Mol. Cells *32*, 227-234, September 30, 2011
DOI/10.1007/s10059-011-1022-6

Molecules and Cells

FVE, an *Arabidopsis* Homologue of the Retinoblastoma-Associated Protein That Regulates Flowering Time and Cold Response, Binds to Chromatin as a Large Multiprotein Complex

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Some genetic studies indicate that plant homologues of proteins involved in chromatin modification and remodeling in other organisms may regulate plant development. Previously, we described an *Arabidopsis* mutant with altered cold-responsive gene expression (*acg1*) displaying a late flowering phenotype, a null allele of *fve*. FVE is a homologue of the mammalian retinoblastoma-associated protein (RbAp), one component of a histone deacetylase (HDAC) complex involved in transcriptional repression, and has been shown to be involved in the deacetylation of the *FLOWERING LOCUS C* (*FLC*) chromatin encoding for a repressor of flowering. In an effort to gain insight into the biochemical functions of FVE, we overexpressed FVE tagged with the hemagglutinin (HA) and FLAG epitope at the N-terminus in *acg1* mutants. The results of physiological and molecular analyses demonstrated that FVE overexpression in *acg1* rescued the mutant phenotypes, including late flowering and alterations in floral pathway gene expression such as *FLC, SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), and *FLOWERING LOCUS T* (*FT*), and also super-induced cold-responsive reporter gene expression. The chromatin immunoprecipitation experiments revealed the amplification of specific DNA regions of *FLC* and *COLD-REGULATED 15A* (*COR15A*), indicating that FVE may bind to the *FLC* and *COR15A* chromatin. Gel-filtration chromatography and the immunoprecipitation of putative FVE complexes showed that FVE forms a protein complex of approximately 1.0 MDa. These results demonstrate that FVE may exist as a multiprotein complex, similar to the mammalian HDAC complex harboring RbAp, to regulate flowering time and cold response by associating with the *FLC* and *COR* chromatin.

INTRODUCTION

The transition to flowering is genetically controlled by several pathways, and involves the integration of environmental cues and the endogenous developmental state of the plant. Five major pathways regulate floral transition (Ausin et al., 2005; Boss et al., 2004; Putterill et al., 2004). These include photoperiod, light quality, ambient temperature cues, gibberellin hormone signaling, and autonomous pathways. Signals from these five flowering regulatory pathways converge into the floral pathway integrator genes, *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), and *LEAFY* (*LFY*), which promote floral transition.

The autonomous pathway promotes flowering by repressing *FLC,* which encodes for a MADS box transcriptional regulator which acts as a floral repressor through the repression of the floral pathway integrators *FT*, *SOC1*, and *LFY* (Boss et al., 2004). Seven autonomous pathway genes, *FCA*, *FLOWERING LOCUS D* (*FLD*), *FLOWERING LOCUS K* (*FLK*), *FPA*, *FVE*, *FY*, and *LUMINIDEPENDENS* (*LD*), have been identified in *Arabidopsis thaliana* (Simpson, 2004). FCA, FPA, and FLK harbor RNA-binding domains and may interact with RNA (Lim et al., 2004; Macknight et al., 1997; Schomburg et al., 2001). FY is a protein partner of FCA and is involved in the 3' processing of mRNA (Simpson et al., 2003). LD is a homeodomain protein (Lee et al., 1994). LD binds to SUPPRESSOR OF FRIGIDA4 (SUF4) in the absence of FRI, thereby preventing SUF4 from acting on *FLC* (Kim et al., 2006). Two components of the autonomous pathway, FLD and FVE, are similar to the components of the mammalian histone deacetylase (HDAC) 1, 2 co-repressor complexes involved in the repression of gene expression via the deacetylation of histone residues (Ausin et al., 2004; He et al., 2003; Kim et al., 2004). FLD, a homologue of human KIAA0601, is similar to the polyamine oxidases and harbors the SWI3p, Rsc8p, and Moira (SWIRM) domains, which are found in the enzymes associated with chromatin remodeling (He et al., 2003).

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Received February 1, 2011; revised May 26, 2011; accepted May 27, 2011; published online June 23, 2011

Keywords: chromatin, flowering, FVE, histone deacetylase, retinoblastoma-associated protein

FVE is a homologue of the yeast protein multicopy suppressor of IRA1 (MSI1) and of the mammalian retinoblastoma-associated proteins RbAp46 and RbAp48 (Qian and Lee, 1995) and is also referred to as AtMSI4 or the nucleosome/chromatin assembly factor group C4 (NFC4) (Ausin et al., 2004; Hennig et al., 2005; Kim et al., 2004). In the *fve* mutants, the *FLC* chromatin was enriched in acetylated histones H3 and H4, thereby indicating that FVE is required for histone deacetylation at the *FLC* chromatin (Ausin et al., 2004). A similar result was obtained with the *fld* mutants, which indicates that both FVE and FLD negatively regulate *FLC* expression via the histone deacetylation of *FLC* chromatin as a component of the HDAC complex (He et al., 2003). It is, however, not currently known whether FVE and FLD are recruited to *FLC* chromatin as a protein complex. We previously isolated an *Arabidopsis* mutant with altered cold-responsive gene expression harboring four copies of a cold responsive C-repeat/dehydration-responsive element (CRT/DRE) fused to the GUS (β-glucuronidase) reporter construct, and determined that *acg1* is a null allele of *fve,* and that FVE negatively regulates the CRT/DRE-binding factor/DRE-binding protein (CBF/DREB) cold response pathway as well as *FLC* (Kim et al., 2004).

In this study, we demonstrate, using chromatin immunoprecipitation and gel-filtration chromatography, that FVE is associated with the promoter and the intron region of the *FLC* chromatin and the promoter, the intron and the exon regions of the *COR15A* chromatin, a representative CBF/DREB target, and forms a protein complex of approximately 1.0 MDa in size. This result indicates that FVE can bind to the *FLC* chromatin as well as the *COR15A* chromatin as a large multiprotein complex, thereby regulating both flowering time and cold response.

MATERIALS AND METHODS

Plant growth conditions

4CRT/DRE-GUS and *acg1* have been described previously (Kim et al., 2002; 2004) and *fve-3* and *fca-9* were obtained from the Arabidopsis Biological Resource Center. We employed the Columbia-0 ecotype in all experiments. *Arabidopsis* was grown on 0.5× Murashige-Skoog (MS) agar media under long-day conditions (16 h light-8 h dark) at 23°C as previously described (Kim et al., 2002), and employed for various measurements. For flowering time analysis, the plants were grown and measured as described previously (Kim et al., 2004). Time to flowering was measured as the number of rosette leaves formed on the main shoot when the main stem had bolted 1 cm.

Plasmid construction and *Arabidopsis* transformation

To generate *FVE*-overexpressing *acg1* plants, the DNA fragment encoding for the full-length FVE protein was PCR-amplified from the plasmid harboring *FVE* cDNA (RINKEN RAFL09-46-N10) using the primers *FVE* F1 (5′-ATGAAAGA AAGTGGGAAGAA-3′) and *FVE* R2 (5′-TTAAGGCTTGGAGG CACAA-3′). The amplified DNA products were subsequently digested with *Bam*HI and *Sac*I, then cloned into the pBluescript II KS (pBSIIKS) (-) vector (Stratagene), yielding pBSIIKS(-)-FVE. The DNA fragment encoding for the HA (amino acids: YPYDVPDYA) and FLAG epitope (amino acids: DYKDDD DKG) were ligated in tandem into pBSIIKS(-)-FVE at the 5′-end of the *FVE* DNA. The *HA:FLAG:FVE* DNA fragment was excised and ligated into the pBI121 (Clontech) vector, yielding Pro_{35S}*:HA:FLAG:FVE*. Pro_{35S}*:HA:FLAG:FVE* was introduced into *acg1* by *Agrobacterium*-mediated transformation. The *acg1* overexpressing *HA:FLAG:FVE* was isolated on the basis of complementation of the late flowering phenotype of the parental-type *acg1*. The homozygous lines were isolated via PCR analysis of the transgene *FVE* and amplified.

RNA-gel blot analysis and immunoblot analysis

RNA-gel blot analysis was conducted with 20 μg of total RNA extracted with TRI reagent (Molecular Research Center) as described previously (Kim et al., 2004). For immunoblot analysis, 50 μg of total proteins extracted by standard procedures (Gusmaroli et al., 2004) were separated on 10% SDS-PAGE and transferred to Immuno-blot PVDF membranes (Bio-Rad, USA), and then detected with ECL™ in conjunction with the Western blotting detection system (GE Healthcare, UK).

Real time RT-PCR analysis for the quantification of *FLC*, *SOC1*, and *FT* expression

Real-time RT-PCR was carried out using a QuantiTect SYBR Green RT-PCR kit (Qiagen) in a Rotor-Gene 2000 real-time thermal cycling system (Corbett Research) as previously described (Jeon et al., 2010). Real-time RT-PCR conditions and primer sequences are shown in Supplementary Table 1.

Analysis of GUS expression

We conducted quantitative GUS assays in tissue extracts by fluorometric measurements of the 4-methylumbelliferone (MU) generated from the β-D-glucuronide precursor, as previously described (Jefferson and Wilson, 1991).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were conducted essentially as previously described (Gendrel et al., 2005). Two-week old plants grown on 0.5× MS agar medium were harvested. Two grams of seedlings were cross-linked in 37 ml of 1% formaldehyde *in vacuo*, washed with distilled water, and ground in liquid N_2. The ground seedlings were added to 30 ml of extraction buffer 1 [0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulphonylfluoride (PMSF)]. The slurry was then filtered through two layers of Miracloth (Calbiochem) and centrifuged for 20 min at 3,000 × *g* at 4°C. The pellet was resuspended in 1 ml of extraction buffer 2 (0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF) and centrifuged for 10 min at 12,000 × *g* at 4°C. The pellet was subsequently resuspended in 300 μl of extraction buffer 3 (1.7 M sucrose, 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 0.15% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF), layered on top of a 300 μl cushion of extraction buffer 3, and centrifuged for 1 h at 12,000 × *g* at 4°C. The chromatin pellet was then resuspended in 500 μl of ice-cold nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 1 mM PMSF), sonicated on ice, and centrifuged for 10 min at 12,000 × *g* at 4°C. The supernatant (300 μl) was subsequently added to 2700 μl of ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) and precleared with equilibrated Protein A-agarose beads (Upstate Biotechnology). After centrifugation, 1 ml of the supernatant was immunoprecipitated with anti-HA antibody (Santa Cruz Biotechnology). The immunoprecipitated DNA was recovered by ethanol precipitation and resuspended in 30 μl of distilled water. PCR analysis was conducted with the immunoprecipitated DNA using Takara Ex-taq polymerase under the following conditions: the mixtures were melted at 94°C for 5 min followed by 30 cycles of 94°C of 30 s, 56°C of 45 s, 72°C of 30 s, and a final 7-minute extension at 72°C for *FLC* and 35 cycles at 94°C for 30 s, 50°C for 45 s, 72°C for 30 s, and a final 7-minute extension at 72°C for *COR15A*. The products were visualized with

ethidium bromide in 2% agarose gel. Quantitative real-time PCR analysis was conducted using SsoFast EvaGreen Supermix (Bio-Rad, USA) on a CFX96 real-time system machine (Bio-Rad, USA) under the following conditions: the mixtures were melted for 5 min at 65°C to 94°C followed by 30 s at 95°C, 2 s at 95°C, 5 s at 56°C for *FLC* and 95°C for 30 s, 95°C for 2 s, and 50°C for 2 s for *COR15A*. The primer sequences used for all ChIP analyses are described in Supplementary Table 2.

Gel-filtration chromatography and immunoprecipitation

Two-week-old *Pro$_{35S}$:HA:FLAG:FVE* plants were harvested after growing on 0.5× MS agar medium. Ground tissues were homogenized in EB1 extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 1mM DTT, 10% glycerol, 0.1% Nonidet P-40) with freshly added protease inhibitors, 1 mM PMSF, protease inhibitor cocktail (3 μg ml^{-1} pepstatin, 3 μg ml^{-1} leupeptin, 5 μg ml^{-1} aprotinin), and the metalloprotease inhibitor, 2 mM o-phenanthrolin hydrochloride monohydrate. The homogenates were microcentrifuged twice for 15 min and the supernatants were filtered through 0.2 μm filters (PALL, USA). For gel fractionation analyses, 100 mg of total proteins were loaded onto a Sephacryl S-300 (HiPrep16/60) gel-filtration column (GE Healthcare, Sweden). The column was then equilibrated with 240 ml of EB1 with final concentrations of 1 mM PMSF and 25 mM β-glycerophosphate. The proteins were eluted in the same buffer at a flow rate of 0.2 ml min^{-1}. All the procedures were carried out at 4°C. One-ml fractions were collected, finishing with 120 fractions. The fractions were concentrated using 20% trichloroacetic acid and washed in acetone at least three times. Equal volumes of each fraction were boiled for 5 min in SDS sample buffer, separated by 10% SDS-PAGE, and transferred onto PVDF Immobilon membranes (Bio-Rad, USA). The elute was incubated overnight with 50 μl of anti-FLAG M2 affinity resin (Sigma) at 4°C on a rotary shaker. The resins were washed three times in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% CA-630, 0.5% sodium deoxycholate, 0.1% SDS) and twice in TBS buffer. The immunoprecipitated proteins were eluted with 3× FLAG peptide (Sigma) and loaded on 12% SDS-PAGE and silver-stained.

MALDI-TOF analysis and database search

The FVE complexes immunoprecipitated with anti-FLAG antibody (Sigma) were loaded on 12% SDS-PAGE and silver-stained. The protein band corresponding to FVE at 60 kDa was enzymatically digested in-gel using modified porcine trypsin as described previously (Shevchenko et al., 1996). Gel pieces were washed in 50% acetonitrile, dried, rehydrated with trypsin (8-10 ng μl^{-1}), and incubated overnight at 37°C. The proteolytic reaction was terminated by the addition of 5 μl of 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. The peptide mixture was concentrated using a SpeedVac concentrator and desalted using C_{18}ZipTips (Millipore), and then the peptides were eluted in 1 to 5 μl of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, after which 1 μl of mixture was spotted onto a target plate. Protein analysis was carried out using a Voyager DE-STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, USA). Mass spectra were obtained in the positive-ion reflector mode with delayed extraction. Spectra were calibrated with angiotensin I (1296.79 Da), adrenocorticotropic hormone (ACTH clip 1-17, 2093.01 Da), and ACTH clip 18-39 (2465.20 Da) as internal standards. The Swiss-Prot database using the MS-Fit search program (http://prospector.ucsf.edu/prospector/mshome.htm) was used to search the MALDI-peptide-mass fingerprinting data.

RESULTS

Overexpression of FVE in *acg1* mutants

In order to determine whether FVE binds to the *FLC* chromatin by the formation of a protein complex, we generated transgenic *Arabidopsis* plants that overexpressed the FVE proteins tagged with the HA and FLAG epitope in tandem at the N-terminus under the control of the CaMV 35S promoter in *acg1* mutants, *Pro$_{35S}$:HA:FLAG:FVE*/*acg1,* and then isolated two independent transgenic lines (N1-11 and N3-3). RNA-gel blot analysis using a *FVE* DNA probe showed that both lines, N1-11 and N3-3, overexpressed the *FVE* mRNA, and higher *FVE* mRNA levels were expressed in N1-11 as compared with N3-3 (Fig. 1A). Immunoblot analysis with antibodies against the HA or FLAG epitope demonstrated that the HA and FLAG-tagged FVE proteins are produced at high levels in *Pro$_{35S}$:HA:FLAG:FVE*/*acg1* *Arabidopsis* plants lacking endogenous FVE proteins (Fig. 1B). The late flowering phenotype of *acg1* was complemented to the wild-type in the N1-11 line and to a significant degree in N3-3, which correlates with FVE expression levels (Figs. 1C-1E). We previously noted markedly elevated *FLC* expression levels in *acg1* with consequent significant reductions in the expression of *SOC1* and *FT* via negative regulation by FLC (Kim et al., 2004). Therefore, we analyzed the expression of *FLC* as well as *SOC1* and *FT* in the N1-11 line by real-time RT-PCR, and demonstrated that *FLC* expression in *acg1* was significantly reduced and that *SOC1* and *FT* expression were increased to almost wild-type levels by FVE overexpression, evidencing a complementation of mutant phenotypes (Fig. 1F). The fact that *FLC* expression was not fully rescued to the wild-type via ectopic expression of *FVE* under CaMV 35S promoter might be attributable to the different levels of tissue expression of *FVE* under the control of the CaMV 35S promoter and that of *FLC,* as, for example, *FLC* is expressed most abundantly in the vegetative apex (Michaels and Amasino, 1999) as opposed to the expression of the GUS reporter gene fused to the CaMV 35S promoter in the larger proportion of phloem-associated cells in roots and stems as compared to leaves (Jefferson et al., 1987). However, the reduced *FLC* expression induced by ectopic *FVE* expression in *Pro$_{35S}$:HA:FLAG:FVE*/*acg1* plants proved sufficient to release the repression of *SOC1* and *FT* by FLC in *acg1* mutants to wild-type levels. In addition, we observed no phenotypes other than flowering time. Finally, we determined that FVE rescued the increased expression of the cold-responsive *GUS* reporter gene in *acg1* with and without the application of cold treatment (Fig. 1G). Collectively, these results demonstrated that FVE can negatively regulate both *FLC* expression and cold-responsive gene expression via CRT/DRE.

Analysis of FVE binding to *FLC* and *COR15A* chromatin by ChIP assays

We then employed ChIP assays to determine whether FVE can be recruited to the *FLC* chromatin. The chromatins of the *Pro$_{35S}$:HA:FLAG:FVE*/*acg1* N1-11 line along with various controls, Col-0, *4CRT/DRE-GUS*, *acg1*, *fve-3*, and *fca-9* plants, were immunoprecipitated with anti-HA antibody. We amplified eight DNA fragments spanning the promoter, the first exon, and the first intron of *FLC* from the precipitated chromatin (Figs. 2A and 2B). Specific DNA fragments were amplified in the promoter region (I), the region close to the translation initiation point (II), and the first intron (IV-VI) of *FLC*. By contrast, we noted no amplification of the DNA fragments in these regions

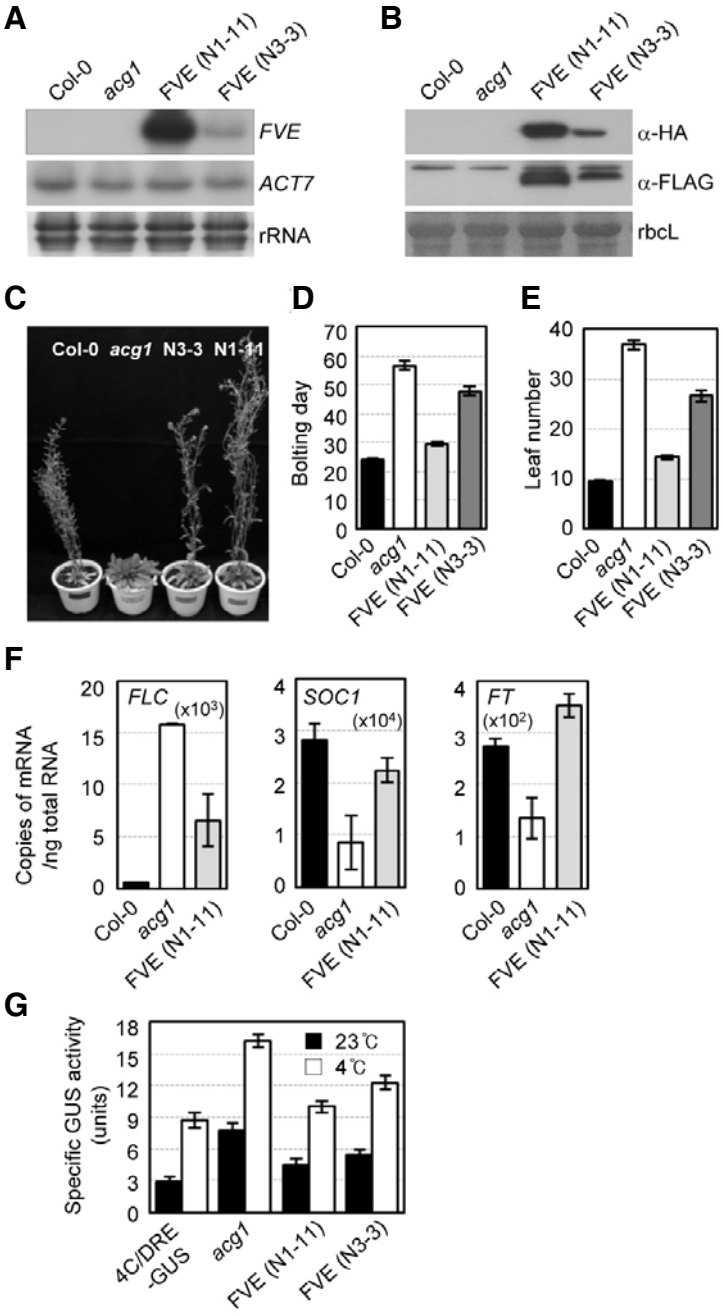

Fig. 1. Overexpression of *HA:FLAG:FVE* in *acg1*. (A) RNA-gel blot analysis of *acg1* overexpressing *HA:FLAG:FVE*. Total RNAs isolated from 10-day-old plants, Col-0, *acg1*, and two lines [FVE (N1-11) and FVE (N3-3)] of Pro_{35S}*:HA:FLAG:FVE*/*acg1*, were subjected to RNA-gel blot analysis with *FVE* or *ACTIN7* DNA probe. (B) Immunoblot analysis of *acg1* overexpressing *HA:FLAG:FVE*. Total proteins extracted from 10-day-old plants, Col-0, *acg1*, and two lines of Pro_{35S}*: HA:FLAG:FVE*/*acg1*, were subjected to immunoblot analysis with anti-HA or anti-FLAG antibody. The rbcL protein encoding for the large subunit of rubisco is shown as a loading control. (C) Eight-week-old Col-0, *acg1*, and *acg1* overexpressing *HA:FLAG:FVE*. Plants were grown for eight weeks under long-day conditions and photographed. (D-E) Bolting day (D) and the rosette leaf numbers (E) of Col-0, *acg1*, and *acg1* overexpressing *HA:FLAG:FVE*. n ≥ 16. (F) Real-time RT-PCR analysis of *FLC*, *SOC1*, and *FT* from Col-0, *acg1*, and *acg1* overexpressing *HA:FLAG:FVE*. Total RNAs isolated from 10-day-old plants, Col-0, *acg1*, and Pro_{35S}*:HA:FLAG:FVE*/*acg1* (N1-11), were quantified by real-time RT-PCR. Copies of the transcripts were plotted per ng of total RNA after the normalization of *ACTIN7* RNA. Multiplication of the number in the brackets inside the graph by the number in the y-axis generates copy numbers of the corresponding transcripts. The mean values and standard errors from biological triplicate experiments were plotted. (G) Fluorometric quantification of the GUS activities of *4CRT/DRE-GUS*, *acg1*, and *acg1* overexpressing *HA:FLAG:FVE*. Ten-day-old plants were used for fluorometric quantification of GUS activities. Specific activities (units) are expressed as the pmol reaction product (4-methylumbelliferone) generated per min per mg of total protein. The data are expressed as the means ± standard errors of three independent treatments. The open column and closed column indicate the sample treated at 4°C for 2 days and the sample treated at 23°C, respectively.

from Col-0, *acg1*, and other mutant plants. In order to eliminate the possibility that the precipitation of the chromatin might be attributable to the overexpression of nonspecific proteins, we employed additional control *Arabidopsis* plants that overexpressed modified green fluorescent protein (EGFP) tagged with the HA epitope in the N-terminus under the control of the CaMV 35S promoter in these ChIP assays. We selected two lines of transgenic Pro_{35S}*:HA:EGFP* plants that exhibited the highest expression of the *HA:EGFP* transcripts among 13 lines generated (data not shown). We carried out quantitative PCR analysis for three genomic DNA regions, in which the most significant amplification of the DNA fragments was assessed without nonspecific PCR products in the ChIP assays (Fig. 2C). We noted significant enrichments in these three DNA regions from the Pro_{35S}*:HA:FLAG:FVE*/*acg1* plants but not from the two Pro_{35S}*:HA:EGFP* lines. (Fig. 2C). These results indicate that FVE is recruited to promoter region I and intron regions V and VI of the *FLC* chromatin.

In the *acg1* mutants, *COR15A* is upregulated and superinduced in response to cold (Kim et al., 2004). Ectopic FVE expression in *acg1* partially complemented the elevated expression of GUS with and without cold treatment through CRT/DRE (Fig.1G), thereby indicating that FVE may be associated with *COR15A* chromatin, like *FLC,* to regulate cold response. To assess this possibility, ChIP was also conducted with eight DNA fragments spanning the promoter to the coding region of *COR15A* (Fig. 3A). The amplification of specific DNA fragments was noted in the promoter regions (II and III) and the

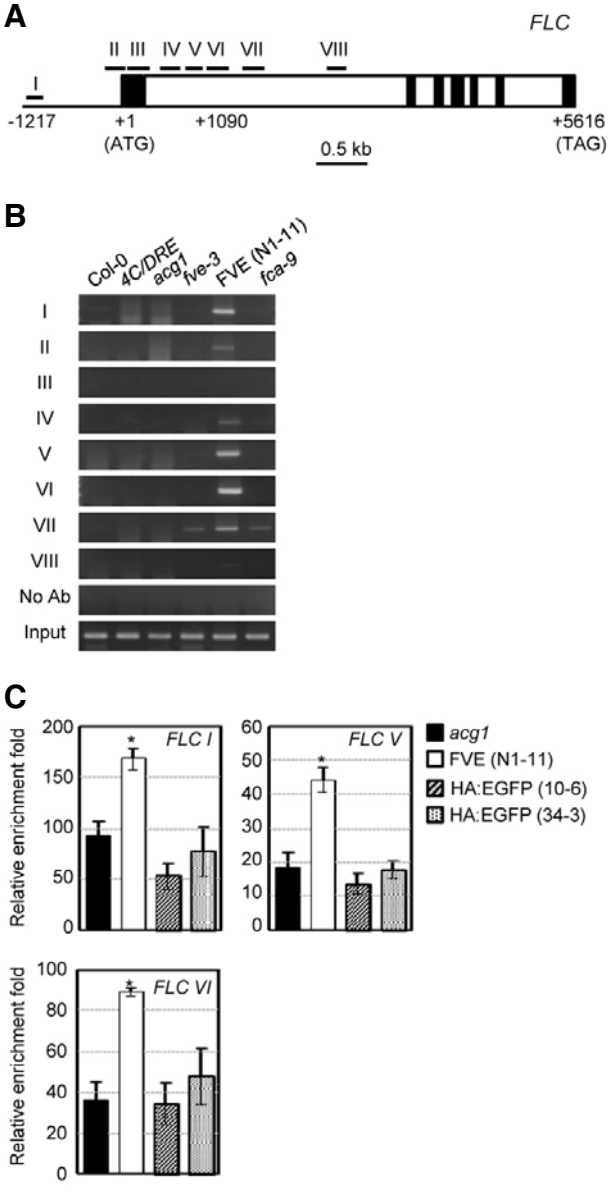

Fig. 2. ChIP analyses of the *FLC* chromatin in *acg1* overexpressing *HA:FLAG:FVE*. (A) Schematic structure of the *FLC* region. I to VIII represent the regions in which FVE binding was evaluated by a ChIP. The translation initiation point is +1. The filled boxes represent exons and the open boxes represent introns. (B) ChIP analyses of FVE binding to the *FLC* chromatin. No Ab indicates ChIP carried out without antibody. The input is the chromatin prior to immunoprecipitation. ChIP analyses were carried out with three biological replicates; representative PCR data are shown. (C) Quantitative PCR analysis of FVE binding to the *FLC* chromatin compared with EGFP. Genomic DNA obtained by ChIP using HA antibody in *acg1* plants overexpressing *HA:FLAG:FVE* or *acg1,* or *HA:EGFP*-overexpressing plants were analyzed by quantitative PCR. Values are expressed as the means of triplicate biological replications with error bars indicating standard errors. *denotes statistically significant changes with $p < 0.05$ compared with the *acg1* control.

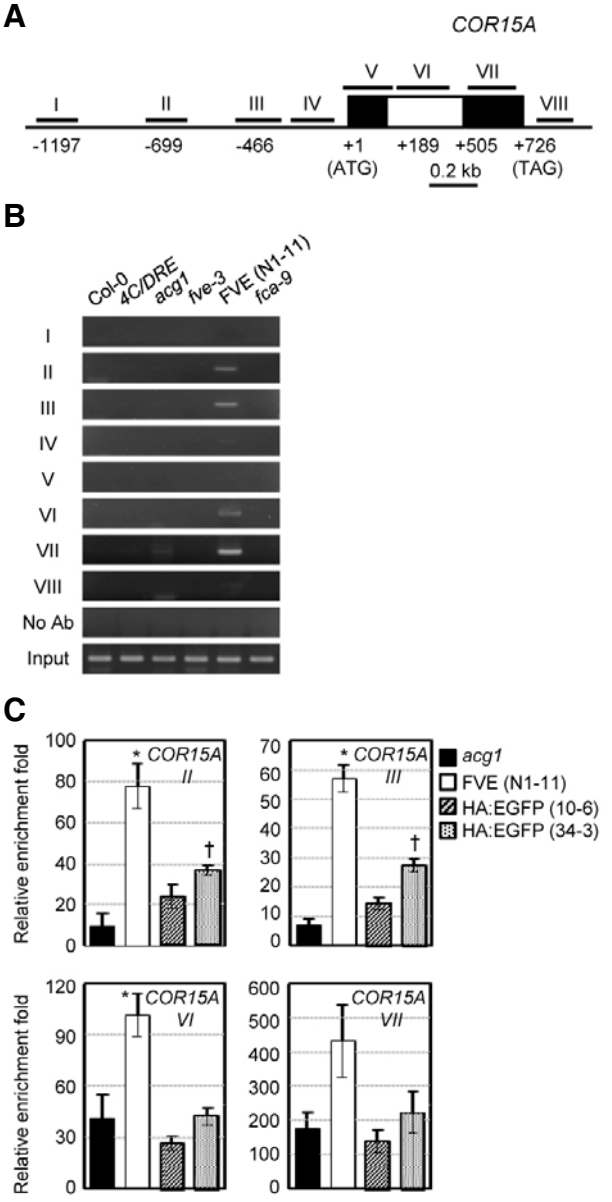

Fig. 3. ChIP analyses of the *COR15A* chromatin in *acg1*-overexpressing *HA:FLAG:FVE*. (A) The schematic structure of the *COR15A* region. (B) ChIP analyses of FVE binding to the *COR15A* chromatin. (C) Quantitative PCR analysis of FVE binding to the *COR15A* chromatin compared with EGFP. Experiments were conducted as described in Fig. 2C.

intron (VI) and the second exon (VII) regions (Fig. 3B). No DNA fragments in these regions were detected in any other control plants. The transgenic *Pro$_{35S}$:HA:EGFP* lines were also employed as additional controls to further verify the binding of FVE to the *COR15A* chromatin. As shown in Fig. 3C, we observed greater enrichment of the DNA fragments in regions II, III, VI, and VII from *Pro$_{35S}$:HA:FLAG:FVE*/*acg1* plants relative to those of the two *Pro$_{35S}$:HA:EGFP* lines upon quantitative PCR analysis of the ChIP assays. These data indicate that FVE is recruited to the *COR15A* chromatin.

Analysis of FVE complex by gel-filtration chromatography

We next attempted to determine, by gel-filtration chromatogra-

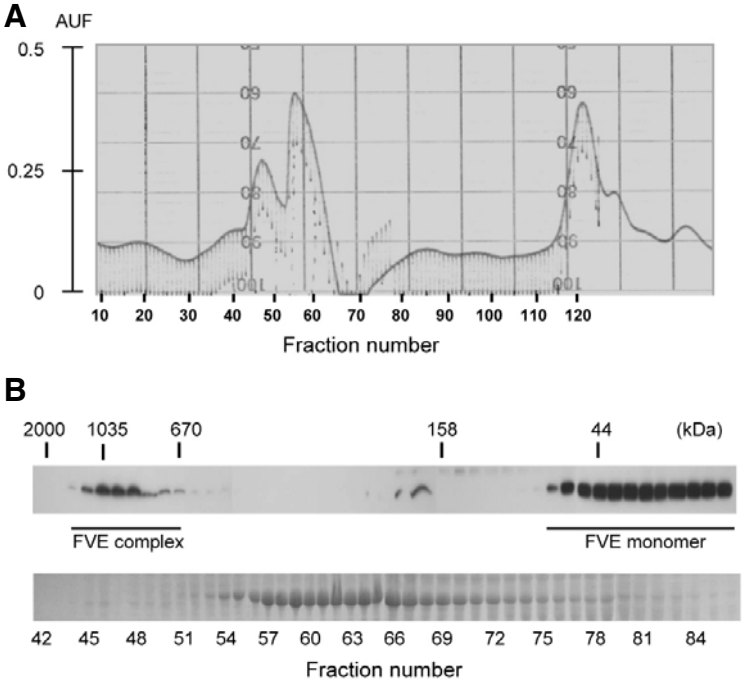

Fig. 4. Gel-filtration chromatography of the total protein extracts from *acg1* overexpressing *HA:FLAG:FVE*. (A) Chromatogram of the total protein extracts from *acg1* overexpressing *HA:FLAG:FVE*. The total proteins from two-week-old *Pro*$_{35S}$*:HA:FLAG:FVE*/*acg1* (N1-11) plants were separated on a Sephacryl S-300 gel filtration column and fractionated at 1 ml. The eluted proteins were monitored at 280 nm. (B) Immunoblot analyses of each fraction received from gel-filtration chromatography. Equal volumes of each fraction were separated by 10% SDS-PAGE and subjected to immunoblot analysis with HA antibody (A). Each fraction was stained by Coomassie blue dye (B).

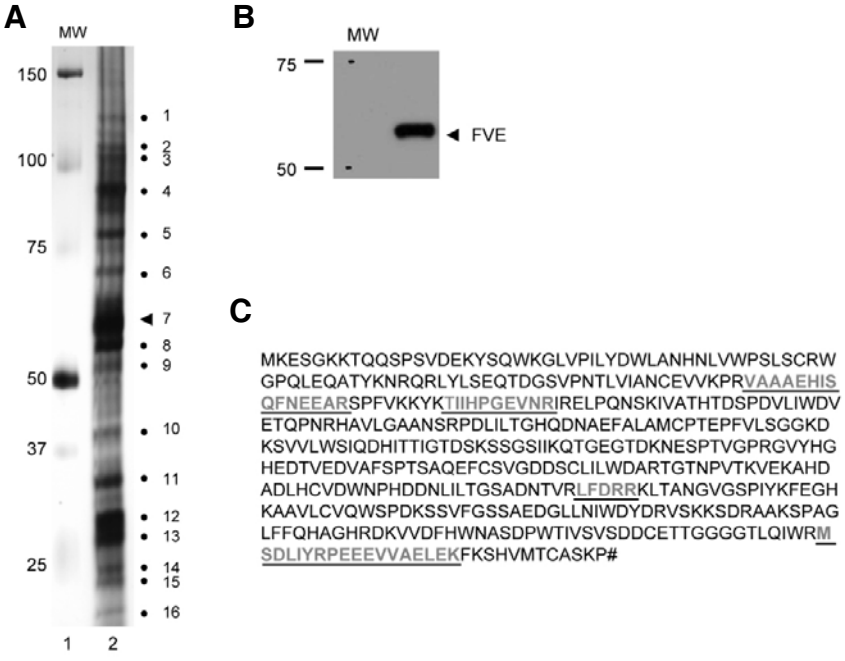

Fig. 5. SDS-PAGE analysis of proteins associated in the FVE complex. (A) SDS-PAGE analysis of the fractions corresponding to the FVE complexes after immunoprecipitation with FLAG antibody. The FVE protein complexes collected by gel-filtration chromatography from 44 to 52 fractions were incubated with 50 μl of anti-FLAG M2 affinity resin, precipitated, and eluted with the 3× FLAG peptide. The elutes (lane 2) were then loaded on 12% SDS-PAGE and silver-stained. The numbers to the left are the sizes of the molecular weight marker proteins (lane 1). Numbers to the right show the apparent molecular weights of the polypeptides detected by silver staining in decreasing order (lane 2). The triangle numbered 7 indicates FVE. (B) Immunoblot analysis of the FVE complexes immunoprecipitated with FLAG antibody. The immunoprecipitated FVE protein complexes were loaded onto 12% SDS-PAGE and subjected to immunoblot analysis with HA antibody. (C) Peptide sequence of FVE detected by MALDI-MS. The FVE complexes immunoprecipitated with FLAG were used for 1-D gel electrophoresis. The number 7 band (A) was identified as FVE. Underlined bold letters indicate the peptides detected by MALDI-MS. The matched peptides represent 11% amino acid sequence coverage.

phy, whether or not FVE forms a multiprotein complex. We extracted total proteins from *Pro*$_{35S}$*:HA:FLAG:FVE*/*acg1* plants that lacked endogenous FVE proteins, but generated high levels of the HA and FLAG-tagged FVE proteins, and conducted gel-filtration chromatography of the extracted proteins followed by immunoblot analyses of each fraction with HA antibody (Fig. 4). The results showed that FVE is eluted at a size of approximately 1.0 MDa in addition to a monomer. The combined fractions corresponding to 1.0 MDa of FVE were pooled from five rounds of gel-filtration chromatography and subjected to immunoprecipitation with FLAG antibody to further verify the presence of FVE in the complex and to detect the polypeptides associated with FVE. After running the immunoprecipitates on SDS-PAGE and silver-staining, 15 polypeptides ranging from 25 to 150 kDa in size (Fig. 5A) were revealed, in addition to an FVE band (indicated by dot number 7, Fig. 5A) by immunoblot

analysis (Fig. 5B). The FVE band was corroborated further by peptide sequencing (bold letters underlined in Fig. 5C). The protein fractions corresponding to 1.0 MDa acquired from the *acg1* plants exhibited no significant bands on SDS-PAGE and silver staining after immunoprecipitation with FLAG antibody (data not shown). These results show that FVE exists as a multiprotein complex in *Arabidopsis*.

DISCUSSION

Hyperacetylation was noted on histones H3 and H4 of the *FLC* chromatin in *fve* mutants, suggesting that FVE might be a component of an HDAC transcriptional co-repressor complex, and negatively regulate the *FLC* chromatin (Ausin et al., 2004). In order to function as a component of the HDAC complex modifying the *FLC* chromatin, FVE should be associated with *FLC* chromatin. To evaluate this model, we overexpressed FVE tagged with the HA and FLAG epitopes in *acg1* mutants lacking endogenous FVE and conducted ChIP assays. Our results showed that FVE can be recruited to the promoter and the first intron of the *FLC* chromatin. The locations of FVE in these regions defined by our ChIP assays are consistent with the hyperacetylation sites of the *FLC* chromatin in *fve* mutants (He et al., 2003). We also demonstrated that FVE is recruited to the *COR15A* chromatin in the promoter regions, the first intron, and the second exon, thus suggesting that FVE regulates flowering time and cold response by modulating the chromatin state. Considering that *COR15A* promoter regions III and IV (Fig. 2) harbor CRT/DRE *cis*-acting elements to which CBF/DREB transcription factors bind (Stockinger et al., 1997), the HDAC complex containing FVE might deacetylate the histone proteins around the regions to which CBF/DREB binds for the regulation of *COR15A* expression.

In mammalian systems, the Rb protein represses the E2F family of transcription factor-mediated transcription by recruiting a histone deacetylase (Luo et al., 1998; Magnaghi-Jaulin et al., 1998). A maize HDAC was also shown to interact physically with Rb-related proteins to repress transcription (Rossi et al., 2003). Mammalian RbAp48 is a component of the HDAC1 recruited by RB (Nicolas et al., 2000). In yeast, the Sin3/RPD3-HDAC complex exists as a large protein complex (larger than 2 MDa in size) (Alland et al., 1997; Hassig et al., 1997; Heinzel et al., 1997; Kasten et al., 1997; Laherty et al., 1997; Nagy et al., 1997; Taunton et al., 1996; Wade et al., 1998; Zhang et al., 1997). The mammalian Sin3 complex consists of at least seven subunits, including HDAC1, HDAC2, RbAp48, RbAP46, and other polypeptides (Zhang et al., 1998). FVE, which is homologous to RbAp46 and RbAp48, interacted with the maize Rb protein in the immunoprecipitation assays, thereby indicating that FVE might be a component of the retinoblastoma-containing complexes in *Arabidopsis* (Ausin et al., 2004). These results, coupled with the fact that FVE is not a specific DNA-binding protein, predicted that FVE should form a multiprotein complex to be recruited to the chromatin. In order to determine whether or not FVE forms a large protein complex, we employed gel-filtration chromatography with total protein extracts harboring FVE tagged with the HA and FLAG epitopes that were overexpressed in *acg1* mutants. Consistent with that prediction, we showed that FVE is a component of a large (1.0 MDa) multiprotein complex. The immunoprecipitation of these complexes with FLAG antibody and SDS-PAGE analysis demonstrated that the complex consists of 16 polypeptides, including FVE. A very recent study showed that FVE functionally interacts with the cullin-RING ubiquitin ligase (CUL4-DDB1) and a CLF-Polycomb Repressive Complex 2 (PRC2) at the *FLC* chromatin, thereby regulating flowering time in *Arabidopsis* (Pazhouhandeh et al., 2011). It will be interesting to determine the molecular identities of the components that form a complex with FVE isolated herein, and to elucidate their biochemical functions, thereby shedding light on the mechanism by which the chromatin state is regulated by histone deacetylation in response to environmental signals and developmental cues.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This work was supported by a grant from the World Class University project funded by the Ministry of Education, Science, and Technology of Korea (grant no. R31-2009-000-20025-0) to J. Kim. We thank Su-Jin Ku and Min-Jung Kim for the plasmid construct, *Pro$_{35S}$:HA:FLAG:FVE* and *Pro$_{35S}$:HA:EGFP* transgenic lines, respectively.

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