A zinc finger protein BBX19 interacts with ABF3 to affect drought tolerance negatively in chrysanthemum

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SUMMARY

Drought is an environmental factor that can severely influence plant development and distribution, and greatly affect the yield and economic value of crops. We characterized CmBBX19, a BBX family subgroup IV member gene, from the transcriptome database of Chrysanthemum morifolium in response to drought stress. Drought stress and ABA treatments downregulated the expression of CmBBX19. We generated CmBBX19-overexpressing (CmBBX19-OX) lines and CmBBX19-suppressing lines (CmBBX19-RNAi), and found that suppressed expression of CmBBX19 led to enhanced drought tolerance compared with the wild-type (WT) controls, while CmBBX19-OX lines exhibited reduced drought tolerance. Downstream gene analysis showed that CmBBX19 modulates drought tolerance mainly through inducing changes in the expression of ABA-dependent pathway genes, including protective protein, redox balance and cell wall biogenesis genes, such as responsive to ABA 18, peroxidase 12, and cellulose synthase-like protein G2. Moreover, CmBBX19 was shown to interact with CmABF3, a master ABA signaling component, to suppress expression of these downstream genes. We conclude that BBX19-ABF3 module functions in the regulation of drought tolerance of chrysanthemum through an ABA-dependent pathway.

Keywords: drought stress, hormone signaling, transcription factors, CmBBX19, CmABF3, abscisic acid, Chrysanthemum morifolium.

INTRODUCTION

Drought stress is a global phenomenon that seriously limits crop production and distribution. Given that plants are mostly sessile, and thus unable to relocate to areas with more water, they have evolved complex morphological, physiological, cellular and molecular level systems to cope with drought stress (Yamaguchi-Shinozaki and Shinozaki, 2006; Yoshida et al., 2014). One of these involves the phytohormone abscisic acid (ABA), which plays a key role in several biological processes, such as plant growth and development, and in adaptive responses to environmental stress. It has been well known that there are two separate, but well-connected, pathways mediating drought stress responses, i.e. the ABA-dependent and independent drought pathways (Yamaguchi-Shinozaki and Shinozaki, 2006; Fujita et al., 2011; Yoshida et al., 2014).

ABA signaling involves three essential core components: (i) ABA receptors known as the pyrabactin resistance (PYR)/pyrabactin resistance-like (PYL)/regulatory component of the ABA receptor (RCAR) proteins; (ii) protein phosphatase 2C (PP2C) proteins; and (iii) sucrose-non-fermenting 1-related protein kinase (SnRK) proteins (Cutler et al., 2010; Raghavendra et al., 2010). In the presence of ABA, PYR1/PYL/RCAR proteins directly inhibit the activity of PP2C proteins, leading to the derepression of SnRK2 isoforms (Raghavendra et al., 2010). These activated SnRK2 proteins then phosphorylate downstream transcription factors, which further trigger the transcription of ABA-responsive genes, such as RAB18, RD29B and ADH1 (Yoshida et al., 2014).

In studies with Arabidopsis thaliana, among the transcription factors that are phosphorylated by SnRK2 proteins during ABA signaling, three have been highlighted for their key involvement in drought stress responses, i.e. ABF2/AREB1, ABF3 and ABF4/AREB2 (Yoshida et al., 2015). These transcription factors activate the transcription of their downstream target genes by binding to an ABA-responsive element (ABRE, PyACGTGG/TG) in the promoter.
(Yamaguchi-Shinozaki and Shinozaki, 2006; Yoshida et al., 2014). It has been well-documented that ABF/AREB proteins are basic leucine zipper (bZIP) transcription factors that can form hetero- or homodimers in the nucleus (Yoshida et al., 2010). However, little is known about their possible interactions with other transcription factors or associated mechanisms related to abiotic stress tolerance.

Another class of proteins that function in abiotic stress tolerance are members of the B-box (BBX) family, a subgroup of the zinc finger proteins with one or two B-box domains in their N terminus (Klug and Schwabe, 1995; Gangappa and Botto, 2014). The B-box domain is responsible for transcriptional regulation and protein interaction (Khanna et al., 2009; Gangappa and Botto, 2014), and BBX family members can be classified into five subgroups based on the number of B-box and CCT domains. BBX proteins also function in light-regulated developmental processes, such as seedling photomorphogenesis, shade avoidance and photoperiod-regulated flowering (Khanna et al., 2009; Gangappa and Botto, 2014; Vaishak et al., 2019). BBX18 represses thermotolerance in A. thaliana (Wang et al., 2013), while BBX24 induces salinity tolerance (Nagaoka and Takano, 2003). Heterologous overexpression of a chrysanthemum (Chrysanthemum morifolium) BBX gene, CmBBX22, in A. thaliana was shown to improve drought tolerance and delay leaf senescence (Liu et al., 2019), and we previously reported that CmBBX24 has dual roles in modulating abiotic stress and flowering time (Yang et al., 2014).

Several studies have investigated the mechanisms by which BBX proteins affect abiotic stress responses. Arabidopsis thaliana BBX18 modulates the expression of a group of heat shock-responsive genes (Wang et al., 2013), while CmBBX24 modulates gibberellin biosynthesis in chrysanthemum (Yang et al., 2014). Moreover, when overexpressed in A. thaliana, CmBBX22 functions in drought tolerance through transcriptional activation of downstream genes in the ABA signaling pathway, such as ABI3 and ABI5 (Liu et al., 2019), suggesting that BBX proteins may mediate ABA signaling. Another example of a study suggesting a relationship between BBX proteins and components of ABA signaling was the observation that BBX21 acts as a suppressor of the ABA control of seed germination through its physical interaction with ABI5 and via binding to the ABI5 promoter (Xu et al., 2014; Kang et al., 2018). Recently, it was also shown that BBX19 acts as a repressor of seed germination by directly regulating ABI5 expression (Bai et al., 2019). However, it has not been determined whether an interaction exists between BBX proteins and components of ABA signaling during responses to abiotic stress, including drought stress.

In present work, we tested the hypothesis that BBX proteins function in drought stress responses through interaction with other transcription factors, and in association with ABA signaling, using the commercially important ornamental plant chrysanthemum as an experimental system. Chrysanthemum is grown worldwide, but drought severely limits its planting areas and productivity, and so elucidating the molecular regulatory mechanisms of drought tolerance has considerable potential valuable for developing water-efficient germplasm.

RESULTS

CmBBX19 expression is downregulated by drought

We identified four unigenes encoding putative BBX group IV subfamily proteins in a chrysanthemum dehydration response transcriptome database (Xu et al., 2013). Of these, UN68402, was downregulated by dehydration treatment (Table S1). Sequence alignment and phylogenetic analysis revealed that the predicted protein contains structural characteristics of BBX group IV from A. thaliana and that it is a homolog of AtBBX18 and AtBBX19 (Figure S1). Accordingly, we named the gene CmBBX19, following the BBX family protein nomenclature system (Khanna et al., 2009).

CmBBX19 expression was evaluated in different organs and relatively high transcript abundance was found in leaves, flowers and stems, but lower levels in roots (Figure 1a). To test the response of CmBBX19 to drought stress, we used quantitative real-time PCR to measure its expression in mature leaves from soil-grown chrysanthemum plants taken over a drought time course. CmBBX19 expression decreased following the application of drought stress, and following severe drought stress (about 11% relative water content of soil), expression levels were 45% of those in well-watered control plants, but then returned to basal levels 1 day after rewatering (Figure 1b).

As endogenous ABA levels tend to rise rapidly in response to drought stress, we next tested whether ABA could affect CmBBX19 transcription. As shown in Figure 1 (c), CmBBX19 expression decreased to 0.2-fold after 1 h of exogenous ABA treatment, suggesting that CmBBX19 may act as a component of the ABA signaling pathway under drought stress.

To confirm whether CmBBX19 functions as a transcription factor, we transiently expressed a CmBBX19-green fluorescent protein (GFP) fusion protein in chrysanthemum protoplasts. The fusion protein was mainly located in the nucleus, but was also detected in the cytoplasm, while the GFP protein alone was present throughout the cell (Figure 1d).

As CmBBX19 contains a typical ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif in its C terminus, it can be considered a transcriptional repressor (Wang et al., 2014). Based on our sequence analysis of the BBX family in chrysanthemum, among the more than 18 BBX family members only

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CmBBX19 has an EAR motif. To determine whether CmBBX19 has transcriptional repression activity, we performed a dual-luciferase transactivation assay. As shown in Figure 1(e), leaves of Nicotiana benthamiana expressing the CmBBX19-GFP construct exhibited much lower relative luciferase activity compared with the control harboring GFP alone, indicating that CmBBX19 is a transcriptional repressor.

**CmBBX19 affects drought stress tolerance**

To determine whether CmBBX19 functions in the regulation of drought stress tolerance, we created 19 CmBBX19-overexpressing chrysanthemum lines (CmBBX19-OX) and 30 CmBBX19-suppressed (RNA interference, CmBBX19-RNAi) lines. We chose two CmBBX19-OX and two CmBBX19-RNAi lines to compare with wild-type (WT) plants. The transcript abundance of CmBBX19 in the transgenic lines was confirmed by quantitative real-time PCR analysis (Figure 2a). We also determined the expression of other members of chrysanthemum BBX group IV in the CmBBX19-RNAi lines and confirmed that only CmBBX19 was silenced (Figure S2).

We also detected the protein levels of CmBBX19-GFP in CmBBX19-OX lines by western blotting analysis. The results showed that substantially higher protein levels were observed in CmBBX19-OX than WT plants (Figure S3b), further confirming the genetic transformation of CmBBX19 at post-transcription level.

To examine the effect of CmBBX19 expression on the tolerance of drought stress, we grew the overexpressing and silenced lines in soil under normal watering conditions, then after 7 days, the plants were treated by withholding water for 30 days, followed by a 5-day recovery process, and the survival rates were determined. Before the drought treatment, compared with the WT, the transgenic lines did not exhibit clear differences in growth. However, after a 30-day drought treatment, the CmBBX19-OX plants and WT plants showed drought-induced damage that was more severe, such as wilted and withered leaves, than did the CmBBX19-RNAi plants (Figure 2c). After a 5-day recovery period, 67% of the WT and 100% of the CmBBX19-RNAi plants survived and showed continued growth of their apical shoots, whereas the survival rate of the CmBBX19-OX plants was 33%, and the surviving plants mostly and subsequently exhibited weak outgrowth of the lateral or basal shoots (Figure 2b).

To understand the physiological mechanisms of drought tolerance that were influenced by CmBBX19, we compared the transpiration rates, stomatal conductance, water loss and photosynthesis rates in transgenic plants and WT plants under drought/dehydration conditions. The transpiration rate, stomatal conductance levels, and photosynthesis rates were significantly higher in CmBBX19-RNAi plants, whereas the water loss rate was significantly lower in CmBBX19-RNAi plants than in the WT control under drought/dehydration conditions (Figure 2d–g). The CmBBX19-OX plants exhibited the opposite effects. These results were consistent with CmBBX19 influencing multiple physiological processes that contribute to drought tolerance.

CmBBX19 modulates the expression of abiotic stress-responsive genes in the ABA-dependent pathway

As described above, we observed that the exogenous ABA treatment downregulated CmBBX19 expression (Figure 1c). Here, we tested whether ABA treatment affects the protein level of CmBBX19 in transgenic plants. The results showed that almost no difference of CmBBX19 protein was observed between ABA treatment and control in CmBBX19-OX plants, indicating that the response of
CmBBX19 to ABA mainly occurs at the transcription level, rather than post-transcriptional level (Figure S3). Therefore, we investigated whether the action of CmBBX19 in response to drought stress was related to the ABA signaling pathway. Specifically, we carried out a large-scale screen for differentially expressed genes between leaves from CmBBX19-RNAi or CmBBX19-OX plants and WT plants, using a RNA-sequencing (RNA-seq) approach. We focused on the expression of genes related to the ABA-dependent and ABA-independent pathways in the transgenic plants compared with WT plants under normal growth conditions. ABA-dependent pathway genes, such as CmRAB18, CmRD29B, CmERD7 and CmLTI65, were downregulated in the CmBBX19-OX plants but upregulated in CmBBX19-RNAi plants, relative to WT. The expression of genes from the ABA-independent pathway, including CmDREB2 and CmDREB5, did not change in either CmBBX19-OX or CmBBX19-RNAi plants (Figure 3).

We then investigated whether the CmBBX19-modulated drought tolerance was associated with changes in ABA biosynthesis by evaluating gene expression and measuring ABA levels in leaves. We did not observe significant differences in ABA content and expression of ABA biosynthesis-related genes, such as CmNCED and CmABA2 among the transgenic lines and WT (Figures S4 and S5). We also tested whether the effect of CmBBX19 on drought tolerance was through transcriptional regulation of key ABA signaling components and downstream responsive genes.
Among the transgenic lines and WT, we did not find a significant difference in the expression of the ABA receptor gene, *CmPYR/PYL/RCAR*, or core ABA signaling genes, such as *CmSnRK2, CmABI3, CmABI4, CmABI5* and *CmABF3* (Figure S5). However, we detected significant differences in the expression of genes involved in downstream ABA signaling that encoded late embryogenesis-abundant (LEA)-like protective proteins, such as *CmRAB18* and *CmRD29B*; that encoded oxidation-reduction proteins, such as peroxidase 12-like (*PRX12*), glutaredoxin-C11-like (*GRXC11*), and C-terminal cysteine residue is changed to a serine 1 (*CXXS1*); that encoded cell wall biogenesis-related proteins, such as cellulose synthase-like protein G2 (*CmCSLG2*). The expressions of these genes above were downregulated in the *CmBBX19*-OX plants but upregulated in *CmBBX19*-RNAi plants, relative to WT (Figure 3 and Table S2).

A recent study reported that overexpression in *A. thaliana* of *CmBBX22*, another chrysanthemum BBX gene family member, can improve drought tolerance through delaying leaf senescence (Liu et al., 2019). Here, we tested the expression of leaf senescence-related genes, such as *CmNYC1* and *CmNYE1*, in transgenic and WT plants and showed that *CmBBX19* did not affect the expression of leaf senescence-related genes (Figure S5b). This indicates that *CmBBX19* functions in drought tolerance through a different regulatory mechanism from that associated with *CmBBX22*.

Based on these findings, we concluded that *CmBBX19* influences drought tolerance mainly through modulating the accumulation of protective proteins, maintaining cellular redox balance, and promoting cell wall biogenesis in the ABA-dependent pathway, rather than by altering ABA biosynthesis and key ABA signaling components at the transcriptional level.

**Figure 3.** Expression of genes related to the abscisic acid (ABA)-dependent or -independent pathway in *CmBBX19*-OX and *CmBBX19*-RNAi chrysanthemum plants. Quantitative real-time polymerase chain reaction analysis was performed to evaluate the expression of each gene. *CmUBIQUITIN* was used as the control gene. Three independent experiments were performed and error bars indicate standard deviation. Letters indicate significant differences according to Duncan’s multiple range test (*P* < 0.05).

*CmBBX19* modulates its downstream genes through interacting with *CmABF3*

To elucidate how *CmBBX19* affects the expression of downstream genes, we analyzed the promoters of LEA protein genes (Figure S6). However, the expression of ABRE cis-elements were enriched in the promoters of LEA-like protective proteins, such as *CmRAB18* and *CmRD29B*; that encoded oxidation-reduction proteins, such as peroxidase 12-like (*PRX12*), glutaredoxin-C11-like (*GRXC11*), and C-terminal cysteine residue is changed to a serine 1 (*CXXS1*); that encoded cell wall biogenesis-related proteins, such as cellulose synthase-like protein G2 (*CmCSLG2*). The expressions of these genes above were downregulated in the *CmBBX19*-OX plants but upregulated in *CmBBX19*-RNAi plants, relative to WT (Figure 3 and Table S2).

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According to previous studies, ABF/AREB proteins contain four conserved regions (C1–C4) and a bZIP domain.
To determine which conserved CmABF3 domain interacted with CmBBX19, we generated multiple truncated forms of CmABF3 (Figure 4b) and found that only C1 interacted with CmBBX19 in a yeast two-hybrid assay (Figure 4c). Previous studies also showed that the C1 domain of AREB1 has transactivation activity (Fujita et al., 2005) and contains a conserved RXXS/T site, which can be phosphorylated by SnRK2 protein kinases (Uno et al., 2000; Furihata et al., 2006). The interaction between CmBBX19 and CmABF3 suggested that CmBBX19 might affect ABA signaling by suppressing the transactivation activity of CmABF3, rather than by interfering with the promoter-binding activity of CmABF3.

We also isolated CmBBX19 and CmABF3 homologs from A. thaliana to test the conservation of the interaction between BBX19 and ABF3 in another species. Yeast two-hybrid and BIFC analyses showed that neither CmBBX19 nor AtBBX19 interacted with AtABF3 (Figure S8), suggesting that the interaction between BBX19 and ABF3 in chrysanthemum is not conserved in A. thaliana.

As BBX19 interacted with ABF3, we investigated whether BBX19 directly affects the expression of downstream genes. Sequence analysis showed that a 455 bp region of the CmRAB18 promoter contains three ABRE motifs (Figure 5a), and in a yeast one-hybrid assay CmABF3, but not CmBBX19, directly bound to the promoter of CmRAB18 (Figure 5b). We used a 50 bp fragment of the CmRAB18 promoter (position –388 to –339) that contains two ABRE motifs as a probe in an electrophoretic mobility shift assay (EMSA), and observed that the ABRE cis-elements in the CmRAB18 promoter were directly bound by CmABF3, but not by CmBBX19.
BBX19 influences chrysanthemum drought tolerance

not by CmBBX19 (Figures 5c and 6a). We also performed EMSA to examine whether CmBBX19 interferes with the binding affinity of CmABF3 to the promoters of its target genes (Figure 6a). We saw that CmBBX19 had no effect on the binding affinity of CmABF3 to the CmRAB18 promoter fragments, even after increasing the concentration of CmBBX19 in the reactions.

CmBBX19 represses CmABF3 activation of CmRAB18 expression

To determine whether CmBBX19 affects ABA-responsive genes by suppressing the transactivation activity of ABF3, we performed a dual-luciferase reporter assay. We fused the CmRAB18 promoter to firefly luciferase (LUC) (proCmRAB18: LUC) to generate a reporter construct, and used 35S:CmABF3, 35S:CmBBX19 and a mutant BBX19 with a Cys-25 to Ser substitution in B box1 (35S:CmBBX19mut) as three different effectors (Figure 6b). After co-transforming 35S:CmABF3 and the proCmRAB18:LUC into N. benthamiana leaves, we observed significantly higher LUC activities than when the empty vector and proCmRAB18:LUC (SK+proCmRAB18) were co-transformed. When we co-transformed 35S:CmABF3, 35S: CmBBX19 and proCmRAB18:LUC (CmABF3 + CmBBX19 + pcmRAB18), significantly reduced LUC activity was observed (Figure 6c). Furthermore, when mutated CmBBX19 replaced CmBBX19, LUC activity was recovered.

To obtain genetic evidence that CmBBX19 interacts with CmABF3, we silenced CmABF3 in the WT and CmBBX19-RNAi backgrounds, using a modified cabbage leaf-curl geminivirus vector (CaLCuV) containing the artificial microRNA-ABF3 (CaLCuV-amiR-ABF3) (Figure 7a). Silencing of CmABF3 in the WT and CmBBX19-RNAi backgrounds exacerbated the wilting symptoms after a 20-day drought stress treatment, and the plants showed a reduced transpiration rate, stomatal conductance and net photosynthetic rate. In the WT, these symptoms were alleviated after a 20-day drought stress treatment, while in the CmBBX19-RNAi backgrounds, they were not alleviated. Similarly, CmABF3 significantly suppressed the decrease of expression levels of drought-responsive genes (Yuan et al., 2018). In particular, both RAP2.1 and OsDRZ1 act as transcriptional repressors, and the expression of genes related to ABA signaling, demonstrating that CmBBX19 functions in an ABA-dependent manner, which is distinct from the previously reported drought-responsive EAR-containing proteins.

CmBBX19 binds to CmABF3 and interferes with CmABF3-activated expression of downstream genes

It has been reported that BBX21 can transcriptionally activate ABI5 gene or interact with ABI5 protein to influence ABA signaling in A. thaliana (Xu et al., 2014; Kang et al., 2018). Regarding BBX19, a previous report showed that BBX19 negatively modulates flowering time through interacting with CONSTANS (CO) to suppress FLOWERING LOCUS T (FT) transcription (Wang et al., 2014). Recently, BBX19 was found to activate expression of ABI5 through directly binding to its promoter to suppress seed germination (Bai et al., 2019).

Interestingly, in the present work, we found that CmBBX19, a chrysanthemum homolog of BBX19, cannot bind to ABI5, but interacts with CmABF3, another key component of ABA signaling. In addition, our current data showed that BBX19 does not interact with ABF3 in A. thaliana, suggesting that the role of the CmBBX19-CmABF3 module in drought tolerance might be chrysanthemum specific. However, similar to the regulatory manner of BBX19 in A. thaliana, CmBBX19 interfered with CmABF3-
dependent transactivation of downstream genes, instead of directly binding to the promoter of those genes.

In conclusion, we suggest a working model (Figure 8) in which CmBBX19 functions as a transcriptional suppressor in the drought stress response through interaction with CmABF3, and suppresses CmABF3 activation of target genes, thereby tightly influencing drought stress responses.

EXPERIMENTAL PROCEDURES

Plant materials and treatments

We took a chrysanthemum cultivar (Chrysanthemum morifolium, cv. Fall Color) as plant material in present study. Plant cultivation was carried out as previously described (Wei et al., 2017). Forty-day-old tissue culture plantlets were transplanted into 9-cm diameter pots filled with a peat/vermiculite (1:1, v/v) mixture and grown in a controlled environment (23 ± 1°C, 40% relative humidity, 100 μmol m⁻² sec⁻¹ illumination and 16 h light/8 h dark).

For the expression determination of CmBBX19 in different chrysanthemum organs, plants were grown under long-day (16 h light/8 h dark) conditions for 6 months.

For different degrees of drought stress treatment, 60-day-old plants were grown in the controlled environment as described above. Watering was stopped after plants were fully watered. Plants in pots were weighed at the start point and periodical time point of the drought stress treatment. Relative water content of the soil for the indicated treatments were 77% (well-watered), 56% (mild drought), 25% (moderate drought), 11% (severe drought) and 0% (drought). Relative soil water content was calculated using the following formula: 

\[ \text{RWC} (\%) = \left( \frac{W_s - W_t}{W_s} \right) \times 100\% \]

where \( W_s \) is the initial soil water content and \( W_t \) the final soil water content.

For ABA treatments, roots of 40-day-old tissue culture plantlets were soaked in a 100 μM ABA solution. A corresponding aqueous solution was used as the control.

Figure 6. CmBBX19 interacts with CmABF3 to repress transcription of CmRAB18.

(a) Analysis of CmABF3 and CmBBX19 binding to the CmRAB18 promoter, based on an electrophoretic mobility shift assay. Purified protein (3 μg) was incubated with 50 μM biotin-labeled probes. For the competition test, purified CmBBX19 protein, at 1- or 10-fold concentrations, was added to the experiment described above. FP, free probe.

(b) Schematic representation of the double-reporter and effector plasmids used in the dual-luciferase reporter assay.

(c-d) The interaction of CmABF3 or CmBBX19 with the CmRAB18 promoter as shown by a dual luciferase (LUC) reporter system. A 445 bp CmRAB18 promoter fragment was used. Constructs used in the assay are shown above. LUC vectors containing the renilla luciferase (REN) gene under the control of the 35S promoter were used as a positive control. Samples were assayed 3 days after infiltration. Representative photographs are shown of firefly luciferase fluorescence signals (c) and relative LUC/REN ratio are shown of normalizing LUC activity to that of REN (d) when the corresponding effectors and reporters were introduced into Nicotiana benthamiana leaves. Three independent experiments were performed and error bars indicate standard deviations. Asterisks indicate significant differences as determined by Tukey’s honestly significant difference method (**P < 0.01).
RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRizol reagent (TaKaRa, Shiga-ken, Japan). cDNAs were synthesized from 1 μl total RNA using the HiScript II One Step reverse transcription-PCR kit (Vazyme, Nanjing, China). Quantitative real-time PCR reactions were performed with an ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The *CmUBIQUITIN* gene (GenBank accession EU862325) was used as the internal control. Expression was normalized to the reference *CmUBIQUITIN* gene using the comparative ΔΔCt method (Livak and Schmittgen, 2001). The primers used for gene expression analysis were listed in Table S3.

Subcellular localization

The *CmBBX19* ORF sequence, without the stop codon, was cloned into the pSuper1300 (GFP-C) vector driven by the Super promoter. Mesophyll protoplasts were prepared from 40-day-old chrysanthemum leaves according to Yoo *et al.* (2007), and 10 μg of pSuper::*CmBBX19-GFP* plasmid, prepared using an OMEGA Plasmid Maxi Kit (OMEGA, Norcross, GA, USA), was introduced into approximately 2 × 10⁴ protoplasts with polyethylene glycol as described in Higuchi *et al.* (2013). Transformed mesophyll protoplasts were observed with an Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) after being cultured for 16–20 h at 22°C. For confocal microscopy, GFP images were obtained with an excitation at 488 nm and emission at 525 nm. The primers used for vector construction were listed in Table S3.

Chrysanthemum transformation

For construction of the overexpression vector, the *CmBBX19* ORF was cloned into the *XbaI* and *SacI* sites of the pBI121 vector (Chen *et al.*, 2003). For RNAi vector construction, a 319-bp sense and antisense fragment of *CmBBX19* containing *XhoI/ClaI* and *XbaI/KpnI* sites were cloned into the pHANNIBAL vector to obtain an intron containing “hairpin” RNA (ihpRNA) construct. The ihpRNA construct with 35S promoter and Nos terminator was then cloned into the binary pART27 vector (Wesley *et al.*, 2001). These recombinant constructs were introduced separately into *Agrobacterium tumefaciens* strain EHA105, and then transformed into chrysanthemum (Hong *et al.*, 2006).

Drought stress treatment

The *CmBBX19-OX*, *CmBBX19-RNAi* and WT plants were transferred to 25-cm pots containing 550 g of a 1:1 (w/v) mixture of peat and vermiculite, with three plants from each line per pot and...
The measurements were conducted with five biological replicates. The photographs were taken to record their phenotypes both before and after treatments.

**Measurements of transpiration rate, stomatal conductance, photosynthesis rate and water loss rate**

Measurements of transpiration rate, stomatal conductance and photosynthesis rate of the top sixth expanded leaf were carried out using an LI-6400XT Portable Photosynthesis System (LI-CORE, Lincoln, NE, USA). Leaves were placed in a chamber at 22°C, 400 µmol mol⁻¹ CO₂ and 300 µmol m⁻² sec⁻¹ illumination. Data were recorded every 2 min once the infrared gas analyzer was stabilized.

The top fifth fully expanded leaves from 60-day-old *CmBBX19-OX, CmBBX19-RNAi* and WT plants were used for the analyses of relative water loss. Fresh weight (FW) of each leaf was measured immediately after detached from a plant. For the dehydration treatment, the desiccation weight (dW) of each leaf was measured at different time points. Finally, the leaf samples were dried at 65°C for 24 h to determine the dry weight (DW). Relative water loss rates were calculated using the following equation: relative water loss rate (%) = (FW – dW)/(FW – DW) × 100 (Fukao et al., 2011). The measurements were conducted with five biological replicates and the results are presented as the means ± SD.

**RNA-seq analysis**

Total RNA was extracted from the top fifth expanded leaf from 60-day-old *CmBBX19-OX, CmBBX19-RNAi* and WT plants using TRizol reagent (TaKaRa). RNA-seq data were processed as described previously (Gao et al., 2016). Briefly, the low-quality reads (Q value <20) and adapters were filtered using Trimmomatic (Bolger et al., 2014). High-quality clean reads were de novo assembled into contigs using the Trinity program and iAssembler (Grabherr et al., 2011; Zheng et al., 2011). The assembled contigs were used in a search against the GenBank non-redundant, UniProt and *A. thaliana* protein databases using BLAST with a cutoff E value of 1e⁻⁵.

**Dual-luciferase reporter assay in *N. benthamiana***

For analysis of *CmBBX19* transcriptional activation, the *CmBBX19* ORF sequence, without the stop codon, was cloned into the pBD-VP16 vector (Han et al., 2016). The fusion constructs were introduced into *A. tumefaciens* strain GV3101, which was then shocked overnight. The *A. tumefaciens* were collected and adjusted to OD₆₀₀ = 1.0 by infiltration buffer. *Agrobacterium tumefaciens* culture harboring *CmBBX19* was mixed with 1/5 volume of *A. tumefaciens* culture containing the reporter vector (Han et al., 2016), and infiltrated into *N. benthamiana* leaves using a needleless syringe.

To determine the **in vivo** interaction between *CmBBX19*/*CmABF3* and the *CmRAB18* promoter, the *CmRAB18* promoter was inserted into the pGreenII 0800-LUC vector, while the *CmBBX19* or *CmABF3* ORF sequences were cloned into the pGreenII 0029 62-SK vector (Hellens et al., 2005). *Agrobacterium*
tumefaciens strain harboring CmBBX19, CmABF3 and CmRAB18 promoter-driven LUC constructs were mixed as 1:1:10 (v/v/v), and infiltrated into N. benthamiana leaves.

The dual-luciferase reporter assay was conducted using the dual-luciferase reporter assay systems (Promega, Madison, WI, USA) and α-luciferin (Promega) as previously described (Gao et al., 2019). The LUC images were taken using an iKon-L936 imaging system (Andor Tech, Belfast, UK). LUC and REN activities were determined using a GloMax 20/20 luminometer (Promega). The primers used for vector construction are listed in Table S3.

**Yeast two-hybrid assays**

The CmBBX19 and AtBBX19 ORF sequences were amplified and separately cloned into the EcoR I/SaI sites of the pGBK7 vector (Louvet et al., 1997). The ORF or ORF fragments of CmABF genes, AtABF genes and AtABF5 were amplified and cloned into the EcoR I/XhoI sites of the pGADT7 vector (Chien et al., 1991). The pGADT7 and pGBK7T7 recombinant plasmids were transformed into yeast strain Y2HGold together and the Y2H assay was conducted using the Matchmaker™ GAL4 two-hybrid system (Clontech, Shiga-ken, Japan). Transformsants were grown on SD/-Trp-Leu plates, and then transferred to SD/-Trp-Leu-His-Ade plates for spot assays. The PCR primers used for vector construction are listed in Table S3.

**BIFC**

Constructs expressing CmBBX19-YFP<sup>N</sup>, CmABF3-YFP<sup>C</sup> or control vectors were introduced into A. tumefaciens strain GV3101, which was then shaken overnight. The A. tumefaciens were collected and adjusted to OD<sub>600</sub> = 1.0 by infiltration buffer. Combinations were co-infiltrated into 4-week-old N. benthamiana leaves using a needleless syringe. The YFP fluorescence was imaged 60 h after infiltration using an Olympus FV1000 confocal laser scanning microscope. The excitation wavelength for YFP was 488 nm and emission wavelength was 525 nm. The primers used for vector construction are listed in Table S3.

**Yeast one-hybrid assays**

To construct bait vector, CmRAB18 promoter fragments were amplified from chrysanthemum genomic DNA and then inserted into the Kpn I/SaI sites of the pAbAi vector (Clontech). For prey construction, the CmABF3 ORF was amplified and then inserted into the EcoR I/XhoI sites of the pGADT7 vector (Clontech). Y1H assays were performed using the Matchmaker™ Gold Yeast One-Hybrid Library Screening System (Clontech). Transformsants were selected and grown on SD/-Ura-Leu plates, and then transferred to SD/-Ura-Leu-A, Aureobasidin A plates for spot assays. The PCR primers used for vector construction are listed in Table S3.

**EMSA**

EMSA was conducted using biotin-labeled probes and a Light Shift Chemiluminescence EMSA kit (Thermo Scientific, Waltham, MA, USA) as previously described (Dai et al., 2012). The CmRAB18 promoter fragment (5′-gAAAAATGACAAGTACCTTTACAgTATAACAATAAATACATATTACgTTC-3′) with a biotin-labeled probe, and the same unlabeled fragment was used as a competitor. *Escherichia coli* Rosetta cells harboring GST-CmBBX19 or GST-CmABF3 recombinant plasmids were incubated at 16°C for 10 h to induce recombinant proteins by adding isopropyl-thio-β-galactoside to a final concentration of 0.4 mM. The recombinant proteins were purified using glutathione Sepharose 4B beads (GE Healthcare, Huston, TX, USA). Purified GST-CmBBX19 and GST-CmABF3 proteins were incubated with 2 nm biotin-labeled probes in a 20 μl reaction mixture. The primers used for vector construction are listed in Table S3.

**Viruses**

For CmABF3 silencing in chrysanthemum, a previously reported virus-based microRNA expression system was used (Tang et al., 2010). The candidate 21-nt mature amiRNA-CmABF3 sequence and primers for amplifying amiRNA-CmABF3 were designed using the Web MicroRNA Designer (http://www3.broadinstitute.org/cgibin/webmi). amiR-CmABF3 was cloned from the pRS300 plasmid into the CalCuV vector, and then introduced into A. tumefaciens strain GV3101. Agrobacterium tumefaciens cells harboring recombinant plasmids were shaken overnight. The A. tumefaciens were collected and adjusted to OD<sub>600</sub> = 1.5 by infiltration buffer. pCVB and CalCuV (control) or CalCuV-CmABF3 were mixed in a 1:1 ratio/w/w, and were placed at 28°C in the dark for 3-4 h before infiltration. Forty-day-old tissue cultured chrysanthemum RNAi and WT plants were vacuumed in infiltration buffer for 3 min under 0.7 MPa, and then washed by deionized water. The plants were placed at 8°C in the dark for 3 days before transplanting in pots containing 50 g of a mixture of 1:1 (v/v) peat and vermiculite and growing under normal conditions (23 ± 1°C, 40% relative humidity, and 16 h light/8 h dark). Before drought stress treatment, the plants were validated by determination of the expression of CmABF3. Two independent experiments were conducted and at least 12 positive plantlets of WT or RNAi lines were used in each experiment.

For drought stress treatment, plants were fully watered and then water was withheld for 20 days. After treatment, the plants were photographed. Transpiration rate, stomatal conductance and photosynthesis rates were monitored as above, before treatment and after 20 days of drought.

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**AUTHOR CONTRIBUTIONS**

BH conceived, designed, and supported the experiments. YX and XZ performed most of the experiments; AP and JZ performed plant cultivation and vector construction; XM and MZ performed protein interaction assays; LC...
performed chrysanthemum transformation; JG and CM provided conceptual advice; YX and BH analyzed the data and wrote the manuscript.

CONFLICT OF INTEREST
The authors have no conflict of interest to declare.

SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article.

Figure S1. Deduced CmBBX19 amino acid sequence analysis.

Figure S2. Expression levels of members of the BBX group IV in wild type (WT) and CmBBX19-RNAi plants.

Figure S3. mRNA and protein levels of CmBBX19 in the overexpression lines.

Figure S4. ABA content in leaves of CmBBX19 transgenic lines and wild type (WT).

Figure S5. Expression of genes related to abscisic acid (ABA) biosynthesis, signaling pathway, and leaf senescence in transgenic CmBBX19-OX or CmBBX19-RNAi chrysanthemum plants.

Figure S6. Distribution of ABRE and G-box motifs in promoters of LEA protein genes, upregulated in CmBBX19-RNAi plants.

Figure S7. Deduced amino acid sequence analysis of CmABF.

Figure S8. Analysis of BBox19-ABF interaction in Arabidopsis thaliana.

Figure S9. Expression of abiotic stress-responsive genes in the ABA-dependent pathway in CmBBX19-RNAi plants.

Table S1. Expression profiles of BBox family subgroup IV genes in the chrysanthemum transcriptome database in response to dehydration.

Table S2. Differentially expressed genes related to abiotic stress tolerance in CmBBX19 transgenic plants.

Table S3. Primers used for vector construction and quantitative real-time PCR analysis.

Methods S1. Sequence analysis.

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