Dysfunction of Chromatin Assembly Factor 1 Induces Shortening of Telomeres and Loss of 45S rDNA in *Arabidopsis thaliana* [W][OA]

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Chromatin Assembly Factor 1 (CAF1) is a three-subunit H3/H4 histone chaperone responsible for replication-dependent nucleosome assembly. It is composed of CAC 1-3 in yeast; p155, p60, and p48 in humans; and FASCIATA1 (FAS1), FAS2, and MULTICOPY SUPPRESSOR OF IRA1 in *Arabidopsis thaliana*. We report that disruption of CAF1 function by *fas* mutations in *Arabidopsis* results in telomere shortening and loss of 45S rDNA, while other repetitive sequences (5S rDNA, centromeric 180-bp repeat, *CACTA*, and *Athila*) are unaffected. Substantial telomere shortening occurs immediately after the loss of functional CAF1 and slows down at telomeres shortened to median lengths around 1 to 1.5 kb. The 45S rDNA loss is progressive, leaving 10 to 15% of the original number of repeats in the 5th generation of mutants affecting CAF1, but the level of the 45S rRNA transcripts is not altered in these mutants. Increasing severity of the *fas* phenotype is accompanied by accumulation of anaphase bridges, reduced viability, and plant sterility. Our results show that appropriate replication-dependent chromatin assembly is specifically required for stable maintenance of telomeres and 45S rDNA.

INTRODUCTION

Chromatin Assembly Factor 1 (CAF1) is a histone chaperone that deposits histones H3/H4 onto DNA in a replication-dependent manner (Smith and Stillman, 1989, 1991). The plant CAF1 is composed of three subunits: FASCIATA1 (FAS1), FAS2, and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) (Kaya et al., 2001), which correspond to Chromatin Assembly Complex 1 (CAC1) to CAC3 in budding yeast (*Saccharomyces cerevisiae*) (Kaufman et al., 1997) or p155, p60, and p48 in humans (Smith and Stillman, 1989; Verreault et al., 1996), respectively. CAF1 displays structural and biochemical activity conservation across eukaryotes, including yeasts (Kaufman et al., 1997), animals (Tyler et al., 1999, 2001), and plants (Kaya et al., 2001).

The large subunit (FAS1/CAC1/p150) binds histones H3/H4 and interacts with DNA polymerase processivity factor, the proliferating cell nuclear antigen (Shibahara and Stillman, 1999). The middle subunit (FAS2/p60/CAC2) is a WD40 repeat protein responsible for protein–protein interactions within CAF1 and further mediates interaction with the Antisilencing function 1 factor stimulating nucleosome assembly by CAF1 and participating in replication-independent nucleosome assembly (Tyler et al., 1999, 2001; Munakata et al., 2000; Sharp et al., 2001; Mello et al., 2002; Tagami et al., 2004), heterochromatin protein

1 (Murzina et al., 1999), methyl binding protein1 (Reese et al., 2003), or the BLM and WRN helicases (Jiao et al., 2004, 2007). The small subunit of CAF1 (MSI1/p48/CAC3) belongs to the MSI-like family of WD40 repeat proteins, which apart from participating in CAF1 take part in a number of complexes involved in chromatin dynamics (Hennig et al., 2005).

CAF1 is required for cell proliferation and early development in animals (Hoek and Stillman, 2003; Ye et al., 2003; Nabatiyan and Krude, 2004; Houlard et al., 2006; Takami et al., 2007) but is not essential for the viability of yeast (Kaufman et al., 1997; Enomoto and Berman, 1998) or *Arabidopsis thaliana* (Kaya et al., 2001) if only FAS1 or FAS2 are disrupted. Due to its involvement in the PRC2-like (Polycomb repressor complex) complexes in *Arabidopsis*, MSI1 is essential for sporophyte, gametophyte, and early seed development (Hennig et al., 2003, 2005; Kohler et al., 2003).

Mutants affecting CAF1 display reduced position-dependent gene silencing at telomeres (Enomoto et al., 1997; Kaufman et al., 1997; Monson et al., 1997), centromeres (Dohke et al., 2008), the silent mating type loci (Enomoto and Berman, 1998; Dohke et al., 2008), and rDNA (Smith et al., 1999). Proliferating cell nuclear antigen and CAF1 association has also been linked to DNA repair, in particular nucleotide excision repair (Gaillard et al., 1996; Martini et al., 1998; Moggs et al., 2000; Mello et al., 2002; Polo et al., 2006), and to major mechanisms of double strand break (DSB) repair, such as homologous recombination (HR) and nonhomologous end joining (Lewis et al., 2005; Linger and Tyler, 2005; Nabatiyan et al., 2006; Song et al., 2007).

Arabidopsis mutants in CAF1 subunits were originally described as causing stem fasciation, abnormal leaf and flower morphology, and organization of apical meristems (Reinholz, 1966; Leyser and Furner, 1992; Kaya et al., 2001). CAF1 is also involved in the regulation of heterochromatinization and maintenance of

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transcriptional gene silencing (Kaya et al., 2001; Kirik et al., 2006; Ono et al., 2006; Schonrock et al., 2006), regulation of endoreduplication (Exner et al., 2006; Kirik et al., 2006; Ramirez-Parra and Gutierrez, 2007), cell differentiation (Exner et al., 2006), cell cycle duration (Ramirez-Parra and Gutierrez, 2007; Abe et al., 2008; Chen et al., 2008), HR (Endo et al., 2006; Kirik et al., 2006), and trichome development (Exner et al., 2006, 2008). Here, we demonstrate that disruption of the two larger subunits of CAF1 in *Arabidopsis*, FAS1 and FAS2, is responsible for telomere shortening and severe loss of 45S rDNA, without affecting other major repetitive sequences in the *Arabidopsis* genome, thereby functionally linking replication-dependent nucleosome assembly to the maintenance of telomeres and rDNA.

RESULTS

Characterization of Plant Lines

To explore the link between DNA replication, chromatin assembly, and telomere maintenance, we collected different mutant alleles of the genes *FAS1* (At1g65470) and *FAS2* (At5g64630) generated in different genetic backgrounds. Mutants carried either a point mutation or a T-DNA insertion, causing partial or complete disruption of *FAS1* or *FAS2* expression (see Supplemental Tables 1 and 2 and Supplemental Figure 1 online). Two different T-DNA insertion alleles named *fas1-4* were described concurrently by Exner et al. (2006) (ecotype Columbia [Col]) and Kirik et al. (2006) (ecotype C24); these are referred to as *fas1-4* and *fas1-4b*, respectively. Although all the *fas* alleles have been morphologically and genotypically described before, we located the truncated T-DNA in *fas1-4b* to exon 1 rather than exon 6 as reported previously (Kirik et al., 2006) and observed no full-length RT-PCR product in *fas1-4b/fas1-4b* (see Supplemental Figure 1 online). However, we detected *FAS1* transcripts originating from the 3′ region relative to the T-DNA insertion site. Since the *fas* phenotype is partially penetrant in heterozygous plants in this line, there is a possibility this mutation may result in dominant-negative forms of FAS1.

Propagation of the *FAS* mutants was accompanied by progressive loss of viability and fertility (see Supplemental Figure 2 online), presumably resulting in sterility in further generations.

Mutations in *FAS1* or *FAS2* Cause Telomere Shortening on All Chromosome Arms and Are Associated with the Formation of Anaphase Bridges

We observed significantly reduced lengths of terminal restriction fragments (TRFs) in *fas1-2* (Nossen [No]), *fas2-1* (Landsberg *erecta* [L*er*]), and *fas2-2* (No) plants; regardless of the original (ecotype-specific) telomere lengths in wild-type plants, which ranged from a median of 3 to 5 kb, the mutant telomere lengths ranged around a median of 1.5 to 2.0 kb (see Supplemental Figure 3 online). To explore the dynamics and possible progressiveness of the telomere shortening, we examined the segregating lines *fas1-4b* (C24), *fas1-4* (Col), and *fas2-4* (Col). Homozygous mutant and wild-type plants were obtained from two heterozygous parents in the line *fas1-4b* and three hetero-

zygous parents in the lines *fas1-4* and *fas2-4*. Homozygous mutants were propagated by selfing, generating homozygous lines. Gradual telomere shortening was observed from generation G2 onwards in mutants *fas1-4b/fas1-4b* (see Supplemental Figures 3B and 3C online), although this line could not be propagated in larger numbers beyond generation G3 due to radical loss of fertility. We were able to propagate the *fas1-4/fas1-4* and *fas2-4/fas2-4* plants for at least five generations. While telomeres of wild-type plants retained median length of 2.7 to 3.0 kb, telomeres in mutants progressively shortened over three to five generations to approximately one-half, yielding telomeres of median length 1.5 to 1.7 kb (Figures 1A and 1B), the shortest being around 700 bp. Progressive shortening is most
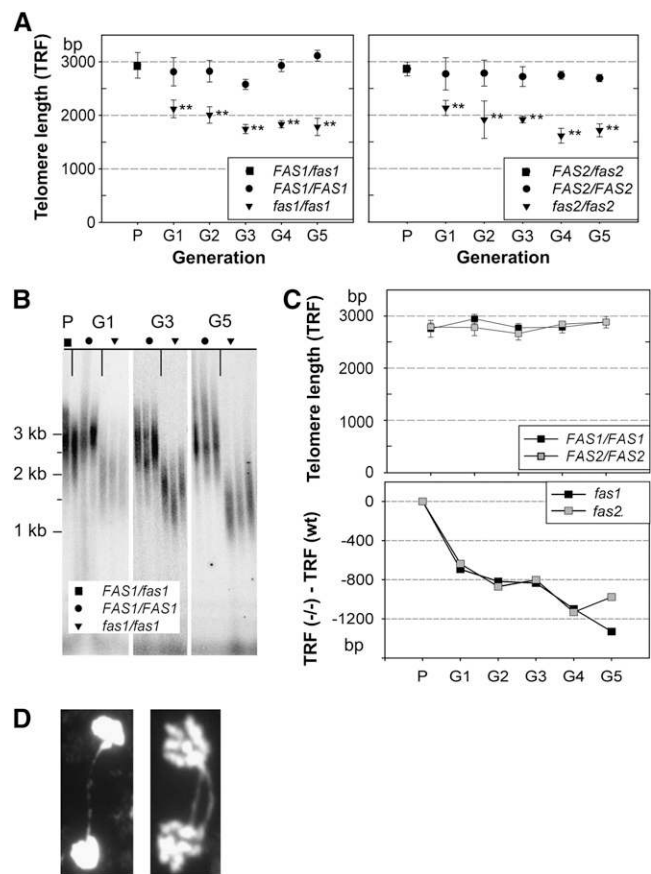

Figure 1. Telomere Length Maintenance in Five Generations of *fas1-4* (Col) and *fas2-4* (Col) Plant Lines.

(A) Absolute telomere lengths in wild-type and mutant plants derived from a common heterozygous parent.
(B) Example of TRF analysis.
(C) Absolute telomere lengths in five generations of wild-type plants (top part); difference between mutant and wild-type telomere lengths (bottom part).
(D) Example of anaphase bridges observed in *fas* mutants. Asterisks indicate significant difference between the mutant and wild type of the same generation based on Student's *t* test: **P < 0.001. Error bars indicate SD. *FAS/FAS* ∼ wt, wild type; *fas/fas* ∼ (−/−), mutant; *FAS/fas*, heterozygote; P, parental plants.

remarkable after the transition from the heterozygous parental generation (P) to generation G1 of homozygous mutant plants when ~800 bp of telomeric DNA per chromosome arm is lost. This is followed in later generations by a slower loss of the telomeric tracts (200 to 300 bp per generation) and homogenization of telomere lengths. These results are consistent for plants bearing either *FAS1* or *FAS2* mutations (Figure 1C). We next used the PCR-based method PETRA (for primer extension telomere repeat amplification) (Heacock et al., 2004) to determine the telomere lengths on separate chromosomal arms with a focus on both arms of chromosomes 2 and 4. In agreement with data from TRFs, the results showed that telomeres on all the chromosome arms studied undergo shortening to a similar extent, regardless of the original wild-type telomere length on that particular arm (see Supplemental Figures 4 and 5 online). Increased formation of anaphase bridges was observed in the later generations of *fas* mutants (3.7%, *n* = 1084 in G5 mutant, compared with 0.3%, *n* = 653 in G5 wild type), indicating inadequate protection of the chromosome termini (Figure 1D).

Shortening of Telomeres in Mutants Is Quickly Reversed by the Presence of a Wild-Type Allele

To understand the nature of the telomere shortening in *FAS* mutants, we crossed (1) the *fas1-4* and *fas2-4* mutant plants in generation G4 with their corresponding wild types and (2) the *fas1/fas1* with the *fas2/fas2* plants in G2 to generate a population of plants heterozygous at both loci. Crossing of *fas* mutant plants with wild-type plants yields a heterozygous population in the generation F1, but the individuals have from one to three populations of telomeres (clearly separated peaks of similar intensi-

ties). The lengths of the separate populations correspond to the telomere lengths of the wild type and mutant parents and/or are intermediate (Figure 2A). In the F2 generation, the length difference between the three populations decreases through elongation of the shortest and the intermediate telomeres. The *fas/fas* plants in F2 always displayed only a single population of medium length telomeres, indicating shortening of the longest telomeres and possibly lengthening of shortest telomeres in F1. Similarly, when *fas1* and *fas2* homozygous mutants are reciprocally crossed, the telomeres elongate immediately in F1 of the heterozygous progeny and reach almost wild-type lengths in F2. Importantly, this cross yields only one population of telomeres that slightly elongates in each generation (Figure 2B).

Mutations in *FAS1* and *FAS2* Induce a Major Progressive Loss of 45S rDNA from Both Loci on Chromosomes 2 and 4, but Other Repeats Are Not Affected

DNA gel dot blot experiments with genomic DNA isolated from three pooled 5-week-old plants revealed a radical loss of the number of 45S rDNA repeats (Figure 3). The copy number reduction is similar in *fas1-4* and *fas2-4* mutants and amounts to ~10 to 20% of the repeats per generation, leaving in G5 ~20% of the repeats in *fas1-4* and 12% in *fas2-4* mutants. There were major losses of the 45S rDNA at the transition from parental generation to G1 (~22% in *fas1-4/fas1-4* and 40% in *fas2-4/fas2-4*). Separate analysis of five individual plants in G2 and G5 indicated that the variability in the amount of 45S rDNA is low within a population of mutants (see Supplemental Figure 6 online). Surprisingly, none of the other analyzed tandem or dispersed repeats (180-bp centromeric repeat, 5S rDNA, retrotransposon
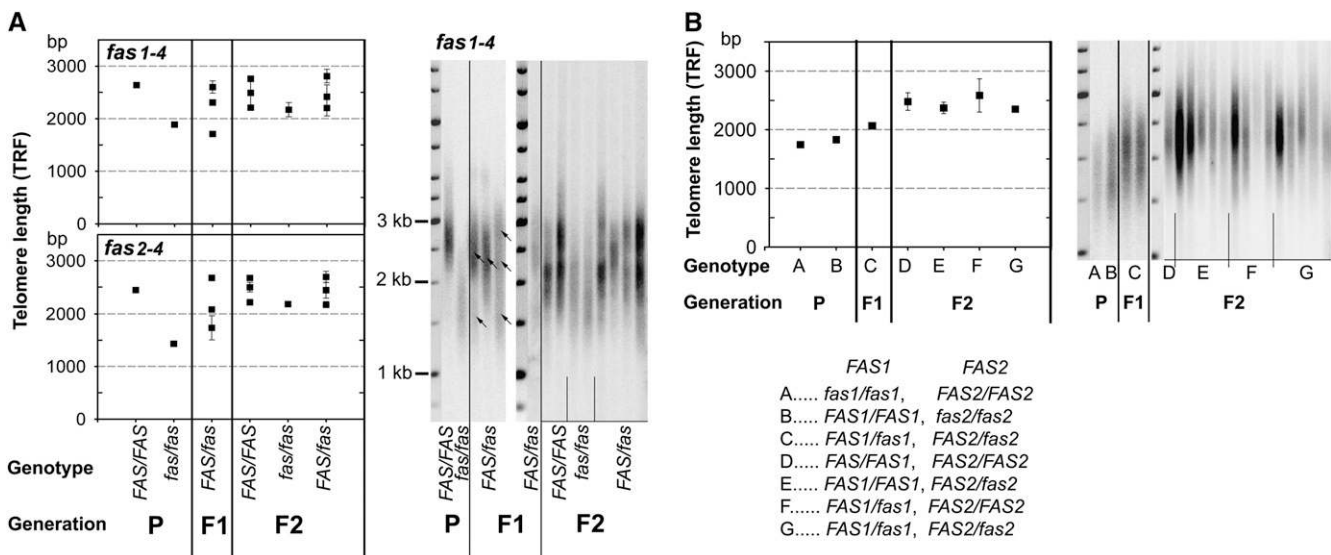

Figure 2. Elongation of Telomeres in the Presence of a Wild-Type Allele.

Telomere lengths and examples of TRF analysis (right in each panel) in two filial generations (F1 and F2) of reciprocal crosses between mutant and wild-type plants in generation G4 **(A)** or between *fas1* and *fas2* mutant plants in generation G2 **(B)**. Error bars indicate SD. Progeny of one parental line is shown. Multiple populations of telomeres defined when clearly separated peaks of similar intensities within one sample appeared (examples indicated by arrows). *FAS/FAS*, wild type; *fas/fas*, mutant; *FAS/fas*, heterozygote; P, parental plants.
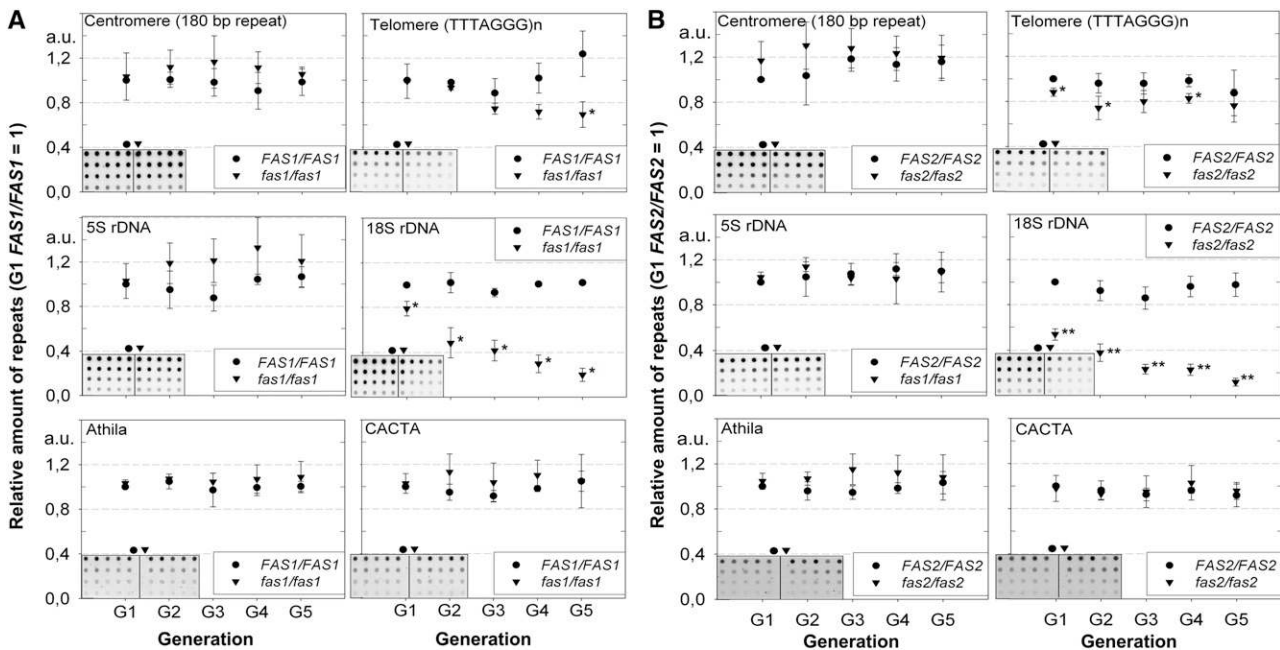
Figure 3. Amounts of Repetitive Sequences in Five Successive Generations of *fas1-4* and *fas2-4* Plants Related to Generation G1 Wild-Type Plants.

Asterisks indicate significant difference between the mutant and wild type of the same generation based on Student's *t* test: *P < 0.05; **P < 0.001. Error bars indicate SD. *FAS/FAS*, wild type; *fas/fas*, mutant. a.u., arbitrary units.

Athila, or transposon *CACTA*) diminished in copy number in the *fas* mutants (Figure 3).

Real-time quantitative PCR (Q-PCR) on leaves of 5-week-old plants confirmed the results for plants of generations G2 and G5. In *fas1-4* and *fas2-4* mutants, the amount of 45S rDNA decreases to ~40 and 36% in G2 and 15 and 8% in G5 of levels found in wild-type plants. Interestingly, the values are higher in seedlings of the same generation; for *fas1-4* and *fas2-4* mutant seedling DNA used in Q-PCR, the values were ~54 and 54% in G2 and 28 and 14% in G5 (Figure 4A).

Fluorescence in situ hybridization signals of 5S rDNA and 25S rDNA in metaphase figures prepared from wild-type and mutant plants from the lines *fas1-4* and *fas2-4* were quantified (*n* = 103 to 108 wild type and 140 to 141 mutant nuclei). Reduced intensity of the 25S rDNA signal in the mutant compared with wild-type nuclei verified the previous results and further showed that the localization and number of the signals are unchanged. The intensity, localization, or number of 5S rDNA signals or F6N15 signals (positive hybridization control) was similar in the mutants and the wild types. The reduced 25S rDNA signal intensity was observed on both of the 45S rDNA loci on chromosomes 2 and 4 (Figures 5A and 5B). In cells where the nucleoli are clearly visible, the majority of the 25S rDNA signal in the wild-type interphase cells forms a knob in the perinucleolar space with multiple weak signals emanating into the nucleolus, while the 25S rDNA signal in the mutant cells is entirely intranucleolar, resembling the intranucleolar portion of the wild-type signal.

We were interested to see whether the loss of 45S rDNA also occurs in mutants of *fas1* and *fas2* in different genetic back-

grounds. We observed reduced amounts of 45S rDNA repeats in *fas1-2* (No), *fas2-1* (L*er*), and a progressive reduction in G1 to G3 *fas1-4b* (C24) mutant plants (Figure 4C; see Supplemental Figure 7 online). Only the *fas2-2* (No) mutants (Gn) displayed a higher amount of 45S rDNA than the wild type (Figure 4C; see Supplemental Figure 7 online). No systematic difference in the amount of centromeric repeats or 5S rDNA was observed in these plants. Based on these results, we suggest that the same phenomenon occurs regardless of ecotype, indicating a common mechanism and effect of the loss of function of the FAS proteins.

Contraction of Telomeric or 45S rDNA Repeats Does Not Affect Levels of Their Transcripts

Since ongoing transcription and the impairment of CAF1 function are expected to cause perturbations in chromatin structure, we performed micrococcal nuclease (MNase) digestion of the nuclei of G4 *fas1-4* and *fas2-4* wild-type and mutant plants (Figure 6B). Ethidium bromide–stained gels of size-fractionated DNA reveal that the bulk nucleosomes had moderately perturbed periodicity in mutant plants compared with the wild type, and this slight difference was observed when DNA gel blots were hybridized with specific probes. However, the experiment did not reveal any significant difference in the level of perturbation between the sequences that change in copy number (telomere and 18S rDNA) and those that do not (180-bp repeat and 5S rDNA).

We further asked whether the reduced copy numbers of 45S rDNA lead to a change in the overall transcriptional level of the 45S rDNA loci. We also questioned whether the presumed
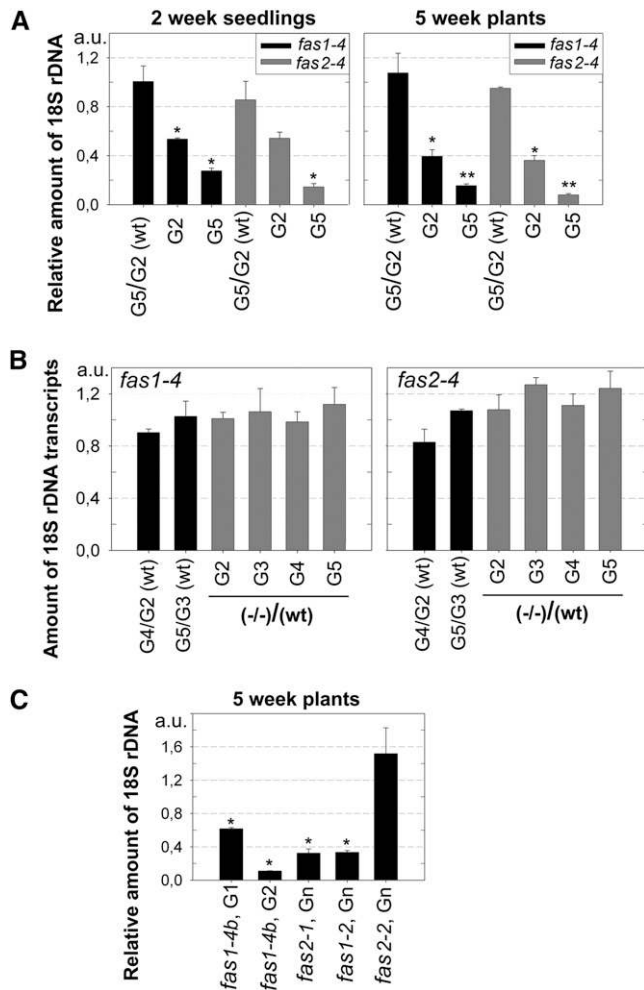
Figure 4. Relative Amount of 45S rDNA in All Studied Plant Lines Estimated by Quantitative PCR.

Amount of repeats in mutants is related to the wild-type plants of corresponding ecotypes where generation of mutant is unknown (Gn) or to segregated wild type of the same generation (G1, G2, and G5). Asterisks indicate significant difference from 1 based on Student's *t* test: *P < 0.05; **P < 0.001. Error bars indicate SD.
(A) Lines *fas1-4* and *fas2-4*, seedlings, and 5-week-old plants.
(B) Relative (mutant/wild type) expression of 18S rDNA in generations 2 to 5 of lines *fas1-4* and *fas2-4*.
(C) Lines *fas1-2*, *fas1-4b*, *fas2-1*, and *fas2-2*, 5-week-old plants.

reduction in telomere silencing (Enomoto et al., 1997; Kaufman et al., 1997; Schonrock et al., 2006) alters the amount of the telomeric repeat-containing RNA, which can negatively regulate telomerase activity and induce replication fork arrest within telomeric DNA (Azzalin et al., 2007; Luke et al., 2008; Schoeftner and Blasco, 2008; Ng et al., 2009). We performed RNA dot hybridization with 18S rDNA and telomeric probes and Q-PCR on RNA isolated from *fas1-4* and *fas2-4* mutant and wild-type seedlings. In the tested generations G2 to G5 of mutant plants, we did not detect any significant progressive or consistent changes in the level of 18S rDNA transcripts (Figure 4B) or in

the abundance of G-rich or the C-rich telomeric sequence transcripts (see Supplemental Figure 8 online).

Neither Telomerase Activity nor the Amount of Extrachromosomal DNA Changes in the FAS Mutants

To find possible mechanism(s) of the selective loss of telomeric and 45S rDNA repeats in the *fas* mutants, we first analyzed the activity of telomerase in vitro. The telomere repeat amplification protocol (TRAP) assay showed no significant difference in the activity of telomerase between the wild-type and mutant seedlings (Figure 6A). Repetitive sequences are naturally prone to recombination events that are documented by the presence of extrachromosomal DNA (eccDNA) derived from these repeats (Cohen et al., 2008). An overall increased level of recombination has been observed in the *fas* mutants (Endo et al., 2006; Kirik et al., 2006; Schonrock et al., 2006; Ramirez-Parra and Gutierrez, 2007), and in yeast, the level of recombination increases at the rDNA loci when enhanced transcription from a reduced number of rDNA copies collides with their replication (Takeuchi et al., 2003). Using neutral-neutral two-dimensional (2D) gel electrophoresis, we analyzed the levels of eccDNA originating either
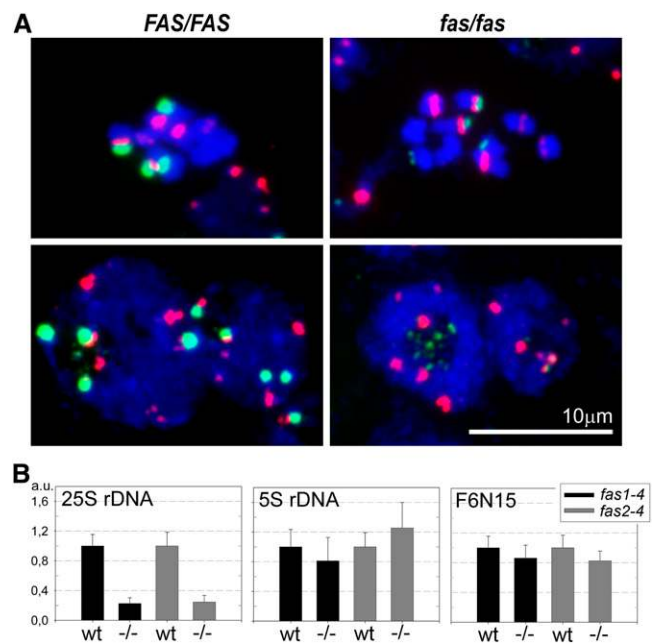

Figure 5. Fluorescence in Situ Hybridization of *fas* Mutants.

(A) Comparison of the 5S (red) and 45S (green) loci in representative G5 *fas1-4* metaphase and interphase nuclei. *FAS/FAS*, wild type; *fas/fas*, mutant.
(B) Quantification of fluorescence intensities of Cy3 (5S rDNA probe) and AF488 (25S rDNA probe) and DEAC (BAC clone F6N15 probe) related to 4',6-diamidino-2-phenylindole in metaphase nuclei. Error bars indicate SD. n(wt) = 103/108, n(−/−) = 140/141 nuclei for *fas1-4* and *fas2-4*, respectively. Wild-type and mutant plants of generation G5 were used. The wild type was arbitrarily set as 1. All values for mutant samples are significantly different from one based on a Student's *t* test: P < 0.001. −/−, Mutant.
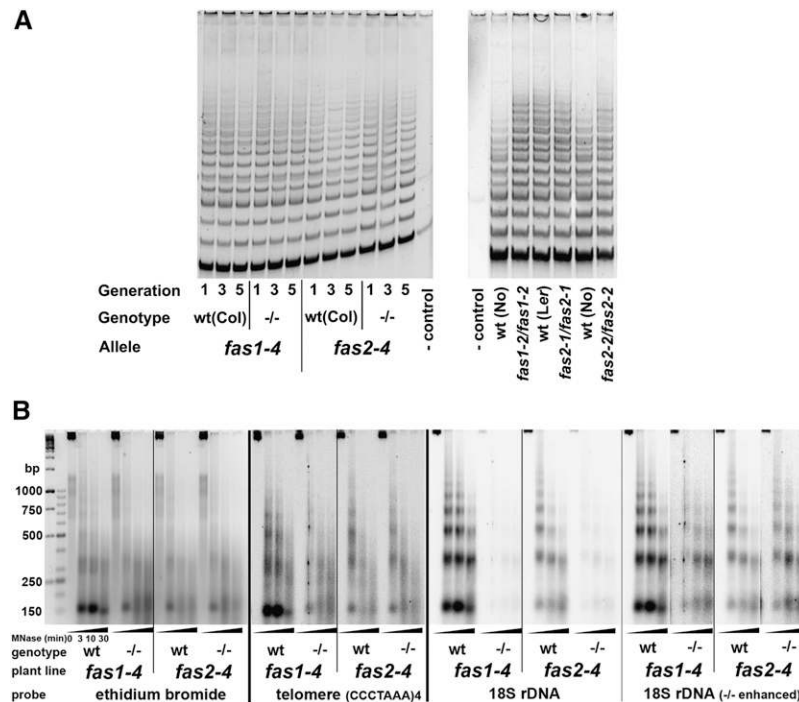
Figure 6. Telomerase Activity and Chromatin Structure in *fas* Mutants.

(A) Telomerase activity in vitro. TRAP performed on all plant lines studied.

(B) MNase assay performed on *FAS1-4* and *FAS2-4* wild-type and mutant plants in generation G4. (−/−), Mutant.

from the telomeric or from 45S rDNA loci to find typical by-products of recombination frequently leading to amplification or contraction of these repeats. Although we were able to detect eccDNAs originating from telomeres and 45S rDNA, we did not observe any difference in their amounts between wild-type and mutant plants (Figure 7).

DISCUSSION

CAF1 Dysfunction Leads to Reduction of Telomeric and 45S rDNA Repeats

We show that mutations in the genes *FAS1* and *FAS2*, encoding two of the three subunits of CAF1 in *Arabidopsis*, cause a progressive reduction of telomeric and 45S rDNA repeats. The results are consistent for both *fas1* and *fas2* mutants, suggesting that the observed phenotype is caused by a dysfunction of CAF1 rather than by a separate involvement of the two subunits in other pathways. The mutations in *FAS1* and *FAS2* are marked by a decrease in median telomere lengths from 3 to 7 kb in the wild type depending on ecotype, to 1.5 to 2 kb in mutants regardless of ecotype (Figure 1; see Supplemental Figure 3 online). The 45S rDNA copy number in *fas1-4* and *fas2-4* (Col) mutants is reduced to 10 to 20% of the wild-type level by the 5th generation of homozygous mutants (Figure 3) implying that ∼57 to 120 copies of the estimated 570 copies per haploid genome (Pruitt and Meyerowitz, 1986) remain. A similar phenomenon with a variable

extent of reduction is observed in different ecotypes of *Arabidopsis*. Apart from telomeres and 45S rDNA, other major repetitive sequences in the *Arabidopsis* genome tested do not display changes in copy numbers. These repeats are as follows: tandemly repeated 5S rDNA and 180-bp centromeric pAL sequences (Round et al., 1997; Heslop-Harrison et al., 1999), major centromeric Ty3/Gypsy-type retrotransposon *Athila* (Pelissier et al., 1995, 1996), and mobile pericentromeric transposon *CACTA1*, which is transcriptionally activated in *fas* mutants (Ono et al., 2006). The propagation of the *fas* mutants is limited by progressive decrease in fertility and viability, but the extent of the phenotypic defects is variable within and between different ecotypes.

Although rDNA and telomeres both represent major tandem repeats, the molecular mechanisms of their loss need not be identical but may reflect the diverse effects of disturbed chromatin assembly in the *fas* mutants on the distinct mechanisms underlying copy number maintenance of these repeats (see below).

Telomere Shortening and Chromosome-End Deprotection

Although increased recombination has been described in the *fas* mutants (Endo et al., 2006; Kirik et al., 2006), several facts argue against the recombination-based mechanism of telomere loss, telomere rapid deletion (Lustig, 2003), in the shortening of telomeres. Recombination at telomeres is downregulated by their folding into a compact nucleoprotein structure (Fajkus et al.,
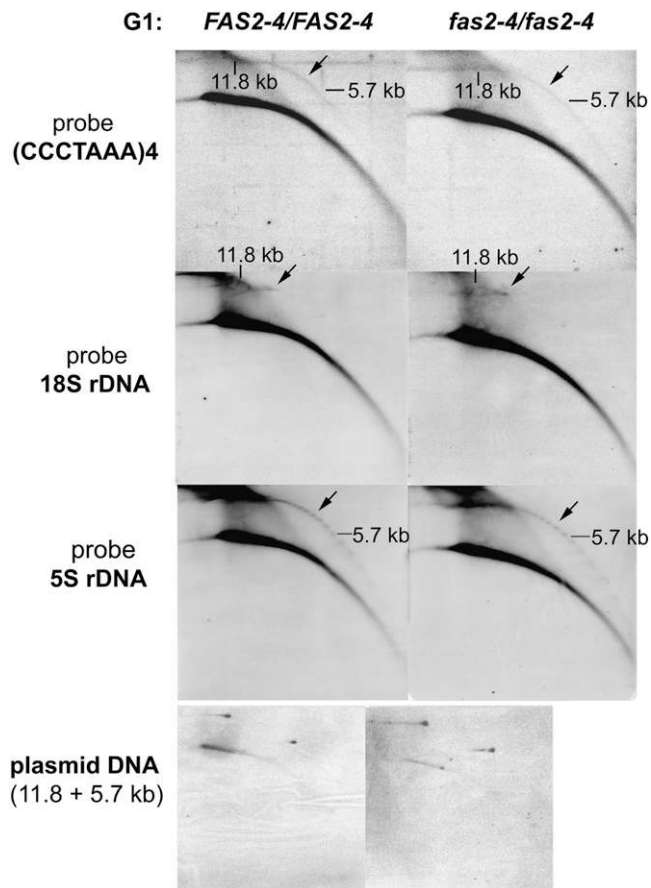
G1:   *FAS2-4/FAS2-4*   *fas2-4/fas2-4*

probe
(CCCTAAA)4

11.8 kb   —5.7 kb   11.8 kb   —5.7 kb

probe
18S rDNA

11.8 kb   11.8 kb

probe
5S rDNA

—5.7 kb   —5.7 kb

plasmid DNA
(11.8 + 5.7 kb)

Figure 7. eccDNA in fas2-4.

eccDNA in generation G1 *fas2-4*: neutral-neutral 2D agarose gel electrophoresis. Arrows point to the eccDNA arc. *FAS/FAS*, wild type; *fas/fas*, mutant.

1995; Fajkus and Trifonov, 2001; Schoeftner and Blasco, 2009; Vrbsky et al., 2010) and by controlled activity of the DNA repair factors (Denchi, 2009). Telomere rapid deletion is stochastic giving rise to heterogeneous telomere lengths (Watson and Shippen, 2007). In the *fas* mutants, however, the telomere lengths remain homogeneous, the dynamics of their shortening is relatively steady and moderate, no increase in the formation of eccDNA originating from telomeres has been detected, and even the shortening observed in the first generation of *fas* mutants (Figure 1C) is less than the estimated net replicative shortening per plant generation in the absence of telomerase or alternative lengthening of telomeres (Ruckova et al., 2008).

In addition to the conventional DNA replication machinery, telomere length is maintained by telomerase (Greider and Blackburn, 1985) in a replication-dependent manner (Fan and Price, 1997; Diede and Gottschling, 1999; Marcand et al., 2000; Qi and Zakian, 2000; Ray et al., 2002). It is possible that under conditions of prolonged and/or incomplete replication and chromatin assembly at telomeres in *fas* mutants, the recruitment of telomerase may be impaired and/or the time slot for its action may be insufficient, resulting in telomere erosion despite the presence of

standard telomerase activity in vitro (Figure 6A). There is faster loss of telomeres on transition from heterozygous parent into G1 (600 to 700 bp) and slower loss (100 to 300 bp) to stabilization in subsequent generations (Figures 1A to 1C). Telomere shortening in later generations may be counteracted by the increased accessibility for telomerase due to shorter telomere lengths that (1) prevent formation of telomerase-repressive t-loop structures and/or (2) enable the more slowly progressing replication machinery to reach telomere ends. Such a scenario could explain the observed shortening of telomeres in yeast CAF1 mutants (Enomoto et al., 1997), whose shorter telomere lengths of ~300 bp may not constitute an obstacle for their full replication.

Reestablishment of permissive conditions for the telomerase activity in heterozygotes could be a possible explanation of the quick reversal of telomere lengths we observed after backcrossing *fas/fas* with *FAS/FAS* plants and crossing *fas1/fas1* with *fas2/fas2* plants (Figure 2). Telomerase has been shown to preferentially act on shorter telomeres (Hemann et al., 2001; Samper et al., 2001; Shakirov and Shippen, 2004; Teixeira et al., 2004; Chang et al., 2007), and telomere length is specific and stable in each *Arabidopsis* ecotype (Riha et al., 2001, 2002; Shakirov and Shippen, 2004; Maillet et al., 2006). This may explain the increase in lengths of the shortest and intermediate telomere lengths and the stable lengths of the longest wild-type telomeres in F2 progeny of the mutant × wild-type crosses (Figure 2A). Altogether, the observed telomere dynamics suggest that telomere maintenance rather than telomere protection is compromised in the *fas* mutants.

The rate of shortening (but not the homogeneous distribution of telomere lengths) and the progressive increase in the severity of the *fas* phenotype resembles telomere shortening and its consequences induced by mutation in telomerase catalytic subunit *TERT* (Fitzgerald et al., 1996; Ruckova et al., 2008). Since the severity of the *fas* phenotype is variable among plants from lines *fas1-4* and *fas2-4* where the rate of telomere shortening is similar and since telomere shortening in the morphologically severe mutants *fas1-4b* is mild, the loss of telomeric tract is not the only contributor to the loss of viability in these plants. However, a fragile telomere stability resulting in telomere deprotection in the *fas* mutants is documented by the presence of anaphase bridges in 3.7% of anaphases in G5 mutants, which in some cases show severe developmental defects and low fertility (<10 seeds collected from one plant). The number of anaphase bridges exceeds the 0.7% observed in the G5 *tert* mutants where rapid increase of anaphase bridges and an onset of developmental defects occur in later generations (Riha et al., 2001). This difference could be attributed to the fast shortening of telomeres from parental generation to G1 *fas* mutants, which may speed up the process of telomere shortening below the threshold of 1 kb, described as the critical telomere length at which the chromosomes get susceptible to fusions (Heacock et al., 2007).

rDNA Loss and Genomic Instability

rDNA copy number is maintained via recombination induced by frequent formation of DSBs at the replication fork barriers within the intergenic spacer of the yeast 35S rDNA (reviewed in

Kobayashi, 2006; Labib and Hodgson, 2007; Tsang and Carr, 2008). Although the mechanism of rDNA copy number maintenance in plants has not been described so far, the replication fork barriers have been documented in plants (Hernandez et al., 1993; Lopez-Estrano et al., 1999), and the intergenic spacer of the 45S rDNA has been identified as a recombination hot spot (Urawa et al., 2001). Whereas the yeast 5S rDNA is subjected to joint copy number regulation as a part of the 35S rDNA locus (Kobayashi, 2006), the separate localization and possibly different regulation of the 45S and the 5S rDNA loci in *Arabidopsis* may explain the selective loss of the 45S and not the 5S rDNA in the *fas* mutants (Figure 3; see Supplemental Figure 7 online). Since dysfunction of CAF1 in plants has been connected to increased numbers of DNA DSBs, increased level of HR, and upregulation of genes involved in HR (Endo et al., 2006; Kirik et al., 2006; Schonrock et al., 2006; Ramirez-Parra and Gutierrez, 2007), it is possible that disruption of a recombination-based maintenance equilibrium may lead to the 45S rDNA loss. However, the bias toward the deterministic loss of the sequences needs to be explained. The fact that we did not observe any increase in the level of eccDNA derived from 45S rDNA loci (Figure 7) may indicate that increased recombination is limited to an early developmental stage (Cohen and Mechali, 2002; Liu et al., 2007). However, since the loss of rDNA in the *fas* mutants seems continuous (Figure 4), and since the presence of eccDNA was detected in adult plants (Figure 7), it is unlikely that recombination-based losses are restricted to early ontogeny. Alternatively, the sequences may not be lost through eccDNA but as other recombination by-products.

The 45S rDNA loss was observed in all the studied lines of *fas* mutants with the exception of the line *fas2-2*, where shorter telomeres (see Supplemental Figure 3 online) but higher amounts of 45S rDNA (Figure 4C; see Supplemental Figure 7 online) were observed in mutants than in the corresponding wild type. Several facts may contribute to this difference: (1) The wild-type and mutant lines are unrelated plants, which may differ in the abundance of rDNA repeats, as was previously reported in other plant species (Rogers and Bendich, 1987a, 1987b). (2) The allele *fas2-2* carries a point mutation that causes a frameshift, resulting in a termination codon 98 amino acids downstream of the mutation (Kaya et al., 2001). We have not detected any alternatively spliced transcripts, and the mutation has been confirmed on the level of cDNA. Thus, we may speculate that the translation results in a protein that is partly functional or whose function is altered by its C-terminal part. Alternatively, ribosome slippage may revert the frameshift, generating a partly functional FAS2.

Reduction of 45S rDNA copy number is more dramatic in the phenotypically more severe *fas2-4* mutants with no detectable transcription of *fas2*, than in *fas1-4* mutants with only reduced expression of *fas1*. A question arises whether rDNA reduction could account for the variable severity of the *fas* phenotype. Only a subset (estimated to 10 to 27% in *Arabidopsis*) of the 45S rRNA genes are transcriptionally active (Grummt and Pikaard, 2003; Pontes et al., 2003). It is plausible that the 10 to 20% of the original rDNA copies maintained by the G5 *fas* mutants can provide sufficient amounts of rRNA, and this is in agreement with the unchanged amount of 18S rRNA in the mutants (Figure 4B). It is therefore unlikely that inadequate transcription of 45S rDNA

could explain the progressive severity of the *fas* phenotype in the five studied generations. Transcriptionally silent copies of the rDNA have been cytologically identified as the condensed rDNA knobs that may be associated with the nucleoli, while the active copies are seen as multiple partly decondensed intranucleolar signals (Leitch et al., 1992; Pontes et al., 2003; Lawrence and Pikaard, 2004). In G5 *fas* mutants, we observed the 25S rDNA as decondensed multiple foci inside the nucleoli, which suggests that the majority of the remaining rDNA copies are transcriptionally active (Figure 5). Further loss of rDNA in the next generations of *fas* mutants can be expected to result in inadequate supply of rRNAs originating from the 45S rDNA loci.

Although most of the rDNA copies are dispensable for normal plant development (Rogers and Bendich, 1987b), low numbers of rDNA repeats have been previously implicated in the phenotype severity and sterility of the *Drosophila melanogaster* mutant *bobbed* (Ritossa, 1968), in release of heterochromatin-induced gene silencing (Paredes and Maggert, 2009), in reduced rDNA condensation and cohesion (Kobayashi and Ganley, 2005), and increased sensitivity to DNA damage when transcribed by RNA polymerase I (Ide et al., 2010). These results support a hypothesis that redundant copies of rRNA genes play an essential role in maintaining genome stability (Kobayashi, 2008). Whether the reduced position-dependent gene silencing observed in the CAF1 mutants and the progressive nature of the *fas* phenotype can be at least partly attributed to the progressive reduction of rDNA copies needs to be further assessed. It is however possible that later generation *fas* mutants present an extreme state where the few 45S rDNA repeats are extensively transcribed, and this may contribute to genome instability.

Numerous questions have been raised and need to be addressed in future, including: (1) What are the mechanisms of rDNA copy number maintenance in plants? (2) What is/are the mechanism/s responsible for the initiation and progress of the selective copy number reduction? (3) What are the consequences of their loss in the *fas* mutants. Studies examining the telomere and rDNA dynamics in plants with combined mutations in *FAS* and other genes regulating telomere and rDNA maintenance or DNA repair are in progress to resolve mechanisms leading to the *fas* phenotype.

METHODS

Plant Material and Genotyping of Mutants

All studies were performed in the following plant lines: *fas1-2* (No) (Kaya et al., 2001), *fas1-4* (Col) (NASC: N828822, SAIL_662_D10, described in Exner et al., 2006), *fas1-4b* (C24) (Kirik et al., 2006), *fas2-1* (Ler) (Leyser and Furner, 1992), *fas2-2* (No) (Kaya et al., 2001), *fas2-4* (Col) [NASC: N533228, SALK_033228 (H) (AE), described in Exner et al., 2006] (see also Supplemental Table 1 and Supplemental Figure 1 online). Lines *fas1-2*, *fas2-1*, and *fas2-2* had been propagated as homozygous mutants for an unknown number of generations and were grown for two more generations in parallel to wild-type plants of ecotypes of the appropriate genetic background. Lines *fas1-4*, *fas1-4b*, and *fas2-4* were obtained as progeny of heterozygous parents and served to segregate wild-type and mutant lines. In *fas1-4* and *fas2-4* lines, three *FAS/fas* parents gave 9 × *FAS/FAS* and 15 × *fas/fas* in three G1 lines, two lines served to give 12 × *FAS/FAS* and 18 × *fas/fas* in G2, and one line of 6 × *FAS/FAS* and 9 × *fas/fas* was

propagated into G3 to G5. In line *FAS1-4b*, two *FAS/fas* parents gave 9 × *FAS/FAS* and 10 × *fas/fas* in G1, 15 × *FAS/FAS* and 13 × *fas/fas* in G2, and 9 × *FAS/FAS* and 7 × *fas/fas* in G3. To obtain the crosses between *fas/fas* (♀) × wild types (♂), three independent lines were crossed and propagated into F1, and one line (total of seven plants) was propagated into F2 (26 plants); in the case of *fas1/fas1* × *fas2/fas2*, one line of plants was observed (total of three plants in F1 and 29 plants in F2). Seeds were surface sterilized and pregrown on half-strength Murashige and Skoog plates for 2 weeks when the plants were put into soil and grown under the conditions of 16 h light, 21°C and 8 h dark, 19°C, 100 μmol/m²·s⁻¹. For genotyping, DNA was isolated using the IRRI method (Collard et al. 2007), and 1 μL of this DNA was used in a PCR reaction using DyNAzyme II DNA polymerase (Finnzymes). Combinations of primers used, their sequences, and sizes of PCR products can be found in Supplemental Tables 2 and 3 online. Genotyping results were confirmed in the higher-purity DNA isolated according to Dellaporta et al. (1983) from the selected plants.

DNA Isolation

DNA was isolated from 2-week-old seedlings or from rosette leaves of 5-week-old plants according to Dellaporta et al. (1983). Quality of DNA was checked, and concentration was determined from 0.8% (w/v) agarose gel stained with ethidium bromide using Gene Ruler 1 kb DNA Ladder (Fermentas) as standard and Multi Gauge software (Fujifilm).

RNA Isolation and Reverse Transcription

RNA was isolated from 100 mg of 2-week-old seedlings using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. RNA was extensively treated with DNase I (TURBO DNA-free; Applied Biosystems/Ambion), concentration was determined using NanoPhotometer (IMPLEN), and quality of RNA was checked on 1% (w/v) agarose gel. cDNA was prepared by reverse transcription of RNAs using M-MuLV reverse transcriptase, Rnase H⁻ (Finnzymes), with random hexamers (Finnzymes) according to the manufacturer's instructions.

Q-PCR

Q-PCR was performed in the same way on cDNA, DNA from 2-week-old seedlings, and DNA from 5-week-old plants using RotorGene3000 (Corbett Life Science). FastStart SYBR Green Master (Rox; Roche) was used with the combination of primers UBQ10f and UBQ10r for ubiquitin (single 186-bp product) and 18Sf and 18Sr for 18S rDNA (single 226-bp product) (see Supplemental Table 3 online). The same cycling conditions were applied to both primer combinations: denaturation 95°C, 10 min; 40 cycles of 95°C/15 s and 60°C/60 s. The SYBR Green I fluorescence was monitored after each extension step at 60°C. The amount of 18S rDNA repeats or transcripts was normalized to ubiquitin. All samples were analyzed in triplicates in three repeated experiments.

Telomere Length Analysis: TRFs

Five hundred nanograms of genomic DNA isolated from leaves of individual 5-week-old plants were digested overnight by Tru1I (*Mse*I) (Fermentas) and separated in 0.8% (w/v) agarose gel at 1.5 V/cm, 16 h, at room temperature. Agarose gels with TRFs were washed in 0.25 M HCl for 15 min and 0.4 M NaOH for 30 min and blotted in 0.4 M NaOH onto Hybond XL membrane (GE Healthcare). TRFs were hybridized overnight at 55°C with telomere-specific probe TR4C (CCCTAAA)₄, end-labeled with [γ-³²P]ATP using T4 polynucleotide kinase (NEB), and visualized using the FLA7000 imager (Fujifilm). Evaluation of product sizes was done using software Multi Gauge with Gene Ruler 1-kb DNA Ladder (Fermentas) as standard. Only samples loaded on the same gel were mutually compared. TRFs were evaluated as the median of the population of telomere lengths, and two or more populations within one plant were distinguished only if the maxima of the individual populations formed clearly separated peaks.

Dot Blot Hybridization

Probes for hybridization were PCR-generated using Phusion High-Fidelity DNA Polymerase (NEB) from genomic DNA with the following sets of primers (for sequences of all primers, see Supplemental Table 3 online): 180-bp centromeric repeat: pALf + pALr (Davison et al. 2007) (single copy 136 bp), 18S rDNA: 18Sf + 18Sr (226 bp), 5S rDNA: 5SLf + 5SLr (single-copy 436 bp), *Athila*: ORF1f + ORF1r (Davison et al. 2007) (126 bp), and *CACTA*: SP15 + SP40 (Kato et al. 2003) (713 bp). The products were inserted into vector pCRII-TOPO (Invitrogen) and sequenced. The probe used as a single-copy gene reference (locus At1g49950 coding for putative telomere binding protein TRB1) was amplified from genomic DNA using primers TRB1fA and TRB1r, inserted into pDONR/Zeo (Invitrogen), and sequenced. Final TRB1 probe was amplified from the vector using primers TRB1fB and TRB1r, generating an 1889-bp product. Probes for hybridization were amplified from the vector using insert-specific primers and radioactively labeled using the DecaLabel DNA labeling kit (Fermentas). Telomeric probe TR4C was labeled as for TRF analysis.

DNA was isolated from 1.2 g of leaves mixed from three 5-week-old plants, 0.4 g each. The variability of the amounts of repetitive sequences was tested on five plants prior to mixing the three plants into one sample. All the experiments were done in three independent biological replicates (plants grown at different times). Genomic DNA (250, 100, 40, and 16 ng) was diluted into 200 μL 0.4 M NaOH and 10 mM EDTA, and samples were denatured at 95°C for 10 min and cooled on ice. They were spotted onto Hybond XL membrane (GE Healthcare) and subjected to hybridization with sequence-specific probes. All membranes were done in triplicates and hybridized first with the TRB1 DNA loading reference probe, washed and hybridized with two other probes in the order of increasing hybridization signal of the particular probes (i.e., pAL < 18S rDNA, TR4C < CACTA, and Athila < 5S). All probes were hybridized in 0.25 M Na-phosphate, pH 7.5, 7% SDS, and 0.016 M EDTA overnight at 65°C and washed with 0.2× SSC + 0.1% SDS, except for At TR4C, which was hybridized at a lower stringency (55°C) and washed with 2× SSC + 0.1% SDS. The signal was evaluated using Multi Gauge absolute signal strength calculated from linear regression lines and normalized to the signal of TRB1. The obtained value of wild-type DNA in generation G1 was arbitrarily set as 1, and all the other values were related to this G1 wild-type sample.

Fluorescence in Situ Hybridization

Mitotic chromosomes were prepared from flower bud pistils of wild-type and mutant plants derived from the same heterozygous parent, which were simultaneously grown under the conditions described above, as described by Mokros et al. (2006). The whole protocol was performed in parallel on mutant and wild-type samples, and the same aliquot of the hybridization probes was used for both types of samples. For visualization of the 45S rDNA loci, an internal 2478-bp (*Eco*RI) fragment of the 25S rRNA gene was used (Kiss et al. 1989). The clone pCT4.2, corresponding to a 500-bp 5S rRNA repeat (M65137), was used for localization of 5S rDNA loci. The probes were directly labeled by AF488-dUTP and Cy3-dUTP, respectively. DEAC-labeled BAC clone F6N15 (GenBank accession number AF069299) was used as an internal hybridization control. The fluorescence in situ hybridization procedures were performed according to Mandakova and Lysak (2008). All images were acquired using the same parameters, and integrated fluorescence intensities were quantified by ISIS imaging software (MetaSystems) in nuclei found in the same cell

cycle phase (metaphase or interphase). Fluorescence intensities of AF488, Cy3, and DEAC were normalized to 4',6-diamidino-2-phenylindole.

MNase Digestion

Two grams of leaves from three 5-week-old plants were used for isolation of nuclei according to Espinas and Carballo (1993), and isolated nuclei were washed and resuspended in 50 mM TrisCl, pH 8, 125 mM saccharose, 3 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM MeSH, 250 μM spermidine, and 100 μM PMSF, divided into four aliquots, and digested with 90 units of MNase (TaKaRa Bio) for 3, 10, or 30 min. After proteinase K treatment and ethanol precipitation, the isolated DNA was separated in 2% (w/v) NuSieve 3-1 agarose (Lonza) gel at 1 V/cm overnight at room temperature. Signals of 18S rDNA or telomeric probe were detected after DNA gel blot hybridization as described.

TRAP

Protein extracts from 2-week-old seedlings were prepared according to Fitzgerald et al. (1996). These extracts were subjected to the TRAP assay as described by Fajkus et al. (1998). TS21 was used as the substrate primer for extension by telomerase and TEL-PR as the reverse primer (see Supplemental Table 3 online) in the subsequent PCR.

2D Agarose Gel Electrophoresis

To probe for eccDNA, 2D agarose gel eletrophoresis was conducted according to modified protocols of Cohen and Lavi (1996), Zellinger et al. (2007), and Navratilova et al. (2008). DNA was isolated from 5-week-old plants as for dot blot hybridization. Five micrograms of genomic DNA with the addition of 5.7- and 11.8-kb plasmids, 5 ng each, were digested with a total of 30 units of Plasmid-Safe ATP-Dependent DNase (Epiceter Biotechnologies) overnight. DNA was precipitated and separated in 0.4% (w/v) agarose gel, 1× TBE at 1 V/cm, 22 h, room temperature in the first direction. Second direction was run in 1% (w/v) agarose, 1× TBE with 0.3 μg/mL ethidium bromide at 4 V/cm, 6 h, at room temperature. The gel was then blotted and hybridized with telomeric TR4C, 18S rDNA, and plasmid probes as described.

Statistical Analysis

All data were statistically analyzed using two-tailed unpaired Student's t test with $\sigma_A \neq \sigma_B$.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative data libraries under the following locus identifiers: *FAS1* (At1g65470) and *FAS2* (At5g64630).

Supplemental Data

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

Supplemental Figure 1. Characterization of Plant Material.

Supplemental Figure 2. Examples of the fas Phenotype.

Supplemental Figure 3. Telomere Length Maintenance in Lines fas1-2, fas1-4b, fas2-1, and fas2-2.

Supplemental Figure 4. Lengths of Telomeres on Individual Chromosome Arms in fas1-4.

Supplemental Figure 5. Lengths of Telomeres on Individual Chromosome Arms in fas2-4.

Supplemental Figure 6. Relative Amount of 45S rDNA in Individual Plants from Generations 2 and 5 of Lines fas1-4 and fas2-4.

Supplemental Figure 7. Relative Amount of Repetitive Sequences in fas1-2, fas1-4b, fas2-1, and fas2-2 Estimated by Dot Blot Hybridization.

Supplemental Figure 8. Relative Expression of Telomeric Repeats in fas1-4 and fas2-4.

Supplemental Table 1. Overview of FAS Mutants Used.

Supplemental Table 2. Genotyping fas Mutants.

Supplemental Table 3. Oligonucleotides Used.

Supplemental Methods.

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