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

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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 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.

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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; Baurle 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 svpl-1 and czz-1 T-DNA insertion lines (SALK_142477 and SALK_026224, respectively, obtained from ABRRC) 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′-GTTTGTGGGACGCACTTTGT-3′ and 5′-GTGATCCCTTGGCACAATAACCG-3′, respectively, whereas plants homozygous for T-DNA insertions in these genes were identified using the respective gene-specific forward primers 5′-CCCATACTGGAAGAGGACCT-3′ and 5′-TGCAAAATCGCTTAACCTGTTGCT-3′. In both cases, we used the T-DNA left border-specific reverse primer 5′-GGCTGAGCGCTGCTTGCAACT-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°C. All plants were maintained under long day conditions of 16 h 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 Xhol–KpnI and SalI 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-ECPF-C1 that expresses free GFP (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 BspHI–Xhol sites of pRTL2 (Restrepo et al., 1990), and an NcoI site was introduced directly before the stop codon of mGal4-VP16. Into the Neo−/Xhol 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 XhoI–Xmal and SalI 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 Sac–Pnu and SalI sites, respectively, of a LexA plasmid pSTT91 (TRP1+, Sutton et al., 2001), and AtCZS and FLD cDNAs were cloned into the SacI 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 μM of 3-aminoo,1,2,4-triazole (3AT).

BiFC

AtSWP1 cDNAs was inserted into the Xhol–KpnI sites of pSAT4-nEGFP-C1 (GenBank accession number DQ168994), and AtCZS cDNA was inserted into the SacI site of pSAT1-EGFP-C1B (GenBank accession number DQ168996). VirE2 was cloned into the BglII–BanHI sites of pSAT4-nEGFP-C1 and pSAT1-nEGFP-C1B. The tested construct pairs were mixed with pSAT-EGFP-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′-ACCCAGAGCCAACTGAC-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 ProtocolScript First Strand cDNA synthesis Kit (New England Biolabs), and PCR-amplified for 28–32 cycles, using primers specific for FLC (forward 5′-AAATTAGAGGCGCAATTACCC-3′, reverse 5′-TAAGTAGGGGAGATGACCC-3′) and ACTIN8 (forward 5′-GTCTGTCACCAATGGTACTGG-3′,
reverse 5'-CCTGCTCTATCATACTCTGC-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'-ATGCTGAAGAGAACAGG-3', reverse 5'-TCAGCTCCTCTGCTCCCAAC-3') and ACTIN-specific (forward 5'-CTTCCTGCTATGTTGCATTCAAGCTGTT-3', reverse 5'-GGCTTACCCCTCGATATTGGTACCGTGT-3') primers. Relative abundance of the FLC mRNAs was normalized to ACTIN mRNA. For the analyses of the swp1-1 and czs-1 mutants, RT-PCR was performed with forward 5'-GGTTTTTGCGAGGCAACTTTG-3' and 5'-TGGAAATGCTACACTTGGTCTG-3', and reverse 5'-CCATCTGGAAAGAGGCGCTT-3' and 5'-GATGCTCTTGCACAAAACC-3' primers which amplified 900-bp products from the respective transcripts; control reactions were performed with ACTIN-specific primers. Due to the presence of overlapping signal (blue-green color) within the cell nucleus (Cutler et al., 2000; Tian et al., 2004) strands and variations in cytosol thickness at the cell cortex, with optical sections through the cell nucleus. As expected, in the same GFP-AtSWP1-expressing cell, CFP was found both in the epidermis together with free cyan spectral variant of GFP (CFP) biolistic delivery of its encoding DNA construct, in the leaf epidermis together with free cyan spectral variant of GFP (CFP) which partitions between the previously described (Johnson et al., 2002) primers specific for the control genes ACTIN2 and TUA3. 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'-AAAAGCTGCAGCTTTCCCTCTTCTGAGTATCG-3' and reverse 5'-TTCCAATGCGATGTCGACTTGGCTTTACGG-3' primers and cloned into the PnR site of pCAMBIA-1300 (GenBank accession number AF234296), and AtCZS was amplified with the forward 5'-ACCGTCTGACCATCTTCAATGCAAGAGAAGATAGA-3' and reverse 5'-TTCCAGCAGGTCACCTCTCAGTCTC-3' primers and cloned into the SacI 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 homozygosity 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-acetate (pH 8.1), 500 mM KAc, and 150 mM MgOAc. Samples were subjected to gene expression analysis via the Affymetrix Arabidopsis ATH1 Genome array that currently queries 24,000 genes. Loban’s GeneTraffic MULTI was used to perform Robust Multi-Channelklp (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 hallmarksof 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 intranuclear 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 chimera 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

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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/dnaxpress). (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, ChromDB 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.
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 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.

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.


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).
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.](image-url)
Finally, we examined the degree of H3K36 dimethylation in the swpl-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 H3H6 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, swpl-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 (Michaelis 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 swpl-1 and czs-1 mutants should delay flowering. Furthermore, vernalization results in a permanent epigenetic suppression of the *FLC* gene (Michaelis and Amasino, 1999; Sheldon et al., 1999); thus, restoration of delayed flowering phenotypes of both swpl-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 methyla-

GenBank accession numbers

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

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