FASCIATA Genes for Chromatin Assembly Factor-1 in Arabidopsis Maintain the Cellular Organization of Apical Meristems

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Received 5 July 2000; revised 21 November 2000; Available online 5 February 2001.

Abstract

Postembryonic development of plants depends on the activity of apical meristems established during embryogenesis. The shoot apical meristem (SAM) and the root apical meristem (RAM) have similar but distinct cellular organization. Arabidopsis FASCIATA1 (FAS1) and FAS2 genes maintain the cellular and functional organization of both SAM and RAM, and FAS gene products are subunits of the Arabidopsis counterpart of chromatin assembly factor-1 (CAF-1). fas mutants are defective in maintenance of the expression states of WUSCHEL (WUS) in SAM and SCARECROW (SCR) in RAM. We suggest that CAF-1 plays a critical role in the organization of SAM and RAM during postembryonic development by facilitating stable maintenance of gene expression states.

Introduction

In higher plants, organogenesis is not limited to embryonic development but continues throughout the life of the plant. During embryogenesis, only the basic body plan is established, with small groups of cells called apical meristems at both ends of the body axis. Apical meristems are almost entirely responsible for postembryonic development to elaborate the plant architecture. The shoot apical meristem (SAM) is responsible for development of the aerial parts of the plant, and the root apical meristem (RAM) is responsible for development of the subterranean root system.

In dicot plants including Arabidopsis, SAM consists of a dome of cells organized into a peripheral zone (PZ) of rapidly dividing cells wherein primordium initiation occurs and a central zone (CZ) of slowly dividing cells that replenish PZ. Superimposed on this are three generative cell layers. The tunica consists of layers 1 and 2 (L1 and L2). L1 and L2 cells, which divide anticlinally, remain clonally distinct, and form the epidermis and mesophyll, respectively. Beneath the tunica, L3 cells of the corpus are characterized by more variable division planes and sizes. L3 cells contribute to pith and vascular tissue (Meyerowitz 1997). Arabidopsis RAM has a regular, rather stereotyped cellular organization. Four distinct types of cell initials (8 cortex/endodermis initials, 16 epidermis/lateral root cap initials, 12 columella root cap initials, and stele initials) are arranged around 4 central cells. A regular invariant pattern of cell division of initials gives rise to progeny that differentiate into specific

cell types in the root and root cap. The central cells divide infrequently and correspond to the quiescent center (QC) (Dolan et al. 1993).

Despite these differences, the basic organization of SAM and that of RAM are similar in terms of having a central region of quiescent or slowly dividing cells. This and other similarities suggest that the differences between SAM and RAM are superimposed upon a fundamentally homologous organization plan (Steeves and Sussex 1989). Recent observations concerning the cellular mechanism of stem cell maintenance in apical meristems further support this view. In RAM, QC controls the behavior of initials, which are mostly unipotent stem cells, via a short-range signal (van den Berg et al. 1997). Similarly, a small population of cells expressing the WUSgene acts as an organizing center to specify the fate of overlying pluripotent stem cells in SAM (Mayer et al. 1998).

Mutations affecting both SAM and RAM may represent lesions in genes required for the fundamental organization of the apical meristem. Such mutants have not been extensively analyzed, and most with defects in the formation and/or maintenance of the apical meristem are specific to either SAM or RAM ([37] and [26]). Only the defective embryo and meristems (dem) mutation in tomato is known to affect both SAM and RAM (Keddie et al. 1998). The Dem gene is required for embryonic pattern formation as well as formation of apical meristems and encodes a novel protein of unknown biochemical function. fas1 and fas2 of Arabidopsis were originally described as mutations causing stem fasciation, abnormal phyllotaxy, and shorter roots ([24] and [32]). Stem fasciation associated with distorted phyllotaxy is an abnormality reported in a variety of plants and has been attributed to defects in SAM (Gorter 1965). Shorter roots indicate defective RAM function during root elongation. Therefore, fas and dem mutations are good candidates for lesions in genes required for the fundamental organization of both types of apical meristems.

Chromatin assembly factor-1 (CAF-1), consisting of three subunits, p150, p60, and p48, was originally purified from a nuclear extract of human cells as a factor which supports the assembly of nucleosomes specifically onto replicating DNA in vitro (Smith and Stillman 1989). Since CAF-1 is associated with newly synthesized histones H3.H4 and localizes at replication foci in proliferating human cells, CAF-1 is thought to be involved in chromatin assembly during DNA replication and DNA repair in vivo ([33] and [49]). We predict that CAF-1 serves to ensure stable propagation of epigenetic states and maintenance of genome integrity by facilitating rapid reformation of chromatin structure after passage of a replication fork. Indeed, yeast cells lacking CAF-1 activity cannot maintain repressed states of gene expression at telomeres and mating type loci ([27] , [11] and[51]).

Here, we show that the FAS1 and FAS2 genes encode two subunits of the Arabidopsis counterpart of CAF-1 and we report the characterization of fas1 and fas2 mutants, novel examples of CAF-1 defective mutants in multicellular organisms. These mutants display severely disturbed cellular and functional organization of both SAM and RAM. They also show a varied pattern of distorted expression of both WUS and SCR, which play key roles in the organization of SAM and RAM, respectively. We suggest that the functional role of the FAS complex is to facilitate stable maintenance of a gene expression state in apical meristems, possibly through its activity as CAF-1, by ensuring stable propagation of epigenetic states through DNA replication

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Results

All of the fas1 and fas2 alleles examined showed essentially the same phenotype, and as no consistent difference was observed between fas1 and fas2 mutants, both will be collectively referred to as fas mutants. As the phenotype of fas mutants at the level of gross morphology has been described ([24] and [40]), we limit our description to apical meristems.

Embryogenesis in fas Mutants

Embryogenesis was not disturbed in fas mutants (Figure 1A and Figure 1B). The morphology of the cotyledons, hypocotyl, and radicle in mature embryos and germinating seedlings was normal. However, the embryonic SAM in some fas mature embryos was slightly broader than that in the wild type (Figure 1C and Figure 1D). In contrast, RAM cellular organization in mature embryos was normal (Figure 1E and Figure 1F).

Full-size image (155K)
High-quality image (162K)

Figure 1.

Embryo Phenotype of fas Mutants

Mature embryo of wild type (En) (A) and fas1-1 (B). Embryonic SAM of wild type (En) (C) and fas1-1 (D). Embryonic RAM of wild type (En) (E) and fas1-1 (F). Arrowheads in (C) and (D) indicate the boundary between cotyledons and SAM. Bars: 100 μ m (A and B) and 50 μ m (C, D, E, and F).

SAM Phenotype of fas Mutants

SAM in fas mutant seedlings was broader and flatter than that in the wild type, and the clear histological distinction between PZ and CZ was lost (Figure 2A–2E). In the wild type, SAM had 6 or 7 outer layers of avacuolate cells (Figure 2A and Figure 2C), whereas only 3 or 4 such layers were observed in fas mutants (Figure 2B and Figure 2D). The regular cell arrangement in L1 and L2 was disrupted in fas mutants (Figure 2B and Figure 2D, compare with 2A, 2C, and 2E). This agrees with a previous report on the SAM phenotype of fas1-1 mutants (Figure 1C in Leyser and Furner, 1992 [not 1B as stated in the legend]). The SAM phenotype of fas mutants varied among individuals for each allele. Thus, SAM organization into functional zones and clonally distinct layers is disrupted in fas mutants. Some seedlings showed various degrees of failure of leaf primordia development (Figure 2G–2I).

Full-size image (684K)
High-quality image (749K)

Figure 2.

Shoot Phenotype of fas Mutants

(A–F) Sections of vegetative SAM. (A) 7-day-old wild type (En), (B) 7-day-old fas1-1, (C) 10-day-old wild type (No), (D) 10-day-old fas2-2, (E) Schematic representation of SAM. Three outer cell layers (L1, L2, and the outermost layer of L3), CZ (yellow), and PZ (green) are marked on the image shown in (C). (F) 11-day-old fas1-1; fas2-2. In wild type (A and C), CZ appears more lightly stained than the surrounding PZ, and three outer layers can be clearly recognized. Bar: 50 μ m.

(G–I) Shoot tip region of seedlings seen from above. (G) 9-day-old fas1-2, (H) 9-day-old fas2-1, and (I) 14-day-old fas2-1. Projections in (H) and (I) are the first pair of leaves. Bar: 0.5 mm.

Reduced Root Growth in fas Mutants

As reported previously (Leyser and Furner 1992), fas plants have shorter roots. This is due to a reduced growth rate (data not shown). Cell division, expansion, and differentiation in Arabidopsis are largely confined to three zones along the apical-basal axis: the RAM, the elongation zone, and the specialization zone. The elongation zone, containing small cells with a dense cytoplasm that are proliferating and expanding, is responsible for root growth ([10] and [38]). In fas roots, the elongation zone was greatly reduced (Figure 3A–D) due to fewer small avacuolate cells in the apical region (data not shown). In addition, cell elongation began at a position more apical in the elongation zone in the fas roots (data not shown).

Full-size image (342K)
High-quality image (370K)

Figure 3.

Root Phenotype of fas Mutants

(A–D) Primary root of 10-day-old seedlings. (A) Wild type (En), (B) fas1-1, (C) wild type (L er), and (D) fas2-1. The region between two arrowheads is the elongation zone. Bar: 0.5 mm.

(E–J) RAM and the apical part of the elongation zone in 6-day-old seedlings. (E) Wild type (En), (F) fas1-1, (G) fas2-2, and (J) fas1-1; fas2-2. (H) and (I) are enlargements of (E) and (F), respectively, showing the RAM region containing QC and initials. Bar: 50 μ m in (E)–(G) and (J).

(K) Schematic representation of RAM. Initials (solid) and their derivatives (shaded) are marked by the same color as follows: green, cortex/endodermis initials; blue, endodermis;

yellow, cortex; orange, stele; pink, epidermis; brown, lateral root cap; red, columella root cap; and white, QC.

(L–R) Starch staining of the primary root tip of 5-day-old seedlings. (L) Wild type (En), (M) fas1-1, (N) wild type (No), (O) fas2-2, and (R) fas1-1; fas2-2. Small arrowheads and arrows in (L) and (N) indicate QC and columella initials, respectively. Arrows in (M), (O), and (R) indicate cells in the region of QC and columella initials. (P) and (Q) are enlargements of (L) and (M), respectively, showing QC (*), columella initials (+), and the first tier of columella cells (arrow). Bar: 50 μ m in (L)–(O) and (R).

RAM Phenotype of fas Mutants

Arabidopsis RAM has a regular cellular organization with four distinct types of initials arranged around central cells in QC (Figures 3E, 3H, 3K) (Dolan et al. 1993). In fas mutants, the regular stereotyped arrangement of the central cells and initials was lost (Figures 3F, 3G, 3I). QC and surrounding initials were difficult to identify unequivocally on the basis of their characteristic positions. However, there was no consistent loss of certain cell types as reported for some mutants (Scheres et al. 1995).

In fas roots, abnormal behavior of initials was observed (see below for SCR::GFP expression). For example, columella initials accumulated starch granules, a specific marker for differentiated columella cells (van den Berg et al. 1995) (Figures 3M, 3O, 3Q, compare with 3L, 3N, and 3P). This, as well as the irregular arrangement of columella cells, suggests that the stem cell state of columella initials is not stably maintained. Since cells in the position of QC also accumulated starch granules, it is evident that QC cells are not stably maintained and are misspecified in these mutants.

fas1; fas2 Double Mutant

Both SAM and RAM organization were perturbed in a similar manner in fas1 and fas2 mutants. Previous genetic analysis, however, could not resolve whether the double mutants were seedling lethal or indistinguishable from parental single mutants (Leyser and Furner 1992). We investigated the phenotype of fas1; fas2 double mutants by combining fas2-2 with either fas1-1 or a FAS1 deletion (see below). In both combinations, double mutants were not seedling lethal as previously suspected. These double mutants were indistinguishable from either of the parental single mutants (Figure 2 and Figure 3).

Identification of the FAS1 Gene

We identified a T-DNA insertion line which failed to complement both fas1-1 and a late-flowering mutation, ft-1 ([22] and [19]). The line has a 75.8 kb deletion at the T-DNA insertion site and a candidate gene located at one end of the deleted region contains a nonsense mutation in both fas1-1 and fas1-2 (Figure 4A). The entire 5′ region up to the 36th base of the third exon of the candidate gene was deleted in the T-DNA insertion line. Based on these results, we concluded that the candidate gene is indeed FAS1. Detailed analysis of the T-DNA insertion line is described elsewhere (Kaya et al. 2000).

Full-size image (518K)
High-quality image (538K)

Figure 4.

Predicted FAS1 and FAS2 Proteins

(A) The deduced amino acid sequence of FAS1. The KER domain is underlined and the ED domain is highlighted. Asterisks indicate mutation sites in fas1-1 (TGG to TGA) and fas1-2 (CAA to TAA).

(B) Schematic alignment of FAS1, hCAF-1 p150, and yeast Cac1p showing the relative positions and amino acid sequence identity (similarity in parentheses) of the KER and ED domains.

(C) The deduced amino acid sequence of FAS2. Seven WD-40 repeats are underlined. Asterisks indicate mutation sites in fas2-1 (TGG to TGA) and fas2-2 (see E).

(D) Schematic alignment of FAS2, hCAF-1 p60, and yeast Cac2p showing the relative position and amino acid sequence identity (similarity in parentheses) of a region containing seven WD-40 repeats.

(E) A frameshift caused by a G to A substitution at the splice acceptor site of the second intron in fas2-2. The affected nucleotide is shown in bold. Arrowheads above the gap indicate the observed splice sites in the wild type and fas2-2. Codons and corresponding amino acid residues are shown. The fas2-2 ORF has an unrelated sequence of 88 amino acid residues after the 76th residue (Gly).

Similarity of the FAS1 Gene Product to the Largest Subunit of CAF-1

The deduced amino acid sequence of the FAS1 protein (Figure 4A) shows significant similarity to the largest subunit of CAF-1 in humans (p150; Kaufman et al. 1995), Drosophila (Bulger et al. 1995), and yeast (Cac1p;[12] and [18]) (Figure 4B). FAS1, hCAF-1 p150, and Cac1p share a highly charged KER domain comprised chiefly of lysine (K), glutamic acid (E) and arginine (R) residues, and an ED domain which is a cluster of glutamic acid (E) and aspartic acid (D) residues. The KER domain is believed to form a coiled-coil structure, and the highly acidic ED region has been suggested to interact with highly basic histones (Kaufman et al. 1995). Because any C-terminal deletion of hCAF-1 tested destroyed all detectable activity in vitro (Kaufman et al. 1995), the two fas1 alleles are likely to represent null alleles.

Identification of the FAS2 Gene by the Candidate Gene Approach

Since CAF-1 is a three-subunit complex and since fas1 and fas2 mutants have very similar phenotypes with the double mutants being phenotypically indistinguishable from both single mutant parents, FAS2 may encode one of the two smaller subunits of CAF-1. A database

search yielded a gene located on chromosome 5 at a position roughly corresponding to that of fas2 (Leyser and Furner 1992). The deduced amino acid sequence of the product of the mub3.9 gene in the P1 clone MUB3 (DDBJ/EMBL/GenBank accession number AB010076) showed similarity to the sequence of hCAF-1 p60. We found a nucleotide substitution in the mub3.9 gene in lines homozygous for fas2-1 or fas2-2 (Figure 4C). Furthermore, a 7.0 kb genomic fragment containing the mub3.9 gene complemented the fas2-2 mutant phenotype (data not shown). We concluded that the mub3.9 gene is indeed the FAS2 gene.

The Predicted FAS2 Gene Product as a Homolog of the Second Largest Subunit of CAF-1

The putative FAS2 protein, hCAF-1 p60 and yeast Cac2p have seven WD-40 repeats implicated in protein–protein interactions (Neer et al. 1994) (Figure 4C and Figure 4D). fas2-1 has a stop codon in the fourth WD-40 repeat resulting in truncation of the polypeptide. fas2-2 has a G to A substitution at the splice acceptor site of the second intron, causing missplicing and a frameshift beginning at the 76th codon (Figure 4E). Both alleles are likely to represent null alleles.

Complex Formation and CAF-1 Activity of the FAS-1, FAS2, and AtMSI1 Products

In terms of subunit composition and in vitro activity, CAF-1 is a well conserved complex in a variety of organisms from yeasts to humans ([43] , [5] , [17] and [18]).
Since FAS1 and FAS2 are single-copy genes as suggested by the results of genomic Southern blot analysis and database searches (H. K. and T. A., unpublished data), FAS1 and FAS2 may represent subunits of the functional counterpart of CAF-1 in Arabidopsis. We tested this hypothesis by investigating complex formation by the FAS1 and FAS2 products and their replication-dependent nucleosome assembly activity in vitro. Among the four known Arabidopsis homologs of yeastMSI1 (Ruggieri et al. 1989), called AtMSI1-AtMSI4 ([1] and [21]), we tentatively chose AtMSI1 as a candidate gene encoding the third subunit, because its product is most similar to hCAF-1 p48 and yeast Cac3p (= Msi1p; [34] and [18]) (data not shown).

To test whether FAS1, His-tagged FAS2 (His-FAS2), and AtMSI1 form a stable complex, the three proteins were produced in an in vitro transcription/translation system. The reaction mixture constituents were then precipitated by either Ni-NTA or a resin. The FAS1 and AtMSI1 products coprecipitated with the His-FAS2 product by Ni-NTA, but not by the resin (Figure 5A). Ni-NTA did not precipitate FAS1 or AtMSI1 product without the His-FAS2 product, indicating that FAS1, His-FAS2, and AtMSI1 form a complex, as do the three subunits of hCAF-1, p150, p60, and p48.

Full-size image (78K)
High-quality image (84K)

Figure 5.

Complex Formation and Replication-Dependent Chromatin Assembly

(A) Complex formation assay. FAS1, His-FAS2, and AtMSI1 products were incubated with either agarose beads (lane 2) or Ni-NTA beads (lane 3) at 4°C for 2 hr. In control experiments, FAS1 and AtMSI1 were incubated with Ni-NTA beads (lanes 5 and 7). Pulled down products were analyzed by polyacrylamide gel electrophoresis using a 12.5% gel. Twenty-five percent of the input for lanes 2 and 3, 5, and 6 was loaded in lanes 1, 4, and 6, respectively.

(B) Supercoiling assay. Post-replicative nucleosome assembly (Shibahara et al. 2000) was performed in the presence of S100 human cell extract with either purified hCAF-1 (lanes 1 and 2) or combinations of reticulocyte lysates containing the FAS1, His-FAS2, and AtMSI1 products (lanes 3–10), as indicated by + or − and by ×1 or ×3 (3-fold volume of reticulocyte lysate as in ×1). Combinations of reticulocyte lysates were incubated at 4°C for 1 hr to allow complex formation before the supercoiling reaction. Unprogrammed reticulocyte lysate was added to equalize the amount of lysate in lanes 3, 5, 7, and 9 and in lanes 4, 6, 8, and 10. Migration positions of form I, Io, and II DNA are indicated. The upper panel shows an autoradiograph of ^{32}P-labeled and replicated DNA, and the lower panel shows an ethidium bromide–stained image of the same gel to show the bulk of the DNA.

(C) Micrococcal nuclease digestion assay. Replicated DNA incubated with either S100 extract and hCAF-1 (lanes 1 and 2), S100 extract and control reticulocyte lysate (mock; lanes 3 and 4), or S100 extract and reticulocyte lysate containing FAS1, FAS2, and AtMSI1 (AtCAF-1; lanes 5 and 6), was digested with 6 Worthington units of micrococcal nuclease at 20°C for the indicated time periods. The DNA was then analyzed on a 2.0% agarose gel. The migration positions of mononucleosome- (mono) and disome-length (di) material are indicated.

The nucleosome assembly activity of the complex (Figure 5A) was then examined using a two-step, post-replicative nucleosome assembly system ([41] and [42]). In Figure 5B, ^{32}P-labeled, newly replicated DNA and unlabeled, nonreplicated DNA from the SV40 replication reaction were incubated with preformed complexes of FAS1, FAS2, and AtMSI1 in the presence of S100 extract to allow nucleosome assembly. The basis of the supercoiling assay in Figure 5B is that the closed circular DNA becomes negatively supercoiled when assembled into nucleosomes in the presence of topoisomerase activity. When increasing amounts of the complex of FAS1, FAS2, and AtMSI1 were added to the reaction, replicated DNA was converted to form I DNA, but nonreplicated DNA remained in a relaxed form (lanes 9 and 10). In contrast, no change in the distribution of topoisomers was observed when either FAS1 or FAS2 or AtMSI1 was removed (lanes 3 to 8).

To confirm that the formation of form I DNA by the FAS proteins was due to nucleosome assembly, we employed the micrococcal nuclease digestion assay. As shown in Figure 5C, replicated DNA incubated with S100 extract and reticulocyte lysate containing FAS1, FAS2, and AtMSI1 proteins was cleaved into a nuclease-resistant ladder of bands, indicating that an array of nucleosomes had been formed on the DNA in a FAS1-, FAS2-, and AtMSI1-dependent manner.

These data showed that FAS1, FAS2, and ATMSI1 formed a complex with replication-dependent nucleosome assembly activity, just as in the case of the p150, p60, and p48

subunits of hCAF-1. We therefore conclude that FAS1, FAS2, and most likely AtMSI1 are functional counterparts of the p150, p60, and p48 subunits of hCAF-1, respectively.

Unstable Maintenance of Expression Patterns of Meristem Regulator Genes in fas Mutants

Recent studies have suggested a functional interdependence between the replication-coupled chromatin assembly mediated by CAF-1 and the inheritance of epigenetically determined chromosomal states (Enomoto and Berman 1998). The plant counterpart of CAF-1, which contains FAS proteins, may contribute to establishing and maintaining the state of gene expression and/or repression in the apical meristems. We tested this by examining the expression pattern of two genes that are expressed in a subset of cells in apical meristems and are involved in the control of stem cells.

The WUS gene is expressed in a small group of cells beneath the third cell layer of the CZ (Figure 6A and Figure 6E) and plays a critical role in maintaining stem cell identity of the overlying cells (Mayer et al. 1998). In fasSAM, the expression domain of WUS expanded laterally, but not uniformly (Figure 6B–6D and Figure 6F). The WUS expression domain also shifted and/or expanded to outer cell layers, and WUS mRNA accumulation was sometimes detected in L2 cells (Figure 6B–6D and Figure 6F). In some extreme cases, weak signals were detected even in L1 cells (Figure 6F). Thus, in fas mutants, the WUS expression pattern was variable as was the histological appearance of SAM.

Full-size image (715K)
High-quality image (772K)

Figure 6.

WUS and SCR::GFP Expression in fas Mutants

(A–F) WUS mRNA accumulation in SAM of 7-day-old seedlings. (A) Wild type (En), (B–D) fas1-1, (E) wild type (No), and (F) fas2-2. (B)–(D) are three sections selected from serial sections of a single SAM to show the full extent of the WUS expression domain. In (F), WUS mRNA accumulation is observed in some L1 cells (arrowhead). Note that WUS expression is excluded from what seems to be an initiating leaf primordium (arrow). Bar: 25 μ m.

(G–R) SCR::GFP expression in roots. (G) Wild type (En), (H-R) fas1-1. (H) shows a normal pattern in a young lateral root. Note that the columella root cap has not yet fully developed. Ectopic expression is observed in nascent cortex cells (large arrowheads in [I]–[K]), and columella initials and the first tier of columella cells (small arrowheads in [I] and [J]). In (J), one cell in the position of the Co/En initial (arrow) does not expressSCR::GFP. In (L), SCR::GFP is not expressed in a group of endodermal cells (arrows) and the entire RAM region. (M)–(O) Three serial sections from a root tip showing the absence of SCR::GFP in QC cells and possibly a Co/En initial (arrows). (P–R) A similar set of three sections from a

root tip showing the absence of SCR::GFP in the region of QC, Co/En initials and daughter cells.

The SCR gene plays a key role in radial patterning of both shoots and roots ([9] and [50]). In roots, SCR is expressed in QC, cortex/endodermal (Co/En) initials, Co/En daughter cells, and endodermis (Figure 6G). Anticlinal division of a Co/En initial gives rise to a Co/En initial and a Co/En daughter cell, which then divides periclinally to generate a cortex and an endodermal cell. SCR is expressed only in the endodermal lineage. In scrmutants, a Co/En daughter cell generates a file of cells with mixed characteristics of the cortex and the endodermis, suggesting that SCR is required for the asymmetric division of Co/En daughter cells to elaborate the radial pattern of roots (Di Laurenzio et al. 1996). We examined the expression of GFP under the control of a 2.5 kb promoter of SCR (SCR::GFP) (Wysocka-Diller et al. 2000) in roots of fas1-1. As in the case of WUSexpression, the pattern varied from root to root. Young lateral roots exhibited the normal pattern of expression (Figure 6H). In some roots, ectopic expression of SCR::GFP was observed in single cells or small groups of cells adjacent to the cells normally expressing SCR::GFP (Figure 6I–6K). These cells showing ectopic expression of SCR::GFP included cells that appeared to be cortex cells, columella initials, and columella daughter cells. The absence of expression was observed in single cells and small groups of adjacent cells including all types of cells that normally express SCR::GFP (Figure 6J and Figure 6M–6O). In extreme cases, the GFP signal was missing from QC, Co/En initials, and Co/En daughter cells, and the cellular arrangement in RAM was highly distorted (Figure 6L and Figure 6P–6R).

Meristem- and Cell-Proliferation-Associated Expression of the FAS1 and FAS2 Genes

Expression of FAS1 in the aerial part of the seedling was examined by in situ RNA hybridization. FAS1 mRNA accumulated primarily in SAM and young leaf primordia (Figure 7A–7C). Expression in SAM was observed in the outer layers of avacuolate cells in both CZ and PZ (Figure 7A). The signal intensity was not uniform, with some single cells showing stronger signals (Figure 7A and Figure 7B).

Full-size image (664K)
High-quality image (710K)

Figure 7.

Expression of FAS Genes

(A–C) Accumulation of FAS1 mRNA in SAM of young seedlings. (A) and (B) 3-day-old Col. Arrows in (B) indicate leaf primordia. A section of SAM of a 4-day-old FAS1 deletion line hybridized with the same probe has no signal (C). Bar: 25 μ m.

(D–H) Accumulation of FAS1 mRNA during late embryogenesis. (D and E) Torpedo stage. The section in (E) was cut obliquely through a plane between two developing cotyledons (perpendicular to the plane of the section in [D]). Strong signals are observed in embryonic

SAM (arrowhead). (F) Late torpedo stage to early bending cotyledon stage. (G) Bending cotyledon stage. Signals are confined to SAM. (H) A mature embryo in a desiccating seed. No signals are observed. Bar: 25 μ m.

(I–N) Histochemical localization of GUS activity in 3-day-old FAS1(1.3)::GUS and FAS2(2.9)::GUS seedlings. (I, K, and M) FAS1(1.3)::GUS. (J, L, and N) FAS2(2.9)::GUS. (K) and (L), and (M) and (N) are enlargements of the SAM region and the root tip of (I) and (J), respectively. Bar: 50 μ m.

(O) Expression of FAS1 (filled squares), FAS2 (filled triangles), histone H4 (open triangles), and the Arabidopsis DEAD box ATPase/RNA helicase gene (AtDRH1) (open circles), and DNA synthesis (open diamonds) in suspension-cultured T87 cells after stimulation of proliferation by subculture. Levels of mRNA in the case of each of the genes are expressed as values relative to those on day 0.

(P) Expression of pFAS1(1.3)::GUS (filled squares) and the neomycin phosphotransferase gene (NPTII) (filled triangles) in tobacco BY-2 cells during the course of the cell cycle. Levels of mRNA are expressed as values relative to those 9 hr after release from aphidicolin. DNA synthesis (open diamonds) and the mitotic index (open circles) are shown.

During late embryogenesis, FAS1 mRNA could be detected until it disappeared in the mature embryo in the dry seed (Figure 7D–7H). In torpedo-stage embryos, expression was limited to the embryonic SAM region, presumptive RAM, developing cotyledons, and vascular primordia (Figure 7D and Figure 7E). Only single cells or small groups of adjacent cells showed accumulation of FAS1 mRNA, and the intensity of the signal varied among positive cells in a given section (Figure 7D–7F). From the late torpedo stage to the bending cotyledon stage, expression of FAS1 became restricted to the embryonic SAM (Figure 7F and Figure 7G).

The expression pattern of β-glucuronidase (GUS) under the control of a 1.3 kb FAS1 promoter (FAS1(1.1)::GUS) was consistent with the mRNA accumulation pattern observed. In young seedlings, GUS activity was detected in SAM, young leaf primordia, the root tip, and the first lateral root primordium at the hypocotyl/root junction (Figures 7I, 7K, 7M). GUS activity in the root tip region was strongest in RAM and the apical part of the elongation zone that contains dividing cells; weak activity was detected in QC cells (Figure 7M). Similar expression of GUS under the control of a 2.9 kb FAS2 promoter that seemed to contain sufficient regulatory elements for the correct expression (FAS2(2.9)::GUS; see Experimental Procedures) was observed (Figures 7J, 7L, 7N).

The nonuniform, patchy pattern of accumulation observed suggests that FAS1 mRNA turns over asynchronously in different cells, consistent with the notion that FAS1 expression is associated with a specific phase of the cell cycle. To test this, the expression of FAS genes was examined in suspension-cultured cells of Arabidopsis after stimulation of cell proliferation. The rate of DNA synthesis and the levels of FAS1 and histone H4 mRNA exhibited similar changes, whereas the increase in FAS2 mRNA levels was slightly delayed (Figure 7O), suggesting the possibility of S phase–preferential expression of FAS1. Analysis of FAS1(1.1)::GUS expression in cell cycle–synchronized tobacco BY-2 cells revealed an increase in the level of FAS1(1.1)::GUS expression before the second peak of DNA synthesis, indicating that the expression is associated with the G1/S transition (Figure 7P).

Discussion

FAS Genes Are Not Required for Embryogenesis

The embryos of fas mutant plants have normal body plans and organ morphology, suggesting that FAS genes are not required for embryogenesis. SAM in some mature fas embryos is slightly enlarged, which may indicate that FAS genes play a role in the development of embryonic SAM, possibly through maintenance of the WUS expression domain (see below).

FAS Genes Are Required for Maintenance of SAM Organization and Function

Maintenance of distinct functional zones (CZ and PZ) and generative cell layers (L1-L3) in SAM is severely disrupted in fas mutants. This and the failure to maintain the proper SAM size and shape and the proper position of primordium initiation suggest that FAS genes play a role in the function of both CZ and PZ by maintaining the proper cellular organization in SAM. This is in contrast to the roles of CLAVATA (CLV) genes, which promote the transition of cells from CZ to PZ for primordium initiation, but are not required for the PZ function per se ([7] and [23]). CLV genes also differ from FAS genes in that they are specific regulators of SAM. Double mutant analysis suggests that FAS and CLV genes function in different pathways (Leyser and Furner 1992).

In fas SAM, the WUS expression domain expanded laterally. Because the group of WUS-expressing cells acts as an organizing center to direct the fate of the overlying stem cells in CZ (Mayer et al. 1998), the expandingWUS expression domain may cause expansion of the stem cell population in CZ to the peripheral region that would otherwise function as PZ. The WUS expression domain also expanded or shifted in the apical direction, and, in some cases, WUS mRNA accumulation was observed even in L1 cells. Thus, maintenance of the stem cell population itself may also be disturbed. Furthermore, the capacity of cells in the L1 and L2 layers to differentiate into specific cell types (epidermis and mesophyll, respectively) may also be disturbed. These notions are consistent with the SAM phenotypes of fas mutants.

In clv mutants, the WUS expression domain laterally expands and shifts one cell layer up, but it remains as two cell layers (Schoof et al. 2000). It has been proposed that the CLV1 receptor kinase (Clark et al. 1997) and the CLV3 peptide ligand (Fletcher et al. 1999) form a regulatory loop with WUS to maintain the WUS expression domain and the CLV3 expression domain, thereby maintaining functional zones in SAM (Schoof et al. 2000). In contrast to clv mutants, in fas SAM, the WUS expression domain expands apically and is locally more than three layers deep. fas also differs from clv in that the expansion is not uniform in either the lateral and or the apical direction. These observations imply that the mechanism of WUS regulation differs between pathways mediated by CLV and FAS (see below).

FAS Genes Are Required for Maintenance of RAM Organization and Function

FAS genes have not previously been implicated in RAM function, but our observations clearly suggest a role for FAS genes in the maintenance of RAM organization and function. RAM formation during embryogenesis and lateral root initiation are not affected in fas mutants, and nascent RAM seems normal (Figure 1) (H. K. and T. A., unpublished data), indicating that FAS genes are not required for RAM formation per se. However, regular cellular organization of RAM is not maintained during subsequent growth in fas mutants. The reduced cell population in the elongation zone in fas roots suggests a

defect in regulating cell proliferation in the elongation zone and/or in the activity of initials that supply cells to the elongation zone.

It has been suggested that the activity of initials is under the influence of a short-range signal from adjacent QC cells, and a long-distance signal from more mature regions of the root ([47] and [48]). In developed fas roots, it is difficult to unequivocally identify QC cells. Cells in the position of QC frequently lose their characteristic marker (SCR::GFP) or exhibit characteristics of differentiated columella cells (starch granules). Therefore, it is likely that FAS genes regulate the activity of initials at least in part via their role in QC cells.

FAS1 and FAS2 Gene Products Are Subunits of the Arabidopsis Counterpart of CAF-1

FAS1 and FAS2 proteins have similarity to the largest two subunits of CAF-1. In the Arabidopsis genome, there are at least four candidate genes for the smallest subunit ([1] and [21]). Among them, AtMSI1 has the highest similarity to hCAF-1 p48 and Cac3p. Based on our observations that FAS1, FAS2, and AtMSI1 form a complex in vitro and that this complex has replication-dependent nucleosome assembly activity in a post-replicative assembly system, we conclude that FAS1, FAS2, and most likely AtMSI1, are subunits of the Arabidopsis counterpart of CAF-1. fas mutants represent the first examples of mutants defective in CAF-1 in multicellular organisms. The phenotypes of fas mutants described above clearly show the role of CAF-1 in postembryonic development of Arabidopsis (also discussed below).

FAS genes are expressed predominantly in apical meristems. FAS1 mRNA accumulation is not uniform over the expression domain with some single cells showing stronger signals. Analysis using Arabidopsis T87 and tobacco BY-2 cells suggests that FAS1 expression is associated with the G1/S transition. This is consistent with the role of CAF-1 in replication-dependent nucleosome assembly.

FAS Genes Facilitate Stable Maintenance of Gene Expression in Apical Meristems

In fas mutants, the WUS expression domain in SAM and the SCR expression domain (monitored as SCR::GFP) in RAM are not stably maintained. The pattern of misexpression is not constant, showing a wide range of variation among individual meristems, and the degree of misexpression tends to become more severe with time. In addition, the change in the expression state is not in one direction. These points are clearly illustrated bySCR::GFP. fas roots, which show ectopic expression or loss of expression, or both, in a single RAM, and the pattern is highly variable among RAMs between plants and even within a single plant. Ectopic expression or loss of expression is restricted to small groups of cells. Cells with ectopic SCR::GFP expression are always adjacent to cells which share a common progenitor and have the proper expression state. These observations may suggest that, in the case of these cells in fas mutants, one or both of the daughter cells after division fail to maintain expression state of SCR::GFP. Since the fas mutations analyzed here are likely to be null alleles for chromatin assembly activity, these varied patterns seem to reflect a stochastic rather than a partial loss-of-function phenotype. Importantly, these phenotypes are quite distinct from those of other mutants, such as clv orshort-root, in which abnormal expression patterns of WUS and SCR::GFP, respectively, are fairly consistent ([15] and [39]).

It is likely that the functional role of the FAS genes is to facilitate stable maintenance of gene expression states in apical meristems. CAF-1 is most likely a physiologically relevant factor

involved in chromatin assembly behind DNA replication forks. We therefore favor the hypothesis that the FAS complex possibly ensures stable propagation of epigenetic states of chromosomes through facilitating chromatin assembly during DNA replication. Rapid re-formation of nucleosomes onto newly replicated DNA by CAF-1 would prevent transcriptional regulators from being targeted to the DNA nonspecifically, thereby preventing random changes in gene expression patterns in daughter cells. Additionally, since mammalian CAF-1 binds to heterochromatin proteins (HP-1), it may promote inheritance of epigenetically silenced chromatin by recruiting proteins that determine localized heterochromatinization of genes after DNA replication (Murzina et al. 1999). The availability of probes for several regulator genes involved in the formation and/or maintenance of either SAM or RAM should enable further testing of this hypothesis.

CAF-1-Mediated Chromatin Assembly Plays an Important Role in Postembryonic Development, but Not in Early Embryogenesis

fas embryos have normal body plans and normal organ morphology, suggesting that a defect in CAF-1-mediated chromatin assembly during DNA replication does not affect early embryogenesis. During embryogenesis inArabidopsis, the zygote gives rise to a mature embryo that consists of 15,000–20,000 cells patterned into apical-basal and radial organization within 14 days (Jürgens 1994). This rather rapid and programmed development is protected within a seed and proceeds autonomously, being little affected by external environmental conditions. We hypothesize that maintenance of epigenetic state during early embryonic development might be ensured by an abundance of transcriptional regulators and other chromatin assembly factors, such as RCAF, which might substitute for CAF-1 (Tyler et al. 1999). In contrast, most postembryonic development in apical meristems proceeds by perceiving and responding to various stimuli. Pluripotent stem cells in apical meristems could differentiate in a stochastic manner. The chromatin states of these pluripotent stem cells may be less committed than in the case of differentiated cells, and these stem cells may contain a variety of transcriptional regulators that could change gene expression upon stimulation. Without rapid assembly of chromatin by CAF-1 behind the DNA replication fork, epigenetic states of parental strands may easily change in a stochastic manner.

Experimental Procedures

Plant Materials

The original alleles of fas1 (Reinholz 1966) from ecotype Enkheim (En) and fas2 (Leyser and Furner 1992) from Landsberg er (L er) were obtained from O. Leyser and renamed fas1-1 and fas2-1, respectively. Four novel EMS alleles, fas1-2 (NoM_2-50-#103/fas), fas1-3 (NoM_2-82-fasV), fas2-2 (NoM_2-96-fasX), and fas2-3 (NoM_2-23-fasW), were isolated from ecotype Nossen (No). The FAS1-FT deletion line, vTAAT26C51 has been described elsewhere (Kaya et al. 2000).

Histological Analysis

Mature embryos from dry seeds were prepared for confocal laser scanning microscopy as described (Running et al. 1995). For SAM observation, seedlings were fixed with FAA, embedded in Technovit 7100 resin (Kulzer, Wahrheim), and stained in 4 μ m sections with Toluidine blue. Whole-mount preparations of mature embryos and RAM were prepared, stained with propidium iodide, and observed using a confocal laser scanning microscope

(Zeiss LSM 410). Starch staining was performed as previously described (van den Berg et al. 1997).

Complementation Test of FAS2

A 7.0 kb fragment of the FAS2 gene (corresponding to the region 49490–56481 of MUB3) was introduced into fas2-2 plants and the phenotype was examined for complementation.

Transcript Analysis of fas2-2

A fas2-2 cDNA was amplified by RT-PCR with the primers FAS2 RT-1 (5′-TAACCGTAGATTTTCACCCGATTTC-3′) and FAS2 RT-2 (5′-TGCTTTTTCTCCTCACCATCAACAG-3′) and sequenced using the FAS2 RT-1 primer.

Expression of FAS1, FAS2, and AtMSI1

cDNA clones were obtained by RT-PCR with total RNA from Arabidopsis T87 cells as the template (Axelos et al. 1992). The primers used were: 5′-AGTATTAATACGACTCACTATAGGAAACAGACACCATGGACGAAGTTTCGACG-3′ and 5′-ACGCGTCGACCAAATAAAGATGGAAACTGTG-3′ for FAS1; 5′-CCGGAATTCATGAAGGGAGGTACGATACAGA-3′ and 5′-ACGCGTCGACAAAAACACTGATGGAAAATGAC-3′ for FAS2; and 5′-GGAATTCCATGGGGAAAGACGAAGAGGAAAT-3′ and 5′-GCGGATCCTGCGCTTTGGATACTAAATAACA-3′ for AtMSI1. A FAS2 cDNA cloned in pBSII (Stratagene) (EcoRI and SalI sites) was digested with EcoRI and XhoI and subcloned into pCITE-4a (Novagen). AnAtMSI1 cDNA cloned in pBSII (EcoRI and BamHI sites) was subcloned into pCITE-2 after digestion with NcoI and BamHI. In vitro transcription/translation reactions were performed using the TNT quick coupled transcription/translation system (Promega) according to the manufacturer's instructions. FAS1 was expressed by in vitro transcription of PCR products with the T7 promoter in the primer sequence followed by in vitro translation.

Complex Formation Assay and Replication-Dependent Chromatin Assembly Assay

In the complex formation assay, the coupled transcription-translation products of FAS1, His-FAS2, and AtMSI1 were mixed with Ni-NTA agarose (Qiagen) in buffer A200 (25 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10% glycerol, 200 mM NaCl, and 0.01% NP-40). After incubation at 4°C for 2 hr, the beads were washed five times with 600 μ l of buffer A200, and the precipitated proteins were then subjected to SDS-PAGE.

Chromatin assembly activity was assayed by a two-step system (Shibahara et al. 2000). Six μ l of the effluent from a spin column was mixed with S100 extract from human 293 cells (56 μ g of protein), topoisomerases I (13 ng)/II (100 ng), and mixtures of reticulocyte lysates containing the products of FAS1, His-FAS2, or AtMSI1 as indicated. The micrococcal nuclease assay was performed as previously described (Shibahara and Stillman 1999)

In Situ RNA Hybridization

Seedlings were fixed with 4% paraformaldehyde in PBS. Eight μ m paraffin sections were hybridized with digoxygenin-labeled probes as described (Braissant and Wahli 1998) with

the modification of M. Heisler. TheFAS1 and WUS probes were antisense strands corresponding to the region 1536–2454 of the FAS1 ORF (DDBJ/EMBL/GenBank accession number AB027229) and to 101–1202 of WUS cDNA (DDBJ/EMBL/GenBank accession number AJ012310), respectively.

Imaging of SCR::GFP Expression

F2 seeds from a cross between the SCR::GFP line (Wysocka-Diller et al. 2000) and fas1-1 were kindly provided by H. Fukaki and P. Benfey. fas1-1 and wild-type siblings carrying the transgene were examined. GFP fluorescence was visualized in whole mounts using a confocal laser scanning microscope (Zeiss LSM 410) with an argon laser. The FITC channel (green: GFP) was overlaid onto the TRITC channel (red: propidium iodide) to permit identification of GFP-positive cells.

FAS1::GUS and FAS2::GUS Transgenic Plants

The β-glucuronidase (GUS) coding sequence in pBI101 (Clontech) was fused downstream of a 1327 bp SspI-HindIII fragment of FAS1 (positions 2656–3982 in F5I14) to generate a translational fusion (FAS1(1.1)::GUS). A similar translational fusion (FAS2(2.9)::GUS) was constructed using a 3070 bp EcoRI-ClaI fragment from FAS2 (positions 49538–52607 in MUB3). The promoter of FAS2(2.9)::GUS is only 50 bp shorter than that in a complementing construct. Transgenic plants (Col) were generated as described (Bechtold et al. 1993). GUS histochemical staining was performed as described (Taoka et al. 1999).

Expression of FAS1 and FAS2 in Suspension-Cultured Cells

Arabidopsis suspension cell line T87 was cultured in 100 ml of JPL medium (Axelos et al. 1992). Four ml of packed cells was transferred to 100 ml of fresh medium every 7 days. Cells were harvested for analysis at 1 day intervals, from day 0 (just before subculture) to day 7. The rate of DNA synthesis was determined as previously described (Ohtsubo et al. 1997). The probes used for RNA blot analysis were PCR-amplified: a 2189 bp fragment of FAS1, a 1135 bp fragment of FAS2, a 201 bp fragment of the Arabidopsis H4 gene H4A748 (Chaboute et al. 1987), and a 135 bp fragment of the Arabidopsis DEAD box ATPase/RNA helicase geneAtDRH1 (Okanami et al. 1998). Information on the primers is available upon request.

Expression Analysis Using Tobacco BY-2 Cells

Expression of pFAS1(1.1)::GUS during the course of the cell cycle was analyzed in tobacco BY-2 cells. Transformation, cell cycle synchronization, and analysis of the expression of transgenes were performed as previously described (Taoka et al. 1999).

Acknowledgements

We thank H. M. O. Leyser, H. Fukaki, P. Benfey, M. Axelos and the RIKEN Genebank, the NSF-supported ABRC, S. Tabata, S. Sato, M. Heisler, M. Aida, M. Tasaka, T. Oyama, S. Ishiguro, and K. Okada for materials and advice, T. Meshi for helpful discussions, and R. Martienssen for comments. This work was supported by Grants-in-Aid for Special Research on Priority Areas (Nos. 08273210, 09262208, 10158207, and 10182101) from the Ministry of Education, Science, Sports, and Culture of Japan (to T. A.), a grant through the “Research for

the Future" Program from the Japan Society for the Promotion of Science (JSPS-RFTF96L00403) (to T. A.), and a grant from the US National Cancer Institute (CA13106) (to B. S.). K. S. was supported by the Career Development Award of the Leukemia and Lymphoma Society.

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