

## ***BBX32* Interacts with *AGL24* Involved in Flowering Time Control in Chinese Cabbage (*Brassica rapa* L. ssp. *pekinensis*)**

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### Abstract

B-box (BBX) zinc finger proteins play critical roles in both vegetative and reproductive development in plants. Many BBX proteins have been identified in *Arabidopsis thaliana* as floral transition regulatory factors, such as *CO*, *BBX7* (*COL9*), *BBX19*, and *BBX32*. *BBX32* is involved in flowering time control through repression of *COL3* in *Arabidopsis thaliana*, but it is still elusive that whether and how *BBX32* directly interacts with flowering signal integrators of AGAMOUS-LIKE 24 (*AGL24*) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (*SOC1*) in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) or other plants. In this study, *B-box-32* (*BBX32*), a transcription factor in this family with one B-box motif was cloned from *B. rapa*, acted as a circadian clock protein, showing expression changes during the circadian period. Additional experiments using GST pull-down and yeast two-hybrid assays indicated that *BrBBX32* interacts with *BrAGL24* and does not interact with *BrSOC1*, while *BrAGL24* does interact with *BrSOC1*. To investigate the domains involved in these protein-protein interactions, we tested three regions of *BrBBX32*. Only the N-terminus interacted with *BrAGL24*, indicating that the B-box domain may be the key region for protein interaction. Based on these data, we propose that *BrBBX32* may act in the circadian clock pathway and relate to the mechanism of flowering time regulation by binding to *BrAGL24* through the B-box domain. This study will provide valuable information for unraveling the molecular regulatory mechanisms of *BrBBX32* in flowering time of *B. rapa*.

**Keywords:** B-box32; bolting; expression pattern; interaction; light; motif

### Introduction

Flowering in plants is mediated by multiple endogenous genetic pathways and multiple exogenous environmental factors to guarantee that it occurs at the most appropriate time of the plant life cycle (He and Amasino, 2005; Bluemel *et al.*, 2015; Song *et al.*, 2015a). Research has shown that many genetic pathways affect the transition from vegetative to reproductive development, such as the photoperiod pathway, the autonomous pathway and the gibberellin (GA) pathway.

The intricate network of these pathways controls the floral transition via transcriptional regulation of several floral integrators, including SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (*SOC1*), SHORT VEGETATIVE PHASE (*SVP*), FLOWERING LOCUS C (*FLC*), FLOWERING LOCUS T (*FT*), LEAFY (*LFY*), FRUITFULL (*FUL*), AGAMOUS-LIKE 24 (*AGL24*) and

CONSTANS (*CO*) (Boss *et al.*, 2004; Parcy, 2004; Yoo *et al.*, 2005; Mateos *et al.*, 2015). The complex networks formed by the interactions of these pathways control the process of flowering through the transcriptional regulation of gene expression in several flowering pathways. *SOC1* (AGAMOUS-LIKE 20) and *AGL24* (AGAMOUS-LIKE 24) are MADS-box genes that share a highly conserved CC (A/T)<sub>6</sub>GG (CArG) box called the MADS-box transcription domain (Shore and Sharrocks, 1995; Michaels *et al.*, 2003; Helliwell *et al.*, 2006; Theißen *et al.*, 2016). *SOC1* has multiple functions as a flowering time signal integrator and a determiner of the floral meristem (Moon J *et al.*, 2003; Melzer *et al.*, 2008). As a downstream target of CONSTANS (*CO*), *SOC1* is directly activated by CONSTANS (*CO*) under long-day (LD) conditions and regulates *LFY* (*LEAFY*) expression in the gibberellic acid (GA) pathway through direct binding to the *LFY* promoter (Seo *et al.*, 2009; Lee and Lee, 2010). *CO* is a central

regulator of the photoperiod pathway, and *FLC* (*FLOWERING LOCUS C*) is an essential factor in both the vernalization and autonomous pathways (Seo *et al.*, 2009; Lee and Lee 2010). Studies in *Arabidopsis* mutants have indicated that *SOC1*, as a key floral activator of several floral induction pathways, plays an important role in controlling flowering time (Lee and Lee, 2010).

*AGL24* is another MADS-box domain transcription factor that acts as a positive regulator, similar to *SOC1* (Liu *et al.* 2008), and studies have shown that the expression levels of *AGL24* and *SOC1* affect each other (Michaels *et al.*, 2003; Valentim *et al.*, 2015). *AGL24* is a close homolog of *SHORT VEGETATIVE PHASE* (*SVP*) and is widely expressed in a variety of tissues such as the vegetative shoot apex, inflorescence, root and leaf. Overexpression of *AGL24* promotes flowering and inflorescence identity (Yu *et al.*, 2002; Yu *et al.*, 2004). Protein interaction assays have shown that *AGL24* can physically interact with *SOC1*, but the mechanisms by which *AGL24* and *SOC1* regulate the floral transition differ (Lee *et al.*, 2000; Hepworth *et al.*, 2002; Sasaki *et al.*, 2017). *FLC* and *FT* both repress *SOC1* expression, while *AGL24* is independent of *FLC* and *FT*; in contrast, *AGL24* is affected by *CO*, while *SOC1* is indirectly regulated by *CO* via an unknown DNA-binding factor (Lee *et al.*, 2000; Hepworth *et al.*, 2002; Xu *et al.*, 2016).

The B-box (BBX) proteins are zinc finger transcription factors that have multiple functions across multicellular species. These proteins contain a highly conserved group of B-box motifs (Huang *et al.*, 2012; Crocco and Botto, 2013). In the model species *Arabidopsis*, the BBX protein family that contains 32 family members divided into five structural groups based on the number of B-box motifs (one or two) in the N-terminal domain and according to the presence or absence of a CCT domain in the C-terminal region (Khanna, *et al.*, 2009). The proteins in group I have two B-box domains (B1 and B2) in N-terminal domain and a CCT (CO/COL/TOC1) domain in the C-terminus. Group II proteins resemble those in group I except for minor differences in the B2 domain. Group III proteins contain only one B1 and one CCT domain, while group IV lacks only the CCT domain, and group V has only a single B1 structure domain in the N-terminus (Khanna *et al.*, 2009). These proteins are key factors in different plant developmental processes. In the regulation of flowering, many B-box (BBX) family signal integrators have been found; *CONSTANS*(CO/BBX1) was the first B-box protein identified from the BBX family in *Arabidopsis* to induce flowering time (Samach *et al.*, 2000).

CO includes two B-box motifs in the N-terminal and one CCT in the C-terminal domain. CO interacts with another B-box protein, B-BOX19, to indirectly regulate *FLOWERING LOCUS T* (*FT*) expression (Valverde, 2011; Wang *et al.*, 2014). In addition, CO-responsive CCAAT-box elements in the *FT* promoter direct the binding of CO, which activates *FT* transcription (Cao *et al.*, 2014). The CO-FT module is highly conserved with different modes of action in many plants. In the LD plant *Arabidopsis thaliana*, CO promotes the expression of *FT*

under inducing long days, but in rice, a short-day (SD) plant, CO acts as a repressor in non-inductive long-day conditions (Suárez-López *et al.*, 2001; Hayama *et al.*, 2003; Andrés and Coupland, 2012). Thus, CO principally regulates the expression of *FT* via a variety of complex mechanisms to affect photoperiodic flowering time. BBX family member genes regulating flowering include not only CO but also some other B-box proteins, such as BBX4 (COL3), BBX6 (COL5), BBX6(COL9), BBX7, BBX18, BBX19, BBX24/SALT TOLERANCE (STO), BBX31 (MiP1a) and BBX32 (EIP6), which have been reported to have distinct, overlapping or antagonistic functions in flowering signalling (Cheng and Wang, 2005; Datta *et al.*, 2006; Hassidim *et al.*, 2009; Weller *et al.*, 2009; Park *et al.*, 2011; Li *et al.*, 2014; Wang *et al.*, 2014).

*Brassica rapa* (AA, 2n=20) is one of important vegetable crops in the world and the flowering time has a great impact on the yield and quality of product organs (Huang *et al.*, 2015; Song *et al.*, 2015b; Liu *et al.*, 2016). The flowering time is an indication of transformation from vegetative to reproductive stage, which depending on low temperature and day length. *SOC1* and *AGL24* are two most significant floral integrator and floral meristems factors (Valentim *et al.*, 2015). Therefore, it is hard to ignore *SOC1* and *AGL24* when studying flowering time control genes. It was reported that a novel flowering regulator factor, BBX32, could regulate flowering in *Arabidopsis* partly through regulating the expression of *FT* (Tripathi *et al.*, 2017). BBX32 may also influence flowering time through heterodimer formation with EMBRYONIC FLOWER1, a vegetative development protein (Park *et al.*, 2011). However, there is little information about the interaction mechanism of BBX32 with the two flowering signal integrator *SOC1* and *AGL24*, and the flowering time interaction mechanism of BBX32 with *SOC1* and *AGL24* needs to be elucidated in *B. rapa*. In this study, we used a homologous cloning method to acquire a BBX protein with one B-box domain in the N-terminal domain and without a CCT domain from an extremely-late bolting Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) line and named this protein Br-BBX32(*BrBBX32*). RT-PCR showed that *BrBBX32* is regulated by the circadian clock may be unaffected by light in *B. rapa*. Protein-protein interaction testing indicated that *BrBBX32* directly interacts with *BrAGL24* via the B-box in the N-terminus but does not interact with *BrSOC1*. However, *BrAGL24* can interact with *BrSOC1*. This is the first time report that BBX32 had been found to interact with *AGL24* in Chinese cabbage. This valuable information may assist in-depth studies to understand the mechanisms of *BBX32* in flowering time control in *B. rapa* and broaden the understanding of the complex regulation network of flowering time.

## Materials and Methods

### *Growth conditions and plant material*

For sequencing and expression analysis of the *BrBBX32*, *BrSOC1* and *BrAGL24* genes, we used Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) line GW-18-19, a Guiyang

(China) local cabbage variety with extremely late bolting. Seeds were sown in soil and grown in a growth chamber (Guiyang, China) for 4 weeks under conditions of 16 h light and 8 h dark (16L:8D) at 25 °C, 50% humidity and 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity for gene cloning. For qualitative and quantitative real-time PCR, seedlings were grown in 12L: 12 D for 4 weeks and transferred to diurnal conditions (12L: 12 D) or continuous light (12L: 12 L) conditions and harvested at intervals (every 12 h for sqRT-PCR and every 6 h for qRT-PCR).

#### RNA extraction and RT-PCR

Total RNA was extracted using the MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China). First-strand cDNA synthesis was performed on 1-2 mg of RNA using the PrimeScript 1st Strand cDNA Synthesis Kit System (TaKaRa). For semi-quantitative RT-PCR (sqRT-PCR) expression analysis we use PrimeSTAR Max DNA Polymerase Mix (TaKaRa) and the *BrTub* gene of Chinese cabbage was used as a sqRT-PCR internal control. The primers are shown in Table 1. A total reaction volume of 20  $\mu\text{L}$  was used for sqRT-PCR, containing 1  $\mu\text{L}$  template

cDNA, 10 pmol each primer, 7  $\mu\text{L}$  sterile water and 10  $\mu\text{L}$  mix. The PCR conditions were initial denaturation at 98 °C for 2 min followed by 32(for *BrBBX32*) or 28(for *BrTub*) cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 15 s, and extension at 72 °C for 15s and a final extension at 72 °C for 5 min. The PCR products were visualized by a Molecular Imager ChemiDoc XRS<sup>+</sup> with Image Lab Software (Bio-Rad, USA) on a 2% agarose gel. Quantitative real-time PCR (qRT-PCR) was used to further study the expression of *BrBBX32*, *BrSOC1*, *BrAGL24*, and *BrTub* (internal control gene). A 2 $\times$ SYBR Fast qPCR Mix (TaKaRa) was used at a final reaction volume of 10  $\mu\text{L}$  with 1  $\mu\text{L}$  template cDNA (100 ng), 1  $\mu\text{L}$  (10 pmol) each primer (forward and reverse) and 2  $\mu\text{L}$  ultra-pure water (ddH<sub>2</sub>O). The qRT-PCR conditions were 5 min denaturation at 94 °C, followed by 25 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 10 s and extension at 72 °C for 15 s. Experiments were independently repeated in three biological replicates.

The data were processed for analysis by Bio-Rad CFX Manager. The primers used for sqRT-PCR and RT-PCR are listed in Table 1.

Table 1. Primers that were used in this study

Primer	Sequence (5'-3')
BBX32-F	CCACCGTGTATTCTCCTTCAATC
BBX32-R	CAACACGTCGATCGATGATACATG
AGL24-F	ACCTAATCTAAGATCCTCCACTC
AGL24-R	CTTGCAATTTGTTTCATCCATCG
SOC1-F	GCTCCTCCGTTAATGCTTGTATAAG
SOC1-R	TGGATACATAACAGAGGCAGTTCAG
BBX32-F- <i>NdeI</i>	GGGTTT <u>CATATG</u> ATGGTGAAACTCTGTGAGCTGTG
BBX32-R- <i>BamHI</i>	CGCGGATCCCTCAAACGTTGTCGTTTTTCGGCC
AGL24-F- <i>NdeI</i>	GGGTTT <u>CATATG</u> ATGGCGAGAGAAAAGAGAAGGAT
AGL24-R- <i>BamHI</i>	CGCGGATCCCTTACCCAAGTTTAAGAGAAGTGTGCG
SOC1-F- <i>NdeI</i>	GGGTTT <u>CATATG</u> ATGGTGAGGGGAAAACCTCAG
SOC1-R- <i>BamHI</i>	CGCGGATCCCTTTCTTGAAGAACAAGGTAACC
AGL24-GstF- <i>BamHI</i>	CGCGGATCCATGGCGAGAGAAAAGAGAAGGAT
AGL24-GstR- <i>XhoI</i>	CGCGCTCGAGTTACCCAAGTTTAAGAGAAGTGTGCG
BBX32-N-R- <i>BamHI</i>	CGCGGATCCAGACGAAGCTGAAGACGAAGACGTT
BBX32-M-F- <i>NdeI</i>	GGGTTT <u>CATATG</u> TGTGGAACGTCTTCTGCTTTCAGCTT
BBX32-M-R- <i>BamHI</i>	CGCGGATCCCGTCCCTCTTGTTTTCCCCACG
BBX32-C-F- <i>NdeI</i>	GGGTTT <u>CATATG</u> GCTTTGGCCGTGGGGAAAACAAGAG
SqBBX32-F	TATCTCCACCTCCGAGCTATC
SqBBX32-R	CGGTTACATCTTCCACCTTCTT
SqTubulin-F	AGGCGTCGTGTGGAAGATAC
SqTubulin-R	TTCTCAGCCTCCTTACGCAC
qBBX32-F	TGCTCGTCTCTGGACCTTAT
qBBX32-R	CACGGCATTGACTCCCTTT
qAGL24-F	AATAGGAGACTGAGGGAGAGAG
qAGL24-R	GAGAAGTGTGCGGAGAAGTCATC
qSOC1-F	AAACTCTTGGGAGAAGGCATAG
qSOC1-R	GAGTCTTTCTTGCTCGGATACA
qTubulin-F	TGAAGGAGCCGAGCTTATTG
qTubulin-R	CACCAAGCGAGTGACATACT

### Molecular cloning and plasmid construction

The PCR mixture used for gene cloning included 10×Buffer for KOD-Plus-Neo, 5 µL; 2 mM dNTPs, 5 µL; 25 mM MgSO<sub>4</sub>, 2 µL; F-primer, 2 µL; R-primer, 2 µL; cDNA, 1 µL; PCR-grade water, 35 µL; KOD-Plus-Neo DNA polymerase, 1 µL (Toyobo, Osaka, Japan). The PCR cycling steps were as follows: predenaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 15 sec, annealing at T<sub>m</sub> °C for 30 sec, and extension at 68 °C for 45 sec. The PCR products were collected by the E.Z.N.A. Gel Extraction Kit (Omega Biotek, USA). The PCR fragments were ligated into the pEASY Blunt Simple Cloning vector (TransGen Biotech, Beijing, China) and transformed into *Escherichia coli* DH5α cells. DNA sequencing was performed after PCR detection (BGI, Beijing, China), and the correct clones were selected as template DNA for the follow-up experiment. Primers were designed with specific restriction enzyme cutting sites for the construction of yeast two-hybrid and glutathione S-transferase (GST) pull-down plasmids (Table 1).

For the yeast two-hybrid assay, the entire coding regions of *BrSOC1*, *BrAGL24* and *BrBBX32* and the domain fragments of *BrBBX32* were sub cloned via PCR with appropriate primer pairs (primers with the restriction enzyme sites *NdeI* and *BamHI*) and successfully ligated into pGADT7 and pGBKT7 to construct the yeast prey plasmids pGADT7-*BrSOC1*, pGADT7-*BrAGL24*, and yeast bait plasmids pGBKT7-*BrSOC1*, pGBKT7-*BrBBX32*, pGBKT7-*BrBBX32-N*, pGBKT7-*BrBBX32-M*, and pGBKT7-*BrBBX32-C*. The same method was used to construct the pGEX4T-1-*BrAGL24*, pCold I-*BrSOC1* and pCold I-*BrBBX32* plasmids for GST pull-down.

### Protein-protein interaction assays

Using the Matchmaker Gold Yeast Two-Hybrid System (Clontech, Mountain View, USA), the recombinant prey plasmids pGADT7-*BrSOC1*, pGADT7-*BrAGL24*, pGADT7-T and pGADT7 were transformed into Y187 yeast; the bait plasmids pGBKT7-*BrSOC1*, pGBKT7-*BrBBX32*, pGBKT7-*BrBBX32-N*, pGBKT7-*BrBBX32-M*, pGBKT7-*BrBBX32-C*, pGBKT7-Lam, pGBKT7-53 and pGBKT7 were transferred into Y2HGold yeast; the positive clones of all yeast plasmids were selected for toxicity tests and autoactivation tests for the bait plasmids; and then, the correct Y187 recombinant yeasts (pGADT7-*BrSOC1/BrAGL24/T*) were selected to blend with the Y2HGold recombinant yeasts (pGBKT7-*BrSOC1/ BrB-Box32/ BrB-Box32-N/ BrB-Box32-M/ BrB-Box32-C/ Lam/53*) at 30 °C for 24 h to produce the corresponding diploid hybrid yeast strains. Y2HGold (pGBKT7-Lam) fused with Y187 (pGADT7-T) and Y2HGold (pGBKT7-53) fused with Y187 (pGADT7-T) served as the negative and positive controls, respectively. After fusion, positive diploid yeast clones were identified on DDO(SD/-Trp/-Leu) medium plates and then transferred to DDO/A(SD/-Trp/-Leu/+AbA) (AbA: Aureobasidin A), QDO(SD/-Trp/-Leu/-Ade/-His) and QDO/+X-α-Gal/+AbA (SD/-Trp/-Leu/-Ade/-His/+x-α-Gal/+AbA) medium plates at 30 °C for 5d. Each experiment was repeated three times. For a detailed protocol, please refer to the Matchmaker Gold Yeast Two-Hybrid System User Manual (Cat. No. 630439, Clontech).

For GST pull-down, the pGEX4T-1-*BrAGL24*, pCold I-*BrSOC1* and pCold I-*BrBBX32* plasmids were transformed into *Escherichia coli* BL21 cells. Selection of BL21 (pGEX4T-1-*BrAGL24*), BL21 (pCold I-*BrSOC1*) and BL21 (pCold I-*BrBBX32*) positive clones was performed overnight at 37 °C with 100 µg ml<sup>-1</sup> ampicillin in 10 ml LB (Luria broth) cultures. The expression of the GST/HIS fusion protein in BL21 (pGEX4T-1-*BrAGL24*), BL21 (pCold I-*BrSOC1*), BL21 (pCold I-*BrBBX32*) was induced at an A600 of 0.6 with a final concentration of 1 mM IPTG (isopropylthio-β-Dgalactoside). BeaverBeads GSH (Beaver Bio code: 70601-5, Suzhou, China) was used to purify the GST recombinant proteins, and for the HIS-tag recombinant proteins, the BeaverBeads IDA-Nickel Kit (Beaver Bio code: 70502) was used. The purified recombinant proteins were used for GST dropdown analysis to verify the interaction. Glutathione Sepharose 4B beads were bound to pGEX4T-1-*BrAGL24* and incubated with His-tagged fusion protein pCold I-*BrSOC1* or pCold I-*BrBBX32* at 4 °C for 6 h. After incubation, the protein complex of the beads was washed with washing buffer, the resin was removed with the elution buffer, and then, tricine-SDS-PAGE was carried out (Liu et al., 2016). The experiment was repeated three times.

## Results

### Sequence analysis of *BrBBX32*, *BrSOC1* and *BrAGL24*

We used homologous cloning to obtain the sequences of *BrB-Box32* (*BrBBX32*), *BrSOC1* and *BrAGL24*, encoding proteins of 230, 213 and 216 amino acids, respectively. The predicted molecular weights were 24.52 kDa for *BrBBX32*, 24.41 kDa for *BrSOC1* and 24.55 kDa for *BrAGL24*. Sequence analysis showed that the *BrBBX32* protein includes a putative B-box domain (amino acids 1-46) in the N-terminus and lacks a CCT domain (Fig. 1), indicating that *BrBBX32* belongs to structural group V. The phylogenetic tree of B-box32 showed that *BrBBX32* has close evolutionary relationships with the B-box32 proteins of *Brassica napus* and *Raphanus sativus* (Fig. 2A). Both *BrSOC1* and *BrAGL24* are typical type II MADS-box genes that encode 4 domains named MADS domain, I domain, K domain, and C domain (Fig. 1).

### Expression of *BrBBX32*

Previous studies have shown that *BBX32* in *Arabidopsis* is a rhythmically regulated gene (Tripathi et al., 2017). However, the sequence analysis of *BBX32* analysis showed only 65% homology between *B. rapa* and *Arabidopsis*. Thus, the expression of *BrBBX32* of Chinese cabbage may be not the same as that in *Arabidopsis*.

To analyze the expression characteristics of *BrBBX32* in Chinese Cabbage, we first employed a semi-quantitative RT-PCR (sqRT-PCR) method to test the expression pattern of *BrBBX32*. Plants were entrained in a cycle regime (12 h of light, 12 h of dark; 12L: 12 D) or a continuous light regime (24 h of light; 12L: 12 L). We harvested samples every 12 hours to determine the relative mRNA expression levels with respect to the time of day.

The sqRT-PCR results show that *BrBBX32* expression is rhythmic, with a weak oscillation under both 12L: 12 D

A

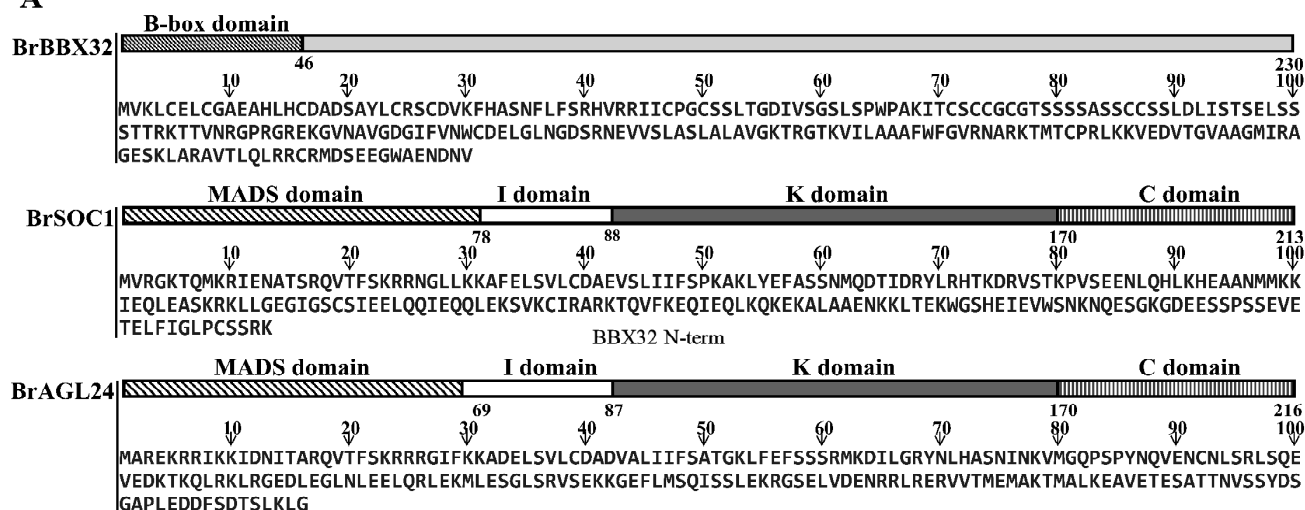
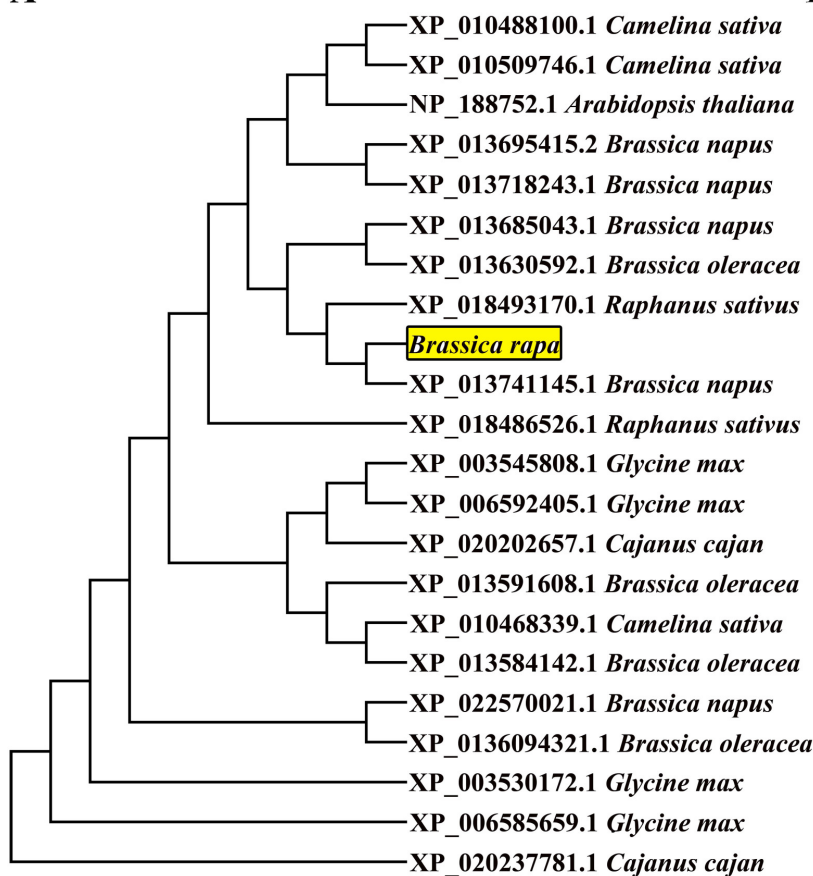


Fig. 1. Alignment of amino acid sequences and predicted domains of BrB-box32 (*BrBBX32*), *BrAGL24* and *BrSOC1*

and 12L: 12 L conditions (Fig. 3A). To further investigate the relative mRNA expression levels of *BrBBX32*, a more accurate method, quantitative real-time PCR (qRT-PCR), was used. For qRT-PCR, we collected samples every 6 hours, and the same results were found: *BrBBX32* is affected by changes in circadian period, with peak expression in the early morning unaffected by light (Fig.3BC), but the

amplitude of the *BrBBX32* expression period is small. Thus, we speculated that *BrBBX32* is a potential weakly circadian-clock-regulated gene independent of light. We also investigated the expression patterns in the organs of mature plants, and we found that the transcripts levels of *BrBBX32* were little different in leaves, flowers and buds, but lower in roots (Fig. 2. B). In *Arabidopsis*, *BBX32* transcript levels

A



B

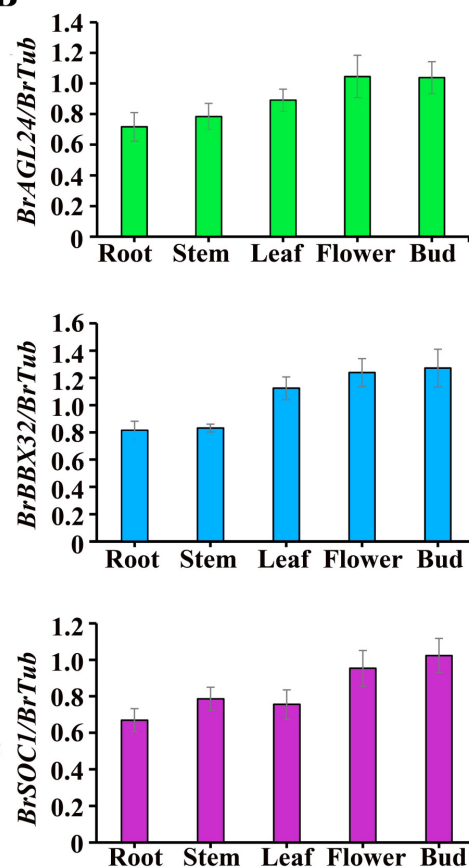


Fig. 2. (A) Phylogenetic analysis of BrB-Box32 and its orthologs. (B) Quantitative RT-PCR analyses of *BrAGL24*, *BrBBX32* and *BrSOC1* in the organs of mature plants. Data are the mean  $\pm$  SD of three replicates

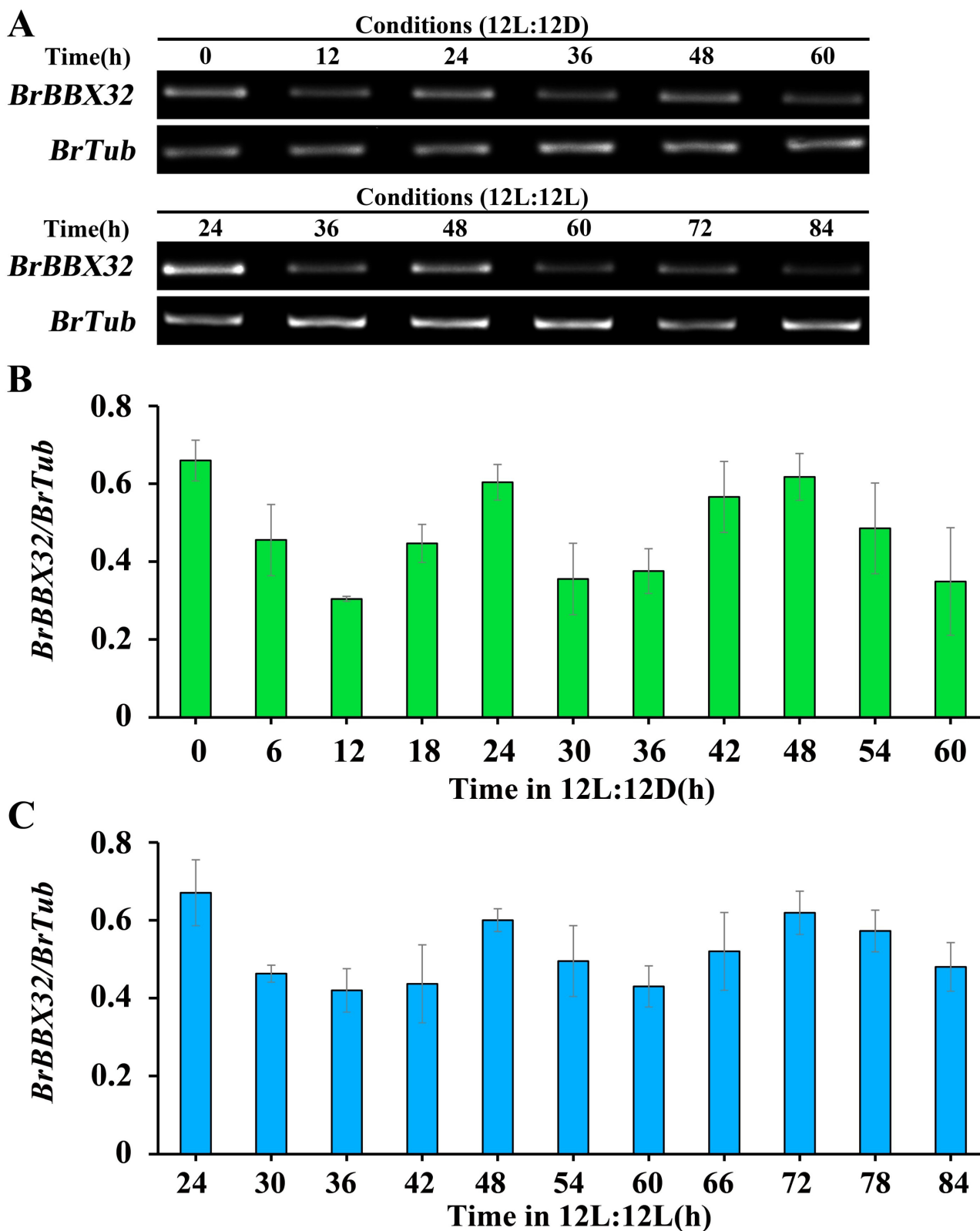


Fig. 3. Expression profiles of *BrBBX32* in *B. rapa* varieties. (A) Expression of *BrBBX32* in different conditions by sqRT-PCR. (B, C) Relative gene expression analysis of *BrBBX32* under the two conditions (12L: 12 D and 12L: 12 L). *B. rapa* seedlings were grown in 12L:12 D for 4 weeks, transferred to 12L:12D conditions or 12L:12L (continuous light) for 60 h and harvested every 12 h for sqRT-PCR or 6 h for qRT-PCR. Experiments were independently repeated in three biological replicates

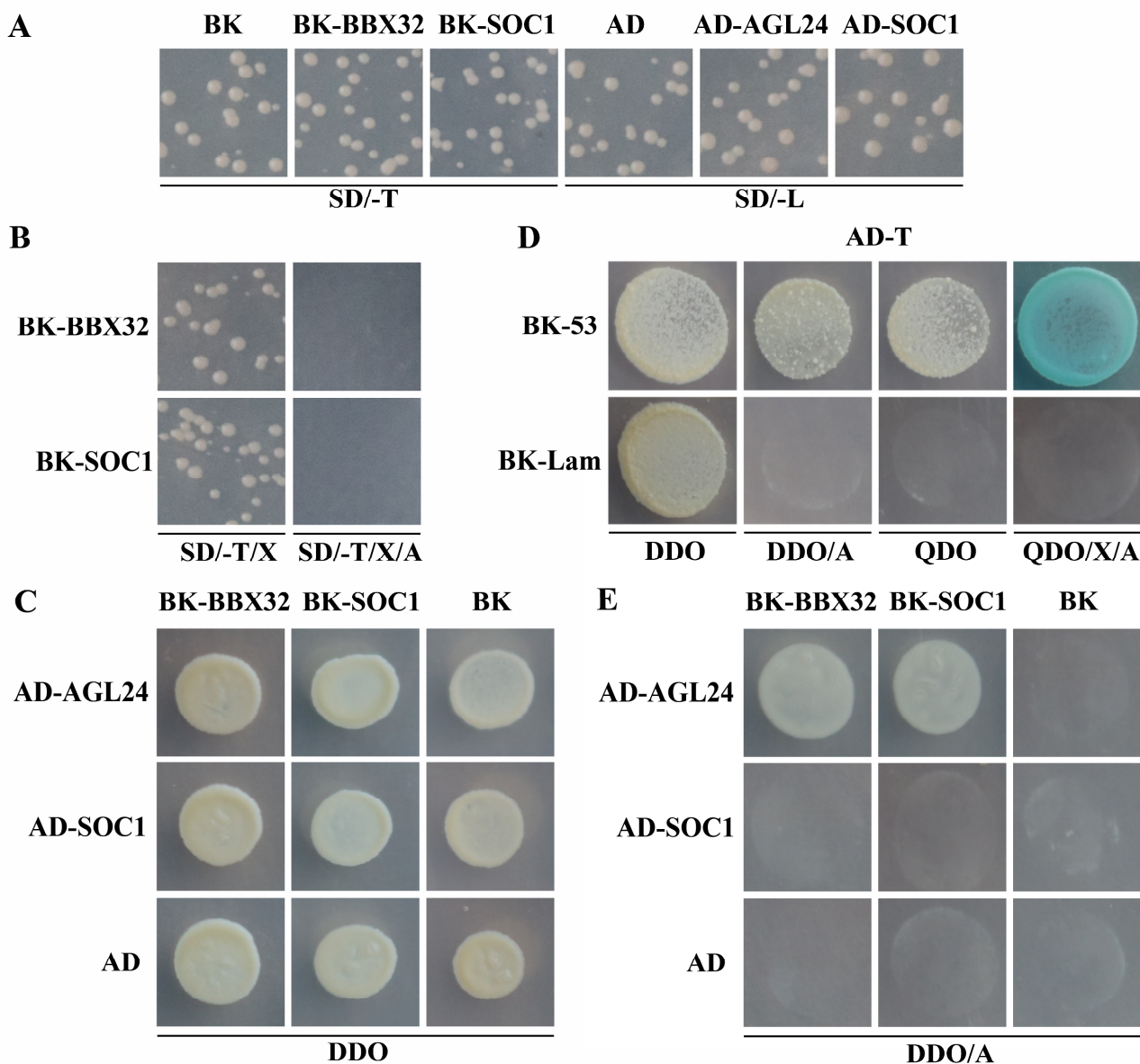


Fig. 4. Yeast two-hybrid assay for *BrBBX32*, *BrAGL24* and *BrSOC1*. (A) Test for toxicity. (B) Test for autoactivation. (C) Diploid yeast on DDO(SD/-Trp/-Leu) media. (D) Positive control and negative control. (E) Diploid yeast in DDO/A(SD/-Trp/-Leu/+AbA) media. BK represents pGBKT7, and AD represents pGADT7. Each experiment was repeated three times

were low in floral organs and high in leaves (Park *et al.*, 2011). The result in *B. rapa* were not the same as those in *Arabidopsis*, which may indicate that *BBX32* is involved in the mechanism producing flowering diversity between *B. rapa* and *Arabidopsis*.

#### Interactions of *BrBBX32* with *BrSOC1* and *BrAGL24*

To determine whether *BrBBX32* interacts with *BrSOC1* or *BrAGL24*, we conducted a yeast two-hybrid assay. For this assay, we tested the toxicity of the recombinant plasmids pGBKT7-*BrBBX32*, pGBKT7-*BrSOC1*, pGADT7-*BrSOC1* and pGADT7-*BrAGL24*. The yeast strains with both empty and recombinant plasmids showed white clones with uniform size and uniform density (Fig. 4A). This result indicated that the recombinant plasmids were non-toxic in yeast. Testing the

bait for autoactivation showed pGBKT7-*BrBBX32* and pGBKT7-*BrSOC1* grew on SD/-Trp and SD/-Trp/+X-a-gal media with no blue colonies (Fig 4B), but not on SD/-Trp/+X-a-gal/+AbA media (Fig 4B); these results showed that the bait plasmids for this experiment could not autoactivate the reporter. After testing toxicity and autoactivation, we fused the strains to create experiments groups as follows: Y187 (pGADT7-*BrSOC1*) × Y2HGGold (pGBKT7-*BrBBX32*), Y187 (pGADT7-*BrAGL24*) × Y2HGGold (pGBKT7-*BrSOC1*) and Y2HGGold (pGBKT7-*BrBBX32*) × Y187 (pGADT7-*BrAGL24*).

The control experiments were listed in figure. All diploid yeast could grow on SD/-Trp/-Leu (DDO) media, indicating successful yeast fusion (Fig. 4C), but only Y187 (pGADT7-*BrAGL24*) × Y2HGGold (pGBKT7-*BrSOC1*) and Y2HGGold (pGBKT7-*BrBBX32*) × Y187 (pGADT7-*BrAGL24*) could grow on SD/-Trp/-Leu/+AbA (DDO/

A) (Fig. 4E), SD/-Trp/-Leu/-Ade/-His (QDO) (Fig. 5A) and produce blue colonies on SD/-Trp/-Leu/-Ade/-His/+x- $\alpha$ -Gal/+AbA (QDO/X/A) plates (Fig. 5B). These findings suggested that *BrBBX32* interacted with *BrAGL24* but did not interact with *BrSOC1*, and *BrAGL24* interacted with *BrSOC1* in *B. rapa*.

To further confirm the interactions between *BrAGL24* and *BrBBX32* or *BrSOC1*, a GST pull-down experiment was established. Recombinant GST-*BrAGL24*, His-*BrBBX32* and His-*BrSOC1* plasmids were established and expressed in *E. coli* and then purified by the BeaverBeads GSH kit. The GST-*BrAGL24* protein was absorbed onto the beads and incubated with His-*BrBBX32* or His-*BrSOC1* protein for 8 h. Subsequent tricine-SDS-PAGE detected both His-*BrBBX32* and GST-*BrAGL24* protein bands or His-*BrSOC1* and GST-*BrAGL24* protein bands (Fig. 5C). These results indicated GST-*BrAGL24* bound to and co-eluted with His-*BrBBX32* or His-*BrSOC1*.

The results of GST pull-down experiments showed that *BrBBX32* can interact with *BrAGL24* and *BrSOC1* can interact with *BrAGL24* *in vitro*. These results were the same as those of the yeast two-hybrid assay.

The results of these two experiments showed that *BrBBX32* could interact with *BrAGL24* in *B. rapa*, while *BrAGL24* could interact with *BrSOC1*, but *BrBBX32* could not interact with *BrSOC1*. AGL24 interaction with SOC1 is also found in *Arabidopsis* and other plants (de Folter et al., 2005).

In this study, we confirmed that AGL24 can interact with SOC1 in *B. rapa*. In addition, we first used yeast two-hybrid and GST pull-down experiments to prove that *BrBBX32* can interact with *BrAGL24*, which has not been reported in *Arabidopsis* or other species. Previous studies have shown that SOC1 and AGL24 are key floral activators in *Arabidopsis*. This study presents the possibility of a new regulator in the flowering regulation network.

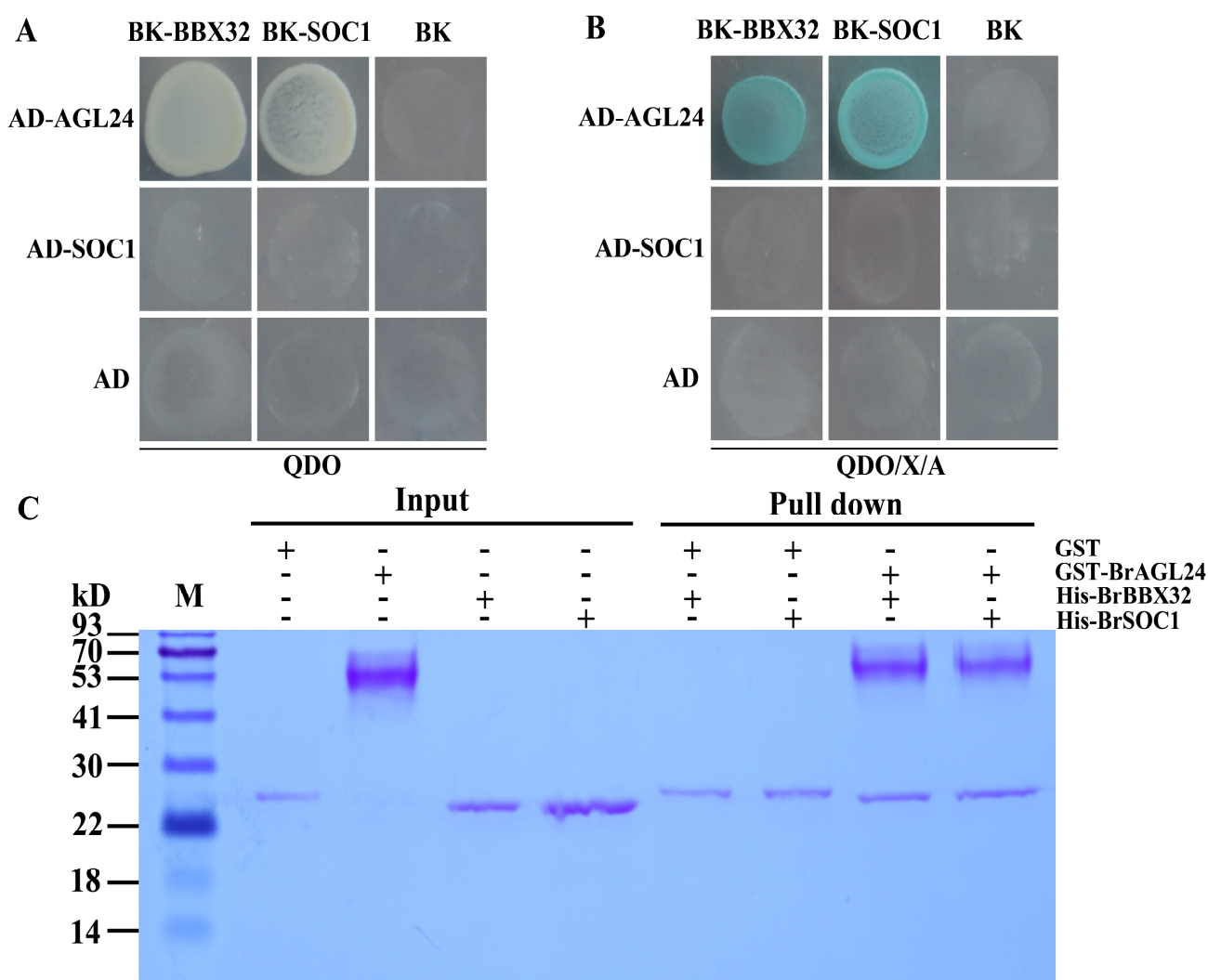


Fig. 5. Yeast two-hybrid and GST pull-down assays for *BrBBX32*, *BrAGL24* and *BrSOC1*. (A) Diploid yeast on QDO(SD/-Leu/-Trp/-Ade/-His) media. (B) Diploid yeast in QDO/X/A (SD/-Leu/-Trp/-Ade/-His/+AbA/+X- $\alpha$ -Gal) media. BK represents pGBKT7, and AD represents pGADT7. (C) Confirmation of *BrBBX32* interaction with *BrAGL24* and *BrAGL24* interaction with *BrSOC1* via GST pull-down assay. *BrBBX32* and *BrSOC1* proteins were generated by cloning into the pCold I vector; *BrAGL24* were cloned into the pGEX-4T-1 vector. Each experiment was repeated three times

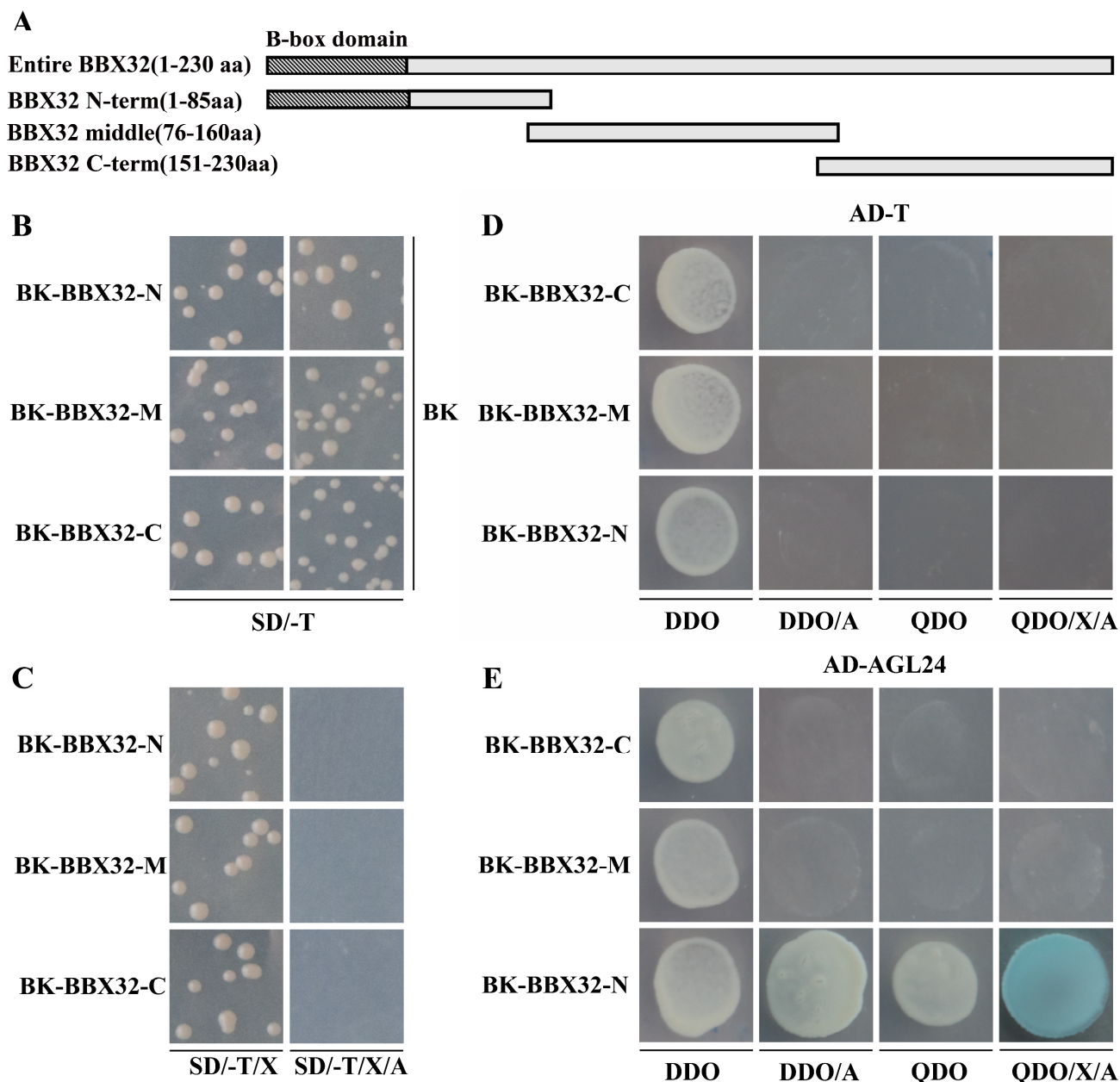


Fig. 6. The domains of *BrBBX32* involved in the interactions with *BrAGL24*. (A) The entire protein structure and fragments of *BrBBX32* used in yeast two-hybrid assays. (B) Toxicity test for the fragments of *BrBBX32*. (C) Autoactivation test for fragments of *BrBBX32*. (D) The control experiment. (E) The direct interactions between the fragments of *BrBBX32* and *BrAGL24* in the yeast two-hybrid assay. BK represents pGBKT7, and AD represents pGADT7; N, N-terminus; M, middle; C, C-terminus. Each experiment was repeated three times

*BrBBX32* physically interacts with *BrAGL24* through a putative B-box domain in the N-terminal domain

The sequence analysis showed that the *BrBBX32* gene from a Chinese cabbage variety contained a putative B-box domain in the N-terminal domain. However, whether the putative B-box domain in *BrBBX32* regulated the protein-protein interactions between *BrBBX32* and *BrAGL24* was unknown.

To identify the regional domains of *BrBBX32* required for interaction with *BrAGL24*, we constructed a set of pGBKT7 vectors including the N-terminal (amino acids 1-85), the middle (amino acids 76-160) and the C-terminal (amino acids 151-230) regions of *BrBBX32* (Fig. 6A), and

the interactions with the entire open reading frame (ORF) of *BrAGL24* were investigated using a yeast two-hybrid assay. After toxicity and autoactivation tests, we found that only the diploid yeast fused to the N-terminal region of *BrBBX32* could grow on DDO/A, QDO and QDO/X/A plates and produce blue colonies on QDO/X/A plates (Fig. 6E). This result means that the N-terminal region of *BrBBX32* is involved in the interaction with the entire ORF of *BrAGL24*. The N-terminal region contains the Box domain, and it is assumed that Box is necessary for this protein interaction. In addition, these results show that the middle and C-terminal domain may not be necessary for protein-protein interaction.

## Discussion

*BrBBX32*, which contains one Box domain, was cloned from Chinese cabbage in this study. This protein is a member of the BBX32 class and a group V member of the BBX TF family, similar to BBX32 in *Arabidopsis* (Khanna et al., 2009). However, although *B. rapa* and *Arabidopsis* both belong to the family Brassicaceae, the homology of BBX32 is only 65% in these two species. Previous research has proven that BBX32 in *Arabidopsis* is a clock gene (Tripathi et al., 2017), and this study provides an identical conclusion regarding *BrBBX32* expression in Chinese cabbage. This similarity suggests that the low homology of BBX32 between *Arabidopsis* and *B. rapa* may not affect the rhythmic mechanism of *BBX32* expression; the differing parts of the protein sequence may not be the key factors involved in time-based gene expression. However, the transcriptional levels of *BBX32* in different organs show a slight difference. In *B. rapa*, *BBX32* is low in the roots and high in other organs (Fig. 2B), while in *Arabidopsis*, *BBX32* is low in floral organs and high in leaves (Park et al., 2011), which means that the transcriptional expression mechanisms of these two species are not completely consistent.

Previous research has proven that BBX32 in *Arabidopsis* interacts with COL3, enabling COL3 to bind to the *FT* promoter and repress its transcriptional regulation of flowering time (Tripathi et al., 2017). BBX32 regulates photomorphogenesis through interacting with BBX21 by suppressing HYPOCOTYL5 (HY5) (Holtan et al., 2011). Additionally, the *BBX32* gene of *Arabidopsis* affects crop productivity in soybean by an unclear molecular mechanism (Preuss et al., 2012). In this study, we have demonstrated that *BrBBX32* interacts with *BrAGL24* and that *BrAGL24* interacts with *BrSOC1* in *B. rapa* by yeast two-hybrid and GST pull-down assays. The interactions between *SOC1* and *AGL24* in *B. rapa* corroborates results from previous studies in *Arabidopsis* (de Folter et al., 2005; Liu et al., 2008; Lee and Lee, 2010). Both *SOC1* and *AGL24* play crucial roles in multiple pathways controlling flowering. Previous studies have shown that the expression levels of *SOC1* and *AGL24* affect each other in plants such as *Arabidopsis*; the expression of *BrBBX32*, *BrAGL24* and *BrSOC1* shared the same trend in flowers, buds and leaves (Fig.2B), and the protein had direct physical relationships in this study, suggesting that the expression of these three genes may also influence each other in *B. rapa*. *BrBBX32* may regulate flowering by direct interaction with *BrAGL24* or regulate the expression of *BrSOC1* by formation of a protein complex with *BrAGL24*. Thus, a model of *BrBBX32-BrAGL24* function in the floral transition in *B. rapa* was proposed. It suggested that *BrBBX32* protein was recruited by the flowering regulators of *BrAGL24* and *BrSOC1*, which directly or indirectly involved in flowering time control. Ternary complex *BrBBX32 /BrAGL24/ BrSOC1* may be involved in regulation of expression of *SOC1* and *AGL24* during flowering time control. Further research is needed to assess the possible formation of a trimeric complex by *BrBBX32*, *BrAGL24*, and *BrSOC1*.

In *Arabidopsis*, another BBX family protein, CONSTANS (CO/BBX1), acts as a key floral factor in the photoperiod pathway, which may directly or indirectly affect the expression of *SOC1* and *AGL24* (Hepworth et al., 2002). Both CONSTANS and BBX32 belong to the BBX family, but their regulatory relationship must be studied in greater depth. Another circadian clock pathway gene, *STO* (*BBX24*), affects the flowering time genes *FLC* and *SOC1* in different pathways (Li et al., 2014; Valentim et al., 2015). *STO* contains two B-box domains and one CCT domain, unlike BBX32; however, both of these genes are regulated by the circadian clock, and there is evidence that they affect *SOC1* directly or indirectly, indicating possible overlaps between these two proteins pathways. Protein sequence analysis showed that the B-box domain structure of *BrBBX32* in *B. rapa* conforming to the structure of the *Arabidopsis* (B-box1: C-X2-C-X7-8-C-X2-D-X-A-X-L-C-X2-C-D-X3-H ) and rice (B-box2: C-X2-C-X8-C-X7-C-X2-C-X4-H-X8-H) proteins (Gangappa and Botto, 2014). Further investigation is necessary to validate the finding that the B-box domain of in N-terminal region of *BrBBX32* is necessary for the interaction between *BrBBX32* and *BrAGL24*. This paper found that the B-box-domain of *BBX32* was the key region that mediated the interactions of *BBX32/AGL24* in *B. rapa*. These data corroborate results from *Arabidopsis* (Park et al., 2011), which indicated a possibility that *BrBBX32* proteins were recruited to their targets via the B-box domain. However, similar results were unreported in other studies. Thus, the regulation mechanisms between BBX32 protein and *AGL24* still need to be further researched. This paper may add a new regulator to the interaction of BBX proteins and MADS domain proteins in flowering regulation, but the mechanisms of action between these two different protein families require more evidence to be completely understood.

## Conclusions

In conclusion, this study provides evidence that the transcripts levels of *BrBBX32*, a gene cloned from *B. rapa* that contains one B-box motif, is modulated by the circadian clock. RT-PCR assays showed that the expression of *BrBBX32*, *BrAGL24* and *BrSOC1* was high in leaves, flowers and buds. The expression levels of these three genes may mutually influence each other. We found that *BrBBX32* is capable of interaction with *BrAGL24* but not with *BrSOC1* and that *BrAGL24* interacts with *BrSOC1*. Additionally, we proved that the B-box domain in the N-terminal region of *BrBBX32* is required for interaction with *BrAGL24*. Therefore, it is probable that BBX32, *AGL24* and *SOC1* belong to a regulatory protein complex that acts to modulate flowering time.

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