CmBBX8 accelerates flowering by targeting CmFTL1 directly in summer chrysanthemum

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Summary
For a flowering plant, the transition from vegetative stage to reproductive growth is probably the most critical developmental switch. In the model plant Arabidopsis thaliana, the product of BBX7, group II member of BBX family, acts to delay floral transition. In this study, a presumed chrysanthemum homolog of a second group gene AtBBX8, designated CmBBX8, had been isolated and characterized. The transcription of CmBBX8 followed a diurnal rhythm as the chrysanthemum floral transition regulator. Overexpression of CmBBX8 accelerated flowering, while its (artificial microRNAs) amiR-enabled knockdown delayed flowering in plants grown under both long- and short-day conditions. Global expression analysis revealed that genes associated with photoperiod were down-regulated in amiR-CmBBX8 lines compared with the wild type, which were verified to be up-regulated in overexpressing lines (OX-CmBBX8) by RT-PCR. A number of in vitro assays were used to show that CmBBX8 targets CmFTL1. Furthermore, the function of CmFTL1 as a floral inducer under long-day conditions was confirmed by the behaviour of engineered summer-flowering chrysanthemum plants. The conclusion is that the BBX8-FT regulatory module is an important determinant of reproductive development in summer-flowering chrysanthemum.

Introduction
The transition from vegetative stage to reproductive growth which is probably the most critical event in the life cycle of a flowering plant is triggered by a variety of both exogenous and endogenous cues (Boss et al., 2004). In Arabidopsis thaliana, four major regulatory pathways (photoperiod, vernalization, gibberellin and autonomous) act to determine floral transition (Mouradov et al., 2002). The photoperiod pathway combines a set of photoreceptors, circadian clock proteins and an output pathway (Simpson and Dean, 2002).

CONSTANS-LIKE 1 (CO, BBX1), a transcription factor belonging to the BBX family of proteins containing a CCT domain (nuclear localization signal), provides a key link between the circadian clock and the floral integrator FT (Andrés and Coupland, 2012). BBXs participate in many plant regulatory networks, ranging from seedling morphomorphogenesis to stress response (Gangappa and Botto, 2014). The A. thaliana BBX proteins have been categorized into five groups, while they all harbour a conserved B-box domain, some members also have a CCT domain (Datta et al., 2006), and BBX4, BBX20, BBX21 and BBX22 can promote light morphogenesis (Chang et al., 2008; Datta et al., 2007). Conversely, BBX18, BBX19, BBX24, BBX25 and BBX32 can inhibit plant photoperiodism (Gangappa and Botto, 2014; Holtan et al., 2011). Overexpression of AtBBX7 (COL9) induces a delay in flowering under long-day (LD) conditions through its repression of FT (Cheng and Wang, 2005; Imtiaz et al., 2015). AtBBX10 (CQL12) delays flowering by repressing CO protein activity (Ordoñez-Herrera et al., 2017), while the functions of BBX family members in the groups II (BBX7-13) are largely unknown in other plants.

In plants, flowering is tightly controlled by the integration of various exogenous and endogenous signals, including light, temperature and the age of the plant. The FT-like clade of plant phosphatidylethanolamine-binding proteins (PEBPs) including a number of important regulators integrates these signals, which regulate photoperiod-dependent flower development in different angiosperm species are now well-known. In the facultative LD plant A. thaliana, the FT transcripts in the leaves are activated by CO only under LD conditions (An et al., 2004). The FT protein is generated in the phloem and transported from thence to the shoot apical meristem (Corbesier et al., 2007; Endo et al., 2018). In the meristem, FT interacts with the bZIP transcription factor FD to promote flowering by activating the expression of floral meristem identity genes such as APETALA1 (Jaeger and Wigge, 2007; Wigge et al., 2005). In rice, a facultative short-day (SD) plant, the initiation of flowering requires the presence of the FT homolog HD3a, whereas under LD conditions, flowering is initiated by the FT homolog RFT1 (Kojima et al., 2002; Komiyama et al., 2009).

Chrysanthemum varieties which flower in the fall act as SD plants, while the summer chrysanthemum flowers under both LDs and SDs (Sun et al., 2017). The chrysanthemum genome contains three FT-like genes: CsFTL3 functions under SD conditions (Oda
et al., 2011), and CmFTL2 is also active during the process of floral transition under SD conditions and much more strongly induced than either CmFTL1 or CmFTL3 by sucrose treatment (Sun et al., 2017); although FTL1 has been suggested to act as an LD florigen analogously to RFT1 in rice, the consequence of its constitutive expression in the SD cultivar cv. ‘Jinba’ implies that it has only weak florigenic activity (Higuchi et al., 2013; Mao et al., 2016). Its function in summer-flowering chrysanthemum cultivars is unknown.

In the current study, the focus was placed on characterizing the gene CmBBX8, which is a subgroup II gene. Its function has not been reported. The experiments showed that the transcription of CmBBX8 followed a diurnal rhythm and that the gene was particularly strongly transcribed in the leaves of vegetative plants. Its gene product was deposited in the nucleus, and the segment of the protein lying between the B-box and the CCT domain was found to have transcriptional activity. In Arabidopsis thaliana, BBX7 and BBX8 have highest homology (Gangappa and Botto, 2014). To our surprise, overexpression of CmBBX8 accelerated flowering, which was opposite to the role played by BBX7 in Arabidopsis. Further analysis showed that CmBBX8 was a floral activator in the photoperiod pathway. It accelerated flowering by targeting CmFTL1 directly to induce its expression. The flowering function of CmFTL1 under LD conditions is further validated with the transgenic summer-flowering cv. ‘Yuuka’. The intention of the research was to improve the understanding of the control of the floral transition in summer-flowering Chrysanthemum, with a view to using molecular breeding for varietal improvement in this valuable ornamental species.

**Results**

**Isolation of chrysanthemum BBX8**

To explore the function of BBX genes in summer chrysanthemum, the BBX8 sequence was isolated from ‘Yuuka’ comprising a 1104 bp open reading frame (ORF), predicted to encode a 367-residue polypeptide. Its deduced polypeptide sequence shared between 54.3% and 97.6% similarity with BBX proteins produced by a range of plant species and included a highly conserved two B-box domain in its N terminus and a CCT domain in its C terminus, a characteristic of BBX group II proteins (Figure 1a). A phylogenetic analysis confirmed its close relatedness with the A. thaliana group II BBXs, most strongly so with AtBBX8 (Figure 1b). On this basis, the gene was designated CmBBX8.

**Transcriptional profiling of CmBBX8 in cv. ‘Yuuka’**

To investigate the potential function of CmBBX8 in regulation of flowering time, its expression in different organs including apical meristem, leaves, stems and roots at vegetative stages with quantitative RT-PCR (qRT-PCR) was evaluated. CmBBX8 was abundantly transcribed in the apical meristem, leaves, stems and roots of cv. ‘Yuuka’ plants sampled at the vegetative stage; the highest abundance of the transcript present was in the leaves (Figure 2a). Whether the transcripts of the CmBBX8 in leaves were under the regulation of a diurnal clock was further investigated. The expression levels of CmBBX8 revealed oscillations, with a peak occurring at about Zeitgeber time 8 or 12 h from light (ZT8 or ZT12) under LD or SD conditions, followed by a second peak 36 or 32 h later (ZT36 or ZT32; Figure 2b). Accordingly, these results showed that CmBBX8 had a diurnal-controlled expression that it responded to day length, as was similarly the case for CmFTL1 (Figure S1).

CmBBX8 localizes to the nucleus and has transactivational activity

To obtain evidence that CmBBX8 acted as a transcription factor, the subcellular localization of CmBBX8 was investigated by transiently expressing in tobacco with a transgene comprising CmBBX8 fused to GFP and driven by the CaMV 35S promoter. In transformed cells, GFP activity overlapped with that of the nuclear marker (Ds3-mCherry), while in cells transformed with the p35S::GFP control plasmid, GFP activity was observed in both the cytoplasm and the nucleus (Figure 3a). As a test to further determine CmBBX8’s capacity for transactivation, the gene was fused to the GAL4-binding domain (BD) and expressed in yeast strain Y2H. The CmBBX8-BD fusion exhibited a stronger level of transcriptional transactivation than did the BD control (Figure 3b).

A deletion analysis to determine which segments of the protein were responsible for its transcriptional activated activity showed that the key segment lay between the conserved B-box and the CCT domain (Figure 3b). Together, these data show that CmBBX8 may regulate the transcripts of downstream genes as a transcriptional activator.

**Overexpression of CmBBX8 accelerates floral transition in cv. Yuuka**

To establish whether CmBBX8 participates in regulating flowering time, a set of 20 transgenic plants in which CmBBX8 was overexpressed (CmBBX8-OX plants) and four in which CmBBX8 was specifically knocked down using an artificial microRNA (amiR-CmBBX8 plants) was obtained. From both transgenic types, three lines were selected (Figure S2 and Figure 4a, b) in order to assess the effect of CmBBX8 on phenotype. The OX plants grown under LD conditions initiated their first involucral primordia 50 days after transplanting, while (wild type) WT plants required 70 days to reach this developmental stage; at this time point, the OX plants had already reached the bud breaking stage, but the WT plants were still at the bud formation stage (Figure 4c). In contrast, amiR plants did not reach the involucral primordium initiation stage until 85 days after transplanting and reached each of the subsequent flowering stages later than WT plants (Figure 4c); in addition, the overexpression was shorter than WT and the amiR lines (Figure S3). CmBBX8-OX also accelerated flowering under SD conditions (Figure S3).

In a previous report, CONSTANS-LIKE 9 (COL9, AtBBX7) was found to delay floral transition in Arabidopsis (Cheng and Wang, 2005; Imtiaz et al., 2015). A. thaliana transgenics harbouring a p35S::CmBBX8 construct flowered earlier than WT plants, different from AtBBX7 (Figure S4). Taken together, these results imply that CmBBX8 plays a role in the regulation of flowering time in both chrysanthemum and Arabidopsis.

**Differential transcription is induced by manipulating the transcription of CmBBX8**

The transcripts of CmBBX8 were regulated by light and diurnal clock, indicating that it is probably involved in the photoperiod pathway of flowering. An RNA-seq analysis was used to identify which genes were differentially transcribed in amiR line #3. The outcome was a set of 9357 up-regulated and 16867 down-regulated genes (Figure S5). Based on the expression pattern of CmBBX8 and its regulation of flowering time, we focused on differentially transcribed genes (DTGs) involved in the flowering pathways. Mainly, three of the differentially expressed genes were likely in the photoperiod pathway (Table S1); based on their
homology with *A. thaliana* sequences, they were designated *CmGI*, *CmPRR7* and *CmEMF2.2* (Figure S6 and Table S1). In addition, the gene *CmFTL1* was more significantly down-regulated than other genes in amiR line #3 (Table S1). A qRT-PCR assay confirmed the down-regulation of *CmGI*, *CmPRR7* and *CmFTL1* in the amiR plants and their up-regulation in OX plants.

![Figure 1](image1.png)

**Figure 1** Characterization of the *CmBBX8* polypeptide sequence. (a) Alignment of the deduced polypeptide sequences of *CmBBX8* with those of other plant BBXs. Red and green lines indicate the Bbox1 and Bbox2 within the conserved B-box domain, the yellow line indicates the conserved CCT domain. (b) A phylogenetic analysis of the *CmBBX8* sequence with other *A. thaliana* BBXs. Bootstrap values indicate the divergence of each branch, and the scale shows branch length.

![Figure 2](image2.png)

**Figure 2** Transcription profiling of *CmBBX8* in cv. ‘Yuuka’. (a) qRT-PCR-based profiling in various parts of plants harvested at the vegetative stage. Letters above the bars indicate significant differences as determined by Tukey’s (honestly significant difference) HSD test (*P* < 0.05). (b) The transcriptional response to varying the photoperiod. The abscissa indicates the sampling time point; white and black horizontal bars below the axis represent light and dark periods, respectively. Values shown are means (*n* = 3), and the error bars represent the SE.
These results indicated that the expression patterns of the DTGs were generally consistent with RT-PCR. Therefore, CmFTL1, a flowering integration factor, could most likely be directly regulated by CmBBX8.

DNA–protein interactions involving CmBBX8
EMSA, LUC and ChiP-qPCR assays were used to characterize DNA–protein interactions involving CmBBX8. According to Gnesutta et al. (2017), the CmFTL1 promoter harbours the CORE element (CCACA) (annexed table). The EMSA suggested that CmBBX8 was able to bind in vitro to the CmFTL1 promoter (Figure 6a), a conclusion further supported by experiments based on both luciferase activity (Figure 6b) and ChiP-qPCR (Figure 6c). These results showed protein–DNA interaction occurred between CmBBX8 and the proximal region CORE element (CCACA) of the CmFTL1 promoter. Taking together, we conclude that CmBBX8 regulates CmFTL1 directly.

CmFTL1 promotes flowering in summer-flowering chrysanthemum grown under LD conditions
Weak floral inducerc activity of CmFTL1 was indicated in the previous reports (Higuchi et al., 2013; Mao et al., 2016). The induction of flowering exerted by CmFTL1 in cv. “Yuuka” plants raised under LD conditions was investigated by observing the behaviour of the OX and amiR plants (Figure 7a, b and Figure S7). WT plants had entered the budding stage by 72 days after transplanting, a stage which had been reached by the OX plants 12 days earlier; meanwhile, the amiR plants were delayed by 20–63 days, depending on the extent of the down-regulation achieved by the amiR transgene (Figures 7c, d and S7). Thus,
these results showed that CmFTL1 can act as floral inducer under LD and play a central role in the flowering time of summer chrysanthemum.

Discussion

CmBBX8 accelerates flowering by binding to the CmFTL1 promoter

The flowering regulation by BBX proteins was studied extensively. CO has long been recognized as a central regulator of the photoperiod-responsive floral transition (Putterill et al., 1995). Some CO-like proteins act as floral activators, while others act as repressors (Kurokura et al., 2017). FvCO and AtCO played a major role in the photoperiodic control of flowering in Fragaria vesca (Kurokura et al., 2017) and Arabidopsis (Putterill et al., 1995), respectively. OsCO3 primarily controlled flowering time by negatively regulating Hd3a and FTL expression under SD conditions in rice (Kim et al., 2008). A number of them have been shown to influence traits unrelated to flowering, such as stem height in alfalfa (Herrmann et al., 2010) and the photoperiodic response of stem elongation in Norway spruce (Picea abies; Holefors et al., 2009). The overexpression of COL5 in A. thaliana plants grown under SD conditions promotes flowering (Hassidim et al., 2009). While CmBBX8 appeared to accelerate flowering by activating the CmFTL1 promoter (Figure 6), AtBBX7 (a homolog of AtBBX8) delays flowering by repressing CO and FT (Cheng and Wang, 2005). The rice homologs OsCOL9 (BBX7) and OsCOL10 (BBX8) encode products which act in a similar way to the A. thaliana ones (Cheng and Wang, 2005; Liu et al., 2016; Tan et al., 2016). It should be noted that OsCOL10 lacks one B-box.

Figure 4  The phenotype of CmBBX8-OX and amiR-CmBBX8 plants grown under LD conditions. (a) A qRT-PCR assay for quantifying the abundance of CmBBX8 transcript in CmBBX8-OX plants. Values shown are means (n = 3). Letters above the bars indicate significant differences as determined by Tukey’s HSD test (P < 0.05). (b) A qRT-PCR assay for quantifying the abundance of CmBBX8 transcript in amiR-CmBBX8 plants. Values shown are means (n = 3). Letters above the bars indicate significant differences as determined by Tukey’s HSD test (P < 0.05). (c) The phenotype and developmental progression of cv. ‘Yuuka’ and the CmBBX8 transgenic lines. Statistics based on >20 seedlings per genotype. Bar = 2 mm.

Figure 5  CmBBX8 regulates the expression of CmFTL1. (a) A qRT-PCR-based transcriptional profiling of flowering time control genes in plants harbouring the p35S::GFP-CmBBX8 transgene grown under LD conditions. (b) A qRT-PCR-based transcriptional profiling of flowering time control genes in plants harbouring the amiR-CmBBX8 transgene exposed to LD conditions. Values shown are means (n = 3). Asterisk above the bars indicate significant differences as determined by Tukey’s HSD test (P < 0.05).
domain, which may contribute to the different flowering function (Tan et al., 2016). CmBBX8 targeted the CmFTL1 promoter at a CORE element close to the gene’s ATG transcription initiation codon, even though there were two other suitable CORE elements lying in the distal region of the promoter (Figure 6c). In the Arabidopsis genome, only ~15% of the CTCTGYTY motifs are bound by the enzyme REF6, and the authors speculated that not only the motifs are necessary for the binding, but also DNA methylation combination with other epigenetic modifications plays a role in the contribution of the binding (Qiu et al., 2019). Therefore, only the element in the proximal region of the CmFTL1 promoter was bound by the CmBBX8 most strongly, which might be due to the local different epigenetic modifications.

Variation on the regulation of flowering in the summer Chrysanthemum ‘Yuuka’

Shortening the vegetative phase is a significant breeding goal of many arable and horticultural crop species improvement programmes. The timing of the switch from vegetative to reproductive growth depends on a balance of floral inducers and anti-floral inducer within the plant, which in turn is strongly influenced by environmental cues such as photoperiod and temperature. A major ‘floral inducer’, FT is discovered in Arabidopsis mutants firstly as the integrator of flowering activator in plant species (Turck et al., 2008). Usually, FT and TSF promote flowering as in Arabidopsis, rice and soya bean (Corbesier et al., 2007; Kim et al., 2013; Komiya et al., 2009; Kong et al., 2010). Indeed, rice never flowers once two FT homologs are inactivated (Komiya et al., 2008). Hd1, the rice CO homolog, acts as a transcriptional repressor of FT under LD conditions, while it activates H'id3a and RFT1 expression under SD conditions (Song et al., 2010). In poplar (Populus trichocarpa), the activation of FT can also be altered in response to photoperiod (Hsu et al., 2011). Besides modulating FT transcripts in response to different environmental factors, there are multiple FT-related genes in many plants (Higuchi, 2018). The products of the two FT paralogs harboured by the beet (Beta vulgaris) genome act antagonistically and interact to determine the plants’ flowering time (Pin et al., 2010). BvFT1 is also involved in vernalization pathway, which provides a flexibility of networks using FT signalling. The Chrysanthemum harbours three FT-like genes, CsFTL1, CsFTL2 and CsFTL3. CsFTL3 acts as a short-day floral inducer, while CsFTL1 expression is repressed in short day (Oda et al., 2011). In autumn-flowering, Chrysanthemum flowering time is majorly regulated by the photoperiod pathway under SD, and the GA signalling pathway predominated for flowering under LD (Dong et al., 2017), while the antiflorigen function of AFT under LD obligates the flowering only under SD (Higuchi et al., 2013). In summer-flowering chrysanthemum, flowering might be promoted by the photoperiod and GA pathways under
SD, and by the T6P and sugar signalling pathways under LD, while the flowering time is delayed by PHYB under LD (Ren et al., 2016).

In our study, it was revealed that CmFTL1 regulated flowering time in cv. ‘Yuuka’ (Figure S7), and CmFTL1 and CmBBX8 harboured the same pattern of response to day length (Figures 2, S1). Moreover, CmBBX8 was able to regulate the expression of CmFTL1 directly under diurnal-controlled daylight (Figures 2, 5, S1, S9). Therefore, these examples of the FT signalling system underline the flexibility available for the regulation of flowering time offered by exploiting the various pathways which rely on FT.

Materials and methods

Plant materials and growing conditions for rhythm expression analysis

Rooted cuttings of Chrysanthemum morifolium cv. ‘Yuuka’ (obtained from Nanjing Agricultural University’s Chrysanthemum Germplasm Resource Preserving Center) were raised in a mixture of perlite, meteorite, and soil, then transplanted into a mixture of peat, soil and perlite and held for two weeks under LD conditions (16-h/8-h photoperiod), a constant temperature of 23 °C and a relative humidity was 40%. When the plants had formed 15–19 fully expanded leaves, they were transplanted into a cabinet supplying either a 16-h or an 8-h photoperiod; in each case, the temperature was maintained at a constant 23 °C.

Quantification of flowering time

The number of days required for the first flower to appear at any node in the main stem was taken as the flowering time (Fehr et al., 1971). Plants were recognized as having entered the early blooming stage (EB stage) when at least 50% of the ray flowers were unfolding (Blanchard and Runkle, 2009). When Ox plants appeared small size bud, apical meristem of amiR plants at flower bud differentiation stage was observed through microscopic. The other flowering stages to be timed were the appearance of the first flower buds, the development of flower buds, the opening of the first flower and full bloom (Yang et al., 2014). Measurements were based on the performance of at least 20 plants per treatment.

The isolation of CmBBX8 and the analysis of its structure and phylogeny

RNA was extracted from cv. ‘Yuuka’ leaf tissue using the TRizol reagent (Invitrogen, New York). The CmBBX8 ORF sequence was amplified using the primer pair CmBBX8-F/-R (annexed table), and the resulting amplicon was inserted into the pMD19-T plasmid (Takara, Beijing, China) for the purpose of sequencing. The domain content of the CmBBX8 sequence was inferred by querying the Conserved Domains database (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The sequences of BBX8 homologs were recovered from GenBank and used to construct a multiple sequence alignment with the help of Clustal X v2.1 software (Aiyar, 2000). The phylogeny of a set of BBX sequences was derived using MEGA v5.05 software (Tamura et al., 2007), applying the neighbour-joining algorithm with 1000 bp replications.

Chrysanthemum transformation

The CmBBX8 and CmFTL1 ORF sequences were amplified using primer pairs (CmBBX8-F/CmBBX8-R; CmFTL1-F/CmFTL1-R) to introduce, respectively, a BamH I and an EcoR I recognition site (CmBBX8(B)-F/CmBBX8(E)-R), and a Kpn I and an Xho I (CmFTL1-
(K)-F/CmFTL1(X)-R) recognition site (annexed table). The resulting pENTR1A-CmBBX8 and pENTR1A-CmFTL1 constructs were digested by Pvu I, in order to apply the LR recombination reaction as a way of constructing the overexpression plasmid pMDC43 (Curts and Grossniklaus, 2003). The pMDC43 vector harbours the reporter gene GFP driven by the CaMV 2 x 35S promoter. The construction methods of pMDC32-amiR-CmBBX8 and pMDC32-amiR-CmFTL1 were followed as reported by Schaab et al. (2006). The amiRNA-containing precursor generated by amplifying miR319 template with pairs of overlapping primers (l/II/IV/II/III/II/II) was inserted into the pENTR1A via its Pst I/BamHI I and Sal I/Wot I sites to apply the LR recombination reaction as a way of constructing overexpression plasmid pMDC32. The OX (pMDC43-CmBBX8 and pMDC43-CmFTL1) and amiR knockdown (pMDC32-amiR-CmBBX8 and pMDC32-amiR-CmFTL1) constructs were introduced into Agrobacterium tumefaciens strain EHA105 and from thence into chrysanthemum using the leaf vacuum method (Simmons et al., 2009). DNA was extracted from selected plants using a rapid plant genomic DNA isolation kit (Sangon Biotech, Shanghai, China) following the manufacturer’s protocol, and used as a template to verify the plants’ transgenic status using the primer pair 35S-F/gene-R, as listed in annexed table.

Floral dip for Arabidopsis thaliana transformation

CmBBX8 was heterologously expressed in A. thaliana by introducing the pENTR1A-CmBBX8 (containing stop codon) construct using the primers CmBBX8 (R)-F/CmBBX8 (E + Z)-R, recombined into the final vector pBCH4 using the LR recombination reaction (Hanano and Goto, 2011). The floral-dip method was used for Agrobacterium-mediated transformation (Clough and Bent, 1998). Afterwards, DNA was extracted by rapid plant genomic DNA isolation kit (Sangon Biotech, Shanghai, China) following the manufacturer’s protocol, and the T1-T3 seedlings were identified in DNA level with primer (35S-F/gene-R) listed in annexed table. The mutant bbx7 (SALK_137167) was obtained from the Arabidopsis Information Resource (www.arabidopsis.org) and identified with primer (LBB1.3/bbx7-F; bbx7-R) in annexed table.

qRT-PCR analysis

RNA was extracted from plant tissue using the TRizol reagent (Invitrogen, New York) and 1 µg aliquot was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Takara, Beijing, China) in the presence of oligo (dT), following the manufacturer’s protocol. A 5 µl aliquot of the resulting cDNA was diluted 20-fold to act as the template of a 20 µl qRT-PCR based on a SYBR Premix Ex Taq™ Kit (Takara, Beijing, China). The qRT-PCR involved an initial denaturation (95 °C2 min), followed by 40 cycles of 95 °C15 s, 60 °C/15 s and 72 °C/15 s. The primer pair (qCmBBX8-F, qCmBBX8-R) used to amplify CmBBX8 cDNA is given in annexed table. Estimates of transcript abundance were based on the mean of three biological replicates and were calculated using the 2−AΔΔCt method (Livak and Schmittgen, 2001). The reference sequence was chrysanthemum EF1α (KF305681.1).

Subcellular localization of CmBBX8

The p35S::GFP-CmBBX8 (pMDC43) construct was introduced into tobacco epidermal cells with Agrobacterium-infiltrated tobacco (Nicotiana benthamiana) leaves (Wang and Zhang, 2012). After holding the samples for 16–30 h at 22 °C, the material was monitored for GFP activity using laser scanning confocal microscopy. Control samples were transformed with an empty pMDC43 empty vector.

Transactivation analysis of CmBBX8

Three segments of the CmBBX8 sequence were considered separately: the segment which encodes the B-box motifs (CmBBX8-B-boxes), the segment which encodes the CCT domain (Cm-CCT) and the lack of CCT domain (Cm-ΔCCT). The three segments were amplified using primers pairs designed to incorporate an EcoR I at one end of the amplicon and a BamHI site at the other end (annexed table), following a BamHI/EcoR I digest and a subsequent DNA ligase reaction. The resulting constructs were transformed into yeast strain Y2H. Yeast cells were cultured on either synthetic drop-out (SD)/Leu− or SD/Terp media for three days at 30°C and then transferred to SD/His− Ade− plates in either the presence or absence of X-Gal. Transactivation was inferred when the yeast cells formed blue colonies.

Transient expression assays in tobacco and luciferase imaging

The CmFTL1 promoter sequence was amplified from a template of cv. ‘Yuuka’ DNA using the primer pair CmFTL1pro-F/CmFTL1pro-R (annexed table) with Xma I/BamHI I and inserted into the pCAMBIA 13812-Luc vector which contains a reporter gene encoding firefly luciferase (kindly provided by Dr. Huazhong Shi, Texas Tech University, Lubbock, TX), Agrobacterium tumefaciens strain GV3101 harbouring pCFTL1::LUC and p35S::GFP-CmBBX8 was grown in infiltration medium (2 m Na3PO4, 50 m MES, 100 m acetosyringone) to an OD600 of 0.5 and then introduced via a syringe into the leaf of a 6- to 7-week-old Nicotiana benthamiana plant. After 48–96 h, a CCD camera was used to observe luciferase activity following the method given by Kost et al. (1995).

Electrophoretic mobility shift assay (EMSA)

The necessary bait 3’-end labelled sequences harbouring putative binding sites in the promoter CmFTL1 by CmBBX8 were synthesized by Generay (Shanghai, China), based on the primer pairs given in annexed table. The subsequent EMSAs were performed using a LightShift™ Chemiluminescent EMSA Kit (Thermo Fisher, New York), following the manufacturer’s protocol. The resulting samples were loaded onto a pre-run native 6.5% polyacrylamide gel, using TBE buffer as the electrolyte. After electro-blotting onto a nylon membrane (Millipore, Darmstadt, Germany) and UV cross-linking (2000J for 5 min), the membrane was incubated in blocking buffer for 30 min and rinsed in washing buffer. Finally, a CCD camera was used to visualize the chemiluminescent signal.

Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed following Bowler et al. (2004). Briefly, 1.5 g of leaf was harvested from 2-week-old seedlings harbouring p35S::GFP-CmBBX8 grown under LD conditions at ZT 8 h (three replicates of each sample from lines #6 and #8) and subjected to formaldehyde cross-linking. The resulting chromatin preparations were purified using a Qiagen Plasmid Extraction Kit (Hilden, Germany), following the manufacturer’s protocol. Subsequent qRT-PCR was performed based on the primer pairs given in annexed table (P1-P6). The ‘fold enrichment method’ was used for qPCR data (Lacazette, 2016). Means were compared using Tukey’s honestly significant difference (HSD) test, with a significance threshold of 0.05.
RNA extraction, transcriptome sequencing and bioinformatic analysis

RNA was extracted from stem apical meristems and leaves of plants grown under LD conditions and sampled at ZT 10 from plants bearing 14 leaves (three replicates of each sample) using an RNA Isolation Kit (Waryong, Beijing, China) following the manufacturer's protocol, and the RNA formed combining equal quantity from each biological replicate was subjected to illumina sequencing at Beijing Genomics institution (Shenzhen, China) using the HiSeq2000 platform. Annotation was based on seven functional databases (KEGG, GO, NR, NT, SwissProt, Pfam and KOG), and the TransDecoder was used to identify the candidate CDS regions. A Q value < 0.05 and FPKM> 0.3 were regarded as the criteria for differential genes referring to the method (Rteam et al., 2014) and fold change calculation method referred to Yang et al. (2014). The three parts (up-regulation, down-regulation and non-DEG) were analysed with scatter diagram in Excel to obtain the volcano plot. The KEGG metabolic pathways, the differential genes involved, are divided into five branches (cellular processes, environmental information processing, genetic information processing, metabolism and organisational systems). Gene ontologies include three functional categories: molecular function, cellular component and biological process. Functional classification was conducted according to the results of differential gene testing. GO classify and KEGG pathway classify method was described by Du et al. (2016) and Jia et al. (2006).

Western blotting

The third leaves (counted back from the apical stem) were sampled from plants grown for 3 weeks. Protein was extracted by Western blotting as described (Han et al., 2005) with GFP and actin antibody (Thermo Fisher, New York), respectively.

Statistical analyses

For statistical significance, Tukey's honestly significant difference (HSD) test was employed. The difference was considered significant at $P < 0.05$ for all qPCR data.

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Conflict of interest

The authors declare that they have no conflict of interests.

Author contributions

J.-F. J. and L.-J. W. designed the experiments; L.-J. W., J.S., L.-P. R., M. Z. and X.-Y.H. performed the experiments. L.-J. W., L. D., F. Z., Z.-Y. G., W.-M. F., S.-M., C., F.-D. C., L.D. and J.-F. J. analysed the data. J.-F. J. and L.-J. W. wrote the manuscript. All authors discussed the results and commented on the manuscript.

References

Ajay, A. (2000) The use of clustal w and clustal x for multiple sequence alignment. In Bioinformatics Methods and Protocols (Misener, S. and Krawetz, S.A. eds), pp. 221–242. Totowa: Humana Press Inc.

An, H., Roussot, C., Suárez-López, P., Corbesier, L., Vincent, C., Péireiro, M., Hepworth, S. et al. (2004) CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. Development, 131, 3615–3626.

Andrés, F. and Coupland, G. (2012) The genetic basis of flowering responses to seasonal cues. Nat. Rev. Genet. 13, 627.

Blanchard, M.G. and Runkle, E.S. (2009) Use of a cyclic high-pressure sodium lamp to inhibit flowering of chrysanthemum and velvet sage. Scientia Hort. 122, 448–454.

Boss, P.K., Bastow, R.M., Mylne, J.S. and Dean, C. (2004) Multiple pathways in the decision to flower: enabling, promoting, and resetting. Plant Cell, 16, 518–531.

Bowler, C., Benvenuto, G., Laflamme, P., Molino, D., Probst, A.V., Tariq, M. and Paszkowski, J. (2004) Chromatin techniques for plant cells. Plant J. 39, 776–789.

Chang, C.J., Li, Y.H., Chen, L.T., Chen, W.C., Hsieh, W.P., Shin, J., Wang, J.N. et al. (2008) LZF1, a HYS-regulated transcriptional factor, functions in Arabidopsis de-etiolation. Plant J. 54, 205–219.

Cheng, X.F. and Wang, Z.Y. (2005) Overexpression of COL9, a CONSTANS-LIKE gene, delays flowering by reducing expression of CO and FT in Arabidopsis thaliana. Plant J. 43, 758–768.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735–743.

Corbesier, L., Vincent, C., Jiang, S., Fornera, F., Fan, Q., Searle, I., Giakountis, A. et al. (2007) FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science, 316, 1030–1033.

Curts, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol. 133, 462–469.

Datta, S., Hettriarchachi, G., Deng, X.-W. and Holm, M. (2006) Arabidopsis CONSTANS-UXE3 is a positive regulator of red light signaling and root growth. Plant Cell, 18, 70–84.

Datta, S., Hettriarchachi, C., Johansson, H. and Holm, M. (2007) SALT TOLERANCE HOMOLOG2, a B-box protein in Arabidopsis that activates transcription and positively regulates light-mediated development. Plant Cell, 19, 3242–3255.

Dong, B., Deng, Y., Wang, H., Gao, R., Stephen, G., Chen, S. and Jiang, J. et al. (2017) Gibberellic acid signaling is required to induce flowering of chrysanthemums grown under both short and long days. Int. J. Mol. Sci. 18, 1259.

Du, J., Li, M., Yuan, Z., Guo, M., Song, J., Xie, X. and Chen, Y. (2016) A decision analysis model for KEGG pathway analysis. BMC Bioinform. 17, 407.

Endo, M., Yoshida, M., Sasaki, Y., Negishi, K., Horikawa, K., Daimon, Y., Kurotani, K. et al. (2018) Re-evaluation of floral inducer transport kinetics with separation of function mutations that uncouple flowering initiation and long-distance transport. Plant Cell Physiol. 59, 1621–1629.

Fehr, W., Caviness, C., Burmood, D. and Pennington, J. (1971) Stage of development descriptions for soybeans, Glycine Max (L.) Merrill 1. Crop Sci. 11, 929–931.

Gangappa, S.N. and Botto, J.F. (2014) The BBX family of plant transcription factors. Trends Plant Sci. 19, 460–470.

Gnesutta, N., Kumiomo, R.W., Swain, S., Chiara, M., Sirwardana, C., Horner, D.S., Holt, B.F. et al. (2017) CONSTANS imparts DNA sequence specificity to the histone fold NF-YB/NF-YC dimer. Plant Cell, 29, 1516–1532.

Han, Y., Jiang, J., Liu, H., Ma, Q., Xu, W., Xu, Y., Xu, Z. et al. (2005) Overexpression of OsSIN, encoding a novel small protein, causes short internodes in Oryza sativa. Plant Sci. 169, 487–495.
Lazacete, E. (2016) A laboratory practical illustrating the use of the ChiP-qPCR method in a robust model: Estrogen receptor alpha immunoprecipitation using MCF-7 culture cells. Biochem Mol Biol Educ. 45, 152–160.

Liu, H., Gu, F., Dong, S., Liu, W., Wang, H., Chen, Z. and Wang, J. (2016) CONSTANS-like 9 (COL9) delays the flowering time in Oryza sativa by repressing the Ehd1 pathway. Biochem. Biophys. Res. Co. 479, 173–178.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2–ΔΔCT method. Methods, 25, 402–408.

Mao, Y., Sun, J., Cao, P., Zhang, R., Fu, Q., Chen, S., Chen, F. et al. (2016) Functional analysis of alternative splicing of the FLOWERING LOCUS T orthologous gene in Chrysanthemum morifolium. Hor. Res. 3, 16058.

Mouradov, A., Cremer, F. and Coupland, G. (2002) Control of flowering time: interacting pathways as a basis for diversity. Plant Cell, 14, 5111–5130.

Oda, A., Narumi, T., Li, T., Kando, T., Higuchi, Y., Sumitomo, K., Fukai, S. and et al. (2011) CsFTL3, a chrysanthemum FLOWERING LOCUS T-like gene, is a key regulator of photoperiodic flowering in chrysanthemums. J. Exp. Bot. 63, 1461–1477.

Ordóñez-Herrera, N., Trimborn, L., Menje, M., Herschel, M., Robers, L., Kaufholdt, D., Hänisch, R. et al. (2017) The transcription factor COL12 is a substrate of the COP1/SPA E3 ligase and regulates flowering time and plant architecture. Plant Physiol. 176, 1327–1340.

Putterill, J., Robson, F., Lee, K., Simon, R. and Coupland, G. (1995) The CONSTANS gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell, 80, 847–857.

Qu, Q., Mei, H., Deng, X., He, K., Wu, B., Yao, Q., Zhang, J. et al. (2019) DNA methylation repels targeting of Arabidopsis REF6. Nat. Commun. 10, 2063.

Ren, L., Liu, T., Cheng, Y., Sun, J., Gao, J., Dong, B., Chen, S. et al. (2016) Transcriptomic analysis of differentially expressed genes in the floral transition of the summer flowering chrysanthemum. BMC Genom. 17, 673.

Rteam, R., Team, R.D.C., Team, R., Team and R. Null, R.D.C.T. (2014) R: a language and environment for statistical computing. Computing 14, 12–21.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N. and Weigel, D. (2006) Highly specific gene silencing by artificial microRNAs in Arabidopsis. Plant Cell, 18, 1121–1133.

Simmons, C.W., VanderGheynst, J.S. and Upadhyaya, S.K. (2009) A model of Agrobacterium tumefaciens vacuum infiltration into harvested leaf tissue and subsequent in planta transient expression. Biotechnol. Bioeng. 102, 965–970.

Simpson, G.G. and Dean, C. (2002) Arabidopsis, the Rosetta stone of flowering time? Science, 296, 285–289.

Song, Y.H., Ito, S. and Imaizumi, T. (2010) Similarities in the circadian clock and photoperiodism in plants. Curr. Opin. Plant Biol. 13, 594–603.

Sun, J., Wang, H., Ren, L., Chen, S., Chen and Ji, F. (2017) CmFTL2 is involved in the photoperiod-and sucrose-mediated control of flowering time in chrysanthemum. Hortic. Res. 4, 17001.

Tamura, K., Dudley, J., Nei and Kumar, S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Tan, J., Jin, M., Wang, J., Wu, F., Sheng, P., Cheng, Z., Wang, J. et al. (2016) OsCol10, a CONSTANS-Like gene, functions as a flowering time repressor downstream of Ghd7 in rice. Plant Cell Physiol. 57, 798–812.

Turck, F., Fornara, F. and Coupland, G. (2008) Regulation and identity of floral inducer: FLOWERING LOCUS T moves center stage. Annu. Rev. Plant Biol. 59, 573–594.

Wang, F.Q. and Zhang, Z.Y. (2012) Interactions among mediator subunits of tobacco by bimolecular fluorescence complementation (BiFC) method. J. Agricul. Biotechnol. 38–47.

Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Loehman, J.U. and Weigel, D. (2005) Integration of spatial and temporal information during floral induction in Arabidopsis. Science, 309, 1056–1059.
Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Differentially transcribed transcription factors identified from the contrast WT vs amiR-CmBBX8 line#3 grown under LD conditions.

Figure S1 Transcriptional profiling of CmFTL1.

Figure S2 Identification of plants harboring a CmBBX8 transgene, based on a PCR assay of gDNA and western blot.

Figure S3 The phenotypic consequences of over-expressing and knocking down CmBBX8 in cv. ‘Yuuka’ plants grown under SD conditions.

Figure S4 The phenotypic effect of expressing the p35S::CmBBX8 transgene in A. thaliana.

Figure S5 The identification of genes differentially transcribed by the knocking down of CmBBX8.

Figure S6 Alignment of selected chrysanthemum genes with their A. thaliana homologs.

Figure S7 Identification and flowering phenotype of plants harboring a CmFTL1 transgene in the background of ‘Yuuka’.

Figure S8 The identification and phenotype of the A. thaliana bbx7 mutant. (a) Identification of bbx7 mutant in Arabidopsis thaliana. (red: 3 12 32 homozygous mutation; yellow: 5 27 heterozygous mutation).

Figure S9 Transcriptional profiling of CmBBX8 under darkness 48 h and illumination 48 h.