The DELLA-CONSTANS Transcription Factor Cascade Integrates Gibberellic Acid and Photoperiod Signaling to Regulate Flowering[1]

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Gibberellin (GA) and photoperiod pathways have recently been demonstrated to collaboratively modulate flowering under long days (LDs). However, the molecular mechanisms underlying this collaboration remain largely unclear. In this study, we found that GA-induced expression of *FLOWERING LOCUS T* (*FT*) under LDs was dependent on CONSTANS (CO), a critical transcription factor positively involved in photoperiod signaling. Mechanistic investigation revealed that DELLA proteins, a group of crucial repressors in GA signaling, physically interacted with CO. The DELLA-CO interactions repressed the transcriptional function of CO protein. Genetic analysis demonstrated that CO acts downstream of DELLA proteins to regulate flowering. Disruption of CO rescued the earlier flowering phenotype of the *gai-t6 rga-t2 rgl1-1 rgl2-1* mutant (*dellap*), while a gain-of-function mutation in *GA INSENSITIVE* (*GAI*, a member of the *DELLA* gene) repressed the earlier flowering phenotype of *CO*-overexpressing plants. In addition, the accumulation of DELLA proteins and mRNAs was rhythmic, and REPRESSOR OF GA1-3 protein was noticeably decreased in the long-day afternoon, a time when CO protein is abundant. Collectively, these results demonstrate that the DELLA-CO cascade inhibits CO/FT-mediated flowering under LDs, which thus provide evidence to directly integrate GA and photoperiod signaling to synergistically modulate flowering under LDs.

To maximize reproductive success and seed production, plants determine the most appropriate time to flower by monitoring internal and external environment changes. In Arabidopsis (*Arabidopsis thaliana*), approximately 180 genes are involved in flowering-time control. These occur in a network of six flowering pathways: the vernalization pathway, the photoperiod pathway, the gibberellin (GA) pathway, the age pathway, the autonomous pathway, and the ambient temperature pathway (Fornara et al., 2010). The photoperiod pathway monitors seasonal changes in day length to regulate flowering time (Fornara et al., 2010; Song et al., 2013). The vernalization and ambient temperature pathways control flowering in response to changes in temperature (Fornara et al., 2010; Kim et al., 2009). The GA, autonomous, and age pathways affect flowering time in response to the internal developmental status (Fornara et al., 2010; Kim et al., 2009).

As a long-day (LD) plant, flowering of Arabidopsis is accelerated by LDs and delayed by short-day (SD) conditions, which controls the photoperiod pathway. Numerous studies have defined the core of the photoperiod pathway comprises GIGANTEA (GI), CYCLING DOF FACTORs (CDFs), CONSTANS (CO), and FLOWERING LOCUS T (FT; Fornara et al., 2010; Kobayashi and Weigel, 2007; Fornara et al., 2009). At the end of the day, light promotes GI interaction with the F-box ubiquitin ligase FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1), increasing its stability under LDs (Song et al., 2014; Sawa et al., 2007). SCF^{FKF1} degrades CDFs, which are a family of transcription factors that repress flowering by down-regulating *CO* expression in the leaves, through the 26S proteasome (Sawa et al., 2007; Imaizumi et al., 2005). CO directly activates transcription of the *FT* gene, which encodes a floral-inductive long-distance signal and thus determines flowering time (Samach et al., 2000; Corbesier et al., 2007). Besides being regulated by the photoperiodic pathway, expression of *CO* is also modulated by the circadian clock, with its mRNA peaking late in the day (Suárez-López et al., 2001). At the posttranscriptional level, CO protein stability is regulated by various signaling pathways. For example, the blue-light photoreceptor Cryptochrome and red/far-red light photoreceptor Phytochrome A stabilize CO in the evening, whereas the red/far-red light photoreceptor

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Phytochrome B facilitates CO degradation in the morning or in darkness (Valverde et al., 2004). In addition, the RING finger E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC1 promotes ubiquitin-mediated proteolysis of CO in darkness (Liu et al., 2008; Jang et al., 2008). Moreover, FKF1, ZEITLUPE, GI, and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 are also involved in CO stability (Song et al., 2012, 2014; Lazaro et al., 2012).

GA are a class of critical plant hormones that function as essential growth regulators to mediate diverse aspects of developmental processes (Sun and Gubler, 2004; Fleet and Sun, 2005; Sun, 2008). Genetic screening has identified several molecular components involved in GA perception and signaling: the GA receptors GA INSENSITIVE DWARF1 (GID1a, b, and c), a group of repressor proteins DELLA (GA INSENSITIVE [GAI], RGA, RGA-LIKE1 [RGL1], RGL2, and RGL3), and the F-box ubiquitin ligase SLEEPY1 (SLY1; Peng et al., 1997; Silverstone et al., 1998; Dill and Sun, 2001; McGinnis et al., 2003; Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006). GID1 and SLY1 (as an SCF^{SLY1} complex) recruit DELLA proteins for ubiquitination and degradation, leading to activation of various transcriptional factors in the presence of GA (Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006; Dill et al., 2004; Harberd, et al., 2009; Claeys et al., 2014). Recently, several studies demonstrated that GA are essential for floral induction under both SDs and LDs. Mutations affecting GA synthesis fail to flower in SDs but show relatively weak late-flowering phenotypes under LDs (Wilson et al., 1992), suggesting that GA play their most important function in flowering under SDs. However, recent studies strongly indicated that GA are also involved in flowering time control under LDs. For example, GA have critical roles in promoting the transcription of *FT*, *TWIN SISTER OF FT* (*TSF*), and *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) genes in response to LDs (Galvão et al., 2012; Porri et al., 2012; Posé et al., 2012). The *gid1a*, *b*, and *c* triple mutant exhibited a remarkably late flowering phenotype under LDs (Griffiths et al., 2006; Willige et al., 2007). Additionally, DELLA proteins directly bind to SPLs and inhibit transcriptional activation of MADS box genes and *miR172* under LDs (Yu et al., 2012).

Accumulating evidence has indicated that the photoperiod and GA pathways coordinate to modulate flowering under LDs (Galvão et al., 2012; Porri et al., 2012; Reeves and Coupland, 2001; Hou et al., 2014; Nguyen et al., 2015). Very recently, Xu et al. (2016) reported that DELLA proteins physically interact with CO, indicating a direct association between the photoperiod and GA pathways. However, the biological significance of the DELLA-CO physical interactions remains largely unclear. Further molecular and genetic investigations are needed to elucidate the exact molecular mechanisms underlying the photoperiod and GA signaling to synergistically modulate flowering. In this study, we found that GA-induced expression of *FT* was compromised in the *co-2* mutant under LDs. DELLA proteins were found to directly interact with the CO transcription factor and repress its transcriptional activity. Additional genetic analysis revealed that CO acts downstream of DELLA proteins and that DELLA represses flowering partially through CO. Thus, we propose that DELLA proteins act as upstream components of CO to modulate flowering under LDs.

RESULTS

GA-Induced Expression of *FT* Is Dependent on CO

Previous studies have indicated that DELLA proteins act as repressors of flowering and that their GA-dependent degradation contributes to induction of flowering under LDs (Galvão et al., 2012). Molecular and genetic analysis has shown that GA and DELLA regulate flowering partially through modulating the expression of *FT* (Galvão et al., 2012; Porri et al., 2012). Consistent with previous studies, we also noticed that *FT* transcripts were strongly reduced in a GA-deficient mutant *ga1* (Columbia-0 [Col-0]), and those reductions can be fully rescued by exogenous GA3 (Fig. 1A; Hou et al., 2014). These findings collectively suggest that GA positively regulates the expression of *FT*; however, little is known of how GA affect *FT* expression. Samach et al. (2000) demonstrated that the CO protein is a crucial positive regulator for *FT* expression under LDs. We thus queried whether GA-induced expression of *FT* under LDs required CO. To test this possibility, we analyzed the expression of *FT* in *co-2* mutant and wild type (Landsberg

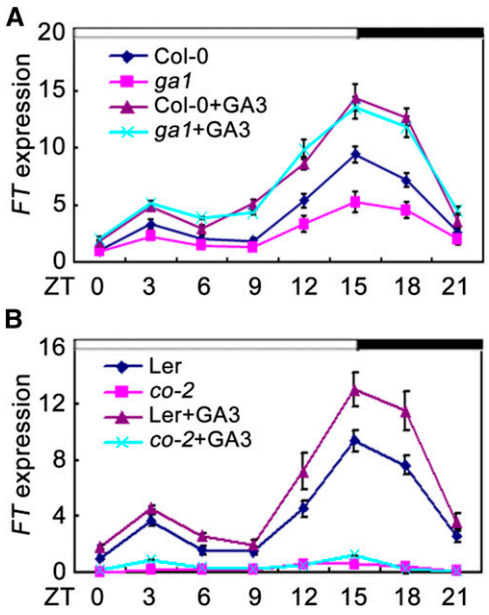

Figure 1. GA-induced expression of *FT*. qRT-PCR analysis of *FT* expression in response to GA3 in *ga1* mutant (A) and in *co-2* mutant (B) under LDs. The *IPP2* gene was used as an internal control. Total RNA was extracted from 10-d-old seedlings. Time is expressed as hours from dawn. Error bars indicate SD from three independent RNA extracts.

erecta [Ler]) in response to GA3. As expected, the expression levels of *FT* were induced by GA3 in wild type; however, exogenous application of GA3 was almost unable to induce *FT* expression in the *co-2* mutant plants (Fig. 1B). These observations indicated that the GA-induced expression of *FT* is dependent on CO.

DELLA Physically Interact with CO

Having confirmed that CO may be involved in GA signaling-regulated flowering, we sought to determine how CO participates in GA signaling. Consistent with previous studies, we also found that *CO* transcription and CO protein abundance were not regulated by the GA pathway (Supplemental Fig. S1; Galvão et al., 2012; Xu et al., 2016). Recently, several transcription factors have been found to physically interact with DELLA repressors to regulate various GA-mediated physiological processes (Yu et al., 2012; Davière et al., 2008; de Lucas et al., 2008; Feng et al., 2008; Hong et al., 2012; Wild et al., 2012). Therefore, we hypothesized that CO may physically interact with DELLA proteins to mediate GA-signaled flowering. To test this possibility, we fused the CO protein with deleted activation domains (deleted amino acids 1-175) to the Gal4 DNA-binding domain of the bait vector (BD-CO-CT), and introduced the full-length coding sequences of DELLA proteins into the prey vector (AD-DELLA). Then, the interactions between CO and DELLA proteins were assayed using the yeast two-hybrid system. As shown in Figure 2A, CO protein interacted with all five DELLA proteins in yeast cells. We also confirmed the interaction of CO with RGA by performing in vitro pull-down assay. The pull-down results showed that the GST-fused CO could retain His-RGA, whereas GST alone could not (Fig. 2B). In agreement with our results, Xu et al. (2016) showed the DELLA protein GAI physically interacts with CO in yeast cells and binds to CO in vitro.

To further determine whether CO interacts with DELLA proteins in plant cells, we used the bimolecular fluorescence complementation (BiFC) assay for analysis. Full-length DELLA proteins were fused to the C-terminal region of yellow fluorescent protein (DELLA-cYFP), and full-length CO protein was fused to the N-terminal region of YFP (CO-nYFP). When CO-nYFP was coinfiltrated with DELLA-cYFP in tobacco (*Nicotiana tabacum*) leaves, strong YFP fluorescence was detected in the nuclei (Fig. 2C; Supplemental Fig. S2). No fluorescence was detected in the negative controls (Fig. 2C; Supplemental Fig. S2). In addition, interaction

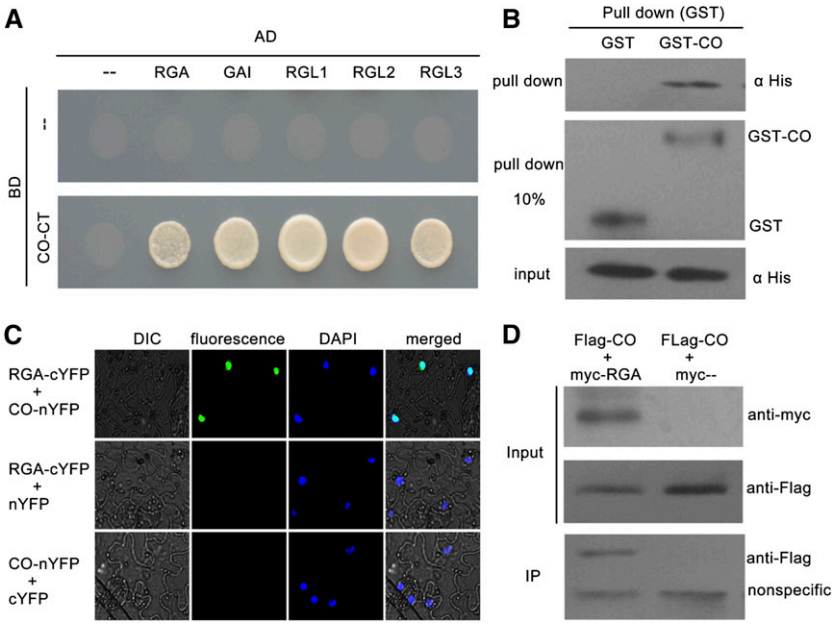

Figure 2. Physical interactions between DELLA proteins and CO. A, Yeast two-hybrid (Y2H) analysis of DELLA-CO interactions. Interaction was indicated by the ability of cells to grow on selective media lacking Leu, Trp, His, and adenine. The Gal4 DNA binding domain (BD) and activation domain (AD) were used as negative controls. The pictures were taken 3 d after incubated at 28°C. B, In vitro GST pull-down assay for CO and RGA interaction. Soluble GST and GST-CO fusion proteins were extracted and immobilized to glutathione affinity resin. Purified GST, GST-CO were incubated with the His-RGA fusion protein from *Escherichia coli* cell lysate for 2 h at 4°C. The interaction was determined by western blot using anti-His antibody. The purified GST and GST-CO were diluted 10 times (pull down 10%) and detected with anti-GST antibody (middle). C, BiFC assay showing the fluorescence complementations of the cYFP fused with RGA and thenYFP fused with CO. 4′,6-Diamidino-2-phenylindole (DAPI) staining marks the nucleus. D, Co-IP assay for CO and RGA interaction. Flag-fused CO and Myc-fused RGA were transiently coexpressed in tobacco leaves. All infected leaves treated with 10 μM MG132 and 20 μM paclobutrazol for 8 h were used for Co-IP. MYC-RGA and MYC tag were immunoprecipitated with anti-MYC M2 agarose beads and detected with anti-FLAG antibodies. Protein input for Flag-CO proteins in immunoprecipitated complexes was also detected and shown.

between RGA and CO was also confirmed by a coimmunoprecipitation (Co-IP) assay in tobacco (Fig. 2D). Consistent with this, Xu et al. (2016) showed three DELLA proteins (RGA, GAI, and RGL1) colocalized with CO in the same nuclear bodies, further supporting the idea that CO interact with DELLA proteins in plant cells. Taken together, these results demonstrated that CO interacts with DELLA proteins in vitro and in vivo, indicating that CO functions as a target of DELLA proteins.

To characterize which domain of DELLA proteins is responsible for interacting with CO, the DELLA protein RGA was divided into N-terminal parts (amino acids 1-199) containing the DELLA motif and C-terminal parts (amino acids 200-587) containing two Leu zipper domains. Moreover, we also deleted the DELLA motif of RGA (deleted amino acids 44-60). The directed yeast two-hybrid analysis revealed that deleting the N-terminal residues or DELLA motif of RGA did not affect the physical interaction with CO (Supplemental Fig. S3). However, deletion of the C-terminal parts of RGA eliminated this interaction (Supplemental Fig. S3). Thus, C-terminal parts, but not the DELLA motif or N-terminal parts of RGA, contribute to the interaction between RGA and CO.

DELLA Inhibit the Transcriptional Function of CO

As transcriptional regulators, DELLA proteins lack a DNA binding domain and have been shown to exert their function mainly by inhibiting transcription factor activity through protein-protein interactions (Yu et al., 2012; Davière et al., 2008; de Lucas et al., 2008; Feng et al., 2008; Hong et al., 2012; Wild et al., 2012). Having ascertained that DELLA proteins directly interact with CO, we hypothesized that DELLA might affect the transcriptional function of CO. To test this possibility, we further performed transient expression assays in Col-0 wild-type Arabidopsis mesophyll protoplasts (Yoo et al., 2007). As *FT* is a direct target of CO (Samach et al., 2000), the *FT* promoter was fused to the *Luciferase* (*LUC*) gene as a reporter (Fig. 3A). The effector constructs had a *CO* or *DELLA* gene driven by the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 3A). Consistent with the previous study (Samach et al., 2000), expression of CO dramatically activated expression of *LUC* driven by the *FT* promoter (Fig. 3, B and C). However, coexpression of RGA or RGL1 with CO repressed *LUC* expression in comparison with expression of CO alone (Fig. 3, B and C). This supported the hypothesis that DELLA proteins affect the transcriptional function of CO. Previous studies have indicated that DELLA proteins are subjected to GA-induced proteolysis in normal conditions, and the DELLA motif is essential for this process (Dill et al., 2001). To further test the effect of DELLA protein abundance on CO transcriptional activity, coexpression of RGAd17 (GA-insensitive form of RGA) or RGL1d17 (GA-insensitive form of RGL1) with CO was performed. Our results indicated that RGAd17 and RGL1d17 displayed a stronger repression of transcriptional activity of CO compared with RGA and RGL1 (Fig. 3, B and C). As negative controls, coexpression of RGA, RGL1, RGAd17, or RGL1d17 with GFP did not significantly affect the LUC/REN ratio in comparison with expression of GFP

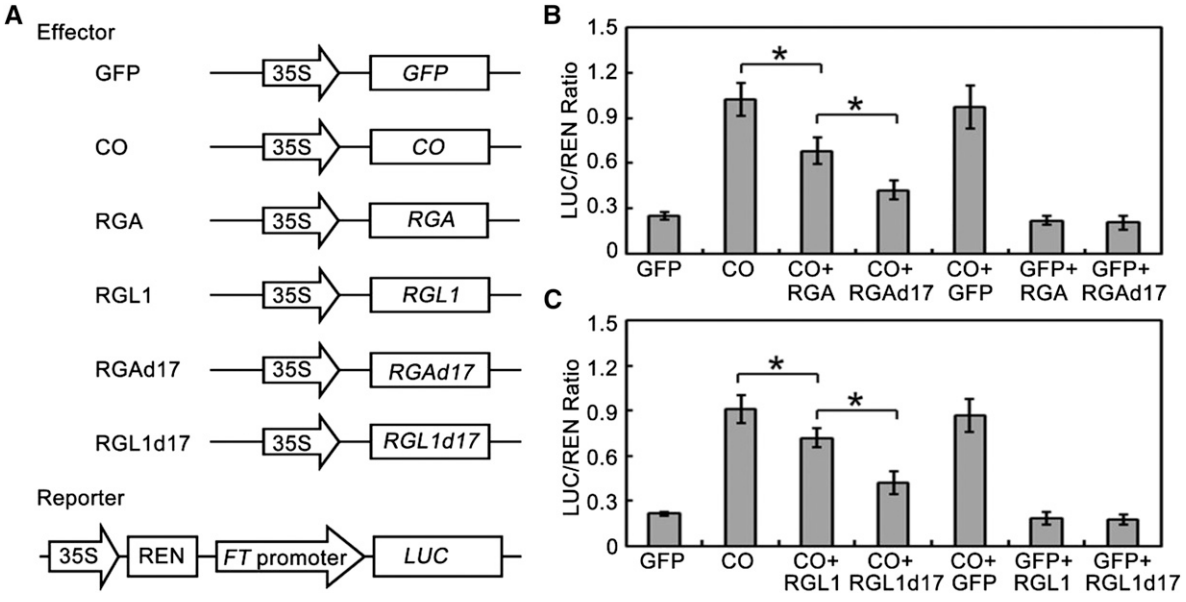

Figure 3. DELLA proteins repress the transcriptional activity of CO. A, Schematic of the reporter and effectors used in the transient transactivation assays. B and C, Transient dual-luciferase reporter assays show that the activation of *FT* expression by CO is repressed by DELLA protein RGA (B) and RGL1 (C). Error bars indicate SD from three biological replicates; statistics by Student's *t* test; $*P < 0.05$.

alone (Fig. 3, B and C). Together, these results suggest that DELLA proteins interact with CO and inhibit its transcriptional function to activate *FT* expression.

DELLA-Repressed Flowering under LDs Requires Functional CO

As DELLA form a complex with CO and affect its transcriptional functions, we further asked whether DELLA genetically interact with CO to mediate GA-regulated flowering. To test this, we analyzed the flowering phenotype of the *gai-t6 rga-t2 rgl1-1 rgl2-1 co-2* mutant plants (*dellap co-2*), which was generated by crossing *gai-t6 rga-t2 rgl1-1 rgl2-1* (*dellap*; L*er*) with *co-2*. Consistent with previous studies, the *dellap* mutant exhibited early flowering, while the *co-2* (L*er*) mutant exhibited late flowering compared with the wild type (Fig. 4, A and B; Supplemental Fig. S4; Yu et al., 2012; Putterill et al., 1995). Interestingly, *dellap co-2* plants flowered with 11.3 ± 1.3 rosette leaves and 4.7 ± 0.7 cauline leaves under LDs, later than both the *dellap* mutant (7.4 ± 0.6 rosette leaves, 2.4 ± 0.5 cauline leaves) and wild type (9.3 ± 0.7 rosette leaves, 2.5 ± 0.5 cauline leaves) but earlier than the *co-2* single mutant plants (17.9 ± 3.1 rosette leaves, 4.8 ± 0.8 cauline leaves; Fig. 4B). Consistent with the flowering phenotype, expression levels of *FT* in *dellap* plants were increased compared with those in the wild type (Fig. 4C). However, *FT* transcripts in the *dellap co-2* plants displayed lower expression levels, similar with that in *co-2* single mutant plants (Fig. 4C). Collectively, these results demonstrated that the flowering time of *dellap co-2* is closer to that of *co-2* and much later than *dellap* and wild type, indicating that the early-flowering phenotype of *dellap* requires functional CO and that DELLA represses flowering partially through CO.

To further elucidate the genetic interaction between DELLA and CO, we generated transgenic plants overexpressing *RGL1* and *CO* (Col-0 background) driven by the CaMV 35S promoter. One line of *35S:RGL1* (line 4) and one line of *35S:CO* (line 3) was selected for further study (Supplemental Fig. S5, A and B). Under LDs, overexpression of *RGL1* resulted in slightly delayed flowering, while plants overexpressing *CO* exhibited a much earlier flowering phenotype compared with the wild type (Fig. 5A; Samach et al., 2000). However, plants constitutively expressing *RGL1* and *CO* simultaneously (genetic crossing of *35S:RGL1* to *35S:CO*) exhibited early flowering similar to *35S:CO* plants (Fig. 5, A and B; Supplemental Figs. S5 and 6), which appears to be in conflict with the notion that DELLA repress CO. If DELLA inhibits the function of CO, one should expect that the earlier flowering phenotype of *35S:CO* should be compromised by *35S:RGL1*. However, our finding that DELLA protein abundance affects CO transcriptional activity (Fig. 3) may help to reconcile these discrepancies. To further determine this, we generated a gain-of-function *gai-1* (Col-0) mutant from the *gai-1* (L*er*) allele (a GA-insensitive mutant) through backcrossing *gai-1* (L*er*) with Col-0 three times. Indeed, overexpression of *CO* in this *gai-1* (Col-0) background (genetic crossing of *35S:CO* to *gai-1* [Col-0]) conferred plants flowering significantly later than *35S:CO* plants under LDs (Fig. 5C; Supplemental Figs S5 and 7). This finding was corroborated by quantitative real-time PCR (qRT-PCR), which showed that *FT* transcripts

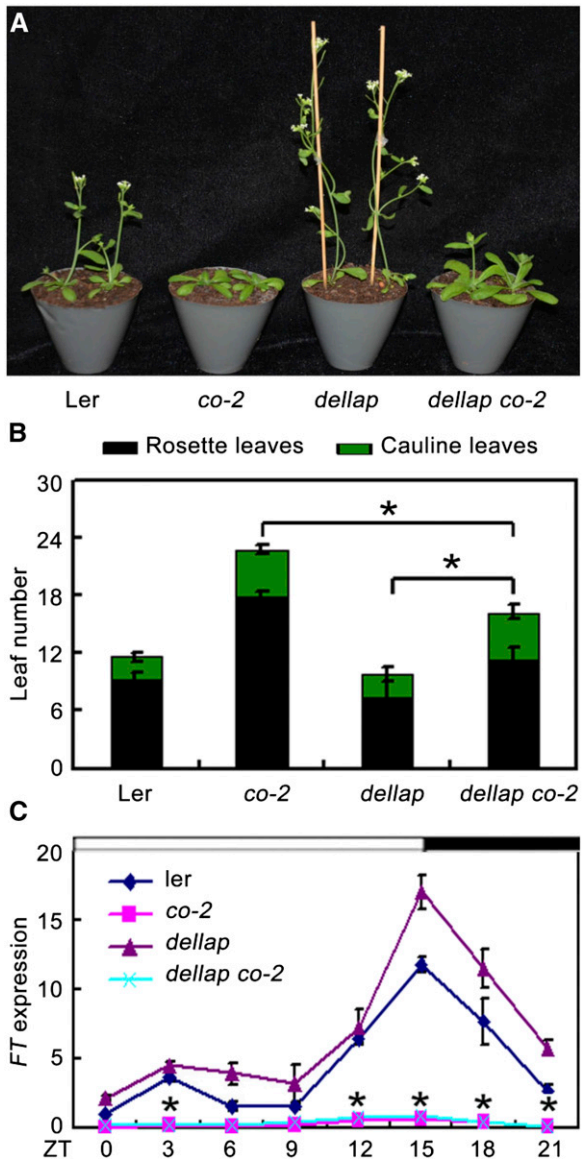

Figure 4. *co-2* partially rescued the earlier flowering phenotype of the *dellap* mutant. A, Plants of L*er*, *co-2*, *dellap*, and *dellap co-2* observed 25 d after germination under LDs. All plants are in L*er* background. B, Flowering time of L*er*, *co-2*, *dellap*, and *dellap co-2* plants under LD conditions. Data are mean of at least 15 plants. Error bars indicate the SD of total leaf number. *Difference between *dellap* and *dellap co-2* is highly significant (Student's *t* test; $P < 0.01$). C, qRT-PCR analysis of *FT* expression in 10-d-old L*er* and various mutant seedlings under LDs. The *IPP2* gene was used as an internal control. Error bars indicate SD from three independent experiments. *Difference between *dellap* and *dellap co-2* is highly significant (Student's *t* test; $P < 0.01$).

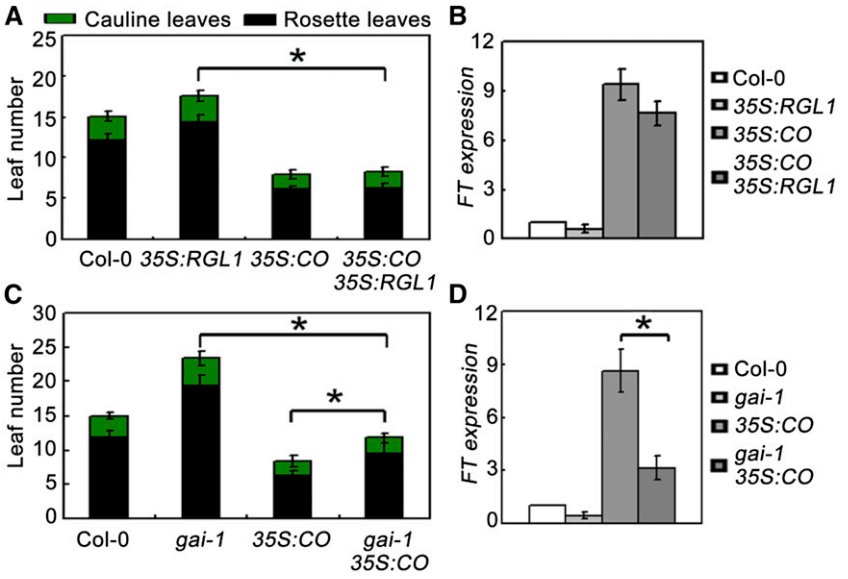

Figure 5. A gain-of-function mutation (*gai-1*) of *GAI* represses the earlier flowering phenotype of *CO*-overexpressing plants. A and C, Flowering phenotypes of various gene types under LDs. All plants are in Col-0 background. Error bars indicate the SD of total leaf number; statistics by Student's *t* test; $^*P < 0.01$. B and D, qRT-PCR analysis of FT expression levels in various plants. The *ACTIN2* gene was used as an internal control. Total RNA was extracted from 10-d-old plants at ZT 16 grown under LD. Error bars indicate SD from three independent RNA extracts; statistics by Student's *t* test; $^*P < 0.05$.

were enhanced in *35S:CO* but reduced in *35S:CO gai-1* plants (Fig. 5D).

Taken together, our results indicate that DELLA represses flowering partially through the CO/FT-mediated pathway.

The Expression of *DELLA* mRNA and Accumulation of RGA Protein Are Rhythmic

Previous studies demonstrated CO acts between the circadian clock and the control of flowering (Suárez-López et al., 2001; Song et al., 2015). Recently, Arana et al. (2011) revealed DELLA proteins participate in controlling rhythmic growth of wild-type hypocotyls under SDs. To examine whether DELLA are rhythmic, their expression profiles were investigated under LDs. qRT-PCR analysis showed that *DELLA* were rhythmically expressed, peaking at the LD afternoon (zeitgeber time [ZT] 12 to 15; Fig. 6A). Consistent with the previous study (Suárez-López et al., 2001), the transcripts of *CO* varied during a 24-h period, showing peak levels between ZT12 and ZT21 (Fig. 6A). Having ascertained that the expression of DELLA mRNA is rhythmic, we further queried whether the accumulation of DELLA protein is also rhythmically regulated. Initially, we analyzed expression profiles of *GFP-RGA* in *proRGA:GFP-RGA* transgenic plants. qRT-PCR analysis showed that *GFP-RGA* was rhythmically expressed, which is similar to the expression profile of endogenous *RGA* (Supplemental Fig. S8). Then, we examined the accumulation pattern of RGA protein in *proRGA:GFP-RGA* transgenic plants. As shown in Figure 6B, the RGA protein was strongly decreased at ZT9, ZT12, and ZT15 under LDs when CO protein was abundant (Fig. 6B; Valverde et al., 2004). Taken together, these results indicate that DELLA proteins were rhythmically regulated at both transcriptional and posttranslational levels.

DELLA and CO Antagonistically Regulate Expression of Multiple Flowering-Related Genes

Acting as two important pathways in flowering time control, the photoperiod and GA pathways have been shown to regulate multiple flowering-related genes, including *SPLs*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *TSF*, and *FRUITFULL* (*FUL*; Porri et al., 2012; Yu et al., 2012; Jung et al., 2012). To further investigate whether DELLA-CO interactions affect expression of these genes, their expression was monitored in *co-2*, *dellap*, and *dellap co-2* mutants by qRT-PCR analysis. As shown in Supplemental Figure S9, their expression was decreased in *co-2* and increased in *dellap* compared with that in the wild type (Supplemental Fig. S9). Furthermore, their transcripts in *dellap co-2*, which were more than those in *co-2*, were significantly less than those in *dellap* (Supplemental Fig. S9). Taken together, these results indicate that DELLA and CO proteins also antagonistically affect the expression of several other flowering-related genes.

DISCUSSION

Recently, numerous studies have demonstrated that the photoperiod and GA pathways act synergistically to promote flowering in response to inductive LDs. For example, Porri et al. (2012) found that GA are required for *FT* and *TSF* expression in the vascular tissue under LDs. Similarly, Galvão et al. (2012) showed that GA regulate flowering through controlling the expression of *FT*, *TSF*, and *SPLs* in a day length-specific manner. Additionally, BOTRYTIS SUSCEPTIBLE1 INTERACTORs (a group of interacting partners of DELLA proteins) interact with CO, which inhibit CO to target *FT* (Nguyen et al., 2015). However, the exact molecular mechanisms underlying interactions of the photoperiod and GA pathways remain limited. Investigating specific crosstalk between these two critical flowering-promoted pathways

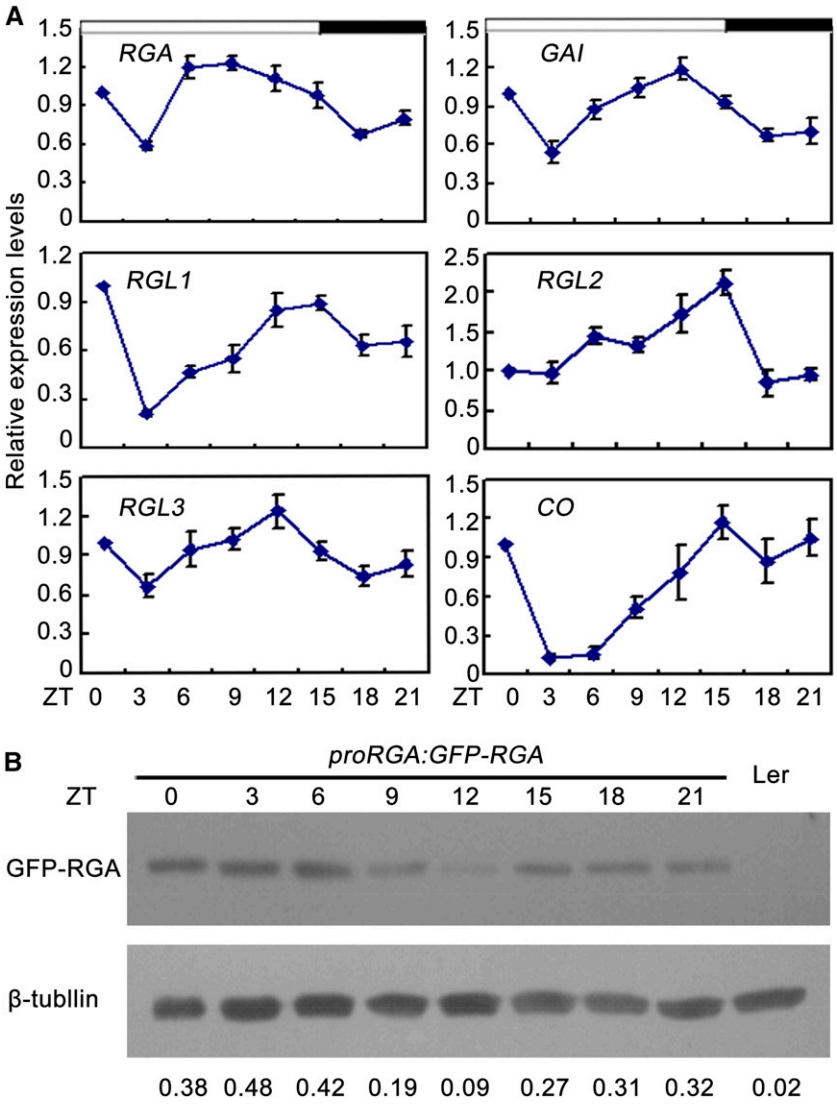

Figure 6. Analysis of DELLA transcript and protein accumulation under LD photoperiod. A, qRT-PCR of *DELLA* and *CO* relative to *IPP2* under LDs. Total RNA was extracted from 10-d-old L*er* seedings. Error bars indicate SD from three independent RNA extracts. B, Western-blot analysis of GFP-RGA protein accumulation. The blots from seedlings of 10-d-old wild type (L*er*) or homozygous *proRGA:GFP-RGA* transgenic plants. An anti-GFP mouse antibody (Sigma-Aldrich) was used to detect GFP-RGA. β-Tubulin was used as an internal control, and GFP-RGA/β-tubulin ratios were calculated and shown. Western-blot experiments were repeated at least three times, and similar results were obtained. Time is expressed as hours from dawn.

will provide new insights into our understanding of floral transition.

As crucial repressors of the GA pathway, DELLA proteins were previously reported to regulate GA-mediated responses through physically interacting with several transcription factors. For example, GLABRA1, PHYTOCHROME INTERACTING FACTORs, MYC2, GLABRA3, ENHANCER OF GLABRA3, Class I TCP, BRASSINAZOLE-RESISTANT1, ETHYLENE INSENSITIVE3, AUXIN RESPONSE FACTOR6, and SPLs, were reported as targets of DELLA proteins to regulate diverse aspects of GA-mediated processes (Yu et al., 2012; Davière et al., 2008, 2014, 2016; de Lucas et al., 2008; Feng et al., 2008; Hong et al., 2012; Wild et al., 2012; Qi et al., 2014; Bai et al., 2012; An et al., 2012; Oh et al., 2014). Furthermore, our study and Xu et al. (2016) found that the B-box protein CO also interacts with DELLA (Fig. 2), indicating that CO may also function as a target of DELLA. Consistently, DELLA inhibits the transcriptional activity of CO to regulate its target gene *FT* (Fig. 3). As CO is a critical regulator of the photoperiod pathway, the DELLA-CO physical interactions may integrate GA and photoperiod signaling to regulate flowering under LDs.

Further genetic analysis demonstrated that CO may act downstream of DELLA proteins to regulate flowering. As shown in Figure 4, the flowering time of *dellap co-2* quintuple mutants, which was earlier than the *co-2* single mutant, was significantly later than that of *dellap* plants, indicating that disruption of CO partially rescued the earlier flowering phenotype of *dellap*. Consistently, the expression of *FT* was remarkably reduced in *dellap co-2* quintuple mutant compared with that in *dellap* (Fig. 4). These results demonstrate that DELLA proteins repress flowering required functional CO. Moreover, a gain-of-function mutation in *GAI* (*gai-1*) repressed the earlier flowering phenotype of *CO*-overexpressing plants (Fig. 5). All those genetic results together with the finding that DELLA proteins repress the transcriptional activity of CO support the notion

that CO acts downstream of DELLA proteins to regulate flowering.

The expression analysis showed that the *FT* expression was abolished in the *dellap co-2* quintuple mutants (Fig. 4), which was similar with that in the *co-2* single mutants, indicating that the GA-promoted FT-mediated flowering pathway is blocked in both *dellap co-2* and *co-2* mutant background. However, phenotypic analysis showed that the flowering time of *dellap co-2* was still earlier than that of the *co-2* single mutant plants (Fig. 4), suggesting that other DELLA-repressed flowering-related targets may contribute to the induction of flowering in *dellap co-2*. Consistently, our expression analysis showed that the expression of multiple flowering-related genes was increased in the *dellap co-2* quintuple mutants compared with that in the *co-2* single mutants (Supplemental Fig. S9). Moreover, Yu et al. (2012) found that DELLA proteins directly interact with SPLs and repress flowering partially through inactivating *miR172* and MADS box genes under LDs. Recently, Li et al. (2016) showed that DELLA proteins act as corepressors to regulate flowering by interacting with FLOWERING LOCUS C. All those previous findings and our results in this study demonstrate a complex regulation for GA- and DELLA-modulated flowering. Future studies are needed to illustrate the relationships among these GA- and DELLA-signaled flowering pathways.

As repressors of GA signaling, DELLA proteins are subjected to GA-induced proteolysis in normal conditions (Dill et al., 2001). In this study, we observed that RGAd17 and RGL1d17 showed a stronger repression of the transcriptional activity of CO than RGA and RGL1 (Fig. 3). Consistent with this, the early flowering phenotype of *35S:CO* was compromised in the *gai-1* (Col-0) background but not in the *35S:RGL1* background (Fig. 5). It is possible that DELLA may repress CO-mediated flowering in a dose-dependent manner. Interestingly, Xu et al. (2016) recently reported that DELLA protein RGA represses the interaction of CO with NF-YB2 in a dose-dependent manner. The results suggest that the tight regulation of DELLA dose may also be critical for flowering. Interestingly, as shown in Figure 6B, we found that the accumulation of RGA protein was rhythmically regulated. RGA was strongly decreased in ZT12 and ZT15, when CO protein was abundant (Fig. 6B; Valverde et al., 2004). It is thus possible that the rhythmically regulated degradation of DELLA in the LD afternoon releases CO and subsequently activates *FT* expression. Consistent with these, the expression of *FT* was strongly induced at the end of the day (Figs. 1 and 4; Suárez-López et al., 2001). Further studies elucidating the mechanisms underlying how DELLA proteins are rhythmically regulated may shed new light on the molecular basis of DELLA- and GA-modulated flowering.

MATERIALS AND METHODS

Materials and Arabidopsis Growth Conditions

The plant hormone GA3 was purchased from Sigma-Aldrich. Taq DNA polymerase was purchased from Takara Biotechnology. Other chemicals were obtained from Shanghai Sangon Biotechnology. Arabidopsis *(Arabidopsis thaliana) dellap* mutant seeds were obtained from Dr. Xingliang Hou (South China Botanical Garden, Chinese Academy of Sciences) and *co-2* seeds were provided by Dr. Hongquan Yang (Shanghai Jiaotong University). Homozygous *dellap* was crossed with *co-2* to generate *dellap co-2* homozygous plants. To generate *CO* and *RGL1* overexpression transgenic plants, full-length cDNAs of *CO* and *RGL1* were cloned into a pOCA30 vector in the sense orientation behind a CaMV 35S promoter (Wang et al., 2015). Plants used in this study were derived from Arabidopsis Col-0 or L*er* ecotypes. Arabidopsis plants were grown in growth chambers at 22°C under LDs (16-h light [100 μE $m^{-2}s^{-1}$]/8-h dark cycle) or SD conditions (8-h light [100 μE $m^{-2}s^{-1}$]/16-h dark cycle). Induction treatments with the plant hormone GA3 was performed as described in Galvão et al. (2012). Primers used for identification of mutants or clones are listed in Supplemental Table S1.

RNA Extraction and qRT-PCR

For real-time RT-PCR analysis, total RNA was extracted from Arabidopsis seedlings using the TriZol reagent (Invitrogen). A total of 1 μg of DNase-treated RNA was reverse transcribed in a 20-μL reaction mixture using Superscript II in accordance with manufacturer's instructions (Invitrogen). Following the reaction, 1 μL of resultant cDNA was used as a template for qRT-PCR, using a SYBR Premix Ex Taq kit (Takara). At least three independent biological samples were conducted for each experiment. The *IPP2* gene was used as an internal control in Figures 1, 4C, and 6A, while the *ACTIN2* gene was used as an internal control in Figure 5, B and D, and Supplemental Figure S9. Primers used for qRT-PCR are listed in Supplemental Table S1.

Yeast Two-Hybrid Assay

The truncated CO CDSs were cloned into the bait vector pGBKT7 and full-length or truncated CDSs of DELLA proteins were cloned into the prey vector pGADT7. Yeast two-hybrid assay was performed as described previously (Hu and Yu, 2014). Primers used for generating various clones for yeast two-hybrid assays are listed in Supplemental Table S1.

BiFC Assay

Full-length CDS of CO was inserted into pFGC-nYFP vector to generate N-terminal in-frame fusions with N-YFP, while DELLA coding sequences were cloned into pFGC-cYFP vector to form C-terminal in-frame fusions with C-YFP (Hu et al., 2013a). All plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105, and infiltration of tobacco (*Nicotiana tabacum*) leaves was performed as described previously (Hu et al., 2013a). Infected tissues were analyzed 48 h after infiltration under a confocal laser-scanning microscope (Olympus). Primers used for clones are listed in Supplemental Table S1.

Co-IP Analysis

For Co-IP assays, the full-length CDS of *CO* or *RGA* was amplified and cloned into tagging vectors behind the single FLAG or MYC tag in the sense orientation behind the CaMV 35S promoter (Hu et al., 2013a). Flag-fused CO and Myc-fused RGA were transiently coexpressed in tobacco leaves. All infected leaves were treated with 10 μM MG132 and 20 μM paclobutrazol (a GA biosynthesis inhibitor) 40 h after infiltration. After 8 h, those leaves were homogenized in an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) Triton X-100, and 1× complete protease inhibitor cocktail (Roche). Then, MYC-fused RGA and MYC was immunoprecipitated using an anti-MYC rabbit antibody (Sigma-Aldrich), and coimmunoprecipitated proteins were detected using an anti-Flag mouse antibody (Sigma-Aldrich). Primers used for clones are listed in Supplemental Table S1.

Pull-Down Assay

Full-length CO and RGA cDNAs were cloned into pGEX-TX-1 (GE Healthcare) and pET-28a (Novagen), respectively. All plasmids were introduced into *Escherichia coli* BL21 cells (TransGen Biotech). GST, GST-CO, and His-RGA protein expression was induced by 0.1 mM isopropyl-β-thiogalactopyranoside. Soluble GST and GST-CO fusion proteins were extracted and immobilized to glutathione affinity resin (Thermo Fisher Scientific). For pull-down assays, His-RGA fusion protein from *E. coli* cell lysate was incubated with the immobilized GST and GST-CO fusion proteins in Pull-down buffer (50 mM Tris-HCl, pH 7.2,

150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1× protease inhibitor cocktail) for 2 h at 4°C. Proteins were eluted in the elution buffer, and the interaction was determined by western blot using anti-His antibody (Sigma-Aldrich).

Transient Transactivation Assay

To generate reporter constructs, a 2,675-bp region upstream of the start codon of *FT* was amplified and cloned into a pGreenII 0800-LUC vector (Hellens et al., 2005). To create the effector constructs, the corresponding cDNAs of RGA, RGL1, RGAd17, and RGL1d17 were amplified and cloned into pGreenII 62-SK vectors (Hellens et al., 2005). All primers used for generating these constructs are listed in Supplemental Table S1. Preparation of Arabidopsis mesophyll protoplasts from wild-type (Col-0) leaves and subsequent transfections were performed as described by Yoo et al. (2007). A dual-luciferase reporter assay system (Promega) was used to measure firefly LUC and renilla luciferase (REN) activities. The *REN* gene under the control of the CaMV 35S promoter and the *LUC* gene were in the pGreenII 0800-LUC vector (Hellens et al., 2005). Relative REN activity was used as an internal control, and LUC/REN ratios calculated.

Protein Analysis

To analyze the accumulation pattern of RGA protein, the *proRGA:GFP-RGA* transgenic plants were grown under LD for 10 d and harvested every 3 h. Total protein was extracted using an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) Triton X-100, and 1× complete protease inhibitor cocktail (Roche). An anti-GFP mouse antibody (Sigma-Aldrich) was used to detect GFP-RGA. β-Tubulin was used as an internal control, and GFP-RGA/β-tubulin ratios were calculated and shown.

Accession Numbers

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: CO (At5g15840), RGA (At2g01570), GAI (At1g14920), RGL1 (At1g66350), RGL2 (At3g03450), RGL3 (At5g17490), FT (At1g65480), SOC1 (At2g45660), SPL3 (At2g33810), SPL4 (At1g53160), SPL5 (At3g15270), FUL (At5g60910), TSF (At4g20370), IPP2 (At3g02780), and ACT2 (At3g18780).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. CO transcription and protein abundance are not regulated by the GA pathway.

Supplemental Figure S2. BiFC assay showing the fluorescence complementations of the cYFP fused with DELLAs and the nYFP fused with CO.

Supplemental Figure S3. C-terminal parts of RGA contribute to the interaction between RGA and CO.

Supplemental Figure S4. Flowering time of L*er*, *co-2*, *dellap*, and *dellapco-2* under LDs.

Supplemental Figure S5. Overexpression lines for RGL1 and CO.

Supplemental Figure S6. Flowering time of Col-0, *35S:CO*, *35S:RGL1*, and *35S:CO 35S:RGL1* plants under LDs.

Supplemental Figure S7. Flowering time of Col-0, *35S:CO*, *gai-1*, and *gai-1 35S:CO* plants under LDs.

Supplemental Figure S8. qRT-PCR of RGA and GFP-RGA expression.

Supplemental Figure S9. Expression of multiple flowering-related genes was coregulated by DELLA and CO.

Supplemental Table S1. Primers used in this study.

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