Plant Molecular Biology Supplemental material

Dokládal L, Benková E, Honys D, Dupľáková N, Lee L-Y, Gelvin SB and Sýkorová E* ***An armadillo-domain protein participates in a telomerase interaction network***

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Materials and Methods

RNA isolation and RT-qPCR analysis of pollen samples

RNA from various *Arabidopsis* pollen developmental stages (Dupl'akova et al. 2016) was isolated using a Plant RNeasy Kit (Qiagen, www.qiagen.com) according to the manufacturer's instructions, and further purified by DNase I treatment (1µl DNase I, RNase free, Ambion). The quality and quantity of RNA were checked by absorbance (NanoDrop 1000 Spectrophotometer) and by electrophoresis through a 1% (w/v) nondenaturing agarose gel in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA). RNA was denatured in a formaldehyde/formamide sample buffer (50% formamide, 2.2 M formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA). First-Strand cDNA was prepared using 1 µg of RNA, M-MLV Reverse Transcriptase, RNase H minus, Point mutant (Promega) and oligo(dT)$_{18}$V. Reactions were supplemented with RNasin Plus RNase Inhibitor (Promega).

Transcript levels relative to a ubiquitin reference gene (*ubi-10*) were analyzed using a 7300 Real-Time PCR System (Applied Biosystems, www.appliedbiosystems.com) or a Roche LightCycler Nano instrument. A 1 µl aliquot of cDNA was added to the 10 µl reaction mix; the final concentration of primers was 0.3 µM. Reactions were performed in triplicate; PCR cycle conditions consisted of 10 min of initial denaturation (95°C) followed by 45 cycles of 20 s at 95°C, 30 s at 60°C, and 1 min at 72°C. SYBR Green I fluorescence was monitored after each extension step. The amount of each respective transcript was determined for two biological replicates using the ΔΔCt method (Pfaffl 2004).

Expression profile of genes encoding interacting proteins

The transcriptome datasets (presented at Fig. 1d) were downloaded from the NASCArray microarray database through the AffyWatch service (Craigon et al. 2004; http://www.arabidopsis.info) and completed by the male gametophyte data originated from the co-author lab (Honys lab, IEB ASCR, Prague; Honys and Twell 2004) and processed as described in Duplakova et al. 2007. All transcriptomic data sets were normalized using freely available dChip 1.3 software (Li and Wong 2001a; Li and Wong 2001b; http://www.softpedia.com/get/Science-CAD/dChip.shtml). The reliability and reproducibility of the analyses were ensured by the use of duplicates in each experiment, the normalization of all arrays to the median probe intensity level, and the use of normalized intensities of

all arrays for calculating of model-based gene-expression values based on the Perfect Match-only model (Li and Wong 2001a; Li and Wong 2001b). For each sample, only probes with the detection call of 'present' and an expression value detection level of 'well above background' (Boolean flag, two- sided t-test) in both replicates were considered to be expressed.

Phenotype of *arm* mutant plants

Root length of 10-day-old seedlings, pollen viability, rosette diameter, leaf number, flowering time, and silique number of mutant plants was compared with soil-grown wild-type (Col-0) plants over three subsequent generations of homozygous *arm-1* plants (examples shown on Supplemental Fig. S3).

Preparation of constructs of human proteins

The PCR product of armadillo repeat-containing protein 6 (ARMC6) encoding isoform 2 (Genebank accession NP_219483.1) was prepared with primers hARMC6.v2_Fw-EcoRI (5'-CCGGAATTCATGGTCTCCAAGCGCATTGC-3') and hARMC6_R+ SpeI (5'-CCGACTAGTTCATGGCGCCAGGTTGCC-3') using KAPA Taq Polymerase (KAPA Biosystems) and 35 PCR cycles of 94°C/20s, 53°C/20s,72°C/1min 20s. The PCR product was digested with *Eco*RI and *Spe*I (New England Biolabs) and ligated into a pBluescript SK vector digested with these same enzymes. After sequencing, the insert was excised from the plasmid using *Eco*RI and *Not*I, gel-purified, and ligated into the vector pTriEx4 (Novagene). Correct ORFs were checked by sequencing. For Gateway cloning (Invitrogen), the primers hARMC6.v2-Fw_pB1 (5'-AAAAAGCAGGCTTCATGGTCTCCAAGCGCATTGC-3') and hARMC6_R+pB2 (5'-AGAAAGCTGGGTCATGGCGCCAGGTTGCC) bearing partial attB sites were used in 35 PCR cycles of 94°C/20s, 56°C/20s,72°C/1min 30s. The PCR product was further amplified using an adaptor PCR protocol and attB1/attB2 primers (Invitrogen). The entry clone in the vector pDONRZeo was sequenced and used for the LR reaction with the vectors pGBKT7-DEST or pDEST17. The constructs TRF2/pGBKT7-DEST and TPP1/pGBKT7-DEST were created using entry clones of TRF2 (amino acids 43-542) and TPP1 (89-544) in pDONRZeo and the destination vector pGBKT7-DEST. Correct reading frames of all destination clones were verified by sequencing.

Protein expression *in vitro*, immunoprecipitation, and detection of human telomerase activity

A construct encoding a c-myc tagged ARMC6 protein, ARMC6/ pGBKT7-DEST, was expressed in 25 µl of a rabbit reticulocyte lysate (RRL). To reconstitute telomerase activity, the construct hTERT-hTR in pBluescript-SK (Bachand et al. 2000) was expressed in 50ul of RRL. Samples were mixed together in reaction buffer (final concentration 25 mM HEPES, pH 7.5/150 mM KCl/5 mM $MgCl_2$/0.1 mM PMSF/2 $\mu g.ml^{-1}$ leupeptin/1 $\mu g.ml^{-1}$ pepstatin/1 mM DTT/0.1% Nonidet P-40), mouse anti-myc antibody (1:8000, Sigma) was added, and the mixture was incubated at 4°C overnight using a rotator. The TPP1/pGBKT7-DEST construct served as a positive control and sterile water as a negative

control. Samples were mixed with 10 µl of magnetic beads (Dynabeads® Protein G, Life Technologies) preincubated in modified reaction buffer (25 mM HEPES, pH 7.5/150 mM KCl/5 mM $MgCl_2$/0.1 mM PMSF/4 µg.ml^{-1} leupeptin, 2 µg.ml^{-1} pepstatin/1 mM DTT/0.2% Nonidet P-40) and washed four times with the same buffer. Two µl of input and unbound fractions were collected for each sample and, together with bound fractions (2 µl of beads), served as templates for the TRAP assay (Sykorova et al. 2003). The samples were incubated in 46 µl of TRAP reaction buffer with 1 µl of 10 µM substrate primer TS21 (Fitzgerald et al. 1996) for 30 min at 30°C, heat denaturated, and the PCR step was started at 80°C by adding a mixture containing 1 µl of 10 µM reverse primer HUTPR29 (Fulneckova et al. 2013) and 2 units of KAPA Taq Polymerase (KAPA Biosystems). TRAP products were separated by electrophoresis through a 12.5% native polyacrylamide gel, stained, and visualized with LAS3000 (Fujifilm).

Analysis of protein-protein interactions of human ARMC6 protein by co-immunoprecipitation

The bait construct TRF2/pGBKT7-DEST was expressed in 25 µl of a RRL, the prey constructs ARMC6/pTriEx4, ARMC6/pDEST17 and RAP1/pHGWA were expressed in 50 µl RRL, and radioactively labeled using ^{35}S-Met. The immunoprecipitation procedure was the same as above. 5 µl of input and unbound fractions were collected for each sample and all fractions including bound fractions (10 µl of beads) were mixed with 2xSDS sample buffer, heated at 80°C for 10 min, and separated by 10% SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane and analysed with FLA5000 (Fujifilm).

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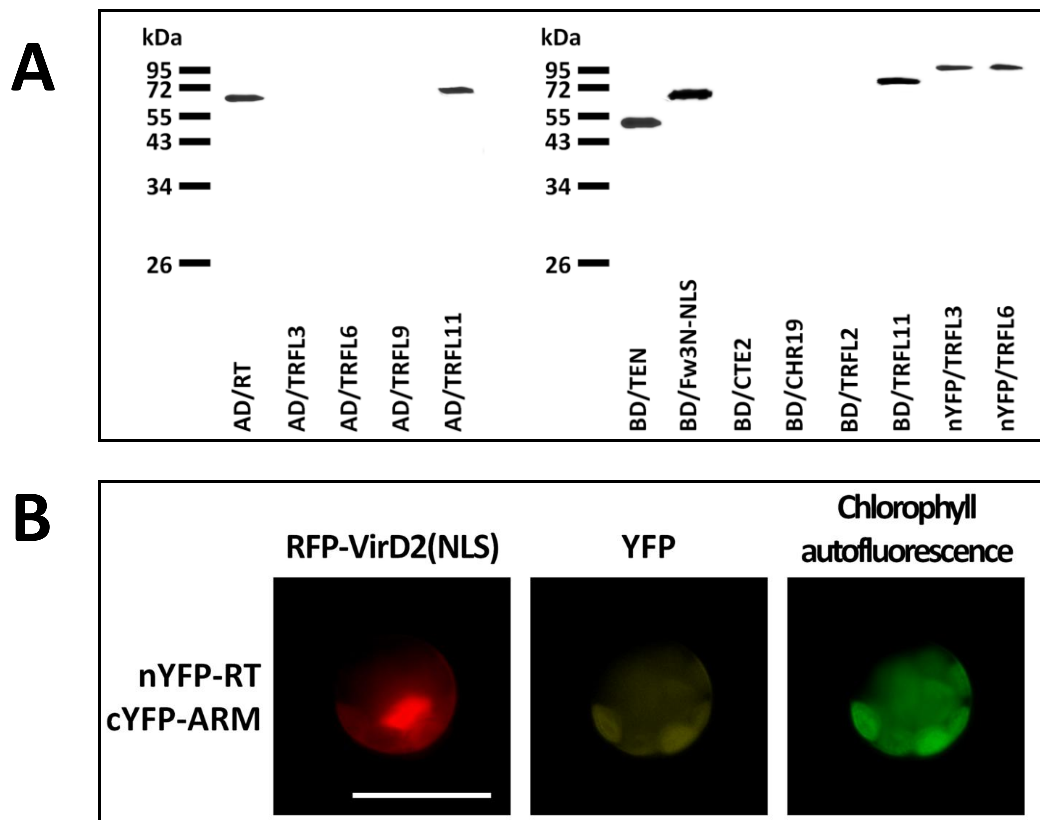
Figures

Supplemental Figure S1. Protein-protein interactions.

A. Protein blot analysis of representative protein constructs tested for interactions using the Y2H and BiFC assays. Constructs encoding TRFL2,-3,-6,-9,-11, CHR19, and TERT fragments (TEN, Fw3N-NLS, RT, CTE2) fused with the GAL4 activation domain (AD, left panel), the GAL4 DNA-binding domain (BD, right panel) or nYFP (right panel) were expressed in the corresponding systems. AD-, BD- and nYFP-fusion proteins were detected by immunoblotting using mouse anti-HA, mouse anti-myc and anti-GFP antibodies targeting the epitope tags of these proteins, respectively.

B. Bimolecular Fluorescence Complementation assay for interaction between the RT domain and ARM BiFC assay in *Arabidopsis* leaf protoplasts shows that the RT domain of AtTERT does not interact with the ARM protein. A mRFP-VirD2-NLS construct was used to indicate the nucleus.

C. Control BiFC reactions with the control protein GAUT10. Control BiFC assays using nYFP- and cYFP-GAUT10 constructs in combination with investigated proteins in tobacco BY-2 protoplasts (on left) and *N. benthamiana* leaves (on right). A mRFP-VirD2-NLS construct was used to indicate the nucleus in the left panels. bar = 20 µm.



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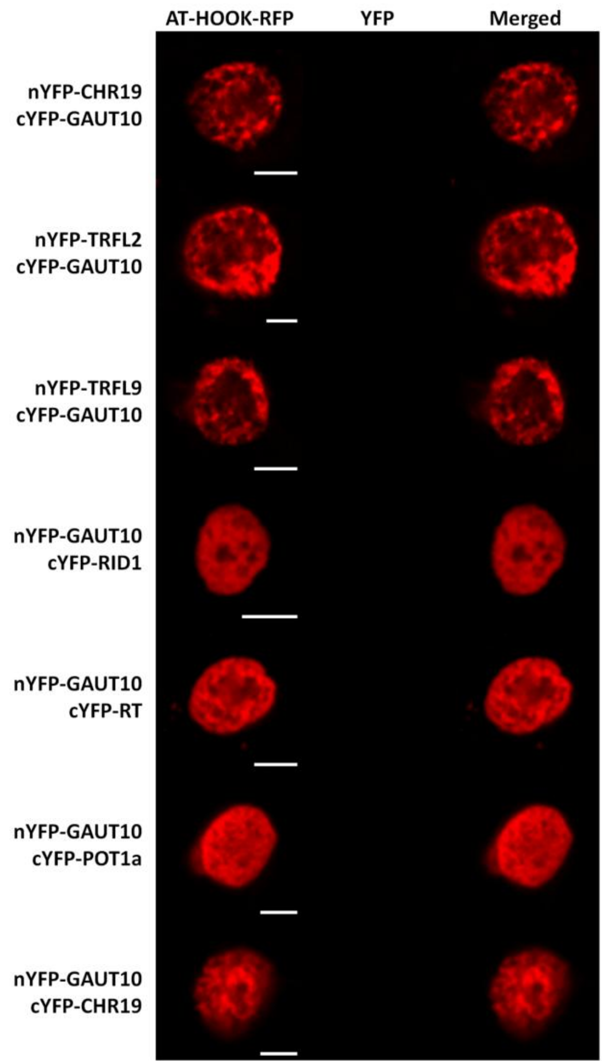
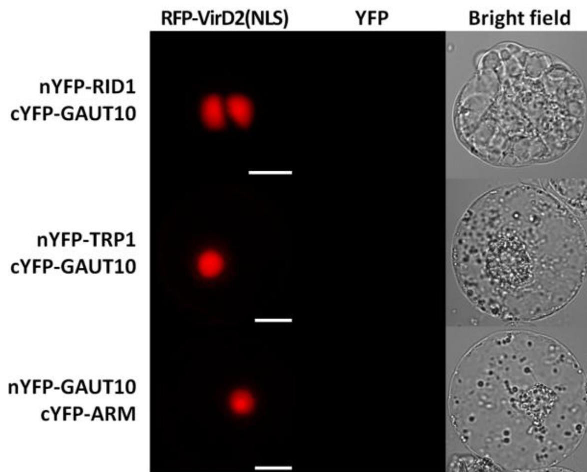
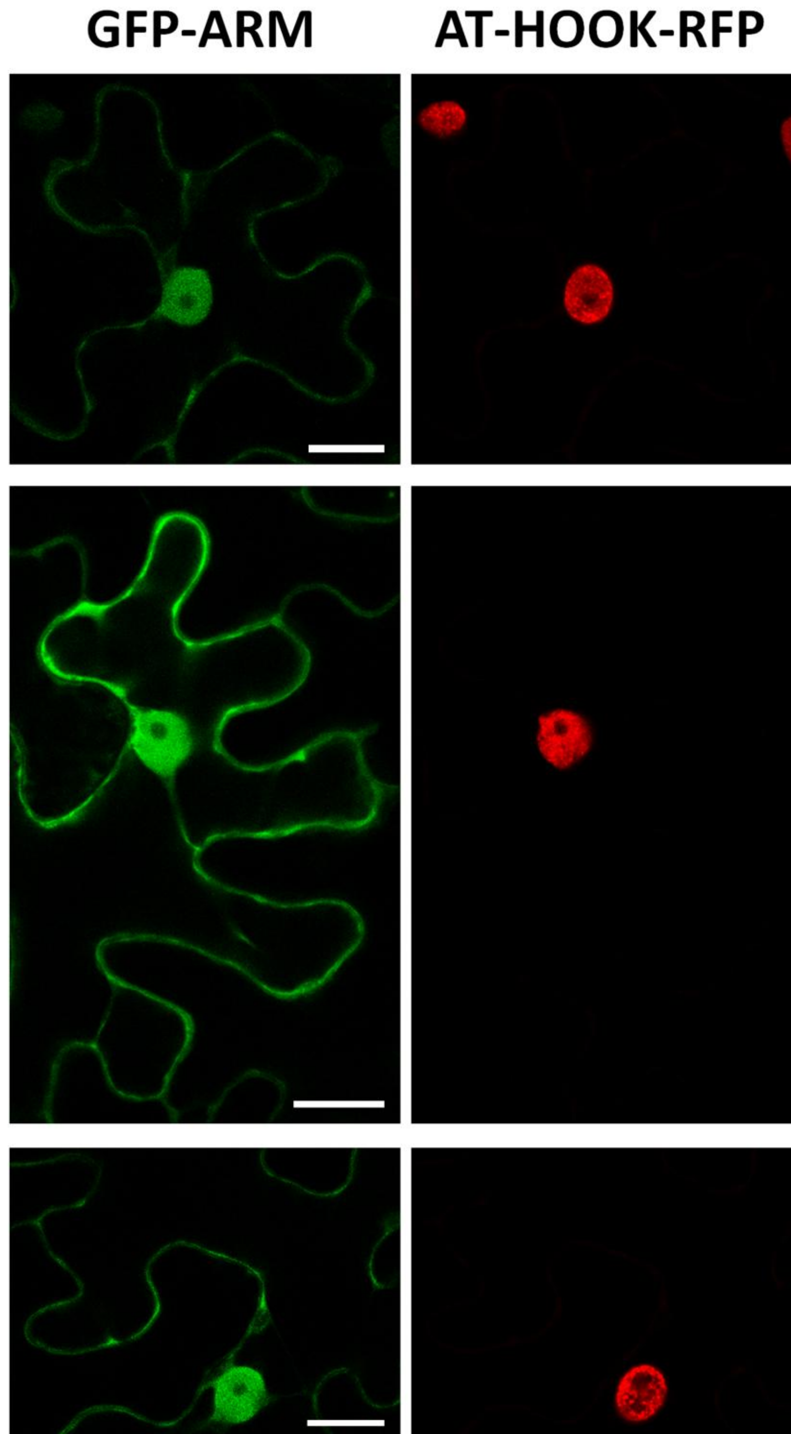
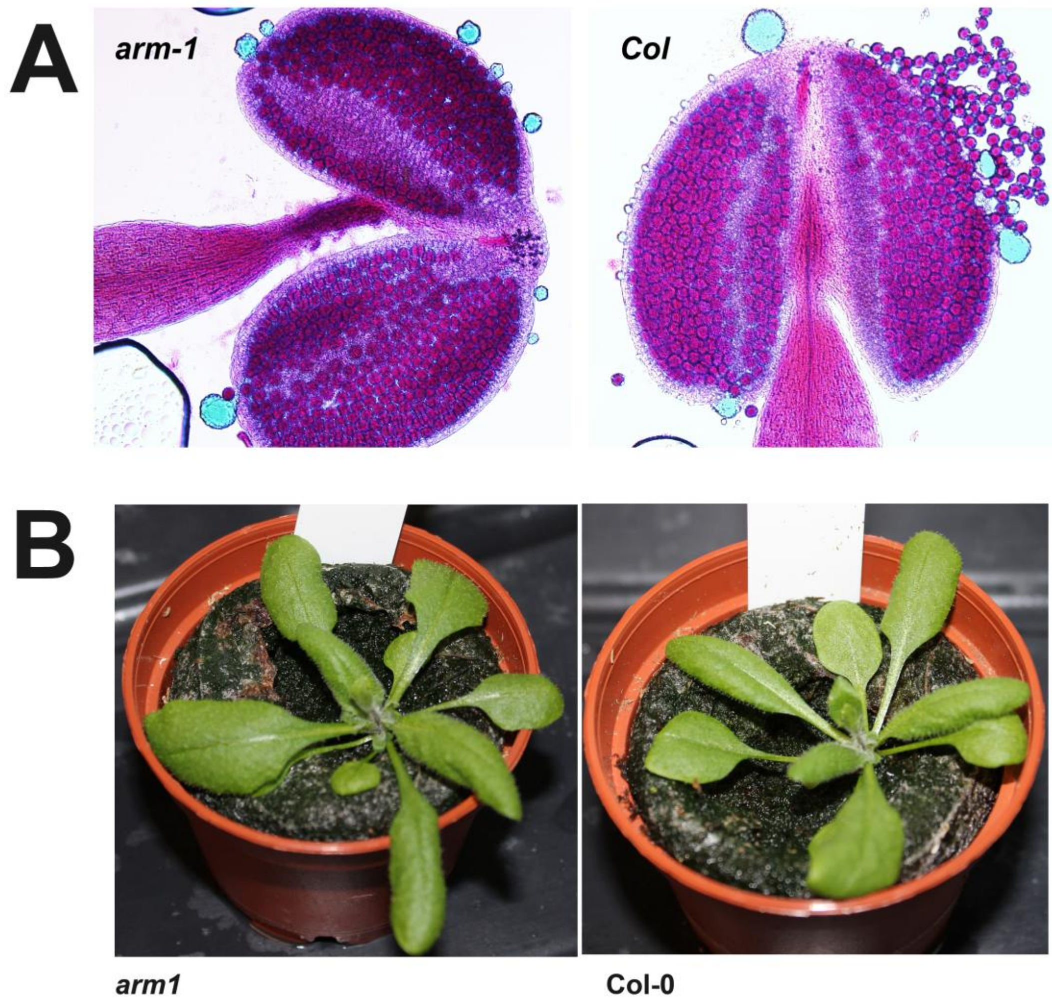




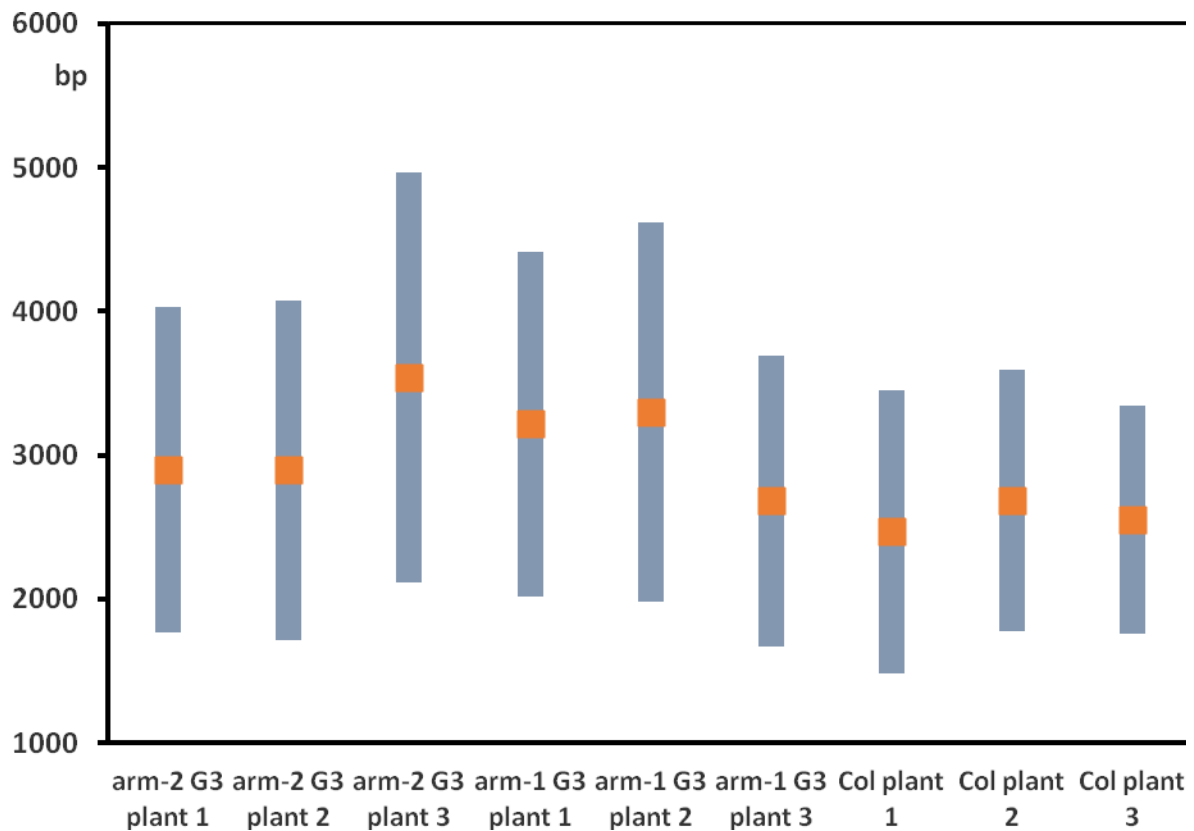
Supplemental Figure S2. Further examples of ARM localization. The GFP-ARM protein (green) showed co-localization with the nuclear marker AT-HOOK-RFP (red) in *N. benthamiana* leaf cells. Agrobacterium cultures with pMDC43::ARM and pK7RWG2::AT-HOOK constructs were used for transient expression in N. benthamiana leaves via syringe infiltration. After 3 days incubation, fluorescence was observed. Bar = 20 μm.



Supplemental Figure S3. Phenotype of *arm* mutants. (A) Viable pollen grains were observed in *arm-1* mutants and wild-type (Col) plants using Alexander's staining. (B) No morphological differences were observed between *arm*-1 mutant and wild-type plants; 23-day-old plants are shown as representatives.

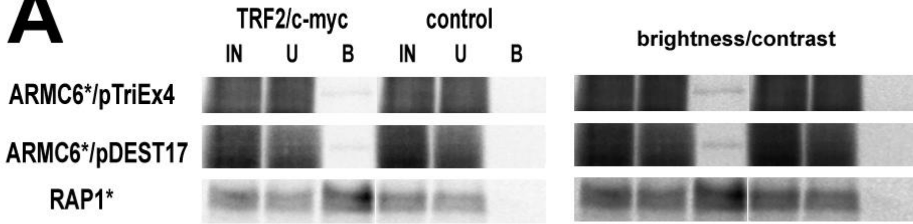


Supplemental Figure S4. Telomere length in *arm* mutants. Telomere lengths in *arm-1* and *arm-2* T-DNA insertion lines were determined by terminal restriction fragment (TRF) Southern blot analysis followed by analysis using the TeloTool software. Although telomeres in both *arm-1* and *arm-2* G3 plants were slightly longer than those in wild-type (Col-0) plants, a paired Student t-test evaluated these changes in telomeres as not significant (the two-tailed P values equal 0.1175 and 0.0751 for *arm-1* and *arm-2* , respectively). TRF signal – blue rectangle, mean value – red square.



Supplemental Figure S5. Analysis of telomerase activity in *arm* mutants. Telomerase activity in 7-day-old seedlings of homozygous *arm-1* and *arm-2* T-DNA insertion lines was compared to wild-type (Col-0) plants using a telomeric repeat amplification protocol (TRAP). Both mutant and wild-type control lines showed positive results that are demonstrated by the ladder of amplified telomerase products. 50 ng of total protein was used in each sample except the negative control (NC, extraction buffer only).

Supplemental Figure S6. Analysis of human ARMC6 protein interactions. (A) The ARMC6 protein interacts with TRF2 protein *in vitro*. The c-myc-tagged TRF2 and radioactively labeled (*) ARMC6 protein construct were produced by a rabbit reticulocyte lysate (RRL) system. Using mouse anti-myc antibodies, the positive interaction between TRF2 and ARMC6 was demonstrated in immunoprecipitated fractions. Input (I), unbound (U) and bound (B) fractions were analyzed. To confirm weak signal from this experiment and to exclude a false positivity, the TRF2-ARMC6 interaction was investigated using ARMC6 constructs expressed independently from two different vectors (pDEST17 and pTriEx4) and we observed the same positive result. On right panels the brightness and contrast of the same picture only was manipulated to highlight weak signals in [B] fractions. Co-immunoprecipitation of radioactively labeled RAP1 protein with c-myc-tagged TRF2 served as a positive control. The experiment was repeated twice. (B) Positive result of the TRAP assay demonstrates that the ARMC6/c-myc protein construct pulls-down human telomerase reconstituted *in vitro* and co-immunoprecipitates telomerase activity. Human telomerase was reconstituted in RRL using hTERT+hTR construct (RRL), mixed with c-myc-tagged ARMC6 protein and subjected to pull-down assay using anti-myc antibodies and protein G magnetic beads. Input (I), unbound (U) and bound fractions (B), were collected for each sample and served as templates for the TRAP assay. Human protein TPP1/c-myc construct served as a positive control and sterile water as a negative control. The experiment was performed three times. (NC), negative control, RRL lysate only.


A
TRF2/c-myc
control
IN
U
B
IN
U
B
brightness/contrast
ARMC6*/pTriEx4
ARMC6*/pDEST17
RAP1*

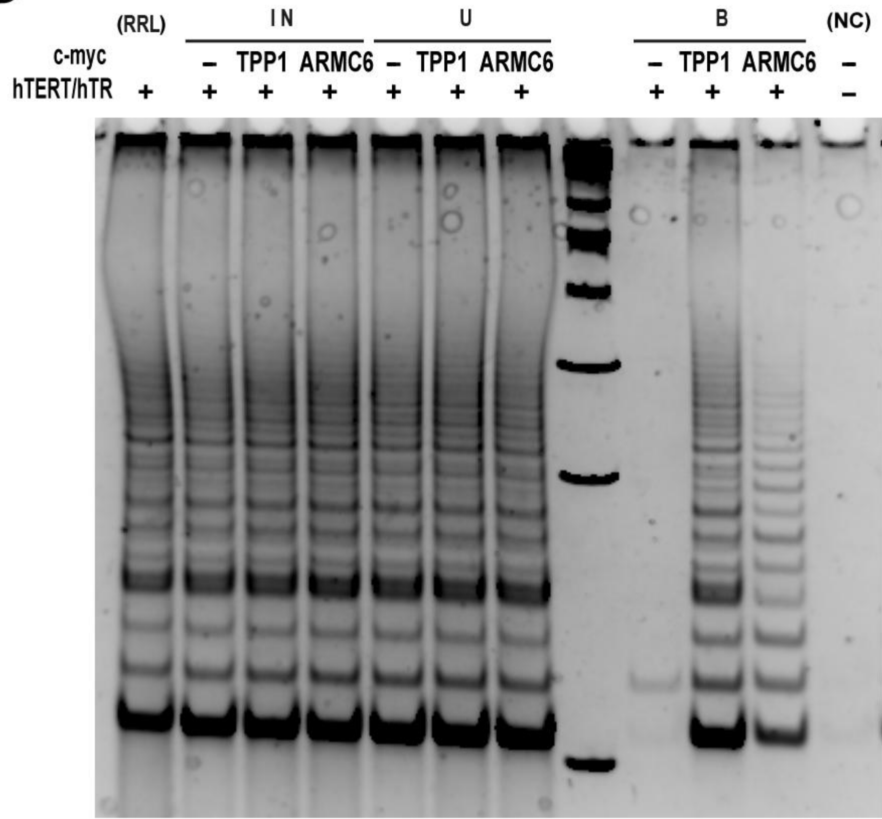

B
(RRL)
IN
U
B
(NC)
c-myc
–
TPP1
ARMC6
–
TPP1
ARMC6
–
TPP1
ARMC6
–
hTERT/hTR
+
+
+
+
+
+
+
+
+
+
–

Table S2. Summary of protein-protein interactions observed among ARM, TERT fragments, CHR19, and *Arabidopsis* Myb-like domain containing proteins using a yeast-two-hybrid assay (Y2H), co-immunoprecipitation (co-IP), and bimolecular fluorescence complementation (BiFC).

	Y2H[a]						co-IP	BiFC					
	BD/AD ARM	BD/AD CHR19	BD/AD TRP1	BD/AD TRFL2	BD/AD TRFL11	BD/AD TRB1,2	TRFL9	ARM	CHR19	TRP1	TRFL 2	TRFL 3,6	TRFL 9
ARM	- / -	n.e. / +	+ / n.e.	n.e. / +	- / -	- / -	-	n.a.	+	+	+	-	+
TERT-RID1	- / ++	n.e. / +	- / n.e.	n.e. / -	(-[b])	(+[c])	-	+	+	(+[c])	n.a.	n.a.	+
TERT-TEN	- / -	n.e. / -	- / n.e.	n.e. / -	(-[b])	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
TERT-FW3N-NLS	- / -	n.e. / -	- / n.e.	n.e. / -	- / -	(+[c])	n.a.	n.a.	n.a.	(+[c])	n.a.	n.a.	n.a.
TERT-RT	- / -	n.e. / -	- / n.e.	n.e. / +	- / -	n.a.	n.a.	-	n.a.	n.a.	+	n.a.	n.a.
TERT-CTE2	+ / n.e.	n.e./n.e.	- / n.e.	n.e./n.e.	- / n.e.	- / n.e.	n.a.	(+[d])	n.a.	n.a.	n.a.	n.a.	n.a.
POT1a	- / -	n.e. / -	- / n.e.	n.e. / -	- / -	n.a.	+	n.a.	n.a.	n.a.	n.a.	n.a.	+
CHR19	+ / n.e.	n.e./n.e.	- / n.e.	n.e./n.e.	- / n.e.	+/ n.e.	-	+	n.a.	n.a.	-	n.a.	+

[a], + interaction using histidine selection, ++ interaction using both histidine and stringent adenine selection, — no interaction observed; [b], negative result also using BiFC in (Majerska et al. 2017); [c], positive results also using BiFC and co-IP in (Schrumpfova et al. 2014); [d], in (Lee et al. 2012); n.e., not expressed; n.a., not analyzed