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Arabidopsis MSI1 is required for epigenetic maintenance of reproductive development

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SUMMARY

WD40 repeat proteins similar to yeast MSI1 are conserved in animals and plants, in which they participate in complexes involved in chromatin metabolism. Although MSI1-like proteins are well characterised biochemically, their function in the development of multicellular eukaryotes is not well understood. We constructed *Arabidopsis* plants in which the AtMSI1 protein level was altered. Strong ectopic expression of AtMSI1 produced no visible altered phenotype, but reduction of AtMSI1 dramatically affected development. The primary shoot apical meristem was unable to develop organs after the transition to flowering. Flowers that developed on floral shoots from axillary meristems experienced a progressive loss of floral morphology, including a reduction in size of the petals and stamens and the development of carpel-like sepals. Ovule development was disrupted in all flowers, resulting in complete female sterility. Molecular analysis of the mutant plants revealed that AtMSI1 is required to maintain the correct temporal and organ-specific expression of homeotic genes, including *AGAMOUS* and *APETALA2*. In contrast, FAS1 and FAS2, which together with AtMSI1 form the chromatin assembly complex CAF-1, are not required for repression of these genes. Therefore, AtMSI1 has specific functions in addition to CAF-1-mediated chromatin assembly. Efficient formation of heterochromatin, but not methylation of centromeric DNA repeats, depends on AtMSI1 presence demonstrating a key role of AtMSI1 in maintenance of chromatin structure.

Key words: *Arabidopsis thaliana*, Chromatin, MSI1, RbAp48, AGAMOUS, Meristem, Ovule

INTRODUCTION

Regulatory decisions that are made during development must be maintained correctly during the life of the organism. In multicellular eukaryotes, developmental programs are controlled largely at the level of transcriptional regulation and modulation of chromatin structure. Chromatin packages DNA into higher-order structures, but also maintains accessibility to DNA for regulatory DNA-binding proteins. Transcriptional activators also facilitate DNA accessibility by increasing chromatin fluidity. In contrast, transcriptional repressors establish and maintain chromatin structures that repress the activity of individual genetic loci or entire chromosome regions. During the last few years considerable insight has been gained into the biochemistry of chromatin metabolism and its role in development of plants and animals (for reviews see Habu et al., 2001; Muller and Leutz, 2001; Verbsky and Richards, 2001; Narlikar et al., 2002; Reyes et al., 2002).

Unlike in most animals, environmental factors strongly affect development of plants. Although the differences in developmental strategies are striking, it now emerges that plants utilise molecular mechanisms similar to those that control animal development (Habu et al., 2001; Verbsky and Richards, 2001; Meyerowitz, 2002). For example, chromatin-modifying proteins such as histone deacetylases, several polycomb-group proteins, like-heterochromatin protein 1, and chromatin assembly factor 1 (CAF-1) are also required for normal plant development (Goodrich et al., 1997; Chaudhury et al., 1997; Grossniklaus et al., 1998; Wu et al., 2000; Tian and Chen, 2001; Gaudin et al., 2001; Kaya et al., 2001). Proteins similar to yeast MSI1 (multicopy suppressor of *ira1*) and to the mammalian retinoblastoma-associated proteins RbAp46/48 are WD40 repeat proteins encoded by small multigene families in most eukaryotes. These conserved proteins are components of complexes involved in chromatin metabolism, including CAF-1, pRb, histone acetyl transferases and histone deacetylases (Qian et al., 1993; Parthun et al., 1996; Verreault et al., 1996; Taunton et al., 1996). CAF-1, a trimeric complex that facilitates deposition of nucleosomes on newly synthesised DNA, has been identified in *Arabidopsis*, mammals, yeast, flies and *Xenopus* (Smith and Stillman, 1989; Bulger et al., 1995; Kaufman et al., 1997; Quivy et al., 2001; Kaya et al., 2001). Biochemical analysis has shown that, similar to their animal counterparts, plant MSI1-like proteins are found in CAF-1 and can bind to plant retinoblastoma-related proteins and histones (Ach et al., 1997; Kaya et al.,

2001; Rossi et al., 2001). The biochemical data have not provided substantial insights into the biological function of MSI1-like proteins. Loss of MSI1 function in yeast does not produce apparent phenotypic alterations under optimal growth conditions. Detailed molecular analysis of *msi1* mutants revealed, however, that silencing of telomeric regions and mating type loci is decreased and cells are more sensitive to UV light (Kaufman et al., 1997). The functional analysis of MSI1-like proteins has proved more difficult in multicellular eukaryotes. The mutation *lin53* in *Caenorhabditis elegans* revealed that *LIN53* encodes a MSI1-like protein, which interacts with the retinoblastoma-like LIN35 protein and is required during vulva formation (Lu and Horvitz, 1998). RNAi-mediated interference with *LIN53* expression caused embryo lethality, suggesting that the gene product has an essential function during development. Additional mutants that affect MSI1-like proteins in other organisms could therefore provide important new insights into the function of this family of WD40 proteins in yeast and multicellular eukaryotes.

Four MSI1-like proteins have been identified in *Arabidopsis* (AtMSI1-4), but only AtMSI1 is most similar to RbAp48 (Ach et al., 1997; Kenzior and Folk, 1998). Similar to MSI1-like proteins from mammals, *Drosophila* and yeast, AtMSI1 and its tomato homologue LeMSI1 are predominantly localised to the nucleus (Quian et al., 1993; Tyler et al.,1996; Ach et al., 1997; Zhu et al., 2000; Bouché et al., 2002). A direct biological role, however, has not been established for either mammalian or plant MSI1-like proteins. Here we show that reducing *AtMSI1* expression in *Arabidopsis* disrupts several aspects of the developmental program. Our results suggest that AtMSI1 function is required during vegetative and reproductive growth and for maintenance of correct homeotic gene expression.

MATERIALS AND METHODS

Plant material and growth conditions

To construct plants in which *AtMSI1* is ectopically expressed, the complete coding sequence was fused to the cauliflower mosaic virus (CaMV) 35S promoter into the binary vector pSLK7292. Tri-parental mating of *Escherichia coli* DH5α cells transformed with the binary plasmids, *Agrobacterium tumefaciens* (strain GV3101/mp90) and *E. coli* helper strain (pRK2013) (Koncz and Schell, 1986) was performed to transfer the plasmid into *Agrobacterium*. *Arabidopsis thaliana* plants (accession Columbia) were transformed by floral dip (Clough and Bent, 1998). T_1 seeds were plated on Murashige and Skoog (MS) medium containing 50 μg/ml kanamycin, and after approximately two weeks the transformed seedlings were transferred to soil. Subsequent generations were grown in the greenhouse under long days (16 hours of light) at 23°C (±1.5°C). Alternatively, Conviron growth chambers with mixed cold fluorescent and incandescent light (230 μmol/m^2/second, 23°C) were used to raise the plants. The *AGAMOUS::GUS* reporter line was kindly provided by Drs Nathaniel P. Hawker and John Bowman (UC Davis, CA). It is similar to the promoter-intron GUS construct described by Sieburth and Meyerowitz (Sieburth and Meyerowitz, 1997) and contains all relevant intragenic sequences. The GUS expression in this line faithfully resembles spatial expression patterns of the endogenous *AG* gene (J. Bowman, personal communication). Seeds of Columbia, Landsberg *erecta* and Enkheim wild-type accession and of *fas1-1* and *fas2-1* (Reinholz, 1966; Leyser and Furner, 1992) mutants were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC On-Line Catalogue). *fas1-1 fas2-1* double mutants were generated by crossing.

Protein gel blot analysis

Protein extracts were prepared from *Arabidopsis* by grinding fresh or frozen tissue in extraction buffer (100 mM Tris, pH 7.5, 500 mM NaCl, 5 mM EDTA, 10 mM EGTA, 10% sucrose, 40 mM β-mercaptoethanol, 0.5 mg/ml Pefablock SC (Roche, Rotkreuz, Switzerland), 1 μg/ml pepstatin, 0.5 μg/ml leupeptin, 40 μg/ml bestatin. Homogenates were centrifuged for 5 minutes at 14,000 ***g*** and 4°C. SDS-PAGE, protein blotting and detection were performed as described previously (Ach et al., 1997). Chemiluminescent detection was performed with ECL (Amersham, Uppsala, Sweden) according to manufacturer's instructions.

Production of AtMSI1-5 fusion proteins

For coupled in vitro transcription and translation reactions, cDNAs of *AtMSI1-5* were cloned in frame with the HA-tag in pGADT7 (Clonetech, Palo Alto, CA). Reactions were performed using a Promega TnT wheat germ extract system (Promega, Madison, WI) according to manufacturer's instructions.

RNA isolation and RT-PCR

RNA was extracted from leaves of 4-week-old plants using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Fractionation of RNA on an agarose gel, transfer to nylon membranes, hybridisation with a random-primed ^{32}P-labelled probe and detection were performed as described previously (Ach et al., 1997). For RT-PCR analysis, 1 μg total RNA was treated with DNase I. Half of the DNA-free RNA (0.5 μg) was reverse-transcribed using an oligo(dT) primer and MMLV reverse transcriptase (Clonetech, Palo Alto, CA), while the remaining RNA was incubated without reverse transcriptase. Aliquots of the generated cDNA, which equalled 50 ng total RNA, were used as template for PCR with gene specific primers (Table 1).

Histological analysis

Flowers or inflorescences were fixed in FAA (3.7% formalin, 5% acetic acid, 50% ethanol) overnight at 4°C, and embedded in Technovit 7100 resin (Kulzer, Wahrheim, Germany). Sections of 5 μm were stained with Toluidine Blue and observed using an Axioplan 2 microscope (Zeiss, Jena, Germany). Tissues were cleared with chloralhydrate after fixation in ethanol:acetic acid (9:1) and observed under differential interference contrast (DIC) optics. Images were recorded with an Axiocam HRC CCD camera (Zeiss, Jena, Germany) and edited with ZeissVision software. For GUS expression analysis, flowers were fixed for 1 hour at 4°C in 90% acetone and washed three times with 50 mM phosphate buffer (pH 7.0) before overnight incubation at 37°C in reaction buffer (0.19 mM 5-bromo,4-chloro,3-indolyl-D-glucuronide, 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 50 mM phosphate buffer, pH 7.2). Tissue was cleared in ethanol, mounted in 50% glycerol and observed with a Zeiss Axioplan microscope.

Cytological analysis

Samples were prepared according to the method of Ross et al. (Ross et al., 1996). Briefly, inflorescences were fixed in ethanol:acetic acid (3:1), washed in water and incubated for 2 hours at 37°C with 0.3% (w/v) each of cellulase, pectolyase and cytohelicase in 10 mM sodium citrate (pH 4.5) to remove cell walls. Nuclei from petals were spread on microscopic slides as described, and stained with 4′,6′-diamidino-2-phenylindole (DAPI). The fluorescence patterns were examined with a Zeiss Axioplan microscope, and images were recorded with an MagnaFire® CCD camera (Optronics, Goleta, CA). Digital images were quantified using ImageJ 1.27Z (W. Rasband, NIH, USA, http://rsb.info.nih.gov/ij/). Total fluorescence was determined for at least 40 representative nuclei. Regions corresponding to chromocentres were selected manually and their emitted fluorescence was quantified.

Table 1. Sequences of gene-specific primers used for RT-PCR

Gene	Forward primer	Reverse primer
AtMSI1	GCACCGCTCTTCACACATTTG	TCGATCCTGCTAAGGTCCCAA
AtMSI2	ATTGGCCACAGCTTCCTCAGA	TTGGAAGACCTCTCCCTCATGG
AtMSI3	TGCGCCATTACATGTCCTGA	TCCCCAACCCTGTTGATATCC
AtMSI4	ACGAAGGTTGAAAAAGCGCA	CGATCAAACAACCGGACAGTG
AtMSI5	GGACAAAGTGGTCGACTTCCA	CCGCCATATCTGCAATGTACC
AG	TCGGACAATTCTAACACCGGA	CCCATCAATTGCCTGTTGG
AP1	TAGGGCTCAACAGGAGCAGT	CCACCCATGTTGAGAAAAGG
AP2	ATTTGGGTTTGTTCGACACC	ATGACTCGGCATTGAGTTCC
AP3	ACCACAACGAAGGAGATCGT	CAAGCTCGTCCAAACACTCA
GAPDHα	TTCTTGGCACCAGCTTCAAT	CTCCCTTGGAAGGAGCTAGG
LFY	ACCAAGGTGACGAACCAAGTATTC	TGGAGAGCGTAACAGTGAACGTAG
SUP	CACCATGGAGCTATGGAGAT	TTGCCATTGTTGAGTAAGAG
WUS	ACAGCATCAGCATCATCATC	TTGGCCATACTTCCAGATGG

Computational methods

Sequences were aligned using CLUSTAL_X (Thompson et al., 1997) with default settings. Using the neighbour-joining algorithm implemented in CLUSTAL_X, an N-J tree was constructed that was corrected for multiple substitutions. The tree was bootstrapped and a graph of the tree was displayed using TreeView software (Page, 1996).

RESULTS

The *Arabidopsis* genome encodes five MSI1-like proteins

Four MSI1-like genes, *AtMSI1-4*, were previously reported from *Arabidopsis* (Ach et al., 1997; Kenzior and Folk, 1998). After analysis of the complete *Arabidopsis* genome (The Arabidopsis Genome Initiative, 2000), we identified a fifth gene (At4g29730) encoding a protein similar to AtMSI1 that we termed *AtMSI5*. Results of RT-PCR analysis (see below) confirmed that *AtMSI5* is expressed.

Phylogenetic analysis of selected MSI1-like proteins from plants, yeast and animals demonstrated that diversification of these proteins occurred independently several times in evolution (Fig. 1). We were unable to identify a clade containing MSI1-like proteins that could be used to predict a specific biochemical function. When proteins from chicken and *Xenopus* were included in the analysis, we obtained similar results and found that the additional sequences grouped more closely with the human proteins RbAp46 and RbAp48, and *Drosophila* p55. In plants, divergence of MSI1-like proteins most likely occurred before the monocot and dicot split, because AtMSI1, AtMSI4 and AtMSI5 have close relatives in the monocot corn (*Zea mays*). AtMSI2/AtMSI3 and AtMSI4/AtMSI5 form pairs of very similar proteins (similarity of 90% and 83%, respectively), suggesting that the proteins are functionally redundant. Since AtMSI1 is sufficiently diverged from the other proteins, we expected that altering expression of *AtMSI1* would likely provide new insights into the function of this WD40 protein.

AtMSI1 is expressed in all plant organs

We analysed the expression patterns of *AtMSI1* to determine if the gene is differentially expressed. RNA blot analysis revealed that *AtMSI1* transcripts are present in all analysed tissues, including etiolated and green seedlings, roots, mature vegetative leaves, cauline leaves, stems, flowers and siliques (Fig. 2A). To correlate the transcript profile with AtMSI1 protein levels, we used an affinity-purified anti-LeMSI1 antiserum (Ach et al., 1997). This antibody is specific because it cross-reacts only with the HA-tagged AtMSI1 fusion protein, but not with the HA-AtMSI2-5 fusion proteins that were synthesised in a coupled in vitro transcription/translation system (Fig. 2B). A control protein blot probed with the anti-HA antibody showed that all five AtMSI proteins were produced in nearly similar amounts (Fig. 2B, upper panel). Protein extracts from several *Arabidopsis* organs and tissues were subsequently separated by SDS-PAGE and analysed by immunoblotting with the anti-LeMSI1 antibody. AtMSI1 protein was clearly detected in seedlings, roots, young and mature leaves, stems, flowers and siliques and the highest accumulation was found in flowers (Fig. 2C).

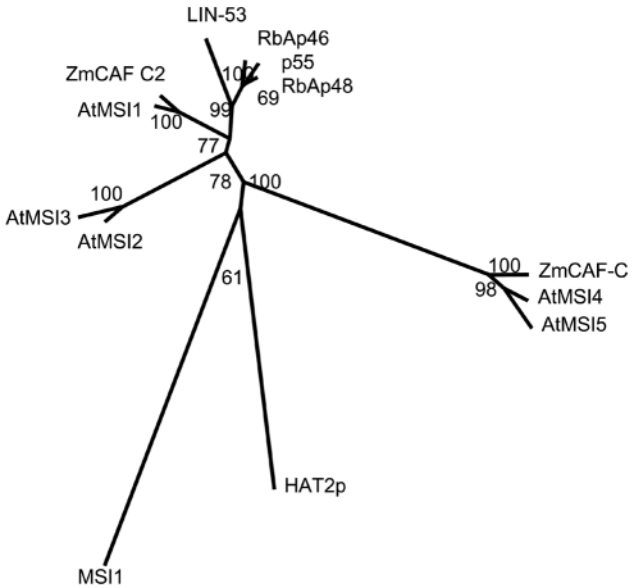

Fig. 1. Phylogenetic relationship of MSI1-like proteins. *Arabidopsis thaliana* AtMSI1-5, maize ZmCAF-C, maize ZmCAF-C2, *Saccharomyces cerevisiae* MSI1, *S. cerevisiae* HAT2p, human RbAp46 and RbAp48, *Drosophila melanogaster* p55 and *Caenorhabditis elegans* LIN-53 were used to generate a multiple alignment and the unrooted phylogenetic tree. Numbers represent bootstrap values of 100 trials.

Constitutive expression of *AtMSI1* sense RNA results in either ectopic protein expression or co-suppression

RNAi-mediated interference with *LIN53* expression in *C. elegans* caused embryo lethality (Lu and Horvitz, 1998), suggesting that the MSI1-like protein has an essential function during development. Ectopic expression of proteins also often provides new insights into their function. In plants, the activity of a transgene may also induce co-suppression, which results in the inactivation of both transgene and endogenous gene. We therefore transformed *Arabidopsis* plants with a construct containing the complete *AtMSI1* coding sequence in the sense orientation under control of the constitutive 35S promoter. The transformation produced 70 kanamycin-resistant *AtMSI1-OE* plants, of which 11 T_1 plants accumulated up to 50-fold higher levels of AtMSI1 (data not shown). Two independently transformed T_1 lines, 1OEa3 and 1OEc2, were characterised in detail. All T_1 plants appeared normal and showed no discernible morphological changes, suggesting that increasing AtMSI1 levels has no adverse effect on *Arabidopsis* development.

Among the progeny of ten of the original 70 T_1 *AtMSI1-OE* plants, including 1OEa3 and 1OEc2, several plants developed a severely stunted phenotype and were sterile. To determine the level of AtMSI1 protein accumulation in this class of *AtMSI1-OE* plants, protein extracts were prepared from rosette leaves of T3 siblings and subjected to gel blot analysis. Fig. 2D shows results from six 1OEa3 plants as an example. In plants that had stunted growth, AtMSI1 protein levels were reduced to less than 10% of control plants (Fig. 2D, lanes 3, 4 and 6). Siblings that appeared normal accumulated either wild-type (lane 2) or strongly increased AtMSI1 levels (lanes 7 and 8). Similar results were obtained for 1OEc2 T3 siblings (data not shown). The reduction of AtMSI1 levels in these plants was most likely the result of co-suppression of transgene and endogenous gene expression (Matzke and Matzke, 1995). Co-suppression of *AtMSI1* was gene specific, as would be expected based on the limited amino acid sequence homology with AtMSI2-5. RT-PCR with cDNA-specific primers demonstrated that mRNA levels of *AtMSI2-5* were not decreased in the *AtMSI1-OE* co-suppressed (subsequently termed *AtMSI1-CS*) plants (Fig. 2E). The strong *AtMSI1* co-suppression phenotype was recovered in 10-30% of the progeny of at least 3 independently transformed plants, and *AtMSI1-CS* plants from lines 1OEc2 and 1OEa3 showed a similar phenotype for all aspects that were subsequently analysed.

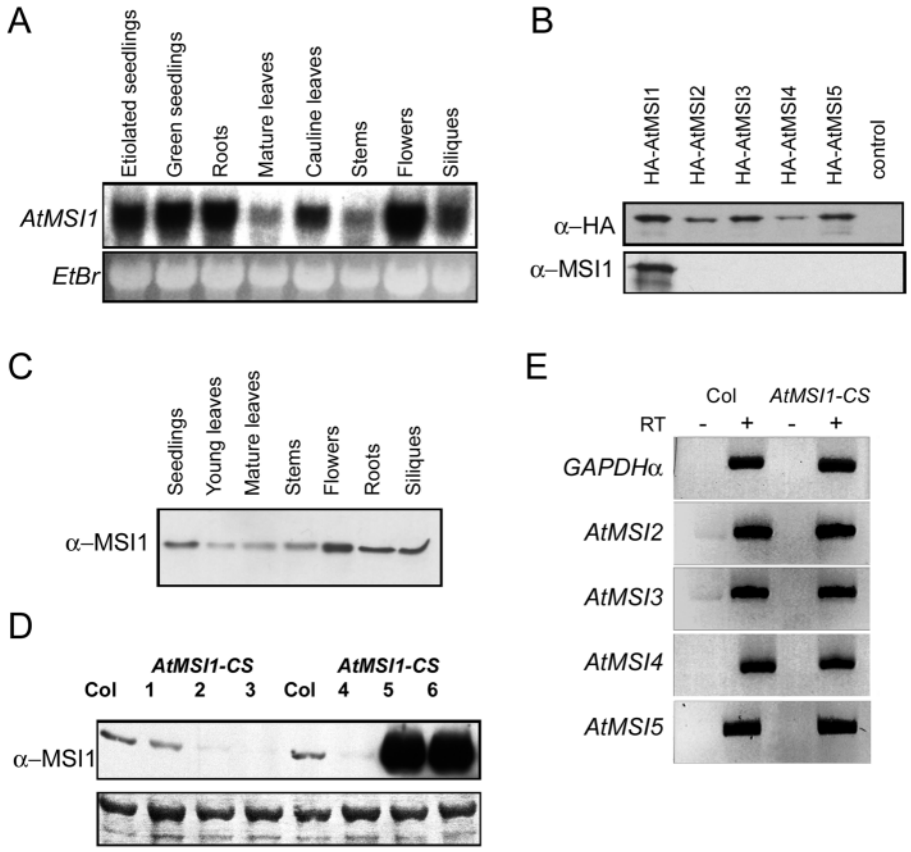

Fig. 2. Expression of *AtMSI1*. (A) Seedlings were grown for 7 days on plates containing MS medium, otherwise plants were grown on soil under a long-day light regime. RNA was extracted from different plant organs and RNA blots containing 10 μg of total RNA were probed with an *AtMSI1* probe (upper panel). The ethidium bromide-stained agarose gel is shown as a loading control (lower panel). (B) HA-tagged AtMSI1-5 were produced in vitro. Immunoblots containing similar fractions of the total reaction mixture were tested with either affinity-purified α-MSI1 or with α-HA antisera. Extract not supplemented with an *AtMSI* cDNA served as control (last lane). (C) Protein extracts were prepared from different plant organs. Leaves were harvested just after emergence from the shoot apical meristem and before they were completely expanded (young leaves) or after many other vegetative leaves had developed (mature leaves). Seedlings were grown on plates containing 50% MS medium. 10 μg protein was loaded in each lane. Blots were probed with affinity-purified, α-MSI1-specific antisera. (D) Protein was extracted from leaves of wild-type control plants (Col) and siblings of a segregating progeny of an *AtMSI1* overexpression (OE) line (1OEa3). Ten μg protein per sample was subjected to immunoblotting with affinity-purified, α-MSI1-specific antiserum (upper panel). Ponceau red-staining of the blot is shown as a loading control (lower panel). (E) RNA was isolated from leaves of wild-type and *AtMSI1-CS* plants before bolting. After treatment with DNaseI, RNA was subjected to reverse transcription in the presence or absence of reverse transcriptase using oligo(dT) primers. PCR with different cDNA-specific primers was performed on aliquots of the produced cDNA.

Co-suppression of *AtMSI1* strongly affects plant development

The rosette leaves of *AtMSI1-CS* plants were irregular in shape, and the normal phylotactic pattern of leaf production from the shoot apical meristem was altered (Fig. 3A,B). After transition to flowering, the primary shoot of wild-type plants elongated and formed cauline leaves, lateral branches and flowers (Fig. 3C, left). In contrast, the primary shoot of *AtMSI1-CS* plants arrested early in

development (Fig. 3C, right and Fig. 3D). After elongating for 5 to 15 mm, some shoots formed one or two aborted floral buds, but more frequently no organs developed except some bract-like structures.

Mature *AtMSI1-CS* plants were bushy and stunted, suggesting that they also had reduced apical dominance (Fig. 3E). Between 1 and 2 weeks after emergence of the aborted primary shoot, secondary shoots developed from axillary meristems. In contrast to the primary shoot, these shoots produced cauline leaves, lateral branches and flowers, although flower morphology was strongly altered. The number of unopened floral buds was increased in *AtMSI1-CS* plants relative to control plants. Unlike wild-type *Arabidopsis* flowers, which consist of sepals, white petals, pollen-producing stamens, and ovule-bearing carpels that are arranged in four concentric whorls (Fig. 4A, left), the flowers of *AtMSI1-CS* plants were deformed. The outer whorl of sepals appeared to be separated from the petals. The carpels were strongly elongated relative to the stamens and protruded from the enclosing petals and sepals (Fig. 4A, right). The morphological alterations that were already detectable in the first flowers on the axillary shots increased in severity in flowers that developed later. While overall morphology of older flowers still resembled that of wild-type flowers (Fig. 4A,B), younger flowers often lacked petals and anthers, the organs of whorls 2 and 3. Sepals developed carpel-like characteristics, including stigmatic surfaces (Fig. 4C). All flowers that developed on the axillary inflorescences were sterile. Reciprocal crosses showed that the sterility of *AtMSI1-CS* plants was caused by maternal defects.

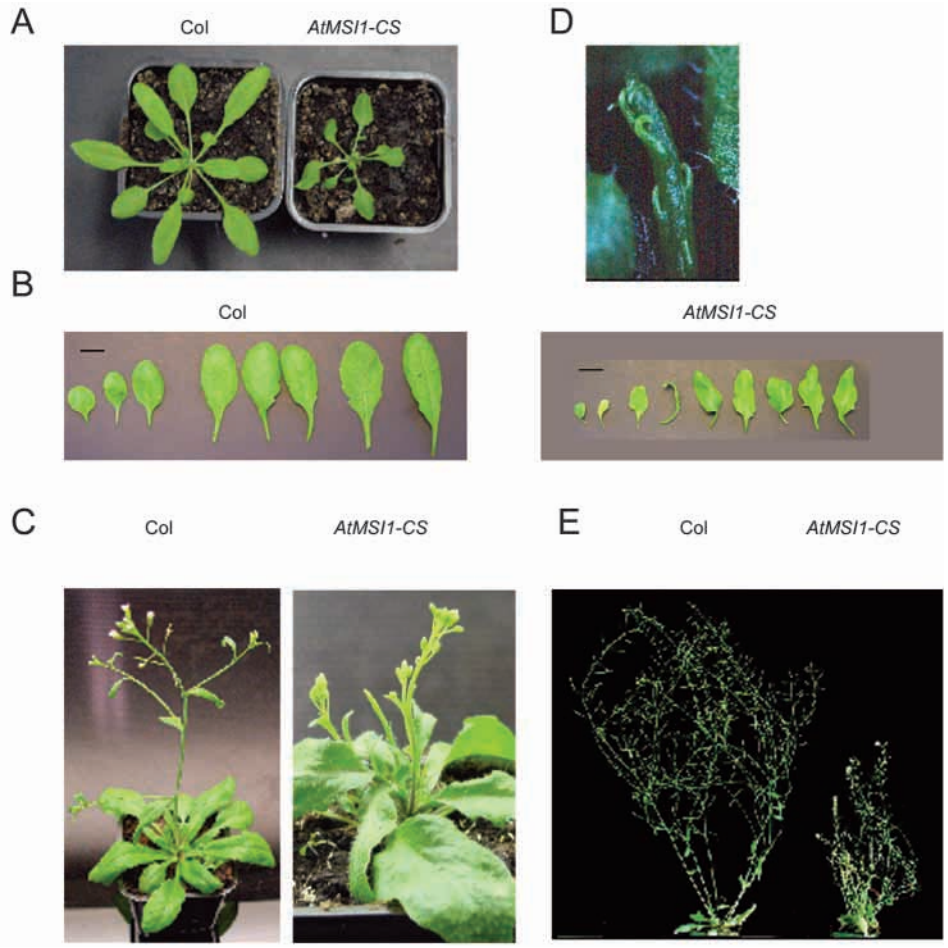

Fig. 3. Developmental alterations in *AtMSI1-CS* plants. (A) Rosettes of 3-week-old wild-type and *AtMSI1-CS* plants. (B) Rosette leaves of wild-type and *AtMSI1-CS* plants at time of bolting (scale bar: 10 mm). (C) Phenotype of the primary inflorescence shoot shortly after bolting in wild-type (Col) and *AtMSI1-CS* plants. (D) Close-up view of an arrested primary inflorescence shoot of an *AtMSI1-CS* plant. (E) Appearance of mature, flowering wild-type and *AtMSI1-CS* plants.

To determine the basis of female sterility, ovules were analysed in *AtMSI1-CS* plants (Fig. 5). Longitudinal sections of early flowers showed that floral organs (sepals, petals, stamens and carpels) initiated normally, but both petals and anthers were shorter than in wild-type flowers (also shown in Fig. 5B). Initiation of ovules on the placenta was similar to wild-type *Arabidopsis* with no gaps (Fig. 5A). Mature wild-type ovules have a curved funiculus, integuments that enclose the nucellus, and a clearly visible embryo sac (Fig. 5B,C,H). Longitudinal sections of *AtMSI1-CS* flowers of a comparable developmental stage revealed that ovule development was severely delayed and the ovules lacked embryo sacs (Fig. 5D). Ovule development appeared to be arrested at megagametogenesis (Fig. 5G). Further examination, including

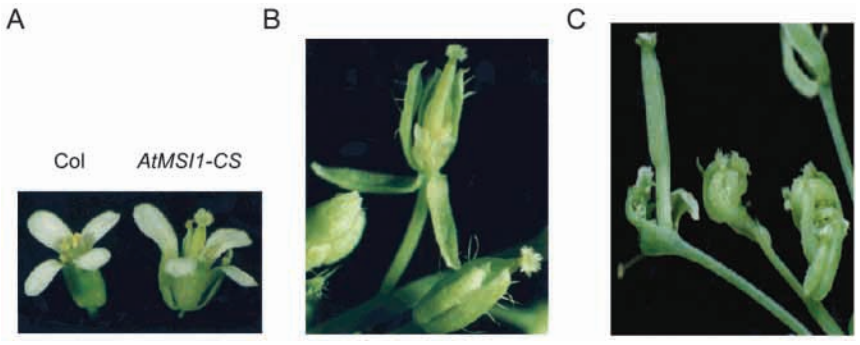

Fig. 4. Flower morphology of *AtMSI1-CS* plants. (A) A flower of a wild-type plant (Col) and a flower formed early on a secondary inflorescence shoot of an *AtMSI1-CS* plant (right). (B) A flower that developed later on the same *AtMSI1-CS* secondary inflorescence shoot. (C) Latest flowers that developed on this *AtMSI1-CS* secondary inflorescence shoot.

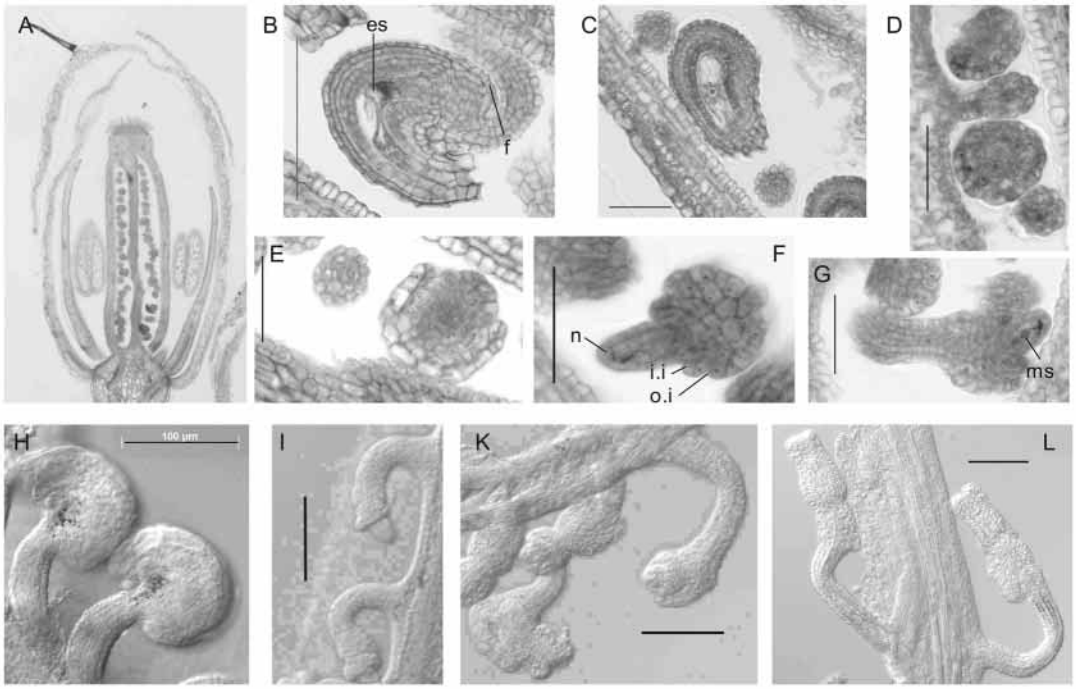

Fig. 5. Irregular ovule development in *AtMSI1-CS* plants. Floral buds of stage 11-12 were either embedded, sectioned and stained with Toluidine Blue or cleared and observed with differential interference contrast (DIC) optics. (A) Section of a floral bud from a *AtMSI1-CS* plant. (B,C) Longitudinal and transverse sections of mature ovules of a wild-type plant. (D-G) Sections of ovules from *AtMSI1-CS* plants. (H) DIC image of a wild-type ovule. (I) DIC images of ovules from an *AtMSI1-CS* plant. (K,L) DIC images of ovules from stage 16 flower from *AtMSI1-CS* plants. Scale bars: 50 µm (C-G,I,K,L) and 100 µm (B,H). es, embryo sac; f, funiculus; i.i, inner integument; ms, megaspore; n, nucellus; o.i, outer integument.

optical sections using DIC optics, revealed that both integuments initiated normally, but outgrowth of integuments was strongly retarded (Fig. 5E,F,I). To determine whether ovule development was arrested with the observed morphological changes or could proceed further, using DIC optics we examined ovules from gynoecia of flowers that had just shed sepals, petals and anthers. Although the inner integument had continued to grow and enclosed the nucellus, the outer integument rarely showed the well-defined asymmetric growth resulting in the anatropy of mature wild-type ovules, and embryo sacs were missing (Fig. 5K,L).

Control of homeotic gene expression is lost in *AtMSI1-CS* plants

The deformed and curled leaves, as well as abnormal flower morphology, in *AtMSI1-CS* plants are reminiscent of mutants in which control of floral homeotic gene expression is disrupted. For example, in *curly leaf* (*clf*) and *methyltransferase 1 antisense* (*met1*) mutants floral homeotic genes become ectopically expressed in leaves. Using RT-PCR, we tested for the presence of *AGAMOUS* (*AG*), *SUPERMAN* (*SUP*), *APETALA1-3* (*AP1-3*), *WUSCHEL* (*WUS*) and *LEAFY* (*LFY*) mRNAs in RNA isolated from leaves of wild-type and *AtMSI1-CS* plants (Fig. 6A). As expected, all genes were expressed in wild-type flowers, and only very low mRNA levels were detected in leaves of wild-type plants. In contrast, *AG* and *AP2* mRNAs accumulated to significantly higher levels in *AtMSI1-CS* leaves than in wild-type leaves, suggesting that the control of their expression was deregulated when AtMSI1 levels are decreased below a certain threshold. No increase in mRNA levels was detected for *WUS*, *AP1*, *AP3*, *SUP* and *LFY* (Fig. 6A and data not shown). In order to test whether aberrant expression of floral homeotic genes is restricted to leaves or also occurs in flowers, we crossed AtMSI1-CS plants with an *AG::GUS* reporter line. Fig. 7A shows GUS-staining patterns for a segregating wild-type plant. As for the endogenous *AG* gene, expression of the reporter is restricted to the inner two whorls. The intermediate *AtMSI1-CS* flower shown in Fig. 7B is characterised by shorter stamens and petals and enlarged sepals. In contrast to wild-type sepals, which did not contain any GUS staining, the carpel-like stigmatic structures at the tip of *AtMSI1-CS* sepals clearly expressed the *GUS* reporter gene.

AtMSI1 was previously reported to function together with FAS1 and FAS2 in the *Arabidopsis* CAF-1 complex (Kaya et al., 2001), and *fas1* and *fas2* single mutants have been described (Reinholz, 1966; Leyser and Furner, 1992). To better understand the functions of the two *FASCIATA* genes relative to *AtMSI1*, we constructed *fas1 fas2* double mutants. The mutant plants were viable and displayed the same set of developmental alterations as the parental single mutants or the *fas1 fas2* double mutant (Kaya et al., 2001). In contrast, none of the characteristic traits of *AtMSI1-CS* plants (Figs 3-5) were observed (data not shown). Similarly, no *AG* or *AP2* transcript could be detected in leaves of *fas1* and *fas2* single or *fas1 fas2* double mutants (Fig. 6B). Together, these results demonstrate that repression of homeotic genes in leaves requires AtMSI1 but is independent of CAF-1 function.

AtMSI1-CS plants contain reduced amounts of heterochromatin

Arabidopsis chromocentres contain highly condensed heterochromatic DNA that consists of centromeric and pericentomeric repeats and rRNA genes (Maluszynska and Heslop-Harrison, 1991; Fransz et al., 2002). Formation of chromocentres requires epigenetic imprints, such as DNA methylation and histone acetylation, because reduced amounts of heterochromatin and dispersion of pericentromeric sequences away from chromocentres were observed in *decreased DNA methylation 1* (*ddm1*) and *met1* mutants (Soppe et al., 2002). Because AtMSI1 most likely participates in specific chromatin-modifying complexes, it could also be required for maintenance of functional heterochromatin. We therefore investigated the structure of nuclei in *AtMSI-CS* plants that had strongly reduced AtMSI1 levels using nuclear spreads and compared them to wild-type nuclei. The general appearance of *AtMSI-CS* interphase nuclei, including nuclear size and number of chromocentres, was not significantly different (Fig. 8). We quantified nuclear fluorescence originating from heterochromatic chromocentres and from the remaining euchromatic regions of nuclei. In wild-type interphase nuclei, euchromatin fluorescence was 2.3 times higher than heterochromatin fluorescence. In contrast, this ratio increased to 4.0 in *AtMSI1-CS* plants (Fig. 8B, right). These observations indicate that recruitment of chromosomal DNA into heterochromatic chromocentres is strongly reduced in the absence of AtMSI1.

DISCUSSION

MSI1-like RbAp48-related proteins have been identified as components of complexes that regulate transcription activity and chromatin structure in mammals, yeast, flies and other eukaryotes, but their functional role in the control of gene expression is not well understood (Qian et al., 1993; Parthun et al., 1996; Verreault et al., 1996; Taunton et al., 1996). In organisms that encode multiple *MSI1*-like genes, it is not clear how much functional overlap exists between the different MSI1-like proteins. *Drosophila* has only one MSI1-like protein (p55), which is a subunit of CAF-1 and is found in complexes with histone deacetylases, histone acetyl transferases, the nucleosome remodelling factor NURF and histone

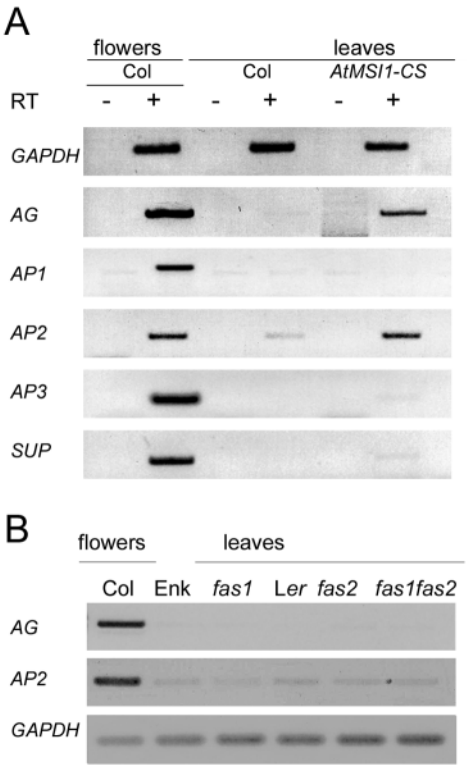

Fig. 6. Ectopic expression of floral homeotic genes in leaves of *AtMSI1-CS* plants. RNA was isolated from (A) floral buds and open flowers of wild-type plants and leaves of wild-type and *AtMSI1-CS*, and (B) flowers of wild-type and leaves of *fas1*, *fas2* and *fas1 fas2* plants before bolting. After treatment with DNAseI, RNA was subjected to reverse transcription in the presence (+) or absence (–) of reverse transcriptase using oligo(dT) primers. PCR with different cDNA-specific primers was performed on aliquots of the produced cDNA (10 ng total RNA from flowers and 50 ng total RNA from leaves).

Fig. 7. Spatial expression patterns of *AGAMOUS* in flowers of *AtMSI1-CS* plants. *AtMSI1-CS* plants were crossed with an *AG::GUS* reporter line. Flowers of segregating wild-type plants carrying the reporter (A) and of *AtMSI1-CS AG::GUS* plants (B) were stained for GUS activity. Shown are (from left to right) whole flowers, stamens (×2), petals (×2) and sepals (×2). Scale bars: 100 μm.

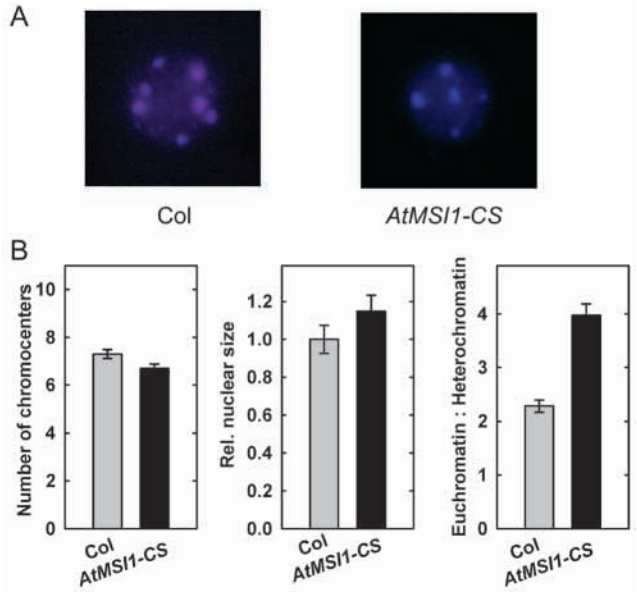

Fig. 8. Reduced amounts of heterochromatin in *AtMSI1-CS* plants. (A) Phenotypes of representative DAPI-stained petal interphase nuclei. Chromocentres are smaller and staining is weaker in *AtMSI1-CS* than in wild-type nuclei. (B) Quantification of observations shown in A. Number of chromocentres (left), relative nuclear size (middle) and the ratio of euchromatin: heterochromatin (right) are plotted for wild type and *AtMSI1-CS*. Shown are the mean±s.e.m. of 40 nuclei each.

methyltransferases (Tyler et al., 1996; Martinez-Balbas et al., 1998; Czermin et al., 2002). Human RbAp46 and RbAp48 appear to be more specialised. RbAp46 interacts with histone acetyltransferase HAT1, and only RbAp48 was reported as a subunit of human CAF-1 (Verreault et al., 1996; Verreault et al., 1998). Similarly, yeast HAT2p is specific for HAT1p, and only CAC3/MSI1 is a component of yeast CAF-1 (Verreault et al., 1996; Kaufman et al., 1997). The MSI1-like proteins that participate in CAF-1 function in different organisms (human RbAp48, *Drosophila* p55, yeast MSI1, AtMSI1), however, are not more closely related to each other than they are related to other MSI1-like proteins. This suggests that functional divergence of these proteins has evolved independently in yeast, mammals and plants. Amino acid sequence comparison also suggests that plant MSI1-like proteins already diverged before the evolutionary split in monocot and dicot plants. It will be interesting to establish the functional significance of multiple MSI1-like proteins in yeast, mammals and plants, as compared to *Drosophila*, in which p55 is found to be associated with several chromatin-modifying complexes.

AtMSI1 is not functionally redundant with AtMSI2-5

Reduced levels of AtMSI1 altered cotyledon and leaf shape, affected meristem function, and reduced fertility, indicating that AtMSI1 function is required during vegetative and reproductive development. This conclusion is consistent with the presence of AtMSI1 in all tissues and organs that were analysed. Moreover, AtMSI1 was expressed most strongly in flowers, which were also most severely affected by reduced AtMSI1 levels in *AtMSI1-CS* plants. RT-PCR with gene-specific primers confirmed that *AtMSI2-5* mRNA levels were not reduced in *AtMSI1-CS* plants. The morphological changes are therefore strictly correlated with the changes in AtMSI1 levels, suggesting that there is only limited functional redundancy between AtMSI1 and the other four MSI1-like proteins in *Arabidopsis*.

AtMSI1 may function in multiple chromatin-modifying complexes

A CAF-1 complex active in chromatin assembly in vitro is also present in *Arabidopsis* and consists of FAS1, FAS2 and AtMSI1, which are similar to CAC1, CAC2 and CAC3 (Kaya et al., 2001). FAS1 or FAS2 are both encoded by single copy genes in *Arabidopsis*. Mutations in these genes cause fasciation as the result of an enlarged shoot apical meristem. Fasciated plants have an altered phyllotaxy and leaf shape, reduced root growth, broadened, flattened or bifurcated stems and increased numbers of flower organs (Reinholz, 1966; Leyser and Furner, 1992). Despite strongly reduced AtMSI1 protein levels *AtMSI1-CS* plants showed only mild symptoms of fasciation. It is possible that the AtMSI1 levels still present in these plants are sufficient to maintain minimal CAF-1 activity for functional chromatin assembly. Alternatively, one of the other MSI1-like proteins in *Arabidopsis* (AtMSI2-5) can substitute for AtMSI1 function in CAF-1 activity. *AtMSI1-CS* plants also have several phenotypic alterations not associated with the fasciation phenotype in *fas1* and *fas2*, and repression of *AG* and *AP2* expression in leaves is lost when AtMSI1 is strongly reduced, but not when FAS1 and FAS2 are absent. Because *Arabidopsis* accessions are known to differ in genetic potential and developmental programs (Alonso-Blanco and Koornneef, 2000), both the *fas1-1* and the *fas2-1* allele were back-crossed into the Columbia accession. However, the different phenotypes of *AtMSI1-CS* and *fasciata* mutants remained independent of the genetic background (data not shown). Therefore, AtMSI1 functions not only in CAF-1 but also in other chromatin-modifying complexes that do not contain the two larger CAF-1 subunits. Studies on MSI1-like proteins in yeast and animals suggest potential biochemical functions involving histone acetylation, deacetylation or methylation and nucleosome remodelling (Tyler et al., 1996; Martinez-Balbas et al., 1998; Tie et al., 2001; Czermin et al., 2002). Protein binding studies revealed that AtMSI1 interacts with the *Arabidopsis* retinoblastoma-related protein (RBR) and histone deacetylase HDA1, and that AtMSI2 and AtMSI3 interact with FAS1 (Heidi Feiler, Lars Hennig, N. S., P. T. and W. G., unpublished data). Together, our data suggest AtMSI1-5 have different functional specificities. While some of the functions depend strictly on AtMSI1, other functions (e.g., CAF-1 mediated chromatin assembly) could also involve AtMSI2-5.

AtMSI1 is required for the maintenance of meristem function

AtMSI1 deficiency affects the shoot apical meristem, and both floral meristems and primordia. The fate of the primary shoot meristem after transition to flowering was strongly dependent on AtMSI1 levels. While leaf development during the vegetative phase was affected but not abrogated, essentially no organs developed on the *AtMSI1-CS* primary shoot after transition to flowering. The appearance of bract-like structures demonstrated that primordia could be initiated by the inflorescence shoot apical meristem, but were unable to

differentiate correctly. Secondary inflorescence shoots arising from axillary meristems of rosette leaves were significantly less affected and only occasionally showed a developmental arrest similar to the primary inflorescence shoot. Although the secondary inflorescence shoots usually gave rise to bracts, flowers and lateral shoots, flowers formed on these shoots displayed a progressive loss of floral morphology. As with leaf development during the vegetative phase, flower organs appeared to initiate normally, but their normal differentiation was disrupted. Since the severity of phenotypes increased with additional rounds of cell divisions, perhaps AtMSI1 is required to maintain the epigenetically controlled developmental pattern of gene expression during cell division.

Sterility of *AtMSI1-CS* plants is caused by defects in ovule development

In wild-type *Arabidopsis* plants, ovule development comprises primordia initiation, specification of identity, pattern formation, morphogenesis and cellular differentiation (Grossniklaus and Schneitz, 1998; Schneitz, 1999). Ovules arise as finger-like protrusions from the placental tissue of the carpel. After polarity has been established along a proximal-distal (PD) axis of symmetry, megasporogenesis begins and the two integuments initiate. Outgrowth of the outer integument shows strong polarity along the adaxial-abaxial (Ad-Ab) axis, which results in anatropy of mature ovules. Similar to leaf and flower development, initiation of ovule primordia and the integuments occurred normally. Loss of AtMSI1 did not affect polarity along the PD and Ad-Ab axis, but prevented further asymmetric growth and megagametogenesis. Several genes whose functions are required during ovule development have been identified already (for review, see Grossniklaus and Schneitz, 1998; Schneitz, 1999). In addition to ovule-specific genes, genes involved in other developmental processes are also required for proper ovule development. Among them *SUP*, *AG*, *WUS* and *SPLAYED* (*SPY*) are noteworthy, since mutants defective in these genes, or lines in which these genes are ectopically expressed, share several morphological alterations with *AtMSI1-CS* plants (Ray et al., 1994; Gaiser et al., 1995; Western and Haughn, 1999; Groß-Hardt et al., 2002; Wagner and Meyerowitz, 2002). In particular, ovule development arrests at similar stages in *spy* mutants and *AtMSI1-CS*. SPY is a SWI/SNF ATPase homolog, which is thought to modify activity of the LFY transcription factor by altering chromatin states, and *AG* is among the genes whose expression depends on LFY and SPY activity (Wagner and Meyerowitz, 2002). Given the phenotypic aspects shared among the mutants, it will be interesting to test possible genetic interactions between *SPY* and *AtMSI1*.

Reduction of AtMSI1 function activates the ectopic expression of homeotic genes that control meristem fate

In *AtMSI1-CS* plants, reduction of AtMSI1 levels disrupts the spatial and temporal control of expression for several homeotic genes that regulate plant development and organ identity. The class C floral organ identity gene *AG*, whose expression is confined to the inner two whorls of flowers in wild-type *Arabidopsis*, was ectopically expressed in leaves of *AtMSI1-CS* plants. *AP2*, a class A floral organ identity gene that is also expressed weakly in leaves (Jofuku et al., 1994; Okamuro et al., 1997), was more strongly expressed in leaves of *AtMSI1-CS* plants than in wild-type plants. Expression of *WUS*, *AP1*, *AP3* and *SUP* was not affected in *AtMSI1-CS* plants, suggesting that loss of AtMSI1 function affects only a selected class of regulatory genes. Aberrant expression of *AG* is not restricted to leaves, and activity of the *AG::GUS* reporter in *AtMSI1-CS* flowers is also consistent with the observed homeotic changes of organ identity. GUS activity was detected in the tips of enlarged sepals that acquired a carpel-like, abnormal identity characterised by stigmatic structures. GUS staining was much weaker in *AtMSI1-CS* leaves and was concentrated along the leaf veins (data not shown). Thus, transcriptional control of both the endogenous *AG* gene and also the reporter transgene depends on AtMSI1 function.

AP2 and *AG* are known to interact antagonistically and reciprocally inhibit their activation in floral whorls (Bowman et al., 1991). Because *AP2* and *AG* expression domains overlap also in whorl 3 and whorl 4 of WT flowers, AP2 and AG gene products are not sufficient for transcriptional repression (Jofuku et al., 1994). Therefore, the simultanous expression of both *AP2* and *AG* in *AtMSI1-CS* leaves is quite conceivable. Current models suggest that homeotic genes, which control the developmental fate of meristems, are controlled by modulation of their chromatin structures and/or methylation status (Conner and Liu, 2000; Jacobsen et al., 2000; Tian and Chen, 2001; Yoshida et al., 2001; Wagner and Meyerowitz, 2002). For example, *AG*, *AP3* and *SUP* are ectopically expressed in mutants such as *clf*, *met1* and others (Finnegan et al., 1996; Goodrich et al., 1997). Thus, it is possible that the higher-order chromatin structure or assembly of specific repressor complexes at promoters of *AG* and *AP2* depend, either directly or indirectly, on AtMSI1 function. Notably, this function is independent of chromatin assembly by CAF-1 because even the *fas1 fas2* double mutant maintains the repressed state of *AG* and *AP2* in leaves.

Reduced AtMSI1 levels alter nuclear chromatin organisation

The functional analysis of AtMSI1 discussed above suggests that the protein may have a role in control of chromatin structure and dynamics. Our cytological analysis of *AtMSI-CS* nuclei subsequently revealed a significant loss of heterochromatin assembly into chromocentres. Recent reports have established a central role of nuclear chromocentres for the organisation of chromosomal DNA, and specific euchromatic loops were detected that extended from the condensed heterochromatin (Fransz et al., 2002; Soppe et al., 2002). In contrast to the DNA in such loops, DNA present in chromocentres was heavily methylated. Similar to our observations in *AtMSI1-CS* nuclei, *ddm1* and *met1*, which both have reduced DNA methylation, also had smaller chromocentres. Methylation is a genomic imprint that is required for the maintenance of heterochromatin (Soppe et al., 2002). Strikingly, both *met1* and *AtMSI1-CS* plants assemble less DNA into chromocentres, ectopically express *AG* and other floral homeotic genes, and share other phenotypic traits (Finnegan et al., 1996; Soppe et al., 2002; this study). We therefore tested if methylation of centromeric repeats, which is strongly decreased in *met1* plants, was affected in *AtMSI1-CS*. Blots of genomic DNA digested with methylation-sensitive or -insensitive restriction endonucleases demonstrated, however,

that centromeric methylation patterns were intact in *AtMSI1-CS* plants (data not shown). In summary, we propose that AtMSI1 is required downstream of DDM1- and MET1-dependent DNA methylation in order to facilitate formation of repressive chromatin structures. Alternatively, AtMSI1 might function in a pathway parallel to DNA methylation. Experiments are in progress to establish the role of AtMSI1 in heterochromatin condensation.

The extent of the phenotypic alterations that resulted from the reduction and loss of AtMSI1 function suggests that AtMSI1 has a fundamental role in development and cellular differentiation. Our view is consistent with the report that RNA-mediated interference (RNAi) of *LIN53* expression, which encodes a protein similar to RbAp48 and MSI1, causes embryonic lethality in *C. elegans* (Lu and Horvitz, 1998). It is important to note, however, that not all developmental processes are similarly affected in plants with reduced AtMSI1 levels. The role of AtMSI1 in specific developmental pathways will be understood better once the significance of its interactions with the retinoblastoma tumour suppressor protein, HDA-dependent transcriptional co-repressors, CAF-1, NURF and perhaps other chromatin-modifying complexes has been analysed in more detail. It will also be important to clarify the function of the other MSI1-like proteins in *Arabidopsis*, which may provide new insights into the biological functions of this class of WD40 proteins in other multicellular eukaryotes as well.

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