

The Cold Signaling Attenuator HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1 Activates FLOWERING LOCUS C Transcription via Chromatin Remodeling under Short-Term Cold Stress in *Arabidopsis*^{CJW}

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Exposure to short-term cold stress delays flowering by activating the floral repressor FLOWERING LOCUS C (FLC) in *Arabidopsis thaliana*. The cold signaling attenuator HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1 (HOS1) negatively regulates cold responses. Notably, HOS1-deficient mutants exhibit early flowering, and FLC expression is suppressed in the mutants. However, it remains unknown how HOS1 regulates FLC expression. Here, we show that HOS1 induces FLC expression by antagonizing the actions of FVE and its interacting partner histone deacetylase 6 (HDA6) under short-term cold stress. HOS1 binds to FLC chromatin in an FVE-dependent manner, and FVE is essential for the HOS1-mediated activation of FLC transcription. HOS1 also interacts with HDA6 and inhibits the binding of HDA6 to FLC chromatin. Intermittent cold treatments induce FLC expression by activating HOS1, which attenuates the activity of HDA6 in silencing FLC chromatin, and the effects of intermittent cold are diminished in *hos1* and *fve* mutants. These observations indicate that HOS1 acts as a chromatin remodeling factor for FLC regulation under short-term cold stress.

INTRODUCTION

Seasonal fluctuations in ambient temperature affect various aspects of plant growth and development. In particular, low temperatures significantly limit plant propagation, reproduction, and geographical distribution. Therefore, plants have evolved a variety of adaptive mechanisms to increase freezing tolerance after exposure to low but nonfreezing temperatures, which is known as cold acclimation (Chinnusamy et al., 2007; Zhu et al., 2007).

During the cold acclimation process, a group of genes encoding transcriptional regulators, such as C-repeat/dehydration-responsive element binding factors (CBFs), is induced, and the encoded proteins activate downstream genes that trigger cold responses. Cold induction of the CBF genes is mediated by INDUCER OF CBF EXPRESSION1 (ICE1), a cold-activated MYC transcription factor (Chinnusamy et al., 2003; Lee et al., 2005). Cold responses occur within minutes, and plants are cold acclimated within a few days after exposure to cold temperatures (Sung and Amasino, 2005; Medina et al., 2011).

Floral transition is profoundly affected in the process of cold acclimation (Chinnusamy et al., 2007; Zhu et al., 2007). The effects of cold stress on flowering time have been investigated more precisely by intermittent cold treatments, in which plants are exposed to 4°C for several hours during the day until flowering (Kim et al., 2004). Intermittent cold treatments delay flowering and induce FLOWERING LOCUS C (FLC) gene expression by approximately threefold (Kim et al., 2004; Seo et al., 2009). By contrast, prolonged exposure to cold temperatures promotes flowering, which is known as vernalization, by suppressing the FLC gene (Sung and Amasino, 2005; Kim et al., 2009). The short-term cold stress response would provide an adaptive strategy that prevents precocious flowering under short-term temperature fluctuations, which often occur during early spring and late autumn. In this regard, the cold stress response apparently differs from vernalization, which mediates a prolonged cold effect to promote flowering.

Consistent with the role of FLC in flowering initiation under cold stress, the flowering of FLC-deficient mutants is not influenced by intermittent cold treatments (Seo et al., 2009). It has recently been shown that cold signaling components that mediate the cold acclimation response, such as ICE1 and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1 (HOS1), are not involved in vernalization (Bond et al., 2011; Lazaro et al., 2012). In addition, transgenic plants overexpressing CBF genes exhibit late flowering with moderate induction of FLC expression (Gilmour et al., 2004; Seo et al., 2009), supporting the signaling linkage between cold response and flowering time.

The effects of cold stress on flowering initiation are also mediated by FVE, which belongs to the autonomous flowering

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pathway. FVE negatively regulates the expression of the *FLC* gene and several cold-responsive genes in *Arabidopsis thaliana* (Kim et al., 2004). Consequently, loss-of-function *fve* mutants show enhanced freezing tolerance and late flowering, and flowering is not delayed by intermittent cold treatments in the mutants (Ausín et al., 2004; Kim et al., 2004).

The FVE protein is an *Arabidopsis* homolog of the human retinoblastoma-associated protein 46/48 that constitutes histone deacetylase (HDAC) corepressor complexes (Ausín et al., 2004; Gu et al., 2011). FVE induces histone deacetylation and transcriptional silencing of various genetic loci, including *FLC*, which are targeted by small interfering RNAs and antisense RNAs associated with HDA6 (Gu et al., 2011; Pazhouhandeh et al., 2011; To et al., 2011). FVE also interacts with a chromatin repressive complex, which is similar to Polycomb repressive complex2 (PRC2), to modulate the deposition of the H3K27 trimethylation (H3K27Me3) that acts as a repressive chromatin mark in *FLC* repression (Pazhouhandeh et al., 2011). Therefore, FVE appears to mediate cold stress signals in the regulation of *FLC* expression by forming multiprotein complexes with various chromatin remodeling factors.

The *Arabidopsis* REALLY INTERESTING NEW GENE finger E3 ligase HOS1 is a negative regulator of cold-responsive genes, such as *CBF* and *COLD-REGULATED* genes (Ishitani et al., 1998; Lee et al., 2001; Dong et al., 2006). Under cold stress, HOS1 triggers the degradation of the ICE1 transcription factor, a direct upstream activator of the *CBF3* gene (Chinnusamy et al., 2003; Dong et al., 2006). Although the inactivation of the *HOS1* gene blocks cold-induced degradation of ICE1, the overexpression of *HOS1* accelerates ICE1 degradation even at normal temperatures (Dong et al., 2006). Interestingly, loss-of-function *hos1* mutants exhibit early flowering, and *FLC* expression is reduced in the mutants (Ishitani et al., 1998; Lee et al., 2001). It is therefore envisioned that HOS1 plays a role in cold regulation of flowering in an *FLC*-dependent manner. Notably, recent studies have shown that HOS1 regulates flowering time in response to ambient temperature changes and intermittent cold treatments (Jung et al., 2012a; Lee et al., 2012). However, it remains largely unknown how HOS1 regulates *FLC* expression.

In this article, we demonstrate that HOS1 regulates *FLC* transcription at the chromatin level, under cold stress, through interactions with FVE and HDA6. HOS1 binds to *FLC* chromatin and inhibits the association of HDA6 with *FLC* chromatin, resulting in *FLC* induction and delayed flowering. However, HOS1 does not degrade either HDA6 or FVE. Our data support the notion that HOS1 plays a role in the modulation of *FLC* chromatin and provide an insight into how cold acclimation response is linked with *Arabidopsis* flowering time control.

RESULTS

HOS1 Regulates *FLC* Transcription in *Arabidopsis* Flowering

To elucidate the molecular mechanisms by which HOS1 regulates *FLC* expression, we isolated two mutant alleles of the *HOS1* gene in Columbia-0 (Col-0) background (Figure 1A). Both

the *hos1-3* and *hos1-5* loss-of-function mutants exhibited early flowering under long days (LDs) (Figure 1B), as observed with the *hos1-1* mutant in C24 background (Ishitani et al., 1998; Lee et al., 2001). The loss-of-function mutants initiated flowering at a total leaf number of 8, whereas Col-0 plants initiated flowering at a total leaf number of 14 under our assay conditions. In addition, *FLC* expression was reduced in the mutants but elevated approximately twofold in *HOS1*-overexpressing transgenic plants (see Supplemental Figure 1 online), showing that HOS1 is correlated with *FLC* expression.

FLC expression is epigenetically regulated in response to various endogenous and environmental cues (He, 2009; Jarillo et al., 2009). We therefore performed chromatin immunoprecipitation (ChIP) assays on *FLC* chromatin using five sets of primer pairs covering the *FLC* genomic DNA (Figure 1C). It was found that H3 acetylation (H3Ac) and H3K4 trimethylation, which are marks for active gene expression (He, 2009; Jarillo et al., 2009), were reduced in the *hos1-3* mutant (Figures 1D and 1E). By contrast, H3K27Me3, which is a repressive chromatin mark (He, 2009; Jarillo et al., 2009), was elevated in the mutant (Figure 1F), which is consistent with the low-level expression of *FLC* in the mutant. These observations suggest that HOS1 regulates *FLC* transcription at the chromatin level.

HOS1 Binding to *FLC* Chromatin Is Induced by Cold Stress

Our data suggested that HOS1 would bind directly to *FLC* chromatin. To examine this, we performed ChIP assays with or without cold treatments, using transgenic plants overexpressing the *HOS1-MYC* gene fusion (*HOS1-ox*), in which the MYC-coding sequence was fused in frame to the 3' end of the *HOS1* gene and driven by the cauliflower mosaic virus (CaMV) 35S promoter. The results showed that HOS1 binds to *FLC* chromatin primarily in the P1, P2, and P3 sequence regions and HOS1 binding is dramatically elevated at 4°C (Figure 2A).

Because the ectopic expression of the *HOS1* gene may cause potential artifacts, we generated a HOS1-specific antibody for the ChIP assays. The epitope for the antibody is a peptide sequence that differs from any other sequence of annotated *Arabidopsis* proteins (Figure 2B). To verify the specificity of the anti-HOS1 antibody, we performed protein gel blot analysis of the *HOS1-ox* transgenic plants. Transgenic plants overexpressing the *MYC-FCA* gene fusion (*FCA-ox*) were used as control (Jung et al., 2012b). Using the anti-MYC antibody, HOS1 and FCA proteins were detected in the transgenic plants. However, protein gel blot analysis using the anti-HOS1 antibody showed that HOS1 proteins were detected in both the *HOS1-ox* and *FCA-ox* transgenic plants, whereas FCA proteins were not detected (see Supplemental Figure 2 and Supplemental References 1 online). In addition, HOS1 proteins were not detected in the *hos1-3* plants (Figure 2B), showing that the anti-HOS1 antibody detects specifically HOS1 proteins.

Next, we conducted ChIP assays using the HOS1-specific antibody with cold-treated plants. The results showed that the HOS1 proteins were enriched in the P1, P2, and P3 sequence regions in the cold-treated plants (Figure 2C), as observed in Figure 2A. To further examine the HOS1 binding to *FLC* chromatin, the MYC-coding sequence was fused in frame to the

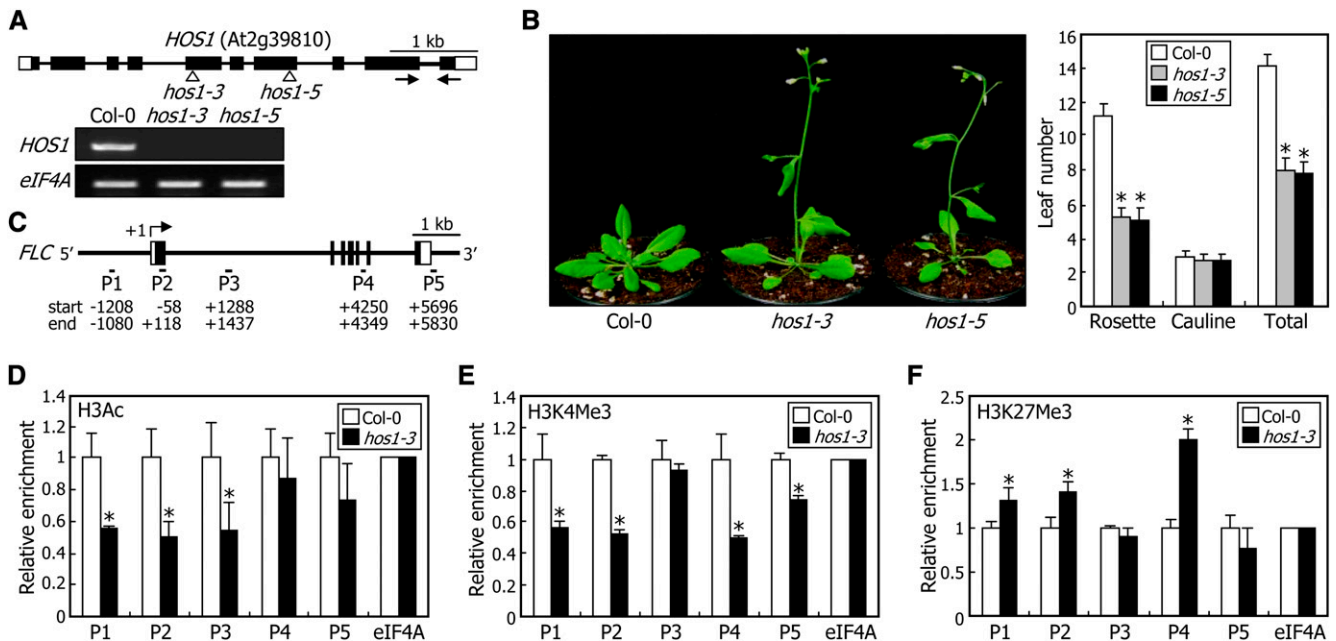


Figure 1. HOS1 Regulates *FLC* Transcription at the Chromatin Level.

(A) Isolation of *hos1* mutants. In the *hos1-3* and *hos1-5* mutants, one copy of the T-DNA element is inserted in the 5th and 7th exons of the *HOS1* gene, respectively (top panel). Black boxes represent exons, and white boxes represent untranslated regions. Black arrows indicate the primer pair used for RT-PCR. *HOS1* expression was examined by RT-PCR using total RNA samples extracted from 10-d-old whole plants grown on MS-agar plates (bottom panel). The *eIF4A* gene was used as the RNA quality control.

(B) Flowering times of *hos1* mutants. Five-week-old plants grown in soil under LDs were photographed (left panel). The leaf numbers of 20 plants were averaged for each plant genotype and statistically treated using a Student's *t* test ($*P < 0.01$) (right panel). Bars indicate *se*.

(C) Structure of the *FLC* gene. The sequence regions marked by P1 to P5 indicate regions examined in the ChIP assays. Numbers indicate residue positions relative to the transcription start site. Black boxes indicate exons, and white boxes denote untranslated regions.

(D) to (F) Relative levels of histone modifications in *FLC* chromatin. ChIP assays were performed using anti-H3Ac (D), anti-H3K4 trimethylation (E), and anti-H3K27Me3 (F) antibodies. Plants grown on MS-agar plates for 10 d under LDs were used for chromatin preparation. An *eIF4A* DNA fragment was used for normalization. Four measurements were averaged for each plant genotype and statistically treated using a Student's *t* test ($*P < 0.01$). Bars indicate *se*.

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3'-end of the *HOS1* gene with the native promoter sequence consisting of ~2.8 kb upstream of the transcription start site (Lee et al., 2001), and the fusion construct was transformed into Col-0 plants. ChIP-quantitative PCR (qPCR) assays of the transgenic plants revealed that HOS1 binding to *FLC* chromatin was detectably elevated in the cold-treated plants (Figure 2D), confirming that HOS1 binds directly to *FLC* chromatin in planta.

We also examined whether the cold induction of HOS1 binding to *FLC* chromatin is associated with *FLC* expression. The *hos1-3* mutant was treated with intermittent cold, and the *FLC* expression was analyzed by quantitative real-time RT-PCR (qRT-PCR). Although *FLC* expression was induced in Col-0 plants after intermittent cold treatments, the inductive effects of intermittent cold disappeared in the *hos1-3* mutant (Figure 2E). Consistent with this observation, flowering initiation was not delayed in the *hos1-3* and *hos1-5* mutants treated with intermittent cold, as observed in the null allele of the *FLC* gene (Figure 2F; Seo et al., 2009; Lee et al., 2012). These observations indicate that the HOS1 binding to *FLC* chromatin induces *FLC* expression under short-term cold stress.

HOS1 Interacts with FVE in the Nucleus

We next determined how HOS1 regulates *FLC* expression. HOS1 is an E3 ubiquitin ligase that triggers the degradation of the ICE1 protein through the 26S proteasome pathway (Dong et al., 2006). Therefore, one possibility is that HOS1 targets the negative regulator(s) of *FLC* expression.

We first examined whether HOS1 interacts with the components of the autonomous flowering pathway that negatively regulate *FLC* expression (He, 2009; Jarillo et al., 2009). Yeast two-hybrid assays showed that HOS1 interacts with FVE (Figure 3A). By contrast, no obvious interactions were detected between HOS1 and other components. To determine the protein domains responsible for the HOS1-FVE interactions, we generated several deletions of the HOS1 and FVE proteins (Figure 3B), and the deletions were coexpressed in *Saccharomyces cerevisiae* AH109 cells. We found that the C-terminal region of the HOS1 protein, covering residues 457 to 927, is responsible for the interaction between HOS1 and ICE1 (Dong et al., 2006), and the C-terminal region of the FVE protein (residues 154 to 507), containing a series of WD40 domains, mediates the

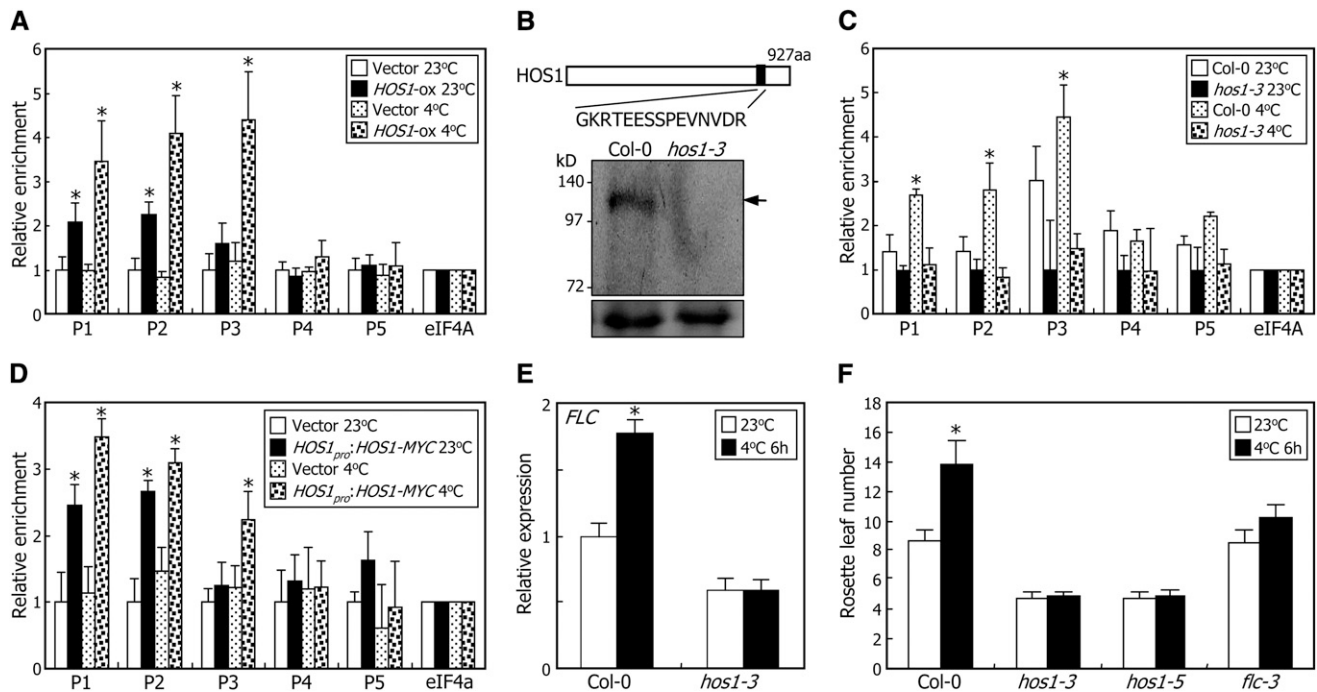


Figure 2. Chromatin Binding of HOS1 Induces *FLC* Expression under Cold Stress.

For the ChIP assays, biological triplicates were averaged for each measurement and statistically treated using a Student's *t* test (**P* < 0.01). Bars indicate *se*.

(A) ChIP assays of HOS1 binding to *FLC* chromatin using MYC-specific antibody. ChIP assays were conducted as described in Figure 1, using an anti-MYC antibody. The *HOS1-ox* transgenic plants grown on MS-agar plates at 23°C for 10 d were further grown at either 23°C or 4°C for 2 d before harvesting plant materials. The genomic DNA sequences examined in the ChIP assays were identical to those described in Figure 1C. Transgenic plants harboring the 6xMYC-pBA vector alone were used as control plants.

(B) Schematic illustration of anti-HOS1 antibody production. A peptide region covering residues 796 to 810 of HOS1 protein was selected for the epitope (black box). Nuclear proteins extracted from Col-0 plants and *hos1-3* mutants were used for examination of anti-HOS1 antibody specificity. HOS1 proteins were immunologically detected using the anti-HOS1 antibody. Arrow indicates endogenous HOS1 protein. Parts of Coomassie blue-stained gels are shown at the bottom as loading control. aa, amino acids.

(C) ChIP assays of HOS1 binding to *FLC* chromatin using HOS1-specific antibody. ChIP assays were conducted as described in Figure 1, using an anti-HOS1 antibody. Chromatin extracts from Col-0 and *hos1-3* plants were used for the ChIP assays. The conditions for the cold treatments were identical to those described in **(A)**.

(D) ChIP assays using *HOS1_{pro}::HOS1-MYC* transgenic plants. ChIP assays were performed using an anti-MYC antibody as described in Figure 1. The conditions for the cold treatments were identical to those described in **(A)**. Transgenic plants harboring the 6xMYC-pBA vector were used as control plants.

(E) Effects of intermittent cold on *FLC* expression. Plants treated with intermittent cold for 6 h at dawn for 15 d after germination were used for extraction of total RNA. Levels of *FLC* mRNA were determined by qRT-PCR. Biological triplicates were averaged for each plant genotype and statistically treated using a Student's *t* test (**P* < 0.01). Bars indicate *se*.

(F) Effects of intermittent cold on flowering time. Plants were treated with intermittent cold for 6 h at dawn, each day until flowering. Rosette leaves of 15 plants were counted for each plant genotype and statistically treated using a Student's *t* test (**P* < 0.01). Bars indicate *se*.

HOS1–FVE interaction (Figures 3C and 3D). The HOS1–FVE interaction was also verified by *in vitro* pull-down assays using a recombinant MBP–FVE fusion protein prepared in *Escherichia coli* cells and using *in vitro*-translated ³⁵S-labeled HOS1 polypeptides (Figure 3E).

To test whether HOS1 interacts with FVE *in vivo*, coimmunoprecipitation assays were performed using transgenic plants overexpressing a *FVE-MYC* gene fusion (*FVE-ox*), in which the MYC-coding sequence was fused in frame to the 3' end of the *FVE* gene under the control of the CaMV 35S promoter. Total protein extracts were immunoprecipitated using an anti-MYC

antibody, and the precipitated protein complexes were analyzed immunologically using an anti-MYC or anti-HOS1 antibody. Whereas a band having the expected size of the HOS1 protein was detected in the protein extracts of the *FVE-ox* transgenic plants, no such band was detected in the extracts of control plants (Figure 3F), indicating that HOS1 interacts with FVE *in vivo*.

Bimolecular fluorescence complementation (BiFC) assays were also employed to examine the HOS1–FVE interaction *in planta*. The *HOS1* gene sequence fused in frame to the 5' end of a gene sequence encoding the C-terminal half of yellow

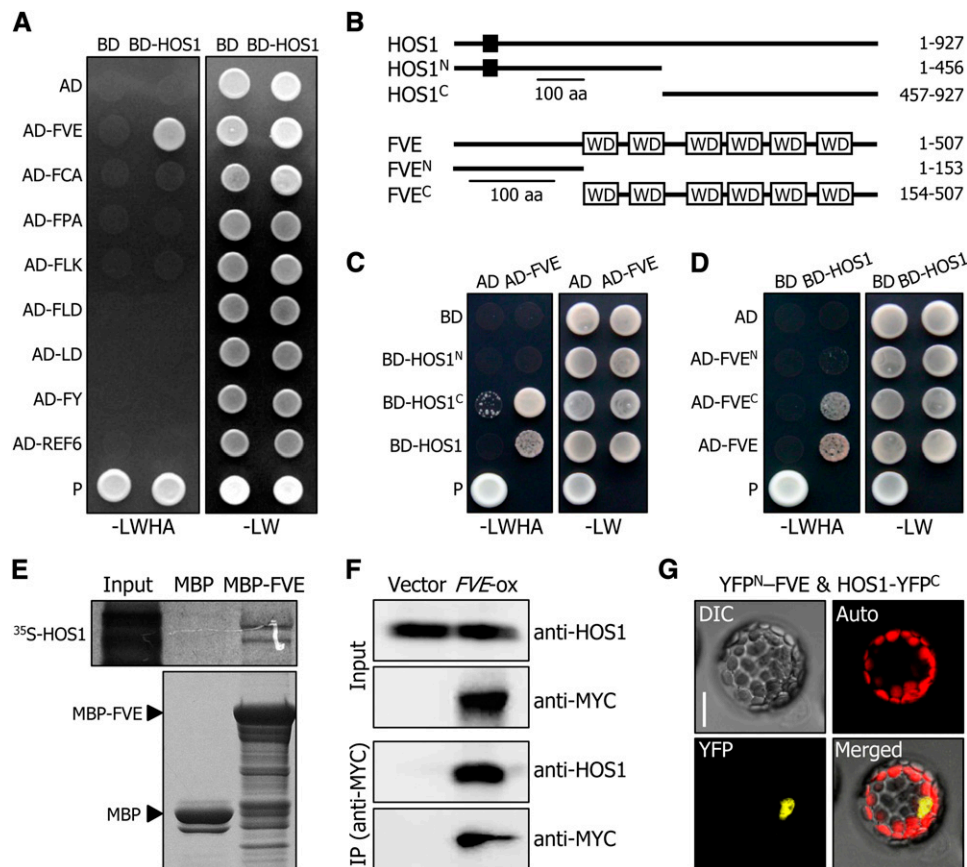


Figure 3. HOS1 Interacts with FVE in the Nucleus.

(A) Interactions of HOS1 with FVE in yeast cells. Autonomous flowering pathway genes and the *HOS1* gene were fused in frame to the GAL4 activation domain (AD)-coding and GAL4 DNA binding domain (BD)-coding sequences, respectively. The HOS1–FVE interactions were assayed by cell growth on selective medium. –LWHA indicates Leu, Trp, His, and Ade dropout plates. –LW indicates Leu and Trp dropout plates. P, positive control.

(B) Deletion constructs of HOS1 and FVE. Numbers indicate residue positions. Black box, REALLY INTERESTING NEW GENE finger domain. WD, WD40 domain. aa, amino acids.

(C) and **(D)** Interactions of HOS1 with FVE in yeast cells. The HOS1 **(C)** or FVE **(D)** constructs were fused with GAL4 BD or GAL4 AD, respectively. Yeast two-hybrid assays were performed as described in **(A)**.

(E) In vitro pull-down assays. Recombinant MBP-FVE fusion and in vitro–translated ^{35}S -labeled HOS1 polypeptides were used (top panel). Part of a Coomassie blue–stained gel is shown (bottom panel). The input represents 5% of the radiolabeled protein used in the assays.

(F) Coimmunoprecipitation assays. Total proteins were extracted from transgenic plants overexpressing a *FVE*-MYC gene fusion (*FVE*-ox) grown on MS-agar plates for 6 d. Protein complexes were immunoprecipitated using an anti-MYC antibody and detected immunologically using an anti-MYC or anti-HOS1 antibody. Transgenic plants harboring the 6xMYC-pBA vector alone were used as control plants.

(G) BiFC assays. The YFP^N-FVE and HOS1-YFP^C constructs were coexpressed transiently in *Arabidopsis* protoplasts and visualized by differential interference contrast microscopy and fluorescence microscopy. Bar = 20 μm .

fluorescent protein (HOS1-YFP^C) and the *FVE* gene sequence fused in frame to the 3' end of a gene sequence encoding the N-terminal half of YFP (YFP^N-FVE) were transiently coexpressed in *Arabidopsis* protoplasts. It was found that HOS1 interacts with FVE in the nucleus (Figure 3G), where chromatin modifications occur (He, 2009; Jarillo et al., 2009).

FVE interacts with DAMAGED DNA BINDING1 (DDB1) proteins, which are core subunits of the E3 ligase complex containing CULLIN4 (CUL4), via the Trp-Asp-X-Arg motif in the C-terminal region of FVE (Pazhouhandeh et al., 2011). We therefore examined whether the Trp-Asp-X-Arg motif is required for the HOS1–FVE interaction. The Arg (R) residue in the Trp-Asp-X-Arg

motif was mutated to a His (H) residue (R323H) (see Supplemental Figure 3A online), a mutation known to strongly reduce the FVE–DDB1 interaction (Pazhouhandeh et al., 2011). Yeast two-hybrid assays showed that the R323H mutation does not disrupt the HOS1–FVE interaction (see Supplemental Figure 3B online), indicating that the HOS1–FVE interaction is structurally different from the FVE–DDB1 interaction.

HOS1 Regulation of *FLC* Chromatin Is Mediated by FVE

FVE regulates *FLC* expression at the chromatin level (Ausin et al., 2004; Pazhouhandeh et al., 2011). We found that HOS1

interacts with FVE and regulates *FLC* expression by binding to *FLC* chromatin. To study how HOS1 is functionally linked with FVE in the chromatin regulation of *FLC* expression, we crossed the early-flowering *hos1-3* mutant with the late-flowering FVE-deficient *fve-4* mutant to produce an *fve hos1* double mutant and examined the flowering phenotypes of the double mutants. The *fve hos1* double mutant flowered later than the *hos1-3* mutant (see Supplemental Figures 4A and 4B online). Consistent with the flowering phenotypes, the levels of *FLC* mRNA were significantly higher in the double mutant than in the *hos1-3* mutant (see Supplemental Figure 4C online). In addition, ChIP assays of *FLC* chromatin revealed that the level of H3Ac in the double mutant was similar to that in the *fve-4* mutant but markedly higher than that in the *hos1-3* mutant (Figure 4A). These observations show that the HOS1-mediated transcriptional regulation of *FLC* expression is at least in part mediated by FVE.

We next examined whether HOS1 binding to *FLC* chromatin is influenced by FVE. ChIP-qPCR assays using an anti-HOS1 antibody revealed that HOS1 binding to *FLC* chromatin is reduced by ~50% in the *fve-4* mutant (Figure 4B), indicating that FVE is required for the efficient binding of HOS1 to *FLC* chromatin.

The level of *FLC* mRNA in the *fve-4* mutant was comparable to that in the *fve hos1* double mutant (see Supplemental Figure 4C online). However, the double mutant flowered earlier than the

fve-4 mutant (see Supplemental Figure 4B online), suggesting that HOS1 regulation of flowering time does not entirely depend on *FLC* gene expression (see Discussion).

FVE Is Not Ubiquitinated by HOS1

Under cold stress, nuclear-localized HOS1 proteins ubiquitinates the ICE1 transcription factor, which is then degraded through the proteasome-mediated pathway (Dong et al., 2006). We found that HOS1 interacts with FVE, suggesting that HOS1 may ubiquitinate FVE.

We first examined whether the stability of the FVE protein is altered by cold treatments. The FVE-ox transgenic plants were exposed to 4°C, and total protein extracts were prepared for protein gel blot analysis. It was found that the levels of FVE protein were uninfluenced by cold treatments (see Supplemental Figure 5A online). We next performed in vivo ubiquitination assays. The ICE1 protein was also included in the assays for comparison. Although a high molecular weight polypeptide band corresponding to the polyubiquitinated form of the ICE1-MYC fusion was detected in the cold-treated transgenic plants overexpressing the ICE1-MYC gene fusion, no polyubiquitinated forms of the FVE-MYC fusion was detected in the cold-treated FVE-ox transgenic plants (see Supplemental Figure 5B online). These observations indicate that FVE is not ubiquitinated by HOS1 under cold stress.

FVE binds to *FLC* chromatin (Jeon and Kim, 2011; Pazhouhandeh et al., 2011). We therefore performed ChIP assays to examine whether cold treatments influence the binding of FVE to *FLC* chromatin. Col-0 plants and FVE-ox transgenic plants were cold treated, and chromatin preparations were subjected to ChIP assays using the primer pairs employed in the ChIP assays to study the binding of HOS1 to *FLC* chromatin (Figure 1C). We found that the cold treatments did not affect the FVE binding to *FLC* chromatin (see Supplemental Figure 5C online), showing that the binding of FVE to *FLC* chromatin was not influenced by HOS1-mediated cold stress signals.

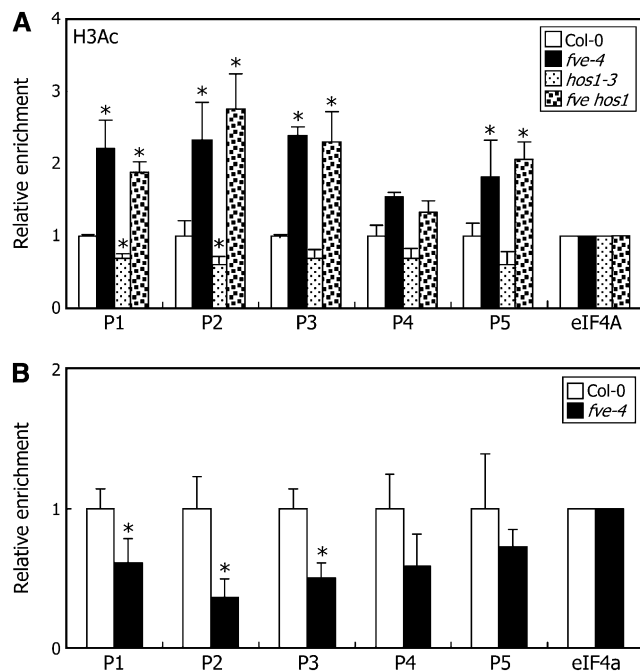


Figure 4. HOS1 Regulation of *FLC* Transcription Requires FVE.

Whole plant materials grown on MS-agar plates at 23°C under LDs for 10 d were used for ChIP assays. Biological quadruplicates were averaged for each measurement and statistically treated using a Student's t test (* $P < 0.01$). Bars indicate se.

(A) Relative levels of H3Ac in *FLC* chromatin. ChIP assays were conducted using an anti-H3Ac antibody.

(B) Binding of HOS1 to *FLC* chromatin in the *fve-4* mutant. ChIP assays were conducted using an anti-HOS1 antibody.

HOS1 Interacts with HDA6 in the Nucleus

FVE acts as an integral component of multiple chromatin-modifying complexes that mediate transcriptional repression (Ausín et al., 2004; Gu et al., 2011). FVE associates with HDA6 to form protein complexes that bind to *FLC* chromatin, leading to transcriptional silencing by histone deacetylation (Gu et al., 2011). Our data indicate that HOS1 binding to *FLC* chromatin is mediated by FVE. We therefore postulated that HOS1 interacts with HDA6 and other HDAC enzymes to modulate their association with *FLC* chromatin.

We first employed trichostatin A (TSA), a potent inhibitor of class I and II mammalian HDACs (Taunton et al., 1996; Richon et al., 2000), to examine whether HDAC activity is related to the HOS1 regulation of *FLC* expression. The levels of *FLC* mRNA were examined in plants grown for 10 d on one-half-strength Murashige and Skoog (MS)-agar plates containing 0.5 μ M TSA. Notably, the low level of *FLC* expression in the *hos1* mutants was recovered to a level comparable to that observed in Col-0 plants after TSA treatments (Figure 5A), indicating that the

HDACs are involved in the HOS1-mediated activation of *FLC* expression.

We next performed yeast two-hybrid assays to examine whether HOS1 interacts directly with HDAC proteins. Of the HDACs assayed, HDA6 and HDA15 interacted strongly with both FVE and HOS1 (see Supplemental Figures 6A and 6B online). The assays using truncated HOS1 forms revealed that the HOS1–HDAC interactions occurred via the C-terminal region of HOS1 (Figure 5B), like the HOS1–FVE interaction (Figures 3B and 3C). HDA5 and HDA9 also interacted moderately with FVE and HOS1 in yeast cells (see Supplemental Figures 6A and 6B online).

The interactions of HDA6 and HDA15 with FVE and HOS1 were also examined by in vitro pull-down assays using in vitro-translated, ^{35}S -labeled HDAC polypeptides and recombinant MBP-FVE or MBP-HOS1 fusion proteins. HDA6 and HDA15 interacted with both FVE and HOS1 (Figure 5C), consistent with the results obtained with yeast cells. We conducted BiFC assays using *Arabidopsis* protoplasts to examine whether the interactions occur in vivo. Strong reconstituted YFP signals were detected in the nuclei of cells coexpressing the YFP^N-FVE and

HDAC-YFP^C fusions as well as the YFP^N-HOS1 and HDAC-YFP^C fusions (Figure 5D), demonstrating that the interactions of HDA6 and HDA15 with FVE and HOS1 occur in the nucleus.

Coimmunoprecipitation assays were also employed to confirm the in vivo interactions of FVE and HOS1 with HDA6 using total proteins extracted from *FVE-ox* or *HOS1_{pro}:HOS1-MYC* transgenic plants. We found that HDA6 proteins were immunoprecipitated by both FVE- and HOS1-MYC proteins coupled with an anti-MYC antibody (Figures 6E and 6F). However, no HDA6 protein was immunoprecipitated from total proteins extracted from control plants expressing only MYC. These observations demonstrate that both FVE and HOS1 physically interact with HDA6 in vivo.

HOS1-Mediated Cold Stress Signals Inhibit HDA6 Binding to *FLC* Chromatin

We found that HOS1 interacts with HDA6, an interacting partner of FVE in the repression of the *FLC* gene (Gu et al., 2011). Therefore, a question was whether HOS1 ubiquitinates HDA6.

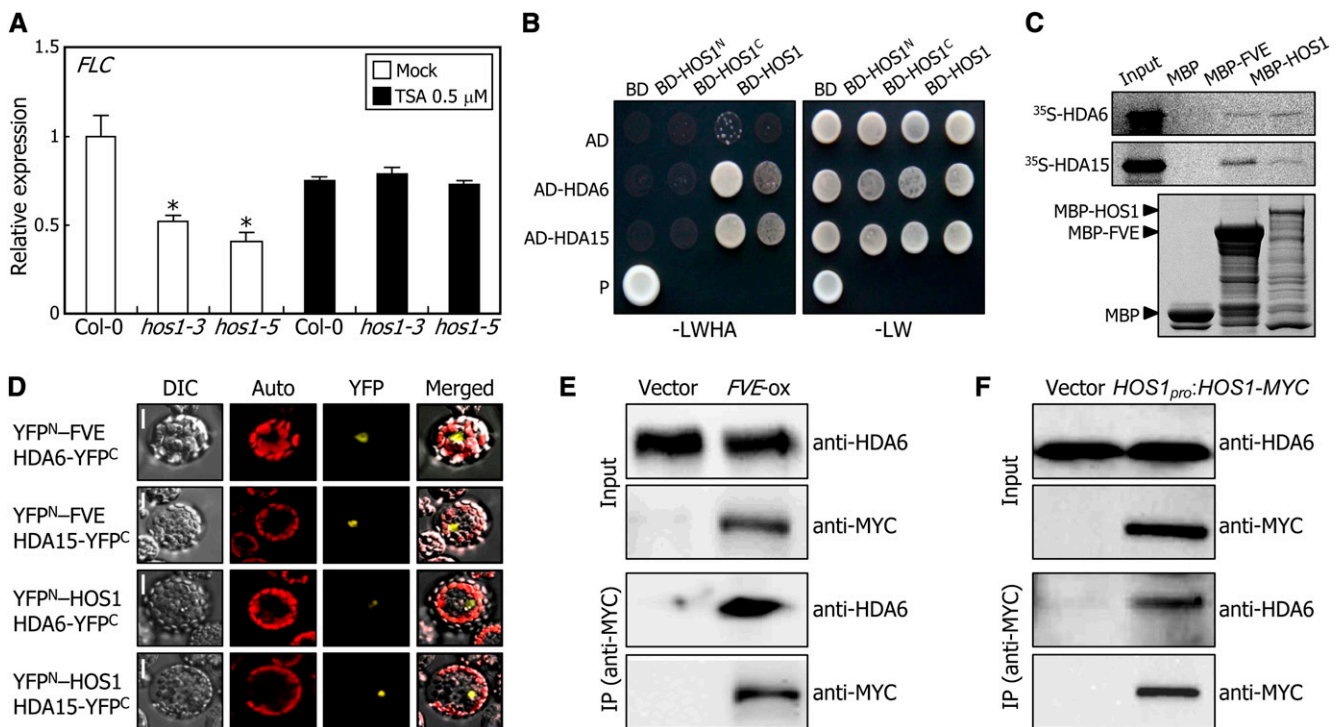


Figure 5. HOS1 Interacts with HDA6 in the Nucleus.

(A) Effects of TSA on *FLC* expression. Plants were grown for 10 d on MS-agar plates containing 0.5 μM TSA. The levels of *FLC* mRNA were determined by qRT-PCR. Biological triplicates were averaged for each plant genotype and statistically treated (Student's *t* test, **P* < 0.01). Bars indicate *se*.

(B) Interactions of HOS1 with HDA6 and HDA15 in yeast cells. The *HDA6* and *HDA15* genes were fused in frame to the GAL4 AD-coding sequence. The HOS1 constructs used were as described in Figure 3B.

(C) In vitro pull-down assays. Recombinant MBP-FVE and MBP-HOS1 fusions as well as in vitro-translated, ^{35}S -labeled HDA6 and HDA15 polypeptides were used (top and middle panels, respectively). Part of a Coomassie blue-stained gel is shown (bottom panel).

(D) BiFC assays. Partial YFP fusion constructs were coexpressed transiently in *Arabidopsis* protoplasts and visualized by differential interference contrast microscopy (DIC) and fluorescence microscopy. Bars = 20 μm .

(E) and **(F)** in vivo interaction of HDA6 with FVE **(E)** and HOS1 **(F)**. Total proteins extracted from the *FVE-ox* and *HOS1_{pro}:HOS1-MYC* transgenic plants grown for 6 d were immunoprecipitated with anti-MYC agarose beads. The FVE, HOS1, and HDA6 proteins were detected immunologically using an anti-MYC or anti-HDA6 antibody.

We examined whether HDA6 is degraded by the HOS1-mediated ubiquitination pathway using transgenic plants overexpressing *HDA6-MYC* gene fusions (*HDA6-ox*). The transgenic plants were exposed to 4°C, and the levels of HDA6-MYC fusion protein were analyzed immunologically using an anti-MYC antibody. The results showed that the levels of HDA6 protein were not influenced by cold treatments (Figure 6A). In addition, no visible ubiquitination of HDA6 was detected under identical conditions (Figure 6B), indicating that HOS1-mediated cold stress signals do not influence the stability of the HDA6 protein.

Both *FVE* and HDA6 bind to *FLC* chromatin (Jeon and Kim, 2011; Pazhouhandeh et al., 2011). We found that HOS1 also binds directly to *FLC* chromatin and interacts with *FVE* and

HDA6. Therefore, one possibility was that HOS1 interfered with the binding of HDA6 to *FLC* chromatin. ChIP assays using the *HDA6-ox* transgenic plants revealed that HDA6 binds to *FLC* chromatin in the P1, P2, and P3 sequence regions of *FLC* genomic DNA (Figure 6C), to which HOS1 also binds (Figures 2A, 2C, and 2D), suggesting that HOS1 may compete with HDA6 for binding to *FLC* chromatin.

To examine this possibility, we transformed the *HDA6-MYC* gene fusion into Col-0 plants and the *hos1-3* and *fve-4* mutants. We identified several *HDA6-ox* transgenic plants suitable for ChIP assays, in which the levels of *HDA6* mRNA and HDA6 protein in both the mutant backgrounds were similar to those in Col-0 background (Figure 6D, left and right panels, respectively).

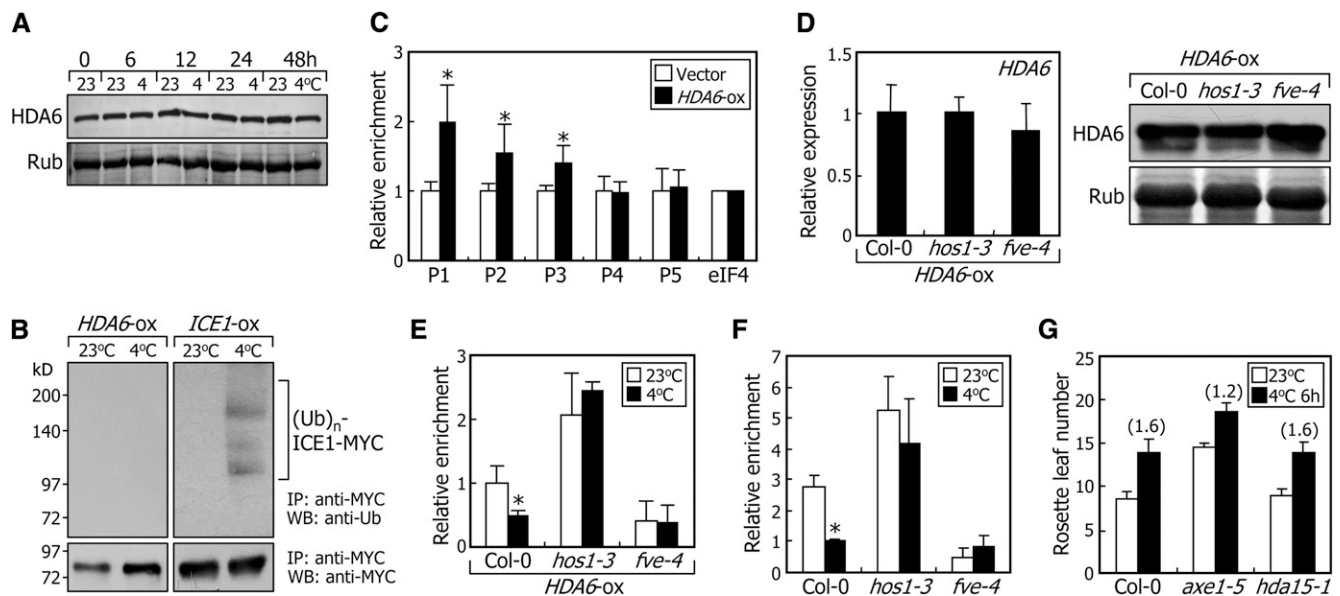


Figure 6. Cold Stress Attenuates Binding of HDA6 to *FLC* Chromatin.

(A) Effects of cold stress on HDA6 protein stability. Ten-day-old transgenic plants overexpressing a *HDA6-MYC* gene fusion under the control of the CaMV 35S promoter (*HDA6-ox*) were exposed to 4°C for the indicated time. Whole plants were harvested for preparation of protein extracts. The levels of HDA6 were compared immunologically using an anti-MYC antibody. Parts of Coomassie blue-stained gels containing the ribulose-1,5-bis-phosphate carboxylase/oxygenase protein are shown at the bottom.

(B) Effects of cold stress on HDA6 ubiquitination. Ten-day-old *HDA6-ox* transgenic plants were used in the ubiquitination assays. Total protein was immunoprecipitated with an anti-MYC antibody and analyzed immunologically using anti-MYC and anti-ubiquitin antibodies. Transgenic plants overexpressing the *ICE1-MYC* gene fusion (*ICE1-ox*) were also analyzed for comparison. IP, immunoprecipitation assay. WB, protein gel blot analysis.

(C) Binding of HDA6 to *FLC* chromatin. The *HDA6-ox* transgenic plants were grown on MS-agar plates for 2 weeks before harvesting of plant materials. ChIP assays were performed using an anti-MYC antibody, as described in Figure 1. Biological triplicates were averaged and statistically treated using Student's *t* test (**P* < 0.01). Bars indicate *se*.

(D) Relative levels of HDA6 protein and *HDA6* mRNA in *HDA6-ox* transgenic plants in different genetic backgrounds. The mRNA levels were examined by qRT-PCRs (left panel). Biological triplicates were averaged and statistically treated (*t* test, **P* < 0.01). Bars indicate *se*. The levels of HDA6 were determined immunologically using an anti-MYC antibody (right panel).

(E) Effects of cold stress on HDA6 binding to *FLC* chromatin. ChIP assays were performed as described in Figure 1, using an anti-MYC antibody. Chromatin extracts from plant materials described in **(D)** were used. Ten-day-old plants grown on MS-agar plates at 23°C were further grown at either 23 or 4°C for 2 d before harvesting of plant materials. The P1 sequence region of the *FLC* chromatin, as shown in Figure 1C, was used in the ChIP assays. Biological triplicates were averaged and statistically treated (*t* test, **P* < 0.01). Bars indicate *se*.

(F) Binding of HDA6 to *FLC* chromatin in the *hos1-3* and *fve-4* mutants. ChIP assays were performed as described in Figure 1, using an anti-HDA6 antibody. Chromatin extracts from Col-0 plants and *hos1-3* and *fve-4* mutants were used. Conditions for cold treatments were identical to those described in **(E)**. Biological triplicates were averaged and statistically treated (*t* test, **P* < 0.01). Bars indicate *se*.

(G) Effects of intermittent cold on flowering time. Intermittent cold treatments and measurements of flowering times were performed as described in Figure 2. The numbers in parentheses refer to the ratios of rosette leaf numbers with and without intermittent cold treatments.

We next conducted ChIP assays of the binding of HDA6 to *FLC* chromatin using the *HDA6-ox* transgenic plants in different genetic backgrounds. At normal temperatures, the HDA6 binding to *FLC* chromatin was lower in the *fve-4* background but higher in the *hos1-3* background compared with that in Col-0 background (Figure 6E). These results are consistent with the high expression of *FLC* and delayed flowering in the *fve-4* mutant as well as the low expression of *FLC* and accelerated flowering in the *hos1* mutant (see Supplemental Figure 4C online). Under cold stress, the association of HDA6 with *FLC* chromatin decreased by ~50% in Col-0 background (Figure 6E). This result is consistent with the elevated *FLC* expression and delayed flowering under cold stress (Figures 2E and 2F). However, the effects of cold stress on the HDA6 binding to *FLC* chromatin were largely dampened in both the *hos1-3* and *fve-4* mutant backgrounds. ChIP assays using a HDA6-specific antibody also showed that the HDA6 binding to *FLC* chromatin was not affected by cold treatments in the *hos1-3* and *fve-4* mutant backgrounds (To et al., 2011; Figure 6F).

ChIP assays of the binding of HDA6 to *FLC* chromatin were also performed using chromatin extracts prepared from *HOS1-ox* transgenic plants. The HDA6 binding to *FLC* chromatin was reduced by ~50% in the *HOS1-ox* transgenic plants (see Supplemental Figure 7 online). By contrast, the binding of HOS1 to *FLC* chromatin was unaffected in the *axe1-5* mutant (see Supplemental Figure 8 online). Altogether, these observations unequivocally demonstrate that cold stress signals influence the HDA6 binding to *FLC* chromatin via FVE and HOS1. It is also evident that HOS1 suppresses the HDA6 binding to *FLC* chromatin.

Our data suggested that HDA6 is involved in the regulation of flowering time via HOS1-mediated cold signaling. To examine this, the *axe1-5* mutant was treated with intermittent cold until flowering. Intermittent cold treatments delayed flowering by ~60% in Col-0 plants but only 20% in the *axe1-5* mutant (Figure 6G), showing that the effects of cold stress on the HDA6 binding to *FLC* chromatin is physiologically important in flowering time control. By contrast, the effects of intermittent cold on flowering initiation were similar in the *hda15-1* mutant and Col-0 plants, showing that the HDA15 function appears somewhat different from that of HDA6 in flowering time control under cold stress.

DISCUSSION

We report a HOS1-mediated molecular mechanism that links cold signaling with the onset of flowering in *Arabidopsis*. In this working scenario, HOS1, which is otherwise a well-known E3 ubiquitin ligase that attenuates cold responses (Dong et al., 2006), acts as a chromatin remodeling factor that regulates the dynamics of *FLC* chromatin under short-term cold stress (Figure 7). Under cold stress, HOS1 binds to *FLC* chromatin in an FVE-dependent manner to limit the chromatin accessibility of HDA6, resulting in activation of *FLC* chromatin and delayed flowering.

HOS1-Mediated Activation of *FLC* Chromatin under Short-Term Cold Stress

Our data depict that cold-activated HOS1 prevents HDA6 from binding to *FLC* chromatin to activate *FLC* expression, perhaps

by destabilizing the FVE-HDA6 complex (Figure 7). This scheme is in agreement with the late-flowering phenotype of the *fve-4* and *axe1-5* mutants as well as the early-flowering phenotype of the *hos1* mutants.

It is remarkable that FVE interacts with HOS1, an E3 ubiquitin ligase that mediates the cold-induced degradation of ICE1 (Chinnusamy et al., 2003; Dong et al., 2006), providing a distinct role of FVE in cold regulation of *FLC* chromatin. HOS1 interacts with HDA6 and binds to specific sequence regions within *FLC* chromatin to which HDA6 also binds. We therefore concluded that HOS1 inhibits the binding of HDA6 to *FLC* chromatin. We propose that the multiple interactions among HOS1, FVE, and HDA6 enzymes serve as a molecular web that integrates cold stress signals into the flowering genetic network at the *FLC* locus.

FVE is a component of a large multiprotein complex consisting of at least 16 protein subunits (Jeon and Kim, 2011). Our data showed that FVE interacts with at least four HDACs: HDA5, HDA6, HDA9, and HDA15. FVE is also associated with several H3K4 demethylases, such as FLD, LDL1, LDL2, and LDL3, which repress *FLC* expression (He et al., 2003; Jiang et al., 2007). Previous data and the data presented here support that FVE serves as a platform for the dynamic assembly of chromatin-modifying complexes involved in the epigenetic regulation of various loci, including the *FLC* gene.

Our data support that FVE acts as a platform for binding of HDA6 and HOS1 to *FLC* chromatin. Given the induction of *FLC* expression and delayed flowering in the *HDA6*-deficient mutant (Gu et al., 2011; Yu et al., 2011), which is in contrast with the effects of *hos1* mutation on *FLC* expression and flowering time, it is unlikely that HOS1 and HDA6 bind to *FLC* chromatin as parts of protein complexes. Instead, they would compete with each other for chromatin binding in response to temperature changes.

FVE interacts with the CUL4-DDB1 E3 ligase complex, which plays a role in photomorphogenesis and flowering time control (Pazhouhandeh et al., 2011). The CUL4-DDB1 complex, in association

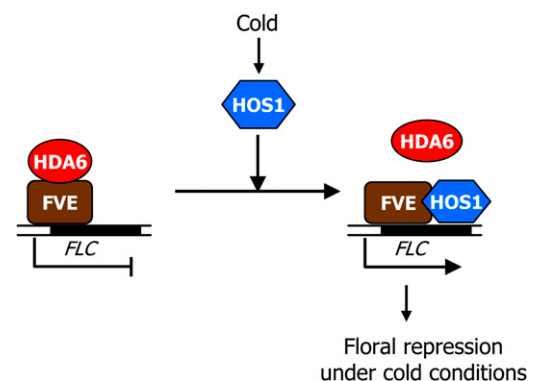


Figure 7. Model of HOS1 Function in *Arabidopsis* Flowering.

Under normal conditions, the FVE-HDA6 complex silences *FLC* chromatin. Under cold stress, HOS1 strongly binds to *FLC* chromatin and interacts with FVE and HDA6 to dissociate HDA6 from *FLC* chromatin, resulting in the activation of *FLC* chromatin and delayed flowering. [See online article for color version of this figure.]

with a CURLY LEAF–containing PRC2-like complex, epigenetically represses the *FLC* and *FT* genes. However, the CUL4-DDB1 enzyme complex does not seem to be related to the HOS1–FVE interaction. First, mutations of the *CUL4* gene induce *FLC* expression (Pazhouhandeh et al., 2011); by contrast, *FLC* expression is repressed in the *hos1* mutants. Second, both CUL4 and HOS1 require FVE for regulating *FLC* expression (Pazhouhandeh et al., 2011; see Supplemental Figure 4 online). However, though the binding of FVE to *FLC* chromatin requires CUL4, binding is not influenced by HOS1-mediated cold stress signals (see Supplemental Figure 5C online). Finally, the Trp-Asp-X-Arg motif of FVE, which is required for the interaction of FVE with DDB1 proteins (Pazhouhandeh et al., 2011), is not essential for the HOS1–FVE interaction (see Supplemental Figure 3B online). It is therefore likely that HOS1 acts independently of the CUL4-DDB1 complex in the transcriptional silencing of the *FLC* gene. Therefore, it is proposed that FVE incorporates two different environmental cues into the flowering genetic networks at the chromatin level: light signals via the CUL4-DDB1 complex and cold stress signals via HOS1.

Our work provides a molecular mechanism by which HOS1, together with FVE and HDA6, regulates *FLC* transcription in response to cold stress. Besides *FLC*, *FLC* gene homologs and several transposable elements, which are silenced by RNA-directed DNA methylation, are targeted by the FVE-HDA6 complex (Gu et al., 2011; Pazhouhandeh et al., 2011; To et al., 2011). Therefore, it is possible that the HOS1-FVE-HDA6 regulatory circuit may also play a role in maintaining the silencing of *FLC* gene homologs and transposable elements. Genome-wide transcript profiling and epigenome analysis of the loss-of-function *hos1* mutant will be greatly helpful for understanding how HOS1 modulates the transcriptional regulation of *FLC* gene in *Arabidopsis*.

HOS1 as a Chromatin Remodeling Factor in Flowering Time Control

HOS1 activation of *FLC* expression is important for suppression of flowering initiation under short-term cold stress. However, the early flowering phenotype of the *hos1* mutants at normal temperatures is not likely to be related to the activities of *FLC*. Although the level of *FLC* mRNA in the *fve-4* mutant was comparable to that in the *fve hos1* double mutant (see Supplemental Figure 4C online), the double mutant flowered earlier than the *fve-4* mutant (see Supplemental Figure 4B online). In addition, mutations of *FLC* and its gene homologs in Col-0 background have minimal effect on flowering at normal temperatures (Ratcliffe et al., 2001, 2003; Scortecci et al., 2001; Figure 2F). It is therefore envisioned that the HOS1-mediated regulation of flowering time is exerted via two distinct mechanisms: modulation of *FLC* chromatin and regulation of yet undefined flowering time gene(s) distinct from *FLC*.

The floral integrator *FT* is a potential target of HOS1-mediated chromatin regulation. The CUL4-DDB1 E3 ligase complex, which interacts with FVE (Pazhouhandeh et al., 2011), is necessary for maintaining the repression of *FT* and *FLC* chromatin (Pazhouhandeh et al., 2011). The CUL4-DDB1 E3 ligase complex also negatively regulates the abundance of CONSTANS (CO) (Chen et al., 2010), an upstream activator of *FT* expression

(Onouchi et al., 2000; Samach et al., 2000). It has recently been reported that HOS1 interacts with CO to regulate CO abundance in photoperiodic flowering (Jung et al., 2012a; Lazaro et al., 2012), supporting the notion that HOS1 influence the protein stability of CO. Alternatively, HOS1 may regulate the status of CO and *FT* chromatin through interactions with FVE and HDACs.

We did not find any evidence for ubiquitination and degradation of HOS1-interacting partners under both normal and cold temperature conditions. However, it is still possible that the E3 ligase activity of HOS1 is involved in cold regulation of *FLC* expression. Most studies of the epigenetic systems mediated by E3 ubiquitin ligases in *Arabidopsis* have been focused on the monoubiquitination of H2A or H2B histone derivatives. H2A monoubiquitination is mediated by PRC1-like E3 ubiquitin ligase complexes (Bratzel et al., 2010). H2B monoubiquitination is regulated by two E3 ligases, HISTONE MONOUBIQUITINATION1 (HUB1) and HUB2 (Cao et al., 2008; Gu et al., 2009). In addition, the CUL4-DDB1 E3 ligase complex facilitates cellular responses to DNA damage by modulating the monoubiquitination of H2A, H3, and H4 (Kapetanaki et al., 2006; Wang et al., 2006). Future investigations include determining whether HOS1 plays a role in the monoubiquitination events of histone proteins and how these biochemical events affect the binding of HDA6 to *FLC* chromatin.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana lines used were in Col-0 background. The *hos1-3* (SALK-069312), *hos1-5* (SAIL-1211-D02), *axe1-5* (Murfett et al., 2001), and *hda15-1* (SALK-004027) mutants were isolated from a pool of T-DNA insertion lines deposited in the ABRC (Ohio State University). The *fve-4* and *flc-3* mutants have been described previously (Kim et al., 2004; Seo et al., 2009). To produce transgenic plants overexpressing the *HOS1*, *FVE*, *HDA6*, *HDA15*, and *ICE1* genes, six copies of the MYC-coding sequence were fused in frame to the 3' ends of the genes and driven by the CaMV 35S promoter using the myc-pBA vector (Seo et al., 2010), resulting in *HOS1-ox*, *FVE-ox*, *HDA6-ox*, *HDA15-ox*, and *ICE1-ox* plants, respectively. To generate *HOS1_{pro}:HOS1-MYC* transgenic plants, a *HOS1* genomic fragment, which contains a 2.8-kb promoter sequence upstream of the transcription start site (Lee et al., 2001), was subcloned into the modified myc-pBA vector, in which the CaMV 35S promoter was deleted. The PCR primers used for subcloning are listed in Supplemental Table 1 online. *Agrobacterium tumefaciens*-mediated *Arabidopsis* transformation was performed according to a modified floral dip method (Clough and Bent, 1998). Plants were grown in soil or on MS-agar plates in a controlled culture room at 23°C under LDs (16-h-light and 8-h-dark cycles).

Flowering Time Measurement and Intermittent Cold Treatment

Plants were grown in soil at either 23 or 4°C under LDs until flowering. The flowering times were determined by counting the number of rosette and cauline leaves at bolting. Fifteen to 20 plants were counted and averaged for each measurement. For intermittent cold treatments, plants were placed at 4°C for 6 h at dawn until flowering.

Analysis of Gene Transcript Levels

Gene transcript levels were examined by either RT-PCR or qRT-PCR. RT-PCR reactions were performed using the Applied Biosystems 2720 Thermal

Cycler to verify absence of *HOS1* gene expression in the *hos1-3* and *hos1-5* mutants. qRT-PCR reactions were performed in 96-well blocks with the Applied Biosystems 7500 real-time PCR system using the SYBR Green I master mix in a reaction volume of 20 μ L. All qRT-PCR assays were performed in biological triplicates using total RNA samples extracted from three independent plant materials treated under identical conditions and gene-specific primer pairs listed in Supplemental Table 1 online. RNA preparation, data processing, and determination of reaction specificities were performed as described previously (Jung et al., 2012b).

Generation of the Anti-HOS1 Antibody

The HOS1-specific antibody was generated against a synthetic peptide covering residues 796 to 810 (GKRTTEESSPEVNVDR) of the *Arabidopsis* HOS1 protein. The synthetic peptide was HPLC purified, conjugated to keyhole limpet hemocyanine, and injected into rabbits over an 8-week period (one primary injection and three boosting injections), as described previously (To et al., 2011). Two rabbits were used for immunization (AbClon). The antiserum obtained was affinity purified to remove non-specific antibodies, increasing sensitivity and reducing background, and used for ChIP assays.

Protein Gel Blot Analysis

The *FVE*-, *HDA6*-, and *HDA15-MYC* gene fusions were overexpressed under the control of the CaMV 35S promoter in Col-0 plants. The resultant *FVE-ox*, *HDA6-ox*, and *HDA15-ox* transgenic plants were grown on MS-agar for 10 d and exposed to cold (4°C) for appropriate time periods. Whole plants were harvested for preparation of total protein extracts. Total proteins were separated by SDS-PAGE and transferred to a Millipore Immobilon-P membrane, and immunological analysis was performed to detect the fusion proteins using an anti-MYC antibody at 1:2000 dilution (Millipore).

Ubiquitination Assays

In vivo detection of ubiquitinated proteins was performed as described previously (Dong et al., 2006; Miura et al., 2011). Ten-day-old transgenic plants overexpressing the *FVE*-, *HDA6*-, *HDA15*-, and *ICE1-MYC* gene fusions were pretreated with 50 μ M MG132 (Sigma-Aldrich), a potent 26S proteasome-specific inhibitor (Lee and Goldberg, 1998), for 24 h and then exposed to cold (4°C) for 15 h. The total protein extract was immunoprecipitated using an anti-MYC antibody coupled to agarose beads and analyzed immunologically using anti-MYC and antiubiquitin antibodies.

ChIP Assays

ChIP assays were performed using 10- or 14-d-old plants grown on MS-agar plates under LDs. Processing of plant materials and qPCR were performed as described previously (Yang et al., 2011). The PCR primers for qPCR were designed to amplify DNA fragments of 100 to 200 bp and listed in Supplemental Table 1 online. An *eIF4A* DNA fragment was used for normalization. To validate the ChIP-qPCR runs in the P1 to P5 sequence regions of *FLC* gene, we sequenced the ChIP-qPCR products (see Supplemental Data Set 1 online) using an Applied Biosystems 3730xl DNA analyzer at the National Instrumentation Center for Environmental Management (Seoul National University).

Protein-Protein Interaction Assays

Protein-protein interactions were examined by yeast two-hybrid assays using the BD Matchmaker system (Clontech) and in vitro pull-down assays, as described previously (Seo et al., 2012). To evaluate whether the

protein-protein interactions occur in vivo, BiFC assays were performed using *Arabidopsis* mesophyll protoplasts, as described previously (Hong et al., 2011). Coimmunoprecipitation assays were also employed for the same purpose, as described previously (Song et al., 2012).

TSA Treatments

Plants were grown for 10 d on MS-agar plates containing 0.5 μ M TSA, a potent inhibitor of class I and II mammalian HDACs (Taunton et al., 1996; Richon et al., 2000). Whole plants were used for extraction of total RNA. The levels of *FLC* mRNA were examined by qRT-PCR.

Statistical Analysis

The statistical significance between two means of measurements was determined using a Student's *t* test with P values < 0.01.

Accession Numbers

Sequence data from this article can be obtained from the Arabidopsis Genome Initiative databases under the following accession numbers: *eIF4A* (At3g13920), *FLC* (At5g10140), *FVE* (At2g19520), *HDA6* (At5g63110), *HDA15* (At3g18520), *HOS1* (At2g39810), and *ICE1* (At3g26744).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. *HOS1* Is Correlated with *FLC* Expression.

Supplemental Figure 2. Examination of Anti-HOS1 Antibody Specificity by Protein Gel Blot Assays.

Supplemental Figure 3. The Trp-Asp-X-Arg Motif Is Not Required for HOS1-FVE Interactions.

Supplemental Figure 4. *FLC* Expression in *five hos1* Double Mutant.

Supplemental Figure 5. Cold Stress Does Not Influence FVE Protein Stability.

Supplemental Figure 6. Interactions of FVE and HOS1 with Histone Deacetylases in Yeast Cells.

Supplemental Figure 7. Binding of HDA6 to *FLC* Chromatin in the *HOS1-ox* Transgenic Plant.

Supplemental Figure 8. HOS1 Binding to *FLC* Chromatin in the *axe1-5* Mutant.

Supplemental Table 1. Primers Used in qRT-PCR, ChIP-qPCR, and Gene Cloning.

Supplemental Data Set 1. Sequencing Analysis of ChIP-qPCR Products.

Supplemental References 1. References for Supplemental Figures.

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AUTHOR CONTRIBUTIONS

C.-M.P. designed the experiments. J.-H.J., J.-H.P., and S.L. performed the experiments. C.-M.P. and J.-H.J. analyzed the data. T.K.T., J.-M.K., and M.S. generated and evaluated the anti-HDA6 antibody. C.-M.P. and J.-H.J. wrote the article.

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