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CONSTANS and ASYMMETRIC LEAVES 1 complex is involved in the induction of *FLOWERING LOCUS T* in photoperiodic flowering in *Arabidopsis*

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Summary

Plants monitor changes in day length to coordinate flowering with favorable seasons to increase their fitness. The day-length specific induction of *FLOWERING LOCUS T* (*FT*) regulated by CONSTANS (CO) is the crucial aspect of photoperiodic flowering in *Arabidopsis thaliana*. Recent studies elucidated some mechanisms of CO-dependent *FT* induction. Here, we demonstrate another mechanism of CO-dependent *FT* regulation. Our results indicate that CO protein regulates *FT* transcription partially by forming a complex with ASYMMETRIC LEAVES 1 (AS1) protein, which regulates leaf development partly by controlling gibberellin (GA) levels. We identified AS1 as a CO-interacting protein in yeast and verified their interaction *in vitro* and *in planta*. We also showed that the *AS1* temporal and spatial expression pattern overlapped with that of *CO*. In addition, *as1* mutants showed GA-independent delayed flowering under different light/dark conditions. *FT* expression levels in the *as1* mutants and the *SUC2:CO-HA/as1* line under long-day and 12-h light/12-h dark conditions were reduced compared to wild-type plants and the *SUC2:HA-CO* line, respectively. Moreover, AS1 directly bound to the specific regions of the *FT* promoter *in vivo*. These results indicate that CO forms a functional complex with AS1 to regulate *FT* expression and that AS1 plays different roles in two regulatory pathways, both of which concomitantly regulate a precise timing of flowering.

Keywords

Arabidopsis; ASYMMETRIC LEAVES1 (AS1); CONSTANS (CO); FLOWERING LOCUS T (FT); Flowering time; Photoperiodic pathway

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Introduction

Plants primarily transition from a vegetative phase to a reproductive phase on a seasonal basis. This transition is precisely controlled by various environmental signals, such as light, temperature, and the availability of water and nutrients, and by endogenous factors, such as differences in developmental stages. In the model plant *Arabidopsis thaliana*, various signaling pathways regulate the flowering response (Baurle and Dean, 2006; Amasino, 2010). Plants sense changes in day length (=photoperiod) to regulate the timing of flowering (Kobayashi and Weigel, 2007; Turck *et al.*, 2008; Song *et al.*, 2010). For *Arabidopsis thaliana*, a longer day accelerates the transition to flowering through the photoperiodic pathway (Kobayashi and Weigel, 2007; Turck *et al.*, 2008). The difference in day length is perceived in the leaf, at a site for the induction of the transmissible floral inductive signals termed florigen (Corbesier *et al.*, 2007; Tamaki *et al.*, 2007). Day-length measurement requires the integration of temporal information provided by the circadian system and light signals perceived by various photoreceptors (Suarez-Lopez *et al.*, 2001; Yanovsky and Kay, 2002; Valverde *et al.*, 2004; Sawa *et al.*, 2007). The prolonged cold exposure known as vernalization, as well as more subtle ambient temperature changes, also influence the timing of flowering (Kim *et al.*, 2009; Amasino, 2010; McClung and Davis, 2010).

Flowering, especially under short-day conditions, is regulated through the action of the phytohormone gibberellins (Eriksson *et al.*, 2006). The autonomous pathway in which mutations affect the timing of flowering under various conditions shares some components with the vernalization pathway (Mouradov *et al.*, 2002; Simpson, 2004; Feng *et al.*, 2011). These pathways converge on regulating the expression of common target genes referred to as the floral integrators, such as *FLOWERING LOCUS T (FT)*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*, and *LEAFY (LFY)* genes (Simpson and Dean, 2002; Hayama and Coupland, 2003; Sung *et al.*, 2003; Amasino, 2005; Parcy, 2005). Having interconnected regulatory networks among these pathways enables plants to precisely coordinate the timing of flowering with the optimal environments. (Mouradov *et al.*, 2002; Vandenbussche and Van Der Straeten, 2004; Baurle and Dean, 2006).

In *Arabidopsis*, photoperiodic flowering is mainly regulated through the function of CONSTANS (CO) and FT proteins (Suarez-Lopez *et al.*, 2001; Valverde *et al.*, 2004; Abe *et al.*, 2005; Wigge *et al.*, 2005). CO, which is a B-box zinc-finger-type transcription factor, accelerates flowering through the induction of *FT* expression in the leaf vasculature (Putterill *et al.*, 1995; Robson *et al.*, 2001; Takata and Goto, 2003; An *et al.*, 2004). Mutations in *CO* and *FT* genes result in delayed flowering under long day conditions, whereas overexpression of these genes accelerates flowering regardless of photoperiods (Samach *et al.*, 2000). The circadian-clock regulated timing of *CO* gene expression and light-dependent stability regulation of CO protein are crucial processes to control photoperiodic induction of *FT* (Suarez-Lopez *et al.*, 2001; Yanovsky and Kay, 2002; Valverde *et al.*, 2004; Sawa *et al.*, 2007; Imaizumi, 2010). FT protein is synthesized in the leaf and translocated to the shoot apex, where it induces the expression of floral identity genes that trigger transcriptional cascades of floral development (Abe *et al.*, 2005; Wigge *et al.*, 2005; Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Mathieu *et al.*, 2007). FT protein is thought to play a major role as a florigen. FT orthologs are also identified as having similar characteristics as a florigen in other plant species such as rice and cucumber (Lin *et al.*, 2007; Tamaki *et al.*, 2007).

Recent advances in *Arabidopsis* research have provided some insights into the molecular mechanisms of CO-dependent *FT* regulation. Through the C-terminal CCT (CONSTANS, CO-LIKE and TOC1) domain, CO protein physically interacts with HEME ACTIVATOR PROTEIN (HAP) components (also known as NUCLEAR FACTOR-Y), HAP3 and HAP5

proteins, that form the trimeric CCAAT-binding transcription factor complex (Wenkel *et al.*, 2006). The loss of several *HAP3* and *HAP5* genes caused late flowering, which resembles the flowering phenotype of the *co* mutants (Laubinger *et al.*, 2006; Kuminoto *et al.*, 2008). In addition, CO potentially interacts with other transcription factors *in vivo*, as it has been shown that CO may bind to TGAGC MOTIF-BINDING FACTOR 4 (TGA4) (Song *et al.*, 2008). Recent results have indicated that CO by itself might also directly bind to the specific *cis*-elements in the *FT* promoter through its CCT domain (Tiwari *et al.*, 2010). These results suggest that CO protein may induce *FT* transcription through various mechanisms. CO also binds to CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and SUPPRESSOR OF PHYA-105 (SPA) proteins, which together form the E3 ubiquitin ligase complex that degrades CO protein in the dark (Laubinger *et al.*, 2006; Jang *et al.*, 2008). This dark-dependent degradation of CO ensures that *FT* is not induced in the dark.

To further understand the molecular mechanisms of CO dependent *FT* induction, we aimed to find additional factors that may participate in *FT* regulation together with CO. We isolated ASYMMETRIC LEAVES 1 (AS1) Myb-type transcription factor as a CO interacting protein. AS1 controls leaf patterning by regulating cell differentiation in part through direct repression of class-1 *KNOTTED*-like homeobox (*KNOX*) gene expression at leaf primordia (Byrne *et al.*, 2000; Hay *et al.*, 2002; Guo *et al.*, 2008; Hay and Tsiantis, 2010). *KNOX* proteins repress the expression of the GA biosynthesis gene, *AtGA20ox1*; thus AS1 indirectly regulates GA biosynthesis (Hay *et al.*, 2002; Hay and Tsiantis, 2010; Ikezaki *et al.*, 2010). The GA signaling pathway is detrimental to proper leaf development (Hay *et al.*, 2002; Hay and Tsiantis, 2010). Reduced GA biosynthesis or GA signaling enhances the phenotype of the *as1* mutants, indicating the possible role of AS1 in the regulation of GA signaling (Hay *et al.*, 2002). *AtGA20ox1* overexpression induces GA overproduction phenotypes including early flowering in SD (Huang *et al.*, 1998; Coles *et al.*, 1999). Conversely, the *ga20ox1* mutation, together with the *ga20ox2* mutation, causes the reduction of GA levels and also late flowering phenotype of the mutants in both LD and SD (Coles *et al.*, 1999; Rieu *et al.*, 2008). These results indicate that AS1 may be involved in the control of flowering time by regulating active GA levels (Ikezaki *et al.*, 2010). In addition to the possible role of AS1 in GA-mediated flowering regulation, our data indicated that AS1 functions as a floral regulator involved in regulation of *FT* mediated by direct interaction with CO in the photoperiodic pathway.

Results

Isolation of AS1 as a CO interacting protein

CO contains two functional domains: two tandem repeats of B-box domains at the N-terminus and the CCT domain at the C-terminus (Khanna *et al.*, 2009). Currently published data demonstrated that the CCT domain is an important domain in terms of regulating CO flowering function. Through the CCT domain, CO interacts with the HAP trimetric transcription factor complex, which regulates *FT* expression (Wenkel *et al.*, 2006), as well as the COP1-SPAs E3 ubiquitin ligase complex, which controls the CO protein stability (Laubinger *et al.*, 2006; Jang *et al.*, 2008; Liu *et al.*, 2008). The B-box domain is also predicted to be involved in the protein-protein interaction (Robson *et al.*, 2001; Torok *et al.*, 2001). In addition, there are five *co* mutant alleles that have mutations in the B-box domains, indicating that the B-box domain may also be important for the CO function (Robson *et al.*, 2001). We previously found that TGA4 transcription factor interacts with the CO B-box domain *in vitro* and in yeast, although the purpose of this interaction remains elusive (Song *et al.*, 2008). Therefore, the role of the CO B-box domain still hasn't been well defined in the regulation of flowering time.

To understand the mechanisms by which CO regulates *FT* transcription, especially through the B-box domains, yeast two-hybrid screening was performed. A truncated CO protein that possesses two B-box domains was used as bait to isolate potential unidentified CO interacting transcription factors. We screened an *Arabidopsis* transcription factor library of roughly 1100 transcription factors (Song *et al.*, 2008) and isolated a clone containing *ASYMMETRIC LEAVES 1 (AS1)* cDNA as a potential interactor of the partial CO peptides (Figure 1a). When AS1 was used as bait, we reciprocally detected the interaction of AS1 with CO in yeast (Figure 1a). To further define the interacting domain of CO with AS1, we tested AS1 interaction with various truncated CO proteins (Figure 1b). As expected, AS1 interacted with the truncated CO proteins that contain two B-box domains (Figure 1c). The truncated CO protein that possesses only the first B-box domain (designated B1) was not sufficient to bind to AS1 (Figure 1c). These results indicate that either the second B-box domain or both B-box domains may be involved in the interaction with AS1.

To verify the interaction observed in yeast, *in vitro* binding assay was performed using a recombinant glutathione S-transferase (GST)-fused full length of CO protein and a full length of AS1 protein. More AS1 protein was precipitated with GST-CO than with GST alone (Figure 1d). We also performed another GST pull down assay to confirm the result shown in Figure 1c, and found that the truncated CO protein that contains only two B-box domains was sufficient for the interaction *in vitro* (Figure 1e). We also tested whether four known *co* mutations, which cause missense mutations in the B-box domains (Robson *et al.*, 2001), affect the interaction with AS1. All four CO variants attenuated the binding to AS1 *in vitro* (Figure S1), further supporting the notion that the B-box domains are important for the interaction with AS1.

We further confirmed the interaction of CO and AS1 by coimmunoprecipitation analysis using the *Nicotiana benthamiana* transient expression system. AS1-HA specifically coimmunoprecipitated with CO-GFP when both AS1-HA and CO-GFP were expressed in tobacco leaves (Figure 1f). In addition, when AS1-GFP and CO-RFP were co-expressed in *Arabidopsis* protoplasts, both GFP and RFP fluorescence co-localized in the nucleus (Figure 2a), indicating that both CO and AS1 exist in the same domain in the nucleus. Together with the results of yeast two-hybrid analysis, these results imply that CO may physically associate with AS1 in the nucleus *in vivo*.

Spatial and temporal expression patterns of AS1

CO expression is restricted in specific tissues and at specific times of the day. *CO* is mainly expressed in leaf phloem tissues (Takada and Goto, 2003; An *et al.*, 2004). The peak expression of *CO* occurs in the late afternoon and at night (Suarez-Lopez *et al.*, 2001), while *CO* protein stabilized only in the afternoon of long days (Valverde *et al.*, 2004). If *CO* interacts with AS1 *in vivo*, we predicted that the spatial and temporal expression patterns of *AS1* might overlap with those of *CO*. To study the spatial expression pattern of *AS1*, we analyzed the GUS expression pattern derived from the *AS1* genomic fragment fused to the β -glucuronidase gene (*AS1:AS1-GUS*). GUS activity was predominantly found in the phloem tissues of leaves, cotyledons, hypocotyls, and roots (Figures 2b and S2) (Iwakawa *et al.*, 2007). The expression patterns were similar within plants grown under various growth conditions (Figure S2). This result indicates that *CO* and *AS1* expression overlaps in leaf vascular tissues.

We then analyzed the temporal expression patterns of *AS1* under long-day (LD) and short-day (SD) conditions. Under these conditions, *AS1* mRNA levels changed throughout the day with an afternoon peak in LD and with a broad night peak in SD (Figure 2c,d). In LD, the *AS1* peak coincides with the daytime peak of *CO* (Suarez-Lopez *et al.*, 2001). The results of the diurnal expression patterns of *AS1* also prompted us to examine whether *AS1* expression

is controlled by the circadian clock. Therefore, we analyzed *AS1* expression under continuous light (LL) conditions. The *AS1* mRNA level oscillated diurnally under LL conditions (Figure 2e), indicating that *AS1* expression is regulated by the circadian clock. Together with the *AS1* spatial expression pattern, our data indicated that *AS1* is largely expressed in the same spatiotemporal domain of *CO*. In addition, these data are consistent with the possibility that *CO* and *AS1* work together.

AS1 is a positive regulator of flowering

Since *CO* regulates flowering time, we investigated the effect of *as1* mutation on flowering time in response to changes in photoperiods. We used *as1-1*, *as1-2*, and a newly identified T-DNA insertion *as1* allele (SAIL 915_A12, designated as *as1-21*, Figure S3) for the analysis. The *as1* mutations had little effect on the timing of flowering in LD (Figure 3a). In SD, all the *as1* mutants examined showed slightly late flowering (Figure 3b). It has been shown that some flowering time mutants and wild-type accessions respond differently to various day-length conditions, and exhibit stronger flowering phenotypes under certain day-length conditions (Giakountis *et al.*, 2010). Therefore, we also tested the *as1* flowering phenotype under various day-length conditions. Under 12-hour light/12-hour dark conditions, all three *as1* alleles exhibited distinct late flowering (Figure 3c). In addition, we found that *as1* showed a late flowering phenotype when the day is shorter than 14 hours (Figure S3g).

AS1 is also thought to be a positive regulator in the GA signaling pathway. The *as1* phenotype is enhanced by the inhibition of GA signaling and/or biosynthesis (Hay *et al.*, 2002). *AS1* directly suppresses the expression of *KNOX* genes (Figure S4a) (Guo *et al.*, 2008), and the *KNOX* transcription factors repress the expression of GA biosynthesis gene, *GA-20 oxidase 1* (*AtGA20ox1*) (Hay *et al.*, 2002; Hay *et al.*, 2010; Ikezaki *et al.*, 2010). Therefore, the loss of *as1* function indirectly reduces *AtGA20ox1* expression and subsequently decreases the rate of active GA biosynthesis (Figure S4b) (Ikezaki *et al.*, 2010).

Because the severe GA-biosynthesis deficient mutant *gal-3* failed to flower specifically in SD, GA plays a crucial role in floral induction especially under SD conditions (Wilson *et al.*, 1992). At least, GA signals regulate the expression of *LEAFY* (*LFY*) (Eriksson *et al.*, 2006). The mutation in *AtGA20ox1* also delays the timing of the floral transition in SD, and the late flowering phenotype was rescued by application of exogenous GA (Coles *et al.*, 1999; Rieu *et al.*, 2008). Therefore, we postulated that the *as1* mutant flowering phenotype described above could be partly caused by changes in the GA signaling levels due to the misexpression of *AtGA20ox1*. To test this possibility, we compared the flowering time of the *as1* alleles with the *AtGA20ox1* mutant, *ga5-3*. The flowering phenotype of the *as1* alleles was similar to that of the *ga5-3* mutant under both LD and SD conditions (Figure 3a,b). Under the 12L/12D conditions, the *ga5-3* displayed a slight late-flowering phenotype, while the *as1* mutants flowered even later than the *ga5-3* plants (Figure 3c). In addition, the application of exogenous GA₃ to the *as1* mutant could not completely rescue the delayed flowering phenotype. On the contrary, the GA₃ application to the *ga5-3* mutant complemented the flowering phenotype observed in the 12L/12D conditions (Figure 3d). This result indicates that, at least under the 12L/12D conditions, *AS1* regulates flowering by both GA-dependent and GA-independent mechanisms.

An *as1* mutation enhances the late flowering phenotype of *co* mutant

Since our biochemical results indicate the presence of the *CO-AS1* complex, we reasoned that if the GA-independent *AS1* flowering function requires functional *CO*, this *AS1* function would not be observed when the *co* mutation is present. To test this possibility we generated an *as1 co* double mutant using the possible *co* null mutant (*co-101*; Takada and

Goto, 2003), which we found that it has a T-DNA insertion in the same position to the null *co-10* allele (data not shown) (Laubinger *et al.*, 2006). When we analyzed flowering phenotype under five different photoperiods (from SD to LD), we found that the *co as1* double mutant always demonstrated a later flowering than the *co* mutant (Figure 4a). When the *co as1* double mutant was grown under the 12L/12D conditions with exogenous GA₃ application, the flowering phenotype of the double mutant was indistinguishable with that of the *co* mutant (Figure 4b). This result shows that the later flowering phenotype of the *co as1* mutant caused by the addition of *as1* mutation was rescued by the treatment of GA application and that the GA-independent *as1* flowering phenotype did not exist when the *co* is mutated. This result implies that the GA-independent AS1 flowering function seems to be dependent on the CO function.

Our flowering experiments demonstrated that AS1 is involved in the GA-dependent flowering time regulation, and that under certain photoperiods AS1 is also involved in the GA-independent flowering time regulation, possibly through the function of CO.

AS1 is involved in the CO dependent regulation of *FT* expression

Given that AS1 likely interacts with CO *in vivo* (Figure 1) and that AS1 may partially regulate flowering through CO function (Figure 4), we hypothesized that the *as1* mutation might also affect the expression level of *FT*, a direct target of CO activity. *FT* transcript levels in the *as1* mutant were slightly lower than those in the wild type plants in LD and 12L/12D (Figure 5b,c), although the *CO* expression in LD between the *as1* mutant and the wild type plants looked identical (Figure 5a). This suggests that AS1 may facilitate CO inducing *FT*. Since CO protein is likely expressed at very low levels, and because CO may be recruited to form various protein complexes (Valverde *et al.*, 2004; Wenkel *et al.*, 2006; Song *et al.*, 2008), we postulated that the endogenous abundance of CO protein could become a limiting factor to analyze the specific role of a potential CO-AS1 complex. To overcome this potential limitation, we utilized transgenic plants in which *CO* expression levels were elevated specifically in phloem tissues where *AS1* is also expressed. To further test whether the *as1* mutation attenuates the ability of CO to induce *FT*, we examined *FT* expression in the *SUC2:CO-HA* transgenic plants with or without the *as1* mutation. For this experiment, the *as1* mutation was introduced into *SUC2:CO-HA* plants by genetic cross, and we confirmed that *CO* expression levels in the transgenic plants with or without the *as1* mutation were similar (Figure 5d). In the *SUC2:CO-HA* plants, the *FT* expression level was drastically higher than that in wild type plants in LD and 12L/12D (Figure 5e,f). In the *SUC:CO-HA/as1-21* line, the *FT* expression level was still higher than that in the wild type; however, it was markedly lower than that in the *SUC2:CO-HA* plants (Figure 5e,f). This result suggests that the elevated *CO* levels (=sensitized conditions) facilitate the depiction of the AS1 contribution to CO dependent *FT* expression. These results indicate that AS1 may assist CO in inducing *FT* under these conditions and this could be a GA-independent role of AS1 in flowering time regulation. We also analyzed whether the difference in *FT* expression between the *SUC2:CO-HA* and *SUC:CO-HA/as1-21* lines caused the difference in flowering time and found that the flowering time of both lines was very similar in LD and 12L/12D conditions (Figure S5), indicating that the *FT* expression levels in both lines are still high enough to induce a similar early flowering.

AS1 binds to *FT* promoter *in vivo*

We have shown that AS1 may form a complex with CO (Figure 1), and that AS1 may participate in CO-dependent *FT* induction (Figure 5). Since CO likely binds to the *FT* promoter directly to regulate *FT* transcription (Tiwari *et al.*, 2010), we examined whether AS1 also binds to the *FT* promoter. We performed chromatin immunoprecipitation (ChIP) analysis using plants expressing functional Hemagglutinin (HA)-tagged AS1 (Figures S3e

and S6; previously described by Theodoris et al., 2003) and examined whether AS1-HA protein was coimmunoprecipitated with the *FT* chromatin (Figure 6a). The *FT* promoter sequences were most highly enriched around amplicons 8 and 11 in the *35S:AS1-HA* line relative to the *as1* mutant (Figure 6a). These contain the regions that CO proteins may associate with the *FT* promoter (Adrian et al., 2010; Tiwari et al., 2010). Our ChIP results indicate a presence of AS1 to the regions adjacent to a transcriptional start site of *FT*.

Since AS1 is a Myb transcription factor, we next analyzed a possible direct binding of AS1 to the *FT* promoter sequences *in vitro*. We detected specific binding of AS1 to the DNA fragment that corresponds to the amplicon 11 region *in vitro* (Figures 6b and S7a). Although we could not successfully identify specific *cis*-elements for AS1 binding, our competition experiments further narrowed down the AS1 binding region to the sequence positions of -371/-272 (Figure 6b). This suggests that the region likely contains an AS1 binding site. Based on the *Arabidopsis* transient expression analysis, this region also participated in the CO-dependent induction of the *FT:GUS* reporter expression, although this region does not contain either the CCAAT-binding sites or the CORE (CO responsive element) sequences (Tiwari et al., 2010). Under our conditions, the presence of CO protein in the reaction did not affect the binding affinity of AS1 to the amplicon 11 sequences (Figure S7b), suggesting that CO may not be required for AS1 to bind the *FT* promoter. Together with our ChIP results, our data indicate that AS1 binds to the region around the transcriptional start site of *FT* *in vivo*.

Discussion

In this manuscript we reported the identification of another CO-interacting protein, AS1. We have shown the evidence that CO directly binds to AS1 (Figure 1). *AS1* diurnal expression is regulated by the circadian clock and its spatiotemporal expression pattern largely overlaps with that of *CO* (Figure 2). The *as1* mutants showed the late flowering phenotype under the 12L/12D and SD conditions, and at least part of the late flowering phenotype is not caused by *as1*-induced alteration of GA biosynthesis (Figure 3). In addition, the *as1* mutation reduced the expression of *FT* without changing *CO* expression (Figure 5). Furthermore, we have demonstrated that AS1 binds to the *FT* promoter *in vivo* (Figure 6). These results indicate that AS1 is involved in the regulation of *FT* transcription by possibly interacting with CO on the *FT* promoter. Since AS1 was originally isolated as an interacting protein to the CO B-box domains, our results also indicate the potential role of the CO B-box domains in the flowering regulation.

The CO B-box domain is involved in the floral regulation

Five out of eight *co* alleles, all of which showed different degrees of the late flowering phenotype, have mutations in the B-box domains (four missense mutations and one deletion) (Robson et al., 2001). This indicates the functional importance of the CO B-box domains for the regulation of photoperiodic flowering. In contrast, most of the known CO interacting proteins that regulate flowering time bind to the CCT domain but not to the B-box domains (Laubinger et al., 2006; Jang et al., 2008; Liu et al., 2008). Thus, the molecular function of the B-box domains still remained elusive. Our results imply that the transcriptional factor AS1 binds to the CO B-box domains and regulates flowering in part through the function of CO. Based on the flowering phenotype of the *as1* mutants, it is improbable that the attenuated binding to AS1 is a sole cause of the *co* mutant phenotypes. We have previously shown that CO may bind to a bZIP transcription factor TGA4 (Song et al., 2008). These results allow us to infer that CO may recruit several different types of transcription factors, other than HAP proteins, that regulate flowering through direct binding to the B-box domains. It would be intriguing to find out what other transcription factors bind to CO B-box domains and when and how the interaction occurs.

AS1 has GA-dependent and -independent roles in the flowering time regulation

Both the photoperiod pathway and the GA pathway contribute to the control of floral induction even under LD conditions (Reeves and Coupland, 2001). Because of the presence of the photoperiod pathway, a defect in the GA pathway by itself does not have a detrimental effect on flowering in LD, but it is necessary for the regulation of flowering when the CO/FT module is also absent (Blazquez and Weigel, 2000; Reeves and Coupland, 2001; Eriksson *et al.*, 2006). Thus there is interdependence between these pathways.

We demonstrated that the *as1* mutants showed the late flowering phenotype when the day was shorter than 14 hours. Under the 12-hour day length, the application of bioactive GA could not completely rescue the *as1* flowering phenotype. In addition, when the *co as1* double mutant was treated with the same amount of GA, the flowering phenotype of the double mutant was indistinguishable with that of the *co* mutant. Moreover, the *co as1* double mutant also showed distinct later flowering than the *co* single mutant. Furthermore, the results of our biochemical analysis indicated that AS1 forms a complex with CO protein. Together with published data regarding the role of AS1 in the GA-dependent pathway (Ikezaki *et al.*, 2010), our results support the notion that AS1 likely has dual roles (GA-dependent and GA-independent) in the regulation of flowering time. One role is regulating the GA levels that affect flowering, and the other is to function as an accessory protein of CO that participates in FT induction under various day-length conditions. FT expression levels in the *as1* mutant are lower than that in wild type plants at least in LD and 12L/12D conditions, although *as1* flowered at the same time as the wild-type plants in LD. This indicates that the FT expression level in the *as1* mutant in LD was still high enough to induce early flowering, which is similar to the wild type plants. Thus, in LD as long as CO is fully active, the contribution of AS1 in photoperiodic flowering seems to be negligible. However, under day-length conditions of 14 hours and shorter, AS1 contributes more to flowering time regulation. Even under SD conditions, the GA-dependent mechanism maintains the basal level of floral induction and AS1 contributes to this mechanism by indirectly regulating the expression of the *AtGA20ox1* gene (Ikezaki *et al.*, 2010). When the day is getting longer, in addition to the AS1-dependent GA-level regulation mechanism, AS1 seemed to facilitate CO to activate FT transcription by potentially direct binding to CO itself. This AS1-dependent CO activation mechanism may enhance the effect of day-length changes on CO activation and subsequently on floral transition especially when CO activity is still relatively low (such as under 12L/12D conditions). Thus AS1 plays a dual role to regulate flowering time under a broad range of photoperiods, and the contribution of AS1 in each pathway changes depending on the day-length conditions. This is a unique example of one protein that is shared between two different pathways that together regulate the same developmental transition to flowering. Our data also indicated that the late flowering phenotype of *co* was partially rescued by the application of GA₃. It still remains to be revealed whether the AS1-CO complex is involved in GA biosynthesis or GA signaling.

In nature, the changes in day length occur gradually throughout the year. Together with other published results (Reeves and Coupland, 2001; Giakountis *et al.*, 2010), our data suggest that as the days lengthen from winter to spring, *Arabidopsis* plants likely utilize both the GA pathway and the photoperiodic pathway to regulate the timing of flowering. Under these conditions, AS1 seems to have an active role in the regulation of flowering in these two different pathways. Having multiple pathways connected to each other to regulate flowering time enables *Arabidopsis* plants to more easily adapt to broader seasonal conditions.

Experimental Procedures

Plant materials and growth conditions

All *Arabidopsis thaliana* plants, WT (Col-0), *asl-1*, *asl-2*, *asl-21*, *ga5-3*, and *co-101*, are in the Col background (Theodoris *et al.*, 2003; Takata and Goto, 2003; Hisamatsu *et al.*, 2005). *asl-1*, *asl-2*, *ga5-3* and *co-101* seeds were gifts from Drs. M. Freeling (University of California, Berkeley), G.T. Kim (Dong-A University), M. Koshioka (National Institute of Floricultural Science), and K. Goto (Research Institute for Biological Sciences), respectively. We also used a new *asl* allele (designated as *asl-21*, ABRC stock number CS841207), which is identified using LB3 (Sessions *et al.*, 2002) and *AS1* gene specific primers (Table S1). For flowering time experiments, plants were grown on soil in 16-h light/8-h dark (16L/8D, LD), 14-h light/10-h dark (14L/10D), 12-h light/12-h dark (12L/12D), 10-h light/14-h dark (10L/14D), and 8-h light/16-h dark (8L/16D, SD) conditions under cool white fluorescent lights (70-90 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Flowering time was measured by counting total leaf number when plants were bolted.

Generation of transgenic plants

For *AS1:AS1-GUS* (*gAS1:GUS*), *AS1:AS1-HA* (*gAS1:HA*), *SUC2:AS1-HA*, and *SUC2:CO-HA* (*SUC2:CO*) constructs, Cauliflower Mosaic Virus 35S promoter in pCambia1303 vector was replaced by either the *AS1* genomic fragment (3805 bp) or the *SUC2* promoter (An *et al.*, 2004). The *AS1* genomic fragment consisting of 2701 bp promoter and *AS1* ORF (1104 bp) was amplified from the BAC clone F13M22 and cloned into the *GUS* coding sequence in the pCambia1303 vector to generate *AS1:AS1-GUS*. The *SUC2* promoter and full-lengths of *AS1* and *CO* cDNA were sequentially inserted in front of the *GUS* coding sequence, generating *SUC2:AS1-GUS* and *SUC2:CO-GUS*. The HA sequences containing a stop codon were inserted in front of the *GUS* gene to generate non-translational fusion with *GUS* for *SUC2:AS1-HA*, *SUC2:CO-HA*, and *AS1:AS1-HA* constructs. For *35S:AS1-HA*, the full-length of *AS1* was introduced into pCambia1303 vector, and then the HA sequences were cloned in front of *GUS*.

Yeast two-hybrid analysis

The experimental procedures of the GATEWAY based yeast two-hybrid assay were based on the manufacturer's protocol (Invitrogen). For the yeast two-hybrid screening, the *CO* cDNA encoding the CO N-terminus (designated as CO Δ C, amino acid positions: 1-130) was introduced in pDEST32 vector to generate the bait construct *BD-CO Δ C*. First, the *BD-CO Δ C* plasmid was transformed into the yeast strain pJ69-A (James *et al.*, 1996). Next, *Arabidopsis* transcription factor (TF) library, which contains about 1100 TF cDNAs in pDEST22 AD vector, was transformed into the yeast cells that possess the *BD-CO Δ C* plasmid. The transformants were selected on SD agar medium lacking Tryptophan (Trp), Leucine (Leu), Histidine (His) for 6 days. Positive colonies were isolated and grown in SD liquid medium lacking Trp, Leu, and His for 2 days, and plasmids were isolated from the yeast cells. The insert DNA fragment was amplified using GAD-F and attB2 adapter primers (Table S1). To confirm the results of the screening, we performed the filter lift β -galactosidase assay.

The full-length of *AS1* cDNA and the *CO* deletion clones were amplified using primers described in Table S1. The PCR products of *AS1* and *CO* fragments were inserted into the pDEST32 bait and pDEST22 prey vectors, respectively. Both bait and prey constructs were co-transformed in pJ69-4A, and the transformants were selected on SD agar medium lacking Trp, Leu, His, and Adenine (Ade). Four to five independent colonies per each combination were transferred into SD liquid medium lacking Trp and Leu. After 2-day incubation, the β -galactosidase activity was quantified using o-nitrophenyl- β -D-galactopyranoside (ONPG).

GST pull down assay

The full-length of *CO* cDNA was cloned into pGEX-5X-1 (GE) vector. pGEX-5X-1 and pGEX-5X-1-*CO* plasmids were transformed in *E. coli* BL21(DE3) strain. GST and GST-*CO* proteins were purified using glutathione-sepharose beads (Sigma). The full-length of *AS1* cDNA was inserted into pDEST17 vector (Invitrogen) to generate 6xHis-*AS1*. ³⁵S-labeled *AS1* proteins were synthesized using pDEST17-*AS1* in the T'nT *in vitro* transcription-translation system (Promega) according to the manufacturer's instructions. Equal amounts (5 µl) of ³⁵S-labeled translation products and the GST-beads were added to 50 µl of binding buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 10 mM EDTA, 2 mM EGTA, and protease inhibitors (complete, Roche)], and incubated at 4°C for 2 h with gentle mixing. After washing the beads with the binding buffer and the proteins bound to the beads were extracted by heating in Laemmli buffer. The extracted proteins and 0.5 µl of ³⁵S-labeled *AS1* (input) were fractionated on the SDS-PAGE gel and subjected to autoradiography.

The full-length and the truncated versions of *CO* were cloned into the GST fusion vector pDEST15 (Invitrogen). All GST- and 6xHis-fused proteins were expressed in *E. coli* BL21 (DE3). GST and GST-fusion proteins were immobilized to the glutathione beads. For *in vitro* GST pull-down assay, 5 µg of total *E. coli* extracts containing 6xHis-*AS1* protein were mixed with GST or GST-*CO* proteins attached to the glutathione-beads in the binding buffer [150 mM NaCl (for Figure 1d,e) or 250 mM NaCl (Figure S1)]. After 2 h incubation at 4°C, the beads were washed with the binding buffer. The proteins attached to the beads were extracted in Laemmli buffer. The *AS1* proteins co-purified with GST-*CO* were detected by western blot using anti-6xHis antibody (PerkinElmer).

Gene expression analysis

To analyze *CO* and *FT* mRNA abundance, plants were grown for 8 or 10 days (LD or 12L/12D, respectively) on the agar media containing half-strength of Linsmaier and Skoog (LS) salts and 2% sucrose and harvested every 3 hours on day 8 or 10. Two µg of total RNA isolated by illustra RNAspin Mini (GE Healthcare) was used for cDNA synthesis using iScript cDNA Synthesis kit (Bio-Rad). Transcripts of *CO*, *FT*, and *IPP2* were measured by quantitative-PCR (Sawa *et al.*, 2007). *IPP2* was used as a control to normalize the amount of cDNA. Sequences of the Q-PCR primers are listed in Table S1.

Intracellular distribution of CO and AS1 in Arabidopsis protoplasts

GFP- and RFP-fusion vectors and the methods for isolating protoplasts and transformation were described previously (Choi *et al.*, 2005). Full-length *AS1* and *CO* cDNAs were fused into *GFP* and *RFP* coding sequences in p326-GFP and p326-RFP vectors to generate *35S:AS1-GFP* and *35S:CO-RFP* constructs, respectively.

Coimmunoprecipitation and western blot

Tobacco (*Nicotiana benthamiana*) transient system was used for the coimmunoprecipitation assay as described in (Baudry *et al.*, 2010). The full-length of *CO* cDNA was cloned into pMDC83 vector (Curtis and Grossniklaus, 2003) by LR recombination reaction (Invitrogen) to generate *CO-GFP* construct. *pCAMBIA1303-35S:AS1-HA* and *pMDC83-CO* plasmids were transformed into the agrobacteria GV3101. Overnight cultured *agrobacterium* carrying *35S:AS1-HA* or *35S:CO-GFP* were individually infiltrated or co-infiltrated into 3-week-old tobacco leaves. After 4 days, tissues were harvested. For coimmunoprecipitation assay, anti-*AS1* antibody were pre-incubated with Protein A agarose (Invitrogen) in binding buffer [100 mM NaCl, 50 mM Tris pH 7.5, 0.2% triton X-100, 1 mM DTT, 50 µM MG132, protease inhibitors, and phosphatase inhibitors (sodium fluoride and sodium orthovanadate)] at 4°C. Approximately 2 mg of protein extracts were added and further incubated for 2 hours with

gentle agitation. Immune complexes were extracted in Laemmli buffer by heating at 90°C for 2 min and subjected to SDS-PAGE.

To detect AS1-HA and CO-GFP proteins, anti-AS1 and anti-CO antibodies were used. The full-length of *CO* cDNA was cloned to pDEST17 vector (Invitrogen) to generate 6xHis-CO. pDEST17-AS1 and pDEST17-CO were introduced into *E. coli* BL21-AI cells to induce 6xHis-AS1 and 6xHis-CO proteins, respectively. His-fusion proteins were purified using Ni-NTA agarose (Qiagen) under denatured conditions according to the manufacturer's manual and used for antibody generation in Rabbit. To purify antibodies, each 6xHis-fusion protein was immobilized on a PVDF membrane and then incubated with Rabbit serum containing anti-AS1 or anti-CO antibody at room temperature for 2 hours. After the membrane was washed five times with 1X TBS at 4°C for 10 min, the antibodies were eluted in 200 mM Glycine pH 2.5 at 4°C for 10 min and then were quickly neutralized by 2 M Tris-HCl pH 8.8. Specificities of anti-AS1 and anti-CO were tested using epitope-tagged overexpression plants (Figure S8).

For western blot, proteins were separated on 10% SDS-PAGE, transferred to a PVDF membrane (Hybond-P, Amersham Biosciences), and probed with anti-GFP (Abcam), anti-HA (Abcam), anti-AS1, or anti-CO antibodies. Immunoreactive proteins were visualized by ECL kits (GE).

Chromatin immunoprecipitation (ChIP) assay

Procedures for the chromatin isolation and immunoprecipitation of protein-chromatin DNA were described previously (Bowler *et al.*, 2004). Briefly, 12-day-old seedlings were vacuum infiltrated with 1% formaldehyde and subsequently 0.125 M glycine and were ground to a fine powder. *Arabidopsis* chromatin pellet was isolated and sonicated in nuclei lysis buffer [50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, 1 mM PMSF, 50 µM MG132, protease inhibitors, and phosphatase inhibitors (Roche)] to shear DNA to approximately 0.5 - 1 kb DNA fragments. The chromatin solution was diluted with ChIP dilution buffer (16.7 mM Tris-HCl pH 8.0, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 1 mM PMSF, 50 µM MG132, protease inhibitors, and phosphatase inhibitors). Protein A agarose beads coated by sheared Salmon sperm DNA (Upstate) were pretreated with anti-AS1 antibody or rabbit serum, which is used as a negative control, and incubated with chromatin solution for 2-3 hours at 4°C. Precipitated DNA was analyzed by quantitative PCR. See Table S1 for the primer sequences used for the amplification of *FT* genomic regions designated as amplicons 1 to 16.

Electrophoretic mobility shift assay (EMSA)

6xHis-AS1 fusion protein used in the GST pull-down assay was used for the EMSA. The crude proteins containing 6xHis-AS1 were extracted in buffer consisting 20 mM HEPES-KOH pH 7.9, 25 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol and protease inhibitors and used without further purification. A method for the EMSA was described previously (Song *et al.*, 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T. FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science*. 2005; 309:1052–1056. [PubMed: 16099979]
- Adrian J, Farrona S, Reimer JJ, Albani MC, Coupland G, Turck F. *cis*-Regulatory elements and chromatin state coordinately control temporal and spatial expression of *FLOWERING LOCUS T* in *Arabidopsis*. *Plant Cell*. 2010; 22:1425–1440. [PubMed: 20472817]
- Amasino R. Vernalization and flowering time. *Curr Opin Biotechnol*. 2005; 16:154–158. [PubMed: 15831380]
- Amasino R. Seasonal and developmental timing of flowering. *Plant J*. 2010; 61:1001–1013. [PubMed: 20409274]
- An H, Roussot C, Suarez-Lopez P, Corbesier L, Vincent C, Pineiro M, Hepworth S, Mouradov A, Justin S, Turnbull C, Coupland G. CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of *Arabidopsis*. *Development*. 2004; 131:3615–3626. [PubMed: 15229176]
- Baudry A, Ito S, Song YH, Strait AA, Kiba T, Lu S, Henriques R, Pruneda-Paz JL, Chua NH, Tobin EM, Kay SA, Imaizumi T. F-box proteins FKF1 and LKP2 act in concert with ZEITLUPE to control *Arabidopsis* clock progression. *Plant Cell*. 2010; 22:606–622. [PubMed: 20354196]
- Baurle I, Dean C. The timing of developmental transitions in plants. *Cell*. 2006; 125:655–664. [PubMed: 16713560]
- Blazquez MA, Weigel D. Integration of floral inductive signals in *Arabidopsis*. *Nature*. 2000; 404:889–892. [PubMed: 10786797]
- Bowler C, Benvenuto G, Laflamme P, Molino D, Probst AV, Tariq M, Paszkowski J. Chromatin techniques for plant cells. *Plant J*. 2004; 39:776–789. [PubMed: 15315638]
- Byrne ME, Barley R, Curtis M, Arroyo JM, Dunham M, Hudson A, Martienssen RA. *Asymmetric leaves1* mediates leaf patterning and stem cell function in *Arabidopsis*. *Nature*. 2000; 408:967–971. [PubMed: 11140682]
- Choi K, Kim S, Kim SY, Kim M, Hyun Y, Lee H, Choe S, Kim SG, Michaels S, Lee I. *SUPPRESSOR OF FRIGIDA3* encodes a nuclear ACTIN-RELATED PROTEIN6 required for floral repression in *Arabidopsis*. *Plant Cell*. 2005; 17:2647–2660. [PubMed: 16155178]
- Coles JP, Phillips AL, Croker SJ, Garcia-Lepe R, Lewis MJ, Hedden P. Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase genes. *Plant J*. 1999; 17:547–556. [PubMed: 10205907]
- Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G. FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science*. 2007; 316:1030–1033. [PubMed: 17446353]
- Curtis MD, Grossniklaus U. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol*. 2003; 133:462–469. [PubMed: 14555774]
- Datta S, Hettiarachchi C, Johansson H, Holm M. SALT TOLERANCE HOMOLOG2, a B-box protein in *Arabidopsis* that activates transcription and positively regulates light-mediated development. *Plant Cell*. 2007; 19:3242–3255. [PubMed: 17965270]
- Eriksson S, Bohlenius H, Moritz T, Nilsson O. GA4 is the active gibberellin in the regulation of *LEAFY* transcription and *Arabidopsis* floral initiation. *Plant Cell*. 2006; 18:2172–2181. [PubMed: 16920780]
- Feng W, Jacob Y, Velez KM, Ding L, Yu X, Choe G, Michaels SD. Hypomorphic alleles reveal *FCA*-independent roles for *FY* in the regulation of *FLOWERING LOCUS C*. *Plant Physiol*. 2011; 155:1425–1434. [PubMed: 21209277]

- Giakountis A, Cremer F, Sim S, Reymond M, Schmitt J, Coupland G. Distinct patterns of genetic variation alter flowering responses of *Arabidopsis* accessions to different daylengths. *Plant Physiol.* 2010; 152:177–191. [PubMed: 19889880]
- Guo M, Thomas J, Collins G, Timmermans MC. Direct repression of *KNOX* loci by the *ASYMMETRIC LEAVES1* complex of *Arabidopsis*. *Plant Cell.* 2008; 20:48–58. [PubMed: 18203921]
- Hay A, Kaur H, Phillips A, Hedden P, Hake S, Tsiantis M. The gibberellin pathway mediates *KNOTTED1*-type homeobox function in plants with different body plans. *Curr Biol.* 2002; 12:1557–1565. [PubMed: 12372247]
- Hay A, Tsiantis M. *KNOX* genes: versatile regulators of plant development and diversity. *Development.* 2010; 137:3153–3165. [PubMed: 20823061]
- Hayama R, Coupland G. Shedding light on the circadian clock and the photoperiodic control of flowering. *Curr Opin Plant Biol.* 2003; 6:13–19. [PubMed: 12495746]
- Hisamatsu T, King RW, Helliwell CA, Koshioka M. The involvement of gibberellin 20-oxidase genes in phytochrome-regulated petiole elongation of *Arabidopsis*. *Plant Physiol.* 2005; 138:1106–1116. [PubMed: 15923331]
- Huang S, Raman AS, Ream JE, Fujiwara H, Cerny RE, Brown SM. Overexpression of 20-oxidase confers a gibberellin-overproduction phenotype in *Arabidopsis*. *Plant Physiol.* 1998; 118:773–781. [PubMed: 9808721]
- Ikezaki M, Kojima M, Sakakibara H, Kojima S, Ueno Y, Machida C, Machida Y. Genetic networks regulated by *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* in leaf development in *Arabidopsis thaliana*: *KNOX* genes control five morphological events. *Plant J.* 2010; 61:70–82. [PubMed: 19891706]
- Imaizumi T. *Arabidopsis* circadian clock and photoperiodism: time to think about location. *Curr Opin Plant Biol.* 2010; 13:83–89. [PubMed: 19836294]
- Iwakawa H, Iwasaki M, Kojima S, Ueno Y, Soma T, Tanaka H, Semiarti E, Machida Y, Machida C. Expression of *ASYMMETRIC LEAVES2* gene in the adaxial domain of *Arabidopsis* leaves represses cell proliferation in this domain and is critical for the development of properly expanded leaves. *Plant J.* 2007; 51:173–184. [PubMed: 17559509]
- Jaeger KE, Wigge PA. FT protein acts as a long-range signal in *Arabidopsis*. *Curr Biol.* 2007; 17:1050–1054. [PubMed: 17540569]
- James P, Halladay J, Craig EA. Genomic libraries and a host strain designed for high efficient two-hybrid selection in yeast. *Genetics.* 1996; 144:1425–1436. [PubMed: 8978031]
- Jang S, Marchal V, Panigrahi KC, Wenkel S, Soppe W, Deng XW, Valverde F, Coupland G. *Arabidopsis* COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. *Embo J.* 2008; 27:1277–1288. [PubMed: 18388858]
- Khanna R, Kronmiller B, Maszle DR, Coupland G, Holm M, Mizuno T, Wu SH. The *Arabidopsis* B-box zinc finger family. *Plant Cell.* 2009; 21:3416–3420. [PubMed: 19920209]
- Kim DH, Doyle MR, Sung S, Amasino RM. Vernalization: winter and the timing of flowering in plants. *Annu Rev Cell Dev Biol.* 2009; 25:277–299. [PubMed: 19575660]
- Kobayashi Y, Weigel D. Move on up, it's time for change -mobile signals controlling photoperiod-dependent flowering. *Genes Dev.* 2007; 21:2371–2384. [PubMed: 17908925]
- Kumimoto RW, Adam L, Hymus GJ, Repetti PP, Reuber TL, Marion CM, Hempel FD, Ratcliffe OJ. The Nuclear Factor Y subunits NF-YB2 and NF-YB3 play additive roles in the promotion of flowering by inductive long-day photoperiods in *Arabidopsis*. *Planta.* 2008; 228:709–723. [PubMed: 18600346]
- Laubinger S, Marchal V, Le Gourrierec J, Wenkel S, Adrian J, Jang S, Kulajta C, Braun H, Coupland G, Hoecker U. *Arabidopsis* SPA proteins regulate photoperiodic flowering and interact with the floral inducer CONSTANS to regulate its stability. *Development.* 2006; 133:3213–3222. [PubMed: 16854975]
- Lin MK, Belanger H, Lee YJ, Varkonyi-Gasic E, Taoka KI, Miura E, Xoconostle-Cazares B, Gendler K, Jorgensen RA, Phinney B, Lough TJ, Lucas WJ. FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. *Plant Cell.* 2007; 19:1488–1506. [PubMed: 17540715]

- Liu LJ, Zhang YC, Li QH, Sang Y, Mao J, Lian HL, Wang L, Yang HQ. COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in *Arabidopsis*. *Plant Cell*. 2008; 20:292–306. [PubMed: 18296627]
- Mathieu J, Warthmann N, Kuttner F, Schmid M. Export of FT protein from phloem companion cells is sufficient for floral induction in *Arabidopsis*. *Curr Biol*. 2007; 17:1055–1060. [PubMed: 17540570]
- McClung CR, Davis SJ. Ambient thermometers in plants: from physiological outputs towards mechanisms of thermal sensing. *Curr Biol*. 2010; 20:R1086–1092. [PubMed: 21172632]
- Mouradov A, Cremer F, Coupland G. Control of flowering time: interacting pathways as a basis for diversity. *Plant Cell*. 2002; 14(Suppl):S111–130. [PubMed: 12045273]
- Parcy F. Flowering: a time for integration. *Int J Dev Biol*. 2005; 49:585–593. [PubMed: 16096967]
- Putterill J, Robson F, Lee K, Simon R, Coupland G. The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell*. 1995; 80:847–857. [PubMed: 7697715]
- Reeves PH, Coupland G. Analysis of flowering time control in *Arabidopsis* by comparison of double and triple mutants. *Plant Physiol*. 2001; 126:1085–1091. [PubMed: 11457959]
- Rieu I, Ruiz-Rivero O, Fernandez-Garcia N, Griffiths J, Powers SJ, Gong F, Linhartova T, Eriksson S, Nilsson O, Thomas SG, Philips AL, Hedden P. The gibberellin biosynthetic genes *AtGA20ox1* and *AtGA20ox2* act, partially redundantly, to promote growth and development throughout the *Arabidopsis* life cycle. *Plant J*. 2008; 53:488–504. [PubMed: 18069939]
- Robson F, Costa MM, Hepworth SR, Vizir I, Pineiro M, Reeves PH, Putterill J, Coupland G. Functional importance of conserved domains in the flowering-time gene *CONSTANS* demonstrated by analysis of mutant alleles and transgenic plants. *Plant J*. 2001; 28:619–631. [PubMed: 11851908]
- Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, Coupland G. Distinct roles of CONSTANS target genes in reproductive development of *Arabidopsis*. *Science*. 2000; 288:1613–1616. [PubMed: 10834834]
- Sawa M, Nusinow DA, Kay SA, Imaizumi T. FKF1 and GIGANTEA complex formation is required for day-length measurement in *Arabidopsis*. *Science*. 2007; 318:261–265. [PubMed: 17872410]
- Sessions A, Burke E, Presting G, Aux G, McElver J, Patton D, Dietrich B, Ho P, Bacwaden J, Ko C, Clarke JD, Cotton D, Bullis D, Snell J, Miguel T, Hutchison D, Kimmerly B, Mitzel T, Katagiri F, Glazebrook J, Law M, Goff SA. A high-throughput *Arabidopsis* reverse genetics system. *Plant Cell*. 2002; 14:2985–2994. [PubMed: 12468722]
- Simpson GG, Dean C. *Arabidopsis*, the Rosetta stone of flowering time? *Science*. 2002; 296:285–289. [PubMed: 11951029]
- Simpson GG. The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of *Arabidopsis* flowering time. *Curr Opin Plant Biol*. 2004; 7:570–574. [PubMed: 15337100]
- Song YH, Song NY, Shin SY, Kim HJ, Yun DJ, Lim CO, Lee SY, Kang KY, Hong JC. Isolation of CONSTANS as a TGA4/OBF4 interacting protein. *Mol Cells*. 2008; 25:559–565. [PubMed: 18587275]
- Song YH, Ito S, Imaizumi T. Similarities in the circadian clock and photoperiodism in plants. *Curr Opin Plant Biol*. 2010; 13:594–603. [PubMed: 20620097]
- Suarez-Lopez P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G. *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. *Nature*. 2001; 410:1116–1120. [PubMed: 11323677]
- Sung ZR, Chen L, Moon YH, Lertpiriyapong K. Mechanisms of floral repression in *Arabidopsis*. *Curr Opin Plant Biol*. 2003; 6:29–35. [PubMed: 12495748]
- Takada S, Goto K. TERMINAL FLOWER2, an *Arabidopsis* homolog of HETEROCHROMATIN PROTEIN1, counteracts the activation of *FLOWERING LOCUS T* by CONSTANS in the vascular tissues of leaves to regulate flowering time. *Plant Cell*. 2003; 15:2856–2865. [PubMed: 14630968]
- Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K. Hd3a protein is a mobile flowering signal in rice. *Science*. 2007; 316:1033–1036. [PubMed: 17446351]

- Theodoris G, Inada N, Freeling M. Conservation and molecular dissection of *ROUGH SHEATH2* and *ASYMMETRIC LEAVES1* function in leaf development. *Proc Natl Acad Sci USA*. 2003; 100:6837–6842. [PubMed: 12750468]
- Tiwari SB, Shen Y, Chang HC, Hou Y, Harris A, Ma SF, McPartland M, Hymus GJ, Adam L, Marion C, Belachew A, Repetti PP, Reuber TL, Ratcliffe OJ. The flowering time regulator *CONSTANS* is recruited to the *FLOWERING LOCUS T* promoter via a unique cis-element. *New Phytol*. 2010; 187:57–66. [PubMed: 20406410]
- Torok M, Etkin LD. Two B or not two B? Overview of the rapidly expanding B-box family of proteins. *Differentiation*. 2001; 67:63–71. [PubMed: 11428128]
- Turck F, Fornara F, Coupland G. Regulation and identity of florigen: *FLOWERING LOCUS T* moves center stage. *Annu Rev Plant Biol*. 2008; 59:573–594. [PubMed: 18444908]
- Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G. Photoreceptor regulation of *CONSTANS* protein in photoperiodic flowering. *Science*. 2004; 303:1003–1006. [PubMed: 14963328]
- Vandenbussche F, Van Der Straeten D. Shaping the shoot: a circuitry that integrates multiple signals. *Trends Plant Sci*. 2004; 9:499–506. [PubMed: 15465685]
- Wenkel S, Turck F, Singer K, Gissot L, Le Gourrierec J, Samach A, Coupland G. *CONSTANS* and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of *Arabidopsis*. *Plant Cell*. 2006; 18:2971–2984. [PubMed: 17138697]
- Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D. Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science*. 2005; 309:1056–1059. [PubMed: 16099980]
- Wilson RN, Heckman JW, Somerville CR. Gibberellin Is Required for Flowering in *Arabidopsis thaliana* under Short Days. *Plant Physiol*. 1992; 100:403–408. [PubMed: 16652976]
- Yanovsky MJ, Kay SA. Molecular basis of seasonal time measurement in *Arabidopsis*. *Nature*. 2002; 419:308–312. [PubMed: 12239570]

Figure 1. CO binds to AS1 *in vitro* and *in planta*

(a) Yeast two-hybrid assay for the CO-AS1 interaction. Serial dilutions of yeast cells harboring different combination of bait and prey constructs were grown on the selection media (SD-Trp, Leu, and His) for the protein-protein interaction assay. For the positive protein-protein interaction control, the HY5 and STH2 interaction (Datta et al., 2007) was used as a reference. (b) Schematic representation of the truncated CO proteins. The constructs containing the truncated CO genes were used in two-hybrid assay shown in (c). (c) Yeast two-hybrid assay to map the AS1 interaction site of CO. Different combinations of truncated CO proteins and the full length of AS1 were expressed in yeast. β-galactosidase (*LacZ*) activities that indicate the strength of the interaction were quantified. Similar mean values were observed in three independent assays. (d) GST pull-down assay for verifying the CO-AS1 interaction. Radioisotope labeled AS1 protein was co-purified with GST-CO protein *in vitro*. (e) *In vitro* binding assay to confirm the interaction domain of CO to AS1 observed in (c). (f) Coimmunoprecipitation assay for the CO-AS1 interaction *in planta*. AS1-HA and/or CO-GFP proteins were transiently expressed in tobacco leaves. AS1-HA proteins were precipitated against anti-AS1 antibody, and the presence of CO-GFP was analyzed. The AS1-HA and CO-GFP proteins were detected by anti-HA and anti-CO antibodies, respectively.

Figure 2. Vascular and diurnal expression of *ASI*

(a) Nuclear localization of AS1-GFP and CO-RFP proteins in *Arabidopsis* leaf protoplast. Scale bar is 10 μ m. (b) Transverse section of a rosette leaf of the *gAS:GUS* plant. P, phloem; X, xylem. A scale bar is 50 μ m. (c-e) Temporal expression profiles of *ASI* in wild-type plants grown in LD (c), SD (d), or continuous light conditions (e). *ASI* expression levels were quantified by real-time PCR. All Data points represent an average of three biological replicates and include error bars (SEM). Open, filled, and grey bars represent light, dark, and subjective dark periods, respectively.

Figure 3. AS1 regulates flowering time under various day-length conditions

(a-c) Flowering time of *as1* alleles under different day-length conditions. Flowering time of *as1* alleles, wild type plants, and *ga5-3* mutant (recently renamed *Atga20ox1*) were measured under LD (a), SD (b), and 12L/12D (c) conditions. At least 16 plants per each line were analyzed, and the means $\pm$ SD are shown in all panels. ** indicates $p \leq 0.01$ calculated using Student's *t* test. (d) Effects of the GA₃ application for flowering time. GA₃ (100 μ M) was sprayed twice a week to the plants grown in 12L/12D conditions until they started to flower. Error bars show SD; $n = 12-18$ plants.

Figure 4. Effect of double mutations in *ASI* and *CO* genes in flowering time

(a) Flowering times of *co-101 as1-21* double mutants under different day-length conditions. At least 18 plants per each line were analyzed. Error bars represent SD (n = 10-14). (b) Application of exogenous GA₃ to 12L/12D-grown plants. Error bars show SD; n = 14-18 plants.

Figure 5. The *as1* mutation reduces the expression of *FT*

Plants were grown in LD (a,b,d,e) and 12L/12D (c,f) conditions. (a-c) Daily *CO* (a) and *FT* (b,c) mRNA expression patterns were examined in WT and *as1* plants. (d-f) Daily *CO* (d) and *FT* (e,f) mRNA expression patterns were examined in WT, *SUC2:CO-HA*, and *SUC2:CO-HA/as1-21* plants. *CO* and *FT* levels were normalized to *IPP2* expression levels. The means $\pm$ SEM of normalized values of three or four independent experiments are shown.