Telomere length deregulation and enhanced sensitivity to genotoxic stress in *Arabidopsis* mutants deficient in Ku70

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The Ku70/80 heterodimer is a critical component of the non-homologous end-joining (NHEJ) pathway and of the telomere cap in yeast and mammals. We report the molecular characterization of the *KU70* and *KU80* genes in *Arabidopsis* and describe the consequences of a Ku70 deficiency. *Arabidopsis KU70/80* genes are ubiquitously expressed and their products form stable heterodimers *in vitro*. Plants harboring a T-DNA insertion in *KU70* exhibit no growth or developmental defects under standard growth conditions. However, mutant seedlings are hypersensitive to γ -irradiation-induced double-strand breaks. Unexpectedly, we found that mutants are hypersensitive to methyl methanesulfonate during seed germination, but lose this sensitivity in seedlings, implying that the requirement for NHEJ varies during plant development. Lack of Ku70 results in a dramatic deregulation of telomere length control, with mutant telomeres expanding to more than twice the size of wild type by the second generation. Furthermore, in contrast to the situation in mammals, chromosome fusions are not associated with a Ku deficiency in *Arabidopsis*. These findings imply that Ku may play a different role in capping plant and animal telomeres.

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Introduction

Double-strand DNA breaks (DSBs) are lethal DNA lesions that can be induced by external assaults such as ionizing radiation and radiomimetic drugs, or by endogenous metabolic processes including DNA replication. Because a single unrepaired DSB can lead to cell-cycle arrest (Sandell and Zakian, 1993), efficient DSB repair mechanisms are essential. In eukaryotes, broken ends can be processed in two pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR) (Critchlow and Jackson, 1998; Hiom, 1999; Thompson and Schild, 2001). In both cases, the break is repaired by 'resealing' the free DNA ends to generate a contiguous molecule.

Ku is a heterodimeric complex of 70 and 80 kDa subunits, critical for the NHEJ pathway. Ku binds with high affinity to broken DNA ends to prevent degradation and facilitate alignment (Walker *et al.*, 2001) for subsequent

ligation by DNA ligase IV. DNA-dependent protein kinase and the ligase IV-associated protein XRCC4 are also required for NHEJ, but their precise roles are unclear (Critchlow and Jackson, 1998). In yeast lacking functional Ku, NHEJ is severely impaired (Boulton and Jackson, 1998; Baumann and Cech, 2000), and mutants show enhanced sensitivity to radiomimetic agents in a *rad52* background (Mages *et al.*, 1996). Mammalian cells or animals deficient in either the Ku70 or Ku80 subunit are similarly radiosensitive and are defective in V(D)J recombination (Featherstone and Jackson, 1999). In addition, Ku-deficient mice exhibit impaired growth and the early onset of senescence (Nussenzweig *et al.*, 1996; Vogel *et al.*, 1999).

While NHEJ is believed to be the major DNA repair pathway in higher eukaryotes, this mechanism is inherently error prone (Kirik *et al.*, 2000). Recombinational repair, in contrast, provides an error-free route for DSB repair and is the preferred pathway in lower eukaryotes (Hiom, 1999). Recent studies suggest that HR also plays an important role in DSB repair in higher eukaryotes during S and G₂, when the homologous sequence from a sister chromatid can be utilized for accurate repair (reviewed in Thompson and Schild, 2001). Moreover, cell cycle-dependent employment of both NHEJ and HR has been reported in vertebrates (Takata *et al.*, 1998), with NHEJ playing the dominant role during G₁, and HR in late S and G₂.

Relatively little is known about DNA repair in plants (Vonarx *et al.*, 1998; Britt, 1999). Two mutants deficient in DSB repair have been described in *Arabidopsis thaliana*. Inactivation of *MIM*, a plant homolog of *Rad18*, reduces somatic recombination rates and increases sensitivity to genotoxic stress (Mengiste *et al.*, 1999). Similarly, disruption of *RAD50* increases sensitivity to methyl methanesulfonate (MMS) and renders mutants sterile (Gallego *et al.*, 2001). To date, no mutants deficient in NHEJ have been described, although *Arabidopsis* orthologs of ligase IV and XRCC4 have been characterized (West *et al.*, 2000).

The natural ends of linear eukaryotic chromosomes are masked from DNA repair and checkpoint machinery by telomeres, higher order nucleoprotein caps. Telomeres consist of short G-rich repeat arrays associated with specific non-nucleosomal telomere-binding proteins (Collins, 2000) that protect the ends from fusions (van Steensel *et al.*, 1998) and control the length of the telomere tract (Smogorzewska *et al.*, 2000). Replication of telomeres is mediated by telomerase, a ribonucleoprotein reverse transcriptase that uses its RNA subunit as a template for addition of telomere repeat sequences by the catalytic subunit, TERT (Nugent and Lundblad, 1998). Proteins involved in DSB repair and checkpoint response are also closely associated with telomeres, and are

Fig. 1. Identification of Ku 70/80 proteins in *Arabidopsis*. (A) Schematic diagram of Ku70 and alignment of the C-terminal DNA-binding domain (SAP) (Aravind and Koonin, 2000). (B) Dimerization of Ku70/80 proteins *in vitro*. Co-immunoprecipitation experiments using recombinant T7-tagged Ku80 or Ku70 and ³⁵S-labeled (asterisk) Ku70 or luciferase are shown. See Materials and methods for details.

essential for telomere maintenance. For example, mutations in the *MRT2* gene, which encodes a *Caenorhabditis elegans* homolog of the yeast DNA checkpoint protein Rad17, result in telomere shortening and chromosome fusions (Ahmed and Hodgkin, 2000). In addition, the DSB repair complex Mre11/Rad50/Xrs2 (Nbs2) is required for telomere maintenance in yeast and humans (Nugent *et al.*, 1998; Ranganathan *et al.*, 2001), and recent studies reveal cell cycle-regulated association of this complex with the human telomere protein TRF2 (Zhu *et al.*, 2000).

The Ku heterodimer also plays a critical role in telomere length homeostasis and, while these proteins are known to interact directly with TRF1, TRF2 and the telomerase RNA subunit (Hsu *et al.*, 2000; Song *et al.*, 2000; Peterson *et al.*, 2001), their contribution to the telomere cap remains enigmatic. Inactivation of *Saccharomyces cerevisiae* *YKU70* or *YKU80* leads to a temperature-sensitive phenotype characterized by shorter telomeres with extended terminal G overhangs (Boulton and Jackson, 1998; Gravel *et al.*, 1998; Polotnianka *et al.*, 1998). Silencing of telomere-proximal genes is also dramatically reduced and telomeres are delocalized from the nuclear periphery (Laroche *et al.*, 1998). The temperature sensitivity of *yku* cells is attributed to a lethal alteration in telomere structure at elevated temperature (Fellerhoff *et al.*, 2000). The telomeres of fission yeast that harbor *ku70* mutations are shorter than wild type and exhibit extensive subtelomeric rearrangements (Baumann and Cech, 2000). However, unlike budding yeast, *Schizosaccharomyces pombe* *ku70*⁻ cells are not temperature sensitive (Manolis *et al.*, 2001). Mammalian cells deficient in Ku display frequent end-to-end chromosome fusions (Bailey *et al.*, 1999; Hsu *et al.*, 2000; Samper *et al.*, 2000; d'Adda di Fagagna *et al.*, 2001). The impact on telomere dynamics in mammals is still unclear as both telomere shortening and lengthening

have been reported (Samper *et al.*, 2000; d'Adda di Fagagna *et al.*, 2001).

As in other eukaryotes, telomeres in plants are maintained by telomerase, and *Arabidopsis* mutants harboring a T-DNA insertion in the TERT gene are characterized by gross cytogenetic and developmental defects when telomeres become critically shortened (Fitzgerald *et al.*, 1999; Riha *et al.*, 2001). A role for the Mre11/Rad50/Xrs2 DNA repair complex at telomeres is implied from analysis of the *Arabidopsis* Rad50 homolog. Plants lacking Rad50 protein are sterile, but show no telomere defects. However, cell cultures derived from these mutants exhibit dramatic telomere shortening and massive cell death. Survivors harbor telomeres that are significantly longer than wild-type (Gallego and White, 2001). Here we investigate the role of NHEJ in plants and describe the consequences of a Ku70 deficiency in *Arabidopsis*. We demonstrate an essential role for Ku in the DNA damage response, and also provide evidence that the NHEJ pathway is not the sole mechanism for repairing DSBs in plants. Finally, we show that although telomere length homeostasis is severely perturbed in mutants lacking Ku, the protective cap on the chromosome terminus remains intact.

Results

Molecular characterization of Ku70 and Ku80 homologs in *Arabidopsis*

A TBLASTN search of the DNA database revealed a likely *Arabidopsis* homolog for Ku70 on chromosome 1 (BAC clone F6I1; DDBJ/EMBL/GenBank accession No. AC051629), which displayed ~30% identity to human Ku70. To obtain a full-length cDNA for the *Arabidopsis* gene, we searched the *Arabidopsis* expressed sequence tag (EST) non-redundant database and found a cDNA clone, FB059c01F (DDBJ/EMBL/GenBank accession No. AV533293; Asamizu *et al.*, 2000), with identity to the 3' end of the *AtKU70* gene. The remainder of the *KU70* cDNA was obtained by RT-PCR and 5' RACE using primers designed from the predicted coding sequence. Our *KU70* cDNA sequence matches a sequence reported in the DNA database during preparation of this manuscript (DDBJ/EMBL/GenBank accession No. AF283759). Overall, the predicted Ku70 protein exhibits 29% identity (45% similarity) to the human Ku70 protein and 20% identity (33% similarity) to the *S.cerevisiae* Yku70. The C-terminus of AtKu70 contains a SAP domain (Aravind and Koonin, 2000), a DNA-binding motif conserved in all characterized Ku70 orthologs except *S.cerevisiae* Yku70 (Figure 1A).

Recently, the complete mRNA sequence of an *Arabidopsis* Ku80-like protein was reported in the DNA database (DDBJ/EMBL/GenBank accession No. AF283758; Y.Adachi, K.Oguchi, K.Tamura and H.Takahashi, unpublished data). *AtKU80* codes for a predicted protein with 22% identity (43% similarity) to human Ku86 and 24% identity (41% similarity) to *S.cerevisiae* Yku80. We used RT-PCR to obtain a full-length cDNA of *AtKU80*.

Since Ku70 and Ku80 exist as a heterodimeric complex in other organisms, we tested whether the putative Ku70 and Ku80 proteins from *Arabidopsis* interact *in vitro* using co-immunoprecipitation experiments. The *AtKU70* and

Fig. 2. Expression analysis of *Ku70/80* mRNA. RNA isolated from a variety of organs was analyzed by northern blot hybridization using a probe derived from the entire *Ku70* cDNA. The blot was stripped and rehybridized with a *Ku80* probe. Arrowheads denote two *Ku80* RNA species that may arise from different 3' end processing events.

AtKu80 coding regions were inserted into a pET28a expression vector to produce recombinant Ku70 and Ku80 proteins either with or without an N-terminal T7 tag. As shown in Figure 1B, untagged ^{35}S -labeled Ku70 protein was precipitated in the presence of unlabeled T7-tagged Ku80 using a T7 antibody (lane 3). In control reactions, no interaction was seen between T7 antibodies and untagged Ku70 (Figure 1B, lane 1), or between Ku80 and luciferase (Figure 1B, lane 7). Furthermore, as reported for mammalian Ku proteins (Osipovich *et al.*, 1997; Cary *et al.*, 1998), we found no evidence for AtKu70 homodimerization (Figure 1B, lane 5).

Northern blot analysis of *KU70* and *KU80* transcripts from total RNA samples detected RNAs of the expected size (~2.3 kb) in all tissues examined (Figure 2; see also Figure 3B). The ubiquitous expression profile of *KU* genes in *Arabidopsis* resembles the profile in mammals (Cai *et al.*, 1994; Koike *et al.*, 1996). In non-photosynthesizing tissues, a slightly higher molecular weight species of *KU80* mRNA was detected (Figure 2). RT-PCR indicated that the two *KU80* RNA species are not products of alternative splicing (data not shown). Instead, we suspect that they result from distinct 3' end mRNA processing events (Hartung and Puchta, 2000), since two putative cleavage/polyadenylation signals are present 100 bp apart in the *KU80* 3'-untranslated region.

Identification of an *Arabidopsis ku70* mutant

To examine the role of the Ku complex in plants, we screened a collection of 60 480 *Arabidopsis* T-DNA insertion lines available at the University of Wisconsin *Arabidopsis* Knock-out Facility (Krysan *et al.*, 1999) and identified a line harboring a disruption in the *KU70* gene (Figure 3A). Southern blot analysis revealed a single site of T-DNA insertion that maps to the 10th intron of *KU70*. The disruption appears to be comprised of three adjacent T-DNA insertions (data not shown).

Northern blot analysis of mutants homozygous for the T-DNA insertion failed to detect *KU70* mRNA (Figure 3B; the arrowhead indicates non-specific hybridization). The level of *KU80* was the same as in wild-type. We confirmed that expression of the full-length transcript was abolished by the T-DNA insertion by performing RT-PCR using primers that flank the T-DNA insertion site. No product was obtained after 35 cycles of PCR amplification

Fig. 3. Disruption of the *Arabidopsis KU70* gene by T-DNA insertion. (A) Schematic diagram of the *AtKU70* gene. The position of a T-DNA insertion is indicated. Boxes = exons; arrowheads = primers used for RT-PCR and genotyping. (B) Northern blot analysis of *KU70/80* expression in mutant and wild-type plants. The same membrane was used for sequential hybridizations with *Ku70* and *Ku80* probes. The arrowhead marks a non-specific hybridization signal. (C) RT-PCR analysis of the disrupted *ku70* allele. Thirty-five cycles of PCR were performed. Under semi-quantitative conditions (30 PCR cycles, 250 ng of RNA), products corresponding to the Ku70 N-terminus were barely detectable in mutants, while the wild-type signal was strong (data not shown).

(Figure 3C, lane 4). This finding excludes the possibility that splicing out the entire T-DNA generates an intact full-length Ku70 transcript. RT-PCR of the *KU70* 5' and 3' coding regions flanking the T-DNA insertion revealed the presence of low abundance transcripts undetectable by northern blotting (Figure 3C, lanes 2 and 6). Sequence analysis of the 3' transcript expressed from a cryptic promoter within the T-DNA (Mengiste *et al.*, 1999) demonstrated the lack of a consensus AUG codon in a reading frame appropriate for translation of the Ku70 C-terminus (data not shown). Taken together, these data argue that Ku function in the mutants is likely to be severely compromised, and possibly null.

Plants homozygous for the T-DNA insertion were propagated for two generations. In striking contrast to mammals deficient in Ku (Nussenzweig *et al.*, 1996), *Arabidopsis ku70* mutants were phenotypically indistinguishable from their heterozygous and wild-type siblings. No defects in vegetative or generative growth and development were detected under standard growth conditions, and mutants were fertile.

Response of *Ku70*-deficient *Arabidopsis* to genotoxic stress

To determine whether disruption of *KU70* alters the response to DNA damage, we germinated seeds from *ku70*^{-/-} and wild-type plants on solid media supplemented with various concentrations of MMS. The germination efficiency of wild-type dropped by ~20% in the presence of 100 p.p.m. MMS. In contrast, the ratio of germinating to

Fig. 4. Response of *ku70* mutants to genotoxic stress. (A) Germination efficiency of wild-type and mutant seeds in media supplemented with various concentrations of MMS. Results from four independent experiments are shown. Bars denote the standard deviation. Concentration units are in p.p.m. (B) Response of seedlings to MMS. Five-day-old seedlings were incubated in media containing different concentrations of MMS (in p.p.m.). (C) Response of seedlings to γ -irradiation. Five-day-old seedlings were subjected to increasing doses of radiation as indicated. For both experiments, plants were photographed 10 days after genotoxic treatment.

non-germinating mutant seeds decreased steadily with increasing concentration of MMS. In 100 p.p.m. MMS, germination was completely abolished (Figure 4A). Surprisingly, a subset of the mutants that germinated in the presence of 75 p.p.m. MMS grew at a rate similar to wild type (data not shown). This observation strongly suggests that Ku-deficient plants are hypersensitive to MMS only during germination. Previous studies demonstrate that seedlings defective in DNA repair or damage checkpoint response are hypersensitive to MMS (Mengiste *et al.*, 1999; Ulm *et al.*, 2001). Therefore, we examined the sensitivity of Ku-deficient plants to MMS treatment. Five-day-old wild-type and mutant seedlings germinated and grown in the absence of MMS were transferred into a liquid medium supplemented with MMS. In this assay, mutant seedlings showed the same response to MMS as wild type (Figure 4B). In our experiments, the toxicity of MMS was higher in liquid media such that growth of both wild-type and mutant plants was completely inhibited in 75 p.p.m. MMS. However, we detected no difference in the growth of wild type and mutants in 50 p.p.m. MMS

Fig. 5. Telomere length analysis of Ku70-deficient plants. (A) TRF analysis of DNA from individual wild-type, heterozygous and first- (G_1) and second- (G_2) generation mutants. Note that the sharp bands in the wild type and heterozygotes are replaced by smears in mutants. (B) Single telomere analysis of wild-type and G_2 *ku70* mutants using a telomere-associated probe derived from the right arm of chromosome 2. The asterisk denotes interstitial telomeric DNA.

(Figure 4B). In addition, 8-day-old mutant seedlings transferred to solid media containing 100 p.p.m. MMS grew as well as the wild type (data not shown). Thus, the MMS hypersensitivity of Ku-deficient mutants is confined to a specific stage in development.

In mammals, Ku-deficient mutants are hypersensitive to ionizing radiation. We examined the response of *Arabidopsis ku70* mutants to ionizing radiation by exposing 5-day-old seedlings to γ -irradiation (Figure 4C). A dose of 50 Gy retarded the growth of mutants, while 100 Gy strongly suppressed development of true leaves and roots. In contrast, wild-type plants treated with this higher dose displayed only minimal growth defects. From these data, we conclude that Ku-deficient seedlings respond differently to genotoxic stresses, as they exhibit wild-type sensitivity to MMS and hypersensitivity to ionizing radiation.

Telomere status and genome stability in Ku-deficient Arabidopsis

We next examined the role of Ku70 in telomere length homeostasis by determining telomere length in individual plants from the first two generations of mutants. Terminal restriction fragments (TRFs) were generated by cleavage with *Tru9I* and analyzed by Southern hybridization with a telomeric probe. Telomeres in the wild-type Wassilewskija (WS) ecotype and plants heterozygous for the *ku* mutation ranged in size from 4 to 8 kb, and were twice the size of telomeres in the Colombia ecotype (Fitzgerald *et al.*, 1999). This finding was not unanticipated, since telomere lengths vary by ~ 2 -fold among different *Arabidopsis* ecotypes (K.Davis and T.McKnight, personal communication). Surprisingly, however, telomeres in first-generation *ku70* homozygous mutants (G_1) extended from 6 to 12 kb (Figure 5A) and, by the second generation (G_2), approached ~ 20 kb. Thus, telomeres in

ku70 mutants are subject to continual extension over successive plant generations.

Two types of experiments were performed to verify that the telomere elongation phenotype was associated with the T-DNA insertion in *KU70*. First, co-segregation analysis was conducted using 70 progeny derived from plants heterozygous for the T-DNA insertion. A total of 19/19 homozygous mutants displayed elongated telomeres, while 51/51 heterozygous and wild-type plants exhibited the wild-type telomere profile (data not shown). Secondly, the telomere phenotype was complemented by ectopic expression of wild-type Ku70. Two complementation constructs, one harboring a full-length genomic clone containing 1.6 kb of putative promoter sequence and the second containing Ku70 cDNA expressed from the cauliflower mosaic virus (CaMV) 35S promoter, were used for complementation of the *ku70* mutant. The two constructs were introduced into plants heterozygous for the T-DNA insertion in *KU70*. In T1, four individuals were identified (two plants for each complementation construct) that were homozygous for the T-DNA disruption in Ku70 and which ectopically expressed wild-type Ku70 mRNA. In each case, ectopic expression of *KU70* prevented the dramatic telomere extension (data not shown). Taken together, these data demonstrate that the telomere phenotype is linked to a disruption in *KU70*.

The discrete banding profile of TRFs in wild-type plants (Figure 5A) implies that telomeres are tightly regulated in the WS ecotype. Probes specific for individual chromosome ends indicated that telomere tracts are maintained within a 1 kb range (Figure 5B; data not shown). Individual telomere tracts are maintained in the same size range in the Colombia ecotype (Riha *et al.*, 2001; K.Riha and D.Shippen, unpublished data). In contrast, telomeres in *ku70* mutants were extremely heterogeneous (Figure 5A). This heterogeneity was especially apparent when TRFs were visualized using probes specific for individual chromosome ends (Figure 5B; data not shown). The 2R probe, for instance, showed a discrete band of 8–9 kb in wild-type plants, and a heterogeneous signal spreading from 8 to ~18 kb in the mutants (Figure 5B).

Tru9I recognizes the sequence TTAA, and cleaves immediately adjacent to the terminal telomere repeat tract. Hence, the longer terminal restriction fragments that arise from the mutants are comprised almost exclusively of TTTAGGG repeats. A preliminary analysis of subtelomeric regions indicated that there was no gross DNA rearrangement (data not shown). Instead, only the terminal telomere repeat tract appears to be modified in Ku-deficient plants.

Inactivation of Ku in mammals results in a high frequency of end-to-end chromosome fusions (Bailey *et al.*, 1999; Samper *et al.*, 2000; d'Adda di Fagagna *et al.*, 2001). Cytogenetic analysis of >700 anaphases in *G*₁ and *G*₂ *ku70* mutants failed to reveal any fusion events. In contrast, in late-generation telomerase-deficient mutants analyzed by the same protocol, up to 45% of the anaphases examined harbored bridges (Riha *et al.*, 2001). We conclude from these data that despite dramatic deregulation of telomere length control, telomeres in Ku70-deficient *Arabidopsis* are still functional and protect chromosomes from end-to-end fusions.

Discussion

Plants face a constant risk of DNA damage due to continuous exposure to a variety of environmental factors, and thus very efficient repair mechanisms must operate to maintain genome integrity. Here we report establishment of a plant model deficient in the NHEJ DNA repair pathway. The Ku70 mutant we characterized carries a T-DNA insertion in the 10th intron located in the middle of the gene. While we could detect no full-length transcripts from the disrupted gene by northern blot analysis or RT-PCR, a low abundance transcript from the N-terminus was detected by RT-PCR. It is unlikely that a truncated protein translated from this RNA would be functional as studies in yeast show that deletion of the extreme C-terminus of Ku70 is essential for both NHEJ and telomere maintenance (Driller *et al.*, 2000). However, we cannot rule out the possibility that a protein derived from the Ku70 N-terminus interferes with some biological function in plants. A binding site for the telomere protein TRF2 is located in the N-terminal half of human Ku70 (Song *et al.*, 2000) and, hence, the presence of a truncated Ku protein could potentially contribute to the unique telomere phenotype associated with *ku70* mutants (see below).

Requirement for the NHEJ pathway varies during plant development

Both HR and NHEJ are implicated in repair of DSBs in eukaryotes, but NHEJ has been assumed to be the major pathway in plants based on the random integration of a foreign DNA into plant genomes (Britt, 1999; Mengiste and Paszkowski, 1999). We directly investigated the contribution of the NHEJ pathway to DSB repair in *Arabidopsis* by exposing plants deficient in Ku to two different genotoxic stresses, γ -irradiation and MMS. Surprisingly, we found that mutant seedlings are hypersensitive to γ -irradiation, but relatively insensitive to MMS. We postulate that these distinct responses reflect the mechanism by which the break is introduced. Ionizing radiation leads to DSBs (as well as a broad spectrum of other DNA lesions) immediately after exposure. In seedlings, the majority of plant cells are in *G*₁, and unrepaired DSBs induced by γ -irradiation would prevent cells from entering S phase. The failure of *Arabidopsis ku70* mutants to produce true leaves following a relatively low dose of γ -irradiation is consistent with a crucial role for NHEJ in DSB repair during *G*₁.

MMS is a monofunctional alkylating agent that produces abasic lesions and single-strand nicks (Helbig and Speit, 1997; Glaab *et al.*, 1999). The damaged site subsequently is converted into a DSB during DNA replication in S phase and leads to fork arrest. Thus, in contrast to γ -irradiation, the DSBs introduced by MMS are S-phase specific. Vertebrate studies indicate that the cell cycle context of a DSB determines whether NHEJ or HR is employed to repair it (Takata *et al.*, 1998). DSBs that arise during DNA replication, such as those introduced by MMS, are repaired through HR (Marians, 2000; Michel, 2000). Consistent with this idea, *Drosophila* mutants defective in HR (*rad54*^{-/-}) are more sensitive to MMS treatment than *ku70*^{-/-} flies (Kooistra *et al.*, 1999). Furthermore, MMS-treated Ku-deficient hamster cells have the

same rate of DNA break rejoining as control cells (Helbig and Speit, 1997).

Recent studies support the notion that HR contributes to DSB repair in plants. *MIM* and *RAD50* are predicted to play a role in HR (Mengiste *et al.*, 1999; Signon *et al.*, 2001), and plants deficient in *MIM* and *RAD50* are sensitive to MMS treatment (Mengiste *et al.*, 1999; Gallego *et al.*, 2001). These results together with data presented here argue that both NHEJ and HR are utilized to repair DSBs in plants and that the cell cycle context of the DSB strongly influences which pathway is chosen for its repair.

One surprising result from our study is that in contrast to Ku-deficient seedlings, mutant seeds are hypersensitive to MMS and germination is reduced dramatically even at very low levels of MMS. We interpret this to mean that the requirement for NHEJ varies throughout plant development. In support of this idea, mouse studies demonstrate that the relative contributions of HR and NHEJ in the repair of radiation-induced DNA damage change during development (Essers *et al.*, 2000). During dehydration and dry storage, plant seeds are subject to extensive DNA damage, and pre-replicative repair occurs immediately after imbibition (reviewed in Vonarx *et al.*, 1998; Whittle *et al.*, 2001). Although germination of Ku-deficient seeds is unaffected under normal conditions, our data suggest that the additional damage incurred by MMS treatment cannot be repaired in the absence of Ku. Since seed viability and DNA integrity decrease during prolonged storage, it will be interesting to examine germination efficiency in the aging seeds of Ku mutants.

***Arabidopsis* Ku is required for telomere length homeostasis, but not for chromosome stability**

The Ku heterodimer is involved in two seemingly paradoxical processes: it mediates resealing of broken DNA ends as a part of NHEJ and it localizes to telomeres, which protect chromosome ends from fusions. A Ku deficiency in mammals leads to massive genome instability and chromosome fusions, which are thought to cause stunted growth and accelerated aging. In contrast, the genome of Ku-deficient *Arabidopsis* is stable, and we could detect no chromosome fusions in >700 anaphases examined. This observation is consistent with the normal growth and development of mutant plants and argues that the telomere capping function in Ku-deficient plants is intact.

The absence of Ku affects telomere homeostasis in yeast to reset telomere tracts to a shorter but stabilized length (Boulton and Jackson, 1998; Gravel *et al.*, 1998; Polotnianka *et al.*, 1998; Baumann and Cech, 2000). However, in *Arabidopsis ku* mutants, telomere length control is deregulated, and telomere tracts steadily increase to over twice the size of wild type by the second generation. Monitoring telomere status in subsequent generations of the mutants will be necessary to determine if and when equilibrium is reached. The loss of telomere length homeostasis in *ku70* mutants is remarkable, since wild-type *Arabidopsis* telomeres are very tightly controlled (Fitzgerald *et al.*, 1999; Zentgraf *et al.*, 2000; Riha *et al.*, 2001; this study). Interestingly, inactivation of telomerase has the opposite effect, reducing telomere length polymorphism and leading to a very slow rate of

telomere shortening (Fitzgerald *et al.*, 1999; Riha *et al.*, 2001).

The longer telomeres in Ku-deficient *Arabidopsis* could result from a perturbation in telomere architecture that increases access of recombination machinery or telomerase. Elevated recombination rates at telomeric and subtelomeric regions are associated with a Ku deficiency in yeast (Polotnianka *et al.*, 1998; Baumann and Cech, 2000). Alternatively, Ku could act as a negative regulator of telomerase through direct interactions with telomerase (Grandin *et al.*, 2000; Peterson *et al.*, 2001) or telomere-binding proteins such as TRF1 and TRF2 (Hsu *et al.*, 2000; Smogorzewska *et al.*, 2000; Song *et al.*, 2000). Intriguingly, recent studies suggest that Ku is a positive regulator of telomerase in yeast (Grandin *et al.*, 2000). The longer telomeres and the absence of chromosome fusions in Ku-deficient plants argue that the function of Ku at telomeres differs among eukaryotes. Analysis of *Arabidopsis tert/ku70* double mutants should not only provide further insight into the role of the Ku complex, but may also help to define the relative contribution of telomerase and recombination to telomere maintenance in higher eukaryotes. These studies are underway.

Materials and methods

Plant growth and assays for sensitivity to genotoxic treatment

Arabidopsis thaliana plants (ecotype Wassilewskija) were grown at 23°C in an environmental chamber under a 16/8 h light/dark photoperiod. Seeds for the germination assay were sterilized in 50% bleach and plated on solid 0.5 BM medium (Mathur and Koncz, 1998) supplemented with 0–100 p.p.m. (v/v) MMS (Aldrich). Five-day-old seedlings were transferred to separate wells of a 24-well plate containing liquid 0.5 BM medium with 0–100 p.p.m. MMS and incubated in a shaker with constant light. To test sensitivity to ionizing radiation, 5-day-old seedlings were exposed to increasing time periods of γ -irradiation from a ^{60}Co source (Theratron 780; Atomic Energy Systems, Canada), for absorbed doses of 10, 50, 100 or 150 Gy. Following irradiation, seedlings were transferred to individual wells of a 24-well plate containing liquid 0.5 BM medium, and incubated in a shaker with constant light.

cDNA synthesis

Total mRNA was extracted from 0.1–0.5 g of plant tissue using Tri Reagent solution (Sigma). To obtain the 5' terminus of *AtKU70*, 5' RACE was performed using the 5' RACE System (Gibco) and nested primers Ku70-8 (5'-TGCGTTTATCTGCTGTC-3') and Ku70-9 (5'-TGAG-CCTTGAGTGACTGAGCAAT-3') according to the manufacturer's protocol. Ku70 and Ku80 cDNAs were synthesized from total leaf RNA using Superscript II reverse transcriptase (Gibco): 5 pmol of Ku70-4 (5'-AAACTCCACCATAAAGTTAGAGCCAGAT-3') or Ku80-2 (5'-GAA-CAAAAAGGGTTGTTCTGA-3') primers were incubated with 2 μg of total RNA in the supplied buffer at 65°C for 5 min. Reverse transcription was carried with 100 U of Superscript II at 42°C for 50 min. RNA was degraded with RNase H (USB). The coding regions of *KU70* and *KU80* were amplified with Ex-Taq polymerase (Takara) from the primer pairs: Ku70-12 (5'-CATATGGCTAGCATGGAATTG-GACCCAGATGATGT-3')/Ku70-6 (5'-CTTTTGTAGCTGAGGCAA-AACGAT-3') and Ku80-1 (5'-TCAGGTTCCATGGCACGAAATCG-GGAGGGTT-3')/Ku80-3 (5'-CGGTACCGTTGTCGACAAACAG-TTACAA-3'). PCR products were cloned into a pCR2.1-TOPO vector (Invitrogen).

Expression analysis of Ku70 and Ku80

A 15 μg aliquot of total RNA was separated in a 1.2% formaldehyde gel and blotted onto a nylon membrane (Sambrook and Russell, 2001). Hybridization with ^{32}P -labeled cDNA probes labeled by random priming was carried out as described (Church and Gilbert, 1984). Expression analysis of the regions flanking the T-DNA insertion in the *KU70* gene was performed using 1 μg of total RNA and the Access RT-PCR system

(Promega) (Fitzgerald *et al.*, 1999). The following primer sets were used: Ku70-12/Ku70-9 specific for the N-terminus; Ku70-15 (5'-CTG-TGGAAGAGCTATCCCAAGTA-3')/Ku70-5 (5'-ACGTCGCATTAGAGCTGATGCTT-3') for the C-terminus and Ku70-7 (5'-GGGAGA-ATTCCTTTATAGT-3') and Ku70-11 (5'-ACTTGGGATAGCTCT-TCCACAGTA-3') flanking the T-DNA insertion (see Figure 3A).

Dimerization assay

Full-length *KU80* cDNA was inserted into the *Bam*HI–*Sal*I restriction sites in the expression vector pET28a (Novagen) to produce a Ku80 fusion protein with an N-terminal His-T7 tag. *KU70* cDNA was cloned in either the *Nhe*I–*Hind*III or *Bam*HI–*Hind*III restriction sites of pET28a, resulting in His-Ku70 or His-T7-Ku70 fusions, respectively. All three constructs and the control vector encoding untagged luciferase were transcribed and translated separately in a TnT-coupled rabbit reticulocyte lysate (Promega) with or without [³⁵S]L-methionine (Amersham). Translation was stopped with cycloheximide (4 ng/μl) before ³⁵S-labeled proteins were mixed with T7-tagged unlabeled proteins in a ratio of 3:1 and incubated at 30°C for 15 min. Proteins were immunoprecipitated with T7 antibodies as described (Bryan *et al.*, 2000). Precipitate and supernatant fractions were analyzed by SDS–PAGE and autoradiography.

Identification of a *ku70* mutant

The α population of T-DNA insertion lines at the University of Wisconsin *Arabidopsis* Knock-out Facility was screened using primers Ku70-1 (5'-ACAACCACTGGCTTATTGACAGCTTTGTT-3') and Ku70-4 according to a protocol available at <http://www.biotech.wisc.edu/Arabidopsis/>. The progeny of a plant heterozygous for a T-DNA insertion in *KU70* were genotyped by PCR using the primers Ku70-2 (5'-TACTACACCAAGCAAGCCGTGATGGTT-3'), Ku70-11 and LB-CD6 (5'-GAA-CATCGGTCTCAATGCA-3') (Fitzgerald *et al.*, 1999). A 939 bp product was generated from the wild-type allele and a 639 bp product from the disrupted allele. Plants homozygous for the *ku70* disruption (referred to as G₁) were self-pollinated to obtain second-generation mutants (G₂).

Telomere analysis

DNA from individual plants was extracted as described (Cocciolone and Cone, 1993). TRF analysis was performed with *Tru*9I (Promega) restriction enzyme and ³²P 5' end-labeled (T₃AG₃)₄ oligonucleotide as a probe (Fitzgerald *et al.*, 1999). Single telomere analysis for the right arm of chromosome 2 (2R) was performed as follows: 1 μg of genomic DNA was digested with *Pvu*II, and DNA was separated by electrophoresis in a 0.7% agarose gel and blotted onto a nylon membrane. A DNA sequence derived from telomere-adjacent region 2R was used as a probe for hybridization (Riha *et al.*, 2001). Anaphase spreads were prepared from pistils as described previously (Riha *et al.*, 2001).

Complementation analysis

Two complementation constructs were prepared. First, a 6.7 kb genomic DNA fragment of the *KU70* gene containing 1.6 kb of putative promoter sequence was amplified by PCR using primers Ku18 (5'-GCCTTT-CACCACGTGCATCAT-3') and Ku21 (5'-ACGTTCTCAGGATC-CTTCAA-3'), cloned into a pDR PCR cloning vector (Qiagen) and then subcloned into a binary vector pCB302 (Xiang *et al.*, 1999). The second complementation construct was prepared by transferring a Ku70 cDNA fragment (see above) into a binary vector, pCBK05 (K.Riha and D.E.Shippen, unpublished data), to allow expression from a 35S CaMV promoter. Both vectors carry the *bar* gene as a selectable marker. Complementation vectors were introduced in *Agrobacterium tumefaciens* strain GV3101. Plants heterozygous for the T-DNA disruption in *KU70* were transformed by the *in planta* method (Bechtold *et al.*, 1993). T1 transformants were selected on 0.5 BM medium supplemented with 20 mg/l of phosphinotricine (Crescent Chemical), genotyped and analyzed for *Ku70* mRNA expression and telomere length as described above.

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