E2F Regulates FASCIATA1, a Chromatin Assembly Gene Whose Loss Switches on the Endocycle and Activates Gene Expression by Changing the Epigenetic Status\(^1\)[C][W][OA]

Elena Ramirez-Parra and Crisanto Gutierrez*

Centro de Biología Molecular “Severo Ochoa,” Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

Maintenance of genome integrity depends on histone chaperone-mediated chromatin reorganization. DNA replication-associated nucleosome deposition relies on chromatin assembly factor-1 (CAF-1). Depletion of CAF-1 in human cells leads to cell death, whereas in Arabidopsis (Arabidopsis thaliana), where it is involved in heterochromatin compaction and homologous recombination, plants are viable. The mechanism that makes the lack of CAF-1 activity compatible with development is not known. Here, we show that the FASCIATA1 (FAS1) gene, which encodes the CAF-1 large subunit, is a target of E2F transcription factors. Mutational studies demonstrate that one of the two E2F binding sites in its promoter has an activator role, whereas the other has a repressor function. Loss of FAS1 results in reduced type A cyclin-dependent kinase activity, inhibits mitotic progression, and promotes a precocious and systemic switch to the endocycle program. Selective up-regulation of the expression of a subset of genes, including those involved in activation of the G2 DNA damage checkpoint, also occurs upon FAS1 loss. This activation is not the result of a global change in chromatin structure, but depends on selective epigenetic changes in histone acetylation and methylation within a small region in their promoters. This suggests that correct chromatin assembly during the S-phase is required to prevent unscheduled changes in the epigenetic marks of target genes. Interestingly, activation of the endocycle switch as well as introduction of activating histone marks in the same set of G2 checkpoint genes are detected upon treatment of wild-type plants with DNA-damaging treatments. Our results are consistent with a model in which defects in chromatin assembly during the S-phase and DNA damage signaling share part of a pathway, which ultimately leads to mitotic arrest and triggers the endocycle program. Together, this might be a bypass mechanism that makes development compatible with cell division arrest induced by DNA damage stress.

Chromatin is the functional template for a variety of key biological processes, such as DNA replication, repair of DNA damage, recombination, and transcription. Chromatin reconstitution involves the association of histone complexes with DNA to form nucleosomes, a step that depends on two major chaperone pathways (Polo and Almouzni, 2006). One is DNA replication independent and relies on the histone gene repressor (HIRA) chaperone and another relies on chromatin assembly factor-1 (CAF-1), which is tightly associated with DNA synthesis events, either semiconservative DNA replication or DNA repair synthesis. In both cases, histones are transferred to these chaperones by the antirepressing factor 1 (ASF1).

The CAF-1 chaperone targets acetylated histone H3/H4 onto newly synthesized DNA, thus allowing de novo assembly of nucleosomes (Smith and Stillman, 1989; Polo and Almouzni, 2006). CAF-1 is a heterotrimeric complex that has been highly conserved during evolution. In yeast, where it is known as chromatin assembly complex (CAC), it consists of Cac1, Cac2, and Cac3 subunits (Haushalter and Kadonaga, 2003; Polo and Almouzni, 2006), whereas in mammalian cells these correspond to p150, p60, and p48 (Smith and Stillman, 1989; Kaufman et al., 1995; Verreault et al., 1996). In Arabidopsis (Arabidopsis thaliana), the three subunits are encoded by the FASCIATA1 (FAS1), FAS2, and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) genes, respectively (Leyser and Furner, 1992; Kaya et al., 2001; Henning et al., 2003).

Yeast cac mutants have underassembled chromatin (Adkins et al., 2004) and are defective in maintaining gene silencing at telomeres and at the mating-type locus (Monson et al., 1997; Enomoto and Berman, 1998). In addition, they show increased genome instability
(Kolodner et al., 2002; Myung and Kolodner, 2003) and sensitivity to double-strand breaks (DSBs), but not to DNA replication stress, dying at the metaphase. This demonstrates that CAF-1 in yeast plays an essential role in DNA repair, in both homologous recombination (HR) and nonhomologous end-joining (NHEJ) pathways, but not in cell cycle progression (Linger and Tyler, 2005).

In multicellular eukaryotes, the physiological relevance of CAF-1 seems to be different. In human cells, CAF-1 defects inhibit nucleosome assembly and activate the S-phase checkpoint, inducing S-phase arrest and causing cell death (Hoek and Stillman, 2003; Ye et al., 2003; Nabatiyan and Krude, 2004; Nabatiyan et al., 2006), suggesting that CAF-1 is essential for cell cycle progression. In Arabidopsis, fas1 and fas2 mutants were isolated in a screening for recessive mutations leading to a distorted meristem structure (Leyser and Furner, 1992). These mutant plants carry loss-of-function mutations in the p150 (FAS1) and p60 (FAS2) subunits of CAF-1, respectively. Their phenotype is visible both in the shoot apical meristem and the root apical meristem, where expression of marker genes such as WUSCHEL (WUS) and SCARECROW (SCR), respectively, is disturbed (Kaya et al., 2001). Whole-genome transcriptomic analysis has revealed that, in spite of pleiotropic developmental defects, only a relatively small amount (<2%) of genes are transcriptionally deregulated in Arabidopsis CAF-1 mutants (Schönrock et al., 2006). However, among those with altered expression, a large proportion of genes belonging to the DNA repair functional category are present (Schönrock et al., 2006), suggesting a functional link of CAF-1 and DNA damage responses. Recently, FAS1, the large subunit of CAF-1, has been implicated in HR in Arabidopsis (Endo et al., 2006; Kirik et al., 2006). CAF-1 has also been implicated in cell differentiation during plant development (Exner et al., 2006) and in complete compaction of heterochromatin, but not in maintenance of the transcriptional repression of heterochromatic genes (Schönrock et al., 2006). In spite of these abnormalities, Arabidopsis CAF-1 mutants are fully viable.

The mechanism that confers viability to CAF-1-deficient Arabidopsis plants, a unique situation in multicellular organisms, is not known. Therefore, it is important to identify the mechanisms that regulate the availability of CAF-1 subunits. We have found that FAS1 is a target of the E2F transcription factor and that the E2F binding sites in the FAS1 promoter play distinct activator and repressor roles. It has recently been proposed that Arabidopsis lacking CAF-1 might have an extended S-phase, but eventually they manage to divide (Schönrock et al., 2006). We have also observed that loss of CAF-1 leads to a reduction in type A cyclin-dependent kinase (CDKA) activity and inhibition of cell division, consistent with approximately a 3.5-fold reduction in cell number in leaves. However, this is compensated by a switch of the endocycle program that occurs shortly after germination and is maintained systemically. Loss of FAS1 is accompanied by up-regulation of the expression of a subset of genes, including RAD51, PARP1, and BRCA1, involved in the G2 DNA damage checkpoint, as well as CYCB1;1, but not Ku70. In all these cases, this is the consequence of selective epigenetic changes in histone H3 acetylation and methylation in their promoters and not of global changes in chromatin remodeling. A similar response was observed in wild-type plants treated with DNA-damaging agents. Together, our data lead us to propose that defects in chromatin assembly during the S-phase and DNA damage signaling share part of the same pathway through changing the epigenetic status of target genes. This response might be a bypass pathway that makes development compatible with cell division arrest induced by DNA damage stress.

RESULTS

FAS1 Is an E2F Target in Vivo

The FAS1 gene presents a cell cycle-regulated expression pattern with an expression peak in the S-phase (Kaya et al., 2001). In addition, a role for FAS1 in the cell cycle has been proposed, although not experimentally demonstrated (Endo et al., 2006; Exner et al., 2006). Members of the E2F/DP family of transcription factors are among the major regulators controlling the expression of cell cycle genes (Gutierrez, 2005; Inzé and De Veylder, 2006). Thus, we looked for the presence of consensus E2F binding sites in the putative promoter region of FAS1. Analysis revealed the presence of two optimal consensus E2F binding sites, TTTGGGCGC (at −345 from the putative ATG) and TTTCCCGCCAAG (at −290), both in reverse orientation, although the second presents a double palindromic site (Fig. 1A). To determine whether these E2F sites can mediate E2F binding, we used electrophoretic mobility shift assay (EMSA) using a 175-bp-long fragment of the FAS1 promoter that contained the E2F binding sequences (Fig. 1B). Recombinant Arabidopsis E2F/HDEL3 protein bound to this probe in a specific and E2F site-dependent manner, as indicated by the competition assay with oligonucleotides containing E2F consensus sites or a mutated version of the consensus. Moreover, both E2F sites (E2F1 and E2F2) seem to be functional in vitro using EMSA assays with purified Arabidopsis E2F/HDEL3 and oligonucleotides containing each E2F site independently.

To address whether FAS1 behaves as a direct E2F target in vivo, we used a chromatin immunoprecipitation (ChIP) approach, taking advantage of the plants that express a hemagglutinin (HA)-tagged version of E2F/HDEL3. Specific immunoprecipitation was carried with an anti-HA antibody, while an anti-Myc antibody was used as a control IgG. ACTIN2 (ACT2), a non-E2F-regulated gene, was used as a control. As shown in Figure 1D (right), ACT2 promoter sequences that do
not contain E2F consensus binding sites were not recovered from the immunoprecipitates with either anti-HA or anti-Myc antibodies. However, promoter fragments of the \( FAS1 \) gene that contain E2F binding sites were specifically amplified from the anti-HA immunoprecipitates of \( E2Ff^{OE} \) extracts. This indicates that \( E2Ff/DEL3 \) can bind directly to the \( FAS1 \) promoter in vivo, likely participating in the regulation of its expression.

To investigate further the regulation of \( FAS1 \) expression by E2F, we used real-time reverse transcription (RT)-PCR analysis to determine the \( FAS1 \) mRNA levels in a set of Arabidopsis plants with altered E2F expression (Fig. 1E). We observed a drastic increase in \( FAS1 \) expression in plants that overexpress the activators \( E2Fa \) and \( E2Fb \) (DeVeylder et al., 2002; Sozzani et al., 2006) and a 3- to 4-fold decrease in \( FAS1 \) expression in plants overexpressing the repressor \( E2Fc^{OE} \) (del Pozo et al., 2002). Furthermore, a dominant negative version of DP, the E2F heterodimeric partner (Ramirez-Parra et al., 2003), also showed decreased \( FAS1 \) expression (Fig. 1E). In addition, \( FAS1 \) expression was also changed in plants with altered levels of the atypical E2F that do not need DP for their activity. Thus, \( FAS1 \) expression was found to be increased in plants that overexpress \( E2Fd/DEL2 \) (M.A. Lopez-Matas, E. Ramirez-Parra, and C. Gutierrez, unpublished data) and \( E2Ff/DEL3 \) (Ramirez-Parra et al., 2004) or that are deficient for \( E2Fe/DEL1 \) (\( e2fe \) plants; Fig. 1E). The opposite effect was observed in plants deficient for \( E2Fd/DEL2 \) (\( e2fd \) plants) or \( E2Ff/DEL3 \) (\( e2ff \) plants; Ramirez-Parra et al., 2004), and in \( E2Fe/DEL1^{OE} \) plants (Vlieghe et al., 2005; Fig. 1E). All these data together suggest complex regulation of \( FAS1 \) gene in vivo by different activator and repressor E2F family members. Thus, it seems that different E2Fs may regulate \( FAS1 \) expression in a concerted and/or tissue-specific manner during development.

Each E2F Binding Site Has a Distinct Role in Regulating \( FAS1 \) Expression

Regulation of the \( FAS1 \) promoter was analyzed in detail using transgenic plants expressing the \( GUS \) (\( uidA \)) reporter gene under the \( FAS1 \) promoter (\( pFAS1^{wt} \); Fig. 2A). At least three independent transgenic lines that consistently gave comparable GUS activity levels (Fig. 2B) were selected for further analysis. In 4-d-old seedlings, we observed high levels of expression in cotyledons, the shoot apical region, and the primary root meristem (Fig. 2, C–E). In 10-d-old seedlings, \( FAS1 \) expression has disappeared in cotyledons, whereas leaf primordia and young leaves showed strong expression (Fig. 2F). These results indicate that \( FAS1 \) expression is controlled by different E2F family members in a tissue-specific manner during development.

RT-PCR. Measurements were normalized to the amount of \( ACT2 \) and then the \( FAS1 \) values made relative to the amount present in wild-type plants. Asterisks indicate the statistical significance of the differences with wild type, applying Student’s t test. **, \( P \leq 0.005 \); *, \( P \leq 0.01 \). [See online article for color version of this figure.]

---

**Figure 1.** E2F-regulated expression of the \( FAS1 \) gene. A, Scheme of the \( FAS1 \) promoter showing the presence of two E2F binding sites at \( -290 \) and \( -345 \) bp from the ATG translation start codon. B, EMSA with recombinant E2F protein (100 ng) and a fragment of the promoter containing the two E2F sites as a probe (bracket). Lane 1, Free probe; lane 2, complex of E2F to the promoter (arrow) is specifically competed out with a 100-fold molar excess of unlabeled oligonucleotide containing a consensus E2F site (lane 3), but not with a mutated oligonucleotide (lane 4). C, EMSA of recombinant E2F protein with oligonucleotide probes containing the sites E2F1 (lanes 1 and 2) and E2F2 (lanes 3 and 4). Specific binding is competed out with the corresponding unlabeled oligonucleotide probes (lanes 2 and 4). D, ChIP analysis of 12-d-old Arabidopsis seedlings using anti-HA antibodies with wild-type or HA-E2F^{OE} plants. Anti-Myc was used as a negative control IgG. Immunoprecipitated genomic DNA was quantified using real-time PCR with primers specific for the indicated promoters using 40 cycles of amplification. The \( ACT2 \) promoter was used as a negative control IgG. E, Expression of the \( FAS1 \) gene in 12-d-old seedlings with altered expression of the six different Arabidopsis E2F or overexpressing a truncated version of DP, analyzed by real-time
expression occurs in proliferative tissues, confirming previous experiments (Kaya et al., 2001). In addition, we found that FAS1 expression disappeared in mature leaves, where it was restricted to the hydatodes at the leaf margin (Fig. 2G). In flowers, FAS1 expression was mainly detected in developing anthers (Fig. 2H). In contrast, mature flowers did not show detectable reporter gene activity, as was also the case in siliques (data not shown). Lateral root primordia and meristems also showed GUS activity (Fig. 2I).

To define the role of the E2F binding sites in FAS1 gene expression, we mutated the E2F binding sequences (Fig. 2A). Thus, mutated versions of the promoter in each E2F binding site (pFAS1-Mut1 and pFAS1-Mut2) or both (pFAS1-Mut12) were fused to the uidA gene. Quantification of GUS activity in transgenic plants carrying the wild type or each of the mutated promoters described in A. Measurements were carried in triplicate using three independent lines. The background level was considered to be that of nontransgenic plants (Col-0). C to I. Histochcmical localization of GUS activity in 4-d-old wild-type whole seedlings (C), details of the shoot (D), the root (E) apical regions, the shoot apical region of 10-d-old seedlings (F), a mature leaf (G), and flowers (H) and lateral roots (I) at different stages of development. J to U. Histochcmical localization of GUS activity in plants carrying the three mutated FAS1 promoters, as indicated. We show here details of the shoot (J, N, R) and root (K, O, S) apical regions of 4-d-old seedlings, the shoot apical region of 10-d-old seedlings (L, P, T), and flowers at different developmental stages (M, Q, U) because these are the locations showing differences in the mutated promoters.

Figure 2. Relevance of E2F sites on expression pattern of FAS1 during Arabidopsis development. A, Scheme of FAS1 promoter constructs containing the two wild-type E2F sites (pFAS1-wt) and point mutations in each of them (pFAS1-Mut1 and pFAS1-Mut2) or both (pFAS1-Mut12) fused to the uidA gene. B, Quantification of GUS activity in transgenic plants carrying the wild type or each of the mutated promoters described in A. Measurements were carried in triplicate using three independent lines. The background level was considered to be that of nontransgenic plants (Col-0). C to I, Histochcmical localization of GUS activity in 4-d-old wild-type whole seedlings (C), details of the shoot (D), the root (E) apical regions, the shoot apical region of 10-d-old seedlings (F), a mature leaf (G), and flowers (H) and lateral roots (I) at different stages of development. J to U, Histochemical localization of GUS activity in plants carrying the three mutated FAS1 promoters, as indicated. We show here details of the shoot (J, N, R) and root (K, O, S) apical regions of 4-d-old seedlings, the shoot apical region of 10-d-old seedlings (L, P, T), and flowers at different developmental stages (M, Q, U) because these are the locations showing differences in the mutated promoters.

mutation of the E2F2 site (pFAS1-Mut2) significantly increased GUS activity (Fig. 2B). Mutation of both sites led to GUS activity levels similar to those of plants carrying the wild-type FAS1 promoter (Fig. 2B). These data suggest that E2F1 functions as an activator, whereas E2F2 functions as a repressor. This set of reporter constructs also allowed us to evaluate the individual contribution of each site to the spatial regulation of FAS1 expression by E2F sites. pFAS1-Mut1 plants, bearing a mutation in the first E2F binding site, showed a drastic decrease in the promoter activity of meristematic and young tissues (Fig. 2, J–L), as well as in developing anthers (Fig. 2M). In contrast, mutation of the second E2F site (pFAS1-Mut2) produced an increase in reporter gene expression (Fig. 2, N–Q). These data confirm that E2F1 is an activator site, but E2F2 is a repressor site. The promoter containing mutations in both E2F binding sites (pFAS1-Mut12) recovered almost wild-type levels of GUS activity (Fig. 2, R–U). Together, our analysis indicates that E2F sites
contribute to regulate the level of FAS1 expression and their elimination did not largely modify the spatial pattern of FAS1 expression.

Loss of CAF-1 Inhibits Cell Division

To define the relevance of CAF-1 during plant growth and development, we decided to analyze the effect of a loss of function of FAS1, which encodes the CAF-1 large subunit. To this end, we chose the fas1-4 allele (SAUL_662_D10), a T-DNA insertion within the sixth intron, in Columbia (Col-0) background (Fig. 3A). This allele has been used recently in developmental studies (Exner et al., 2006) and is useful for comparison purposes with other alleles identified in the En and C24 ecotypes. In our conditions, homozygous fas1-4 plants showed <3% of the full-length FAS1 mRNA levels and expressed a truncated product that lacks the domains required for interaction with proliferating cell nuclear antigen (PCNA) and other CAF subunits (data not shown). Arabidopsis fas1-4 plants showed phenotypes similar to those previously described for other fas1 alleles (Leyser and Furner, 1992; Kaya et al., 2001; Exner et al., 2006), such as reduced growth, altered floral organ structure, and reduced fertility associated with smaller siliques, among others (Supplemental Fig. S1). However, they are not as dwarfed as recently described for a fas1 mutation in the C24 ecotype (Kirik et al., 2006).

The fas1-4 mutant leaves are dentate, smaller, and narrower than the wild type (Fig. 3A). Microscopic visualization of the adaxial leaf epidermis revealed the presence of very large cells in mature fas1-4 leaves (Fig. 3B). It has recently been reported that the fas1-4 mutation produces an increase in cell size in the leaf epidermis and that mutant leaves do not reveal any obvious alteration of the internal histology (Exner et al., 2006). Thus, we wanted to analyze other aspects of leaf development that were not addressed previously. First, we determined whether this cell size phenotype was present in the different leaf cell layers. As shown in Figure 3B, fas1-4 leaves contained larger cells in both epidermal cell layers as well as in the mesophyll. Then, taking advantage of the coordination of cell proliferation and differentiation (Beemster et al., 2005), we asked when during leaf development this cell size phenotype appeared. Thus, we carried out a kinematic study in leaves 1 and 2 by measuring leaf and cell sizes and estimating the cell number at various developmental stages spanning from the leaf primordial stage (9 d after sowing) through leaf maturity (35 d after sowing). This analysis clearly demonstrated that the fas1-4 adaxial leaf epidermis contained approximately 3.5-fold fewer, but approximately 2-fold larger, cells than the wild type (Fig. 3C).

The presence of larger cells in plants is frequently, although not always, associated with an increase in the nuclear ploidy produced by successive rounds of endoreplication (Kondorosi et al., 2000; Larkins et al., 2001; Sugimoto-Shirasu and Roberts 2003). A characteristic feature of leaf development is that a developmentally regulated switch from cell division to the endocycle occurs (Boudolf et al., 2004; Castellano et al., 2005). Thus, we carried out a detailed analysis of ploidy levels during leaf development. We found that the switch to the endocycle program is altered in fas1-4 plants. Kinematic analysis revealed that it occurred early during leaf development (Fig. 3D). Moreover, similar behavior was found for fas1-1 leaves (En ecotype; data not shown). A rough correlation between trichome nuclear ploidy and branch number has also been observed (Hülskamp et al., 1999; Castellano et al., 2004; Desvoyes et al., 2006). We observed that approximately 45% of fas1-4 trichomes developed more than four branches, in clear contrast to the wild-type situation, where approximately 3% of trichomes contain more than three branches (Supplemental Fig. S2). This branching phenotype correlates well with the size of 4,6-diamino-phenylindole (DAPI)-stained trichome nuclei (Supplemental Fig. S2), strongly suggesting that fas1-4 leaf trichomes have developed an extended endoreplication program. Flow cytometry measurements in other organs indicated that an increased ploidy phenotype was consistently observed in cotyledons and even flowers, which do not normally have a significant proportion of >4C nuclei, whereas it is less apparent in roots (Supplemental Fig. S2). Therefore, we conclude that loss of FAS1 leads to a systemic increase in the endoreplication level.

Therefore, one important question was to establish when during plant development stimulation of the endocycle switch occurs. Thus, we analyzed early stages of embryo development and confirmed that a significant fraction of fas1-4 seeds aborted, but those that survived contain embryos with a rather normal appearance (Supplemental Fig. S1). Upon imbibition of the dry seed, the developing embryo undergoes a fast proliferative stage and then, approximately 24 to 36 h after germination, a fraction of cells initiates their endocycle program (Masubelele et al., 2005). The switch to the endocycle program was also advanced in the young fas1-4 seedlings, although in this case the effect was less pronounced than during leaf development (Fig. 3E).

CDKA Activity Is Reduced in CAF-1-Deficient Plants

Together, our data, as well as those of others, point to significant inhibition of cell division in fas1-4 plants not only during leaf development, but also in all organs and, interestingly, since very early after germination. A good indication of alterations in cell cycle progression could be derived from measurements of CDKA activity. We found that extracts of fas1-4 seedlings have a significant reduction in total CDKA activity measured as kinase activity of p13suc1-bound material on histone H1 as substrate (Fig. 4A). However, the total amount of CDKA revealed with an anti-PSTAIRE antibody was similar both in wild-type and fas1-4
extracts (Fig. 4A). These results are consistent with a decrease of cell proliferation activity in the mutant.

To investigate the cell division phenotype further, we determined by real-time RT-PCR the mRNA levels of a series of cell cycle marker genes (Menges et al., 2005). We found that expression of CYCD2;1 and CYCD3;1, or CYCA2;1 and CYCA3;1, typical of the S-phase, did not show significant changes in 14-d-old fas1-4 leaves as was also the case with CDKB1;1 and KNOLLE typical markers of G2 and M, respectively (Supplemental Fig. S3). However, a subset of genes active in the S-phase, such as histone H4, histone H3.1a, and histone H3.1b, or CYCB1;1, a G2/M marker, were up-regulated in the mutant fas1-4 (Col-0) plants (Supplemental Fig. S3), in agreement with the microarray data of fas1-1 (En) and fas2-1 (Landsberg erecta

Figure 3. Effects of loss of FAS1 on cell division, cell size, and endoreplication. A, Scheme of the FAS1 locus showing the position of the T-DNA insertion site in the fas1-4 allele (SAIL 662_D10; Col-0 ecotype). Gray boxes represent the exons. Rosette leaves of 35-d-old wild-type and fas1-4 mutant plants. B, Cell size analysis of the adaxial, mesophyll, and abaxial leaf epidermal layers of wild-type and fas1-4 mutants (21 d after sowing; leaves 1 and 2) analyzed by phase-contrast microscopy (left and middle). Bar corresponds to 50 μm. Cell size (log μm²) distributions in each layer are shown on the right. Note that, in all cases, cells of fas1-4 leaves are larger than the wild type. C, Kinematic analysis of leaf (1 and 2) development from the young proliferating stage (day 9) until maturity (day 35). Average leaf blade size (mm²) and average cell size (μm²) are given in the top and middle images, respectively (bars are the SD in each case). Measurements were carried out in the central region of at least seven leaves in each case (n ≥ 500 cells). The estimated cell number in the adaxial epidermis per leaf at the indicated developmental time is presented in the bottom image. D, Ploidy distribution of wild-type and fas1-4 leaf 1 and 2 nuclei at the indicated developmental times. Average values of each nuclear DNA content class (± SD) are presented. Asterisks denote the statistical significance of the differences between wild type and fas1-4. * P ≤ 0.01; ** P ≤ 0.005. E, Ploidy distributions of wild-type and fas1-4 seedlings at early stages after germination (0–4 d after germination [dag]). Quantification and statistical analysis was described in D. [See online article for color version of this figure.]
Loss of FAS1 Provokes Removal of Epigenetic Silencing Marks of Transcribed Genes

Cytological studies revealed that Arabidopsis CAF-1 mutants have reduced heterochromatin content, indicating that CAF-1 is required for heterochromatin formation. Nevertheless, DNA methylation of pericentromeric repeats was normal, indicating that CAF-1 is not required for maintenance of DNA methylation (Schönrock et al., 2006). Immunolocalization experiments have shown that dimethylation of the K9 residue of histone H3 (H3K9me2), a typical mark of heterochromatin in Arabidopsis (Fischer et al., 2006; Franz et al., 2006; Fuchs et al., 2006), remains enriched in heterochromatic chromosome centers of fas1 nuclei (Kirik et al., 2006; Schönrock et al., 2006). These data have been used in support of the notion that chromatin in fas1 mutants presents an open conformation.

We first determined whether fas1-4 plants contain global changes in the amount of acetylated histones H3 and H4, and of H3K9me2. Western blotting with the corresponding antibodies demonstrated that this was not the case (Fig. 5A). Then we investigated by ChIP experiments whether local changes in these epigenetic modifications could explain the changes in the expression of a subset of genes in fas1-4 plants. We found that the promoters of genes up-regulated in fas1-4 plants, such as histone H4, CYCB1;1, RAD51, and BRCA1, but not Ku70, whose expression is not altered, were enriched in both acetylated histones H3 and H4 and deprived of H3K9me2 (Fig. 5, B and C). In these experiments, the ACT2 gene, whose expression did not change either in fas1-4 plants, was used as a control. Therefore, these data suggest that epigenetic modifications typical of actively transcribed, euchromatic genes are introduced constitutively as a consequence of FAS1 loss.

Changes in Histone Modifications Are Locus Specific and Preferentially Located within the Promoter Region

One possibility is that these changes in epigenetic marks of constitutively active genes in fas1-4 plants are due to global open conformation of chromatin over large chromosomal regions. To address this, we determined by ChIP analysis the epigenetic status of the promoters of genes around those actively transcribed in fas1-4 and covering approximately a 20-kb-long chromosomal region. We focused here on two of them, histone H4 (At5g59690) and BRCA1 (At4g21070). As summarized in Figure 6, A and B, the genes flanking these two targets did not change significantly in fas1 compared to the wild type (Schönrock et al., 2006). Our data clearly showed that the promoters of genes flanking the histone H4 and BRCA1 genes did not differ in their histone modifications comparing fas1-4 and wild-type plants. In contrast, the target H4 and BRCA1 genes were enriched for acetylated histone H3 and H4, whereas they contain decreased amounts of H3K9me2 (Fig. 6, A and B).

We also determined the epigenetic status in a region covering from approximately 2 kb upstream of the putative ATG to approximately 300 bp inside the coding region of both H4 and BRCA1 genes. Our ChIP experiments clearly showed that only a few hundred base pair upstream of the ATG appeared to be affected in the H3/H4 acetylation and H3K9me2 status (Fig. 6, C and D). Together, these results indicate that loss of FAS1 promotes changes in the epigenetic status of the promoter region of a subset of target genes, which span a relatively small region, likely associated with the transcription start site, but excluding the coding region. These changes are sufficient to explain the up-regulation of these genes in response to the loss of FAS1.

fas1-4 Mutation Causes Hypersensitivity to Both DNA Replication Stress and DNA Damage

It has been reported that viability of budding yeast (Saccharomyces cerevisiae) cac1 and cac2 mutants is not compromised after treatments that produce DNA replication stress, but they are hypersensitive to treatments producing DSBs (Linger and Tyler, 2005). Also,
fas1-1 and fas2-1 seedlings are hypersensitive to the alkylating agent methylmethane sulfonate (Takeda et al., 2004). Thus, to investigate whether the fas1-4 mutation had a similar consequence, we used aphidicolin, a DNA polymerase α-inhibitor, and hydroxyurea (HU), a ribonucleotide reductase inhibitor that reduces the dNTP pool, as DNA replication stress-inducing agents, and zeocin, a radiomimetic drug of the bleomycin family, as a DSB-inducing treatment.

Wild-type and fas1-4 mutant seeds were germinated on control plates (Murashige and Skoog, day 0), grown for 4 d, and then maintained in the same plates (control) or transferred to other plates (treated) containing HU (1 mM) or aphidicolin (12 μg/mL), as described (Culligan et al., 2004). The fas1-4 mutant seedlings show a certain degree of root growth inhibition in the absence of external DNA damage (Fig. 7A), consistent with alterations of the root apical meristem in the fas1-1 mutants (Kaya et al., 2001). To eliminate these intrinsic experimental differences, we quantified the effect of DNA-damaging treatments on the relative root growth (control versus treated, as indicated in Fig. 7A). We found that root growth of fas1-4 seedlings was more sensitive to treatment with either zeocin or aphidicolin, although growth reduction was also observed at late times during HU treatment (Fig. 7A). Therefore, we conclude that Arabidopsis seedlings respond differently than budding yeast to the loss of FAS1 because the fas1-4 mutation produced hypersensitivity to both DNA replication stress-inducing (aphidicolin) and DSB-inducing (zeocin) treatments. Up-regulation of the CYCB1;1, RAD51, PARP1, and BRCA1 genes, which was constitutively activated in fas1-4 plants early after germination (see Fig. 4C), but not of the Ku70 gene, was maintained later during seedling growth (Fig. 7B). Furthermore, after DNA-damaging treatments, expression of these genes was increased to higher levels in the fas1-4 seedlings, in particular after treatment with aphidicolin (Fig. 7B).

**DNA-Damaging Treatment Partially Phenocopies the fas1-4 Phenotype**

The plant response to the loss of FAS1 in terms of impairment in cell cycle progression and up-regulation of G2 checkpoint genes is reminiscent of that of a wild-type plant to DNA-damaging treatments. Thus, we analyzed the response to treatment with zeocin, known to produce DSBs, in terms of ploidy, activation of G2 checkpoint genes, including CYCB1;1, and modifications of their epigenetic marks.

Using flow cytometry analysis of nuclei extracted from wild-type leaves with or without treatment with zeocin, we found that chronic zeocin treatment significantly increased the ploidy level in the leaves, in particular when the damaging treatment is initiated early during leaf development (10-d-old seedlings). At this time, cells are still in the proliferative phase (leaves 3 and 4) or have just initiated their endocycle.
program (leaves 1 and 2; Fig. 8A). We also measured the ploidy profiles in the roots of zeocin-treated seedlings and found that it was less pronounced than in leaves, although with a significant decrease of 2C nuclei and an increase of 4C nuclei (Supplemental Fig. S4), consistent with effects on cell cycle progression. It is worth noting that this result is similar to the relative smaller effect of the \textit{fas1-4} mutation on the ploidy level in roots compared to leaves (see Fig. 2B; Supplemental Fig. S2). This may reflect a distinct response of different organs to DNA damage or lack of \textit{FAS1}.

We wanted to confirm the up-regulation of \textit{CYCB1;1} using the \textit{CYCB1;1:GUS} translational reporter line (Colon-Carmona et al., 1999). We found that high \textit{CYCB1;1} levels accumulate shortly after treatment of wild-type plants with zeocin (Fig. 8B). It should be kept in mind that, in \textit{fas1-4} seedlings without damaging treatment (Fig. 4B) or in wild-type plants after damaging treatment, other mitotic cyclins are up-regulated in root and aerial organs (Supplemental Fig. S5; Chen et al., 2003; Schönrock et al., 2006). Finally, we determined by ChIP experiments the epigenetic status of a set of genes that are up-regulated in response to zeocin treatment and also in a constitutive manner in \textit{fas1-4} plants. We found that the promoter regions, close to the putative ATG, of histone H4, \textit{CYCB1;1}, \textit{RAD51}, and \textit{BRCA1}, but not of \textit{Ku70} and the control \textit{ACT2} genes, were enriched for both acetylated H3 and H4 and deprived of H3K9me2 (Fig. 8C). Thus, the parallelism between the response to DNA-damaging agents and the loss of function of \textit{FAS1} is striking in all the hallmarks studied, including triggering of the endocycle program, up-regulation of a similar subset of genes, and changes in their epigenetic code.

**DISCUSSION**

Chromatin needs to be reconstituted in each DNA replication round to maintain the correct functionality in a variety of cellular processes, including DNA replication fidelity, DNA repair, recombination, and gene transcription. At the biochemical level, the primary function of CAF-1 is to transfer histone H3/H4 complexes to DNA in a DNA replication-dependent manner. Nevertheless, its functional relevance in different cellular processes and in the context of development is highly organism dependent. Here, we have identified \textit{FAS1} as a target of the E2F transcription factors and found that the presence of chromatin assembly factors is required to prevent unscheduled activation of G2 DNA damage checkpoint genes. Upon loss of \textit{FAS1}, selective epigenetic changes in the histone acetylation and methylation status occur in the promoters of target genes. Concomitantly, CDKA activity is reduced and cell cycle progression is...
Figure 6. Effects of fas1-4 on histone modifications in chromosomal domains and within a locus. A, An approximately 20-kb region of chromosome 5 containing the histone H4 (At5g59690) gene that is up-regulated in fas1-4 plants. ChIP assays show that promoter of At5g59690 gene is hyperacetylated and hypomethylated in fas1-4 plants. The promoters of neighboring genes (At5g59680 and At5g59700), whose expression did not change, remain unaffected. Quantification was carried out as described in Figure 5B. B, The same ChIP assay shown in A was analyzed for a region of chromosome 4 spanning approximately 21 kb and containing the BRCA1 (At4g21070) and flanking genes. C and D, Fine mapping of the histone acetylation and methylation status of histone H4 and BRCA1 loci. ChIP assays were performed using antibodies against tetra-acetylated-H4 (H4ac), diacetylated-H3 (H3ac), and H3K9me2.
impaired. However, instead of resulting in irreversible cell cycle arrest and cell death, as occurs in human cells, a switch to the endocycle program occurs, thus making compatible FAS1-induced defects with development. The reduction in CDKA activity is somehow paradoxical because it occurs together with an increase in the ploidy level. One possibility to explain this observation is that the overall level of CDK activity required for the G2/M transition might be relatively much higher than that required for the G1/S transition. However, detailed understanding of this particular aspect would require further experimentation.

The availability of FAS1 is cell cycle regulated, showing a peak in the S-phase (Kaya et al., 2001. We have found that members of the E2F family of transcription factors participate in the transcriptional regulation of the FAS1 gene. From the FAS1 expression pattern in plants with altered E2F levels, it is not possible to simply make a correlation with either activator or repressor E2F members. This suggests a complex regulatory network in which different combinations of E2F may affect FAS1 expression, possibly in a cell type-dependent manner. This idea of complexity in E2F regulation has a precedent in the data obtained in mRNA-profiling experiments of plants with increased levels of E2Fa, considered a typical activator (Vlieghe et al., 2003; Vandepoele et al., 2005). These studies identified genes whose expression was activated as well as genes whose expression was repressed, although, in both cases, some of them contain E2F binding sites in their promoters. However, it seems clear that the two E2F binding sites in the FAS1 promoter are not redundant and they play distinct roles. Based on our deletion analysis, we conclude that one of them is required for enhanced expression (site 1) and the other acts as a repressor (site 2). Similar complex behaviors of the E2F-mediated expression of target genes with more than one E2F binding site have been previously observed (for review, see Ramírez-Parra et al., 2007). The two E2F binding sites in the Nicotiana tabacum RNR2 promoter are involved in upregulation at the G1/S transition, but one of them acts as a repressor when cells are outside the S-phase (Chabouté et al., 2000). The two E2F binding sites in the Nicotiana benthamiana PCNA gene contribute to the repression of the promoter in mature leaves. However, one E2F site counters the repression activity of the second E2F site in young leaves (Egelkruit et al., 2001, 2002). However, in the case of rice (Oryza sativa) PCNA, potential E2F binding sites mediate its transcriptional activation in dividing cells, but they do not participate in its repression in terminally differenti-
Mutations in topoisomerase genes also produce an endoreplication phenotype (Sugimoto-Shirasu et al., 2005) as occurs in animal cells with topoisomerase inhibitors (Cortés et al., 2004). Loss of CAF-1 in yeast (cac mutants) produces defects in the maintenance of silencing at particular genomic locations, such as telomeres or the mating-type HML locus (Monson et al., 1997; Enomoto and Berman, 1998), and genome instability (Kolodner et al., 2002; Myung and Kolodner, 2003). The latter is likely linked to the increased sensitivity of budding yeast to treatment with DSB-inducing agents (Linger and Tyler, 2005). In human cells, loss of CAF-1 activity by eliminating the function of the p150 or the p60 subunits inhibits nucleosome assembly, induces S-phase arrest, and causes cell death (Hoek and Stillman, 2003; Ye et al., 2003; Nabatiyan and Krude, 2004; Nabatiyan et al., 2006). In addition, CAF-1 has been involved in transcriptional silencing (Tchenio et al., 2001). In plants, mutations in the FAS1 or the FAS2 genes cause pleiotropic developmental defects, including fasciation, dwarfism, flower abnormalities and reduced fertility.

Figure 7. G2 DNA damage checkpoint phenotype of fas1-4 mutants. A, Kinematic analysis of root growth of wild-type and fas1-4 seedlings germinated in Murashige and Skoog medium during 4 d and then transferred to plates containing 10 μg/mL zeocin, 12 μg/mL aphidicolin, or 1 mM HU. Experiments were done in duplicate and at least 40 seedlings were used in each treatment. The relative root growth of untreated (control) versus treated seedlings is presented for wild-type and fas1-4 samples. B, Expression levels of CYCB1;1 and DNA damage response marker genes in 14-d-old seedlings treated with 10 μg/mL zeocin or 12 μg/mL aphidicolin, determined by real-time RT-PCR. Values are the average (± SD) of at least three assays performed on different cDNAs. Control refers to untreated wild-type and fas1-4 plants. [See online article for color version of this figure.]

De Veylder, 2006). Mutations in topoisomerase genes also produce an endoreplication phenotype (Sugimoto-Shirasu et al., 2005) as occurs in animal cells with topoisomerase inhibitors (Cortés et al., 2004).

Loss of CAF-1 in yeast (cac mutants) produces defects in the maintenance of silencing at particular genomic locations, such as telomeres or the mating-type HML locus (Monson et al., 1997; Enomoto and Berman, 1998), and genome instability (Kolodner et al., 2002; Myung and Kolodner, 2003). The latter is likely linked to the increased sensitivity of budding yeast to treatment with DSB-inducing agents (Linger and Tyler, 2005). In human cells, loss of CAF-1 activity by eliminating the function of the p150 or the p60 subunits inhibits nucleosome assembly, induces S-phase arrest, and causes cell death (Hoek and Stillman, 2003; Ye et al., 2003; Nabatiyan and Krude, 2004; Nabatiyan et al., 2006). In addition, CAF-1 has been involved in transcriptional silencing (Tchenio et al., 2001). In plants, mutations in the FAS1 or the FAS2 genes cause pleiotropic developmental defects, including fasciation, dwarfism, flower abnormalities and reduced fertility.

Figure 8. DNA damage induces endoreplication and gene-specific epigenetic changes in wild-type Arabidopsis plants. A, Ploidy profiles (left) and nuclear DNA ploidy distribution (right) of nuclei from leaves 1 and 2 and 3 and 4 were determined 10 d after initiation of continuous treatment of 5-d-old seedlings with or without zeocin (10 μM). B, Expression of the CYCB1;1:GUS marker gene (Colon-Carmona et al., 1999) in wild-type seedlings with or without induction of DNA damage. GUS activity was detected 24 h after treating 13-d-old plants with zeocin (10 μg/mL). C, ChIP assays of 15-d-old seedlings after 10-d treatment with or without zeocin (10 μM), using antibodies specific for acetylated histones H3 (H3ac), H4 (H4ac), and methylated histone H3 (H3K9me2). Semi-quantitative PCR was used to estimate the relative enrichment of genomic fragments corresponding to each promoter. The number of total cycles was calculated as in Figure 5. The results shown are representative of at least three independent assays.
retarded root growth, and both root and shoot meristem disorganization (Leyser and Furner, 1992; Kaya et al., 2001). This situation, quite different from that in human cells and yeast, may be due, at least in part, to redundancy with other histone chaperones (e.g. ASF1).

Genome-wide expression analysis has shown that CAF-1 is involved in regulating the transcription of genes acting during the late S-phase when heterochromatin is replicated (Schönrock et al., 2006). These authors also found that genes belonging to the DNA repair category, such as BRCA1, RAD51, PARP1, among others, are up-regulated in fas1 and fas2 mutants. These genes are characteristic of the G2 DNA damage checkpoint and some of them directly participate in HR events. Consistent with this role of CAF-1, intrachromosomal HR appears to be drastically increased in plants carrying mutations in the FAS1 gene (Endo et al., 2006; Kirik et al., 2006). It has been proposed that these genes are regulated differently in the C24 ecotype, where the fas1-4 mutation was identified, than in the En or Ler ecotypes, the background of the fas1-1 and fas2-1 mutations, respectively. This might well be the case because in the fas1-4 (Col-0 ecotype; Exner et al., 2006; this work) and fas1-2 and fas2-1 (En and Ler ecotypes, respectively; Schönrock et al., 2006) plants, significant overexpression of G2 checkpoint genes is detected. Interestingly, expression of the Kiu70 gene, a component of the G1 checkpoint pathway, was not altered, reinforcing the idea that defects in CAF-1 likely result in activation of a G2 checkpoint.

It is worth noting that CYCB1;1, but not other mitotic cyclins, are up-regulated after DNA-damaging treatment (Chen et al., 2003). A similar response is observed after aphidicolin-induced replication stress, which is not displayed in the atr mutants, defective in the G2 checkpoint (Culligan et al., 2004). Interestingly, mutations in the TOUSLED (TSL) gene, which encodes a kinase responsible for activating the ASF1 histone chaperone, produce constitutive activation of CYCB1;1, but not of CYCB1;2 (Ehsan et al., 2004). Likewise, plants carrying a mutation in the BRUSHY1/TONSOKU/MGOUN3 (BRU1/TSK/MGO3) gene, which encodes a chromatin stabilization protein, have phenotypes remarkably similar to fas1 mutants (Suzuki et al., 2004; Takeda et al., 2004; Guyomard et al., 2006), including disturbed meristems and increased HR. Interestingly, the bru1/ tsk/mgo3 mutant also has increased expression of PARP1 (Takeda et al., 2004) and of CYCB1;1, as well as a slightly increased ploidy level (Suzuki et al., 2005). Thus, a significant body of circumstantial evidence strongly suggests that both DNA damage and chromatin defects converge at the G2 checkpoint.

Conflicting reports have appeared regarding the role of CAF-1 in maintenance of silent chromatin states. Schönrock et al. (2006) showed that transcriptional repression of genes located in heterochromatic regions, which are usually silent in the wild type, still persists in the absence of CAF-1. In addition, they showed that CAF-1 is dispensable for maintenance of DNA methylation. In another report, transcriptional silencing of the endogenous CACTA transposon is relieved in fas mutants (Ono et al., 2006), although this transcriptional activation was relatively weak, infrequent, and stochastic. We have found that transcriptional activation of a set of euchromatin genes is not due to global changes in chromatin organization over large chromosomal regions. Instead, changes in the epigenetic marks (H3 and H4 acetylation and H3K9 methylation) of a relatively small region in the putative promoter, close to the ATG site and consistent with up-regulation of selected genes, are maintained in fas1-4 mutant plants. Interestingly, the same type of histone modifications as well as similar locus specificity occur in wild-type plants after a DNA-damaging treatment, suggesting that the G2 checkpoint signaling pathway is shared, at least in part, by the response to a loss of FAS1. It has recently been reported that, in the absence of external damage, fas1 mutant plants contain an increased amount of DSBs (Endo et al., 2006), suggesting that the cellular response to a loss of FAS1, and likely defects in CAF-1-mediated chromatin assembly during the S-phase, is transduced through the G2 DNA damage checkpoint (Fig. 9). Another possibility is that altered chromatin organization during DNA replication is recognized by specific DNA damage-sensing complexes. This already has a precedent in human cells where damaged DNA structures are recognized by a BRCA1-associated genome surveillance complex, a multiprotein complex that includes MSH2, MSH6, MLH1, ATM, BLM, RAD50-MRE11-NBS1, and the RFC proteins (Wang et al., 2000). The

Figure 9. Simplified model that accounts for the proposed link between E2F regulation of FAS1, CAF-1-mediated chromatin assembly during S-phase, G2 DNA damage checkpoint, and endoreplication control. Loss of CAF-1 in Arabidopsis constitutively activates a G2 DNA damage checkpoint and changes the epigenetic status of a subset of genes, inhibits mitotic progression, and promotes the switch to the endocycle program.
downstream consequence is that progression to mitosis is inhibited. However, the cellular phenotype of \textit{fas1} mutants strongly suggests that, in the context of a developing plant, G2 arrest is not permanent and/or irreversible, but, instead, a physiological switch to the endocycle program occurs. In this way, unscheduled triggering of endocycles may act as a bypass pathway that makes development compatible with cell division arrest triggered by chromatin alterations or DNA damage. Bypass of mitosis by switching on the endocycle program has been reported as a consequence of local treatments of the shoot apical meristem with oryzalin, an inhibitor of mitotic progression (Grandjean et al., 2004). Therefore, it would be important to identify the molecular links between DNA replication-dependent chromatin assembly, the G2 checkpoint, recombination events, and the endoreplication potential in survival. This may contribute to understanding whether these pathways are part of a more general mechanism that contributes to the high growth plasticity of plants in response to environmental challenges.

**MATERIALS AND METHODS**

**Growth of Arabidopsis Plants**

For standard growth conditions, Arabidopsis (\textit{Arabidopsis thaliana} ecotype Col-0) seeds were sown on 0.5\% Murashige and Skoog salt medium (Duchefa), supplemented with 1\% Suc and 1\% agar, pH 5.8, and grown with a 16-h-light/8-h-dark cycle at 21°C. HU (500 \text{mM} stock in water; Sigma), aphidicolin (12 \text{\mu g/mL} stock in dimethyl sulfoxide; Sigma), and zeocin (Invitrogen) were added to Murashige and Skoog plates, as required, at the concentrations described in the text and figure legends. For experiments using aphidicolin, all control and aphidicolin plates contained an equivalent amount (0.05\%) of dimethyl sulfoxide to allow direct comparison. The Arabidopsis T-DNA insertion lines SALK\textsubscript{049751} (\textit{fas1-4}) were obtained from the Arabidopsis Biological Resource Center (ABRC) and homozygous plants were selected for further analysis.

**RNA Extraction, RT-PCR, and Real-Time RT-PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen) and RT was carried out with the ThermoScript RT system (Invitrogen). The LightCycler system with FastStart DNA Master SYBR Green I (Roche) was used for quantitative real-time RT-PCR. The concentration of actin (\textit{ACT2}) mRNA in each sample was determined to normalize for differences of total RNA amount. Data were derived from two independent cDNA preparations and PCR analysis carried out in triplicate. To avoid amplification of contaminating genomic DNA, primers were designed for scanning exon-exon junctions. Sequences of primers used are specified in Supplemental Table S1.

**EMSA and ChIP Assays**

EMSA were carried out as described (Ramirez-Parra et al., 2004) using purified MBP-AE2F2 and a \textit{FAS1} promoter fragment (−370 to −195 relative to the ATG start codon) or double-stranded oligonucleotides containing E2F sites, E2F1 (5\’-CTTGATGTCGGCCGAAAACAGATGATAT-3\’) and E2F2 (5\’-AACCTTGGCCGGAAAATCAACGGAC-3\’) and their complementary sequences as probes.

ChIP assays and data analysis were carried out basically as previously described (Ramirez-Parra et al., 2004), treating plants with 10 \text{mM} sodium butirate (Sigma) during 4 h prior to fixation and using anti-HA (Roche), anti-Myc 9E10 (Santa Cruz Biotechnology), anti-tetra-AcH4 (Upstate), anti-di-AcH3 (Upstate), and anti-di-MetK9H3 (Upstate) antibodies. Sequences of primers used are specified in Supplemental Table S2.

**Microscopy**

For light microscopic analysis, leaves were incubated in 95\% ethanol at 90°C for 5 min and in lactophenol (25\% [w/v] phenol, 25\% lactic acid, 25\% glycerol, and 25\% water) overnight at room temperature. Samples were mounted on slides and analyzed by Nomarski microscopy. For kinetic cell analysis, at least 500 cells taken from 10 different leaves were photographed with a digital CoolSnap FX camera (Roper Scientific) mounted in an Axio- \textit{skop2} plus microscope (Zeiss) and processed with ImageJ software (National Institutes of Health). Nuclear visualization was done by staining with DAPI (0.1 \text{\mu g/mL}) for 1 h. Samples were washed and analyzed by fluorescence microscopy using an Axioskop2 plus microscope (Zeiss) and the images captured with a digital CoolSnap FX camera (Roper Scientific). For scanning electron microscopy, a FEI QUANTA 200 microscope was used in ambient mode or low vacuum conditions with unfixed material.

**Kinematic Analysis of Root Growth**

For analysis of root growth, root length was measured every 72 h after germination for a period of 14 d during which seedlings were grown in a vertical position. Length was basically determined as described (De Veylder et al., 2001). Digitized images from scanning were processed using ImageJ software.

**Flow Cytometry Measurements**

Leaves, cotyledons, roots, or dissected seedlings after germination were chopped and resuspended in cold nuclear isolation buffer (Galbraith et al., 1991). This crude preparation of isolated nuclei was filtered through 60-\text{\mu m} nylon mesh, treated with RNase A (100 \text{\mu g/mL}), and stained with propidium iodide (50 \text{\mu g/mL}; Sigma). At least 10\textsuperscript{3} isolated nuclei were analyzed with a FACSCalibur flowcytometer (BD Bioscience).

**Pull-Down Experiments, in Vitro Kinase Assays, and Western-Blot Analysis**

For protein expression, \textit{Escherichia coli} BL21 (DE3) cells were grown to an OD\textsubscript{600} of 0.6 at 37°C and induced by adding 0.5 \text{mM} isopropyl\textsubscript{1-H}-\beta-D-galactosidase for 3 h. His-p13\textsubscript{AcH} protein was purified with nickel-nitrotetrazic acid agarose. CDKA\textsubscript{\textit{p13AC}} complexes were purified using protein extracts of wild-type and \textit{fas1}-4 seedlings (12 d old). Protein extracts, pull-down experiments with p13\textsubscript{AcH} beads (20 \text{\mu L}), and in vitro kinase assays, using histone H1 (1 \text{\mu g}) as substrate, were done essentially as described (Boniotto and Gutierrez, 2001). Proteins were fractionated on a 12\% SDS-PAGE gel and the phosphorylated histone H1 was detected by autoradiography. Total protein and p13\textsubscript{AcH}-affinity purified proteins were fractionated on 12\% SDS-PAGE gels and blotted onto Immobilon-P membranes (Millipore). CDKA protein was detected with rabbit polyclonal anti-PSTAIRE antibodies (Santa Cruz) diluted to 1:1,000. Secondary peroxidase-conjugated goat anti-rabbit (Santa Cruz) antibodies were diluted to 1:12,000 and revealed with ECL detection reagents (Amersham).

**Construction of Mutated Promoter Sites and Detection of GUS Activity**

For expression analysis, 780 bp of the genomic region containing the \textit{FAS1} promoter were fused in-frame to the \textit{GUS} coding sequence in a pB1013.2 vector (Jefferson et al., 1987) and used for transformation of Arabidopsis (\textit{pFAS1::GUS} plants). Site-directed mutagenesis of E2F binding sites was performed using the QuickChange kit (Stratagene). Histochemical detection of GUS activity was done using 5-bromo-4-chloro-3-indolyl-b-D-galactosidase (Jefferson et al., 1987). Fluorometric quantification of GUS activity was done using 10-d-old seedlings. Total protein was extracted in GUS buffer (50 \text{mM} phosphate buffer, pH 7.0, 10 \text{mM} \text{MgCl}_2, 0.1 \text{mM} EDTA, 0.1\% SDS, 0.1\% Triton X-100). Samples were assayed using 1 \text{mM} 4-methylumbelliferyl-b-D-glucurononanodisucrose acid (Sigma) at 37°C, and, after 60 min, the reaction stopped with 0.2 \text{mM} Na\textsubscript{2}CO\textsubscript{3}. Fluorescence was measured using a SLM-AMINCO luminescence spectrometer (AMINCO-BOWMAN) and normalized to the amount of total protein in each sample.

**Supplemental Data**

The following materials are available in the online version of this article.
Supplemental Figure S1. Reproductive phenotype of fas1-1.
Supplemental Figure S2. Nuclear ploidy of fas1-1.
Supplemental Figure S3. Expression of cell cycle genes.
Supplemental Figure S4. Effect of zeocin on ploidy in roots.
Supplemental Figure S5. Effect of bleomycin and mitomycin C on cell cycle genes.
Supplemental Table S1. Oligonucleotides for RT-PCR.
Supplemental Table S2. Oligonucleotides for ChIP.

ACKNOWLEDGMENTS

We thank C. Vaca for technical help, the ABRC for T-DNA seeds, P. Doerner for CYCB1;1:uidA plants, J.L. Miclo for the wild type (En) and fas1-1 mutant seeds, L. De Veylder and D. Inze for the E2Fa and E2Fb/DDEL1 overexpressor plants, R. Cella for the E2Fb overexpressor plants, and L. Tormo (MNCN-CSIC) for her help with scanning electron microscopy. We also thank J.A. Tercero, J. Difeilley, and E. Martinez-Salas for comments.

Received December 18, 2006; accepted March 1, 2007; published March 9, 2007.

LITERATURE CITED


