Arabidopsis B-BOX32 interacts with CONSTANS-LIKE3 to regulate flowering

Prateek Tripathi[a], Marcela Carvallo[b,1], Elizabeth E. Hamilton[b,2], Sasha Preuss[c], and Steve A. Kay[a,3]

[a]Department of Neurology, University of Southern California, Los Angeles, CA 90089; [b]Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093; and [c]Monsanto Company, St. Louis, MO 63167

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Plants have the ability to respond to seasonal environmental variations by monitoring day length to initiate flowering. The transition from vegetative to the reproductive stage is the critical developmental switch in flowering plants to ensure optimal fitness and/or yield. It has been previously reported that B-BOX32 (*BBX32*) has the potential to increase grain yield when ectopically expressed in soybean. In the present study, we performed a detailed molecular characterization of the *Arabidopsis* B-box domain gene *BBX32*. We showed that the circadian clock in *Arabidopsis* regulates *BBX32* and expressed in the early morning. To understand the molecular mechanism of *BBX32* regulation, we performed a large-scale yeast two-hybrid screen and identified CONSTANS-LIKE 3 (COL3)/BBX4 as one of its interacting protein partners. Using different genetic and biochemical assays, we have validated this interaction and shown that COL3 targets *FT* in the presence of BBX32 to regulate the flowering pathway. Based on these findings, we hypothesized that this *BBX32-COL3* module could be an additional regulatory mechanism affecting the reproductive development in *Arabidopsis* that could be translated to crops for increased agricultural productivity.

BBX32 | Flowering | Constans-like | *Arabidopsis* | circadian rhythms

Many organisms prepare for seasonal changes by integrating day-length and growth and developmental information. Thus, having a robust mechanism for day-length measurement to thrive in the face of seasonal variations and geographic constraints represents an adaptive advantage for plants. For example, in agriculture, premature flowering affects the overall fitness of the plant, which could lead to dramatic downstream events resulting in loss of crop productivity. The mechanism of flowering has been elucidated through studies describing different physiological and genetic phenotypes in the model species *Arabidopsis thaliana* (1–3). The mechanism for how plants perceive seasonal variations via sensing the light period, and light quality and further coordination of the network of signaling pathways, has been clearly outlined (1, 3–6). *Arabidopsis*, a facultative long-day plant, flowers earlier in long days than in short days, in coordination with the circadian clock and photoperiod (7–10). The molecular mechanism of day-length measurement is comprised of the circadian regulation of CONSTANS (*CO*) gene expression and the light regulation of CO protein stability and activity (11). FLOWERING LOCUS T (FT) protein, expressed in long days, acts as a floral integrator, relaying the signal from the light-sensing leaves via the phloem to the shoot apical meristem (SAM), where flowering is initiated (3, 11). This process, however, occurs only under long-day conditions, when the repression of *CO* by CYCLING DOF FACTORS1 and 2 (CDF1 and CDF2) is relieved. This derepression is mediated primarily by the interaction of the clock-regulated proteins GIGANTEA (GI) and FLAVIN-BINDING, KELCH REPEAT F-BOX1 (FKF1); GI and FKF1 form a light-dependent complex near the end of long days and targets the CDFs to the proteasome via polyubiquitination (10). CO and its interacting partners such as ASYMMETRIC LEAVES 1 (AS1) bind to the *FT* promoter via the C-terminal CCT [CO, CONSTANS-LIKE (COL), and TIMING OF CAB EXPRESSION1 (TOC1)] domain (12, 13). The TARGET OF EAT1 (TOE1) protein binds to the *FT* promoter near the CO-binding site, leading to the reduction of CO activity during the morning. TOE1 interacts with FKF1 in the afternoon, competitively interfering with the FKF1–CO interaction and stabilizing CO (11). Taken together, these results suggest that *CO* may induce *FT* expression through different regulatory mechanisms at different times of day to fine-tune photoperiodic flowering. The *CO-FT* module is highly conserved across different crop species such as rice, barley, maize, tomatoes, sunflowers, and sugar beet with a different mode of action (3). However, the mechanism of *CO-FT* action varies in short days (3). Temperature and geographical locations are also reported to affect the photoperiodic flowering pathway thus affects the seasonal flowering (3). Thus, the integration of geographical and environmental cues makes the whole photoperiodic flowering regulatory framework complex.

In *Arabidopsis*, zinc finger transcription factors (TFs) account for ∼15% of the total genome and play a critical role in plant growth and development (14, 15). The B-BOX (BBX) family members are zinc finger TFs that contain 32 family members. The TF family is divided into five groups (I–V) based on one or two B-BOX motifs involved in protein–protein interaction and the presence or absence of a CCT domain (15). BBX proteins exist in species from algae, mosses, animals, and plants and are functionally diverse (15, 16). The founding and best-characterized member of the B-box family is CO. Studies have reported that other members of the BBX family such as COL3/BBX4, BBX6, BBX7 (COL9), BBX19, BBX24/SALT TOLERANCE (STO), microProtein1a (MiP1a)/BBX31 and BBX32/EMBRYONIC FLOWER-1 INTERACTING PROTEIN6 (EIP6) also affect flowering with distinct mechanisms (17–22).

Significance

Clock genes have been shown to be important in regulating many key agronomic traits. Therefore, identifying new players in this interconnected clock network will provide novel strategies toward developing new crop varieties. Our study identifies CONSTANS-LIKE 3 (COL3) as a critical protein-binding partner for B-BOX32 (BBX32) action in *Arabidopsis*. The discovery of the interaction with COL3 provides molecular clues as to how BBX32 exerts its effects on growth and yield. It also implicates COL3 as an integral protein-binding partner that can be used in combination with BBX32 for increased productivity. This regulatory pathway could be applied as an efficient strategy for genetic manipulation in crops for increased agricultural productivity.

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[1]Present address: Hologic, San Diego, CA 92121.

[2]Present address: Monsanto Company, St. Louis, MO 63167.

[3]To whom correspondence should be addressed. Email: stevekay@usc.edu.

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It was suggested that *BBX32* may play a critical role at the interface of light and the circadian clock in soybean (23). Interestingly, overexpression of the *Arabidopsis BBX32* has been shown to significantly increase grain yield in soybean (23), implicating the role of this family of TFs in plant reproductive fitness. In the present study, we dissected the molecular mechanism of *BBX32* action in *Arabidopsis*. We show that *BBX32* is regulated by the circadian clock, regulates flowering and hypocotyl growth, and directly interacts with COL3. We provided various supporting genetic and biochemical evidence to establish this interaction and proved that *FT* is a direct target of COL3. Thus, this study proposes a role for *BBX32* in the flowering pathway and a possible mechanism of action.

Results

***BBX32* Is Regulated by the Circadian Clock.** To understand the relationship between the *Arabidopsis BBX32* (*At3g21150*) and the clock, we first evaluated the rhythmic expression pattern of *BBX32* in diurnal conditions (12 h light and 12 h dark cycles; 12L:12D) and free-running conditions (continuous light; LL). We performed a time course experiment with samples harvested every 3 h and determined the relative mRNA expression level by using quantitative RT-PCR (qPCR). Our results show that *BBX32* expression is rhythmic under both diurnal and free-running conditions with the peak expression in the early morning (Fig. 1 *A* and *B*). To confirm *BBX32* clock regulation, its expression in mutants of CIRCADIAN CLOCK ASSOCIATED1 (*cca1-1*), *toc1-4*, and EARLY FLOWERING3 (*elf3-3*) was checked (24–26). Relative mRNA expression of samples harvested every 4 h showed expression of *BBX32* was altered, indicated by the loss of robust oscillations in *elf3-3* and almost arrhythmic with increased expression level in *toc1-4* mutants (Fig. S1*A*). In *cca1-1* mutant, expression of *BBX32* appears to be altered with a short-period phenotype (Fig. S1*A*). These observations suggest a quantitative change in the pace of the clock, but to better understand the detail mechanism, further investigation is needed. *BBX32* has been shown to alter *CCA1*, LONG ELONGATED HYPOCOTYL (*LHY*), and *TOC1* gene expression in soybean (23); thus, *CCA1*, *LHY*, and *TOC1* expression was checked in *Arabidopsis* plants overexpressing BBX32. Flag-tagged *BBX32* overexpression line (*BBX32-OX*) (Fig. S1*B*) harvested every 4 h in 12L:12D conditions showed altered and reduced expression of *CCA1* and *LHY* and a modest increase in *TOC1* levels in *Arabidopsis* (Fig. S1*C*). Bioluminescence assays performed with the *BBX32* overexpression line containing a *LHY* promoter luciferase fusion (*LHY::LUC*$^+$) under LL showed that when overexpressed, *BBX32* lengthens the period by ~2 h (Fig. 1 *C* and *D*). To determine whether this alteration in promoter activity is also evident at the mRNA level, we checked the endogenous expression of *LHY* in *BBX32-OX* under LL over 2 d. We observed a shift in the *LHY* expression compared with wild type, confirming that *BBX32* regulates the clock in *Arabidopsis* (Fig. 1*E*). We also generated artificial microRNA lines (*BBX32-AMI*) (Fig. S2*A*). These *BBX32-AMI* lines were transformed with the *LHY* reporter, and bioluminescence assays were performed to assess *LHY* promoter activity under LL and a modest period lengthening was observed (Fig. 1*F* and Fig. S2*B*). Furthermore, clock-regulated phenotypes such as flowering time and hypocotyl growth were examined. *BBX32-OX* flowered late under both long (16L:8D) and short (8L:16D) day conditions at 22 °C compared with wild type (Fig. S2*C*). *BBX32-AMI* plants also have a late flowering phenotype compared with the wild type under both long- and short-day conditions (Fig. S2*C*). The delayed flowering and circadian phenotypes of *BBX32-AMI* could be due to the redundancy among the TF family and/or feedback regulation. Also, *BBX32-OX* showed long hypocotyl growth in long and short days (Fig. S2*D*). Thus, taken together, these data indicate that BBX32 is a morning gene under clock regulation, and it suggests that *BBX32* may regulate the clock and may have a role in flowering and hypocotyl growth.

BBX32 Physically Interacts with COL3. Protein–protein interaction is a key functional aspect of this TF family's regulatory mechanism. B-box proteins, including BBX32, have been shown to interact with SALT TOLERANCE HOMOLOG2/BBX21 to regulate photomorphogenesis by acting antagonistically on ELONGATED

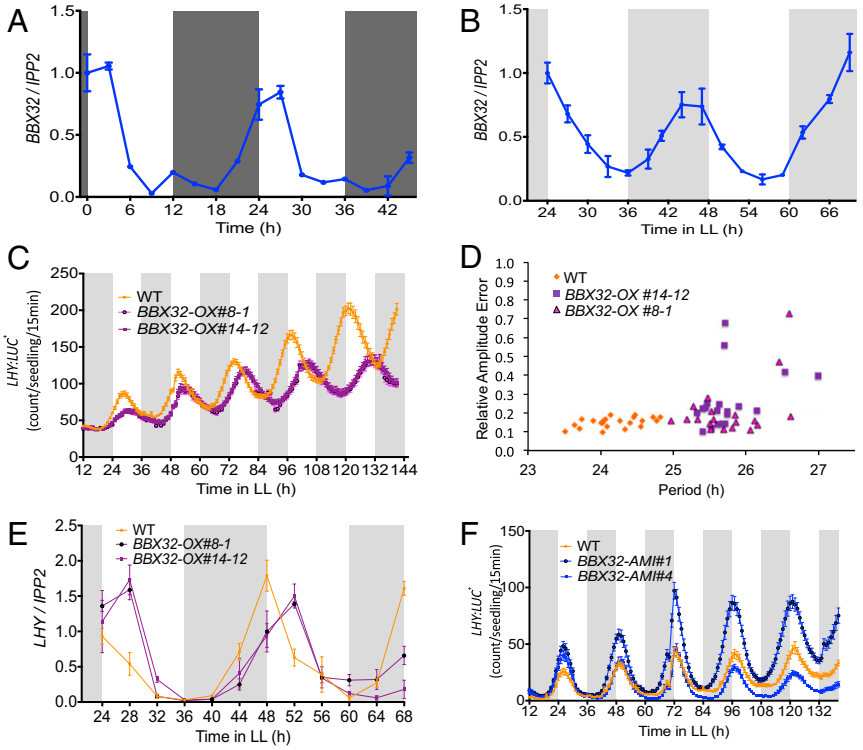

Fig. 1. Gene expression and phenotype analysis of *BBX32*. Relative gene expression analysis of *BBX32* under diurnal conditions (12L:12D) (*A*) and free running conditions (LL) (*B*) at 22 °C. Wild-type (Col-0) seedlings were grown in 12L:12D for 7 d (*A*) and 12L:12D for 6 d and transferred to continuous light (LL) and harvested 24–68 h later (*B*). Error bars represent the SEM of biological triplicates. Experiments were independently repeated three times, each time with two biological replicates per genotype. (*C*) *BBX32-OX:LHY-LUC*$^+$ activity under LL. Six-day-old seedlings entrained in 12L:12D were monitored for 5–7 d under LL. Values are shown as mean ± SEM; $n = 20$. (*D*) Relative amplitude error of WT and *BBX32-OX* seedlings imaged for 5 d under LL conditions calculated by using FFT-NLLS; $n = 20$. (*E*) Relative mRNA expression of *LHY* in WT and *BBX32-OX*. Six-day-old WT and *BBX32-OX* seedlings were grown in 12L:12D and transferred to continuous light (LL) and harvested 24–68 h later. (*F*) *BBX32-AMI:LHY-LUC*$^+$ activity under LL. Six-day-old seedlings entrained in 12L:12D were monitored for 5–7 d under LL. Values are shown as mean ± SEM; $n = 20$. Experiments were repeated three times independently.

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HYPOCOTYL5 (HY5) (27). To gain a deeper insight into the molecular function of *BBX32*, we performed a high-throughput yeast two-hybrid (Y2H) screen by using BBX32 as bait (Dataset S1). One of the promising interactors in our Y2H screen was COL3 (*At2g24790*). Further analysis confirms that the N terminus of COL3 interacts with BBX32 (Fig. 2*A*). COL3 is also a BBX TF family member and is temporally coexpressed with *BBX32* (Fig. S3*A*). GFP-tagged overexpression of *COL3* (*COL3-OX*) (Fig. S3*B*) also shows a late flowering phenotype in long days compared with the wild type similar to what was observed for *BBX32-OX* (Fig. S3*C*).

To validate this interaction and evaluate its subcellular localization, we performed a bimolecular fluorescence complementation (BiFC) assay. For this assay, the *35S:BBX32:YFP* construct with YFP tag fused either to the N or C terminus were generated. Similar to COL3, BBX32 localized to the nuclear speckles (Fig. S4*A*) (26). Therefore, to confirm the interaction of BBX32 and COL3 *in planta*, the N-terminal half of YFP was fused to BBX32, and the C-terminal half of YFP was fused to COL3. Both constructs were then transiently expressed in tobacco (*Nicotiana bethamiana*). Reconstituted YFP fluorescence was observed in the nucleus, confirming that BBX32 and COL3 are colocalized and interacts *in planta* (Fig. 2*B*). Together these results indicate that both proteins possess a similar localization pattern and phenotype, which suggests that these proteins may form a complex to regulate flowering.

To further validate this interaction in vivo, coimmunoprecipitation assays were performed in tobacco and *Arabidopsis*. For this assay, a BBX32 C-terminal fusion construct (*BBX32 OX-HFC*) and a COL3-GFP fusion construct (*COL3 OX-GFP*) were generated (Fig. S4*B*). COL3-GFP specifically coimmunoprecipitated with BBX32-HFC when both *BBX32 OX-HFC* and *COL3 OX-GFP* were expressed in tobacco leaves (Fig. 2*C*). In *Arabidopsis,* COL3-GFP also immunoprecipitated with BBX32-HFC, confirming that BBX32 physically interacts with COL3 in vivo (Fig. 2*D*).

Genetic Interaction and Physiological Characterization of Flowering Time. To understand the functional relationship and genetic interaction between *BBX32* and *COL3* and their role in the regulation of flowering, we investigated whether the late flowering phenotype of *BBX32* depends on *COL3*. We generated *BBX32-OX* in Wassilewskija (WS) background along with *BBX32-OX* and *BBX32-AMI* lines in a *col3* mutant background (*BBX32-OX_col3* and *BBX32-AMI_col3*) (Fig. S1*B*). Homozygous lines were obtained

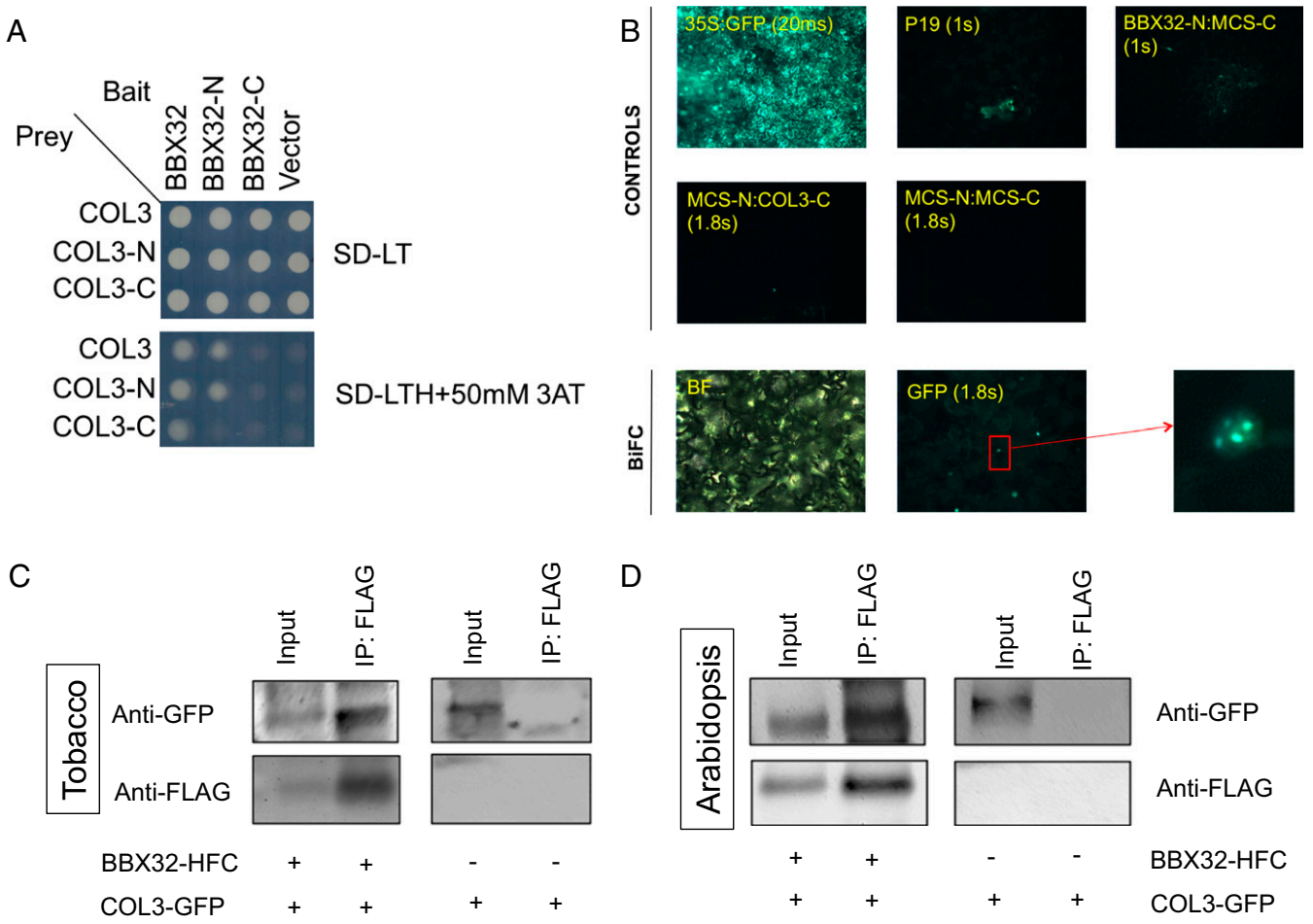

Fig. 2. BBX32 physically interacts with COL3. (*A*) Y2H assay between BBX32 and each of COL3, COL3-N, and COL3-C. These experiments were repeated twice. (*B*) BiFC in tobacco. COL3-YFP^{C} interacts with BBX32-YFP^{N} in the nucleus and localized to nuclear speckles. *35S:GFP,* p19 and empty vector (MCS-N MCS-C) were used as negative controls. B32-N MCS-C represents the fusion of BBX32 on N terminus alone; MCS-N COL3-C represents the fusion of COL3 on C terminus alone (*Materials and Methods*). Enlarged image of the nuclei shows the size and number of speckles. BF, bright field. (*C* and *D*) Co-Immunoprecipitation (Co-IP) in tobacco and *Arabidopsis*, respectively. Immunoprecipitations (IPs) were performed on tobacco leaves (3 dpi) and 10-d-old *Arabidopsis* seedlings grown in long days (16L: 8D) at 22 °C. Tissues were harvested 1 h after dawn. IP is performed by using anti-FLAG antibody and COL3 was coimmunoprecipitated with anti-GFP antibody. A 5% input was used. Western blots were performed on 10% (wt/vol) precast gels (Bio-Rad), and experiments were repeated at least five times.

for each construct, and flowering time analysis was performed in long days (16L:8D) at 22 °C (Fig. 3*A*). Regardless of the genetic background, *BBX32* produces a late flowering phenotype when overexpressed (Fig. 3*A*). Also, *COL3* gene is required for alterations in BBX32 mRNA levels to alter flowering time, because both *BBX32-OX_col3* and *BBX32-AMI_col3* showed an early flowering phenotype similar to *col3* (Fig. 3*A*) compared with wild type (in this case, WS). Moreover, epitope-tagged double mutants overexpressing both *BBX32* and *COL3* (*BBX32-OX: COL3-OX*) were generated (Fig. S5*A*), and flowering time was assessed (Fig. 3*B*). The *BBX32-OX:COL3-OX* plants were late flowering compared with wild type (Fig. 3*B*). Taken together, our data suggest that the late flowering observed in lines with altered levels of *BBX32* depends on *COL3*.

CO and *FT* are two major genes regulating the flowering pathway in *Arabidopsis* under long days. Hence, we checked the expression of these two genes in *BBX32-OX* and *BBX32-AMI* lines. No significant change in *CO* expression was observed in *BBX32-OX*, *BBX32-AMI*, and *BBX32 AMI_col3* lines (Fig. S5 *B* and *C*). However, *FT* levels were reduced in *BBX32-OX* and *BBX32-AMI* lines (Fig. S5*B*). Thus, we reasoned that the late flowering phenotype could be *FT* mediated. We next checked the expression of *FT* in our epitope-tagged single and double mutants of *BBX32* and *COL3* harvested every 4 h under long-day conditions (16L:8D) at 22 °C. Our data distinctly show that lines that flowered earlier have more *FT* expression, whereas the late flowering lines have less *FT* expression (Fig. 3 *C* and *D*).

***FT* Is a Target of COL3.** Because COL3 is a TF and *col3* exhibits an early flowering phenotype and increased levels of FT mRNA, we reasoned that the *FT* promoter could be a target of COL3 regulation. To answer this question, we used a modified yeast-one hybrid (Y1.5H) and chromatin immunoprecipitation (ChIP) assays. In our Y1.5H, we evaluated the binding of COL3 to the *FT* promoter with or without the presence of BBX32. The concept underlying this assay was to express the protein of interest (BBX32) with an activation domain and evaluate the activation of the candidate DNA (*FT*) regions fused to a reporter in the presence and/or absence of the interacting partner (COL3) in the yeast system. This assay will allow us to determine whether the interaction between these two proteins is required for the activation of the target.

Six fragments (FR1–6) of the *FT* promoter were integrated into the yeast strain YM4271 (Fig. 4*A*). Individually, neither BBX32 nor COL3 was sufficient to bind and activate *FT*. However, coexpressing the BBX32 activation domain fusion with COL3 resulted in successful binding and activation of *FT* FR1 and FR4. To further quantify this observed activation, we measured the β-galactosidase activity with FR1 and observed a fourfold induction of the reporter gene activity (Fig. 4*B*). FR4 did not pass our threshold of ≥twofold. These data suggest that COL3 directly binds to *FT* promoter in the presence of BBX32, at least in a heterologous system.

To confirm the binding of COL3 to the *FT* promoter in vivo, we also performed a ChIP assay by using plants expressing functional GFP-tagged *COL3* (*COL3-OX*) (Fig. S4*B*) and determined whether COL3-GFP protein was able to immunoprecipitate the *FT* promoter region of the chromatin (Fig. 4*C*). The immunoprecipitated DNA was highly enriched in the *FT* promoter regions denoted as amplicons 1 and 4 in *COL3-OX* line relative to *35S:GFP* (Fig. 4*C*). These amplicons contain the regions to which CCT domain proteins may associate with the *FT* promoter (12, 28). Moreover, ChIP experiments were also performed in plants expressing a functional Flag-tagged *BBX32* (*BBX32-OX*) in wild type and (*BBX32 OX_col3*) in *col3* mutant background (Fig. S4*B*). The immunoprecipitated DNA was enriched in the *FT* promoter regions (amplicons 1 and 4) in *BBX32-OX* but not in *BBX32-OX_col3* (Fig. 4*D*). Our ChIP data indicates the presence of COL3 in the region adjacent to the transcriptional start site of *FT*.

Discussion

We have performed characterization of *BBX32*, a group V member of BBX TF family, at the molecular level (15). The unique architecture of *BBX32* with only one B-box domain known for protein–protein interactions made BBX32 an interesting candidate to study further (21). Also, previous studies in soybean have shown that the *Arabidopsis BBX32* has a high potential for increasing crop productivity (23). However, the molecular mechanism of *BBX32* regulation in soybean or *Arabidopsis* was not known. Thus, we performed a detailed molecular study in *Arabidopsis* and found that

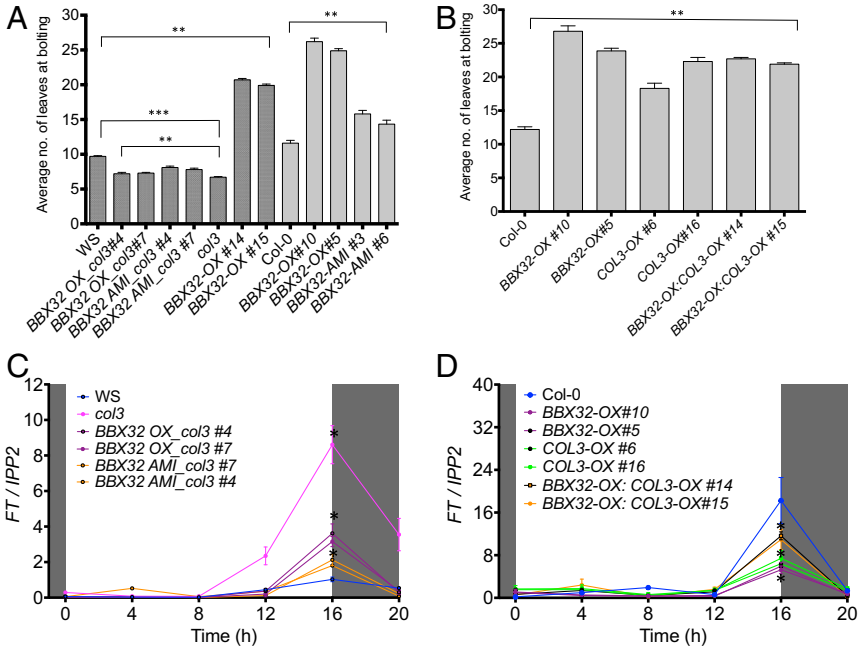

Fig. 3. Flowering time analysis and expression of *FT* in overexpression and higher order mutant lines. (*A* and *B*) Flowering time analysis was performed on plants grown in 16L:8D cycles at 22 °C for different epitope-tagged single and double transgenic plants. Shaded bars represent mutant lines in WS background. Data shows average number of leaves at bolting, and error bar represents SE ($n = 12$). ***$P \leq 0.0001$; one-way ANOVA (*A*) and **$P \leq 0.001$; unpaired *t* test (*B*). (*C* and *D*) Relative expression analysis of *FT* in all epitope-tagged single and double mutants. Experiments were performed on 7-d-old seedlings grown in 16L:8D cycles at 22 °C. Error bars represent the SEM of biological triplicates. Experiments were independently repeated three times, each time with two biological replicates per genotype. Unshaded and shaded area represents light and dark period, respectively.

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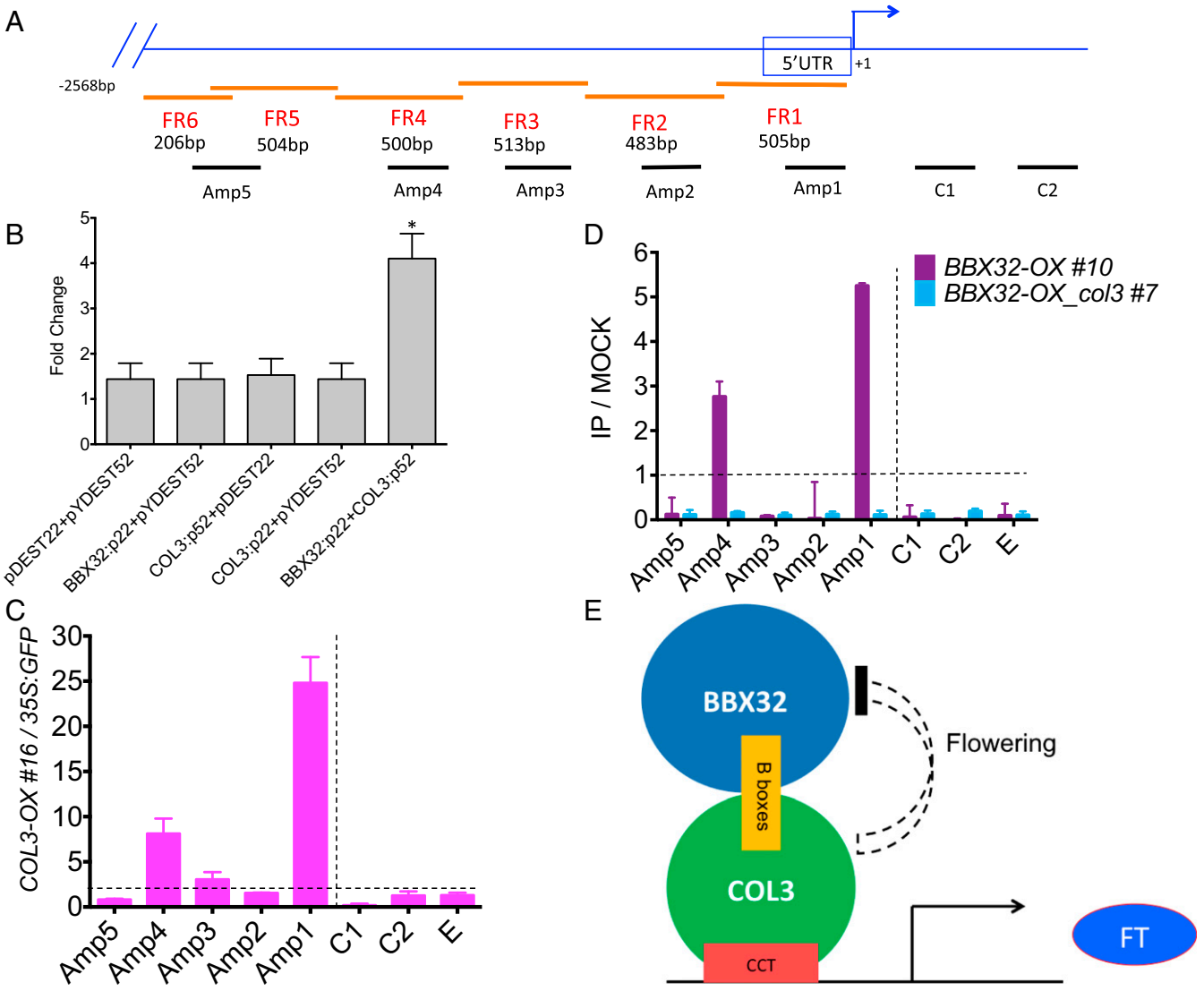

Fig. 4. COL3 targets *FT* to regulate flowering. (*A*) Schematic representation of the *FT* gene and amplified promoter regions used for yeast and ChIP assays. FR (1–6) represent fragments cloned in yeast and Amp (1–5) represent amplicons amplified for ChIP assay. C1 and C2 represent regions of locus-control used in ChIP as described (10). (*B*) β-galactosidase activity in yeast FR1. The fold change of β-galactosidase activity of each construct was calculated by normalizing over control vectors. Bars represent the average of two biological replicates, and error bars represent the SEM. $^{**}P < 0.001$ (unpaired *t* test). (*C* and *D*) Chromatin immunoprecipitation (ChIP) analysis of GFP-tagged *COL3-OX #16* and Flag-tagged *BBX32-OX #10* on the *FT* promoter. The 10-d-old GFP-tagged *COL3-OX #16*, *35S:GFP*, Flag-tagged *BBX32-OX #10*, and Flag-tagged *BBX32 OX_col3 #7* seedlings were grown in 16L:8D cycle at 22 °C and harvested at 2–3 h after dawn. The ratio of specific enrichment in the GFP-tagged *COL3-OX #16* samples and that in the *35S:GFP* sample is represented (*C*) whereas FLAG-tagged *BBX32-OX #10*; FLAG-tagged *BBX32 OX_col3 #7* and that in mock (no antibody) (*D*) in each region were plotted. Experiments were independently repeated three times, each time with two biological replicates. C1 and C2 are the control regions and E (*UBQ10*) is a nonlocus control as described (10). Dotted line represents no enrichment. (*E*) Model representing mechanism of action of *BBX32* in regulating flowering.

BBX32 participates in the regulation of flowering. With enhanced agronomical trait(s) such as flowering, *BBX32* can play an important role in plant growth and development, thus contributing as one among other plausible mechanism(s) for the increase in broad acre yield in soybean. We have demonstrated that *BBX32* is a clock gene with an early morning expression peak. We have established that COL3 interacts with BBX32, and possess similar expression patterns and phenotypes. Moreover, our data corroborates results from previous studies, which implicated the B-BOX domain of BBX32 in the formation of heterodimers with other members of the family to modulate their activities (27). Hence, this BBX32–COL3 interaction is indispensable in defining its mechanism of action. We have also shown that COL3 targets *FT* and affects its regulation of flowering time in *Arabidopsis*. Thus, our proposed model is the interaction of BBX32 with COL3 enables COL3 to bind to the *FT* promoter and represses its transcription (Fig. 4*D*), but the detailed mechanism needs further investigation.

The broad acre yield observed in soybean upon ectopic expression of *Arabidopsis BBX32* suggests that it might have pleiotropic effects on growth parameters (23). This *BBX32-COL3* module suggests they potentially modulate growth parameters via flowering time regulation. Despite the variation of genome complexity and domestication, *BBX32-COL3* could hold its merit to explain the enhanced agronomical trait(s) in soybean. It could be an additional regulatory mechanism, which facilitates soybean flowering in a range of photoperiods. Also, *BBX32* is in phase with the photoperiod-sensitive gene, *COL3*. Whether this relation of clock

and flowering pathway via *BBX32* is direct or indirect, with or without the involvement of *CO* needs further investigation. The phenotype of the *BBX32-OX:COL3-OX* suggest that the *COL3* could be a part of the bigger complex involving other "modifiers" including *COL3* that leads to architectural changes in overall plant growth as observed in soybean. At the same time, the possibility of B-BOX proteins being recruited to their targets via common DNA binding motif facilitating diverse effects in different plants cannot be ruled out either. Also, recent studies with BBX19 and MiP1a (*BBX31*) showing attenuation of CO activity add a new layer to the flowering regulation by BBX proteins (20, 22).

Thus, the involvement of BBX proteins in flowering suggests their potential use in transgenic crops to obtain desirable agronomic trait. This study describes the molecular function of two B-BOX family members whose physical interaction result in a late flowering phenotype. In fact, this present study provides the direct primary evidence of translational approach on how *BBX32* exerts an effect on growth and yield, which could be a profitable strategy to generate early or late flowering plants depending on production requirements or climatic and geographic limitations. Early flowering could be a favorable trait for crops cultivated primarily for seeds whereas late flowering could be an advantage when total biomass is the goal of production (29). This *BBX32-COL3* module could apply to other crop species and can potentially result in crop improvement.

Materials and Methods

Plant Materials and Growth Conditions. Overexpression and artificial microRNA *Arabidopsis* lines were generated in the Col-0 background, whereas the higher order mutants were generated in *col3* background by using *A. tumefaciens*-mediated transformation (floral dip) (30). *BBX32-OX:COL3-OX* double transgenic plant was generated by crossing Flag-tagged *BBX32-OX* to GFP-tagged *COL3-OX*. The *col3* mutant was in WS background (18) hence the WS is used as wild type when single mutant and higher order mutants were used in the experiments. For flowering time analyses, seeds were sown in soil (Sunshine Mix), fertilized once in a month (15–16–17 Peat Lite Special; Everris) and grown in a growth chamber (Conviron) with long-day conditions of 16 h light and 8 h dark (16L:8D) at 22 °C and 50% humidity. The total leaf number was counted at the time of bolting. For hypocotyl measurement, seeds after 3-d stratification were grown on 0.5× MS (no sucrose) vertical plates under short-day conditions (8L:16D) in a growth chamber (Percival) at 80 μE intensity at 22 °C. The hypocotyls were measured by using ImageJ software after scanning the seedlings on day 6. For RNA isolation, 7-d-old seedlings grown in growth chambers (Percival) with lights at 60–80 μE intensity were harvested and immediately frozen in liquid nitrogen and stored in −80 °C. All of the phenotyping experiments were repeated at least three times with similar results, using two independent transgenic lines and data shown for one representative line.

Details. Details of molecular cloning and constructs, luciferase imaging and data analysis, yeast one-hybrid assay, Y2H, bimolecular fluorescence complementation, coimmunoprecipitation, chromatin immunoprecipitation, and gene expression analyses are provided in *SI Materials and Methods* and Table S1.

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