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Increased frequency of homologous recombination and T-DNA integration in *Arabidopsis* CAF-1 mutants

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Chromatin assembly factor 1 (CAF-1) is involved in nucleo some assembly following DNA replication and nucleotide excision repair. In *Arabidopsis thaliana*, the three CAF-1 subunits are encoded by *FAS1*, *FAS2* and, most likely, *MSI1*, respectively. In this study, we asked whether genomic stability is altered in *fas1* and *fas2* mutants that are lacking CAF-1 activity. Depletion of either subunit increased the frequency of somatic homologous recombination (HR) *in planta* ~40-fold. The frequency of transferred DNA (T-DNA) integration was also elevated. A delay in loading histones onto newly replicated or repaired DNA might make these DNA stretches more accessible, both to repair enzymes and to foreign DNA. Furthermore, *fas* mutants exhibited increased levels of DNA double-strand breaks, a G2-phase retardation that accelerates endoreduplication, and elevated levels of mRNAs coding for proteins involved in HR—all factors that could also contribute to upregulation of HR frequency in *fas* mutants.

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Introduction

In eukaryotic cells, the genomic DNA is highly compacted into chromatin through assembly with histone and nonhistone proteins. In proliferating cells, the bulk of the chromatin is assembled during DNA replication in the S phase of the cell cycle (Krude and Keller, 2001; Tyler, 2002; Verreault, 2003). Replication-specific nucleosome assembly is mediated by histone chaperones such as chromatin assembly factor 1 (CAF-1). CAF-1 was originally purified from nuclear extracts of human cells as a factor that supports the assembly of nucleosomes specifically onto replicating DNA *in vitro* (Smith and Stillman, 1989). CAF-1 mediates the first step of nucleosome assembly, that is, the deposition of H3/H4 histones onto replicating DNA (Smith and Stillman, 1989, 1991; Shibahara and Stillman, 1999; Tagami *et al*, 2004). CAF-1 is also involved in nucleosome assembly after nucleotide excision repair (NER; Ridgeway and Almouzni, 2000). CAF-1 is evolutionarily conserved, and homologs have been described in yeast, insects, plants, and vertebrates. In yeast, CAC1, CAC2, and CAC3 are counterparts of the p150, p60, and p48 subunits of human CAF-1. Despite the important role played by CAF-1 in nucleosome assembly during DNA synthesis, yeast *cac* mutants did not yield a lethal phenotype. However, increased UV sensitivity (Kaufman *et al*, 1997; Game and Kaufman, 1999), impaired gene silencing at telomeres (Monson *et al*, 1997; Enomoto and Berman, 1998) and at mating loci (Enomoto *et al*, 1997), and gross chromosomal rearrangements (Myung *et al*, 2003) were reported in such mutants. In higher eukaryotes, on the other hand, evidence for a more essential *in vivo* function of CAF-1 is accumulating. Ectopic expression of a dominant-negative form of the p150 subunit of CAF-1 caused severe early developmental defects in *Xenopus laevis* (Quivy *et al*, 2001), and induced S-phase arrest, accompanied by DNA damage and S-phase checkpoint activation, in human cells (Ye *et al*, 2003). Knockdown of the p60 subunit of CAF-1 by RNAi in human cells led to induction of cell death in proliferating, but not in quiescent, cells (Nabatiyan and Krude, 2004).

In *Arabidopsis thaliana*, the CAF-1 subunits corresponding to the human subunits p150, p60, and p48 are encoded by *FAS1*, *FAS2*, and, most likely, *MSI1*, respectively (Kaya *et al*, 2001; Henning *et al*, 2003). *fas1* and *fas2* mutants of *Arabidopsis* were originally described as mutations causing stem fasciation, and abnormal phyllotaxy, leaf shape, root growth, and flower organ number (Reinholz, 1966; Leyser and Furner, 1992). These mutants display severely disturbed cellular and functional organization of both shoot apical meristem (SAM) and root apical meristem (RAM). They also show a varied pattern of distorted expression of both *WUSCHEL* and *SCARECROW*, which play key roles in the organization of SAM and RAM, respectively (Kaya *et al*, 2001). Thus, CAF-1 appears to be important for the maintenance of plant developmental gene expression patterns. Other than these alterations in postembryonic development, these mutants are viable.

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We speculated that delayed assembly of histones, expected as a consequence of a lack of CAF-1 activity, might lead to enhanced genomic instability in *fas* mutants. In this study, we used two different assays to measure DNA instability in a chromatin context: somatic homologous recombination (HR) and integration of the transferred DNA (T-DNA) of *Agrobacterium tumefaciens*. HR is a process that is used for precise DNA repair in somatic plant cells, whereas in meiosis it is used to generate novel distribution of genetic material between maternal and paternal chromosomes (Schuermann *et al*, 2005). T-DNA is a widely used tool for genetic engineering and plant insertional mutagenesis (Galbiati *et al*, 2000). Although we currently have very limited knowledge of the roles played by plant genes and proteins during this process, T-DNA integration is considered to use a nonhomologous end-joining (NHEJ)-related mechanism (Zupan *et al*, 2000; van Attikum *et al*, 2001; Friesner and Britt, 2003). Analysis of flanking sequence tags (FSTs) revealed that integration events are progressively less frequently observed towards the centromere (Brunaud *et al*, 2002), and also that about 40% of integration events occur in genes. These data suggested that chromatin structure can prevent T-DNA integration.

In this study, we detected an ~40-fold increase in the frequency of HR, as well as increased T-DNA integration, in *fas* mutants. To aid further understanding of these findings, we analyzed the transcription of DNA repair genes, the generation of DNA double-strand breaks (DSBs), and cell cycle progression in *fas* mutants. The results presented here suggested that delayed chromatin assembly could lead to prolonged exposure of not yet chromatinized DNA to enzymes capable of repairing DNA by either HR or NHEJ in plants. In addition, induced DNA DSBs and enhanced transcription of genes involved in HR at S phase could stimulate HR.

Results

The frequency of HR is strongly elevated in fas mutants

We used an HR repair assay that allows recombination events to be visualized and scored by histochemical staining for a reconstituted recombination substrate locus (Swoboda *et al*, 1994; Schuermann *et al*, 2005). The constructs used as substrates for HR contain parts of the β-glucuronidase (GUS) gene in direct or inverted orientation (Figure 1A), which can recombine to reconstitute a functional *GUS* gene. *fas1-2* (ecotype Nossen) and *fas2-1* (ecotype Landsberg *erecta* (Ler)) plants were crossed to *Arabidopsis* lines (ecotype Columbia (Col)) carrying such recombination substrates, and F3 progeny plants were monitored for lines homozygous for the recombination reporter construct as well as for the *fas1* and *fas2* mutation. Mutants in either *fas1* or *fas2* resulted in around 40-fold more GUS recombination spots than in wild-type plants (Figure 1B–E), independent of the relative orientation of the truncated recombination target sequences, and regardless of which CAF-1 subunit was mutated. To confirm that the difference in HR frequency was not due simply to the heterogeneous genetic background of the parent plants, we analyzed HR frequency of FAS2 RNAi knockdown plants as well as a T-DNA tagging mutant of FAS2 (*fas2-4*), both of which are in a Col background. Both these plant lines also showed enhanced HR frequency compared to wild-type Columbia (Supplementary Figure S1).

T-DNA integration is enhanced in fas mutants

The mechanism of *in vivo* integration of T-DNA into plant DNA represents a special case of a NHEJ process. NHEJ is the main pathway used by higher eukaryotic organisms to repair DSBs in DNA. This repair mechanism is usually accomplished with concomitant changes at the junction sequence, and is thus error prone (Lees-Miller and Meek, 2003).

The efficiency of T-DNA integration can be assessed using a root tumorigenesis assay (Nam *et al*, 1999). Root transformation with *Agrobacterium* A208 results in large green tumors on the roots. Indeed, increased numbers of tumors were observed on roots of *fas1* and *fas2* mutants infected with *Agrobacterium* compared to roots of the respective wild-type ecotypes (Figure 1F and G). When we analyzed transient expression of GFP following *Agrobacterium*-mediated root transformation, no difference in GFP expression between wild-type and *fas* mutants was observed (data not shown), suggesting that CAF-1 depletion does not increase T-DNA transmission from *Agrobacterium* to plant nuclei.

It is interesting to note that ecotypes naturally more refractory to T-DNA integration, such as Ler and, especially, Nossen, reacted more strongly to the mutation than Col, which is already very sensitive to T-DNA integration in the wild-type context.

Enhanced transcription of genes involved in HR in fas mutants

The results of the two experiments described above could be linked to defects in nucleosome assembly according to various, not necessarily mutually exclusive, scenarios: (i) the expression of repair enzymes is upregulated, (ii) there is an increased level of breaks in the DNA that can be repaired by HR and NHEJ activities, and (iii) repair enzymes, due to a lack, or delayed assembly, of nucleosomes, could have easier access to breaks in DNA in need of repair.

To test the hypothesis that an increased level of transcription of repair enzymes might contribute to enhanced rates of HR and T-DNA integration, we measured the transcription levels of several such genes by real-time PCR. As shown in Figure 2A, *AtRAD51*, which plays a central role in HR repair (Doutriaux *et al*, 1998), was found to be upregulated 4–5-fold in *fas* mutants. Transcription of *AtRAD54* (a homolog of the yeast *RAD54* gene, K Osakabe *et al*, 2006) was slightly induced in the mutants, whereas transcription of genes commonly grouped in the NHEJ pathway (*AtKU70*, *AtKU80*, and *AtLIG IV*) was unchanged, as was transcription of *AtLIG I*, which encodes a DNA replication factor. The results of Western blot analysis also indicated that the amount of AtKu70 protein remained constant in *fas* mutants (Supplementary Figure S2).

Interestingly, the same genes that were transcriptionally upregulated in the *fas* mutants (*AtRAD51* and *AtRAD54*; Figure 2A) were also found to have a higher steady-state level of transcription in wild-type *Arabidopsis* plants exposed to γ-irradiation (Figure 2B).

Increased level of DNA DSBs in fas mutants

The experiments described above suggested the increased presence of DNA DSBs in *fas* mutants. To directly monitor the extent of DSBs in the DNA of wild-type and *fas* mutants, we attempted to quantify DNA DSBs by comet assay (Menke *et al*, 2001), which can indicate DNA damage in individual

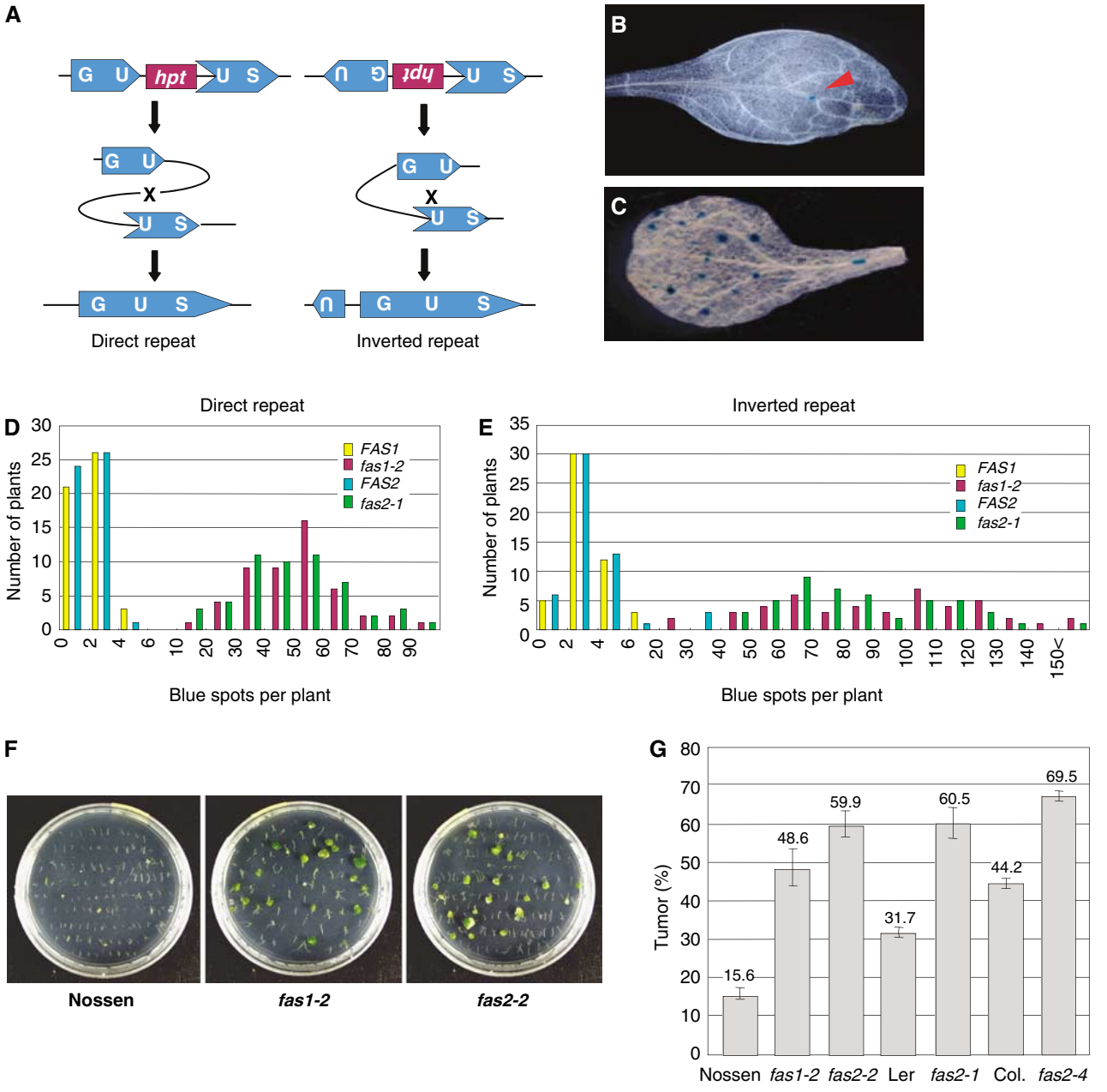

Figure 1 Genomic flexibility, as measured by intrachromosomal HR and T-DNA integration, is increased in *fas* mutants. (**A**) Recombination marker constructs. The β-*glucuronidase* (*GUS*; *uidA*) sequences have an overlap (indicated by 'U') either in the direct (left) or inverted (right) orientation. Recombination (indicated by 'x') between the two overlapping sequences produces a functional *GUS* gene. (B, C) Visualization of recombination events by histochemical GUS staining of leaves from a *FAS1* control (**B**), and a *fas1-2* plant homozygous for the inverted repeat-type recombination reporter (*GU-US/GU-US*) (**C**). An arrowhead in (B) indicates GUS-positive cells. (D, E) Frequency distribution histograms showing the proportions of plants with a given number of blue GUS spots in the direct repeat (**D**) and inverted repeat (**E**) populations. (F, G) Mutants *fas1-2* and *fas2-2* (ecotype Nossen), *fas2-1* (ecotype Ler), and *fas2-4* (ecotype Col), and the corresponding wild-type plants were inoculated with *A. tumefaciens*. (**F**) Plates showing growth of roots and tumors of Nossen, and its mutants *fas1-2* and *fas2-2*, photographed 1 month after infection. (**G**) Efficiency of T-DNA integration as represented by the percentage of root segments that produced tumors. Error bars indicate standard error (s.e.). Data (means ± s.e.) were taken from 10 plants of each type.

cells. A schematic representation of a comet assay is shown in Figure 2F. The amount of DNA in the comet tail separated from intact nuclear DNA in an electric field correlates with the number of breaks in the nuclear DNA (Menke *et al*, 2001). Figures 2C–E show typical comet assays of wild-type *Arabidopsis* nuclei with different amounts of DNA damage. As shown in Figure 2G, a small but significant increase in the number of DSBs was observed in *fas* mutants compared to wild-type plants. Severely damaged and fragmented nuclei (Figure 2E) were found more frequently in *fas* mutants (12.8 and 9.1% of the total number of cells counted in *fas1-2* and *fas2-2*) than in wild-type (1.7% of total cells counted) but such nuclei were not included in the statistical analysis. Therefore, the relative level of DNA DSBs in *fas* mutants should be greater than that shown in Figure 2G.

One of the earliest known responses to DSB induction is the phosphorylation of thousands of molecules of the histone

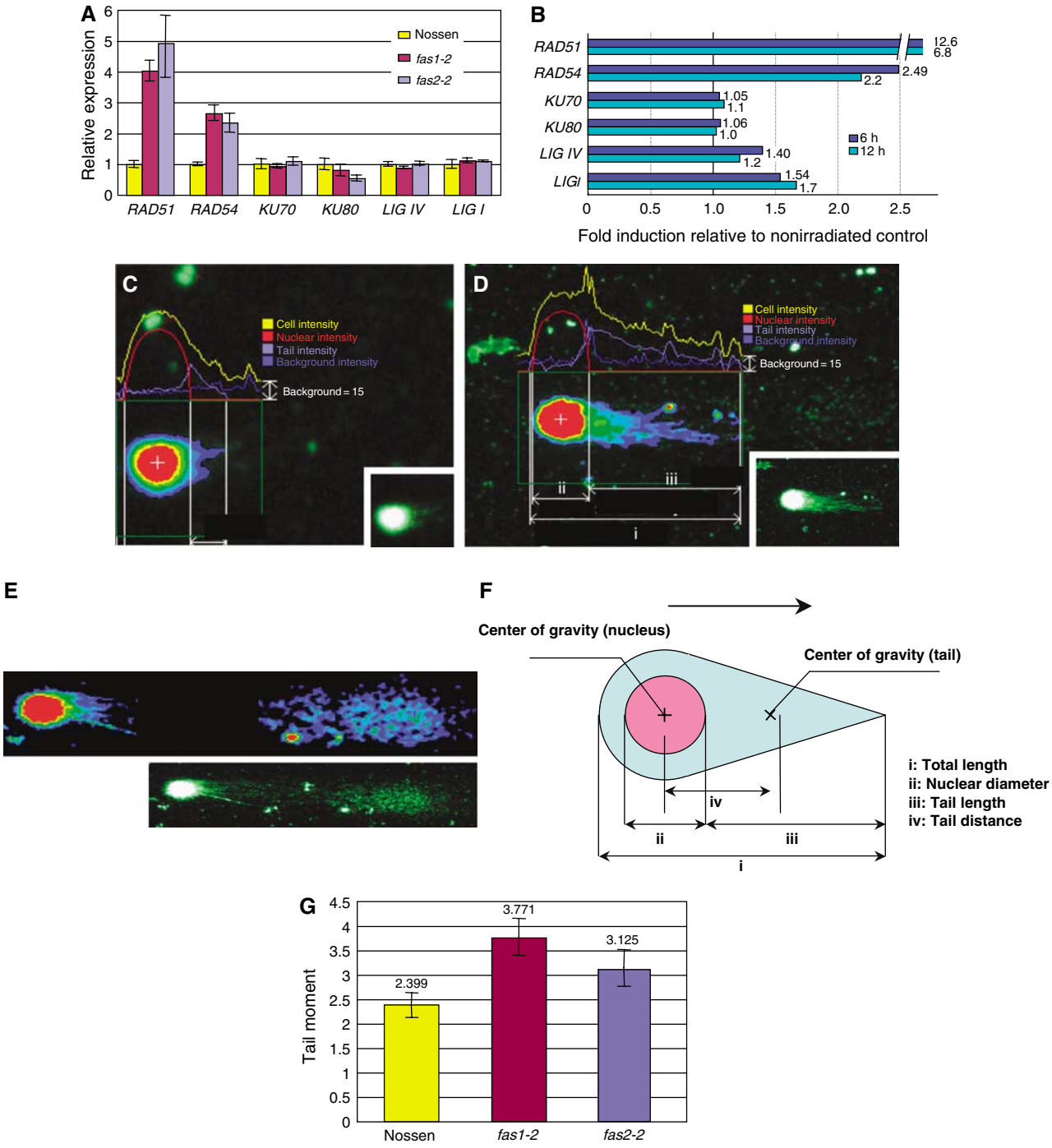

Figure 2 Elevated transcription of HR repair genes, and increased DNA damage in *fas* mutants. (**A**) Transcript levels of the repair genes indicated in *fas1-2* and *fas2-2* mutants relative to wild-type Nossen, as determined by real-time quantitative PCR. Error bars indicate standard error (s.e.). (**B**) Transcript levels of the repair genes indicated at 6 and 12 h after γ-irradiation in wild-type Nossen as determined by microarray analysis. (C–E) Comet images of intact and fragmented nuclear DNA from wild-type Nossen. (**C**) An almost intact nucleus (short tail). (**D**) A damaged nucleus (long tail). (**E**) A severely damaged nucleus (long fragmented tail). Original images are shown as white colored comets in the right corner panels of (C) and (D), and in the lower panel of (E). DNA intensity is indicated by gradation of color. (**F**) Schematic representation of a comet. (**G**) Statistical analysis of a comet assay. The level of DNA DSBs in the nucleus is represented as the tail moment, defined as the product of comet tail length and the fraction of total DNA in the tail (see Materials and methods). Error bars indicate the s.e. values of analyzed cells.

variant H2AX at the site of the break (Rogakou *et al*, 1998). In *Arabidopsis*, induction of phosphorylated H2AX (known as γ-H2AX) in an irradiation-dose-dependent manner, and its subsequent disappearance through DNA DSB repair have been demonstrated (Friesner *et al*, 2005). Quantification of γ-H2AX in wild-type and *fas* mutants by Western blot analysis revealed a small induction of γ-H2AX in *fas* mutants compared to wild-type (1.3-fold in *fas1-2* and 2.1-fold in *fas2-2*) (Supplementary Figure S3), thus supporting the comet assay data.

fas mutants show increased sensitivity to DNA-damaging treatments

fas mutants were expected to be more sensitive to DNA-damaging stresses compared to wild-type plants due to the intrinsically higher level of DNA DSBs. Thus, we next investigated the γ-ray sensitivity of *fas1* and *fas2* mutants. As shown in Figures 3A–C, root growth of *Arabidopsis* seedlings was inhibited by γ-irradiation in a dose-dependent manner. This inhibition was greater in *fas* mutants than in wild-type plants, regardless of ecotype. We also assessed the development of true leaves in γ-irradiated plants (Figure 3D). *fas1* and *fas2* mutants exhibited increased yellowing of the cotyledons, resulting in death, following 600 Gy irradiation. In contrast, true leaves from wild-type plants did emerge. In *fas1-2*, 60, 20, 20, 10, and 0% of plants produced true leaves after 200, 300, 400, 500, and 600 Gy of γ-irradiation. In *fas2-2*, 70, 60, 50, 30, and 20% of plants produced true leaves after 200, 300, 400, 500, and 600 Gy of γ-irradiation. Under our experimental conditions, all plants could produce true leaves after 200–600 Gy of γ-irradiation in wild-type Nossen. Similarly, *fas1* and *fas2* mutants are more sensitive to UV-C irradiation than wild-type plants (see Supplementary Figure S4).

Cell cycle regulation in fas mutants

An essential step in the completion of S phase of the cell cycle is the reassembly of histone onto newly replicated DNA. CAF-1 is involved in this process. To analyze the effect of CAF-1 depletion on cell cycle progression, we analyzed the proportion of cells in each phase of the cell cycle by flow cytometry. Nuclei from the true leaves of 9-day-old seedlings showed an increase in 4C and a decrease in 2C cells in *fas1-2* and *fas2-2* mutants (Figure 4A and B). These results suggest an increased frequency of nuclei in G2/M phase in *fas* mutants.

To further investigate cell cycle progression in *fas* mutants, we analyzed transcription of cyclin genes using microarrays (Figure 4C). In this assay, transcription of the mitotic cyclin *AtCYCB1;1* (At4g37490, B-type cyclin gene) was drastically

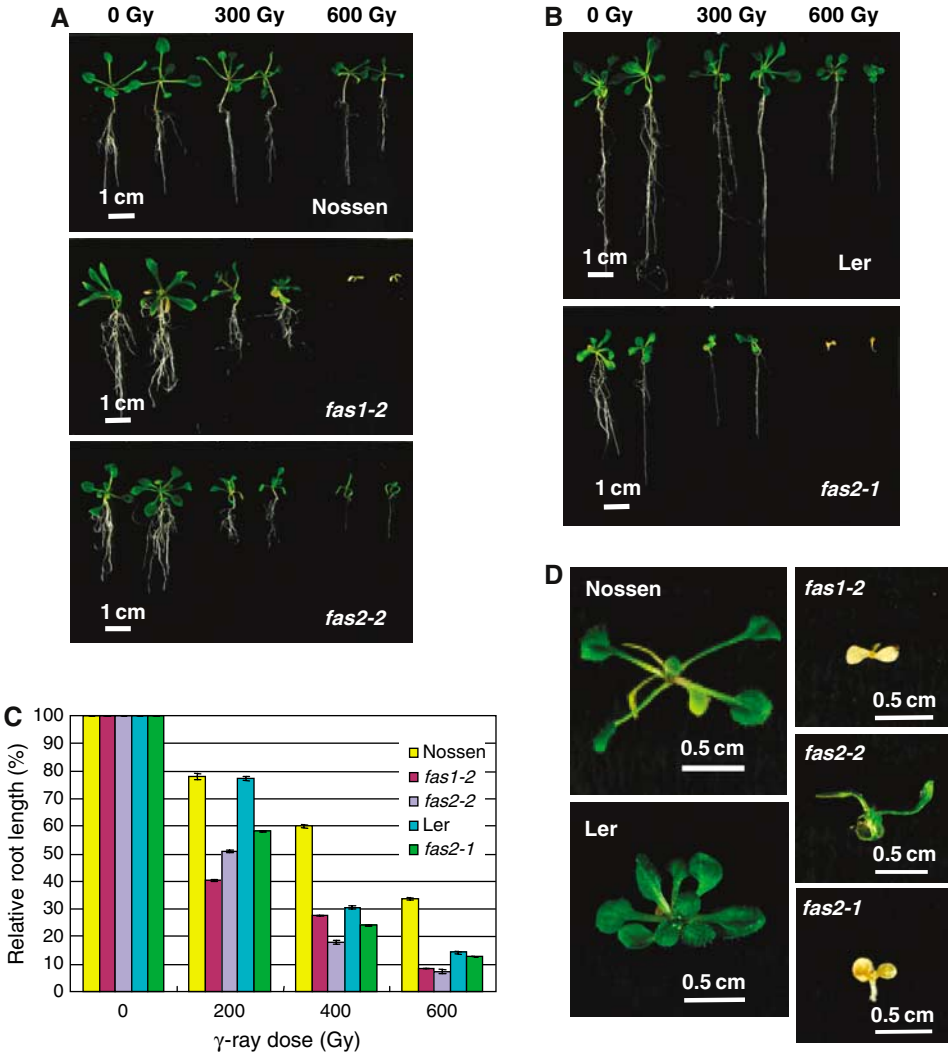

Figure 3 *fas* mutants show increased sensitivity to DNA-damaging treatments. (A, B) Phenotype of seedlings following exposure to the doses of γ-irradiation is indicated. (**A**) Wild-type Nossen (top), Nossen background mutants *fas1-2* (middle) and *fas2-2* (bottom). (**B**) Wild-type Ler (top) and Ler background mutant *fas2-1* (bottom). (**C**) Relative root growth of *fas1-2*, *fas2-2*, and *fas2-1* mutants and wild-type plants after exposure to the doses of γ-irradiation indicated. The average root length of nonirradiated plants in each case was taken as 100%. Error bars indicate s.e. Data are means ± s.e., and represent the results of three independent experiments. (**D**) Development of true leaves of *fas1-2*, *fas2-2*, and *fas2-1* mutants and the corresponding wild-type plants after γ-irradiation (600 Gy).

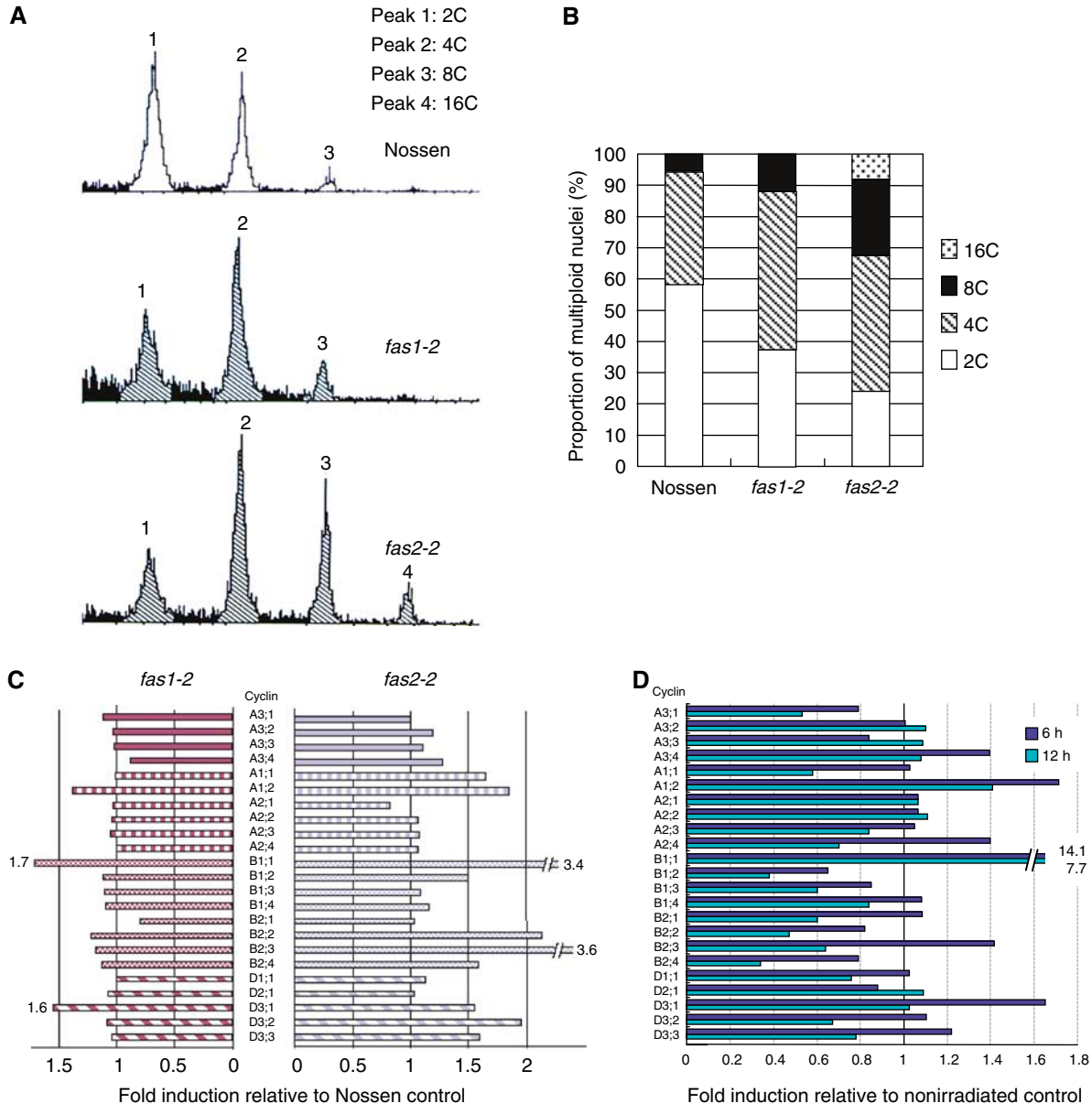

Figure 4 Aberrant cell cycle regulation in *fas* mutants. (**A**) Flow cytometric analysis of true leaves in 9-day-old Nossen, and Nossen background *fas1-2* and *fas2-2* mutants. (**B**) Calculated proportion of multiploid cells in 9-day-old Nossen, and Nossen background *fas1-2* and *fas2-2* mutants. (**C**) Transcription of cyclin genes in *fas1-2* and *fas2-2* mutants as determined by microarray analysis. (**D**) Transcript levels of cyclin genes 6 and 12 h after γ-irradiation in wild-type Nossen as determined by microarray analysis.

increased in *fas* mutants. The *Arabidopsis* mitotic cyclin CYCB1;1 product is reported to accumulate only around the time of the G2/M transition (Doerner *et al*, 1996; Shaul *et al*, 1996). Interestingly, expression of this cyclin was also strongly induced by γ-irradiation (Figure 4D), suggesting the crucial role of a G2 retardation for DNA DSB repair following γ-irradiation.

To confirm the results of microarray analysis and to further investigate the effects of CAF-1 depletion on cell cycle regulation, especially tissue-specific effects, we examined the expression of *AtCYCB1;1*::*GUS*. Mitotic cyclin turnover requires a short peptide motif known as the 'destruction box' (King *et al*, 1996). Transcriptional and post-translational regulation together restricts the accumulation of mitotic cyclins to G2 and M phase of the cell cycle. *Arabidopsis* Col plants transformed with *AtCYCB1;1*::*GUS* (Colon-Carmona *et al*, 1999) were crossed with *fas* mutants. Figure 5 shows an example of *AtCYCB1;1*::*GUS* expression in *fas2-4* and wild-type Col. As shown in Figures 5A–C, cells expressing GUS activity appeared sporadically only in the root tip of wild-type Col. In contrast, significantly large numbers of cells in the stems, flower buds, leaves, and root tips of the *fas2-4* mutant showed strong GUS activity (Figure 5D–F). Enhanced expression of *AtCYCB1;1*::*GUS* was also detected regardless of ecotype and of which CAF-1 subunit was mutated (Supplementary Figure S5).

Discussion

Hyper-recombination of genomic DNA in fas mutants

In the present study, mutants in either *fas1* or *fas2* exhibited around 40-fold more recombination events than observed in

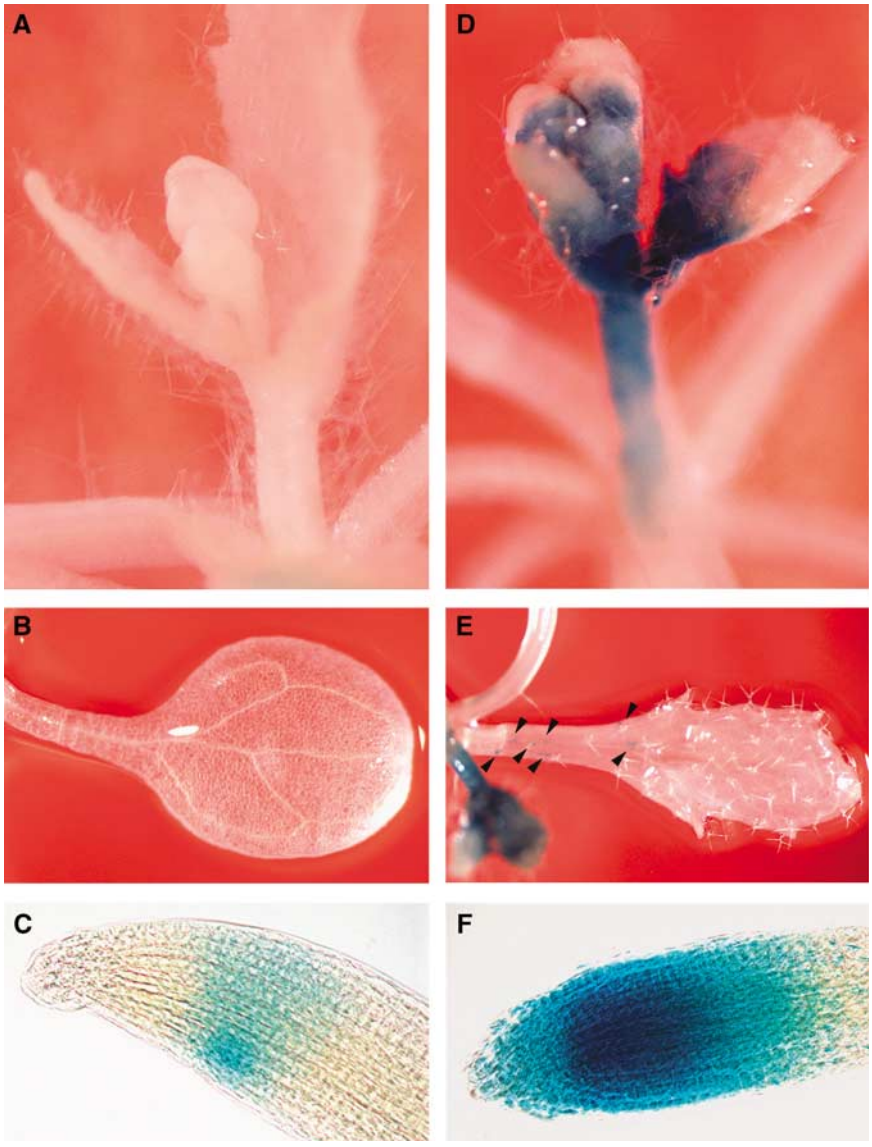

Figure 5 Histochemical assay of the *AtCYCB1;1*::*GUS* reporter. GUS staining patterns of Col (**A–C**) and Col background *fas2-4* mutant (**D–F**) are shown. (A, D) Close-up of flower buds. (B, E) GUS staining of true leaves. Arrowheads in (E) indicate GUS-positive cells in a *fas2-4* leaf. (C, F) Close-up of root tips.

wild-type plants (Figure 1A–E). We interpret this to mean that it is the complete CAF-1 complex, and not any one individual subunit, that maintains HR at a low level in wild-type plants. In this context, stimulated intrachromosomal recombination was reported in a yeast *cac1* mutant (Prado *et al*, 2004), but in this case the increase was only 2–3-fold. These differences could be connected to the fact that HR efficiency in yeast is already high under normal conditions. In vertebrate systems, histone H3 comes in two major forms, histone H3.1 and histone H3.3. The former is loaded onto replicated DNA via CAF-1, whereas H3.3 replaces H3.1 in a postreplicative pathway via HIRA (Tagami *et al*, 2004). Interestingly, yeast has only the H3.3 version of histone H3 (Ahmad and Henikoff, 2002), hence CAF-1 might not be essential in yeast. The relative importance of CAF-1 between plants and yeast could thus result in a difference in the ratio of HR enhancement. It will be of interest to investigate HR in vertebrates with a similar assay.

CAF-1 was reported to be involved in the maintenance of epigenetic state in yeast (Monson *et al*, 1997; Enomoto and Berman, 1998). Therefore, the increased number of GUS spots observed in *fas* mutants could be explained by the release of transcriptional gene silencing (TGS) of the *GUS* gene. However, based on the following arguments, we judge the increased number of GUS spots observed in *fas* mutants to be due mainly to hyper recombination of the recombination substrate: we tested two different loci for indication of the hyper-recombination phenotype and both showed the same behavior. A hygromycin-resistance gene (*hpt*) was located between the two disrupted (but partially overlapping) *GUS* gene fragments (see Figure 1A) and the plants used in this assay showed stable hygromycin resistance. Hence, silencing of the *GUS* locus is unlikely. Furthermore, recently it has been reported that TGS of a silent *GUS* transgene was partially, but not totally de-repressed in *fas* mutants (Ono *et al*, 2006). These data thus concur with our prediction that *fas* mutants indeed exhibit an increased level of genome

instability, as measured by intrachromosomal HR. A mutation in the yeast linker histone *HHO1* led to an increased frequency of HR (Downs *et al*, 2003); partial depletion of histone H4 gave a similar phenotype (Prado and Aguilera, 2005). As CAF-1 assembles histones H3 and H4 following DNA replication (Smith and Stillman, 1989; Shibahara and Stillman, 1999; Tagami *et al*, 2004), depletion of CAF-1 and partial loss of histone H4 could enhance HR by similar mechanisms.

Increased accessibility of the T-DNA/protein complex to genomic DNA in fas mutants

Using a root tumorigenesis assay, we detected increased T-DNA integration in *fas* mutants (Figure 1F and G). As T-DNA integration occurs mainly through non-HR mechanisms, it is most likely that NHEJ proteins are required for the process of T-DNA integration. In fact, *ku80*-mutant *Arabidopsis* plants are defective in T-DNA integration in somatic cells, whereas Ku80-overexpressing plants exhibit increased susceptibility to *Agrobacterium* infection (Li *et al*, 2005). However, real-time PCR analysis failed to detect increased transcription of NHEJ pathway genes (*AtKU70*, *AtKU80*, and *AtLIG IV)* in *fas* mutants (Figure 2A). Furthermore, the protein level of Ku70 was unchanged in wild-type and *fas* mutants (Supplementary Figure S2). Therefore, the increased levels of T-DNA integration observed in *fas* mutants might reflect an increased level of genome instability rather than enhanced expression of NHEJ pathway genes.

The root tumorigenesis assay has been successfully used for the isolation of mutants resistant to *Agrobacterium*-mediated transformation in a population of T-DNA-mutagenized *Arabidopsis* plants (Mysore *et al*, 2000; Zhu *et al*, 2003). In this assay, loss-of-function mutants of histone H2A, histone deacetylases, histone acetyl transferase, and other chromatin-modifying proteins showed resistance to *Agrobacterium*-mediated transformation. These results indirectly suggest the importance of chromatin structure in T-DNA integration.

Taken together, the results of enhanced HR and T-DNA integration in *fas* mutants suggest that the condensed chromatin state in wild-type plants could maintain HR and T-DNA integration at a low level by preventing easy access of HR repair proteins and T-DNA to genomic DNA. CAF-1 is known to be involved in nucleosome assembly (Verreault, 2000; Krude and Keller, 2001; Mello and Almouzni, 2001). We postulated that rapid nucleosome formation would be disturbed, at least locally, in the absence of CAF-1. This would leave replicated DNA naked and easily accessible for a longer period of time. Recently, Schonrock *et al* (2006) reported reduced heterochromatin in *fas* mutants. Furthermore, Costa and Shaw (2006) showed, via three-dimensional fluorescence *in situ* hybridization on intact root epidermal tissue, that most nuclei of root epidermal cells in *fas2* mutants were in an open chromatin state.

Factors enhancing HR in fas mutants

In addition to the enhanced accessibility of HR repair proteins to the site of DNA damage, various other scenarios can be envisaged to explain the increased frequency of HR in *fas* mutants. We found enhanced transcription of the HR genes *AtRAD51* and *AtRAD54* as well as the presence of increased numbers of DNA DSBs in *fas* mutants (Figure 2A and G). Schonrock *et al* (2006) also reported increased expression of several HR-related genes including *AtRAD51* in *fas* mutants. In our experiment, the same set of genes that was transcriptionally upregulated in *fas* mutants was also found to have a higher steady-state level of transcription in wild-type *Arabidopsis* plants exposed to γ-irradiation, which induces DNA DSBs (Figure 2B). These results are consistent with the increased level of DNA DSBs observed in *fas* mutants (Figure 2G).

In fact, a dominant-negative human p150 mutant also induced S-phase arrest, accompanied by increased DNA damage (Ye *et al*, 2003). On the other hand, a robust transcriptional induction of HR genes has never been reported, even after γ-irradiation, in yeast and vertebrates (Haaf *et al*, 1995; Mercier *et al*, 2001). This fact suggests that if there is any correlation between the enhanced HR observed in yeast and induction of HR factors, it might involve translational or post-translational events. The enhanced γ-ray (Figure 3A–D) and UV-C sensitivity (Supplementary Figure S4) observed in *fas* mutants might also be due to intrinsic DSBs. Besides intrinsically induced DNA DSBs, a defect in NER repair activity in *fas* mutants could also contribute to this UV-sensitivity phenotype.

Furthermore, a small increment of phosphorylated H2AX (γ-H2AX) was also detected in *fas* mutants (Supplementary Figure S3). As induction of γ-H2AX following ionising radiation exposure—with protein levels increasing with irradiation dose—has been reported in *Arabidopsis* (Friesner *et al*, 2005), the increased amount of γ-H2AX found in *fas* mutants indicates an increased level of DSBs. Considering the function of CAF-1, which is involved in nucleosome assembly following DNA replication, increased DSBs in *fas* mutants were induced in the small proportion of the cells that are dividing. In mammals, γ-H2AX is induced in an ATM-dependent manner in response to DSBs, whereas γ-H2AX is induced in an *ATR*-dependent manner in situations where DNA synthesis is blocked. Interestingly, in *Arabidopsis*, both ATM and ATR contribute to ionizing radiation-induced γ-H2AX formation (Friesner *et al*, 2005). It will be of interest to investigate the roles of ATM and/or ATR on the enhanced induction of γ-H2AX in *fas* mutants.

As both enhanced expression of HR genes and induction of DNA DSBs have been reported to be involved in upregulation of HR (Reiss *et al*, 1996, 2000; Shalev *et al*, 1999; Molinier *et al*, 2005), the elevated levels of HR gene expression found in *fas* mutants could be explained by these factors. Therefore, the small increase in the number of DNA DSBs together with the relatively low enhancement of HR gene expression can partially explain the elevated levels of HR in *fas* mutants.

Flow cytometric analysis showed an increased proportion of 4C nuclei in *fas* mutants (Figure 4A and B). Our microarray analysis showed an increased transcription level of *AtCYCB1;1*, expressed only around the time of G2/M in *fas* mutants and in γ-irradiated wild-type plants (Figure 4C and D). Histochemical analysis of plants transformed with an *AtCYCB1;1::GUS* construct confirmed that a large number of cells remain at the pre-M phase in proliferating cells of *fas* mutants compared to wild-type plants (Figure 5A–F, Supplementary Figure S5). Schonrock *et al* (2006) also showed enhanced expression of *AtCYCB1;1::GUS* in *fas* mutants. These results indicate that the enhanced expression of *AtCYCB1;1::GUS* observed in *fas* mutants was probably

the result of a G2 retardation. Furthermore, γ-irradiation-induced G2 arrest was also reported in *Arabidopsis* using an *AtCYCB1;1*::*GUS* reporter assay (Culligan *et al*, 2004).

The reported ploidy results could mean either that cells spend more time in G2 or that endoreduplication is accelerated. According to the expression data of mitotic cyclin, AtCYCB1;1, a severe delay in the cell cycle at the postreplicative (G2) phase in *fas* mutants might contribute to the ploidy results.

Several recent reports hint at the direct or indirect mediation of DNA repair by cell cycle regulators. It was reported that transcription and expression of cyclin A1 in mice was induced by γ-irradiation, and that cyclin A1 and A2 enhance DNA DSB repair by HR (Müller-Tidow *et al*, 2004). Moreover, a direct involvement of cyclin-dependent kinase (CDK) in HR has been reported. Esashi *et al* (2005) reported CDK-dependent phosphorylation of BRCA2, which interacts directly with the essential recombination protein Rad51 in cultured human cells. An involvement of CDK in the recruitment of Rad51 to the site of DNA DSBs has also been shown in *Saccharomyces cerevisiae* (Aylon *et al*, 2004; Ira *et al*, 2004). Further analysis of cell cycle regulation in *fas* mutants will reveal the cross-talk of cell cycle progression and regulation of HR in plants.

In the vertebrate system, CAF-1 depletion induces DNA DSBs at S phase, resulting in S-phase arrest and cell death (Ye *et al*, 2003; Nabatiyan and Krude, 2004). In contrast, *fas* mutants grow to maturity. This means that DNA DSBs generated in *fas* mutants, most probably during S phase, must be repaired before M phase. This fact also supports the presence of a G2 retardation in *fas* mutants. G2 retardation might make the chromatin structure of *fas* mutants relatively open, allowing enhanced HR and increased T-DNA integration. Interestingly, transcription of the *AtRAD51* gene has been reported to be upregulated not only by DNA damage stress but also during S phase of the cell cycle (Doutriaux *et al*, 1998). Furthermore, it has been reported that DNA DSBs induced during late S–G2 phase are preferentially repaired by HR in vertebrate and yeast (Reski, 1998; Dronkert *et al*, 2000).

Summarizing the above discussion, in *fas* mutants, delayed chromatin assembly at S phase could lead to prolonged exposure of not yet chromatinized DNA to enzymes capable of repairing DNA by HR. In addition, induction of DNA DSBs and enhanced transcription of genes involved in HR might occur during S phase and stimulate HR in *fas* mutants. In this communication, we have discussed the effects of CAF-1 depletion in plants on nucleosome assembly following DNA replication. However, CAF-1 is also known to be involved in coupling of nucleosome assembly to NER (Ridgeway and Almouzni, 2000). With respect to the connection between NER and HR in *Arabidopsis*, a T-DNA-mediated knockout of the *Centrin2* gene involved in the NER pathway led to a hyper-recombinogenic mutant (Molinier *et al*, 2004). Thus, a direct or indirect involvement of the NER pathway in the upregulation of HR and T-DNA integration remains to be investigated.

A delay in, or downregulation of, chromatin assembly seems to enable improved recruitment of repair enzymes to sites of damage as well as easy access of T-DNA to the plant genomic DNA. These studies thus demonstrate that nucleosome structures maintain recombination repair activities at a low level, necessitating chromatin remodelling functions such as that proposed for the AtINO80 protein involved in controlling HR (Fritsch *et al*, 2004). Also in yeast, remodelling and decompaction of chromatin is essential for efficient DNA repair (van Attikum *et al*, 2004; Morrison *et al*, 2004; Prado *et al*, 2004; Tsukuda *et al*, 2005).

During submission of our manuscript, Kirik *et al* (2006) reported enhanced intrachromosomal HR and increased ploidy level in a C24 background *fas1* mutant, *fas1-4*. However, in contrast to our data and those of Schonrock *et al* (2006), nearly normal transcription levels (1.5-fold) of *AtRAD51* were observed in *fas1-4*. This difference might be attributed to different experimental conditions, differences in the mutants, or differences in the ecotypes used.

Materials and methods

Plants

fas1-2 (Kaya *et al*, 2001) and *fas2-2* (Kaya *et al*, 2001) from ecotype Nossen, *fas2-1* (Leyser and Furner, 1992) from ecotype Landsberg *erecta* (Ler), and *fas2-4* (H Kaya and T Araki, unpublished data) from ecotype Columbia (Col) were used in this study. *fas2-4* was found as SALK_033228 in the searchable database of T-DNA insertion sequences established by the Salk Institute Genome Analysis Laboratory. *fas2-4* also shows some morphological changes seen in other *fas* mutants. The position of insertion of the T-DNA in *fas2-4* is shown in Supplementary Figure S1 (A).

Recombination assays

Recombination reporter lines (Gherbi *et al*, 2001) 1406 (direct repeat-type line), 1415 (inverted repeat-type line); ecotype Col were used in this study. The two GUS reporter lines were crossed with *fas1-2* (ecotype Nossen) and *fas2-1* (ecotype Ler), respectively. F3 seeds from plants homozygous for the GUS recombination reporter and homozygous for the mutant *fas1-2* (*GU-US/GU-US*, *fas1-2/fas1-2*) or *fas2-1* (*GU-US/GU-US*, *fas2-1/fas2-1*) allele were stained for GUS activity. Plants homozygous for the GUS recombination reporter and either homozygous or heterozygous for *FAS1* (*GU-US/GU-US*, *FAS1/FAS1* or *FAS1/fas1-2*) or *FAS2* (*GU-US/GU-US*, *FAS2/FAS2* or *FAS2/fas2-1*) were used as controls. Histochemical GUS staining was performed as described previously (Schuermann *et al*, 2005). The number of spots, each indicating a recombination event, on each seedling was then determined visually under a dissecting microscope. For each line, 50 seedlings were analyzed. Initial visual identification of *fas1-2/fas1-2* and *fas2-1/fas2-1* homozygote seedlings was based on the fact that they had unusual shoots (Kaya *et al*, 2001). The *FAS1* and *FAS2* genotypes of individual plants were then confirmed by DNA sequencing.

T-DNA integration assay

For the root tumorigenesis assay, *A. tumefaciens* A208 (Sciaky *et al*, 1978) was grown at 28°C in YEB medium supplemented with 10 mg/l Rifampicin. *A. thaliana* seeds were surface sterilized and germinated on solidified MS medium. Roots of 3-week-old plants were cut and infected with *A. tumefaciens* as previously described (Nam *et al*, 1999). After 2 days co-cultivation, root segments were transferred to solidified MS medium containing 100 mg/l Timentin to remove agrobacteria. The number of tumors was counted 1 month after infection. The number of root segments analyzed is as follows: Nossen, 551; *fas1-2*, 715; *fas2-2*, 833; Ler, 631; *fas2-1*, 718; Col., 1115; *fas2-4*, 857. We repeated this experiment more than three times. Methods of transient GFP expression are described in Supplementary Materials and methods.

RNA isolation

Total RNA prepared from 4-week-old seedlings (without the roots, i.e. true leaves with cotyledon and hypocotyls) using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used for microarray and real-time PCR analysis. Four-week-old sterile seedlings of wild-type plants (ecotype Nossen) were irradiated with 100 Gy of γ-rays at a dose rate of 300 Gy/h. At 6 and 12 h after irradiation, samples were frozen in liquid nitrogen and immediately ground for total RNA isolation (RNeasy Plant Mini Kit).

Real-time PCR
Reverse transcription–PCR was performed as described previously (Osakabe *et al*, 2002). Detailed condition of real-time PCR and primer sequences used in this experiment are described in Supplementary Materials and methods.

Microarray analysis
An Arabidopsis 2 Oligo Microarray kit (Agilent Technology, CA, USA) was used in this study. Detailed conditions for these experiments are described in Supplementary Materials and methods.

Comet assay
Twelve-day-old seedlings (as defined above) were used for the comet assay. The experimental conditions used were described previously as N/N protocol (Menke *et al*, 2001). A CCD camera was used to capture images of SYBR green-stained comets. Signal quantification was performed using Comet analyzer Software (YOUWORKS, Japan) under conditions excluding severely damaged nuclei. Intensity of DNA is shown in graded colors. DNA DSBs are represented as the 'tail moment' (Olive *et al*, 1990), which is defined as tail distance × (sum of tail intensity/sum of cell intensity). For individual parameters, see Figure 2D and F. We analyzed 40–70 nuclei/line and the experiment was repeated at least three times.

γ-Ray sensitivity tests
Relative root growth after γ-irradiation was analyzed as described by Harlow *et al* (1994) with minor modifications. Detailed condition for γ-ray sensitivity tests are described in Supplementary Materials and methods.

More than 20 plants each were used in the DNA damaging, γ-ray sensitivity, UV sensitivity, and comet assays. These experiments were repeated at least three times.

Flow cytometric analysis
True leaves of 9-day-old seedlings were chopped in an extraction buffer (Galbraith *et al*, 1983) with 2.5 mg/ml DAPI. The filtered nuclei (filter, 30 μm) were subjected to flow analysis with laser excitation at 357 nm.

Histochemical assay of AtCYCB1;1::GUS reporter
Arabidopsis Col plants transformed with *AtCYCB1;1::GUS* (Colon-Carmona *et al*, 1999) were crossed with *fas* mutants and wild-type plants of corresponding ecotypes. Sterile 4-week-old plants containing the *AtCYCB1;1::GUS* reporter gene and homozygous for the *fas1* and *fas2* mutation (*fas1/fas1* and *fas2/fas2*) and either homozygous or heterozygous for *FAS1* (*FAS1/FAS1* or *FAS1/fas1*), or *FAS2* (*FAS2/FAS2* or *FAS2/fas2*), were used for histochemical GUS staining.

Accession number
The microarray data comparing wild-type plants versus *fas* mutants and nonirradiated wild-type plants versus γ-irradiated wild-type plants are available at GEO (http://www.ncbi.nlm.nih.gov/geo/) with the accession numbers GSM112793, GSM112794, GSM112815, and GSM112816.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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