이학석사학위논문

애기장대에서 FRI 단백질 복합체에 의한 개화 및 꽃기관 형성 조절에 관한 연구

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이 논문을 이학석사 석사학위논문으로 제출함

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ABSTRACT

A study on the FRI complex–mediated regulation of flowering and floral morphogenesis in *Arabidopsis thaliana*

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Flowering of winter annual *Arabidopsis* is prevented before winter, but the prolonged winter cold, called vernalization, accelerates flowering of the plants in response to environmental cues next spring. The key regulator of such flowering behavior is *FLOWERING LOCUS C* (*FLC*), which represses flowering but is suppressed by vernalization. The expression of *FLC* is transcriptionally activated by FRIGIDA–complex (FRI–C) which includes FRIGIDA LIKE1 (FRL1), FRIGIDA ESSENTIAL1 (FES1), FLC EXPRESSOR (FLX) and SUPPRESSOR OF FRIGIDA4 (SUF4). However, it is not known how vernalization affects the activity of FRI–C when FRI–C is not needed any longer. Here we show that the components of FRI–C are affected at neither transcriptional level nor translational level by vernalization. Instead, we show that the FRI–C structure is changed and *FLC* gene loop is disrupted by

vernalization. Before vernalization, FRI-C forms a large protein complex, ca 1 MDa size but after vernalization, a subcomplex containing SUF4, ca 200kDa size, is dissociated from the large complex. Additionally, we show that *fri* and *suf4* mutant fails to produce *FLC* gene loop. Taken together, we propose that FRI and SUF4 are required for *FLC* looping and the disruption of the *FLC* gene loop caused by SUF4 subcomplex dissociation is a molecular basis of inability to activate *FLC* after vernalization.

Functions of FRI-C for *FLC* regulation is clearly elucidated, such that FRI-C activates FLC transcription with the general transcription factors and FRI-associated proteins. Among the components of FRI-C, SUF4 binds the promoter of *FLC*, FES1 and FLX provide the activity of transcriptional activation, and FRI acts as a scaffold protein to recruit not only all the components of FRI-C but also basal transcription factors and chromatin remodeling factors such as SWR1 complex. However, if the components of FRI-C have other function has not been addressed. In this study, we show that SUF4 and FES1, the two components of FRI-C, have functions in the development of flower, especially developing the right number of floral organs. When the tissue specificity of FRI-C were analyzed, we found that the components of FRI-C is highly expressed in flower tissues. Furthermore, flowers of *suf4 fes1* double mutants produce extra floral organs, demonstrating that *SUF4* and *FES1* regulate floral organ number. Consistent with this, the expressions of genes regulating floral organ numbers such as *CLAVATA (CLV), PERIANTHIA (PAN), ULTRAPETALA1 (ULT1)* and *PETAL LOSS (PTL)* were altered in *suf4 fes1* double mutants. Chromatin immunoprecipitation analysis shows that SUF4 is enriched at the

promoter region of *CLV1, CLV3, PAN, ULT1* and *PTL*, suggesting that SUF4 directly regulates these genes. Taken together, we propose that the components of FRI-C regulates the number of floral organs by regulating the genes determining floral organ numbers.

In this study, we disclose that FRI-C regulates *FLC* expression by controlling gene loop. We also show that the components of FRI-C regulate floral organ numbers. From these results, we suggest that the FRI-C is a multi-functional protein regulating various developmental processes.

Keywords : flowering, vernalization, gene loop, floral organ patterning

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ABBREVIATIONS

3'	Three prime end of DNA fragment
5'	Five prime end of DNA fragment
AG	*AGAMOUS*
AP1, 2	*APETALA1, 2*
ARP6	*ACTIN RELATED PROTEIN 6*
CLF	*CURLY LEAF*
CLV1, 3	*CLAVATA 1, 3*
Col	Columbia
EFS	*EARLY FLOWERING IN SHORT DAYS*
ERA1	*ENHANCED RESPONSE TO ABA 1*
ETT	*ETTIN*
FES1	*FRIGIDA ESSENTIAL1*
FLC	*FLOWERING LOCUS C*
FLX	*SUPPRESSOR OF FRIGIDA 4*
FRI	*FRIGIDA*
FRL1	*FRIGIDA LIKE 1*
FT	*FLOWERING LOCUS T*
H3K9me2	*histone H3 lysine 9 di-methylation*
LD	*LUMINIDEPENDENCE*
LFY	*LEAFY*
LHP1	*LIKE-HETEROCHROMATIN PROTEIN 1*

MAF1, 2	*MADS AFFECTING FLOWERING 1, 2*
MSI1	*MULTICOPY SUPRESSOR OF IRA 1*
PAN	*PERIANTHIA*
PTL	*PETAL LOSS*
RNA Pol II	RNA polymerase II
SAM	Shoot apical meristem
SEP	*SEPALLATA*
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
SUF4	*SUPPRESSOR OF FRIGIDA4*
SUP	*SUPERMAN*
SWN	*SWINGER*
SWR1-C	SWR1 complex
TAF14	TBP-associated factor 14
UFO	*UNUSUAL FLORAL ORGANS*
ULT1	*ULTRAPETALA*
UTR	Untranslated region
VIN3	*VERNALIZATION INSENSITIVE3*
VRN2	*VERNALIZATION2*
WUS	*WUSCHEL*

Chapter I. FRI complex regulates *FLC* expression by controlling gene loop.

1.1 INTRODUCTION

1.1.1 Vernalization mediated flowering pathway.

Since flowering at right season is critical for the reproductive success, plants have evolved complex genetic networks to control flowering time in response to environmental cues and endogenous signals (Michaels and Amasino, 2000; Amasino, 2004; Schmitz and Amasino, 2007; Amasino, 2010).

In terms of flowering characteristics, Arabidopsis ecotypes are classified into summer annuals and winter annuals (Sung and Amasino, 2004a; Wollenberg and Amasino, 2012). Summer annuals flower relatively early whereas winter annuals delay flowering until next spring. The prolonged winter cold makes the winter annuals competent to flower in response to spring cues, which is called vernalization (Minorsky, 2002; Sung and Amasino, 2004a).

Through genetic analyses of natural variation in Arabidopsis, two major genes, *FRIGIDA (FRI)* and *FLOWERING LOCUS C (FLC)* have been identified to control the flowering characteristics of winter annuals (Napp-Zinn, 1979; Clarke and Dean, 1994; Lee et al., 1994; Gazzani et al., 2003a). *FRI* encoding a protein with coiled-coil domain is required for the strong activation of *FLC*

transcription (Choi et al., 2011a). *FLC* encoding a MADS box transcription factor is a strong flowering repressor and causes late flowering by directly repressing the expression of the two flowering pathway integrators, *FT* and *SUPPRESSOR OF CONSTANS1* (*SOC1*) (Rouse et al., 2002; Chiang et al., 2009; Kimura et al., 2015). Thus, Summer annuals such as Col and L *er* have a mutation in either *FRI* and/or *FLC*, thus show early flowering phenotype. (Sheldon et al., 1999).

1.1.2 The mechanisms of transcriptional regulation of *FLC* before vernalization.

The expression of *FLC* is regulated by diverse mechanisms and genetic flowering pathways (Michaels and Amasino, 2000; Lempe et al., 2005; Schmitz and Amasino, 2007). For example, *FRI* causes strong activation of *FLC* whereas vernalization suppresses the expression in winter annuals (Choi et al., 2011b). In contrast, the so called autonomous flowering pathway represses *FLC* in summer annuals which containing *fri* mutation. Henceforth, the mutation in the autonomous pathway causes increased expression of *FLC* and late flowering, which can also be suppressed by vernalization (Soppe et al., 1999; Michaels and Amasino, 2000, 2001; Rouse et al., 2002). Thus, vernalization overrides activating function of FRI and FRI overrides repressing function of autonomous pathway. The genes involved in the autonomous pathway for flowering are mostly chromatin modifiers or RNA processing factors (Kim et al., 2009).

For strong activation of *FLC*, several factors regulating transcription and histone modification or chroma tin remodeling are required (Sung and Amasino,

2004a, b; Schmitz et al., 2008; Tamada et al., 2009; Choi et al., 2011b). For example, the mutations of the components of PAF1 (RNA polymerase associated factor 1) complex such as *vernalization independence 3* (*vip3*), *vip4, vip5* cause reduced expression of *FLC* and early flowering, indicating that histone H3 methylation of *FLC* chromatin mediated by the PAF1 complex is required for the full activation (Krogan et al., 2003; Kim et al., 2009). In addition, Arabidopsis trithorax group, *ATX1*, *ATX2*, *ATXR3* and *ATXR7*, the histone methyltransferases which are required for H3K4 methylation, are also required for the strong activation of *FLC* in winter annuals (Jiang et al., 2009; Tamada et al., 2009; Yun et al., 2012). Moreover, *EARLY FLOWERING IN SHORT DAYS* (*EFS*), involved in the methylations both at H3K4 and H3K36, is required for the *FLC* activation (Kim et al., 2005; Ko et al., 2010). A chromatin remodeling complex, SWR1 complex, which has a role for the histone replacement from H2A to H2AZ, is also necessary for full activation of *FLC* (Choi et al., 2007; Deal et al., 2007). Additionally, RNA-processing factors, HUA2, SERRATE and mRNA cap-binding protein, CBP80/ABH1, also have functions in the *FLC* activation (Bezerra et al., 2004; Doyle et al., 2005). Such reports suggest that the transcription of *FLC* is regulated by myriads of transcriptional regulators.

1.1.3 *FLC* is regulated by vernalization pathway through epigenetic regulation.

Vernalization progressively downregulates *FLC* and eventually silence the *FLC* chromatin in a stepwise manner (Song et al., 2013). Initially prolonged cold increases the transcript level of a noncoding RNA, *COOLAIR*, which induces

reduction of *FLC* expression (Swiezewski et al., 2009). Then, the expressions of *VERNALIZATION INSENSITIVE3* (*VIN3*), encoding a plant homeodomain (PHD) protein, and another noncoding RNA, COLDAIR, are induced (Sung and Amasino, 2004; Heo and Sung, 2010). COLDAIR interacts with Polycomb Repressive Complex 2 (PRCs) and mediates the trimethylation of H3K27 position in the *FLC* chromatin. VIN3 acts as a component of PHD –PRC2 and is necessary for the establishment of *FLC* silencing (De Lucia et al., 2008). Finally, the LIKE HETEROCHROMATIN PROTEIN1, a homolog of metazoan HETEROCHROMATIN PROTEIN1, binds to H3K27me3 and maintaining the silencing of *FLC* (Sung et al., 2006).

Recently, another factor involved in the repression of *FLC* was found. *FLC* has a gene loop with interaction between 5' and 3' region before vernalization. This loop is disrupted in the early phase of vernalizat ion, suggesting that it is related to vernalization–mediated *FLC* suppression (Crevillen et al., 2013). Loop disruption may expose the promoter of COOLAIR, thus causing transcriptional activation of COOLAIR, which contribute to reduction of *FLC* expression. This disruption of *FLC* loop is mediated by BAF60, a subunit of SWI/SNF chromatin remodeling complex (Jegu et al., 2014). *BAF60* knock down lines show late flowering behavior, suggesting that the disruption of *FLC* loop is required for *FLC* regulation.

1.1.4 Vernalization causes changes of FRI complex.

While such complicated processes of epigenetic silencing during vernalization proceed, it is largely unknown what happens for the transcriptional activator, FRI-C. FRI-C is composed of at least five components, FRI, FRL1, FES1, FLX and SUF4 (Choi et al., 2011). Among the components, SUF4 binds the promoter of *FLC*, FES1 and FLX provide the activity of transcriptional activation, and FRI acts as a scaffold protein to recrui t not only all the components of FRI-C but also basal transcription factors and chromatin remodeling factors such as SWR1 complex. Recently, it has been reported that FRI protein is degraded during vernalization, thus modulates flowering time (Hu et al., 2 014). However, we have observed that the level of FRI protein as well as SUF4 protein is not decreased during vernalization. In addition, even though FRI or SUF4 is not reduced, the plants still show perfect vernalization response, meaning accelerated flow ering by vernalization. Here, we report that the structure of FRI-C is changed by vernalization, thus SUF4 subcomplex is dissociated from the large complex of FRI-C. Consistent with this, we show that *fri* and *suf4* mutant fails to produce *FLC* gene loop between the promoter and 3 ' -UTR which is disrupted by vernalization. We propose that FRI and SUF4 are required for *FLC* looping and that the dissociation of SUF4 subcomplex causes the disruption of *FLC* gene loop and is a molecular basis of transc riptional inactivation of *FLC* during vernalization.

1.2 MATERIALS AND METHODS

1.2.1 Plant materials and growth condition.

The wild type used in this study was Col: *FRI*^{SF2} (*FRI*-Col) strain that *FRI* from San Feliu is introgressed into Col-0. All the flowering time mutants used have been described previously (Michaels et al., 2004; Choi et al., 2005; Stinchcombe et al., 2005; Kim et al., 2006). Seeds were stratified on 0.65% phytoagar containing half-strength Murashige and Skoog salts for 3 days at 4 ℃ for homogeneous germination. For vernalization treatment, the seedlings were incubated for 40 days at 4℃ under short day conditions. Afterwards, plants were grown in long day conditions (16h light / 8h dark) under white fluorescent lights at 22℃. Flowering time was measured by counting the number of rosette leaves from at least 20 plants.

1.2.2 Development of Transgenic lines

For producind *pFRI:myc:FRI fri* and *pSUF4:SUF4:FLAG* transgenic lines, endogenous promoters and epitope tagged genomic DNA of *FRI* and *SUF4* were cloned into modified pPZP211 vector. The *myc-FRI* construct was transformed into Col-0 and *SUF4-FLAG* construct was transformed into *FRI*-Col. Transgenic lines were selected by proper antibiotics and were confirmed by PCR. *SUF4 : FLAG vin3-4* and *SUF4 : FLAG suf3* were generated by genetic crosses, and stable homozygous F3 transgenic plants were selected and used for gel filtration analyses.

1.2.3 RNA extraction and RT-PCR

RNA extraction was performed as described previously (Choi et al., 2005). Total RNA was extracted from whole seedlings and cDNA was used for amplification of specific genes. *Tubulin* was used as an internal gene expression control. The gene specific primers used to detect the transcripts are listed in I-table 1.

1.2.4 Immunoblotting assay

Total proteins were extracted from *myc - FRI* and *SUF4 - FLAG* transgenic lines using gel filtration buffer (20mM Tris-HCl pH7.4, 200mM NaCl, 10% glycerol), followed by filtering using 0.2um syringe filter. Protein was separated by electrophoresis on a 9% SDS-polyacrylamaide gel and then transferred to Polyvinylidene difluoride (PVDF) membrane. Primary myc antibody (1:2000; Cell signaling) or FLAG antibody (1: 10000, Sigma) was used and anti-rabbit or anti-mouse secondary antibody (1:5000; Cell signaling) was used for detection. Signals were detected by LAS 4000.

1.2.5 Gel filtration assay

1.5mili-grams of total protein extracts from *myc - FRI* and *SUF4 - FLAG* transgenic plants grown for 11 days was injected in Superdex 200 10/300 GL column (Amersham Biosciences). After fractionation by the AKTA fast protein liquid chromatography system (FPLC), 16 tubes containing 0.5ml protein were

collected. Proteins in each fraction were precipitated by acetone, immnuo blotted by methods mentioned above.

1.2.6 Co-IP analysis

For Co-IP, we generated double transgenic lines of *myc - FRI SUF4 - FLAG* obtained from the genetic crosses between *myc - FRI* and *SUF4 - FLAG*. Total proteins were extracted using gel filtration buffer added 100mM PMSF and immunoprecipitated overnight using FLAG antibody (5ul/ml, Sigma), followed by incubation with protein A sepharose for 4 hours at 4 ℃ under gentle rotation. Beads were washed 5 times with gel filtration buffer, and the protein was eluted at 95 ℃ for 10min in 2X sample buffer (120mM Tris- Cl pH6.8, 10% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.4mg/ml bromophenol blue). Total proteins which were not immunoprecipitated were used as input control. Eluted proteins and input controls were separated by 9% SDS -PAGE, transferred and probed by myc primary antibody (5000:1, Cell signaling). Signals detected by anti-mouse secondary antibody were digitalized by image J program.

1.2.7 Chromatin IP assay (ChIP assay)

For ChIP assay, we used nuclear extracts from *myc - FRI* and *SUF4 - FLAG* transgenic lines. 4-grams seedlings grown under long day condition for 11 days were cross-linked for 20min by 1% formaldehyde solution added 1mM PMSF. Nuclear isolated by nuclear isolation buffer(0.25M Sucrose, 15mM PIPES pH6.8,

5mM MgCl**2**, 60mM KCl, 15mM NaCl, 1mM CaCl, 0.9% Triton X-100) was purified, immunoprecipitated overnight at 4℃ and incubated with protein sepharose A for 4 hours at 4℃. Beads were washed 5 times using TE buffer (1mM EDTA, 10mM Tris-Cl pH8.0). The antibody against myc or FLAG was used for immunoprecipitation, and DNA was pre cipitated by phenol/chloroform/isoamylalcohol solution. ChIP products were used for amplification of *FLC* genomic fragments by quantitative real -time PCR. *Tubulin* was used as normalization control. Used primers are listed in I-table 1.

1.2.8 Chromatin conformation capture assay (3C assay)

3C assays were performed according to previous reported protocols with some modifications (Hagege et al., 2007; Louwers et al., 2009; Crevillen et al., 2013). 2-grams of seedling were cross-linked with 2% formaldehyde in PBS buffer for 20min. Nuclei isolated by nuclei isolation buffer (10mM Tris-HCl pH8.0, 250mM sucrose, 1mM MgCl**2**, 5mM KCl, 40% glycerol, 0.25% Triton X-100) were washed and treated with 0.2% SDS at 65℃ for 30min. To sequester SDS, 2% Triton X-100 was added and the nuclei was incubated at 37℃ for 30min. Digestions were performed at 37℃ with 600U BamHI (NEB, #R0136T) and BglII (NEB, #R0144M) for 16 hours. For inactivation of restriction enzyme, 1.6% SDS was added and incubated at 65℃ for 20min. SDS was sequestered with 1% Triton X-100. Ligations were performed using 350U T4 DNA ligase (Takara, 2011B) at 16℃ for 5hours, followed at room temperature for 30min. After reverse crosslinking, DNA was precipitated by phenol/chloroform/ isoamylalcohol solution. 3C products were amplified by their interaction

frequency. To normalize concentration of 3C products, the primer set (LC) which does not include any restriction enzyme site was used. We used control plasmid containing full *FLC* sequences for normalization of different primer efficiency. Each PCR experiment included triplicates of all DNA samples. Relative interaction was calculated according to protocol previous reported (Hagege et al., 2007).

Table 1. List of primers used in this study.

Oligo name	5' to 3' sequences
FLC pro 2 F	GCACAGCTCCGAGTGTTACTG
FLC pro 2 R	CTCGGAGTGGGTGAAACTGA
FLCp 6QF	GAGTGGAGGTTCTTTCTGCAA
FLCp 6QR	AAGACAAGATTGCCACGTGTA
FLC body 1 F	AAATATCTGGCCCGACGAAG
FLC body 1 R	TCCTCAGGTTTGGGTTCAAG
FLC body 3 F	CCAACCTCTTTGGTACGGATCT
FLC body 3 R	TTGACCAACATGGCCAAACT
FLC body 5 F	AGACTGCCCTCTCCGTGACT
FLC body 5 R	AAAGCAGCACGGTTGTTCTC
FLC 9Q-2_F	CTCTGATTCAGCCTACAAGATG
FLC 9Q-2_R	TGAAATTGCTGGTTAGCTTCGG
3C_Ib	CGTGCTCGATGTTGTTGAGT
3C_IId	TTGGTTTCCTTGAAGGTTGTG
3C_IIId	TGGCCAAGAGACTTTGTGTG
3C_IVd	CTTCCGTAGTTCCGTCATCC
3C_Vd	AAGGCAACACAAACTTTCTCG
3C_VId	TGTCCTTGGCAAGAAAGAGG
LC_F	TTCATTTAGCAACGAAAGTGAAAAC
LC_R	TTGTGTTTTGAAGACAAGATTGC
pTUB F Q	ACAAACACAGAGAGGAGTGAGCA
pTUB R Q	ACGCATCTTCGGTTGGATGAGTGA

1.3 RESULTS

1.3.1 *myc-FRI* transgenic lines show late flowering and strong response to vernalization like winter annual wild type.

To elucidate FRI complex -mediated flowering repression, we generated *myc*-tagged *FRI* transgenic lines, namely, *pFRI:myc:FRI fri*. We introduced a construct which contained *myc*-tagged genomic *FRI* sequences under the control of endogenous promoter into Columbia -0 (Col-0), a *fri* null mutant. We selected four transgenic lines, *myc-FRI* #1, #2, #3 and #11, showing late flowering phenotype. To determine whether our transgenic lines have functional *FRI*, we measured flowering time with and without vernalization treatment. As reported, *FRI*-Col showed late flowering, and it produced about 70 leaves when flowering. The vernalization accelerated flowering such that about 20 leaves were produced when flowering in winter annual wild type (Figures 1B). Similarly, the four *myc*-tagged transgenic lines displayed similar late flowering before vernalization, and the vernalization accelerated flowering of *myc-FRI* transgenic lines. Our data indicates that the introduced transgene is functional. Also, *myc-FRI* is fully functional such that it delays flowering and late flowering is suppressed by vernalization similar to winter annuals (Figures 1A).

Figure 1

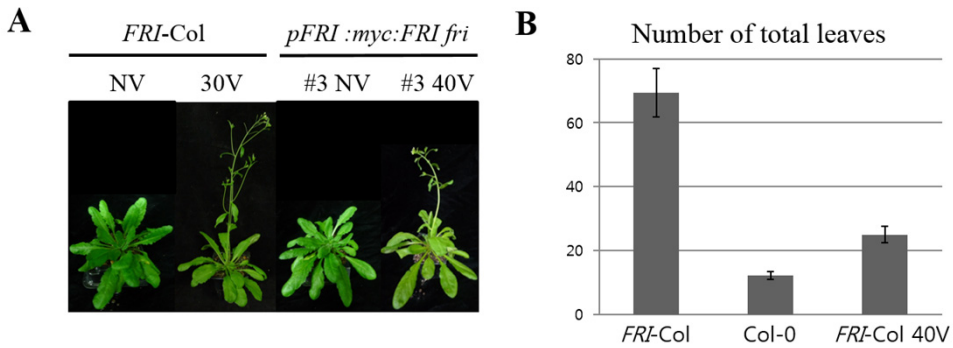


Figure 1. Flowering phenotype and vernalization response of *myc-FRI* transgenic lines.

(A) Vernalization effects on flowering of *FRI*-Col and *myc-FRI* transgenic line grown under Long day conditions (16h light/8h dark). NV; non-vernalization, 30V; 30 days of vernalization, 40V; 40 days of vernalization.

(B) Number of total leaves of *FRI*-Col, Col-0, *FRI*-Col affected by vernalization. Plants were grown under long day conditions (16h light/8h dark). 40V: 40 days of vernalization. Flowering time measured by counting rosette leaves from at least 20 plants.

1.3.2 Levels of the components of F RI complex are n ot changed vernalization.

We analyzed if vernalization affects the expression of FRI-C. We performed RT-PCR using extracts from whole seedlings. Five components of FRI -C, *FRI, SUF4, FRL1, FLX* and *FES1*, showed similar levels of RNA transcripts before and after vernalization (Figures 2A). This result suggests that vernalization does not affect on the transcript level of the components of FRI -C

Because the genetic and biochemical data showed that SUF4 binds *FLC* promoter and regulates the expression of *FLC* by recruiting transcription activator, we regarded SUF4 as indispensable component for FRI -C function. To elucidate whether proteins of FRI complex are affected by vernalization, we checked the protein levels of FRI and SUF4. We generated *FLAG*-tagged *SUF4* transgenic lines, namely, *pSUF4:SUF4:FLAG* by inserting a vector containing endogenous promoter and *FLAG*-tagged genomic *SUF4* sequences into *FRI*-Col. After selecting transgenic lines showing similar late flowering as FRI -Col, we checked the protein levels. When we examined protein levels of FRI and SUF4 for *myc-FRI* and *SUF4-FLAG*, both FRI and SUF4 protein levels were not reduced by vernalizaiton (Figure 2B). Rather than that, we found that the FRI protein level was increased steadily by vernalization, but su ch increase was recovered to non-vernalized level after returned to room temperature. Similar to the level of FRI protein, SUF4 protein level was slightly increased by prolonged cold. After vernalization, increased SUF4 protein level was recovered. Taken together, it suggests that levels of FRI and SUF4 are not affected by vernalization both in their transcripts and protein levels. Meanwhile, it has been

reported that FRI protein is degraded after vernalization by 26S proteasome (Hu et al., 2014). In contrast, FRI-C is not reduced by vernalization in our experiments. This difference might be due to the difference in the vector used or vernalization conditions. Our results suggest that the expression of FRI complex is not affected by vernalization.

Figure 2

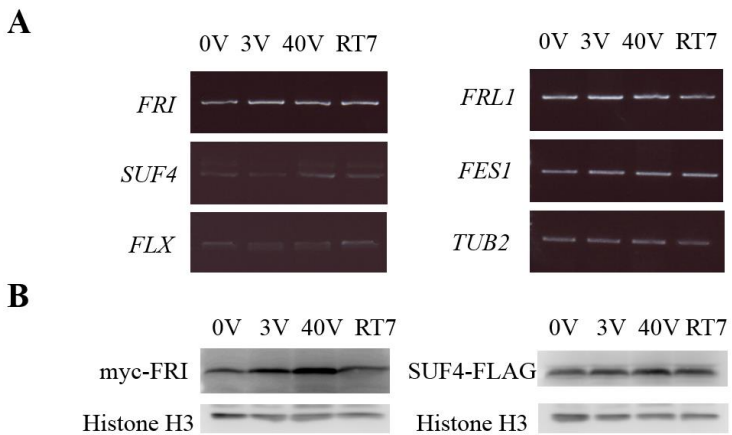


Figure 2. Expression of the components of FRI complex.

(A) The transcript levels of the components of FRI complex during vernalization. Total RNA was extracted from whole seedlings of *FRI*-Col. 0V; non-vernalization, 3V; 3 days of vernalization, 40V; 40 days of vernalization, RT7; 7 days in room temperature after 40 days of vern alization. *Tubulin* was used as normalized control. This figure is contributed by Juhyun Kim.

(B) The protein levels of FRI and SUF4 during vernalization. Total proteins extracted from whole seedlings of *myc-FRI* and *SUF4-FLAG* transgenic lines were detected by immuno blotting. Histone H3 was used as loading control. This figure is contributed by Juhyun Kim.

1.3.3 Interaction of FRI and SUF4 is decreased by cold.

Previously, gel filtration analysis showed that FRI forms about 1MDa protein complex with FRI-associated proteins such as SUF4, FES1, FLX, FRL1, YAF9, EFS and TAF14 (Choi et al., 2011b). To confirm that FRI complex forms the large complex with SUF4, we performed gel filtration analyses using *myc*-tagged *FRI* lines and *FLAG*-tagged *SUF4* lines. Without vernalization, the protein complex including SUF4 was detected in about 670kD a protein fraction, ranging from 400 to 1000kDa (Figures 3B). We confirmed that the size of the protein complex detected for SUF4-FLAG is similar size with that for *myc-FRI*. It suggest that SUF4 is included in the large complex of FRI (Figures 3A).

We wondered if the stability of FRI-C is affected by vernalization. During vernalization, the size of FRI complex detected by myc antibody was not changed significantly by vernalization, indicating that FRI exists mostly as a component of the large complex throughout vernalization (Figures 3A). By contrast, FLAG-tagged SUF4 was detected in about 158kDa size fraction after 3-days cold, suggesting that SUF4 is dissociated from the large complex of FRI (Figures 3B). Interestingly, such dissociated SUF4 was returned to the large complex when we checked SUF4 using samples returned to room temperature after 3 days of cold. Additionally, to determine the restorability of SUF4, we performed the similar experiments in extended cold, namely, 7days of cold. As a result, in 7 days of cold condition, the size of the protein complex including SUF4 was about 158kDa and then, in return to room tem perature, SUF4 backed again to the large complex of FRI (Data not shown). Our data indicates that

SUF4 is dissociated from the large complex of FRI, affected by short cold, but SUF4 is capable of binding to the large complex after cold.

However, when we performed gel filtration analysis in full vernalization condition, SUF4 could not be restored to the large complex (Figures 3B). As we expected, SUF4 was detected in about 158kDa size of fraction with full vernalization. However, when the samples are returned to room temperature after vernalization, the protein complex containing SUF4 was detected in 158kDa protein fraction. Our results support that FRI complex is changed in its structure by short cold, and this change cannot be recovered after full vernalization.

Based on this finding, we tested the *in vivo* interaction between FRI and SUF4 by co-IP assay to examine whether the interaction is affected by vernalization. Total proteins were extracted using double transgenic lines of *myc-FRI SUF4-FLAG* obtained from the genetic crosses between *myc-FRI* and *SUF4-FLAG* transgenic lines, and the proteins were immunoprecipitated with FLAG antibody. Precipitated proteins were immunoblotted using myc antibody. As previously shown, the interaction of SUF4 with FRI was observed. During vernalization, the interaction efficiency of FRI and SUF4 was not changed (Figures 3E). To analyze efficiency of interaction, we digitalized the interaction signal compared to input control, using image J program. The digitalized data showed that the interaction was decreased by cold, thus it confirms that SUF4 is dissociated from the FRI-C (Figures 3E).

To define further the mechanism of SUF4 dissociation, we observed the size of protein complex including SUF4 in *vin3-4* and *suf3* mutant backgrounds. VIN3, a component of plant polycomb repressive complex, is the most important for suppression of *FLC* during vernalization. The expression of *VIN3* is increased during vernalization, playing a critical role in the repression of *FLC*. In *vin3-4* mutant background, SUF4 which was included in a large complex before cold treatment was detected in a small complex with 3 -days of cold (Figures 3C). Our observation indicates that SUF4 can exist as a component of FRI-C without *VIN3* and that the dissociation of SUF4 is independent from *VIN3*. SUF3, a homolog of components of an ATP-dependent chromatin-remodeling SWR1 complex, have an important function in transcriptional activation of *FLC*. Previous study showed SUF3 is capable of binding to SWC6 *in vivo*, supporting that SUF3 recruits SWR1 complex to *FLC* gene (Choi et al., 2011b). Whereas SUF4 is included in the large complex of FRI before vernalization, in *suf3* background, the size of complex including SUF4 was detected in a small size of fraction even without vernalization (Figures 3D). This result suggests that interaction between FRI and SUF4 depends on existence of *SUF3*, in other words, SUF4 cannot be comprised of FRI-C without *SUF3*.

Figure 3

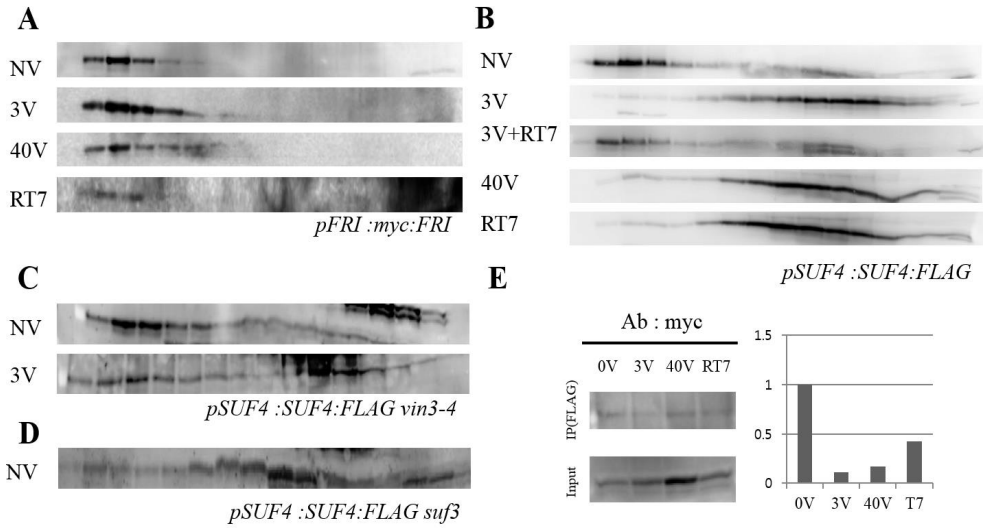

A
NV
3V
40V
RT7
pFRI :myc:FRI
B
NV
3V
3V+RT7
40V
RT7
pSUF4 :SUF4:FLAG
C
NV
3V
pSUF4 :SUF4:FLAG vin3-4
D
NV
pSUF4 :SUF4:FLAG suf3
E
Ab : myc
0V 3V 40V RT7
IP(FLAG)
Input
1.5
1
0.5
0
0V 3V 40V T7

Figure 3. *In vivo* interaction of FRI and SUF4 is affected by vernalization .

(A to D) An immunoblot of the gel filtration fractions from Superdex 200 column. Total protein extracted from epitope tagged transgenic lines was used for gel filtration and the 16 tubes of fraction were collected and immunoblotted using proper antibody. NV; non-vernalization, 3V; 3 days of vernalization, 3V+RT7; 7 days in room temperature after 3 days of cold, 40V; 40 days of vernalization, RT7; 7 days in room temperature after 40 days of vernalization. (B) was contributed by Juhyun Kim.

(E) *In vivo* interaction between myc-FRI and SUF4-FLAG detected by Co-IP assay. Protein extracted from *myc-FRI SUF4-FLAG* was immunoprecipitated by FLAG primary antibody. Input protein which were not immunoprecipitated and the eluted protein were immunoblotted using myc antibody. Relative signal of interaction was digitalized by image J program.

1.3.4 FRI and SUF4 bind *FLC* promoter and the proximal region to 3' UTR.

In general, physical interaction between transcription activators and promoter makes high gene expression (Hahn and Young, 2011). Consistent with this, FRI-C binds *FLC* promoter region and activates transcription by recruiting general transcription factor before vernalization (Choi et al., 2011b). However, how vernalization affects function and structure of FRI-C is unknown. To address this question, we performed ChIP assay using *myc:FRI* and *SUF4:FLAG* lines. Firstly, we monitored an enrichment of FRI and SUF4 at *FLC* promoter. As expected, both FRI and SUF4 were enriched approximately twofold at the proximal region of the *FLC* promoter before vernalization (Figures 4A, 4B). This result confirms previous report that FRI-C regulates *FLC* expression by binding *FLC* promoter. Interestingly, these enrichments were maintained after vernalization treatment, thus suggesting that binding of FRI-C to *FLC* promoter is not affected by vernalization.

Additionally, we examined enrichment at three distant regions from promoter, body3, body5 and 9Q-2 (Figures 4A). Body3 is included in the first intron of *FLC* and both Body5 and 9Q-2 are proximal region of 3' UTR. Both FRI and SUF4 were not enriched at body3 regardless of vernalization treatment. However, the enrichments at body5 was identified in both FRI and SUF4 (Figures 4B, 4C), and such enrichments were maintained even after vernalization. Our data suggest that FRI and SUF4 bind not only *FLC* promoter but the proximal region of 3' UTR regardless of vernalization. It is noteworthy that FRI-C binds at *FLC* even when FRI complex is not needed for *FLC* expression.

Figure4

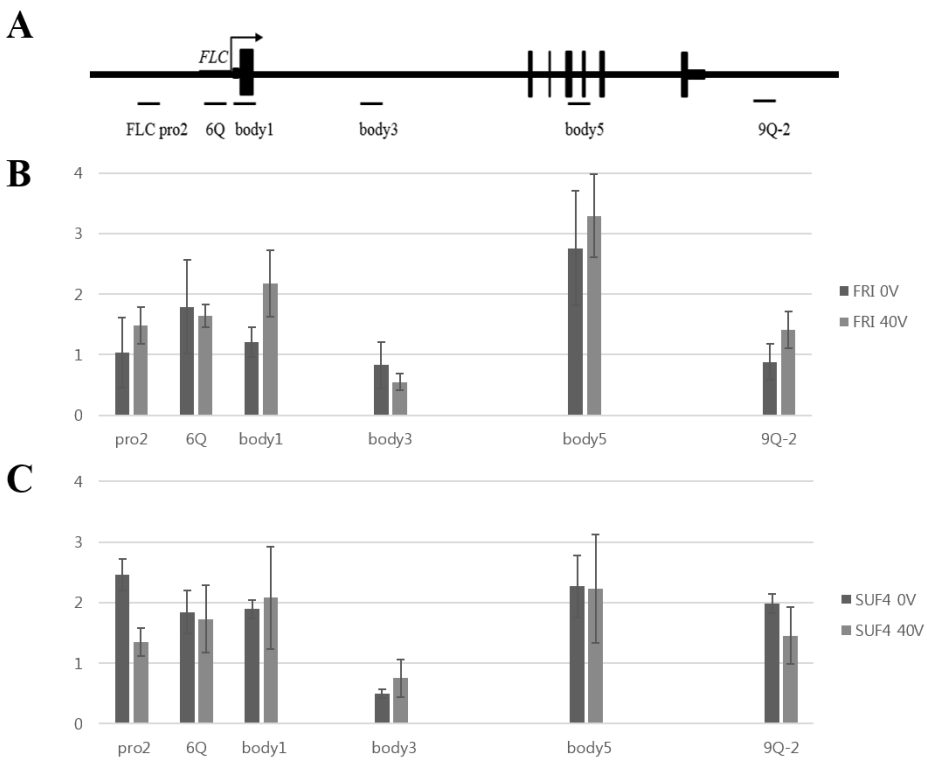


Figure 4. Chromatin IP of FRI and SUF4 on the *FLC* gene.

(A) Locations of primers used for quantitative real-time PCR

(B) and (C) The enrichments of FRI and SUF4 at FLC regions. Nuclear extracts from *myc-FRI* and *SUF4-FLAG* transgenic lines were used and immunoprecipitated by proper antibody for overnight. The relative enrichments of FRI and SUF4 were detected by quantitative real-time PCR. Left bars indicate the enrichment of without vernalization and right bars indicate th e enrichment after vernalization. *Tubulin* was used as normaliz ied control.

1.3.5 FRI and SUF4 affect gene loop formation at *FLC*.

Recently, a gene loop-mediated transcriptional regulation has been elucidated in yeast and *Drosophila*. In general, the gene loop built by promoter and 3' UTR has a function for high gene expression. *FLC* also has such a gene loop which is disrupted in the early phase of vernalization (Dean et al., 2013). Because the enrichments of FRI and SUF4 were identified at promoter region and the proximal region of 3' UTR, we hypothesized that FRI complex affects formation of *FLC* loop.

To identify whether FRI-C affects *FLC* loop formation, we performed chromosome conformation capture assay (3C assay) using *fri, suf4* mutants and *FRI*-Col. 3C assay is based on cross-linking to capture interactions of chromatin. As shown in figure 5A, *FLC* gene can be divided into 6 fragments by BamHI and BglII restriction enzymes. By using tandem primers, ligated fragments can be detected by their interaction frequency. The relative interaction of anchor region with other regions is estimated by quantitative real-time PCR. We used fragment 1, included promoter and the first exon, as an anchor.

As reported previously, the interaction frequency detected between fragment 1 and 5 was much higher than that of others. Additionally, the interaction between fragment 1 and 3 was observed in high frequency in our experiment. Our data show that the promoter of *FLC* physically interacts with 3' UTR in *FRI*-Col. By contrast, the decreased interaction frequency between fragment 1

and 5 was detected in *fri* and *suf4* mutants (figures 5B). From this, we suggest that the *FLC* loop is formed in low frequency without FRI and SUF4. Taken together, our results indicate that the physical interaction between promoter and 3' UTR in *FLC* is mediated by FRI and SUF4, in other words, FRI-C affects gene loop formation of *FLC*.

Figure 5

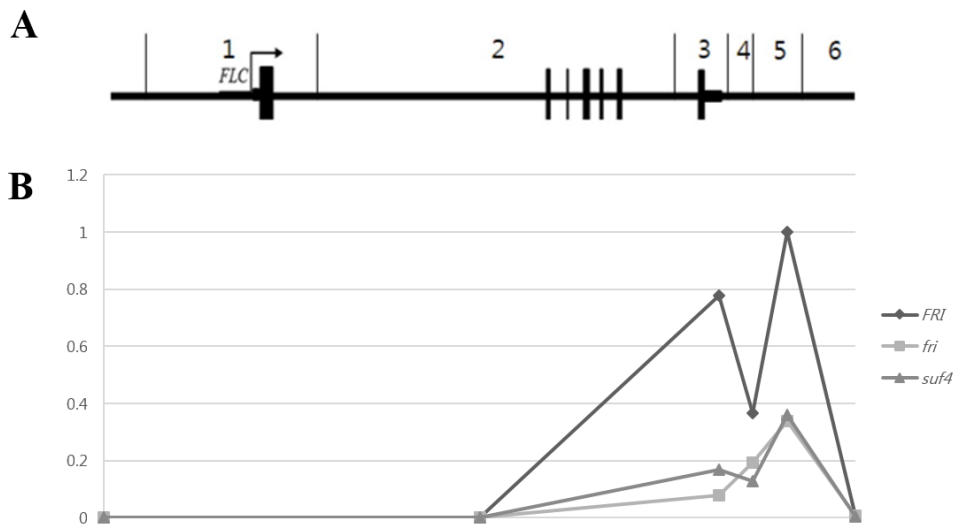


Figure 5. *FLC* loop formation is affected by FRI and SUF4.

(A) FLC gene fragments restricted by BamHI and BglII. Fragment 1 was used as anchor region and the relative interaction with other fragments (2−6) was detected by quantitative real−time PCR.

(B) Relative interaction of fragment 1 with 2−6 detected by chromosome conformation capture assay. Interaction frequency was calculated according to previous report (Hagege et al., 2007). LC primer was used for normalization of 3C products and *FLC* plasmid DNA was used for normalization of primer efficiency. Primers used in this study are listed in I−table 1.

1.3.6 After vernalization, the changes of FRI complex may lead *FLC* loop disruption.

An obvious function of FRI-C for transcriptional activation of *FLC* is clearly elucidated. However, how vernalization affects the structure and biochemical function of FRI-C is not revealed. To address this question, we analyzed structure and behavior of FRI-C during vernalizatio n. ChIP assay supports that FRI-C binds not only the promoter but the proximal region to 3 ' UTR regardless of vernalization. 3C assay data show that FRI and SUF4 is required to form *FLC* loop. Thus, we suggest that FRI -C mediates *FLC* loop formation which is required for full activation of *FLC* befofe vernalization (Figures 6A).

Because SUF4 is dissociated in very early phase of vernalization, SUF4 dissociation seems earlier event than loop disruption. Thus, we propose that the interaction between FRI and SUF4 is decreased by SUF4 dissociation, thus disrupting *FLC* loop during vernalization (Figures 6B). Such disruption of *FLC* loop makes gene body be revealed to polycomb repressive complex, including VIN3. Although FRI-C located in the *FLC* chromatin, FRI-C may lose its function with SUF4 dissociation. By a series of histone modification, *FLC* chromatin seems to be silenced during vernalization.

Figure 6

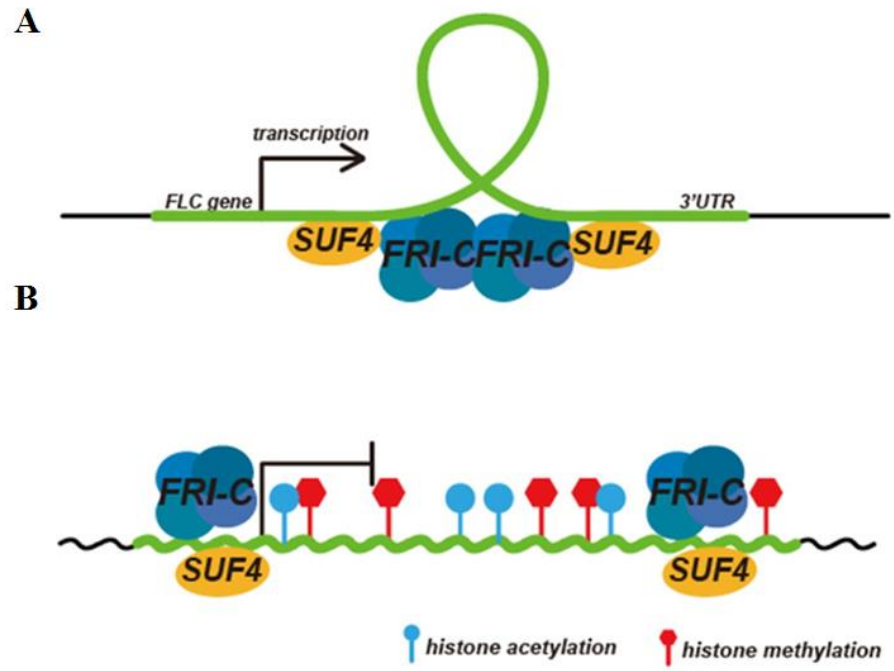


Figure 6. Model of FRI complex−mediated *FLC* gene looping.

(A) Without vernalization, FRI complex −mediated *FLC* looping contributes to FLC activation. In this condition, FRI complex is stable and located at *FLC* promoter and the proximal region of 3' UTR.

(B) During vernalization, the interaction between FRI and SUF4 is decreased, and it may lead disruption of *FLC* loop. Afterwards, *FLC* is repressed by epigenetic regulators during vernalization.

1.4 DISCUSSION

Vernalization requirement in *Arabidopsis* is responsible to elevated *FLC* expression (Michaels and Amasino, 2001). To date, a variety of genes involved in activating *FLC* have been identified. *FRI*, encoding coiled-coil domain, is a strong transcriptional activator of the *FLC* (Johanson et al., 2000; Gazzani et al., 2003b; Choi et al., 2009; Geraldo et al., 2009). It mostly acts as a scaffold protein to make protein-complex with transcriptional activators and the FRI-associated components (Choi et al., 2011b)

For full activation of *FLC*, accumulation of active epigenetic marks is required such as histone H3 lysine 4 methylation (H3K4me) and H3K36me, which are involved in active chromatin state (Ausin et al., 2004; Jiang et al., 2009; Tamada et al., 2009; Yun et al., 2012). H3K4 methylation at *FLC* is established by *Arabidopsis TRITHORAX* genes *ATX1* and *ATX2* and H3K36 methylation is accumulated by *EARLY FLOWERING IN SHORT DAYS (EFS)* (Soppe et al., 1999). The interaction of FRI-C with EFS has been reported, which explains the FRI complex-mediated *FLC* activation by H3K4 and H3K36 methylation (Michaels et al., 2004; Noh et al., 2004; Ko et al., 2010) Altogether, the function of FRI-C has been regarded as transcriptional activation of *FLC* through recruiting general transcription factors.

The mechanism of FRI-mediated *FLC* upregulation is clearly identified. However, molecular behaviors and biochemical functions of FRI-C affected by vernalization are not elucidated. Here, we monitored the molecular activity of

FRI-C during vernalization to identify function of FRI-C in cold condition.

During vernalization, the expression of *FLC* is gradually repressed by a series of histone modification (Amasino, 2004; Sung and Amasino, 2004a, b; Kramer and Hall, 2005). H3K27me3 marks are accumulated at the *FLC* chromatin by PRC2 complex (Kohler and Grossniklaus, 2002; Choi et al., 2009). Therefore, *FLC* fully silenced by epigenetic regulators exists in heterochromatin state during vernalization. In such condition, we observed tha t FRI-C was not affected in transcriptional and translational levels. Our finding suggests that FRI-C, the strong activator of *FLC*, presents even when *FLC* is suppressed.

A similar case has been observed in humans, in which the human heat shock factor HSF-1 remains in a latent state in the cytoplasm (Jolly et al., 2002). They mostly exist in latent state, which means that it can induce their target genes again or that it can be degraded in response to extracellular signals (Baldwin, 1996; Darnell, 1997; May and Ghosh, 1997; Baltimore, 1998). Factors determining the destiny of transcriptional activators are not fully understood, but potential functions of transcription factors may be possible reason for remaining.

The expression of FRI-C can be understood in the same context. FRI-C is normally expressed during vernalization, suggesting that FRI-C remains in latent state to induce *FLC* again or other target genes. Indeed, we found SUF4 and FES1, components of FRI-C can regulate floral organ numbers (II-Figures 2). Our data support that FRI-C have potency to regulate the various

developmental process, thus elucidating the reason why FRI-C is not degraded.

However, for *FLC* activation, FRI-C seems to be functionally incompetent, because FRI-C undergoes a great change of structure during vernalization. In detail, SUF4 is dissociated from the FRI-C in cold condition (Figures 3). Among previously identified components of FRI-C, SUF4, C2H2-type zinc finger protein, plays a critical role in FRI-C function that is binding at the *FLC* promoter (Kim et al., 2006; Choi et al., 2009). Also, SUF4 has physical interactions with FRI, LD and SWC6 of SWR1-C, thus indicating that SUF4 of FRI-C can recruit chromatin remodeling factor into the FLC promoter (Choi et al., 2011b). Specific loading of FRI-C at the *FLC* promoter cause H2A.Z acetylation by SWR1-C, contributing to transcriptional activation of *FLC*. Therefore, SUF4 dissociation may cause functional incompetence of FRI-C for *FLC* activation.

Notwithstanding malfunction of FRI-C, the direct interaction between *FLC* and FRI-C was found during vernalization. It is noteworthy that FRI-C binds not only to the *FLC* promoter but also to the proximal region of 3' UTR (Figures 4). Our previous study showed that SUF4 directly binds to the cis-element (5'-CCAAATTTTAAGTTT-3') located in the proximal region of *FLC* promoter (Choi et al., 2011b). Here, the additional enrichment of FRI and SUF4 was observed at the proximal region of 3' UTR, suggesting that FRI-C may be involved in formation of chromatin conformation.

The loop formation of *FLC* would likely be required for transcription, and the

disruption of the loop may cause *FLC* repression by epigenetic regulators during vernalization (Crevillen et al., 2013). Recently, a factor which regulates *FLC* gene disruption is identified. The disruption of *FLC* loop is mediated by BAF60, a subunit of SWI/SNF chromatin remodeling complex, thus inducing *FLC* repression (Jegu et al., 2014).

However, the mechanism of loop formatio n is not elucidated. Here, we found that the loop formation was decreased in *fri* and *suf4* mutants (Figures 5). In *FRI*-Col, high interaction frequency is detected between 5′ region and 3′ region of *FLC*. By contrast, 5′-3′ interaction of *FLC* is disrupted in *fri* and *suf4* mutants. Our results indicate that the physical interaction between promoter and 3' UTR in *FLC* is mediated by FRI and SUF4. From this, we propose that FRI-C can activate *FLC* expression by forming loop structure.

Chromatin loops regulating gene transcription have been studied deeply in yeast, fruit flies and mammalian cells. Among several chromosomal conformation, intrachromosomal looping, called 5' -3' gene looping, has a function for transcriptional activation (Kadauke and Blobel, 2009; Bohn and Heermann, 2010; Clauvelin et al., 2012; Bernardo et al., 2014). Such type of looping is dependent on RNA pol II, improving transcription efficiency (Sexton et al., 2009). *FLC* loop is detected by interaction of 5' and 3' end of chromatin, suggesting that this 5' -3' gene looping may enhance *FLC* transcription. Thus, we suggest that FRI complex -mediated 5' -3' gene looping may lead full activation of *FLC* before vernalization.

Occasionally, loop formation is mediated by boundary elements, such as CCCTC-binding factor (CTCF) in *Drosophila* (Holwerda and de Laat, 2013). The boundary elements provide boundary between histone active marks and repressive marks, thus it prevent histone repressive marks to being spread (Heger et al., 2012; Herold et al., 2012; Kornblihtt, 2012; Holwerda and de Laat, 2013; Tark-Dame et al., 2014). In *Drosophila,* CTCF regulates transcription by providing such boundary. CTCF is highly conserved among *Drosophila*, mouse and human, whereas homologs of CTCF cannot be found in Arabidopsis.

By sequence analyses, we found that SUF4 is similar to CTCFs. Because most CTCFs have C2H2 type zinc finger domains, amino acid sequence identities of *SUF4* compared to mouse *CTCF* were relatively high, suggesting that SUF4 can act as boundary elements.

The *SUF4* gene encodes ZP207 class zinc finger protein, including a nuclear localization signal, two C2H2 -type zinc finger domains and a Pro -rich domain (Kim et al., 2006; Kim and Michaels, 2006). C2H2-type zinc finger domains are mainly used in DNA binding (Najafabadi et al., 2015; Persikov et al., 2015). Although CTCF in mouse and human have 11 zinc-finger domains, a single zinc-finger domain is enough to bind DNA (Dathan et al., 2002). SUF4 have two zinc-finger domains, which may enhance affinity to DNA binding (Klug and Schwabe, 1995).

Whereas zinc-finger domain is composed of 60 amino acids, pro-rich domains

are 211 amino acids, accounting for more than half of SUF4. Pro-rich domains are found in many transcription factors involved in transcriptional activation. Most protein interaction modules recognize very short sequence of amino acids, including Pro-rich domains(Kay et al., 2000; Zhou, 2006). Thus, Pro-rich domain of SUF4 may be critical to interact with other transcription factors.

The domain similarities of *SUF4* with *CTCF* suggests that it can have additional function to regulate transcription such as gene looping. The binding sites of SUF4 are promoter and the proximal 3 ' UTR of *FLC*, supporting that SUF4 can functions as boundary elements, which regulate transcription by providing boundary between active histone marks and repressive histone marks. In cold condition, SUF4 is dissociated from FRI-C, and then *FLC* loop is disrupted by vernalizaiton. If SUF4 acts as boundary elements, SUF4 dissociation may lead to the disruption of the *FLC* loop. Thereafter, H3K27me3 deposited around the *FLC* can be spread to the *FLC* whole chromatin, causing *FLC* to be silenced.

Taken together, we propose a model for the *FLC* regulation by FRI complex - mediated gene looping. Before vernalization, FRI- C, including FRI and SUF4, forms *FLC* loop at the 5' and 3' regions to activate *FLC* transcription. In the absence of the interaction of FRI and SUF4, *FLC* looping is decreased. SUF4 may provide boundary between active chromatin and repressive chromatin. In short cold condition, SUF4 is dissociated from FRI, thus FRI cannot activate *FLC* expression. SUF4 dissociation may lead the disruption of the *FLC* loop, then *FLC* chromatin is repressed by spread ing of repressive marks during

vernalization. Therefore, we suggest that the disruption of the *FLC* gene loop caused by SUF4 dissociation is a mole cular basis of transcriptional silenc ing of *FLC* after vernalization.

Chapter II. Components of FRI complex regulate floral organ number.

2.1 INTRODUCTION

2.1.1 Flower development governed by floral organ identity genes.

Arabidopsis flowers can be separated into four territories, namely whorls, which have specific organs. Four sepals are developed in the most outer territory, the first whorl, and then four petals are developed in the second whorl. Inner two whorls produce reproductiv e organs. In the third whorl, six stamens are produced, and such male reproductive organs have critical role in the production of pollen. The fourth whorl, the center of flower, is occupied by two carpels, which are the female reproductive organs (O'Maoileidigh et al., 2014b; O'Maoileidigh et al., 2014a).

Development of floral organs in proper position, namely, floral patterning, is completed by floral organ identity genes. To date, molecular and genetic analyses have shown the mechanisms of floral patterning. Particularly, ABC model has provided a basis for understanding development of floral organs. In ABC model, there are three classes of genes regulating development of four floral organs (Lohmann et al., 2001; Lohmann and Weigel, 2002; Sablowski, 2015). A class genes, including *APETALA1 (AP1)* and *AP2*, have functions in development of sepals and petals (Ripoll et al., 2011; Krogan et al., 2012; Han

et al., 2014). B class genes such as *AP3* and *PISTILLATA (PI)* regulate petals and stamens formation (Jing et al., 2015). Lastly, C class genes, including *AGAMOUS (AG)*, control formation of reproductive organs such as stamens a nd carpels (Michaels et al., 2003; Das et al., 2009; Liu et al., 2011; O'Maoileidigh et al., 2013). ABC model suggests that the perfect floral patterning is completed by coordination of each class of genes. Recently, E class genes determining all the floral organ patterning are identified. *SEPALLATA (SEP)* encoding MADS domain protein regulates development of petals, stamens, and carpels by interacting with all classes of genes (Jetha et al., 2014; Puranik et al., 2014). In addition to floral identity genes mentioned above, *UNUSUAL FLORAL ORGANS (UFO), SUPERMAN (SUP)* and *LEAFY (LFY)* are considered as upstream regulators of ABCE genes (Takeda et al., 2004; Siriwardana and Lamb, 2012; Cheng et al., 2013; Engelhorn et al., 2014)

2.1.2 Mutants producing flowers with extra number of floral organs.

Genetic analyses have identified a number of mutants showing abnormal floral organs. In particularly, several genes determining the number of organs are disclosed. *CLAVATA 1 (CLV1)* and *CLV3*, the genes mainly regulating shoot apical meristem, show enlarged meristem and increa sed floral organs when they are mutated (Clark et al., 1995; Clark et al., 1996). The *clv1* and *clv3* mutants fail to develop normal floral organs, but the additive effects were not found in *clv1 clv3* double mutants, suggesting that *CLV1* and *CLV3* act in the same pathway controlling flower development. Additionally, mutants of *ULTRAPETALA (ULT1), PERIANTHIA (PAN), ENHANCED RESPONSE TO*

ABA 1 (ERA1) and *ETTIN (ETT)* produce flowers which have extra organs, indicating that these genes are required to develop normal numbers of floral organs (Running and Meyerowitz, 1996; Sessions et al., 1997; Chuang et al., 1999; Ziegelhoffer et al., 2000; Fletcher, 2001; Carles et al., 2005; Kelley et al., 2012; Monfared et al., 2013)

By genetic and physiological analyses, genetic interactions of them are revealed. *PAN* regulates floral organ development redundantly with *ULT1* and *ETT*, suggesting that these genes act on the same genetic pathway . In the different pathway from this, *CLV*s and *ERA1* play a role independently in normal number of floral organ development (Running and Meyerowitz, 1996 ; Fletcher, 2001)

2.1.3 Mutants producing flowers with decreased number of floral organs

By contrast, *PETAL LOSS (PTL)*, a trihelix transcription factor, shows decreased number of petals when it is mutated (Kaplan-Levy et al., 2014; O'Brien et al., 2015). *PTL* is expressed in the boundary between the first and the second whorl, thus considered as a boundary gene. Most flowers of *ptl* fail to develop petals, indicating that *PTL* is necessary for petal formation. *WUSHEL(WUS)*, a homeobox gene controlling stem cells, regulates floral patterning by upregulation of *AG* expression (Liu et al., 2011). Mutations in *WUS* cause abnormal flower which have less floral organs than wild -type.

2.1.4 Components of FRI complex regulate floral organ number.

To date, functions of FRI-C for *FLC* activation is clearly elucidated. However, the additional function of FRI -C has not been reported. Here, we show that SUF4 and FES1, the components of FRI-C, have functions for developing normal numbers of floral organs in addition to the regulation of *FLC*. Previously, the functions of SUF4 and FES1 have been elucidated as components of FRI -C. SUF4, a C2H2 type zinc finger protein, regulates *FLC* expression by interacting with *FLC* promoter (Kim et al., 2006). FES1, encodes C3H1-type zinc finger protein, contributes to stabilize structure of FRI -C (Schmitz et al., 2005).

When the tissue specificity of FRI- C were analyzed, a strong expression of FRI complex was found in flower tissues. Interestingly, flowers of *suf4 fes1* double mutants produce extra floral organs, demonstrating that SUF4 and FES1 are important regulators of flower development. In present study, we found direct bindings of SUF4 at *CLV1, CLV3, PAN, ULT1* and *PTL* promoter regions. Taken together, our data pro pose that the components of FRI-C can regulate floral organs number by interaction with floral identity genes.

2.2 Materials and methods

2.2.1 Materials and growth condition

The wild type used in this study was Col: *FRI* (Sf2) strain and all flowering time mutants used have been described previously (Michaels et al., 2004; Choi et al., 2005; Schmitz et al., 2005; Kim et al., 2006). Seeds were stratified on 0.65% phytoagar containing half-strength Murashige and Skoog salts for 3 days at 4℃ for homogeneous germination. Afterwards, Plants were grown in long day conditions (16h light/8h dark) under white fluorescent lights at 22 ℃. The percentage of flowers which have altered numbers of floral organs was measured by counting flowers from at least 30 plants.

2.2.2 Immunoblotting assay

Total proteins were extracted from the several tissues of *myc-FRI* and *SUF4-FLAG* transgenic lines using protein extraction buffer (50mM Tris-Cl pH7.5, 100mM NaCl, 10mM MgCl2, 1mM EDTA, 10% glycerol) added 1mM PMSF, 1mM DTT and 1X Complete protease inhibitor (Roche), followed by several centrifugation. Protein was separated by electrop horesis on a 9% SDS-PAGE and then transferred to PVDF membrane. Appropriate primary anti-myc (1:2000; Cell signaling) or anti-FLAG (1: 10000, Sigma) antibody was used and anti-rabbit or anti-mouse secondary antibody (1:5000; Cell signaling) was used for detection. Signals were detected by LAS 4000.

2.2.3 RNA extraction and RT-PCR

RNA extraction was performed as described previously (Choi et al., 2005). Total RNA was extracted from flower tissues of Col, *suf4, fes1* and *suf4 fes1* and cDNA was used for amplification of specific genes. *Tubulin* was used as normalization control. Relative expressions of s pecific genes were measured by RT-PCR. The gene specific primers used to detect the transcripts are listed i n II-table 1.

2.2.4 Chromatin IP assay

For ChIP assay, we used nuclear extracts from flowers of *SUF4-FLAG* transgenic lines. 2-grams flowers grown under long day condition were cross-linked for 20min by 1% formaldehyde solution added 1mM PMSF. Nuclear isolated by nuclear isolation buffer(0.25M Sucrose, 15mM PIPES pH6.8, 5mM MgCl**2**, 60mM KCl, 15mM NaCl, 1mM CaC l, 0.9% Triton X-100) was purified, immunoprecipitated overnight at 4 ℃ and incubated with protein sepharos e A for 4 hours at 4℃. Beads were washed 5 times using TE buffer (1mM EDTA, 10mM Tris-Cl pH8.0). The antibody against FLAG was used for immunoprecipitation, and DNA was pre cipitated by phenol/chloroform/isoamylalcohol solution. ChIP products were used for amplification of specific gene fragments by quantitative real-time PCR. *Tubulin* was used as normalization control. Used primers are listed in II-table 1.

Table 1. List of primers used in this study.

Oligo name	5' to 3' sequences
ERA1 real F	CCTCGGCTTGCAGAGATATG
ERA1 real R	TCTCTGAATCCACCGTCAGG
ULT1 real F	AGGAGGTTCAGGCTGAGGAG
ULT1 real R	CACCTTCCTGCTCCCTCTCT
PTL real-2 F	GATAGTGTTCATGGTTTTCATC
PTL real-2 R	CTCCTCTTTTTCCTTCTACTAC
qCLV1-F	GGATACATCGCCCCAGAGT
qCLV1-R	TCCAAATTCACCAACAGGTTT
qCLV3-F	CGTTCAAGGACTTTCCAACC
qCLV3-R	TCATGTAGTCCTAAACCCTTCGT
PAN real F	AGCCGGAAATGGTGTCTTT
PAN real R	GCATCCACTAGAACGCGTAG
ARF3 QF	GAGATTCCAGAGGGTCTTGC
ARF3 QR	AAATTCTGTTCCTTTGAAGCG
qWUS-F	ACAAGCCATATCCCAGCTTCA
qWUS-R	CCACCGTTGATGTGATCTTCA
PAN-ChIP F	CAGTTAAAAGCAAATCATAAGCC
PAN-ChIP R	GGGAGTATGTTCATATCTTTC
PAN UTR-ChIP F	GGCTTTTGATAATACGTAAGAT
PAN UTR-ChIP R	GGTTGGATCTTACCTTGGTG
ULT1-ChIP F	TTCGAATATTTATAACCACCATC

ULT1-ChIP R	GGGAGTGAACTTATGGATTC
ULT1 UTR-ChIP F	GACACAACCCTATTCCTCAAC
ULT1 UTR-ChIP R	TCACCTACATAACATTGTATTG
PTL ChIP F	GAACATCACGGCCAAACCT
PTL ChIP R	AAAGCAACATCATCACTGCAAA
PTL-UTR ChIP F	AATGAATGTAGGTATACTCGGTGATGT
PTL-UTR ChIP R	TGGACACATGCATGCCATAA
CLV1-ChIP F	CCATCGTTGCTATATGTCGTT
CLV1-ChIP R	GGGAATAAAGGTTTAGGTTCTC
CLV1 UTR-ChIP F	CCTGCAAGATCAGCAGATGG
CLV1 UTR-ChIP R	CGCCAAAATACTTTTATGTGTTC
pTUB F Q	ACAAACACAGAGAGGAGTGAGCA
pTUB R Q	ACGCATCTTCGGTTGGATGAGTGA

2.3 Results

2.3.1 FRI and SUF4 are highly expressed in flower tissues.

Aspects of mechanisms of *FLC* regulation by FRI-C have been elucidated. FRI forms about 1MDa size complex with FRI-associated proteins such as SUF4, FES1, FLX, FRL1, YAF9, EFS and TAF14 (Choi et al., 2011b)Such a large complex of FRI regulates *FLC* expression by interacting with general transcription factors. In elucidating the effects of vernalization on FRI- C, we found that FRI-C is normally expressed even after vernalization.

Thus, we hypothesized that FRI-C can be a regulator in controlling other functions. To identify other functions of FRI-C, we examined protein expressions of FRI and SUF4 in several tissues, including whole seedlings, rosette leaves, cauline leaves, stems, siliques and flower tissues. We used transgenic lines which inserted an epitope tagged vector contained endogenous promoter and full length of genomic DNA, namely, *pFRI:myc:FRI fri* and *pSUF4:SUF4:FLAG*. Protein expression detected by myc antibody was found high in whole seedlings but was rarely found in the leaves, stems and the siliques. Interestingly, in flower tissues, myc-FRI was expressed high, suggesting that FRI might have specific function in flower development (Figures 1A).

Because SUF4 is a key component of FRI complex, we checked SUF4 expression also. We monitored expression of the SUF4 in several tissues

including flowers, using total protein extracts of *SUF4-FLAG* transgenic lines. Similar to the results of FRI, the protei n was strongly detected by FLAG antibody in the whole seedlings but was rarely expressed in the leaves and siliques. Additionally, the weak expression of SUF4 was detected in the stems. In flower tissues, the SUF4 protein was observed in higher levels than other tissues, indicating that FRI and SUF4 proteins are translated strongly in flower tissues (Figures 1B). Our data showing that FRI -C is highly expressed in flower tissues than th e other tissues may support FRI -C have a role in regulating flower develo pment.

Figure 1

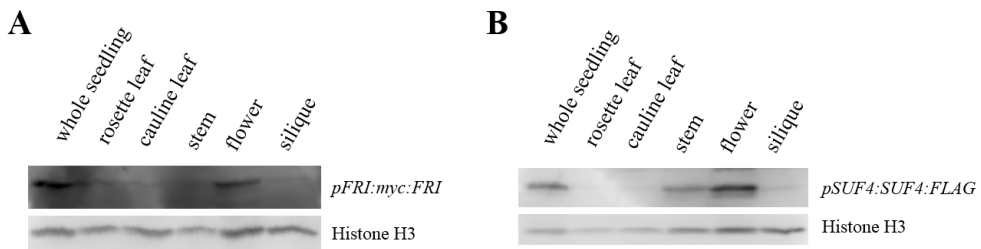


Figure 1. Protein levels of FRI and SUF4 in flowers.

(A) and (B) Immunoblotting analyses of *myc−FRI* and *SUF4−FLAG* transgenic lines. Total protein was extracted from whole seedling, rosette leaves, cauline leaves, stems, flowers and siliques. Histone H3 was used as loading control. This figures are contributed by Juhyun Kim.

2.3.2 *suf4 fes1* double mutants have abnormal number of floral organs.

To expand the understanding of functions of FRI-C on flower development, we observed the mutants of the compone nts of FRI-C such as *suf4, fes1, frl1* and *flx*. From the genetic and biochemical analyses, it is demonstrated that the components of FRI-C has differential functions in the *FLC* regulation.

Particularly, SUF4 and FES1 are considered as critical components. Because SUF4, included in FRI-C, binds the *FLC* promoter and recruits general transcription factors (Kim et al., 2006; Choi et al., 2011b). FES1, encoding zinc finger protein, leads to stabilization of FRI-C (Schmitz et al., 2005). Because components of FRI-C are necessary for the function of *FLC* regulation, all the mutants of the components of FRI-C showed early flowering behavior similar to summer annuals, confirming that these genes are required to repress flowering (Data not shown.).

However, other developmental and morphological defects cannot be observed in these mutants. Our observation showed that most flowers have four sepals, four petals, six stamens and two carpels in wild type (Figures 2A). When *SUF4* is mutated, most flowers developed in *suf4* mutants had identical morphology to wild type (Figures 2B), but some flowers showed abnormal number of sepals and stamens (Figures 2E). The flowers which have defects on stamens number were detected in wild type also, thus we consider such defect was originated from developmental noise. Likewise, *fes1, frl1* and *flx* mutants did not cause any noticeable defects of floral organ number (Figures 2C). Our findings

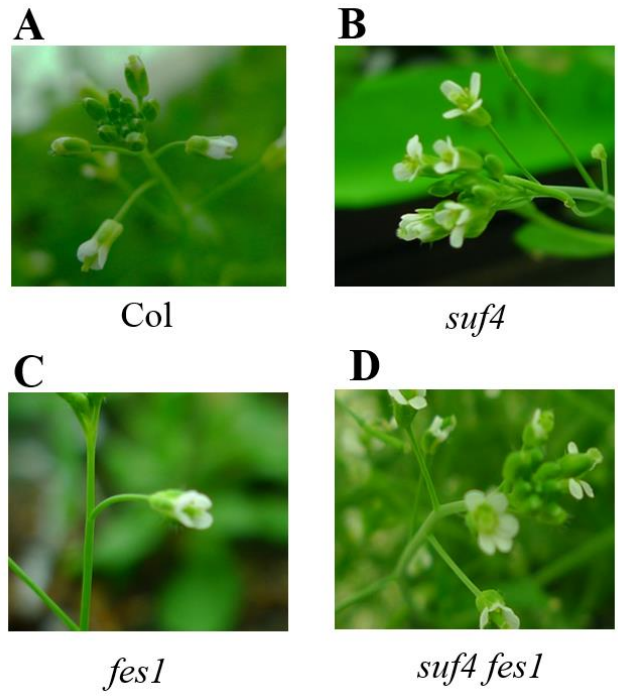
suggest that mutations in *suf4, fes1, frl* and *flx* do not affect morphological development in Arabidopsis.

Because single mutants did not result in marked phenotype, we generated double mutants to identify whether they have genetic redundancy for functions in flower development. Interestingly, When both *SUF4* and *FES1* were mutated, some flowers which produce extra floral organs were observed (Figures 2D).

Particularly, a significant number of flowers showed an increase in petal number. Whereas ten percents or less of flowers have abnormal number of sepals and stamens in *suf4* mutants, about forty-percents of flowers in *suf4 fes1* displayed extra petals and sepals. Also, more than half of flowers in *suf4 fes1* fail to develop normal number of stamens (Figures 2E).

Meanwhile, defects on carpels number were not observed in FRI-C mutants. In the samples observed, the increased organ numbers of *suf4 fes1* petals and sepals were noticeably above those of the single mutants. Our results indicate that *SUF4* and *FES1* act redundantly on development of floral organs. We concluded from these results that SUF4 an d FES1, key components of FRI -C, have redundant function for regulation of floral organs number, thus suggesting that *SUF4* and *FES1* regulate not only *FLC* expression but flower development.

Figure2


A
B
Col
suf4
C
D
fes1
suf4 fes1

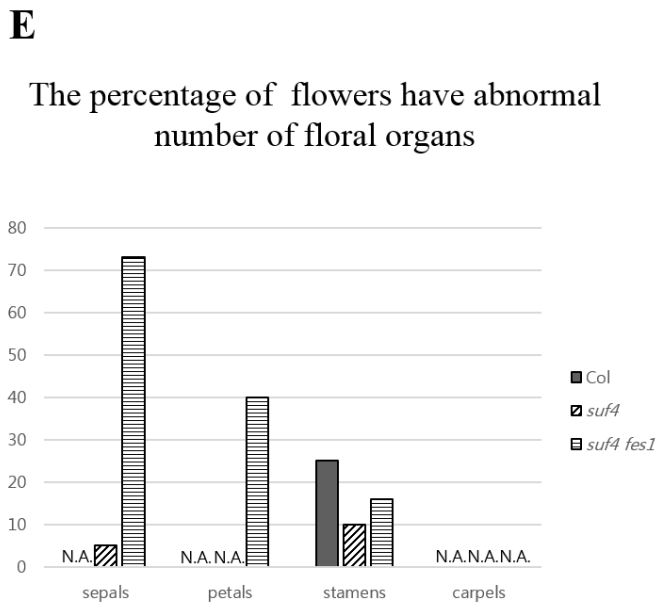

E
The percentage of flowers have abnormal number of floral organs
80
70
60
50
40
30
20
10
0
N.A.
N.A. N.A.
N.A. N.A. N.A.
sepals
petals
stamens
carpels
Col
suf4
suf4 fes1

Figure 2. Phenotypes of *suf4 fes1* double mutants.

(A) to (D) Flowers of Col, *suf4, fes1* and *suf4 fes1.* Plants were grown under long day conditions at 22℃

(E) The percentage of flowers have abnormal number of floral organs in Col, *suf4, suf4 fes1.* The percentage of flowers have alter ed number of floral organs was measured by counting flowers from at least 30 plants.

2.3.3 *CLV1, ULT1* and *PAN* are decreased and *PTL* is increased in flowers of *suf4 fes1.*

To elucidate the mechanisms of flower regulation by *SUF4* and *FES1*, we investigated whether the expressions of genes regulating development of floral organs are altered in our mutants by quantitative PCR. To date, many of genes determining floral organ number are defined as *CLV1, CLV3, ULT1* and *PTL* (Clark et al., 1995; Carles et al., 2005; Landau et al., 2015; O'Brien et al., 2015). We used cDNA obtained from flower tissues of Col −0, *suf4, fes1* and *suf4 fes1* to examine the expre ssions of candidate genes. Candidates of the genes were classified by phenotype of mutants.

Mutations in *CLV1, CLV3, ULT1, ERA1, PAN* and *ETT* mainly increase floral organ numbers, whereas mutations in *WUS* and *PTL* show decreased floral organs number. When *CLV1* or *CLV3* is mutated, flowers have enlarged SAM and extra organs in all whorls. Because *clv1 clv3* does not show additive effects, genetic analyses elucidate that *CLV1* and *CLV3* regulate meristem development on same pathway (Clark et al., 1995). Similarly to *CLV*s, *ULT1* regulates all the floral organs, but the function of *ULT1* is more apparent dominant in the first and second whorls. *PAN* is involved in the first, second and third whorl patterning, thus most flowers of *pan* show increased number of sepals and petals, and have decreased number of stamens (Chuang et al., 1999).

If *suf4 fes1* have genetic interaction with the genes of which mutations have

extra floral organs, they might show decreased expression in the *suf4 fes1*. As we expected, except for *ERA1*, the transcriptions of *CLV1, CLV3, ULT1, PAN* and *ETT* were down-regulated in *suf4 fes1* compared to wild type. *ERA1* is expressed as similar as wild type, suggesting that *ERA1* does not affect defective phenotypes of *suf4 fes1*. The expressions of *CLV3* and *ETT* were decreased in *suf4* and such decrease was more severe than in *fes1*. However, the additive decrease of expressions were not found in *suf4 fes1*, indicating that the development of extra organs of *suf4 fes1* is less related to *CLV3* and *ETT* (Figures 3).

In *CLV1* and *ULT1*, decreased expressions were observed in *suf4*, whereas the expressions detected in *fes1* were similar to that of wild type. To elucidate the development of extra floral organs of *suf4 fes1* double mutants, we examined whether the more strong decrease of expression is found in *suf4 fes1*. Thus, we checked the transcript levels of the genes regulating floral organ number in *suf4 fes1* compared to single mutants. *CLV1* and *ULT1* transcribed in *suf4 fes1* were found in lower level than in *suf4* single mutants, suggesting that *CLV1* and *ULT1* are involved in morphological phenotype of *suf4 fes1*. Similarly, a noticeable decrease of *PAN* expression was observed in *suf4 fes1* compared to *suf4* and *fes1* single mutants (Figures 3). Our results suggest that *CLV1, ULT1* and *PAN* are strong candidates for genetic interaction partner of *SUF4* and *FES1*, because they showed remarkably decreased expression in *suf4 fes1* than in single mutants.

In contrast to genes mentioned above, mutations in *WUS* and *PTL* displayed

decreased organ number, thus, we expected that *WUS* and *PTL* expression is increased in *suf4 fes1*. However, decreased expression of *WUS* was detected in *suf4* and *suf4 fes1*, indicating that the phenotype of *suf4 fes1* is not due to low expression of *WUS*. However, high expression of *PTL* was observed in *suf4 fes1*, suggesting that *PTL* is involved in the disrupted floral patterning of *suf4 fes1*. It is noteworthy that *PTL* was expressed stably in Col-0, *suf4* and *fes1* (Figures 3). From these results, we predict that the defects shown only in *suf4 fes1* might be related to *PTL* regulation.

Figure 3

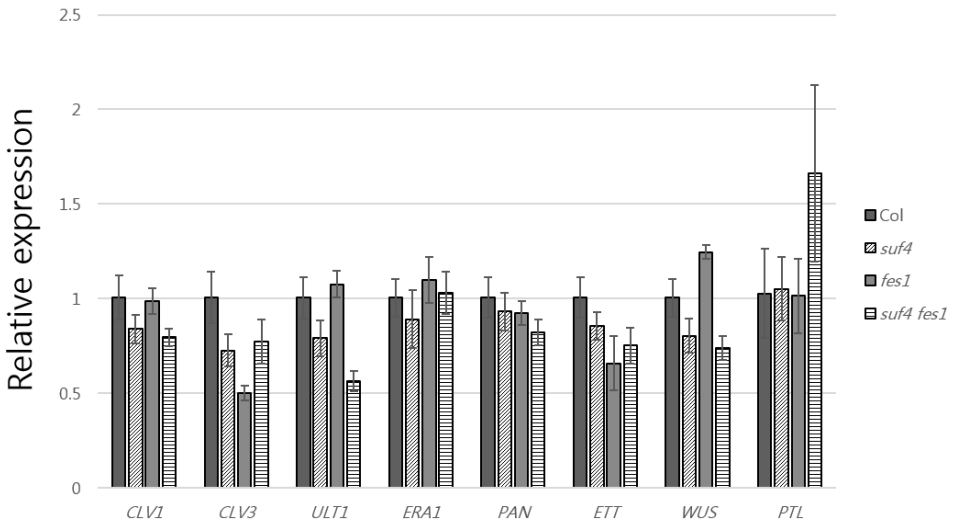


Figure 3. Gene expression analyses in flowers of *suf4 fes1*.

Gene expression analyses of Col, *suf4, fes1,* and *suf4 fes1* Total RNA extracted from flowers and cDNA was used for detection of specific gene expression. Primers used in this study were listed in table 1.

2.3.4 SUF4 binds *CLV1, ULT1, PAN* and *PTL* genes.

Based on the expression analyses, we performed chromatin immunoprecipitation assay to identify the direct interaction of SUF4 with *CLV1, ULT1, PAN* and *PTL* genes. We used DNA extracted from *SUF4-FLAG* transgenic lines to detect relative enrichment o f SUF4 compared to *tubulin*. We examined enrichment of SUF4 at two separated regions of promoter. SUF4 was enriched approximately twofold at the proximal region and distal region of transcription start site of *CLV1* and *ULT1*, indicating that SUF4 binds promo ter of *CLV1* and *ULT1* directly. Also, a small enrichment of SUF4 was observed at the proximal region of *PAN* promoter, whereas SUF4 was not enriched at the distal region of *PAN* promoter.

From this, we considered that SUF4 interacts with the promoter of *PAN* in low frequency. Similarly, SUF4 was enriched about 7 -fold at the proximal region of the *PTL* promoter. By contrast, no enrichment was detected at the distal region, thus demonstrating that SUF4 can bind at TSS site of *PTL* specifically (Figures 4).

Therefore, our results showed that SUF4 can bind the promoters of *CLV1, ULT1, PAN* and *PTL*, thus suggesting that direct interaction between SUF4 and floral organ identity genes functions for floral patterning in Arabidopsis.

Figure 4

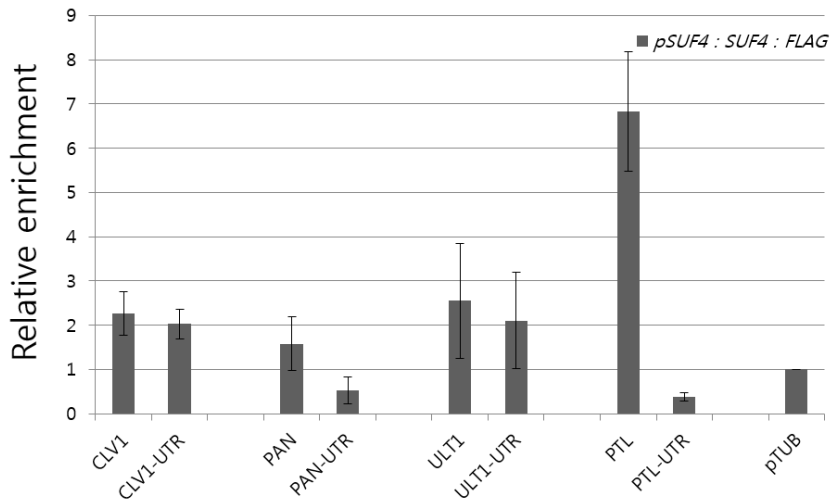


Figure 4. Chromatin IP of SUF4 on *CLV1, ULT1, PAN* and *PTL* genes.

The relative enrichment of SUF4-FLAG at target genes. 11 days old plants were used for ChIP analysis using FLAG antibody (Sigma). Left bars indicate the enrichment at the proximal region to transcription start site. Right bars indicated the enrichment at the distal region to transcription start site. *pTUB* primer was used as normalized control.

2.4 Discussion

FRI complex, a strong transcriptional activator of *FLC*, is mainly comprised of FRI, SUF4, FES1, FLX and FRL1 (Choi et al., 2011b). Here, the effects of mutations in FRI-C on Arabidopsis are examined. *fri* mutants, namely Col-0, have no obvious developmental defects. Similar to *fri* mutant, other single mutation of each component of FRI-C showed no significant phenotype in development. And also, most of double mutants showed no developmental defections. However, some of *suf4 fes1* mutants had increased number of sepals and petals in their flowers. From these, we suppose that SUF4 and FES1 had additional function in floral development.

SUF4, a C2H2-type zinc finger protein, facilitates specific binding to DNA (Kim et al., 2006). Previously, it has been reported that SUF4 activates *FLC* transcription by recruiting FRI-C and general transcription factors to the *FLC* chromatin (Choi et al., 2011b). FES1, a CCCH-type zinc finger protein, has known to be essential for the transcriptional activation of *FLC* (Schmitz et al., 2005). Except for *FLC* activation, additional functions of *SUF4* and *FES1* have not been suggested yet. Through genetic analyses, we found that *SUF4* and *FES1* have a critical role in floral development. While *suf4* and *fes1* single mutants showed no developmental defects, *suf4 fes1* double mutants formed increased number of sepals and petals. In the first and second whorls, five or six organs were observed and in the third whorl, from four to eight stamens were found. Our observation suggests that *SUF4* and *FES1* can regulate the numbers of floral organs and they act redundantly in flower development.

Additionally, the shoot apical meristems (SAM) of *suf4 fes1* were enlarged compared to wild-type (Data not shown.). We observed the SAM of mature embryos by confocal scanning laser microscopy. The average width of SAM for *suf4 fes1* is about 1.6 fold larger than that of Col-0 (Data not shown). Similarly, *clv3,* a mutant show increased floral organs, had enlarged SAM about three fold larger than wild type (Clark et al., 1995). Although the enlargement of SAM in *suf4 fes1* is less than that of *clv3*, disrupted regulation of SAM in *suf4 fes1* may be related to the development of extra organs.

To obtain further understanding of the mechanism regulating floral organ numbers in *suf4 fes1*, we investigated genetic interactions with other genes regulating floral organ numbers. In *suf4 fes1*, *CLV1, ULT1, PAN* and *PTL* expressions were altered compared to wild type and single mutants, supp orting that these genes may be responsible for the abnormal phenotype of *suf4 fes1*. *clv1-1* mutants show increased number of sepals and stamens, suggesting that increase of sepals in *suf4 fes1* can be due to low expression of *CLV1* (Clark et al., 1995). *CLV3* is a gene acting in the same pathway with *CLV1*. *clv3* mutant shows increase in all floral organs (Clark et al., 1995). Similar to *clv3*, *ult1* has extra floral organs in all whorls (Fletcher, 2001), indicating that the functions of *CLV3, ULT1, SUF4* and *FES1* are overlapped in regulation of sepals and petals number.

When *PAN* is mutated, most flowers have five sepals, five petals, five stamens and two carpels (Chuang et al., 1999; Das et al., 2009). Because flowers of *suf4 fes1* showed increased number in sepals and petals and altered number of

stamens, there seems to be significant correlation between *pan* and *suf4 fes1*.

Lastly, *PTL* regulates second whorl organs, displaying loss of petals when it is mutated. *PTL* was highly expressed in *suf4 fes1*, suggesting that overexpressed *PTL* affects development of extra petals in *suf4 fes1*.

ChIP assay showed that SUF4 was enriched at *CLV1, ULT1, PAN* and *PTL* promoters, indicating that SUF4 can bind *CLV1, ULT1, PAN* and *PTL* genes. It is noteworthy that SUF4 regulates not only *FLC* but also other genes by direct binding. These results elucidate that the physical interaction between SUF4 protein and promoters of target gen es may regulate floral organ numbers.

Based on *suf4 fes1* mutant phenotypes and the ChIP analyses, we propose that SUF4 and FES1, two components of FRI-C, act redundantly on 1-3 whorl patterning by regulating *CLV1, ULT1, PAN* and *PTL*.

In future, phenotype analyses of triple mutants may allow the elucidation of the genetic pathway among these genes. If triple mutants with *clv1, ult1* and *pan,* generated from *suf4 fes1* double mutant, show additive effects, *SUF4* and *FES1* act on different pathway of flower development from these genes. Similarly, if *suf4 fes1 ptl* triple mutant rescues extra organ phenotypes of *suf4 fes1*, *SUF4* and *FES1* play a role for flower patterning independently from *PTL*. Once the mechanism of flower development regulation by SUF4 and FES1 is revealed, it may be the first example of additional function of the components of FRI complex.

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ABSTRACT IN KOREAN

식물은 주변의 환경 변화에 적응하고, 번식하기 위해 진화적으로 특별한 개화 기작을 발달시켰다. 애기장대의 경우 여름종과 겨울종에서 온도에 따라 다른 개화 반응을 보이는데, 애기장대 여름종은 계절에 상관없이 조기 개화가 이루어 지지만, 겨울종의 경우 추운 겨울 동안에는 꽃이 피는 것을 억제하고 있다가 이듬해 봄이 되면 개화가 시작되는 개화 지연반응을 보인다. 이러한 반응을 춘화라고 하는데, 여기에는 전사활성 인자 *FRIGIDA (FRI)*와 개화 억제 인자 *FLOWERING LOCUS C (FLC)*가 관여한다. 겨울종 애기장대에서 FRI가 다른 요인들과 복합체를 이뤄 *FLC*의 전사를 활성화 시킴으로써 개화를 억제하고 있다가, 겨울이 시작되면 *FLC*가 다른 후성 억제 인자들에 의해 발현이 억제되고, 그에 따라 이듬해 봄이 되면 개화가 이루어지게 된다.

본 연구에서 *FLC*가 억제되는 겨울 조건에서 FRI 복합체가 정상적으로 발현하는 것을 확인하였고, 저온 조건에서 FRI 복합체의 구성 요소 중 하나인 SUF4가 분리되는 것을 확인하였다. 유전자의 구조를 확인하는 실험을 통해 FRI와 SUF4가 *FLC* 유전자 루프 구조 형성에 영향을 끼쳐 겨울이 오기 전 *FLC*의 전사를 활성화 시키는 데에 기여하고, SUF4 분리에 의한 FRI 복합체의 구조 변화가 *FLC* 루프 구조의 붕괴를 매개하여 *FLC*의 전사 억제에도 관여할 것이라는 모델을 세우게 되었다.

또한 FRI 복합체가 *FLC* 활성에 대한 기능을 잃었음에도 불구하고, 세포 내에 남아있는 이유를 찾던 중에 FRI와 SUF4 단백질이 애기장대의 꽃 기관에서 높게 발현하는 것을 발견하였고, FRI 복합체의 구성 요소인 SUF4와 FES1이 없는 돌연변이에서 꽃받침과 꽃잎의 개수가 증가된 꽃을 생성하는 것을 확인하였다. 이들 꽃에서 *CLV1,*

*ULT1, PAN, PTL*과 같은 꽃 기관의 수를 결정하는 유전자들의 발현이 증가 혹은 감소되어있는 것을 확인하였고, 이를 통해 SUF4와 FES1이 FRI 복합체의 구성요소로써 *FLC*의 전사 활성 기능 외에 정상적인 수의 꽃 기관 발달에도 기능함을 알게 되었다.

*FLC*의 전사 활성 기능을 하는 것으로 알려져 있던 FRI 복합체가 구조 변화를 통해 *FLC* 유전자 구조 변화를 매개하여 *FLC*의 전사 억제에도 관여하고, FRI 복합체의 구성요소들이 정상적인 꽃 기관 발달에도 기능함을 발견하였으며, 이러한 결과들은 FRI 복합체 기능의 다양성과 애기장대에서의 또 다른 기능에 대한 가능성을 제시한다는 데에 본 연구의 의의가 있다.