Ribonucleotide Reductase Regulation in Response to Genotoxic Stress in Arabidopsis[1][W]

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Ribonucleotide reductase (RNR) is an essential enzyme that provides dNTPs for DNA replication and repair. Arabidopsis (*Arabidopsis thaliana*) encodes three *AtRNR2-like catalytic subunit* genes (*AtTSO2*, *AtRNR2A*, and *AtRNR2B*). However, it is currently unclear what role, if any, each gene contributes to the DNA damage response, and in particular how each gene is transcriptionally regulated in response to replication blocks and DNA damage. To address this, we investigated transcriptional changes of 17-d-old Arabidopsis plants (which are enriched in *S*-phase cells over younger seedlings) in response to the replication-blocking agent hydroxyurea (HU) and to the DNA double-strand break inducer bleomycin (BLM). Here we show that *AtRNR2A* and *AtRNR2B* are specifically induced by HU but not by BLM. Early *AtRNR2A* induction is decreased in an *atr* mutant, and this induction is likely required for the replicative stress checkpoint since *rnr2a* mutants are hypersensitive to HU, whereas *AtRNR2B* induction is abolished in the *rad9-rad17* double mutant. In contrast, *AtTSO2* transcription is only activated in response to double-strand breaks (BLM), and this activation is dependent upon AtE2Fa. Both TSO2 and E2Fa are likely required for the DNA damage response since *tso2* and *e2fa* mutants are hypersensitive to BLM. Interestingly, *TSO2* gene expression is increased in *atr* versus wild type, possibly due to higher *ATM* expression in *atr*. On the other hand, a transient ATR-dependent *H4* up-regulation was observed in wild type in response to HU and BLM, perhaps linked to a transient *S*-phase arrest. Our results therefore suggest that individual *RNR2-like catalytic subunit* genes participate in unique aspects of the cellular response to DNA damage in Arabidopsis.

In the first step of the DNA damage response, DNA lesions or replication inhibition must be detected. In mammals, activation of this response involves at least two master regulatory kinases, ataxia telangiectasia mutated (ATM) and ATM and Rad3 related (ATR), which have specific functions in response to genotoxic stress (McGowan and Russell, 2004). ATR is required for initiation of replicative stress response, and is activated by single-stranded DNA, present at stalled replication forks or persisting repair intermediates. In contrast, ATM plays a major role in response to DNA double-strand breaks (DSBs), as ATM is directly activated by protein bindings to broken DNA ends. Though DNA damage pathways are conserved among eukaryotes, the transcriptional response induced by genotoxins is primarily regulated in yeast (*Saccharomyces cerevisiae*) by the ATR ortholog MEC1, whereas this response is primarily ATM dependent in mammals (Elkon et al., 2005). Similarly, the DSB transcriptional response is regulated by ATM in Arabidopsis (*Arabidopsis thaliana*), as determined by complete transcriptome analyses (Culligan et al., 2006; Ricaud et al., 2007), while the ATR-mediated response to replicative stress was only partially characterized (Culligan et al., 2004). However, these experiments employed very young Arabidopsis plantlets ranging from 5 to 7 d postgermination (Culligan et al., 2004, 2006; Ricaud et al., 2007). The DNA damage response is also controlled by checkpoint proteins that lead to specific cell-cycle arrests as well as changes in the chromatin structure at the site of DNA damage. For instance, Arabidopsis ATR regulates a G2-phase cell-cycle checkpoint, in response to DNA damage and replication inhibitors (Culligan et al., 2004). In addition, the

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replication inhibitor hydroxyurea (HU), which inhibits ribonucleotide reductase (RNR)-dependent production of dNTPs for DNA synthesis, appears to induce a novel G1 checkpoint in 5-d-old plantlets (Culligan et al., 2004). Other checkpoint proteins were also identified in Arabidopsis, such as AtRAD17 and AtRAD9 (Heitzeberg et al., 2004) that are epistasic in the DSB response. An ATM-dependent transcriptional regulation of *AtRAD17* was also shown in response to γ-irradiation (Culligan et al., 2006; Ricaud et al., 2007).

RNR regulation is of particular interest since it provides the dNTP pool needed for DNA replication and DNA repair. RNR is a heterodimeric enzyme composed of two R1 regulatory and two R2 catalytic subunits. Eukaryotic cells have developed several surveillance mechanisms to regulate RNR activity in response to genotoxic stress to ensure balanced dNTP pools for high-fidelity DNA repair. In yeast, the two genes encoding the catalytic subunits (*RNR2* and *RNR4*) as well the gene encoding the regulatory subunit (*RNR3*) are induced through Mec1-dependent Rad53 signaling in response to DNA damage (Elledge et al., 1993; Huang and Elledge, 1997; Mulder et al., 2005; Fu and Xiao, 2006). Mammals also express *R2* and an alternative *R2* termed *p53R2*. While the former paralog is not induced by DNA damage, *p53R2* is activated by p53 in an ATM/CHK2-dependent manner (Tanaka et al., 2000).

In contrast with yeast and mammals, regulation of the small *RNR* multigene family in response to geno-

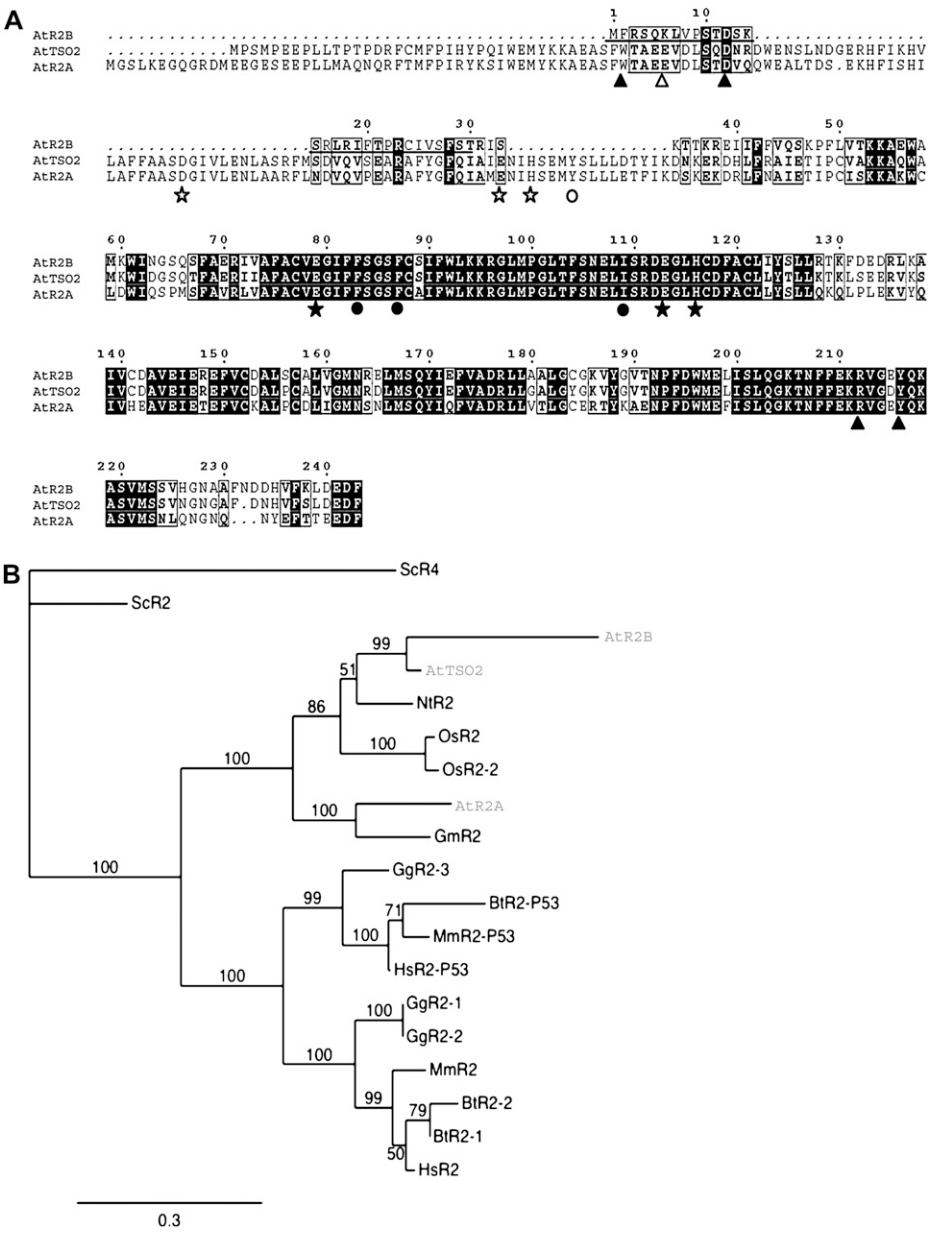

Figure 1. Characterization of the AtR2 proteins in Arabidopsis. A, Alignment of the Arabidopsis R2 proteins was performed as described in Supplemental Materials and Methods S1. Numbering of amino acids starts with the first Met of the R2B sequence. Identical amino acids are boxed in black and amino acids with similar physical properties are boxed in white. Functional sites were reported for the sequences as follows: triangles for residues involved in the interaction of R2 with R1, stars for residues required for iron binding and the subsequent generation of the (Fe)2-Y cofactor required for catalysis, and dots for the residues needed for tyrosyl radical. In R2B, R2 residues changed or lost are indicated by white symbols and a putative mitochondrial signal sequence is underlined. B, Phylogenetic tree of R2 proteins. The tree was constructed as described in Supplemental Materials and Methods S1. The scale indicates the evolutionary distance (number of substitution per site). The relevant protein sequences were downloaded from The Arabidopsis Information Resource database for Arabidopsis sequences (TSO2, AT3G27060; AtR2A, AT3G23580; AtR2B, AT5G40942), from Genpept for yeast (ScR2, AAA34988; ScR4, AAB72236), tobacco (NtR2, CAA63194), mouse (MmR2, NP_033130.1; MmR2-P53, NP_955770.1), *B. taurus* (BtR2-1, XP_584910.2; BtR2-P53, XP_607398.2; BtR2-2), *Gallus gallus* (Gg, GgR2-1 ENSGALP00000030966, GgR2-2 ENSGALP00000026474, GgR2-3 ENSGAL-P000000258083), rice (OsR2, BAD46182.1; OsR2-2, NP_00105668.1), human (HsR2-P53 NP_056528; HsR2 NP_001025), and *Glycine max* (GmR2, AAD32302.1).

toxic stress in plants is not yet fully understood. For example, among the three Arabidopsis genes encoding the small subunit (*AtTSO2*, *AtRNR2A*, and *AtRNR2B*; Wang and Liu, 2006), *AtTSO2* was shown to be strongly induced by ionizing radiation (IR; Culligan et al., 2006; Ricaud et al., 2007) or bleomycin (BLM) plus mitomycin C (MMC; Chen et al., 2003). By contrast, *AtRNR1* encoding the large subunit is up-regulated in the DSB response and upon UV-B irradiation (Culligan et al., 2004, 2006; Ricaud et al., 2007). *NtRNR1a* and *NtRNR1b* genes are induced by HU in proliferating tobacco (*Nicotiana tabacum*) cells but are differentially expressed with a high and low induction of *NtRNR1a* and *NtRNR1b*, respectively (Chabouté et al., 2002; Lincker et al., 2004). In addition, *NtRNR1a* is also up-regulated by UV-C, and E2F cis-elements present on its promoter are important to drive its specific induction (Lincker et al., 2004). However, functional studies showing the direct implication of E2F in the RNR DNA damage response have not yet been demonstrated in Arabidopsis. In addition to the partial characterization of *AtRNR* gene response to DNA damage, recent data showed that the *tso2-rnr2a* double mutant displays genomic instability with selective induction of DNA repair genes, and is hypersensitive to UV-C (Wang and Liu, 2006). However no clear link was established between RNR induction and DNA damage signaling.

The aim of this article is to characterize the *RNR* gene response to the replication-blocking agent HU and the DSB inducer BLM in plants at 17 d postgermination, expressing high levels of the *S*-phase H4 marker gene. Based on our results, we provide (1) evidence for a specific induction of *AtRNR* genes with respect to genotoxins, (2) functional analyses of *rnr* mutants linked to specific sensitivity to genotoxins, and (3) evidence for the *AtTSO2* DNA damage response controlled by AtE2Fa. In addition, we highlight a differential *AtTSO2* DSB response in the *atr* mutant, which is dependent upon growth stage and *H4* histone gene expression.

RESULTS

The *RNR* Gene Family in Arabidopsis: *RNR2* Gene Diversity Is Conserved through Evolution

The diversity of *RNR2* genes in mammals and yeast is linked to specific gene expression in response to genotoxins. To determine the evolutionary link between R2 proteins, we conducted a phylogenetic analysis using Arabidopsis, yeast, and mammal R2 proteins.

Arabidopsis (var. Columbia, ecotype Columbia-0 [Col-0]) genome contains three *RNR2* genes, *AtTSO2* (At3g27060), *AtRNR2A* (At3g23580), and *AtRNR2B* (At5g40942), encoding R2 catalytic subunits (TSO2, R2A, and R2B; Wang and Liu, 2006). However only one *RNR1* gene, termed *AtRNR1* (At2g21790), encodes the R1 regulatory subunit. Alignment of the R2-encoded proteins revealed that AtR2B is truncated in the N-terminal region and some residues involved in the catalytic function of the enzyme are missing compared to AtR2A or AtTSO2 (Fig. 1A). Among the 15 amino acids important for enzyme function (Chabouté et al.,

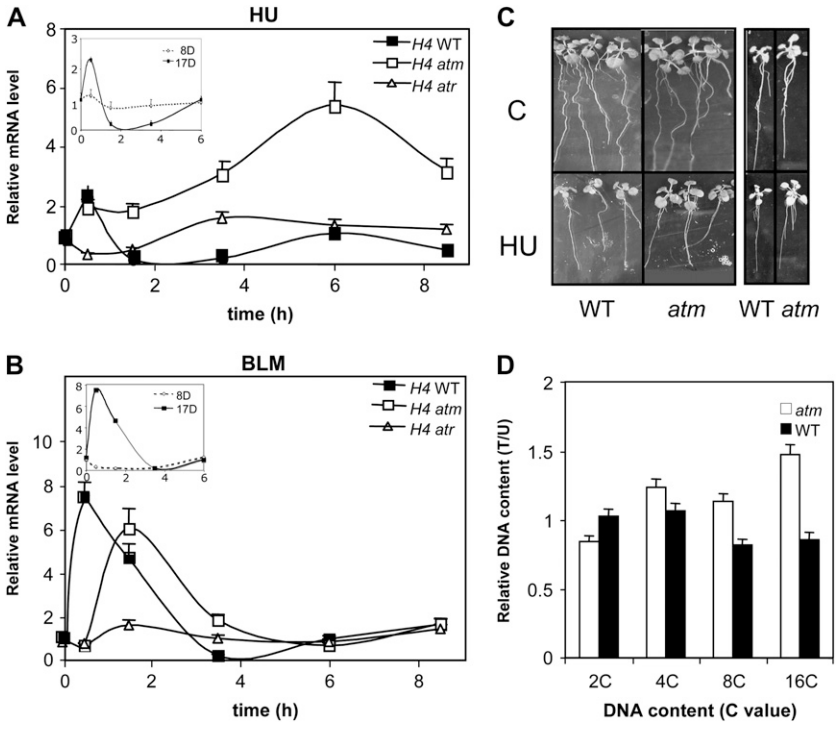

Figure 2. *AtH4* expression in response to genotoxins. Seventeen-day-old plantlets were either treated with HU (1 mM; A) or BLM (10^{-6} M; B). Relative mRNA levels (treated/nontreated) were evaluated using *18S* as a standard. Analyses were performed in wild type (WT), *atm*, and *atr* at different time points during genotoxic treatment. SDS are indicated. Small inset graphs were included for comparison of *H4* expression between 8- and 17-d-old wild-type plantlets. C, Wild type and *atm* were grown on Murashige and Skoog medium during 8 d and transferred to medium without (control: C) or with HU (1 mM) and left to grow for 8 d. Two independent experiments are presented. D, Relative DNA content in 17-d-old *atm* and wild-type plantlets untreated (U) or treated (T) with HU (1 mM).

1998) highlighted in Figure 1A, five residues are either modified into nonconservative amino acids or absent in the N-terminal half of R2B: one residue (white triangle) involved in the association with R1, three residues (white star) required for iron binding and the subsequent generation of the $(Fe)_2$-Y cofactor required for catalysis, and one residue (white dot) needed for tyrosyl radical. Similarly in yeast R4 (Wang et al., 1997), five functional residues are changed: one residue involved in the interaction with R1, three residues involved in the iron center, and one residue providing the tyrosyl radical (Huang and Elledge, 1997).

Arabidopsis R2 proteins were phylogenetically compared to other known R2 proteins (Fig. 1B). These proteins are divided into two families, one with R2B and TSO2 proteins, and the other with R2A protein. In rice (*Oryza sativa*), two *R2* genes have been identified belonging to the same family as for the two R2 proteins (R2 and R4) in yeast (*Saccharomyces cerevisiae* [Sc]; Wang et al., 1997). In contrast, in mammals (*Bos taurus* [Bt], mouse [Mm], and human [Hs]), the two R2 proteins (R2-R2p53) have diverged into two separate families (Tanaka et al., 2000). Even in the same family, significant divergence is apparent between R2 members as for R2B and TSO2 in Arabidopsis or R2 and R4 in yeast.

Phenotypic Characterization of the Response to Genotoxic Stress

We previously showed that *RNR* gene expression is primarily induced during *S*-phase where DNA replication occurs (Chabouté et al., 2000, 2002). According to this, we investigated whether there are differential *AtRNR* responses to DNA damage in plantlets enriched in *S*-phase cells, characterized as having high

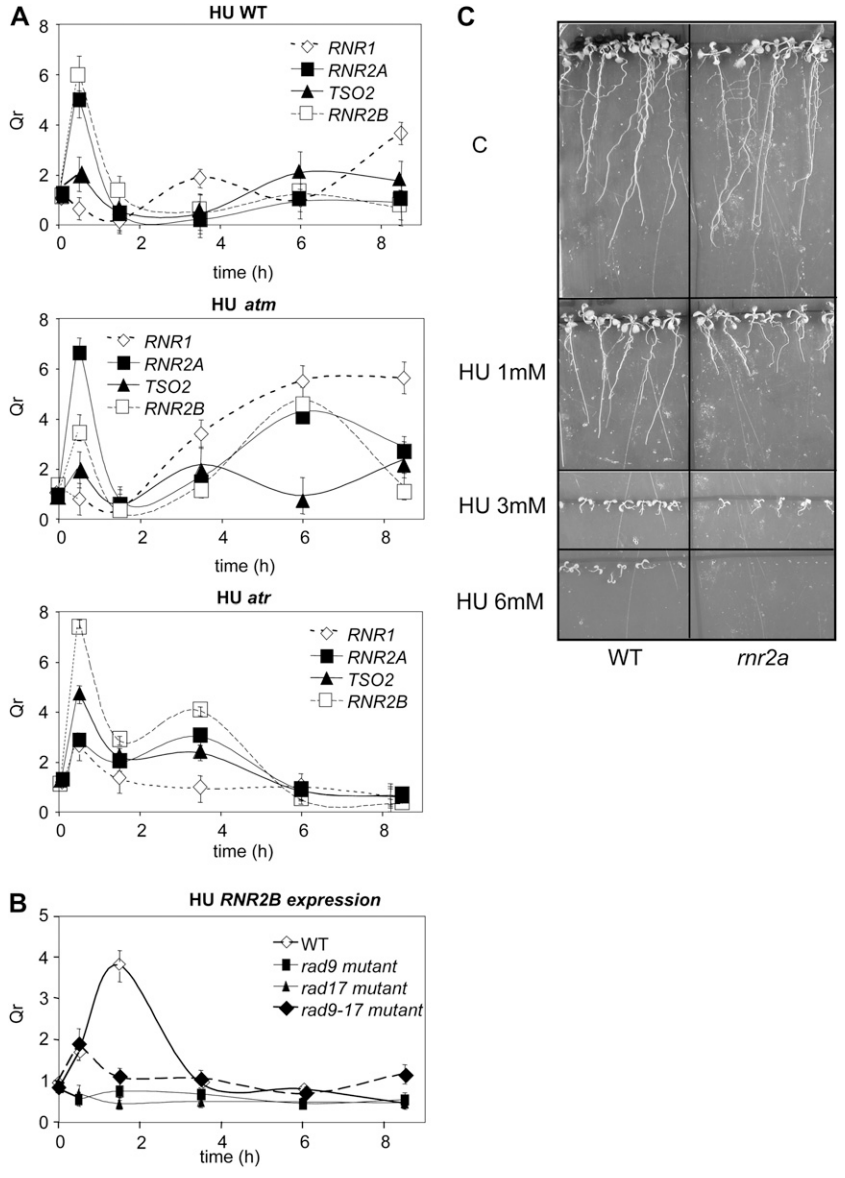

Figure 3. *RNR* gene response to HU in Arabidopsis plantlets. A, Seventeen-day-old plants treated with HU (1 mM). Gene expression was evaluated in wild type (WT), *atm*, and *atr* at different time points during genotoxic treatment. Relative expression of the four *AtRNR* genes (*AtRNR1* [white diamonds], *AtTSO2* [black triangles], *AtRNR2A* [black squares], *AtRNR2B* [white squares]) was quantified by RT-quantitative PCR (as described in Supplemental Materials and Methods S1) performed on plantlets RNAs. B, HU response of *AtRNR2B* performed as in A in wild type (white diamonds), *rad9* (black squares), *rad17* (black triangles), and *rad9-17* mutants (black diamonds). SDS are indicated. C, Test of hypersensitivity of the *rnr2a* and wild-type plantlets to increasing concentrations of HU (1, 3, and 6 mM). Plants were compared 15 d after germination on HU versus control plants (C).

H4 histone gene expression (Reichheld et al., 1995, 1998; Meshi et al., 1998). Studies of the DNA damage response in Arabidopsis plants have typically only included young seedlings (Culligan et al., 2004, 2006; Molinier et al., 2005; Ricaud et al., 2007) in which *H4* gene expression was considerably lower than in older plantlets (17-d-old plantlets; Supplemental Fig. S1A). In this plant developmental context, where endoreduplication level was also higher (Supplemental Fig. S1B), we wanted to determine if *H4* gene expression is affected by genotoxins. To address this, we analyzed *H4* (At5g59970) mRNA levels in 17-d-old plants treated with HU or BLM. HU blocks DNA replication by inhibiting RNR-dependent production of dNTPs required for DNA synthesis, while BLM primarily induces DSBs through generation of oxidative damage. The relative mRNA levels (treated versus untreated plants) were evaluated by semiquantitative PCR, using *18S* RNA as a standard. For comparison, we tested if these expression pattern modulations were similar in *atm* and *atr* to determine ATM- and ATR-dependent effects on the cell cycle gene.

HU treatment of wild-type plants (Fig. 2A), resulted in a rapid *H4* gene induction (0.5 h, 2.3-fold) that was reduced in *atr* (Fig. 2A), but not in *atm* where expression increased continuously until 8 h. Interestingly, HU (1 mM) sensitivity tests revealed no difference in the root growth between wild type and *atm*, but enhanced leaf development was observed in *atm* (Fig. 2C). This latest phenotype may be accounted for by a stimulation of endoreduplication processes in the leaves of HU-treated *atm* plants due to the continuous transcriptional activation of H4 that we observed (Fig. 2A). To test this hypothesis, we analyzed endoreduplication level in the leaves of wild-type and *atm* plants treated with HU or untreated. Indeed, fluorescence-activated cell sorting analyses revealed a higher relative DNA content (treated versus untreated plants) in *atm* compared to wild-type plants, notably for the 16C DNA content (Fig. 2D). However, this result was never observed in 8-d-old plantlets (data not shown).

Seventeen-day-old wild-type plants treated with BLM (Fig. 2B) showed a strong induction of *H4* gene expression (7.5-fold) after 0.5 h. This induction was delayed in *atm* and considerably reduced in *atr*. By contrast, we never observed any up-regulation of *H4* gene upon HU or BLM treatments in younger plantlets (8-d-old; Fig. 2, A and B, small inset graphs). We suggest that this differential response is linked to plant development, with a lower level of *H4* gene expression in 8-d-old plantlets compared to 17-d-old plantlets without genotoxins. Taken together, our results highlight an early transient *H4* induction in response to HU and BLM treatments and that this induction is ATR dependent.

RNR Replicative Stress Response

Similar to the regulation of *H4*, *AtRNR2A*, and *AtRNR2B* displayed an early induction (0.5 h, approximately 5- to 6-fold) in HU-treated wild-type plants (Fig. 3A). In contrast, no *AtTSO2* induction was observed whereas *AtRNR1* induction was delayed (>8 h).

In HU-treated *atm* plants, only the early induction of *AtRNR2B* was decreased, showing that functional ATR cannot maintain the maximal induction observed in wild-type plants. However, *AtRNR2A*, *AtRNR2B*, and *AtRNR1* displayed a late reinduction (6 h) that was lost in *atr* (Fig. 2A). This late HU-mediated induction may reflect *S*-phase-specific gene transcription for endoreduplication process controlled by ATR in the absence

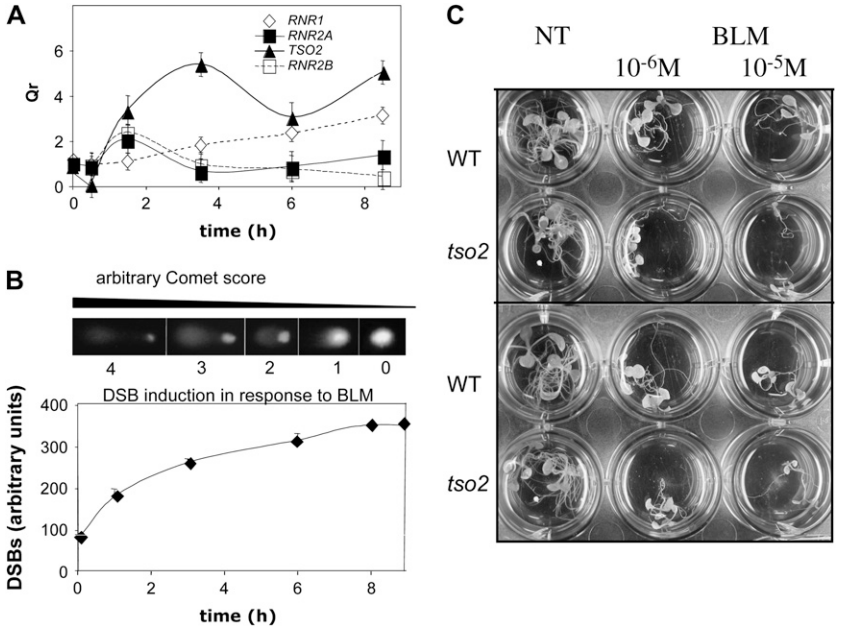

Figure 4. BLM response of *AtRNR* genes. A, Expression of *RNR* genes in 17-d-old wild-type plants treated continuously with BLM (10^{-6} M). Relative expression of the four *AtRNR* genes (*AtRNR1* [white diamonds], *AtTSO2* [black triangles], *AtRNR2A* [black squares], *AtRNR2B* [white squares]) was quantified by RT-quantitative PCR (as described in Supplemental Materials and Methods S1) performed on plantlet RNAs. SDS are indicated. B, Quantification of DSBs in Arabidopsis plantlets by neutral comet assay in response to BLM (10^{-6} M). DNA damage arbitrary units were used for each experiment. C, BLM sensitivity test performed with wild type and *tso2*. Eight-day-old plantlets were transferred to liquid Murashige and Skoog media containing 10^{-6} and 10^{-5} M BLM and allowed to grow for an additional 8 d. Two independent experiments are presented. WT, Wild type.

of ATM, as previously suggested for *AtH4* gene regulation.

In contrast, the early induction of *AtRNR2A* was decreased in *atr* when grown on HU. To understand the physiological relevance of *RNR2A* induction, we analyzed the hypersensitivity of the *rnr2a* mutant to HU. The mutant plants grew slower compared to wild type at low HU concentration (1 mM) and died or did not germinate at higher concentrations (3 and 6 mM, respectively; Fig. 3C), demonstrating the importance of R2A in the replicative stress response in Arabidopsis. Since HU-dependent *AtRNR2B* induction was not affected in *atr* (Fig. 2A), we investigated the role of the AtRAD9 and AtRAD17 checkpoint proteins, which we showed to be involved in the replicative stress response according to their high sensitivity to HU (Supplemental Fig. S2A). HU *AtRNR2B* induction was lost in the single *rad9* and *rad17* mutants as well as in the *rad9/rad17* double mutant (Fig. 3B), highlighting the RAD9/RAD17-mediated induction of *AtRNR2B* in the replicative stress response. In contrast, *AtTSO2* was up-regulated in *atr*, possibly through a transcriptional derepression.

These data suggest that each subunit employs a unique expression pattern in response to the HU-induced *S*-phase checkpoint, and this expression is sometimes dependent upon functional ATR, ATM, or RAD9/RAD17.

RNR BLM Response

Although neither *AtRNR2A* nor *AtRNR2B* were induced in wild-type plants treated with BLM (Fig. 4A), we observed under the same conditions a significant *AtTSO2* induction from 1.5 to 8.5 h of treatment. The *AtRNR1* gene was also induced at 6 to 8.5 h, but to a lesser degree than *AtTSO2*, and displayed different kinetics of induction. Although *AtTSO2* induction was maximal at 3.5 and 8.5 h of the BLM treatment, this could reflect differences in the time course of DSB induction. To determine this, we employed a neutral comet assay (Fig. 4B). This assay shows that generation of DSBs increase exponentially up to about 3.5 h, and reaches a plateau to approximately 8.5 h.

Similar to γ-irradiated young plantlets (5- to 8-d-old; Culligan et al., 2006; Ricaud et al., 2007), an ATM dependency of *AtTSO2* and *AtRNR1* gene expression was observed in 17-d-old BLM-treated plants (Sup-

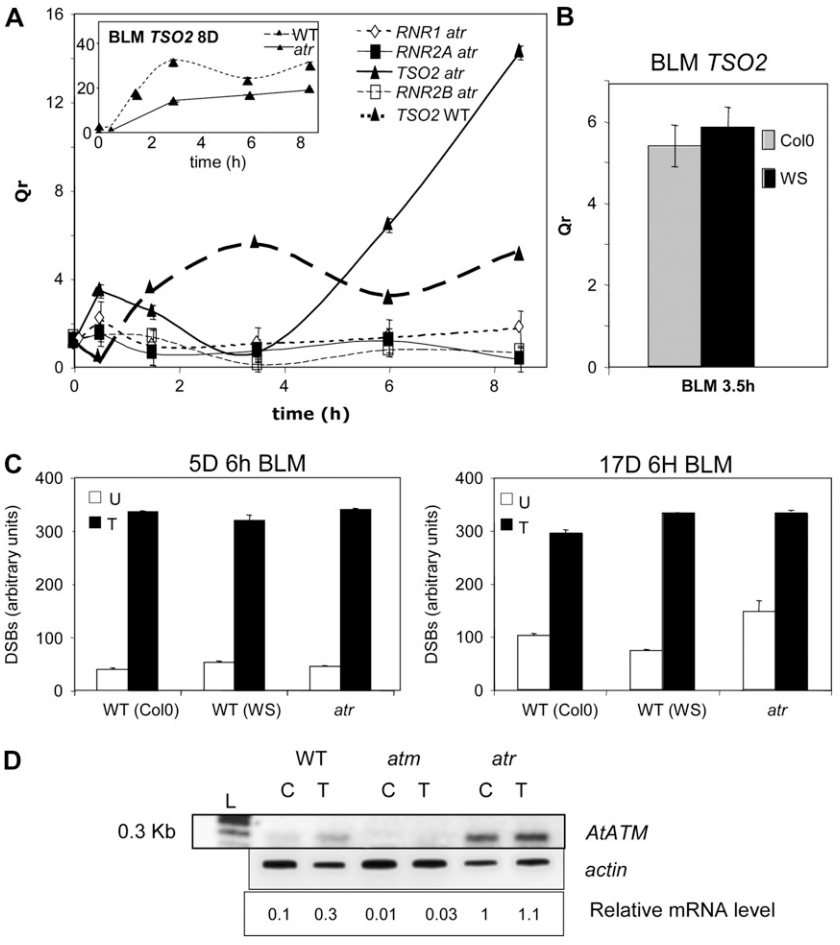

Figure 5. Specific BLM response of *TSO2* in *atr*. A, *AtRNR* gene expression in *atr* plants treated with BLM. *TSO2* expression in wild type (WT) and *atr* from 8-d-old plantlets is shown for comparison in the inset graph. B, *TSO2* expression evaluated after a 3.5-h BLM treatment in 17-d-old wild-type plants (Col-0 and Ws ecotypes). SDS are indicated. C, DSBs were quantified in 5- and 17-d-old plantlets in untreated plants (U) or plants submitted to a 6-h BLM treatment (T). Three independent experiments were performed and SDS are indicated. All differences were considered significant when $P < 0.05$. D, *AtATM* expression was evaluated by semiquantitative PCR in 17-d-old plantlets treated (T) or not (C) with BLM. Experiments were performed in wild-type, *atm*, and *atr* plants. DNA ladder (L) is indicated as well as the size of the expected amplicon. Actin was used as a standard and relative *AtATM* mRNA level is indicated.

plemental Fig. S2B). To demonstrate that *TSO2* induction is related to its involvement in the DSB response, we analyzed the sensitivity of *tso2* mutants to BLM. Their sensitivity was higher compared to wild-type plants as BLM concentration was increasing (Fig. 4C). Therefore the specific *TSO2* up-regulation induced by BLM suggests that TSO2 is involved in the response to DSBs.

Specific BLM-Induced Expression of *TSO2* in *atr*

In *atr* plants treated with BLM, *TSO2* displays biphasic gene induction both early (0.5 h) and late (6–8.5 h; Fig. 5A). This late up-regulation was significantly (3-fold) higher than in wild type (Fig. 5A), and interestingly was never observed in younger material (5- to 8-d-old) treated with BLM (Fig. 5A, left border) or γ-irradiated (Culligan et al., 2006; Ricaud et al., 2007). Although the *atr* mutation is in a different wild-type ecotype background (Wassilewskija [Ws]), the maximal *AtTSO2* induction upon BLM treatment was similar in wild-type Col-0 and wild-type Ws (Fig. 5B).

To explain the DSB response in *atr*, we hypothesize that more DSBs may occur in 17-d-old plants compared to younger seedlings. Therefore, we quantified the DSBs in wild-type and *atr* plantlets from 5 and 17 d postgermination using the neutral comet assay. In the absence of BLM (Fig. 5C), we observed no DSB content difference between wild type and *atr* in 5-d-old plantlets but a significant DSB increase (at least 1.5 more) was revealed in *atr* compared to wild type (Col-0 and Ws) in 17-d-old plants. This observation suggests that more genomic instability occurs in *atr* at this developmental stage without BLM treatment. After a 6-h BLM treatment, no significant difference was observed between wild type and *atr* either in 5- or 17-d-old plants (Fig. 5C), perhaps due to a saturated response from large amounts of DSBs generated. As *AtTSO2* induction is ATM dependent, we checked if this induction in *atr* may be due to *ATM* up-regulation. Indeed, a

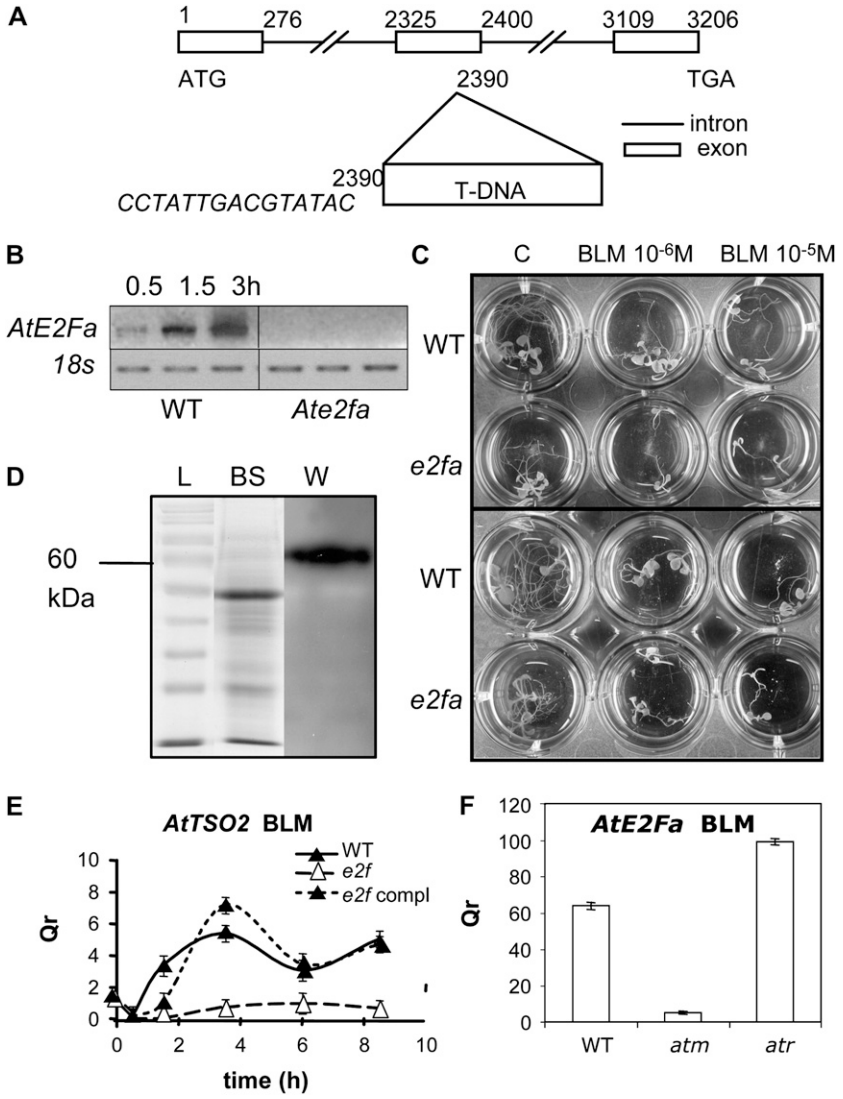

Figure 6. Analysis of the *Ate2fa* mutant. A, Schematic representation showing the position of the T-DNA at the *AtE2Fa* locus. Open rectangles represent the first (1) and last (13) exons of the gene as well as exon (10) where the T-DNA insertion occurred. Connecting lines represent introns. ATG and STOP codons are indicated and exons borders are numbered according to their location in the gene. The sequence flanking the left border (left flanking sequence tag) of the T-DNA is indicated in italics. B, Wild-type (WT) and *e2fa* plantlets were treated with BLM (10^{-6} M) for 0.5, 1.5, and 3 h. *AtE2Fa* expression was evaluated using primers E2FaFW2/RW2. *18S* was used as a standard. C, Analysis of the hypersensitivity to BLM for *e2fa* and wild-type plantlets. Eight-day-old plantlets were grown in presence of BLM (10^{-6}, 10^{-5} M) for an additional 8 d. C are control plants. Two independent experiments are presented. D, Analysis of the rescued mutant *e2fa* overexpressing an Etag fusion of AtE2Fa. Homozygous lines were selected and western experiments were performed on plant extracts using an anti-Etag antibody (lane W). Blue staining of the gel is presented (BS) as well as a M_r ladder (L). E, *AtE2Fa* expression was analyzed in response to BLM (10^{-6} M) by RT-quantitative PCR in wild type (thick line), *e2fa* mutant (broken line), and rescued mutant (dotted line). F, *AtE2Fa* expression in wild-type, *atm*, and a*tr* plantlets in response to a 6-h BLM treatment. SDS are presented. Specific primers used in the experiments as well as *E2Fa* genomic positions of the primers used to genotype the *e2fa* mutant are indicated in the Supplemental Table S1.

significant induction of ATM was observed in control (C) or BLM-treated *atr* plants (T) from 17 d postgermination (Fig. 5D), but not in younger plantlets (data not shown). Taken together these data suggest that genomic instability is increased in *atr* likely due to an up-regulation of *AtATM* in 17-d-old plantlets. Compared to *atr* control plants, the level of *ATM* mRNA is considerably lower in control wild-type plants but in these plants, a 3-fold induction was observed in response to BLM. This may explain the discrepancy observed in the up-regulated expression of *TSO2* between *atr* and wild type upon BLM treatment.

AtTSO2 Induction Is Controlled by AtE2Fa in the ATM-Mediated BLM Response

We determined the transcriptional regulation of *AtTSO2* and observed similar increased levels between *AtTSO2* promoter activity (2-fold) and *AtTSO2* mRNA (Qr = 3, Fig. 4A) after a 1.5-h BLM treatment (Supplemental Fig. S3D), suggesting that *AtTSO2* is regulated at the transcriptional level in the DSB response. It has been shown that *AtE2Fa* was induced after BLM plus MMC treatment (Chen et al., 2003) and therefore this transcription factor may be a good candidate for controlling *AtTSO2* gene induction in response to BLM treatment.

To test this, we analyzed a T-DNA insertion line for the gene *AtE2Fa*. The T-DNA insertion occurs in exon 10 of the gene and the sequence of the left-border flanking sequence tag given by GABI-Kat GenBank was confirmed by sequencing the PCR product (Fig. 6A). Southern analysis showed only one T-DNA insertion that contained a deletion of approximately 700 bp on the right border (data not shown). To confirm that we had null mutant lines, *AtE2Fa* gene expression was analyzed by reverse transcription (RT)-PCR in a homozygous mutant compared to the wild type in 17-d-old plantlets: No expression was observed in the mutant line (Fig. 6B, Supplemental S3A), whereas *AtE2Fa* induction increased until 3 h in the wild type. Homozygous *e2fa* $-/-$ plants presented no obvious growth phenotype, but when treated with BLM (10^{-6}, 10^{-5} M), they proved more sensitive to BLM than the wild type (Fig. 6C). Besides their BLM sensitivity, *e2fa* mutants failed to show any induction of the *AtTSO2* gene upon BLM treatment (Fig. 6E). To determine that the lack of *TSO2* induction is due to the E2Fa mutation, we genetically complemented the T-DNA insertion line with a TAG (Etag) fusion of AtE2Fa. A protein of the correct expected size was detected in western experiments using an antibody directed against the Etag epitope (Fig. 6D). In the complemented mutant (showing a BLM sensitivity similar to wild type; Supplemental Fig. S3B), *AtTSO2* expression was rescued in response to BLM (Fig. 6E). This demonstrates the E2Fa-mediated *AtTSO2* induction in response to BLM, probably through binding of the E2Fa transcription factor on its target cis-elements present on the *TSO2* promoter (Supplemental Fig. S3C). In addition, *AtE2Fa* gene induction was lost in *atm* upon BLM treatment but increased in *atr* (Fig. 6F). Thus, our data suggest that the ATM-mediated transcriptional activation of *AtE2Fa* is needed to regulate the cellular response to DSBs.

DISCUSSION

AtRNR2 Genes Are Differentially Expressed in Response to Genotoxins

We have shown here a differential transcriptional response of the three *AtRNR2* genes: *AtTSO2* is only induced by DSBs and therefore may constitute a transcriptional marker of the DSB response. However, *AtRNR2A* and *AtRNR2B*, which are induced in response to HU but not DSBs, represent transcriptional markers of replicative stress (Fig. 3A). As HU is a direct inhibitor of RNR, it is possible that a simple feedback regulation mechanism, independent of single-stranded DNA induction, may occur. The physiological response of *rnr2a* to HU but not to BLM (data not shown) suggests activation of replicative stress signaling. In contrast, the hypersensitivity of *tso2* to BLM but not to HU (data not shown) indicates activation of DSB signaling. A differential gene expression was also observed for *RNR1* genes in response to DNA damage in tobacco. Indeed, among the *NtRNR1* small multigenic family, we showed a strong induction of the *NtRNR1a* gene in response to HU compared to *NtRNR1b* (Chabouté et al., 2002; Lincker et al., 2004).

Ultimately, it appears that through evolution, *RNR* genes have evolved to fulfill specific functions, notably in DNA repair. Indeed, *p53R2* was shown to be induced by both UV and γ-radiation in humans (Tanaka et al., 2000). Alternatively in yeast, induction of *RNR2* and *RNR4* genes was observed upon various stresses such as γ-radiation (Gasch et al., 2001), HU, and UV (Aboussekhra et al., 1996; Huang and Elledge, 1997). Our data suggest that the specific induction of *AtRNR2* genes in response to genotoxic stress, may suggest that

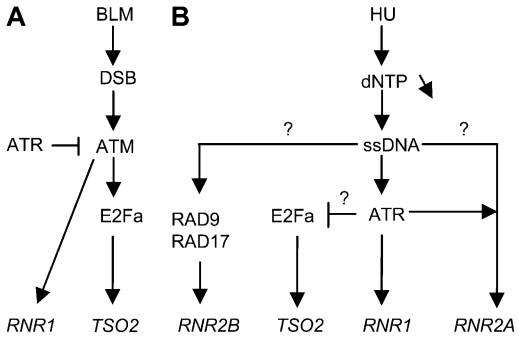

Figure 7. Model of *AtRNR* gene regulation in response to genotoxins BLM (A) and HU (B) in 17-d-old plantlets. Arrows indicate activation, whereas T-bars represent repression.

these genes have unique roles in Arabidopsis DNA repair.

The Transcription Factor AtE2Fa Is Regulated by ATM and ATR in the DNA Damage Response

Through evolution, *RNR* gene expression is tightly controlled in response to DNA damage. In yeast, *RNR* induction is achieved through derepression of CRT1 and CRT10 under the control of the MEC1/DUN1 pathway in response to IR (Huang et al., 1998; Fu and Xiao, 2006). In mammals, some DNA repair genes are controlled by the p53 transcription factor: through *RAD51* repression to regulate homologous recombination (Arias-Lopez et al., 2006) or *p53R2* induction to produce dNTPs (Tanaka et al., 2000). More recently, the E2F7 and E2F8 transcriptional repressors were shown to act upstream of E2F1, thereby influencing the capacity of cells to initiate a DNA damage response in mammals (Panagiotis Zalmas et al., 2008). In contrast, the AtE2Fa transcriptional activator regulates the expression of *AtTSO2* in the Arabidopsis DSB response (Fig. 6E) as well as that of a subset of DNA repair genes harboring E2F elements in their promoters such as *AtRAD51* (Supplemental Fig. S4) and *AtBRCA1* (data not shown). These E2F target genes are also coexpressed in the DNA repair network (http://atted.jp) including 16 genes such as *AtPARP1*, *AtRPA-like*, *AtPOL2a*, and *AtRAD17*, and are also ATM-dependent induced in the DSB response (Culligan et al., 2006; Ricaud et al., 2007). As for *AtTSO2*, the ATM-mediated induction of *AtE2Fa* may be required for the specific induction of these genes. In contrast, the lack of *AtTSO2* induction in the HU response in wild type might be due to the decreased *AtE2Fa* expression that we observed (Supplemental Fig. S6A). In addition, as *AtTSO2* is up-regulated in *atr* by HU, we cannot exclude a down-regulation of *AtE2Fa* mediated by ATR, perhaps leading to no *AtTSO2* induction in wild type. Similar results were obtained for the *AtFAS1* gene encoding the chromatin remodelling factor with HU treatment, with no induction in wild type but an up-regulation in *atr* (Supplemental Fig. S6B).

This reveals a diversity of mechanisms controlling *RNR* gene expression between animals and plants in response to DNA damage. Since *AtTSO2* as well as *AtRAD51* or *AtFAS1* are also cell-cycle regulated and target of AtE2Fa (Doutriaux et al., 1998; Vandepoele et al., 2005; Wang and Liu, 2006; Ramirez-Parra and Gutierrez, 2007), this may involve specific coregulators of AtE2Fa controlled by the ATM and ATR pathways in the DNA damage response.

Model of *AtRNR* Regulation Linked to Plant Growth and *H4* Gene Expression in Response to Genotoxins

In the absence of a functional ATR in 17-d-old plantlets, where *H4* gene expression is high, *AtTSO2* gene induction is considerably higher compared to wild type (Fig. 5A) as well as for *AtRAD51* (data not shown). A similar expression pattern was observed with IR in 17-d-old plants (Supplemental Fig. S5) but not in 5-d-old plants (Culligan et al., 2006; Ricaud et al., 2007). Compared to BLM, the IR response was increased and occurred earlier in the kinetics. This difference may be due to the fact that BLM is a chemical that needs to be activated before generating DSBs (Liang et al., 2002). *AtRNR1* and *AtH4* genes are also developmentally regulated in response to HU: These genes are not induced in very young plantlets (Culligan et al., 2004; Fig. 2A for H4), however are significantly up-regulated in wild-type 17-d-old plantlets (Figs. 2 and 3A), but not in *atr*. Therefore in older plants enriched in endoreduplicated cells, ATR might be important to control the replicative stress response of *AtRNR1* and *AtH4* genes.

Taken together, these results highlight a complex regulation of *RNR* genes in the ATM-ATR DNA damage network. On one hand (Fig. 7A), *AtTSO2* expression is controlled by an ATM-mediated induction of *AtE2Fa* in the DSB response that may be negatively controlled by ATR. Indeed, *TSO2* expression appears to be also repressed by ATR in the HU response, probably through the down-regulation of *AtE2Fa* (Fig. 7B). On the other hand the HU response of *AtRNR2B* is controlled by RAD9/RAD17 and *AtRNR2A* partly controlled by ATR (Fig. 7B). However, the specific HU response of *AtRNR2B* is decreased in *atm* but lost in the *rad9/rad17* double mutant, suggesting that ATM may also interfere in the replicative stress response but probably not in the same pathway as RAD9/RAD17. Since the *rnr2a* mutant is less sensitive than *atr* to HU (data not shown), R2A and ATR are probably not acting in the same pathway.

Interestingly, we observed that the lack of a functional ATM stimulates a late up-regulation of *H4* gene as well that of *RNR1*, *RNR2A*, and *RNR2B* genes in response to HU. This may be connected with a developmentally controlled program leading to enhanced endoreduplication. Such a process may require an ATR-dependent DNA replication checkpoint as recently suggested for the function of MIDGET in the topoisomerase VI complex (Kirik et al., 2007). In addition, the transient *H4* up-regulation by HU or BLM may also correspond to a transient *S*-phase arrest mediated by ATR and linked to the *S*-phase checkpoint that was never described in younger plantlets (Fig. 2, A and B). This highlights the plasticity of plants in the control of the cell cycle in response to DNA damage throughout development.

MATERIALS AND METHODS

Arabidopsis Lines and Plant Growth Conditions

Our experiments were performed in various mutants that were already characterized: the *atr-3* −/− and *atm-2* −/− null mutants (Garcia et al., 2003; Culligan et al., 2004), the *rad9-1* and *rad17-1* mutants (Heitzeberg et al., 2004), as well as the *tso2-1* −/− and *rnr2a-1* −/− ethyl methanesulfonate mutants

(Wang and Liu, 2006). Seeds were surface sterilized for 10 min in the SET solution (sodium hypochlorite solution [0.4%], ethanol [80%], and Triton X-100 [0.05%]) and rinsed twice in ethanol. Seeds were sown on nylon membrane (SEFAR NITEX 03–37/24) for a subsequent transfer to different media. Arabidopsis (*Arabidopsis thaliana*) plants were grown on Murashige and Skoog (Duchefa, MO 221), pH 5.7, 1% Suc, stabilized with 1.2% Bacto-agar (Difco) for vertical growth.

HU and BLM Treatments

Seventeen days postgerminated plantlets were used in our experiments. They were grown on Murashige and Skoog medium during 17 d and transferred to plates without (control plants) or with genotoxins (1 mM HU or 10^{-6} M BLM) for 8.5 h in growth chamber. Plants were harvested after 0.5, 1.5, 3.5, 6, and 8.5 h, then frozen in liquid nitrogen.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from plant seedlings with TRIzol (Invitrogen SARL) according to the manufacturer's instructions. After treatment by Deoxyribonuclease I (Fermentas, UAB), RNAs were stored at −80°C. One microgram of total RNA was then reverse transcribed with the Improm-II Reverse transcriptase (Promega Corporation) using random hexamers as primers.

Real-Time Semiquantitative PCR and RT-PCR Assays

Amplification was performed with 1 μL of cDNA in a final volume of 25 μL with the qPCR MasterMIX Plus for SYBER Green I with fluorescein (Eurogentec), and gene-specific primers (Supplemental Table S1). As a reference for PCR quantification, the *18S ribosomal* RNA gene was amplified with specific primers (Supplemental Table S1), but the cDNA was diluted 20-fold more in the PCR reaction. Three quantifications were performed for each sample as described (Supplemental Materials and Methods S1; RT-quantitative PCR). RT-PCR was monitored in 25-μL reactions using GoTaq FexI DNA polymerase (Promega Corporation), 1 μL of cDNA, and the specific primers (Supplemental Table S1). As a reference for PCR quantification, either *actin2* or *18S ribosomal* primers were used (Supplemental Table S1). Equal volumes of PCR products were analyzed on agarose gels and visualized by ethidium bromide staining. Band intensity was quantified using the software program Quantity One (Bio-Rad).

Mutant Analysis

A T-DNA insertion line for the gene *AtE2Fa* was available in GABI-Kat genebank (line 348E09, see http://www.gabi-kat.de/db/showseq.php gene=At2g36010). This line was screened for homozygous plants by segregation on selective medium and then by genotyping as recommended by the GABI Web site with a gene-specific primer and a T-DNA-specific primer.

E2Fa-Etag Constructs

The open reading frame of the *AtE2Fa* cDNA (At2g36010) sequence was amplified by PCR from total cDNA using the specific primers RW2 and FW2 (Supplemental Table S1) and cloned in TOPO vector. A *Xma*I-*Not*I DNA fragment was cloned in frame with Etag in pNEX-1 vector, under the control of the 35 s promoter (kindly provided by Dr. J.-L. Evrard, IBMP). A second digestion was then performed with *Eco*RI and *Hin*dIII for cloning into pGreen0029 vector. Finally, a GV3101 *Agrobacterium* strain containing the pSOUP plasmid was transformed with the pGreen0029 vector and used for floral-dipping transformation. AtE2Fa migrates with an apparent molecular mass of 66 kD and the addition of the 16 amino acids of Etag does not modify this migration pattern.

AtTSO2 Promoter GUS Construct and GUS Quantification

A *Pst*I-*Xba*I DNA fragment extending from 1,078 bp upstream and 21 bp downstream of the ATG from *TSO2* genomic sequence was cloned into the *Pst*I-*Xba*I restriction sites of the binary vector pBI101. Plasmid construct was introduced into *Agrobacterium tumefaciens* strain GV3101 and used to transform Arabidopsis. Ten independent transgenic lines were obtained. Quantification of GUS activity was carried out using the Tropix GUS Light kit (Applied Biosystems) as described (Chabouté et al., 2002).

Plant Protein Extracts and Western Experiments

Plant protein extracts were performed as described (Lincker et al., 2006) and a monoclonal antibody raised against Etag epitope (GE Healthcare, Europe GmbH) was used in western-blot experiments.

Comet Assays

About 20 17-d-old plantlets were incubated with or without BLM and were frozen in liquid nitrogen and stored at −80°C. DSBs were evaluated using neutral comet assay as described (Menke et al., 2001). Dry agarose gels were stained with 15 μL ethidium bromide (5 μg/mL) and were used for evaluation with a Nikon E800 fluorescence microscope. DNA damage in each comet tail was evaluated as described (Collins, 2004), assigning an arbitrary value (0–4) according to the comet size. In each experiment, the sum of 100 comet scores corresponds to arbitrary DNA damage unit. The mean value of four independent slides was presented.

Flow Cytometry Analyses

Fresh plants were chopped with a sharp razor blade in CysStain-UV-ploidy medium and analyzed as described by the manufacturer, using a Cyflow-R ploidy analyzer (Partec). Five independent experiments were performed.

Supplemental Data

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

Supplemental Figure S1. A, *H4* gene expression; B, DNA content in 8- and 17-d-old plantlets.

Supplemental Figure S2. A, Hypersensitivity of *rad* mutants to HU; B, ATM-dependent expression in response to BLM.

Supplemental Figure S3. A and B, Characterization of *e2fa* mutant; C and D, analysis of *TSO2* promoter.

Supplemental Figure S4. E2Fa-regulated expression of *RAD51* in response to BLM.

Supplemental Figure S5. IR response of *TSO2* and *RAD51*.

Supplemental Figure S6. Gene regulation in HU response.

Supplemental Table S1. List of primers.

Supplemental Materials and Methods S1. RT-qPCR and bioinformatics.

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