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ScienceDirect
Developmental Biology 303 (2007) 259–269
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

C2H2 zinc finger-SET histone methyltransferase is a plant-specific chromatin modifier

Alexander Krichevsky [a,*], Helen Gutgarts [a], Stanislav V. Kozlovsky [a], Tzvi Tzfira [b], Ann Sutton [a], Rolf Sternglanz [a], Gail Mandel [c], Vitaly Citovsky [a]

[a] Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794-5215, USA
[b] Department of Molecular, Cellular and Developmental Biology, The University of Michigan, Ann Arbor, MI 48109, USA
[c] Howard Hughes Medical Institute and Department of Neurobiology and Behavior, State University of New York, Stony Brook, NY 11794, USA

Received for publication 12 September 2006; revised 20 October 2006; accepted 6 November 2006
Available online 10 November 2006

Abstract

Histone modification represents a universal mechanism for regulation of eukaryotic gene expression underlying diverse biological processes from neuronal gene expression in mammals to control of flowering in plants. In animal cells, these chromatin modifications are effected by well-defined multiprotein complexes containing specific histone-modifying activities. In plants, information about the composition of such co-repressor complexes is just beginning to emerge. Here, we report that two *Arabidopsis thaliana* factors, a SWIRM domain polyamine oxidase protein, AtSWP1, and a plant-specific C2H2 zinc finger-SET domain protein, AtCZS, interact with each other in plant cells and repress expression of a negative regulator of flowering, *FLOWERING LOCUS C* (*FLC*) via an autonomous, vernalization-independent pathway. Loss-of-function of either AtSWP1 or AtCZS results in reduced dimethylation of lysine 9 and lysine 27 of histone H3 and hyperacetylation of histone H4 within the *FLC* locus, in elevated *FLC* mRNA levels, and in moderately delayed flowering. Thus, AtSWP1 and AtCZS represent two main components of a co-repressor complex that fine tunes flowering and is unique to plants.

Keywords: Chromatin remodeling; Histone modification; Gene repression

Introduction

Polyamine oxidase (PAO)-containing co-repressor complexes represent one of the major regulators of gene expression in animal cells (Jepsen and Rosenfeld, 2002). The main components of these co-repressor complexes include: KIAA0601, a SWIRM domain PAO-like protein which, in mammalian cells, is a histone lysine demethylase (LSD1) (Shi et al., 2004), G9a (Roopra et al., 2004), a SET [Su(var)3-9, Enhancer-of-zeste, Trithorax (Peters et al., 2003; Tachibana et al., 2001)] domain protein with a histone methyltransferase (HMT) activity (Tachibana et al., 2001), ZNF217, a zinc finger protein (You et al., 2001) with still unknown function, histone deacetylases (HDAC), and CoREST co-repressor (Andres et al., 1999). One of the major effects of PAO-containing co-repressor complexes is transcriptional gene silencing via post translational modifications of the core histones. Among such modifications, the most commonly found in the silenced genetic loci include general histone deacetylation, methylation on lysines 9 (K9) and 27 (K27) of histone H3 (Cao et al., 2002; Jenuwein and Allis, 2001; Rea et al., 2000), and demethylation on lysine 4 (K4) of histone H3 (Shi et al., 2004). These histone modifications, therefore, represent specific marks which determine the epigenetic state of the chromatin. In turn, the chromatin state determines the cell fate during such diverse developmental events as acquisition of neuron-specific traits in mammals (Ballas et al., 2005; Lunyak et al., 2004), and determination of flower timing in plants (He and Amasino, 2005).

Unlike the animal co-repressor complexes, knowledge about plant PAO-containing co-repressor complexes is virtually non-existent. Only recently, an existence of a gene repression

* Corresponding author. Fax: +1 631 632 8575.
E-mail address: akrichevsky@notes.cc.sunysb.edu (A. Krichevsky).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.11.012

mechanism involving a PAO-like protein has been demonstrated by an observation that an *Arabidopsis* homolog of KIAA0601/LSD1 termed FLOWERING LOCUS D (FLD) represses the *FLOWERING LOCUS C* (*FLC*), a negative regulator of flowering (reviewed by Amasino, 2005; Bäurle and Dean, 2006; He and Amasino, 2005; Noh and Noh, 2006; Schubert et al., 2005; Sung and Amasino, 2005), by histone deacetylation (He et al., 2003); the molecular partners of FLD involved in its repressor activity, however, remain largely unknown. Here, we identified two components of an *Arabidopsis* co-repressor complex, a SWIRM domain PAO protein AtSWP1 and its cognate plant-specific C2H2 zinc finger-SET domain HMT, AtCZS, and showed that they interact with each other in plant cells and repress expression of *FLC*. Loss-of-function of either AtSWP1 or AtCZS resulted in hyperacetylation of H4 and substantial demethylation of H3K9 and H3K27 within the *FLC* locus, in elevated levels of the *FLC* transcripts, and, consequently, in delayed flowering.

Materials and methods

Plants

Wild-type and *swp1-1* and *czs-1* T-DNA insertion lines (SALK_142477 and SALK_026224, respectively, obtained from ABRC) were derived from the Columbia (Col) ecotype of *Arabidopsis thaliana*. *Arabidopsis* plants carrying the *GUS* reporter transgene under the mGal4-VP16-inducible *Gal4-UAS* promoter were obtained from Dr. J. Haseloff (University of Cambridge, UK; see http://www.plantsci.cam.ac.uk/Haseloff). For PCR analyses, *Arabidopsis* genomic DNA was extracted using the DNeasy Plant Kit (Qiagen). For PCR-based identification of plants carrying wild-type *AtSLP1* or *AtCZS*, we utilized gene-specific forward primers 5′-GTTTTGGCGAGGCAACTTGGT-3′and 5′-GATGTCCTTGCACAAA-ACCGC-3′, respectively, whereas plants homozygous for T-DNA insertions in these genes were identified using the respective gene-specific forward primers 5′-CCCATCTGGAACAGAGGGCTT-3′and 5′-TGCAAATCGC-TAACCGTTGCT-3′. In both cases, we used the T-DNA left border-specific reverse primer 5′-GCGTGGACCGCTTGCTGCAACT-3′ as described (Alonso et al., 2003) (http://signal.salk.edu/cgi-bin/tdnaexpress). Plants were grown in soil (for phenotypic characterization; one plant per pot, at least 20 plants per each experimental condition) or on Gamborg's B5 (Sigma)/0.1% sucrose medium (for RNA extractions and ChIP analyses) in an environment-controlled chamber at 22–24°C. All plants were maintained under long day conditions of 16 h white light (70–80 μmol photons m^{-2} s^{-1}) and 8 h dark. For vernalization, 3-day-old seedlings were maintained for 44 days at 4°C with dim light (He et al., 2003), prior to shifting them to the standard growth conditions.

Bombardment and nuclear import

AtSWP1 or AtCZS cDNAs (GenBank accession number NM_104961.3 or DQ104398, respectively) were cloned into the *Xho*I–*Kpn*I or *Sal*I sites, respectively, of pSAT6-EGFP-C1 (Tzfira et al., 2005). Each of the resulting constructs (25 μg) expressing the GFP-AtSWP1 or GFP-AtCZS fusion was mixed (1:1 w/w) with pSAT-ECFP-C1 that expresses free CFP (Tzfira et al., 2005), adsorbed onto 10 mg of 1-μm gold particles (Bio-Rad, CA) and bombarded at 150–200 psi into the leaf epidermis of greenhouse-grown *Nicotiana benthamiana* plants using a Helios gene gun (PDS-1000/He, Bio-Rad). After incubation for 24 h at 22–24°C, the bombarded tissues were viewed under a Zeiss LSM 5 Pascal confocal laser scanning microscope.

Transcriptional repression assay

Coding sequence of mGAL4-VP16 (obtained from Dr. J. Haseloff) was cloned into the *Bsp*HI–*Xba*I sites of pRTL2 (Restrepo et al., 1990), and an *Nco*I site was introduced directly before the stop codon of mGal4-VP16. Into the *Nco*I–*Xba*I sites of the resulting construct, we inserted the coding sequences of AtSWP1, AtCZS, or nopaline-specific *Agrobacterium* VirE2 (Tzfira et al., 2001), resulting in mGal4-VP16-AtSWP1, mGal4-VP16-AtCZS, and mGal4-VP16-VirE2 fusions. In all experiments, DNA fragments were amplified by PCR using a high fidelity *Pfu* DNA polymerase (Stratagene) and verified by DNA sequencing. These constructs were bombarded into the leaf epidermis of the *Gal4-UAS-GUS Arabidopsis* plants. To monitor the efficiency of bombardment, 1/10 volume of gold particles carrying pSAT6-EGFP-C1 that expresses free GFP (Tzfira et al., 2005) was added to the bombardment mixture; on average, the efficiency of transformation varied by less than 10–15% between each experiment. For coexpression of free AtSWP1 and AtCZS, their cDNAs were cloned as *Xho*I–*Xma*I and *Sal*I fragments, respectively, into the corresponding sites of pSAT6-MCS (Tzfira et al., 2005), and the resulting constructs were mixed (1:1 w/w) with the mGal4-VP16-encoding construct. After incubation for 24 h at 22–24°C, GUS activity was assayed histochemically as described (Nam et al., 1999) for 12–18 h, followed by chlorophyll extraction in 75% ethanol for 12–18 h using 75% ethanol, and the leaves were observed under a Leica MZ FLIII stereoscope. In each plant, multiple leaves were bombarded, and each experiment was repeated at least four times.

Yeast two-hybrid assay

AtSWP1 and *AtCZS* cDNAs were cloned into the *Sal*I–*Pst*I and *Sal*I sites, respectively, of a LexA plasmid pSTT91 (TRP1+, Sutton et al., 2001), and *AtCZS* and *FLD* cDNAs were cloned into the *Sal*I site of pGAD424 (LEU2+, Clontech). *Arabidopsis* cDNA library and VirE2 in pGAD424, as well as human lamin C and topoisomerase I in pSTT91 were described previously (Ballas and Citovsky, 1997; Tzfira et al., 2001, 2002). Protein interaction, indicated by histidine prototrophy, was assayed in the *Saccharomyces cerevisiae* strain TAT7 [L40 (Hollenberg et al., 1995)-*ura3*] (SenGupta et al., 1996) by growing cells for 3 days at 30°C on a leucine-, tryptophan- and histidine-deficient medium in the presence of 10 mM of 3-amino-1,2,4-triazole (3AT).

BiFC

AtSWP1 cDNAs was inserted into the *Xho*I–*Kpn*I sites of pSAT4-nEYFP-C1 (GenBank accession number DQ168994), and AtCZS cDNA was inserted into the *Sal*I site of pSAT1-cEYFP-C1(B) (GenBank accession number DQ168996). VirE2 was cloned into the *Bgl*II–*Bam*HI sites of pSAT4-nEYFP-C1 and pSAT1-cEYFP-C1(B). The tested construct pairs were mixed with pSAT-ECFP-C1 (2:2:1 w/w), bombarded into *N. benthamiana* leaves, incubated for 48 h at 22–24°C, and observed under a confocal microscope. Experiments were repeated at least three times, with the entire bombarded leaf area examined in each experiment.

Rapid amplification of cDNA ends (RACE) PCR

The *AtCZS* cDNA clone initially identified in the two-hybrid library screen lacked its 5′-terminal sequence which was then amplified from the *Arabidopsis* cDNA library (Ballas and Citovsky, 1997) by 5′-RACE PCR (Frohman et al., 1988) using a *AtCZS*-specific reverse primer (5′-ACCACGAGAGCCAACTA-GAC-3′) and a set of forward primers corresponding to the *Arabidopsis* genomic sequence located upstream of the identified *AtCZS* sequence and spaced 200–250 bp apart from each other. The largest amplified fragment was sequenced, and this information was used to PCR-amplify the full length *AtCZS* cDNA clone from the same library.

RT-PCR and quantitative real-time PCR analyses

For RT-PCR, total RNA from 2-week-old seedlings was isolated with TRI-reagent (Molecular Research Center), treated with RNase-free DNase (DNA-free kit, Ambion), and 500 ng of the purified DNA-free RNA was reverse-transcribed with ProtoScript First Strand cDNA synthesis Kit (New England Biolabs), and PCR-amplified for 28–32 cycles, using primers specific for *FLC* (forward 5′-AAAGTAGCCGACAAGTCACC-3′, reverse 5′-TAAGTAGTGGGAGAGT-CACC-3′) and *ACTIN8* (forward 5′-GTCTGTGACAATGGTACTGG-3′,

reverse 5′-CCTGCTTCATCATACTCTGC-3′), which generated 546-bp and 1,073-bp products from the corresponding transcripts, respectively. Both primer pairs were designed to amplify across introns to rule out residual contamination with genomic DNA; the lack of such contamination was also demonstrated by control PCR without reverse transcription. RT-PCR products were detected by ethidium bromide staining of agarose gels. Quantitative real-time PCR utilized the same procedure as RT-PCR, and was performed in an ABI PRISM 7700 Sequence Detector using iQ SYBR-green supermix (Bio-Rad) and *FLC*-specific (forward 5′-ATGCTGAAAGAAGAGAACCAGG-3′, reverse 5′-TCAGCTTCTGCTCCCACATG-3′) and *ACTIN4*-specific (forward 5′-CTCCTGCTATGTATGTTGCCATTCAAGCTGTTC-3′, reverse 5′-GCGT-AACCCTCGTAGATTGGTACCGTGT-3′) primers. Relative abundance of the *FLC* mRNAs was normalized to *ACTIN* mRNA. For analyses of the *swp1-1* and *czs-1* mutants, RT-PCR was performed with forward 5′-GTTTT-GGCGAGGCAACTTGGT-3′ and 5′-TGCAAATCGCTAACCGTTGCT-3′, and reverse 5′-CCCATCTGGAACAGAGGGCTT-3′ and 5′-GATGTCCTTG-CACAAAACCGC-3′ primers which amplified 900-bp products from the respective transcripts; control reactions were performed with *ACTIN8*-specific primers (forward 5′-ACCTTGCTGGTCGTGACCTT-3′, reverse 5′-GATCCCGTCATGGAAACGAT-3′), amplifying a 632-bp product.

ChIP

ChIP experiments performed as described (Johnson et al., 2002). Briefly, 2-week-old seedlings were fixed with formaldehyde, chromatin was isolated, shared by sonication, and immunoprecipitated using antibodies (Upstate Biotechnology) against dimethyl-histone H3 Lys9 (#07-212), dimethyl-histone H3 Lys27 (#07-322), dimethyl-histone H3 Lys36 (#07-369), tri- and dimethyl-histone H3 Lys4 (#07-473), acetyl-histone H4 (#06-598), and total histone H3 (#06-755). The cross-links were heat-reversed (65°C), DNA purified on spin columns (GFX Kit, Amersham) and amplified by 38–45 cycles of PCR with primers specific for the *FLC* region−260 to+65 involved in *FLC* repression (Bastow et al., 2004; He et al., 2003; Sheldon et al., 2002) (forward 5′-CGGTA-CACGTGGCAATCTTGTC-3′ and 5′-GAGAAGGTGACTTGTCGGCTAC-3′) and with the previously described (Johnson et al., 2002) primers specific for the control genes *ACTIN2* and *Ta3*. PCR products were resolved on agarose gels and detected by staining with ethidium bromide.

Genetic complementation of the swp1-1 and czs-1 mutants

The full-length *AtSWP1* and *AtCZS* transgenes were produced as described (Li et al., 2005; Tian et al., 2004) and contained the native gene promoter, coding region with introns, and the 3′ UTR sequences; specifically, based on the size of the intergenic regions of these genes predicted from the complete *Arabidopsis* genome sequence (The Arabidopsis Genome Initiative, 2000), we included 1 kb upstream of the translation initiation codons and 0.5 kb downstream of the STOP codon in our constructs. *AtSWP1* was amplified from the wild-type *Arabidopsis* genomic DNA with the forward 5′-AAAACTGCAGCTTTTCCTTCTTCTGA-GATC-3′ and reverse 5′-TTCCAATGCATTGGCTGCAGCTTCGATTCGGTT-CTTACGG-3′primers and cloned into the *Pst*I site of pCAMBIA-1300 (GenBank accession number AF234296), and *AtCZS* was amplified with the forward 5′-ACGCGTCGACCATTCATAATCCAGAAGAAGATAAG-3′ and reverse 5′-TTCCGCGGCCGCTATGGCCGACGTCGACAGATTCAATCCT-TCCAAAGAGTTTC-3′primers and cloned into the *Sal*I site of pCAMBIA-1300. The resulting binary constructs were introduced into *A. tumefaciens* EHA105 strain, used to transform the mutant plants by flower dipping (Kim et al., 2003), and 3–5 hygromycin-resistant T2 transformants were selected, self-crossed to homozygocity for the tested transgene, and 20–50 resulting plants were analyzed for timing of flowering.

Microarray hybridization and analysis

Microarray analysis was performed at the Genomic Informatics Center (University of Rochester Medical School, Rochester, NY) under the supervision of Dr. Andrew Brooks. Total RNA was purified from the 2-week-old wild-type and mutant seedlings as described above, reverse transcribed to cDNA followed by addition of an initiation site for T7 RNA polymerase at the 3′ end. cRNA was generated from 1 μg of the modified cDNA using biotinylated UTP and CTP and fragmented (20 μg from each sample) for 35 min at 94°C in 200 mM Tris–actetate (pH 8.1), 500 mM KOAc, and 150 mM MgOAc. Samples were subjected to gene expression analysis via the Affymetrix *Arabidopsis* ATH1 Genome array that currently queries 24,000 genes. Iobion's GeneTraffic MULTI was used to perform Robust Multi-Chip Analysis (RMA) that is a median polishing algorithm used in conjunction with both background subtraction and quantile normalization approaches. Data were analyzed by Statistical Analysis of Microarrays (SAM) (http://www.fgc.urmc.rochester.edu).

Results

AtSWP1, a SWIRM-PAO protein involved in gene repression

The *Arabidopsis* genome encodes four homologs of the animal KIAA0601/LSD1 protein (Shi et al., 2004). One of them, which we designated AtSWP1 (AGI code At1g62830), shows the highest degree of homology (34.7% identity and 25% similarity) to KIAA0601/LSD1. AtSWP1 is a ~93-kDa protein containing two major conserved domains that represent the hallmarks of the KIAA0601/LSD1 protein family (Fig. 1A): a 101 amino acid residue-long N-proximal SWIRM domain found in a number of chromatin-regulating proteins (Aravind and Iyer, 2002) and a 429 residue-long PAO domain.

We examined whether consistent with its potential co-repressor function, AtSWP1 is a nuclear protein with an inhibitory effect on gene expression. To determine its subcellular localization in plant cells, AtSWP1 was tagged with green fluorescent protein (GFP) and transiently expressed, following biolistic delivery of its encoding DNA construct, in the leaf epidermis together with free cyan spectral variant of GFP (CFP) which partitions between the cell cytoplasm and the nucleus, conveniently visualizing and identifying both of these cellular compartments. Fig. 1B shows that GFP-AtSWP1 was imported into the plant cell nucleus, displaying a predominantly intra-nuclear accumulation as determined by confocal microscopy with optical sections through the cell nucleus. As expected, in the same GFP-AtSWP1-expressing cell, CFP was found both in the cytoplasm – displaying the characteristic transvacuolar strands and variations in cytosol thickness at the cell cortex (Cutler et al., 2000; Tian et al., 2004) – and in the nucleus; the combined image of GFP and CFP fluorescence showed overlapping signal (blue-green color) within the cell nucleus (Fig. 1B). Because the predicted size of the GFP-AtSWP1 fusion protein (~120 kDa) is substantially larger than the size exclusion limit of the nuclear pore (reviewed by Dingwall and Laskey, 1991; Garcia-Bustos et al., 1991; Meier, 2005; Pemberton and Paschal, 2005), its accumulation within the nucleus must result from the active process of nuclear import.

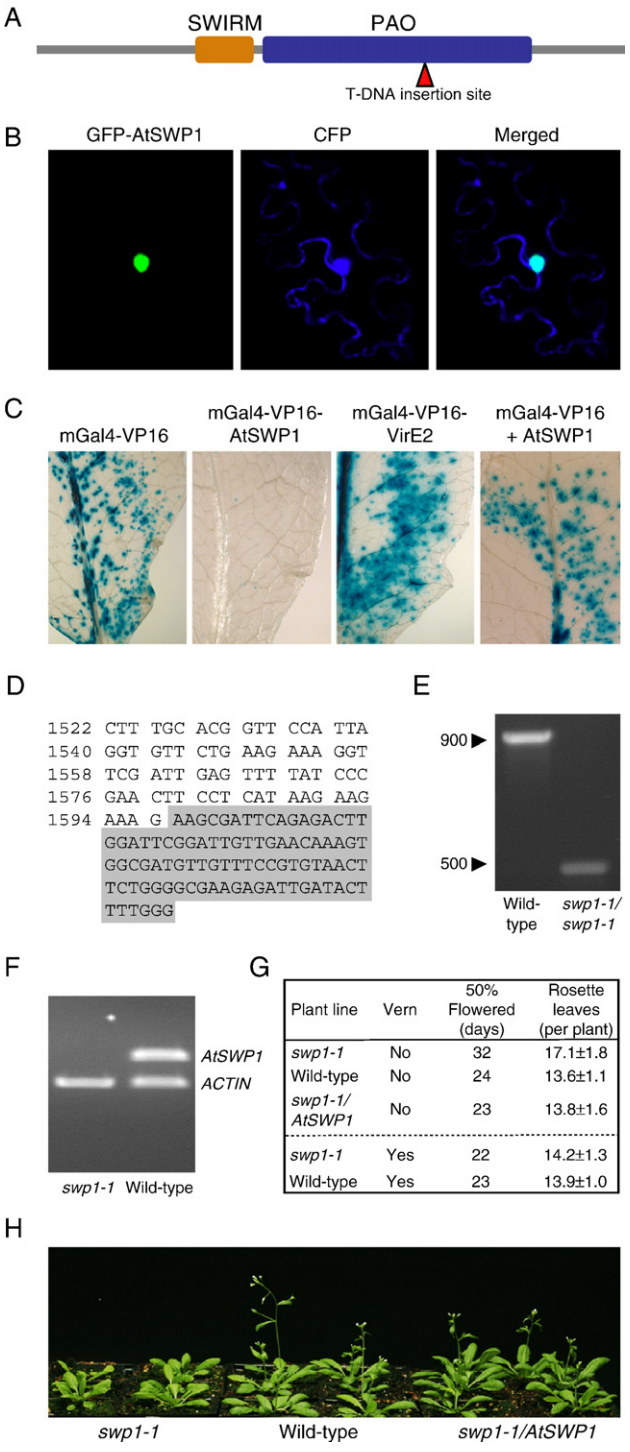
To examine the effect of AtSWP1 on gene expression, we adapted for use *in planta* an assay based on inhibition of transcriptional activation; previously, this approach has been utilized to study a yeast H3 histone methyltransferase (HMT) Set2 (Strahl et al., 2002). AtSWP1 was fused to a chimeric transcriptional activator containing the yeast GAL4 DNA binding domain [mGal4, modified for optimal activity in *Arabidopsis* (Haseloff, 1999)] fused to the VP16 transcriptional activator from *Herpes simplex virus*, and transiently expressed

by bombardment of its encoding construct into *Arabidopsis* plants that carry a β-glucuronidase (GUS) reporter transgene driven by a mGal4-VP16-inducible Gal4-UAS promoter (http://www.plantsci.cam.ac.uk/Haseloff/Home.html). Each tested construct was co-bombarded with another vector that constitutively expresses GFP; essentially identical levels of GFP expression were observed in all experiments (data not shown), confirming equal and consistent efficiencies of the transformation procedure. Fig. 1C shows that expression of mGal4-VP16 induced high levels of GUS activity detected as indigo-blue histochemical staining. In contrast, the reporter gene was virtually not expressed in the presence of mGal4-VP16 tethered to AtSWP1 (Fig. 1C). This inability to induce gene expression was specific for AtSWP1 because mGal4-VP16 fused to a comparably large (~70 kDa), but unrelated protein, VirE2 of *Agrobacterium* (reviewed by Duckely and Hohn, 2003; Ward and Zambryski, 2001), efficiently activated GUS expression. No inhibition of GUS activity was observed when mGal4-VP16 was coexpressed with free AtSWP1, indicating that AtSWP1 has to be recruited to the target gene for inhibition of expression (Fig. 1C). In control experiments, no GUS activity was detected in wild-type *Arabidopsis*, i.e., plants lacking the GUS reporter transgene, in the presence of mGal4-VP16 and in transgenic plants in the absence of mGal4-VP16 (data not shown).

To investigate the role of AtSWP1 in plant development, we identified an *Arabidopsis* mutant, designated *swp1-1*, from the Salk collection (Alonso et al., 2003) with a T-DNA insertion in the coding sequence of the *AtSWP1* gene (Fig. 1D) and demonstrated that the homozygous *swp1-1* line (Fig. 1E) did not express the *AtSWP1* mRNA (Fig. 1F). The major phenotypic characteristic of the *swp1-1* mutant was its delayed flowering and increased numbers of rosette leaves (Figs. 1G, H), which are associated with late flowering (He et al., 2003; Zhao et al., 2005), suggesting that the delay in flowering was caused by repression of meristem transition from the vegetative to reproductive stage. That this phenotype was indeed due to the mutation in the *AtSWP1* gene was confirmed by its genetic complementation with a transgene corresponding to the full-length genomic sequence of *AtSWP1* with its native regulatory elements. The resulting transgenic plants displayed the wild-type timing of flowering and numbers of rosette leaves (Figs. 1G, H). The delayed-flowering phenotype of the *swp1-1* plants was reversed by vernalization (cold treatment) (Fig. 1G), which is diagnostic of autonomous-pathway flowering mutants (Simpson et al., 1999).

AtCZS, an AtSWP1-interacting protein with a co-repressor function

To begin identification and characterization of the protein components of the AtSWP1-containing potential co-repressor



Plant line	Vern	50% Flowered (days)	Rosette leaves (per plant)
swp1-1	No	32	17.1±1.8
Wild-type	No	24	13.6±1.1
swp1-1/AtSWP1	No	23	13.8±1.6
swp1-1	Yes	22	14.2±1.3
Wild-type	Yes	23	13.9±1.0

Fig. 1. AtSWP1 is a nuclear transcriptional repressor involved in regulation of flowering. (A) Domain structure of AtSWP1 and location of the mutagenic T-DNA insertion in the *swp1-1* mutant. (B) Nuclear import of GFP-AtSWP1 in *N. benthamiana* leaf epidermis; coexpressed free CFP identifies the cell nucleus and outlines the expressing cell. GFP signal is in green, CFP signal is in blue, and overlapping GFP and CFP signals are in blue-green. Images are single confocal sections. (C) AtSWP1 tethered to mGal4-VP16 inhibits mGal4-VP16-induced expression of GUS reporter in *Arabidopsis* leaves. (D) Mutagenic T-DNA insertion in the *swp1-1* line. *AtSWP1*-T-DNA right border integration junction sequence is shown, in which the T-DNA sequence is shaded, and the reading frame and nucleotide positions of the *AtSWP1* mRNA are indicated. (E) PCR analysis of the homozygous *swp1-1* line. (F) RT-PCR analysis detects no *AtSWP1* mRNA-specific product in the *swp1-1* mutant. The wild-type *AtSWP1* gene and its allele containing the mutagenic T-DNA are represented by 900-bp and 450-bp PCR products, respectively (http://signal.salk.edu/cgi-bin/tdnaexpress). (G, H) Loss of AtSWP1 function leads to delayed flowering in the *swp1-1* mutant, which is genetically reversed by the wild-type *AtSWP1* gene and counteracted by vernalization. Time to flowering for vernalized plants was measured after the completion of the vernalization period.

complexes, we used the yeast two-hybrid system to screen an *Arabidopsis* (ecotype Columbia) cDNA library (Ballas and Citovsky, 1997; Tzfira et al., 2001) with AtSWP1 as bait. These experiments identified a cDNA clone that encoded a ~125-kDa protein with three N-proximal C2H2 zinc finger domains and a C-terminal SET domain flanked by cysteine-rich PreSET and PostSET sequences (Fig. 2A); thus, we designated this protein AtCZS (accession numbers DQ104397, DQ104398). Importantly, although AtCZS contains domains conserved in a large number of eukaryotic proteins, it does not have overall sequence homologs in the *Arabidopsis* genome, potentially representing a single gene; furthermore, AtCZS had no homologs in non-plant organisms, but it was conserved between diverse plant species, such as *Arabidopsis*, maize, and rice (Fig. 2B).

Next, we examined the subcellular localization of AtCZS tagged with GFP. Fig. 3A shows that GFP-AtCZS accumulated in the cell nucleus, colocalizing with the nuclear portion of the coexpressed free CFP. Thus, both AtCZS and AtSWP1 (see Fig. 1B) localize to the same subcellular compartment, which is consistent with their interaction with each other.

The interaction between the protein product of the full-length *AtCZS* cDNA and AtSWP1 was demonstrated in yeast and *in planta*. In the yeast two-hybrid system, the AtCZS–AtSWP1 interaction was detected by cell growth in the absence of histidine (Fig. 3B). This interaction did not occur with diverse negative controls, i.e., lamin C (Fig. 3B) and topoisomerase I (data not shown), known to detect false positives in yeast two-hybrid assays (Bartel et al., 1993; Hollenberg et al., 1995), and an unrelated protein, the *Agrobacterium* VirE2 (Fig. 3B). Interestingly, AtCZS did not interact with FLD (Fig. 3B), which shares sequence similarity with AtSWP1 (He et al., 2003), suggesting high specificity of the AtSWP1 recognition by AtCZS.

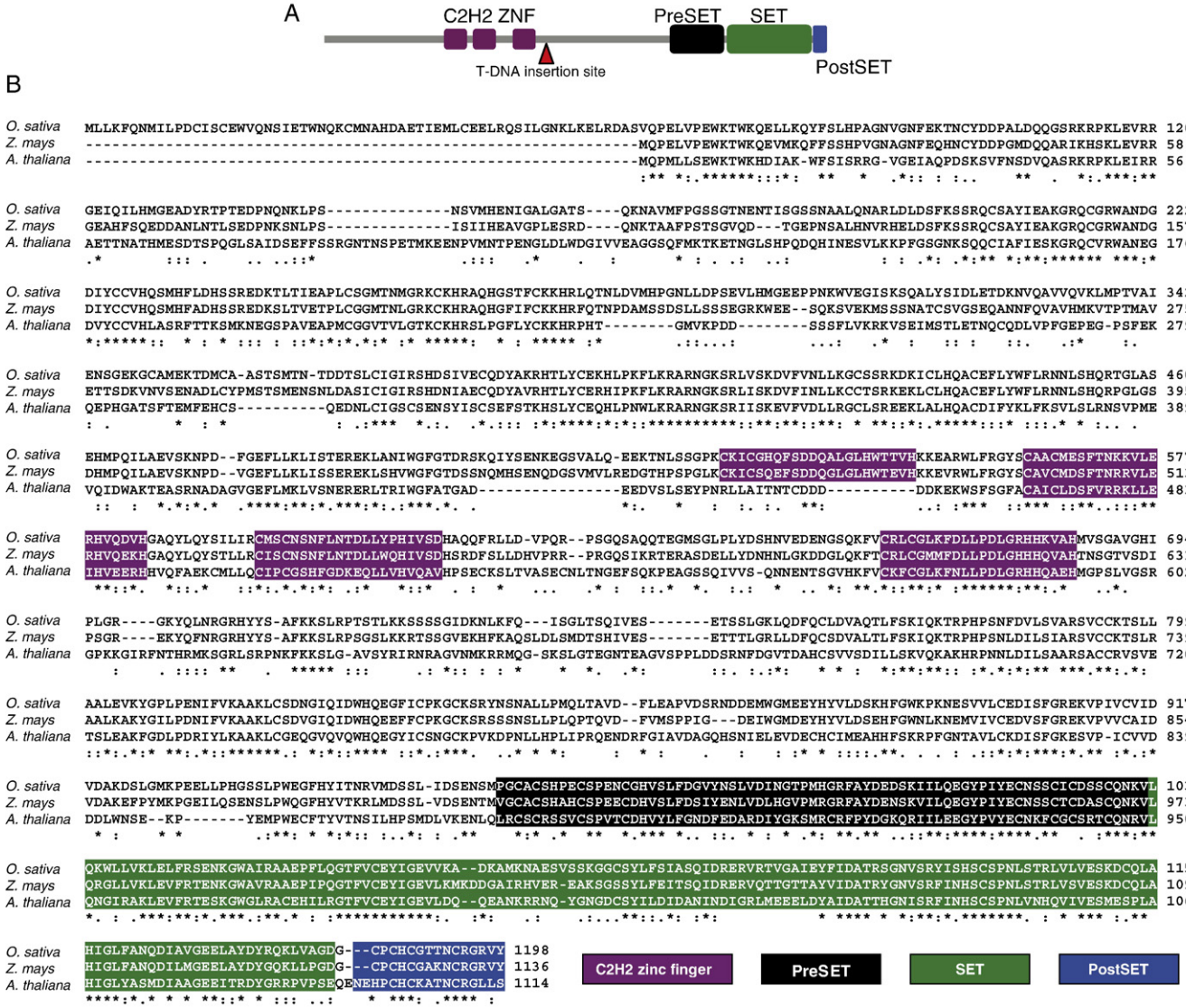


Fig. 2. Sequence comparison between dicot and monocot CZS proteins. (A) Domain structure of AtCZS and location of the mutagenic T-DNA insertion in the *czs-1* mutant. (B) Amino acid sequence alignment of AtCZS with the SDG706/117 proteins of rice (*Oryza sativa*, CromDB ID number LOC_Os02g47900) and maize (*Zea mays*, ChromDB ID number MCG4656) was performed by ClustalW (ver. 1.82) at EMBL-EBI using the default settings. Symbols designations: "*" identical residues, ":" conserved substitutions, "." semi-conserved substitutions. Conserved domains are highlighted by different colors as indicated.

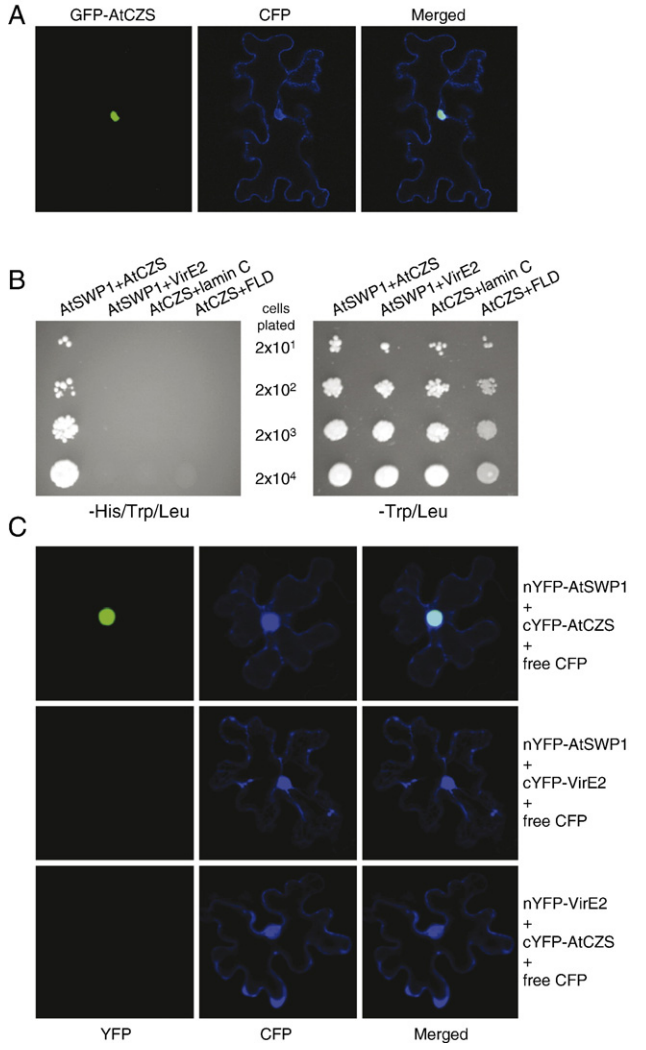
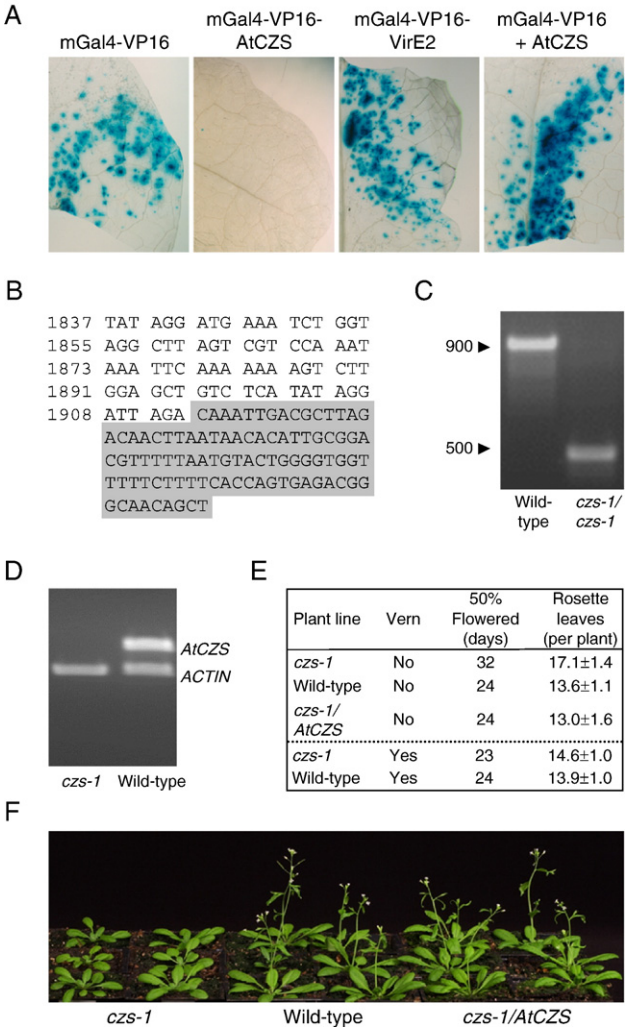


Fig. 3. AtCZS is a nuclear protein that interacts with AtSWP1. (A) Nuclear import of GFP-AtCZS in *N. benthamiana* leaf epidermis; coexpressed free CFP identifies the cell nucleus and outlines the expressing cell. (B) AtCZS specifically interacts with AtSWP1 in the two-hybrid system. (C) The BiFC assay for AtCZS–AtSWP1 interaction *in planta*; coexpressed free CFP identifies the cell nucleus and outlines the expressing cell. GFP or YFP signal is in green, CFP signal is in blue, and overlapping GFP/YFP and CFP signals are in blue-green. Images are single confocal sections.

In planta, the AtCZS–AtSWP1 interaction and the subcellular location of the interacting proteins were determined using a bimolecular fluorescence complementation (BiFC) assay (Hu et al., 2002). In this approach, a molecule of yellow spectral variant of GFP (YFP) is separated into two portions, N-terminal (nYFP) and C-terminal (cYFP), neither of which fluoresces when expressed alone, but the fluorescence is restored when nYFP and cYFP are brought together as fusions with interacting proteins expressed *in planta*; the location of the plant cell nucleus and the cell outlines are visualized by coexpression of free CFP (Lacroix et al., 2005; Li et al., 2005; Tzfira et al., 2004). Fig. 3C shows that cYFP-tagged AtCZS interacted with nYFP-AtSWP1 in the nuclei of living plant cells, resulting in reconstruction of the YFP fluorescence which colocalized with the nuclear CFP signal. In negative control experiments, no YFP signal was detected following coexpression of cYFP-AtCZS or nYFP-AtSWP1 with nYFP-VirE2 or cYFP-VirE2, respectively (Fig. 3C); also, we observed no interaction between cYFP-AtCZS and nYFP-FLD (data not shown).

That AtCZS interacts with AtSWP1 suggests that these two proteins may function in the same co-repressor complex; in this scenario, AtCZS would inhibit gene expression, and a mutant in the *AtCZS* gene would phenocopy the *swp1-1* mutant. The effect of AtCZS on gene expression was investigated by the inhibition of transcriptional activation assay in the experimental design utilized for the analysis of AtSWP1 (see Fig. 1C).



Plant line	Vern	50% Flowered (days)	Rosette leaves (per plant)
czs-1	No	32	17.1±1.4
Wild-type	No	24	13.6±1.1
czs-1/AtCZS	No	24	13.0±1.6
czs-1	Yes	23	14.6±1.0
Wild-type	Yes	24	13.9±1.0

Fig. 4. AtCZS is a transcriptional repressor involved in regulation of flowering. (A) AtCZS tethered to mGal4-VP16 inhibits mGal4-VP16-induced expression of GUS reporter in *Arabidopsis* leaves. (B) Mutagenic T-DNA insertion in the *swp1-1* line. *AtSWP1*-T-DNA right border integration junction sequence is shown, in which the T-DNA sequence is shaded, and the reading frame and nucleotide positions of the *AtSWP1* mRNA are indicated. (C) PCR analysis of the homozygous *swp1-1* line. The wild-type *AtCZS* gene and its allele containing the mutagenic T-DNA are represented by 900-bp and 450-bp PCR products, respectively (http://signal.salk.edu/cgi-bin/tdnaexpress). (D) RT-PCR analysis detects no *AtCZS* mRNA-specific product in the *czs-1* mutant. (E, F) Loss of AtCZS function leads to delayed flowering in the *czs-1* mutant which is genetically complemented by the wild-type *AtCZS* gene and reversed by vernalization. Time to flowering for vernalized plants was measured after the completion of the vernalization period.

Specifically, we tested the ability of AtCZS fused to mGal4-VP16 to inhibit the mGal4-VP16-induced expression of the *GUS* reporter gene. Fig. 4A shows that AtCZS tethered to mGal4-VP16 was unable to induce *GUS* expression in plant tissues whereas an unrelated, control protein VirE2 fused to mGal4-VP16 or the untethered AtCZS coexpressed with free mGal4-VP16 had no effect on the inducer activity of mGal4-VP16.

Next, a homozygous insertional mutant in the *AtCZS* locus was obtained from the Salk collection (Alonso et al., 2003) and designated *czs-1* (Figs. 4B, C). This mutant line did not produce the *AtCZS* transcript (Figs. 4D), and its relevant phenotypic features, i.e., delayed flowering and elevated number of the rosette leaves, were almost identical to those of the *swp1-1* line (compare Figs. 4E, F to Figs. 1G, H). This phenotype was genetically complemented by transgenic expression of the full-length genomic *AtCZS* sequence from its native regulatory elements (Figs. 4E, F). Similarly to AtSWP1, AtCZS most likely functions in an autonomous pathway because vernalization reversed the *czs-1* mutant phenotype (Fig. 4E).

The putative AtSWP1/AtCZS complex represses the FLC gene by H3K9 and H3K27 methylation and H4 hypoacetylation

A cDNA microarray analysis performed on mRNA isolated from 2-week-old seedlings of the *swp1-1* and *czs-1* lines identified a number of upregulated genes (data not shown), one of which, *FLC*, represented a known major regulator of flowering (Michaels and Amasino, 1999). De-repression of *FLC* in the mutant lines was confirmed by RT-PCR (Fig. 5A) and quantified by real-time RT-PCR analyses (Fig. 5B), which demonstrated 2–5-fold increase in the *FLC* mRNA levels. *FLC* repression is known to be mediated by histone deacetylation in autonomous regulation pathways (Ausín et al., 2004; He et al., 2003) and by H3K9 and H3K27 dimethylation during vernalization (Bastow et al., 2004; Sung and Amasino, 2004). The protein components of the *FLC* repression machinery that effect these histone modifications, especially during the autonomous events, are still poorly understood.

Potentially, AtSWP1 and AtCZS are involved in such repression of *FLC* by histone modification. AtCZS represents an especially promising candidate for chromatin-modifying activity because it contains a SET domain found in all proteins known to function as HMTs and to participate in histone methylation (reviewed in Springer et al., 2003). Different SET domains methylate different lysine residues of histones; for example, the SET1 domain is involved in H3K4 methylation

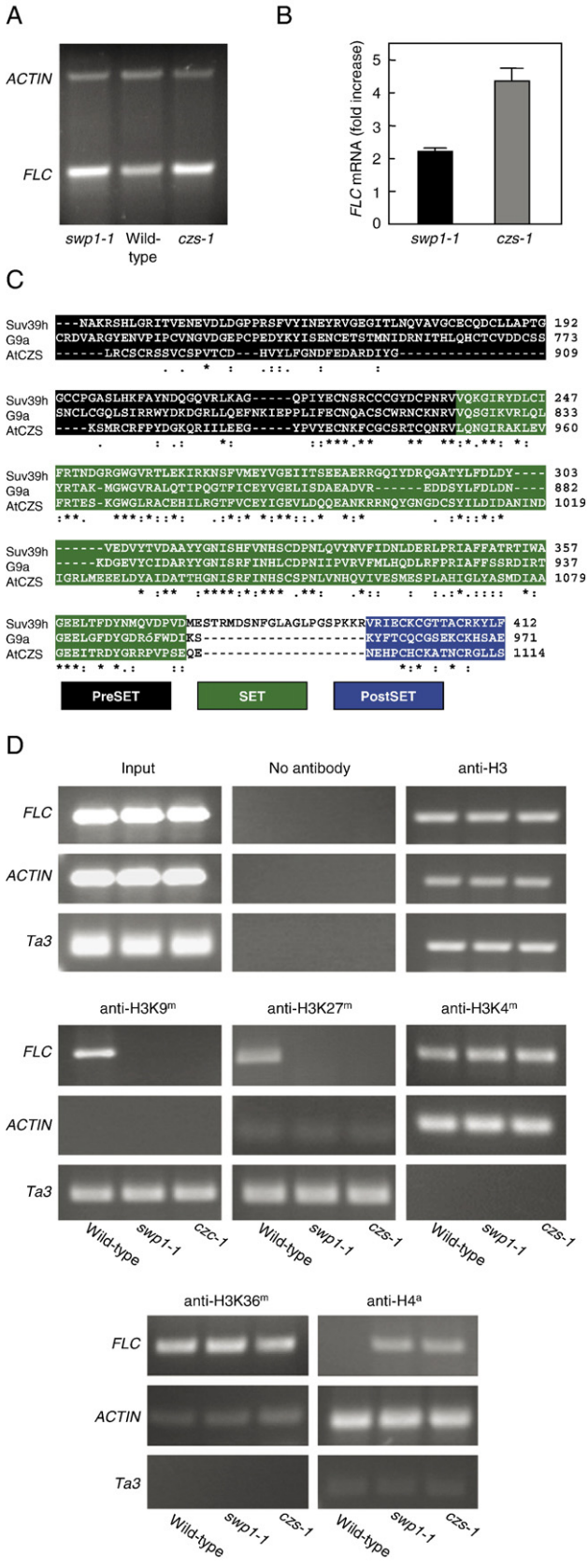


Fig. 5. AtSWP1 and AtCZS repress the *FLC* gene via histone modification. (A) RT-PCR analysis of *FLC* de-repression in the *swp1-1* and *czs-1* mutants. (B) Quantitative real-time RT-PCR analysis of *FLC* de-repression in the *swp1-1* and *czs-1* mutants. (C) Amino acid sequence alignment of the AtCZS SET domain with the corresponding sequences of the human G9a (GenBank accession number CAA49491) and mouse Suv39h proteins (GenBank accession number AAB92225) was performed by ClustalW (ver. 1.82) at EMBL-EBI using the default settings. Symbols designations: "*" identical residues, ":" conserved substitutions, "." semi-conserved substitutions. (D) ChIP analysis of histone modifications on the *FLC* regulatory sequences in the *swp1-1* and *czs-1* mutants. "Input" refers to chromatin sample processed without immunoprecipitation, "no antibody" indicates a sample processed without primary antibody.

(e.g., Briggs et al., 2001) whereas the SET2 domain mediates methylation of H3K36 (e.g., Strahl et al., 2002). Alignment of the SET domain of AtCZS with other SET domain proteins with known histone methylation revealed a homology to the human G9a protein and to the mouse Suv39h protein (Fig. 5C), both of which belong to the class V of SET domain proteins characterized by the PreSET–SET–PostSET structure (Springer et al., 2003) and known to act as HMTs with specific selectivities for H3K9 and/or H3K27 (Peters et al., 2003; Tachibana et al., 2001). Thus, we used chromatin immunoprecipitation (ChIP) to examine the potential epigenetic effects of AtSWP1 and its cognate AtCZS on the *FLC* gene. This analysis was performed on the *FLC* sequence involved in the repression of this gene (Bastow et al., 2004) for the wild-type, *swp1-1*, and *czs-1* plants using antibodies specific to dimethyl K9 H3, dimethyl K27 H3, and acetylated H4.

Fig. 5D shows that, relative to the wild-type plants, both *swp1-1* and *czs-1* mutants showed a reduction in H3K9 and H3K27 dimethylation and hyperacetylation of H4. Control experiments confirmed equal input of chromatin, lack of non-specific signal in the absence of primary antibodies, and equal amounts of total H3 histone associated with *FLC*; in addition, actively expressed *ACTIN* (Zhao et al., 2005) was associated with hyperacetylated H4, but not with dimethylated H3K9 and H3K27 whereas the silenced *Ta3* transposon (Zhao et al., 2005) was marked by dimethylated H3K9 and H3K27, but not by hyperacetylated H4 (Fig. 5D). The effects of the *swp1-1* and *czs-1* mutations on H3K9 and H3K27 methylation and H4 acetylation were reversed in plants genetically complemented by the wild-type copies of the corresponding genes (data not shown). Noteworthy, the relative degree of H4 hyperacetylation in both *swp1-1* and *czs-1* mutants was lower than that in an insertional mutant of the *FLD* gene; specifically, while the ChIP signal corresponding to the acetylated H4 could be detected already after 25–30 amplification cycles (data not shown and He et al., 2003), the comparable intensity of the ChIP signal in *swp1-1* and *czs-1* plants was achieved only after 40 cycles (Fig. 5D). These differences are consistent with a more severe late-flowering phenotype of the *fld* mutants (He et al., 2003).

Next, we investigated whether or not the *swp1-1* and *czs-1* mutations affect H3K4 methylation which – unlike H3K9 and H3K27 methylation marks known to associate with inactive chromatin – is diagnostic of active chromatin (e.g., Cao et al., 2002; Jenuwein and Allis, 2001; Lachner and Jenuwein, 2002; Rea et al., 2000). Fig. 5D illustrates that no obvious differences were detected between both mutants and the wild-type plants in the degree of H3K4 dimethylation. That the *czs-1* plants exhibited no decrease in H3K4 di/trimethylation indicates the lack of the HMT activity of AtCZS toward H3K4. Furthermore, the lack of alterations in the degree of H3K4 methylation in the *swp1-1* plants suggests that AtSWP1, which is homologous to the mammalian LSD1 that demethylates H3K4 (Shi et al., 2004), may not possess such a demethylase activity; this notion is consistent with our inability to observe H3K4-specific demethylation with purified recombinant AtSWP1, although parallel experiments with purified LSD1 resulted in clear demethylation of H3K4 (data not shown).

Finally, we examined the degree of H3K36 dimethylation in the *swp1-1* and *czs-1* mutants. Similarly to H3K4, methylation of H3K36 is associated with euchromatin (Cao et al., 2002; Jenuwein and Allis, 2001; Lachner and Jenuwein, 2002; Rea et al., 2000), and it has been shown to activate the *FLC* gene, while reduction in this methylation following loss-of-function of the corresponding HMT, *SDG8*, induced early flowering (Zhao et al., 2005). Because loss-of-function of AtSWP and AtCZS in the corresponding mutants exhibit the delayed, rather than accelerated, timing of flowering, we did not expect to see alterations in the H3H36 methylation in these plant lines. Indeed, Fig. 5D shows that the levels of dimethylated H3K36 were comparable between both mutants and the wild-type plants. That we observed no alterations in the H3K4 and H3K36 methylation marks in the mutant plants is consistent with the sequence homology of AtCZS to known H3K9 and/or H3K27-selective HMTs (see Fig. 5C) (Peters et al., 2003; Springer et al., 2003; Tachibana et al., 2001).

Collectively, our results suggest that AtCZS, which contains the PreSET–SET–PostSET hallmark motif of functional HMTs (Springer et al., 2003), methylates H3K9 and H3K27. Because AtCZS interacts with AtSWP1 within plant cells, we propose that these proteins function in a AtSWP1/AtCZS co-repressor complex.

Discussion

We identified two *Arabidopsis* nuclear proteins, AtSWP1 and AtCZS, which most likely represent the components of a novel co-repressor complex involved in regulation of expression of plant genes, among them the flowering regulator, *FLC*. We also showed that insertional mutations, *swp1-1* and *czs-1,* in the *AtSWP1* and *AtCZS* genes result in late flowering phenotypes. Thus, it makes biological sense that *FLC* represents one of the target genes of *AtSWP1* and *AtCZS*: *FLC* is a negative regulator of flowering (Michaels and Amasino, 1999), and its suppression by AtSWP1 and/or AtCZS is expected to promote flowering in wild-type plants whereas the lack of such suppression in the *swp1-1* and *czs-1* mutants should delay flowering. Furthermore, vernalization results in a permanent epigenetic suppression of the *FLC* gene (Michaels and Amasino, 1999; Sheldon et al., 1999); thus, restoration of delayed flowering phenotypes of both *swp1-1* and *czs-1* mutants to the wild-type timing of flowering by this biological inactivation of *FLC* indicates that the phenotypes of these mutants depend on the presence of the *FLC* function.

Regulation of *FLC* is very complex, involving three main mechanisms: *FRIGIDA* (*FRI*) gene-dependent positive regulation which delays flowering, vernalization-induced negative regulation which promotes flowering, and autonomous pathways which also down-regulate *FLC* and promote flowering; because vernalization permanently represses FLC, it overrides the effects of the other two regulatory systems (reviewed by Amasino, 2005; Bäurle and Dean, 2006; He and Amasino, 2005; Noh and Noh, 2006; Schubert et al., 2005; Sung and Amasino, 2005). These regulatory pathways involve numerous and diverse protein components, many of which have been

identified in recent years (e.g., Gendall et al., 2001; He et al., 2004; Kim et al., 2005; Levy et al., 2002; Martin-Trillo et al., 2006; Mylne et al., 2006; Sheldon et al., 2006; Sung and Amasino, 2004; Sung et al., 2006; Zhao et al., 2005). Although one major common factor between the *FLC* regulatory mechanisms is alteration in the state of chromatin, the chromatin modifying proteins involved in these events remain largely unexplored. Specifically, *FLC* activation is known to associate with H3K4 and H3K36 methylation marks (He et al., 2004; Kim et al., 2005; Wang et al., 2006; Zhao et al., 2005) whereas its repression involves methylation of H3K9 and H3K27 (Bastow et al., 2004; Sung and Amasino, 2004; Wang et al., 2006). However, while the HMTs that mediate the H3K4 and H3K36 methylation – i.e., the SET domain-containing EFS (Kim et al., 2005) and SDG8 proteins (Zhao et al., 2005), respectively – have been recently identified, HMT(s) responsible for the H3K9 and H3K27 methylation of the *FLC* chromatin are unknown. Our present data shed light on some of these elusive activities.

ChIP analyses of the *swp1-1* and *czs-1* mutants indicated that the putative AtSWP1/AtCZS complex is involved in histone deacetylation and H3K9 and H3K27 methylation. By analogy to animal SWIRM-PAO proteins (Jepsen and Rosenfeld, 2002) and one of their plant homologs FLD (He et al., 2003), the AtSWP1/AtCZS co-repressor complex is expected to recruit histone deacetylases (HDACs) to the target *FLC* gene. On the other hand, because AtCZS contains a highly conserved SET domain characteristic for HMT enzymes (Springer et al., 2003), we suggest that this component of the presumed complex, may function as HMT that generates H3 K9 and K27 methylation marks in the target gene chromatin.

AtCZS, the first HMT homolog shown to induce silencing of the *FLC* gene via chromatin remodeling by an autonomous pathway, may function, together with its binding partner AtSWP1, as one of the regulators of flower timing in *Arabidopsis*. Interestingly, the effect of AtSWP1 and AtCZS on flowering – with respect to the length of its timing delay and number of rosette leaves – is less dramatic than of FLD, another autonomous regulator of *FLC* (He et al., 2003). Potentially, the proposed AtSWP1/AtCZS co-repressor complex may represent a fine-tuning mechanism that modulates flowering in the presence of FLD. This idea of distinct functionalities for these two protein homologs, AtSWP1 and FLD, is consistent with the ability of only AtSWP1, but not FLD, to interact with AtCZS.

That HMTs with the characteristic domain structure of AtCZS are found both in dicotyledonous (e.g., *Arabidopsis*) and monocotyledonous plants (e.g., rice and maize), but not in animals or fungi suggests that AtCZS has evolved prior to divergence of dicots from monocots, but after the divergence of plants from other eukaryotes. Indeed, the AtSWP1/AtCZS co-repressor complex possesses several intriguing characteristics that set it apart from its known animal counterparts. For example, while the animal homolog of AtSWP1, LSD1, is a H3K4 demethylase (Shi et al., 2004), AtSWP1 appears not to possess this enzymatic activity. Also, although the domain structure of AtCZS is unique to plants, its functionality in mammalian PAO-containing complexes (Jepsen and Rosenfeld, 2002) is most closely represented by a zinc finger protein ZNF217 (You et al., 2001) and a PreSET–SET–PostSET HMT G9a that methylates H3K9 and H3K27 (Tachibana et al., 2001). Thus, AtCZS likely combines the functionalities of two different animal co-repressor complex components in a single molecule. It is tempting to speculate that the plant-specific features of AtSWP1 and AtCZS, taken together with many more unique characteristics of plant gene regulatory systems, may, at least in part, underlie perhaps one of the most fascinating differences between plants and animals: while in adult animals, "growth and morphogenesis cease" with most cells dividing, but terminally differentiated, "plant morphogenesis and growth continues throughout the lifetime of the organism" with most cells retaining "totipotency to generate the entire plant" and dedifferentiate (Loidl, 2004).

On the other hand, the basic features of histone modification, i.e., deacetylation and H2K9 and H3K27 methylation, affected by the putative AtSWP1/AtCZS co-repressor complex are similar to those induced by animal co-repressors, such as the CoREST-HDAC or REST/CoREST complexes (reviewed by Jepsen and Rosenfeld, 2002; Lunyak et al., 2004). Potentially, the mechanism by which histone methylation within the *FLC* chromatin results in suppression also parallels histone methylation-mediated gene repression in non-plant systems; for example, in animal and yeast cells, genes are repressed by binding of HP1 proteins to H3K9 methylated sites (reviewed by Lachner and Jenuwein, 2002), and epigenetic silencing of *FLC* also requires a plant homolog of HP1, LHP1 (Mylne et al., 2006; Sung et al., 2006). Also by analogy to animal co-repressor complexes the core components of which often control multiple pathways (reviewed by Jepsen and Rosenfeld, 2002), the putative AtSWP1/AtCZS complex may participate in regulation of cellular functions in addition to the timing of flowering; evidentially, this notion is supported by multiple upregulated genes detected by microarray analyses of the *swp1-1* and *czs-1* mutant plants (data not shown).

GenBank accession numbers

AtCZS genomic sequence, DQ104397; *AtCZS* cDNA sequence, DQ104398.

Acknowledgments

We thank Dr. Nurit Ballas for creative ideas and stimulating discussions. The work in the VC laboratory is supported by grants from NIH, NSF, USDA, BARD, BSF, and CDR-USAID, the RS laboratory is supported by NIH grant GM28220. GM is an investigator of the HHMI.

References

Alonso, J.M., et al., 2003. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science 301, 653–657.

Amasino, R.M., 2005. Vernalization and flowering time. Curr. Opin. Biotechnol. 16, 154–158.

Andres, M.E., et al., 1999. CoREST: a functional corepressor required for regulation of neural-specific gene expression. Proc. Natl. Acad. Sci. U. S. A. 96, 9873–9878.

Aravind, L., Iyer, L.M., 2002. The SWIRM domain: a conserved module found in chromosomal proteins points to novel chromatin-modifying activities. Genome Biol. 3 (RESEARCH0039.1–0039.7).

Ausín, I., et al., 2004. Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat. Genet. 36, 162–166.

Ballas, N., Citovsky, V., 1997. Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. Proc. Natl. Acad. Sci. U. S. A. 94, 10723–10728.

Ballas, N., et al., 2005. REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. Cell 121, 645–657.

Bartel, P., et al., 1993. Elimination of false positives that arise in using the two-hybrid system. BioTechniques 14, 920–924.

Bastow, R., et al., 2004. Vernalization requires epigenetic silencing of *FLC* by histone methylation. Nature 427, 164–167.

Bäurle, I., Dean, C., 2006. The timing of developmental transitions in plants. Cell 125, 655–664.

Briggs, S.D., et al., 2001. Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. Genes Dev. 15, 3286–3295.

Cao, R., et al., 2002. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298, 1039–1043.

Cutler, S.R., et al., 2000. Random GFP::cDNA fusions enable visualization of subcellular structures in cells of *Arabidopsis* at a high frequency. Proc. Natl. Acad. Sci. U. S. A. 97, 3718–3723.

Dingwall, C., Laskey, R.A., 1991. Nuclear targeting sequences—A consensus? Trends Biochem.Sci. 16, 478–481.

Duckely, M., Hohn, B., 2003. The VirE2 protein of *Agrobacterium tumefaciens*: the Yin and Yang of T-DNA transfer. FEMS Microbiol. Lett. 223, 1–6.

Frohman, M.A., et al., 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. U. S. A. 85, 8998–9002.

Garcia-Bustos, J., et al., 1991. Nuclear protein localization. Biochim. Biophys. Acta 1071, 83–101.

Gendall, A.R., et al., 2001. The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in *Arabidopsis*. Cell 107, 525–535.

Haseloff, J., 1999. GFP variants for multispectral imaging of living cells. Methods Cell Biol. 58, 139–151.

He, Y., Amasino, R.M., 2005. Role of chromatin modification in flowering-time control. Trends Plant Sci. 10, 30–35.

He, Y., et al., 2003. Regulation of flowering time by histone acetylation in *Arabidopsis*. Science 302, 1751–1754.

He, Y., et al., 2004. PAF1-complex-mediated histone methylation of *FLOWERING LOCUS C* chromatin is required for the vernalization-responsive, winter-annual habit in *Arabidopsis*. Genes Dev. 18, 2774–2784.

Hollenberg, S.M., et al., 1995. Identification of a new family of tissue-specific basic helix–loop–helix proteins with a two-hybrid system. Mol. Cell. Biol. 15, 3813–3822.

Hu, C.D., et al., 2002. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol. Cell 9, 789–798.

Jenuwein, T., Allis, C.D., 2001. Translating the histone code. Science 293, 1074–1080.

Jepsen, K., Rosenfeld, M.G., 2002. Biological roles and mechanistic actions of co-repressor complexes. J. Cell Sci. 115, 689–698.

Johnson, L., et al., 2002. Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. Curr. Biol. 12, 1360–1367.

Kim, J.Y., et al., 2003. Developmental regulation and significance of KNOX protein trafficking in *Arabidopsis*. Development 130, 4351–4362.

Kim, S.Y., et al., 2005. Establishment of the vernalization-responsive, winter-annual habit in *Arabidopsis* requires a putative histone H3 methyl transferase. Plant Cell 17, 3301–3310.

Lachner, M., Jenuwein, T., 2002. The many faces of histone lysine methylation. Curr. Opin. Cell Biol. 14, 286–298.

Lacroix, B., et al., 2005. The VirE3 protein of *Agrobacterium* mimics a host cell function required for plant genetic transformation. EMBO J. 24, 428–437.

Levy, Y.Y., et al., 2002. Multiple roles of *Arabidopsis VRN1* in vernalization and flowering time control. Science 297, 243–246.

Li, J., et al., 2005. Uncoupling of the functions of the *Arabidopsis* VIP1 protein in transient and stable plant genetic transformation by *Agrobacterium*. Proc. Natl. Acad. Sci. U. S. A. 102, 5733–5738.

Loidl, P., 2004. A plant dialect of the histone language. Trends Plant Sci. 9, 84–90.

Lunyak, V.V., et al., 2004. REST and peace for the neuronal-specific transcriptional program. Ann. N. Y. Acad. Sci. 1014, 110–120.

Martin-Trillo, M., et al., 2006. *EARLY IN SHORT DAYS 1 (ESD1)* encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates *FLC* accumulation in *Arabidopsis*. Development 133, 1241–1252.

Meier, I., 2005. Nucleocytoplasmic trafficking in plant cells. Int. Rev. Cytol. 244, 95–135.

Michaels, S.D., Amasino, R.M., 1999. *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell 11, 949–956.

Mylne, J.S., et al., 2006. LHP1, the *Arabidopsis* homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of *FLC*. Proc. Natl. Acad. Sci. U. S. A. 103, 5012–5017.

Nam, J., et al., 1999. Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*. Mol. Gen. Genet. 261, 429–438.

Noh, B., Noh, Y.S., 2006. Chromatin-mediated regulation of flowering time in *Arabidopsis*. Physiol. Plant. 126, 484–493.

Pemberton, L.F., Paschal, B.M., 2005. Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic 6, 187–198.

Peters, A.H., et al., 2003. Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol. Cell 12, 1577–1589.

Rea, S., et al., 2000. Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406, 593–599.

Restrepo, M.A., et al., 1990. Nuclear transport of plant potyviral proteins. Plant Cell 2, 987–998.

Roopra, A., et al., 2004. Localized domains of G9a-mediated histone methylation are required for silencing of neuronal genes. Mol. Cell 14, 727–738.

Schubert, D., et al., 2005. Epigenetic control of plant development by Polycomb-group proteins. Curr. Opin. Plant Biol. 8, 553–561.

SenGupta, D.J., et al., 1996. A three-hybrid system to detect RNA–protein interactions *in vivo*. Proc. Natl. Acad. Sci. U. S. A. 93, 8496–8501.

Sheldon, C.C., et al., 1999. The *FLF MADS* box gene: a repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. Plant Cell 11, 445–458.

Sheldon, C.C., et al., 2002. Different regulatory regions are required for the vernalization-induced repression of *FLOWERING LOCUS C* and for the epigenetic maintenance of repression. Plant Cell 14, 2527–2537.

Sheldon, C.C., et al., 2006. Quantitative effects of vernalization on *FLC* and *SOC1* expression. Plant J. 45, 871–883.

Shi, Y., et al., 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119, 941–953.

Simpson, G.G., et al., 1999. When to switch to flowering. Annu. Rev. Cell Dev. Biol. 15, 519–550.

Springer, N.M., et al., 2003. Comparative analysis of SET domain proteins in maize and *Arabidopsis* reveals multiple duplications preceding the divergence of monocots and dicots. Plant Physiol. 132, 907–925.

Strahl, B.D., et al., 2002. Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. Mol. Cell. Biol. 22, 1298–1306.

Sung, S., Amasino, R.M., 2004. Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature 427, 159–164.

Sung, S., Amasino, R.M., 2005. Remembering winter: toward a molecular understanding of vernalization. Annu. Rev. Plant Biol. 56, 491–508.

Sung, S., et al., 2006. Epigenetic maintenance of the vernalized state in *Arabidopsis thaliana* requires LIKE HETEROCHROMATIN PROTEIN 1. Nat. Genet. 38, 706–710.

Sutton, A., et al., 2001. A novel form of transcriptional silencing by Sum1-1 requires Hst1 and the origin recognition complex. Mol. Cell. Biol. 21, 3514–3522.

Tachibana, M., et al., 2001. Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity

and specific selectivity to lysines 9 and 27 of histone H3. J. Biol. Chem. 276, 25309–25317.

The *Arabidopsis* Genome Initiative, 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408, 796–815.

Tian, G.W., et al., 2004. High-throughput fluorescent tagging of full-length *Arabidopsis* gene products in *planta*. Plant Physiol. 135, 25–38.

Tzfira, T., et al., 2001. VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. EMBO J. 20, 3596–3607.

Tzfira, T., et al., 2002. Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis VIP1* gene. Proc. Natl. Acad. Sci. U. S. A. 99, 10435–10440.

Tzfira, T., et al., 2004. Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. Nature 431, 87–92.

Tzfira, T., et al., 2005. pSAT vectors: a modular series of plasmids for fluorescent protein tagging and expression of multiple genes in plants. Plant Mol. Biol. 57, 503–516.

Wang, J., et al., 2006. Nonadditive regulation of FRI and FLC loci mediates flowering-time variation in *Arabidopsis* allopolyploids. Genetics 173, 965–974.

Ward, D.V., Zambryski, P.C., 2001. The six functions of *Agrobacterium* VirE2. Proc. Natl. Acad. Sci. U. S. A. 98, 385–386.

You, A., et al., 2001. CoREST is an integral component of the CoREST-human histone deacetylase complex. Proc. Natl. Acad. Sci. U. S. A. 98, 1454–1458.

Zhao, Z., et al., 2005. Prevention of early flowering by expression of *FLOWERING LOCUS C* requires methylation of histone H3 K36. Nat. Cell Biol. 7, 1156–1160.