Disruption of an RNA helicase/RNAse III gene in Arabidopsis causes unregulated cell division in floral meristems

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SUMMARY

Arabidopsis thaliana floral meristems are determinate structures that produce a defined number of organs, after which cell division ceases. A new recessive mutant, carpel factory (caf), converts the floral meristems to an indeterminate state. They produce extra whorls of stamens, and an indefinite number of carpels. Thus, CAF appears to suppress cell division in floral meristems. The function of CAF is partially redundant with the function of the CLAVATA (CLV) and SUPERMAN (SUP) genes, as caf clv and caf sup double mutants show dramatically enhanced floral meristem over-proliferation. caf mutant plants also show other defects, including absence of axillary inflorescence meristems, and abnormally shaped leaves and floral organs. The CAF gene was cloned and found to encode a putative protein of 1909 amino acids containing an N-terminal DExH/DEAD-box type RNA helicase domain attached to a C-terminal RNaseIII-like domain. A very similar protein of unknown function is encoded by a fungal and an animal genome. Helicase proteins are involved in a number of processes, including specific mRNA localization and mRNA splicing. RNase III proteins are involved in the processing of rRNA and some mRNA molecules. Thus CAF may act through some type of RNA processing event(s). CAF gives rise to two major transcripts of 2.5 and 6.2 kb. In situ hybridization experiments show that CAF RNA is expressed throughout all shoot tissues.

Key words: Flower development, Meristem determinacy, RNaseIII, Helicase, Arabidopsis thaliana, carpel factory (caf)

INTRODUCTION

Shoot apical meristems of angiosperms are the ultimate source of plant aerial structures. These meristems initiate leaves, axillary meristems, and flower meristems, while continually maintaining a pool of undifferentiated cells (Steeves and Sussex, 1989). In Arabidopsis thaliana the shoot apical meristem is indeterminate, with floral meristem formation continuing until senescence. Several genes affecting shoot meristem function have been described in Arabidopsis. Mutations in the CLAVATA (CLV) genes (CLV1, CLV2 and CLV3) lead to an over-proliferation of meristem cells (Clark et al., 1993, 1995; Alvarez and Smyth, 1994; Kayes and Clark, 1998; Fletcher et al., 1999), while mutations in the SHOOTMERISTEMLESS (STM), and WUSCHEL (WUS) loci cause defects in meristem maintenance (Barton and Poethig, 1993; Laux et al., 1996).

The Arabidopsis floral meristem differs from the shoot apical meristem both in the type of organs produced (floral meristems produce sepals, petals, stamens and carpels) and in its determinate nature. Several genes have been shown to affect floral meristem identity, and mutations in these genes lead to a partial conversion of the flower meristem into a shoot meristem. In particular, LEAFY (LFY) and APETALAI (API) mutations produce some loss of floral meristem identity and a partial loss of determinacy, and double mutant combinations of LFY and API lead to a greatly enhanced loss (Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993). In addition, several mutations affecting apical meristem structure affect floral meristem structure in the same way, with clv1, clv2, and clv3 mutations resulting in over-proliferation and partial loss of determinacy in the floral meristem, and mutations in WUS and STM resulting in a loss of organs, especially in the inner whorls.

Other genes have been shown to affect floral meristem determinacy while not affecting apical meristem development. Mutations in SUPERMAN (SUP) lead to increased number of stamens and carpels, while AGAMOUS (AG) mutants show many extra whorls of sepals and petals (Bowman et al., 1991, 1992; Schultz et al., 1991).

Most of these genes have been cloned; some are putative transcription factors while others seem to be involved in cell to cell signaling processes. AG and API encode MADS-Box type transcription factors (Yanofsky et al., 1990; Mandel et al., 1992), SUP encodes a zinc-finger protein (Sakai et al., 1995), STM and WUS encode homeodomain proteins (Long et al.,
1996; Mayer et al., 1998), LFY encodes a new type of transcription factor (Pardy et al., 1998), CLV1 encodes a transmembrane leucine-rich repeat receptor kinase (Clark et al., 1997), and CLV3 may encode its ligand (Fletcher et al., 1999). Though it seems likely that these genes act through regulation of downstream genes involved in the control of cell division and differentiation functions, none of these target genes nor the specific mechanisms involved are known.

We have identified the CARPEL FACTORY (CAF) gene, which plays a role in floral meristem determinacy. caf mutant flowers show extra stamens and carpels, reminiscent of sup mutants, but also show organ morphogenesis defects. The CAF gene encodes a protein with similarities to both DEXH/DEAD-box type RNA helicases and RNaseIII proteins, suggesting a mechanism for control of floral meristem proliferation. The Schizosaccharomyces pombe and Caenorhabditis elegans genomes both contain a gene coding for a protein highly similar to that encoded by CAF, indicating that CAF-type RNase III-helicase proteins may play a role in many eukaryotic organisms.

**MATERIALS AND METHODS**

**Mutant isolation and analysis**

The caf mutant was isolated in a screen of mutants generated by Agrobacterium tumefaciens-mediated transformation of Arabidopsis thaliana seeds of ecotype Wassilewskija (Feldmann, 1992). Phenotypic and genetic analysis of caf was carried out both in the Wassilewskija background and in a line of caf that had been backcrossed to the Ler ecotype five times. The phenotype was similar in both backgrounds. The caf phenotype did not seem to be qualitatively different when grown at 16°C, 23°C, or 29°C. All of the reported analyses of this mutant was carried out at 23°C. Plants were sown in a 1:1 mixture of vermiculite:perlite:soil, grown under constant illumination, and watered periodically with a dilute solution of Miracle Grow (20:20:20) plant fertilizer. Scanning electron microscopy (SEM) was performed as described by Bowman et al. (1989). Confocal laser scanning microscopy was performed as described by Running et al. (1995). Negatives and slides were scanned and digitized using Nikon Coolscan slide scanner. Brightness, contrast and color balance were adjusted using Adobe Photoshop 3.0 and figures were printed using a Kodak 8300 digital printer.

**CAF gene cloning**

Genomic DNA was extracted from plants homozygous for caf, partially digested with Sau3AI, partially filled with dGTP and dATP, cloned into the partially filled XhoI site of lambdaGem-11 (Promega), and packaged with Gigapack II Gold packaging extracts (Stratagene). The cDNA library used was constructed from floral mRNA (Weigel et al., 1992). The longest cDNA (cDNA8) contained 2558 bp of half of the full cDNA sequence. Three separate rounds of 5'-PCR-RACE were performed using a kit (GIBCO-BRL) as outlined in the manufacturer's instructions.

A CAF-containing cosmid clone (CosA) was obtained by screening a Wassilewskija genomic DNA library constructed in a pOCA18 derivative which confers hygromycin resistance to transgenic plants (obtained from the Arabidopsis Biological Resource Center; Olszewski et al., 1988; Schulz et al., 1995). The CosA clone was transformed into the Agrobacterium tumefaciens strain ASE, and whole plants were transformed using the vacuum-infiltration method (Bechtold et al., 1993). Transgenic plants were then crossed to caf mutant plants, and F3 populations of these crosses were selected for lines which were homozygous for kanamycin resistance (and hence were homozygous for the caf mutation), and which were segregating for hygromycin resistance (3:1, hygromycin resistant:hygromycin sensitive). Within these populations, all of the hygromycin-resistant plants showed a wild-type phenotype (an example is shown in Fig. 7B) while all of the hygromycin-sensitive plants showed the caf mutant phenotype, showing that CosA complements caf. Since CosA contained not only the caf gene, but also an adjacent pyrophosphatase gene whose 3' end is very close to the T-DNA, there remained a remote possibility that the pyrophosphatase gene was also affected by the T-DNA, and that this could contribute to the caf phenotype. To rule out this possibility, we showed by RNA blot analysis that the size and abundance of a 1 kb transcript homologous to the pyrophosphatase gene was unaffected in the caf mutant. Furthermore, DNA sequence analysis showed that the pyrophosphatase gene in the caf mutant has a sequence which is identical to that of wild-type WS plants. Thus, neither the structure nor expression of the pyrophosphatase gene are affected in caf.

For mapping, an EcoRI restriction fragment length polymorphism (RFLP) detected with CosA was mapped in a Ler × Columbia mapping population (Chang et al., 1988). For secondary confirmation, a HinCII RFLP contained within the helicase domain of the CAF gene was mapped in a Niederzenz X Columbia mapping population. A similar map position was obtained.

Southern blot analysis showed that the T-DNA insertion at the CAF locus is complex, consisting of multiple tandem copies of the T-DNA. The junction between the CAF gene and the T-DNA was determined by cloning and sequencing a 6.5 kb EcoRI fragment of genomic DNA from a lambda clone isolated from the caf genomic library. This clone contained a region of CAF corresponding to the RNaseIII-like domain, up to an amino acid 1836 of CAF (see insertion site shown in Fig. 7A), adjacent to 493 bp of sequence homologous to the right border of the T-DNA followed by 1684 bp of sequence homologous to the vector pBR322, followed by the Tm903, OCS3', and Tm5-1'2' portions of the T-DNA (Feldmann, 1992).

**CAF RNA expression**

To ensure that our DNA probes were specific for the CAF gene, we tested two probes corresponding to the 5' and 3' ends of CAF, on Southern blots. The N-terminal helicase probe was a 1809 bp PCR product corresponding to positions 475-2283 in the 5815 bp cDNA. The primers used to generate this probe were JP237 (5'AGATGGAACCTGACCTGAACTAT), and JP236 (5'AATGTTTGCCACGACCGTCTT). The C-terminal RnaseIII probe was a 1838 bp PCR product corresponding to positions 3930-5767 in the cDNA. The primers used to generate this were JP121 (5'GTTTTCTTCCACCTGAACTA) and JP131 (5'CTCATCATCGTGAAAGAGATA). These probes were hybridized to filters containing genomic DNA cut with the restriction enzymes EcoRI, XhoI, BglII, HindIII, HincII, or EcoRV. Only the fragments predicted from the CAF genomic sequence were detected. These Southern blots and the northern blots shown in Fig. 9 were prepared, hybridized, and washed under high stringency conditions as previously described (Chang et al., 1988).

In situ hybridization experiments were carried out using sense or antisense RNA probes synthesized with T7 or T3 polymerase from cDNA8 plasmid DNA that had been linearized with EcoRI or XhoI. The remainder of the in situ procedure was done according to Drews et al. (1991), with modifications by Sakai et al. (1995). These in situ hybridization experiments were carried out twice independently, with similar results. One of the experiments was carried out both in the presence and absence of 500 µg/ml polyuridylic acid (Pharmacia Biotech, product number 27-4440), to ensure that the poly(A) tail on cDNA was not causing an increase in the background hybridization. No difference in signal was observed between the poly(U) plus or minus treatments.
RESULTS

carpel factory floral determinacy defects

During a screen of T-DNA mutagenized lines (Feldmann, 1992) for mutants with abnormal flower development, we found a recessive mutant with indeterminate flowers. The mutant gene was named CARPEL FACTORY (CAF). caf flowers show a lack of floral determinacy that is evident in both the third and fourth whorls. Wild-type Arabidopsis flowers produce four organ types, each occupying a separate whorl or concentric ring (Fig. 1A). The first whorl consists of four sepals, the second whorl contains four petals, the third whorl has six stamens, and the fourth or inner whorl is composed of two carpels which later fuse to form the gynoecium. caf flowers contain a roughly normal number of sepals, petals, and stamens in the first three whorls (Fig. 1B). However, most caf mutant flowers contain one or two extra stamens interior to the third whorl stamens (Fig. 1C). Occasionally, flowers contain multiple whorls of stamens (up to 40 stamens/flower have been observed) (Fig. 1D). These defects are reminiscent of the sup mutant phenotype (Schultz et al., 1991; Bowman et al., 1992).

The caf gynoecium is severely affected. While wild-type flowers produce a gynoecium consisting of two fused carpels (Fig. 1E), caf flowers typically consist of a gynophore (a region of internode elongation between the third and fourth whorl), and several sterile unfused carpels (Fig. 1F). Usually, additional growth can be seen interior to the first whorl of carpels, forming structures which resemble carpels, carpelloid filaments, or filaments with no obvious floral character (Fig. 1G). Occasionally, staminoid structures are also present amongst the carpels (Fig. 1G). These ectopic organs are produced at the flanks of a visible group of undifferentiated cells in the center of the caf floral meristem (Fig. 1H-J).

Scanning electron microscopy of floral meristems shows that the region of cells in the fourth whorl is larger in caf flowers than in wild type (Fig. 2). Whereas wild-type floral meristems terminate in the development of two carpels (Fig. 2A), caf fourth whorl cells continue to proliferate and give rise to additional primordia on the flanks of an indeterminate meristem (Fig. 2B,C).

carpel factory leaf and floral morphogenesis defects

caf affects the shape of most plant organs. As early as 9 days after development, caf seedlings can be recognized by the thinner

![Fig. 1.](image-url)
shape of the cotyledons and rosette leaves and a decrease in the overall size of the seedling (Fig. 3A,B). Mature rosette and cauline leaves are also thinner than wild type (Fig. 3C). Secondary meristems, which normally arise from the axils of rosette leaves and cauline leaves, are almost always absent in caf plants (Fig. 3D-G). Decapitating plants does not induce the development of these meristems, suggesting that these meristems are missing or non-functional and not merely inactive. Roughly 10% of the leaves show severe defects. Some leaves are reduced to filamentous structures which resemble the petiole or midrib tissue, but which lack leaf blade character (Fig. 3H). Intermediate leaves are also produced that have patches of blade tissue on the adaxial surface of a filamentous structure resembling the midrib (Fig. 3I). SEM analysis confirms that these filamentous structures have surface features similar to wild-type petioles or midribs (lack of stomata and cells in regular files) (not shown).

The first formed flowers are sometimes highly reduced (Fig. 3J). Some consist of floral pedicels and very few sepal-like organs, while others are reduced to filamentous structures. The reduced flowers are primarily observed within the first 20 flowers.

Some of the caf morphological defects can be traced to early defects in the development of meristems. Scanning electron microscope (SEM) analysis of 14-day-old wild-type and caf plants revealed that a small percentage of the developing caf floral meristems were smaller than wild-type ones (Fig. 4A,B). These smaller primordia probably correspond to the reduced flowers observed on more mature plants. Flowers produced by 30-day-old plants (after elongation of the inflorescence stem) showed fewer defects than those from 14-day-old plants (Fig. 4C,D). We also noted a slight increase in the size of the caf apical meristem relative to wild type (Fig. 4A-D). This size difference was confirmed by confocal analysis (Fig. 4E,F).

caf sepal develop in roughly the correct number and position, but are narrower than wild-type sepals (Fig. 1A,B). SEM analysis shows that the sepals are narrower than in wild type at the earliest stages of development (Fig. 4C,D). Though most caf petals are similar to wild-type petals (Fig. 1A,B), occasionally they are reduced to filamentous structures (not shown). All stamens contain a reduced number of pollen sacs. Approximately 80% of stamens have an anther consisting of two pollen sacs instead of the wild-type number of four, while the remaining 20% of stamens consist of a filament with no

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**Fig. 2.** Wild-type and caf fourth whorl development. The three panels show a wild-type (wt) floral meristem at stage 7 (Smyth et al., 1990), which has terminated in the development of two carpel primordia (A), and two caf floral meristems (B,C) showing excess cells in the center of the flower after the initiation of carpel primordia. In all three panels, the white arrow indicates the center of the flower, and the black arrow, a stamen. Bar in A (for A-C) is 10 \( \mu \text{m} \).

**Fig. 3.** Morphological defects of caf mutants. Nine-day-old wild-type (A) and caf (B) seedlings photographed at the same magnification. (C) Mature rosette leaves from wild type (bottom) and caf (top). Rosettes of wild type (D) and caf (E). White arrows show the axil of a rosette leaf and a stem. An axillary meristem has developed in this position in wild type but not in caf. Wild-type (F) and caf (G) cauline leaves showing an axillary meristem in wild type but not in caf. (H) Filament on a caf plant in a position normally occupied by a cauline leaf. (I) caf cauline leaf showing two patches of blade tissue on the adaxial surface of a filamentous structure resembling the midrib. The arrow shows the midrib region lacking the leaf blade. (J) Reduced flowers produced during the early development of caf inflorescences. White arrow shows a flower consisting of a pedicel and one filamentous organ.
anther at all (Fig. 5A-C). Normal anthers containing 4 pollen sacs have not been observed. Some stamen primordia are much smaller than normal (see Fig. 2B). These smaller primordia may give rise to the antherless stamens observed in mature flowers. The structures produced in the fourth whorl of caf flowers are usually carpelloid or filamentous. The carpelloid organs range in phenotype from relatively normal but unfused carpels (Fig. 5D) to filamentous organs tipped with stigmatic tissue (Fig. 5E). While wild-type meristems were 75±0 µm in diameter, caf meristems were 84.6±3.0 µm (numbers are mean ± standard error where n=5). Bar in E (for E and F) is 50 µm.

Genetic interactions of caf with mutations affecting floral meristem patterning

We constructed double mutants of caf with several mutations affecting floral organ identity, apetala2 (ap2) mutations affect the identity of the first and second whorl organs. The ap2-1 mutation converts the first whorl organs to leaves and the second whorl organs to staminoid petals (Bowman et al., 1989). The ap2-1 caf double mutant has a phenotype similar to that of caf single mutants, except that it has a floral organ identity phenotype similar to that of ap2-1 mutants (not shown). pistillata (pi) mutations affect the identity of the second and third whorl organs, converting the petals and stamens to sepals and carpels (Bowman et al., 1989). The pi caf double mutant shows an additive phenotype, exhibiting morphological characteristics and meristem over-proliferation defects of caf mutants but the floral organ identity phenotype of pi mutants (not shown). ag mutations affect the identity of the third and fourth whorl organs and cause floral indeterminacy, such that ag flowers produce sepals in the first whorl, petals in the second and third whorls, then a new flower showing the repeating pattern (sepals, petals, petals)n (Fig. 6A; Bowman et al., 1989). The ag caf double mutant has a phenotype similar to caf single mutants throughout most of the plant. However, interior to the first whorl sepals ag-1 caf double mutant flowers contain only petals (Fig. 6B). This phenotype is reminiscent of sup ag double mutant flowers (Bowman et al., 1992; Schultz et al., 1991), which also contain only petals in the inner whorls. In summary, the double mutants of caf with the floral homeotic mutants ap2-1, pi-1, and ag-1 show a largely additive phenotype; that is, they display the floral meristem over-proliferation phenotype...
characteristics of the caf mutant, but also show floral organ misspecification in a manner similar to that seen in the floral homeotic single mutants.

We also combined the caf mutation with other mutations that affect floral organ number. sup mutant flowers display extra cell proliferation in the developing third whorl, resulting in the production of an excess number of stamens (Fig. 6C; Bowman et al., 1992; Schultz et al., 1991). sup-1 caf double mutant flowers show greatly enhanced gynophore development and floral indeterminacy (Fig. 6D). After producing the organs in the first three whors (arrow), double mutant flowers produce many staminoid, carpelloid, and leaf-like organs. Thus, SUP and CAF appear to be partially redundant in their control of floral determinacy. The clv3-2 mutant has an enlarged apical meristem and enlarged floral meristems that produce an excess number of organs in all of the floral whors (Fig. 6E; Clark et al., 1995). Additionally, clv3-2 plants often produce extra whors of carpels interior to the fourth whorl (Clark et al., 1995). clv3-2 caf exhibit an essentially additive interaction with respect to vegetative development. clv3-2 caf plants have enlarged apical meristems (similar to clv3-2 single mutant plants) and produce few secondary meristems (similar to caf single mutants) (not shown). However, clv3-2 caf flowers exhibit an enhanced floral indeterminacy phenotype, producing a bouquet of stamens, carpels, and filaments (Fig. 6F).

**CAF cloning**

caf was isolated from a screen of Agrobacterium T-DNA insertion mutants (Feldmann, 1992). After 3 back crosses of caf to wild-type Landsberg erecta (Ler) plants, a single kanamycin resistance locus cosegregated with the caf phenotype, suggesting that CAF was tagged with a T-DNA element. To isolate plant sequences flanking the T-DNA, we generated a caf genomic lambda library and screened for clones containing homology to the right border of the T-DNA. A clone was isolated that contained homology both to the right border and to plant genomic DNA. Southern blot analysis showed that this plant DNA cosegregated with the caf phenotype. Plant DNA flanking the T-DNA insertion was used to probe a cDNA library, and twelve hybridizing clones were found. One of these clones (cDNA8; 2.5 kb in length) was sequenced, and found to contain a long ORF, followed by a 76 base pair 3′ untranslated region, and a short poly(A) tail. PCR analysis showed that of the other 11 cDNA clones, three had 5′ ends which were at a very similar position, while eight were somewhat shorter. To determine the 5′ end of the CAF RNA, we performed 5′ RACE and found additional sequences that extended the ORF. Two additional rounds of RACE were performed using primers progressively closer to the 5′ end of the full length RNA. The resulting predicted RNA of 5815 nucleotides (Accession number, AF187317) contains an ORF of 1909 amino acids (Figure 7A). An in-frame stop codon in the genomic sequence nine nucleotides upstream of the predicted RNA sequence suggests that the first methionine shown in Fig. 7 is the start of translation (see Accession number AC007323 for the complete genomic sequence).

A cosmig clone (CosA) with homology to CAF was isolated and transformed into wild-type plants, and the resulting transgenic plants were crossed to caf mutant plants. This cosmig fully complemented the caf mutant floral phenotype (Fig. 7B), as well as all other caf phenotypes (not shown). To determine the genomic sequence of CAF, three adjacent EcoRI fragments were subcloned from CosA and sequenced. These fragments contained 8603 bp of genomic sequence comprising the entire CAF coding region and sequences upstream and downstream. The sequence predicts that the CAF coding sequence is interrupted by 19 relatively small introns. One of the fragments contained the junction between the putative CAF promoter and the cosmig cloning vector. The distance from the vector junction to the 5′ end of the predicted RNA sequence was 675 bp. Since this cosmig complements caf, this suggests that all of the relevant 5′ promoter sequences are contained within this 675 bp region. 48 bp downstream from the 3′ end of the CAF cDNA sequence

![Fig. 6. agamous-1 caf, superman-1 caf, and clavata3-2 caf double mutants.](image)
was a sequence identical to a 3' EST clone with homology to pyrophosphatase (EST Z29202), suggesting that there are only 47 bp between the 3' end of the CAF gene and the 3' end of the adjacent gene. Using restriction fragment length polymorphism mapping, we mapped the CAF gene to the top of chromosome one (Fig. 7C).

The T-DNA insertion in the caf mutant lies in the 19th exon of the CAF gene, and thus separates most of the CAF gene and the 3' end of the adjacent gene. Using restriction fragment length polymorphism mapping, we mapped the CAF gene to the top of chromosome one (Fig. 7C).

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**Similarity of CAF with helicase and RNAse III like sequences**

Analysis of the predicted CAF sequence with PSORT (Nakai and Kanehisa, 1992) suggests that CAF is a nuclear protein, which contains two putative bipartite nuclear localization signals (underlined in Fig. 7A). The sequence of CAF shows extensive similarity along most of its length to a predicted protein of 1374 amino acids in *Schizosaccharomyces pombe* (C8A4.08C) and a predicted protein of 1822 amino acids in *Caenorhabditis elegans*, which contains a transgenic copy of the wild-type CAF gene and exhibits a wild-type phenotype. (C) Map position of the reading frame extension conferred by the T-DNA. (B) Flower from a localization sequences. Also shown is the position of the T-DNA insertion in the centimorgans (cM) and the log likelihood value (LOD; Chang et al., 1988). GenBank accession no. AF187317.

**Fig. 7. The CARPEL FACTORY gene.** (A) Predicted amino acid sequence of the CAF protein. The two underlined regions are putative nuclear localization sequences. Also shown is the position of the T-DNA insertion in the caf-1 mutant. Above the insertion site is the predicted open reading frame extension conferred by the T-DNA. (B) Flower from a caf homozygote which contains a transgenic copy of the wild-type CAF gene and the 3' end of the adjacent gene. Using restriction fragment length polymorphism mapping, we mapped the CAF gene to the top of chromosome one (Fig. 7C).
Fig. 8. Similarity of CAF with helicase and RNase III like sequences.
(A) Schematic of the domain structure of CAF and two similar predicted
sequences from S. pombe (CA4.08C; Accession no. Q09884) and C.
elegans (K12H4.8; Accession no. P34529). Hatched box denotes an N-
terminal CAF sequence with a high content of charged residues that is
not found in the other two proteins. Stippled boxes denote the
ATPase/Helicase domains. Vertically hatched boxes denote the RNaseIII-
like domains, which are duplicated in all three predicted proteins. Black
boxes denote the double stranded RNA binding domains, which are
CAF (Caf; residues 239-779) was duplicated in CAF but not in C8A4.08C or K12H.8. Lines denote regions that show homology to each other, but not to other known sequences. The number of amino acids (aa) present in each domain is noted above each segment. Diagram is drawn to scale. (B) The N-terminal helicase-like domain of CAF (Caf; residues 239-779) was aligned with similar domains present in the predicted C8A4.08C protein of S. pombe (Spo; Accession no. Q09884, residues 2-479) and the predicted K12H.4 protein of C. elegans (Ce1; Accession no. P345529; residues 3-509), and other predicted helicase-like proteins from Archaeoglobus fulgidus (Afu; Accession no. 266258; residues 6-476), Methanococcus jannaschii (Mja; Accession no. 2127880; residues 11-471), Methanobacterium thermoautotrophicum (Mth; Accession no. 2622572; residues 7-478), S. cerevisiae (Sce; Accession no. P40562; residues 77-624), and C. elegans (Ce2; Accession no. 2662588; residues 283-822 and Ce3; Accession no. 1667297; residues 361-854). Also included in the alignment are the Drosophila melanogaster protein VASA (Vas; Accession no. P09052; residues 258-597) and the mouse protein elf-4A1 (Elf; Accession no. FMS44A; residues 29-364). A consensus sequence of seven domains (I-VI) present in these proteins is shown above the alignment and numbered as in Gorbelenya et al. (1989). □ denotes the hydrophobic residues (LL,VM,F,Y,W); ○ denotes the charged or polar residues (S,T,D,E,N,Q,K,R). Asterisks mark positions where at least 10 of the 11 sequences matched the consensus. Identical residues are boxed in columns where at least 3 of the residues are identical. The (20 aa) and (49 aa) notations denote either 20 or 49 amino acids, which were present in only one protein and were removed from the alignment. (C) Similarity of CAF to the conserved regions of the catalytic domains of RNaseIII-like proteins. The duplicate RNaseIII-like domains of CAF ([Caf1; residues 1345-1518]/[Caf2; residues 1561-1707]), C8A4.08C ([Spo1; residues 900-1038]/[Spo2; residues 1085-1233]), K12H.8 ([Ce11; residues 1295-1501]/[Ce12; residues 1557-1717]), and RNC_AEEL (Accession no. 001326) ([Crm1; residues 1-110]/[Crm2; residues 164-271]) were aligned with similar domains present in E. coli RNaseIII (Eco; Accession no. P05797; residues 8-128), S. cerevisiae RNaseIII (Rnt; Accession no. Q02555; residues 206-331), the Pac1 gene of S. pombe (Pac; Accession no. P22192; residues 138-262), and the predicted ATFC3 protein from A. italicana (Ath; Accession no. Z97338; residues 56-195). Identical residues are boxed in columns where at least 3 of the residues are identical. Asterisks mark conserved positions that are sites of inactive point mutations in E. coli RNaseIII (Court, 1993). (D) Double stranded RNA binding domains (dsRNABds) of CAF. Two putative dsRNABds of CAF ([ca2; residues 1733-1796]/[ca2b; residues 1831-1906]) were aligned with similar domains found in C8A4.08C (Spo2; residues 1256-1341), K12H.8 (Ce12; residues 1745-1808), RNC_AEEL (Crm2; residues 298-373), E. coli RNaseIII (Eco; residues 155-225), S. cerevisiae RNaseIII (Rnt; residues 361-437), and the Pac1 gene of S. pombe (Pac; residues 286-356). Identical residues are boxed in columns where at least 3 of the residues are identical. Asterisks denote positions where the residues are highly conserved in a variety of additional dsRNABds (Kharrat et al., 1995). Arrows indicate the position of the T-DNA insertion in the second dsRNAbd of CAF (ca2B). All alignments were performed using PILEUP and PRETTYPLOT [Genetics Computer Group, (1991), Madison, Wisconsin], using a gap creation penalty of 3.0 and a gap extension penalty of 0.05.
for the conserved residues DExH and DEAD, with x representing any amino acid (Gorbalenya, et al., 1989). Fig. 8B shows an alignment of CAF and the related sequences along with eIF-4A and VASA, two RNA helicase proteins of the DEAD box type. CAF contains sequence similarity within all seven of the most conserved domains present in these DEAD/DExH proteins (Fig. 8B; Gorbalenya et al., 1989). These include domains I and II which are present in a wide variety of ATPases, and domains III through VI which are specific to helicases. Mutation analysis has shown these domains to be important for ATPase and/or helicase activity (reviewed by Gorbalenya and Koonin, 1993).

The middle portion of the CAF protein (amino acids 780-1344) shares dispersed sequence identity with the above mentioned S. pombe (C8A4.08C; 21% identity) and C. elegans (K12H4.8; 18% identity) sequences, but is not similar to other known proteins. However, CAF and these two related proteins contain a C-terminal domain with sequence similarity to each other (average of 31% identity), to several bacterial and yeast RNase III proteins (Nashimoto and Uchida, 1985; Elela et al., 1996; Iino et al., 1991; Xu et al., 1990) and to predicted proteins from a number of species, including A. thaliana, S. pombe and C. elegans (Fig. 8C). CAF, C8A4.08C, K12H4.8 and another predicted protein from C. elegans (RNC_CAEEL) each contain adjacent duplicated domains with similarity to the RNaseIII catalytic domain (Fig. 8A, C). These sequences contain two absolutely conserved residues which are the sites of inactivating point mutations in E. coli RNase III (Court, 1993). At the extreme C terminus, these proteins contain domains similar to double stranded RNA binding domains (dsRNAbds) found in RNaseIII and in a variety of additional RNA binding proteins (Fig. 8D; Kharrat et al., 1995). CAF contains a duplication of this dsRNAbd, which is not found in any of the other known RNaseIII-like proteins (Fig. 8A,D). The most C-terminal of these putative dsRNAbds is disrupted by the insertion of the T-DNA in the caf mutant, indicating that this sequence is necessary for wild-type CAF function.

**CAF RNA expression**

To test whether CAF is a single copy gene in Arabidopsis, or is a member of a family of closely related genes, we performed DNA blot analysis with probes from both the helicase and RNaseIII domains. Under high stringency conditions, both...
probes detected only the restriction fragments predicted by the CAF genomic sequence (see Materials and Methods). These results show that CAF is single copy in the Arabidopsis genome, and that under these conditions, the probes used are specific for the CAF gene.

To analyze the expression pattern of CAF, RNA blot analysis was performed with probes to both the helicase and RNaseIII domains. The helicase probe detected an RNA of approximately 6.2 kb, consistent with predictions from the cDNA and genomic sequences (Fig. 9A). However, the RNaseIII domain detected two RNA species, a 6.2 kb RNA and an additional 2.5 kb RNA (Fig. 9B). Thus CAF produces two predominant RNA species, one apparently encoding the full 1909 amino acid protein containing both the helicase and RNaseIII like domains, and one containing only the RNaseIII domain. Neither the 6.2 kb nor the 2.5 kb RNA species were detected in RNA extracted from the caf mutant. Instead, two larger transcripts of approximately 4.8 kb and 8.7 kb were present (Fig. 9A,B), presumably because transcription of the mutant caf gene extends into the T-DNA and adds approximately 2.4 kb to the size of each of the two major RNA species. The abundance of these aberrant caf mutant RNAs is similar to that of the wild-type CAF RNAs.

Results from RNA blot and RT-PCR experiments indicate that these two transcripts are present in vegetative tissue (leaves and stems) from 2-week old plants, and present in inflorescence tips from 4-week old plants (data not shown), suggesting that CAF RNA may be expressed ubiquitously throughout the shoot. To confirm these results and to test whether CAF could be expressed in a cell layer-specific manner, in situ hybridization experiments were performed (Fig. 10). A low level of CAF RNA was found in all cells of the apical and floral meristems, and in the flowers, cauline leaves and stems. Thus CAF RNA is expressed evenly throughout most shoot tissues. This expression pattern is consistent with the caf mutant phenotype, in that most shoot tissues show some defect in the mutant.

**DISCUSSION**

The caf mutant was initially chosen for study because of its dramatic defect in floral meristem determinacy. caf mutants exhibit over-proliferation of the floral meristem, such that flowers contain excess numbers of stamens and carpels. The caf mutant phenotype is distinct from that of the ag mutants, which also show reduced floral determinacy. Whereas ag mutations cause a reiteration of the floral program so that flowers show a repeating pattern of sepals and petals, caf flowers show unregulated cell division in the center of the flower, but show normal floral organ identity. caf mutants are also defective in other aspects of plant development. caf mutant plants lack nearly all of the axillary inflorescence meristems normally found in the wild type, and possess abnormally shaped cotyledons, leaves, sepals, stamens and carpels. Thus the wild-type CAF gene plays a role in specifying the determinate growth of the floral meristem, but also functions in the specification of axillary meristems and in the morphogenesis of organs.

It seems possible that the morphogenesis defects seen in the caf mutant could be explained as a secondary consequence of the defect in caf apical and floral meristem structure, in which case, one might expect that CAF activity could be localized to the meristems. However, we find that CAF RNA is evenly expressed throughout most shoot tissues, including apical and floral meristems, floral organs, stems and leaves. CAF is expressed in actively dividing cells at roughly the same level as in differentiated cells. Thus it seems more likely that CAF may act in flowers to pattern meristem determinacy, and act separately in other tissues to promote the proper development of axillary meristems, leaves and floral organs. However, it is still possible that the different floral and vegetative abnormalities found in caf are mechanistically related. For instance, one could interpret many of the vegetative and floral phenotypes as being losses in cell division control, with too much cell division giving rise to the extra carpels and too little cell division contributing to a lack axillary meristems and the reduced size of leaves, flowers and anthers.

The mutant allele of caf that we have studied, which is the only mutant allele known, seems unlikely to be a complete loss-of-function mutation. First, northern blot experiments show that the CAF RNA is expressed at a normal level in the caf mutant. Second, the T-DNA insertion in caf is predicted to prematurely truncate the CAF protein at amino acid number 1836, replacing the last 73 amino acids of CAF with eight amino acids from the T-DNA. This would leave 96% of the CAF protein intact in the mutant. Third, in several mutant screens carried out in our laboratory for mutations affecting floral structure, we have failed to find additional caf alleles. Thus, it is possible that stronger alleles of caf might have a lethal phenotype, or have a phenotype that would not have been detected in our screens. Given that CAF homologues exist in fungi and animals, it seems likely that CAF may carry out a conserved cell biological function (since plants and animals arrived independently at multicellularity), and therefore that CAF could be an essential gene, strong mutations in which might lead to a lethal phenotype. Thus the caf mutation described here may be a weak allele which uncovers CAF’s role in the regulation of cell division in floral meristems.

The genetic interactions of caf with other floral mutations support a role for CAF in controlling floral meristem proliferation. The double mutant phenotypes of caf combined with the floral homeotic mutants ap2-1, pi-1, and ag-1 are roughly additive. However, caf shows a synergistic interaction in combinations with two other mutations, sup-1 and clv3-2, which increase cell proliferation in floral meristems, but which do not seem to directly control organ identity. Epistasis was not found in the double mutant combinations of caf with sup or clv3 mutations, suggesting that CAF may, at least in part, control meristem activity through a mechanism somewhat different than that of SUP or CLV3.

The caf mutant phenotype resembles that of the sup mutants, in two ways. First both types of mutations cause an increase in the number of stamens and carpels (Gaiser et al., 1995; Jacobsen and Meyerowitz, 1997). Second, in an ag mutant background, both mutations cause a conversion of the inner whorl sepals to petals. The caf and sup mutations differ greatly however in their effects outside of the flower. Whereas the caf mutation affects the morphogenesis of most of the organs of the shoot, the sup mutations have no detectable effect on the development of non-floral tissues.

The N terminus of the predicted CAF protein is similar to DEAD/DExH type helicase/ATPase proteins from a number of organisms. CAF and these related proteins (all of unknown
RNaseIII domains may act together to process RNAs, with the end maturation, or snRNA processing. The helicase and in processes such as mRNA stability, mRNA splicing, rRNA 3' RNA helicase proteins and RNaseIII proteins, and considering processing enzyme. Extrapolating from the known functions of RNaseIII proteins, it seems likely that CAF acts as an RNA function than the bacterial and yeast RNaseIII proteins.

helicase domains, suggest that CAF may have a different results in the mutant phenotype. The altered structure of oskar RNA translation (Webster et al., 1997 and references therein), and is required for proper localization and translation of nanos RNA (Gavis et al., 1996). Both eif-4A (in combination with another factor, eif-4B) and VASA have been shown to possess ATP-dependent RNA helicase activity in vitro (Rozen et al., 1990; Liang, et al., 1994). Other helicase-related proteins are known to be involved in mRNA splicing (reviewed by Kramer, 1996). For instance, PRP2, a DEAH type protein, is thought to activate the precatalytic spliceosome (Kim and Lin, 1996). RAD3 is a DEAH family helicase-like protein, which acts in DNA repair and has DNA-dependent ATPase and DNA helicase activity (Harosh and Deschavanne, 1991). Another DEAD protein appears to regulate entry into mitosis in Schizosaccharomyces pombe (Warbrick and Glover, 1994). Other functions ascribed to helicase proteins include RNA processing (Kressler et al., 1997), mRNA stability (Most and Dreyfus, 1994), and mRNA degradation (Anderson and Parker, 1996). Given the diverse functions of helicase proteins, it is difficult to predict a specific function for the helicase domain of CAF; however, it is likely that this domain participates in nucleic acid dependent ATPase activity and may act to unwind RNA or DNA.

The C-terminal portion of CAF and the related S. pombe (C8A4.08C) and C. elegans (K12H4.8) proteins contain homology to RNaseIII proteins. Relative to the RNaseIII proteins from bacteria and yeast, these proteins have a duplication of the putative RNase catalytic domain, followed by either one or two double stranded RNA binding domains (dsRNAbds). RNaseIII proteins cleave specific regions of RNA that form base-paired stem-loop structures (reviewed by Court, 1993). Though RNaseIII cleaves only particular RNAs, the sequence requirements for this specific cleavage are unknown. In E. coli, RNase III is involved in the processing of ribosomal precursor RNAs and certain mRNA molecules (Court, 1993). In S. cerevisiae, RNaseIII proteins are known to process rRNAs and small nuclear RNAs (Elela et al., 1996; Channeau et al., 1997; Rotondo et al., 1995). Unlike other known RNaseIII proteins, including the homologous C. elegans and S. pombe proteins, CAF contains a second putative dsRNAAbd. This putative dsRNAAbd is essential for wild-type CAF function since disruption of this sequence by the insertion of the T-DNA of the gene and another that initiates in the middle. This would be consistent with the observation that smaller transcripts with the CAF protein itself could participate in such a cleavage reaction. However, analysis of the RNA structure in the caf mutant suggests that the CAF RNA processing still occurs, since both a large and a small transcript accumulate (Fig. 9). Thus, if CAF does process its own transcript, the second dsRNAAbd probably does not play a role in this process, as this is absent in the caf mutant. A third alternative is that the CAF gene contains two promoters, one that initiates at the beginning of the gene and another that initiates in the middle.

Why does mutation of the putative CAF RNA processing enzyme result in the observed loss of control of cell division seen in caf mutant floral meristems? One possibility is that CAF is an mRNA processing enzyme, and that the caf mutant phenotype results from the misregulation of one or more specific mRNAs, at least one of which plays a major role in the control of floral meristem determinacy. CAF would likely affect the processing of additional RNAs as well, which are important in other processes throughout the plant, such as those needed for the proper specification of the normal number of pollen sacs in anther, for the specification of the axillary inflorescence meristems, and for the proper shape of most organs of the shoot.

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