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# Analysis of protein binding characteristics among *Arabidopsis* BBX protein family

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**Abstract**

Plants have evolved various mechanisms of adjusting their diurnal and seasonal growth and development in response to variations in day length and light quality. This plasticity is facilitated by intricate regulatory networks that comprise transcription factors, whose expression is modulated by the activity of photoreceptors. In *Arabidopsis*, B-box (BBX) transcription factors, which contain one or two Zn-ligating B-box motifs in their N-termini, serve as key mediators of light signaling for photomorphogenesis, shade avoidance, and photoperiodic flowering. While multiple BBX proteins may function as a single regulatory unit, the binding networks that form among members of the BBX family have not been extensively investigated. Here, we have demonstrated that the homodimerization of two B-box motifs containing CONSTANS protein (BBX1), which regulates light signaling and is the most extensively characterized among all BBX proteins, requires at least three B-box motifs. Therefore, the number of B-box motifs may significantly influence heterodimerization among BBX family members. An interactome analysis of all 32 known B-box family members revealed that the binding affinity between group III and V proteins with only one B-box motif is relatively weaker than that observed among other group members. In fact, the group V proteins BBX26 and BBX27 rarely interact with other BBX members. Taken together, the results of this study emphasize the importance of the B-box motif in network formation among BBX proteins and provide insights into investigating the various signaling pathways mediated by these networks.

**Keywords** B-box protein, CONSTANS, Florigen, *FLOWERING LOCUS T*, Flowering time, Heterodimer, Homodimer, Transcription factor

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## Introduction

Plants constantly monitor light information to synchronize their physiology with the variations in their environment [1, 2]. Changes in day length and light quality determine developmental processes such as flowering time [1, 2]. In *Arabidopsis*, the B-box (BBX) transcription factor (TF) CONSTANS (CO/BBX1) conveys light information for the induction of *FLOWERING LOCUS T* (*FT*) gene that encodes a major component of florigen [3–5]. The day length-dependent regulation of *FT* expression by CO protein is a crucial aspect of seasonal flowering [1, 2]. The blue light photoreceptor FKF1 modulates the expression pattern of *CO* gene, whose transcripts highly accumulate under light in long-day conditions (LDs) but remain very low during the day in short-day conditions (SDs) [6]. This enables

*Arabidopsis* plants to induce *FT* highly in LDs and consequently promote flowering in these conditions [7].

B-box and CCT [CO, CONSTANS-LIKE (COL), and TIMING OF CAB EXPRESSION 1 (TOC1)] are highly conserved domains in CO. Both serve as binding sites for protein–protein interactions [8, 9]. The activity and stability of CO protein for *FT* induction are modulated by its interacting partners [2, 7, 10–13]. The coiled motif-containing proteins SUPPRESSOR OF PHYA1 (SPA1) and CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) interact with CO probably through the C-terminal CCT domain and degrade it during the night [14, 15]. The CCT domain of CO binds to the *FT* promoter along with nuclear factor (NF) proteins [16–18]. In addition, the stability of CO during the daytime is controlled by photoreceptors that interact with it. However, the domain responsible for the formation of this complex has not yet been identified. The red light photoreceptor phytochrome B (phyB) coupled with HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1) and the blue light photoreceptor ZEITLUPE (ZTL) degrade CO, whereas the ZTL homolog FKF1 stabilizes it [2, 19–22]. The N-terminal B-box domain of CO binds to other classes of TFs such as *octopine synthase (ocs)*-element-binding factor 4 (OBF4) and ASYMMETRIC LEAVES 1 (AS1) [23, 24]. Additionally, the two B-box motifs in the domain are required for homodimerization as well as heterodimerization with other B-box proteins. BBX19, BBX28, BBX30, and BBX31 contain only one or two B-box motifs and lack the CCT domain. They form protein complexes with CO, which prevents *FT* induction under LDs at 22 ℃ [12, 25, 26]. The splicing variant of CO lacking the CCT domain interacts with full-size CO and promotes its degradation [10].

Like CO, BBX proteins form heterodimeric complexes with other BBX members, likely via B-box motifs [13]. Several BBX proteins share the same interacting partner and have similar functions, indicating that functional redundancy exists among BBX proteins [9, 13]. BBX proteins participate in various signaling pathways regulating photomorphogenesis, shade avoidance, and flowering. Nevertheless, the functions of many BBX proteins are unknown [13]. Hence, it is necessary to identify the binding networks among BBX proteins in order to predict and clarify the roles of BBX family members. Here we performed an interactome analysis on all BBX proteins and established that the proteins form homodimer or heterodimer complexes, with a preference for those containing more B-box motifs.

## Materials and methods

### Subcellular localization and bimolecular fluorescence complementation (BiFC) assays

Full-length *CO*, *CO 1–132* variant, *BBX30*, and *BBX31* cDNAs were subcloned into the BiFC vectors pDEST-VYNE(R)$^{GW}$ and pDEST-VYCE(R)$^{GW}$ [27] and the constructs *35S:N-YFP-CO 1–132*, *35S:N-YFP-BBX30*, *35S:N-YFP-BBX31*, *35S:C-YFP-CO*, and *35S:C-YFP-CO 1–132* were generated. CO, the CO 1–132 variant, BBX31, and BBX31 were expressed under the control of the CaMV 35S promoter. For subcellular localization, nucleotide sequences encoding the full-length CO, Coβ truncated at the C-terminal, BBX28, and BBX31 were cloned into pMDC43 vector to create a GFP C-terminus fusion [28]. *Arabidopsis* mesophyll protoplast transfection was conducted and a tobacco transient expression system was utilized to analyze subcellular localization and protein–protein interactions by BiFC. The procedures used for protoplast isolation were previously described [29]. Tobacco plants grown for 3 weeks under long-day conditions (LDs) were used for the infiltration. The plasmids were transformed into *Arabidopsis* mesophyll protoplasts and tobacco leaves via polyethylene glycol (PEG)-mediated transformation and *Agrobacterium*-mediated infiltration, respectively, as previously described [30, 31]. Green fluorescent protein (GFP) and yellow fluorescent protein (YFP) were viewed and imaged under a fluorescence microscope (AX70; Olympus Corp., Tokyo, Japan).

### Yeast two-hybrid and β-galactosidase assays

For the yeast two-hybrid assays, cDNA encoding full-length CO, the CO deletion derivatives CO 1–254, CO 1–132, CO 133–373, CO 1–105, CO 1–82, CO 1–62, and CO 63–105, and the N-termini of the *co* mutant alleles *co-2* 1–132, *co-3* 1–132, *co-4* 1–132, and *co-6* 1–132 were amplified by polymerase chain reaction (PCR) and subcloned into pDEST22 or pDEST32 vectors to generate the GAL4 activation domain (AD) and the GAL4 DNA-binding domain (BD) fusion constructs, respectively. The N-terminal region of the BBX protein containing the complete B-box domain was used to elucidate the binding networks among BBX proteins while avoiding false positive selection resulting from self-transactivation. The bait (BD fusion) and prey (AD fusion) plasmids were co-transformed into pJ69-4A via the standard lithium acetate method. The procedures used for the yeast two-hybrid assays (including heat shock-mediated transformation and transformant selection) and the β-galactosidase assays were previously described [24]. After transformation, yeast cells were grown on various types of SD agar media including those (1) lacking leucine (Leu) and tryptophan (Trp), (2) lacking Leu, Trp, and histidine (His) in the presence of 0.1 mM, 0.5 mM, 1 mM, or 5 mM 3-amino-1,2,4-triazole (3-AT), or (3) lacking Leu, Trp, His, and adenine (Ade). Protein–protein interactions were confirmed through β-galactosidase

activity and quantified with *o*-nitrophenyl-β-*D*-galactopyranoside (ONPG).

### In vitro pull-down assays

The procedures used to construct protein expression vectors, determine protein expression in bacteria, and execute the GST pull-down assays were previously described [24]. The cDNAs of full-length *CO* and the *co* mutant alleles were introduced into pDEST15 and pDEST17 vectors to generate expression cassettes for GST and His fusion proteins, respectively. The nucleotide sequences encoding the CO 1–132 and CO 1–62 proteins were subcloned into pDEST15 vector. GST and GST-fused proteins were expressed in *E. coli* and were purified. GST and GST-fused proteins were then incubated at 4 °C for 2 h with whole crude extract containing the His-fusion proteins expressed in *E. coli*. The proteins pulled down with the GST-fusion proteins were detected by western blotting against anti-6XHis antibody.

## Results and discussion

### B-box motifs are important for CO homodimerization

CO can form a complex with its splicing variant COβ lacking the CCT domain [10]. Hence, we hypothesized that the binding motif for CO homodimerization resides in the B-box domain. To test this idea, truncated versions of CO, namely, CO 1–132 and CO 133–373, which contain two B-box motifs and the remainder of the CO including the CCT domain, respectively, were used for protein–protein interaction analysis in yeast (Fig. 1A). As expected, the β-galactosidase assays revealed that the CO 1–132 variant interacted with the same variant but not with the CO 133–373 variant, supporting our hypothesis that CO forms a homodimer through the B-box domain (Fig. 1B). Therefore, we ruled out the CCT domain as a candidate for homodimerization. We then investigated whether both B-box motifs participate in CO homodimerization. To this end, we constructed deletion derivatives of the B-box domain encoding proteins containing two B-box motifs (CO 1–105) or only a single B-box motif (CO 1–82, CO 1–62, and CO 63–105) (Fig. 1C). We observed no dimerization between single B-box containing proteins (Fig. 1D), suggesting that a single B-box motif may not be sufficient for binding to a protein with a single B-box motif. To investigate the number of B-box motifs required for CO homodimerization, we further analyzed complex formation between one and two B-box containing CO derivatives. Interestingly, we found that the one B-box motif variants, CO 1–82, CO 1–62, and CO 63–105, were able to bind to the CO 1–132 protein with two B-box motifs, although the binding affinity between the one and two B-box variants was relatively weaker than that between the two B-box variants (Fig. 1E, F). These results suggest that CO homodimerization and CO heterodimerization with other BBX proteins may require at least three B-box motifs between the two proteins. To validate the interactions detected in yeast, we conducted in vitro pull-down assays using glutathione S-transferase (GST)-fused CO 1–373, CO 1–132, CO 1–62, and 6XHistidine-tagged CO 1–373 (6XHis-CO 1–373) proteins. The results showed that more 6XHis-CO 1–373 proteins were precipitated in the presence of GST-CO, GST-CO 1–132, and GST-CO 1–64 proteins than in the presence of GST alone (Fig. 1G), thereby confirming the results obtained from the yeast two-hybrid assays. We also verified B-box motif-mediated homodimerization in vivo through bimolecular fluorescence complementation (BiFC) assays (Fig. 1H). The CO 1–132 protein fused with the N- or C-terminus fragments of enhanced yellow fluorescent protein (eYFP) was co-expressed in *Arabidopsis* mesophyll protoplasts. The reconstituted eYFP signal was detected in the nucleus, resembling homodimerization between full-length CO proteins [10].

### The importance of B-box-mediated dimerization in flowering time regulation

Four *co* mutant alleles that cause single amino acid substitutions in the B-box domain have relatively low binding affinity for AS1 and are attributed to delayed flowering phenotypes [8, 24]. These observations suggest that CO dimerization through the B-box domain is important to its role in flowering time regulation. Therefore, we investigated whether B-box mutations also affect CO homodimerization by performing yeast two-hybrid assays coupled with the β-galactosidase activity measurement.

(See figure on next page.)

**Fig. 1** CO forms homodimers via B-box motifs. **A**, **C**. Schematic representations of truncated CO proteins. **B**, **D**, **F**. Yeast two-hybrid assays mapping minimum binding motifs required for CO homodimerization. Various combinations of indicated truncated CO proteins were expressed in yeast. LacZ (β-galactosidase) activity was quantified and represented interaction strength. Similar means were obtained for all three independent assays. **E**. Yeast cells were grown on SD agar media (1) lacking Trp (T), Leu (L), and His (H) in the presence of 5 mM 3-AT (denoted as SD -TLH + 5 mM 3AT) or (2) lacking T, L, H, and Ade (A) (denoted as SD -TLAH). **G**. GST pull-down assays confirming interactions in yeast. GST-fused full-length CO (CO 1–373) and truncated CO proteins were pulled down along with 6XHis-fused CO 1–373. Proteins were visualized by western blotting against anti-GST and anti-6XHis antibodies. **H** In vivo CO 1–132 variant homodimerization to validate interactions observed in vitro and in yeast. The YFP N-terminal fragment (N-YFP) and the YFP C-terminal fragment (C-YFP) were fused with CO 1–132 variant. N-YFP and C-YFP fusion constructs constitutively expressing N-YFP-CO 1–132 and C-YFP-CO 1–132 were transfected into *Arabidopsis* mesophyll protoplasts. DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear staining

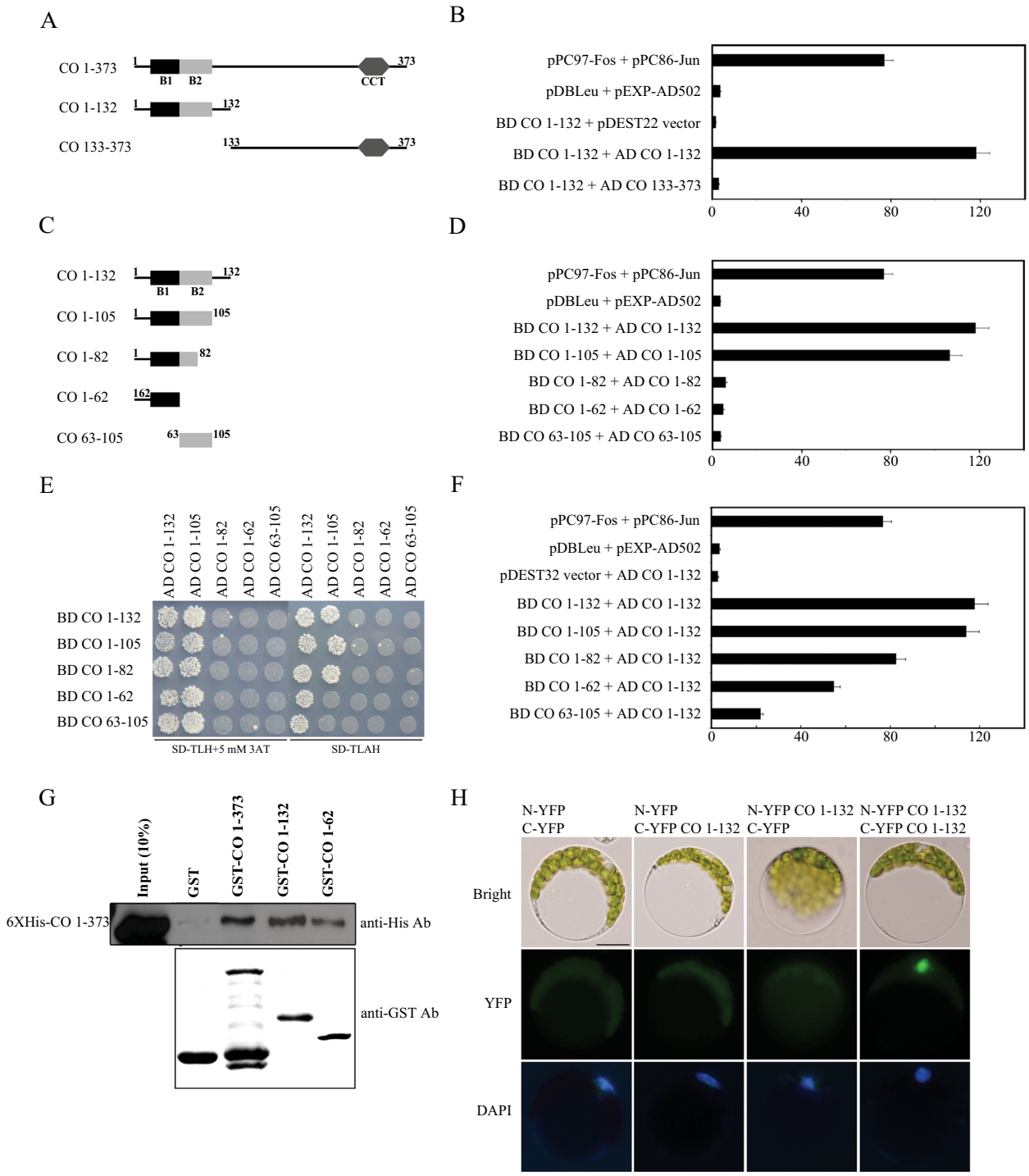


**Fig. 1** (See legend on previous page.)

The N-terminus region (amino acids 1–132) of the B-box mutants was used to minimize the intrinsic transcriptional activation potential in yeast (Fig. 2A). Our results showed weakened binding affinities between the *co-4* 1–132 (A71T) and *co-6* 1–132 (A37V) variants (Fig. 2B). Moreover, homodimerization of the *co-2* 1–132 (R59H) and *co-3* 1–132 (H91Y) variants was abolished (Fig. 2B). Notably, the *co-4* 1–132 proteins exhibited the

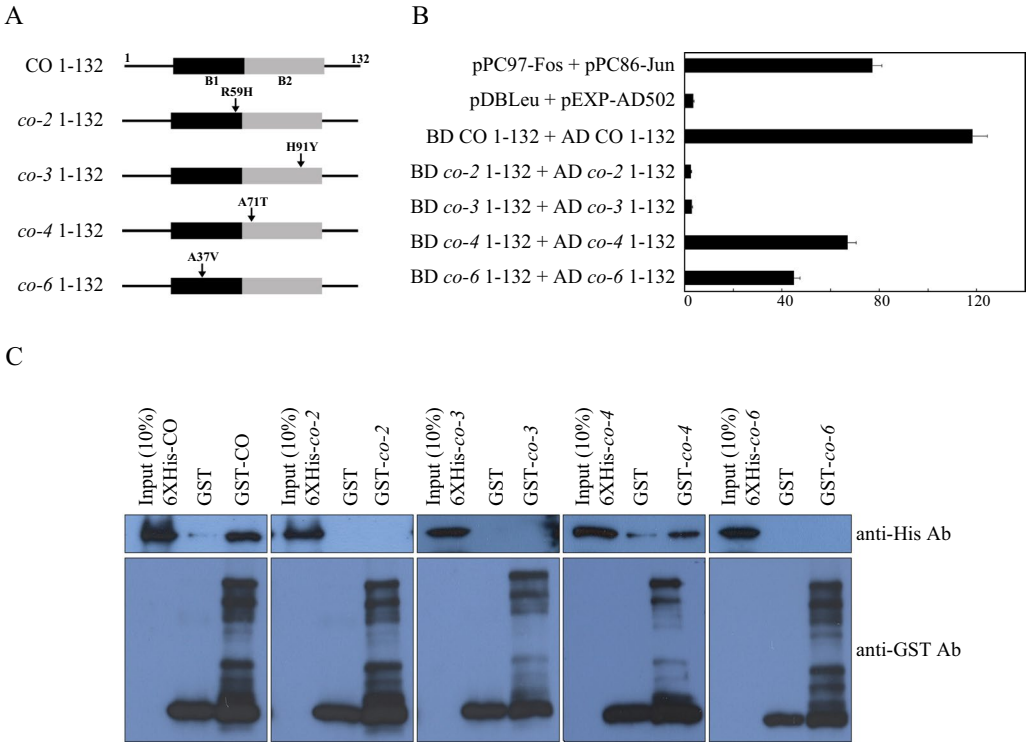


**Fig. 2** Effect of B-box mutations on CO homodimerization. **A**. Schematic illustration of *co* mutant alleles. Sequences of the N-terminal regions of the *co* mutant alleles *co-2*, *co-3*, *co-4*, and *co-6* were amplified using the mRNA extracted from each mutant allele and introduced into the pDEST22 and pDEST32 vectors. **B**. Yeast two-hybrid assays coupled with β-galactosidase measurement. **C**. In vitro binding assays. Full-length *co* mutant cDNA was introduced into the pDEST15 and pDEST17 vectors to generate the GST and 6XHis fusion proteins, respectively. Presence or absence of the interactions was confirmed through western blotting against anti-GST and 6XHis antibodies

highest binding affinity among all the variants (Fig. 2B). To validate the observed changes in homodimerization associated with CO mutant forms, we performed GST pull-down assays using full-length versions. The *co-2* (R59H), *co-3* (H91Y), and *co-6* (A37V) mutants did not homodimerize, while weaker homodimerization was observed for the *co*-4 (A71T) mutant compared to the wild-type version of CO (Fig. 2C). This trend is consistent with the results presented in Fig. 2B. Considering the phenotypes of the *co* mutant alleles, these findings suggest that homodimerization may play a crucial role for CO function in flowering and for its complex formation with other classes of TF proteins.

### Interactome analysis of BBX proteins

Our data proposed the possibility that the number of B-box motifs may be critical to the interactions among BBX family members (Fig. 1). To test this possibility, we analyzed the binding networks of all 32 BBX proteins in yeast. Because 24 BBX proteins showed intrinsic transcriptional activation potential in yeast, we used truncated versions of the proteins that contained the complete B-box domain. To differentiate the binding strength, we classified it into three levels based on the degree of growth observed in the selective medium. Strong interactions were characterized by growth on SD agar medium lacking leucine (Leu), tryptophan (Trp), and histidine (His) in the presence of 10 mM 3-amino-1,2,4-triazole (3-AT) or on SD agar medium lacking Leu, Trp, His, and adenine (Ade). Moderate interactions were characterized by growth on SD agar medium lacking Leu, Trp, and His in the presence of 1–5 mM 3-AT. Weak interactions were characterized by growth on SD agar medium lacking Leu, Trp, and His in the presence of 0.1–0.5 mM 3-AT. Based on these criteria, we finally identified 111 strong, 164 moderate, and 44 weak interactions among 1024 protein pairs (Fig. 3A, B). Additionally, 17 BBX proteins exhibited strong binding affinities in their homodimerization (Fig. 3A, B). Unexpectedly, some group III and V proteins containing a single B-box motif were able to form homodimers (Fig. 3A, B). The group III proteins had the lowest rate of interaction with other group members (Fig. 3B). Conversely, the group V proteins demonstrated the highest and most robust frequency of interaction with other group members, particularly with those possessing two B-box motifs (Fig. 3B). BBX26 and BBX27

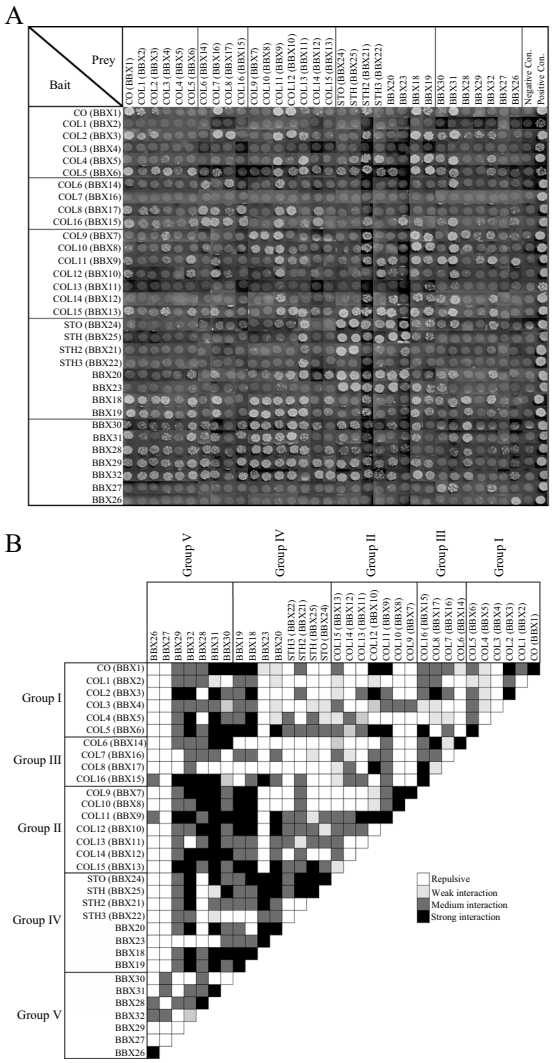


**Fig. 3** Protein–protein interactions among 32 B-box proteins. **A**. Analysis of 1024 protein–protein interactions was performed using a 96-well format system. BD and AD fusion constructs were individually transformed into yeast. To determine and distinguish interaction strengths, yeast cells were spotted and grown on SD agar media (1) lacking T, L, and H in the presence of 0.1–0.5 mM 3-AT, (2) lacking T, L, and H in the presence of 1–5 mM 3-AT, (3) lacking T, L, and H in the presence of 10 mM 3AT, or (4) lacking T, L, A, and H. Positive pPC97-Fos/pPC86-Jun and pDBLeu/pEXP-AD502 combinations were used as positive and negative interaction controls. **B**. BBX family dimerization map. Strong, moderate, and weak interactions are indicated by black, dark gray, and light gray, respectively

in the group V rarely formed heterodimeric complexes with proteins in other groups (Fig. 3B). Furthermore, the group IV proteins with two B-box motifs displayed the highest rate of homodimerization and formed the most heterodimer complexes with other members within their group (Fig. 3B). These findings suggest that having more B-box motifs may increase the likelihood of binding between BBX proteins. In addition to BBX19, BBX28, BBX29, BBX30, and BBX31, which are known to form in vivo protein complexes with CO, BBX9, BBX10, and BBX18 were identified as potential candidates for CO interaction proteins (Fig. 3B).

BBX family proteins containing one or two B-box motifs are considered as a group of the key transcription factors that are regulated by light and transduce light signaling in plant growth and development [13]. Multiple BBX proteins participate in a common signaling pathway through cooperative and antagonistic interactions, serving as a regulatory unit [9, 13]. To investigate signaling networks involving BBX proteins, we analyzed the protein-binding properties of CO, which was the first identified BBX protein, as a proxy for understanding the formation of homodimeric and heterodimeric complexes mediated by B-box motifs. The biological significance of CO homodimerization remains unclear, while its heterodimerization with other known B-box proteins has been shown to negatively influence CO function under LDs at 22 °C [12, 25, 26]. Our study on the interaction between CO and *co* mutant forms, which contain single amino acid substitutions in the B-box domain, suggests that homodimerization through B-box motifs may be essential for the role of CO in flowering time regulation (Fig. 2) [8]. One possible explanation for this is that a CO homodimer can directly bind to CO-responsive elements (COREs) on the *FT* promoter through its CCT domain. However, CO recruitment to the promoter largely relies on complex formation with DNA-binding transcription factors [2, 16–18, 24, 32, 33]. This possibility is supported by observations that heterodimerization of CO with BBX proteins, which possess only one B-box motif and lack the CCT domain, inhibits the function of CO in *FT* activation [12, 25, 26]. These findings suggest that a heterodimer composed of CO and a BBX protein carrying the CCT domain is capable of binding to the *FT* promoter, while a heterodimer formed by CO and a BBX protein lacking the CCT domain might be unable to bind to the promoter. Given that CCT domains in other BBX proteins can activate the expression of a gene that contains COREs in its promoter [17], interactions among CCT-containing proteins could facilitate their binding to target loci. Our interactome analysis identified 93 combinations of homodimerization and heterodimerization among group I, II, and III proteins possessing the CCT domain, with 22 strong, 45 medium, and 26 weak interactions (Fig. 3B).

Our BiFC assay showed that interactions between CO 1–132 variants lacking the CCT domain occurred in the nucleus (Fig. 1H). In addition, homodimerization of COβ, a CO splicing variant, and interactions of CO with COβ, BBX28, BBX29, BBX30, and BBX31,

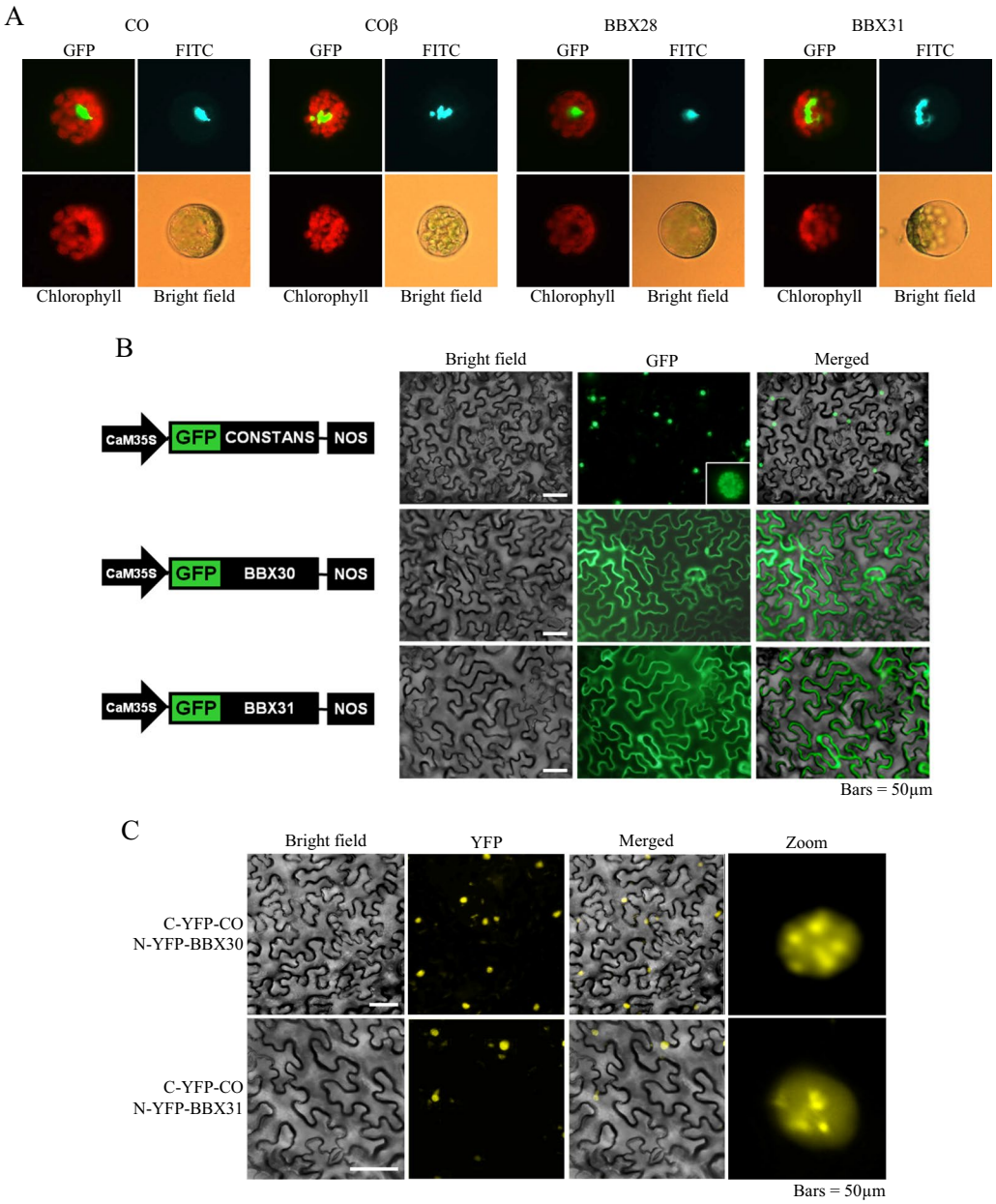


**Fig. 4** Subcellular localization of BBX proteins. **A**. GFP fusion proteins were constitutively expressed in *Arabidopsis* mesophyll protoplasts. **B**. Schematic representation of the GST-fused BBX protein expression cassettes (left panel). GFP signals in tobacco leaf epidermal cells. Scale bars = 50 μm. **C.** *In planta* interactions between CO and (1) BBX30 and (2) BBX31. The N-YFP fragment was fused with CO while the C-YFP fragment was fused with BBX30 and BBX3. The C-YFP-CO/N-YFP-BBX30 and C-YFP-CO/N-YFP-BBX31 combinations were co-expressed in tobacco leaves. The YFP signals indicate that CO interacts with BBX30 and BBX31 in the nucleus. Zoom images show nuclear speckle formation. Scale bars = 50 μm

all of which lack the CCT domain, also occurred in the nucleus [10, 12, 25, 26, 34]. Since the nuclear localization signal (NLS) is present in the CCT domain [8], it remains unclear how CO-interacting BBX proteins are transported to the nucleus. One possible scenario is that CO binds to BBX proteins in the cytosol and enters the nucleus with them. Our subcellular localization experiments using GFP-tagged proteins in protoplasts showed that COβ and BBX31 were present both inside and outside the nucleus, while GFP signal for BBX28 was detected only in the nucleus, as previously reported (Fig. 4A) [12]. In addition, BBX30 and BBX31 were located in the cytosol and nucleus in tobacco epidermal cells of leaves (Fig. 4B). However,

their interactions with CO only occurred in the nucleus (Fig. 4C) [26], indicating that these BBX proteins are translocated into the nucleus independently of CO. This suggests that a BBX protein with one B-box motif is part of a large complex that includes multiple BBX proteins and another family of transcription factors with NLSs. Several observations support this possibility; for example, multiple BBX proteins are in the same signaling pathway and share a binding partner belonging to another family of transcription factors [9, 13]. Furthermore, group V BBX proteins with one B-box motif strongly and more frequently form heterodimer complexes with group I, III, and IV proteins with two B-box motifs (Fig. 3B).

In conclusion, our findings provide insight into novel signaling networks in which BBX proteins play important roles in light-mediated regulation of plant growth and development.

### Abbreviations

| | |
|---|---|
| AD | Activation domain |
| BBX | B-box protein |
| BD | Binding domain |
| BiFC | Bimolecular fluorescence complementation |
| CCT | CO, CONSTANS-LIKE (COL), and TIMING OF CAB EXPRESSION 1 |
| CO | CONSTANS |
| COL | CONSTANS-LIKE |
| CORE | CO-responsive element |
| FT | FLOWERING LOCUS T |
| GFP | Green fluorescence protein |
| HOS1 | HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 |
| LDs | Long-day |
| ONPG | *o*-Nitrophenyl-β-*D*-galactopyranoside |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| phyB | Phytochrome B |
| SDs | Short-day |
| TF | Transcription factor |
| TOC1 | TIMING OF CAB EXPRESSION 1 |
| YFP | Yellow fluorescence protein |

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Not applicable.

### Author contributions
SYS, HK, SGW, and YHS performed the experiments and collected the data. SYS, HK, and YHS analyzed the data. JCH and YHS conceptualized the research, interpreted the data, and wrote the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials
The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

## Declarations

### Competing interests
The authors declare that they have no competing interests.

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