The rice *FLATTENED SHOOT MERISTEM*, encoding CAF-1 p150 subunit, is required for meristem maintenance by regulating the cell-cycle period

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Abstract

We isolated flattened shoot meristem (fsm) mutants in rice that showed defective seedling growth and died in the vegetative phase. Since most fsm plants had flat and small shoot apical meristems (SAMs), we suggest thatFSM is required for proper SAM maintenance. FSM encodes a putative ortholog of Arabidopsis FASCIATA1 (FAS1) that corresponds to the p150 subunit of chromatin assembly factor-1 (CAF-1). FSM is expressed patchily in tissues with actively dividing cells, suggesting a tight association of FSM with specific cell-cycle phases. Double-target in situ hybridization counterstained with cell-cycle marker genes revealed that FSM is expressed mainly in the G_1 phase. In fsm, expressions of the two marker genes representing S- and G_2- to M-phases were enhanced in SAM, despite a reduced number of cells in SAM, suggesting that S- and G_2-phases are prolonged in fsm. In addition, developmental events in fsm leaves took place at the proper time, indicating that the temporal regulation of development occurs independently of the cell-cycle period. In contrast to the fasciated phenotype of Arabidopsis fas1, fsm showed size reduction of SAM. The opposite phenotypes between fsm and fas1 indicate that the SAM maintenance is regulated differently between rice and Arabidopsis.

Keywords: Flattened shoot meristem; Rice; Apical meristem; CAF-1; Cell cycle; Fasciata1

Introduction

In higher plants, all above- and below-ground organs are produced from shoot apical meristems (SAMs) and root apical meristems (RAMs). These meristems maintain their pluripotency and continue to produce new organs and tissues throughout the vegetative and reproductive phases. Thus, the mechanism of how the SAM is properly maintained is of primary importance in developmental plant biology.

Several genes that participate in the maintenance of SAM have been identified in Arabidopsis. SHOOT MERISTEMLESS (STM) and WUSCHEL (WUS) were identified from their mutants with perturbed SAM formation and/or maintenance ([Barton and Poethig, 1993] and [Laux et al., 1996]). STM (Long et al., 1996) is homologous to maize KNOTTED1 (Hake et al. 1989) and rice OSH1 (Matsuoka et al., 1995). These genes are expressed in indeterminate cells and are estimated to be required for SAM maintenance and formation ([Jackson et al., 1994] , [Long et al., 1996] and [Matsuoka et al., 1995]). WUS encodes a homeodomain protein and is predicted to act as a transcription factor (Mayer et al., 1998). WUS is expressed in a small group of cells beneath the third cell layer of the CZ (central zone) of SAM in wild type, and plays a crucial role in the maintenance of stem cell identity of the overlying cells (Mayer et al., 1998). CLV genes were identified from their mutants showing enlarged SAM phenotypes ([Clark et al., 1993] and [Clark et al., 1995]), indicating that the CLV genes function to restrict the size of the stem cell population. In addition, WUS promotes CLV3 expression, while CLV3 limits WUS expression ([Brand et al., 2000] and [Schoof et al., 2000]), indicating thatCLV3–WUS interaction controls the size of stem cell population in SAM.

Another issue in meristem maintenance is how the cell cycle is associated with SAM maintenance, since tightly regulated cell divisions in SAM help guarantee the regular and on-going formation of leaf primordia and stems. Concerning this issue, FASCIATA1 (FAS1) and FAS2 in Arabidopsis are good candidate genes for regulating SAM and RAM maintenance through its effects on cell-cycle progression (Kaya et al., 2001). FAS1 andFAS2 were originally characterized from their mu tant phenotypes: fasciated stems associated with SAM enlargement, abnormal phyllotaxy, dentate and narrow leaves, and altered floral development (Leyser and Furner, 1992). They also show disturbed cellular organization in SAM and RAM, and unstable expression patterns of WUS and SCR that regulate SAM and RAM functions respectively, suggesting that FAS1 and FAS2 are required for the stable maintenance of specific gene expression in apical meristems (Kaya et al., 2001). In addition to previously reported defects, Exner et al. (2006) reported that fas1 and fas2 also have defects in hypocotyl growth, ploidy level, and leaf hair differentiation, and Kirik et al. (2006) reported that a new fas1 allele having T-DNA insertion exhibits reduced apical dominance, severely retarded growth, and reduced organ size. Therefore, FAS1 and FAS2 play pleiotropic roles in plant development as well as SAM and RAM maintenance.

FAS1 and FAS2 encode the p150 and p60 subunits of CAF-1, respectively (Kaya et al., 2001). CAF-1 consists of three subunits, p150, p60, and p48, and it was originally identified as a component required for in vitro replication-dependent chromatin assembly in human cell nuclear extract (Smith and Stillman, 1989). CAF-1 interacts with the de novo histone H3 and H4 and mediates their deposition onto replicating DNA ([Ridgway and Almouzni, 2000] , [Smith and Stillman, 1991] and [Tagami et al., 2004]). In Arabidopsis, FAS1 (p150), FAS2 (p60), and AtMSI1 (p48) were co-immunoprecipitated in vitro, and a FAS1–FAS2–

AtMSI1 complex had nucleosome assembly activity in vitro (Kaya et al., 2001). In addition, fas1 fas2 double mutants were indistinguishable from either of the single mutants. Therefore, these three proteins constitute a functional CAF-1 complex ([Kaya et al., 2001] and [van Nocker, 2003]). Although CAF-1 is essential for viability in vertebrates ([Houlard et al., 2006] and [Quivy et al., 2001]), fas1, fas2 ([Kaya et al., 2001] and [Kirik et al., 2006]) and yeast mutants in CAF-1 subunits ([Game and Kaufman, 1999] and [Kaufman et al., 1997]) were viable, suggesting functional diversity of CAF-1 among taxa. Several reports have shown that CAF-1 is required for cell-cycle progression. Depletion of the p150 subunit of CAF-1 in human cells leads to S-phase accumulation and delayed DNA replication (Hoek and Stillman, 2003). The chicken DT40 B-cell line with depleted CAF-1 p150 or p60 shows delayed S-phase progression (Takami et al., 2007). Thus, CAF-1 is tightly associated with cell-cycle progression in animals. In plants, several works suggested CAF-1's role on cell-cycle progression ([Exner et al., 2006] , [Kaya et al., 2001] and [Ramirez-Parra and Gutierrez, 2007]). However, the correlation between the defects in cell-cycle progression caused by CAF-1 loss of function and SAM maintenance is poorly understood.

Here we report recessive mutants, flattened shoot meristem (fsm), which exhibit a flat SAM and eventual SAM loss in the vegetative phase. We show that FSM has a high similarity to Arabidopsis FAS1. The SAM ofArabidopsis fas1 was fasciated and correlated with the expanded and variable expression patterns of WUS (Kaya et al., 2001). In contrast, fsm SAM had reduced number of cells correlated with attenuated expression ofOSH1. Eventually most of fsm plants resulted in lethality in the vegetative phase. FSM is expressed in a patchy pattern in tissues in which cell divisions are active. We also suggest that FSM is expressed exclusively in the G_1-phase of the cell cycle. In addition, in spite of a reduced number of cells in the SAM, fsm showed increased levels of histone H4 and cdc2Os3 (Oryza;CDKB2;1) expression in the SAM, suggesting that fsm has prolonged S- and G_2-phases. Therefore, FSM is predicted to regulate proper development in rice including apical meristem maintenance by regulating the durations of the S- and G_2-phases of the cell cycle through its chromatin assembly activity. Furthermore, from the quite opposite SAM phenotypes between Arabidopsis fas1 and rice fsm, we suggest that the mechanisms of the SAM maintenance might be diverged between eudicots and grass family.

Materials and methods

Plant materials

During the course of screening for mutants defective in meristem maintenance, fsm-1 was identified as a single-gene recessive mutant from an M_2 population of rice (Oryza sativa L.) cv Kinmaze mutagenized with N-methyl-N-nitrosourea. Two other alleles, fsm-2 and fsm-3, were found from Tos17 insertion lines of cv. Nipponbare (http://www.sciencedirect.com/science?_ob=RedirectURL&_method=externObjLink&_locator=url&_issn=00121606&_origin=article&_zone=art_page&_plusSign=%2B&_targetURL=http%253A%252F%252Ftos.nias.affrc.go.jp%252F). Mutant and wild type plants were grown in MS medium (Murashige and Skoog, 1962) containing 3% sucrose and 0.3% gelangum or in a paddy field or pots under natural conditions.

Paraffin sectioning

Mature embryos, leaves, shoot apices, and roots were fixed in a 1:1:18 solution of formaldehyde, acetic acid, and 50% ethanol (FAA). They were dehydrated in a graded

ethanol series and replaced with xylene, and embedded in Paraplast Plus (McCormic Scientific, MO). The embedded samples were sectioned 8 μm thick using rotary microtome and stained with Delafield's hematoxylin or 0.05% toluidine blue.

Scanning electron microscopy (SEM)

Samples were fixed in FAA for 12 h, dehydrated in a graded ethanol series and substituted with 3-methyl-buthyl-acetate. The samples were critical-point dried, sputter-coated with platinum, and observed under a scanning electron microscope (S-4000; Hitachi Ltd., Tokyo) at an accelerating voltage of 10 kV.

In situ hybridization

Samples were fixed in 4% paraformaldehyde and embedded in Paraplast Plus after dehydration and replacement with xylene as described above. Paraffin sections were prepared 8 μm thick and applied to slide glasses coated with aminopropylytriethoxy silane (APS; Matsunami Glass Ind., Osaka, Japan). Digoxigenin-labeled antisense probes were prepared from the cDNAs of histone H4, cdc2Os3, FSM, and OSH1. We also prepared biotin-labeled antisense probes from the cDNAs of histone H4. For single-target in situ hybridization, we followed the method of Kouchi and Hata (1993). For double-target in situ hybridization, we carried out hybridization, posthybridization washes, and blocking according to the method of Kouchi and Hata (1993), except that we used a hybridization solution that contained both DIG–RNA and biotin–RNA probes. For the detection of biotin-labeled probe, we performed tyramide signal amplification (TSA) according to the manufacturer's instruction for its TSA biotin system (Perkin Elmer, Waltham, MA). After the blocking washes, slides were incubated for 1 h at room temperature with 1:100 diluted horseradish peroxidase-labeled streptavidin. After three washes in TNT wash buffer (0.1 M Tris–HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20), biotinyl tyramide solution at the manufacturer's recommended dilution was applied to the sections. After three washes in the TNT wash buffer, 1 mg/mL fluorescein-conjugated streptavidin (Invitrogen) in PBS was applied to the sections for at least 1 h. The fluorescent signals were developed in the dark. After the pre-detection of signals for the biotin-labeled probe, the slides were incubated for 1 h at room temperature with 1:1000 diluted alkaline phosphatase-conjugated anti-DIG antibody solution (Roche) for the detection of DIG-labeled probe. After three washes in a buffer (0.1 M Tris–Cl pH 8.0, 0.1 M NaCl, 10 mM $MgCl_2$), the HNPP/Fast Red TR solution (Roche) was applied to the sections. The sections were incubated for 30 min at room temperature in the dark. The slides were washed in fresh water and mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen). The hybridization signals were observed with fluorescence microscopy (BZ-8000; Keyence Co., Tokyo, Japan).

To estimate the frequency of histone H4 and cdc2Os3 signals in SAM, we first counted the number of SAM cells in the median section. Next we counted the number of histone H4 or cdc2Os3 signals in the SAM and divided the number of signals by the number of SAM cells. Ten SAMs were examined for each gene in wild type and fsm plants.

Map-based cloning

For map-based cloning of the FSM gene, we crossed FSM/fsm-1 (O. sativa L. subsp. japonica auct) with cv Kasalath (O. sativa L. subsp. indica Kato). For the rough mapping, we selected 14 fsm plants from the F2 population. Using multiple restriction

fragment length polymorphism, sequence-tagged sites, and cleaved-amplified polymorphic sequence (CAPS) markers, the FSM locus was mapped around 155 cM on the long arm of chromosome 1 between two CAPS markers R1764 and S10526 (Fig. S1). Fine mapping using 116 F_2 plants confined the FSM locus within 300-kb region covered by the three BAC clones B1078G07, P0696G06, and P0674H09. The Rice Genome Automated Annotation System (Sakata et al., 2002) predicted about 100 genes in this region. Among the 100 genes, we searched for a gene with annotations that suggested abnormalities in cell divisions and SAM maintenance, and found a putative FAS1 gene (Fig. S1).

For complementation test, the 12.5-kb Apa1 fragment, including the FSM candidate and 4.5-kb direct upstream of initiation codon, was cloned into binary vector pPZP2H-lac and introduced into fsm-1 homozygotes by the Agrobacterium tumefaciens-mediated transformation method (Hiei et al., 1994).

To determine the putative FSM cDNA sequence, we extracted total RNA from wild type plants, reverse transcribed the RNA, and performed PCR with the primers C1F3 (5′-TGAACATCGCCATTTCCCAG-3′) and C6R (5′-CTTGCTGCTGCAGACCATTGT-3′) to amplify the 3 kb fragment including putative start and termination codons. Next we cloned and sequenced the PCR product. Exon–intron structures were deduced by comparing the cDNA sequence to the genomic sequence.

Phylogenetic analysis

Multiple sequence alignments were performed using ClustalW (EMBL-EBI, http://www.sciencedirect.com/science?_ob=RedirectURL&_method=externObjLink&_locator=url&_issn=00121606&_origin=article&_zone=art_page&_plusSign=%2B&_targetURL=http%253A%252F%252Fwww.ebi.ac.uk%252FTools%252Fclustalw%252Findex.html) program. The phylogenetic tree was constructed based on the well-conserved D/ED domain by the neighbor-joining method using ClustalW and TreeView (Page, 1996) programs.

Flow cytometric analysis

Mature leaves of wild type and fsm were cut into small pieces in 430 µL of extraction buffer (CyStain® UV Precise P, Partec, Münster, Germany), incubated for 30 min at room temperature, filtered through a 20-µm mesh, mixed with 1.6 mL of nuclear staining buffer (Partec) and incubated further for 30 min at room temperature. The samples were analyzed with a Partec Ploidy Analyzer (Partec). For quantification, the results of three independent measurements were averaged.

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under Accession Numbers At1g65470 (FAS1), BC067093 (H. sapiens CAF-1 p150), BC053740 (M. musculus CAF-1 p150), andABA10486 (Zea mays fasciata 1-like protein).

Results

Phenotypes in the embryo and vegetative phases

fsm-1 was first identified by its dwarf and apparently weak seedlings that died frequently during vegetative phase (Fig. 1A). We also identified the other two alleles, fsm-2 and fsm-3, from the screening in Tos17 insertion lines after the identification of the FSM gene ([Hirochika, 2001] and [Hirochika et al., 1996]). Since fsm-1 and fsm-2 showed similar and severer phenotypes than fsm-3, we used mainly fsm-1 in the following experiments. Although fsm-1, fsm-2, and fsm-3 showed a high germination rate (more than 90%), fsm seedlings began to die around 7 days after germination (DAG); at 10 DAG, about 20% of fsm-1 and fsm-2 plants and 5% of fsm-3 plants died. Finally, most of the fsm plants (> 90%) died before the reproductive phase. Although almost all main shoots died within 1 month, a few tillers eventually survived to the reproductive phase.

Full-size image (204K)
High-quality image (895K)

Fig. 1.

Phenotypes of fsm. (A) One-week-old seedlings of wild type, fsm-1, fsm-2, and fsm-3. (B and C) Mature embryos of wild type (B) and fsm-1 (C). sm, SAM; ra, radicle. (D and E) Embryonic SAM of wild type (D) andfsm-1 (E). White lines show boundaries of cells in the SAM. (F and G) Cross sections of third leaves in wild type (F) and fsm-1 (G). Arrowheads indicate bulliform cells. (H and I) Enlarged view of midriv in wild type (H) and fsm-1 (I). (J) Leaf emergence rate in wild type and fsm-1. (K and L) Formation of large vascular bundles (arrowheads) in P2 leaf primordium of wild type (K) and fsm-1 (L). (M and N) Onset of ligule protrusion (arrows) in P3 leaf primordium of wild type (M) and fsm-1 (N). Bars = 3 cm in panel A, 200 μm in panels B, C, F, G, K, and L; 50 μm in panels D, E, H, and I.

Since seedling abnormalities of fsm were observed from the germination stage, we examined embryos. The mature wild type embryo has three leaf primordia on the flank of a conical-shaped SAM and a radicle (Figs. 1B and D). In contrast, the fsm mature embryos had only one or two leaf primordia and aberrant SAM comprising a reduced number of cells (Figs. 1C and E). In addition, large cells were frequently observed in the SAM and other tissues of fsm (Figs. 1D and E). The fsm radicle was short and small (Figs. 1B and C).

Cross sections of mature fsm leaves revealed that bulliform cells, which were formed on the adaxial epidermis of the leaf blade between two flanking vascular bundles in the wild type (Fig. 1F, arrowheads), became enlarged and covered a broader region of the adaxial epidermis (Fig. 1G). In the vascular bundles, phloem and xylem cells were not well-differentiated (Figs. 1H and I). Although the growth of shoots was suppressed, the leaf emergence rate (plastochron) of fsm was comparable to that of the wild type (Fig. 1J). Also, developmental events in fsm leaves, such as vascular bundle initiation and ligule protrusion, took place at the proper timing; large vascular bundles were first visible in the P2 leaf primordium (Figs. 1K and L, arrowheads), with ligule protrusion in the P3 leaf primordium (Figs. 1M and N, arrowheads).

SAM phenotypes

Because most of the fsm plants stopped new leaf production and died during vegetative development, it is thought that fsm has a defect in the maintenance of SAM. To confirm this, we observed shoot apices of vegetative plants. In the wild type, SAM was dome-shaped throughout the vegetative phase (Figs. 2A, C, and F). On the other hand, in fsm, SAM at 7 DAG was quite flat and consisted of reduced number of cells (Figs. 2B and E). At 30 DAG, most of the SAMs of primary shoots were rudimentary (Fig. 2D). The SAMs were frequently lost in the main shoot (Fig. 2H) or rudimentary even in tillers that survived after the primary shoot aborted (Fig. 2G).

Full-size image (127K)
High-quality image (599K)

Fig. 2.

Shoot apex of fsm. (A) One-week-old wild type. (B) One-week-old fsm-1. (C) One-month-old wild type. (D) One-month-old fsm-1. (E) The number of cells in 1-week-old SAM of wild type and fsm-1. (F and G) Scanning electron micrographs of 45-day-old shoot apices in wild type (F) and fsm-1 (G). (H) The shoot apex of one of the dead fsm-2 plants. (I and J) The expression of OSH1 in wild type (I) and fsm-1 (J) shoot apices in 7-day-old seedlings. Arrowheads indicate SAM or vestige of SAM. P1, P2 indicate P1 and P2 leaf primordia, respectively. Bars = 50 μm in panels A–D; 100 μm in panel I and J; 200 μm in panel H.

To reveal the course of SAM disappearance, we examined the expression of OSH1, a knotted1-type homeobox gene that marks indeterminate cells in SAM and is thought to be required for SAM maintenance (Fig. 2I). In the shoot apices of 7 DAG fsm seedlings, the expression of OSH1 was attenuated in SAM (Fig. 2J). This shows that indeterminate cells of fsm SAM are lost at the vegetative stage. Thus, fsm has defects in the maintenance of indeterminate cells, resulting in the loss of SAM and finally in the abortion of the plants.

Root phenotypes

In addition to the above-ground organs, fsm showed abnormalities in the roots. The rice root system consists of seminal, crown and lateral roots (Itoh et al., 2000). At 7 DAG, the length of seminal roots and the number of crown roots were reduced in fsm (Figs. 3A and B). This suggests that fsm is also defective in the maintenance of RAM.

Full-size image (122K)
High-quality image (529K)

Fig. 3.

Root and reproductive development in fsm. (A) Length of seminal root at 10 days after germination in wild type and fsm-1. (B) The number of crown roots at 10 days after germination in wild type and fsm-1. (C and D) Longitudinal section of root apex in wild type (C) and fsm-1 (D). Initial cells, epidermis, and central stele are marked by asterisks, gray, and white outlines of its cells, respectively. (E and F) Cross sections of roots in wild type (E) and fsm-1 (F). (G) Mature plants of wild type (left) and fsm-1 (right) at flowering stage. (H) Spikelet of wild type. (I) Spikelet of fsm-1. (J) Flower of wild type. (K) A typical flower of fsm-1. (L) Severe spikelet offsm-1 showing increased number of palea and lemma. (M) Severe flower of fsm-1. Stamens and pistils are immature. pa, palea; le, lemma; st, stamen; pi, pistil. Bars = 100 μm in panels E and F; 20 cm in panel G.

The inner structure of roots was examined by sectioning. In wild type RAM, the cell lineage in the apical-proximal direction is quite regular. Cell lineages of the epidermis, cortex, and central stele converged to four common initial cells (only two initial cells marked by asterisks in Fig. 3C are visible in one longitudinal section) through a regular pattern of cell divisions (Fig. 3C). That is, each initial cell contributes to the three tissues. Notably, in fsmroots, two initial cells aligned in the longitudinal direction were identified in one section, with each initial cell contributing to only one or two tissues (Fig. 3D). This aberrant cell lineage suggests that the original initial cell may eventually abort, and another cell functions as a new initial cell.

Irregular cell files were also observed along the radial axis, suggesting abnormal patterns of cell division (Figs. 3E and F). In cross sections just above the root apex of the wild type, regular layer structures from the circumference inward are conserved; that is, the epidermis, cortex, endodermis, pericycle, and a central core of vascular tissue are radially formed (Fig. 3E). In fsm, however, the radial layer structure was disrupted, and aberrant cells and a wide variation in cell size were observed (Fig. 3F). In addition, cell and tissue differentiation was disrupted. Cortex and epidermal cells seemed parenchyma-like, and cell differentiation in the vascular bundle was incomplete. These observations show that fsm has defects in RAM maintenance and cellular organization in roots.

Phenotypes in reproductive phase

The majority of fsm plants died before flowering, but only a few plants showed reproductive development. The mature fsm plants at the flowering stage were dwarfed, and the leaves were short and narrow as in the early vegetative phase (Fig. 3G). The panicle (inflorescence) of fsm was quite small (4–6 cm long), about one third of the wild type panicle, and comprised reduced numbers of primary branches and spikelets. Several vestiges of primary branch meristems were observed, indicating that maintenance of the primary branch meristems was impaired in fsm. The lemma and palea in fsm spikelets were narrow (Figs. 3H and I). Flowers were almost normal except that the stamens and pistils were underdeveloped (Figs. 3J and K). Only one panicle of fsm-1 showed severe spikelet phenotypes with altered number of floral organs (Figs. 3L and M).

Identification of the FSM gene

To investigate the molecular function, we isolated the FSM gene by a map-based cloning strategy. The FSM locus was mapped around 155 cM region on the long arm of chromosome 1 (Fig. S1). Because a homolog ofArabidopsis FAS1, whose mutant phenotypes partially resemble those of fsm, is positioned in this region, we examined the putative FAS1 sequence

in the fsm. Genome sequencing revealed that fsm-1 had one base deletion of C3273 in the putative FAS1 gene (Fig. 4A). This deletion causes a frame shift and generates a stop codon 27 amino acids downstream (Figs. 4A and B). To clarify whether the candidate gene represented FSM, we performed a complementation test by introducing a 12.5-kb genomic fragment containing the candidate gene into fsm-1 homozygous plant. This fragment rescued the mutant seedling phenotypes (Fig. S2). In fsm-2 and fsm-3, one copy of Tos17 was inserted in this gene (Figs. 4A and B). Accordingly, we conclude that this gene is FSM. We cloned the full-length cDNA and found that it consists of 2823 bp, and the protein comprises 940 amino acid residues (Fig. 4B). A comparison of the genome and cDNA sequences revealed that the gene is composed of 12 exons (Fig. 4A). The fsm-1 has one base deletion in the sixth exon, and fsm-2 and fsm-3 haveTos17 insertions in the first and fifth exon, respectively (Fig. 4A).

Full-size image (176K)
High-quality image (676K)

Fig. 4.

Structure of FSM gene. (A) Exon–intron structure. Boxes indicate exons. Mutation sites of the three fsm alleles are indicated. (B) Deduced amino acid sequence of FSM. Single line corresponds to KE domain and double lines to D domain. Locations of the mutations in each allele are shown. The asterisk means stop codon. (C) Comparison of FAS1, FSM, and hCAF-1 p150 proteins.

hCAF-1 p150 and FAS1 share a KER domain that mainly comprises lysine (K), glutamic acid (E), and arginine (R), and an ED domain that is a cluster of glutamic acid (E) and aspartic acid (D) (Kaya et al., 2001). We performed a protein motif search using the PROSITE database (Hulo et al., 2006) and found that FSM has a KEQ domain (322–462) mainly comprising lysine (K), glutamic acid (E), and glutamine (Q), which seems to correspond to the KER domain. We also found a D domain (599–702), consisting of a cluster of aspartic acid (D), which seems to correspond to the ED domain (Figs. 4B and S3). Thus, hCAF-1 p150, FAS1, and FSM share a KE domain that seems to correspond to the KER domain previously reported (Kaya et al., 2001) and a D/ED domain (Fig. 4C). Between FSM and FAS1, and FSM and hCAF-1 p150, the amino acid identity in the KE domain was 47% and 25%, respectively, while that in the D/ED domain was 75% and 56%, respectively (Fig. 4C). The KE and D/ED domains are considered necessary for chromatin assembly, because deletion of one of these domains destroys chromatin assembly activity (Kaufman et al., 1995). The fsm-1 and fsm-2 lack KE and/or D domains, and thus would represent null alleles, whereas fsm-3 occasionally transcribes KE and D domains (data not shown), and should be a weak allele.

In the course of BLAST search, we found another protein (Os07g0273400) that shows similarity to the FSM protein in KE and D domains in rice (Fig. S3). We detected weak expression for this gene by RT–PCR (data not shown). Phylogenetic analysis revealed that the homologues of p150 subunit of CAF-1 were resolved into two groups, one containing

FAS1, FSM, and maize homologue for FAS1 (ABA10486), and other containing human and mouse CAF-1 p150, and Os07g0273400 (Fig. S4). Considering the facts that single fsm mutation caused severe abnormal phenotype and that Os07g0273400 appears in a distinct clade of the phylogenetic tree (Fig. S4), FSM is likely the orthologous protein for FAS1 of Arabidopsis, corresponding to the CAF-1 p150 subunit, and the functional redundancy with Os07g0273400 is less likely.

To investigate whether the other two CAF-1 subunits (p60 and p48) exist in rice, we performed BLAST search using Arabidopsis amino acid sequences corresponding to p60 (FAS2) and p48 (MSI1) (Kaya et al., 2001). For FAS2, we found only one homologous protein (Os08g0108200) with high similarity. For MSI1, we found two proteins, Os03g0640100 and Q6ASS7. Our phylogenetic analysis with MSI1 and related proteins revealed that Os03g0640100 and one protein (Q8W514) of maize were resolved in the same branch as MSI1 of Arabidopsis (Fig. S5), suggesting that Os03g0640100 is the orthologous protein corresponding toArabidopsis MSI1. From these results, homologous proteins with high similarity to the three subunits of CAF-1 exist in rice genome, and thus, CAF-1's biochemical function would be conserved between rice andArabidopsis.

Expression pattern of the FSM gene

To gain more insight into FSM function, we performed in situ hybridization in wild type plants. In 7-day-old embryos, FSM was expressed in a patchy pattern in leaf primordia, the coleoptile, and the radicle (Fig. 5A). In 7 DAG roots, FSM expression was detected in a patchy pattern in the cell division zone, and was reduced in the cell elongation zone (Fig. 5B). In 14 DAG shoot apices, FSM was expressed abundantly in SAM and leaf primordia, but less abundantly in mature leaves (Fig. 5C). In flowers, FSM was expressed in stamen, carpel and ovule primordia (Fig. 5D). Therefore, FSM is expressed in tissues in which cells are actively dividing such as leaf primordia, the cell division zone of roots, and floral organ primordia. This expression pattern is consistent with pleiotropic phenotypes in fsm in that cellular organizations in apical meristems, leaves, roots, and floral organs were impaired and strongly suggests that FSM is associated with cell division.

Full-size image (101K)
High-quality image (451K)

Fig. 5.

In situ localization of FSM transcripts in wild type. (A) Longitudinal section of 7-day-old embryo. (B) Longitudinal section of 1-week-old root tip. (C) Longitudinal section of 2-week-old shoot apex. Arrowhead indicates SAM. (D) Longitudinal section of spikelet at the stage of ovule formation. sm, SAM; ra, radicle; st, stamen; ca, carpel; ov, ovule. Bars = 200 μm in panel A; 100 μm in panels B–D.

FSM is expressed mainly in the G_1 phase of the cell cycle

In situ hybridization experiments showed that FSM is expressed in a patchy pattern and more abundantly in actively dividing cells, and thus it is plausible that FSM is associated with a specific cell-cycle phase. To elucidate the correlation between FSM and cell-cycle progression, we performed a double-target in situ hybridization counterstained FSM with two cell-cycle marker genes histone H4 representing the S-phase and cdc2Os3expressed in the G_2- to M-phases (Umeda et al., 1999). Expressions of histone H4 (Fig. 6A) and FSM (Fig. 6C) were mutually exclusive in most cells, and only a few cells coexpressed both genes (Fig. 6E, arrowheads), indicating that the major expression phase of FSM is not the S-phase but to a small extent might extend to S-phase. An examination of cdc2Os3 (Fig. 6B) and FSM (Fig. 6D) expression in the same section showed that these genes were expressed in distinct cells (Fig. 6F), indicating that FSM is not expressed in the G_2- to M-phase. We also counterstained the samples with DAPI and found that the cells at mitosis had no FSM signals (Fig. 6G). Thus, FSM expression is confined mainly to the G_1-phase although extending partially to the S-phase. These results are consistent with a previous report that Arabidopsis FAS1 is associated with the G_1/S transition (Kaya et al., 2001).

Full-size image (93K)
High-quality image (400K)

Fig. 6.

Double-target in situ hybridization in 1-week-old wild type shoot apex with FSM counterstained with histone H4 or cdc2Os3. (A) Expression of histone H4. (B) Expression of cdc2Os3. (C and D) Expression of FSM in the same sections used in panels A and B, respectively. (E) Overlaid picture of panels A and C. Arrowheads indicate cells (yellow) co-expressing histone H4 and FSM. (F) Overlaid picture of panels B and D. The white circles in panels A to F indicate SAM region. (G) Enlarged overlaid picture of FSM expression (red) and DAPI staining (blue). The arrow shows a metaphase nucleus with no FSM expression. Bars = 100 μm.

fsm causes enhanced expression of cell-cycle marker genes

Because FSM is associated with the cell cycle (G_1-phase), it is plausible that the cell-cycle pattern could be abnormal in fsm. we examined the expression of the above-mentioned cell-cycle marker genes histone H4 andcdc2Os3 in fsm shoot apices. It is noteworthy that fsm accumulated histone H4 and cdc2Os3 transcripts in a larger number of cells in both SAM and leaf primordia (Figs. 7C and D) than in the wild type (Figs. 7A and B). This indicates that in fsm shoot apices, more cells are in the S- to G_2-phase than in wild type examples. To quantitatively confirm this, we calculated the frequency of cells expressing these two genes in SAM. The frequency of cells expressing either gene was about 3-fold larger in fsm than in the wild type (Figs. 7G and H). A simple interpretation of these results would be that cell divisions are enhanced in fsm SAM. However, the number of cells in SAM was severely reduced and resulted in the loss of SAM (Fig. 2). Accordingly, we consider that cell-cycle progression is impaired in fsm.

Full-size image (99K)
High-quality image (403K)

Fig. 7.

Double-target in situ hybridization in 1-week-old wild type and fsm-1 shoot apices with histone H4 counterstained with cdc2Os3: (A, C, and E) wild type; (B, D, and F) fsm-1. (A and B) Expression of histone H4. (C and D) Expression of cdc2Os3 using the same sections in panels A and B, respectively. (E) Overlaid picture of panels A and C. (F) Overlaid picture of panels B and D. (G) The frequency of histone H4 signals in shoot apex. (H) The frequency of cdc2Os3 signals in shoot apex. (I and J) Metaphase cells (arrowheads) stained with DAPI and expressing cdc2Os3 in wild type (I) and fsm-1 (J). The white circles in panels A to F indicate SAM region. Bars = 100 μm.

Enhanced histone H4 and cdc2Os3 expression in fsm may indicate that the specificities of these gene expressions are lost and proper cell-cycle progression is blocked. We therefore examined the relationship between histone H4 and cdc2Os3 expressions in the fsm shoot apex by double-target in situ hybridization. We also counterstained the samples with DAPI to detect cells in the metaphase and the anaphase. The overlaid pictures of histone H4 and cdc2Os3 showed that histone H4 and cdc2Os3 were expressed in distinct cells in both the wild type and fsm-1 (Figs. 7E and F). In wild type, the expression of cdc2Os3 extended to the metaphase (Fig. 7I) as was suggested previously (Umeda et al., 1999), and even in fsm, the expression of cdc2Os3 was observed in the metaphase cells (Fig. 7J). These results suggest that in fsm, despite the increased expression level of histone H4 and cdc2Os3, these genes are transcribed in a normal mutually exclusive sequential order.

Arabidopsis fas1 enhances endoreduplication in mature leaves (Kirik et al., 2006) and hypocotyls (Exner et al., 2006). To measure the DNA content in fsm nuclei, we performed flow cytometric analysis using mature leaves. In contrast to fas1, leaf cells maintained 2C-level DNA content, and almost no endoreduplication was detected in fsm leaves (Fig. S6).

The above results indicate that in fsm, histone H4 and cdc2Os3 are not misexpressed and that fsm cells complete cell divisions successfully. In this situation, the only conceivable interpretation is that increased histone H4 andcdc2Os3 signals reflect the changes in the relative lengths of S- and G_2-phases in a cell cycle. Prolonged S- and G_2-phases could cause a long cell-cycle period in SAM, which would result in an insufficient supply of indeterminate cells that are used for leaf primordium formation and eventually result in the loss of the SAM.

Discussion

In this study, we investigated fsm mutants that showed lethality during the vegetative stage due to defects in SAM maintenance, and revealed that FSM is orthologous to Arabidopsis FAS1 that encodes CAF-1 p150 subunit. Interestingly, fsm showed considerably different

phenotypes from Arabidopsis fas1. The present results raise three issues to be discussed: causes of phenotypic difference between fsm and fas1, FSM function in relation to cell division, and the relation of cell proliferation rate and developmental timing.

Loss of function of CAF-1 p150 subunit causes opposing phenotypes between rice and Arabidopsis

fsm plants exhibited pleiotropic phenotypes, some of which are shared with Arabidopsis fas1 plants including aberrant organization of apical meristems, aberrant organ size and morphology, stem fasciation and growth retardation (Leyser and Furner, 1992). Since both fsm and fas1 commonly form small leaves and short roots with aberrant RAM organization ([Leyser and Furner, 1992] and [Kaya et al., 2001]), leaf and root phenotypes are considered to be fundamentally comparable between the two species. These phenotypes would reflect directly prolonged cell-cycle period. Thus, the opposite phenotypes are mainly associated with SAM maintenance.

The opposite phenotypes in SAM maintenance, loss of fsm SAM versus continuation of fas1 SAM, might have been caused by the differences in the regulatory mechanisms of SAM maintenance between Arabidopsis and grass species. Some evidence for the difference in SAM maintenance between the grass family and other eudicots is suggested in previous reports. clavata 1 (clv1), clv2, and clv3 mutants of Arabidopsis show drastic SAM enlargement in vegetative phase ([Clark et al., 1993] , [Clark et al., 1995] and [Kayes and Clark, 1998]). However, equivalent mutants in maize and rice show little or no phenotype in vegetative phase ([Suzaki et al., 2004] , [Suzaki et al., 2006] and [Taguchi-Shiobara et al., 2001]), although some reports indicate that CLAVATA-like genes function even in vegetative phase ([Bommert et al., 2005] and [Chu et al., 2006]). Thus, their effects on vegetative SAM of grasses do not seem to be critical. Also, the expression patterns of FON1 (rice) and TD1 (maize) in SAM are different from that of the corresponding gene CLV1 in Arabidopsis ([Bommert et al., 2005] and [Suzaki et al., 2004]). In addition, the expression pattern of WUS (Mayer et al., 1998) was different from that of OsWUS (Nardmann and Werr, 2006). Furthermore, recent publications from two groups revealed that the mutants of rice and maize in ta-siRNA biogenesis pathway show stronger phenotypes in their SAM initiation and maintenance than those of Arabidopsis ([Hunter et al., 2003] , [Nagasaki et al., 2007] ,[Nogueira et al., 2007] , [Peragine et al., 2004] and [Xie et al., 2005]). These studies reinforce the idea that even orthologous genes of rice and Arabidopsis may have distinct functions and that organization and regulatory mechanisms of the SAM may differ considerably between the two species. These differences might lead to distinct, and in some cases opposite responses to increases in cell-cycle length.

Another possibility is that the relation between cell proliferation and leaf initiation differs between the two species. In rice fsm, plastochron is not affected in spite of decreased cell proliferation, suggesting uncoupling between cell proliferation rate and developmental timing. However, consistent understanding about the relation between cell proliferation and developmental timing has not been obtained to date as to be discussed later. Accordingly, it is possible that the extent of dependence of developmental timing on cell proliferation varies with species and may cause different mutant phenotypes, although the leaf initiation rate in fas1 is not known.

FSM is required for proper cell-cycle progression and cell proliferation

Double-target in situ hybridization experiments using histone H4 and cdc2Os3 indicated that FSM is expressed mainly in the G_1-phase. This estimation is roughly consistent with the previous report suggesting that FAS1 is associated with the G_1/S transition by experiments using Arabidopsis T87 and tobacco BY-2 cell lines (Kaya et al., 2001). One of the functions of CAF-1 is to assemble histones H3 and H4 following DNA replication ([Shibahara and Stillman, 1999] , [Smith and Stillman, 1989] and [Tagami et al., 2004]). Therefore, the p150 subunit of CAF-1 transcribed in the G_1-phase might be stable through the S-phase.

fsm showed enhanced frequency of histone H4 and cdc2Os3 expression but the number of SAM cells was reduced. We also showed by double-target in situ hybridization that histone H4 and cdc2Os3 are expressed in distinct cells in both fsm and the wild type. Together with the fact that almost no endoduplication occurred in fsm, these results suggest that these genes are transcribed in a normal exclusive sequential order in a cell cycle, and that cell cycle/cell division is going on normally in fsm except for long S- and G_2-phases in a cell cycle. Consistent with these results, a chicken DT40 B-cell line with depleted CAF-1 p150 or p60 was recently reported to delay S-phase progression (Takami et al., 2007). Our study suggests that fsm has a long G_2-phase as well as a long S-phase. A long cell-cycle period would result in the reduced cell division frequency that gave rise to the reduced number of cells in SAM.

The fsm mutation caused increased expression of the signals of histone H4 and cdc2Os3 genes. The question is how FSM affects these genes. It is reported that the epigenetic states of histone H4 and CycB1;1 are changed in fas1–4, which results in upregulation of these genes (Ramirez-Parra and Gutierrez, 2007). Thus, the up-regulation of histone H4 and cdc2Os3 in fsm might be caused by changes in the epigenetic states of these genes. The next question is how FSM affects the epigenetic states of these genes. The defect in histone H3/H4 assembly during DNA replication would lead to a prolonged S-phase. If the prolonged S-phase causes a longer duration of the loosened chromatin state, it could change the epigenetic states of histone H4 and cdc2Os3. Thus, loss of FSM function that causes defect in chromatin reconstruction could epigenetically alter the expressions of cell-cycle-related genes.

Small size or loss of SAM, small leaf size, and truncated roots can be explained by the reduced number of cells supplied by meristems due to a long cell-cycle period. fsm SAM with an insufficient supply of cells are eventually consumed by leaf primordia as seen in the loss of visible SAMs in dead plants. Large cells frequently observed in fsm leaves and SAM would also result from compensation for the reduced number of cells as a result of a long cell-cycle period. Long cell-cycle periods may also cause aberrant cell division patterns, which might result in distorted cell files in many tissues; cell-division arrest in one cell might induce extra cell division in one or more adjacent cells, and thus disturb regular cell files.

Developmental timing is not affected by the elongated cell-cycle period

Despite the fact that defects in cell proliferation affected cell differentiation and morphogenesis in SAM and leaves, plastochron was not significantly affected in fsm. In addition, developmental events of leaves such as vascular bundle formation and ligule formation occurred at the proper time. These results suggest that developmental timing is not affected by the alteration of the cell-cycle period. This suggestion is contrary to the case of rice pla mutants. In pla1 and pla2 mutants, leaf development (maturation) is accelerated, and cell proliferation in the SAM is enhanced (Kawakatsu et al., 2006). Since both genes are

expressed in leaf primordia but not in SAM (Kawakatsu et al., 2006), it is probable that developmental timing of leaves affects the leaf initiation rate and cell proliferation in the SAM. Although the cell-cycle period in pla has not been examined, regulation of developmental timing may be upstream of the regulation of cell proliferation and the cell-cycle period.

The relationships between cell division rate and developmental timing have been discussed in several studies. In tobacco plants with a lower frequency of cell division, the developmental timing including the leaf initiation rate and the transition from juvenile to adult phase was not affected (Hemerly et al., 1995). In Arabidopsis, the overexpression of KRP2, one of the seven CKI genes, inhibited cell division and leaf expansion rate, whereas the temporal pattern of development was not affected (De Veylder et al., 2001). These studies suggest an independent regulation of the cell division rate and developmental timing. In contrast, overproduction of Arabidopsis CYCD2 and CYCD3 in tobacco plants resulted in rapid leaf initiation and accelerated development towards maturity as well as enhanced cell division ([Boucheron et al., 2005] and [Cockcroft et al., 2000]). This report indicates a positive correlation between the cell division rate and developmental timing. Although cell-cycle periods in these studies are not clear, interdependency between cell proliferation and developmental timing may vary with species or corresponding genes. Although the relation has not yet been well understood to date, our study on fsm suggests that the cell-cycle period does not affect developmental timing, and this independency seriously affects SAM maintenance when one of the two components is impaired at least in rice.

Here we revealed that FSM encoding the CAF-1 p150 subunit plays a critical role in the maintenance of apical meristems by regulating cell-cycle progression in rice. CAF-1's role on development seems to be more critical in rice than that performed in Arabidopsis. We also showed that the balance between cell division and the timing of differentiation is crucial for proper plant development.

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