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Regulatory mechanism of flowering time by the COP10-DET1-DDB1 complex in *Arabidopsis thaliana*

By

MIN YOUNG KANG

FEBRUARY, 2015

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY
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UNDER THE DIRECTION OF DR. NAM-CHON PAEK
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
BY THE COMMITTEE MEMBERS
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ABSTRACT

Most plants use day length to determine the optimal timing of flowering. Arabidopsis thaliana is a facultative long-day (LD) plant that flowers early in LD and late in short day (SD) conditions. The COP10-DET1-DDB1 (CDD) complex delays flowering and mutants of CDD components (i.e., det1-1, cop10-4, and ddb1a-2) flower early in LD, SD, or both. However, the regulatory functions of these CDD components remain unknown. Here we examine the molecular mechanisms by which the CDD complex represses flowering, using det1-1 mutants, which flower much earlier in SD. The det1-1 mutation alters the rhythm of mRNA abundance of FKF1, but not GI; this
causes their peaks to overlap during daytime in SD, leading to daytime expression of \textit{CO}. In addition, DET1 and COP10 interact with GI, and GI binding affinity to the \textit{FT} promoter increases in \textit{det1-1} mutants, suggesting that the CDD complex restricts GI function, which directly promotes \textit{FT} expression independently of \textit{CO}. Moreover, we found that DET1 induces \textit{FLC} expression possibly via histone modification of H3K4 and H3K27 by interaction with CUL4-CDD-MSI4/FVE. These results indicate that the CDD complex acts in both photoperiod and autonomous pathways to delay expression of floral integrators \textit{FT} and \textit{SOC1}. Consistent with this, the early flowering of \textit{det1-1} mutants disappears in the \textit{ft-1 soc1-2} background. Thus, we propose a model that DET1, likely in CDD form, suppresses flowering under SD by repressing daytime expression of \textit{FKF1} and GI binding activity to the \textit{FT} promoter, and also by epigenetically inducing expression of \textit{FLC}.

**Key words:** DET1; COP10; GI; \textit{FT}; \textit{FLC}; MSI4; Flowering time; Circadian dysfunction

**Student number:** 2007-23121
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INTRODUCTION

The appropriate timing of flowering is tightly linked to the success of reproduction in higher plants. The transition from vegetative to reproductive development is determined by intrinsic genetic programs and by various environmental factors, mainly day length and temperature. In particular, photoperiod provides a major cue for controlling flowering time through highly regulated genetic pathways, and light perception enables plants to synchronize initiation of flowering with seasonal changes in photoperiods (de Montaigu et al., 2010).

In Arabidopsis thaliana, several signaling components are involved in the regulatory circuit promoting photoperiodic flowering, including GIGANTEA (GI), CONSTANS (CO), and FLOWERING LOCUS T (FT) (Kardailsky et al., 1999; Park 1999; Suárez-López P et al., 2001). FT integrates multiple flowering pathways and FT protein is an essential component of florigen, the floral stimulus that moves long-distance from an induced leaf to the shoot apex and causes flowering (Kardailsky et al., 1999; Corbesier et al., 2007). CO directly regulates
expression of *FT* mRNA and CO mediates between the circadian clock and the control of flowering. CO is stabilized in the light and degraded in darkness by ubiquitin-mediated proteolysis (Suárez-López P et al., 2001; Valverde et al., 2004). Under LD photoperiods, *CO* expression coincides with light at the end of the day, and daytime expressed *CO* promotes *FT* expression to induce flowering. Under SD, however, as the peak time of *CO* transcriptional expression occurs after dusk, CO protein becomes unstable at dark, resulting in failure to induce *FT* expression (Yanovsky and Kay, 2002; Valverde et al., 2004; Liu et al., 2008; Jang et al., 2008).

GI, together with FLAVIN-BINDING, KELCH REPEAT, F-BOX PROTEIN 1 (FKF1), is an essential factor for the timing of *CO* expression. GI and FKF1 form complex to regulate *CDF1* (*CYCLING DOF FACTOR*), a key *CO* repressor. Under LD conditions, GI and FKF1 are expressed with the same peaks in the afternoon, and GI-FKF1 complex is recruited to the *CO* chromatin and in turn degrades CDF1 to activate *CO* expression (Sawa et al., 2007; Sawa et al., 2008). The diurnal expression of GI and FKF1 overlap less in SD than in LD, leading to minimal formation of the GI-FKF1 complex (Sawa 2008). This indicates that GI acts as a flowering inducer with FKF1 in the *CO-*
FT pathway in SD conditions. CO-independent flowering pathways have also been well studied; GI can directly activate FT expression by binding to its promoter region (Sawa and Kay 2011), indicating that GI has the ability, either directly or indirectly, to induce FT transcription in the photoperiod pathway. In addition to flowering time regulation, GI has a distinctive role in the circadian clock; GI is required to maintain circadian rhythms of LHY (LATE ELONGATED HYPOCOTYL) and CCA1 (CIRCADIAN CLOCK ASSOCIATED) genes, which are core factors of the plant circadian clock (Park et al., 1999; Mizoguchi et al., 2005). On the other hand, LHY and CCA1 control GI rhythm by binding to the promoter of GI (Niwa et al., 2007; Lu et al., 2012). In this process, DET1 acts as a critical transcriptional corepressor in the circadian clock (Lau et al., 2011). These reports present that GI has important dual function in regulation of circadian rhythms and promotion of flowering time in the photoperiodic pathway.

In addition to regulation by the photoperiod pathway, genes involved in the autonomous and vernalization pathways also control FT expression. FLOWERING LOCUS C (FLC) has a central place in the autonomous/vernalization pathway and directly regulates FT and SOC1 expression by binding to their promoters (Samach et al., 2000;
Hepworth et al., 2002; Searle et al., 2006). FLC expression is affected by many chromatin remodelers. MSI4/FVE, which belongs to the autonomous pathway, negatively regulates FLC expression via histone deacetylation of the FLC locus (Aisin et al., 2004). The MSI4/FVE protein is evolutionarily conserved in animals and plants. It is a homolog of Retinoblastoma-Associated Protein 46/48 (RbAp46/48) in human (Hennig et al., 2005). In the recently paper, MSI4/FVE interacts with DDB1 and HDA6, and mediates transcriptional silencing by histone modification of H3K4me3 (Gu et al., 2011) and H3K27me3 (Pazhouhandeh et al., 2011). This indicates that MSI4/FVE plays a significant role in FLC expression by making a complex with various chromatin remodeling factors.

DET1 was first identified among the CONSTITUTIVE PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA (COP/DET/FUS) gene family (Chory et al., 1989). Arabidopsis det1-1 mutant has de-etiolated phenotype under dark conditions, and has pleiotropic phenotype under light conditions, including increased anthocyanin, decreased chlorophyll, dwarf, and photoperiod-insensitive early flowering (Chory et al., 1989; Pepper and Chory, 1997). DET1 forms a complex with COP10 and DAMAGED DNA BINDING PROTEIN 1
(DDB1) to promote the activity of ubiquitin-conjugating enzymes (E2) for repression of photomorphogenesis in the ubiquitination pathway (Suzuki et al., 2002; Yanagawa et al., 2004). DET1 also acts as a pacemaker to adjust the period length of the circadian rhythm (Millar et al., 1995), possibly through the LHY-CCA1-DET1-COP10 interaction (Lau et al., 2011). DET1 acts as a flowering repressor, because det1-1 mutants flower slightly early under long day (LD) and extremely early under short day (SD) conditions (Pepper and Chory 1997). Despite recent advances in the understanding of DET1 function, the molecular mechanism causing early flowering in det1-1 mutants remains elusive.

Here we demonstrate that DET1 delays flowering by modulating the circadian rhythm and reducing GI activity in the photoperiod pathway. DET1 also epigenetically regulates FLC in the autonomous pathway. These effects, in turn, lead to reduced levels of FT and SOC1 transcripts. These findings provide new insights into how DET1 dynamically suppresses FT expression in both photoperiod and autonomous pathways to delay flowering under non-inductive SD conditions in Arabidopsis.
MATERIAL AND METHODS

Plant materials and growth conditions

All the *Arabidopsis thaliana* lines used in this study have Columbia (Col-0) genetic background. Flowering-time mutants were obtained from the Arabidopsis Biological Resource Center (USA), except for *det1-1* and *cop10-4*, which were kindly provided by Xing Wang Deng. *cry2-1* (CS3732), *gi-1* (CS3123), *soc1-2* and *ft-1* (Moon *et al.*, 2005), *fkf1-t* (Cheng and Wang 2005), and *co-101* (Takada and Goto 2003) were used for genetic analysis. To create double and triple mutants, F₁ heterozygotes were obtained by crossing the *det1-1* mutant as the female plant with other flowering-time mutants as pollen donors. To select correct transformants, the plants showing morphological phenotype of *det1-1* were first isolated among F₃ plants, and flowering-time mutations were finally confirmed by PCR-based genotyping. Plants were grown on soil at constant 22°C under white fluorescent light conditions (90-100 μmol m⁻²s⁻¹) in LD (16 h light/8h dark) and SD (10 h light/14h dark) or SD (8 h light/16h dark).
Analysis of flowering time

The bolting date was measured as the number of days from seed sowing to opening of the first flower and by counting the total number of rosette leaves at bolting. Data were obtained from three experimental replications (20 to 60 plants per replication).

RNA preparation and quantitative real-time PCR analysis

Tissue samples were collected every 3 h from 3-week-old seedlings. Total RNA was extracted with the plant RNA extraction kit (Macrogen). For each sample, 2 μl of total mRNA was reverse-transcribed using M-MLV reverse transcriptase (Promega). The level of the transcripts was measured by real-time PCR, using GoTaq qPCR Master Mix (Promega) and the Light cycler 2.0 instrument (Roche). Each PCR was repeated at least three times using biologically independent samples. The amount of each RNA level was determined using specific primers. The primers used for real-time PCR are listed in Table 2. For RNA of 18T, Tissue samples were collected every 2 h during daytime and every 3 h during
nighttime from 10-day-old seedlings under SD of 18T. Each PCR was repeated at least three times using biologically independent samples. The amount of each RNA level was determined using specific primers. The primers used for real-time PCR are listed in Table 1.

**Yeast two-hybrid assays**

The full-length cDNAs of *DET1, GI, PHYA, PHYB, CCT1, CCT2, CO, FKF1, FLC, SVP, TEM1*, and *TEM2* were amplified from wild-type total RNA using RT-PCR. GI was divided into three parts: GI N-terminal (aa 1-507), GI middle (aa 401-907), and GI C-terminal (aa 801-1173) regions. The PCR products were cloned into pGBKTK7 and pGADT7 vectors (MATCHMAKER GAL4 TWO-hybrid system 3, Clontech) to get the bait and prey clones. For the interaction study, plasmids containing fusion proteins were transformed into *Saccharomyces cerevisiae* AH109 and grown on media lacking adenine, leucine, histidine, and tryptophan. Galactosidase activity assays were performed according to the manufacturer’s protocol.
In Vivo Pull-down assays

TAP-DET1 and TAP-GFP were from Xing Wang Deng. pGI:GI-HA gi-2 det1-1 was obtained by crossing pGI:GI-HA gi-2 and det1-1. For DET1-GI binding assays, TAP-DET pGI:GI-HA gi-2 and TAP-GFP pGI:GI-HA gi-2 plants were grown on MS medium in SD (8h light/16h dark) for 10 days and then vacuum infiltrated for 7~10 min in 1X MS (Duchefa) liquid media supplemented with 50 mM MG132 (Sigma) for proteasome inhibitor treatment. After that, plants were incubated for 10 h under light conditions. These plants were homogenized and were isolated; total protein extract buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl$_2$, 1 mM EDTA (pH 8.0), 10% glycerol, 1 mM PMSF, 1 mM DTT]. These experiments were performed with IgG beads for TAP-IP. After washing, the immunoprecipitated fractions were analyzed by immunoblotting. The TAP-DET1 and GI fusion proteins were detected by using anti-HA antibody.

Bimolecular fluorescence complementation assays
Each cDNA of COP10, GI, ELF3, ELF4, DET1, and MSI4 was cloned into the BiFC gateway vectors (Citovsky et al., 2006) to examine their in vivo interactions. For partial YFP tagged ELF4, COP10, DET1, and MSI4 constructs, the cDNA of the gene was obtained by RT-PCR from WT (Col-0) plants and fused into four BiFC plasmid sets, such pSAT5-DEST-cEYFP(175-end)-C1(B) (pE3130), pSAT5(A)-DEST-cEYFP(175-end)-N1 (pE3132), pSAT4(A)-DEST-nEYFP(1-174)-N1(pE3134), and pSAT4-DEST-nEYFP(1-174)-C1 (pE3136), respectively. Partial YFP-tagged ELF3 and GI constructs were previously described (Yu et al., 2008). Each pair of recombinant plasmids encoding nEYFP and cEYFP fusions was mixed 1:1 (w/w), co-bombarded into onion epidermal layers using a DNA particle delivery system (Biolistic PDS-1000/He, BioRad), and incubated on MS solid media with MG132 (50 mM) for 16-24 h at 22°C under light or dark incubation, followed by observation and image analysis using a confocal laser scanning microscope (Carl Zeiss LSM710).

**Chromatin immunoprecipitation assay**

For the ChIP assay, Col-0, pGI:GI-HA gi-2, and pGI:GI-HA gi-2 det1-1
plants were grown for 10 days under SD (8 h light/16h dark) conditions and collected at ZT8. The samples were cross-linked with 1% formaldehyde, ground to powder in liquid nitrogen, and then sonicated (Cho et al. 2012). The sonicated chromatin complexes were bound with anti-HA antibody (ab9110, Abcam) for immunoprecipitation. The amount of DNA fragment was analyzed by quantitative real-time PCR (qPCR) using specific primers. *UBI10* was used as internal standard for normalization. The primers used for qPCR are listed in Table 2. For another ChIP assay, Col-0 and *det1-1* plants were grown for 14 days under SD (8 h light/16h dark) conditions and collected at ZT8. For immunoprecipitation, we used the anti-trimethyl H3K4 (07-473, Millipore), anti-trimethyl H3K27 (07-449, Millipore), and anti-acetyl H3 (06-599, Millipore). *FUSCA* and *Ta3* were used as internal standards for normalization (Pazhouhandeh et al., 2011). Experiments were performed with three biological repeats.

**Accession codes**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following
accession numbers: DET1, At4g10180; COP10, At3g13550; GI, At3g13550; FKF1, At1g68050; CO, At5g15840; FT, At1g65480; SOC1, At2g45660; PHYA, At1g09570; PHYB, At2g18790; CRY1, At4g08920; CRY2, At1g04400; SVP, At2g22540; TEM1, At1g25560; TEM2, At1g68840; TOE1, At2g28550; MSI4, At2g19520; FUS3, At3g26790; VRN2, AT4G16845; VIL1, AT3G24440; PIE1, AT3G12810; SNZ, AT2G39250; DCL1, AT1G01040; SE, AT2G27100; FLC, AT5G10140.
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### FLC promoters

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RESULTS

The det1 mutation alters the expression modes of flowering-time genes

To examine flowering-time regulation by the CDD complex, we used cop10-4 and det1-1 weak mutants, as null cop10 and det1 mutants are lethal. The cop10-4 and det1-1 mutants, and ddb1a-2 null mutants flower early (Chory and Peto 1990; Suzuki et al., 2002; Pazhouhandeh et al., 2011). To study the molecular mechanism by which the CDD complex functions in floral repression, we examined the plant shape and rosette leaf number at bolting of det1-1 and cop10-4 mutants (Figure 1). The two weak mutants were smaller and flowered earlier than wild type (WT), and the effect of det1-1 seemed to be more severe than that of cop10-4 or ddb1a-2 (Pazhouhandeh et al., 2011); det1-1 mutants developed to be smaller and flowered earlier than cop10-4 mutants in LD and SD. It indicates that DET1 acts as a floral repressor in SD and have a key role in maintaining the photoperiod sensitivity of flowering-time redulation in Arabidopsis. We thus chose det1-1 mutants
for molecular-genetic study to uncover the floral repressor function of the CDD complex.

The *det1-1* mutation causes period-shortening of clock-regulated gene expression; the internal circadian periods of *CAB2:LUC* (encoding a luciferase) expression in *det1-1* mutants were approximately 18 h and 21 h in continuous dark and light conditions, respectively (Millar *et al.*, 1995). To verify whether circadian-period shortening causes the extremely early flowering of *det1-1* mutants in SD (Table 2 and Figure 1), we examined if the flowering-time defect can be recovered when *det1-1* mutants were entrained in SD (light:dark = 1:2) under reduced diurnal cycles, i.e. environmental time period (T) = SD of 24T (8-h light:16-h dark), 21T (7-h light:14-h dark), and 18T (6-h light:12-h dark). Although both reduced diurnal cycles of 21T and 18T delayed flowering compared to normal cycles of 24T, *det1-1* mutants still flowered much earlier than WT under 24T (Figure 2). These data strongly suggest that internal period-shortening in *det1-1* mutants partially contribute to very early flowering under SD.

To investigate why *det1-1* mutants flower extremely early in SD (Table 2 and Figure 3), we next analyzed the expression modes of floral inducers by measuring the phases and amplitudes of *GI, FKF1, CO, FT,*
and \textit{SOC1} mRNA abundance, in WT and \textit{det1-1} mutants grown in SD (Figure 4). We collected plant tissues of 3 week-old WT and \textit{det1-1} mutants grown under SD conditions, in time course of every 3 hours. In WT, \textit{GI} expression peaks at ZT6 (zeitgeber time; 6 h after dawn) during daytime, but the peaks of \textit{FKF1} and \textit{CO} expression occur at ZT9 and ZT12 during nighttime, respectively, resulting in no \textit{FT} expression (Sawa \textit{et al.}, 2007). In \textit{det1-1} mutants, expression modes of \textit{FKF1}, \textit{GI}, \textit{CO}, \textit{FT}, and \textit{SOC1} are also rhythmic and \textit{GI} expression is not significantly altered compared with WT (Figure 4). However, the waveforms of \textit{FKF1} and \textit{CO} expression shifted 3 h and 6 h earlier than those in WT (Figure 4B and C). Accordingly, the peaks of \textit{GI}, \textit{FKF1}, and \textit{CO} expression occurred at ZT6 during daytime in SD. Thus, it appears that the daytime expression of \textit{CO} and light-stabilized \textit{CO} (Figure 4C) can activate \textit{FT} expression in \textit{det1-1} mutants under SD (Figure 4D). The waveform and peak time of \textit{SOC1} expression were not altered, but the mRNA abundance increased (Figure 4E), possibly due to daytime expression of \textit{CO} and/or increased expression of \textit{FT} (Figure 4C and D; Lee \textit{et al.}, 2000; Samach \textit{et al.}, 2000; Yoo \textit{et al.} 2005). To investigate a cause of delayed flowering in \textit{det1-1} mutant under reduced diurnal cycles, we further analyzed the expression
modes of *FKF1* and *CO* under SD of 18T (Figure 5B and C). In both WT and *det1-1* mutants, *FKF1* expression peaks the same time at ZT9 during nighttime and *CO* expression abundance is low during daytime. These data strongly suggest that internal period-shortening in *det1-1* mutants partially contribute to very early flowering under SD. However, although changed *FKF1* and *CO* expression modes, expressions of *FT* and *SOC1* mRNA are still expressed in *det1-1* mutants under SD of 18T (Figure 5D and E). Also, *FLC* mRNA expression level was still remarkably upregulated in *det1-1* mutants (Figure 5F). These results indicate that flowering is activated by other pathway(s) in *det1-1* mutant. Thus, we speculated that *det1-1* mutants flower extremely early in SD due to partially alterations in the *FKF1-GI-CO* module, which is attributed to a circadian defect, as reported previously (Lau et al., 2011).
Figure 1. Flowering-time phenotypes of det1-1 and cop10-4 mutant plants under LD and SD conditions.

(A) Phenotypes of wild-type (WT, Columbia-0 ecotype) and the mutant plants. Plants were grown at 22°C under cool-white fluorescent light (90-100 μmol m⁻²s⁻¹) in LD (16-h light:8-h dark) and SD (10-h light:14-h dark) conditions, and photographed at 2 to 4 days after bolting. Scale bars = 2 cm.

(B) Rosette leaves number of Col-0 and flowering mutants grown under LD (16-h light:8-h dark) and SD (10-h light:14-h dark) conditions (Table 1). Flowering time was measured as the number of
rosette leaves at bolting. Means and standard deviations were obtained from at least 20 plants.
Figure 2. Flowering time of *det1-1* mutants under reduced diurnal cycles.

(A) Effect of reduced diurnal cycles on the flowering time of *det1-1* mutants. Plants were entrained in SD (light [L]:dark [D] = 1:2) of 24 h (24T = 8L:16D), 21 h (21T = 7L:14D), and 18 h (18T = 6L:12D). T represents environmental time period. Means and standard deviations were obtained from at least 20 plants. Col-0 means Columbia-0 ecotype (wild type). (B) Phenotypes of *det1-1* mutants after bolting under SD of 24T, 21T, and 18T. Plants were grown at 22-24°C under cool-white fluorescent light (90-100 μmol m⁻² s⁻¹). Scale bars = 2 cm.
**DET1 is involved in both photoperiodic and autonomous pathways**

To test whether the activation of the *FKF1-GI-CO-FT* module in SD is responsible for the early flowering phenotype of *det1-1* mutants, we next examined the flowering-time phenotypes of the double mutants that combined *det1-1* with late-flowering mutations such as *cry2-1*, *fkf1-t*, *gi-1*, *co-101*, *ft-1*, and *soc1-2* (Figure 3 and Table 2). The *cry2-1 det1-1* double mutants flowered much earlier than the *cry2-1* single mutants in both LD and SD, suggesting that *DET1* acts downstream of *CRY2*. The *fkf1-t det1-1* and *co-101 det1-1* double mutants exhibited intermediate flowering time phenotypes compared to *fkf1-t*, *co-101*, and *det1-1* single mutants in both LD and SD, suggesting that although daytime expression of *CO* contributes to early flowering in SD, *det1-1* mutants can flower early in the absence of CO activity in both photoperiod conditions. In *gi-1 det1-1* and *ft-1 det1-1* mutants, the early-flowering effect of *det1-1* was almost abolished by *gi-1* or *ft-1* in both LD and SD (Figure 3 and Table 2), indicating that GI and FT play major roles in the *DET1*-mediated flowering pathway.

As *SOC1* expression is regulated by both photoperiod and
autonomous pathways (Hepworth et al., 2002), we further tested whether DET1 is also involved in the autonomous pathway using the soc1 mutant. We found that soc1-2 det1-1 double mutants showed intermediate flowering-time phenotypes in both LD and SD; also, in det1-1 ft-1 soc1-2 triple mutants, the early flowering effect of det1-1 completely disappeared (Figure 3 and Table 2). These results suggest that the regulation of flowering time by DET1 does not entirely depend on the FT-mediated photoperiod pathway, but also depends on the SOC1-mediated autonomous pathway. Thus, we further examined the expression of FLC, a major gene in the autonomous pathway, in det1-1 mutants. We found that the FLC mRNA abundance is very low in det1-1 mutants under SD (Figure 4F) and VIN3-LIKE 1 (VIL1), REDUCED VERNALIZATION RESPONSE 2 (VRN2), and PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1) mRNA abundance are not big different although it is slightly changed (Figure 6), suggesting that DET1 induces FLC expression to repress FT and SOC1. Taking these results together, we concluded that DET1 acts as a floral repressor in both the photoperiod and autonomous flowering pathways.
Figure 3. Flowering-time phenotypes of det1-1 and cop10-4 mutant plants under LD and SD conditions.

Genetic analysis to show epigenetic relationship between det1-1 and other flowering mutants using double (A) and triple mutants (B). Rosette leaves number of Col-0 and flowering mutants grown under LD (16-h light:8-h dark) and SD (10-h light:14-h dark) conditions in (A), and LD (16-h light:8-h dark) and SD (8-h light:16-h dark) conditions in (B) (Table 2). Flowering time was measured as the number of rosette leaves at bolting. Means and standard deviations were obtained from at least 20 plants.
Table 2. Effect of *det1* mutation on flowering time in different mutant backgrounds

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Figure 4. Effect of det1-1 mutation on GI, FKF1, CO, FT, SOC1, and FLC expression under SD.

(A-F) The expression of GI (A), FKF1 (B), CO (C), FT (D), SOC1 (E), and FLC (F) genes was analyzed in Col-0 and det1-1 mutants by real-time PCR using 3-week-old plants. Plants were grown at 22°C under SD (8-h light:16-h dark) conditions, and plant tissues were harvested every 3 hours. ACT2 expression was used for normalization. The results are mean values from three biological repeat experiments. Error bars indicate standard error of the mean. (G) Comparison of GI protein stability between pGI:GI-HA and pGI:GI-HA det1-1 mutants under SD.
conditions. The plant tissues were collected every 2 hours during daytime and every 4 hours during night-time using 3-week-old seedlings. GI protein was detected with anti-HA. RPT5 expression was used for normalization. Means and standard deviations were obtained from three biological replicates.
Figure 5. Effect of det1-1 mutation on GI, FKF1, CO, FT, SOC1, and FLC expression under 18T (6-h light:12-h dark).

(A-F) The expression of GI (A), FKF1 (B), CO (C), FT (D), SOC1 (E), and FLC (F) genes was analyzed in Col-0 and det1-1 mutants by real-time PCR using 10-day-old plants. Plants were grown at 22°C under SD (6-h light:12-h dark) conditions, and plant tissues were harvested every 2 hours during day-time and every 3 hours during night-time. ACT2 expression was used for normalization. Means and standard deviations were obtained from three biological replicates.
Figure 6. Effect of det1-1 mutation on VIL1, VRN2, and PIE1 expression under SD.

(A-C) The expression of VIL1 (A), VRN2 (B), and PIE3 (C) genes was analyzed in Col-0 and det1-1 mutants by real-time PCR using 3-week-old plants. Plants were grown at 22°C under SD (8-h light:16-h dark) conditions, and plant tissues were harvested every 3 hours. ACT2 expression was used for normalization. Means and standard deviations were obtained from three biological replicates.
**DET1 and COP10 interacts with GI in vivo**

In the photoperiod pathway, GI expression is not significantly altered in det1-1 mutants (Figure 4A) but the gi-1 mutation nearly abolished the early flowering effect of det1-1 in gi-1 det1-1 double mutants (Figure 3 and Table 2), which induces us to postulate that DET1 regulates GI at the post-translational level. Thus, we examined whether DET1 negatively regulates GI stability in transgenic plants expressing a tagged GI protein (pGI:GI-HA gi-2 and pGI:GI-HA gi-2 det1-1, see Methods) using anti-HA antibody. We collected plant tissues of 3 week-old the transgenic plants at intervals of 2 hour for daytime and 4 hour for nighttime under SD conditions. Our results revealed that det1-1 mutants had no significant alteration in the rhythmic accumulation of GI protein in SD (Figure 4G), indicating that the det1-1 mutation does not affect GI stability.

The CDD complex interacts with LHY and CCA1 and they regulate the circadian rhythms of expression of clock-regulated genes (Lau et al., 2011). This raises the possibility that the CDD complex negatively regulates GI activity through the physical interactions of CDD complex components and GI. To examine this, we first performed
yeast two-hybrid assays, which revealed that DET1 interacts with the N-terminal region of GI (aa 1-507) and COP10 interacts with the full-length, N-terminal, and C-terminal regions of GI (aa 801-1173) although DET1 did not interact with full-length GI (Figure 7). To examine the DET1 and other relative genes of circadian clock, we performed yeast two-hybrid assays, which revealed that DET1 does not interact with the light-input components PHYA, PHYB, CRY1 C-terminus (CCT1), or CRY2 C-terminus (CCT2), or floral inducers CO or FKF1 (Figure 9). To test the in vivo interaction of DET1 and GI, we performed bimolecular fluorescence complementation (BiFC) assays. Using a transient expression assay in onion epidermal cells, we detected the reconstituted YFP fluorescence in the nucleus when nYFP-DET1 and GI-cYFP plasmids were co-transformed. nYFP-ELF3 and cYFP-ELF4 plasmids served as a positive control (Fig. 8A). To further confirm the DET1-GI interaction in vivo, we tested whether GI and DET1 co-immunoprecipitate from transgenic plants expressing tagged proteins. To that end, we sampled the p35S:TAP-DET1 pGI:GI-HA gi-2 and p35S:TAP-GFP pGI:GI-HA gi-2 (a negative control) transgenic plants at ZT8 in SD, and used the antibodies for the TAP tag to pull down DET1. We found that HA-GI co-immunoprecipitated with TAP-
DET1, but not with TAP-GFP (Figure 8B). Also we checked interaction of GI and COP10, which forms complex with DET1 for repression of photomorphogenesis (Suzuki et al., 2002). To test the in vivo interaction of COP10-GI, we performed bimolecular fluorescence complementation (BiFC) assays. Using transient expression assays in onion epidermal cells, we detected reconstituted YFP fluorescence in the nucleus only when the nYFP-GI and cYFP-COP10 plasmids were co-transformed (Figure 8A). These results indicate that the CDD complex physically interacts with GI in the nucleus.
**Figure 7. DET1 and COP10 directly interact with GI in yeast.**

Interaction of DET1-GI was tested by the yeast two-hybrid assay. The baits was full-length DET1. For prey, GI was divided into three pieces: N-terminal (N; 1-507), middle (M; 401-907), and C-terminal (C; 801-1173). Gal4 indicates a positive control. Empty pGBK7 (BD) and pGADT7 (AD) vectors were the negative control. SD medium (-LWHA; lacking tryptophan, leucine, histidine, and adenine) was used to select for the interaction between bait and prey proteins. β-galactosidase activity assays were performed according to the manufacturer’s protocol. Means and standard deviations were obtained from three biological replicates.
Figure 8. DET1 and COP10 directly interact with GI in vivo.

(A) BiFC analysis of the interactions of nYFP-DET1/GI-cYFP and cYFP-COP10/nYFP-GI in the nucleus of onion epidermal cell. nYFP-ELF3 and cYFP-ELF4 plasmids served as a positive control. For negative control, empty nYFP, empty cYFP, GI-cYFP, nYFP-DET1, and cYFP-COP10 were used. Scale bar = 50 μm. (B) Coimmunoprecipitation of DET1 and GI. Total protein was extracted from 2-week-old seedlings of p35S:TAP-DET1 pGI:GI-HA gi-2 and p35S:TAP-GFP pGI:GI-HA gi-2. IgG beads were used for the pull-
down. An anti-HA antibody was used for GI-HA protein band. p35S:TAP-GFP pGI:GI-HA gi-2 plants served as a negative control. The upper panel is a coimmunoprecipitated sample, and the middle panel is input sample for GI-HA protein. The lower panel is input samples of p35S:TAP-GFP and p35S:TAP-DET1, respectively.
Figure 9. DET1 does not interact with relative genes of circadian clock in yeast two-hybrid.

DET1 does not interact with PHYA, PHYB, CCT1, CCT2, CO, and FKF1 in yeast two-hybrid assays. DET1 was used as bait in pGBK vector. For preys, PHYA, PHYB, CCT1, CCT2, CO, and FKF1 were used. Gal4 indicates a positive control. Empty pGBK7T (BD) and pGADT7 (AD) vectors were a negative control. SD medium (-LWHM; lacking medium of tryptophan, leucine, histidine, and adenine) was used for selection of the interaction between bait and prey proteins. β-galactosidase activity assays were performed according to the manufacturer’s protocol. Means and standard deviations were obtained from three biological replicates.
DET1 negatively regulates GI activity in a CO-independent pathway

As the det1-1 mutation does not alter GI mRNA expression (Figure 4A) or GI protein levels, but does cause early flowering in SD (Figure 4G), we hypothesized that in the photoperiod pathway, DET1 negatively regulates GI activity, which directly upregulates FT expression through a CO-independent pathway (Sawa and Kay 2011). To test whether the GI-FT module is affected by the det1-1 mutation, we performed chromatin immunoprecipitation (ChIP) assays, using the pGI:GI-HA gi-2 and pGI:GI-HA gi-2 det1-1 seedlings entrained in SD, to test whether det1-1 affects the ability of GI to bind to the FT promoter. Plant tissues were collected from 10-day-old seedlings at ZT8. To detect relative enrichment of the promoter regions, we used primers for six genomic regions of the FT promoter, as described previously (Sawa and Kay 2011). When the GI binding affinity to the FT promoter regions was compared, the amplicons close to the 5’-untranslated region (UTR) were significantly more enriched in det1-1 mutants (Figure 10B). This result strongly supports the notion that DET1 plays an important role in the suppression of FT transcription by preventing
GI binding to the *FT* promoter, and thus contributing to late flowering in SD conditions. FLC, SVP, TEM1, and TEM2 bind to the regions near 5’UTR in the *FT* promoter. In each single mutant, *FT* transcriptional expression is increased similar to the level of *det1-1* mutant (Lee et al. 2007; Castillejo and Pelaz 2008; Searle et al. 2006). To investigate the interaction of DET1 and these repressors, we performed yeast two-hybrid, which revealed that DET1 does not interact with FLC, SVP, TEM1, and TEM2 (Figure 11). In addition, in the *CO*-independent pathway, GI indirectly activates *FT* expression by up-regulating *microRNA 172* (*miRNA172*) which suppresses the expression of *SNZ* and *TOE1*, acting as *FT* repressor (Jung, Seo et al. 2007, Mathieu, Yant et al. 2009). We thus examined mRNA levels of *SNZ* and *TOE1* in *det1-1* mutants to examine whether compromised GI activity caused by *det1-1* mutation also affects the GI-miR172 module. It revealed that the expression levels of *SNZ* and *TOE1* were decreased by *det1-1*. Also, *DCL1* and *SE*, miRNA processing enzymes, were down regulated in *det1-1* mutants (Figure 12). These results suggest that processing of miRNAs would be regulated by GI. Taking these results together, we propose that DET1-COP10 complex is involved in downregulating the *FT* expression both by direct regulation of GI.
binding capacity to the \textit{FT} promoter and by indirect upregulation of \textit{FT} repressors in \textit{CO}-independent pathway.
Figure 10. DET1 mediates GI binding affinity to the FT promoter.

(A) Gene structure of FT and the amplicon regions for the ChIP assay. Six amplicon locations (I, II, III, IV, V and VI) are shown. (B) FT promoter binding affinity of GI in the det1-1 mutant, relative to the wild type. All samples were harvested at ZT8 under SD (8-h light:16-h dark) conditions. Chromatin isolated from these samples was immunoprecipitated with anti-HA. Relative enrichment in Col-0, pGI:GI-HA gi-2, and pGI:GI-HA gi-2 det1-1 are shown. Data are from an average of three independent experiments. UBIQUITIN 10 (UBI10) was used as a negative control. Black, gray, and white boxes represent Col-0, pGI:GI-HA gi-2, and pGI:GI-HA gi-2 det1-1, respectively. Asterisks indicate statistically significant differences compared to
pGI:GI-HA as determined by Student’s t-test (*P<0.05 and **P<0.01, respectively).
Figure 11. DET1 and COP10 do not interact with repressors of FT in a yeast two-hybrid assay.

DET1 and COP10 do not interact with FLC, SVP, TEM1, and TEM2 in yeast two-hybrid assays. DET1 and COP10 were used as bait in pGBK vector. For preys, FLC, SVP, TEM1, and TEM2 were used. Gal4 indicates a positive control. Empty pGBK7 (BD) and pGADT7 (AD) vectors were a negative control. SD medium (-LWHM; lacking medium of tryptophan, leucine, histidine, and adenine) was used for selection of the interaction between bait and prey proteins. β-galactosidase activity assays were performed according to the manufacturer’s protocol. Means and standard deviations were obtained from three biological replicates.
Figure 12. Effect of *det1-1* mutation on *SNZ, TOE1, DCL1* and *SE* expression under SD.

(A-D) The expression of *SNZ* (A), *TOE1* (B), *DCL1* (C) and *SE* (D) genes was analyzed in Col-0 and *det1-1* mutants by real-time PCR using 3-week-old plants. Plants were grown at 22°C under SD (8-h light:16-h dark) conditions, and plant tissues were harvested every 3 hours. *ACT2* expression was used for normalization. Means and standard deviations were obtained from three biological replicates.
DET1 epigenetically regulates \( FLC \) expression to delay flowering time in SD

In the autonomous pathway, \( FLC \) is a key floral repressor that is epigenetically controlled and downregulates the transcription of \( FT \) and \( SOC1 \) (Michaels and Amasino 1999, 2001; Michaels et al., 2005). As the transcript levels of \( FT \) and \( SOC1 \) are upregulated in \( det1-1 \) mutants under SD (Figure 4D and E), and \( FLC \) expression is almost absent in \( det1-1 \) mutants entrained in SD (Figure 4F), we reasoned that DET1 also functions to delay flowering in the autonomous pathway by upregulating \( FLC \) mRNA levels. A previous report showed that the CDD complex interacts with CUL4 (Chen et al., 2006), and the DDB1-CUL4 complex interacts with MSI4/FVE to induce \( FLC \) transcription (Pazhouhandeh et al., 2011). Thus, we asked if COP10 or DET1 interacts with MSI4 to form a CUL4-CDD-MSI4 complex for \( FLC \) induction. To test this notion, we examined the \textit{in vivo} interactions of MSI4-DET1 and MSI4-COP10 by BiFC assay (Figure 13). Strong YFP fluorescence was detected in the nucleus when the plasmids expressing DET1-nYFP/cYFP-MSI4 or nYFP-MSI4/COP10-cYFP were co-transformed, indicating that these CDD components interact with MSI4,
which directly binds to the *FLC* promoter to repress *FLC* expression.

Since MSI4 binds to the *FLC* promoter for histone modification of H3K27me3 and H3K4me3 at the *FLC* locus (Pazhouhandeh *et al.*, 2011, Gu *et al.*, 2011), we further examined the histone methylation levels on the *FLC* locus using anti-H3K27me3 and anti-H3K4me3 antibodies in the WT and *det1-1* mutants. The ChIP analysis revealed that *det1-1* mutants maintained higher levels of H3K27me3 and lower levels of H3K4me3 at the *FLC* locus than did WT (Figure 14), although H3Ace level is not changed (Figure 15), which is consistent with histone modification states observed in the early-flowering *hos1-3* mutants (Jung *et al.*, 2013). Taking these results together, we concluded that CUL4-CDD<sup>MSI4</sup> complex contributes to late flowering in SD by acting as an inducer of *FLC* expression through histone modification on the *FLC* locus in the autonomous pathway.
Figure 13. DET1 interacts with MSI4 and regulates histone methylation of the FLC locus.

(A) BiFC analysis of the interaction between MSI4 and DET1 in onion epidermal cells. For negative controls, nYFP/cYFP-MSI4 and DET1-nYFP/cYFP were used. (B) BiFC analysis of the interaction between MSI4 and COP10 in onion epidermal cells. For negative controls, nYFP-MSI4/cYFP, and nYFP/COP10-cYFP were used. Scale bar = 50 μm.
Figure 14. DET1 interacts with MSI4 and regulates histone methylation of the FLC locus.

Relative levels of histone modifications on the FLC locus were examined by ChIP analysis using H3K4me3 and H3K27me3 antibodies in Col-0 and det1-1. The top of the panel represents the FLC gene structure and the region used for primers (I, II and III) in the ChIP-quantitative PCR analyses. Chromatin was prepared from 14-day-old seedlings grown under SD (8-h light:16-h dark). FUSCA3 (FUS3) was used for the normalization of the quantitative PCR analysis. Means and standard deviations were obtained from three biological replicates. Asterisks indicate statistically significant difference compared to Col-0 as determined by Student’s t-test (*P < 0.05).
Relative levels of histone modifications on the \textit{FLC} locus were examined by ChIP analysis using H3Ace antibodies in Col-0 and \textit{det1-1}. The top of the panel represents the \textit{FLC} gene structure and the region used for primers (I, II and III) in the ChIP-quantitative PCR analyses. Chromatin was prepared from 14-day-old seedlings grown under SD (8-h light:16-h dark). \textit{FUSCA 3} (\textit{FUS3}) was used for the normalization of the quantitative PCR analysis. Means and standard deviations were obtained from three biological replicates.

Figure 15. DET1 interacts with MSI4 and regulates histone acetylation of the \textit{FLC} locus.
Figure 16. Working model of DET1 for flowering regulation in *Arabidopsis*. DET1, likely in CDD complex form, suppresses *FT* and *SOC1* expression via multiple routes in the photoperiod and autonomous pathways. In the photoperiod flowering pathway, DET1, possibly in a complex with CCA1 and LHY, functions in floral repression by modulating GI-mediated floral induction at the transcriptional and post-translational levels during daytime under SD. First, DET1 blocks FKF1-GI complex formation by repressing daytime
expression of *FKF1* to suppress daytime *CO* expression in a *CO*-dependent pathway. Second, DET1 represses the function of daytime-expressed GI by preventing GI from binding to the *FT* promoter in a *CO*-independent pathway. In the autonomous pathway, DET1 modulates trimethylation of *FLC* chromatin to induce *FLC* expression. Collectively, this model proposes that DET1 delays flowering by modulating circadian rhythmicity of *FKF1* and reducing GI activity in the photoperiod pathway and by epigenetic regulation of *FLC* in the autonomous pathway, which in turn leads to the reduced level of *FT* and *SOC1* transcripts. Genes and proteins are represented as rectangles and ovals. Dotted lines represent possible relationships that require further study.
DISCUSSION

DET1 is involved in repression of photomorphogenesis in the ubiquitination pathway (Suzuki et al., 2002; Yanagawa et al., 2004; Nixdorf and Hoecker 2010), light-response regulatory pathway (Pepper and Chory 1997), and circadian period (Millar et al., 1995; Lau et al., 2011). However, the function of DET1 in the regulation of flowering time remains elusive. In this study, we provide several pieces of evidence showing how DET1 regulates the suppression of FT and SOC1 expression to delay flowering under SD conditions. The det1-1 mutation caused altered rhythmic expression of FKF1 (Figure 4B), compromised GI activity (Figure 10), and affected epigenetic silencing of FLC expression (Figure 14), all of which led to reduced FT transcript levels. Thus, we propose a model of the regulatory role of DET1 in the repression of FT expression via both CO-dependent and -independent pathways (Figure 16).

In this study, we showed that gi-1 and ft-1 partially suppressed the early flowering of det1-1 mutants and DET1 directly interacts with GI in vitro and in vivo (Figure 7 and 8). However, DET1 does not interact
with other circadian-clock components, such as PHYA, PHYB, CRY1 C-terminus (CCT1), or CRY2 C-terminus (CCT2), CO, and FKF1 (Figure 9), indicating that DET1 has a unique role in the post-translational regulation of GI in the photoperiod pathway. In addition, we found that COP10 also interacts with GI in yeast two-hybrid and BiFC assays (Figure 7 and 8). These in vitro and in vivo results show that the CDD complex interacts with GI for negative regulation of flowering. A previous study revealed that EARLY FLOWERING 4 (ELF4), one of the circadian-clock components (Doyle et al., 2002), acts downstream of GI (Kim et al., 2012). ELF4 represses GI binding to the CO promoter to control flowering, (Kim et al., 2013). Our results revealed that co-101 det1-1 mutants showed intermediate flowering-time phenotypes, but in det1-1 ft-1 mutants, the early flowering phenotype of det1-1 almost disappeared under LD (Figure 3), indicating that DET1 function mainly depends on FT for regulation of flowering. Thus, we hypothesized that DET1 regulates GI binding to the FT promoter to suppress flowering. We showed that GI binding activity to the FT promoter significantly increased in the det1-1 background (Figure 10). This result indicates that FT expression is repressed by DET1 via direct regulation of GI affinity for binding to the
COP1 interacts with GI to control GI stability (Yu et al., 2008). Also, COP1 directly interacts with COP10, possibly with the CDD complex, to repress photomorphogenesis (Suzuki et al., 2002). Thus, this indicates that the CDD complex functions with COP1 in flowering regulation, although we have no direct evidence because the det1-1 cop1-4 double mutant was lethal (Data not shown, Ang and Deng, 1994). However, our findings revealed that DET1 does not directly regulate GI stability (Figure 4G), while COP1 interacts with GI in the presence of ELF3, leading to GI degradation (Yu et al., 2008). Therefore, although DET1 and COP1 have very similar mutant phenotypes and post-translational behavior, they function by distinct molecular mechanisms in the regulation of GI function.

Other negative regulators of FT transcription, including FLC, SVP, TEM1, and TEM2, bind to the regions near the 5`UTR of FT. In each single mutant, FT mRNA expression increases to levels similar to those seen in det1-1 mutants (Lee et al., 2007; Castillejo and Pelaz 2008; Searle et al., 2006). Notably, SVP, TEM1, and TEM2 interact with GI to regulate FT expression, although the regulatory function of their interaction is not clearly understood (Sawa and Kay 2011). Therefore, it
is possible that DET1 may be involved in the function of these $FT$ repressors. To investigate the possibility that the CDD complex positively regulates the $FT$ repressors, we examined the interaction of DET1 and COP10 with these four $FT$ repressors by yeast two-hybrid assays, indicating that CDD complex independently regulates $GI$-$FT$ cascade from these known $FT$ repressors (Figure 11). It has been reported that GI regulates the TARGET OF EAT1 ($TOE1$) through microRNA172 ($m$i$R$172) to repress $FT$ expression in the $CO$-independent pathway (Jung et al., 2007). We thus examined mRNA levels of $TOE1$, $SCHNARCHZAPFEN$ (SNZ), $DICER-LIKE 1$ (DCL1), and $SERRATE$ (SE) in $det1$-$1$ mutants as another example affected by GI in the $CO$-independent pathway. It revealed that the mRNA expression levels of $TOE1$, $SNZ$, $DCL1$, and $SE$ were downregulated in $det1$-$1$ mutants comparing WT (Figure 12). Taking these results together, we propose that the CDD complex negatively regulates $FT$ expression through GI protein in the $CO$-independent pathway.

In addition, we revealed that DET1 regulates the expression of $FLC$, a key component in the autonomous pathway. We showed that the $FLC$ mRNA level decreased remarkably and histone modification levels of H3K4 and H3K27 were altered in $det1$-$1$ mutants (Figure 4F
and 14), as observed in the early flowering *hos1-3* mutants (Jung *et al.*, 2013). Furthermore, we found that in addition to DDB1 among three component of the CDD complex, DET1 and COP10, also interact with MSI4/FVE, which represses *FLC* expression in the autonomous pathway (Jung *et al.*, 2013). A recent study showed that MSI4/FVE interacts with HDA6 and directly binds to the *FLC* locus to repress *FLC* transcription in chromatin remodeling (Gu *et al.*, 2011). Therefore, further analysis is necessary to reveal whether the CUL4-CDD-MSI4/FVE complex acts together with HDA6 in epigenetic silencing of *FLC*. *FLC* negatively regulates not only *FT* but also the downstream factor *SOC1*, which encodes a MADS box transcription factor (Lee and Lee, 2010). In genetic analysis, *ft-1* showed almost-complete epistasis to *det1-1*. By contrast, in *det1-1 ft-1 soc1-2* triple mutants, the early flowering effect of *det1-1* was completely suppressed; consistent with this, *SOC1* expression was downregulated in *det1-1* mutants (Table 2, Figure 3 and 4E). This supports the notion that DET1 suppresses both *FT* and *SOC1* via promoting *FLC* expression in the autonomous pathway.

The *det1-1* mutants flowered extremely early and this phenotype was partially suppressed in the *fkf1-t* and *co-101* mutant background.
(Figure 3 and Table 2), which implies that DET1 may function independent of the FKF1-CO module. However, we found that the peak of the FKF1 circadian rhythm was shifted 3 h earlier, while that of GI showed a slight, non-significant shift to the earlier time in det1-1 mutants (Figure 4A and B). In previous studies, the circadian phase of TOC1 shifted 4 h earlier in det1-1 mutants and the circadian shift of GI was much less than that of TOC1 on the first day under continuous light conditions (Lau et al., 2011). A similar pattern of gene expression was observed in lhy-12 mutants, in which the peak time of TOC1 expression clearly shifted earlier, but the GI peak did not shift (Mizoguchi et al., 2002). In lhy cca1 double mutants, the expression phase of TOC1, GI, and FKF1 is more severely shifted (Mizoguchi et al., 2002; Niwa et al., 2007). This suggests that DET1 likely functions in a complex with LHY/CCA1 to regulate circadian rhythms of evening genes such as FKF1, TOC1 and GI. In addition, the early flowering effect of the lhy-11 cca1-1 double mutant was completely suppressed in the gi-3 background (Mizoguchi et al., 2005), implying that DET1, likely in a complex with LHY/CCA1, regulates GI binding affinity to the FT promoter to repress flowering. To reveal whether LHY/CCA1 participate in the mechanism of DET1-mediated regulation of GI
activity, further analysis is needed, such as analysis of det1-1 lhy double mutants.

Notably, the early-flowering and photoperiod-insensitive phenotypes of det1-1 are quite similar to those of lhy-11 cca1-1 double mutants, whereas the pattern of circadian gene expression in det1-1 is similar to that of lhy-12 single mutant (Green and Tobin 1999; Mizoguchi et al., 2002; Niwa et al., 2007). This discrepancy suggests that the floral repression function of DET1 is suppressed in another pathway independent of the LHY/CCA1-mediated photoperiod flowering pathway in det1-1 mutants. Indeed, the expression level of FLC, a key gene in the autonomous pathway, was remarkably downregulated in det1-1 (Figure 4F), whereas no significant difference in FLC expression was observed in either CCA1-OX plants or lhy cca1 mutants, compared to the wild type (Fujiwara et al., 2008; Lu et al., 2012). Thus, we reason that DET1 regulates circadian rhythms of evening genes such as FKF1, TOC1 and GI, possibly in a complex with LHY/CCA1, in the photoperiod flowering pathway, and DET1 also regulates FLC expression independently of the LHY/CCA1 complex in the autonomous pathway to repress flowering.

Circadian expression of FKF1 and GI is critical to promote
flowering. In LD, the peaks of expression of $FKF1$ and $GI$ coincide in the afternoon, which enables the formation of maximum amounts of $FKF1$-$GI$ complex, leading to floral promotion by inducing daytime expression of $CO$ (Sawa et al., 2007). In SD, however, $FKF1$ expression does not coincide with $GI$ because $GI$ expression peaks about 3 h earlier than the peak of $FKF1$ expression (Sawa et al., 2007). Interestingly, in $det1-1$ mutants under SD, the peak time of $FKF1$ expression coincides with that of $GI$ expression (Figure 4A and B). This coincident expression increases $FKF1$-$GI$ complex formation and thereby induces daytime $CO$ expression even under SD. In $det1-1$ mutants, the $FKF1$ peak time shifted to an earlier phase compared with the wild type. This result suggests that DET1 regulates circadian expression of $FKF1$ especially under SD, and the early shift of $FKF1$ expression appears to partially contribute to early flowering and the photoperiod insensitive phenotype of $det1-1$ mutants.

Examining these data, we propose a model for the molecular mechanism by which DET1 represses flowering in non-inductive SD conditions. In wild type, the absence of $FT$ expression under SD can be explained by the incongruity of peak expression of $FKF1$ and $GI$; $GI$ peaks in the late afternoon but $FKF1$ peaks at night, leading to reduced
CO and FT expression during the daytime. As GI also directly induces FT expression in a CO-independent pathway, we wondered why GI, which expresses in the late afternoon (Sawa et al., 2007, Sawa and Kay 2011), is not capable of inducing FT expression under SD. In this study we found that DET1 suppresses the induction of FT transcript by repressing GI binding to the FT promoter (Figure 10). Therefore, we reasoned that DET1 suppresses flowering by blocking FT expression via two photoperiod pathways under SD. First, DET1 blocks formation of the FKF1-GI complex by repressing daytime-expression of FKF1, which eventually suppresses CO and FT expression in a CO-dependent pathway. Second, DET1 represses the function of daytime-expressed GI by restraining GI from binding to the FT promoter in a CO-independent pathway. In other words, DET1 functions in floral repression by modulating GI-mediated floral induction at the transcriptional and post-translational levels during daytime under SD. This model is further supported by genetic analysis showing that gi-1 and ft-1 are partly epistatic to det1-1 (Figure 3), indicating that DET1 mainly regulates flowering via GI. Therefore, it is likely that COP1 degrades GI and CO proteins during the night (Jang et al., 2008, Yu et al., 2008) and DET1 suppresses GI action during the day to inhibit flowering under SD.
In conclusion, we propose a new function of DET1 as a floral repressor in the photoperiodic and autonomous flowering pathways (Figure 16). DET1 suppresses FT expression by modulating the circadian rhythmicity of FKF1 in a CO-dependent pathway; DET1 also controls GI activity and epigenetically regulates FLC in a CO-independent pathway. DET1 may act in a complex with COP10 and DDB1 in these floral induction pathways, and CCA1 and LHY are likely to be involved in CDD function in the photoperiod pathway. Further analysis of the involvement of the LHY/CCA1 complex in the DET function will provide more insights into the mechanisms of CDD-mediated floral regulation.
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국문 초록

식물에게 낮의 길이는 적절한 개화시기를 결정하기 위한 중요한 요소 중의 하나이다. 애기장대는 장일조건에서 꽃이 빨리 피고 단일조건에서 꽃이 늦게 피는 장일 식물이다. 광형태 형성 억제자로 알려진 DE-ETIOLATED 1 (DET1)의 돌연변이체인 det1-1 은 장일조건에서는 약간 이른개화를 보이지만, 단일 조건에서 매우 빠른 개화시기를 보인다. 즉, DET1 은 광주기 둔감성 개화시기를 보이며 개화를 지연하는 개화억제자로서 작용하고 있음을 알 수 있었다. 또한, 하루 시간을 24 시간이 아닌 18 시간으로 줄여서 개화시기 결정해 보았을 때 det1-1 돌연변이체의 개화시기가 느려지는 것을 확인하였으며 이것은 det1-1 돌연변이체의 조기 개화의 한 가지 원인이 생체리듬 장애 때문임을 밝혔다. 그러나 아직까지 DET1 이 개화에 미치는 정확한 영향에 대해서는 잘 알려져 있지 않다. 조기 개화의 정확한 원인 분석을 위해 광주기성 경로에 관련된 유전자의 돌연변이체들과의 교배를 통해 유전학적 분석을 하였다. 그
결과, DET1 이 광주기성 경로뿐만 아니라 자발적 경로에서도 작용하고 있음을 알 수 있었다.

DET1의 개화억제자로서의 역할을 밝히기 위해 분자유전학적 분석을 하였다. 단일조건에서 매우 이른 조기개화를 보이는 det1–1 돌연변이체에서 GI mRNA 발현양과 패턴에는 변화가 크게 없지만 FKF1의 mRNA 발현 패턴이 낮시간의 증가하는 현상을 보였다. 이로 인해 낮시간의 GI–FKF1 복합체 형성이 증가하고 결국 CO mRNA 발현양이 증가하였다. 이 증가된 CO는 개화 조절에 메인 유전자로 알려진 FT mRNA 발현을 유도하여 개화를 촉진하게 된다. DET1 단백질은 GI 단백질과 직접 결합하여 GI 단백질의 FT 프로모터 결합정도를 억제하여 FT mRNA 발현을 저해시킨다는 것을 발견하였다. 또한, DET1은 MSI4/FVE와 결합하여 H3K4와 H3K27 메틸레이션을 통해 자발적 경로에서 중요한 작용을 하는 유전자로 알려진 FLC mRNA 발현을 유도한다는 것을 확인하였다. 이 모든 기능을 수행함에 있어서 DET1과 함께 CDD 복합체를 형성한 다고 알려진 COP10 또한 같은 일을 수행하고 있음을 GI와 MSI4 단백질과의 상호결합을 통해 보여주었다. 이런 실험결
과를 토대로 광주기성 경로에서는 CDD 복합체가 FKF1 발현 패턴을 조절하고 GI 단백질과 결합을 통해 GI 단백질의 FT 프로모터에 결합하는 정도를 억제하며 자발적 경로에서는 MSI4/FVE 단백질과 결합하여 후생유전학적 조절을 통해 FLC 발현을 유도하여 FT와 SOC1 발현을 억제하는 개화 역제자로서의 역할을 하는 새로운 모델을 제시하였다.

주요어: DET1, COP10, GI, MSI4, FT, FLC, 개화, 생체시계, 광주기성 경로, 자발적 경로
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