ATR cooperates with CTC1 and STN1 to maintain telomeres and genome integrity in *Arabidopsis*

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ABSTRACT **The CTC1/STN1/TEN1 (CST) complex is an essential constituent of plant and vertebrate telomeres. Here we show that CST and ATR (ataxia telangiectasia mutated [ATM] and Rad3-related) act synergistically to maintain telomere length and genome stability in *Arabidopsis*. Inactivation of ATR, but not ATM, temporarily rescued severe morphological phenotypes associated with *ctc1* or *stn1*. Unexpectedly, telomere shortening accelerated in plants lacking CST and ATR. In first-generation (G1) *ctc1 atr* mutants, enhanced telomere attrition was modest, but in G2 *ctc1 atr*, telomeres shortened precipitously, and this loss coincided with a dramatic decrease in telomerase activity in G2 *atr* mutants. Zeocin treatment also triggered a reduction in telomerase activity, suggesting that the prolonged absence of ATR leads to a hitherto-unrecognized DNA damage response (DDR). Finally, our data indicate that ATR modulates DDR in CST mutants by limiting chromosome fusions and transcription of DNA repair genes and also by promoting programmed cell death in stem cells. We conclude that the absence of CST in *Arabidopsis* triggers a multifaceted ATR-dependent response to facilitate maintenance of critically shortened telomeres and eliminate cells with severe telomere dysfunction.**

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INTRODUCTION

A critical function of telomeres is to protect natural chromosome ends from DNA damage. The protective cap that defines the chromosome terminus consists of telomere-binding proteins that associate with the double-stranded region, the single-stranded 3′ G-rich extension (G-overhang), or that bridge these two domains. The best-characterized telomere-capping complexes are shelterin in vertebrates and Cdc13/Stn1/Ten1 (CST) in budding yeast. The six-member shelterin complex spans both the double- and single-strand regions of the telomere (Palm and de Lange, 2008). Within shelterin, TRF2 and POT1 play leading roles in chromosome end protection (van Steensel *et al.*, 1998; Baumann and Cech, 2001). The CST complex associates exclusively with the G-overhang (Lin and Zakian, 1996), forming a heterotrimeric complex with structural similarity to replication protein A (RPA; Gao *et al.*, 2007; Sun *et al.*, 2009). A null mutation in any CST component is lethal, whereas other alleles trigger massive degradation of the telomeric C-strand, causing grossly extended G-overhangs (Nugent *et al.*, 1996; Grandin *et al.*, 1997, 2001). Deletion of either the Stn1 or Ten1 orthologue in fission yeast leads to catastrophic loss of telomeric DNA and end-to-end chromosome fusions (Martín *et al.*, 2007).

CST was recently discovered in plants and vertebrates (Song *et al.*, 2008; Miyake *et al.*, 2009; Surovtseva *et al.*, 2009). STN1 and TEN1 are sequence homologues of the budding and fission yeast proteins (Song *et al.*, 2008; Miyake *et al.*, 2009; Price *et al.*, 2010). The third member of the complex, conserved telomere maintenance component 1 (CTC1), is not a sequence homologue of Cdc13, although it shares functional similarities. Like Cdc13, CTC1 physically interacts with STN1, as well as with lagging-strand replication machinery (Casteel *et al.*, 2009; Miyake *et al.*, 2009; Surovtseva *et al.*, 2009; Price *et al.*, 2010). In addition, CTC1 in complex with STN1 and TEN1 binds single-stranded DNA, but in a sequence-independent manner (Miyake *et al.*, 2009).

Ctc1 or Stn1 knockdown in human cells results in an increase in G-overhang signal, sporadic loss of telomeric DNA, and aberrant chromatin bridges (Miyake *et al.*, 2009; Surovtseva *et al.*, 2009). Recent studies reveal that mutations in *CTC1* underlie the rare

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Abbreviations used: ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3-related; CST, CTC1/STN1/TEN1; CTC1, conserved telomere maintenance component 1; DDR, DNA damage response; PETRA, primer extension telomere repeat amplification; RAM, root apical meristem; TF-PCR, telomere fusion PCR; TRAP, telomere repeat amplification protocol; TRF, terminal restriction fragment.

human genetic disorder Coats plus, characterized by neurological and gastrointestinal defects (Anderson *et al.*, 2012). Coats plus patients also exhibit shortened telomeres and evidence of an ongoing DNA damage response (Anderson *et al.*, 2012). The major function for vertebrate CST may be related to DNA replication and repair and not to chromosome end protection per se (Linger and Price, 2009; Price *et al.*, 2010; Giraud-Panis *et al.*, 2010). Recent studies show that *Xenopus* CST is required to prime single-stranded DNA (ssDNA) for replication (Nakaoka *et al.*, 2011). In addition, genetic data argue that CST and shelterin act in distinct pathways to promote telomere integrity in human cells. When both Stn1 and Pot1 are depleted, a synergistic increase in telomere dysfunction–induced foci is observed (Miyake *et al.*, 2009).

CST plays a pivotal role in protecting plant telomeres. Although *ctc1*- and *stn1*-null mutants are viable, they suffer dramatic telomere shortening, end-to-end chromosome fusions, increased G-overhangs, and elevated extrachromosomal telomeric circles, indicative of aberrant telomere recombination (Song *et al.*, 2008; Surovtseva *et al.*, 2009). Genetic analysis of *Arabidopsis thaliana STN1* and *CTC1* confirms that these two components act in the same pathway for chromosome end protection (Surovtseva *et al.*, 2009). Unlike vertebrates, *Arabidopsis* harbors only a subset of shelterin components, and thus far, none of these is required for chromosome end protection (Watson and Riha, 2010). Moreover, *Arabidopsis* encodes three POT1-like proteins, which associate with telomerase instead of the telomere (Surovtseva *et al.*, 2007; Cifuentes-Rojas *et al.*, 2011). Thus CST appears to function as the major telomere protection complex in plants (Price *et al.*, 2010). CST is also likely to play a role in DNA replication in *Arabidopsis*, given its interaction with DNA polymerase α (Price *et al.*, 2010) and the results of vertebrate studies described earlier.

When telomere integrity is compromised due to loss of essential capping proteins or prolonged inactivation of telomerase, the unprotected chromosome terminus triggers a cellular DNA damage response (DDR) that is mediated by the phosphoinositide-3-kinase–related protein kinase ATM (ataxia-telangiectasia mutated) or ATR (ATM and Rad3-related; Sabourin and Zakian, 2008). ATM primarily responds to double-strand breaks, whereas ATR is activated by excessive single-stranded DNA (Nam and Cortez, 2011). As expected for telomere duplex binding components, TRF2 in vertebrates suppresses activation of ATM (Denchi and de Lange, 2007), whereas the single-strand–binding proteins mouse Pot1a (Denchi and de Lange, 2007), chicken Pot1 (Churikov *et al.*, 2006), and yeast Cdc13 (Garvik *et al.*, 1995; Ijpma and Greider, 2003; Hirano and Sugimoto, 2007) suppress an ATR-dependent DDR.

ATR and ATM are also required to maintain normal telomeres. Neither ATM nor ATR has been shown to affect telomerase enzyme activity levels in yeast or vertebrates (Sprung *et al.*, 1997; Chan *et al.*, 2001; McNees *et al.*, 2010), but in yeast both kinases are implicated in the recruitment of telomerase to chromosome ends. In *Schizosaccharomyces pombe*, Tel1 (ATM) and Rad3 (ATR) are required for Ccq1-mediated interaction with telomerase (Moser *et al.*, 2009, 2011). Similarly, in budding yeast Mec1 (ATR) and Tel1 (ATM) were both proposed to phosphorylate Cdc13 as a prerequisite for telomerase recruitment (Tseng *et al.*, 2006), although this finding is now controversial (Gao *et al.*, 2010). Nevertheless, a number of studies show that Tel1 facilitates the preferential recruitment of telomerase to critically shortened telomeres (Arneric and Lingner, 2007; Bianchi and Shore, 2007; Sabourin *et al.*, 2007) and stimulates telomerase repeat addition processivity on these chromosome ends (Chang *et al.*, 2007). Analysis of the ATR-deficient Seckel mouse indicates that although ATR is not required for telomerase recruitment to short telomeres (McNees *et al.*, 2010), it suppresses telomere fusions and the formation of fragile sites triggered by replication fork stalling in highly repetitive telomere repeat arrays (Martínez *et al.*, 2009; Sfeir *et al.*, 2009; McNees *et al.*, 2010).

Many key components of DDR are conserved in plants, but there is considerable divergence in cell cycle–regulated responses relative to vertebrates (Dissmeyer *et al.*, 2009). For example, ATM- and ATR-null mutations are not lethal in plants (Garcia *et al.*, 2003; Culligan *et al.*, 2004), and there is substantial overlap in the two pathways (Culligan *et al.*, 2004; Friesner *et al.*, 2005; Furukawa *et al.*, 2010). Moreover, plants are extraordinarily tolerant to genome instability, an outcome that may reflect the presence of undifferentiated stem cell niches in the shoot and root apical meristems. Meristematic cells allow for continual growth and tissue differentiation, blunting the effect of DNA damage in somatic tissue. Ionizing radiation, for instance, can induce cell cycle arrest in meristems but not in somatic cells (Hefner *et al.*, 2006).

Although mutation of either ATM or ATR has no effect on telomere length homeostasis in *Arabidopsis* (Vespa *et al.*, 2005), these kinases act synergistically with telomerase to maintain the telomere tract (Vespa *et al.*, 2005, 2007). Plants doubly deficient in ATM and TERT, the telomerase catalytic subunit, experience an abrupt, early onset of genome instability compared with *tert* single mutants (Vespa *et al.*, 2005). Analysis of individual telomere tracts showed that ATM prevents stochastic deletional recombination events, allowing cells to maintain similar telomere lengths on homologous chromosome arms (Vespa *et al.*, 2007). ATR makes a more immediate contribution to telomere maintenance than ATM (Vespa *et al.*, 2005). From the outset, telomeres in double *atr tert* mutants shorten at a greatly accelerated pace relative to *tert*, so that telomere dysfunction occurs in the third generation of the double mutant, compared with the sixth generation of *tert*.

Here we employ a genetic approach to investigate how CST components interface with ATM and ATR to promote telomere integrity and genome stability in *Arabidopsis*. We demonstrate a pivotal role for ATR in the response to CST abrogation that leads to programmed stem cell death. We also show that the combined absence of ATR and CST results in catastrophic loss of telomere tracts in a biphasic manner. The second, more severe phase of telomere shortening coincides with strong down-regulation of telomerase activity. These findings indicate that ATR and CST act synergistically to maintain genome integrity and telomere length homeostasis.

RESULTS

Loss of ATR rescues morphological defects in CST mutants.

To explore the role of ATR and ATM in plants lacking CST, we crossed *ctc1* or *stn1* heterozygotes to *atr* and *atm* mutants. F1 plants heterozygous for both mutations were self-crossed, and offspring were used for analysis. As previously reported (Garcia *et al.*, 2003; Culligan *et al.*, 2004; Vespa *et al.*, 2005), *atm* (Figure 1A) and *atr* (Figure 1B) mutants were phenotypically indistinguishable from wild type. In contrast, *ctc1* and *stn1* mutants exhibited serious morphological defects (Song *et al.*, 2008; Surovtseva *et al.*, 2009), including fasciated inflorescence bolts and flowers (Figure 1C, arrowheads, and Supplemental Figure S1, white arrows), irregularly spaced siliques (Figure 1C, arrows, and Supplemental Figure S1), and small, curved leaves. Although *ctc1* and *stn1* mutants always display morphological abnormalities, the expressivity of the mutant alleles is somewhat variable, with some individuals showing more severe phenotypes than others (Song *et al.*, 2008; Surovtseva *et al.*, 2009). Both *ctc1 atm* and *stn1 atm* double mutants displayed the same

FIGURE 1: Loss of ATR rescues the morphological defects of *ctc1* mutants. The morphology of *ctc1* mutants in the presence or absence of ATM or ATR is shown. (A) The phenotype of a *ctc1 atm* double mutant (right) resembles that of the *ctc1* single mutant. (B, C) Morphological defects of *ctc1* mutants are largely rescued when ATR is lost. Arrowheads indicate fasciated stems and flowers; arrows indicate irregular phyllotaxy. Images of second-generation (G2) *ctc1 atr* mutants are presented showing an intact plant (D) with curved, small leaves or malformed flowers (E) bearing a curved pistil and stamen and petal deficiency.

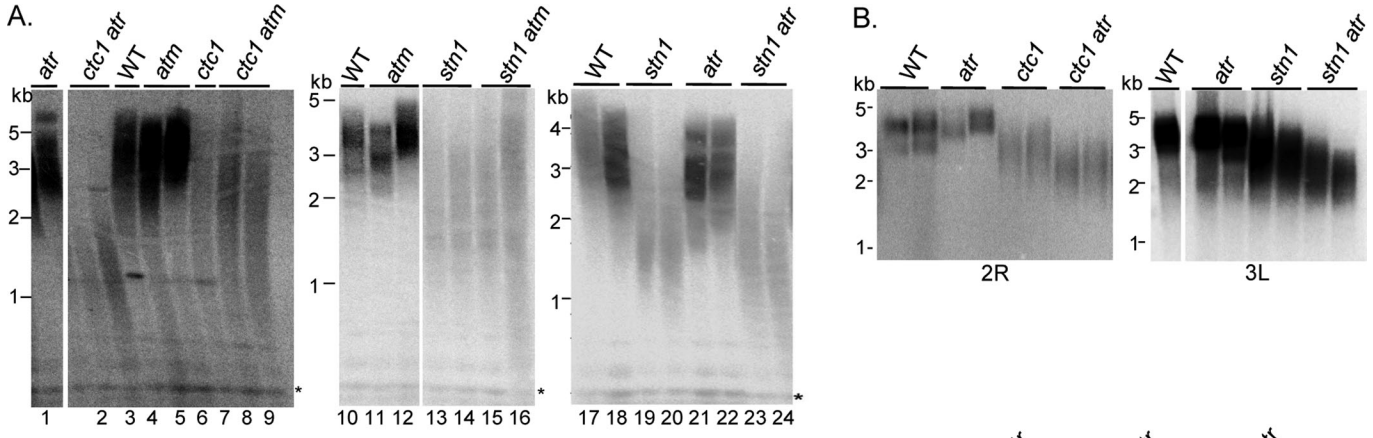
range of growth defects as *ctc1* (Figure 1A) or *stn1* mutants (Supplemental Figure S1A). In contrast, *ctc1 atr* and *stn1 atr* mutants showed only minor perturbations in morphology—mainly irregularly spaced siliques. Approximately 30% of the double mutants appeared like wild type (Figure 1, B and C, and Supplemental Figure S1B). The apparent rescue of morphological defects in *ctc1 atr* and *stn1 atr* mutants is consistent with the conclusion that CST protects against ATR activation.

The improvement of morphological deficiencies in *ctc1 atr* mutants was only temporary. Second-generation (G2) *ctc1 atr* mutants showed severe developmental defects, and most died before bolting (Figure 1, D and E). Many of the phenotypes associated with G2 *ctc1 atr* resembled G1 *ctc1* mutants (Surovtseva *et al.*, 2009). Defects included curved, misformed leaves and severe floral abnormalities, such as missing anthers, curved pistils, open carpels with seeds exposed, and petals that were green like sepals (Figure 1E). We conclude that ATR alters plant growth in response to CST abrogation.

ATR facilitates telomere length maintenance in the absence of CTC1 or STN1

The morphological rescue seen in CST mutants lacking ATR argues that ATR is activated by telomere dysfunction. Given the role of ATR in telomere maintenance in telomerase mutants (Vespa *et al.*, 2005), we considered the possibility that ATR also contributes to telomere maintenance in plants lacking CST. Bulk telomere length was monitored using terminal restriction fragment (TRF) analysis. As previously reported (Vespa *et al.*, 2005), telomere tracts in *atr* and *atm* were similar to wild type (Figure 2A, lanes 1, 4, and 6), whereas G1 *ctc1* telomeres were shorter and more heterogeneous (Figure 2A, lane 7). The absence of ATM did not affect telomere length in G1 *ctc1* mutants (Figure 2A, lanes 8 and 9). In both G1 *ctc1* and G1 *ctc1 atm* mutants, telomeres ranged from 1 to 5 kb, with a peak signal at 2 kb. In contrast, telomeres were consistently shorter in G1 *ctc1 atr* mutants than in G1 *ctc1* (Figure 2A, lanes 2, 3, and 7), with some signals trailing below 1 kb (peak, 1.5 kb). Similar findings were obtained with G1 *stn1 atm* (Figure 2A, lanes 13–16) and G1 *stn1 atr* mutants (Figure 2A, lanes 19, 20, 23, and 24).

Primer extension telomere repeat amplification (PETRA) was used to precisely measure telomere length on individual chromosome arms. In this assay, wild-type telomeres range from 2 to 5 kb and typically appear as one to three bands, depending on the chromosome arm (Figure 2B; Heacock *et al.*, 2004). As with bulk telomere analysis, PETRA showed that the telomere profiles of *atr* (Figure 2B) and *atm* (Supplemental Figure S2, A and B) were similar to that of wild type, whereas telomeres from G1 *ctc1* and G1 *stn1* migrated as a broad smear ranging from 1.5 to 4 kb (Figure 2B). PETRA confirmed that telomere tracts were similar in G1 *ctc1* and G1 *ctc1 atm* mutants (Supplemental Figure S2A). In contrast, telomeres in G1 *ctc1 atr* mutants were shorter by an average of 300 base pairs



C.

Average Telomere Length (kb)						Avg. Difference from WT	Avg. Difference from *atr*	Avg. Difference from *ctc1*
Chromosome Arm:	1L	2R	4R	5L	Avg.			
WT	4.57	5.20	3.37	3.21	4.09	0.00	-0.52	0.76
atr	4.68	5.72	4.36	3.67	4.61	0.52	0.00	1.28
ctc1	3.81	4.56	2.67	2.29	3.33	-0.76	-1.28	0.00
ctc1 atr	3.57	4.05	2.54	1.92	3.02	-1.07	-1.59	-0.31

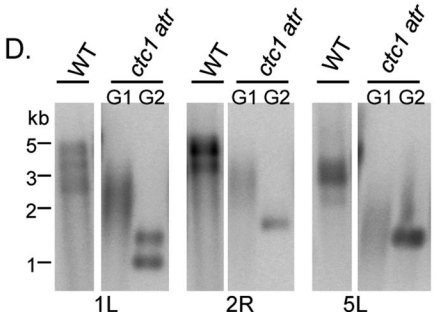


FIGURE 2: ATR, but not ATM, contributes to telomere length maintenance in *ctc1* and *stn1* mutants. (A) TRF analysis of *ctc1* crosses to *atr* and *atm* (lanes 1–9) and *stn1* crosses to *atm* (lanes 10–16) and *atr* (lanes 17–24). (B) PETRA results for the 2R telomere in *ctc1 atr* mutants and the 3L telomere in *stn1 atr* mutants. (C) Quantification of telomere lengths from *ctc1 atr* PETRA analysis shown in B. Telomere length was calculated by subtracting the distance of the subtelomeric primer binding site relative to start of the telomere repeat array from the PETRA value. For all genotypes, n = 4. (D) Parent–progeny PETRA analysis of telomeres in G1 and G2 *ctc1 atr* mutants. Asterisk indicates interstitial telomeric repeats used as a loading control.

compared with G1 *ctc1* mutants (Figure 2, B and C). The same result was obtained for *stn1* mutants in both *atm*-deficient (Supplemental Figure S2B) and *atr*-deficient (Figure 2B) backgrounds. Hence, ATR, but not ATM, contributes to telomere length maintenance when CST is compromised.

We examined the status of the G-overhang in G1 *ctc1 atr* mutants using in-gel hybridization. This assay detects single-stranded, G-rich telomeric DNA either at the extreme chromosome terminus or within the double-stranded telomere region, if gaps are present in the C-strand. As previously reported (Surovtseva *et al.*, 2009), *ctc1* single mutants showed enhanced G-overhang signals, threefold to sixfold greater than wild type (Supplemental Figure S3). G-Overhang status was wild type in *atr* mutants. Furthermore, the loss of ATR did not exacerbate the G-overhang phenotype in *ctc1* mutants (Supplemental Figure S3). We conclude that ATR does not play a significant role in G-overhang maintenance, and further that *ctc1 atr* mutants do not carry extensive sections of incompletely replicated telomeric C-strand DNA.

Because G2 *ctc1 atr* mutants have much more severe morphological defects than G1 *ctc1 atr* (Figure 1, D and E), we were prompted to examine telomere length in G2 double mutants using PETRA. Telomere tracts in G2 *ctc1 atr* were much shorter (up to 1 kb) than their G1 parents (Figure 2D). This attrition is more than three times greater than the telomere shortening in G1 *ctc1 atr* mutants versus their *ctc1* siblings (300 base pairs; Figure 2, A–C), and more than two times higher than G2 *stn1* mutants versus their G1 parent (~400 base pairs; unpublished data). In conjunction with telomere shortening, the profile of telomere fragments switched from heterogeneous, smeary bands in the G1 *ctc1 atr* parents to very homogeneous, sharp bands in the G2 *ctc1 atr* offspring (Figure 2D). PETRA assays conducted with five generations of *atr* mutants revealed no change in telomere length (Supplemental Figure S2C), confirming that the telomere maintenance defect in *ctc1 atr* mutants reflects a synergistic effect of both ATR and CST dysfunction. These data further indicate that ATR contributes to telomere maintenance in a biphasic manner. In the first generation of a CST deficiency, ATR makes a modest contribution to telomere maintenance. However, the prolonged absence of ATR in plants lacking CST leads to a much more dramatic loss of telomeric DNA.

Inactivation of ATR down-regulates telomerase enzyme activity

A profile of shorter, more homogeneous telomere tracts is consistent with a defect in telomerase-mediated telomere maintenance (Riha *et al.*, 2001; Kannan *et al.*, 2008). Thus one explanation for the enhanced rate of telomere loss in G2 *ctc1 atr* mutants is that telomerase can no longer act on dysfunctional chromosome ends. To investigate this possibility, we used the quantitative telomere repeat amplification protocol (Q-TRAP) to measure telomerase enzyme activity levels in consecutive generations of *ctc1 atr* mutants. As expected (Song *et al.*, 2008; Surovtseva *et al.*, 2009), telomerase activity was robust in G1 and G2 *ctc1* and *stn1* seedlings and indistinguishable from wild-type samples (Figure 3 and data not shown). Wild-type levels of telomerase activity were also detected in G1 *atr* mutants. Unexpectedly, however, telomerase activity declined by ~15-fold in G2 *atr* mutants (Figure 3). This decrease persisted in subsequent plant

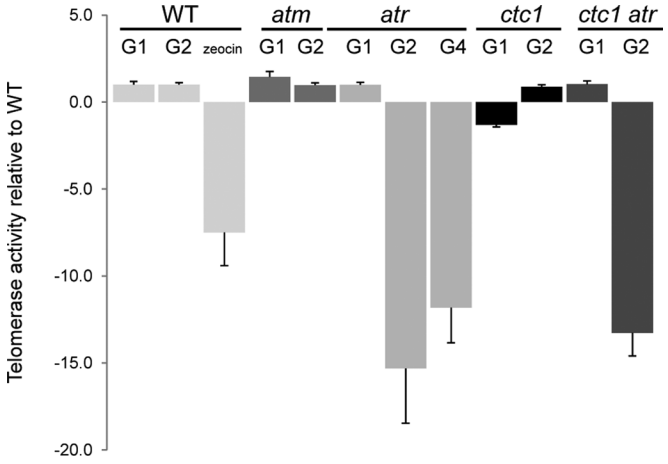


FIGURE 3: ATR stimulates telomerase activity. Quantitative TRAP results for first-generation (G1), second-generation (G2), and fourth-generation (G4) mutants of different genotypes are shown. Q-TRAP was also performed on wild-type seedlings treated with 20 μM zeocin for 3 d. All samples were from flowers except G2 *atr,* G2 *ctc1*, and G2 *ctc1 atr*, which were from seedlings. Telomerase activity is plotted relative to wild type. For zeocin-treated seedlings, telomerase activity is relative to untreated-wild type seedlings. Error bars represent SD. n = 2 for all genotypes except G1 WT, n = 5; zeocin-treated WT, n = 6; G1 *ctc1*, n = 4; G2 *atr*, n = 3; and G4 *atr*, n = 4.

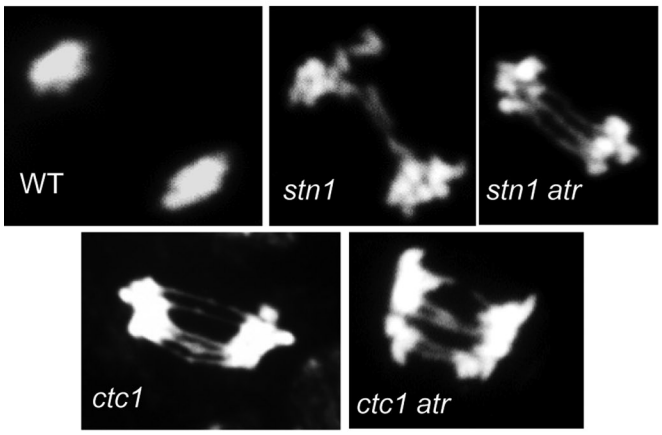
generations, with G4 *atr* mutants also exhibiting dramatically reduced enzyme activity. A similar decrease in TRAP activity was not observed in *atm* deficient plants (Figure 3). The reduction in telomerase activity was not confined to a specific developmental stage; Q-TRAP data obtained from both seedlings and flowers gave similar results (Figure 3). Of note, Q-TRAP revealed the same level of enzyme activity in G1 *ctc1 atr* mutants as in wild-type plants, and enzyme activity in G2 *ctc1 atr* decreased by the same amount as in G2 *atr* (Figure 3). Hence, loss of ATR, and not CTC1, leads to decreased telomerase activity.

In yeast and vertebrates, disruption of ATR causes genome wide replicative stress (Nam and Cortez, 2011), suggesting that the stimulus for reduced telomerase activity in G2 *atr* mutants might be accumulating genome damage. To investigate whether genotoxic stress triggers a decrease in telomerase activity, wild-type seedlings were treated with zeocin, which induces double-strand breaks. Q-TRAP revealed ~7.5-fold reduction in telomerase in treated seedlings versus controls (Figure 3). This observation suggests that the repression of telomerase activity in G2 *atr* mutants may reflect the activation of a DDR triggered by replicative stress. Taken together, these results show that the dramatic loss of telomeric DNA in G2 *ctc1 atr* mutants correlates with an abrupt decline in telomerase enzyme activity.

ATR suppresses the formation of end-to-end chromosome fusions in CST mutants

Catastrophic loss of telomeric DNA in *ctc1* and *stn1* mutants coincides with the onset of telomere fusions (Song *et al.*, 2008; Surovtseva *et al.*, 2009). Dysfunctional telomeres are recruited into chromosome fusions through the nonhomologous end-joining (NHEJ) pathway, which is activated by ATM and indirectly by ATR (Denchi and de Lange, 2007; Deng *et al.*, 2009). Therefore we used telomere fusion PCR (TF-PCR) to ask whether the accelerated telomere shortening in plants lacking CST and ATR correlates with an increased incidence of telomere fusions. TF-PCR uses primers specific to unique subtelomeric sequences on each chromosome arm to

A.



B.

Genotype	Anaphases		
	Total	Bridges	Ratio %
WT	69	0	0%
stn1	369	76	21%
ctc1	166	38	23%
atm	202	0	0%
atr	454	6	1%
stn1 atm	137	27	20%
stn1 atr	501	288	57%
ctc1 atr	234	125	53%
stn1 atm atr	323	225	70%

FIGURE 4: End-to-end chromosome fusions increase in plants lacking CST and ATR. (A) Cytology of anaphases from pistils from G1 plants of the genotypes indicated. Spreads are stained with DAPI. (B) Quantification of anaphase bridges from cytology in A.

amplify junctions of covalently fused telomeres. For these studies, DNA from mature G1 mutants was analyzed. As expected, telomere fusions were not observed in wild type or *atr* (Supplemental Figure S4, B and D) or *atm* (Supplemental Figure S4, A and C) mutants. In contrast, massive chromosome end-joining events, represented by abundant heterogeneous smears, were associated with the loss of CTC1 (Supplemental Figure S4, A and B) or STN1 (Supplemental Figure S4, C and D). When either ATR (Supplemental Figure S4, B and D) or ATM (Supplemental Figure S4, A and C) was absent in *ctc1* or *stn1* mutants, TF-PCR products were still detected.

TF-PCR provides an indication of whether telomeres are prone to end-joining reactions, but it does not give quantitative information about the number of chromosome fusions. To obtain a quantitative assessment of telomere joining events, we monitored the incidence of anaphase bridges in mitotically dividing cells using conventional cytology (Figure 4A). As described previously (Song *et al.*, 2008; Surovtseva *et al.*, 2009), bridged chromosomes were detected in the floral pistils of G1 *ctc1* and *stn1* mutants (23 and

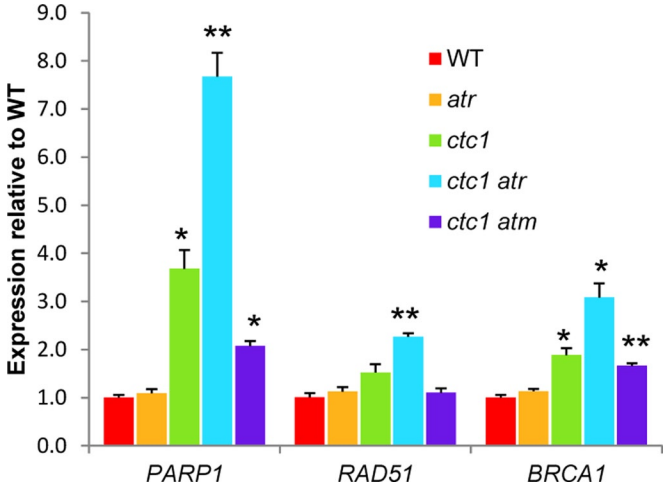


FIGURE 5: Loss of CTC1 activates a transcriptional response, which is alleviated by ATR. Quantitative RT-PCR results are shown for the DDR transcripts *PARP1*, *BRCA1*, and *RAD51* in floral organs. Expression levels are relative to wild type, and data for first-generation (G1) mutants are shown. For each genotype, n = 3, except for *ctc1 atm*, n = 2. *p < 0.05 relative to wild type; **p < 0.005 relative to wild type (Student's *t* test). Error bars represent SEM.

21% of all anaphases, respectively), compared with few or none in wild type and *atr* and *atm* mutants (Figure 4B). The loss of ATM did not alter the percentage of anaphase bridges in *stn1* mutants. Conversely, there was a dramatic increase in the incidence of anaphase bridges in G1 *stn1 atr* (57%) and G1 *ctc1 atr* (53%) relative to *stn1* and *ctc1* (Figure 4B). Remarkably, 70% of anaphases in the triple G1 *stn1 atr atm* mutants contained bridged chromosomes (Figure 4B). Thus an ATR- and ATM-independent mechanism can promote fusion of dysfunctional telomeres. The increased incidence of chromosome bridges suggests that ATR inhibits telomere fusion in CST mutants.

ATR attenuates the transcriptional response to DNA damage in plants lacking CTC1

The role of ATR in repressing telomere fusions, together with the accelerated telomere shortening and morphological disruptions in CST mutants, argues that loss of CST triggers an ATR-mediated DDR. To investigate this possibility, we monitored the expression of several transcripts implicated in the DDR (*RAD51*, *BREAST CANCER SUSCEPTIBILITY 1* [*BRCA1*]) and (*poly* [*ADP-ribose*] *polymerase 1* [*PARP1*]; Doucet-Chabeaud *et al.*, 2001; Lafarge and Montané, 2003; Yoshiyama *et al.*, 2009). Quantitative real-time (RT)-PCR was performed using cDNA made from first-generation (G1) *ctc1* flowers. Expression of both *PARP1* and *BRCA1* was significantly up-regulated in *ctc1* mutants compared with wild type (3.7- and 1.9-fold, respectively; Figure 5). In addition, *RAD51* expression was 1.5 times higher in *ctc1* mutants (Figure 5), but the difference was not statistically significant. These results suggest that the CST complex protects against a DDR.

We next asked whether ATM or ATR is necessary to initiate a transcriptional response in plants lacking CST, since in *Arabidopsis*, the response to double-strand breaks is mostly mediated by ATM, but ATR is also required (Friesner *et al.*, 2005). In *ctc1 atm* mutants, *PARP1* and *BRCA1* transcripts were above wild-type levels (2.1 and 1.7 times wild type, respectively) but were slightly less abundant than in *ctc1* mutants. This finding suggests that ATM contributes to the activation of a DNA repair transcriptional program in *ctc1* mutants. A more dramatic change in transcript level was observed in plants doubly deficient in CTC1 and ATR. Expression of all three DDR genes was significantly elevated in *ctc1 atr* mutants relative to wild type, *atr*, or *ctc1* (Figure 5). Compared to wild type, *ctc1 atr* mutants showed a 7.7-fold increase in *PARP1* expression, a 2.3-fold increase in RAD51, and a 3.1-fold increase in BRCA1. Thus ATR curbs the transcriptional response to loss of CTC1. This observation is consistent with ATR-mediated suppression of chromosome fusions.

ATR promotes programmed cell death in ctc1 mutants

ATR is implicated in programmed cell death signaling in *Arabidopsis* (Fulcher and Sablowski, 2009; Furukawa *et al.*, 2010). To further explore the role of ATR in plants lacking CST, we monitored stem cell viability in root apical meristems (RAMs) of seedlings, using propidium iodide (PI) staining (Figure 6A). PI is a membrane-impermeable dye that is excluded from live cells. In dead cells, PI passes through the cell membrane and binds nucleic acids. The limited biomass of young seedlings precluded genotyping to identify G1 double mutants so early in their development. Therefore we examined the RAM in their progeny, G2 *ctc1 atr* mutants. As expected, PI staining was not associated with the RAM in wild-type seedlings (Figure 6A, ii). Similarly, G2 *atr* seedlings showed no PI staining (Figure 6A, iii). In contrast, strong PI staining was observed in G2 *ctc1* RAM (Figure 6A, iv) or G2 *stn1* RAM (Figure 6A, v), consistent with activation of a robust DDR. We next asked whether ATR is responsible for cell death in CST mutants (Figure 6A, vi). Strikingly, the number of PI positive cells in G2 *ctc1 atr* dropped to an average 1.75 cells/root, compared with 5.75 and 4.35 cells/root for *stn1* and *ctc1*, respectively (Figure 6A, vi, and B). A subset of mutant seedlings (25% in *stn1*, 35% in *ctc1*, and 67% in *ctc1 atr*) had no PI-positive cells. The short roots from these plants had a high density of root hairs and no obvious RAM (Figure 6A, vii and viii). We speculate that in such plants, epithelial precursor cells may be able to differentiate, but other cell types have been eliminated from the RAM or have differentiated inappropriately. These mutant roots are remarkably similar to gamma-irradiated *lig4* roots, where RAM cells are arrested (Hefner *et al.*, 2006). Taken together, these data indicate that ATR activation leads to programmed cell death in plants lacking CST. Furthermore, we speculate that the decrease in PCD in *ctc1 atr* mutants leads to an accumulation of cells exhibiting DDR and increased number of end-to-end chromosome fusions.

DISCUSSION

CST protects telomeres from activating ATR

A key function of intact telomeres is to prevent the chromosome terminus from eliciting a cellular DDR that leads to end-to-end chromosome fusions and genome-wide instability. Here we show that the *Arabidopsis* CST prohibits the activation of ATR-mediated DDR. We find that the absence of CTC1 results in elevated levels of DDR transcript expression and programmed cell death in the RAM. The sacrifice of stem cells by programmed cell death is a common response to DNA damage in plants (Fulcher and Sablowski, 2009; Furukawa *et al.*, 2010) and has obvious benefits for organismal viability. Several observations support the idea that ATR-mediated programmed cell death reduces genome instability in CST mutants. First, expression of DDR transcripts increases in *ctc1 atr* mutants compared with *ctc1* mutants. Second, the incidence of chromosome fusions increases in *ctc1 atr* mutants. Finally, plants lacking core components of CST display severe morphological abnormalities as a consequence of profound genome instability (Song *et al.*, 2008; Surovtseva *et al.*, 2009), and these phenotypes are largely rescued by a deficiency in ATR but not ATM. The rescue is only temporary,

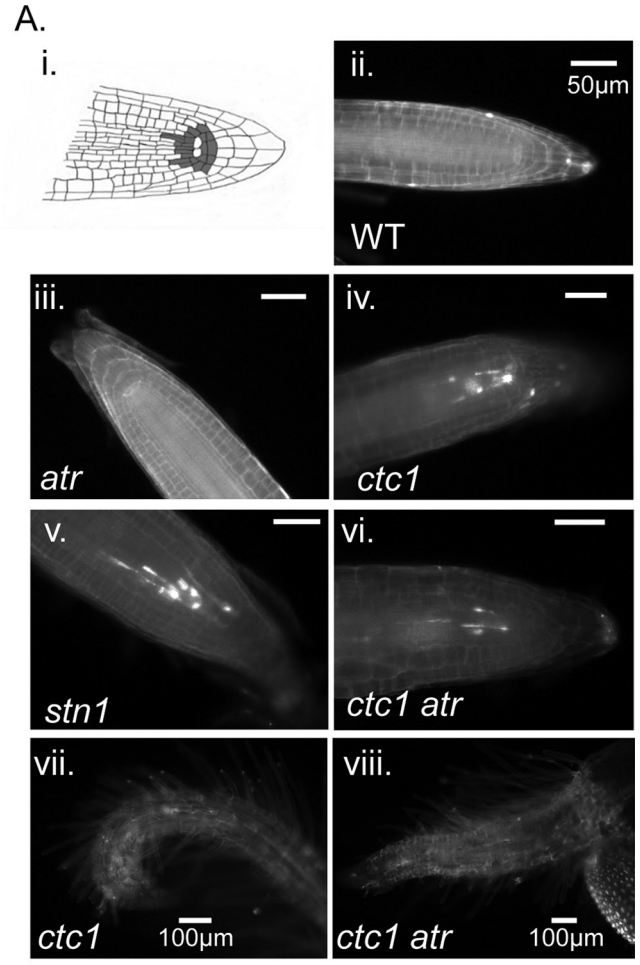


FIGURE 6: ATR activates programmed cell death of the root apical meristem (RAM) of *ctc1* mutants. (A) Representative images of G2 seedling root tips stained with propidium iodide (PI). (i) Diagram of a root tip. Stem cells and adjacent daughter cells are shaded gray. White cells in the RAM center are quiescent center cells. WT (ii) and *atr* (iii) roots are PI negative, but the RAMs of *ctc1* (iv) and *stn1* (v) mutants have numerous PI-positive (dead) cells. (vi) Fewer PI-positive cells are present in *ctc1 atr* mutants. (vii and viii) A subset of *ctc1* or *stn1* roots were PI negative but displayed severe morphological defects. (B) Quantification of PI-positive cells in different genetic backgrounds. The average number of PI-positive cells per root tip is shown. *stn1* (n = 12), *ctc1* (n = 17), *ctc1 atr* (n = 12). *$p < 0.05$ (Student's *t* test). Error bars represent SEM.

however, and in the next generation (G2), *ctc1 atr* mutants suffer even more devastating developmental defects than G2 *ctc1* single mutants. This observation is consistent with checkpoint bypass, resulting in the accumulation of DNA damage when ATR is lost in *ctc1* mutants. We postulate that the failure to initiate programmed cell death allows *ctc1 atr* cells with dysfunctional telomeres to continue cycling until rampant genome instability leads to developmental arrest (Figure 7B).

While this article was under review, Amiard *et al.* (2011) published a study that verifies and complements our findings concerning the role of CST in suppressing an ATR-mediated DDR. These authors show an ATR-dependent induction of γH2AX at telomeres in *Arabidopsis ctc1* mutants, consistent with our transcriptional data showing induction of DDR transcripts in response to loss of CTC1. They also demonstrate that ATR and ATM repress formation of anaphase bridges and promote PCD in *ctc1* mutants. They conclude, as do we, that ATR maintains genome stability in CST mutants (Amiard *et al.*, 2011).

Together these *Arabidopsis* studies highlight the complexity of the DDR in plants and show that multiple, overlapping mechanisms are harnessed to detect and to process dysfunctional telomeres. For example, the increased incidence of telomere fusions in plants lacking CST and ATR could reflect survival of cells with profound telomere dysfunction due to checkpoint bypass, as well as a contribution of ATR in facilitating maintenance of short telomeres (see later discussion). Of note, telomere fusions accumulate even in the absence of both ATM and ATR when CST is compromised (Amiard *et al.*, 2011; this study). A third PIKK family member in vertebrates—DNA-dependent protein kinase catalytic subunit (DNA-PKcs)—functions in NHEJ (Lieber *et al.*, 2003) and could potentially serve as backup mechanism to trigger telomere fusion. Plants lack an obvious DNA-PKcs orthologue, and thus the ATR/ATM–independent response elicited by telomere dysfunction is unknown. Further complicating matters, uncapped telomeres engage both canonical and no-canonical DNA repair pathways in *Arabidopsis*. Critically shortened telomeres fuse in the absence of two core NHEJ repair proteins, Ku70 and ligase IV (Heacock *et al.*, 2007), and in plants lacking Ku as well as Mre11 (Heacock *et al.*, 2004). In humans, an alternative end-joining pathway, which uses PARP1 and DNA ligase III, is activated if the canonical DNA-PKcs/Ku pathway is nonfunctional (Audebert *et al.*, 2004; Wang *et al.*, 2006). It is unknown whether PARP1 plays a similar role in plants, but it is an intriguing possibility, given the induction of *PARP1* expression in *ctc1* and *ctc1 atr* mutants (Figure 5).

Cooperation of CST and ATR in telomere maintenance

Figure 7 presents a model summarizing the multifunctional roles of ATR at *Arabidopsis* telomeres. The data presented here showing a central role for ATR in the response to CST abrogation provide additional support for the proposal that CST binds single-stranded DNA at the chromosome terminus in multicellular organisms (Miyake *et al.*, 2009; Surovtseva *et al.*, 2009; Figure 7A). Although our findings do not specifically address whether CST directly contacts the G-overhang, they are consistent with this conclusion and with the present model that single-strand telomere-binding proteins protect the chromosome terminus by excluding RPA from the G-overhang (Gong and de Lange, 2010; Flynn *et al.*, 2011).

Our results show that CST and ATR cooperate in the maintenance of telomeric DNA. We found that inactivation of ATR, but not ATM, accelerates the attrition of telomeric DNA at telomeres lacking CST. Multigenerational analysis of *ctc1 atr* mutants demonstrated that ATR makes a biphasic contribution to telomere length homeostasis. Our data indicate that in the first generation of a CST

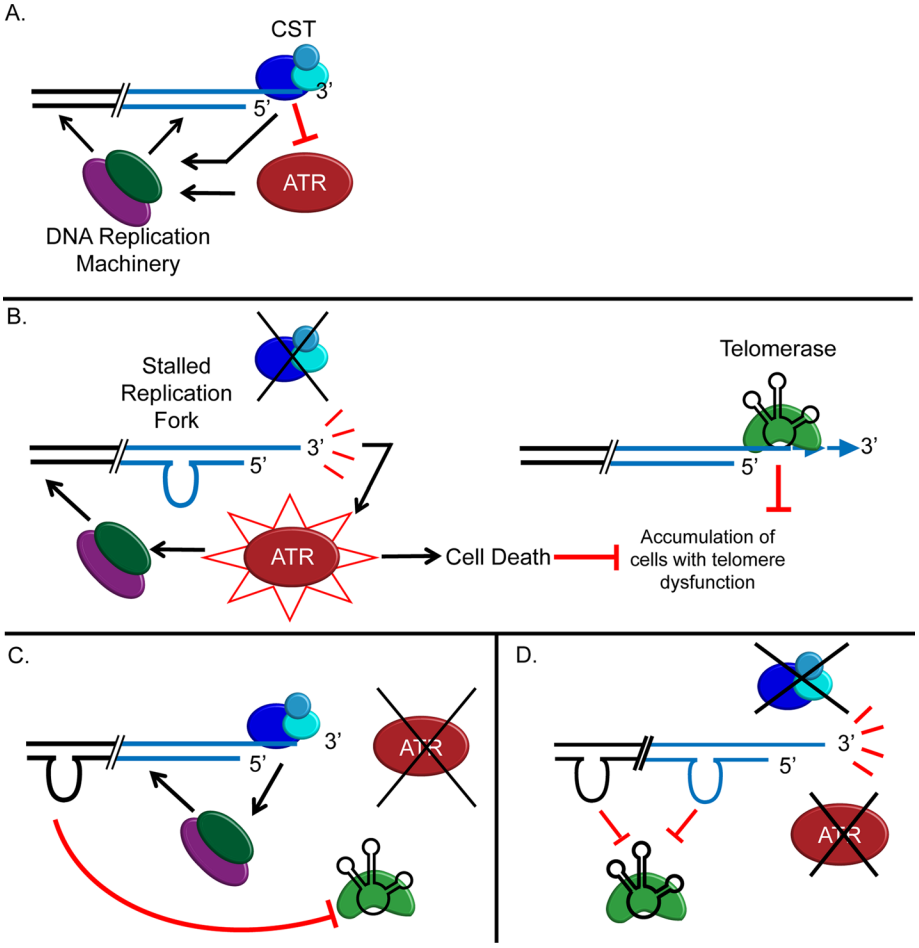


FIGURE 7: Model depicting CST and ATR cooperation in maintaining telomeric DNA and genome integrity in *Arabidopsis*. (A) In wild-type plants, CST interacts with the 3′ overhang to protect the chromosome terminus from telomere shortening, end-to-end chromosome fusions (Song *et al.*, 2008; Surovtseva *et al.*, 2009), and activation of ATR-dependent DDR (this study). ATR facilitates replication fork progression. Similarly, CST is believed to stimulate replication fork restart within the telomeric duplex via interaction with DNA polymerase alpha (Price *et al.*, 2010; Nakaoka *et al.*, 2011). Telomeric DNA is represented by blue lines. (B) Plants lacking CST activate ATR-dependent DDR, initiating programmed cell death in stem cell niches. Replication fork progression is perturbed in the telomeric duplex, contributing to the loss of telomeric DNA. Telomerase action delays the onset of complete telomere failure. (C) Accumulating replicative stress in *atr* mutants triggers an ATR-independent DDR that results in telomerase inhibition. Telomeres in the wild-type size range can be maintained. (D) Catastrophic telomere shortening occurs in plants lacking both CST and ATR due incomplete replication of the duplex and failure of telomerase to act on critically shortened telomeres. See the text for details.

deficiency, the role of ATR is relatively minor. Telomeres are ~300 base pairs shorter in *ctc1 atr* mutants than when ATR is intact. However, in the next generation, telomere shortening is much more aggressive, and up to 1 kb more telomeric DNA is lost. We hypothesize that this biphasic response reflects two distinct contributions of ATR in promoting telomere maintenance (Figure 7, B and C).

Emerging data indicate that ATR and CST cooperate to facilitate DNA replication through the telomeric duplex (Price *et al.*, 2010; Stewart and Price, personal communication). ATR is activated in response to replication fork stalling (Verdun *et al.*, 2005; Miller *et al.*, 2006) and specifically suppresses telomere fragility derived from incomplete replication (Martínez *et al.*, 2009; Sfeir *et al.*, 2009; McNees *et al.*, 2010). Of note, mammalian chromosomes depleted of CTC1 or STN1 display multiple telomere signals, consistent with telomere fragile sites (Price *et al.*, 2010). CST is proposed to participate in replication fork restart via its interaction with DNA polymerase-α (Casteel *et al.*, 2009; Price *et al.*, 2010). Consistent with this model, *Xenopus* CST is required for priming replication of ssDNA (Nakaoka *et al.*, 2011). Taken together, these findings indicate CST and ATR cooperate in relieving replicative stress within the telomere duplex (Figure 7, B and C). When both CST and ATR are compromised, replication fork stalling is increased (Figure 7D), triggering double-strand breaks and, in turn, deletion of telomeric DNA.

Replicative stress may account for the modest increase in telomere shortening in G1 *ctc1 atr* mutants. Although the extent to which ATR and CST modulate replication of the telomeric duplex in plants is unknown, preliminary data suggest that the contribution of these two components could be less significant in plants than in vertebrates. In human cells lacking CST, a small fraction of G-rich, telomeric single-stranded DNA signal is resistant to exonuclease treatment (Surovtseva *et al.*, 2009; Miyake *et al.*, 2009), consistent with incomplete replication of internal telomeric DNA tracts. Parallel analysis in *Arabidopsis* failed to detect exonuclease-resistant, G-rich, single-stranded DNA (Surovtseva *et al.* 2009), suggesting that CST acts primarily at the extreme chromosome terminus. We also found no increase in G-rich, single-stranded DNA in *ctc1 atr* mutants relative to *ctc1*, implying that loss of ATR does not trigger massive replication fork stalling in CST mutants.

Telomerase and ATR

What accounts for the abrupt and dramatic loss of telomeric DNA in G2 *ctc1 atr* mutants? We propose that this delayed response reflects telomerase inhibition triggered by prolonged ATR inactivation. Depletion of ATR in mice leads to extensive chromosome fragmentation, and a null mutation is embryonic lethal (Brown and Baltimore, 2000; de Klein *et al.*, 2000). In contrast, plants lacking ATR are viable, fully fertile, and morphologically wild type (Culligan *et al.*, 2004). Although no overt genome instability is associated with ATR depletion in *Arabidopsis*, we speculate that accumulating replicative stress elicits a hitherto unrecognized DDR, one consequence of which is telomerase repression (Figure 7C). In support of this hypothesis, we showed that the genotoxin zeocin inhibits telomerase activity in wild-type seedlings. Strikingly, telomerase activity is unaffected in plants lacking CST, indicating that telomere dysfunction does not inhibit telomerase. Sustained repeat incorporation onto compromised chromosome ends would be advantageous if it delays the onset of complete telomere dysfunction. Of note, *ctc1 tert* telomeres shorten more rapidly than in either single-mutant background (Boltz and Shippen, unpublished data), arguing that telomerase continues to act on telomeres in the absence of CST.

Although the molecular basis for this ATR-independent pathway of DNA damage–induced telomerase repression is unknown, such a response reduces the potential for telomerase to act at sites of DNA damage, thereby limiting the chance of inappropriate telomere formation. A variety of mechanisms have been reported in yeast and vertebrates to restrain telomerase action following genotoxic stress (Schulz and Zakian, 1994; Kharbanda *et al.*, 2000; Wong *et al.*, 2002; Makovets and Blackburn, 2009). The extent to which all of these pathways are conserved bears further investigation.

Finally, it is curious that despite the strong inhibition of telomerase in plants lacking ATR, telomere length homeostasis is unperturbed in the five generations of mutants we monitored (Vespa *et al.*, 2005; this study). One possibility is that DNA damage triggers a qualitative change in telomerase behavior, which is detected in our Q-TRAP assay as a quantitative change in activity. Repeat addition processivity (RAP) is not a property of *Arabidopsis* telomerase that can be assessed in our PCR-based TRAP assay. However, RAP of telomerase influences, and is influenced by, telomere length (Lue, 2004). Telomerase RAP is dramatically altered in human cancer cells, depending upon whether telomeres are within the normal range or are artificially shortened (Zhao *et al.*, 2011). Similarly, the RAP of yeast telomerase is enhanced at critically shortened telomeres in an ATM-dependent manner (Chang *et al.*, 2007). Thus it is conceivable that a crippled telomerase in *atr* mutants is sufficient to maintain telomeres already in the wild-type range but lacks the capacity to act efficiently on critically shortened telomeres in *ctc1* mutants, thereby enhancing the pace of telomere attrition.

MATERIALS AND METHODS

Plant lines and growth conditions

Mutant *Arabidopsis thaliana* lines and genotyping have been previously described. The alleles used were *ctc1-1* and *ctc1-3* (Surovtseva *et al.*, 2009), *stn1-1* (Song *et al.*, 2008), *atr-2* (Culligan *et al.*, 2004), and *atm-2* (Garcia *et al.*, 2003). Crosses were made with plants heterozygous for *ctc1* or *stn1* and homozygous mutant for *atr* or *atm*. F1 plants were genotyped to identify plants that were heterozygous for both alleles. These were self-crossed, and F2 siblings were used for analysis. Plants were grown on soil at 22°C under 16-h light/8-h dark conditions. For experiments using seedlings, seeds were sterilized in 50% bleach with 0.1% Triton-X 100 and then plated on Murashige and Skoog (MS) medium with 0.7% agar (Caisson Labs, North Logan, UT). Plates were placed in the dark at 4°C for 2–4 d and then moved to long day conditions.

For zeocin treatment, seeds were treated as described. When seedlings were 5–7 d old, they were transferred to liquid MS culture either with or without 20 μM zeocin (Invitrogen, Carlsbad, CA). Seedlings were grown in the dark for 3 d and then harvested for protein extraction.

Quantitative RT-PCR

Total RNA was extracted from G1 flowers using the E.Z.N.A. Plant RNA kit with on-column DNaseI digestion (Omega Bio-Tek, Norcross, GA). To make cDNA, 2 μg of RNA was used with the qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). cDNA was diluted 1:4 in 10 μg/ml yeast tRNA (Sigma-Aldrich, St. Louis, MO), and 1 μl was used in each quantitative PCR. The SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) was used following manufacturer's recommendations. Reactions were run on a Bio-Rad CFX96 thermal cycler using 58°C primer annealing and 10-s extension. RNA from at least three individual plants was used for each genotype, and two replicates were run for each reaction. The raw amplification data were imported into LinRegPCR (Ruijter *et al.*, 2009) using the default settings. The window of linearity and Cq threshold were calculated for each amplicon group. The resulting Cq values, which had been adjusted for the mean PCR efficiency for each amplicon, were used for calculation of expression levels.

For each run, we measured three reference genes (*GAPDH*, *TIP41L*, and *At4G26410*) reported by Czechowski *et al.* (2005). The geometric mean of the three reference genes was used to calculate expression levels by the ΔΔCt method. Expression levels for each genotype were averaged and compared with that of wild type.

Primers sequences were 5′-TGCATCCATTAAGTTGCCCTGTG-3′ and 5′-TAGGCTGAGAGTGCAGTGGTTC-3′ for *BRCA1* (At4G21070), 5′- ATGCTACTCTGGCACGGTTCAC-3′ and 5′-AGGAGGAGCTATTCGCAGACCTTG-3′ for *PARP1* (At4G02390), and 5′-CGAGGAAGGATCTCTTGCAG-3′ and 5′- GCACTAGTGAACCCCAGAGG-3′ for *RAD51* (At5G20850).

Telomere length measurement, in-gel hybridization, TF-PCR, and TRAP

Genomic DNA was extracted from whole plants or seedlings using 2x CTAB buffer (Vespa *et al.*, 2005) with slight modification. Plant extracts were incubated for 1 h at 50°C, and all mixing was done by inverting tubes rather than vortexing. TF-PCR and PETRA (Heacock *et al.*, 2004) and TRF (Fitzgerald *et al.*, 1999) were conducted as previously reported. For all three assays, products were detected by Southern blot with a $[^{32}P]$5′-end-labeled $(TTTAGGG)_4$ probe. A $[^{32}P]$5′-end-labeled $(CCCTAAA)_3$ probe was used for in-gel hybridization as described previously (Surovtseva *et al.*, 2009). Telomere lengths from PETRA analyses were calculated using QuantityOne software (Bio-Rad). For lanes with multiple bands, the average size was calculated. Protein extracts from 5- to 7-d-old seedlings were used for quantitative TRAP as previously described (Kannan *et al.*, 2008).

Propidium iodide staining and cytogenetics

Five- to seven-day-old G2 seedlings were gently removed from MS plates and placed in 10 μg/ml propidium iodide solution diluted in water for 10 min at room temperature in the dark. Seedlings were then transferred to water. Roots and shoots were separated, and roots were mounted on slides in water. *Arabidopsis* chromosome spreads were prepared from pistils as described (Riha *et al.*, 2001). The spreads were mounted on slides with Vectashield Plus 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). All slides were visualized with a Zeiss (Thornwood, NY) Axioplan2 epifluorescent microscope using a rhodamine filter for PI slides and a DAPI filter for chromosome spreads. ImageJ (Abramoff *et al.*, 2004) was used to adjust the brightness and contrast of images.

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