atr homozygous alleles are phenotypically wild-type in growth (of the root and shoot) and development (leaf and flower development, seed set and viability) under standard conditions. Thus, ATR is not essential for normal somatic development in Arabidopsis. By contrast, ATR is an essential gene in animals; ATR knockout mice die early in embryogenesis (Brown and Baltimore, 2000; de Klein et al., 2000), whereas conditional knockout human cell lines divide only a few times before dying (Cortez et al., 2001; Brown and Baltimore, 2003). Because ATR and ATM are paralogs known to play partially overlapping roles in animal cells (Abraham, 2001), it is possible that ATM could compensate for ATR deficiency in Arabidopsis. To test this hypothesis, we established atr atm double mutants and found no obvious phenotypic differences (in vegetative growth and development) when compared with wild-type or single-mutant plants, indicating that neither ATR nor ATM play an essential role during normal vegetative (somatic) growth.

The double mutants, however, were completely sterile (Figure 2). atr mutant plants are fertile and produce normal gametes; pollen staining for viable spores revealed no differences versus
Figure 1. Phylogenetic Analysis, Structure, and T-DNA Insertions of AtATR.

(A) The neighbor-joining tree of Rad3-like protein sequences was performed as described (Culligan et al., 2000). Numbers above each branch represent the number of times the branch was found in 1000 bootstrap replicates. Arabidopsis sequences are in bold. No DNA-PKcs (DNA–protein kinase catalytic subunit)-like sequences were identified in the Arabidopsis genome.

(B) Overview of the AtATR gene, positions of atr-1, atr-2, and atr-3 T-DNA insertions, and its corresponding cDNA structure. Numbered gray boxes indicate exons of AtATR; the positions of the T-DNA insertions are denoted as triangles. The cDNA structure (bottom) shows the corresponding highly conserved domains found in ATM, ATR, TOR, and FKBP12-rapamycin-associated protein sequences, including the kinase domain denoted as a black rectangle (PI3Kc). The cDNA sequence was confirmed by isolating and sequencing independent cDNAs of AtATR (ecotype Ws) using RT-PCR. The resulting sequence predicts 17 exons that encode a protein of 2702 amino acids.

(C) Structure of the atr-3 T-DNA insertion. Capital letters denote T-DNA insertion sequence. Corresponding amino acids are shown above the DNA sequence. The left-border sequence encodes 10 new amino acids within the AtATR reading frame before a premature stop (boxed as TGA). The approximate size of the entire insertion is 6 kb.

(D) Partial alignment of the kinase domain where atr-3 is inserted: 1 denotes a 30-bp deletion (equivalent to 10 amino acids) caused by the insertion, and 2 denotes the position of the catalytic Asp required for kinase function.
the wild type, and the siliques were full (normal seed set). atm mutant plants, however, are partially sterile because of abundant chromosomal fragmentation during meiosis, resulting in a reduction in viable pollen and reduced seed set (Garcia et al., 2003). We suggest that AtATM and AtATR play partially redundant roles during meiosis in Arabidopsis.

AtATR Is Required for Full Transcriptional Induction of the Large Subunit of RNR, AtRNR1

In the budding yeast S. cerevisiae, Mec1 (the ATR ortholog) regulates expression of RNR at both the transcriptional and posttranscriptional levels (Zhao et al., 2001). Null alleles of mec1 are lethal, but this lethality is suppressed by mutations in genes that negatively regulate RNR (Huang et al., 1998; Zhao et al., 1998), such as the transcriptional repressor Crt1 (Huang et al., 1998) or the inhibitor of RNR activity Smi1 (Zhao et al., 1998). Derepression of RNR transcription in response to DNA damage is dependent on Mec1 and the downstream kinase Dun1, which indirectly induces an inhibitory phosphorylation of Crt1 (Elledge et al., 1992; Zhao and Rothstein, 2002). Whether Mec1 or ATR also play a role in the normal cell-cycle specific regulation of RNR transcription in unchallenged cells remains unclear.

We therefore wanted to determine if RNR transcription is regulated in an AtATR-dependent manner in challenged and/or unchallenged plants. We searched GenBank and identified the Arabidopsis homolog of RNR1, the large subunit of RNR (accession number AF092841). Transcription of RNR1 has already been shown to be regulated in a cell-cycle dependent manner in tobacco (Nicotiana tabacum) (Chabouté et al., 1998, 2002), and transcription is induced by DNA damage in this plant (M.E. Chabouté, personal communication). We probed RNA gel blots with AtRNR1 and found that AtRNR1 transcript is reduced in atr mutants, down ~40% versus the wild type under standard conditions (Figure 3), suggesting that ATR is required to support normal expression of RNR1. Plants treated with chronic doses of UV-B light, sufficient to inhibit but not eliminate growth, display a level of RNR1 transcript similar to that observed under standard (UV-free) conditions, and again atr plants exhibit reduced levels of RNR1 transcript. Interestingly, plants treated with acute doses of UV-B light displayed a transient inhibition of AtRNR1 transcript (at 2 h postirradiation), followed by a return to standard condition levels at 6 h postirradiation. This UV-induced suppression is not ATR dependent, though again, the overall level of RNR1 transcript is reduced (Figure 3).

Because we used HU (an inhibitor of RNR activity) as a DNA replication-blocking agent (see below) to characterize the phenotypic effects of the atr mutant, we wanted to determine the effects of HU treatment on RNR expression in whole plants. Surprisingly, in the presence of HU, RNR1 expression is the same in both the wild type and atr (Figure 3) with levels similar to the wild type under standard conditions. This suggests that there are additional ATR-independent levels of RNR1 regulation in Arabidopsis and is consistent with previous studies showing that HU induces RNR expression in plants (Chabouté et al., 1998).
Arabidopsis atr Mutants Are Mildly Sensitive to Acute Doses of γ-Radiation but Proficient in γ-Induced G2 Arrest

Conditional mutants of ATR/Mec1 in mammalian cells and S. cerevisiae, as well as Rad3 mutants in S. pombe, are hypersensitive to γ-radiation (Jimenez et al., 1992; Cliby et al., 1998; Wright et al., 1998). Mutants of ATM/scTel1, including those in Arabidopsis, are also hypersensitive to γ-radiation (Sedgwick and Boder, 1991; Garcia et al., 2003). This sensitivity is generally interpreted as a failure of checkpoint responses to double-strand breaks (Abraham, 2001) but may also involve a failure to induce DNA repair genes (Garcia et al., 2003). To determine whether ATR plays a role in γ-resistance in Arabidopsis, we challenged wild-type, atr, and atm lines with sublethal doses of γ-radiation. In contrast with atm mutants, atr lines exhibited only mild sensitivity to γ-radiation. The root length of atr was reduced by ~30% versus the wild type 7 d after seeds were γ-irradiated at 200 Gray (Gy), whereas atm root length was reduced by >95% (Figure 4A).

To determine the role of Arabidopsis ATR as a cell-cycle checkpoint regulator in response to γ-irradiation, we established homozygous wild-type and atr mutant lines carrying a cyclinB1 promoter coupled to β-glucuronidase (PcyclinB1::GUS) reporter construct. This construct harbors a GUS gene fused to a mitotic destruction sequence and a cyclinB1 promoter (Colon-Carmona et al., 1999). The GUS fusion gene is expressed upon entry into G2 (via the cyclinB1 promoter), and the fusion protein is degraded just before anaphase during mitosis. GUS-positive cells stain blue in G2/early M, allowing us to visually monitor the progression of the cell cycle in root meristems (Preuss and Britt, 2003). To determine G2-specific arrest (versus post-G2 mitotic arrest), we further stained root tips with 4′,6-diamidino-2-phenylindole (DAPI) to visualize mitotic nuclei. In response to 400 Gy of γ-radiation, atr mutants showed only a slightly altered cell-cycle response when compared with the wild type; both wild-type and atr meristematic cells arrest in G2 (DAPI staining revealed no accumulation of mitotic figures) but differ slightly in the timing of the response. For instance, at 8 h postirradiation, atr root tips accumulated more G2 cells versus the wild type, but at 1 d postirradiation the root tips accumulated less G2 cells versus the wild type (Figure 4B). This suggests that Arabidopsis ATR is not absolutely required for the G2 checkpoint response to acute doses of γ-radiation but does influence its rate of induction and persistence.

Arabidopsis atr Mutants Are Hypersensitive to Replication-Blocking Agents

hsATR, scMec1, and spRad3 deficiencies are also associated with hypersensitivity to replication blocking agents. For example, yeast mec1 mutants fail to undergo the G2 arrest normally induced by stalled replication and instead proceed directly into mitosis. Consequently, mutant cells accumulate single-strand gaps and double-strand breaks, leading to cell death (Weinert et al., 1994). In animals, this failure to arrest induces apoptosis (Brown and Baltimore, 2000; Cortez et al., 2001). To determine whether ATR is required for plant cell-cycle responses to replication blocks, we challenged wild-type and homozygous atr mutant plants with the replication-blocking agents HU (1 mM), aphidicolin (12 μg/mL), and UV-B light. All of these agents inhibit replication in fundamentally different ways. HU inhibits RNR, thus depleting available dNTPs for DNA polymerases. Aphidicolin is an inhibitor of the replicative DNA polymerases δ and ε, and UV-B light creates physical replication blocks (e.g., cyclobutane pyrimidine dimers) in DNA. We chose to focus our analysis on root meristems because we and others (Wiedemeier et al., 2002) found that both HU and aphidicolin inhibit root growth in wild-type plants, suggesting that these agents are absorbed by the root. We gerninated wild-type and homozygous atr seeds on control MS agar plates or on plates containing either HU or aphidicolin, selecting a dose of each agent that had mild but perceptible effects on growth of the wild-type root. Both the wild type and atr grew at very similar rates on control plates. On plates containing 1 mM HU, wild-type root growth was reduced ~40%, whereas the atr root growth was reduced >95% (Figures 5A and 5D) and produced abnormally long root hairs that were densely clustered at the root tip. Moreover, lateral root growth initiated much earlier in atr on HU plates compared with control plates. The shoot was reduced in size and produced anthocyanins (stress-induced pigments), perhaps as an indirect effect of the lack of root growth. The same differences between the wild type and atr (40% versus >95% growth inhibition, overproduction of root...
hairs and lateral roots) were observed when germinated on plates containing 12 μg/mL of aphidicolin or when 5-d-old seedlings were transferred to plates containing HU or aphidicolin, starting 2 d after transfer (Figures 5C and 5D). Furthermore, chronic doses of UV-B light had a similar phenotypic effect on atr roots compared with the wild type (Figure 5B); the mutant was also retarded (to a lesser extent at this dose) in its root growth and formed root hairs at the tip of the root. Thus, three treatments that block replication but differ in the mechanism of inhibition produced similar gross phenotypic effects in atr roots.

AtATR Regulates a G2 Checkpoint in Response to Replication Blocks

Mec1 and ATR are required for the G2 checkpoint response to replication blocks in fungi and mammals, respectively (Melo and Toczyski, 2002). To determine whether ATR performs a similar function in plants, we GUS and DAPI stained homozygous wild-type and atr mutant lines carrying the PcyclinB1:GUS reporter construct (described above) after growth on HU and aphidicolin. As shown in Figure 6, the wild type and atr displayed typical patterns of PcyclinB1:GUS expression on control plates (~10 to 20 blue cells per root tip), representing the fraction of G2/early M cells present during normal growth. By contrast, the meristematic cells of aphidicolin treated wild-type plants displayed a substantial increase in the number of PcyclinB1:GUS-expressing cells and perhaps in the density of their staining (Figure 6). Using DAPI staining to visualize mitotic nuclei, we observed no significant aphidicolin-induced accumulation of mitotic figures in either wild-type or atr meristematic cells, thus ruling out post-G2 mitotic arrest as a likely cause for the PcyclinB1:GUS accumulation. This indicates that the dividing cells of wild-type plants experiencing aphidicolin-induced replicational stress spend a greater fraction of their cell cycle in G2 and that atr plants are defective in this G2 arrest (Figure 6).
In contrast with the aphidicolin response, HU did not induce G2-phase arrest in wild-type roots. In fact, a decline in the fraction of G2 cells was observed from day 4 to 6 (as measured by the lack of $\text{P}_{\text{cyclinB1}}:\text{GUS}$ staining; Figure 6). The cells are presumably arresting in G1- or S-phase because no increase in mitotic figures was observed via DAPI staining. This response also occurred in $\text{atr}$ root meristems but appeared to be slightly accelerated.

To confirm the $\text{P}_{\text{cyclinB1}}:\text{GUS}$ analysis, we employed semi-quantitative RT-PCR, with primers specific to the $\text{cyclinB1}$ gene (which does not amplify the $\text{P}_{\text{cyclinB1}}:\text{GUS}$ transcript), on untreated, HU-, or aphidicolin-treated wild-type and $\text{atr}$ root tips. As shown in Figure 7, $\text{cyclinB1}$ gene expression is reduced $\sim 20\%$ (relative to untreated control root tips) in the wild type when grown in the presence of HU but is elevated nearly threefold in the presence of aphidicolin, consistent with our $\text{P}_{\text{cyclinB1}}:\text{GUS}$ experimental conclusion that HU induces G1- or S-phase arrest and aphidicolin induces G2 arrest. Furthermore, aphidicolin-treated $\text{atr}$ root tips display no enhancement of $\text{cyclinB1}$ transcript in response to aphidicolin, again suggesting the lack of G2 arrest in $\text{atr}$.

To distinguish between G1- or S-phase arrest in wild-type HU-treated roots, we employed RT-PCR analysis of the S-phase specific gene $\text{Histone H4}$. In plants, the expression of $\text{Histone H4}$ has been shown to be strongly induced upon entry into S-phase and maintained through G2 (Reichheld et al., 1995; Menges and...
Aphidicolin, but Not HU, Induces Cell Death in atr

To further characterize the different cellular responses to HU and aphidicolin, we DAPI stained wild-type and atr root tips (time points and treatments as above) to observe the integrity of the nuclei. As seen in Figure 8A, wild-type roots had very organized meristems with round and slightly diffuse nuclei, and they retained this morphology on HU and aphidicolin. However, atr exhibited different cellular morphologies on both HU and aphidicolin. The cell files of atr meristems grown on HU were disorganized and the cells enlarged, but the nuclei were similar in appearance to the wild type (round and slightly diffuse). All cells up to the root cap had a defined nucleus (Figure 8A). The meristems of atr grown on aphidicolin also were disorganized, but the appearance of the nuclei differed from that observed in either the wild type (all conditions) or atr grown on HU. Starting on day 4, the meristematic nuclei near the tip became condensed, and DAPI staining was reduced relative to atr grown on HU or the wild type (all conditions). By day 6, most cells in the meristematic zone of aphidicolin-treated roots had completely lost their nuclei, leaving only a blue haze (Figures 8A and 8B). This indicates that atr mutant cells undergo nuclear degradation (cell death) when treated with aphidicolin, but not HU, at dose levels of both agents that inhibit wild-type growth by ~40%. Although some cell death also can be seen at higher doses of HU (data not shown), these higher doses inhibited wild-type growth >95%. This suggests that (1) the aphidicolin-induced AtATR-dependent G2 arrest plays a critical role in protecting the genome, and (2) that additional factors, such as the G1 arrest induced by HU, may rescue atr meristems from massive genomic instability induced by progression through M-phase in the absence of complete replication.

DISCUSSION

ATR plays a central role in the cell’s response to DNA damage by activating cell-cycle checkpoints that allow time for DNA repair. We have shown that Arabidopsis atr mutants are viable and normal under standard growth conditions but are hypersensitive to replication blocks and mildly sensitive to γ-radiation. Furthermore, we have shown that AtATR is required for the G2-phase checkpoint in response to replication blocks but is not absolutely required in the G2-arrest response to double-strand breaks (using acute doses of γ-radiation). The differences seen in response to HU, aphidicolin, and γ-irradiation suggest that additional checkpoints also play important roles in the cell-cycle and cell-death phenotypes observed.

AtATR Plays a Relatively Minor Role in γ-Resistance

At 400 Gy of γ-radiation, the atr mutant is similar to the wild type in root growth and its ability to recover from γ-radiation treatments. By contrast, atm is not able to recover from γ-radiation doses as

Figure 6. GUS Staining of Root Tips.

Five-day-old wild-type and atr-3 seedlings (carrying Pcyclind1-GUS) were transferred to aphidicolin (Aph), or HU, or control (n.t.) plates, and root growth was measured every other day after transfer for 8 d (Figure 5D). Individual wild-type and atr plants were harvested at each time point for GUS staining of root tips. Wild-type and atr were grown for 5 d on MS plates and then transferred to control plates or to plates containing 1 mM HU or 12 μg/mL of aphidicolin. These experiments were repeated several times with very similar results; thus, representative pictures are shown. GUS stained root tips from 2, 4, and 6 d after transfer are shown. At least 12 individual plants were harvested and stained at each treatment and time point. Two representative examples are shown for each treatment.
Wild-type and atr plants were grown as described in Figure 6. Root tips were excised (1 to 2 mm from the root cap) from wild-type and atr plants 4-d after transfer for RNA extraction, cDNA preparation, and RT-PCR analysis. Twenty-five microliters of each RT-PCR sample was loaded on an ethidium bromide–stained agarose gel, and representative gels are shown. All RT-PCR products were between 400 bp and 850 bp in size. The RT-PCR product of eIF4A (eukaryotic initiation factor) was employed as a standard for RT-PCR amplification. Numbers below each gel band denote its relative expression (first normalized to eIF4A) to the wild-type untreated (n.t.) sample. Standard deviations for two replicates are shown in parentheses. Aph, aphidicolin.

Figure 7. Semiquantitative RT-PCR Analysis of G2-Phase and S-Phase–Specific Genes.

It is clear from both yeast and animal studies that ATR plays a critical role in the G2 checkpoint response to replication blocks. Many of these studies have employed HU and/or aphidicolin as model agents for replication blocks. HU or aphidicolin treatment of wild-type yeast and animals cells (at concentrations that allow S-phase progression) results in G2 arrest. In ATR-deficient mammalian cells, progression into mitosis (without G2 delay) leads to caspase-independent chromosomal fragmentation, which in turn induces an apoptotic response (Brown and Baltimore, 2000). We have shown that atr plants are hypersensitive to the growth-inhibitory effects of HU and aphidicolin. The G2 arrest (as measured by \( P_{\text{cycB1-GUS}} \)) in wild-type root meristems on aphidicolin and the concomitant lack of G2 arrest in atr root meristems indicates that ATR is required for the G2-phase checkpoint response to replication blocks. The lack of wild-type G2 arrest in response to HU, however, was surprising. Because the fundamental difference between HU and aphidicolin in blocking S-phase replication is that HU-treated cells are reduced in dNTPs, this implies that the G1 arrest is activated by a novel checkpoint responsive to low dNTPs. Previous studies in human cells demonstrated a p53-dependent G1 arrest in response to low ribonucleotide levels, but not deoxynucleotide levels (Linke et al., 1996). The response to low dNTPs may help to prevent plant cells from initiating S-phase DNA replication in the absence of sufficient dNTP pools. Because phosphate is a relatively immobile and limiting nutrient in soil (Abel et al., 2002), this checkpoint could also represent a plant-specific response that helps direct root growth away from phosphate-depleted regions of soil, eliminating growth of root meristems in phosphate-depleted regions, while promoting the formation of other lateral root meristems elsewhere along the root.
Figure 8. DAPI-Stained Root Tips from Wild-Type and atr Grown as in Figure 6.

(A) Shown are root tips day 4 and day 6 after transfer.

(B) Close-up of 6 d after transfer to aphidicolin wild-type (left) and atr (right) DAPI-stained root tips.
The cell death observed in atr in response to aphidicolin, but not HU, suggests that the G1 (dNTP-dependent) checkpoint may rescue atr from cell death. However, atr mutants are hypersensitive to the effects of HU on the growth of the root, a fact that may appear paradoxical given the hypothesis that this line is proficient in the G1 arrest induced by this agent. For this reason, we remind the reader that the HU-treated wild-type roots are growing, though at a slightly reduced rate. In other words, the cells are progressing through the entire cell cycle, although they are spending a relatively longer fraction of that cycle in G1. We thus propose the following model for Arabidopsis cell-cycle checkpoint regulation in response to HU. HU treatment induces two independent checkpoints in Arabidopsis: one in G1 in response to low dNTPs (AtATR independent) and one in G2 in response to replication blocks (AtATR dependent). HU-treated wild-type and atr meristematic cells arrest in G1 in response to low dNTP pools, thus retarding their entry into S-phase. The G1 checkpoint is eventually relieved after enough dNTPs accumulate to permit entry into S-phase, and some fraction of cells leak through into S-phase. The resulting S-phase–induced depletion of dNTPs in the presence of HU then results in a requirement for AtATR-dependent G2 checkpoint activation. The inability of atr to activate this G2 checkpoint results in defective DNA replication and meristematic failure.

By contrast, aphidicolin-treated atr cells do not invoke the G1 checkpoint because they have normal levels of dNTPs and enter S-phase without delay. atr cells then fail to arrest in G2 and proceed into mitosis with incompletely replicated genomes, perhaps to a much larger degree than HU-treated atr cells. This results in a majority of the daughter G1 cells having an intolerable amount of single-strand gaps and double-strand breaks, leading to meristematic failure and cell death.

The cell death seen in atr was unexpected. Mutants in DNA repair/maintenance genes in Arabidopsis, such as DNA ligase IV, ERCC1, and telomerase, do not exhibit obvious cell death, even in response to treatment with DNA damaging agents (Riha et al., 2001; Friesner and Britt, 2003; Hefner et al., 2003). A prevailing hypothesis is that plants, which have no p53 homolog, lack DNA damage–induced cell death (apoptosis). We believe the atr response to aphidicolin might be an example of programmed cell death, as indicated by the fact that all cells in the root tip die, including normally nondividing cells (e.g., epidermal cells), suggesting that the signal for the cell death is diffusible. Alternatively, it is possible that the cells surrounding the dying meristem are recruited to reconstitute the missing meristem, and this induction of the cell cycle induces their death. In this case, death might simply be the direct result of the chromosomal fragmentation induced by progression through M-phase with an incompletely replicated genome. In any event, it is clear that replication-induced checkpoints play an important role in protecting cells from the consequences of replicational stress.

**AtATR-Dependent and AtATR-Independent Regulation of AtRNR1 Transcription**

The regulation of RNR in yeast is well defined and involves transcriptional and posttranslational control (i.e., inhibitory protein–protein interactions, modification of protein localization, and allosteric regulation). For instance, the yeast RNR1 (large subunit) gene is expressed during vegetative growth, upregulated in late G1 and early S, and further upregulated in response to DNA damage (Elledge et al., 1992). The expression patterns of several RNR genes have been characterized in tobacco, including an RNR1-like gene (Chaboté et al., 1998, 2002). The reduced level of RNR1 transcript (Figure 3) we observed in atr versus the wild type, in the absence of DNA damaging agents or with chronic UV-B light, suggests that RNR1 is transcriptionally maintained in Arabidopsis through both AtATR-dependent and AtATR-independent mechanisms. Our analysis employing HU on whole seedlings (Figure 3) further indicate that RNR1 is regulated transcriptionally, and independently of AtATR, via a feedback-type mechanism that boosts RNR1 transcripts when dNTPs are low.

The rapid reduction of AtRNR1 transcript in response to acute doses of UV-B in wild-type and atr plants suggests an additional mode of transcriptional regulation for AtRNR1 in response to DNA damage that is independent of AtATR. In yeast, RNR upregulation in response to DNA damage is rapid, occurring within 30 min after treatment (Elledge and Davis, 1990; Elledge, 1993). It was thus surprising that under our experimental conditions, an acute dose of UV-B light caused a rapid reduction of AtRNR1 transcript within 2 h after UV-B treatment, followed by a return to nontreatment control levels within 6 h after UV-B treatment. This response could represent a process by which inhibition of AtRNR expression leads to low dNTP levels and a concomitant arrest of the cell cycle during DNA damage, possibly through the G1 checkpoint described above. A decrease in RNR expression might also be expected to inhibit the potentially mutagenic actions of bypass polymerases, which require high levels of dNTPs for activity (Johnson et al., 2001; Chabes et al., 2003). One plausible model for this mode of regulation would be that immediately after experiencing acute DNA damage, cells initially try to eliminate as much of the damage as possible via error-free pathways. Initial downregulation of dNTPs would inhibit the inherently mutagenic bypass polymerases and contribute to the arrest of the cell cycle. Return to normal levels of dNTPs then allows the cell cycle to progress and enables the cell to utilize bypass polymerases as needed to complete replication. This model is suggestive of the SOS response in Escherichia coli, wherein error-free repair mechanisms are induced early, and error-prone translesion synthesis is induced later (Goodman and Woodgate, 2000; Rangaragan et al., 2002) in response to acute induction of DNA damage.

**AtATR Is Required for the Completion of Meiosis in the Absence of AtATM**

Given the partial sterility of atm mutant plants and nonsterility of atr, the observation of complete sterility of the atr atm double mutant suggests that ATR can partially complement ATM function during meiosis. Although it is not clear whether ATR has a distinct meiotic function or if ATR is simply filling in for the lack of ATM (or vice versa), our experiments do at the very least suggest that ATR is present and functional during meiosis in Arabidopsis. In mammalian cells, in situ localization studies have shown that both ATM and ATR are localized to meiotic chromosomes.
Further studies using atr and atm single and double mutant plants will allow us to better understand the role of ATR in plant meiosis, possibly leading to additional insights into ATR function during animal meiosis.

**Conclusions**

We have shown that plants encode an ortholog of ATR and that its role in G2 checkpoint regulation is similar in many respects to its yeast and mammalian counterparts. The presence of ATM and other downstream effectors of the cell cycle and DNA repair, including BRCA1 (Lafarge and Montane, 2003) and a putative CHK1 (S.B. Preuss, K. Culligan, and A. Britt, unpublished data) for example, further suggest that G2 cell-cycle checkpoint responses to DNA damage and replication blocks are regulated in a similar fashion in plants and animals. This is not to say that plant responses to DNA damage and replication blocks are identical to that of animals; perhaps the most significant difference between plants and animals is the lack of a p53-dependent programmed cell death response to persisting DNA damage. Plants may encode an analogous pathway for the degradation of loosely compromised cells, as evidenced by the cell death observed in atr, but this is probably a response to massive disruption of DNA replication coupled to uninhibited progression through the cell cycle. Perhaps because plants do not die from cancer, a cell death response to minor levels of DNA damage (such as those that occur spontaneously and persist in repair defective mutants) would be counteradaptive. Because of this insensitivity (versus animals) to persisting DNA damage, plants may ultimately serve to be a robust model system for the study of many fundamental processes in cell-cycle regulation and DNA repair pathways in eukaryotes (for review, see Hays, 2002).

**METHODS**

**Isolation of Mutants**

The atr-1 allele (ecotype Ws) was isolated from the Arabidopsis Knockout Facility as described (Krysan et al., 1999). The atr-2 allele (ecotype Columbia: SALK_032841) was identified using the Salk Signal-AL Web site (http://signal.salk.edu), and the seeds obtained from the ABRC. The atr-3 allele (ecotype Ws) was identified using the FLAG database (Genoplante; http://genoplante-info.infobiogen.fr/).

**Growth of Arabidopsis**

For standard growth conditions, seeds were sown on 1× MS (GBICO, Cleveland, OH) phytosafe (Sigma, St. Louis, MO) agar, pH 5.8, and grown under cool-white lamps filtered through Mylar at an intensity of 100 to 150 μmol/m²/s with a 24-h day light cycle at 21°C. HU (Sigma; 500 mM stock in water) and aphidicolin (ICN Biomedicals, Basingstoke, UK; 20 μg/mL stock in DMSO) were added to MS plates (as above) to concentrations described in the text and figure legends. For experiments using aphidicolin, all control and HU plates contained an equivalent amount (0.05%) of DMSO to allow direct comparison.

**UV-B and γ-Irradiation of Arabidopsis**

For chronic doses of UV-B light, plants were sown on MS plates and grown with a 24-h light cycle under cool-white lamps, supplemented with UV-B light using a medium wave UV-B lamp (Spectronics, Westbury, NY; peak intensity 312 nm) filtered through three sheets of cellulose acetate (Golden State Plastics, Sacramento, CA; thickness 0.12 mm) to eliminate the UV-C (<280 nm) fraction of emitted light. The cellulose acetate was replaced every 3 d. The dose rate of UV-B was 2.5 J/m². For acute doses of UV-B, 5-d-old seedlings were irradiated with a UV trans-illuminator (Fisher, Houston, TX), peak output at 305 nm, filtered through a single cellulose acetate sheet. UV-B intensities were measured with a UV-B radiometer with a peak response at 310 nm and a 50% response at 280 and 340 nm (UV Products, San Gabriel, CA). For γ-radiation experiments, Arabidopsis seeds and plants were irradiated using a Cs source (Institute of Toxicology and Environmental Health, University of California, Davis). Seeds were imbibed in water at 4°C for 3 d, irradiated, and then immediately placed on MS agar plates for germination and placed in the growth chamber; plants were grown on MS plates (for 5 d), irradiated, and then immediately returned to the growth chamber.

**Preparation of RNA and RNA Gel Blotting**

Total RNA was prepared from 7-d-old seedlings using an RNeasy plant mini kit (Qiagen, Valencia, CA). RNA was blotted onto positively charged nylon membranes (BriteStar-Plus; Ambion, Austin, TX) and probed with a 32P radioactively labeled cDNAs. This 761-bp fragment was generated with PCR primers RNR1 (5'-AATGAGTGAGGAACTCTC-3') and RNR3 (5'-CTCAAGCACAATTA-GCGTAG-3'). The resulting bands were visualized on a Storm 860 PhosphorImager and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Semiquantitative RT-PCR**

Wild-type and atr Arabidopsis plants were sown on MS agar plates, grown for 5 d, then transferred to MS plates (n.t.). MS plates plus 1 mM HU (+HU), or MS plates plus 12 μg/mL of aphidicolin (+Aph) for 4 d of additional growth. All plants were verified for their respective phenotypes described above. Approximately 1 to 2 mm of root tips, from >200 individual plants of each treatment, were excised and pooled together for RNA extraction (RNeasy mini-prep; Qiagen). RNA samples were DNase treated (DNase set; Qiagen) and quantitated. To produce cDNA for RT-PCR, 2 μg of total RNA from each condition described above was reverse transcribed, employing random hexamer primers, with a Superscript first-strand synthesis kit (Stratagene, La Jolla, CA) in a 40-μL reaction according to the manufacturers protocol. RT-PCR was performed in 50 μL reactions using Ex-Taq polymerase (Takara Bio, Otsu, Shiga, Japan), 2 μL of cDNA, and the following gene-specific primers: elf4A-1 (5'-CTCTTGCAACTTCTGGCTTCTTCTTT-3'), elf4A-5 (5'-TCATAGATCTGGTCTGGAAAC-3'), CycB1-2 (5'-GTCCGCTTTCT-TCTTAGAAGCTCTTCT-3'), CycB1-6 (5'-GCGCTCATTACACTCTCAAA-CAG-3'), H4-5 (5'-ATGCTGGTCTGGAAAGGGA-3'), H4-8 (5'-ACGAATGGTCGTTTCCATAG-3'), cATR-1 (5'-GACGGCCAAAAGAATCATT3'), and cATR-3 (5'-GCGCCCGTGAAAGTGGTGTTT-3'). All primers (except Histone H4) spanned intron-exon boundaries to eliminate the possibility of amplifying contaminating genomic DNA. PCR-cycle parameters for all primer pairs were 20 s at 94°C, 20 s at 62°C, and 45 s at 72°C. Primer pairs were first tested for log-linear amplification using 18, 21, 24, 27, and 30 cycles of PCR. The optimized number of cycles for each primer pair was: elf4A, 23 cycles; cycB1, 27 cycles; Histone H4, 29 cycles; and ATR, 27 cycles. PCR products from ethidium bromide-stained gels were quantified using ImageQuant software as above.

**GUS and DAPI Staining**

GUS staining was performed as described (Jefferson et al., 1987; Preuss and Britt, 2003). DAPI staining was performed by fixing roots in a solution (Keegan et al., 1996; Moens et al., 1999). Further studies using atr and atm single and double mutant plants will allow us to better understand the role of ATR in plant meiosis, possibly leading to additional insights into ATR function during animal meiosis.
of ethanol and acetic acid (3:1). The roots were then washed twice in PBS, pH 7.4, and stained in PBS containing 2.5 μg/ml of DAPI (Molecular Probes, Eugene, OR) for 20 min. The roots were then washed in PBS two times and mounted on slides in 50% glycerol. Slides were visualized on a Zeiss Axioshot microscope, and photographs were taken with a Zeiss MC100 camera. DAPI staining was visualized with a standard UV fluorescence filter set.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AB040133 and AF092841.

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ATR Regulates a G2-Phase Cell-Cycle Checkpoint in *Arabidopsis thaliana*

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