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Increased telomere length and hypersensitivity to DNA damaging agents in an *Arabidopsis KU70* mutant

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ABSTRACT

We have identified a putative homologue of the *KU70* gene (*AtKU70*) from *Arabidopsis thaliana*. In order to study its function in plants we have isolated an *A.thaliana* line that contains a T-DNA inserted into *AtKU70*. Plants homozygous for this insertion appear normal and are fertile. In other organisms the *KU70* gene has been shown to play a role in the repair of DNA damage induced by ionising radiation (IR) and by radiomimetic chemicals such as methylmethane sulfonate (MMS). We show that *AtKU70*$^{-/-}$ plants are hypersensitive to IR and MMS, and thus the *AtKU70* gene plays a similar role in DNA repair in plants as in other organisms. The *KU70* gene also plays a role in maintaining telomere length. Yeast and mammalian cells deficient for Ku70 have shortened telomeres. When we studied the telomeres in the *AtKU70*$^{-/-}$ plants we found unexpectedly that they were significantly longer (>30 kb) than was found in wild-type plants (2–4 kb). We propose several hypotheses to explain this telomere lengthening in the *AtKU70*$^{-/-}$ plants.

INTRODUCTION

In a cell the presence of double strand breaks (DSB) in the DNA can be catastrophic. Repair of such damage, often caused by ionising radiation (IR) or DNA replication errors, is essential for the maintenance of genetic integrity and progression of the cell through the cell cycle. Cells have developed two major pathways for the repair of DSB, homologous recombination (HR) and non-homologous recombination (NHR). The predominant pathway of DSB repair in cells (HR or NHR) varies between organisms and cell type. For instance, lower eukaryotes and bacteria generally utilise HR, whereas in higher eukaryotes NHR is the dominant repair pathway. Our knowledge of the proteins involved in both HR and NHR has come to a large extent from the yeast *Saccharomyces cerevisiae*. Many genes involved in NHR in this organism have been identified, including *MRE11*, *RAD50*, *XRS2*, *LIF1*, *NEJ1*, *LIG4*, *YKU70* and *YKU80* (reviewed in 1,2). The Ku70 and Ku80 proteins form a heterodimer, which in mammalian cells form a ternary complex with the DNA-PK_{cs} protein. In yeast, however, this protein is not present. The Ku heterodimer is probably an ancient structure as homologues of *KU70* and *KU80* have also been found in bacteria (3). The Ku heterodimer binds tightly and specifically to a variety of DNA end structures, suggesting that it serves as a primary sensor of broken chromosomal DNA (4). Once bound, Ku may recruit other factors to the break, for instance via its interactions with the ligase IV/XRCC4 complex (5) and Mre11 (6). Human or mouse cell lines lacking Ku70 are defective in DSB repair and are hypersensitive to IR and radiomimetic agents such as methylmethanesulfonate (MMS) (7). Mice lacking Ku70 or Ku80 are also proportional dwarfs (40–60% the size of normal littermates) (8–10) and have defects in V(D)J recombination (11). Furthermore, cells or animals lacking Ku70 have also been found to have shortened telomeres (reviewed in 12).

As part of our investigation into the role of plant proteins in DNA repair we have cloned an orthologue of the *KU70* gene from the model dicotyledonous plant *Arabidopsis thaliana* (*AtKU70*). In order to study the function of this gene, we have identified an *A.thaliana* line containing a T-DNA insertion in the *AtKU70* gene. Plants homozygous for the T-DNA insertion in *AtKU70* are phenotypically normal but hypersensitive to DNA damaging agents. Furthermore, when we investigated the length of the telomeres in such plants we found that rather than being shortened they were much longer than found in wild-type *Arabidopsis* plants. The data show that *AtKU70* plays a role in DNA repair and telomere maintenance in *Arabidopsis*. Plants seem to be able to tolerate mutations in NHR genes much more readily than mammalian cells. This makes plants an ideal system to investigate the roles of NHR genes in multicellular eukaryotes by studying lines containing multiple mutations in NHR genes as has been done in yeast previously.

MATERIALS AND METHODS

Identification of the *AtKU70* gene and isolation of a mutant plant line

The 1.8 kb *AtKU70* cDNA was amplified from a cDNA population synthesised from seedling total mRNA using the primers AKP (CATGCCATGGAATTGGACCCAGATGATGTTTTC) and AK3′ (CCCTCGAGGGTTATTTACCAA-

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TGTGAGTCAGAATC) which contain the *AtKU70* stop and start codons respectively (underlined), and cloned into pGEM-T Easy (Promega) for sequencing. The Arabidopsis Knockout Facility (AKF, WI) was screened using the *AtKU70* specific primers AK1 (CTCACCATTTGTTACGACGAGAAAGGTAT) or AK6 (GAGGAACAGCCATTGACTCTCTCGATAA) and a T-DNA specific primer JL-202 (13). The screening primers were designed as suggested (http://www.biotech.wisc.edu/NewServicesAndResearch/Arabidopsis/Guidelines.asp). A 3026 bp PCR product was produced using AK1 + JL-202 on pooled DNA. These primers were used in subsequent rounds of PCR screening until individual positive plants were identified. To map the insertion point of the T-DNA right border (RB) end, PCR was done on DNA from the *AtKU70-1* line using the primers JL-100 (TCCGCAGCGTTATTATAAAATGAAAGTAC) and AK6. The 4 kb PCR fragment produced was then sequenced. For the DNA blots, 4 µg total DNA isolated using the Nucleon Phytopure plant DNA extraction kit (Amersham Life Science) was digested overnight with *Eco*RI according to the manufacturer's instructions. The probe used was a 1871 bp PCR fragment of the *AtKU70* ORF generated using primers AK1 + AK3 (GGCGGTACTACACCTCCGAGATTGG) and was labelled with [α-^{32}P]dCTP. Northern blots were done using 8 µg total RNA isolated using the Rneasy Plant Mini Kit (Qiagen), probed with a 1.8 kb fragment of the full length *AtKU70* cDNA labelled with [α-^{32}P]dCTP. The blots to measure telomere length were done as reported previously (14).

Hypersensitivity of *AtKU70*$^{-/-}$ plants to IR and MMS

Hypersensitivity to MMS was performed as described previously (15). Seeds from *AtKU70-1*$^{-/-}$ plants were germinated for 4 days on 1/2 MS plates and transferred to 1/2 MS liquid medium containing MMS (Sigma). The concentrations tested were 0, 0.006, 0.008 and 0.01% MMS. The seedlings were scored after 3 weeks growth. For X-ray sensitivity, 4-day-old seedlings in liquid 1/2 MS were irradiated with the stated dosage of X-rays at 6 Gy/min using a 225 SMART X-ray machine as a source (Andrex SA, Copenhagen, Denmark), operated at 200 kV and 4 mA with a 1 mm aluminium filter. Plants were scored after 3 weeks further growth.

RESULTS

Isolation of an *AtKU70* mutant

A partial length soybean cDNA (AW598752) showing high homology with the yeast *KU70* sequence was found in a BLAST search of all higher plant sequences. This putative *KU70* gene from soybean was then used to screen the available *A.thaliana* sequences and a single copy gene (*AtKU70*) was identified (AT1G16970). The *AtKU70* cDNA was then amplified and sequenced. Comparison of the cDNA with the genomic sequence showed that this gene contains 18 exons. In Figure 1 the Ku70 proteins from *Arabidopsis*, yeast and humans are aligned.

In order to study the role of *AtKU70* in plants, a screen was performed at the Arabidopsis Knockout Facility as described (13) to identify plant lines containing a T-DNA insertion in *AtKU70*. After several rounds of screening we identified four individual plants that gave a PCR product specific for a T-DNA insertion in *AtKU70*. A DNA blot was done on the four positive plants obtained (Fig. 2B). Three of the four plants (lanes 2, 3 and 5) were found to be heterozygous for the T-DNA insertion in *AtKU70*, as they showed both the original band from the *AtKU70* locus plus an additional band. Interestingly, one plant (lane 4) only gave the mutant band on the DNA blot. This plant was thus homozygous for the T-DNA insertion in *AtKU70*. A detailed characterisation of the T-DNA insertion in the *AtKU70* gene is shown in Figure 2D. The left border (LB) end of the T-DNA was mapped by sequencing the PCR fragment generated using the primers JL-202 + AK1. The LB end was integrated into intron 10 of the *AtKU70* gene and had lost 9 bp during integration. The RB end of the T-DNA was mapped using the primers JL-100 + AK6. Sequencing of this PCR fragment showed the RB end had been heavily truncated. Approximately 4 kb had been lost from the T-DNA RB end. This resulted in loss of most of the *NPTII* ORF that is located on the T-DNA near the LB. We found that 234 bp of the 5′ end of the *NPTII* ORF were linked to exon 10 of the *AtKU70* gene (accession number AF283759, nucleotide 1038). Based on computer predictions, due to the T-DNA insertion a truncated AtKu70 protein of 351 amino acids (full length protein, 720 amino acids) may be produced. The last 22 amino acids of the truncated protein (LASHDSRAASSWSSFRHRTGRS*) are derived from the T-DNA before a stop codon is encountered. However, on a northern blot we were unable to detect a truncated *AtKU70* mRNA in the plants homozygous for the T-DNA insertion in *AtKU70* (*AtKU70*$^{-/-}$), while an mRNA of the predicted size was detectable in wild-type seedlings (Fig. 2C). Therefore, we conclude that the T-DNA insertion in *AtKU70* gives a 'null' phenotype. The T-DNA integrated into the *AtKU70* gene does not carry a functional *NPTII* gene. However, the *AtKU70* mutant line is resistant to the antibiotic kanamycin. The kanamycin resistant phenotype of the *AtKU70* mutant line must therefore result from additional T-DNA copies. Indeed, a DNA blot on the mutant line using a NPTII probe showed that the line contains four T-DNA copies in total (data not shown). Mice lacking Ku70 have a dwarf phenotype so we were therefore interested in examining the phenotypes of *AtKU70*$^{-/-}$ plants. These plants showed no obvious phenotypic differences with wild-type plants. Wild-type and mutant plants were similar in size, flowered at the same time and were fertile (data not shown).

AtKU70 deficient plants are hypersensitive to MMS and X-rays

Yeasts and mammalian cell lines lacking Ku70 are sensitive to DNA damaging agents such as the radiomimetic chemical MMS and X-rays (16,17). The sensitivity of *AtKU70*$^{-/-}$ and wild-type plants to these agents was compared. Seedlings (4 days old) were transferred to liquid medium containing MMS and grown for a further 3 weeks. For X-ray sensitivity, seedlings in liquid medium were exposed to the stated X-ray dosage and also then allowed to grow undisturbed for a further 3 weeks. As shown in Figure 3, *AtKU70*$^{-/-}$ plants are hypersensitive to both MMS and X-rays. A similar phenotype has also recently been reported in plants in which the *AtRAD50* gene, also necessary for NHR, is inactivated (18). This suggests that, as in other organisms, the Ku70 protein in

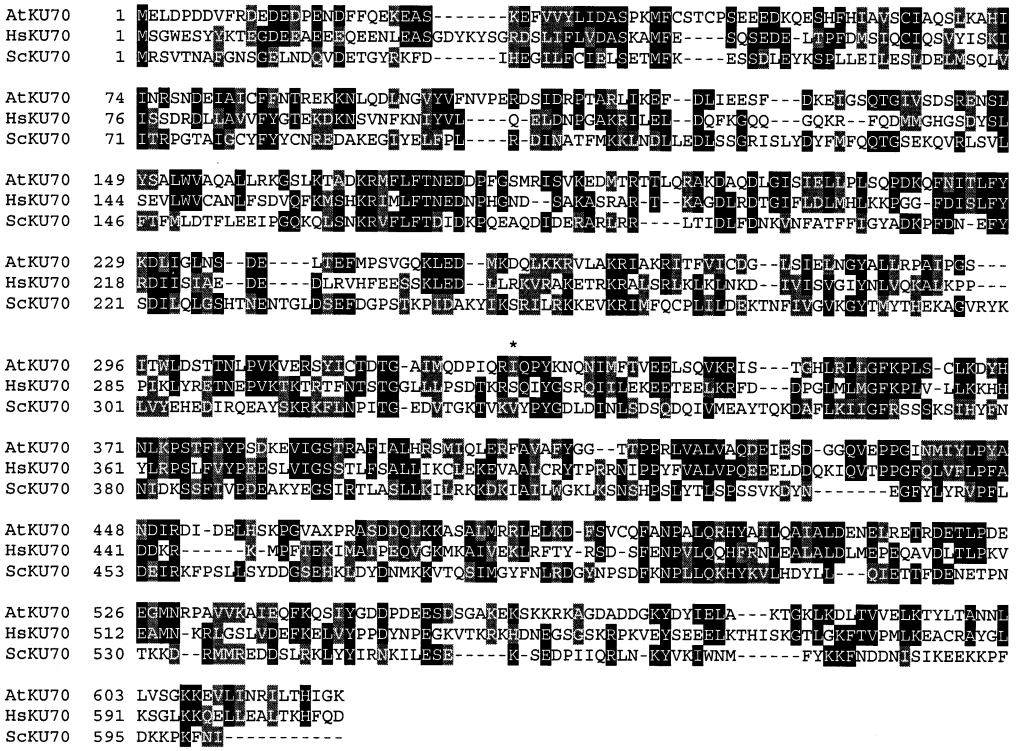

Figure 1. The sequences of the *A.thaliana* (AtKU70), *Homo sapiens* (HsKU70) and *S.cerevisiae* (ScKu70) proteins were aligned using the ClustalW alignment program. Identical residues are marked with black shading, similar residues with grey shading. The position at which the AtKu70 protein is truncated by the T-DNA insertion is indicated with an asterisk. The AtKu70 protein shares 28% identiity with the HsKu70 protein and 16% identity with the ScKu70 protein, respectively.

plants is involved in DNA repair processes. The MMS hypersensitivity phenotype was seen to co-segregate with the T-DNA insert in *AtKU70*. For this experiment, *AtKU70-1*$^{-/-}$ plants were crossed with the wild-type and 130 F_2 seedlings were tested for their hypersensitivity to MMS (0.008% v/v). In total, 28 MMS hypersensitive seedlings were found. PCR was performed on 12 of these, all of which were homozygous for the T-DNA insertion at *AtKU70*. As a control, PCR was also performed on 12 randomly selected seedlings that showed wild-type MMS sensitivity. All these plants contained an intact *AtKU70* locus ($\chi^2 = 24$, df = 1, $P < 0.0001$). This highly statistically significant result shows that the MMS hypersensitivity is strongly linked to the T-DNA insertion in *AtKU70*.

Lengthened telomeres in *AtKU70*$^{-/-}$ plants

Telomeres are specialised structures at the ends of chromosomes that are essential for genomic stability. Telomeres are made up of short G-rich repeats conforming to the consensus sequence Tx(A)Gy. The length of the telomeres varies between organism and cell type. In *Arabidopsis*, telomeres are 2–4 kb in size and consist of repeats of the sequence $TTTAGGG_n$. An *Arabidopsis* line deficient for the telomerase reverse transcriptase subunit (*AtTERT*) has been described (19). When several generations of *AtTERT*$^{-/-}$ plants were grown, a decrease of 500 bp of the telomeres per generation was observed. With this in mind, we measured the length of the telomeres in six successive generations of selfed *AtKU70*$^{-/-}$ plants to study any possible telomere shortening over several generations. Total DNA from 12-day-old seedlings was isolated and digested with the restriction enzyme *Mbo*I. This enzyme generates a series of heterogeneous terminal restriction fragments (TRFs) which are detected on the DNA blot as a classic telomere smear of 3–6 kb after hybridisation with a telomere probe $[TTTAGGG]_6$. The results are shown in Figure 4. To our surprise, we saw that the telomeres in the *AtKU70*$^{-/-}$ plants were longer (>30 kb) in comparison to wild-type plants. This lengthening was apparent from the first generation of plants tested and remained stable in the subsequent generations. The telomere signal appears stronger in the *AtKU70* mutant plants, but this is probably due to reduced migration of the DNA fragments through the agarose gel (0.8%). In *AtTERT*$^{-/-}$ plants the smear of heterogeneous TRFs is absent. It is replaced by a series of discrete bands corresponding to individual chromosome ends. A telomere smear, and therefore a heterogeneous mix of TRFs, is still apparent in the *AtKU70*$^{-/-}$ plants. We can therefore conclude that the mutation in *AtKU70* seems to be affecting all the telomeres in the cell in a similar way.

CONCLUSIONS

In this study we describe the isolation and characterisation of the *KU70* gene from the model plant *A.thaliana*. In order to study the function of this gene in plants we isolated a plant line in which the *KU70* gene was inactivated through a T-DNA insertion. Plants homozygous for this mutation were viable but

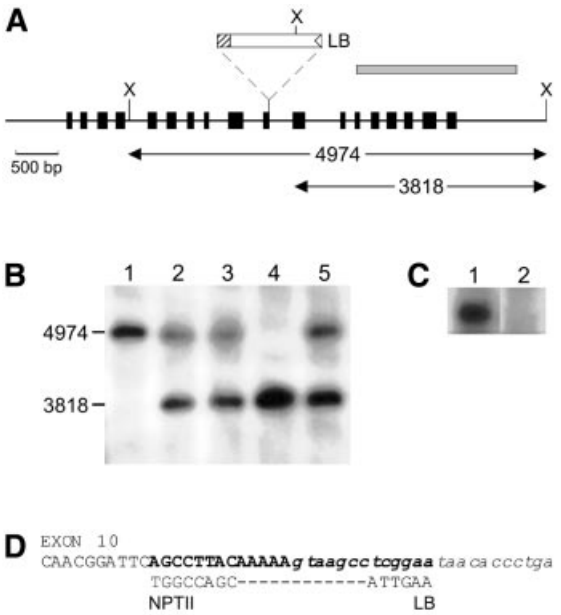

Figure 2. (**A**) Genomic organisation of the *AtKU70* locus with the insertion point of the T-DNA indicated. The exons of *AtKU70* are shown as boxes. The probe used for the DNA blots was a 1871 bp PCR fragment amplified using the primers AK1 + AK3 and is represented as a shaded box. X, *Xho*I restriction sites. LB, T-DNA left border. The truncated *NPTII* ORF on the T-DNA is shown as a hatched box. (**B**) DNA blot. Lane 1, wild-type (Ws) seedlings; lanes 2–5, individual plants containing a T-DNA in *AtKU70*. (**C**) Northern analysis: 8 µg total RNA from 12-day-old seedlings was blotted and probed using the 1.8 kb *AtKU70* cDNA. Lane 1, Ws; lane 2, *AtKU70*$^{-/-}$ seedlings. (**D**) Integration site of the T-DNA. Upper line, the sequence of exon 10 of the *AtKU70* gene is shown in uppercase letters. Intron 10 is indicated in lowercase italics. The sequence deleted due to the T-DNA insertion is in bold. Middle line, the ends of the T-DNA are shown. The left T-DNA border (LB) had lost 9 bp from its end and was integrated into intron 10. The T-DNA right border (RB) end was heavily truncated (~4 kb lost) resulting in part of the *NPTII* ORF being fused to *AtKU70* exon 10.

lacked the *KU70* mRNA. No obvious differences in growth were observed between the *AtKU70*$^{-/-}$ plants and wild-type plants. Germination, plant size, flowering and senescence were unchanged. However, the plants did show a clear hypersensitivity to the DNA damaging agents MMS and X-rays. Therefore, as in other organisms in which the *KU70* gene is mutated, it seems necessary in *Arabidopsis* for the repair of DNA damage.

The mouse is the only other multicellular organism in which the effects of inactivation of the *KU70* or *KU80* genes on the animal have been studied in detail. Mice lacking either of the genes are dwarf but fertile and have immuno-deficiencies due to defects in V(D)J recombination (9,10). Why do we not observe a dwarf phenotype in the plant line lacking AtKu70? The dwarf phenotype of these mice may not be linked with defects in DNA damage repair or V(D)J recombination given that mice lacking another component of the DNA-PK complex, the DNA-PK$_{cs}$ subunit, are defective in DNA repair, show severe immuno-deficiency but are normal in size (20). Thus, mammalian Ku70 must have another function that is necessary for normal development. Plant Ku70 may lack this function. Mammalian Ku70 is able to regulate transcription of other genes (21). Changed levels of transcription of these genes may be responsible for the mouse dwarf phenotype.

How can we explain that the telomeres of *A.thaliana* became much longer when the *AtKU70* gene was mutated? In the cell, telomere length is thought to be governed by a homeostasis mechanism that operates via the influence of positive and negative factors on the enzyme telomerase. The reverse transcriptase telomerase is able to add telomere sequences to the end of chromosomes. In mammals telomerase is developmentally regulated in different cell types, and shows high expression in reproductive tissues but is inactivated in somatic tissue. One consequence of this inactivation in somatic tissue is that telomeres shorten during each cell division due to the end replication problem. This has led to the speculation that telomere shortening may be linked to cellular senescence. In plants, telomerase activity in different tissues has also been measured. The highest activity is found in proliferating tissues such as meristems, but telomerase activity is low or undetectable in non-meristematic tissues such as leaves (19,22). Telomere homeostasis in yeast has been found to be controlled by many factors including telomeric binding proteins (Rap1p, Rif1p, Rif2p and Cdc13p), components of telomerase, telomeric chromatin-associated proteins (Est1p and Est2p) and proteins involved in non-homologous end joining (NHEJ; YKu70p, YKu80p, Mre11p, Rad50p and Xrs2p). Yeast cells deficient for components of the NHEJ pathway for DNA repair have shortened telomeres (12). However, the effects of NHEJ proteins on telomere length are not always conserved between organisms. For instance, mammalian cells lacking Ku70 do have shortened telomeres (23) while mutations in Ku86, the human Ku80 homologue, or DNA-PK$_{cs}$ do not affect telomere length (24–26). The fact that the *AtKU70* mutation results in lengthened telomeres is unique and suggests that plants have a crucially different mechanism for telomere homeostasis. One possible role of AtKu70 at *Arabidopsis* telomeres may be its action on proteins that inhibit telomere elongation. In mammalian cells the telomere binding proteins TRF1 and TRF2 have been identified. These proteins are necessary for maintaining shortened telomeres. Cells expressing a truncated dominant negative form of the TRF1 protein have longer telomeres (27). In mammalian cells the Ku heterodimer binds to the telomere DNA binding proteins TRF1 and TRF2 (28,29). These proteins effect Ku localisation to the telomeres and are thought to inhibit telomere elongation by promoting the formation of a t-loop whereby the telomere is looped backwards so that the single-stranded tail invades duplex telomeric sequences, making it inaccessible to telomerase (30). In *Arabidopsis* a telomere binding protein (AtTRP1) homologous to TRF1 has been characterised (31). The AtKu70 protein may normally bind to AtTRP1 and regulate its proposed negative effect on telomere length. The fact that we observe that all the telomeres in the cell appear to be lengthened argues for such a model in which the telomere homeostasis has been affected.

Another hypothesis is that *Arabidopsis* may have different, recombination-based pathways for alternative lengthening of telomeres (ALT) (32,33). Evidence for this has been found in several studies. First, the effect of the *AtRAD50* gene on telomere length has been reported (14). Plants lacking *AtRAD50* are sterile, but do not show changes in their

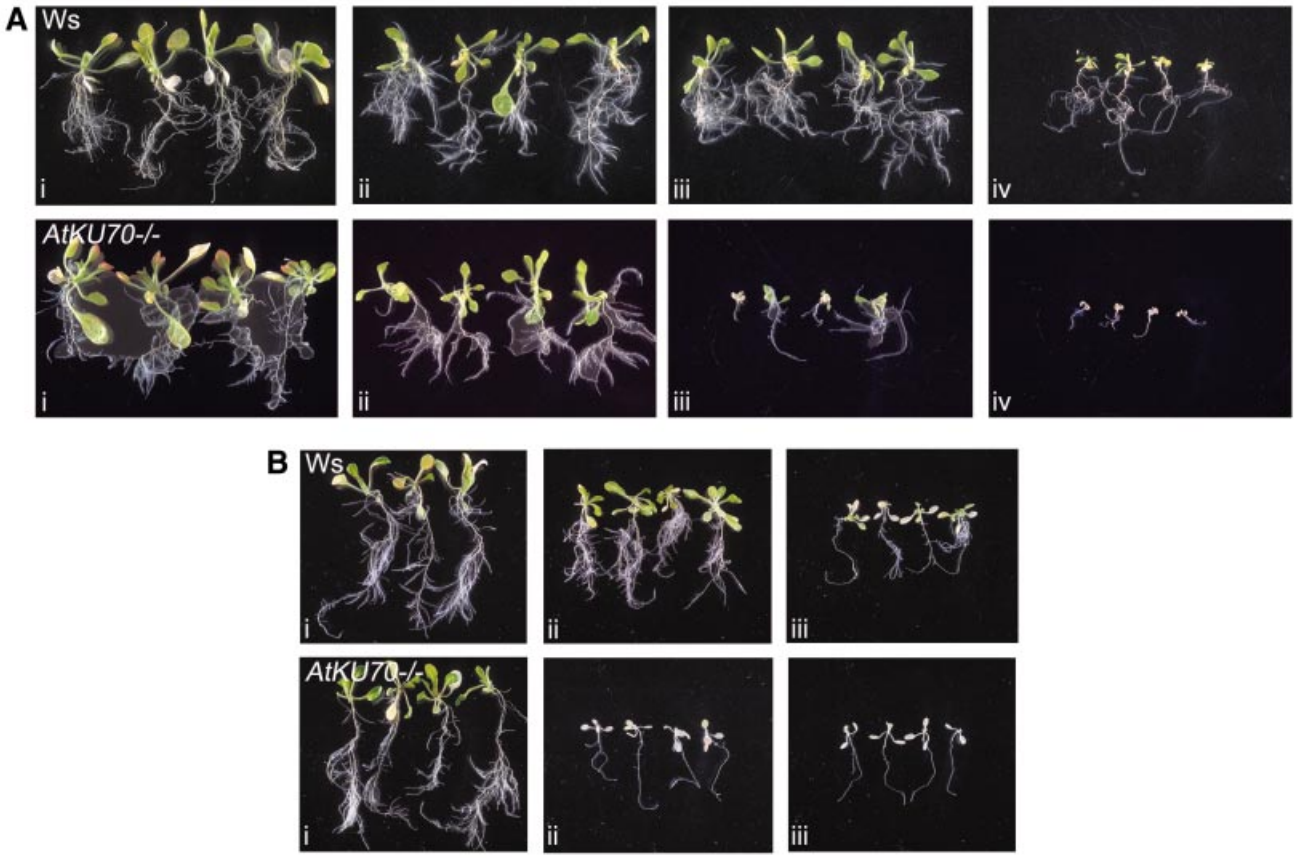

Figure 3. (**A**) MMS sensitivity of *AtKU70*$^{-/-}$ plants. Upper row, wild-type plants (ecotype Ws). Lower row, *AtKU70*$^{-/-}$ plants. MMS concentrations: i, 0%; ii, 0.006%; iii, 0.008%; iv, 0.01%. (**B**) X-ray sensitivity of *AtKU70*$^{-/-}$ plants. Upper row, wild-type plants (ecotype Ws). Lower row, *AtKU70*$^{-/-}$ plants. Seedlings were exposed to: i, 0 Gray; ii, 80 Gray; iii, 100 Gray.

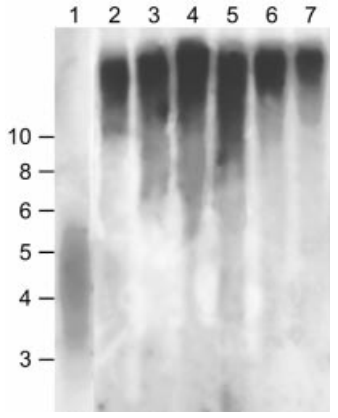

Figure 4. Lengthened telomeres in *AtKU70*$^{-/-}$ plants. Lane 1, wild-type (ecotype Ws); lanes 2–7, successive generations of *AtKU70*$^{-/-}$ plants. The fragment sizes are shown in kilobase pairs.

telomeres. However, cell cultures derived from such plants have no detectable telomeres after 8 weeks growth. The cell culture then undergoes a crisis from which only a fraction of the cells survive. The survivors have longer telomeres compared with wild-type cells. The data suggest that cultured *Arabidopsis* cells are able to maintain their telomeres by an ALT mechanism that does not require *AtRAD50*. Secondly, lengthened telomeres also have been detected in *Arabidopsis* plants lacking telomerase (34) suggesting that such an ALT mechanism also may be telomerase independent. Plants lacking telomerase show a stochastic shortening, and in some cases lengthening, of telomeres per generation. Telomeres that reach a critical minimum length may be subject to lengthening by recombination-based mechanisms. The AtKu70 protein may be involved in ALT mechanisms, for instance by preventing recombination between different telomeres, or AtKu70 may function as a regulator of the ALT pathways. It is also possible that AtKu70 achieves telomere lengthening in a more direct manner. For instance, the AtKu70 protein may also interact directly with telomerase, as has been suggested in yeast (35), perhaps down-regulating its activity. It will therefore be very interesting to study the telomeres of an *Arabidopsis* plant lacking both telomerase and AtKu70. Whatever the reason for the lengthened telomeres in *AtKU70*$^{-/-}$ plants, the phenotype is stable over the six generations tested. Moreover, no phenotypic differences were observed between the different generations, suggesting that longer telomeres do not influence plant development.

In conclusion, we have demonstrated that *AtKU70* plays a role in DNA repair and telomere maintenance in *Arabidopsis*. Plants seem to be able to tolerate mutations in NHR genes much more readily than mammalian cells. This makes plants

an ideal system to investigate the roles of NHR genes in higher eukaryotes by studying lines containing double and triple mutations in NHR genes as has been done in yeast previously.

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