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RESEARCH ARTICLES

The Chromatin Assembly Factor Subunit FASCIATA1 Is Involved in Homologous Recombination in Plants

Angela Kirik,[a,1] Ales Pecinka,[b,2] Edelgard Wendeler,[a] and Bernd Reiss[a,3]

[a] Department of Plant Developmental Biology, Max-Planck-Institut für Züchtungsforschung, 50829 Cologne, Germany
[b] Department of Cytogenetics, Institute of Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany

DNA replication in cycling eukaryotic cells necessitates the reestablishment of chromatin after nucleosome redistribution from the parental to the two daughter DNA strands. Chromatin assembly factor 1 (CAF-1), a heterotrimeric complex consisting of three subunits (p150/p60/p48), is one of the replication-coupled assembly factors involved in the reconstitution of S-phase chromatin. CAF-1 is required in vitro for nucleosome assembly onto newly replicated chromatin in human cells and *Arabidopsis thaliana*, and defects in yeast (*Saccharomyces cerevisiae*) affect DNA damage repair processes, predominantly those involved in genome stability. However, in vivo chromatin defects of *caf-1* mutants in higher eukaryotes are poorly characterized. Here, we show that *fasciata1-4* (*fas1-4*), a new allele of the *Arabidopsis fas1* mutant defective in the p150 subunit of CAF-1, has a severe developmental phenotype, reduced heterochromatin content, and a more open conformation of euchromatin. Most importantly, homologous recombination (HR), a process involved in maintaining genome stability, is increased dramatically in *fas1-4*, as indicated by a 96-fold stimulation of intrachromosomal HR. Together with the open conformation of chromatin and the nearly normal expression levels of HR genes in the mutant, this result suggests that chromatin is a major factor restricting HR in plants.

INTRODUCTION

Chromatin provides the framework for DNA replication, transcription, and DNA damage repair, including recombination (Krude, 1999; Ridgway and Almouzni, 2001; Green and Almouzni, 2002; Maser and DePinho, 2002; Haushalter and Kadonaga, 2003). The first step in postreplicative chromatin synthesis is the assembly of histones into nucleosomes, and this process is initiated by the deposition of histones H3 and H4 onto newly replicated DNA. Two major histone assembly complexes are involved in this step, replication-coupled chromatin assembly factor (RCAF) and chromatin assembly factor 1 (CAF-1). RCAF consists of anti-silencing factor 1 (ASF-1) and histones H3 and H4. CAF-1 is a heterotrimeric complex that consists of three subunits, p150, p60, and p48. In association with histones H3 and H4, it is also known as chromatin assembly complex. *ASF-1* was originally identified in yeast as a gene that caused the derepression of transcriptional silencing when overexpressed. CAF-1 was found in human cell extracts as a component essential for the assembly of nucleosomes onto newly replicated DNA. CAF-1 is evolutionarily conserved, and the genes encoding the corresponding p150, p60, and p48 subunits, *CHROMATIN ASSEMBLY COMPLEX1* (*CAC1*), *CAC2*, and *CAC3*, also exist in yeast (Ridgway and Almouzni, 2000, 2001; Haushalter and Kadonaga, 2003) and in plants (Kaya et al., 2001; Hennig et al., 2003). A direct interaction with proliferating cell nuclear antigen links CAF-1 function tightly to DNA replication, tethering this complex directly to the growing replication fork. In yeast, the ASF-1 and CAF-1 assembly complexes have redundant functions, because defects in either complex alone do not abolish replication-coupled chromatin assembly.

Defects in both complexes cause sensitivity to DNA damage, linking replication-coupled chromatin assembly to DNA damage repair. In particular, in yeast, defects in CAF-1 and ASF-1 cause genome instability by promoting gross chromosomal rearrangements (GCRs) (Kolodner et al., 2002; Myung and Kolodner, 2003). In contrast with the viability of *cac* mutants in yeast, defects in CAF-1 in human cells cause cell cycle arrest and DNA damage, demonstrating the prominent role of CAF-1 in postreplicative chromatin assembly in mammalian cells (Hoek and Stillman, 2003; Ye et al., 2003; Nabatiyan and Krude, 2004). These significant differences between organisms raise the question of which role CAF-1 might play in chromatin establishment and the control of homologous recombination (HR) in plants.

The role of chromatin in plant HR is of special interest because the frequencies of gene targeting are low, despite the high efficiency of extrachromosomal HR (reviewed in Reiss, 2003). This fact suggests that chromatin may play a role in the inhibition of HR.

A major role of chromatin in recombination and DNA damage repair in general is further suggested by the fact that chromatin remodeling activities participate in these processes. Furthermore,

[1] Current address: Universität zu Köln Botanik III, Gyrhofstrasse 15, 50931 Cologne, Germany.
[2] Current address: Gregor Mendel Institute for Molecular Plant Biology, Dr. Bohr-Gasse 3, A-1030 Vienna, Austria.
[3] To whom correspondence should be addressed. E-mail reiss@mpiz-koeln.mpg.de; fax 49-221-5062213.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Bernd Reiss (reiss@mpiz-koeln.mpg.de).
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major recombination proteins interact directly or indirectly with chromatin, suggesting that chromatin, and not only DNA, is the substrate for eukaryotic recombination activities (Alexiadis and Kadonaga, 2002; Alexeev et al., 2003; Fritsch et al., 2004; van Attikum et al., 2004; Schuermann et al., 2005). These findings suggest a need for the removal or loosening of chromatin before the repair machinery can access damaged DNA.

The *fasciata1* (*fas1*) and *fas2* mutants (Leyser and Furner, 1992; Kaya et al., 2000, 2001) are affected in the genes encoding the *Arabidopsis thaliana* homologs of the p150 and p60 subunits, respectively. We have identified a new mutant affected in the gene encoding the p150 subunit that causes major developmental abnormalities. Further characterization revealed severe chromatin defects that affected both heterochromatin and euchromatin. These changes in chromatin structure are likely to be the cause of the significant stimulation of HR that was found in the mutant. Because a similar defect in yeast barely stimulates HR, our data suggest that important differences between yeast and *Arabidopsis* exist in the control of HR by chromatin structure.

RESULTS

Identification of *fas1-4*

A new population of T-DNA–tagged transformants was produced using the *Arabidopsis* C24 ecotype. A recessive mutant with a strong visible phenotype was detected in this population. Early shoot branching suggested reduced apical dominance. More importantly, the mutant was severely retarded in growth (Figure 1A), and all organs, leaves (Figure 1B), stems, roots, inflorescences (Figure 1C), siliques (Figure 1D), and flowers (Figure 1E) were severely reduced in size and showed developmental abnormalities. Cross sections through mature leaves (Figure 1G) showed that leaf morphology was heavily affected. The regular pattern of cell files was distorted, and the number of cells in cross section was drastically reduced. However, the thickness of the leaf was only slightly altered, because the remaining cells were significantly larger. Despite the severity of the phenotype, plants grew to maturity, flowered, and set seed, albeit with drastically reduced yield (data not shown). Molecular analysis of the mutant

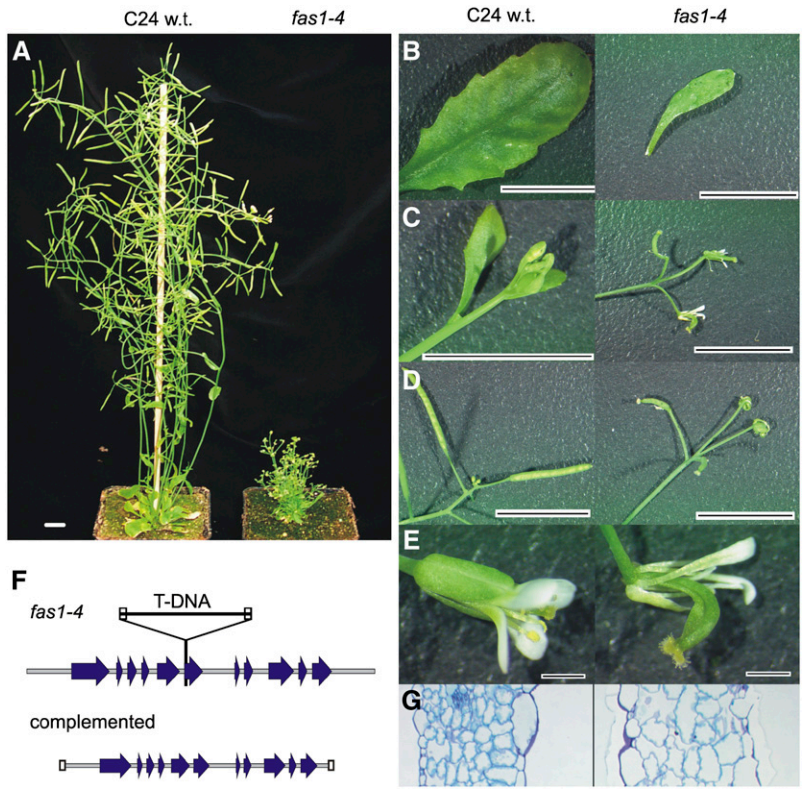

Figure 1. The *fas1-4* Mutant.

(A) Visible phenotypes of C24 wild-type plants and *fas1-4* at the stage of seed set. Mutants (right) are considerably smaller than C24 wild-type plants (left).

(B) to **(E)** Magnifications show the reduced size and developmental abnormalities in leaves **(B)**, inflorescences **(C)**, siliques **(D)**, and flowers **(E)**. Bars = 1 cm in **(A)** to **(D)** and 1 mm in **(E)**.

(F) Structure of the *FAS1* gene as deduced from published cDNA and genomic sequences. Exons are represented by arrows; the position of the T-DNA insertion in the sixth exon in the mutant *FAS1* gene is indicated (top). The *FAS1* construct (bottom) used for complementation by *Agrobacterium tumefaciens*–mediated transformation of the mutant constitutes the entire gene, including the upstream region. Small boxes indicate T-DNA borders.

(G) Toluidine blue–stained cross sections through mature leaves like those shown in **(B)**.

showed that a T-DNA had inserted in the *FAS1* gene, the *Arabidopsis* homolog of the gene encoding the p150 subunit of CAF-1 (Kaya et al., 2001). Because mutants affected in this gene were described previously as *fas1* mutants (Leyser and Furner, 1992; Kaya et al., 2000, 2001), the mutant was named *fas1-4*. The insertion consists of a truncated copy of the original tagging vector from which large portions are absent (data not shown). This insertion interrupts the sixth exon approximately in the middle of the *FAS1* coding region (Figure 1F). Although *fas1-4* is allelic to established *fas1* mutants, it has a different developmental phenotype. The previously described *fas1* alleles show serrated leaves, fasciation with broad stems, and abnormal phyllotaxy. These growth abnormalities have been explained by the destabilization of meristem identity gene expression states that lead to defects in the shoot and root apical meristems (Kaya et al., 2001).

The developmental alterations observed in *fas1-4* seem not to be restricted to meristems, suggesting that a defect affecting all cells contributed to the visible phenotype. The mutation segregated as a single, recessive Mendelian trait, suggesting that additional, unlinked mutations that might have contributed to the phenotype are absent in the mutant. In addition, a wild-type gene copy including the putative promoter region (Figure 1F) reverted the phenotype of homozygous mutants completely back to the wild type, confirming that the T-DNA insert in the *FAS1* gene had caused this mutation. At present, we do not know what caused the phenotypic differences between the other *fas1* alleles and *fas1-4*. The nature of the T-DNA insertion present in *fas1-4* suggests that this mutant is a loss-of-function mutant; however, the other alleles contain either nonsense mutations or a large 5′ terminal deletion and are thus thought to have also lost the function of the p150 subunit of CAF-1 (Kaya et al., 2001). It is possible that differences in the genetic backgrounds of the mutants might have contributed to the differences in phenotype.

fas1-4 Affects Recombination

Mutations in yeast in any of the *CAF-1* subunit homologs lead to moderate sensitivity to UV light (Kaufman et al., 1997). Epistasis analysis with yeast mutants defective in repair genes suggested a role for CAF-1 in postreplicative DNA damage repair, but not necessarily HR (Game and Kaufman, 1999). To analyze UV light sensitivity in *fas1-4*, root tips of pregrown seedlings were irradiated with an increasing dose of UV light. Growth of *fas1-4* roots was moderately inhibited, indicating that the mutant is slightly hypersensitive to UV light (data not shown). This result indicated that defects in CAF-1 also affect DNA damage repair in *Arabidopsis*.

The analysis of intrachromosomal homologous recombination (ICHR) is an established method to probe HR in plants (Reiss, 2003; Schuermann et al., 2005). To analyze whether HR is affected in the mutant, we used an in planta version of this assay, a powerful tool to visualize ICHR directly in whole plants (Swoboda et al., 1994). The system is based on a recombination reporter gene consisting of two overlapping defective fragments of the β-glucuronidase (*GUS*) gene inserted in inverted orientation into the genome (Figure 2A). ICHR creates a functional *GUS* gene that is detected in whole plants by histochemical staining for GUS activity. Heterozygous *fas1-4* plants were crossed to N1IC4 651 (Puchta et al., 1995), a line of the C24 ecotype harboring the N1IC4 recombination reporter gene, and ICHR in progeny plants was analyzed by staining for GUS activity. With this assay, we detected, on average, 96-fold more recombination events per seedling in homozygous *fas1-4* mutants than in N1IC4 651 (Figure 2B). The number of recombination sectors was increased in roots, stems, cotyledons, and leaves, indicating that ICHR was stimulated in all parts of the plant. In addition, both large and small blue-stained sectors were observed, indicating that recombination had occurred early as well as late in plant development, as described for the N1IC4 651 reporter line (Figures 2C and 2D). In the homozygous mutants complemented with the wild-type *FAS1* gene, recombination levels declined to near wild-type levels (Figure 2B), confirming that the defect in *fas1-4* had caused the increase of recombination frequencies.

fas1-4 has a strong developmental phenotype that leads to a reduction in size of all organs, and in vitro–grown mutant seedlings have drastically reduced, narrow true leaves (Figure 2E). This growth defect likely affects the number and generations of cells needed to establish the seedling. Because the efficiency of ICHR is defined by the number of events per number of genomes and generations (Swoboda et al., 1994), the number of recombination sectors per seedling determined as described above may not directly reflect the efficiency of ICHR in the mutant. To address this point, digital imaging was used to determine the surface area of a representative sample of GUS-stained mutant and wild type seedlings, as shown in Figures 2E and 2F. This analysis showed that *fas1-4* seedlings have 81% of the surface area of wild-type seedlings, because a value of 13 ± 2 mm^2 was obtained for the mutant and 16 ± 5 mm^2 was obtained for the wild type. The number of cells in these seedlings is likely to be reduced, because a reduction of cell number (42% of the wild-type level) was observed in mature leaves. However, nuclear DNA content is duplicated (see below). Therefore, the number of genomes represented in the surface area of the mutant may be 84% of the wild-type value. Taking the reduction in surface area together with the reduction in the number of genomes represented in this area, mutant seedlings contain 68% of the genomes of the wild type. Therefore, based on the number of recombination events per seedling, the data are likely to have underestimated the genome-based ICHR frequency; in reality, the mutation may stimulate ICHR by 140-fold.

fas1-4 Affects Heterochromatin

To address the question of whether *fas1-4* has a role in the formation of heterochromatin, nuclei from mature leaves were isolated and analyzed by microscopy. In *Arabidopsis* nuclei, highly condensed heterochromatic DNA is present as visible chromocenters that consist of centromeric and pericentromeric repeats and rRNA genes (Fransz et al., 2002). Confocal microscopy showed that 4′,6-diamidino-2-phenylindole (DAPI)–stained interphase nuclei from mature wild-type (Figure 3A) and *fas1-4* (Figure 3B) leaves had basically the same appearance, as observed previously with *fas1-1* (Takeda et al., 2004), although chromocenters in *fas1-4* mutant nuclei tended to be more blurred. Epifluorescence microscopy of fixed, spread, and DAPI-stained nuclei, as well as quantitative evaluation (Soppe et al., 2002), revealed that the chromocenters in mutant nuclei

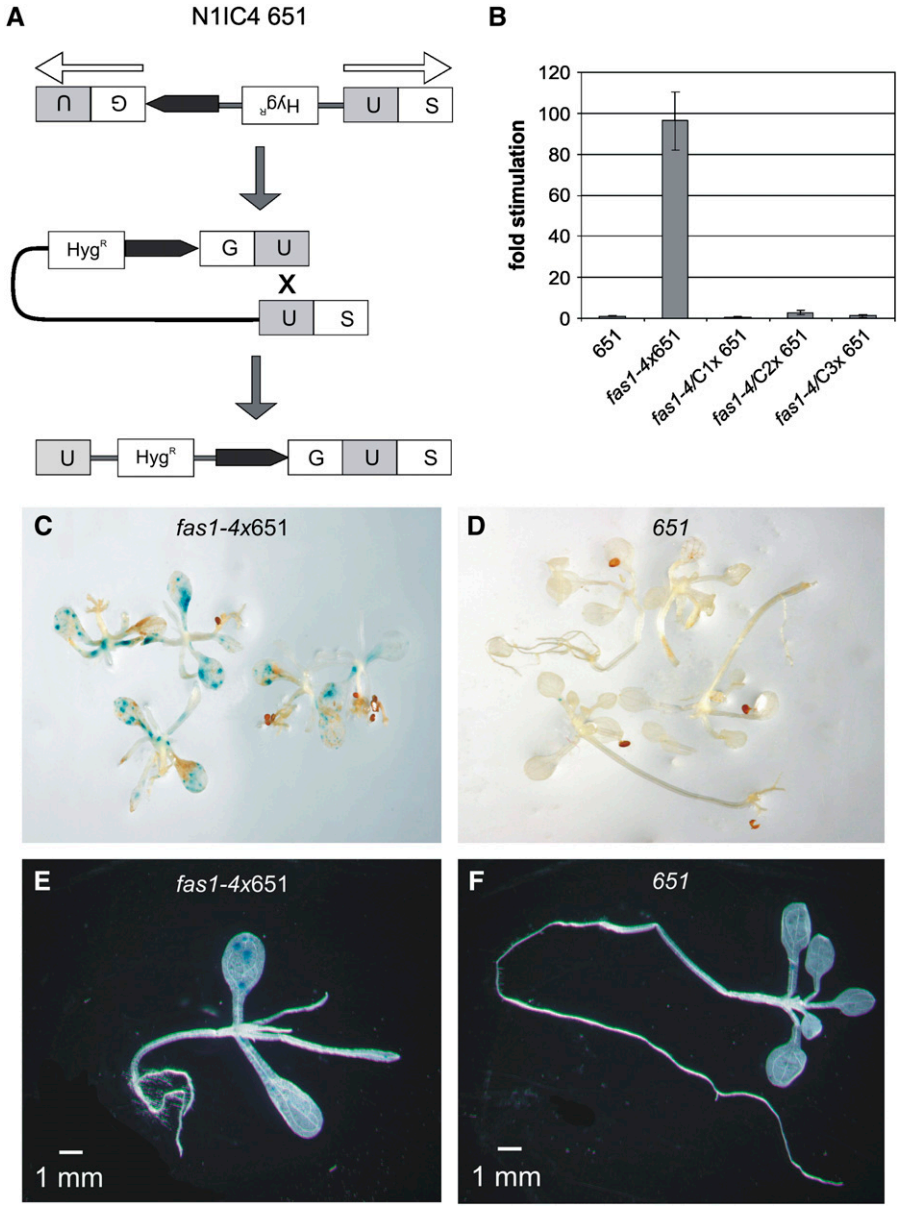

Figure 2. HR Is Stimulated in *fas1-4*.

(A) The HR reporter of line N1IC4 651. The line harbors two complementary half genes (UG, US) of the *GUS* reporter gene as an inverted repeat separated by a functional hygromycin resistance gene (Hyg^R). The open arrows indicate the orientation of the repeats. The black arrows symbolize the cauliflower mosaic virus promoter driving the GUS gene. ICHR generates an intact *GUS* gene.

(B) Quantitative analysis of ICHR in *fas1-4*, the wild type, and complemented mutants. The number of GUS-positive sectors in N1IC4 651, *fas1-4*×N1IC4 651, and three independent complementation lines (*fas1-4*/C1×651, *fas1-4*/C2×651, and *fas1-4*/C3×651) was counted and the data normalized and plotted. On average, *fas1-4* seedlings showed a 96-fold (minimum, 85-fold; maximum, 108-fold) increase in the number of blue sectors over the reporter line N1IC4 651. The number of recombination events in mutants complemented with the *FAS1* gene is comparable to that of the N1IC4 651 reporter line. Data are means of three experiments, and error bars show SD.

(C) and **(D)** Typical patterns of staining for in vivo *GUS* expression obtained for *fas1-4*×N1IC4 651 **(C)** and N1IC4 651 **(D)**.

(E) Close-up view of a *fas1-4*×N1IC4 651 seedling showing the narrow true leaves and the global growth reduction of the mutant.

(F) An N1IC4 651 seedling with well-developed true leaves is shown for comparison.

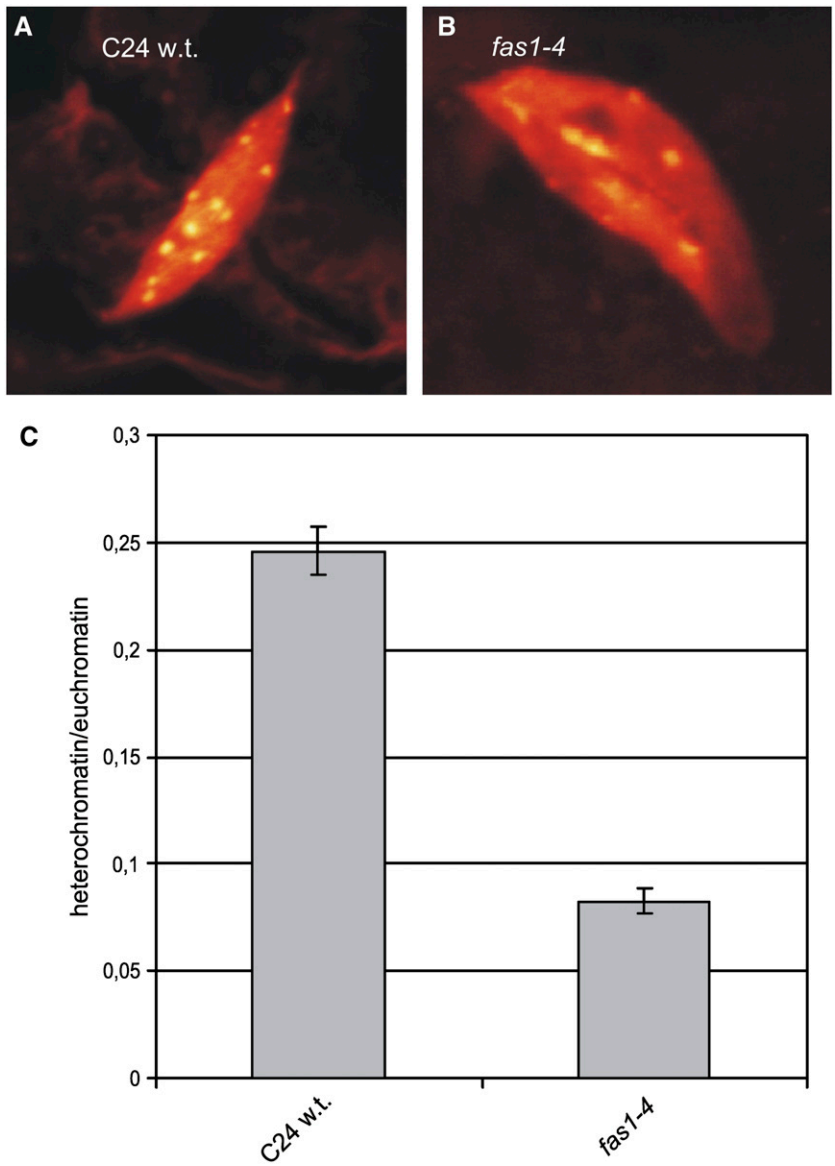

Figure 3. *fas1-4* Causes Loss of Heterochromatin.

(A) and **(B)** Images of DAPI-stained wild-type **(A)** and *fas1-4* **(B)** nuclei of mature leaves visualized by confocal microscopy. The chromocenters in the majority of wild-type nuclei have sharply defined boundaries. By contrast, the boundaries in *fas1-4* nuclei are more blurred.

(C) The size of chromocenters is reduced in *fas1-4*. Nuclei from wild-type and *fas1-4* leaf cells were prepared, stained with DAPI, and spread. The staining intensity in chromocenters and the remaining nuclear area was quantified by digital imaging, and the ratios were calculated. Because chromocenters represent mainly heterochromatin and the remaining staining represents euchromatin, values are expressed as the ratio of heterochromatin to euchromatin. Data are means of three experiments, and error bars show SD.

contained threefold less DNA relative to the entire nucleus than those in wild-type nuclei (Figure 3C). This result indicated a reduced amount of heterochromatin as well as a dispersion of pericentromeric DNA. A similar phenotype has been observed in other mutants, such as in hypomethylated mutants (Soppe et al., 2002), in *bru I*, in which a new protein linking DNA damage repair and gene silencing is downregulated (Takeda et al., 2004), and in *msi1*, a mutant defective in one of the *Arabidopsis* homologs of the p48CAF-1 subunit (Hennig et al., 2003). In addition, like *msi1* and *bru1*, *fas1-4* had no effect on centromeric methylation (Figure 4), although these methylation patterns are regarded as the genomic imprint required for the maintenance of heterochromatin. Because methylation is not affected, both genes appear either to act downstream of DNA methylation processes participating in the establishment of repressive chromatin or to operate in as yet unknown pathways.

Very recently, the CAF-1 defect in *fas-1-1* was also shown to lead to a reduction of heterochromatin, with no effect on

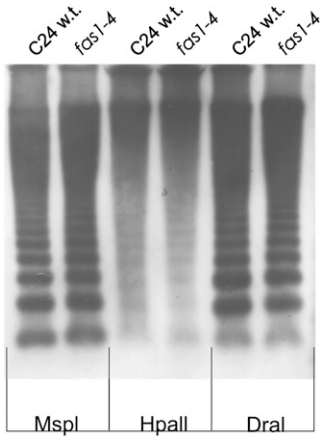

Figure 4. Methylation of Centromeric Heterochromatic DNA Is Unaffected in *fas1-4*.

Genomic DNA isolated from wild-type and *fas1-4* leaves was digested with the methylation-sensitive enzyme *Hpa*II, its methylation-insensitive isoschizomer *Msp*I, and the methylation-insensitive enzyme *Dra*I. All three restriction enzymes release a similar fragment from the *Arabidopsis* centromeric repeat. After separation by gel electrophoresis, the DNA was blotted onto nylon membranes and hybridized with a 180-bp centromeric repeat fragment (Vongs et al., 1993).

centromeric DNA methylation as well (Schonrock et al., 2006). Because blurriness of chromocenters was not observed in *fas1-1* (Takeda et al., 2004) and the reduction of heterochromatin was less pronounced than in *fas1-4*, the latter is likely to be a stronger allele, in accord with the more pronounced visible phenotype of the mutant.

fas1-4 Causes an Open Chromatin Conformation

Chromatin can exist in a closed conformation, a tightly packed structure that is considered to be inactive and inaccessible to proteins interacting with DNA, or in an open conformation, which is the form of actively expressed genes. These two conformations differ in their accessibility to exogenously added DNase (Hebbes et al., 1994).

To analyze whether the mutation had affected such aspects of chromatin, DNase I sensitivity assays were used to probe total chromatin. In nuclei prepared from mature plants, *fas1-4* total genomic DNA was considerably more sensitive to DNase I digestion than nonmutant DNA (Figure 5A), indicating a general effect on chromatin. To specifically analyze the effect of CAF-1 deficiency on euchromatin, the DNase sensitivity of defined loci was tested. The *fas1-4* mutant and the reporter line N1IC4 651 share an identical genetic background, ecotype C24. An *Eco*RI restriction fragment length polymorphism (RFLP) that exists between Columbia (Col-0) and Landsberg *erecta* (Schaffner, 1996) was also found between Col-0 and C24 and was used to exclude any experimental ambiguities in these assays by including nuclei isolated from Col-0 as an internal reference. The pARMS2, pARMS5, and pARMS7 plasmids were originally developed for mapping and encode multiple chromosomal markers that detect such RFLPs (Schaffner, 1996). The sequences in these three plasmids represent seven chromosomal loci in four of the five *Arabidopsis* chromosomes. The corresponding mapping positions on the *Arabidopsis* physical map (Fabri and Schaffner, 1994; Schaffner, 1996) are shown in Figure 5B. In addition, the exact chromosomal positions of three of them were determined by DNA sequencing and alignment to the *Arabidopsis* chromosomal sequence at The Arabidopsis Information Resource (http://arabidopsis.org). These markers are shown in Figure 5B in gray. All genomic loci, particularly the sequenced loci, correspond to single-copy sequences in euchromatic regions of the *Arabidopsis* genome. All three sequenced loci correspond to intergenic regions (pARMS2, positions 86,483 to 86,820 in accession number U93215; pARMS5, positions 126,620 to 127,220 in accession number AL161584; pARMS7, positions 48,335 to 49,135 in accession number AB011482).

For the assay, Col-0 nuclei were mixed with *fas1-4*×N1IC4 651 or N1IC4 651 (both C24) nuclei and digested together with DNase I. The extracted DNA was then digested with *Eco*RI, and the genomic sequences were analyzed by DNA gel blotting (Figure 5C). An identical pattern of signal loss over time indicated that the three loci were equally sensitive to DNase I in N1IC4 651 and Col-0. By contrast, in *fas1-4*×N1IC4 651, C24-specific fragments disappeared faster than Col-0 fragments, indicating a considerably higher sensitivity to DNase I in the mutant. Moreover, the *GUS* gene, as an essential part of the recombination reporter in N1IC4 651, was also hypersensitive to DNase I in *fas1-4* (Figure 5D). Because chromatin in general and seven defined loci in particular are hypersensitive to DNase I and also because heterochromatic regions were reduced in size, CAF-1 deficiency is likely to affect all aspects of chromatin and seems to generally promote a more open structure of chromatin. Furthermore, because the nuclei were isolated from mature leaves consisting mostly of nonproliferating cells, global DNase I hypersensitivity indicates that the mutation has a sustainable effect on chromatin structure that is not likely to be restricted to the replicative or early postreplicative phase but to persist over the entire cell cycle.

fas1-4 Has No Effect on Interhomolog Association

Chromatin in interphase nuclei is highly organized, allowing the identification of individual chromosomes by fluorescence in situ hybridization (FISH). DNA repair by somatic homologous recombination requires physical interaction between allelic sequences (i.e., either homologous chromosomes or sister chromatids). Therefore, we analyzed whether the increased HR frequency in *fas1-4* might be reflected in a generally increased frequency of somatic pairing between homologs in diploid interphase nuclei. Two randomly chosen nonrepetitive euchromatic regions corresponding to BAC clones F18C1 and MGL6 from chromosome 3 were analyzed by FISH (Figure 6A). The homologs in somatic interphase *Arabidopsis* cells paired with a frequency of 4 to 5% (150 nuclei analyzed), which is the statistically predicted frequency of random association (Pecinka et al., 2004). The same pairing frequency (186 nuclei analyzed) was observed in *fas1-4*, indicating that somatic homologous pairing is not enhanced in the mutant. Therefore, CAF-1–dependent chromatin structures do not seem to play a role in the pairing of homologs in somatic cells.

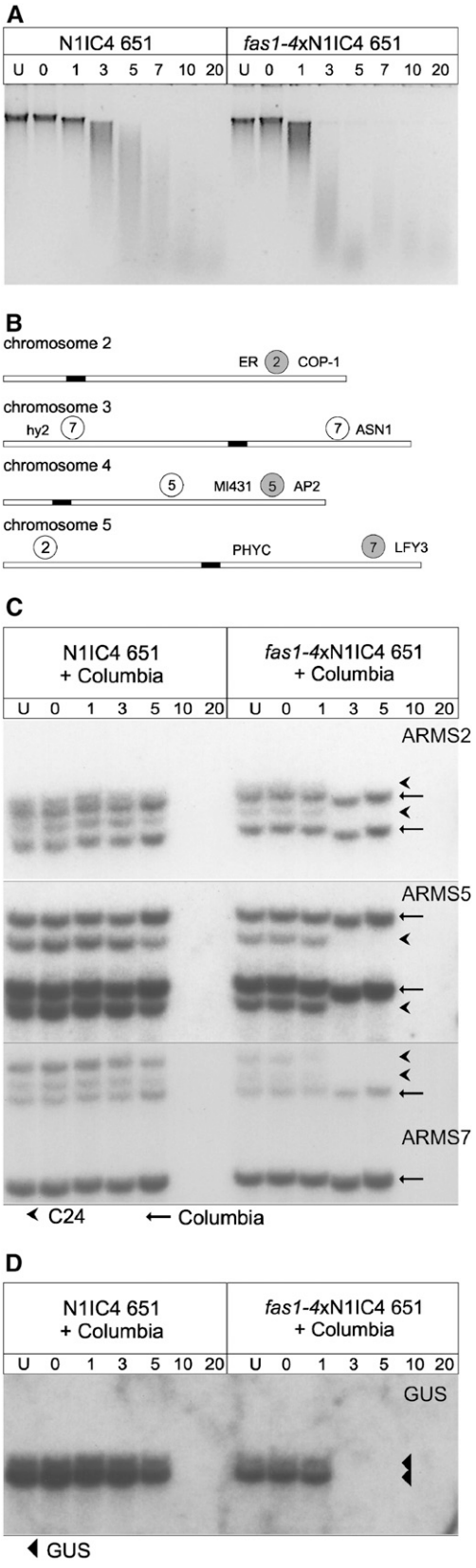

Figure 5. *fas1-4* Affects Chromatin Conformation.

(A) Chromatin is globally more sensitive to DNase I in *fas1-4*. Nuclei were isolated from N1IC4 651 and *fas1-4*×N1IC4 651 and treated with DNase I for the times indicated (in minutes; U = untreated control). DNA was then extracted and separated by agarose gel electrophoresis.

(B) Scheme of *Arabidopsis* chromosomes 2, 3, 4, and 5. The positions of the RFLP markers (numbers in open circles) in pARMS2, pARMS5, and pARMS7, the positions of markers determined by sequence alignment (numbers in gray circles), and chromosomal landmark markers are shown. The centromere is shown as a closed bar.

(C) Euchromatic regions in *fas1-4* are hypersensitive to DNase I. Nuclei from N1IC4 651 and *fas1-4*×N1IC4 651 mixed with nuclei from Col-0 were digested with DNase I for the times indicated (in minutes; U = untreated control). The DNA was extracted, digested with *Eco*RI, separated by gel electrophoresis, blotted onto a nylon membrane, and probed with ARMS2, ARMS5, and ARMS7. All chromosomal loci are considerably more sensitive to DNase I in *fas1-4*×N1IC4 651 than in N1IC4 651.

(D) The recombination reporter locus is hypersensitive to DNase I in *fas1-4*. The membrane used in **(C)** was reprobed with a *GUS* gene fragment.

fas1-4 Affects the Endopolyploidy Level

The *fas1-4* mutation had caused a severe developmental phenotype that suggested that all cells are affected. The same defect in yeast or human cells causes a delay in S and G2/M phases (Prado et al., 2004) or arrest in S phase (Hoek and Stillman, 2003; Ye et al., 2003; Nabatiyan and Krude, 2004), respectively. To analyze whether the *fas1-4* mutation affected such aspects, a flow cytometric analysis was performed. However, *Arabidopsis* is an endopolyploid plant, and the leaf consists of a mixture of diploid and endopolyploid cells (Larkins et al., 2001; Sugimoto-Shirasu and Roberts, 2003). Therefore, polyploidization may overlap with cell cycle defects.

The flow cytometric analysis showed that the mutation significantly affected the DNA content of nuclei isolated from mature, fully developed leaves. The population of wild-type C24 leaf cells contained predominantly 2C, 4C, and fewer 8C nuclei. By contrast, mutant leaves contained fewer 2C than 4C and 8C nuclei and, in addition, a clear 16C fraction (Figure 6B). The duplication of the nuclear DNA content in nearly all mutant cells suggests that the mutation had caused one additional cycle of endoreduplication in all or almost all leaf cells, regardless of their state of endopolyploidy. Therefore, the mutation in *Arabidopsis* affects endoreduplication. Because endopolyploidy is caused by endoreduplication or the duplication of entire genomes in the absence of cytokinesis, endopolyploid cells progress no further through the cell cycle. Therefore, the mutation could have caused premature cell differentiation, in accord with the visible phenotype.

The function of CAF-1 in replication-coupled chromatin assembly and the fact that the same defect causes S-phase delay in yeast and S-phase arrest in human cells suggests that the mutation also affects the cell cycle. If so, the phenotype of *fas1-4* is less severe than the same defect in human cells, because defects in CAF-1 in human cells cause S-phase arrest and cell death. In particular, the viability of *fas1-4* indicates that mutant cells can progress completely through the cell cycle, indicating that the *fas1* defect in *Arabidopsis* does not result in cell cycle arrest in proliferating cells. This observation suggests differences

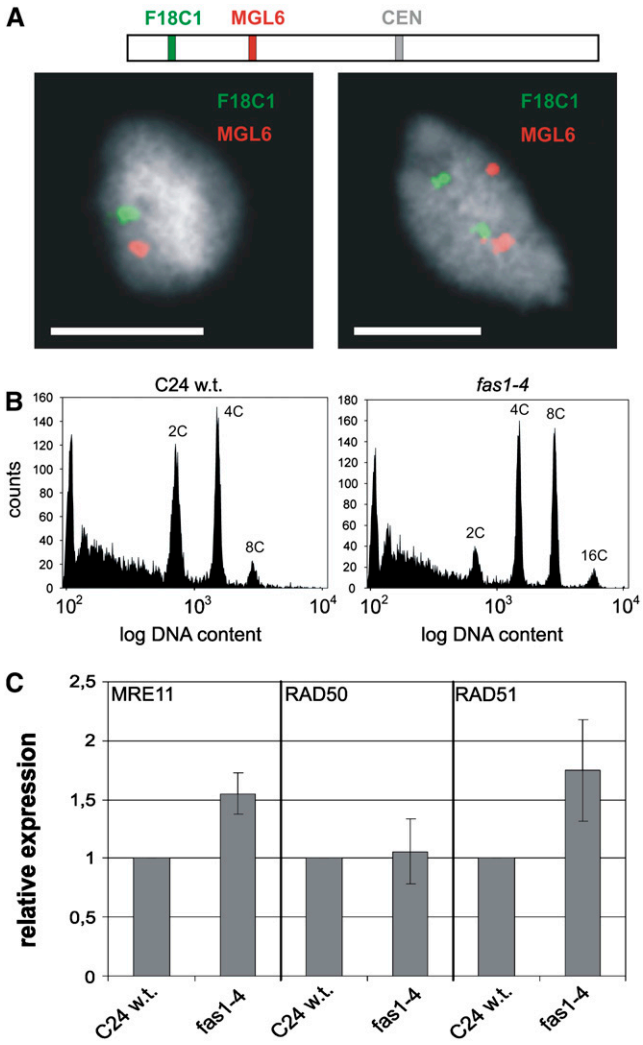

Figure 6. Effects of *fas1-4* on Nuclear DNA Content, Homolog Pairing, and HR Gene Expression.

(A) The frequency of homolog pairing remains unaffected in *fas1-4*. Examples of 2C leaf nuclei of the C24 wild type from a single-point pairing analysis by FISH. The scheme of chromosome 3 (top) indicates the position of the BAC sequences used for analysis. The left panel shows simultaneous homologous pairing of two distant segments (F18C1 and MGL6), and the right panel shows complete separation. Bars = 5 μm.

(B) Flow cytometric DNA content histogram of C24 wild type (left) and *fas1-4* (right) nuclei. During sorting, the number of nuclei was plotted against DNA content. Although most wild-type leaf nuclei have a content of 2C and 4C, this ratio is shifted toward 4C and 8C in *fas1-4* leaf nuclei. Correspondingly, the cell cycle value, indicating the mean number of endoreduplication cycles per nucleus (Barow and Meister, 2003), is shifted from 0.716 in C24 to 1.419 in *fas1-4*, indicating duplication of the DNA content in *fas1-4* cells.

(C) Relative transcript levels of representative genes involved in HR in *fas1-4*. The results of a quantitative RT-PCR analysis after quantification and normalization to wild-type expression levels are shown for *Arabidopsis RAD50*, *MRE11*, and *RAD51*. The data are means of three experiments, and error bars show SD.

between these organisms in the importance or function of the S-phase checkpoint, in accord with such differences in the mechanisms linking cell cycle control and HR (discussed in Li et al., 2004).

fas1-4 Does Not Cause a Significant Stimulation of HR Gene Expression

To analyze whether HR gene expression was affected in *fas1-4*, the transcription of representative genes was analyzed. RAD50 and MRE11 participate in early steps of recombination, being involved in DNA damage recognition and processing of double strand breaks, whereas RAD51 is a typical representative of the HR pathway. The *RAD50* and *MRE11* genes are important recombination and checkpoint transducer genes in yeast (Myung et al., 2004), but little is known about the transducer function of these genes in plants. *Arabidopsis RAD50* does not seem to be transcriptionally activated by DNA damage (Gallego et al., 2001), and no data are available for *MRE11*. However, *Arabidopsis RAD51* is significantly stimulated by γ radiation (Doutriaux et al., 1998) and UV light (Ries et al., 2000). The steady state transcript levels of *RAD50*, *MRE11*, and *RAD51* in leaves of mature *fas1-4* and wild-type plants were analyzed by quantitative RT-PCR. *RAD50* was not affected, whereas the expression of *MRE11* and *RAD51* was increased (Figure 6C). However, the increase in expression was small, varied between experiments, and was within the limits of resolution of our quantitation system. Therefore, these data suggest that the *fas1-4* mutation did not cause a dramatic effect on the expression of genes involved in HR.

However, *RAD51* expression was found to be increased in *fas1-1* in a genome-wide analysis of gene expression patterns in *fas1*, *fas2*, and *msi* mutants (Schonrock et al., 2006). This difference between that study and our data might be attributable to different experimental conditions or to differences in the mutants. However, it is also quite possible that DNA damage repair genes are regulated differently in different ecotypes, because significant differences in gene expression patterns were also observed between ecotypes (Schonrock et al., 2006).

DISCUSSION

ICHR in the *fas1-4* mutant in *Arabidopsis* is stimulated nearly 100-fold. This stimulation is by far the strongest effect on ICHR of all chromatin mutants analyzed in plants to date (*mim*, twofold [Hanin et al., 2000]; *bru1*, fourfold [Takeda et al., 2004]). In addition, this number exceeds the stimulation of ICHR at the N1IC4 651 recombination reporter locus induced by genotoxic stress with UV_{254} and methyl methane sulphonate (1.75- to 3.75-fold induction) but is comparable to that of higher concentrations of the radiomimetic bleomycin (30-fold induction) (Heitzeberg et al., 2004). As observed with *mim*, our data suggest that chromatin is involved directly in this effect. However, chromatin plays a role in a multitude of biological processes; thus, chromatin defects may cause pleiotropic effects.

One possibility is an effect on silencing. *caf-1* mutations relieve silencing in yeast (Haushalter and Kadonaga, 2003), human cells (Tchenio et al., 2001), and *Arabidopsis* (Kaya et al., 2001). However, although the N1IC4 651 recombination reporter locus

(Puchta et al., 1995) is transgenic and, therefore, relief of silencing could have contributed to the observed increase in the number of GUS expression–positive sectors in the mutant, such an effect is unlikely. There is no indication for silencing of this particular locus (Puchta et al., 1995), and the hygromycin resistance gene, which is an integral part of the reporter locus, is expressed throughout in all plants, because these plants are fully resistant to hygromycin. In addition, the sectors of GUS expression representing recombination events were evenly distributed in the mutant, a pattern that is not compatible with the partial and stochastic relief of silencing described for *fas1* mutants (Ono et al., 2006).

Another possibility is the regulation of genes involved in HR. However, we have found that none of the three representative genes analyzed was greatly stimulated in expression. One of them, *RAD51*, plays a key role in HR and is significantly induced by DNA damage such as γ radiation (Doutriaux et al., 1998) and UV light (Ries et al., 2000). However, although *RAD51* was strongly induced, chronic UV light damage stimulated ICHR by 10-fold (Ries et al., 2000). In comparison, *RAD51* was induced 1.5-fold at best in *fas1-4*, whereas ICHR was stimulated nearly 100-fold. In addition, a defect in *RAD51C*, a *RAD51* paralogue likely to act in the same pathway as RAD51, reduced ICHR only twofold (Abe et al., 2005). These data suggest that induction of *RAD51* expression is insufficient to account for the magnitude of stimulation in ICHR observed in the mutant. However, such mechanisms might have contributed, considering the accumulation of small effects over time and the fact that regulation at the protein level may be more important than at the level of transciption. In addition, the mutation could have affected the regulation of other genes that could have affected ICHR in *fas1-4*. In a microarray-based genome-wide analysis of CAF-1 mutants (Schonrock et al., 2006), upregulation of DNA repair genes was found; however, the data provide no clear indication of which particular pathway might have been activated, leaving open a mechanistic explanation for the induction of ICHR in *fas1-4*.

As described for *asf-1* mutants and histone depletion in yeast, DNA damage induced in the replicative phase could generate recombinogenic substrates and thus mechanistically explain the stimulation of ICHR (Prado et al., 2004; Prado and Aguilera, 2005). However, compared with *fas1-4*, a *cac1*Δ mutation in the yeast homolog of *FAS1* stimulated ICHR by only threefold (Prado et al., 2004). Therefore, DNA damage alone seems unlikely to explain the dramatic stimulation observed in *fas1-4*. However, given the tight linkage of CAF-1 function to replication, a delay or defect in replication and their consequences, such as stalled replication forks and the generation of free or single-stranded DNA, may form the basis of the stimulatory effect. Replication defects may also form the basis for the additional cycle of endoreduplication induced in the mutant. A replication defect may cause a severe cell cycle delay that inhibits cell proliferation and consequently may prevent the normal establishment of tissues. The failure to establish normal tissue may have triggered endoreduplication as the driving force to increase cell size. Because cell size and endoreduplication are tightly linked in plants (Sugimoto-Shirasu and Roberts, 2003), such a mechanism to compensate for cell loss is quite conceivable. The additional cycle of endoreduplication might also be the direct cause of the increase in ICHR. However, endoreduplication is a common phenomenon in plants, and there is no precedent for such a mechanism. Nevertheless, mechanisms operating during the transition from mitosis to the endocycle that have yet to be detected can be envisioned.

The DNase I hypersensitivity of chromatin suggests a direct involvement of chromatin. Chromatin can exist in a closed or an open conformation, representing the repressed or active state of chromatin, respectively. Open chromatin contains hyperacetylated core histones and is hypersensitive to DNase I. This form is typical for chromatin with transcriptional competence and is believed to be enabled for transcriptional activation (Hebbes et al., 1994). Therefore, the open conformation can be considered the form of chromatin that is more accessible to proteins interacting with DNA, and DNase I sensitivity is believed to reflect such chromatin states. *fas1-4* could induce an open conformation in *Arabidopsis*. This conformation could facilitate the access of recombination proteins to DNA, thus circumventing the need for chromatin remodeling before repair. Therefore, the stimulation of HR could be the direct consequence of this change in conformation.

fas1-4 and *cac1*Δ (Prado et al., 2004) stimulate ICHR to significantly different extents in *Arabidopsis* and yeast, respectively. This difference is unlikely to be attributable to differences in the chromatin assembly complexes themselves, because the *Arabidopsis*, yeast, and human CAF-1 complexes have biochemically comparable activities, at least in vitro (Kaya et al., 2001). In addition, like *fas1-4* in *Arabidopsis*, the *cac1*Δ mutation in yeast (Adkins and Tyler, 2004) and dominant negative mutants or small interfering RNA transformants in human cells (Tchenio et al., 2001; Ye et al., 2003; Nabatiyan and Krude, 2004) cause DNase hypersensitivity of chromatin, indicating that all mutations cause similar chromatin defects. Therefore, a different function of CAF-1 in the reestablishment of chromatin in different organisms is also unlikely. In addition, the mild effect of the *cac1*Δ mutation on HR in yeast is unlikely to be attributable to the highly active HR present in yeast, because HR can be stimulated further in this organism (e.g., by partial depletion of histone H4 that increases ICHR by 20-fold) (Prado and Aguilera, 2005). Moreover, *asf-1* and *cac* mutations in yeast appear to predominantly stimulate GCRs through imprecise recombination pathways such as nonhomologous end joining (NHEJ) and have a much lesser effect on HR.

In particular, a *cac1*Δ mutation increases GCRs by >2 orders of magnitude (Myung et al., 2003), whereas HR is increased by only threefold (Prado et al., 2004). Because HR is stimulated by almost 2 orders of magnitude in *fas1-4* in *Arabidopsis*, yeast may differ from plants in the role of chromatin in the control of HR.

Linker histones such as the yeast HHO1p are involved in higher order structures that depend on chromatin condensation. HHO1p-dependent chromatin structures in yeast suppress HR but have no effect on NHEJ, mutants show no detectable phenotype, recombination gene expression is not altered, and global DNase sensitivity of chromatin is not affected (Downs et al., 2003). Partial depletion of histone H4 stimulates ICHR by >20-fold, has no effect on recombination gene expression, and does not change the sensitivity of chromatin to DNase (Prado and Aguilera, 2005). Because HR in yeast is stimulated in the

absence of detectable alterations of the chromatin structure but NHEJ is affected predominantly by mutations causing such alterations, it is likely that these two processes are controlled by different structures of chromatin, one affecting the accessibility of chromatin to DNase and the other not.

The *fas1-4* mutation in *Arabidopsis* causes global DNase hypersensitivity of chromatin together with a significant stimulation of HR. Therefore, in *Arabidopsis*, in contrast with yeast, the closed conformation of chromatin appears to be involved in the repression of HR. The cause of the difference between yeast and *Arabidopsis* is unknown, but chromatin in *Arabidopsis* could generally be more restrictive to recombination reactions and thus also contribute more significantly to the control of HR. In future work, it will be interesting to analyze whether or not other aspects of homologous recombination are affected in the mutant. The analysis of gene targeting, a tool urgently needed for precision engineering of plant genes, will be especially important.

METHODS

Identification and Characterization of *fas1-4* Plants

A population of *Arabidopsis thaliana* ecotype C24 plants was transformed with pAC106 (GenBank accession number AJ537513) by vacuum infiltration (Bechtold et al., 1993) and screened for mutants with visible phenotypes. The genomic DNA flanking the T-DNA insertion of *fas1-4* was amplified by inverse PCR using primers specific for the sulfonamide resistance gene of pAC106 and sequenced. Database searches using FASTA (GCG, Wisconsin Computer Group package) identified the tagged gene as the *Arabidopsis* homolog of the gene encoding the p150 subunit of CAF-1 (Kaya et al., 2001). A genomic fragment comprising the entire *FAS1* gene including the putative promoter region was amplified from Col-0 genomic DNA by long-template PCR (Roche Expand high fidelity kit) and inserted into pM001 (Reiss et al., 1994) after verification of the identity of the fragment by DNA sequencing. This construct was used to transform heterozygous *fas1-4* plants by vacuum infiltration. Transformants were selected on methotrexate, and individuals homozygous for *fas1-4* were identified by segregation analysis and DNA gel blotting. Complementation analysis was by visual inspection of the morphological phenotype.

Flow cytometric analysis of leaf nuclei was as described (Barow and Meister, 2003). For UV light sensitivity assays, seedlings were grown in sterile culture on nutrient agar medium on plates tilted into a vertical position as described (Dolan et al., 1993). After germination and initiation of the primary root, seedlings were covered with aluminum foil and the roots were irradiated with increasing UV_{254} doses (0, 250, 500, 750, and 1000 mJ [Stratalinker; Stratagene]) and returned to normal growth conditions. The increase in root length was measured after 3 d. Plates were returned to a horizontal position, and seedlings were grown until phenotypes were visible.

DNA methylation assays with the *Arabidopsis* 180-bp centromeric repeat were as described (Vongs et al., 1993).

Recombination Assays

Heterozygous *fas1-4* plants were selected on sulfadiazine and crossed to homozygous N1IC4 651 plants (Puchta et al., 1995) to result in *fas1-4*×N1IC4 651. Individuals carrying both markers were selected on sulfadiazine and hygromycin, and the seeds of these individuals were used to assay recombination. Seeds were sown on agar nutrient medium containing hygromycin, and seedlings were grown to the four-leaf stage. Homozygous *fas1-4* individuals were selected by their typical visual phenotype at this stage: narrow first true leaves. Seedlings were stained by whole plant staining as described (Puchta et al., 1995), and the number of spots was counted with a stereomicroscope.

Analysis of Recombination Gene Expression

Total RNA was extracted as described (Markmann-Mulisch et al., 1999), and cDNA was synthesized using Superscript II (Invitrogen) and random primers according to the manufacturer's instructions. Competitive RT-PCR was performed with the following gene-specific primers (5′-ACACTTCGAGTACTTGTTGCAACTG-3′ and 5′-GACGAAGAATTTCAATGGCTTTAAC-3′ for *MRE11*; 5′-CAGACCTTTAACCCTAATCGTCGGC-3′ and 5′-GAAGCCTTTTGCGTCAACTGAAATG-3′ for *RAD50*; and 5′-TGACATCAATACAAGTGAAGGG-3′ and 5′-CGCAAGTAGATGGTTCAGC-3′ for *RAD51*) and an *Arabidopsis* 18S rDNA primer and 18S rDNA competimer mixture as internal standard as described by the manufacturer (Ambion Quantum kit) after adjustment of the PCR to the linear range and the strength of the 18S rRNA signal to the strength of the gene-specific signal. PCR products were separated by agarose gel electrophoresis and stained with SYBR Green (Molecular Probes), and bands were quantified using a Kodak DC290 camera and Kodak 1D image analysis software.

Microscopic Analysis

Leaf discs were stained with DAPI, and nuclear structures were visualized by confocal microscopy as described (Maluszynska and Heslop-Harrison, 1991). For quantitative analysis of euchromatin and heterochromatin, nuclei were prepared, fixed on a slide, and stained with DAPI, and the staining intensity in chromocenters and the remaining nuclear area was determined using fluorescence microscopy (Soppe et al., 2002) and digital imaging (Kodak EDAS 290 and Kodak 1D analysis software). For the homolog pairing assays, *Arabidopsis* BACS F18C1 and MGL6 (GenBank accession numbers AC11620 and AB022217, respectively) were labeled and used for FISH on DAPI-stained 2C leaf nuclei as described (Pecinka et al., 2004). The surface area of ethanol-fixed, spread seedlings was determined after photography in the dark field by digital imaging (Image Pro Plus). Thin cross sections through leaves were prepared using standard techniques, fixed in acrolein, drained with ethanol, embedded in Technovit 7100 (Kulzer), and stained with 0.05% toluidine blue.

DNase I Sensitivity Assays

Nuclei were isolated from mature plants, an amount corresponding to 3 μg of DNA was digested with 0.05 units of DNase I (Roche) at 37°C for the times indicated (Figures 3A, 3C, and 3D), and the DNA extracted as described (van Blokland et al., 1997). For direct analysis, the DNA was separated by agarose gel electrophoresis and stained with ethidium bromide. For DNA gel blot analysis, nuclei prepared from Col-0, *fas1-4*×N1IC4 651, and N1IC4 651 plants were mixed before DNase I digestion as indicated and digested as described above. After extraction, the DNA was digested with *Eco*RI, and fragments were separated by agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized with radioactively labeled probes as described (Markmann-Mulisch et al., 2002).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: positions 86,483 to 86,820 in pARMS2, U93215; positions 126,620 to 127,220 in pARMS5, AL161584; positions 48,335 to 49,135 in pARMS7, AB011482; *FAS1* (AT1G65470.1), AB027228; *RAD51* (AT5G20850.1), U43528; *RAD50* (AT2G31970.1), AF168748; *MRE11* (AT5G54260.1), AJ243822; and *RAD51C* (AT2G45280.1), AB062456.

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