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Research article

The heterologous expression in *Arabidopsis thaliana* of a chrysanthemum gene encoding the BBX family transcription factor CmBBX13 delays flowering

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ABSTRACT

Members of the B Box (BBX) family of proteins are known to be important for directing the growth and development of the *Arabidopsis thaliana* plant. Here, an analysis of a newly isolated chrysanthemum gene encoding a BBX family member implied that it was a likely ortholog of *AtBBX13*. The gene (designated *CmBBX13*) was most actively transcribed in the leaves and stem apex. *CmBBX13* transcription was arrhythmic under either continuous darkness or continuous light, so the observed diurnal variation in its transcription appeared not to respond to the circadian clock. The outcome of transiently expressing *CmBBX13* in onion epidermal cells suggested that the CmBBX13 protein localized to the nucleus. Both a yeast- and a protoplast-based assay showed that the protein has transactivational activity. When *CmBBX13* was constitutively expressed in *A. thaliana*, flowering was delayed under both short and long day conditions. The presence of the transgene also down-regulated a number of genes known to promote flowering, including *APETALA1* (*AP1*), *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), *FLOWERING LOCUS T* (*FT*) and *FD*, while simultaneously up-regulating the floral inhibitor-encoding genes *FLOWERING LOCUS C* (*FLC*) and *TARGET OF EAT 2* (*TOE2*). The data suggested that CmBBX13 regulates flowering time independently of the photoperiod pathway.

1. Introduction

The switch from vegetative to reproductive growth is a major event in a plant's life cycle. The timing of this switch is determined by the plant's integrated response to various endogenous and exogenous signals (Anusha and Markus, 2011), and the genetic components which underlie this response have been exhaustively identified in the model species *Arabidopsis thaliana* (Andres and Coupland, 2012). Many of these genes act as transcription factors (Chen et al., 2012), a very large class of proteins encoded by over 1,500 genes in *A. thaliana,* 45% of which belong to gene families which are plant-specific (Riechmann et al., 2000).

The so-called zinc finger proteins belong to one of the most important class of plant transcription factors involved in the regulation of growth and development (Urszula et al., 2017). This class also includes the BBX proteins, the characteristic feature of which is the presence of one or two B Box motifs, thought to co-ordinate protein-protein interactions (Gangappa and Botto, 2014). A phylogenetic analysis of 214 BBX proteins harbored by four green algae species, one moss species, one lycophyte species and three monocotyledonous and three dicotyledonous higher plants has shown that B-box consensus sequences have retained a common and conserved domain topology (Crocco et al., 2013). The 32 members of the *A. thaliana* BBX family have been classified into five groups according to the number of B Box and CCT domains which they contain (Khanna et al., 2009): the members of groups I (AtBBX1 through AtBBX6) and II (AtBBX7 through AtBBX13) all include two B Box domains and one CCT domain, while differing with respect to their B2 domain (Chang et al., 2008); members of group III

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Abbreviations

AP1	APETALA1
CO	CONSTANS
COL	CONSTANS-like
FLC	FLOWRING LOCUS C
FT	FLOWERING LOCUS T
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CO 1
TOE2	TARGET OF EAT 2
ZT	Zeitgeber time

(AtBBX14 through AtBBX17) harbor just one B1 and one CCT domain; those in group IV (AtBBX18 through AtBBX25) have two B Box domains but no CCT domain; and those in group V (AtBBX26 through AtBBX32) have one B Box but no CCT domain (Gangappa and Botto, 2014). The rice, tomato and pear genomes harbor, respectively 30 (Huang et al., 2012), 29 (Chu et al., 2016) and 25 (Cao et al., 2017) *BBX* members. Several *A. thaliana* BBXs have been shown to participate in the regulation of flowering. CO (BBX1) controls the expression of *FT,* a prominent floral inducer (Suárez-López et al., 2001). The over-expression of both *BBX4* and *BBX32* delays flowering (Datta et al., 2006; Park et al., 2011), while that of *COL5* (*BBX6*) accelerates it under short day (SD) conditions (Hassidim et al., 2009). BBX7 delays flowering by repressing *CO* and *FT* (Cheng and Wang, 2005). The rice *Hd1* gene, which is an important determinant of flowering time, is an ortholog of *AtBBX1* (Masahiro et al., 2000). The over-expression of both *OsBBX5* and *OsBBX27* induces late flowering under SD conditions (Kim et al., 2008; Lee et al., 2010). OsBBX14 acts as a floral repressor under both SD and long day (LD) conditions (Bai et al., 2016). The group II members *AtBBX7, 8, 11* and *13* are all inducible by low temperature (Winter et al., 2007), while the over-expression of *AtBBX2*4 has been reported to enhance tolerance to salinity stress (Nagaoka and Takano, 2003).

Chrysanthemum is a short day plant, but in order to meet the demands of a growing market, its year-round production is commercially desirable. Some progress has been made in understanding the genetic basis of the photoperiod dependency of its flowering. The *FT* homolog *CsFTL3* has been cloned from the wild chrysanthemum *C. seticuspe* (Oda et al., 2012), while a second homolog *ClFT* has been isolated from *C. lavandulifolium* (Fu, 2014). The products of both of these genes have been shown to influence floral transition in plants under SD conditions. A single point mutation in the product of *CmFTL3* caused a detectable effect on the flowering phenotype (Sun et al., 2018). According to Mao et al. (2016) *CmFTL1* encodes a florigen, while the product of *CmFTL2* also promotes flowering (Sun et al., 2017). CsAFT appears to inhibit the interaction between CsFTL3 and CsFDL1, thereby negatively impacting flowering (Higuchi et al., 2013). The heterologous expression in *A. thaliana* of *ClCRY1a* and *ClCRY1b*, both of which encode a cryptochrome, affects floral transition (Yang et al., 2017), while that of *ClCOL5* accelerates flowering (Fu et al., 2015). Genes acting in either the gibberellin (Yang et al., 2014) or the age (Wei et al., 2017) pathways have also been identified in chrysanthemum. Although it has been shown that CmBBX24 influences both flowering time and abiotic stress tolerance (Yang et al., 2014), the functions of other members of the CmBBX family remain largely unexplored. Here, the isolation of *CmBBX1*3 has allowed an analysis of the function of its product; the major finding was that when *CmBBX13* was constitutively expressed in *A. thaliana,* flowering time was delayed independently of day length.

2. Materials and methods

2.1. Plant materials and growing conditions

The *Chrysanthemum morifolium* cultivar (cv.) 'Yuuka' was used throughout the study. Plants raised from cuttings were grown at a constant temperature of 23 °C and a relative humidity of 70% under a 16 h photoperiod provided by artificial illumination (light intensity: 100 μmol·m-2·s-1). Experiments involving *A. thaliana* were based on the Col-0 ecotype. Putatively transgenic seedlings were germinated on half strength Murashige and Skoog (1/2MS) medium agar, vernalized by exposure for three days to 4 °C in the dark, then held for a further 14 days at 23 °C and a relative humidity of 70% under a 14 h photoperiod provided by artificial illumination (light intensity: 100 μmol·m-2·s-1). Transgenic plants were selected by challenging them with 25 mg/L hygromycin, and surviving plants were potted into 1:3 (v/v) mixture of soil and vermiculite soaked in liquid 1/2 MS, thereafter the plants were grown under a constant temperature of 23 °C, a relative humidity of 40% and a 16 h photoperiod provided by artificial illumination (light intensity: 100 μmol·m-2·s-1). To assay the effect of photoperiod on the transcription of *CmBBX13* in chrysanthemum, plants which had developed 19 fully expanded leaves were exposed to either a 16 h or an 8 h photoperiod for 48 h, and their leaves were then sampled at four hourly intervals over a period of 48 h. For a free running experiment, plants were exposed to 3 days of either continuous darkness or continuous light, and the abundance of *CmBBX13* transcript was monitored over a period of 48 h.

2.2. RNA isolation and transcriptional profiling

Total RNA was isolated from plant tissue using an RNAprep pure Plant Kit (TIANGEN, Beijing, China) and reverse transcribed into cDNA using M-MLV reverse transcriptase (TaKaRa, Tokyo, Japan), following the manufacturer's protocol. The cDNAs were used as the template in 20 μL quantitative real time PCRs (qRT-PCRs) based on a SYBR Premix ExTaq™ Kit (Takara). Each reaction was represented by three technical replicates and three biological replicates. The reference sequences were chrysanthemum *EF1α* (KF305681) and *AtActin2* (*At3g18780*). Relative transcript abundances were calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Primer sequences are given in Table S1.

2.3. Isolation of CmBBX13 and characterization of its sequence

The *CmBBX13* open reading frame (ORF) was amplified from a template of cDNA prepared from leaves of cv. 'Yuuka' using the primer pair *CmBBX13*-F/-R (Table S1). The resulting amplicon was inserted into the pMD19-T plasmid (TaKaRa) for sequencing. The *CmBBX13* sequence has been deposited in GenBank under accession number KP963935. The alignment of the predicted CmBBX13 polypeptide sequence with that of related polypeptides was carried out using Clustal X v2.1 software implemented in the BioEdit Sequence Alignment Editor (www.mbio.ncsu.edu/BioEdit/page2.html). The sequences of the homologs Cse_sc011467.1_g010.1 (isolated from *C. seticuspe*) (Hirakawa et al., 2019) and Cluster-9838.72124 (isolated from *C. nankingense*) (Song et al., 2018) were also included in the alignment. The sequences of seven group II and four group III *A. thaliana* BBXs were downloaded from the TAIR database (www.arabidopsis.org/). Subsequent phylogenetic analyses were conducted using MEGA v6 software (Tamura et al., 2013), employing the neighbor-joining method in conjunction with a Poisson correction and complete deletion. The resulting dendrogram was validated by a bootstrap test comprising 1,000 replicates.

2.4. Construction of a CmBBX13 transgene and its transformation into A. thaliana

The *CmBBX13* ORF sequence (lacking its stop codon) was amplified from a template of cDNA using the primer pair *CmBBX13*-35AA-SalI-F/NotI-R (Table S1), and the amplicon was introduced into the pENTR1A vector (Invitrogen, Carlsbad, CA, USA) via its *Sal* I and *Not* I recognition sites. The transgene was then moved into pDEST-35AA-SRDX, using LR clonase, as recommended by the manufacturer, transformed into

Agrobacterium tumefaciens strain EHA105, and from thence into *A. thaliana* using the floral dip method (Clough and Bent, 1998). T1 progeny were germinated on solidified 1/2MS plates containing 25 mg/L hygromycin. The plants selected for transcriptional profiling were in the T3 generation.

2.5. Sub-cellular localization of CmBBX13 expression

The *CmBBX13* ORF - lacking its stop codon, and attached to *Xho* I and *Xba* I restriction site - was fused at its 3' terminus to the *GFP* sequence using the pORE-2 × 35S-R4 vector (Catherine et al., 2007). The sequences of the relevant primer pair (*CmBBX13*-R4-*Xho* I–F/*Xba* I-R) are listed in Table S1. The vector pORE-2 × 35S-R4, which harbors *2 × 35S-GFP*, was used as a control. The transgenes were transiently expressed in onion epidermal cells using biolistic transformation (Vasil et al., 1992). The p*35S::D53-RFP* transgene (Zhou et al., 2013) was co-transformed to act as a nuclear marker. After culturing the material overnight on MS medium in the dark, GFP and RFP fluorescence was captured using confocal laser scanning microscopy.

2.6. Transactivational activity analysis

The *CmBBX13* ORF represented in the *pENTR1A-CmBBX13* construct was transferred into pDEST-GBKT7 (Clontech, Mountain View, CA, USA) using LR clonase (Invitrogen) to generate the construct BD-CmBBX13. This transgene was transformed into yeast strain Y2H, which carries the *LacZ* and *HIS3* reporter genes. The plasmids pCL1 and pGBKT7 were used as, respectively, the positive and a negative control. Yeast cells were cultured on either synthetic drop-out (SD)/Leu- or SD/Trp-media for 3 days at 30 °C, then transferred to SD/His-Ade- plates in either the presence or absence of X-Gal. The development of blue pigment was assessed after 8 h. The *pENTR1A-CmBBX13* plasmid was also

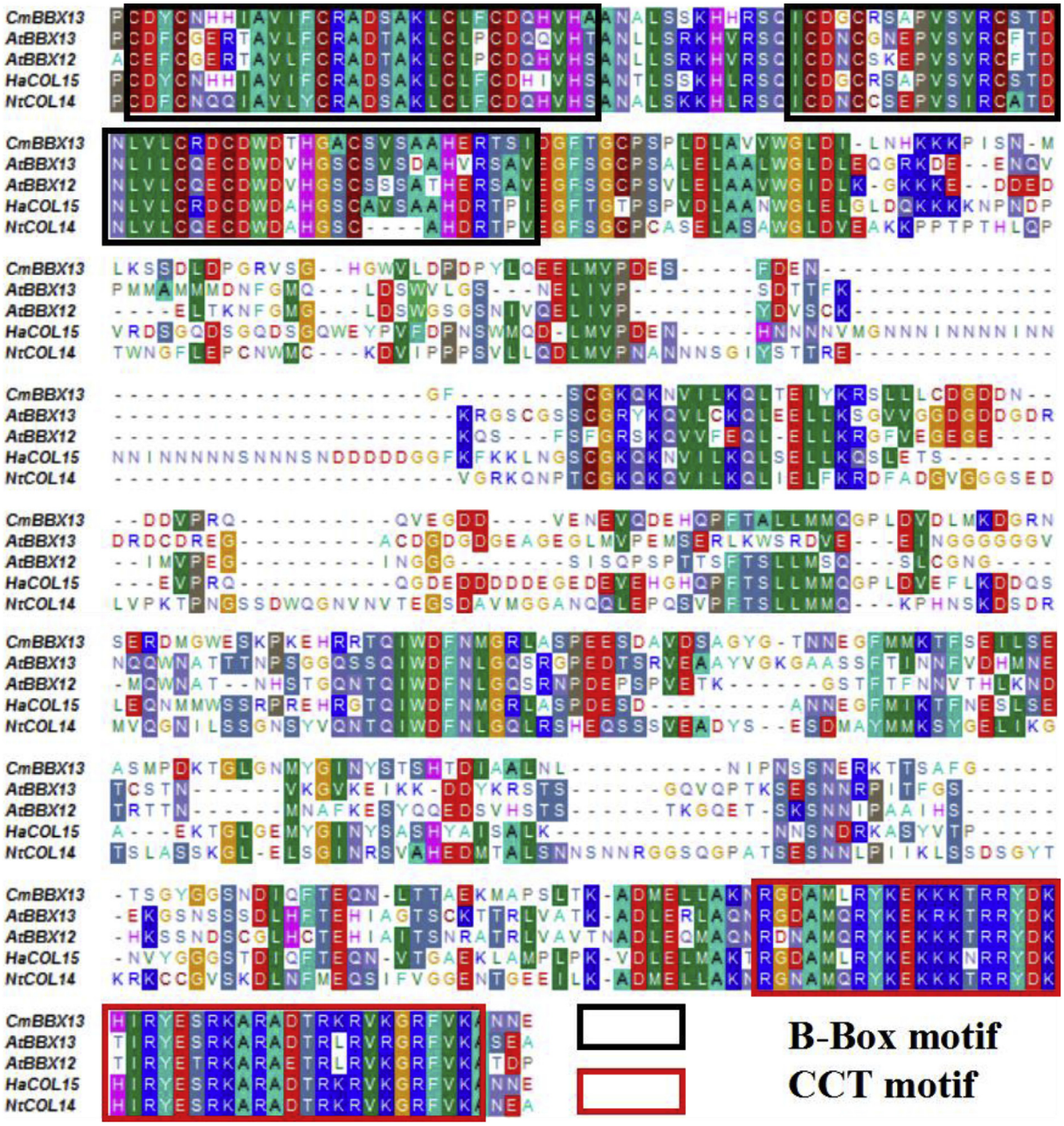

Fig. 1. Alignment of the CmBBX13 sequence with those of related polypeptides. The relevant database accession numbers are *A. thaliana* AtBBX12: AT2G33500, *A. thaliana* AtBBX13: AT1G28050, sunflower HaCOL15: OTG17268, tobacco NtCOL14: AIE45494.1.

recombined with *p35S::GAL4DB* to generate *p35S::GAL4DB-BBX13* via the LR reaction (Invitrogen). *A. thaliana* protoplasts were recovered and transfected following a published protocol (Yoo et al., 2007). A 5.0 μg aliquot of either *p35S::GAL4DB-AtARF5*, *p35S::GAL4DB* or *p35S::GAL4DB-CmBBX13* was combined with a 5.0 μg aliquot of 5X GAL4-LUC for the purpose of transfection. Luciferase activity was assessed as described elsewhere (Song et al., 2013). Three independent experiments were performed.

3. Results

3.1. Isolation of CmBBX13

The length of the *CmBBX13* ORF isolated from chrysanthemum 'Yuuka' was 1308 nt, and the gene was predicted to encode a 435 residue protein. The sequence included two B Boxes at its N terminus and a CCT domain at its C terminus (Fig. 1). A phylogenetic analysis implied that the protein belonged to a set of highly conserved angiosperm sequences, and specifically to those classified as group II BBXs. The chrysanthemum polypeptide shared homology with both Cse_sc011467.1_g010.1 and Cluster-9838.72124. The closest matching protein encoded by *A. thaliana* was AtBBX13 (Fig. 2). On this basis, the chrysanthemum gene was designated *CmBBX13*.

3.2. Transcription profiling of CmBBX13 in chrysanthemum

CmBBX13 transcript was detected in the roots, stems, leaves and apical buds of plants sampled during vegetative growth. The abundance of the transcript was highest in the leaves and the stem apex (Fig. 3). In plants raised under SD, several peaks of transcription were observed, while in those exposed to LD, the level of transcription was low for the first 24 h, then peaked twice during the lit period, in contrast to the behavior of *CmCO*, which exhibited a transcriptional peak only during the dark period (Fig. 4a). The transcription of the gene was arrhythmic in plants exposed to either continuous darkness or continuous light, and the abundance of the transcript was similar in both conditions (Fig. 4b). The suggestion was that the diurnal variation in the transcription of *CmBBX13* was unresponsive to the circadian clock.

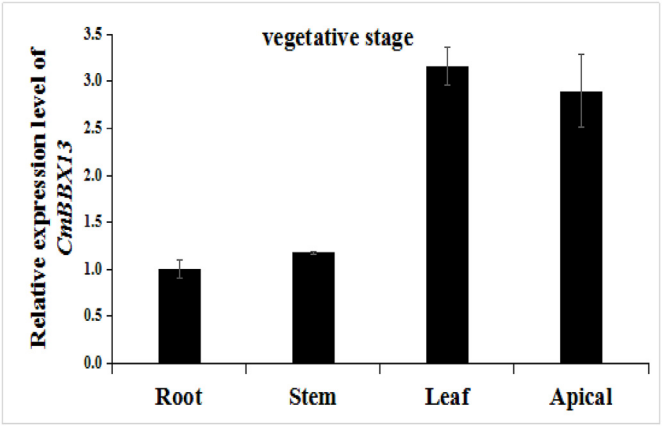

Fig. 3. Topological variation in *CmBBX13* transcript abundance. Abundances shown in the form mean ± SE (n = 3). Plants were sampled at ZT 2.

3.3. Sub-cellular localization of CmBBX13 protein

The pattern of expression of *GFP* in transiently transformed onion epidermal cells was used to deduce the sub-cellular localization of CmBBX13 *in planta*. In cells transformed with only *GFP,* fluorescence was observed in both the cytoplasm and nucleus, while in those transformed with the *CmBBX13-GFP* transgene, activity was detected only in the nucleus, overlapping with the distribution of RFP signal emitted by the D53 nuclear marker gene (Fig. 5).

3.4. Transactivational assay of CmBBX13 in yeast cells

Yeast cells transformed with the control pCL1 plasmid were able to grow freely on SD/His-Ade- medium and turned blue, while cells transformed with the plasmid pGBKT7 were unable to grow. Cells transformed with the BD-CmBBX13 construct were able to grow, implying that CmBBX13 possesses transactivational activity (Fig. 6A). When the *p35S::GAL4DB-CmBBX13* construct was co-transfected along with the luciferase 5X GAL4-LUC plasmid into *A. thaliana* mesophyll

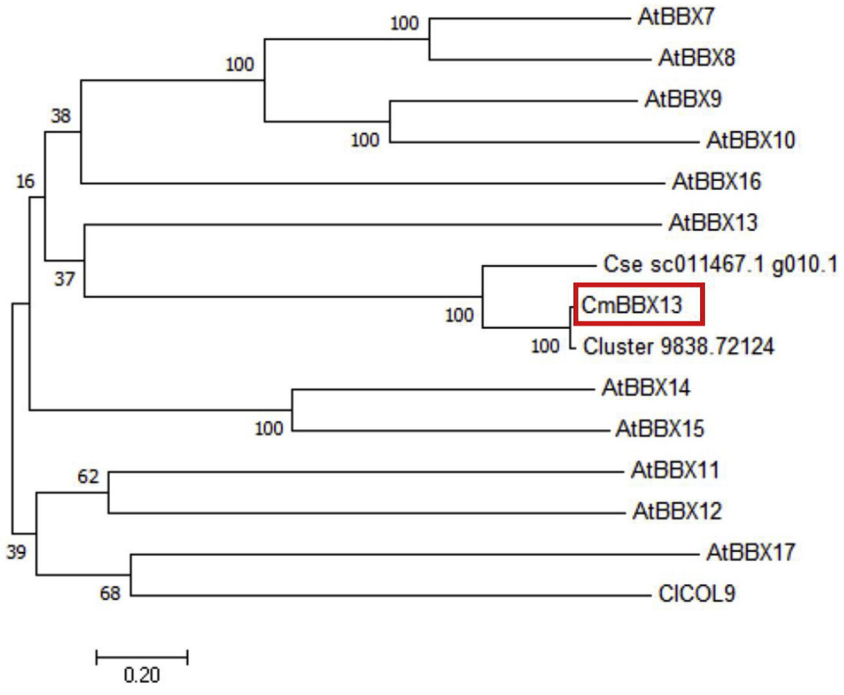

Fig. 2. The phylogeny of CmBBX13. The bar (0.20) indicates branch length. The relevant TAIR or GenBank accession numbers are AtBBX7: AT3G07650, AtBBX8: AT5G48250, AtBBX9: AT4G15250, AtBBX10: AT3G21880, AtBBX11: AT2G47890, AtBBX12: AT2G33500, AtBBX13: AT1G28050, AtBBX14: AT1G68520, AtBBX15: AT1G25440, AtBBX16: AT1G73870, AtBBX17: AT1G49130, *C. lavandulifolium* ClCOL9 (Fu et al., 2015), *C. seticuspe* Cse sc011467.1 g010.1 (Hirakawa et al., 2019), *C. nankingense* Cluster-9838.72124 (Song et al., 2018).

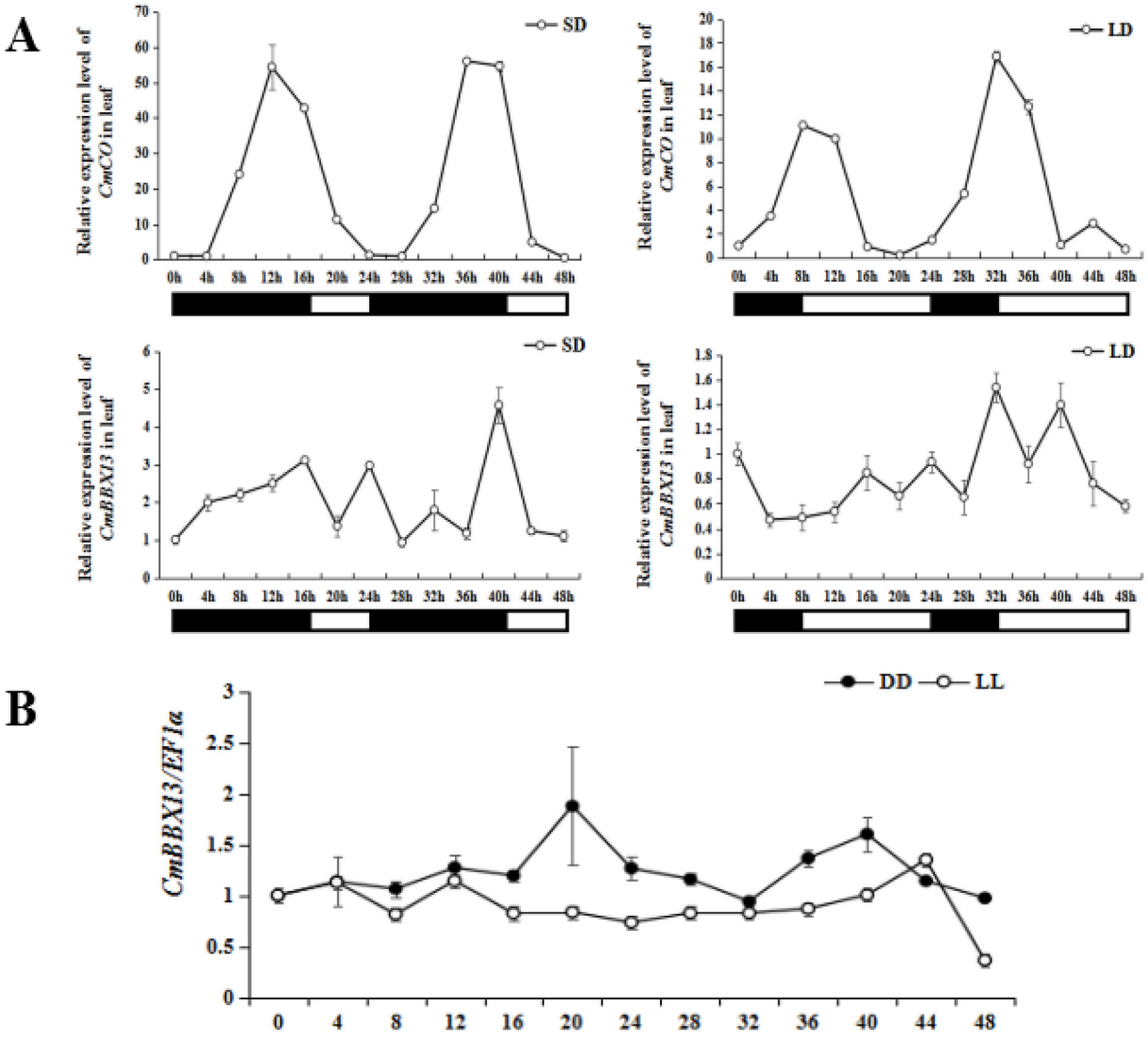

Fig. 4. Transcriptional profiling of *CmBBX13* in the chrysanthemum leaf. a The effect of photoperiod on *CmBBX13* and *CmCO* transcription. Abundances shown in the form mean ± SE ($n = 3$). The empty rectangles under the x axis represent lit periods and the filled ones unlit periods. b Temporal variation in *CmBBX13* transcription in plants exposed to continuous dark or continuous light. Plants were grown for 3 days under either continuous dark (DD; filled circles) or continuous light (LL; open squares), after which transcription was monitored over 48 h.

protoplasts, the observation was that luciferase activity was higher than in the control cells harboring p*35S::GAL4DB*. The conclution was that CmBBX13 functions as a transcriptional activator.

3.5. The constitutive expression of CmBBX13 delays flowering in A. thaliana

A number of *A. thaliana* lines were engineered to constitutively express *CmBBX13* (Fig. 7a). These remained vegetative after wild type plants had started to flower, whether the plants were grown under SD or under LD conditions (Fig. 7b). The transgenic plants developed a

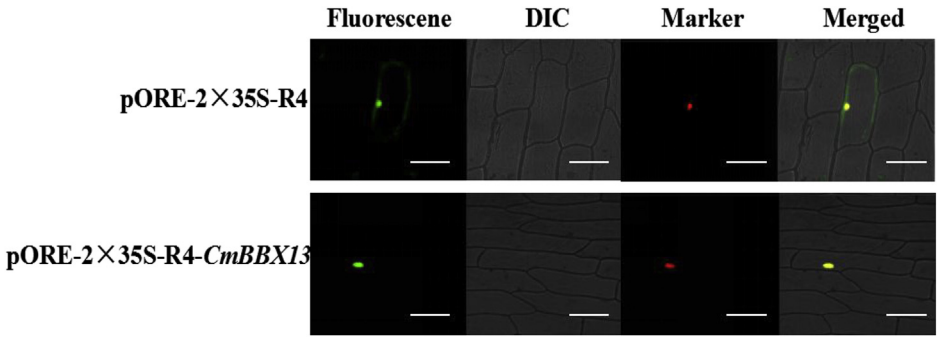

Fig. 5. Sub-cellular localization of *CmBBX13* expression in transiently transformed onion epidermal cells. The p*35S::D53-RFP* transgene was used as a nuclear marker. Fluorescene: image captured in the GFP channel, DIC: image captured under bright light, Marker: image captured in the red channel, Merge: an overlay of the GFP and DIC images. Bar: 0.1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

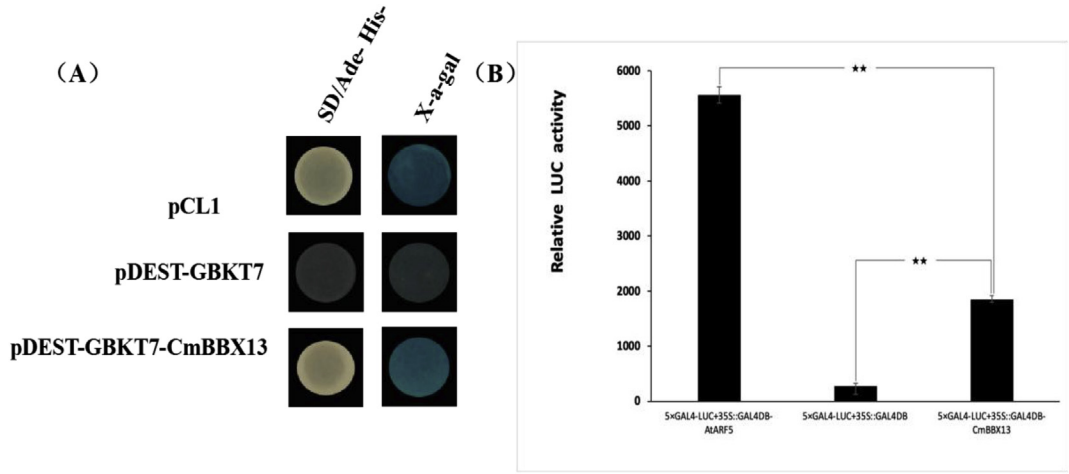

Fig. 6. Transactivational activity of CmBBX13. a An analysis based on expression in yeast. The pCL1 and pGBKT7 plasmids were used as, respectively, the positive and negative control. b Relative luciferase activity in *A. thaliana* mesophyll protoplasts after transfection with *p35S::GAL4DB-CmBBX13*. Asterisks indicate means differing significantly according to a *t*-test (**: $P < 0.05$).

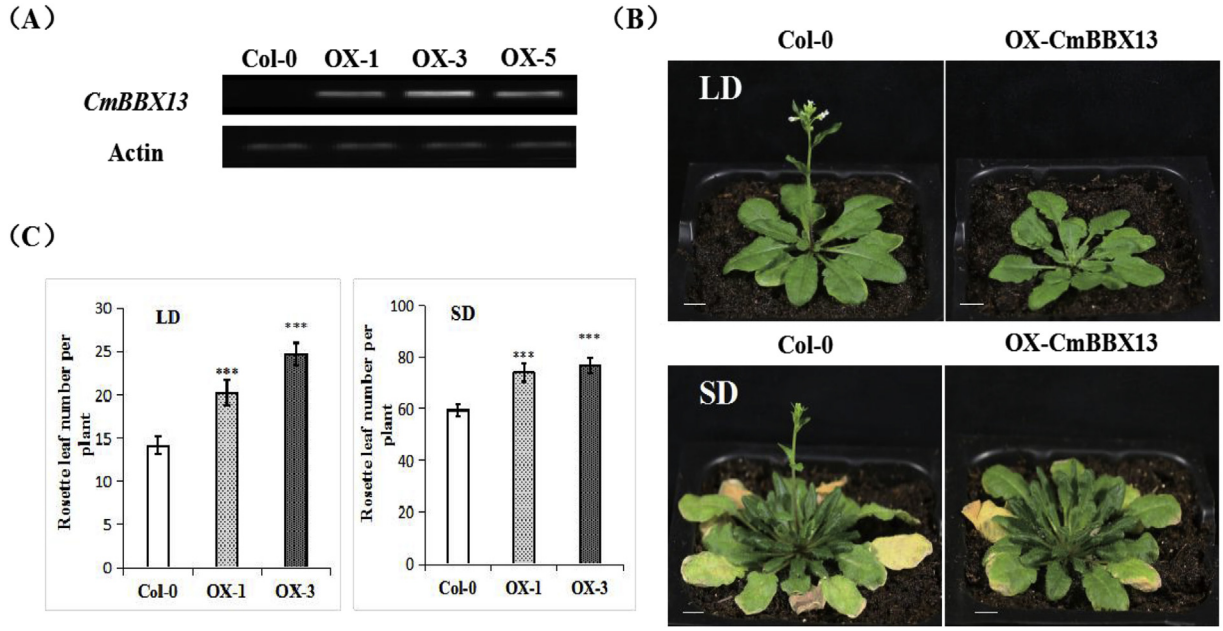

Fig. 7. The phenotype of constitutive expressors of *CmBBX13* in *A. thaliana*. a RT-PCR-based identification of constitutive expressors of *CmBBX13* in *A. thaliana*. *AtActin2* was used as the reference sequence. b Constitutive expressors of *CmBBX13* flowered later than wild type plants independent of the photoperiod. Bar: 1 cm. c Rosette leaf number of constitutive expressors of *CmBBX13* and wild type plants at the time of flowering. Data shown in the form mean ± SE ($n \geq 30$). Asterisks indicate means differing significantly according to a *t*-test (***: $P < 0.001$).

higher number of rosette leaves under both light regimes (Fig. 7c). When the transcription of key flowering control genes was assayed in plants grown under LD conditions, the abundance of *AP1*, *SOC1*, *FD* and *FT* transcript proved to be substantially lower in the constitutive expressors than in wild type plants and vice versa for *FLC* and *TOE2* (Fig. 8).

4. Discussion

BBX transcription factors represent an important component of the regulatory machinery controlling growth and development (Gangappa and Botto, 2014). One of the major developmental events in a plant's life cycle is the switch from vegetative to reproductive growth, and the participation of BBXs in this process was implied by the delay to flowering induced by heterologously expressing *CmBBX13* in *A. thaliana*. The mechanistic basis of this effect is uncertain, but it was clear that the genes encoding a number of key flowering promoters were down-regulated, while at the same time, certain flowering inhibitor genes were up-regulated. The product of *CO* has long been known to regulate the timing of flowering in response to the photoperiod (Mouradov et al., 2002; Simpson and Dean, 2002). This gene belongs to a family of transcription factors which feature both B Boxes and CCT domains (Putterill et al., 1995; Strayer et al., 2000; Robson et al., 2001). The chrysanthemum *BBX* family member described here encoded a protein featuring two B Boxes and one CCT domain, which implies its close relationship to the products of group II *BBX* genes, and in particular to *AtBBX13* (Figs. 1 and 2). The function of AtBBX13 remains unknown in *A. thaliana,* as does that of its homolog in rice. The CCT domain is thought to be important for localizing expression to the nucleus (Jang et al., 2008; Datta et al., 2008; Robson et al., 2001; Yan et al., 2011), which proved to be the site of *CmBBX13* expression (Fig. 5). Most CO-like proteins have been shown to act as

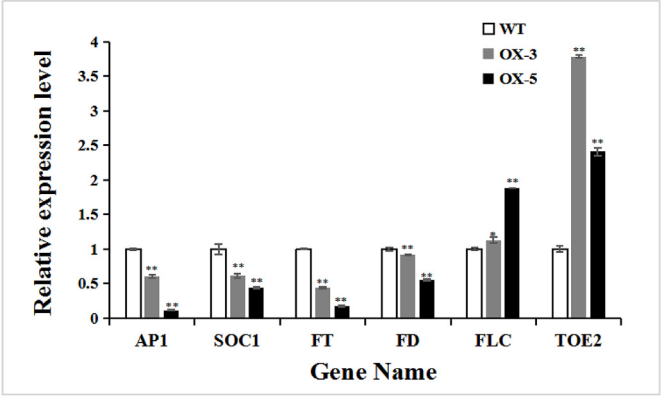

Fig. 8. The transcription of key genes regulating the flowering of *A. thaliana* measured in plants constitutively expressing *CmBBX13*. Asterisks indicate that the mean performance of transgenic and wild type plants differed significantly according to a *t*-test (*: P < 0.1; **: P < 0.05).

transcriptional activators of downstream genes (Tiwari et al., 2010; Sheng et al., 2016), and the yeast- and protoplast-based transient transformation assays both suggested that CmBBX13 possessed transactivational activity (Fig. 6). It is therefore probable that *in planta,* CmBBX13 functions as a transcriptional activator of various downstream genes.

Flowering involves an interaction between many genes, which then act together to create a complex and delicate regulatory network (Sheng et al., 2016). Multiple members of the CO/COL group of proteins act as flowering time regulators in both *A. thaliana* and rice (Valverde, 2011). The transition from vegetative to reproductive growth reflects the outcome of a quantitative balance between repressors and activators (Wang et al., 2014). It has been suggested that CO/COL proteins are able to directly target FT and SOC1 (Huang et al., 2011; Kardailsky et al., 1999; Samach et al., 2000). Here, transcriptional profiling of plants exposed to LD conditions showed that *AP1*, *FT* and *SOC1* were all down-regulated in *A. thaliana* plants constitutively expressing *CmBBX13* (Fig. 8). It has been noted elsewhere that COL9 delays flowering by repressing *CO*, thereby altering the abundance of *FT* and *SOC1* transcript (Cheng and Wang, 2005). COL12 acts as a substrate of COP1/SPA E3 ligase, and delays flowering by down-regulating *FT* in LD-grown plants, while COL12 represses the activity rather than the abundance of CO (Ordoñez-Herrera et al., 2018). *COL5* over-expression is known to promote flowering, but does not influence the abundance of *CO* transcript (Hassidim et al., 2009). Meanwhile, the over-expression of *AtBBX24/STO* not only activates *FT* and *SOC1*, but also suppresses *FLC* (Li et al., 2014), and the over-expression of *CmBBX24* has the effect of delaying flowering (Yang et al., 2014). The summer-flowering cv. 'Yuuka' blossoms earlier under SD than under LD conditions (Ren et al., 2016). Unlike *CmCO*, *CmBBX13* transcription did not appear to be governed by the circadian clock (Fig. 4), but it delayed flowering in *A. thaliana* (Fig. 7). Some *A. thaliana* genes not under the control of the circadian clock have the similar regulatory effect over flowering. For example, *pie1-1* (*PHOTOPERIOD INDEPENDENT EARLY FLOWERING1*) mutants flower early under both SD and LD conditions (Yoo and Rechard, 2003); the normal transcription of *CO* under LD conditions requires the presence of the histone-binding protein MSI1, but in the partially complemented *msi1* mutant *msi1-tap1,* flowering is delayed under both LD and SD conditions because of the reduced expression of *CO* (Bouveret et al., 2006; Steinbach and Hennig, 2014); TOE proteins binding to the activation region of CO and inhibiting CO activity also delay flowering under both SD and LD conditions (Zhang et al., 2015); the *flc* mutant exhibits an increased abundance of *FT* and *SOC1* transcript, leading to early flowering under both SD and LD conditions (Mahrez et al., 2016). The present experiments have established that plants over-expressing *CmBBX13* experienced a delay in their flowering independent of the photoperiod (Fig. 7) and that both *TOE2* and *FLC* were up-regulated (Fig. 8). The major conclusion is that CmBBX13 functions as a negative regulator of flowering independently of the photoperiod pathway.

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Authors and contributors

JJ and QP designed the experiments. QP, PC, FH, LR and HC performed all experiments and analyzed the data. QP and JJ wrote the manuscript. ZG, WF, SC and FC checked the experiments. We thank Dr. Yuehui He (Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, China) for the analysis of flowering phenotypes. All the authors have read and approved the manuscript.

Declaration of competing interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.019.

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